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# Sustainable Recovery of Plant Lipase From Pequi Peel (*caryocar brasiliense camb*.) in Aqueous Two-Phase Systems: An Optimization Approach

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## ABSTRACT

While plant residues represent promising low-cost enzyme sources, their efficient recovery remains a challenge for industrial biocatalysis. This study aimed to optimize thepurification of lipases extracted from pequi peel (*Caryocar brasiliense Camb*.) using aqueous two-phase systems (ATPS) composed of polyethylene glycol (PEG) 1500 g/mol and potassium phosphate at pH 7.0. The effects of extract, salt, and PEG concentrations (% m/m), and temperature on lipase partitioning were systematically evaluated using a full factorial ($2^4$) and a central composite rotational design (CCRD). The optimized ATPS condition obtained through the response surface methodology was 15% PEG, 13.7% salt, 11.5% extract at 25 °C, in which, maximum enzyme partition coefficient ($K_e$) 0.096, selectivity ($S$) 0.0005, theoretical recovery (%$Y$) 97.3%, and purification factor (PF) 3.52 were achieved, confirming that pequi peel lipases preferentially partitioned into the salt-rich phase. These findings highlight pequi peel as a sustainable and underexplored source of lipases with potentially catalytic properties, while demonstrating that optimized ATPS enables efficient and eco-friendly enzyme recovery for industrial biocatalysis.

## 1 | Introduction

Due to the widespread applicability of enzymes in various industrial processes, this market has developed steadily and shows promising growth prospects for the coming years. According to Global Market Insights [1], the global enzyme market was valued at $14.09 billion in 2024 and is projected to reach $24.39 billion by 2032 with a CAGR of 7.1% over the forecast period. With the growing demand for ecological and sustainable solutions, enzymes, natural catalysts, have won the market in industries such as food and beverages, biofuels, and detergents as a more sustainable alternative to traditional chemical processes [2].

Among these enzymes, lipases (triacylglycerol hydrolases, E.C. 3.1.1.3) belong to the hydrolase family and are responsible mainly for catalyzing the hydrolysis of ester bonds in triacylglycerols, as well as promoting ester synthesis and modification reactions such as esterification, transesterification, interesterification, acidolysis, aminolysis, alcoholysis, and glycerolysis [3, 4]. Thus, lipases acting as important biocatalysts and play significant roles in various industrial sectors, including the food industry, oleochemistry, pharmaceutical, formulation of detergents, synthesis of biosurfactants, treatment of effluents, among others [5, 6]. It is estimated that the market for lipases reached US$606.80 million in 2024 and could reach US$985.54 million by 2032, with a CAGR

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of 6.28% during this period, supported by growing applications in the food and pharmaceutical industries [7].

Currently, lipases can be obtained from a wide range of microorganisms, plants, and animal sources. Microbial lipases remain the most extensively studied and the primary commercial source due to their high stability, low production costs, availability, and versatility [8, 9]. Despite this, there is a growing interest in alternative sources of enzymes since properties such as substrate specificity, catalytic kinetics, and stability can vary depending on the enzyme's origin [10].

Plant-derived enzymes have several advantages, including low-cost extraction and purification through simple techniques, renewable sourcing, the potential use of agro-industrial residues, and distinctive catalytic mechanisms [11]. Plant by-products represent promising and sustainable sources of high-value biomolecules, acting as natural reservoirs of enzymes and other metabolites of industrial and biotechnological interest [12]. Therefore, in plants, lipases are mainly found in the leaves, oils, stems, latex, and seeds of oil-bearing crops and cereals [13] playing two main biological functions: providing energy through the hydrolysis of stored seed oils and acting as a defense mechanism, offering antifungal protection against phytopathogens. Reported plant sources of lipases include pequi almond [3], palm oil [14], rice bran [15], papaya latex [16], castor seed [17], and rubber seed [18]. Although native plant materials generally exhibit low enzyme yields, the diversity of plant lipase origins offers structural and functional variability that may lead to effective biocatalysts for industrial applications [19, 20].

Research focused on optimizing the recovery and purification of enzymes from underexplored plant sources is valuable from a biotechnological perspective, particularly when it emphasizes achieving high yields and purity at bottom costs. In our previous study, we have reported the potential of the pequi shell (*Caryocar brasiliense Cambess*), which is considered an agricultural by-product, as a valuable source of lipases [21]. Many other studies also demonstrate the technological potential of this part of the fruit, as a source of many compounds of industrial interest [22–30].

To maximize the practical use the pequi shell lipases and facilitate their large-scale application for industrial purposes, it is essential to develop efficient and cost-effective concentration and purification strategies that can ensure high enzyme recovery and maintain optimal catalytic performance while keeping low operational costs.

Aqueous two-phase system (ATPS) is an alternative liquid-liquid extraction technique to the organic solvents-based conventional ones that has acquired great importance and progressive success in the extraction and purification of biocompounds of technological interest, such as proteins, enzymes, carbohydrates, flavonoids and other bioactive compounds, antibiotics, nucleic acids, and DNA [31–42].

Among the main advantages of ATPS are high biocompatibility with biological materials, low interfacial tension, fast phase separation, low operating cost, reduced environmental impact, and ease of process scaling [43, 44]. As described in the literature, ATPS have been successfully applied to the separation and purification of lipases from both conventional and non-conventional sources [29, 39, 44–46]. The central factor that arouses growing interest in this technique is the high proportion of water in the phases, which favors the separation of target compounds under mild conditions, preserving their structural stability and biological activity [44].

Because it is a low-cost process, high productivity, simplicity, relatively fast separation, possibility of large-scale application, process integration capacity, and great environmental appeal [41, 47, 48], this technique presents the feasibility of industrial application. This shows the importance of research aimed at better understanding this technique and optimizing the partitioning process to scale up at an industrial level.

Thus, the present study aimed to optimize the partition of lipases extracted from pequi bark (*Caryocar brasiliense Camb.*) using two-phase aqueous systems (ATPS), aiming to obtain an adequate enzymatic fraction for future biotechnological applications.

## 2 | Material and Methods

### 2.1 | Preparation of the Extract

The extract was obtained from pequi peels. The fruits were purchased at the free market of Salinas-MG (Brazil). After peeling, the peels were sanitized in chlorinated water (200 ppm) for 15 min, then rinsed in distilled water, cut to approximately 3 cm x 3 cm x 1 cm, and dried in an air-circulation oven (SL 102; Solab) at 50 °C for 18 h. After drying, they were ground in a knife mill (Willey EDB-5; DeLeo, POA) with a grain size smaller than 9 mesh.

To obtain the enzymatic extract, the methodology described by Santos et al. [30] was followed. In centrifuge tubes, 1 g of pequi bark flour was solubilized with 10 g of sodium phosphate buffer solution, 0.1 M, pH 8.0. The mixture was agitated by vortices and placed in an ultrasonic bath for 43 min. After this time, it was centrifuged at 4 °C and 3250 G, and the supernatant was filtered with the aid of gauze.

### 2.2 | Determination of Protein Content

The total protein concentration of the enzymatic extract and the other steps of the experiment was determined using the method proposed by Bradford [49]. The analytical curve was constructed using bovine serum albumin (BSA) as the standard, at concentrations ranging from 0.1 to 1.0 mg/mL (supplementary material), with measurements taken at a wavelength of 595 nm.

### 2.3 | Determination of Enzymatic Activity of the Extract

The hydrolytic activity of the lipases of the extract and purification steps was determined according to the methodology proposed by Winkler and Stuckmann [50] with modifications. The synthetic substrate used was p-nitrophenyl palmitate (p-

NPP). The reaction medium is called solution C, composed of solutions A and B in the ratio of 1:9, respectively.

-Solution A: 162.0 mg of p-NPP dissolved in 30.0 mL of isopropanol.

-Solution B: 9.0 g of Triton X-100 added 0.90 g of Arabic gum, dissolved in 450 mL of Tris-HCl 0.09 M buffer, pH 8.0.

Solution C (0.5 mL) was incubated at 37 °C for 10 min in a thermostatic bath. Then, 50 µL of the extract and 450 µL of the extractor solution (sodium phosphate buffer 0.1 M, pH 8) were added and kept under these conditions for 15 min. To interrupt the reaction, samples were heated to 100 °C for 7 min, and then 0.5 mL of ethylenediaminetetraacetic acid (EDTA) disodium salt solution 0.1 M was added.

