



Production and Partial Purification of Pectinase from a Mutant of *Penicillium* EZ-ZH390 in Solid State Fermentation

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Abstract

Background and Aims: the biotechnological potential of pectinolytic enzymes from fungi has drawn a great deal of attention from various researchers worldwide. Various agro-industrial wastes such as wheat bran, sugarcane bagasse, coffee pulp, lemon peel and apple pomace have been explored for the microbial production of pectinase

Material and Methods: This study describes pectinase production using *Penicillium* sp. EZ-ZH390 in solid-state fermentation by Plackett–Burman design (PBD) and one-factor-at-a-time (OFAT) methodology. Four variables (moisture content, inoculum size, particle size, MgSO₄ 7H₂O), which showed the most effective factors by the PBD analysis. Moisture and MgSO₄ 7H₂O had the most positive impact on pectinase production further optimized using the OFAT method.

Results and Discussion: The optimization results indicated that a maximal pectinase activity of 3825 U/gds was achieved at, 85% initial moisture content and using 0.1% (w/v) MgSO₄ 7H₂O, particle size smaller than 0.7 mm and 10⁸ spores/mL in the fermentation medium. The results showed a 1.92-fold increase in pectinase activity of *Penicillium* sp. EZ-ZH390 under the optimized conditions. Orange peel can be considered as low-cost substrate for pectinase production. Ethyl alcohol and acetone at different concentrations were then used for ethanol-precipitated enzyme. Results showed that pectinase was partially purified 14.37-fold using 60% ethyl alcohol. The optimum pH and temperature of the crude and partially purified enzyme were 5, 55 °C and 5, 45 °C respectively.

What is “already known”:

- Fungal pectinases have wide industrial uses; agro-wastes like orange peel support SSF production.
- Downstream processing costs 50–90% of total; simple purification methods are needed

What this article adds:

- *Penicillium* sp. EZ-ZH390 on orange peel yielded 3825 U/gds pectinase after PBD and OFAT optimization.
- 60% ethanol precipitation gave 14.37-fold partial purification; optima pH 5, 55°C crude, 45°C purified.

1. INTRODUCTION

Solid-state fermentation (SSF) has been defined as the fermentation process that involves a solid matrix and is carried out in the absence or near absence of free water. However, the substrate must possess enough moisture to support growth and metabolism of the microorganism. The solid matrix could be either the source of nutrients or simply a support impregnated by the proper nutrients that allows the development of the microorganisms [1]. SSF processes are particularly interest to industrial applications by higher volumetric productivity and different microbial metabolites such as antibiotics, biopesticides, aromas, hormones, and enzymes, compared with submerged fermentations (SmF). Furthermore, SSF offers lower operating costs and investment outlays, as well as less downstream processing than SmF. In spite of these promising features, comparatively few applications of SSF are currently running at an industrial scale mainly due to unsolved technological and scale-up problems [2].

Pectinase is an enzyme that hydrolyzes pectic substances present in agricultural and food commodities [3]. Based on the pectin degradation mechanism they are classified mainly into pectin esterase, pectin lyase and polygalacturonase (PG). Among them, PG hydrolyzes α -1,4 glycosidic linkages of polygalacturonase acid chain and releases galacturonic acid residues [4]. Most pectic enzyme preparations are used in the fruit processing industry and pectic enzymes alone account for about one quarter of the world's food enzyme production. Fungal pectinases are among the most important industrial enzymes and are of great significance with wide range application in textile processing, treatment of pectic wastewaters, papermaking and coffee and tea fermentations. Therefore, the biotechnological potential of pectinolytic enzymes from fungi has drawn a great deal of attention from various researchers worldwide. Various agro-industrial wastes such as wheat bran, sugarcane bagasse, coffee pulp, lemon peel and apple pomace have been explored for the microbial production of pectinase [5]. Downstream processing is essential for

the purification of the targeted enzyme prior to specific commercial application [4]. The total costs of downstream processing are around 50–90% of the total process costs. Studies on recovery and purification strategies using simple and low-cost processes, but enabling high recovery and purification of the enzyme, are important from the industrial point of view. The increase in the purity of enzyme preparations without impacting its final cost to a large extent may contribute to expand industrial applications of these enzymes, improving the final quality of various products that can benefit from enzyme technology [6].

