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Pectin Degradation in Fruit Juices by Pectinase from *Meyerozyma* sp. VITPCT75 Isolated from *Phyllanthus emblica*

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Abstract

This study aimed to identify and characterize a pectinase-producing novel yeast from the fermented juice of *Phyllanthus emblica* and apply the enzyme for fruit juice clarification. Among the five pectinase-producing yeasts, isolate-1 exhibited the highest pectinase activity and was further used in this study. Based on morphological, physiological, and 18SrRN Aanalyses, isolate-1 was recognized as a new strain sharing 99% sequence homology with other *Meyerozyma* strains and was thus designated as *Meyerozyma* sp. VITPCT75. The strain produced pectinase optimally at a temperature and pH of 25°C and 7, respectively. Maximum pectinase production was observed after 4-days incubation. The enzyme exhibited optimum activity at the temperature of 25 °C and pH 7.0. The enzyme was more stable at a temperature and pH of 20 °C and 7, respectively. Storage stability studies revealed that the enzyme was stable at -20 °C. The cell-free supernatant was partially purified using ammonium sulfate and solvent precipitation. Acetone at a concentration of 20% assured an adequate partial purification. The molecular weight of pectinase was determined as 6 kDa. The enzymatic metal ion preference-related studies revealed that Ca^2z, Kz, Cu^2z, Fe^2z, and Ba^2z ions enhanced, Ni^2z ions moderately inhibited, and Mn^2z ions intensely inhibited the enzymatic activity. Neither Na^+ and Mg^{2+} ions nor EDTA affected the enzyme activity. When subjected to fruit juice clarification, the enzyme significantly reduced the viscosity of the juice.

Keywords: Pectinase, Pectin, *Meyerozyma* sp., *Phyllanthus emblica*, Psychrophilic

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INTRODUCTION

Pectinases (EC 3.2.1.15) (polygalaturonases) are hydrolytic enzymes that cleave the β-1,4 glycosidic bonds linking galacturonic acids in pectin present in the plant cell walls[1,2]. Pectinases play a vital role in the usual ripening processes of certain fruits by altering the nature of pectin substances[3,4]. They are extensively used in the food industry, plant tissue maceration, degumming of natural fibers, textiles, and wastewater treatment[5,6]. In the food industry, they are used for fruit juice clarification, pesticide fermentation, and essential oil extraction[7]. Moreover, pectinases improve liquefaction, clarification, filterability, color release, and flavor compounds from the fruits into boost wine quality[8]. Pectinases of microbial origin are classified into enzymes with depolymerizing and saponifying activities. Polymethyl galacturonases, pectin lyases, pectate lyases, and polygalacturonases are depolymerizing enzymes, whereas pectin esterases are saponifying enzymes[9].

Microbes are the best enzyme sources, allowing an economical advantage with low resource consumption, fewer by products, no toxicity, no requirement of rare and expensive elements, and low emissions involving no social and political issues[10,11]. Microbial enzymes are preferred in the industry due to their wide spectrum of working conditions, better performance, and feasibility for scale-up by genetic modifications[12]. Traditionally, pectinases are obtained from yeast, filamentous fungi, and bacteria. Fungi commonly produce a mixture of enzymes and release methanol as a by product. Yeasts are always preferred for pectinase production as they are unicellular, exhibit simple growth requirements, and are inducer-independent with the ease of genetic manipulations[13,14]. Above all, they do not secrete pectin methylesterase and, hence, pectinases could preferably be used to clarify fruit juices and wine without the risk of methanol fermentation[15,16]. Yeasts generally exhibit short growth and fermentation cycles and are GRAS organisms. Therefore, they could be used safely and efficiently in the food industry. Yeasts could also be immobilized and used for continuous fermentation.