The analytical curve was constructed using p-nitrophenol palmitate as a standard, with readings performed at the wavelength of 410 nm. A unit of lipase ($U$) is defined as the amount of enzyme that releases 1 µmol of p-nitrophenol per mL per minute under the described conditions. Equation 1 was used to calculate the enzymatic activity.

$$U/mL = \frac{C_{pN} \cdot FD \cdot V_T}{V_E \cdot t} \quad (1)$$

where $U$ is the unit of enzymatic activity; $C_{pN}$ is the concentration of p-nitrophenol (µmol) released per mL; FD is the dilution factor; $V_T$ is the total reaction volume; $V_E$ is the volume of enzyme extract; $t$ is the reaction time (min).

According to Equation 2, the specific enzymatic activity (U/mg) was obtained by dividing the enzymatic activity ($U$) by the protein concentration ($P$).

$$AE = \frac{U}{P} \quad (2)$$

The relative activity was calculated based on the highest value of specific activity obtained, which was considered as 100%. The other values were expressed as percentages relative to this pattern.

## 2.4 | Determination of the Equilibrium Data

### 2.4.1 | Binodal Curves

The binodal curves were determined by titration by turbidimetry, described by ALBERTSSON [51]. For the preparation of the systems, concentrated solutions of PEG polymers 1500 g/mol (60% m/m) and a solution of potassium phosphate monobasic/dibasic pH 7.0 (30% m/m) were used. The procedure consisted of weighing 1.00 g of PEG stock solution in test tubes, on an analytical balance (M254A, Bel Engineering, Piracicaba, Brazil). The tubes containing PEG solution were kept in a thermostatic bath (Tecnal, Te- 184, Piracicaba, Brazil) with an accuracy of 0.1 °C until thermal equilibrium was reached at the curve construction temperature (10 °C or 25 °C). Aliquots were added from the stock solution of salt (potassium phosphate) until the turbidity of the system (biphasic solution) and the volume of salt used was recorded. The system was titrated with distilled water until the turbidity disappeared, and the amount of water used was recorded to calculate the concentration of components for the construction of the curve. The empirical equation suggested by Hu et al. [52] was used to adjust the binary curves:

$$X_{SAL} = exp\left(a + b(X_{PEG})^{0,5} + cX_{PEG} + d(X_{PEG})^2\right) \quad (3)$$

where $X_{SAL}$ and $X_{PEG}$ are the mass fraction compositions of potassium phosphate and PEG 1500 g/mol, respectively; $a$, $b$, $c$, and $d$ are constants obtained from the regression of experimental data.

### 2.4.2 | Tie-Lines

The tie-lines (TL) were obtained using the gravimetric method described by Merchuk et al. [53]. This method consists of selecting a point above the biphasic region through which the mooring line is drawn. The global points used in the formation of the TLs correspond to the concentrations of the system components determined in each condition of the experimental planning carried out for partition optimization.

The known compositions of solutes were added in graduated centrifuge tubes, from the mass of the stock solutions of monobasic/bibasic potassium phosphate, PEG 1500 g/mol, and water. The mixture was vigorously stirred and centrifuged (SP LABOR, model Sp-701, Presidente Prudente, Brazil) at 3250G for 10 min to accelerate phase separation. The tubes were kept at rest for 12 h in a B.O.D oven at constant temperature (10 °C or 25 °C) to achieve the equilibrium of the phases. All solutions were prepared using an analytical scale of M254A (Bel Engineering, 80 Piracicaba, Brazil) with an accuracy of 0.0001 g. Once equilibrium was reached, the phases were carefully collected with syringes. After separation, the phases were weighed. The concentration of each component in the collected phases was estimated by applying the lever rule to the relationship between the top phase's mass composition and the system's total composition. To determine the components of the mooring line, the system of four equations (Equations 4–7) and four unknowns ($W_{SALT}{}^t$, $W_{SALT}{}^b$, $W_{PEG}{}^t$, $W_{PEG}{}^b$) was solved [52] with the aid of the solver command Microsoft Excel:

$$W_{SALT^t} = \exp\left(a + b(W_{PEG^t})^{0,5}\right) + cW_{PEG^t} + d(W_{PEG^t})^2 \quad (4)$$

$$W_{SALT^b} = \exp\left(a + b(W_{PEG^b})^{0,5}\right) + cW_{PEG^b} + d(W_{PEG^b})^2 \quad (5)$$

$$\frac{W_{SALT^t} - W_{SAL^M}}{W_{SALT^M} - W_{SALT^b}} = \frac{m_b}{m_t} \quad (6)$$

$$\frac{W_{PEG^M} - W_{PEG^t}}{W_{PEG^b} - W_{PEG^M}} = \frac{m_b}{m_t} \quad (7)$$

where $W_{SALT}{}^t$, $W_{PEG}{}^t$, $W_{SALT}{}^b$, $W_{PEG}{}^b$, $W_{SALT}{}^M$, $W_{PEG}{}^M$ are the mass fraction compositions of salt and PEG in the top, bottom, and mixture phases, respectively. And $m_i$ and $m_s$ are the bottom and top phase mass, respectively.

**TABLE 1** | Levels of independent variables and coded values for the full factorial experiment.

| Independent variable (factor) | Coded factor | Level | |
|---|---|---|---|
| | | −1 | 1 |
| % PEG 1500 | A | 15.0 | 17.5 |
| % Potassium phosphate solution | B | 12.0 | 13.5 |
| % Extract | C | 5.0 | 10.0 |
| Temperature (°C) | D | 10.0 | 25.0 |

The tie-line length (TLL) was obtained according to Equation 8.

$$TLL = \sqrt{[\Delta C_1]^2 + [\Delta C_2]^2} \tag{8}$$

The slope of the tie-line (STL) was determined by Equation 9.

$$STL = \frac{\Delta C_1}{\Delta C_2} \tag{9}$$

where $\Delta C1$ and $\Delta C2$ correspond to component 1 ($C1$) and component 2 ($C2$) concentration differences in the top and bottom phases, respectively, expressed as% m/m.

## 2.5 | Partition Optimization

### 2.5.1 | Full Factorial (FC)

A complete factorial planning was carried out $2^4$ (Table 1), studying the following variables: PEG concentration 1500 g/mol (15.0 and 17.5% m/m), potassium phosphate buffer concentration pH 7.0 (12.0 and 13.5% m/m), percentage of extract in substitution to ATPS water (5.0 and 10% m/m) and temperature (10°C and 25 °C).

### 2.5.2 | Central Composite Rotational Design (CCRD)

To determine the ideal conditions for the partition of the lipases-rich extract, after the results obtained by the full factorial, experimental planning was carried out using CCRD ($2^2$). The independent variables studied were the concentration of extract and the concentration of monobasic/bibasic potassium phosphate (Table 2) as a function of the response variables enzyme partition coefficient ($K_e$), total protein partition coefficient ($K_p$), Gibbs free energy of enzymatic transfer ($\Delta G_e$), Gibbs free energy of protein transfer ($G_p$), selectivity ($S$), theoretical recovery ($\%Y$), and the purification factor (PF). After the full factorial analysis, the PEG 1500 concentration was set at 15% and the temperature at 25 °C for the CCRD study.

To predict ideal conditions, the experimental data were analyzed using an empirical model of second-order polynomial regression (Equation 10), using SAS Studio software (student version, SAS Institute Inc., Cary, North Carolina, USA).

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_1^2 + \beta_3 X_2 + \beta_4 X_2^2 + \beta_5 X_1 X_2 \tag{10}$$

where $Y$ are the predicted values ($K_e$ and $K_p$); $\beta_0$ is the intermission; $\beta_i$, $\beta$ii, and $\beta_{ij}$ are regression coefficients for linear, quadratic, and interaction terms, respectively; $X_i$ and $X_j$ are independent variables that affect the response.

