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Application of Aqueous Two-Phase System in the Extraction of Invertase Enzyme from Potato Tubers Using PEG8000/Potassium Phosphate

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Abstract

An aqueous two-phase system (ATPS) is a clean alternative for traditional aqueous-organic solvent extraction systems. This technique has proven to be highly effective for extracting and separating mixtures of biomolecules. The present investigation involved the extraction of the invertase enzyme from potato tubers utilizing ATPS. The system applied was: Polyethylene glycol 8000 combined with dipotassium diphosphate (PEG8000/PPH). The study investigated the impact of five factors, namely temperature, PEG8000 concentration, dipotassium phosphate concentration, pH, and the addition of sodium chloride (NaCl) or magnesium sulphate (MgSO4) as a catalyst, on the recovery percentage (%Rec) and partition coefficient (KE) of the invertase of the invertase enzyme in ATPS over the study period. The system achieved a maximum recovery (%Rec) of 87.52% and a maximum partition coefficient (KE) of 7.01 at a temperature of 10°C, with a PEG8000 concentration of 1.5 g/10 ml, a dipotassium phosphate concentration of 2.4 g/10 ml, and a pH of 10. After the addition of neutral salts, the system achieved a maximum %Rec of 91.9% and a maximum KE of 11.34 under the same optimal conditions, and a concentration of MgSO4 of 1 g/10 ml.

Keywords: *Aqueous two-phase system; Enzymes; plant source; Potato tuber.*

1. Introduction

Certain features of bioproducts are lost during their extraction from downstream utilizing commercial techniques like liquid-liquid extraction (also known as the aqueous-organic system). The use of an aqueous two-phase system (ATPS) is an environmentally friendly and bioproduct-safe alternative [1], [2]. The ATPS was accidentally discovered by Martinus Willem Beijerinck in 1896, but its practical application was not implemented until the 1970s [1], [2]. In recent years, interest in ATPS systems and their research has become broader. They include several types of chemicals, such as polymer-polymer or polymer salt systems that can be combined, to perform several functions [3]. This process is preferred over alternative extraction processes due to its ability to bear the cost, its suitability to the environment, and its versatility in extracting a wide range of compounds [3], [4]. Water contributes to the stabilization and separation of biomolecular structures in the two phases of ATPS [5], [6], [8], while other liquid-liquid extraction processes can cause damage to biological products due to harsh process conditions and the use of organic solvents [4], [5]. ATPS is a combination of two distinct materials. They may include either a salt and a polymer, such as dipotassium phosphate (K_2HPO_4) and PEG, or two polymers, such as PEG and dextran. In order to form two phases, the polymer/salt concentration must be

higher than the critical concentration, below which the two phases cannot be achieved at lower concentrations. Critical concentrations depend on the molecular weight of the polymer and the type of salt [5], [7]. However, there are biphasic systems, consisting of short-chain alcohols and ionic liquids [7], [9], [10]. Researchers have examined several forms of ATPS to understand and analyze their ability to separate, eliminate, and disinfect molecules and biomolecules [9]. Although ATPS have many advantages, there is complexity in the partitioning of biomolecules in these systems, making it difficult to predict their behavior. In addition, the interaction between the system and biomolecular factors, such as pH and temperature, is of great importance [11], [12].