The present study investigated the production of pectinase by *Penicillium* sp. EZ-ZH390 (a native strain) in solid-state conditions employing orange peel as substrate [7]. Moreover, the optimum conditions for high pectinase activity were established.

2 . MATERIALS AND METHODS

2.1. Materials

Orange peels were provided by a local market in Tehran, province of Iran, and dried at 60-65 °C in a convection oven for 72 h. The peels were then ground and stored in plastic bags at room temperature until use.

2.2. Microorganism and Culture Conditions

The local filamentous fungus, *Penicillium* sp. EZ-ZH390 (a mutant of *Penicillium* sp. EZ-ZH190, which had been isolated from a tea farm in northern Iran) was used in this research [7]. The stock culture was activated in potato dextrose agar medium at a temperature of 30°C for 96 h. More details about the composition of the mineral solution, spore preparation and counting procedure, exact inoculum units, moisture calculation and adjustment, fermentation conditions corresponding to the final optimization, definition of U/gds, protein concentration assay, and the volume/buffer used for resuspension of precipitated proteins are described elsewhere [7].

2.3. Pectinase Production by *Penicillium* sp. EZ-ZH390 in SSF

Ground orange peels were placed in 250 mL



Erlenmeyer flasks, sterilized at 121°C for 20 min and hydrated with sterile solution of minerals to suitable moisture level. This sterile fermentation medium was inoculated with 1 mL spore suspension and incubated to produce the enzymes by SSF. After fermentation, the fermented material was mixed with 5 mL distilled water per gram of fermented material, stirred at 150 rpm [8] for 30 min. Then this suspension was centrifuged at 10000 g for 15 min and the supernatant, including pectinase enzyme, was stored at 4 °C until required for subsequent enzymatic analysis.

2.4. Assay of Pectinase Activity

Pectinase activity was assayed by measuring the release of polygalacturonic acid using the 3,5-dinitrosalicylic acid (DNS) reagent [3]. Galacturonic acid monohydrate is used as the standard. At first the solution of 0.5% (w/v) pectin substrate solution in 0.2 M acetate buffer at pH 5.5 is prepared. Then 250 µL of pectin substrate and 250 µL of enzyme solution were added to test tube and incubated for 10 minutes at 45°C. Then poured with 500 µL of DNS reagent was added, and it was boiled for 15 min. After boiling, it was cooled and added 4 mL of distilled water was added, shaken well, and finally the absorbance was measured by spectrophotometer at 540 nm [9]. One unit of pectinase activity (U) was defined as the amount of enzyme that releases 1 µmol of galacturonic acid per minute under assay conditions [3], U/gds denotes units per gram of dry substrate.

2.5. Experimental Design

For screening and optimization purposes, the fermentation experiments were performed at flask level (250 mL volume) using the orange peel inoculated with 1 mL spore suspension and supplemented with various nutrients and conditions according to the experimental design. The physical parameters such as moisture, fermentation time, temperature, inoculum size, particle size, and medium components including magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) and phosphate as dipotassium hydrogen phosphate (K_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) were evaluated as per the design specifications (Table 1). All experiments were performed in three replicates

and the results were reported as mean values. The experimental design and statistical analyses were carried out using MODDE 7 software.

2.5.1. Plackett–Burman Design (PBD)

A two-level PBD experimental matrix was set up to identify the significant factors of pectinase production. The PBD provides a linear model where only main effects are taken into account. In this study, seven independent variables were selected, namely moisture, fermentation time, particle size, temperature, inoculum size, and medium components of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and phosphate as K_2HPO_4 and KH_2PO_4 . The concentration range of each nutrient was based on various studies. Table 1 shows the design matrix of various components with coded values: low (−1) and high (+1), while Table 2 represents their actual values.

2.5.2. One-factor-at-a-time (OFAT) design

At this point, pectinase production was optimized using the OFAT method. By screening stage effective variables were selected to optimize the production of enzymes.

2.6. Pectinase Partial Purification

Partial purification was carried out using acetone or ethanol with different concentrations of (50, 70 and 90% v/v) and (50, 60 and 70% v/v), respectively. They were added slowly to the enzyme solution and incubated in the refrigerator (4°C) for 4 hours. The precipitated solution was centrifuged at 10000 g for 10 min at 4°C. Then it was used to determine enzyme features.

