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Process Integration for the Disruption of *Candida guilliermondii* Cultivated in Rice Straw Hydrolysate and Recovery of Glucose-6-Phosphate Dehydrogenase by Aqueous Two-Phase Systems

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Abstract Remaining cells of *Candida guilliermondii* cultivated in hemicellulose-based fermentation medium were used as intracellular protein source. Recovery of glucose-6-phosphate dehydrogenase (G6PD) was attained in conventional aqueous two-phase systems (ATPS) was compared with integrated process involving mechanical disruption of cells followed by ATPS. Influences of polyethylene glycol molar mass (M_{PEG}) and tie line lengths (TLL) on purification factor (PF), yields in top (Y_T) and bottom (Y_B) phases and partition coefficient (K) were evaluated. First scheme resulted in 65.9 % enzyme yield and PF of 2.16 in salt-enriched phase with clarified homogenate (M_{PEG} 1500 g mol^{-1}, TLL 40 %); Y_B of 75.2 % and PF_B of 2.9 with unclarified homogenate (M_{PEG} 1000 g mol^{-1}, TLL 35 %). The highest PF value of integrated process was 2.26 in bottom phase (M_{PEG} 1500 g mol^{-1}, TLL 40 %). In order to optimize this response, a quadratic model was predicted for the response PF_B for process integration. Maximum response achieved was PF_B=3.3 (M_{PEG} 1500 g mol^{-1}, TLL 40 %). Enzyme characterization showed G6P Michaelis-Menten constant (K_M) equal 0.07–0.05, $NADP^+$ K_M 0.02–1.98 and optimum temperature 70 °C, before and after recovery. Overall, our data confirmed feasibility of disruption/extraction integration for single-step purification of intracellular proteins from remaining yeast cells.

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Introduction

Currently, there is an increased scientific and commercial interest in the development of feasible strategies to convert renewable raw materials (e.g., rice straw, sugar cane bagasse) into conventional or new products, driven by environmental concerns [1]. Different species of yeast have been used in bioprocess based on hydrolysates of raw material as cultivation medium, being *Candida guilliermondii* and *Pichia stipitis* some of those able to convert D-xylose, present in the hemicellulose fraction of biomass, into xylitol and ethanol, respectively [1, 2]. Therefore, if these cells were not disposable, they could represent a potential source of important intracellular enzymes such as xylose reductase, xylitol dehydrogenase [3], and glucose-6-phosphate dehydrogenase [2]. Previously, a work was published to elucidate the behavior of key enzymes of xylose metabolism in the xylitol production by *C. guilliermondii* grown in hemicellulose hydrolysate and the feasibility of an integration process of xylitol production and glucose-6-phosphate dehydrogenase extraction from biomass [4].

Glucose-6-phosphate dehydrogenase (G6PD, EC1.1.1.49, β-D-glucose-6-phosphate; NADP oxido-reductase) is an intracellular enzyme of pentose phosphate pathway. This pathway has three important functions: the production of reducing equivalents in the form of NADPH for anabolic activities; the production of pentose phosphates necessary for nucleotide biosynthesis as well as the entry of pentoses into the glycolytic pathway. The properties of oxido-reductase enzymes considerably increased their importance and application. Owing to its high catalytic activity and substrate specificity, G6PD has been extensively used as reagent in varied enzymatic assays aiming determinations of ATP and hexose concentrations [5]. Besides, using G6PD in enzyme-based biosensor would be a good option due to advantages as specific measurement of target analyte in complex matrices (e.g., blood, food products, and environmental samples) [6, 7]. However, the potential application of G6PD requires an efficient strategy to its recovery from remaining yeast cells of fermentation medium and, although particularly abundant in *Saccharomyces cerevisiae*, a baker or brewer yeast, *C. guilliermondii* could be a feasible option of this enzyme source.

The purification of target products from fermentation broth and other biological feedstock still remains as a bottleneck. Traditional procedures including salt and organic precipitation, filtration, dialysis and chromatography methods or a combination of these are expensive, time consuming and difficult to scale-up. In addition, depending on the type and usage of the target product, the excessive number of steps will compromise the recovery process. During the last decade, several work using biphasic systems were performed to recover various biological materials, mainly enzymes including α-amylase, papain, glyceraldehyde 3-phosphate dehydrogenase, penicillin acylase, xylanase, and xylose reductase [8–14]. Partitioning in aqueous two-phase systems (ATPS) is recognized as an attractive and powerful technique for primary separation and purification step in overall enzyme/protein recovery. Separation in such system can be accomplished without sophisticated equipment and presents low cost phase forming materials. In areas such as extractive fermentations, ATPS also allows process integration, of both product formation (e.g., extracellular enzymes) and purification into a single-step process, facilitating instant recovery of biomolecules as they are formed [15, 16]. Conversely, recovery of intracellular enzyme must be preceded by its isolation after cell disruption and extract

clarification, adding two steps to the downstream process. It may be noted that, in addition to standardized disruption method, ATPS seems to be an ideal technology where clarification, concentration and partial purification of the target product can be integrated to meet specific objectives that are not efficiently achieved by discrete unit operations [17].

Despite of several advantages of ATPS and due to the multiple factors that influence it, experiments are necessary to find an optimal system for a target product recovery [17, 18]. Statistical design of experiments can be used to identify the main parameters that significantly affect ATPS [16]. Although ATPS has been extensively studied, there are few works about process integration using systems that include separation and partial purification of intracellular products. A representative model for the recovery of a *S. cerevisiae* (baker's yeast) intracellular enzyme by downstream process integration showed feasible [10]. In this sense, the present study deals with the pre-purification of a highly valued product obtained from *C. guilliermondii* grown in rice straw hemicellulose hydrolysate aiming to fulfill the requirements of biorefinery industry. Therefore, the main objective of this study was to develop a simple and optimized downstream process to recover glucose-6-phosphate dehydrogenase from remaining cells of fermentation broth. The evaluation of the recovery by conventional ATPS was compared with the integrated ATPS, accomplishing disruption, clarification and recovery in a single step.

