Enhancing Sweetness of Natural Juices Using Partially Purified Invertase from Prune Plum Fruit

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Abstract: This study extracted, purified, and immobilized invertase from prune plum (Prunus domestica L.) fruit to increase sweetness by hydrolysis of sucrose and improve the taste of natural orange juice. Invertase (INV) purification process, including the use of ammonium sulfate precipitation at 40-80%, followed by DEAE-cellulose and Sephacryl S-200 chromatography. At the end of purification, the specific activity of INV was 12.84 U/mg, with a 6.83-fold purification and a final yield of 20.41%. INV was immobilized on sodium alginate beads with 86% efficiency. The immobilized invertase (Im-INV) worked best and was most stable at pH 5 and 45°C, and it was stable between 25 and 50°C. It kept full activity for 14 d at 4°C, then dropped to 35% after 30 d. The enzyme also showed strong operational stability, keeping full activity after 17 reuses and 81% activity after 30 reuses. The optimal conditions to increase the sweetness in natural juice are to incubate 100 mL of orange juice with 1 g of Im-INV at 45°C for 30 min.

1 INTRODUCTION

Invertase (INV) (β-fructofuranosidase) is particularly important in the food industry. It breaks down sucrose into glucose and fructose, producing invert sugar, which offers higher sweetening power and different functional properties than sucrose. As a result, this enzyme is widely used in this industry [1], [2]. It is usually extracted from microbial sources such as yeasts and fungi; however, plant sources are also an important source of this enzyme [3]. Numerous studies have shown the feasibility of extracting and purifying INV from various plants [4]. Purification usually depends on precipitation, ion exchange, and gel filtration [5]. These methods remove associated proteins and increase the enzyme's specific activity [4]. Despite the industrial importance of free enzymes, several operational challenges exist, including difficulty in recovering and reusing [6]. Therefore, studies have focused on immobilization techniques as a sustainable method to improve the thermal, chemical, operational, and reusability stability of the enzyme [1]. Alginate beads are among the most commonly used immobilization carriers in food applications due to their ease of preparation, low cost, and non-toxicity. Additionally, they can encapsulate the enzyme within a gel matrix, maintaining its activity and allowing the diffusion of substrates and products [7]. The breakdown of sucrose into glucose and fructose, which directly affects the perception of sweetness [2]. Recent studies have shown that sweetness perception in beverages is closely linked to sugar composition, with fructose having a higher sweetening power than sucrose [8]. Furthermore, the balance of sweetness and flavor plays a key role in consumer acceptance of beverages, and enzymatic modification of sugar content offers a promising direction for food product development [9]. However, studies using INV extracted from plant sources to improve the perception of sweetness in natural juices are still limited. Therefore, this study aims to use Im-INV to increase sweetness by hydrolyzing sucrose in natural orange juice.

2 MATERIALS AND METHODS

2.1 INV Extraction

INV was extracted from prune plum (P. domestica L.) fruit according to Liu et al. [4], using 0.1 M sodium phosphate buffer pH 7 containing 1 mM EDTA, β-

mercaptoethanol, and benzamidine. Extraction was performed at a 1:5 (w/v) (sample: buffer) ratio using a homogenizer for 5 min. The mixture was then filtered and centrifuged at 8000 × g for 30 min at 4°C, and the precipitate was discarded. The supernatant, representing the main source of INV, was collected as the crude enzyme extract.

2.2 INV Activity

The activities of INV and Im-INV were determined according to the method described by Sanjay and Sugunan [2]. A 0.5 mL aliquot of INV solution or 0.5 g of Im-INV on sodium alginate gel beads was mixed with 1.5 mL of 10% (w/v) sucrose solution under gentle stirring at 25°C for 30 min. One mL of the reaction product (after removal of Im-INV beads) was then mixed with 5 mL of 3,5-dinitrosalicylic acid (DNS) reagent, heated to boiling for 5 min, and cooled to room temperature. Absorbance at 500 nm was used to estimate the amount of reducing sugars as glucose equivalents, using a glucose standard curve. The enzyme activity unit (U) was defined as the amount of enzyme that releases 1 µmol of reducing sugar (glucose equivalent) per minute under experimental conditions. INV activity was used only during purification, whereas characterization and application were limited to Im-INV. A blank containing sucrose solution without enzyme was treated under identical experimental conditions and used to correct for any non-enzymatic reducing sugars or background absorbance. INV activity calculations were performed using the following (1)-(4):

$$\mu mol\ reducing\ sugar = \frac{C\ (mg/mL)\ \times Vassay\ (mL)\ \times 1000}{180.16}. \quad (1)$$