Studies have already focused on yeast-derived pectinases of several genera as *Saccharomyces, Cryptococcus, Kluyveromyces, Aureobasidium, Rhodotorola*, and *Candida*[17,20]. Alkaline pectinases are generally produced by bacteria, filamentous fungi, and yeasts[21,22]. Yeast-derived pectinases are commonly active at acidic pH of 4–6, although certain pectinases are also active at neutral pH [23,24]. Fruit dumped soil and spoilt refrigerated fruits are also good sources of pectinolytic microorganisms. In a recent study, pectinase-producing microorganisms were isolated from coffee bean samples[25]. In another report, pectinase-producing *Bacillus* sp. was isolated from cassava waste[26]. At present, research efforts are focused on the identification of novel microbial pectinases with desirable biochemical and physicochemical characteristics from diverse ecosystems for commercial applications, considering the potential applications of pectinases in the food sector. The present study was carried out to isolate and characterize pectinase-producing novel yeast strains from fermented *Phyllanthus emblica* (gooseberry) juice to explore the potential of the produced enzymes in fruit juice clarification. To the best of our knowledge, this is the first report of a novel, pectinase-producing yeast strain *Meyerozyma* sp. derived from *Phyllanthus emblica* fruits. This study describes partial purification and characterization of pectinase and its ability to clarify fruit juices.

MATERIALS AND METHODS

Materials

D-Galacturonic acid sodium salt was procured from Sigma Chemicals. The culture media ingredients were purchased from Hi-Media, Mumbai. The protein molecular weight markers were purchased from Merck. All other chemicals used were of analytical grade.

Pectinase-producing yeast strain isolation and identification

Phyllanthus emblica fruits were obtained from a local market and authenticated by a botanist. Well-matured and ripe fruits were selected, surface-sterilized, cut into pieces, crushed, and incubated at room temperature for 4 days for natural fermentation. The fermented juice thus obtained was used to screen for pectinase-producing yeast, which was screened based on the utilization of pectin as the only carbon source in the screening medium, yeast extract peptone

pectin agar (YPPA) containing (g/l) yeast extract 10, peptone 20, pectin 20, and agar 20[27]. Fermented fruit juice samples were serially diluted in sterile water, and aliquots were plated on YPPA using the spread plate technique. The plates were incubated at 25 °C for 48 h. Yeast colonies with a characteristic creamy appearance were selected and sub-cultured on YPP, isolate-1 was identified as a potential pectinase-producing yeast based on the pectinase assay and selected for further study. Species identification of isolate-1 was identified based on its morphological, physiological, and molecular features. The 18 S rRNA gene of the strain was partially amplified using universal ITS primers, and the 5.8 rRNA gene was completely amplified. The sequences were deposited at NCBI with accession number MH217580, and further studies were performed using the NCBI server (http://www.ncbi.nlm.nih.gov) using the BLAST (BLASTN) tool, and a phylogenetic tree was created.

Pectinase assay and protein estimation

The production medium for pectinase was composed of (g/l) yeast extract 10, peptone 20, and pectin 20, and pH7. The sterilized medium was seeded with active inoculum (10%) and incubated at 25 °C under shaking conditions (180 rpm) for four days. The culture fluid was centrifuged at 8000 rpm for 15 min. and 4 °C) to obtain the supernatant for further study. Polygalacturonase/ pectinase activity was calculated by measuring the concentration of reducing sugar using the modified dinitrosalicylic acid(DNS) method using pectin as the substrate[28]. Pectinase acts on pectin and releases galacturonic acid, which reduces DNS to amino nitrosalicylic acid with λmax of 540 nm. D-galacturonic acid was used as the standard in the assay. One unit of pectinase activity was specified as the quantity of enzyme needed to liberate 1µM galacturonic acid per ml per min under prescribed conditions of the assay. Protein was estimated by Lowry's method using BSA as a standard[29].

Partial pectinase purification

The protein fraction of the supernatant was enriched by precipitation using ammonium sulfate, acetone,and ethanol. Ten milliliters of the supernatant was exposed to 20, 30, 40, 50, 60, 70, and 80% saturation and centrifuged (10000 rpm, 20 min, and 4 °C) to derive the precipitates, which were then dissolved in 1ml of sodium phosphate buffer (pH 7). Pectinase and specific activity were evaluated at all saturation points. To purify the enzyme further by ion-exchange chromatography, a pH survey was performed, and it was observed that a pH of 6.5 was ideal for pectinase in the DEAE Sephadex ion exchanger. A DEAES ephadex (20 cm × 1 cm) column was pre-equilibrated with 0.01 M sodium phosphate buffer (pH 6.5). Five milliliters of the acetone precipitate was injected, and the column was rinsed with buffer continuously (flow rate of 60 ml h^{-1}). The bound protein fractions were eluted with NaCl(linear gradient, 0.02–1.0 M). Fractions rich in pectinase were combined, enriched further, and stored at -20 °C.

