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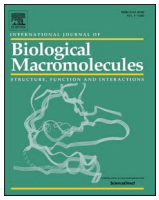

Pectinase bioprocessing: Unlocking the potential of *Bacillus vallismortis* MH 10 for detox juice enhancement

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ABSTRACT

The utilization of pectinases for juice clarification is well established; however, their application in complex Detox juice blends containing both pectin-rich and low-pectin fruits and vegetables remains underexplored. This study aimed to (i) optimize the activity of pectinase derived from the halotolerant bacterium *Bacillus vallismortis* MH 10, (ii) assess its effectiveness in clarifying two distinct detox juice formulations, and (iii) evaluate its impact on juice yield, total phenolic content (TPC), and antioxidant activity. A concentrated "Detox" beverage was developed containing bottle gourd in one formulation and apple in the other, with cucumber, ginger, carrot, lemon, and mint common to both. Optimization of pectinase activity was performed using Central Composite design (CCD). Subsequently, the Box-Behnken design (BBD) was employed to optimize enzyme-assisted juice extraction, resulting in significant improvements in juice yield, TPC, and antioxidant capacity measured by DPPH and ABTS assays. Under optimized conditions, pectinase treatment enhanced juice yield by up to 25 %, increased TPC by 40 %, and improved antioxidant activity by 35 % in both Detox juice formulations. These findings provide valuable insights for the beverage industry, demonstrating that the application of *B. vallismortis* MH 10 pectinase can substantially enhance the nutritional and functional qualities of detox juices during production.

1. Introduction

The pursuit of a healthy diet has led to an increasing interest in functional beverages enriched with bioactive compounds [1]. In this context, "detox" juices or blends of fruits, vegetables, and other health-promoting ingredients have gained popularity for their purported health benefits [2]. Plant-based liquid refreshments usually contain beneficial bioactive compounds such as flavonoids, lignans, phenolic acids, and phytosterols [3]. Bioactive components derived from plants through metabolism, known as secondary metabolites, exhibit promising therapeutic properties, particularly in terms of antioxidative effects [4]. For instance, apple and its products contain secondary metabolites with bioactive properties, such as polyphenols, along with varying amounts of dietary fiber [5]. In addition to polyphenols, saponins, which are classified as glucosides, are present in various vegetables, especially gourd vegetables [6]. Among other dietary additives, ginger possesses inherent preservative qualities and offers a multitude of health advantages. These include anti-inflammatory, anti-emetic, anti-allergic, anti-mutagenic, anti-ulcer, hypoglycemic, and bactericidal properties [7]. Mint is well known not only for its diverse culinary applications but also for its several therapeutic benefits. Its main ingredients, such as menthol, menthone, and different flavonoids support gastrointestinal health while exhibiting potent antioxidant and antibacterial properties. Scientists have also reported that mint contributes to inhibiting infectious growth, reducing oxidative stress, and improving immune function. For instance, menthol relieves gastritis and bloating by comforting the smooth muscles of the digestive tract. The gastroprotective and anti-inflammatory properties of mint are attributed to its high polyphenol content [8]. Extraction of juice from fruit and vegetable puree and pulp commonly contains many structural components, such as pectin-protein

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or pectin complexes, which make it challenging to get a clear juice with good yield. Consequently, sophisticated procedures are needed to improve clarity and juice quality overall [9].

In this context, enzymatic treatment of fruits and vegetables enhances juice extraction efficiency and quality [10]. The application of biotechnological methods to incorporate microbial enzymes into industrial processes provides substantial advantages, requiring minimal energy and financial input [11]. Pectinase improves juice yield and quality by breaking pectin content [12]. Primarily, pectinases function by cleaving glycosidic bonds and transforming polygalacturonic acid into galacturonic acid [13]. Pectinases, derived from bacteria, fungi, and plants, are comprehensively utilized across various industrial sectors for the transformation of pectin-containing constituents, such as in fruit juice production, textile manufacturing, wastewater treatment, paper production, oil processing, cotton fiber treatment, and coffee and tea preparation [14].

Pectinolytic enzymes degrade pectic compounds, facilitate in the aggregation of suspended particles, and enhance juice clarity [15]. As a result, fruit juices exhibit higher levels of total soluble solids (TSS), sugars, acidity, and color. The duration required for effective pectinolytic liquefaction of fruit pulp depends on several factors, including the kind of fruit, incubation temperature, and the concentration of enzyme employed. Therefore, finding the optimum conditions is crucial for efficient enzymatic processing [16]. Although pectinases are well known clarification agent for single fruit or vegetable juice products, their application in multifaceted detox juice combinations, particularly those comprising both pectin-rich and low-pectin fruits and vegetables remains comparatively underexplored. This highlights a noteworthy gap in the knowledge regarding the application of pectinase, particularly in multi-ingredient beverages. However, the diverse plant materials in these juice blends frequently hinder the efficient extraction and conservation of bioactive compounds. Although pectinases produced by bacteria have been studied for their role in clarifying fruit juices, enzymatic extraction of assorted fruit and vegetable juices using pectinases from *B. vallismortis* remains unexplored. The production of pectinase from *B. vallismortis* MH 10 has recently been reported [17], demonstrating its potential for such applications. This study was designed to optimize and evaluate the application of pectinase from the halotolerant *B. vallismortis* MH 10 in enhancing detox juice extraction, addressing gaps in the enzymatic processing of mixed fruit-vegetable preparations. This study primarily aimed to (i) characterize pectinase activity of *B. vallismortis* MH 10 strain through CCD, a response surface methodology (RSM) tool ideal for multifactor optimization; (ii) assess the influence of pectinase preparation from *B. vallismortis* MH 10 on juice yield and antioxidant capacity in two detox juice recipes: one with bottle gourds and the other on apples, both accompanied by cucumber, ginger, carrot, lemon, and mint. This contrast allowed systematic investigation of enzymatic treatment proficiency in both low- and high-pectin juice systems, reflecting the diversity found in commercial detox drinks. Using BBD, the study further refined the extraction conditions to improve juice yield, TPC, and antioxidant capacity. The application of pectinase was proposed to boost the functional properties and yield of the juices.

2. Materials and methods

2.1. Production of pectinase

B. vallismortis MH 10 was retrieved from the departmental culture collection and cultured to produce pectinase. Inoculum (10 %) was added to mineral salt media containing 1 % *Cressa cretica* biomass as a substrate and incubated at 50 °C for 26 h. After incubation, the medium was centrifuged at 3000 ×*g* for 20 min to obtain the cell-free culture supernatant (CFCS) for subsequent analysis. The dinitrosalicylic acid method was used to perform the pectinase assay [18].

2.2. Statistical approach for characterization of pectinase

CCD was applied using Minitab 18 software within the RSM framework to optimize pectinase activity. The design evaluated the effects and interactions of pH, temperature, substrate concentration, and reaction time at three levels (Table S1), with ranges determined from preliminary experiments. This approach enabled the precise identification of optimal conditions, significantly enhancing enzyme activity and ensuring a robust, reproducible protocol. Pectinase assays were conducted according to the CCD experimental matrix.

2.3. Stability across different pH levels and buffer systems

The CFCS was analyzed in buffers of variable pH levels at 50 °C for 3 h, with samples collected at 30-min intervals to assess remaining enzyme activity using specific assay conditions, including buffers like 50 mM sodium citrate (pH 4.8), 50 mM Tris –HCl (pH 7.7 and 8.5), 50 mM sodium bicarbonate (pH 9.6 and 10.5), and 50 mM potassium chloride (pH 12.5). The same range of buffers was used to assess the stability of the enzyme and perform the enzyme assay.