2.7. Effect of pH and Temperature

Different buffer systems were used across the pH range: acetate buffer for pH 2.6–5.0 and phosphate buffer for pH 5.5–7.0, at 0.1 and 0.2 mol/L. Optimum pH and temperature of crude pectinase and partially purified pectinase was analyzed by carrying out assays at different pH ranging from 2.6 to 7.0 using different buffers (acetate and phosphate) of 0.1 and 0.2 mol/L concentration at 45° C and at various temperatures (25°C to 75°C) at optimum pH. Relative activity was expressed as a percentage of the maximum pectinase activity.

Table 1. PBD for screening the major factors affecting pectinase production.

Serial	Temperature (°C)	Moisture (%)	Particle size (mm)	Inoculum size (spore/mL)	MgSO ₄ 7H ₂ O(%)	K ₂ HPO ₄ + KH ₂ PO ₄ %	Time (day)	Pectinase Activity U/gds
1	1	-1	-1	1	-1	1	1	306±7.05
2	1	1	-1	-1	1	-1	1	1185±21.05
3	1	1	1	-1	-1	1	-1	585±25.93
4	-1	1	1	1	-1	-1	1	842±65.00
5	1	-1	1	1	1	-1	-1	561±20.25
6	-1	1	-1	1	1	1	-1	1337±84.48
7	-1	-1	1	-1	1	1	1	378±6.08
8	-1	-1	-1	-1	-1	-1	-1	121±75.00

Table 2. The factors and their coded levels used in the PBD.

Serial number	Variable	Low (-1)	High (+1)
1	Tempreture °C	22	30
2	Moisture %	65	75
3	Particle size (mm)	0.7-1	1.4-2
4	Inoculum (spore/mL)	106	108
5	MgSO ₄ 7H ₂ O%	0	0.04
6	*K ₂ HPO ₄ % +KH ₂ PO ₄ %	0	5
7	Time (day)	3	5

*Concentration of K₂HPO₄% is equal of KH₂PO₄%



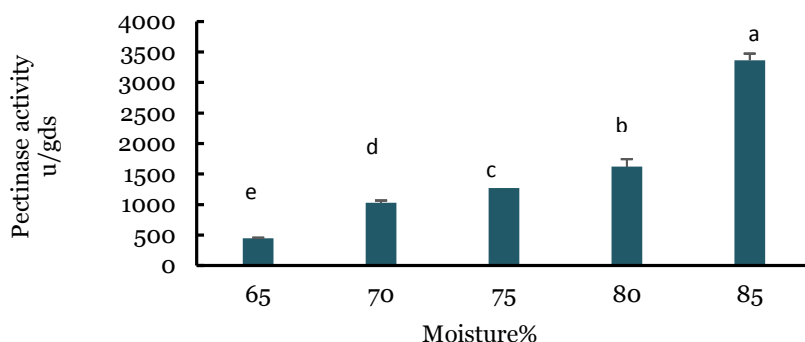


Fig 1. Pectinase activity in different moisture %. Data are presented as mean $\pm$ standard deviation (SD) ($n = 3$). Different lowercase letters indicate statistically significant differences ($p < 0.05$) based on Duncan's test.

3. RESULTS AND DISCUSSION

3.1. Evaluation of Variables Affecting Pectinase Production

PBD experiment showed that moisture content, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, inoculum size, particle size were the most effective factors, respectively (the results not shown) on pectinase activity. Moisture and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ had the most positive impact on pectinase production. So, the effect of higher moisture content and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ was studied in the optimization stage. The highest inoculation content (10^8 spores/mL) was used in the optimization phase due to its positive effect on the production of pectinase. More was not common in similar researches (spores/mL), 5×10^7 [5], 5.8×10^7 [10], 10^8 [11] 2×10^7 [12], 10^6 [3] and may lead to a large number of microbes that competed for food in the substrate and reduced enzyme production. Some researchers also pointed higher inoculum may produce excessive biomass and deplete substrate from the nutrients that is necessary for product formation [13]. Substrate particle size also had a considerable negative effect on the production of pectinase. In other words, the smaller particle size of orange peel increased enzyme production. Yee et al. [14] reported that smaller particles increased the surface area for microbial action, facilitating bioconversion, advantageous for heat transfer and exchange of oxygen and carbon dioxide between air and the solid surface. Larger particles gave higher respiration and aeration efficiency, but limited surface for microbial action, and water-holding capacity in large particles was very low. Ruiza et al., [11] reported that total protein and fat for smaller particles were higher and nitrogen

