



Separation of invertase enzyme from tomato using aqueous two-phase system: PEG1500/sodium citrate and PEG4000/sodium citrate

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Abstract

An aqueous two-phase system (ATPS) is a highly useful, effective, simple, and economically friendly technique for the biomolecules recovery. ATPS is created by mixing two polymers or a polymer and salt above their critical concentration in water. Invertase is an enzyme that hydrolyzes sucrose into glucose and fructose, making it highly crucial for the food industry. Because the ATPS can be applied in mild conditions, in a water-rich phase, and is biocompatible with bioproducts, this method was used to extract the invertase enzyme from tomato pulp. Specific activity and protein concentration of the enzyme were found to be 10.87 U/mg and 9.44 mg/mL, respectively. In this research, two ATPS systems were used: polyethylene glycol 1500/sodium citrate (PEG1500/SC) and polyethylene glycol 4000/sodium citrate (PEG4000/SC). Five parameters were studied to obtain the maximum amount of invertase from tomato: temperature, pH, concentrations of PEG and salt, and supporting neutral salt (NaCl and MgCl₂). The maximum percent recovery (%Rec) of invertase and its partition coefficient (K_E) were 84% and 5.25 respectively, at a temperature of 10 °C, pH 10, and concentrations of PEG1500 and SC of 1.8 g/10 ml each for system-1 (PEG1500/SC) without adding a supporting neutral salt, whereas for system-2 (PEG4000/SC), the optimum operating conditions were at a temperature of 10 °C, pH of 10, and concentrations of PEG4000 and SC of 1.65 g/10 ml and 1.5 g/10 ml respectively, where the maximum %Rec and K_E were 89.8% and 8.8, respectively, without adding supporting neutral salt. Because system-2 provides better results than system-1, it is considered to improve the enzyme recovery by adding NaCl or MgCl₂ as supporting neutral salts, where the %Rec increased to 95.8% and 96.7% for the NaCl and MgCl₂ additions, respectively.

Keywords: Aqueous two-phase system; Enzymes; plant source; extraction.

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1- Introduction

The aqueous two-phase system (ATPS) provides a quick, economical, and scalable method for biomolecule purification through selective phase partitioning without solid-phase interactions [1]. Depending on the density and viscosity differences between the phases, two phases are produced when two components (usually two polymers or a polymer and a salt) are diluted in a buffer (usually water) at a particular concentration and left to stand for several hours (a centrifuge may be used to facilitate separation) [2]. This technique aids in the separation of biomolecules while lowering contaminants and maintaining the stability and activity of enzymes [3]. Proteins and nucleic acids were separated using early aqueous two-phase extractions, which were later modified to separate particular biomolecules in polymer-polymer systems [4]. ATPS can be quite effective for the extraction and purification of biomolecules, including enzymes, which are essential in several industries, such as chemical, pharmaceutical, food, and biotechnology [5, 6]. By managing the “dirty work” of clarity, concentration, and initial purification in a single, elegant step, ATPS is a

potent research tool in labs for examining biomolecular interactions, carrying out initial purifications, and significantly lowering downstream processing costs. [5, 7].

Proteins, sometimes called enzymes, are catalysts made up of long chains of amino acids that are linked together by peptide bonds [8]. They attach perfectly to the substrate to initiate any reaction [9]. The substrate concentration and other environmental conditions, such as pH and temperature, can change the activity of enzymes, which have different roles [10, 11].

Microbes, plants, and animals are just a few sources of enzymes [8]. This commission sorts enzymes into groups based on their structure, function, and reaction type [9]. Enzymes can help with many health problems, but they can also help those who not get enough nutrients [12]. Invertase (β fructofuranosidase) is one of these enzymes that is particularly significant to industry because it breaks down sucrose into glucose and fructose, which are used to manufacture drinks, sweets, and bioethanol [13, 14]. Invertase acts as a hydrolyzing enzyme, but also has transferase activity, especially at high sucrose



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concentration [15, 16]. Invertase has been discovered from a wide range of living organisms such as microorganisms, plants, and animals [17, 18]. Historically, invertase was derived from microbial sources such as *Saccharomyces cerevisiae* [19-21]. However, due to their cost-effectiveness, availability, and safety, plant sources are becoming increasingly favored [22, 23], and may be even cheaper, especially in Iraq, where the price of a kilogram of tomato reaches \$ 0.1 or less.

Potential sources of invertase include tomatoes (*Solanum lycopersicum*), which have a high metabolic activity and are widely grown in agriculture. Using ATPS to extract invertase from tomatoes is a practical and effective method for high recovery and purification of the useful enzyme [24-27]. This study investigated the postextraction activity, specific activity, recovery yield, and partitioning behavior of invertase in a PEG-salt-based ATPS. polyethylene glycol (PEG) was used in this study because it is inexpensive and biocompatible [7].

This study aims to illustrate the use of Iraqi tomatoes as a sustainable, plant-derived invertase source for industrial use. The primary aim of this study is to find the optimum operating conditions required to extract and partially purify invertase from tomatoes via an aqueous two-phase system (ATPS) using response surface methodology (RSM) as the experimental design.

Invertase enzyme can be isolated from multiple sources: from Baker's yeast using polyethylene glycol/MgSO₄ ATPS [28]. Yeast invertase is predominantly intracellular and requires harsh mechanical homogenization or chemical lysis before ATPS. Lysis floods the crude mixture with debris, nucleic acids, and native intracellular proteins that accumulate at the interface, creating a thick, unseparated emulsion layer that traps invertase, reduces mass transfer efficiency. Invertase extracted from potato using PEG4000/Na₂SO₄ ATPS [29]. Crude potato extracts contain two obstacles for invertase extraction: free starch granules that physically damage the extraction phases, and active polyphenol oxidases which oxidize into reactive quinones that covalently bind to invertase, causing browning, structural disruption, and severe loss of enzyme activity. Invertase was also extracted from tomato using PEG3000/NaSO₄ with KCl as the support salt [30]. NaSO₄ is an inorganic salt that can change enzyme activity, stability, and recoverability, compared with the present study, which used an organic salt (sodium citrate), which is soft, biocompatible, has gentler interaction, and enhances enzyme stability [31].

