



Recovery and purification of cellulolytic enzymes from *Aspergillus fumigatus* CCT 7873 using an aqueous two-phase micellar system

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Abstract

Purpose: In this study, an aqueous two-phase micellar system (ATPMS), formed by the non-ionic surfactant Triton X-114, was used to investigate the partitioning of cellulolytic enzymes produced by the filamentous fungus *Aspergillus fumigatus* CCT 7873.

Methods: Performance of the ATPMS on the partitioning of CMCase (activity on carboxymethyl cellulose) and FPase (activity on filter paper) was investigated by varying the temperature (35, 40, 45, 50, 55, 60, and 65 °C), enzyme crude extract concentration (20, 40, 60, and 80% w/w), and Triton X-114 concentration (2, 4, 6, and 8% w/w) and by adding different inorganic salts (NaCl, CaCl₂, MgSO₄, and MnSO₄) in the system.

Results: An ATPMS formed with 8% (w/w) Triton X-114 and 40% (w/w) enzymatic crude extract at a system temperature of 55 °C was most favorable for partitioning the tested enzymes. Under these conditions, a purification factor for CMCase and FPase of 10.89 and 0.65 was reached, respectively. The addition of inorganic salts changed the distribution of enzymes. Of these, CaCl₂ contributed to a higher distribution coefficient (50.0), whereas for FPase, the presence of MnSO₄ in the system improved the purification factor to 3.94.

Conclusion: The highest values obtained for the yield and purification factors demonstrate that ATPMS is an interesting option for recovering and purifying cellulolytic enzymes.

Keywords: *Aspergillus fumigatus*, Cellulases, Micelles, Liquid-liquid extraction, Downstream processing

Introduction

Brazil is a large producer of agro-industrial products, and thus it generates a lot of lignocellulosic residues (Machado et al. 2010). In general, these residues are rich in cellulose, hemicelluloses, and lignin. Additionally, they are a cheap and abundant (Castilho et al. 2000). Cellulose can be used to produce value-added products, such as second-generation ethanol. In this case, pretreatment

and fermentation processes are followed by a distillation step in order to obtain the product (Sousa et al. 2009). Also, depending on the microorganism and the type of fermentation used, important products such as enzymes and pigments can be obtained mainly by using solid-state fermentation (SSF). Using SSF, microorganism growth is carried out in the absence or near absence of free water, occurring on a solid surface (Ruiz et al. 2012). In general, this approach leads to a more concentrated extract, i.e., the metabolites produced are more concentrated, and also has a lower energy demand compared to the classical submerged fermentation. However,

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process variables, such as temperature and pH, are more difficult to control when SSF is used.

Among the enzymes that can be produced using ligno-cellulosic residues, cellulases play a key role in products such as textile, chemicals, and animal feed. Cellulases consist of a group of endo- and exo-enzymes acting synergistically in order to convert cellulose into glucose (Gan et al. 2003; Chandra Kalra et al. 2010). On the other hand, large-scale enzyme production, as well as the production of other biomolecules, depends on the techniques used during the purification protocol. Obviously, the final use dictates the purification degree needed for the biomolecule. Additionally, downstream processing represents up to 80% of the total cost of the process (McCreath et al. 1995, Sousa Júnior et al. 2016). Thus, integrative techniques play a key role in downstream processing. One such technique involves aqueous two-phase micellar systems (ATPMS). This is a liquid-liquid extraction technique that uses surfactants with a concentration higher than the critical micelle concentration (CMC), i.e., a concentration at which the surfactant does not occur as a monomer but rather as a micelle-structure. Additionally, the formation of micelles is related to the equilibrium of intermolecular forces, including Van der Waals, hydrophobic, steric, and electrostatic ones (Liu et al. 1998). Therefore, once these self-assembling aggregates are achieved, a biphasic system is built (Liu et al. 1996), which can be exploited for biomolecule separation/purification. In this case, this system is advantageous since it is able to maintain the activity of the biomolecules, i.e., it is environmentally friendly. The migration of the biomolecules depends on their miscibility to the micelle-rich phase or to the micelle-poor phase (Ramelmeier et al. 1991). The ATPMS is based on the use of a surfactant in order to form two immiscible phases, a bottom phase that is rich in micelles and an upper phase that is poor in micelles (for the systems shown here). The formation of the two phases is usually implemented by changing the temperature, also known the cloud point, which is used to determine the coexistence curve (Rangel-yagui et al. 2004). In these systems, the temperature, along with parameters such as pH, surfactant concentration, and the presence of salts, can induce the two-phase formation (Ooi et al. 2011). The main surfactant used is a nonionic one, such as Triton X-114 (Wang et al. 2013).