## 2.6 | Determination of Partition Parameters

Equation 11 describes how the partition coefficient of total proteins ($K_p$), which is the relationship between the equilibrium concentration (mg/mL) of the total protein in the top phase ($C_t$) and bottom phase ($C_b$), was determined.

$$K_p = \frac{[C]_t}{[C]_b} \tag{11}$$

Equation 12 describes how the partition coefficient of the lipases ($K_e$) was determined by the ratio between the specific enzymatic activity (AE) in the top phase ($t$) and the AE in the bottom phase ($b$) of each system.

$$K_e = \frac{[AE]_t}{[AE]_b} \tag{12}$$

The selectivity ($S$) was calculated as the ratio between the partition coefficient of lipases ($K_e$) and the partition coefficient of total protein ($K_p$) by Equation 13.

$$S = \frac{K_e}{K_p} \tag{13}$$

To select the ATPS with the best extraction capacity of the proteins studied, the system's theoretical recovery ($\%Y$) was calculated using Equation 14 (PICÓ et al., 2006).

$$\%Y = \frac{100}{1 + (1/rK_e)} \tag{14}$$

where $r$ is the ratio between the volumes of the top phase.

The PF was defined as the ratio between specific enzyme activity ($AE$) in the enzyme's preferential partitioning phase ($[AE]_{phase}$) and in the extract ($[AE]_{ext}$).

$$PF = \frac{[AE]_{phase}}{[AE]_{ext}} \tag{15}$$

## 2.7 | Estimation of the Free Energy of Gibbs in the Partition

By studying the substrate partition between the phases of a two-phase aqueous system, the spontaneity of the transfer process can be better understood based on the thermodynamic parameter called free energy of Gibbs transfer ($\Delta G$), which is calculated by the classical relation of thermodynamics:

$$\Delta G\,(kJ/mol) = -RTlnK \tag{16}$$

where R is the universal gas constant (8314 $10^{-3}$ kJ/mol/K), $T$ is the system temperature (K), and $K$ is the partition coefficient

**TABLE 2** | Variables and levels studied for optimization of extraction by experimental design (CCRD).

| Independent variable (factor) | Level | | | | |
|---|---|---|---|---|---|
| | **−1.41** | **−1** | **0** | **1** | **1.41** |
| % Extract | 3.55 | 5.00 | 8.50 | 12.00 | 13.45 |
| % Potassium phosphate | 11.38 | 12.00 | 13.50 | 15.00 | 15.62 |

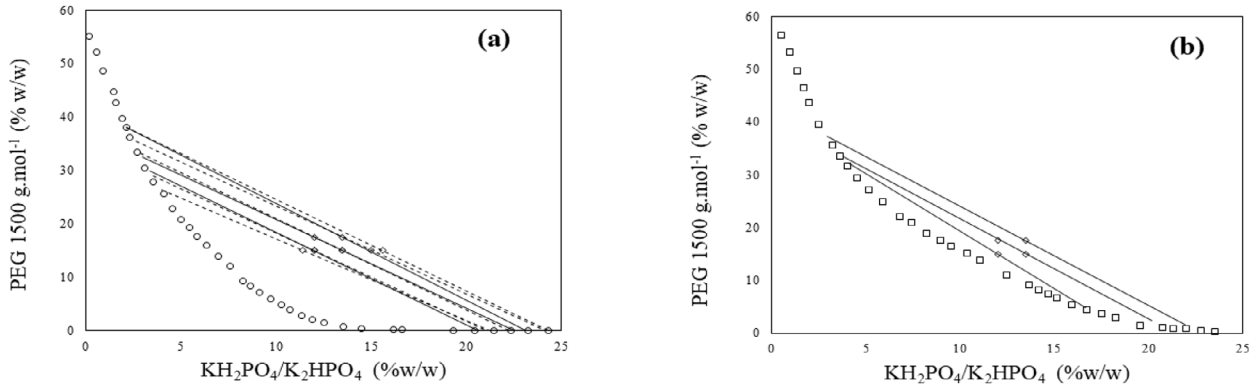



**FIGURE 1** | PEG 1500 g/mol + $KH_2PO_4/K_2HPO_4$ + water formed phase diagrams. (a) 25 °C (°) and (b) 10 °C (□); (—) FC Tie-lines (—) CCRD Tie-lines; (◊) global points.

**TABLE 3** | Adjusted parameters and coefficient of determination ($R^2$) obtained for the PEG systems 1500 g $mol^{-1}$ + $KH_2PO_4/K_2HPO_4$ + water at temperatures of 10 °C and 25 °C by the equation of Hu et al. (2003).

| Temperature (°C) | *a* | *b* | *c* | *d* | $R^2$ |
|---|---|---|---|---|---|
| 10 | −0.2126 | −4.7202 | 2.7421 | −48.2073 | 0.9974 |
| 25 | −0.5181 | −1.0454 | −11.7863 | −89.0815 | 0.9987 |

of enzymatic activity (*Ke*) or the partition coefficient of total proteins ($K_p$). Thus, the Gibbs free energy of enzymatic transfer ($G_e$) and the Gibbs free energy of protein transfer ($G_p$) were determined.

## 3 | Results and Discussion

### 3.1 | Equilibrium Data

Figure 1 represents the equilibrium data for the ATPS determined on this study for further recovery of the lipases from pequi peel extract.

It was observed that the concentrations of the global points used in the experimental designs (FC and CCRD) are within the biphasic regions at the ATPS of 10 °C and 25 °C.

Table 3 presents the values obtained for the parameters a, b, c, and d, as well as $R^2$, for the systems studied. Based on the values of $R^2$, it can be concluded that the empirical equation adjusted satisfactorily to the experimental data.

The equilibrium compositions of the top and bottom phases, the length (*TLL*) and the slope (*STL*) of the TLs of the ATPS composed by PEG 1500 g/mol, monobasic/bibasic potassium phosphate and water at 10 °C and 25 °C are presented in Table 4.

From the data in Table 4, it is possible to observe that the concentrations of PEG in the bottom phase and salt in the top phase are very small, with PEG concentrations almost zero in the bottom phases of the systems at 25 °C. The increase in temperature promotes greater attraction among PEG molecules because they become more hydrophobic, thus reducing the affinity of water for them and consequently, the interaction between PEG molecules and water [54, 55].

In systems at 10 °C, the TLL is smaller. According to Pirdashti et al. (2019), the increase in temperature promotes an increase in *TLL* and the *STL* values, as observed in this study. This occurs due to the increased hydrophobicity of the polymer, promoting the spontaneous diffusion of water from the top phase (rich in polymer) to the bottom phase (rich in salt), that is, the relationship between temperature and the size of the biphasic region in the systems of this study is associated with the endothermic nature of the phase separation process [55].

From the data in Table 4, it is possible to observe that the concentrations of PEG in the bottom phase and salt in the top phase are very small, with PEG concentrations almost zero in the bottom phases of the systems at 25 °C. The increase in temperature promotes greater attraction among PEG molecules because they become more hydrophobic, thus reducing the affinity of water for

**TABLE 4** | Mass fractions (% m/m) for systems formed by PEG 1500 g/mol ($W_{peg}$), monobasic/bibasic potassium phosphate ($W_{salt}$), and water ($W_{water}$) in experimental planning.

| TL | *TLL* | *STL* | Overall composition (%) | | | Top phase (%) | | | Bottom phase (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $W_{peg}$ | $W_{salt}$ | $W_{water}$ | $W_{peg}$ | $W_{salt}$ | $W_{water}$ | $W_{peg}$ | $W_{salt}$ | $W_{water}$ |
| 1 | 30.14 | −2.17 | 15.00 | 12.00 | 73.00 | 32.26 | 4.06 | 63.68 | 4.88 | 16.66 | 78.46 |
| 2 | 34.98 | −1.89 | 15.00 | 13.50 | 71.50 | 33.26 | 3.82 | 62.92 | 2.36 | 20.20 | 77.44 |
| 3 | 34.98 | −1.89 | 17.50 | 12.00 | 70.50 | 33.26 | 3.82 | 62.92 | 2.36 | 20.20 | 77.44 |
| 4 | 40.51 | −1.88 | 17.50 | 13.50 | 69.00 | 37.36 | 2.95 | 59.69 | 1.58 | 21.95 | 76.46 |
| 5 | 34.53 | −1.73 | 15.00 | 12.00 | 73.00 | 29.98 | 3.35 | 66.67 | 0.07 | 20.62 | 79.31 |
| 6 | 37.93 | −1.66 | 15.00 | 13.50 | 71.50 | 32.51 | 2.95 | 64.54 | 0.03 | 22.54 | 77.43 |
| 7 | 37.93 | −1.66 | 17.50 | 12.00 | 70.50 | 32.51 | 2.95 | 64.54 | 0.03 | 22.54 | 77.43 |
| 8 | 43.52 | −1.82 | 17.50 | 13.50 | 69.00 | 38.15 | 2.14 | 59.72 | 0.02 | 23.12 | 76.86 |
| 9 | 31.47 | −1.52 | 15.00 | 11.40 | 73.60 | 26.36 | 3.96 | 69.67 | 0.05 | 21.24 | 78.70 |
| 10 | 33.83 | −1.65 | 15.00 | 12.00 | 73.00 | 28.97 | 3.52 | 67.51 | 0.06 | 21.08 | 78.86 |
| 11 | 38.48 | −1.71 | 15.00 | 13.50 | 71.50 | 33.27 | 2.84 | 63.90 | 0.03 | 22.24 | 77.73 |
| 12 | 41.47 | −1.64 | 15.00 | 15.00 | 70.00 | 35.45 | 2.62 | 61.93 | 0.01 | 24.16 | 75.83 |
| 13 | 44.10 | −1.72 | 15.00 | 15.60 | 69.40 | 38.12 | 2.14 | 59.74 | 0.01 | 24.33 | 75.66 |