2.4. Estimation of Half-life and central point of thermal-inactivation (T_m) of pectinase

The CFCS was incubated at various temperatures (40–80 °C with intervals of 10 °C) without substrate to determine the remaining enzyme functionality over time. The data of the remaining enzyme activity were plotted against time to determine the T_m, indicating the time at which the enzyme lost half of its activity. The half-life ($t_{1/2}$) of an enzyme is the duration required for its activity to decrease by 50 % at a given temperature. In this investigation, the CFCS enzyme was exposed to 55 °C, and pectinase activity was monitored over time, showing a decrease in activity with longer heat exposure, with the initial activity set at 100 %.

2.5. Estimation of pectinase activity at refrigeration temperature (2–4 °C) and 37 °C

Pectinase stability in the cell-free culture supernatant (CFCS) was examined at two temperatures: 37 °C and refrigerator temperature (2–4 °C). The samples were separated and stored at these temperatures to assess enzyme activity over time. The activity of the untreated samples was taken as 100 %.

2.6. Sample preparation for detox drink

For juice extraction, different vegetables, fruits, and herbs such as cucumber, ginger, apples, bottle gourd, carrot, lemon, and mint (Table S2) were sourced from a local market in Karachi, Pakistan. They were then separated into distinct batches of three repetitions. Prior to processing, all fruits underwent a thorough inspection for signs of damage, followed by washing and packing in plastic bags before storage in a freezer.

2.7. Juice extraction and pectinase treatment

To create detox juice, a reference method was used as reported earlier [19] with some modifications. The ingredients, inspected for bugs and damage, were first soaked and washed with tap water to remove any surface impurities and then chopped into small pieces. The measured ingredients were categorized into two groups, Recipe 1 (R1) and Recipe 2 (R2) (their compositions are detailed in Table S2). In both categories, 250 g of mixed vegetables and fruits were homogenized at a consistent rotation and duration using a commercial blender (Panasonic, Malaysia) with 400 mL of locally sourced purified bottled water. The resulting juices from R1 and R2 were transferred into screw-capped bottles. An electric eggbeater (WestPoint) was used to ensure the

uniform mixing of the semi-solid and liquid components of the juice. A 20 mL portion of detox juice was transferred to 100 mL sterilized screw-cap bottles every 45 s. For each experiment involving both recipes, 20 mL of juice was processed according to the Box–Behnken design (BBD), as outlined in Table 2. The purpose of BBD matrix was to evaluate interaction of pectinase units, reaction time, and temperature, reflecting the widespread application of pectinase in food processing. This study investigated the impact of pectinase units (IU mL^{-1}), reaction time (min), and temperature (°C) on the production and free-radical scavenging activity of detox juices. The levels of these variables were determined through preliminary experiments. A water bath was used to maintain the temperatures necessary for pasteurization and enzymatic treatment. The samples were heated at 90 °C for 5 min to inactivate pectinase. Following this, all bottled juices were pasteurized at 90 °C for 10 s and then stored in a refrigerator at 4 °C. A heat-inactivated pectinase control was included in all experiments to account for non-enzymatic changes in juice yield and antioxidant properties. All juice extraction and enzymatic treatment experiments were performed in triplicate, with each replicate representing an independent batch of fruit and vegetable preparations.

2.8. Analytical methods

2.8.1. Determination of juice yield

The yield (%) of Detox juice after enzymatic treatment was calculated using the following equation [20].

$$\text{Percent (\%) yield} = m_{2\times C}/m_{1\times(100-w)} \times 100\%$$

where m1 was weight of the vegetable and fruit smash (g), m2 was weight of the vegetable and fruit (g), C represents the concentration of solvable solid compounds in the liquid, % (w/w) and w denotes the moistness of the initial vegetable and fruit (%).

2.8.2. Estimation of total soluble solids (°Bx)

The total dissolved solids in the detox drink were measured using an Antago RX-5000 digital refractometer (Japan), calibrated to 0°Bx using distilled water. The results are expressed in degrees Brix (°Bx), indicating the percentage of dissolved solids.

2.8.3. Antioxidant study of detox juice

The antioxidant properties of detox juice were assessed by measuring the TPC and evaluating its free radical scavenging activity through DPPH and ABTS assays.

2.8.3.1. Total phenolic extraction. The withdrawal of total phenolics was performed using a modified procedure from [20]. In this process, 5 mL of each recipe 1 and 2 sample was mixed separately with 20 mL of 1.2 M HCl in 50 % methanol and incubated at 60 °C. The mixture was shaken for 2 h in the dark. Following incubation, the extracts were centrifuged at 3000 ×*g* for 15 min. The supernatant was then collected and stored at −20 °C for subsequent analysis of the total phenolic content and antioxidant capacity.

2.8.3.2. Estimation of total phenolic contents. The total phenolic content in the detox preparation was measured using the Folin–Ciocalteu (FC) assay, based on the method described by Nguyen & Nguyen [20] with slight modifications. Briefly, 100 μL of Detox juice was mixed with 2 mL of distilled water and 200 μL of Folin–Ciocalteu reagent (Sigma-Aldrich). Subsequently, 600 μL of 20 % sodium carbonate was added to the solution. After thorough blending, the volume was adjusted to 5 mL. The solution was incubated at 50 °C for 30 min. A UV–visible spectrophotometer was used to quantify the absorbance at a wavelength of 725 nm. Gallic Acid (Merck) standard solutions (0.01 to 0.05 mg mL^{-1}) were prepared by solubilizing it in a 1.2 M hydrochloric acid solution synthesized in absolute methanol (Merck) (v/v). The phenolic content was expressed as milligrams of gallic acid equivalents per 100 mL of the juice formulation.

2.8.3.3. Assessment of antioxidant capacity using DPPH for free radical scavenging. The assessment of DPPH free radical scavenging was performed in accordance with the methodology outlined by Ivanovic et al. [20] with slight changes. A 0.033 % DPPH solution (1 mL) was combined with 1 mL of juice extract in a test tube and incubated in the dark for 30 min at room temperature. This procedure was repeated three times and a UV–Visible spectrophotometer was used to measure absorbance at 517 nm with methanol as the blank control. The IC_{50} value of each juice extract was determined. The percentage of free radical scavenging activity was calculated using the following formula:

$$\text{Percent Scavenging activity} = \text{Absorbance of control} - \text{Absorbance of sample}/\text{Absorbance of control} \times 100$$

2.8.3.4. Assessment of free radical scavenging activity by ABTS. The ABTS reducing activity assay, as described previously [22] with some changes, was used to assess the antioxidant potential in the prepared detox juice. Detox juice (100 μL) samples were pooled with 5 mL of 7 mM ABTS and allowed to react at ambient temperature for 7 min. Absorbance was measured at 734 nm against the blank. The IC_{50} value for each juice extract was calculated. The percentage ABTS scavenging activity was calculated using the same formula as that used for the DPPH assay.

Table 2
Box-Behnken design to study the impact of pectinase enzyme on the juice production and free-radical scavenging activity of detox juice (R1 & R2).