Where: C: Reducing sugar concentration from the standard curve (mg/mL glucose equivalent); Vassay: The volume of the reaction mixture (2 mL) represents the sugar released (the volume of the reaction mixture before adding DNS); 180.16=Molecular weight of glucose (mg/mmol); ×1000 to convert mmol to µmol.

$$INV\ activity\ \left(\frac{U}{mL}\right) = \frac{\mu mol\ of\ reducing\ sugar}{t\ (min) \times V_E\ (mL)}. \quad (2)$$

Where: *VE* = Enzyme volume (0.5 mL); *t* = Time (30 min).

$$Im - INV\ activity\ \left(\frac{U}{g}beads\right) = \frac{\mu mol\ of\ reducing\ sugar}{t\ (min) \times Vi\ (g)}. \quad (3)$$

Where: *Vi* = Im-INV mass of beads (0.5 g); *t* = Time (30 min).

$$Relative\ activity\ (\%) = \left(\frac{Ai}{Amax}\right) \times 100\ . \quad (4)$$

Where: Ai = Im-INV activity (U/g beads); Amax = Highest recorded Im-INV activity in the experiments.

2.3 Protein Estimation

Protein concentration was estimated according to the Bradford [10] method using bovine serum albumin (BSA) as the standard and measured at 595 nm.

2.4 INV Purification

The enzyme was concentrated by ammonium sulfate precipitation at 40-80% according to the method described by Amin et al. [5]. The precipitate was collected by cooling centrifugation at 8000 × g for 30 min. The precipitate was dissolved in 20 mM sodium acetate buffer at pH 5, dialyzed for 24 h in the same buffer, and then freeze-dried. The ion-exchange and gel filtration technique described by Alam et al. [11] was then modified by loading 5 mL of enzyme solution (10 mg/mL) onto a DEAE-cellulose column (30 × 1.5 cm, instead of 24 × 2.1 cm). The column was equilibrated with 20 mM sodium acetate buffer at pH 5 to optimize enzyme charge and binding efficiency. After washing with twice the column volume of the same buffer to remove unbound proteins, the enzyme was eluted with a linear sodium chloride gradient from 0 to 1 M (instead of 0.05 to 0.3 M) with total gradient volume 350 mL at a rate of 3.5 mL/fraction and a flow rate of 42 mL/h using a peristaltic pump. Fractions showing high INV activity were collected based on both enzyme activity and absorbance at 280 nm, then concentrated to 5 mL (5 mg/mL) by freeze-drying and loaded onto a 60 × 1.5 cm Sephacryl S-200 column, replacing the 100 × 0.9 cm Sephadex G-75 column. Then, the separated fractions were collected in 20 mM sodium acetate buffer at pH 5, at 3 mL/fraction and a flow rate of 18 mL/h (replacing the 0.9 mL/h) and then freeze-dried after fraction collection.

2.5 INV Immobilization

NV was immobilized by the entrapping method following the methods of Al-Soufi [12] and Arruda and Vitolo [7], with minor modifications by mixing 2 mL of INV solution of 10 mg/mL with 5 mL of a 2.5% (w/v) sodium alginate solution under gentle stirring at 20 rpm for 5 min at 4°C. The mixture was then added dropwise to a 2% (w/v) calcium chloride solution to form gel beads with diameters of 3-4 mm. The beads were incubated in the calcium chloride solution for 30 min at 4°C to ensure complete gelation and stabilization of the gel structure. The formed beads were collected and washed with 0.1 M sodium acetate buffer at pH 4.0 to remove any unbound enzyme from the surface. The enzyme-containing

beads were stored in the same buffer at 4°C. The activity of the entrapped INV was measured immediately after immobilization in units (U/g beads) based on the amount of reducing sugars formed under the given reaction conditions.