1–4: TL of the ATPS at 10 °C in the concentrations of the global points of the full factorial.
5–8: TL of the ATPS at 25 °C in the global full factorial point concentrations.
9–13: TL of the ATPS at 25 °C in the global point concentrations of the rotational central composite design.

them and consequently, the interaction between PEG molecules and water [54, 55].

## 3.2 | Partition Optimization

### 3.2.1 | Full Factorial Planning

The full factorial (Table 5) was applied to test the statistical significance ($p < 0.05$) of the variables involved in the process of partitioning the lipases in the extract.

The tests were based on the response variable, the lipase partition coefficient ($K_e$), due to interest in the partition of proteins with lipolytic activity. It was observed that the $K_e$ value was lower than 1.00 for almost all the systems studied, except for Treatment 7, indicating that the lipase presented greater affinity with the bottom phase for the systems studied. Salt can influence the partition behavior of proteins in ATPS, as it affects the phase compositions, the electrostatic potential of the system, and the hydrophobic interactions of the proteins. Dissociated ions with charges opposite to that of the protein tend to attract it toward the phase enriched with those specific ions, which may explain the higher affinity of the lipase for the bottom phase [56].

The low of significance ($p < 0.05$) of the extract and salt (potassium phosphate) concentration factors indicates that they influence the lipase partition process of the pequi peel extract in the ATPS concentration range evaluated by the *F* test (Table 6). Therefore, the values of the PEG concentration of 1500 g/mol (15.0%) at 25 °C were fixed for subsequent partition optimization studies. These values were chosen based on the premise of reducing the amount of system-forming material and energy to reach and maintain the process temperature.

**TABLE 5** | Experimental design of full factorial design for partition coefficient ($K_e$).

| Treatment | Variables | | | | $K_e$ |
|---|---|---|---|---|---|
| | *T* (°C) | % Extract | % PEG | % Salt | |
| 1 | 10.00 | 5.00 | 15.00 | 12.00 | 0.275 ± 0.004 |
| 2 | 10.00 | 5.00 | 15.00 | 13.50 | 0.138 ± 0.021 |
| 3 | 10.00 | 5.00 | 17.50 | 12.00 | 0.030 ± 0.011 |
| 4 | 10.00 | 5.00 | 17.50 | 13.50 | 0.049 ± 0.007 |
| 5 | 10.00 | 10.00 | 15.00 | 12.00 | 0.452 ± 0.032 |
| 6 | 10.00 | 10.00 | 15.00 | 13.50 | 0.013 ± 0.002 |
| 7 | 10.00 | 10.00 | 17.50 | 12.00 | 1.156 ± 0.098 |
| 8 | 10.00 | 10.00 | 17.50 | 13.50 | 0.145 ± 0.020 |
| 9 | 25.00 | 5.00 | 15.00 | 12.00 | 0.010 ± 0.001 |
| 10 | 25.00 | 5.00 | 15.00 | 13.50 | 0.034 ± 0.011 |
| 11 | 25.00 | 5.00 | 17.50 | 12.00 | 0.085 ± 0.030 |
| 12 | 25.00 | 5.00 | 17.50 | 13.50 | 0.078 ± 0.009 |
| 13 | 25.00 | 10.00 | 15.00 | 12.00 | 0.480 ± 0.121 |
| 14 | 25.00 | 10.00 | 15.00 | 13.50 | 0.531 ± 0.127 |
| 15 | 25.00 | 10.00 | 17.50 | 12.00 | 0.827 ± 0.057 |
| 16 | 25.00 | 10.00 | 17.50 | 13.50 | 0.834 ± 0.111 |

**TABLE 6** | Analysis of variance for the experimental design of the full factorial design in the optimization of the partition of lipases in the extract.

| Effects | Degrees of freedom | Quadratic mean | $p^a$ |
|---|---|---|---|
| Temperature | 1 | 0.048 | 0.369 |
| % Extract | 1 | 1.749 | <0.0001* |
| % PEG | 1 | 0.202 | 0.072 |
| % SALT | 1 | 0.279 | 0.037* |
| Total | 4 | | |

[a]$p$: Probability ($p < 0.05$) of significance for the tested factors.
*:significant effect.

**TABLE 7** | Experimental design of the factorial scheme within the central composite rotational design for the evaluation of partitioning parameters.

| | Variables | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| T | % E | % S | *Kp* | *Ke* | $\Delta G_e$ (kJ/mol) | $\Delta G_p$ (kJ/mol) | *S* | *%Y* | *PF* |
| 1 | 5.00 | 12.00 | 9.74 | 1.64 | −1159.68 | −5326.15 | 0.1791 | 32.63 | 0.13 |
| 2 | 5.00 | 15.00 | 43.56 | 0.30 | 2820.17 | −8573.23 | 0.0062 | 80.94 | 0.66 |
| 3 | 12.00 | 12.00 | 15.29 | 0.20 | 3114.80 | −6819.62 | 0.0214 | 81.82 | 0.81 |
| 4 | 12.00 | 15.00 | 21.78 | 0.10 | 5499.35 | −7313.96 | 0.0035 | 90.69 | 1.20 |
| 5 | 3.55 | 13.50 | 3.97 | 1.74 | −1305.99 | −3351.86 | 0.4039 | 39.90 | 0.28 |
| 6 | 13.45 | 13.50 | 73.59 | 0.03 | 8321.61 | −9796.72 | 0.0005 | 97.30 | 3.52 |
| 7 | 8.50 | 11.38 | 9.47 | 0.70 | 853.61 | −5622.77 | 0.0639 | 50.80 | 0.56 |
| 8 | 8.50 | 15.62 | 23.27 | 0.20 | 3645.86 | −7572.83 | 0.0093 | 87.41 | 0.95 |
| 9 | 8.50 | 13.50 | 32.96 | 0.23 | 3417.97 | −8078.32 | 0.0060 | 82.89 | 1.18 |
| 9 | 8.50 | 13.50 | 28.93 | 0.37 | 4250.86 | −8307.18 | 0.0057 | 87.35 | 1.33 |
| 9 | 8.50 | 13.50 | 28.54 | 0.29 | 2891.55 | −7028.98 | 0.0037 | 79.49 | 0.89 |

*Note: T*: treatment; *E*: extract; *S*: sodium phosphate; *Kp*: total protein partition coefficient; $K_e$: enzymatic partition coefficient; $\Delta G_e$: Gibbs free energy of enzymatic transfer; $\Delta G_p$: Gibbs free energy of protein transfer; *S*: selectivity; *%Y*: theoretical recovery; and PF: purification factor.

### 3.3 | Central Composite Rotational Design (CCRD)

After executing the full factorial experimental design, a central composite rotational design (CCRD) was carried out using only the significant factors in the partitioning process (Table 7). In this experiment, the enzymatic partition coefficient (*K*e), the total protein partition coefficient (*K*p), the Gibbs free energy of enzymatic transfer ($\Delta G_e$), the Gibbs free energy of protein transfer ($\Delta G_p$), the selectivity (*S*), the theoretical yield (*%Y*), and the PF were evaluated as a function of the variation in the extract concentration and monobasic/dibasic potassium phosphate, at pH 7.0 and 25 °C.