Materials and Methods

Chemicals

Poly(ethylene glycol)/PEG 400, 1000, and PEG 1500 g mol^{-1} were purchased from Sigma-Aldrich (Brazil). Salts and all other chemicals were of analytical grade.

Rice Straw Hemicellulose Hydrolysate and *C. guilliermondii* Cultivation

Rice straw hemicellulose hydrolysate was prepared by acid hydrolysis, centrifuged and then concentrated in an evaporator (Precision Scientific, USA) under vacuum at 65 °C to obtain a xylose content of approximately 100 g L^{-1}. Carbon source concentration of 100 g L^{-1} is elevated and could be detrimental to the cell, during cultivation. As defined in previous work [2], the concentration of xylose in medium was 30 g L^{-1}. After cultivation, cells of *C. guilliermondii* FTI 20037 (ATCC 201935) were harvested by centrifugation (2000×*g*, 20 min), washed with 0.071 M Tris-HCl buffer, pH 7.5, centrifuged and resuspended in the same buffer solution for the attainment of approximately 11 g L^{-1} cell suspensions which were kept at −18 °C until used [2].

Preparation of Cell-Free Extract

Cells were disrupted in a vertical bead mill, 1.0 L capacity. A volume of 100 mL of 11 g L^{-1} cell suspension and 100 mL of glass beads (0.5 mm Ø) were added to reactor camera. Agitation was reached using a mechanical apparatus with 30 W potency (RW 20n, IKA®) under the following conditions: 400 rpm, 25 min, and 25±1 °C. Temperature during disruption was assured by refrigerated water circulation internally the reactor jacket. After disruption, glass beads were separated by filtration under vacuum using a flask with sinterized glass

membrane, and cell homogenate was subject to centrifugation at 2200×*g*, 15 min, and 4 °C for cell debris removal. The obtained cell-free extract was analyzed and applied in purification assays of G6PD using ATPS.

Aqueous Two-Phase System Preparation: Conventional and Integrated Recovery Process

Phase systems were prepared in a 20-mL graduated tube by adding appropriate amounts of stock solutions of phase forming components, polymer, and salt solutions, to reach the final concentration necessary for each assay (total mass of 10 g). For conventional ATPS, aliquots of 1 mL of cell-free extract or unclarified extract were added to the system in all assays and final mass was completed with deionized water. The tubes were stirred in a vortex and immersed in a thermostat water-bath, at 25 °C for 2 h until phase separation. For the integrated recovery process, ATPS were composed of 1.0 mL of *C. guilliermondii* suspension (11 g L^{-1}) and phase-forming components were added in a graduated tube to reach 10 g of total mass. Glass beads were also added, in a proportion of 1:1 of cell suspension. Tubes were stirred in a vortex during 5 min, with ice-bath intervals of 30 s. Then, glass beads were separated from liquid fraction after filtration, and this fraction was returned to tube. All system components were thoroughly mixed and phases were separated by settling for 2 h at the temperature of the experience (25±1 °C) in a water bath. Phase volumes were measured and total protein concentration and G6PD activity assayed. To take into account the interference of system components (PEG and phosphate) on these analytical techniques, all systems were analyzed against blanks of the same composition using distilled water instead of enzymatic extract. Each system was assayed in triplicate.

Enzyme Activity and Total Protein Concentration

G6PD activity was determined by the reduction of $NADP^+$ cofactor using spectrophotometer at 340 nm. The reaction medium was composed of: 600 μL of 0.071 M Tris-HCl buffer (pH 7.5) added with $MgCl_2$ (35 mM); 5 μL of $NADP^+$ (0.131 M); 10 μL of G6P (500 mM), and 100 μL sample. One activity unit (U) of G6PD was defined as the amount of enzyme to catalyze the reduction of 1 μmol of cofactor/min. The molar extinction coefficient used to calculate the enzyme activity was 6220 (M^{-1} L^{-1} cm^{-1}). Specific activities were based on total protein results. Total protein concentration was determined according to the method described by Bradford [19].

Determination of Tie Line Lengths

Binodal curves were determined by titration method [20] using stock solutions of poly(ethylene glycol) of molar mass 400, 1000, and 1500 of approximately 50 % (*w*/*v*) and a salt solution of K_2HPO_4/NaH_2PO_4 40 % (*w*/*v*), pH 7.5. TLLs were originated by systems localized on bifasic regions of binodal curves. Water content analysis was done by lyophilization and phosphate by sample calcination in a muffle at 400 °C during 2 h. Tie line lengths were calculated, following the equation:

$$\mathrm{TLL} = \sqrt{[C(P_1) - C(P_2)]^2 + [C(Q_1) - C(Q_2)]^2} \quad (1)$$

Where: $C(P)$ is the polymer concentration P in one of the phases, $C(Q)$ is the salt concentration in one of the phases, and numbers 1 and 2 defined both phases, top and bottom [20].

Experimental Design and Statistical Analysis

The influence of the variables molar mass of PEG and TLL over G6PD recovery using aqueous two-phase systems was investigated by a 2^2 full-factorial design with three repetitions at the center point and a 2^2 central composite design (only for process integration). The real and coded values of the variables for the experimental designs are given in Table 1. Statistical significance of the variables was determined at the 5 % probability level ($p<0.05$). The data obtained from the 2^2 central composite design were fitted to second-order polynomial equation. Statistical significance of the regression coefficients was determined by Student's test, and the proportion of variance explained by the model was given by R^2, the determination coefficient. Assays to validate the optimal extraction conditions were carried out in triplicate. Statistical analysis of the data and determination of conditions able to maximize primary recovery results (for process integration) were performed using the software Statistica (version 6.0) and *Design-Expert* (version 8.0).