2.6 Immobilization Efficiency

The immobilization efficiency (IE) was estimated using the method of Al-Soufi [12], as in (5):

$$\text{Immobilization efficiency (IE)}(\%) = \left(\frac{A_o - A_s}{A_o}\right) \times 100. \quad (5)$$

Where: A_o = Total enzyme activity before immobilization (in the initial immobilization solution); A_s = Total residual activity in the supernatant + washings after immobilization.

2.7 Im-INV Characterization

The optimum pH for the activity of Im-INV was estimated using a 0.1 M buffer solution at pH from 3 to 6.5 with sodium-acetate, 7 to 9 with sodium-phosphate. The optimum temperature for the activity of Im-INV was estimated between 30 and 80°C. The pH and thermal stability were estimated by incubating the Im-INV for 30 min [13]. All measurements were carried out under assay conditions of INV activity (2.2)

2.8 Storage Period and Reuse

The Im-INV storage period for 30 d and 30 cycles of reuse was estimated at 4°C based on residual activity [12].

2.9 Juice Preparation

Oranges (Citrus sinensis L.) were obtained from local markets in Baghdad. The juice was extracted using an electric blender, filtered, and then centrifuged at 8000 × g for 15 min at 4°C. Then, the precipitate was removed, and the supernatant was retained for use in this study as clarified juice [12].

2.10 Optimal Conditions of Sucrose Hydrolysis

The determination of the optimal time, enzyme amount, and temperature for the relative sucrose hydrolysis (%) by Im-INV was done by incubating 100 mL of orange juice with 0.5 to 2 g of Im-INV at 30 to 60°C for 15 to 60 min. The reaction was monitored by estimating Im-INV activity [2]. To correct for any interference from the juice components, a control sample (blank) of juice at the same concentration without Im-INV, under the same incubation conditions, was prepared. The percentages of progress at other times were determined relative to it using (6). All treatments were conducted in triplicate, and the average of the resulting readings was calculated.

$$\text{Relative sucrose hydrolysis (\%)} = \frac{\text{glucose (mg) released at the step of the reaction}}{\text{glucose (mg) released at the final time of the reaction (60 min)}} \times 100. \quad (6)$$

The expressed apparent values (%) represent the progress of the reaction depending on the amount of reducing sugars released under the same experimental conditions, and do not represent the absolute conversion of sucrose, due to the complex nature of the juice and the possibility of interference between its components and the colorimetric measurement.

3 RESULTS AND DISCUSSION

3.1 INV Purification

INV from the prune plum fruit was purified Table 1 through Precipitation with $(NH_4)_2SO_4$, DEAE-Cellulose (Fig. 1), and Sephacryl S-200 Figure 2.

The purification indicated that the crude extract of INV from prune plum fruit had a total activity of 3160 U and a specific activity of 1.88 U/mg.

Table 1: Purification steps of INV from the prune plum (P. domestica L.) fruit.

Step	Volume (mL)	Protein (mg/mL)	Enzyme activity (U/mL)	Specific activity (U/mg)	Total activity (U)	Fold purification	Yield (%)
Crude extract	250	6.4	12.64	1.88	3160	1	100
Precipitation 40-80% $(NH_4)_2SO_4$	20	13.87	78.31	6.29	1566.2	3.35	49.56
DEAE-Cellulose	21	3.48	38.97	11.19	818.37	5.95	25.89
Sephacryl S-200	18	2.79	35.83	12.84	644.94	6.83	20.41