The enzyme targeted in this research is called invertase, also called beta-fructofuranosidase, which is a glycoprotein whose function is to break down the end parts of beta-fructofuranoside molecules [13]. It is one of the important enzymes in food processing and is responsible for the convert of sucrose by producing D-fructose and D-glucose. It operates best at pH 4.5 and is stable at 50°C [13]. Invertase is often used in the food industry to convert sucrose into fructose syrup. It is also used in the manufacture of sucrose biosensors and in the paper, pharmaceutical and cosmetic industries [14, 15]. Many plants, including potato, carrot, tomato, tobacco, sugarcane, and bamboo, are major plant sources of invertase production [16], [17]. In addition, there is research showing the presence of invertase in several yeasts, including Saccharomyces cerevisiae [20], Candida utilis [21], Pichia anomola [22], and a few fungi, mostly Aspergillus niger and Neurospora sp. [23] – [25]. The ideal pH for acidic invertase is usually between 3.5-5.0, while the alkaline pH is 7.0-8.0 [18], [19]. Fresh potatoes contain high invertase activity, which has been shown to increase during cold storage [26], suggesting that potato tubers are a useful source for research into invertase characterization and purification. Although various methods exist to purify invertase from different plant sources, these techniques are considered time-consuming and costly [26] – [29]. Albertson invented it in the early 1950s to distinguish amongst proteins, nucleic acids, and cells [30]. This technology has advantages such as efficiency, provides rapid separation with little denaturation, high mass transfer, specific partitioning, and low cost. As a result, it finds application in various biotechnology sectors [31], [32].

The aim of this study is to determine the optimal operating parameters for extracting the invertase enzyme from native Iraqi potato tubers utilizing the Taguchi method as an experimental design.

The factors studied that effected on the extraction of invertase from potatoes were: temperature (10 – 40 °C), pH (8 – 10), concentration of potassium phosphate and PEG8000 (1.5 – 2.4 g/10 ml), and concentration of the support NaCl or MgSO4 (0 – 2 g/10 ml).

2. Materials and Experimental Work

2.1. Materials

Dipotassium phosphate (K_2HPO_4), copper sulphate ($CuSO_4$), and sodium carbonate (Na_2CO_3) were obtained from Central Drug House Ltd., India, and sodium chloride (NaCl) was supplied from Alpha Chemika, India. Sucrose and Polyethylene Glycol (PEG8000), 3,5-dinitrisalicylic acid (DNS), sodium hydroxide (NaOH) and bovine serum albumin (BSA) were purchased from HIMEDIA Laboratories Pvt. Ltd., India. Folin reagent from Sisco Research Laboratories Pvt. Ltd. (Srl) in India. As for potatoes, they were purchased from local markets in Iraq. High-quality reagents were used in the analysis. Deionized and double-distilled water, which was obtained from the laboratories of the Department of Biochemical Engineering, was used in this study.

2.2. Experimental Work

2.2.1. Crude Extract Preparation

Fresh potatoes were cleaned with distilled water after removing their peels well. After that, 20 grams of potatoes were weighed, cut into small pieces, and mixed in 50 millimoles of cold citrate buffer with a pH of 4 for one minute at a temperature of 4 degrees Celsius. The mixture was filtered through five layers of cheesecloth, and the remaining solids were separated by centrifugation at 5000 rpm for ten minutes at 4°C. The supernatant layer was taken and considered as the crude enzyme extract. The specific activity of the crude extract was checked and it was 20.11 units/mg. Figure 1 shows the experimental procedure that was performed in the laboratory.

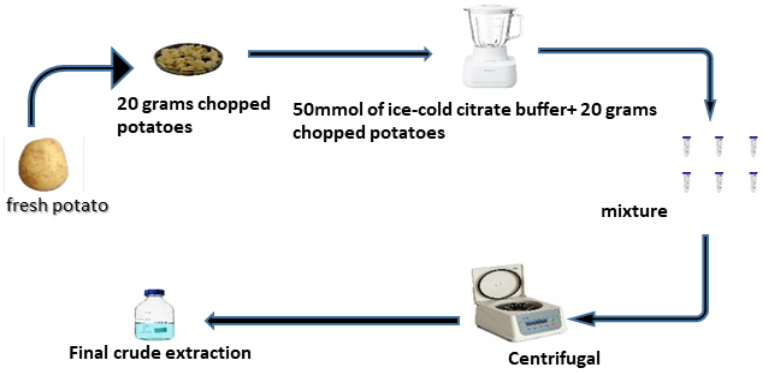

Fig.1. Experimental setup Lab work progress.