Determination of ATPS Responses

The partition coefficient is defined as the ratio of the protein concentration or enzyme volumetric activity in the top phase (P_t and A_t) to that in the bottom phase (P_b and A_b), respectively:

$$K_P = \frac{P_t}{P_b} \quad \text{or} \quad K_E = \frac{A_t}{A_b} \tag{2}$$

The purification factor was calculated as the ratio of the specific activity in the top or bottom phase to the specific activity in the cell extract prior partition (A_i):

$$\mathrm{PF} = \left(\frac{A_{t/b}}{C_{t/b}} \right) \times \left(\frac{A_i}{C_i} \right) \tag{3}$$

Where $C_{t/b}$ and C_i are total protein concentrations expressed as milligrams per liter in the top or bottom phase and initial extract, respectively.

The enzyme activity yield was determined as the ratio of total activity in the top or bottom phase to that in initial extract and expressed as percentage:

$$Y = \left(\frac{A_{t/b} \times V_{t/b}}{A_i \times V_i} \right) \times 100 \tag{4}$$

Where $V_{t/b}$ and V_i are the volumes of the top or bottom phase and the initial extract, respectively.

Table 1 2^2 full-factorial design for G6PD recovery in conventional and integrated processes using ATPS

Process variables[a]		Conventional process[b]												Integrated process[b]					
TLL (% *w*/*w*)	PEG (MM)	Clarified homogenate						Unclarified homogenate						Disruption/extraction					
		K_E	K_P	PF_T	PF_B	Y_T (%)	Y_B (%)	K_E	K_P	PF_T	PF_B	Y_T (%)	Y_B (%)	K_E	K_P	PF_T	PF_B	Y_T (%)	Y_B (%)
30 (−1)	400 (−1)	–	–	1.04	–	93.9	–	3.63	1.62	1.91	0.85	134.3	14.1	0.94	0.48	0.67	0.35	48.2	13.1
40 (+1)	400 (−1)	–	–	1.01	–	79.4	–	6.67	1.24	1.11	0.21	88.6	4.9	0.60	0.37	0.50	0.32	29.2	12.1
30 (−1)	1500 (+1)	0.11	0.51	0.32	1.49	9.2	74.4	0.12	0.72	0.43	2.56	14.7	109.9	0.74	1.71	0.52	1.23	46.6	45.3
40 (+1)	1500 (+1)	0.07	0.83	0.18	2.16	7.1	65.9	0.07	0.64	0.28	2.32	9.4	71.4	1.36	4.60	0.67	2.26	67.5	28.3
35 (0)	1000 (0)	0.36	1.55	0.43	1.91	27.6	51.3	0.07	1.37	0.15	2.90	8.6	75.2	0.84	4.18	0.42	2.12	46.6	28.3

Studied responses (dependent variables): enzyme yield in top phase ($Y_{T;}$ %), enzyme yield in bottom phase ($Y_{B;}$ %), purification factor in top phase (PF_T), purification factor in bottom phase (PF_B), enzyme partition coefficient (K_E), and protein partition coefficient (K_P)

[a] Real and coded values

[b] Conventional process with clarified and unclarified homogenate, and integrated process (disruption-extraction) were carried out by an aqueous two-phase systems using the same independent variables (real and coded value)

G6PD Kinetics Parameters and Optimum Temperature Characterization

The effect of substrate and cofactor concentrations on enzyme activity was evaluated with crude and purified G6PD (before and after recovery in integrated process, respectively). The G6P (substrate) concentrations used were 5, 10, 25, 30, 50, 75, 150, 250, 300, 500, 2500, 7500, 10,000, and 12,500 μM; and $NADP^+$ (cofactor) were 150, 350, 500, 750, 1000, 1250, 1500, and 1750 μM. G6PD activity was determined as previously described. The results were treated according to the method of Lineweaver and Burk [21] in order to calculate the K_M and V_{max} values. The temperature effect was determined by incubating the G6PD solutions, before and after recovery, at temperatures ranging between 30 and 80 °C (increments of 5 °C). Samples were measured for G6PD activity.

Results and Discussion

Phase Diagrams and Tie Line Lengths

The use of aqueous two-phase polymer/polymer or polymer salt systems in bioprocessing research has been well-documented [18, 22]. Compared with other systems, polyethylene glycol (PEG)/salt have been studied extensively considering some advantages as low viscosity and low cost [22]. In this study, PEG/phosphate system was chosen to assess the partition behavior of glucose-6-phosphate dehydrogenase (G6PD), being the basic/acid pair of salt composed of K_2HPO_4/NaH_2PO_4 due to a greater solubility in comparison to Na_2HPO_4/KH_2PO_4 or a pair containing the same cations. The use of different molar ratios of phosphate salts of potassium and sodium do not affect binodal curve position. In addition, the high solubility of this system allows its use in salt stock solutions of elevated concentrations, required for industrial purpose [23].

Figure 1 shows the phase diagrams based on polyethylene glycol (PEG)/phosphate system used to calculate the tie line lengths (TLL) values aiming to evaluate G6PD purification in ATPS, which PEG concentrations varied in a range of 16 to 35 % (*w*/*v*) and phosphate concentration was kept constant at 12 % (*w*/*v*). As reported by Albertsson [20], ATPSs located close to the binodal curve exhibited certain sensitivity to changes in the system composition. In this sense, longer TLL (30, 35, and 40 %) were applied in the experimental study. The regions delineated by the curves demonstrated a two-phase zone, where top phase is polymer-rich and bottom phase is salt-rich. Under the curve, also nominated as uniform region, no phase separation occurred.