The enzyme is affected by high temperatures (>40°C), reducing its catalytic activity and stability [32]. The same applies to an acidic environment (pH < 4) and base environment (pH > 10) [33]. The critical concentrations of PEG and salt required to achieve a two-phase system for PEG1500/SC and PEG4000/SC were determined in an experimental laboratory. Therefore, the factors that affected the extraction of invertase from tomato were as follows: temperature (10 – 40 °C), pH (5 – 10), concentration of material for system-1, PEG1500/SC, (1.8

– 2.4 g/10 ml), concentration of material for system-2, PEG4000/SC, (1.5 – 2.1 g/10 ml), and the supporting agent (NaCl or MgCl₂: 0.1 – 1.5 g/10 ml).

The novelty of this research lies in the specific formulation (PEG4000/Sodium citrate and PEG 1500/Sodium citrate combined with neutral salts additives like NaCl and MgCl₂) and broader parameters optimization (such as temperature, pH, PEG and salt concentrations) applied to a local plant source plant (Iraqi tomatoes), resulting in a significantly higher recovery yield than previously reported methods for this specific enzyme.

2- Materials and experimental work

2.1. Materials

Central Drug House Ltd. and Alpha Chemika, two Indian companies, provided the sodium citrate (Na₂CO₃), Hydrochloric acid (HCl), sodium chloride (NaCl), and manganese chloride (MgCl₂). We purchased polyethylene glycol (PEG1500, PEG4000), sucrose, 3,5-dinitrisalicylic acid (DNS), sodium hydroxide (NaOH), sodium potassium tartrate (C₄H₄KNaO₆·4H₂O), copper sulfate (CuSO₄·5H₂O), and bovine serum albumin (BSA) from HIMEDIA Laboratories Pvt. Ltd. in India. The folin reagent is sourced from Sisco Research Laboratories Pvt. Ltd. (Srl) in India. Regarding fresh ripe tomatoes, they were bought from Iraqi local marketplaces. The analysis was conducted using high-quality reagents. For this investigation, deionized and double-distilled water from the Department of Biochemical Engineering's labs was utilized.

2.2. Experimental work

• Crude extract preparation

Procedure of preparing tomato invertase extract:

1. 500 g of local fresh ripe tomatoes (*Solanum lycopersicum*) is washed by distilled water, removing the peels and seeds, and the fruit was cut into small pieces.
2. Flash-freeze in liquid nitrogen and grind to a fine powder using a pre-chilled mortar and pestle.
3. Add the powdered tissue to chilled extraction buffer at a ratio of approximately 1:2 to 1:3 (w/v). A commonly used buffer is: 0.1 M sodium acetate buffer (pH 5.0) for acid invertase.
4. Homogenize thoroughly and keep on ice for 15–30 minutes.
5. Filter the homogenate through 2 layers of cheesecloth to remove cell debris.
6. Centrifuge the filtrate at 5000 rpm for 20 minutes at 4°C.
7. Collect the supernatant as the crude enzyme extract containing soluble invertase.
8. The extract should be stored at –18°C until use [34, 35]. Fig. 1 shows the experimental procedure of preparing tomato invertase extract.

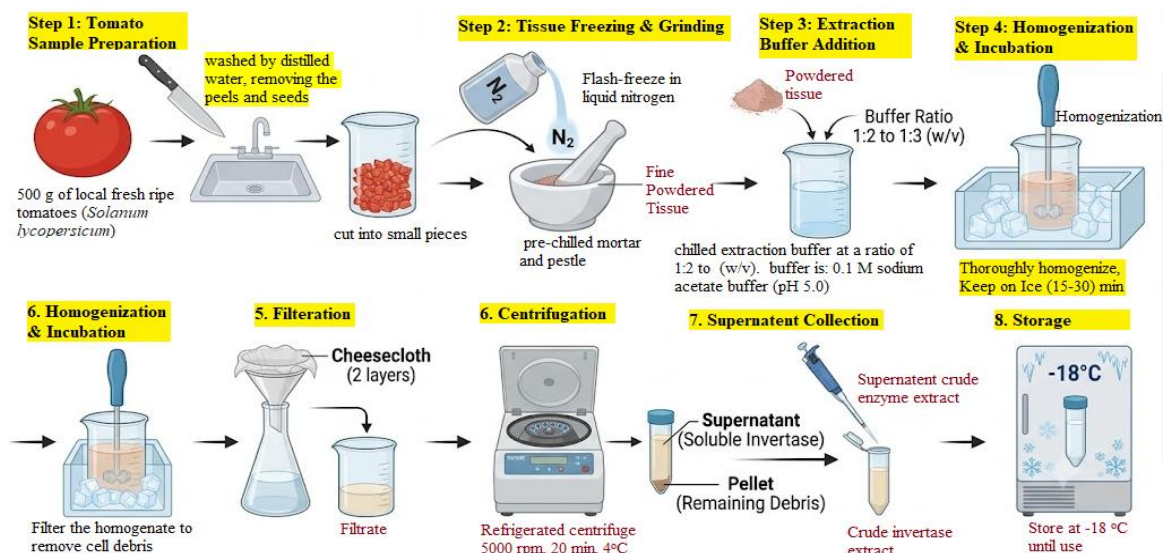


Fig. 1. Experimental procedure of preparing tomato invertase extract

2.3. Analytical methods

2.3.1. Invertase activity determination

By using the 3,5-dinitrosalicylic acid (DNS) method, the reduced sugar that was produced was ascertained by measuring the enzymatic activity using the starting rates of the sucrose hydrolysis reaction method. 0.2 mL of 0.5 M sucrose, 0.6 mL of sodium acetate buffer (0.2 M, pH 5.0), and 0.2 mL of enzyme solution made up the assay mixture, which was incubated for 30 minutes at 37 °C. One milliliter of DNS reagent was then added, and it was cooked for ten minutes in a boiling water bath.

Following cooling to room temperature, the concentration of reducing sugars was determined using spectrophotometry at 540 nm. The mean results of the triplicate assay are used to display the data for each invertase activity determination [36].

2.3.2. Protein assay

The protein concentration was calculated using the Lowry method [37] which included the following solutions:

- Solution I: Dissolve Na_2CO_3 (2 g) in 100 mL of 0.1 M NaOH.
- Solution II: Add 0.5 g of copper sulfate and 1.0 g of tartrate to 100 mL of distilled water.
- Solution III (alkaline copper tartrate): Mix 50 mL of reagent I and 1 mL of reagent II.
- Solution IV (Folin-Ciocalteu reagent): Dilute the commercial (2N) reagent 1:1 with distilled water.
- Solution V was bovine serum albumin (BSA): Standard protein assay. Mix 0.2 mL of the protein sample (5-100 μg) with 1 mL of reagent III. Incubate for 10 minutes at room temperature. Add 0.1 mL of reagent IV and mix immediately. After 30 minutes, measure the absorbency at 500 nm.