Experimental parameters				Recipe 1[a]				Recipe 2[a]			
Run order	Reaction time (min)	Enzyme units (IU mL^{-1})	Temp °C	Juice yield (%)	TPC (mg/100 mL)	ABTS (IC_{50})	DPPH (IC_{50})	Juice yield (%)	TPC (mg/100 ml)	ABTS (IC_{50})	DPPH (IC_{50})
1	60	5	45	41.2	27.1	100	20	95.0	0	100	50
2	240	5	45	67.7	22.7	100	15	82.1	20.9	100	20
3	60	15	45	75.7	34.1	100	30	82.4	32.9	100	70
4	240	15	45	85.7	22.7	80	10	111.4	27.2	100	50
5	60	10	40	56.2	22.8	100	50	73.3	17.7	100	60
6	240	10	40	65.6	22.5	90	30	104.0	22.5	85	40
7	60	10	50	74.4	28.0	100	70	95.7	19.3	95	40
8	240	10	50	98	25.6	100	70	111.4	36.6	100	30
9	150	5	40	42.9	17.4	90	40	94.3	14.8	90	100
10	150	15	40	86.1	31.6	100	75	93.1	19.5	80	30
11	150	5	50	65.8	24.8	100	50	113.6	0	100	45
12	150	15	50	85.8	34.35	75	55	101.6	17.2	100	100
13	150	10	45	96.3	45.1	60	25	97.1	25.9	100	40
14	150	10	45	96.6	41.7	60	30	99.3	23.9	100	40
15	150	10	45	96.5	44.4	50	25	97.4	23.6	100	40

[a] Insignificant standard deviation.

2.9. Statistical analysis

BBD was employed to optimize the juice-processing variables, including enzyme dosage, incubation time, and agitation speed. Each factor was tested at three levels, with ranges determined from preliminary trials. The BBD efficiently evaluated interactions among variables while minimizing the number of experiments. The data obtained from the BBD experiments were analyzed using analysis of variance (ANOVA) in the Minitab 18 software. Results are presented as the mean ± standard deviation (SD) of three replicates, and differences were considered statistically significant at $p < 0.05$. This optimization led to improved juice yield, total phenolic content, and antioxidant activity.

3. Results and discussion

3.1. Statistical optimization of pectinase activity

Considering enhanced pectinase production by *B. vallismortis* MH 10, its cell-free culture supernatant (CFCS) was characterized to determine the ideal conditions for pectinase activity (Table 1). The study investigated the influence of five factors at three different levels: pH (5–10), duration of reaction (10–40 min), pectin concentration (0.3–2.2 %), NaCl concentration (1–3 %), and temperature (35 °C–65 °C) (Table S1). CCD was applied within the RSM framework [23]. The analysis determined the optimal levels for these parameters, with the model's R^2 value of 87.40 %, indicating its effectiveness and precision. A high R^2 value close to 100 % indicates strong model reliability [23]. The regression equation in coded units for pectinase activity (IU mL^{-1}) as a function of temperature (A), reaction time (B), pH (C), NaCl concentration (D), and pectin concentration (E) is:

$$\begin{aligned} \text{IU mL}^{-1} = {} & 33.23 + 51A - 60B - 7.5C + 100D + 45.5E\ 29.7A^2 \\ & - 19.13B^2 - 24.63C^2 - 12.8D^2 - 15.73E^2\ 2.48AB \\ & + 41.25AC - 4.2AD + 12.6AE + 37.13BC + 12.6BD \\ & - 6.72BE - 13.75CD + 3.68CE - 6.44DE^{**} \end{aligned}$$

where A, B, C, D, and E represent the coded values of temperature, reaction time, pH, NaCl concentration, and pectin concentration, respectively.

The impact of interactions between the independent variables on pectinase activity was evaluated using a Pareto chart (Fig. 1) and contour plots (Fig. 2). Pareto chart illustrating the standardized effects of independent variables (pH, temperature, substrate concentration, and reaction time) on pectinase activity, as determined by CCD. The y-axis represents the magnitude of the effect for each variable. The chart reveals that pH had a significant positive influence on pectinase activity, whereas the effects of other variables were comparatively less pronounced. The identification of pH as the most influential factor, supported by the visualization of optimal conditions through contour plots, provides a robust basis for selecting enzyme treatment parameters. It has been reported that pH affects enzyme activity by influencing the charge distribution on both the substrate and enzyme molecules, which impacts substrate binding and catalysis [24].

Temperature is another crucial factor for enzyme activity and stability [25]. This study revealed that pectinase from *B. vallismortis* MH 10 exhibited maximum activity at 50 °C. Thermophilic enzymes are advantageous in industrial pretreatments because of their stability at high temperatures and their eco-friendliness compared to toxic chemicals [26]. However, in the food processing industry, enzymes that function at mesophilic or sub-mesophilic temperatures are favored to preserve the nutritional and sensory qualities of products [27].

The software predicted optimized conditions for pectinase activity, including 0.8 % substrate concentration, 2.7 % sodium chloride concentration, 10 min reaction time, temperature of 50 °C, and pH 8. Experimental validation under these conditions resulted in an enzyme activity of 29 IU mL^{-1}, which closely matched the predicted value of 28 IU mL^{-1}. This strong agreement confirms that CCD effectively identified the optimal parameters for maximizing pectinase activity. The precise alignment between predicted and observed values highlights the robustness and reliability of RSM in modeling complex interactions among variables and fine-tuning enzyme activity conditions. Moreover, the use of CCD reduced the number of experimental trials required, demonstrating its efficiency in optimizing bioprocess parameters with high accuracy [28]. CCD analysis revealed that the optimal conditions

Table 1
Central Composite design (CCD) for assessing elements influencing pectinase activity.

Experiment Order	pH	Temperature (C°)	Conc of Pectin (%)	Conc of NaCl (%)	Duration of Reaction (min)	Pectinase activity (IU mL^{-1})[a]
1	7	47	1.64	1.8	22	12.4
2	7	53	1.36	1.8	22	10
3	7	47	1.36	1.8	28	11
4	7	53	1.64	1.8	28	13
5	8	47	1.36	1.8	22	23
6	8	53	1.64	1.8	22	32.88
7	8	47	1.64	1.8	28	30.12
8	8	53	1.36	1.8	28	35.22
9	7	47	1.36	2.2	22	12
10	7	53	1.64	2.2	22	12.98
11	7	47	1.64	2.2	28	13.56
12	7	53	1.36	2.2	28	13
13	8	47	1.64	2.2	22	23
14	8	53	1.36	2.2	22	27
15	8	47	1.36	2.2	28	30
16	8	53	1.64	2.2	28	37.51
17	7.5	50	1.5	2	25	25.12
18	7.5	35	1.5	2	25	20.2
19	7.5	65	1.5	2	25	16.65
20	7.5	50	1.5	2	10	23.11
21	7.5	50	1.5	2	40	34.96
22	5	50	1.5	2	25	5
23	10	50	1.5	2	25	42
24	7.5	50	1.5	1	25	34.66
25	7.5	50	1.5	3	25	36
26	7.5	50	0.8	2	25	24.56
27	7.5	50	2.2	2	25	40.2

[a] Insignificant deviation.