SDS-PAGE

The molecular size of pectinase from isolate-1 was assessed by SDS-PAGE using a 12% polyacrylamide gel[30]. Carbonic anhydrase (29 kDa), ovalbumin (43 kDa), bovine serum albumin (66 kDa), and phosphorylase-B (97.4 kDa) were used as molecular weight markers.

pH effect and stability

The effect of pH on pectinase activity was ascertained by performing the assay at various pH values(4.5–9). For this experiment, 0.05 M acetate, phosphate,and Tris-HCl buffers (pH 4.5–6, 6–7,

Table 1. Morphological and sugar fermentation characteristics of *Meyerozyma* sp. VITPCT75

S. No.	Test	Observation
1	Simple staining	Oval budding yeast cells
2	Lactophenol cotton blue staining	Oval budding yeast cells
3	Sugar fermentation	
	i). Glucose	Positive with gas
	ii). Sucrose	Positive with gas
	iii). Lactose	Negative

Table 2. Partial purification of pectinase from *Meyerozyma* sp. VITPCT75

Purification steps	Enzyme Volume (ml)	Pectinase Activity (Units/ml/min)	Protein (mg/ml)	Total activity (Units)	Total protein (mg)	Specific Activity (Units /mg of protein)	Yield (%)	Fold purification
Crude extract	100	13.73	0.28	1373	28	49.03	100	1
Ammonium sulphate precipitate	5	22.32	0.06	111.6	0.3	372	8.12	7.58
Acetone precipitate	5	26.79	0.07	133.95	0.35	382.71	9.75	7.80
Ethanol precipitate	5	24.72	0.07	123.6	0.35	353.14	9.00	7.20

and 8–9, respectively) were used. The stability of pectinase at different pH values was analyzed by keeping the enzyme suspensions in the pH range of 6–8 for up to 16 h. Aliquots were collected at 0.5, 1, 2, 4, 8, and 16 h, and the pectinase activity was calculated at pH 7.

Temperature effect and thermal stability

The effect of temperature on pectinase activity was investigated by incubating the reaction mixture in the range of 15–80 °C. The stability of the enzyme at different holding temperatures was scrutinized by incubating the enzyme suspensions at 20, 30, 40, and 50 °C. Specimens were obtained at 0.5, 1, 2, 4, 8, and 16 h, and the pectinase activity was calculated at pH 7 and 25 °C.

Storage stability

The storage stability of pectinase was evaluated at storage temperatures of 25 °C (room temperature), 4°C (cold), and -20 °C(frozen). The suspension was held at the respective temperatures at a constant pH7 for 10 days. Representative specimens were acquired at 48 h intervals, and the remaining pectinase activity was quantified.

Metal ion and inhibitor effects

The effects of metal ions and specific inhibitors on pectinase were reviewed by exposing the suspension to representative metal ions and specific inhibitors at 25 °C for 1h with appropriate controls. Persisting pectinase activity was then determined.

Fruit juice clarification

The food industry is the largest consumer of pectinases, where the enzyme is used to degrade suspended pectin in order to improve the quality and efficiency of fruit juices. To appraise the suitability of pectinase from isolate-1, a study on its ability to identify representative fruit juices was undertaken. Fresh fruits were procured from the local market, and raw juice was obtained after tissue grinding and pressing. Partially purified pectinase from isolate-1, containing 10 units/ml/min of enzyme, was used in this study. Fruit juice (50ml of fruit juice was mixed with 10ml of enzyme extract and incubated at 25 °C for 1h along with the control (without enzyme) to allow pectinase to degrade pectin. After incubation, the mixture was kept in a boiling water bath for 10 min and centrifuged at 8000 rpm for 15min at 4 °C. The

supernatant was saved, and its viscosity was measured in a Tech-ED viscometer[7, 31]. The results were then recorded.

The experiments were performed in duplicates. Pectinase assays were performed in duplicate and repeated twice. The values represented in the tables and graphs indicate the arithmetic means.