Create a series of solutions with known BSA concentrations from the stock solution (0 – 100 $\mu\text{g}/\text{mL}$).

Mix 0.5 mL of each standard tube with 5 mL of solution III and let the tubes stand at room temperature for 10 min. Immediately add 0.5 mL of diluted solution IV to each tube and mix rapidly. The tubes were incubated in the dark at room temperature for 30 min. A deep blue color will be created. The absorbency of each tube was measured at 540 nm. The BSA standard curve using the Lowry technique is displayed in Fig. 2.

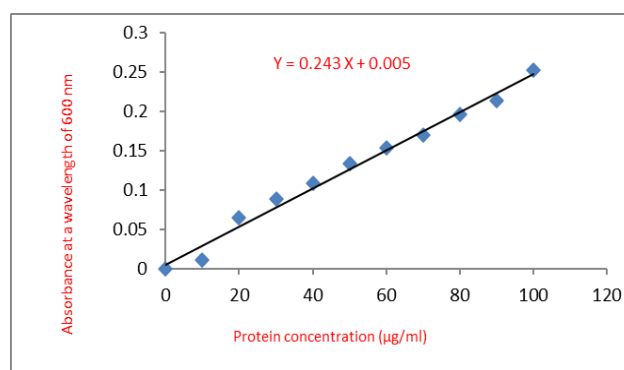


Fig. 2. BSA standard curve

Specific activity and protein concentration of the enzyme were found to be 10.87 U/mg and 9.44 mg/mL, respectively. One unit (U) of invertase activity is the amount of enzyme that releases 1 μmol of reducing sugar per minute under the assay conditions.

2.3.3. Partitioning of invertase in aqueous two-phase system

The experimental study revealed that PEG should be mixed with the crude extract to obtain a higher extraction rate. The minimum concentrations of sodium citrate and PEG that give two-phase system were obtained from the experimental laboratory, where the first system comprised sodium citrate stock solutions (1.8-2.4 g) per 10 mL of distilled water and (1.8-2.4 g) of PEG1500 per 10 mL of crude extract. The second system consists of sodium

citrate stock solutions (1.5-2.1 g) per 10 ml of distilled water and (1.5-2.1 g) of PEG4000 per 10 ml of crude extract. Using a vortex, the two solutions of each system were mixed for 2 min. The combination can be left for 2-3 h to separate into the two phases by gravity; however, to accelerate the separation of the two phases, centrifugation was performed for 5 min at 5000 rpm. The PEG and salt solutions were in the upper and bottom phases, respectively. The raffinate phase (R_f) is the phase in which the enzyme is lost, and the extraction phase (Ex) is the phase in which the enzyme obtained, and is present in equilibrium following extraction. A Pasteur pipette was used to gently separate the top and bottom phases. The sizes of various phases were measured. Enzyme analysis and protein quantification were performed on the phases. Partition parameters, including recovery percentage, partition coefficient, and specific activity, were computed using Eqs. 1, 2, and 3 below. These equations show that the partition coefficient is the ratio of the enzyme concentration in the extraction phase to the concentration in the raffinate phase after the system reaches equilibrium [38-40].

$$K_P = \frac{C_{Ex}}{C_{Rf}} \quad (1)$$

$$K_E = \frac{A_{Ex}}{A_{Rf}} \quad (2)$$

or

$$K_E = \frac{\% Rec}{1 - \% Rec} \quad (3)$$

Where, K_P is the protein content of the invertase enzyme or the partition coefficient (K_E), C_{Ex} and C_{Rf} are the protein concentrations in mg/ml for the extract and raffinate phase, respectively, and U/ml is the enzyme activity. To determine how well the purification process works, the enzyme's activity, specifically the enzyme-specific activity (SA, in U/mg protein), the recovery percentage, and the enzyme's partition coefficient (K_E) must be measured.

Furthermore, calculations were performed with the provided Eq. 4 [41].

$$\text{Invertase activity } \left(\frac{U}{ml} \right) = \frac{A \times V}{\epsilon \times t \times v} \quad (4)$$

Where: A: absorbency at 540 nm, V: Total volume of reaction mixture in (2 ml), ϵ : Enzyme constant (0.01), t: incubation time (30 min), v: volume of crude used in (0.2 ml).

$$SA = \frac{\text{Invertase activity}}{C_{Rf}} \quad (5)$$

$$\text{Recovery \%} = \left(1 - \frac{A_{Rf}}{A_{in}} \right) \times 100 \quad (6)$$

Where A_{in} is the initial activity of the enzyme in U/ml.

3- Results and discussions

The specific activity of crude extract and protein content were determined to be 10.87 U/mg and 9.44 mg, respectively. The number of experiments was designed by the response surface methodology (RSM) approach, Central Composite Design method, using Minitab V17.0 software, where 4 levels for each parameter were selected. Table 1 and Table 2 display the number of experiments for system-1 (PEG1500/sodium citrate) and system-2 (PEG4000/sodium citrate), respectively, with their parameters as well as the recovery per cent (%Rec) and partition coefficient (K_E) of the invertase enzyme as the response of the experiments. The results show that the %Rec of enzyme preferentially correlated with the low temperature, high pH, and low concentrations of SC and PEG1500 for system-1, where the higher %Rec was 84% corresponding to K_E equal to 5.25 at $T = 10^\circ\text{C}$, $\text{pH} = 5$, and the concentrations of SC and PEG1500 = 1.8 g/10 ml each. The parameters of system-2 have a similar effect on %Rec except for the SC concentration, where %Rec prefers a middle SC concentration. The higher value of %Rec was 89.8% corresponding to K_E equal to 8.8 at $T = 10^\circ\text{C}$, $\text{pH} = 5$, and the concentrations of SC and PEG4000 = 1.8 g/10 ml and 1.5 g/10 ml, respectively.

Table 1. Number of experiments of the system 1 (PEG1500/SC) with their results of %Rec and K_E .