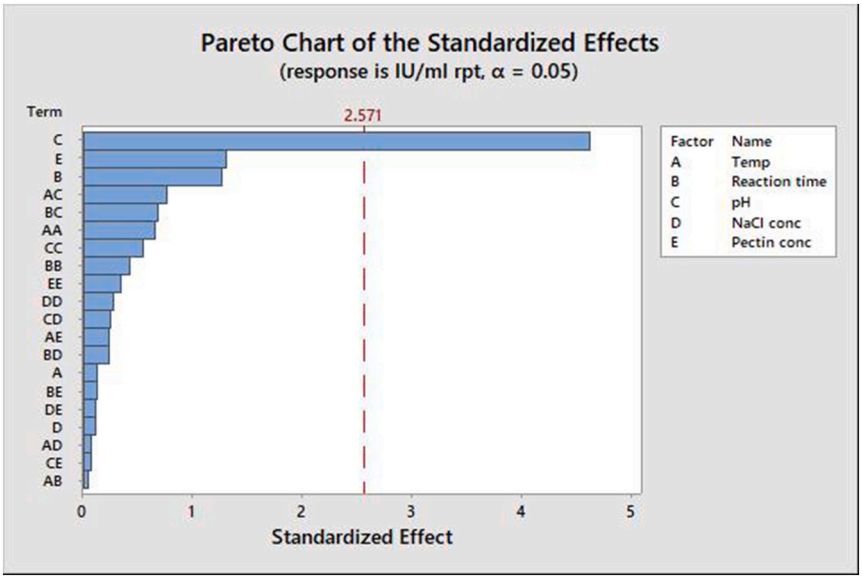

Fig. 1. Response surface plot illustrating the interactive effects of temperature and pH on pectinase activity (IU mL^{-1}) as determined by Central Composite design (CCD).

for pectinase activity in *B. vallismortis* MH 10 were pH 8.0, 50 °C, 0.8 % substrate concentration, and a 10 min reaction time. Under these conditions, maximum pectinase activity reached 12.8 ± 0.4 IU mL^{-1}, which was significantly higher than activity observed at suboptimal conditions ($p < 0.01$, ANOVA). The enzyme retained over 80 % of its activity after 3 h at pH 8 and 50 °C, indicating robust stability that is suitable for juice processing (Table 1).

3.2. Stability in presence of varying pH and buffers

Enzyme stability at different pH levels is essential for industrial applications. This study investigated how pH affects pectinase activity by conducting enzyme assays with substrates in buffers ranging from pH 4.8 to 12.5 (Fig. 3). This demonstrates that the pectinase from *B. vallismortis* MH 10 is most active and stable in alkaline buffer systems, especially within the pH range of 8.5 to 10.5. This suggests that the enzyme is well-suited for applications requiring robust activity under alkaline conditions, such as the enzymatic treatment of Detox juice blends with higher pH. The observed pH-dependent activity profile is critical for optimizing process parameters to maximize enzyme efficiency in industrial juice processing. Variation in pH can influence the enzyme function reversibly or irreversibly. Reversible effects are due to the protonation of amino acids, while irreversible effects result from changes in key amino acid groups that alter the enzyme structure. Irreversible modifications reduce enzyme activity, whereas reversible changes typically do not [29]. This study found that pectinase exhibited peak activity at pH levels of 7.7 and 8.5, with a slight decline at alkaline pH levels of 12 and 13. Similarly, other research showed that *B. sonorensis* ADCN, *B. licheniformis* PKC4, and *Paenibacillus lactis* PKC5 had optimal enzyme activity at pH 8.0, with decreased activity at higher pH levels [30]. This study also highlighted the noteworthy enzyme activity achieved with 50 mM Tris-HCl buffer at pH 10 (Fig. 3).

3.3. Evaluation of mid-point of thermal inactivation (T_m)

Maintaining precise temperature control is essential for maximizing the yield during a process. It is important to note that exposing proteins to high temperatures can result in denaturation, adversely impacting their catalytic performance. Therefore, ensuring the sustained stability and catalytic efficacy of enzymes throughout the processing period is crucial. In this study, it was noted that pectinase obtained from *B. vallismortis* MH 10 retained 90 % of its activity after exposure to a temperature of 50 °C for 30 min. Moreover, the enzyme retained 68 % of its functional capacity at an elevated temperature of 60 °C. Later, a continuous decline in enzymatic activity was observed, which allowed for the evaluation of pectinase's thermal stability, revealing a melting temperature (Tm) of 50 °C. Alqahtani et al. (2022) documented corresponding results, noting a decline in pectinase activity following a peak at 58 °C; this reduction in enzymatic activity was ascribed to thermal denaturation due to the disruption of non-covalent interactions. In this study, after 1 h of exposure at 50 °C, the enzyme maintained complete activity, with 60 % residual activity observed at 58 °C. Prior investigations have indicated that the majority of pectinase enzymes sourced from bacteria exhibit optimal activity within the temperature range of 40–50 °C [32]. A consistent decrease in activity led to the establishment of a T_m of 55 °C for the pectinase released from *B. vallismortis* strain MH 10 (Fig. 4).

3.4. Evaluation of half-life ($t_{1/2}$) at 50 °C

The half-life ($t_{1/2}$) of pectinase from *B. vallismortis* MH 10 was determined at a temperature of 50 °C. As exposure to heat increased, a decrease in pectinase activity was noted. The activity of crude pectinase was set at 100 %. Assessment of the half-life at 50 °C generated a value of 90 min (Fig. 5). When exposed to 50 °C for up to 100 min, the enzyme maintained ~20 % of its activity, but it became completely inactive after 120 min. Another study reported a half-life of nearly 15 min at temperatures of 60 and 70 °C [33].

3.5. Estimation of half-life ($t_{1/2}$) at 4 °C and 37 °C

To estimate the appropriate storage conditions, the time taken for the enzyme to lose half of its activity was evaluated at temperatures of 4 °C and 37 °C. A reduction in the effectiveness of pectinase as time progressed was noted and visually depicted. The initial effectiveness of the untreated enzyme was considered 100 %. The analysis showed that the enzyme maintained stability at 37 °C for up to 18 h. In contrast, at 4 °C, the enzyme preserved 50 % of its activity for >36 h. [31] also indicated that pectinase exhibited 60 % stability at 4 °C over a 24 h period. In contrast, [34] reported a total loss of pectinase activity after 60 min. The reduction in pectinase activity observed during storage at different temperatures may be attributed to unfolding of the enzyme or autolytic