Table 3. Effect of metal ions, detergents and specific inhibitors on pectinase

	Concentration	Relative Activity (%)
Metal Salts		
$CaCl_2$	1Mm	120
$CaCl_2$	10Mm	177
$MnCl_2$	1Mm	12
$CoCl_2$	1mM	125
NiCl2	1mM	78
NaCl	10mM	126
NaCl	1mM	106
KCl	1mM	131
$CuSO_4$	1mM	130
$FeSO_4$	1mM	136
$MgCl_2$	1mM	96
$BaCl_2$	1mM	138
Sodium citrate	1mM	125
EDTA	1Mm	131
EDTA	10mM	144
Detergents		
Tween -20	1% (V/V)	100
Hydrogen peroxide	1% (V/V)	108
Specific Inhibitors		
PMSF	10mM	116
β-mercapto ethanol	% (V/V)	100

RESULT AND DISCUSSION

Pectinase-producing yeast strain isolation and identification

Five yeast strains were isolated from the fermented *Phyllanthus emblica* fruit juice. Isolate-1 was identified as a potential pectinase-producing strain based on the DNS assay with pectinase activity of 13.73 U/ml/min and specific activity of 49.03 U/mg of protein at 25 °C and pH 7 using pectin as a substrate. Table 1 and Figs. 1 and 2 show the morphological and biochemical characteristics of isolate-1. The NCBI NBLAST analysis of 18S and 5.8S rRNA gene sequences showed 99% homology with other *Meyerozyma* sp. In the phylogenetic tree (Fig. 3), the strain lies in a separate branch, representing a novel strain. Therefore,this strain was designated as *Meyerozyma* sp. VITPCT75.

Partial pectinase purification

The cell-free supernatant was better concentrated at 20% saturation by all three applied methods (i.e., ammonium sulfate,

Table 4. Clarification of fruit juices by pectinase

S. No.	Fruit juice	Kinematic Viscosity (v) $m^2 Sec^{-1}$ Test	Control
1.	Orange	67.4	75
2.	Apple	65	72.4
3.	Lemon	67.8	76
4.	Gooseberry	66.8	74

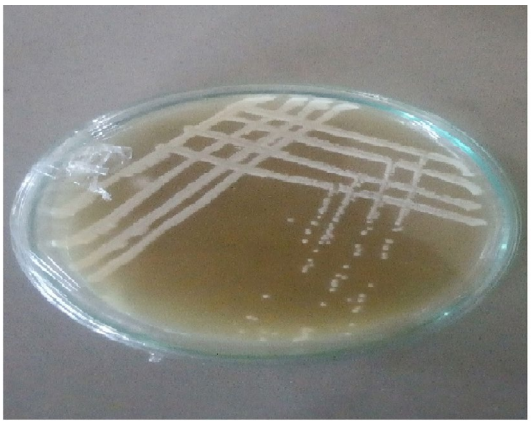

Fig. 1. *Meyerozyma* sp. VITPCT75 on YPPA

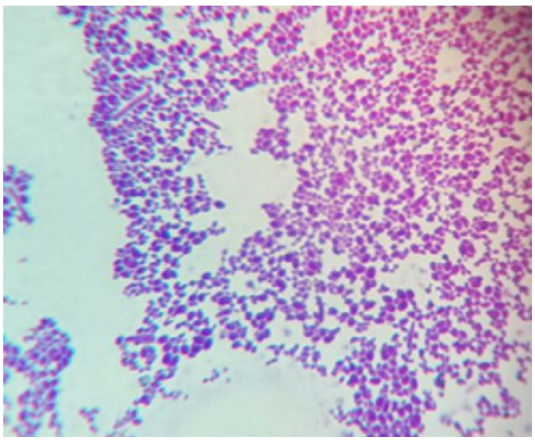

Fig. 2. Microscopic appearance of *Meyerozyma* sp. VITPCT75

acetone, and ethanol precipitation) but resulted in a low yield of 8.1–9.7% and fold purification in the range of 7.2-7.8 (Table 2). The low pectinase yield could be attributed to its low molecular weight of 6 kDa,shown by SDS-PAGE, and high solubility[32]. Alternatively, ultrafiltration using a 5-kDa molecular weight cut-off membrane could be used to concentrate the enzyme in