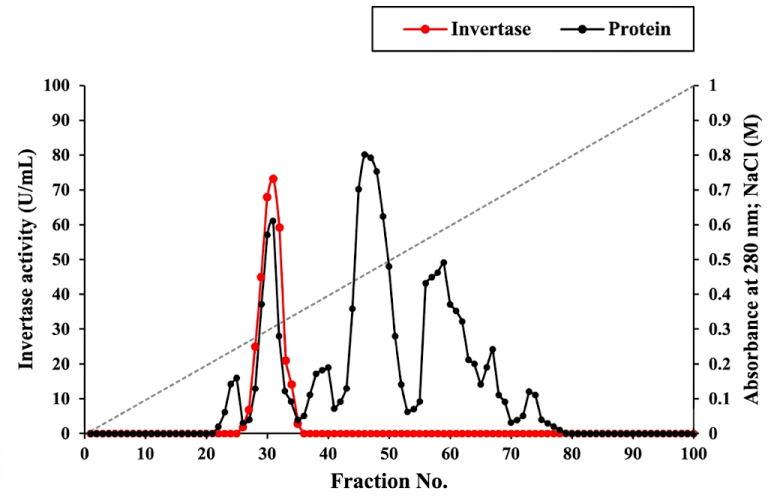

Figure 1: Purification of INV from prune plum (P. domestica L.) fruit by DEAE-Cellulose.

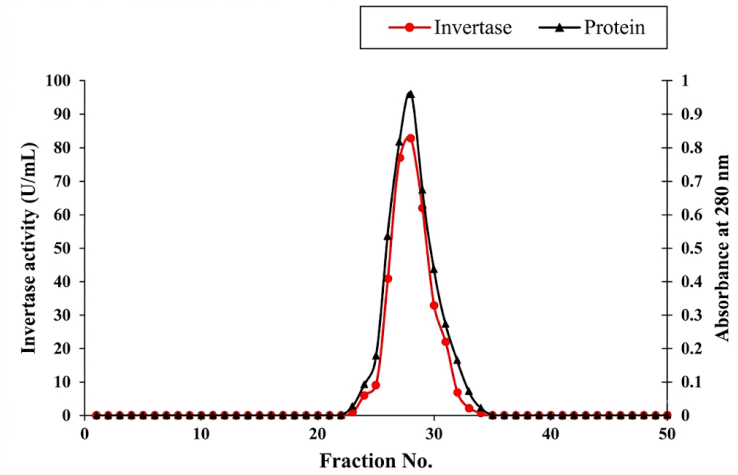

Figure 2: Purification of INV from prune plum (P. domestica L.) fruit by Sephacryl S-200.

As purification progressed, the specific activity gradually increased, reaching 12.84 U/mg after Sephacryl S-200, a 6.83-fold purification, and a yield of 20.41%.

Previous studies indicate that the purification fold of INV often ranges from (4-10) times, with a final yield ranging from (15-40%), depending on the number of purification steps and the nature of the enzyme source, which indicates that the values obtained in this study fall within the scientifically acceptable limits [3], [14]. This indicates a gradual increase in INV relative to contaminating proteins. This trend demonstrates the effectiveness of the purification steps in removing a large number of contaminating proteins and non-enzymatic constituents [15]. This result can be reinforced by previous studies on this enzyme, which have shown a similar gradual increase in specific activity with a decrease in yield due to loss of enzyme activity during the purification steps, as observed in multi-step purification systems, including precipitation and chromatographic techniques [3]. Decreases in enzyme activity of up to 30-60% have been recorded during multiple purification steps as a result of partial loss of the enzyme or a change in its spatial structure during precipitation and chromatographic separation processes [14].

The precipitation with $(NH_4)_2SO_4$ resulted in a 3.35-fold purification and a yield of 49.56%. The DEAE-Cellulose increased the specific activity by 5.95-fold purification and the yield of 25.89%. The Sephacryl S-200 then increased the specific activity by 6.83-fold purification and the yield of 20.41%, with a single, nearly identical peak for enzyme and protein activity representing the target INV, which indicates minimal overlap of non-target proteins.

From an analytical perspective, these results can be interpreted as follows: ammonium sulfate precipitation is a prevalent initial step in enzyme purification to reduce sample volume while increasing enzyme concentration. However, it may cause partial loss of enzyme activity due to partial denaturation or incomplete re-dissolution [14]. While ion exchange is considered an effective purification step due to its high selectivity for separating proteins that are similar in size but different in net charge, similar increases in the purification fold have been reported when using a DEAE column fold (5-8) times when purifying INV from Saccharomyces cerevisiae [16]. Furthermore, gel filtration is used to separate proteins and remove impurities based on molecular weight. This technique is particularly effective in the final polishing step of enzyme purification by column chromatography [15]. This technique has shown high efficiency in achieving accurate final separation, with high purity levels exceeding 80-90% in several studies that adopted gel filtration as a final step in enzyme purification [3], consistent with findings of regarding the effectiveness of gel filtration in removing both low and high-molecular-weight concomitant proteins.