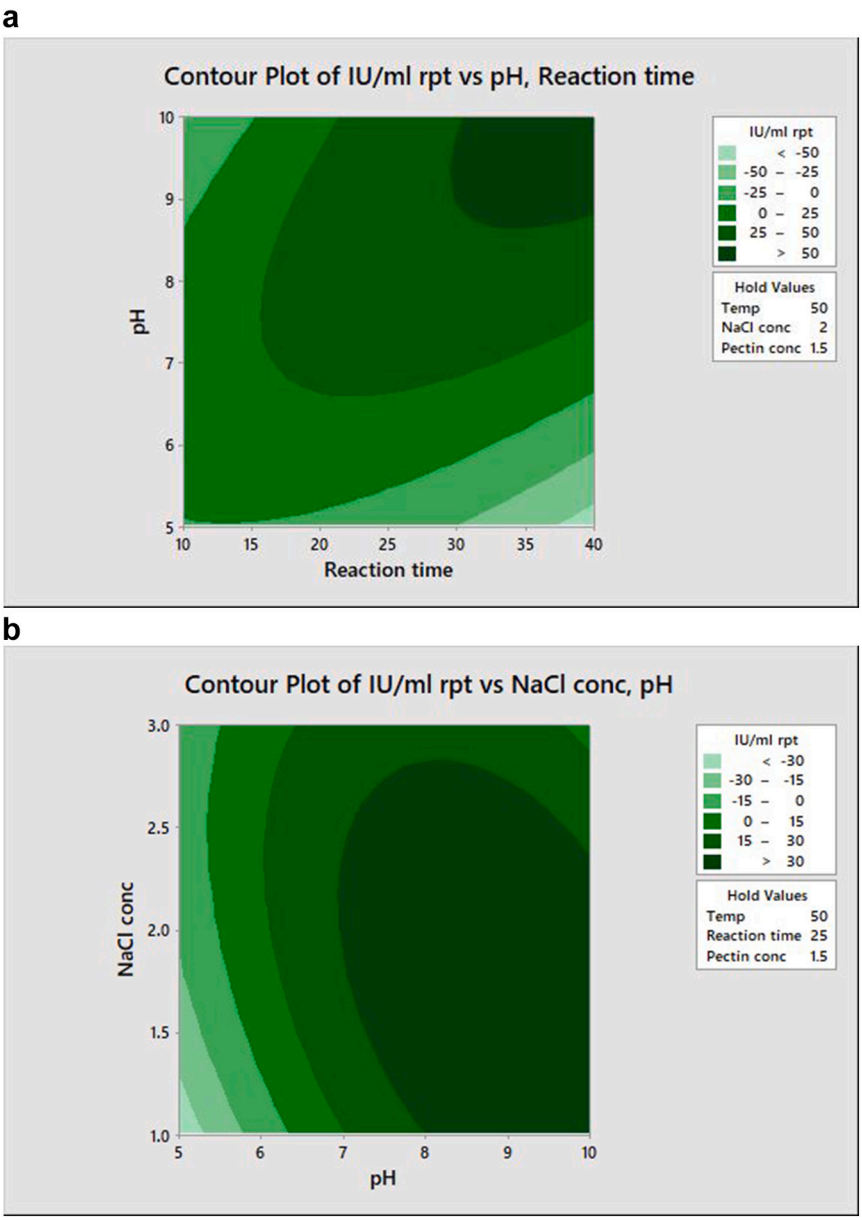

Fig. 2. Contour plot (a) pH and reaction time (b) IU mL^{-1} and Pectin conc, pH.

denaturation processes. The "shelf life" of an enzyme describes the period during which it retains its catalytic function under different storage conditions [35]. Our research findings showed that the half-life of the enzyme at 4 °C and 37 °C was 36 h and 18 h, respectively (Fig. 6).

3.6. Juice yield and total phenolic content

This research utilized two combinations (recipe 1 and recipe 2) that incorporated established antioxidant vegetables, fruits, and herbs [20,36–39].

Recipe 1 consisted of bottle gourd, lemon, carrot, ginger, cucumber, and mint, whereas in recipe 2, an equivalent amount of apple replaced the bottle gourd, with all other ingredients remaining the same. Juice yield and total phenolic content were optimized using BBD, with their responses modeled by second-order polynomial regression equations in coded variables. For example, the juice yield (Y) was calculated as follows:

$$Y = 85.2 + 4.1A + 2.3B + 1.8C - 1.2AB - 0.9AC + 0.7BC - 2.1A2 - 1.5B2 - 1.0C2Y = 85.2 + 4.1A + 2.3B + 1.8C - 1.2AB - 0.9AC + 0.7BC - 2.1A2 - 1.5B2 - 1.0C2$$

where AA, BB, and CC denote the coded pectinase units, reaction time, and temperature, respectively. ANOVA of juice recipe 1 showed the model's significance for juice yield, with F-values of 23.79, 8.53 and p-values of 0.001(Tables S3 and S4). The coefficient of determination (R^2) showed strong correlations between the predicted and observed responses, ranging from 87.1 % for juice yield. Various interactions between the two variables were visually depicted through contour plots (Figs. S1 and S2). Longer reaction times (240 min) tended to result in higher yields, such as 67.7 %, 85.7 %, and 98 %. This phenomenon

Table 3
Response optimization of detox juice recipes 1 (bottle gourd-based) and 2 (apple-based) using Box-Behnken design (BBD) at 50 °C.

Response parameters at temperature of 50 °C		Enzyme units (IU mL^{-1})	Reaction time (min)	(Predicted value)	(Experimental value)[a]
Recipe 1 (Bottle gourd)	JUICE YIELD (%)	8.3	176	93.6	95
	TPC (mg/ 100 ml)			33	28.9
	ABTS (IC_{50})			82.7	85.2
	DPPH (IC_{50})			65	63
Recipe 2 (Apple)	JUICE YIELD (%)	15	216	113	109
	TPC (mg/ 100 ml)			26	20.5
	ABTS (IC_{50})			102	96
	DPPH (IC_{50})			93.7	90.3

[a] Insignificant standard deviation.

resembled that identified in previous research on Jamaican pulp and asparagus [40]. The Pareto chart (Fig. 7a) indicated that enzyme units significantly affected juice yield, and the optimal conditions for maximum yield were a reaction time of 150 min, enzyme units of 10 IU mL^{-1}, and a temperature of 45 °C. Higher enzyme units and longer reaction times generally improve juice yield. Application of *B. vallismortis* MH 10 pectinase under optimized conditions resulted in a significant increase in juice yield from 65 % to 82 % for the bottle gourd blend and from 70 % to 88 % for the apple blend. The application of enzymes in juice processing resulted in a substantial rise in extraction yield compared to using only pressing, leading to a significant increase in juice yield, as supported by previous research [10,41–43]. This occurrence was similar to that noted in previous research on Jamaican pulp and asparagus [20,44]. For instance, in recipe 2, the process yielded 41.2 % juice with a reaction time of 60 min, 5 IU mL^{-1} pectinase, and 45 °C (Table 2). The highest yield of 98 % was achieved with 240 min of reaction time, 10 IU mL^{-1} pectinase, and 50 °C temperature expressing the stable nature of the enzyme. These results demonstrate the efficacy of the enzyme in both low-pectin (vegetable) and high-pectin (fruit) materials. This enhancement can be attributed to enzymatic treatment facilitating the breakdown of complex carbohydrates, which in turn

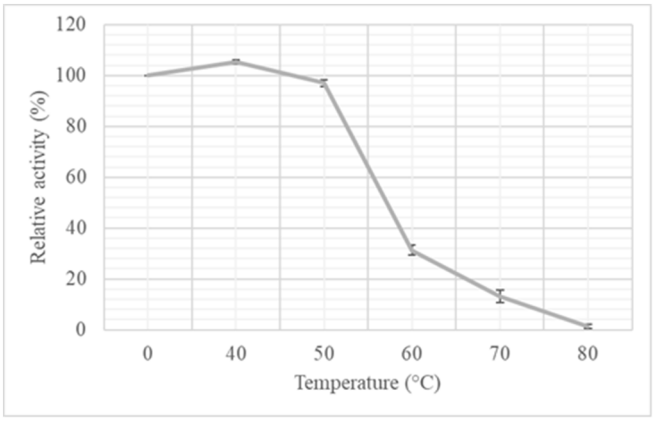

Fig. 4. T_m of pectinase by *B. vallismortis* MH 10. Pectinase activity is expressed as residual activity, with 100 % pectinase activity corresponding to 23 IU mL^{-1}.