In this context, due to the importance of reducing the costs of downstream processing by exploiting integrative techniques, this study investigated the partitioning behavior of cellulases using an ATPMS formed by a nonionic surfactant (Triton X-114), inorganic salts, and crude extract at different concentrations. The coexistence curves, i.e., the formation of a cloud point as a function of the surfactant concentration, were obtained

for every condition assayed. There are few studies in the literature that have applied ATPMS to recover and purify cellulolytic enzymes, such as CMCCase (activity on carboxymethyl cellulose) and FPase (activity on filter paper).

Material and methods

Chemicals

Bovine serum albumin (BSA), the nonionic surfactant Triton X-114, and carboxymethyl cellulose (CMC) were acquired from Sigma-Aldrich (Ohio, USA). A low molecular weight marker was acquired from GE healthcare (Sweden). The inorganic salts CaCl_2 , MgSO_4 , MnSO_4 , and NaCl were of analytical grade. Ultrapure water was obtained from a Milli-Q system.

Sugar cane bagasse

Sugar cane bagasse was acquired from Estivas sugar plants (Rio Grande do Norte-Brazil). After an initial screening, the bagasse was washed and dried using a tray dryer at 70 °C for 5 days. Then, it was milled (Willye, TE-680, Tecnal, São Paulo/Brazil) and sifted through a 20-mesh sieve. The main composition of the sugar cane bagasse, in percentages, consisted of cellulose (39.25 ± 5.49), hemicellulose (25.20 ± 1.13), and lignin (18.82 ± 0.01). The material used in this study was the same material reported by Oliveira et al. (2018).

Microorganism and inoculum

The filamentous fungus *Aspergillus fumigatus*, isolated from coconut shells (identified and registered at the André Tosello Foundation (Campinas/Brazil) as CCT 7873), was used to produce the cellulolytic enzymes and was kept in the Biochemical Engineering Laboratory of the Federal University of Rio Grande do Norte (Natal/Brazil). For the inoculum, *A. fumigatus* CCT 7873 was transferred to potato dextrose agar (PDA) medium and incubated at 30 °C for 5 days. Spore propagation to SSF was carried out by transferring 1.0 mL of Tween 80 (0.2% v/v) containing microorganisms from the Petri plate and incubating it in a Biochemical Oxygen Demand (BOD) machine (Model: TE-394/I, TECNAL-São Paulo/Brazil) at 30 °C for 7 days. The spore concentration was determined using a Neubauer counting-chamber. The spore concentration used as an inoculum for the SSF experiments was 1×10^6 spores/gram of solid medium (Coelho et al. 2001).

Semi-solid fermentation (SSF) and crude enzymatic extract

The cellulolytic crude enzymatic extract was produced by the filamentous fungus *A. fumigatus* CCT 7873 by SSF using sugarcane bagasse as a substrate (50% moisture with water activity of 0.973) and adding nutrient

salting solution (pH 4.5) according to Urbanszki et al. (2000). After 120 h of cultivation, the crude extract was harvested from the flasks. In this case, 35 mL acetate buffer (200 mM, pH 5.0) was added to 5.0 g fermented medium using a glass rod. Next, enzyme extraction was carried out using acetate buffer under shaking (Tecnal, TE-421, São Paulo/Brazil) for 30 min at 160 rpm and 30 °C (Coelho et al. 2001). Then, the extract was filtered and centrifuged for 10 min at 2000 rpm and 20 °C. The supernatant containing the cellulolytic enzymatic extract was stored and used in the ATPMS.

Cloud point determination

The ATPMS cloud point was determined by visual identification, i.e., by observing the system conditions in which the first turbation occurred. The determination of the cloud point for the solution containing the surfactant was carried out as described by Watanabe and Tanaka (1978). It consisted of the visual identification of the temperature at which a given surfactant solution with a known concentration became cloudy. The mixtures were added to conical tubes and transferred to a water bath. The temperature was incremented by 0.1 °C stepwise every 20 min.