Subsequently, regression analysis was performed (Table 8) to determine the equations of the partitioning parameters as a function of the significant independent variables.

*Note:* $K_p$: is the total protein partition coefficient; $K_e$: is the enzymatic partition coefficient; $\Delta G_e$: is the Gibbs free energy of enzymatic transfer; $\Delta G_p$: is the Gibbs free energy of protein transfer; *S*: is the selectivity; *%Y*: is the theoretical recovery; PF: is the purification factor; *E*: is the extract percentage; and *S*: is the salt percentage.

Based on statistical significance ($p < 0.05$), the data were evaluated through analysis of variance (ANOVA) using the *t*-test (supplementary material) for each response variable ($K_e$, $K_p$, $\Delta G_e$, $\Delta G_p$, *S*, *%Y*, or PF), and all models were found to be significant.

The derived equation for studying the lipase partition coefficient ($K_e$) indicated optimal concentrations of 13.68% salt and 11.46% extract. Substituting these values into the equation yielded a $K_e$ value of 0.096. This result is consistent with the empirical data in Table 7, where $K_e$ values lower than 1.0 suggest that the lipases preferentially migrated to the bottom phase of the ATPS [38]. This trend is visually confirmed in the response surface plot of $K_e$ (Figure 2), demonstrating the enzymes' greater affinity for the salt-rich phase. Such behavior may be related to the hydrophilic nature of the lipases present in the pequi peel extract, favoring their partitioning into the more polar salt phase [57].

**TABLE 8** | Regression equations relating partitioning parameters as a function of monobasic/dibasic potassium phosphate concentration (%) and extract concentration (%).

| Parameters | Equations |
|---|---|
| $K_P$ | $\hat{Y} = 1.13491 + 2.93705[E]$ |
| $K_e$ | $\hat{Y} = 12.579 - 1.347E - 0.697[Sal] + 0.0609[E][s] + 0.0224[E]^2$ |
| $G_e$ | $\hat{Y} = -64424 + 746.47699[E] + 8270.17845[s] - 274.65902[s]^2$ |
| $G_P$ | $\hat{Y} = 4020.6095 - 325.254[E] - 617.1309[s]$ |
| S | $\hat{Y} = 1.0386 - 0.14643[E] - 0.02179[s] + 0.00699[E]^2$ |
| $\%Y$ | $\hat{Y} = -815.222 + 39.364[E] + 96.4442[s] - 1.8394[E][s] - 0.5705[E]^2 - 2.6655[s]^2$ |
| $PF$ | $\hat{Y} = -43.4353 + 0.2031[E] + 6.26956[s] - 0.022745[s]^2$ |

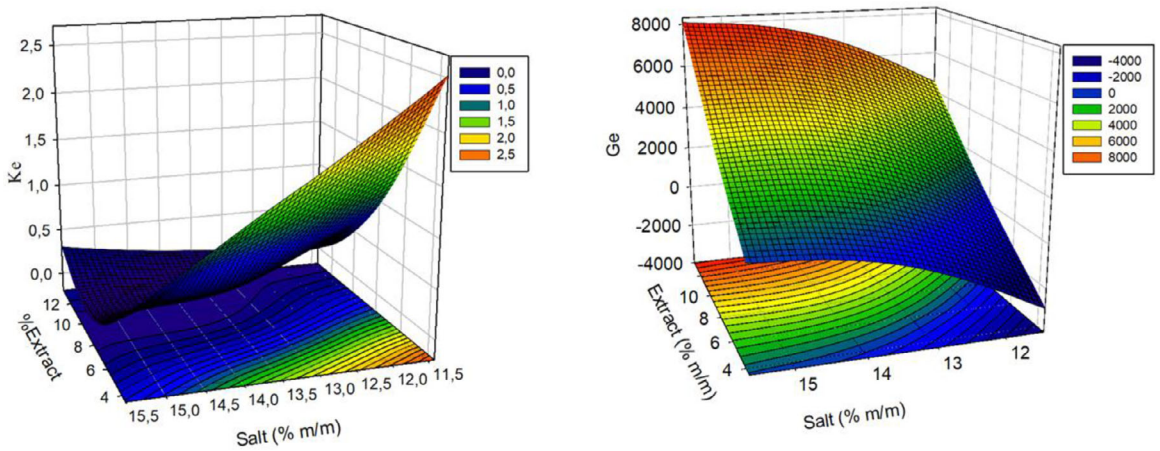



**FIGURE 2** | Response surface for the lipase partition coefficient ($K_e$) and Gibbs free energy of lipase transfer ($G_e$) as a function of potassium phosphate and extract concentrations in the composition of the aqueous two-phase system (ATPS).

This result can also be attributed to the increased solubility of proteins promoted by the salting-in effect of the saline solution, in which dissociated ions enhance the solvation layer around the enzyme [58]. Furthermore, the reduced partitioning into the PEG-rich phase is associated with the so-called excluded volume effect. In this context, polyethylene glycol and lipase compete for the available interstitial spaces, and the high molecular weight of the polymer creates steric hindrance, limiting the biomolecule's access to this phase and favoring its exclusion [38]. A similar behavior was observed by Silva et al. [59] when optimizing a system composed of PEG 1500 and potassium phosphate for the purification of a crude lipase extract produced by *Yarrowia lipolytica* IMUFRJ 50.682. Nascimento et al. [38] also observed the same behavior when purifying lipases extracted from pequi seeds using aqueous systems composed of PEG/2-propanol/ammonium sulfate. Tang et al. [60], while studying aqueous systems with different alcohols and salts for lipase recovery, found that in all systems, the enzyme preferred the salt-rich phase. Akther et al. [61] observed a similar behavior when adding NaCl to aqueous systems composed of Triton X-100 and xylitol for lipase separation. The system demonstrated high selectivity for lipase, thereby increasing its purity. This can be attributed to stronger binding affinities between the salt ions and the lipase residues than those between the contaminant proteins and PEG [62]. This selectivity is crucial for the efficiency of the purification process, indicating that the optimized conditions favor the selective recovery of lipases.

The graph of the Gibbs free energy of lipase transfer ($G_e$) (Figure 2) indicates that the enzymes partitioned into both phases, with a greater tendency toward the bottom phase (positive region of the $G_e$ axis). The derived *G*e equation analysis revealed that the optimal potassium phosphate concentration is 15.05%. It was also observed that the extract concentration influences *G*e linearly, with the best concentration to maximize this parameter being 13.45%, resulting in a *G*e value of 7871.35 kJ/mol. These results and the partition coefficient (*K*e) value confirm that the developed ATPS favors the migration of lipases to the bottom phase. The positive $G_e$ value reinforces that this process is thermodynamically favorable, indicating that the studied conditions are efficient for the selective separation of lipolytic enzymes in the pequi peel extract.

From the intercept value of the linear equation for $K_p$ (1.1349), it can be inferred that proteins migrate to the top phase regardless of the concentrations of the ATPS components. The Gibbs free energy of total protein transfer ($G_p$) results justify the behavior observed in $K_p$. According to the obtained equation, within the

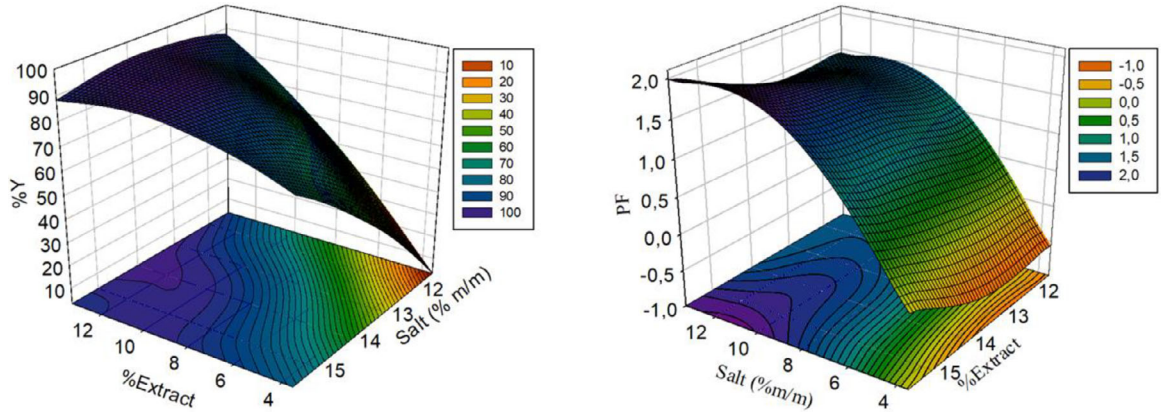



**FIGURE 3** | Response surface for theoretical recovery (%*Y*) and purification factor (PF) as a function of potassium phosphate concentration and extract concentration in the composition of the aqueous two-phase system (ATPS).

studied concentration range, increasing the system component concentrations resulted in increasingly negative Gibbs free energy values, indicating a stronger tendency of proteins to migrate to the top phase. Experimental data confirm this behavior, showing that contaminant proteins are concentrated in the phase opposite to the lipases, as presented in Table 7. A similar behavior was observed by Lee et al. [62] when purifying lipase in an ionic liquid-based ATPS, reporting a *K*p of 1.6, suggesting that the contaminant proteins concentrated in the phase opposite to that of the lipase. This result aligns with the physicochemical nature of the system, where the PEG-rich phase is more hydrophobic, favoring the partition of proteins with lower affinity for water, while the bottom phase, rich in salt, is more polar and favors retention of hydrophilic enzymes such as lipases [38, 57].