Run No.	T °C	pH	SC, g/10ml	PEG1500, g/10ml	%Rec	K_E
1	17.5	6.25	2.25	1.95	44.8	0.81
2	17.5	6.25	2.25	2.25	42.33	0.73
3	32.5	6.25	1.95	1.95	44.7	0.81
4	17.5	6.25	1.95	1.95	46.42	0.87
5	17.5	8.75	2.25	1.95	68.46	2.17
6	32.5	6.25	2.25	2.25	40.38	0.68
7	25	7.5	2.1	2.1	55.25	1.23
8	17.5	8.75	1.95	2.25	66.97	2.03
9	17.5	8.75	2.25	2.25	65.86	1.93
10	25	7.5	2.1	2.1	54.87	1.22
11	17.5	6.25	1.95	2.25	43.76	0.78
12	32.5	8.75	2.25	1.95	66.82	2.01
13	17.5	8.75	1.95	1.95	69.92	2.32
14	25	7.5	2.1	2.1	55.62	1.25
15	32.5	8.75	1.95	1.95	67.86	2.11
16	32.5	8.75	1.95	2.25	65.43	1.89
17	25	7.5	2.1	2.1	55.2	1.23
18	32.5	6.25	2.25	1.95	43.44	0.77
19	32.5	6.25	1.95	2.25	42.1	0.73
20	32.5	8.75	2.25	2.25	63.21	1.72
21	25	7.5	2.1	2.1	55.7	1.26
22	25	7.5	1.8	2.1	56.42	1.29
23	25	7.5	2.1	1.8	58.3	1.4
24	25	7.5	2.1	2.4	52.87	1.12
25	25	5	2.1	2.1	37.8	0.61
26	25	10	2.1	2.1	78.13	3.57
27	25	7.5	2.4	2.1	54.3	1.19
28	10	7.5	2.1	2.1	57.24	1.34
29	40	7.5	2.1	2.1	52.13	1.09
30	25	7.5	2.1	2.1	55.15	1.23

Table 2. Number of experiments of the system 2 (PEG4000/SC) with their results of %Rec and K_E .

Run No.	T °C	pH	SC, g/10ml	PEG4000, g/10ml	%Rec	K_E
1	17.5	6.25	1.95	1.65	50.1	1
2	17.5	6.25	1.95	1.95	50.7	1.03
3	17.5	6.25	1.65	1.65	51.4	1.06
4	25	7.5	1.8	1.8	59.7	1.48
5	32.5	6.25	1.65	1.95	52.2	1.09
6	32.5	6.25	1.65	1.65	47.6	0.91
7	32.5	8.75	1.65	1.65	65.8	1.92
8	17.5	8.75	1.95	1.65	71.4	2.5
9	17.5	6.25	1.65	1.95	49.1	0.96
10	32.5	8.75	1.95	1.95	67.4	2.07
11	32.5	8.75	1.65	1.95	67.6	2.09
12	25	7.5	1.8	1.8	59.7	1.48
13	25	7.5	1.8	1.8	60.3	1.52
14	32.5	6.25	1.95	1.65	50.8	1.03
15	17.5	8.75	1.65	1.95	71.1	2.46
16	25	7.5	1.8	1.8	60.3	1.52
17	17.5	8.75	1.65	1.65	70.2	2.36
18	17.5	8.75	1.95	1.95	69.7	2.3
19	32.5	8.75	1.95	1.65	66.6	1.99
20	32.5	6.25	1.95	1.95	58.4	1.4
21	25	7.5	1.5	1.8	55.5	1.25
22	25	5	1.8	1.8	49.2	0.97
23	25	7.5	1.8	2.1	58.3	1.4
24	25	7.5	2.1	1.8	55.8	1.26
25	40	7.5	1.8	1.8	56.4	1.29
26	10	7.5	1.8	1.8	61.7	1.61
27	25	7.5	1.8	1.5	60.9	1.56
28	25	7.5	1.8	1.8	59.5	1.47
29	25	7.5	1.8	1.8	60.4	1.53
30	25	10	1.8	1.8	82.8	4.81

3.1. Temperature effect

The experimental results indicate that the temperature has a moderate influence on the %Rec during extraction from tomato via an aqueous two-phase system (ATPS), as shown in Fig. 3, Fig. 5, and Fig. 7 for system-1, and Fig. 4, Fig. 6, and Fig. 9, where the %Rec and then K_E of invertase decrease as the temperature increases from 10 to 40 °C. This phenomenon occurs because heat alters the proteins, particularly invertase. The catalytic activity of invertase depends on a certain 3D structure, and high temperatures cause irreversible denaturation by breaking hydrogen bonds, hydrophobic contacts, and van der Waals forces [42]. Frequently less thermostable than its microbial equivalent plant-derived. At higher temperatures, the enzyme becomes slightly unstable or has decreased solubility, which reduces its ability to migrate to the salt phase. Molecular mobility is reduced at low temperatures, thereby maintaining the enzyme's native shape and increasing yield. Proteolysis during heated extraction might cause invertase yields to decrease by 30-70%. The heat alters proteins, particularly invertase [25]. Because invertase is a glycoprotein in plant sources, such as tomato, with significant hydrophilic surface character due to glycosylation, at higher temperatures, the PEG phase creates stronger hydrophobic interactions with hydrophobic regions on the invertase surface, thereby retaining the enzyme in the upper PEG phase rather than permitting it to move into the salt-rich phase [43]. This improved contrast increases the K_E and, consequently, the yield in a single extraction

step. The interfacial tension between the phases can change at lower temperatures, affecting protein adsorption and partitioning. This can encourage migration into the desired phase for a variety of enzymes, including invertase [44, 45].

The invertase will become less stable if it is heated excessively during its removal from ATPS. These findings demonstrate that low to moderate temperatures are ideal for the extraction of invertase from tomatoes utilizing a PEG-based aqueous two-phase system. These results support earlier studies on plant and microbial invertase, emphasizing the need to maintain an ideal temperature to maintain enzyme activity throughout extraction and purification.

These results are in agreement with Madhusudhan, and Raghavarao [46]; and Kadhim, and Hameed [47].

3.2. pH effect

Fig. 3, Fig. 5, and Fig. 7 for system-1, and Fig. 4, Fig. 6, and Fig. 9 for system-2, show the effect of pH on the %Rec. The effect of pH on system-1 is almost similar to that of system-2 to a large extent. As shown from these figures, the pH has more effect among the other four parameters, where the %Rec and then K_E increase with the increase of pH from 5 to 10, which was indicative of the strong pH- dependence of the enzymatic activity and distribution of the protein surface charge. The lowest recovery of enzyme occurred at an acidic pH of 5.0, suggesting that a higher proton concentration makes the enzyme less stable and less likely to move into the PEG-rich phase.