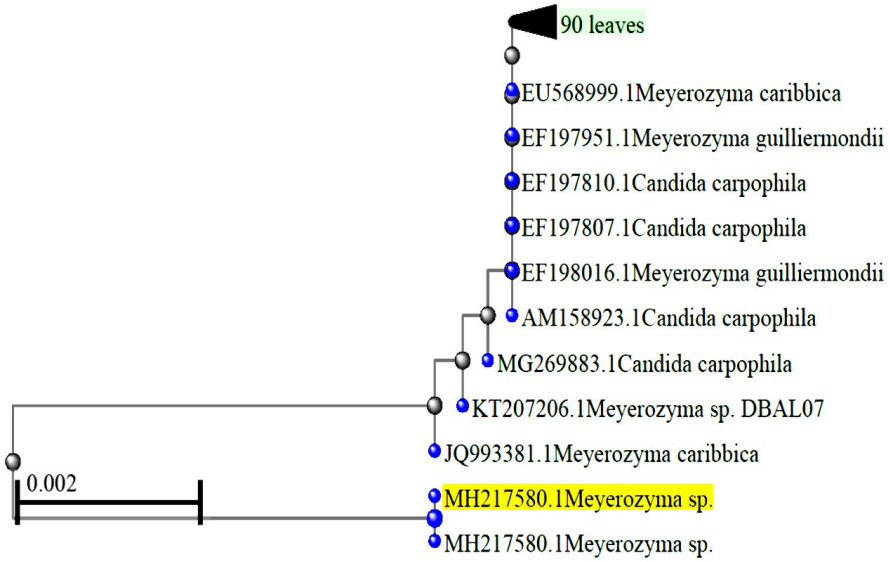

Fig. 3. Phylogenetic tree of *Meyerozyma* sp. VITPCT75 (MH217580)

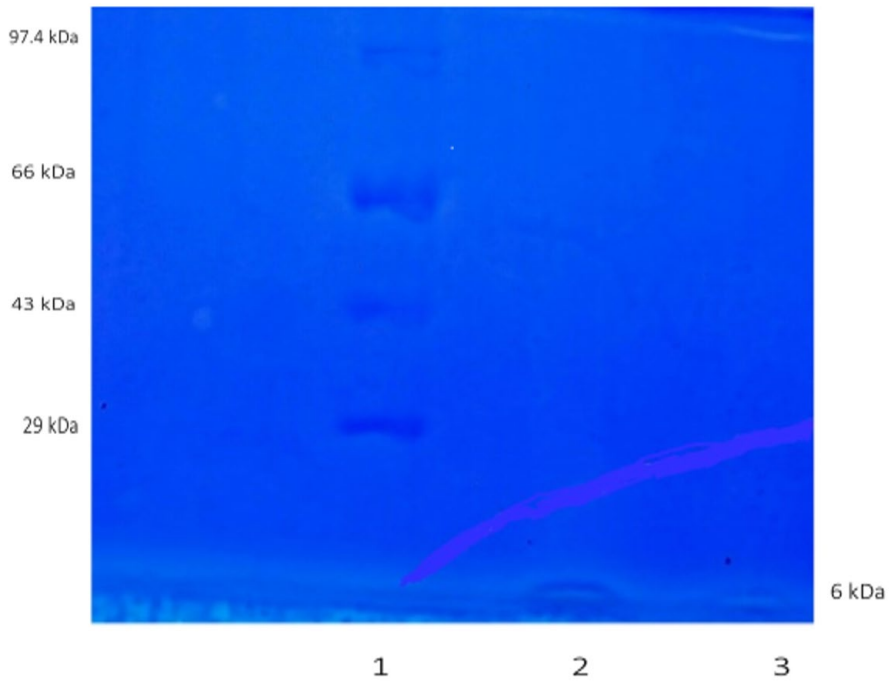

Fig. 4. SDS-PAGE of partially purified pectinase from *Meyerozyma* sp. VITPCT75(Lane-1: Protein Molecular weight marker, Lane-2: Acetone precipitate and Lane-3: Ethanol precipitate)

order to achieve a better yield. However, as indicated by the specific activity and SDS-PAGE, solvent precipitation at a saturation of 20% eliminated most protein present in the cell-free extract. Moreover, for large-scale purification, ultrafiltration remains costly and cumbersome due to clogging, especially when cell-free extracts are used directly. Clients from the industrial sector, the end-users of technical enzymes, always prefer feasible and economical processes. Food industry is no exception, mostly applying enzymes obtained from GRAS organisms due to their safety. Hence, *Meyerozyma* sp. VITPCT75-derived pectinase could be used for pectin degradation in fruit juices after solvent precipitation.