It can be observed that binodal curve shifts toward lower PEG concentration with increasing PEG molar mass (M_{PEG}), leading to an augmentation of the two-phase region at the experimental pH and considered salt concentration. This means that at higher M_{PEG}, lesser amounts of this polymer is necessary for phase separation and, consequently, TLL obtainment. Therefore, PEG presenting higher molar mass could be the best choice based on effective process cost reduction.

Primary Recovery of G6PD from Conventional and Integrated Processes Using ATPS

A 2^2 full-factorial design with different combinations of TLL and polyethylene glycol molar mass (M_{PEG}) was performed to study the partition behavior of glucose-6-phosphate

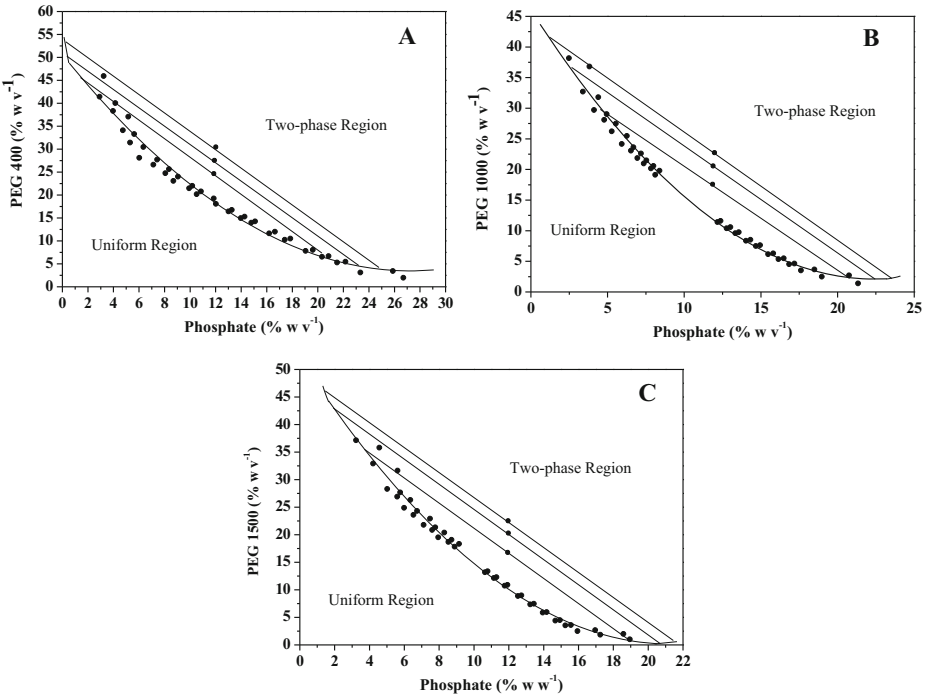

Fig. 1 Phase diagrams of systems composed by K_2HPO_4/NaH_2PO_4 phosphate buffer and PEG 400 (**a**), PEG1000 (**b**), and PEG1500 (**c**) at room temperature and pH 7.5. The phosphate buffer (salt) and PEG aqueous systems are designated by a binodal curve which separates the two-phase area from the single-phase zone. The *lines* in the binodal curve are nominated tie line lengths which presents values of 30, 35, and 40 % (*w*/*v*)

dehydrogenase (G6PD) in conventional and integrated processes using ATPS. Assays were separated according to extraction-disruption conditions into: conventional extraction process using ATPS with clarified homogenate, conventional extraction process using ATPS with unclarified homogenate and integration of extraction and disruption processes. The purification factor (PF), yields in top (Y_T) and bottom (Y_B) phases and partition coefficient (K) were used as response and the results are summarized in Table 1. It can be noticed that partitioning behavior of soluble proteins and enzyme G6PD were distinct in recovery assays carried out with clarified homogenate, unclarified homogenate or during integrated process.

The K represents the relationship between target molecule concentration into top phase and bottom phase, after equilibrium of both phases. This value is usually applied only to evaluate the extension of target molecule separation in ATPS. In general, it is desirable that partition coefficients of total proteins and target enzyme present different values, which could suggest that purification was reached. K value above 1.0 denotes that proteins and enzyme are preferably distributed to the upper phase [24]. As can be seen in Table 1, estimated values of K to G6PD (K_E) from clarified homogenate, were lower than those of proteins (K_P), although both were favorably transferred to the salt-enriched phase (bottom phase), leading to a decrease in partition coefficient. Calculation of K_E and K_P of PEG 400 and TLL 30 or 40 % (*w*/*v*) systems were not possible. In this case, total protein content and enzyme activity were too small making difficult the evaluation of its value by the selected method.

The partitioning behavior of bioproducts, such as proteins, is strongly influenced by PEG molar mass, M_{PEG} [17, 20]. This behavior is related to hydrophobic interaction between PEG chains and hydrophobic surface of protein. In general, increasing M_{PEG} produces an elevation of the exclusion effect due to a more compact conformation of such polymer; thus, the partition of protein into the top phase becomes hindered [25]. In assays with unclarified homogenate, the effect of size exclusion had a stronger influence, justifying the attained results: enzyme preference for the salt-enriched phase owing to M_{PEG} augmentation. Conversely, when using M_{PEG} of 400 g mol^{-1}, a volume exclusion effect might have not taken place, since there was space enough for the enzyme in the top phase of the systems as observed with the highest values of K_E (3.63 and 6.67) and K_P (1.62 and 1.24). In addition, proteins tend to become negatively charged when the pH of the system is higher than their isoelectric point and are preferentially partitioned to the top PEG-rich phase. Our results are in agreement with the findings of other authors [26, 27]. Silva and co-authors experiments results indicated that larger polymer size creates a repulsive effect on fibrinolytic protease partition, leading to a decrease in the coefficient partition [28]. Aside this, it was verified that the partitioning of G6PD for integrated process showed no common behavior due to the fact that the volume exclusion effect could not be considered satisfactory. In the case of PEG 400 g mol^{-1}, the decreasing trend observed at high TLL values might be attributed to the saturation of the volume available for proteins to be accommodated in the upper phase together with some fragments of cell produced from the disruption, despite of pH value. In particular, the effect of the negatively charged surface of G6PD was enhanced by cell fragments that remained at interphase and, thereby, preventing G6PD and protein migration to the bottom phase even increasing M_{PEG} (K_E=1.36 and K_P=4.60 in PEG 1500 g mol^{-1} and TLL 40 %).