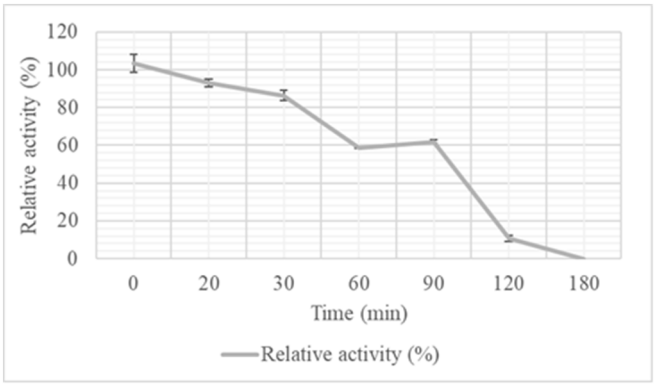

Fig. 5. Thermal stability of pectinase at 50 °C. Pectinase activity is presented as relative activity, with 100 % enzyme activity corresponding to 22 IU mL^{-1} at 50 °C.

improves processing efficiency and increases juice yield [45]. Natural phenolic compounds, ubiquitous throughout the plant kingdom, are key contributors to the antioxidant properties of many food sources [46]. The bioactive compounds in ginger, such as gingerols, along with carrot and cucumber, have shown antioxidant properties in various studies [47,48]. Polyphenolic constituents present in botanical dietary sources such as fruits, vegetables, and cereals are some of the most potent antioxidants. Consequently, it is imperative to quantify their polyphenolic

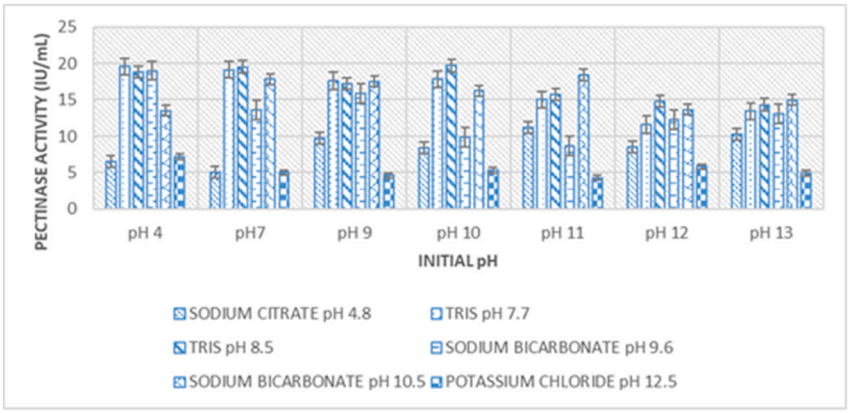

Fig. 3. Pectinase activity (IU mL^{-1}) of *B. vallismortis* MH 10 in various buffer systems.

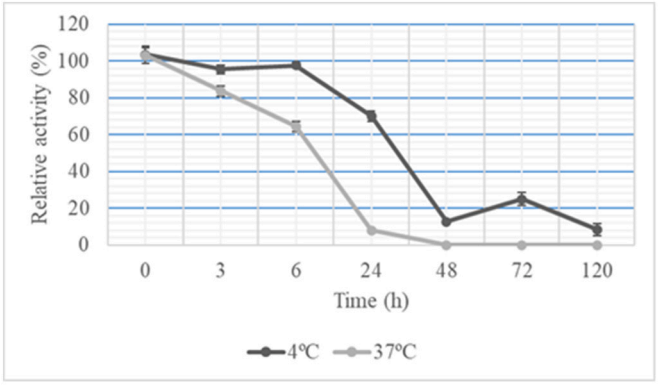

Fig. 6. Pectinase half-life at 4 °C and 37 °C. Pectinase activity is represented as relative activity (100 % enzyme activity = 24 IU mL^{-1}).

content and evaluate their antioxidant efficacy [49]. In R1, the total phenolic content ranged from 0 to 36 mg GAE/100 mL. The F-Value of 8.53, and the p-value of 0.015 indicated that the design was statistically significant (Table S5). The juice yield increased after 240 min, a trend witnessed across different temperature and pectinase concentration combinations (Fig. 7b). A comparable result was observed in a previous study on the yield of mulberry juice [20]. For recipe 2, enzyme units significantly contributed to explaining TPC variability (Table S6). The quadratic and interaction terms did not show significant effects, although there were marginal indications of non-linear and interactive effects for enzyme units and reaction time. Extended reaction times generally resulted in a reduction in juice yield while simultaneously increasing TPC. This suggests that an elongated reaction time aids in releasing more phenolic compounds but may negatively influence total juice yield (Fig. 8). Nguyen & Nguyen (2018) also observed a parallel trend of improved TPC and reduced yield when employing enzymes at a temperature of 50 °C. This phenomenon could be linked to the breakdown of pectin in the vegetable or fruit tissue's inner lamella by

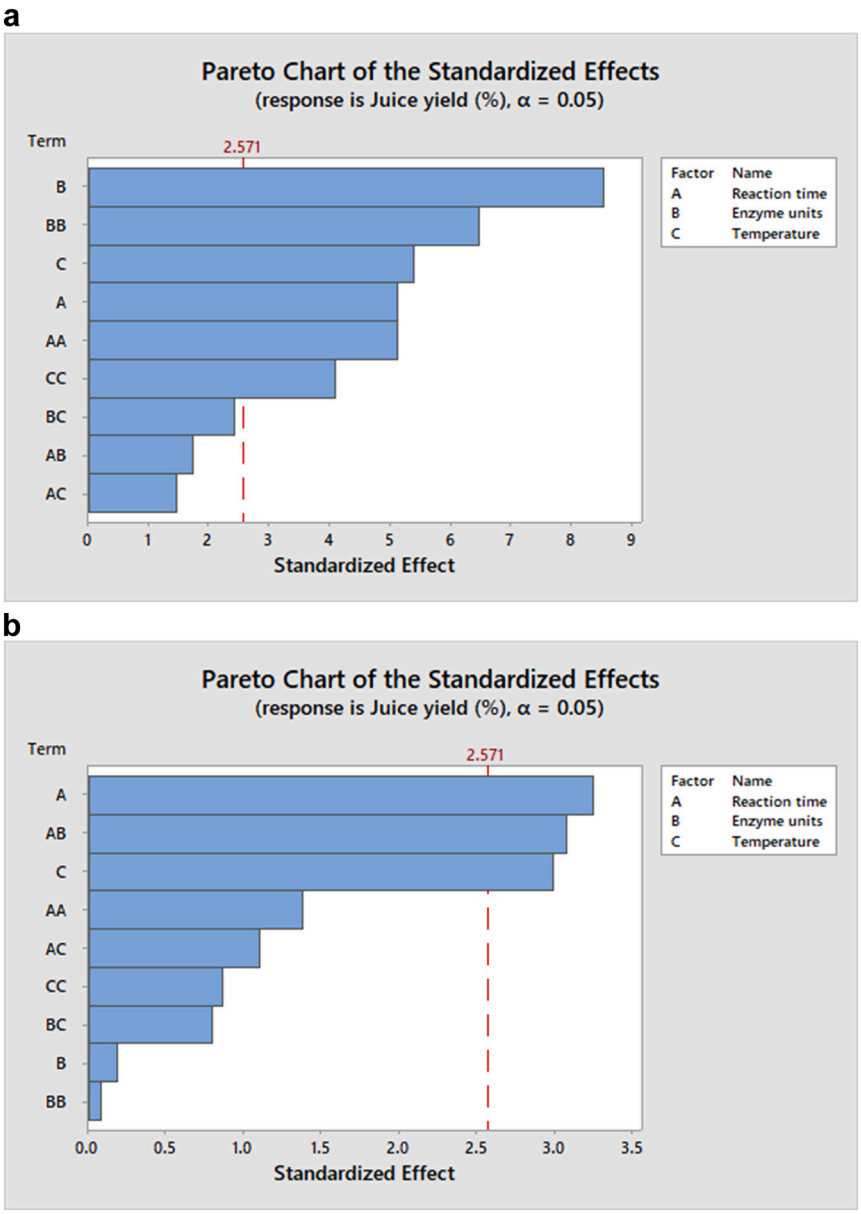

Fig. 7. Pareto chart for juice yield (a) Recipe 1 (b) Recipe 2. (A = pectinase units; B = reaction time; C = temperature; AB, AC, BC = interaction between two variables; AA, BB, CC = quadratic (squared) effect of a single variable).