The reason is that invertase typically has an isoelectric point (PI) around 4.5 – 5, at which its overall net charge is zero, and the protein is often at its least soluble, and electrostatic repulsion is minimal [48]. As pH increased above the PI, the solution is basic, and the protein will have a net negative charge, leading to it being repelled by the slight negative potential of the PEG-phase and is electrostatically attracted toward the salt-rich phase. On the other hand, the PEG phase can behave as a relatively hydrophobic polymer; as invertase becomes more charged (more hydrophilic), its affinity for the PEG phase decreases, and it preferentially partitions into the more polar, salt-rich phase [49]. In the salt-phase, multivalent salt anions (citrate) can interact favorably with positively charged patches still remaining on the enzyme surface, stabilizing it in the salt-phase. These results are in agreement with Yücekan and Önal [30]; Soares et al [50]; and Grilo, et al, [51].

3.3. Effect of sodium acetate concentration

For system-1, the sodium citrate (SC) concentration has little effect on %Rec and then K_E as shown in Fig. 3, Fig. 5, and Fig. 8. Invertase generally has a higher affinity for the more polar, salt-rich environment due to favorable electrostatic interactions and hydrogen bonding as well as the differences in polarity, charge distribution, and specific molecular interactions within the two highly

aqueous environments [52]. This is because the invertase enzyme has a relatively hydrophilic environment, favoring the salt-rich aqueous phase over the PEG, which is a hydrophobic polymer [53]. The salt-rich phase is preferred by the enzyme because PEG tends to exclude proteins, and can be a poorer hydration environment for them, while the salt-rich phase can better maintain protein hydration/solubility and may be favored by charge and ion specific influences under the selected pH and salt conditions. However, the partitioning process preferred the low concentration of SC since the high concentration may cause denaturation or aggregation of enzyme and better structure integrity of the enzyme can be maintained at a lower ionic strength.

For system-2, the effect of SC on the %Rec is shown in Fig. 4, Fig. 6, and Fig. 10. The results show that the %Rec increases with increasing SC concentration up to 1.8 g/10 ml, then decreases with increasing SC, i.e., the moderate concentration is favorable. The interpretation of this phenomenon is that the low molecular weight of PEG forms a less hydrophobic phase, requiring minimal salt to induce phase separation. When the molecular weight of PEG increases, it will create a more hydrophobic phase, needing higher salt concentrations to achieve phase separation due to reduced excluded volume effects [54].

The previous studies show that the recovery of invertase from plants by ATPS is preferred at low to moderate concentrations of salt [30, 55].

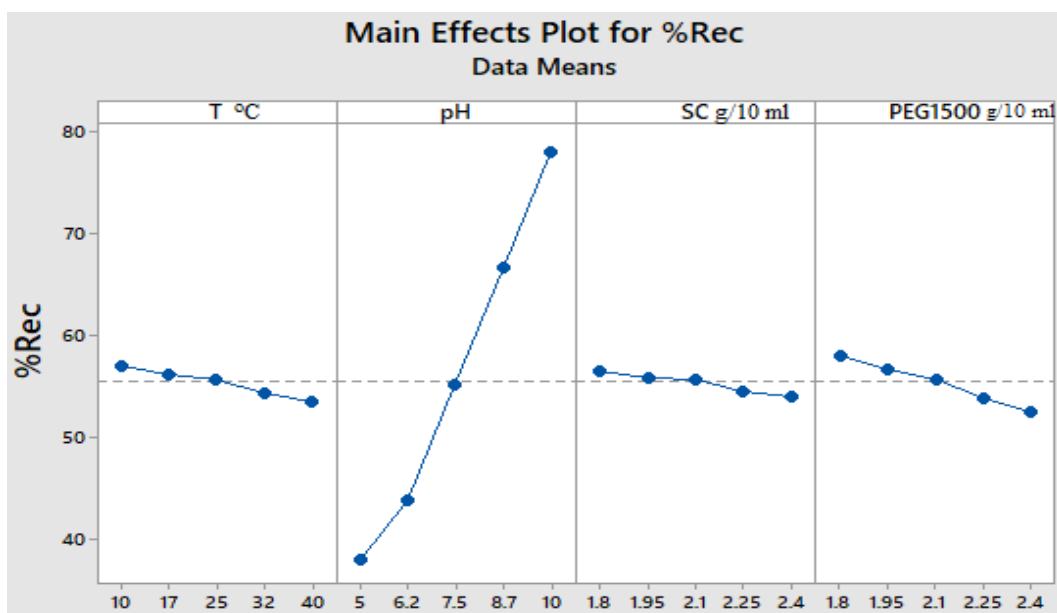


Fig. 3. Effect of temperature, pH, concentrations of PEG1500 and SC on the %Rec for the system-1

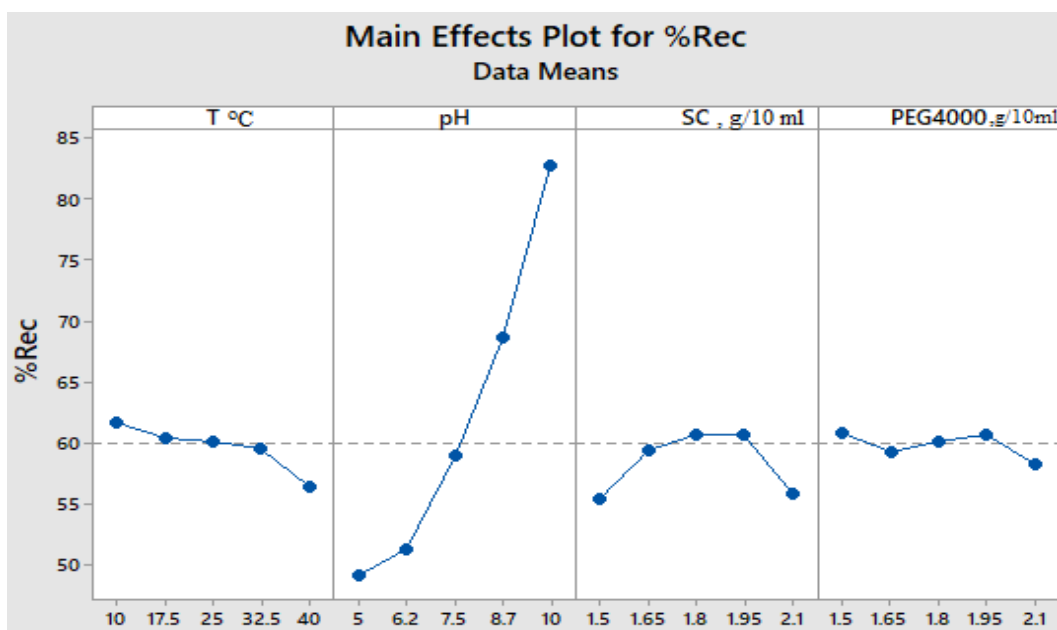


Fig. 4. Effect of temperature, pH, concentrations of PEG4000 and SC on the %Rec for the system-2