The derivation of the selectivity (*S*) equation resulted in an optimal extract concentration of 10.47% to achieve the maximum *S* value. For a fixed PEG concentration, increasing the salt concentration in the overall composition increases the TLL (Figure 1) and the *S* value, confirming the linear influence of salt concentration. Therefore, when the phases exhibit well-defined properties, the biomolecule tends to partition into the phase to which it has greater affinity, enhancing the system's selectivity.

The best experimental values of selectivity (0.0005) and theoretical recovery (%*Y*) (97.30%) were obtained under the same ATPS condition, with 13.45% extract and 13.50% potassium phosphate. Based on the derived %*Y* equation (Table 8), the calculated optimum was 93.76%, corresponding to extract and salt concentrations of 11.95% and 13.99%, respectively. The behavior of theoretical recovery (%*Y*) and PF is shown in Figure 3. These results are consistent with the findings of Padilha and Osório [39], who studied the partitioning of *Burkholderia cepacia* lipase in different systems, observed the highest efficiency (98%) using PEG 1500 and phosphate salt at pH 6.0. These findings reinforce the effectiveness of the system proposed in this study, demonstrating that specific extract and salt concentrations can maximize enzyme recovery while maintaining high selectivity.

Figure 3 also illustrates the variation of the PF as a function of the component concentrations in the ATPS. Based on the PF equation presented in Table 8, it is observed that the potassium phosphate concentration exerts a more significant influence on this parameter than the extract concentration. From the analysis of the equation, the maximum PF point was obtained with 13.78% potassium phosphate and the highest viable extract concentration in the system, corresponding to 10.47%. These results indicate that lipase purification is favored under conditions of high salt concentration combined with an optimal amount of biomolecule, reinforcing the importance of simultaneously optimizing these factors to achieve maximum efficiency in the enzymatic separation process.

## 4 | Conclusion

The integrated application of full factorial design and central composite rotational design (CCRD) allowed the determination of optimal conditions for the partitioning of lipases present in the pequi peel extract using ATPS. The results indicated a tendency of the lipases to migrate to the bottom phases of the system. The best responses for performance parameter such as enzymatic partition coefficient (*K*e), theoretical recovery (%*Y*), and PF were obtained with an estimated composition of 15% PEG 1500 g/mol, 13.7% potassium phosphate (monobasic/dibasic) at pH 7.0, 11.5% pequi peel extract, and 59.8% water. Meanwhile, the best *K*p, *G*p, and *G*e parameter values were achieved with extract concentrations above 13.45% and salt concentrations above 15%. These results highlight the effectiveness of the proposed system in the selective separation and concentration of lipases, reinforcing the technique's potential for sustainable industrial applications, particularly in the utilization of plant residues as enzymatic sources. The optimization of ATPS conditions proved essential for maximizing partitioning efficiency, reducing enzymatic losses, and increasing process selectivity, making it more competitive and environmentally viable. This approach enhances the recovery of high-value-added biomolecules and contributes to the development of more sustainable and economically attractive bioprocesses (Supporting Information).

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### Conflicts of Interest

The authors declare no conflicts of interest.

### Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

### References

1. Global Market Insights, "Enzymes Market Size—By Product (Proteases, Lipases, Carbohydrases, Polymerases & Nucleases, Phytases, Catalyses), Application (Food & Beverage, Processed Food, Dairy, Bakery, Confectionary) & Forecast, 2023 –2032" https://www.gminsights.com/industry-analysis/enzymes-market 2023.

2. Data Bridge Market Research, "Global Enzymes Market Size, Share, and Trends Analysis Report—Industry Overview and Forecast to 2032", https://www.databridgemarketresearch.com/reports/global-enzymes-market Chemical and Materials, 2025.

3. P. A. Nascimento, A. N. Alves, and K. A. dos Santos, "Optimization of Lipase Extraction From Pequi Seed (*Caryocar brasiliense* Camb.)," *Journal of Food Processing and Preservation* 45 (2021): 1–8, https://doi.org/10.1111/jfpp.15616.

4. A. D. S. Pereira, A. H. de Souza, J. L. Fraga, P. Villeneuve, A. G. Torres, and P. F. F. Amaral, "Lipases as Effective Green Biocatalysts for Phytosterol Esters′ Production: A Review," *Catalysts* 12 (2022): 88.

5. Mordor Intelligence, "Lipase Market Size & Share Analysis—Growth Trends & Forecasts (2025 - 2030)," https://www.mordorintelligence.com/industry-reports/lipase-market 2025.

6. Global Marketing Insights, "Microbial Lipase Market Source (Fungi and Bacteria), by Form (Powder and Liquid), By Application (Cleaning Agents, Animal Feed, Dairy Products, Bakery Products, And Confectionery Products), and Region—Global Forecast to 2026," https://www.gminsights.com/industry-analysis/microbial-lipase-market 2025.

7. Fortune Bussines Insights, "Lipase Market Size, Share & Industry Analysis, By Source (Microbial and Animal), By Application (Food & Beverage {Bakery, Dairy, Confectionery, Beverages and Others}, Nutraceuticals, Animal Feed, and Others), and Regional Forecast" 2025–2032, Food Additives & Ingredients, https://www.fortunebusinessinsights.com/lipase-market-110345, 2025.

8. L. Casas-Godoy, F. Gasteazoro, S. Duquesne, F. Bordes, A. Marty, and G. Sandoval, "Lipases: An Overview," *Lipases and Phospholipases: Methods and Protocol 18353* (2018):3–38, 10.1007/978-1-4939-8672-9_1.

9. D. Bharathi and G. Rajalakshmi, "Microbial Lipases: An Overview of Screening, Production and Purification," *Biocatalysis and Agricultural Biotechnology* 22 (2019): 101368, https://doi.org/10.1016/j.bcab.2019.101368.

10. P. Anbu, S. C. B. Gopinath, B. P. Chaulagain, and T. Lakshmipriya, "Microbial Enzymes and Their Applications in Industries and Medicine 2016," *BioMed Research International* 2017 (2017): 1–3.

11. A. N. Alves, P. A. Nascimento, and R. D. C. I. Fontan, "Extraction of Protease from Ora-Pro-Nobis (*Pereskia aculeata* Miller) and Partial Purification in Polyethylene Glycol + Sodium Phosphate Aqueous Two-Phase System," *Journal of Food Processing and Preservation 46* (2022): e16266, https://doi.org/10.1111/jfpp.16266.

12. N. Yeasmen, M. H. R. Bhuiyan, and V. Orsat, "Unravelling Scientific Research Towards the Green Extraction of Phenolic Compounds from Leaves: A Bibliometric Analysis," *International Journal of Food Science & Technology* 56 (2021): 4893–4906, https://doi.org/10.1111/ijfs.15215.

13. N. B. Melani, E. B. Tambourgi, and E. Silveira, "Lipases: From Production to Applications," *Separation & Purification Reviews* 49 (2020): 143–158.

14. M. H. Mohd Din, A. Nair, M. Masomian, M. S. Mohamad Ali, and R. N. Z. R. A. Rahman, "Heterologous Expression and Characterization of Plant Lipase LIP2 from Elaeis Guineensis Jacq. Oil Palm Mesocarp in *Escherichia coli*," *Catalysts* 11 (2021): 244.