2.2.2. Aqueous Two-Phase Partitioning

From the experimental study, it was found that the dipotassium phosphate should be mixed with the crude extract to obtain a greater extraction rate, so the system consists of dipotassium phosphate stock solutions (1.5-2.4 g) per 10 ml of crude extract and (1.5-2.4 g) of PEG8000 per 10 ml of distilled water. The two solutions were mixed for two minutes and using a vortex. To obtain the two phases, the mixture can be left for 2-3 hours under the influence of the force of gravity, but for speed, the mixture was separated into two phases using centrifugation at 6000 rpm for 10 minutes. The upper phase was the PEG8000 solution, and correspondingly the lower phase was the salt solution. After extraction, in equilibrium, there was the raffinate phase (Rf), which is called the phase that loses the enzyme, while the phase that gains the enzyme is called the extraction phase (Ex). On the basis of previous studies, the selected system parameters were temperature, pH, concentrations of polymer (PEG800), and dipotassium phosphate [33] – [37]. The upper phase was carefully isolated from the lower phase using a Pasteur pipette. Measurements of the sizes of distinct phases were taken. Portions of the phases were examined for protein quantification and enzyme analysis. Partition parameters, such as partition coefficient, recovery percent, and specific activity, were calculated using formulas derived from previous studies [38] – [40]. Protein content (K_P) or partition coefficient (K_E) is the definition of the invertase partition coefficient in two-phase aqueous systems. This coefficient indicates the ratio of the enzyme concentration in the extraction phase to the ratio of the concentration of the raffinate phase when the system is in equilibrium, as shown in Eqs. 1, 2 and 3 [41].

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

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

$$\text{OR} \quad K_E = \frac{\%\,Rec}{1-\%\,Rec}, \quad \ldots(3)$$

Where C_{Ex} and C_{Rf} are the total protein concentrations in mg/ml of the extract and raffinate phases respectively,

A_{Ex} and A_{Rf} are the enzyme activities in U/ml of the extract and raffinate phases, respectively.

In order to assess the efficacy of the purification process, it is necessary to measure the activity of the enzyme, specifically referred to as the enzyme-specific activity (SA, expressed in U/mg protein), recovery % and partition coefficient of enzyme (K_E), additionally, the calculations were performed based on the provided equations [42].

$$\text{Invertase activity (U/ml)} = \frac{A \times V}{€ \times t \times v}, \quad \ldots(4)$$

Where:

A: absorbency at 540 nm

V: Total volume of reaction mixture in (2 ml)

€: Enzyme constant (0.01)

t: incubation time (30 min)

v: volume of crude used in (0.2 ml)

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

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

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

2.2.3. Determining Invertase activity

Miller's method for determining invertase activity in plants was described by Miller in 1959. The assay mixture used in this method includes 0.8 ml of substrate, 50 mmole sucrose in citrate buffer with a pH of 4.5, and 0.2 ml of enzyme diluted to the desired concentration. Then, at 37°C, the mixture was incubated for 30 minutes. Next, the reaction was terminated by adding 1 ml of DNS reagent (3,5-dinitrosalicylic acid) and heating for five minutes. The solution was then cooled to ambient temperature using an ice bath and the amount of reducing sugars was measured using

spectrophotometry at a wavelength of 540 nm [43]. One unit of invertase activity is defined as the amount of enzyme that converts 1 micromole of sucrose into glucose within one minute, at a temperature of 37°C and a pH of 4.5.

2.2.4. Protein Concentration Determination

The Lowry method was applied to calculate protein concentration [40], including the solutions that were used to determine the protein:

- Solution (1): 2% Na_2CO_3
  In 500 ml of 0.1M NaOH, 10 g of Na_2CO_3 were dissolved.
- Solution (2): 2% Sodium Potassium tartrate
  In a small amount of distilled water (e.g. 20 ml), 2 g of sodium and potassium tartrate were dissolved, then the volume was completed to 100 ml by distilled water.
- Solution (3): 1% $CuSO_4$
  1 g of $CuSO_4$ was dissolved in 100 ml of distilled water.
- Solution (4):
  It was prepared immediately by mixing 98 ml of solution (1) with 1 ml of solution (2) and 1 ml of solution (3).
- Solution (5): Bovine Serum Albumin (BSA)
  The solution was prepared by gradually dissolving 0.01 g of BSA in distilled water and then adjusting the volume to 100 ml using distilled water.
- Solution (6): Folin-reagent
  1 ml of Folin was diluted in 2 ml of distilled water (1:2 v/v).