Aqueous two-phase micellar system

The cellulolytic enzymes were partitioned using an ATPMS composed of Triton X-114 at concentrations of 2, 4, 6, and 8% (w/w). Additionally, we investigated the impact of temperature (35, 40, 45, 50, 55, 60, and 65 °C), extract concentration (20, 40, 60, and 80%), and inorganic salts (NaCl, CaCl₂, MgSO₄, and MnSO₄), all at

5.0% (w/w), on the partitioning of the cellulolytic enzymes using ATPMS. The pH of the system was kept at 5.0. For the formation of a micellar system, a given quantity of Triton X-114, enzyme extract, and salt was added to a centrifugation tube (15 mL) for a final mass of 5.0 g. Deionized water was used to complement the final mass. The tube was shaken for 1 min and phase separation occurred by settling it for 3 h in a water bath at a given temperature. The phase volume (top and bottom) was measured, and sampling of the phases was performed for quantification of enzymatic activity (CMCase and FPase) and total protein, which permitted the calculation of the partition coefficient, yield, and the purification factor for the cellulases.

Partition coefficient (*K*), yield (*Y*), and purification factor (PF)

The partition coefficient (*K*) for the enzymes in the ATPMS was calculated according to Eq. (1):

$$K = \frac{A_t}{A_b} \quad (1)$$

where *A_t* is the enzymatic activity in the top phase (micelle-poor phase) and *A_b* is the enzymatic activity in the bottom phase (micelle-rich phase).

The enzyme yield (*Y*) in the top phase was obtained as shown by Eq. (2):

$$Y = \left(\frac{A_t V_t}{A_i V_i} \right) \times 100 \quad (2)$$

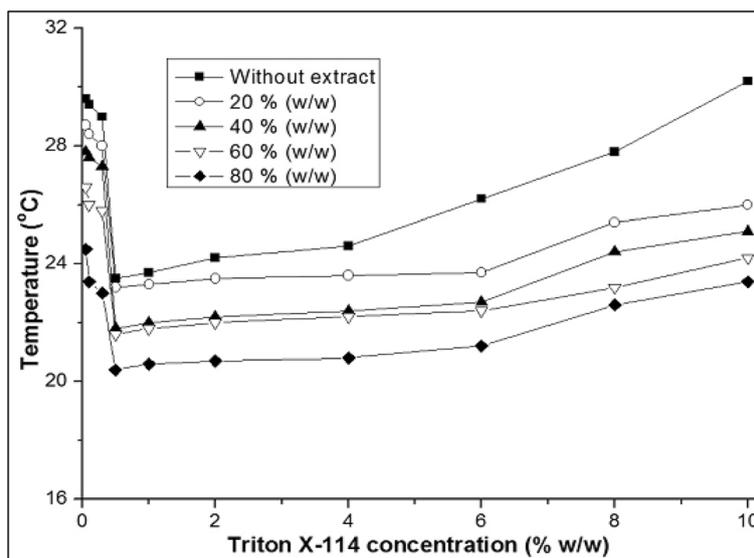


Fig. 1 Coexistence curves for the aqueous two-phase micellar system (ATPMS) with varying enzymatic extract (0 to 80% (w/w)) and Triton X-114 (2, 4, 6, 8, and 10% (w/w)) concentrations at different temperatures

where V_t and V_i are the top and initial volumes, respectively.

The purification factor was estimated with regard to the specific activity as described in Eq. (3):

$$PF = \frac{A_t/C_t}{A_i/C_i} \quad (3)$$

where C_t and C_i are the total protein at top phase and the initial concentration in the fermented broth, respectively.

Cellulase activity (CMCase and FPase) and total protein quantification

The CMCase activity was determined by adding 0.5 mL of enzyme extract to 0.5 mL of CMC 4% (w/w) in citrate buffer (50.0 mM, pH 4.8), according to Ghose (1987).

The enzymatic reaction was performed for 10 min at 50 °C. The mixture was heated in a water bath for 10 min at a temperature adjusted to 50 °C. The reaction was stopped by boiling and the formation of reducing sugars was analyzed in a spectrophotometer (Thermo Spectronic) using the 3,5-dinitrosalicylic acid method (Miller 1959) with D-glucose as standard. A unit (U) of CMCase was defined as the enzyme quantity necessary to produce 1.0 μmol of D-glucose per minute under the assay conditions. The quantification was performed in duplicate and the results shown are the means.