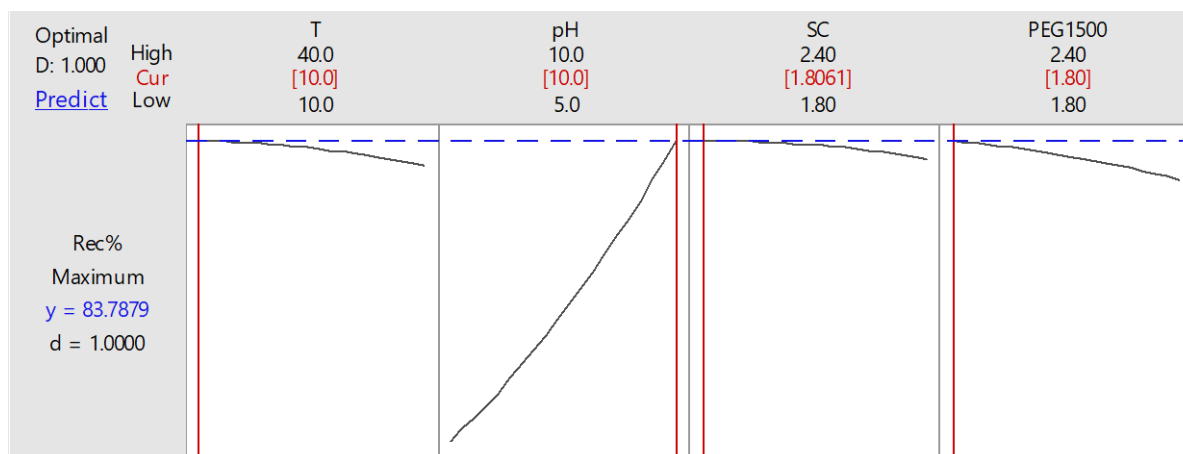


Fig. 5. Optimum operating conditions of system-1 (PEG1500/SC) that give maximum %Rec

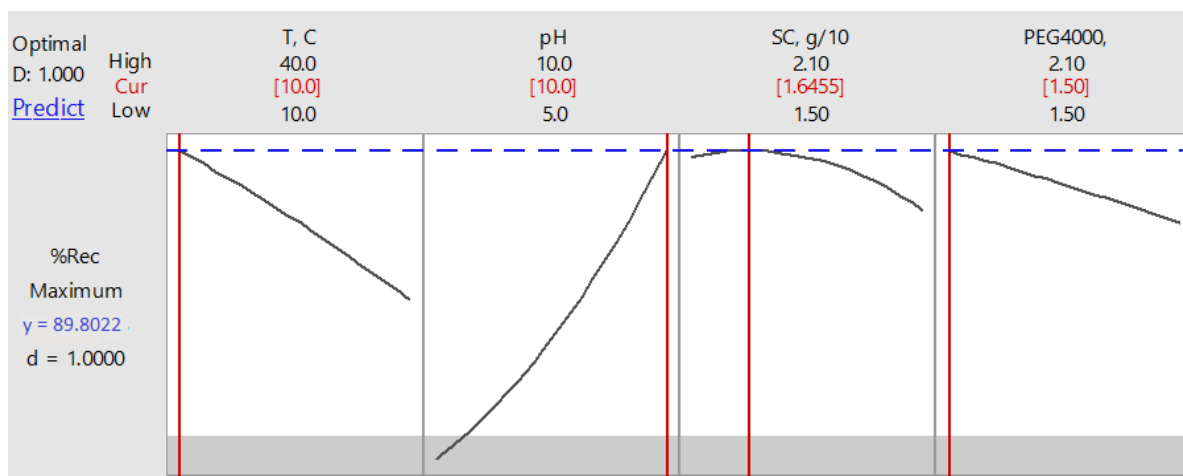


Fig. 6. Optimum operating conditions of system-2 (PEG4000/SC) that give maximum %Rec

3.4. Effect of PEG concentration

The concentration of polyethylene glycol (PEG) in an aqueous two-phase system significantly influences the recovery efficiency of invertase from tomato. For system-1, where the molecular weight of PEG is 1500, the experimental results show that the enzyme can be quickly and stably transported into the salt-rich phase at low PEG concentrations (1.8 g/10 ml) as shown in Fig. 3, Fig. 5 and Fig. 8. This is because the PEG concentration influences the physicochemical characteristics of ATPS. Higher concentrations may affect protein partitioning because they usually increase the density and viscosity of the PEG phase. Invertase may partition less at higher PEG concentrations due to increased hydrophobicity of the top phase (e.g., denaturation, aggregation, and unfavorable interactions) [56]. Invertase can maintain stability and partition more successfully into the intended phase when PEG concentrations are lower because they lessen these effects and enhance mass transfer [57]. Because of the severe gradients and shear stresses at the interface, the PEG concentration can denature the invertase enzyme. Invertase does not require a powerful driving force because it is already hydrophilic. A lower PEG concentration is sufficient for equilibrium to accomplish phase separation and transfer invertase into the salt phase.

This lower concentration also reduces viscosity, lowering the amount of time the enzyme is exposed to the denaturing surfaces.

In system-2, where the molecular weight of PEG is 4000, the requirement of PEG is lower to obtain the two-phase. Fig. 4, Fig. 6, and Fig. 10 show the effect of PEG4000 concentration on the %Rec of invertase. In this system, the PEG concentration has various and unclear effects on the %Rec, but the better %Rec is obtained at low concentrations of PEG also as system-1. Previous researches have demonstrated analogous trends, indicating that moderate PEG concentrations enhance the stability and separation efficiency of tomato invertase. It is important to carefully control the PEG content during ATPS extraction to get a lot of products and keep the enzyme's functional integrity [57].