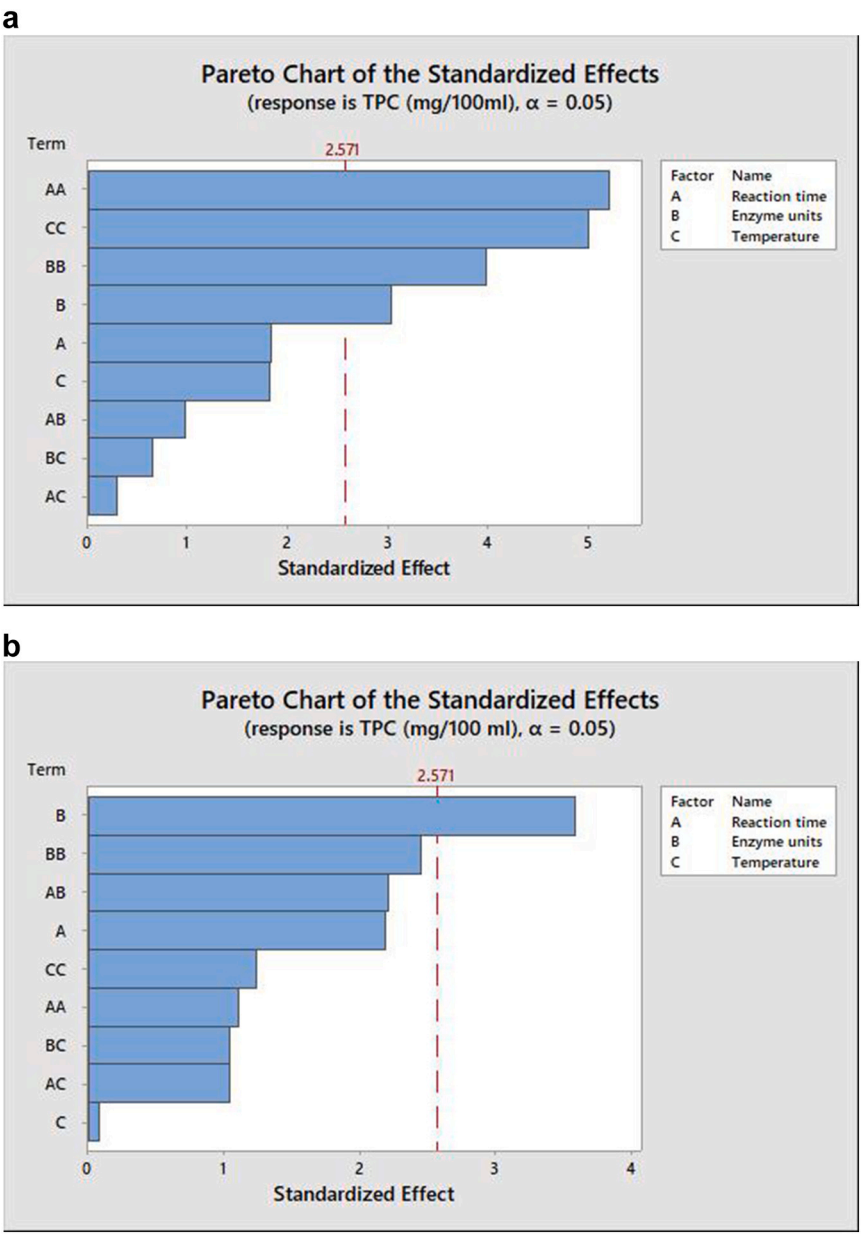

Fig. 8. Pareto chart for Total phenolic content (a) Recipe 1 (b) Recipe 2. (A = pectinase units; B = reaction time; C = temperature; AB, AC, BC = interaction between two variables; AA, BB, CC = quadratic (squared) effect of a single variable.)

pectinases, enhancing the extraction of antioxidants from the cell cytoplasm [50].

3.7. Statistical analysis of antioxidant capacities (DPPH and ABTS radical scavenging activities)

Antioxidants play a crucial role in mitigating, regulating, and averting oxidation processes, which may result in the deterioration of food quality or facilitate the onset and progression of inflammatory diseases [51]. A range of assays with different mechanisms can be used to evaluate the antioxidant potential. In this study, the antioxidant properties of both unprocessed and enzyme-treated Detox drinks were assessed by measuring their DPPH and ABTS radical scavenging activities, expressed as percent inhibition. The enzymatic treatment resulted in a higher total phenolic content in the detox drink, which was associated with an improved free radical scavenging ability (Table 4). Similar increases in antioxidant capacity have been observed when pectinase was used in the production of various fruit juices, such as pomegranates and blackcurrants [20,52]. The antioxidant activities of the Detox juices, as measured by DPPH and ABTS radical scavenging