By Lowry's method the protein concentration was determined [40]. The solutions that had been prepared previously were used and the protein was determined as follows: From the original concentration, the required concentration of BSA 100 µg/ml was prepared. To each tube 4.0 ml of solution 4 was added, then left for 10 minutes. Then, 0.4 ml of folin reagent (solution 6) was added to each tube, shaken well, and then left for 30 minutes. The absorbance was recorded at 600 nm, and tube 1 was used as the blank tube. Figure 2 shows the standard curve of BSA using the Lowry method.

3. Results and Discussions

The protein content and specific activity of the crude extract were measured as 5.11 mg and 20.11 U/mg, respectively.

The Minitab V17.0 was used to design the set of experiments using the Taguchi method, where each parameter had four levels. The study initially focuses on studying the effect of four factors: temperature, pH, and the concentration of PEG8000 and K_2HPO_4 (PPH). After establishing the ideal conditions that produce the highest extraction rate, (NaCl or $MgSO_4$), which are natural salts, were introduced to monitor their effect. Tables 1 shows the number of experiments with the parameters and the results of recovery percent (%Rec) and partition coefficient (K_E).

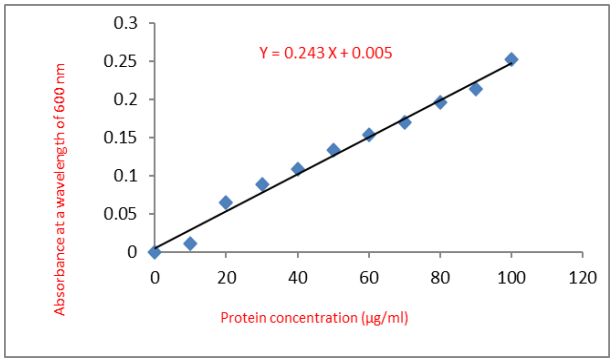

Fig. 2. BSA Standard curve

3.1. Temperature Impact

As the temperature increases, enzymes undergo changes in their conformation, stability, and conformation. Therefore, the temperature was set between 10 – 40oC. Next, by dividing the enzyme activity at different temperatures by the total enzyme activity and then multiplying the result by 100, the relative activity percentage was calculated. The effect of temperature on the invertase extraction rate as well as its partition coefficient was studied as shown in Figures 3 and 4a. Without adding the neutral salts (NaCl or $MgSO_4$), it is clear that the percentage of %Rec and KE recorded the lowest percentage at 40°C, where it reached 71.4% and 2.49, respectively, and they increased between 10 and 30 degrees Celsius, where the highest values were recorded, which were 75.4% and 3.06 for %Rec and KE, respectively. The effect of temperature on the compositional behavior is complicated by the interaction between its component parts, namely electrostatic and hydrophobic forces. The results show a positive comparison with previous reports. Typically, invertase derived from different plants show the highest level of activity at 37 °C [44] – [46]. Studies on the effect of temperature on invertase activity and stability have shown that these properties are strongly influenced by the location of the enzyme and the temperature. For example, soluble invertase

extracted from wheat germ demonstrated maximum activity at 37 ºC and maintained 60% of its activity after exposure to 50 ºC for 4 minutes [47]. Furthermore, Singh and Knox (1984) reported notable differences in the thermal stability of different forms of plant invertase [45].

Table 1,
Number of experiments of the system (PEG8000/PPH) according to Taguchi method with their results of %Rec and K_E.