Molecular mass determination

Figure 4 shows the SDS-PAGE results of acetone and ethanol concentrations, as well as the purified sample from ion-exchange chromatography. The molecular weight of pectinase from *Meyerozyma* sp. VITPCT75 was calculated to be approximately 6 kDa. The molecular mass of pectinase from various sources is reportedly in the range of 20–45 kDa[33–35]. This is the first report of a very-low-molecular-weight pectinase (6 kDa) from *Meyerozyma* sp. VITPCT75. The authenticity of the results was further validated by LC-MS. Low-molecular-weight proteins are always preferred for industrial applications due to the ease of production, purification, and handling they allow without loss of activity. Moreover, enzyme immobilization, genetic cloning, and modifications could be performed on them without significant difficulties.

Stability and pH optimum

The enzyme showed an optimum activity at pH 7 (Fig. 5a) with a very sharp activity decrease at pH8. In contrast,it exhibited 62% of activity at

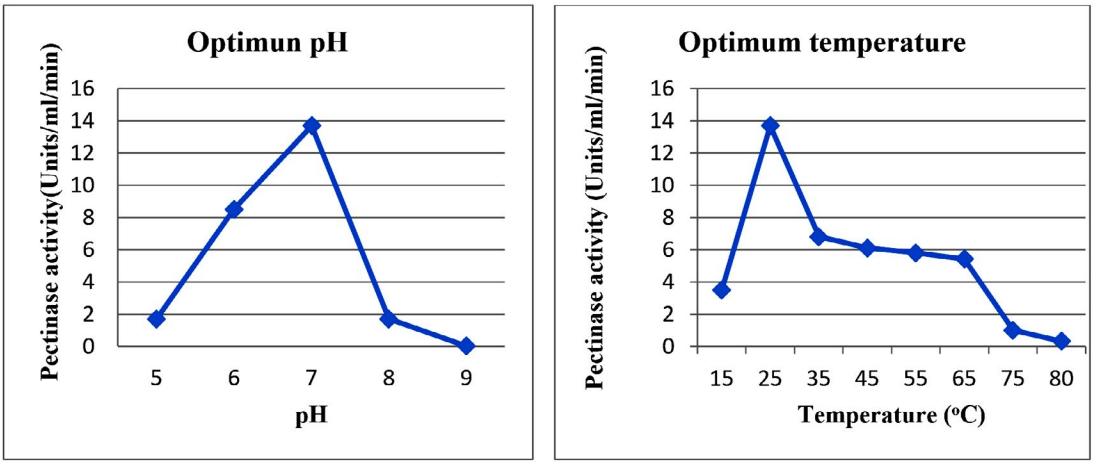

Fig. 5. Optimum temperature (a) and pH (b) of pectinase from *Meyerozyma* sp. VITPCT75

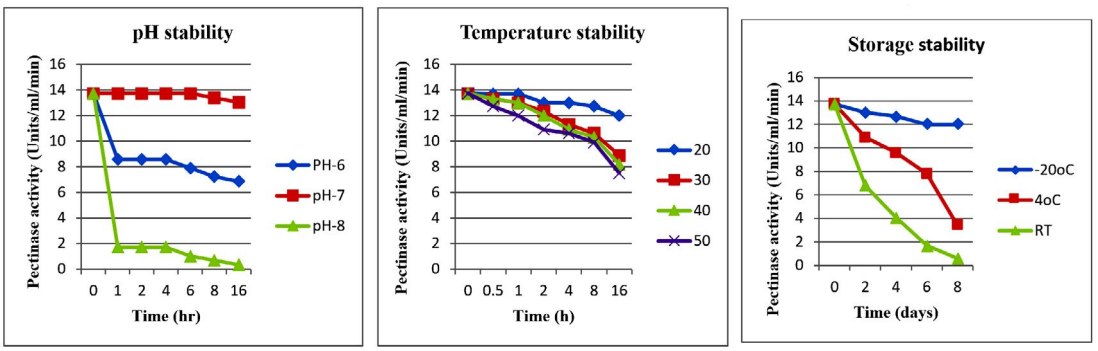

Fig. 6. Temperature (a), pH (b) and storage (c) stability of pectinase from *Meyerozyma* sp. VITPCT75

pH6. This was in good agreement with the fact that yeast-derived pectinases are often active at an acidic pH[36–38]. Pectinase from *Saccharomyces cerevisiae* with optimum activity at pH 7 was reported in an earlier study[7]. Studies on pectinase pH stability showed that the enzyme retained a maximum activity of 95% after 16 h of incubation at pH7 (Fig. 6A). The activity reduced significantly to its maximum at pH 6 but remained roughly the same, indicating that the enzyme could be used successfully in pectin clarification in low-acidic fruit juices such as melon, papaya, carrot, and tomato.