G6PD purification factor was also influenced by M_{PEG} and TLL (Table 1). Purification factor in polymer enriched phase (PF_T) were higher in assays with PEG 400 decreasing with TLL increase. This suggests that even with low molar mass of PEG, the extension of TLL is associated with high polymer concentration, favoring the transfer of the enzyme to the bottom phase. Conversely, it can be noticed an improvement of those values in the bottom phase (PF_B) due to an increase in M_{PEG}. This behavior could be also explained taking into account the estimated molecular weight of activated G6PD dimer of approximately 132 kDa, which means that partitioning is much more sensitive to M_{PEG} for recovery of proteins higher than 50 kDa and that smaller molecules seem to remain in the top phase [29]. Under experimental conditions evaluated, the best value of purification factor was reached at unclarified homogenate in the salt-enriched phase (PF_B=2.90), being accompanied by an elevated yield of 75.2 % when using PEG of 1000 g mol^{-1} and TLL of 35 % (*w*/*v*).

As observed in all assays, G6PD yield varied from 7.1 to 134.3 % in the top phase and from 4.9 to 109.9 % in the bottom phase. It is possible to obtain low G6PD yields, since the enzyme tends to migrate to one phase instead of another or remains in the interphase. In fact, modification in the phase density causes a tendency in precipitate accumulation in the interphase or sediment formation [11].

In this study, although all assays had been performed in a single phosphate concentration, 12 % (*w*/*v*), the augmentation of TLL configured an increase over PEG concentration and the association with cell fragments could probably have modified enzyme solubility. On the other hand, yield values higher than 100 %, like some estimated to G6PD values, have frequently been reported for enzyme extraction using ATPS, presumably due to alteration of the structure of enzyme active site in the presence of PEG or elimination of inhibitors initially present in hemicellulose hydrolysate, hence favoring enzymatic activity [14]. In comparing the

conventional and integrated ATPS, it is essential to follow with a statistical analysis of the integrated process for evaluating the extent of its viability.

Statistical Analysis of Enzyme Partitioning of Integrated Process

Currently, process integration is of great practical and economic interest to bioproduct manufacture [16]. Direct product capture has been achieved by integrating clarification and recovery of intracellular enzymes with expanded bed adsorption (EBA), which is time and cost consuming [30]. Alternatively, early processing steps, such as cell disruption and clarification, could be operated in aqueous two-phase systems. However, the efficiency of the process integration could be closely related to several factors that affect partitioning behavior and enzyme activity of the homogenate in ATPS. The development of a purification procedure requires the variation of factors to get the optimum intracellular protein recovery, namely polymer concentration, polymer molar mass, pH, and temperature system. Other authors analyzed stability of G6PD, and their results allowed setting the parameters temperature and pH system in 25 °C and 7.5, respectively [5, 24]. Therefore, we used a 2^2 central composite design to identify which ATPS composition (independent variables such as M_{PEG} and TLL) is able to maximize G6PD purification factor and yield in the bottom phase (PF_B, Y_B) of integrated process (Table 2). In this study, we also aimed to determine a mathematical model to define enzyme purification. This response was chosen by the fact that higher values of PF were obtained in the salt-enriched phase, suggesting no increase of purity in the polymer enriched phase, as observed in average values of PF_T (<1.0).

It should be noticed that the most significant effect on the purification factor was the positive ones exerted by PEG molar mass, independently of TLL, which highlights that this parameter was improved by increases in this independent variable. In addition to the PF, another important dependent variable is the yield (%) that provides the total quantity of enzyme that can be obtained

Table 2 Responses of 2^2 central composite design for G6PD recovery assays using extraction-disruption process (integrated ATPS process)

Assay	Coded variables		Real variables		PF_B	Y_B
	TLL (%)	PEG (MM)	TLL (%)	PEG (MM)		
1	−1	−1	30	400	0.35	13.1
2	+1	−1	40	400	0.32	12.1
3	−1	+1	30	1500	1.23	45.3
4	+1	+1	40	1500	2.26	28.3
5	−1	0	25	1000	2.95	39.0
6	+1	0	40	1000	2.80	25.9
7	0	−1	35	400	0.41	17.5
8	0	+1	35	1500	3.31	40.9
9	0	0	35	1000	2.35	33.3
10	0	0	35	1000	2.10	28.2
11	0	0	35	1000	1.90	23.3

Studied responses (dependent variables): enzyme yield in bottom phase (Y_B; %) and purification factor in bottom phase (PFB)

in the phases. This parameter is related to the technological viability determination of downstream process [31]. It was verified a negative effect of TLL on the activity yield, while M_{PEG} was positive. As a consequence, the highest value of this response (Y_E=45.3 %) was obtained at the highest M_{PEG} (1500 g mol^{-1}) and the lowest TLL (30 %).