source could play an important role as a nutrient. So, substrate particles smaller than 0.7 mm were selected. Other factors had no significant effect on pectinase production.

3.2. Optimization

As mentioned in the screening stage, from investigated variables, moisture and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ were considered to optimize pectinase production. The first five levels of initial moisture content, 65, 70, 75, 80, and 85%, were selected (based on no free water appearing). The concentration of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in all of them was constant and equal to 0.08% (w/v). Highest enzyme activity ($p \leq 0.05$) was observed at a moisture level of 85% (Fig. 1). Moisture content is a critical factor in SSF processes because this variable has the main influence on growth, biosynthesis and secretion of different metabolites. Lower moisture content causes a reduction in solubility of nutrients in the substrate, a low degree of swelling, and high-water tension. However, the optimal value of moisture content depends on both the microorganism and the solid matrix used [10]. In similar study, four fungal strains, namely *Aspergillus niger* BTL, *Fusarium oxysporum* F3, *Neurospora crassa* DSM 1129 and *Penicillium decumbens*, were grown at optimum pH and at four different initial moisture contents and three strain means *A. niger* BTL, *F. oxysporum* F3, *P. decumbens*, produced highest enzyme at a moisture level 90% [10]. In the next stage, *Penicillium* sp. EZ-ZH390 was grown at optimum initial moisture content and at five different concentration of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, namely 0.04, 0.06, 0.08, 0.1 and 0.12% w/v.

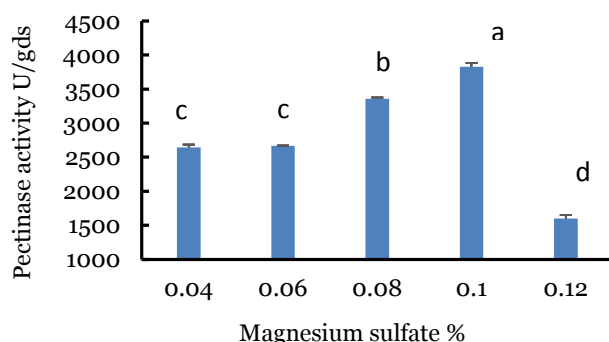


Fig 2. Pectinase activity in different MgSO₄ 7H₂O contents. Data are presented as mean $\pm$ SD (n = 3). Different lowercase letters indicate statistically significant differences ($p < 0.05$) based on Duncan's test

According to Fig. 2 there is no significant difference ($p > 0.05$) between 0.04 and 0.06%, due to metal low concentration. Generally, by increasing the concentration of MgSO₄ 7H₂O to 0.1%, enzyme production increased, but by increasing to 0.12%, enzyme production decreased which is possibly related to several possible causes, including ionic strength, osmotic effects, sulfate effects, or nutrient imbalance. Banu et al. [15], in a similar study for the production of pectinase by *Penicillium chrysogenum* used 0.123% magnesium concentration and observed a reduction of about 25% enzyme. They reported that

the concentration of 0.05% magnesium was appropriate for enzyme production.

3.3. Partial Purification of the Enzyme using Ethanol and Acetone

As can be seen in Table 3, the concentrations of 50% ethanol and acetone were not able to purify the enzyme. It is obvious that ethanol with a concentration of 60%, v/v, was the most appropriate and precipitated out the most active pectinase enzyme more than the other precipitants. Esquivel and Voget [16] in a similar study on partial purification of pectinase, showed a fold purification of 4.5 with 60% ethanol precipitation. Partial purification of pectin lyase of *A. niger* showed a fold purification of 1.3 with 60% ethanol precipitation, results of 1.68 and 2.12-fold purification of PG from *A. niger* MTCC 3323 and *Mucor circinelloides*, respectively by precipitation with 60% ethanol [6]. Some researcher explained the preference for 60% ethanol over other precipitants because of the elimination of inhibitors during the extraction [6]. Pan et al. [17] attribute this phenomenon to the elimination of inhibitors and/or to the fact that high salt and/or high protein concentrations may help to maintain the protein conformation in an active form.