15. C. Yu, L. Zheng, and M. Cheng, "Purification, Identification, Characterization and Catalytic Mechanism of Two Lipases from Rice Bran (*Oryza Sativa*)," *LWT* 140 (2021): 110693.

16. I. Rivera, M. Robles, J. C. Mateos-Díaz, A. Gutierrez-Ortega, and G. Sandoval, "Functional Expression, Extracellular Production, Purification, Structure Modeling and Biochemical Characterization of Carica Papaya Lipase 1," *Process Biochemistry* 56 (2017): 109–116, https://doi.org/10.1016/j.procbio.2017.02.009.

17. F. Tavares, P. R. Sackser, C. E. Borba, and E. A. Silva, "Rapid Enzymatic Hydrolysis of Crambe Oil Catalyzed by castor Seeds Lipases," *Industrial Crops and Products* 171 (2021): 113890.

18. N. F. Ahmad Bahrin, H. Veny, and S. Z. Che Mad, "Investigation on Enzymatic Activity of Rubber Seed as Source of Plant Lipase," *Malaysian Journal of Chemical Engineering and Technology* 3 (2020): 45–50, https://doi.org/10.24191/mjcet.v3i2.10938.

19. K. C. Santos, D. M. J. Cassimiro, and M. H. M. Avelar, "Characterization of the Catalytic Properties of Lipases from Plant Seeds for the Production of Concentrated Fatty Acids from Different Vegetable Oils," *Industrial Crops and Products* 49 (2013): 462–470, https://doi.org/10.1016/j.indcrop.2013.05.035.

20. S. Sankar and K. Ponnuraj, "Less Explored Plant Lipases: Modeling and Molecular Dynamics Simulations of Plant Lipases in Different Solvents and Temperatures to Understand Structure-Function Relationship," *International Journal of Biological Macromolecules* 164 (2020): 3546–3558, https://doi.org/10.1016/j.ijbiomac.2020.08.227.

21. K. A. dos Santos, I. de Carvalho Batista Muniz, and P. A. Nascimento, "Optimization of Extraction Conditions and Characterization of Lipases From Pequi (*Caryocar Brasiliense* Camb.) Fruit By-Product," *Waste Biomass Valorization* 15 (2024): 5527–5537.

22. D. P. Leão, B. G. Botelho, L. S. Oliveira, and A. S. Franca, "Potential of Pequi (*Caryocar brasiliense* Camb.) Peels as Sources of Highly Esterified Pectins Obtained by Microwave Assisted Extraction," *LWT* 87 (2018): 575–580, https://doi.org/10.1016/j.lwt.2017.09.037.

23. M. Mariani, V. R. de Oliveira, R. Faccin, A. D. O. Rios, and J. G. Venkze, "Elaboração e Avaliação de Biscoitos Sem Glúten a Partir de Farelo De arroz e Farinhas De arroz e De soja," *Brazilian Journal of Food Technology* 18 (2015): 70–78, https://doi.org/10.1590/1981-6723.6514.

24. L. de Pinho, P. N. S. Souza, E. Macedo Sobrinho, A. C. de Almeida, and E. R. Martins, "Atividade Antimicrobiana de Extratos Hidroalcoolicos das Folhas de alecrim- pimenta, Aroeira, Barbatimão, Erva Baleeira e Do Farelo Da Casca de pequi," *Ciência Rural* 42 (2012): 326–331, https://doi.org/10.1590/S0103-84782012005000003.

25. J. R. Filho, I. B. S. Lustosa, R. M. M. Sampaio, M. D. R. Motreira, V. D. de Morais, and V. S. S. de Sousa, "Melhoria Do Valor Nutricional Do Brownie Utilizando Farinha Do Mesocarpo Externo Do Pequi (*Caryocar Brasiliense* Camb)," *Motricidade* 14 (2018): 196–204.

26. L. B. Rocha, A. M. Melo, S. L. A. Paula, S. A. M. Nobre, and I. N. Abreu, "Gallic Acid as the Major Antioxidant in Pequi (*Caryocar brasiliense* Camb.) Fruit Peel," *Revista Brasileira De Plantas Medicinais* 17 (2015): 592–598, https://doi.org/10.1590/1983-084X/14_062.

27. B. D. S. Siqueira, L. D. Alves, P. N. Vasconcelos, C. Damiani, and M. S. Soares Júnior, "Pectina Extraída de Casca de Pequi e Aplicação em geleia light de manga," *Revista Brasileira De Fruticultura* 34 (2012): 560–567, https://doi.org/10.1590/S0100-29452012000200030.

28. M. S. Soares Júnior, P. Z. Bassinello, M. Caliari, R. C. dos Reis, D. B. C. L. Lacerda, and S. N. Koakuzu, "Development and Chemical

Characterization of Flour Obtained from the External Mesocarp of 'Pequizeiro' fruit," *Ciência e Tecnologia de Alimentos* 30 (2010): 940–948.

29. P. A. Nascimento, A. N. Alves, and K. A. Santos, "Optimization of Lipase Extraction from Pequi Seed (Caryocar Brasiliense Camb," *Journal of Food Processing and Preservation* 45 (2021): 1–12, https://doi.org/10.1111/jfpp.15616.

30. K. A. dos Santos, I. B. D. C. Muniz, and P. A. Nascimento, "Optimization of Extraction Conditions and Characterization of Lipases from Pequi (*Caryocar Brasiliense* Camb.) Fruit By-Product," *Waste Biomass Valorization* 15 (2024): 5527–5537.

31. Z. Cheng, H. Song, and X. Cao, "Simultaneous Extraction and Purification of Polysaccharides from Gentiana Scabra Bunge by Microwave-Assisted Ethanol-Salt Aqueous Two-Phase System," *Industrial Crops and Products* 102 (2017): 75–87, https://doi.org/10.1016/j.indcrop.2017.03.029.

32. K. Y. Chong, R. Stefanova, J. Zhang, and M. S.-L. Brooks, "Aqueous Two-Phase Extraction of Bioactive Compounds from Haskap Leaves (*Lonicera caerulea*): Comparison of Salt/Ethanol and Sugar/Propanol Systems," *Separation and Purification Technology* 252 (2020): 117399, https://doi.org/10.1016/j.seppur.2020.117399.

33. C. Li, M. Gao, and W. Zhu, "Recent Advances in the Separation and Purification of Lactic Acid From Fermentation Broth," *Process Biochemistry* 104 (2021): 142–151, https://doi.org/10.1016/j.procbio.2021.03.011.

34. M. González-Amado, A. P. M. Tavares, M. G. Freire, A. Soto, and O. Rodríguez, "Recovery of Lactose and Proteins from Cheese Whey With Poly(ethylene)Glycol/Sulfate Aqueous Two-Phase Systems," *Separation and Purification Technology* 255 (2021): 117686, https://doi.org/10.1016/j.seppur.2020.117686.

35. S. Jamshidi and G. Pazuki, "Effect of Hybrane Hyperbranched Polymer Additive on Partitioning of Cephalexin Antibiotic in Aqueous Biphasic Systems," *Journal of Molecular Liquids* 259 (2018): 48–54, https://doi.org/10.1016/j.molliq.2018.03.011.

36. M. Lyu, B. Meany, J. Yang, Y. Li, and M. Zheng, "Toward Complete Resolution of DNA/Carbon Nanotube Hybrids by Aqueous Two-Phase Systems," *Journal of the American Chemical Society* 141 (2019): 20177–20186, https://doi.org/10.1021/jacs.9b09953.

37. F. Mashayekhi, A. S. Meyer, S. A. Shiigi, V. Nguyen, and D. T. Kamei, "Concentration of Mammalian Genomic DNA Using Two-Phase Aqueous Micellar Systems," *Biotechnology and Bioengineering* 102 (2009): 1613–1623, https://doi.org/10.1002/bit.22188.

38. P. A. Nascimento, A. N. Alves, and K. A. dos Santos, "Partitioning of Pequi Seed (*Caryocar brasiliense* Camb.) Lipase in Aqueous Two-Phase Systems Composed of PEG/2-propanol + Ammonium Sulfate + Water," *Brazilian Journal of Chemical Engineering* 38 (2021): 957–965, https://doi.org/10.1007/s43153-021-00154-x.