In order to verify the effect of each operational variable on the responses, Pareto charts were plotted, according to Fig. 2. In this figure, bars extending beyond the vertical line corresponded to the effects statistically significant at 95 % confidence level.

Graphical analysis of the effects of the independent variables and their related interactions showed that only PEG molar mass had a positive effect on both PF and enzyme yield in the bottom phase (Y_B), which means that these parameters were improved by increases in the independent variable. The exception was the interaction of M_{PEG} (quadratic), which presented a negative effect on PF_B. Its negative value points out that higher $M_{PEG,}$ in addition to lower PEG concentration, suggesting a decrease in TLL, promotes a maximum value of PF_B, although TLL had shown no significant effect on studied responses. These results are similar to the work of Sales and co-authors [16] that demonstrated, by statistical analysis, a significant effect of PEG concentration (negative) and the interaction of PEG molar mass (positive) with sodium sulfate concentration for fibrinolytic activity response in the bottom phase, during an integrated process production and extraction of the fibrinolytic protease from *Bacillus* sp. UFPEDA 485.

According to Farrugia et al. [26], there is an interaction between PEGs of low molar mass and the protein surface, which is conducted by van der Waals forces, thus favoring the protein transfer to the PEG enriched phase. In a different manner, with higher $M_{PEG,}$ the exclusion effect prevails, pushing the protein to the salt-enriched phase (bottom phase). These findings are in accordance with our results, and, although excluded volume had demonstrated a relationship with PEG concentration, in the current study, it was mainly due to the presence of cell debris during integrated process.

A regression analysis was carried out to fit a mathematical model to the experimental data, aiming to identify an optimal region for the given response. This analysis allowed determination of quadratic model, describing variation of the dependent variable PF_B as a function of the independent variables, TLL (X_1) and M_{PEG} (X_2), according to the final equation in terms of coded factors:

$$PF_B = 2.15 + 0.25X_1 + 0.89X_2 + 0.37X_1X_2 + 0.29{X_1}^2 - 1.19{X_2}^2$$

Analysis of variance demonstrated that the model was established with a coefficient of determination (R^2) equal to 0.85, meaning a close agreement between the experimental results and those predicted by the model. The lack of fit of the model was also evaluated, being found a ρ-value >0.05 (not significant).

The purification factor values of G6PD were presented in detail as a contour plot, in Fig. 3. As it can be observed, within the variable range investigated, PF_B reached their maximum value at M_{PEG} higher than 1000 g mol^{-1} and decreased below this molar mass threshold. Nevertheless, apart from the importance of PEG features, PF_B increased with extreme values of TLL. Probably, the variation constraint of salt concentration during TLL attainment reduced the effectiveness of integrated process optimization.

The model for response PF_B predicted a maximum value of 2.8±1.0 with an M_{PEG} of 1500 g mol^{-1} and a TLL of 40 %. To confirm the optimum operating conditions established for integrated process, a G6PD extraction run for model validation was carried out, reaching an average PF_B value of 3.5. Certainly, results prove that the model adjusted to experimental data, describing studied region for purification factor.

Maximum response achieved in this study was PF_B=3.3 for M_{PEG} 1500 g/mol and TLL 40 % with an enzyme activity yield of 41 %. Comparable values of PF were reported in the literature, aiming the recovery of different enzymes using conventional ATPS procedure, besides to higher yield values. For example, *Bacillus subtilis* α-amylase was recovered with PF=2.0 and *Y*=90 % by PEG/citrate system and from *Aspergillus oryzae* CBS 819.72 with PF=3.0 and *Y*=67 % [8, 17], *Candida mogii* xilose reductase with PF=1.5 and *Y*=105 % by