In this context, the final yield (20.41%) is within acceptable limits for multi-step enzyme purification processes, where a gradual decrease in yield is often observed alongside an increase in purity. This represents a critical trade-off between practical efficiency and analytical accuracy. The aim of purification is not only to maximize recovery but also to obtain an enzyme of sufficient purity for studying its biological properties and practical applications [14], especially when the purified enzyme is intended for downstream applications such as immobilization or industrial use.

3.2 Immobilization Efficiency

The IE of INV with sodium alginate beads was 86%. It was within the range of other literature, such as Zhou et al. [17], which demonstrated 85% to 90% with cellulose. Al-Soufi [12] states that it is 93% with bentonite. Tanriseven and Doğan [18] reported it at 87% with calcium alginate. This result is an important indicator of how suitable the immobilization conditions are based on the support nature with limited mass transfer resistance within the beads, the binding mechanism, such as entrapment within the gel matrix and the compatibility between the support surface properties and the enzyme's three-dimensional structure, minimizing conformational changes and activity loss as well as the strong binding affinity of INV to a wide range of supports [6], [19]. This immobilization efficiency is a crucial factor in designing food enzyme systems, as it provides better operational stability, reduced enzyme waste, and increased economic viability [1]. Therefore, the success of INV immobilization depends on maintaining functional performance in addition to the structural binding to the support surface, particularly under repeated use and storage conditions [20], [21].

3.3 pH

The results in Figure 3 show that the Im-INV had the highest relative activity (%) at pH 5. At pH 3 and 12, the activities were 76% and 12%, respectively. Also, (Fig. 4) shows that the enzyme was stable across a pH range of 3 to 6, with relative activities of 82% and 87%, respectively.

The stability ranges between pH 3-6, reflecting the ability of the Im-INV to maintain its activity over a wider range than the soluble INV, which is attributed to the role of immobilization in limiting conformational changes in the tertiary structure [19]. It is likely that the immobilization within the alginate support provides a microenvironment that maintains the stability of amino acid charges at the active site of Im-INV, thus reducing the effect of pH changes [6]. These results align with several previous studies indicating that the optimal range for Im-INV activity is between pH 4 and 5.5. Zhou et al. [17] reported that cellulose-Im-INV showed the highest activity at pH 5. The coincidence of the highest enzyme activity with the relative stability range indicates that optimal operating conditions not only enhance catalytic efficiency but also ensure appropriate structural stability of the Im-INV during application.

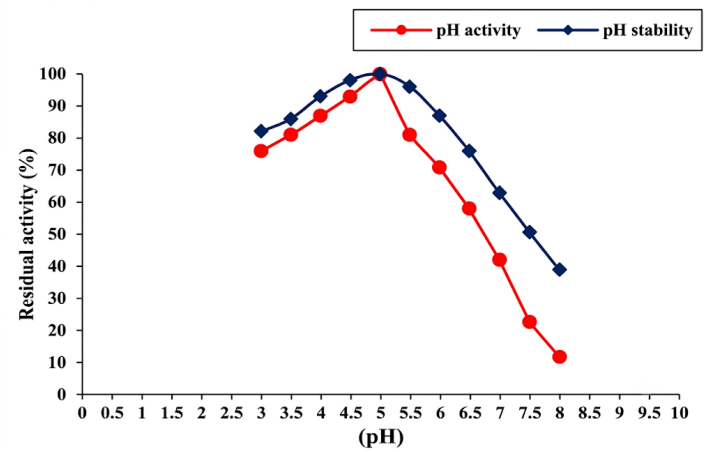

Figure 3: The optimum pH of activity and stability for Im-INV from prune plum (P. domestica L.) fruit on sodium alginate gel beads.