No.	Temp ºC	pH	K_2HPO_4 (PPH) g/10 ml	PEG8000 g/10 ml	% Rec	K_E
1	10	8	1.5	1.5	74.68	2.94
2	10	8.7	1.8	1.8	72.94	2.69
3	10	9.4	2.1	2.1	74	2.84
4	10	10	2.4	2.4	79.58	3.89
5	20	8	1.8	2.1	66.29	1.96
6	20	8.7	1.5	2.4	68.19	2.14
7	20	9.4	2.4	1.5	83.38	5.01
8	20	10	2.1	1.8	76.58	3.26
9	30	8	2.1	2.4	72.46	2.63
10	30	8.7	2.4	2.1	70.88	2.43
11	30	9.4	1.5	1.8	71.51	2.51
12	30	10	1.8	1.5	85.91	6.09
13	40	8	2.4	1.8	74.99	2.99
14	40	8.7	2.1	1.5	71.67	2.52
15	40	9.4	1.8	2.4	69.93	2.32
16	40	10	1.5	2.1	68.35	2.15

3.2. pH Impact

Using hydrochloric acid or sodium hydroxide the pH was adjusted. In many industrial applications, pH stability is another important measure when selecting enzymes as biocatalysts. The pH value range is determined from 8.0 to 10.0 for the PEG8000/K_2HPO_4 system at room temperature. In Figures 3 and 4a, the effect of pH changing from 8 - 10 recorded increasing in a %Rec in the range (72.2% - 77.8%), which indicates that more enzymes will be recovered from the crude extract. The lowest percentages recorded for %Rec and KE were 70.9% and 2.43, respectively, at pH 8.7. These results are consistent with those of [48] – [50]. To interfere this phenomenon, in general, bioproducts can be divided into two phases based on the differences in their charges and surface characteristics. The aqueous solution's pH can cause this to alter. An electric force is created when protein molecules with a positive net charge attempt to yank electrons from other proteins. However, the electric force is reduced and the proteins obtain a net negative charge when the pH is higher than the pH-indicator or potential Isoelectric point (PI); otherwise, it becomes positive. No net charge is realized when the pH and pI values are the same [51].

3.3. Impact of Potassium Phosphate Concentration

In a two-phase system, the concentration of potassium phosphate salt (PPH) used is an important factor that greatly affects enzyme partitioning. From experience, the amount of PPH in the solution has an effect on the amount of enzyme extracted into the ATPS and how strongly it breaks down the ATPS. According to [49], [50], researchers found that in the PEG/salt system the recovery rate and partition coefficient increase with increasing salt concentration. When examining Figures 3 and 4b, it is clear that the high salt content concentration, enhances the extraction process, and increases the partition coefficient. An increasing the salt concentration from 1.5 g/10 mL to 2.4 g/10 mL resulted in an increase in %Rec of enzyme from 70.8% to 77% and thus an increase in partition coefficient from 2.42 to 3.34, as shown in Figures 3 and 4.

3.4. PEG Concentration Impact

The concentration of PEG8000 was further studied in terms of its ability to form the phase. This research revealed that this concentration significantly affects the rate and regularity of the invertase enzyme partitioning, influencing the amount of recoverable invertase as shown in Figure 3 and 4b. When the concentration increased from 1.5 g/10 ml to 2.1 g/10 ml, %Rec of the invertase enzyme decreased from 79% to 72.4%. These results are consistent with [52], suggesting that an increase in PEG8000 concentration decreases %Rec. The proteins in the ATPS migrate from the lower phase to the upper phase, automatically increasing the partition coefficient. The polymer and enzyme interact differently; occasionally, the contact is more hydrophobic, allowing the proteins to separate more readily. The protein's ability to recover is hindered and the partition coefficient (K_E) is significantly reduced when the PEG concentration is elevated [53].