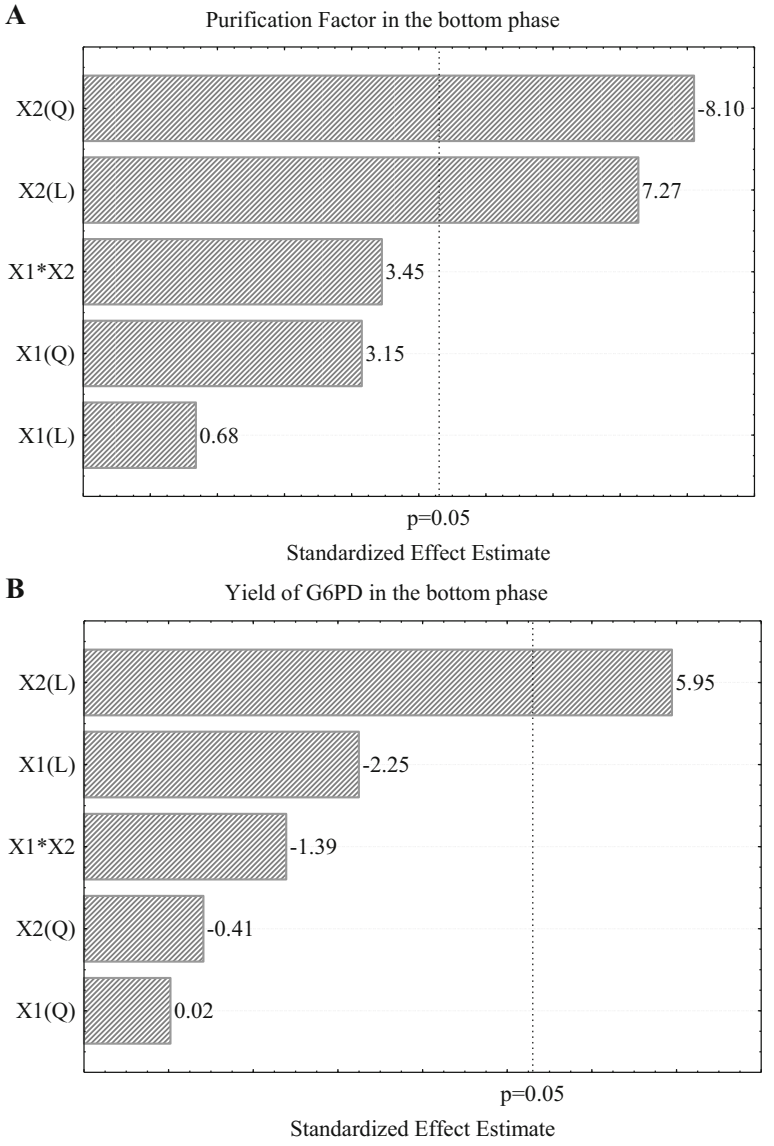

Fig. 2 Pareto Chart for the effects of the variables Tie line length (*X1*) and PEG molar mass (*X2*) on G6PD recovery during extraction-disruption process using purification factor (**a**) and enzyme yield in bottom phase (**b**) as studied responses. The letters (*L*) and (*Q*) correspond to the effects of variables of degree linear and quadratic, respectively

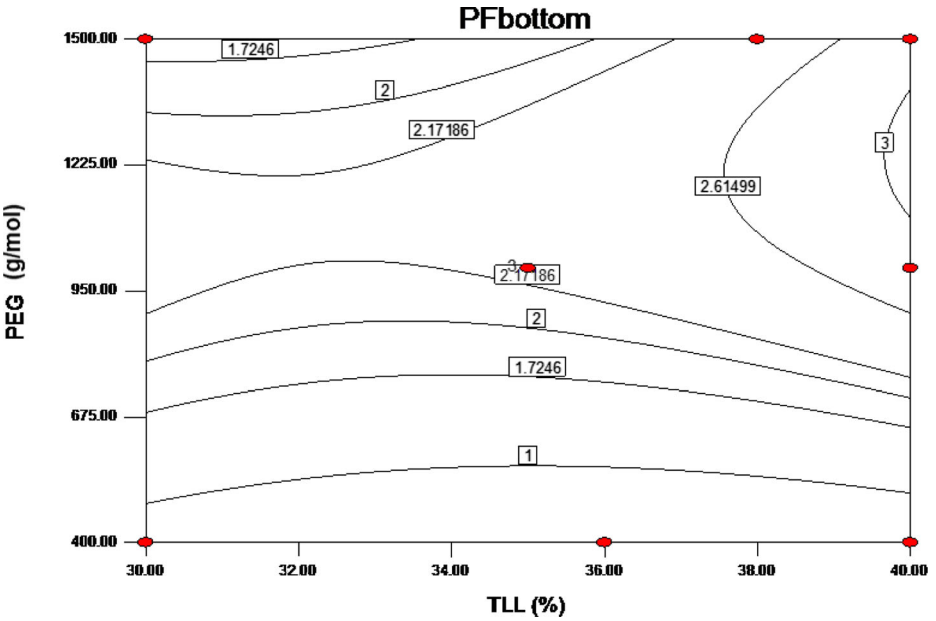

Fig. 3 Countor plot described by the model for the optimization of G6PD purification factor in the bottom phase of extraction-disruption process

PEG1000 and Na/*K* phosphate and TLL 34 [14], *Debaryomyces hansenii* xilose reductase with PF=3.1 and *Y*=131 % by PEG4000/sodium sulfate and TLL 25 [32] and protease with PF=4.2 and *Y*=131 % [33]. In contrast to similar PF, a decrease in the yields of integrated process may be attributed to a direct effect of the simultaneous extraction and disruption upon the final ATPS composition, and its phase separation, in addition to the impact on the efficiency of cell breakage.

G6PD purification from cells of various animals, vegetables and microorganisms species has been reported in the literature by using different purification methods, but mainly by the use of expensive chromatographic methods. Among previous trials of G6PD purification, a few reports listed the data of purification efficiency on each separation step. In one case of G6PD purification from bovine lens [34], the purification factor was increased about 8.3 times but the enzyme recovery yield was decreased to 28 % through the four purification steps which were first lenses disruption followed by two cycles of centrifugation (for debris removal) and chromatography using 2′,5′-ADP-sepharose 4B. In another case, an alternative process for the recovery of G6PD from *S. cerevisiae* reached a purification factor and enzyme yield of 2.2 and 115 %, respectively, using continuous counter-current liquid-liquid extraction by reversed micelles after yeast cell disruption and homogenate clarification [35]. Compared with those data, it is clearly demonstrated that the G6PD was recovered with a satisfactory purification factor and yield, mainly because of the cell disruption and ATPS integration into a single step and process time reduction (Table 3). We assumed that, in spite of the homogenate contains a variety of soluble and insoluble contaminants, this procedure was very simple, and it could be a good alternative to intracellular product recovery.

Table 3 Comparison of integrated and standard protocols for G6PD recovery from *Candida guilliermondii* cultivated in rice straw hydrolysate

Parameter in bottom phase	Integrated protocol	Standard protocol	
	Disruption/extraction	Clarified homogenate	Unclarified homogenate
Yield (%)	40.9	65.9	75.2
Purification factor	3.31	2.16	2.90
Unit operation after cell cultivation	Centrifugation	Centrifugation	Centrifugation
	Cell disruption/extraction in ATPS	Cell disruption/centrifugation	Cell disruption/extraction by ATPS
		Extraction by ATPS	
Step numbers	2	4	3

Kinetic Properties of G6PD and Optimum Activity Temperature Before and After Recovery in Integrated Process