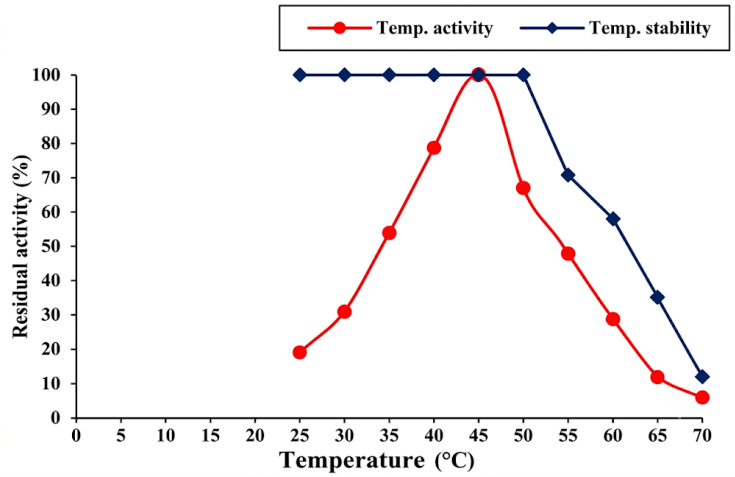

Figure 4: The optimum temperature (°C) of activity and stability for Im-INV from prune plum (P. domestica L.) fruit on sodium alginate gel beads.

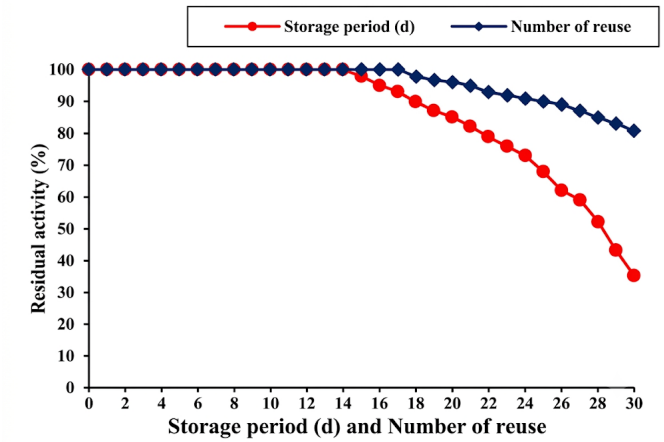

Figure 5: Effect of storage period (d) at 4°C and the number of reuses for Im-INV from prune plum (P. domestica L.) fruit on sodium alginate gel beads.

Additionally, a study by Bayramoglu [6] demonstrated that Im-INV on magnetic particles enhanced the enzyme's stability with high activity retention at pH 5. The results of this study show that a low pH is optimal for Im-INV activity, consistent with work on catalytic properties at acidic conditions [17]. Additionally, the immobilization will help the enzyme to stabilize in the acidic environment [19]. This is attributed to reducing the flexibility of the enzyme's three-dimensional structure and to protecting it from sharp changes in ionization state in an acidic environment [6]. Conversely, the decline in activity at alkaline pH values above 6 is due to changes in amino acids' ionization at the active site and a reduction in the efficiency of substrate binding to the active site of Im-INV [1]. These results confirm the suitability of the Im-INV for acidic food applications, such as fruit juices, where the operating pH is within the optimal range for activity and stability.

3.4 Temperature

The results in Figure 4 show that the relative activity (%) of the Im-INV increased, reaching a maximum of 100% at 45°C. This indicates that this degree represents the optimal activity, as increased kinetic energy promotes the formation of the enzyme-substrate complex up to this point. At 25 and 70°C, the enzyme showed activities of 19% and 6%, respectively. The decrease at 25°C is attributed to a decrease in kinetic energy and weak collisions of molecules, while the decrease at 70°C is due to thermal structural changes in the enzyme's three-dimensional structure [17]. Regarding stability, the immobilized enzyme maintained full stability (100%) across a broad temperature range of 25-50°C. This reflects a wide range of operational thermal stability of the Im-INV and enhances its suitability for food applications [21].