39. G. S. Padilha and W. R. Osório, "Economic Method for Extraction/Purification of a *Burkholderia cepacia* Lipase with Potential Biotechnology Application," *Applied Biochemistry and Biotechnology* 189 (2019): 1108–1126, https://doi.org/10.1007/s12010-019-03041-x.

40. P. F. Requejo, P. Velho, E. Gómez, and E. A. Macedo, "Study of Liquid–Liquid Equilibrium of Aqueous Two-Phase Systems Based on Ethyl Lactate and Partitioning of Rutin and Quercetin," *Industrial & Engineering Chemistry Research* 59 (2020): 21196–21204, https://doi.org/10.1021/acs.iecr.0c02664.

41. K. A. dos Santos, R. da Costa Ilhéu Fontan, and L. S. Santos, "Partitioning of Amylase Produced by *Aspergillus niger* in Solid State Fermentation Using Aqueous Two-Phase Systems," *Process Biochemistry* 94 (2020): 116–125, https://doi.org/10.1016/j.procbio.2020.03.028.

42. K. J. Lanjekar and V. K. Rathod, "Response Surface Methodology for Optimisation of Glycyrrhizic Acid Extraction From *Glycyrrhiza glabra* in the Aqueous Two-Phase System," *Indian Chemical Engineer* 65 (2023): 38–50, https://doi.org/10.1080/00194506.2022.2085194.

43. J. A. Penido, A. B. Mageste, P. L. Martins, and G. M. D. Ferreira, "Surfactant as Selective Modulator in the Partitioning of Dyes in Aqueous Two-Phase Systems: A Strategy for Separation," *Journal of Molecular Liquids* 293 (2019): 111501, https://doi.org/10.1016/j.molliq.2019.111501.

44. I. D. C. B. Muniz, R. M. de Oliveira, and A. N. Alves, "Partition of Porcine Pancreatic Lipase in ATPS Composed of PEG/Salt and Alcohol/Salt: A Thermodynamic Study," *Brazilian Journal of Chemical Engineering* 40 (2023): 873–883, https://doi.org/10.1007/s43153-022-00269-9.

45. T. Carvalho, P. V. Finotelli, R. C. F. Bonomo, M. Franco, and P. F. F. Amaral, "Evaluating Aqueous Two-Phase Systems for *Yarrowia Lipolytica* Extracellular Lipase Purification," *Process Biochemistry* 53 (2017): 259–266, https://doi.org/10.1016/j.procbio.2016.11.019.

46. V. Ramakrishnan, L. C. Goveas, N. Suralikerimath, C. Jampani, P. M. Halami, and B. Narayan, "Extraction and Purification of Lipase from *Enterococcus faecium* MTCC5695 by PEG/Phosphate Aqueous-Two Phase System (ATPS) and Its Biochemical Characterization," *Biocatalysis and Agricultural Biotechnology* 6 (2016): 19–27, https://doi.org/10.1016/j.bcab.2016.02.005.

47. N. B. Melani, E. B. Tambourgi, and E. Silveira, "Lipases: From Production to Applications," *Separation & Purification Reviews* 49 (2020): 143–158.

48. P. L. Show, C. P. Tan, and M. Shamsul Anuar, "Extractive Fermentation for Improved Production and Recovery of Lipase Derived from *Burkholderia cepacia* Using a Thermoseparating Polymer in Aqueous Two-Phase Systems," *Bioresource Technology* 116 (2012): 226–233, https://doi.org/10.1016/j.biortech.2011.09.131.

49. M. M. Bradford, "A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding," *Analytical Biochemistry* 72 (1976): 248–254, https://doi.org/10.1016/0003-2697(76)90527-3.

50. U. K. Winkler and M. Stuckmann, "Glycogen, Hyaluronate, and Some Other Polysaccharides Greatly Enhance the Formation of Exolipase by Serratia marcescens," *Journal of Bacteriology* 138 (1979): 663–670, https://doi.org/10.1128/jb.138.3.663-670.1979.

51. P.-Å. Albertsson, "Partition ofcell particles and macromolecules in polymer two-phases systems", *Advances in Protein Chemistry* 24 (1970): 309–341, https://doi.org/10.1016/S0065-3233(08)60244-2.

52. M. Hu, Q. Zhai, Z. Liu, and S. Xia, "Liquid−Liquid and Solid−Liquid Equilibrium of the Ternary System Ethanol + Cesium Sulfate + Water at (10, 30, and 50)°C," *Journal of Chemical & Engineering Data* 48 (2003): 1561–1564, https://doi.org/10.1021/je0301803.

53. J. C. Merchuk, B. A. Andrews, and J. A. Asenjo, "Aqueous Two-Phase Systems for Protein Separation," *Journal of Chromatography B: Biomedical Sciences and Applications* 711 (1998): 285–293, https://doi.org/10.1016/S0378-4347(97)00594-X.

54. M. Hishida, R. Kanno, and T. Terashima, "Hydration State on Poly(ethylene glycol)-Bearing Homopolymers and Random Copolymer Micelles: In Relation to the Thermoresponsive Property and Micellar Structure," *Macromolecules* 56 (2023): 7587–7596.

55. A. C. de Oliveira, F. H. B. Sosa, M. C. da Costa, E. D. S. M. Filho, and R. Ceriani, "Study of Liquid-Liquid Equilibria in Aqueous Two-Phase Systems Formed by Poly (ethylene glycol) (PEG) and Sodium Thiosulfate Pentahydrate (Na2S2O3.5H2O) at Different Temperatures," *Fluid Phase Equilibria* 476 (2018): 118–125, https://doi.org/10.1016/j.fluid.2018.07.035.

56. K. Akhter, I. Zaheer, A. Bibi, U. A. Awan, A. Rasheed, and Z. Anjum, "Lipase Separation From a New Source of Bacillus Cereus NC7401 Using a Cost-Effective and Eco-Friendly Approach of Surfactant/Xylitol Biphasic System," *Process Biochemistry* 157 (2025): 291–299, https://doi.org/10.1016/j.procbio.2025.08.001.

57. A. I. S. Brígida, F. S. Buarque, and V. L. R. Nogueira, "Partial Purification of Crude Lipase Extract From *Yarrowia Lipolytica*: Precipitation, Aqueous Two-Phase Systems (ATPS), and Immobilization Methods," *Cleaner Chemical Engineering* 6 (2023): 100105.

58. M. Anvari, "Extraction of Lipase From Rhizopus Microsporus Fermentation Culture by Aqueous Two-Phase Partitioning," *Biotechnology*

*& Biotechnological Equipment* 29 (2015): 723–731, https://doi.org/10.1080/13102818.2015.1042406.

59. J. D. C. Silva, P. R. L. de França, and T. S. Porto, "Optimized Extraction of Polygalacturonase From *Aspergillus Aculeatus* URM4953 by Aqueous Two-Phase Systems PEG/Citrate," *Journal of Molecular Liquids* 263 (2018): 81–88, https://doi.org/10.1016/j.molliq.2018.04.112.

60. D. Y. Y. Tang, D.-T. Chu, and L. T. Nhi-Cong, "Investigation and Screening of Mixed Microalgae Species for Lipase Production and Recovery Using Liquid Biphasic Flotation Approach," *Journal of the Taiwan Institute of Chemical Engineers* 142 (2023): 104646, https://doi.org/10.1016/j.jtice.2022.104646.

61. K. Akhter, I. Zaheer, A. Bibi, U. A. Awan, A. Rasheed, and Z. Anjum, "Lipase Separation from a New Source of Bacillus Cereus NC7401 Using a Cost-Effective and Eco-Friendly Approach of Surfactant/Xylitol Biphasic System," *Process Biochemistry* 157 (2025): 291–299, https://doi.org/10.1016/j.procbio.2025.08.001.

62. S. Y. Lee, I. Khoiroh, J. A. P. Coutinho, P. L. Show, and S. P. M. Ventura, "Lipase Production and Purification by Self-Buffering Ionic Liquid-Based Aqueous Biphasic Systems," *Process Biochemistry* 63 (2017): 221–228, https://doi.org/10.1016/j.procbio.2017.08.020.

## Supporting Information

Additional supporting information can be found online in the Supporting Information section.

**Supporting File**: slct71994-sup-0001-SuppMat.docx.