To fulfill the studies in regard to purification process, kinetic parameters and optimum activity temperature were determined for crude and recovered G6PD. The recovered enzyme was obtained in ATPS integrated process optimum conditions (TLL 40 % and PEG 1500 g mol^{-1}) (Fig. 4a). For the substrate, G6PD showed Michaelis-Menten constant (K_M) values equal to 0.07 and 0.05 mM of G6PD, while displaying maximum velocity (V_{max}) values of 34.8 and 19.1 U L^{-1}, before and after recovery, respectively. These results indicate that the affinity of G6PD for G6P was not altered by the integrated process. On the other hand, cofactor $NADP^+$ showed K_M of 0.02 and 1.98 mM and V_{max} of 69.2 and 52.4 U L^{-1} for crude and recovered G6PD, respectively. Ulusu et al. [34] studied the purification of glucose-6-phosphate dehydrogenase from bovine lens by affinity chromatography and observed that the purified enzyme had K_M of 0.008 and 0.035 mM for $NADP^+$ and G6P, respectively. Hasmann et al. [5] showed that Michaelis constant for G6PD of *S. cerevisiae* was almost independent of the presence of cell debris (K_M=49.3–49.4 μM), although these results were not in accordance with ours. Nevertheless, the present data are in good agreement with those of G6PD from *Aspergillus aculeatus* (75 μM) [36].

The effect of temperature on G6PD was investigated. Since the optimum temperature for an enzyme assay is the maximum temperature at which the biological catalyst exhibits a constant activity over a period as long as assay time [34], the optimum temperature to volumetric activity of G6PD, in both cases, was 70 °C, as can be seen in Fig. 4b. At 30 °C, similar temperature recommended by the literature as ideal for G6PD catalytic dosage [37], enzyme activity was 110 U L^{-1} before recovery and 57 U L^{-1} after process integration. Temperature promoted a G6PD activity augmentation until 70 °C (571 and 160 U L^{-1}, respectively), being the values drastically reduced after that. As reported in other studies [5, 35], and despite G6PD have been obtained from mesophilic microorganisms (*C. guilliermondii* in present study and *S. cerevisie* in cited works), this intracellular enzyme is relatively stable to temperature.

Conclusions

This study demonstrated that simultaneous disruption and aqueous two-phase extraction can achieve the primary recovery of intracellular proteins from yeast cultivated in rice straw

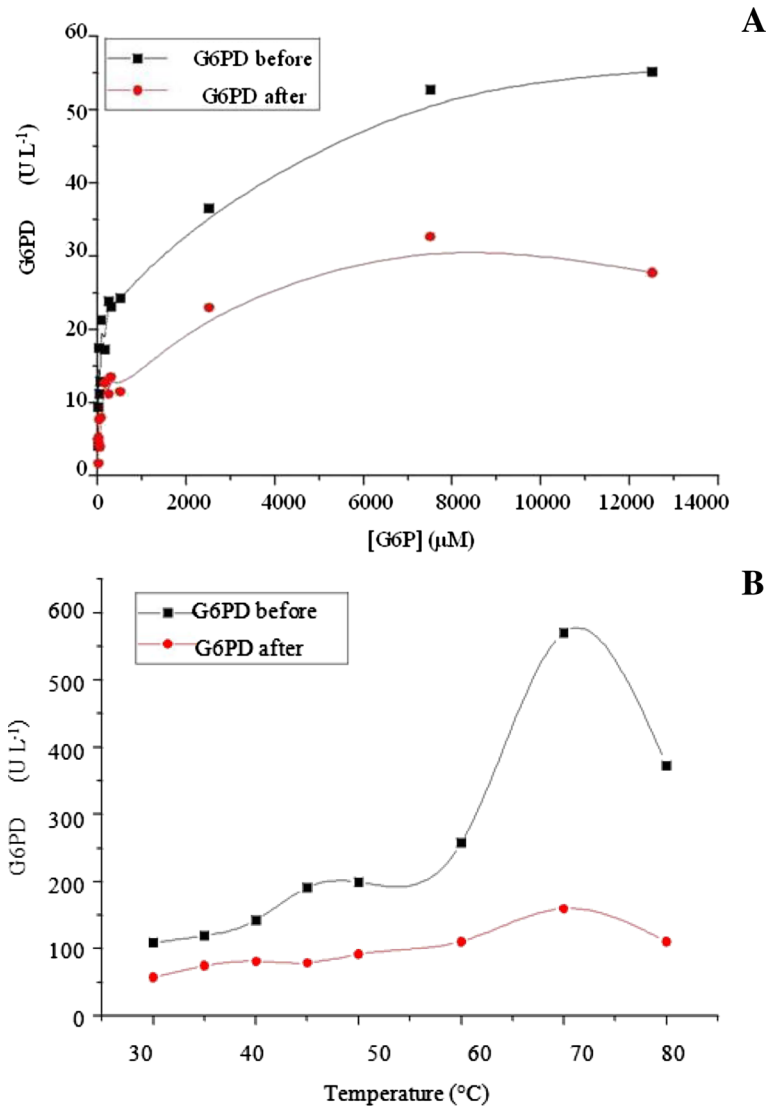

Fig. 4 Variation of G6PD activity as a function of substrate concentration, G6P (**a**) and to the temperature (**b**) before and after recovery in integrated process

hemicellulose hydrolysate, in order to add value to overall bioconversion process. Process integration strategy followed by establishment of operating conditions promoted in situ primary recovery of G6PD with a significant degree of purification regarding the reduction of bulk protein and elimination of cell debris in a single operation. However, further studies are required to improve the yield beyond the current 41 %, without impairing fractionation and product purity. Probably, the present method could yield larger amounts of G6PD if combined with an efficient expression system in genetically modified microorganism cell. Owing to the simplicity and effectiveness, ATPS demonstrated to be feasible for a single-step purification of protein, suggesting a cost reduction of downstream process.

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