Table 3. Partial purification of enzyme by acetone and ethanol.[†]

	Acetone or ethanol concentration (%)	Total protein content (mg/l)	Protein recovery (%)	Pectinase activity (U/gds)	Specific activity (U/mg protein)	Purification fold
Crud	0	18.69 $\pm$ 0.77	100	1989	106.42	1
Acetone	50	0	0	0	0	0
	70	2.20 $\pm$ 0.99	11.77	1345.5 $\pm$ 177.48	611.59	5.74
	90	2.27 $\pm$ 0.18	12.14	1460 $\pm$ 0	643.17	6.04
Ethanol	50	0	0	0	0	0
	60	1.05 $\pm$ 0	5.6	1607.5 $\pm$ 130.81	1530	14.37
	70	4.69 $\pm$ 0	25	1061.5 $\pm$ 40.30	226.33	2.12

[†] It should be noted that the corresponding supernatants were analyzed. Both precipitated and soluble fractions allow a proper mass balance and determination of enzyme recovery.

3.4. Effect of pH and temperature

The crude pectinase showed maximum activity at pH 5 and 55 °C temperature (Fig. 3A). The partially purified pectinase showed maximum activity at pH 5 and 45 °C (Fig. 3B). The results showed that crude and purified enzymes had different activities in different pH values or temperatures. This was because of the presence of impurities in the crude enzyme solution. Also, other factors, such as removal of stabilizing components or different enzyme populations, could contribute. The partially purified pectin methylesterase exhibited maximum activity at a temperature of 50 °C and pH 4.6 [13]. Exo PG from *Aspergillus* species and endo PG from *Mucor rouxii* are exhibited maximum activity at 50 and 35 °C, respectively. It is reported that optimum pH 5.5 for pectinase activity from thermo-tolerant *Aspergillus* sp. [18]. Differences in activity may reflect not only pH but also changes in buffer identity and ionic strength. This should be considered as a limitation when interpreting the pH profile. Pectin from citrus fruit and the metal ion (Co²⁺) were the best substrate and inducer to enhance pectinase yield, respectively [19]. *Aspergillus cervinus* ARS2 has been reported [20]. A report shows that pectinase immobilized on sponge includes promising potentials as a clarifying catalyst in fruit-juice industries [21]. A citrus circular bioeconomy by utilizing orange peels to produce pectinase and subsequently using pectinase to clarify orange juice [22]. An overview of the recent advancements and limitations of next-generation pectinases [23].

4. CONCLUSIONS

This research showed isolate of *Penicillium* sp. EZ-ZH390 has considerable potential in the production of pectinase. A preliminary characterization was conducted for pH and temperature optima. These experiments provide useful preliminary information but do not establish pH stability, thermal stability,

kinetic parameters, substrate specificity, or effects of metal ions/inhibitors. Orange peels, inexpensive agro-residues, were shown to serve as a possible source for the production of pectinase using a novel fungus of *Penicillium* sp. EZ-ZH390 after optimizing the SSF medium composition and conditions by the PBD and OFAT.

method. A 1.92-fold enhancement in enzyme production was achieved compared to the unoptimized fermentation conditions. Partial purification of pectinase with 60% ethanol precipitation showed a fold purification of 14.37. Further study should be conducted for scale-up, economic analysis, downstream processing costs, or comparison with alternative substrates under equivalent conditions.

5. DECLARATIONS

5.1. Conflict of interest: The authors have no relevant financial or non-financial interests to disclose.

5.2. Acknowledgments: Not applicable.

5.3. Funding: No funding was received for conducting this study.

5.4. Author contributions: All authors contributed to the conception and development of the study, interpretation of the results, and critical revision of the manuscript

5.5. Ethical considerations: All ethical principles were adhered to in conducting and writing this article.

5.6. Data availability: In accordance with the principles of transparency and open research, we declare that all data and materials used in this study are available upon request.