3.5. Effect of neutral salts

To improve the efficiency of recovery of the invertase enzyme from tomato, it can add neutral salts to the total mixture of ATPS [58]. In this research sodium chloride (NaCl) and magnesium chloride (MgCl₂) were used, and the study of these two salts was considered in the system-2, which showed better results than system-1. Fig. 11 shows the effect of NaCl and MgCl₂ in the concentration

range (0.25 – 1.5 g/10 ml) on the %Rec. The concentration ranges of these salts based on previous studies that extract the biomaterials [59]. Adding these salts up to 1 g/10 ml increased the %Rec from 89.3 to 95.8 and from 89.4 to 96.7% for the NaCl and MgCl₂ respectively. This improvement by adding the neutral salts causes an increase the ionic strength of the system, and creates a small but significant electrical potential difference between the PEG-phase and the SC salt-phase which is called the interfacial potential, and this enhances better separation between the two phases [60]. Also, these neutral salt can modify the electrostatic interactions between the enzyme and the components of the ATPS [61]. MgCl₂ has divalent cations, perhaps having a more pronounced effect on the electrostatic shielding, obtaining better separation of enzyme compared with NaCl which is a monovalent salt.

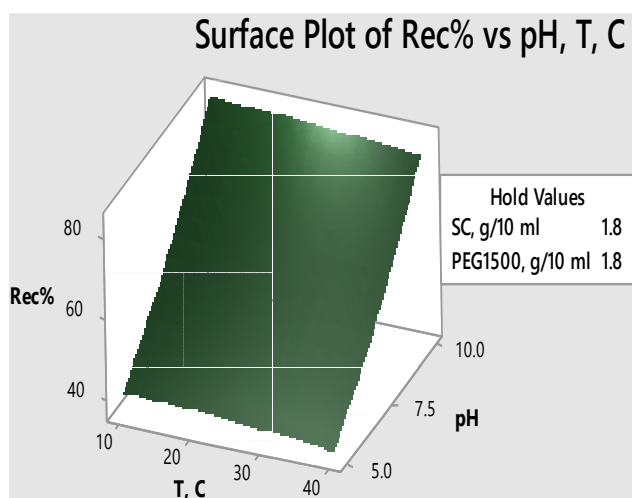


Fig. 7. Effect of temperature and pH on the %Rec for the system-1 at PEG1500 and SC 1.8 g/10 ml each

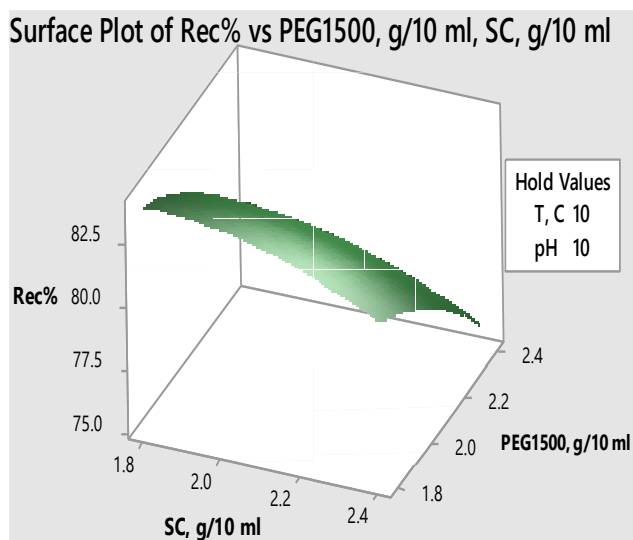


Fig. 8. Concentration effect of PEG1500 and SC on the %Rec for the system 1 at T = 10 °C and pH 10, in 3D plot

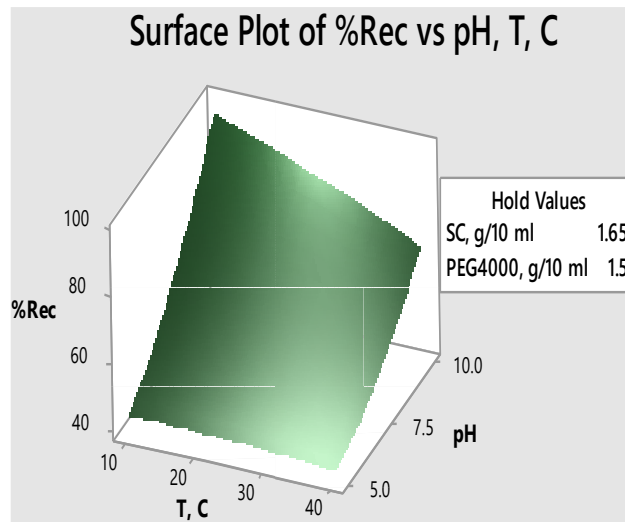


Fig. 9. Effect of temperature and pH on the %Rec for the system-2 at PEG4000 1.95 g/10 ml, and SC 1.8 g/10 ml

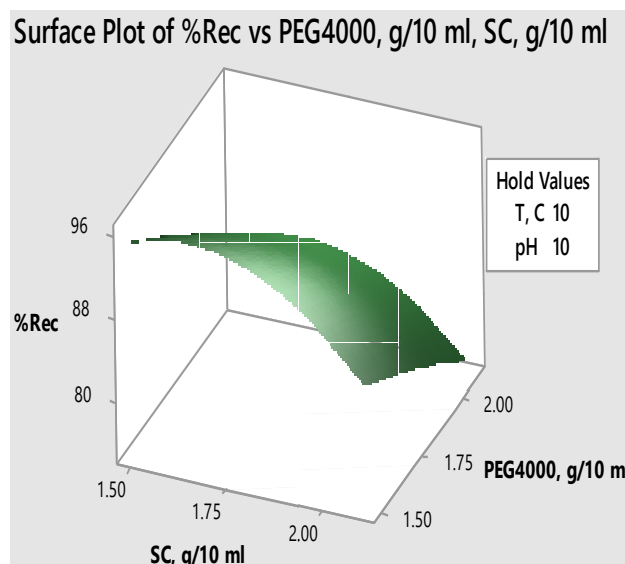


Fig. 10. Concentration Effect of PEG4000 and SC on the %Rec for the system 2 at T = 10 °C and pH 10