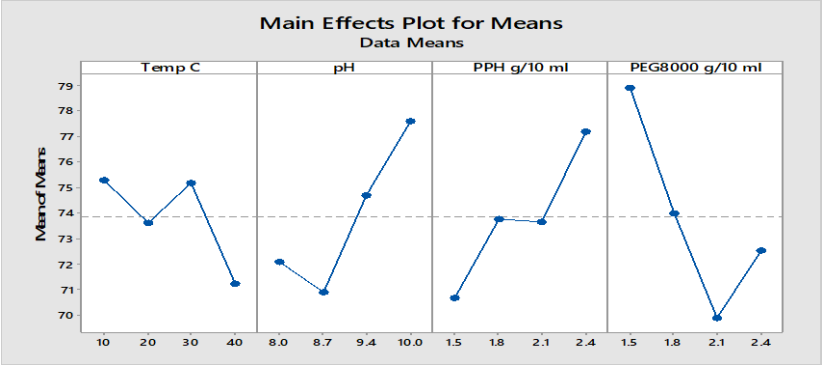

Fig. 3. Impact of (temperature, pH, concentrations of PEG8000 and K_2HPO_4) on the %Rec.

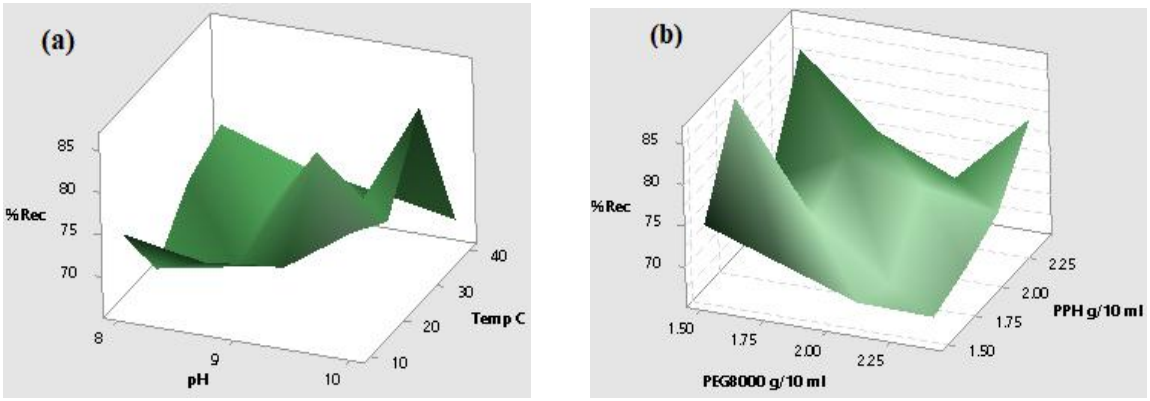

Fig. 4. Effect the parameters on the %Rec of the invertase enzyme in 3 dimensions. (a): effect of temperature and pH; (b) Concentration effect of PPH salt and PEG8000.

3.5. Impact of NaCl and $MgSO_4$

As the last step, supporting agents were added to show their effect and to enhance protein partitioning and its recovery rate. The hydrophobic phase contains proteins with a higher concentration of hydrophobic anions or cations, and the opposite is also true [49]. Figure 5 shows the effect of adding of two different types of NaCl and $MgSO_4$ salts. These results are obtained under optimal conditions: temperature = 10 ºC, pH 10, PEG8000 concentration = 2.4 g/ 10 ml, and K_2HPO_4 concentration = 2.4 g/10 ml. These salts enhance the separation process due to differing hydrophobicity of their ions. One phase preferentially concentrates the more hydrophobicity ions, whereas another phase accumulates less hydrophobic ions [54], [55]. Figure 5 clearly illustrates that the %Rec and then K_E varies greatly with the addition of NaCl and $MgSO_4$. The %Rec increases from 87 to 89.4% for NaCl and from 87.2 to 91.9% for $MgSO_4$ as the salt concentration increases from 0 to 1 g/10 ml. Beyond this point, %Rec and then K_E begin to decline as the

salt concentration continue to increase. The results obtained are consistent with those of [56], [57].