5.7. Using Artificial Intelligence chatbots: Not applied at all

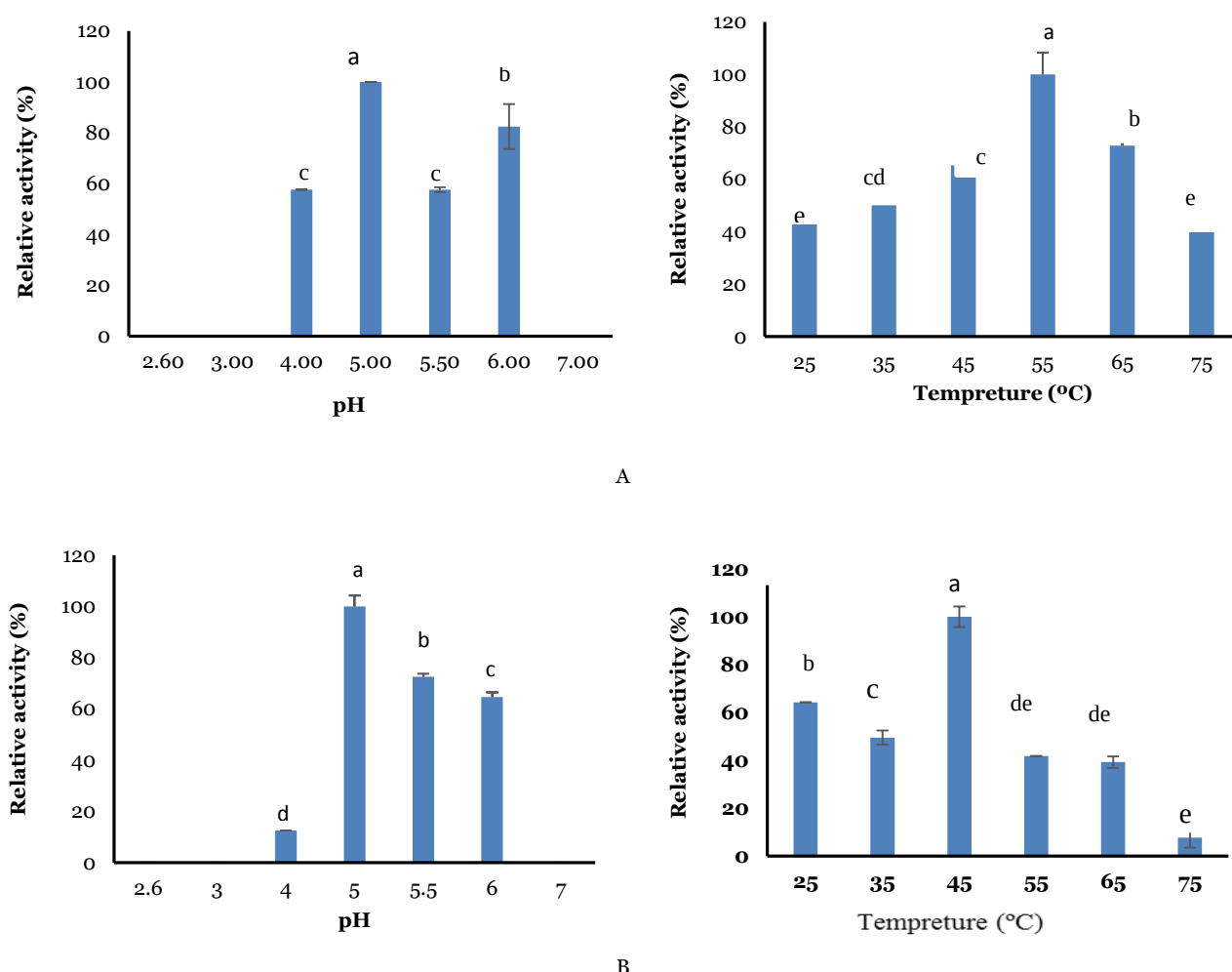


Fig 3. Effect of pH and temperature on A) crude and B) purified pectinase activity. Data are presented as mean $\pm$ SD (n = 3). Different lowercase letters indicate statistically significant differences ($p < 0.05$) based on Duncan's test.

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