These results are generally consistent with the literature. Dokuzparmak [3] indicated that Im-INV within polymer matrices improved its thermal stability and extended its operational lifespan compared to the free enzyme. Similarly, Hakkoymaz and Mazı [21] demonstrated that Im-INV maintained high stability at moderate temperatures, making it suitable for analytical and food applications such as juice processing. This behavior is typical for enzymes restricted within polymeric supports [3].

The temperature increases kinetic energy until the optimal level of activity is reached (45°C), which enhances the collision rate between the enzyme and the substrate and the formation of the enzyme-substrate complex [17]. The immobilization contributes to the enzyme's thermal stability by reducing the enzyme's structural flexibility and restricting its molecular movement within a controlled environment provided by alginate gels [20]. This effect is attributed to its ability to stabilize the three-dimensional structure, which helps maintain the efficiency of the active site and reduces the possibility of losing activity [19].

Conversely, the decrease in relative activity is attributed to gradual changes in the enzyme's three-dimensional structure induced by high temperatures, leading to disruption of the active site or partial loss of catalytic capacity, which leads to partial or irreversible damage to enzymatic activity [19]. This confirms the efficiency of the Im-INV for use in industrial and food applications [21].

3.5 Storage Period and Reuse

The results Figure 5 showed that the Im-INV maintained its full relative activity (100%) for 14 d of storage at 4°C. This indicates a high storage stability for Im-INV. After this period, Im-INV activity gradually declined, decreasing to 35% of the initial activity after 30 d of storage. This suggests the appearance of gradual limitations due to structural changes or diffusion restrictions over time [19]. Regarding the reusability of the immobilized enzyme, the results indicated that the enzyme retained its full activity (100%) over the first 17 reuses. This shows the high operational stability for Im-INV. Afterward, the activity decreased, reaching 81% by cycle 30. This value is an industrially acceptable limit for applications of immobilized enzymes [3].

The results show that immobilization protects the enzyme from losing activity over time. This effect is mainly attributed to the physical entrapment of the enzyme within the alginate support [6]. Furthermore, this demonstrates the treatment's ability to reduce conformational changes in the three-dimensional structure of INV during storage [19], and its high tolerance for reuses under repeated operational cycles without significant loss of Im-INV activity [1]. These results are consistent with several previous studies on the stability of Im-INV during storage, as studies by Dokuzparmak [3] and Hakkoymaz and Mazı [21] found that Im-INV retained a high percentage of its activity after prolonged storage at low temperatures. Waifalkar et al. [20] noted that Im-INV on magnetic particles could be reused for up to 20 reuses without significant activity loss, while Dokuzparmak [3] showed that Im-INV retained about 80% of its activity after 20 reuses. However, the activity

declined after the third week of storage, which can be attributed to factors such as leakage of enzyme molecules from the immobilization matrix, and progressive damage to the active site during repetitive continuous reactions [20]. In addition, there is a possibility of internal diffusion resistance within the gel support during continuous reuse [19].

3.6 Optimal Conditions of Sucrose Hydrolysis

As illustrated in Figure 6, the rate of sucrose hydrolysis steadily increased with longer reaction times up to 30 min, when a notable rise in reducing sugars was seen. However, extending the time to 45 and 60 min did not result in a significant increase in the hydrolysis rate. This behavior indicates that the reaction is approaching a quasi-steady state according to Michaelis-Menten kinetics, i.e., a dynamic equilibrium between the formation and breakdown of the enzyme-substrate complex, where the system reaches substrate saturation, resulting in a stable reaction rate despite an increase in incubation time [21]. As the reaction proceeds, the enzyme-substrate interaction gradually decreases due to a decrease in the substrate and an increase in the accumulation of reaction products (glucose and fructose), which may cause partial inhibition of enzyme activity or bring the system closer to equilibrium [1].

An increase in the Im-INV amount (g) Figure 7 causes the rate of sucrose hydrolysis to increase up to 1g. However, extending the amount to 1.5 and 2g did not result in a significant increase in the hydrolysis rate.

This is attributed to an abundance of active sites and substrate at the beginning of the reaction, but the absence of substrate will affect the reaction's progress as a rate-limiting factor [1], [21].