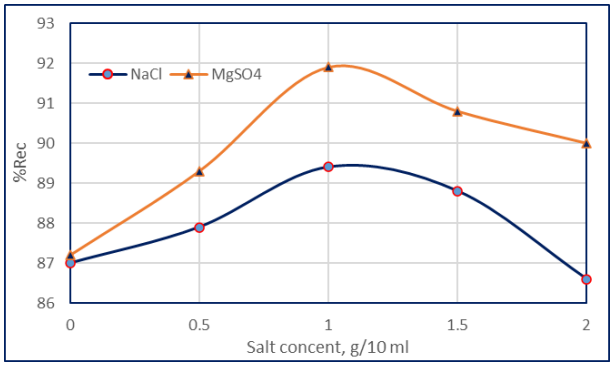

Fig. 5. Impact concentration of NaCl and MgSO4 on the %Rec and KE.

4. Conclusions

Through the results obtained, the system was found to be highly effective in purifying and extracting invertase when preparing both PEG8000 (1.5 g/10 mL) and K_2HPO_4 (2.4 g/10 mL) at pH 10 and a temperature of 10 ºC. In addition, the inclusion of $MgSO_4$ at a concentration of 1 g/10 ml further enhances the process. ATPS offer a cost-effective, safe, and straightforward method for protein purification, demonstrating superior efficiency to other conventional techniques. The distribution of invertase in PEG8000/PPH system demonstrated that the plant enzyme can be extracted to the concentrated phase with the salt at the lower phase. This finding indicates that PEG concentration, type and concentration of salt, temperature, pH, and the use of natural salts as catalysts influenced the partitioning of invertase significantly. As a result, the enzyme recovery rate and partition coefficient were 91.9% and 11.34, respectively. An extremely high %Rec suggests that no more than one stage is required for efficacy recovery

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تطبيق النظام المائي ثنائي الطور في استخلاص إنزيم الإنفرتيز من درنات البطاطس باستخدام فوسفات البوتاسيوم/PEG8000

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المستخلص

يعد النظام المائي ثنائي الطور (ATPS) بديلاً نظيفًا لأنظمة استخلاص مذيبات المياه العضوية التقليدية. أثبتت هذه التقنية فعاليتها العالية في استخلاص وفصل مخاليط الجزيئات الحيوية. تضمن البحث الحالي استخلاص إنزيم الإنفرتيز من درنات البطاطس باستخدام ATPS النظام المطبق: هوالبولي إيثيلين جلايكول ٨٠٠٠ مع ثنائي فوسفات البوتاسيوم . (PEG8000/PPH) بحثتُ في هذه الدراسة تأثير خمسة عوامل:هي درجة الحرارة، وتركيزPEG8000 ، وتركيز فوسفات ثنائي البوتاسيوم، والأس الهيدروجيني، وإضافة كلوريد الصوديوم (NaCl) أو كبريتات المغنيسيوم (MgSO4) كمحفزعلى نسبة الاسترداد (Rec%)، ومعامل الفصل (KE) لإنزيم الإنفرتيز في ATPS خلال فترة الدراسة حقق النظام أعلى قدر من الاسترجاع (Rec%) بنسبة ٨٧,٥٢٪ وأعلى معامل فصل (KE) قدره ٧,٠١ عند درجة حرارة ١٠ درجات مئوية، مع تركيز PEG8000 قدره ١,٥ جم/١٠ مل، وتركيز فوسفات ثنائي البوتاسيوم ٢,٤ جم/ ١٠ مل، ودرجة حموضة ١٠. بعد إضافة الأملاح المحايدة، حقق النظام أعلى نسبة استرداد Rec% وقدره ٩١,٩٪ وأعلى قيمة لمعامل الفصل KE وقدره ١١,٣٤ في ظل نفس الظروف المثالية، وتركيز MgSO4 قدره ١ جم/١٠ مل.