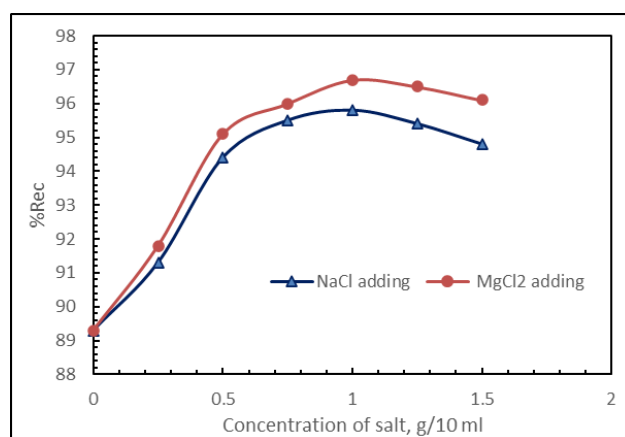


Fig. 11. Effect of adding of neutral salts to ATPS of system-2 on the %Rec at optimum operating conditions of T = 10 °C, pH 10, and concentrations of SC and PEG4000 1.8 and 1.5 g/10 ml, respectively

4- Conclusion

The results of this research showed the effectiveness of ATPS, type (PEG/salt) in the recovery of the invertase enzyme from tomato fruit. The enzyme preferred the salt-rich phase over PEG phase resulting in high recovery. In contrast to harsher separation techniques, the ATPS approach offers simultaneous extraction and partial purification in a moderate, aqueous environment, lowering the danger of enzyme denaturation. System-2 (PEG4000/SC) was slightly more efficient than system-1 (PEG1500/SC). The optimum operating conditions of system-1 were at a temperature of 10 °C, pH of 10, and concentrations of PEG1500 and SC of 1.8 g/10 ml each, where the maximum values of %Rec and K_E reached 84% and 5.25, respectively, whereas the maximum values of %Rec and K_E for system-2 were 89.8% and 8.34, respectively, at optimum operating conditions of a temperature, pH, and concentrations of PEG4000 and SC of 10 °C, 10, and 1.5 and 1.65 g/10 ml, respectively. Adding the supporting agent (NaCl or $MgCl_2$) to the ATPS mixture raised the possibility of enzyme recovery, as the $MgCl_2$ provided more support than NaCl.

Nomenclature

Symbol	Definition	SI Units
A	Absorbency at 540 nm	nm
C_{ex}	Protein concentrations of the extract phase	mg/ml
C_{Rf}	Protein concentrations of the raffinate phase	mg/ml
E_x	Extraction phase	
K_E	Partition coefficient	
K_p	Protein content of the invertase enzyme	
R_f	Raffinate phase	
SA	Enzyme-specific activity	U/mg
t	Incubation time	min
V	Total volume of reaction mixture in (2 ml)	ml
v	Volume of crude used in (0.2 ml)	ml
ϵ	Enzyme constant (0.01)	

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فصل انزيم الأنفرتيز من الطماسة باستخدام نظام ثنائي الطور المائي: (بولي إثيلين كلايكول ١٥٠٠ / صوديوم ستريت) و (بولي إثيلين كلايكول ٤٠٠٠ / صوديوم ستريت)

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الخلاصة

يُعدّ نظام الطورين المائيين (ATPS) تقنيةً فعّالةً وبسيطةً واقتصاديةً لاستخلاص الجزيئات الحيوية. يُحضر نظام ATPS بمزج بوليمرين أو بوليمر وملح بتركيز أعلى من تركيزهما الحرج في الماء. يُعدّ إنزيم الأنفرتيز إنزيمًا يُحلّل السكرز إلى جلوكوز وفركتوز، مما يجعله بالغ الأهمية في صناعة الأغذية. ونظرًا لإمكانية تطبيق نظام الطورين المائيين في ظروف معتدلة، وفي طور غني بالماء، وتوافقه الحيوي مع المنتجات الحيوية، فقد تم استخلاص إنزيم الأنفرتاز من لب الطمطم باستخدام هذه الطريقة. وُجد أن النشاط النوعي للإنزيم وتركيز البروتين يبلغان ١٠,٨٧ وحدة/ملغ و ٩,٤٤ ملغ/مل على التوالي. في هذا البحث، استُخدم نظامان من أنظمة الطورين المائيين: بولي إثيلين جليكول ١٥٠٠/سترات الصوديوم (PEG1500/SC) وبولي إثيلين جليكول ٤٠٠٠/سترات الصوديوم (PEG4000/SC). دُرست خمسة عوامل للحصول على أقصى كمية من إنزيم الأنفرتيز من الطمطم: درجة الحرارة، ودرجة الحموضة، وتركيزات بولي إثيلين جليكول والملح، والملح المتعادل الداعم (كلوريد الصوديوم وكلوريد المغنيسيوم). بلغت أعلى نسبة استخلاص (%Rec) للإنزيم الأنفرتاز ومعامل التوزيع (K_E) الخاص به ٨٤% و ٥,٢٥ على التوالي، عند درجة حرارة ١٠ درجة مئوية، ودرجة حموضة ١٠، وتركيزات PEG1500 و SC تبلغ ١,٨ جم/١٠ مل لكل منهما للنظام ١ (PEG1500/SC) دون إضافة ملح متعادل داعم، أما بالنسبة للنظام ٢ (PEG4000/SC)، فكانت ظروف التشغيل المثلى عند درجة حرارة ١٠ درجة مئوية، ودرجة حموضة ١٠، وتركيزات PEG4000 و SC تبلغ ١,٦٥ جم/١٠ مل و ١,٥ جم/١٠ مل على التوالي، حيث بلغت أعلى نسبة استخلاص (%Rec) ومعامل التوزيع (K_E) ٨٩,٨% و ٨,٨ على التوالي، دون إضافة ملح متعادل داعم. نظرًا لأن النظام ٢ يعطي نتائج أفضل من النظام ١، فقد أخذ بنظر الاعتبار لتحسين استعادة الإنزيم عن طريق إضافة NaCl أو $MgCl_2$ كملاح محايدة داعم، حيث زادت نسبة الاستعادة إلى ٩٥,٨% و ٩٦,٧% بعد إضافة NaCl و $MgCl_2$ على التوالي.

الكلمات الدالة: نظام ثنائي الطور مائي، إنزيمات، مصدر نباتي، استخلاص.