Temperature optimum and stability

Meyerozyma sp. VITPCT75-derived pectinase displayed an optimum activity at 25 °C, and it reduced to half at 35 °C (Fig. 5B). The activity was maintained at approximately 45% in the temperature range of 35–65 °C and reduced to zero at 80 °C. These results suggested that the enzyme could be used at relatively high temperatures, provided that the maximum activity was not a major constraint. Further studies reported on optimum pectinase activity within the range of 25–30 °C [7,39]. The temperature stability results showed that the enzyme retained maximum activity of 95 and 90% after 2 h of incubation at 20 and 30 °C, respectively (Fig. 6B). The activity gradually decreased after this point. The results visibly indicated that the enzyme could be used for a prolonged time with minimal inactivation.

Storage stability

Pectinase retained 95 and 79% of its maximum activity after 2 days of incubation at -20 and 4 °C, respectively, and 87 and 24% after 8 days at the same storage temperatures (Fig. 6C). However, significant decrease in its activity could be observed when it was stored at 25 °C. These results demonstrated that the enzyme could be stored successfully at -20 °C and also at 4 °C if the storage at freezing temperature would not be affordable.

Metal ion and enzyme inhibitor effects

$Ca^{2}z$, Kz , $Cu^{2}z$, $Fe^{2}z$, and $Ba^{2}z$ ions led to an increase in the pectinase activity. $Ni^{2}z$ ions moderately, whereas $Mn^{2}z$ ions strongly inhibited its activity. Neither Na^{+} and Mg^{2+} ions nor EDTA exhibited any enzyme inhibitory effect (Table 3). $Ca^{2}z$ ions reportedly play a vital role in pectinase confirmation maintenance[40]. The discrepancy in the pectinase metal ion preference indicated that the enzyme might have differential flexibility at the active site[25]. Table 3 shows that the enzyme was not inhibited by detergents. Stability in the presence of these agents is an important parameter that promotes the use of enzymes in different industries. Previous reports also stated that pectinases are stable in the presence of surfactants [25,41,42]. Pectinase from *Meyerozyma* sp. VITPCT75 was not inhibited by the serine-specific inhibitor phenyl methyl sulfonyl fluoride, resulting in the absence of a serine residue at the active site.

Fruit juice clarification

Pectin is a methoxylated galacturonic acid polymer present in the cell walls of plant cells. Pectin remains suspended in fruit juices, resulting in unwanted haziness, viscosity, and turbidity. Depectinization of fruit juice using pectinases is very common to improve the filterability, storage, and consumer satisfaction of fruit juices. In the current study, the effect of pectinase from *Meyerozyma* sp. VITPCT75 on pectin degradation and subsequent reduction in the viscosity of fruit juices was undertaken; the results are displayed in Table 4. The enzyme treatment resulted in a significant decrease in viscosity compared to that of the control. These results were in agreement with other reports on pectinases in clarifying fruit juices[7,43,44]. Consequently, the enzyme could be used in the food industry for the depectinization of fruit juices. However, further studies on the byproducts and toxicity should be conducted to ascertain their potential use in industries.

CONCLUSION

This study revealed that the new yeast strain *Meyerozyma* sp. VITPCT75, isolated from *Phyllanthus emblica*, is a potential source of pectinase. The enzyme was partially purified by acetone precipitation. The molecular weight of pectinase was 6 kDa. The enzyme displayed optimum temperature and pH of 25 °C and 7 and exhibited competitive stability in the close to optimum temperature and pH ranges. Pectinase activity was not affected by any metal ions and was not inhibited by detergents. Pectinase from *Meyerozyma* sp. VITPCT75 was not inhibited by PMSF, indicating the absence of serine at the active site. The enzyme significantly reduced the viscosity of representative fruit juices, suggesting that the enzyme could be used in the food industry for

the depectinization of fruit juices. Further studies on byproducts, toxicity, and LC-MS analysis were undertaken in our laboratory.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

None

AUTHOR'S CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable

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