The Im-INV activity increases with temperature until reaching an optimal point at 45°C. However, extending the temperature to 50, 55, and 60°C did not result in a significant increase in the hydrolysis rate Figure 8.

Identifying this optimal temperature is important in industry because it helps balance reaction rate and enzyme stability [3]. Mass transfer limitations within alginate granules also likely play a role in influencing the reaction rate, especially when reaction time or enzyme quantity is increased, due to diffusion restrictions of the substrate and products within the support [1], [21].

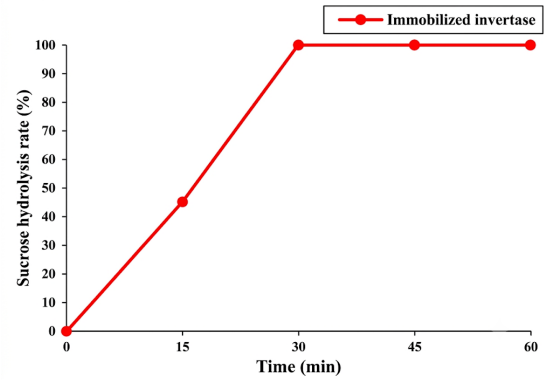

Figure 6: Effect of the time (min) on sucrose hydrolysis rate (%) by Im-INV from prune plum (P. domestica L.) fruit on sodium alginate gel beads.

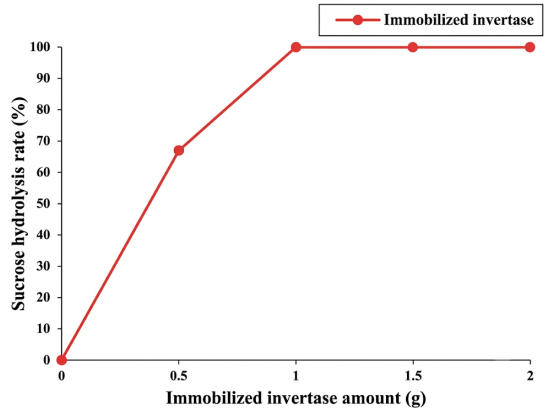

Figure 7: Effect of the amount (g) of Im-INV from prune plum (P. domestica L.) fruit on sodium alginate gel beads on the sucrose hydrolysis rate (%).

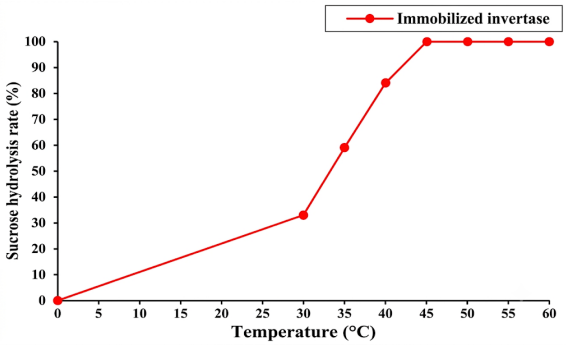

Figure 8: Effect of temperature (°C) on sucrose hydrolysis rate (%) by Im-INV from prune plum (P. domestica L.) fruit on sodium alginate gel beads.

4 CONCLUSIONS

This study demonstrates that invertase (INV) can be successfully extracted from prune plum (Prunus domestica L.) fruit, purified, and effectively immobilized on sodium alginate beads. The immobilized enzyme (Im-INV) retained high catalytic activity over a broad range of pH values and temperatures, exhibited good storage stability, and maintained its activity after repeated reuse. Furthermore, Im-INV efficiently hydrolyzed sucrose in natural orange juice, resulting in improved sweetness and higher sensory acceptability without introducing undesirable flavor changes. These findings demonstrate that enzyme immobilization enhances the operational stability and practical applicability of invertase while preserving its catalytic performance. Therefore, the developed Im-INV system represents a promising and sustainable approach for enzymatic processing in the food industry, particularly for the production of naturally sweetened fruit beverages and other sucrose-containing food products. Future studies may focus on large-scale production, long-term operational performance, and the application of this immobilization strategy to other food-processing systems.

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