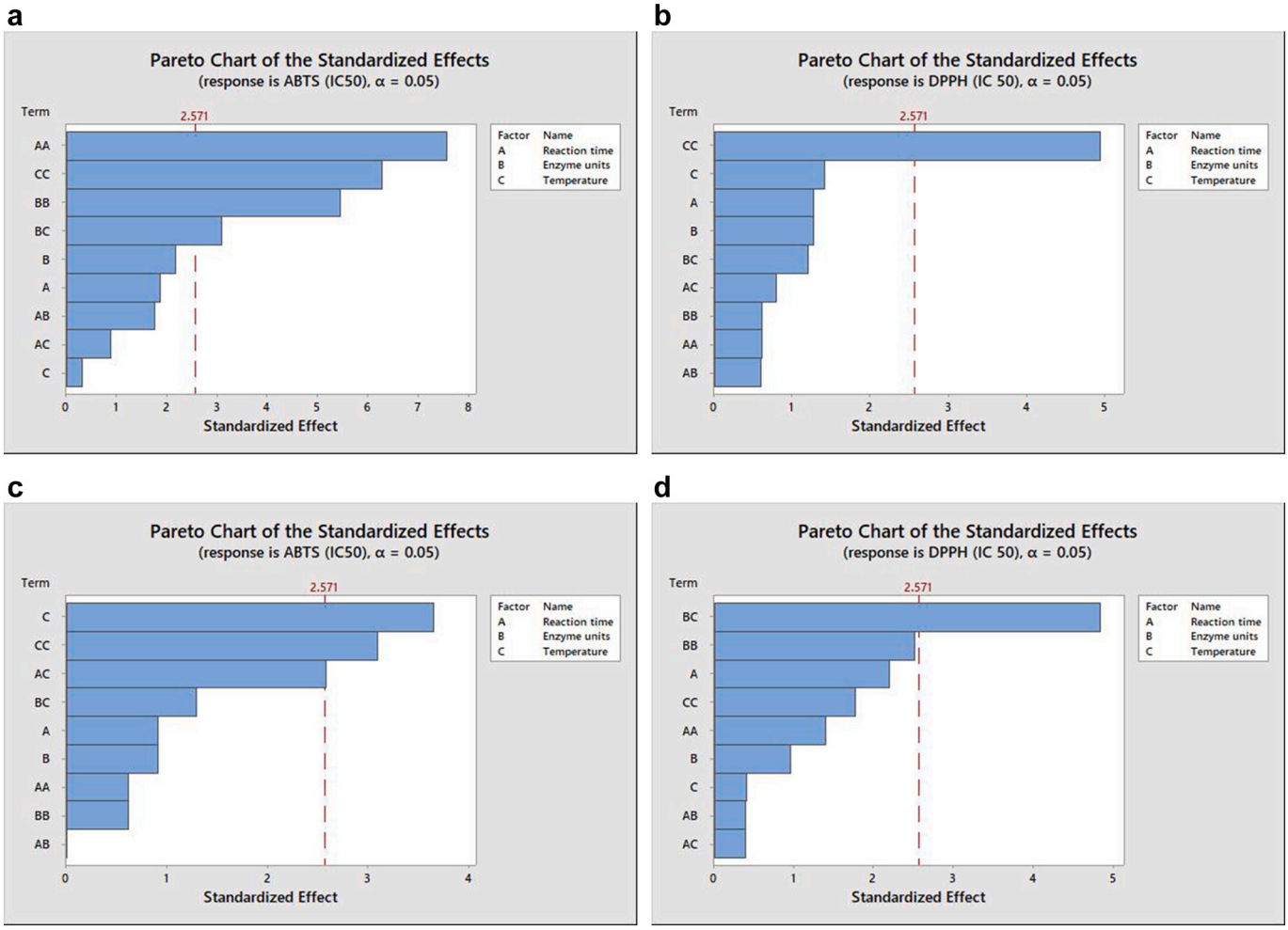

Fig. 9. Pareto chart for ABTS and DPPH (Recipe 1 = a, b and Recipe 2 = c,d).

assays, were displayed using the BBD. The regression equations for the coded variables for DPPH and ABTS activities are as follows:

$$\begin{aligned}\text{DPPH} &= 72.5 + 3.8A + 2.1B + 1.5C - 1.0AB - 0.8AC + 0.6BC - 1.7A2 - 1.2B2 - 0.9C2\text{DPPH} \\ &= 72.5 + 3.8A + 2.1B + 1.5C - 1.0AB - 0.8AC + 0.6BC - 1.7A2 - 1.2B2 - 0.9C2\text{ABTS} \\ &= 68.3 + 4.0A + 2.5B + 1.7C - 1.1AB - 0.9AC + 0.7BC - 1.8A2 - 1.3B2 - 1.0C2\text{ABTS} \\ &= 68.3 + 4.0A + 2.5B + 1.7C - 1.1AB - 0.9AC + 0.7BC - 1.8A2 - 1.3B2 - 1.0C2\end{aligned}$$

where AA, BB, and CC represent the coded values of the pectinase units, reaction time, and temperature, respectively. These models indicated significant linear, interactive, and quadratic effects of the process parameters on antioxidant capacity.

The ANOVA of the Detox juice recipe 1 revealed that the design was statistically significant for both free radical scavenging assays (ABTS and DPPH), with F-values of 14.65 and 3.78, respectively, accompanied by associated p-values of 0.004 and 0.007 (Tables S7-S10). Additionally, the coefficient of determination (R^2), a statistical measure that quantifies the degree of correlation between the predicted and observed outcomes, exhibited values of 96.3 % for ABTS and 87.1 % for DPPH, signifying a robust correlation. Contour diagrams were generated from the regression coefficients to elucidate the interactive influences of the variables (Figs. S3 and S4). This implies that the model can account for a significant percentage of the total variance. The Pareto chart (Fig. 9) highlighted that enzyme units significantly influenced juice yield, while reaction time and temperature had a substantial impact on ABTS and DPPH scavenging activities. Moreover, the interaction between temperature and duration, as well as between temperature and pectinase units, individually and together, played a crucial role in determining antioxidant activity. The observed decline in DPPH concentration subsequent to the interaction with the samples indicated that electron transfer was the predominant mechanism [53]. The quantity of antioxidants required to drop the initial DPPH concentration by 50 %, denoted by $IC_{50,}$ functions as a widely employed benchmark for assessing the antioxidant potency of the specimens [54]. Therefore, [55] documented that a lower IC_{50} value signifies an amplified level of antioxidant efficacy. Comparing the IC_{50} values for recipe 1 revealed that extended reaction durations led to decreased IC_{50} values, demonstrating increased

antioxidant potency. In contrast, the DPPH assay value of lemon skin extract was 20.01 % [56]. In a distinct investigation on a 50:50 mixture of cucumber and carrot juice, it was perceived that this mixture exhibited the highest total antioxidant capacity, with a DPPH activity around 59 % [57], which is comparatively lesser than that of IC_{50} (20 %) found in recipe 1 with bottle gourd.

The ABTS assay is utilized as an alternative approach for evaluating the antioxidant properties of fruits. DPPH and ABTS methods were employed by Suárez et al. [58] to measure the overall antioxidant activity of apple juices. The ABTS decolorization assay is deemed appropriate for assessing antioxidants that are hydrophilic in nature [59]. Previously, the antioxidant capacity of different samples under various reaction conditions was evaluated by determining IC_{50} values through ABTS assay [60,61]. It was noted that prolonging the reaction time and pectinase units, while lowering the temperature, led to a decrease in the ABTS (IC_{50}) value, reflecting an improved antioxidant potential. Notably, pectinase units were identified as a significant factor influencing ABTS IC_{50} values, whereas temperature and duration did not exhibit a noteworthy impact. The scavenging activity of ABTS values were found to range from 39.59 % to 82.79 % documented for well-preserved wild peaches [62].

Upon completion of the Box-Behnken design (BBD), an experiment aimed at optimizing responses was executed for both recipes. The developed model identified the optimal conditions, which involved a temperature of 50 °C, pectinase units (8.3 and 15), and reaction durations of 176 min for recipe 1 and 216 min for recipe 2. These parameters were designed to maximize juice yield, TPC, and antioxidant activity (ABTS and DPPH). Notably, the actual experimental data closely aligned with the projected values (Table 3) when operating under these optimized conditions.

4. Conclusion

In the present study, the pectinase produced by *B. vallismortis* MH 10 exhibited substantial enzymatic activity between 40 and 60 °C, with optimal performance at 50 °C. This thermal stability makes it well-suited for industrial use, especially in the production of fruit and vegetable juices. Besides its thermal stability, the enzyme performed best at a pH of 8.5, and remained stable across a broad pH range (4–12), indicating its flexibility in various processing settings. To assess its practical application, pectinase was applied in mixed fruit and vegetable detox juice recipes. The best results for recipe 1 were achieved at 50 °C, with 8.3 enzyme units, and a reaction time of 176 min, while for recipe 2, the optimal conditions were 50 °C with 15 enzyme units over 216 min leading to augmentations in juice yield, total phenolic content (TPC), and improved release of polyphenolic compounds that significantly contribute to antioxidant activities. These results underline the functional versatility of pectinase produced by *B. vallismortis* 10 and its potential use in the juice-processing industry. Future research could explore its interactions with other hydrolyzing enzymes and its wider applications in evolving functional foods.

CRediT authorship contribution statement

Masooma Hassan: Methodology, Funding acquisition. **Uroosa Ejaz:** Formal analysis, Data curation. **Wissal Audah Alhilfi:** Formal analysis, Data curation. **Mohammed Alorabi:** Funding acquisition, Data curation. **Syed Arsalan Ali:** Resources, Formal analysis. **Syed Muhammad Ghufran Saeed:** Resources, Data curation. **Muhammad Sohail:** Writing – review & editing, Supervision, Resources, Conceptualization.

Ethical approval

Not applicable.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2025.145165.

Data availability

Data will be made available on request.

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