The FPase activity was quantified by adding 0.5 mL of the enzymatic extract to 1.0 mL of citrate buffer (50.0 mM, pH 4.8) containing filter paper strip (Whatman No. 1) (1.0 cm × 6.0 cm) according to Ghose (1987). The mixture was heated in a water bath for 60 min and the formation of reducing sugars was analyzed in a

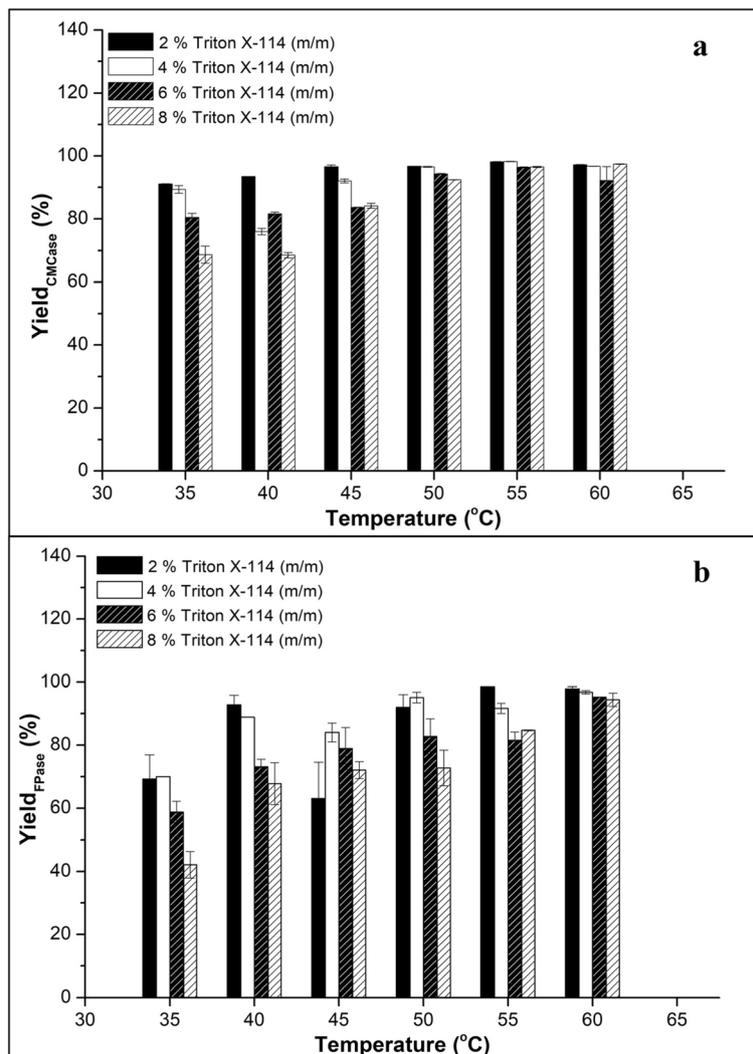


Fig. 2 CMCase (a) and FPase (b) yields obtained during partitioning in the ATPMS using different Triton X-114 concentrations and changing the temperature from 35 to 65 °C at 40% enzymatic extract concentration

spectrophotometer (Thermo Spectronic) using the 3,5-dinitrosalicylic acid method (Miller 1959), with D-glucose as a standard. A unit (U) of FPase was defined as the enzyme quantity necessary to produce 1.0 μmol of D-glucose per minute under the assay conditions. The quantification was performed in duplicate and results shown are the means.

The total protein content was determined at 595 nm according to Bradford (1976). Bovine serum albumin (Sigma-Aldrich, Ohio, USA) was used as a standard. The quantification was performed in triplicate and the results shown are the means.

Polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis using denaturing condition (SDS-PAGE) was performed using 10% (w/v) acrylamide according to Laemmli (1970). The protein bands were stained using

silver nitrate. A low molecular protein weight marker (GE healthcare, Sweden) was used in order to estimate the molecular mass of the proteins.

Zymogram

A zymogram was obtained by adapting the method proposed by Takenaka et al. (1999). In summary, the samples were dialyzed overnight and concentrated (1.0 mg/mL), and were then submitted to a PAGE by adding 0.2% CMC to a gel prepared with Tris-HCl (1.5 M, pH 8.8) for 3 h at 4 °C.

In order to observe the cellulolytic activity, the gel was shaken and incubated using sodium citrate buffer (50.0 mM, pH 4.8) at 50 °C for 1 h. Next, the gel was washed using distilled water, and was then immersed and shaken in congo red dye solution (0.1% v/v) for 20 min and

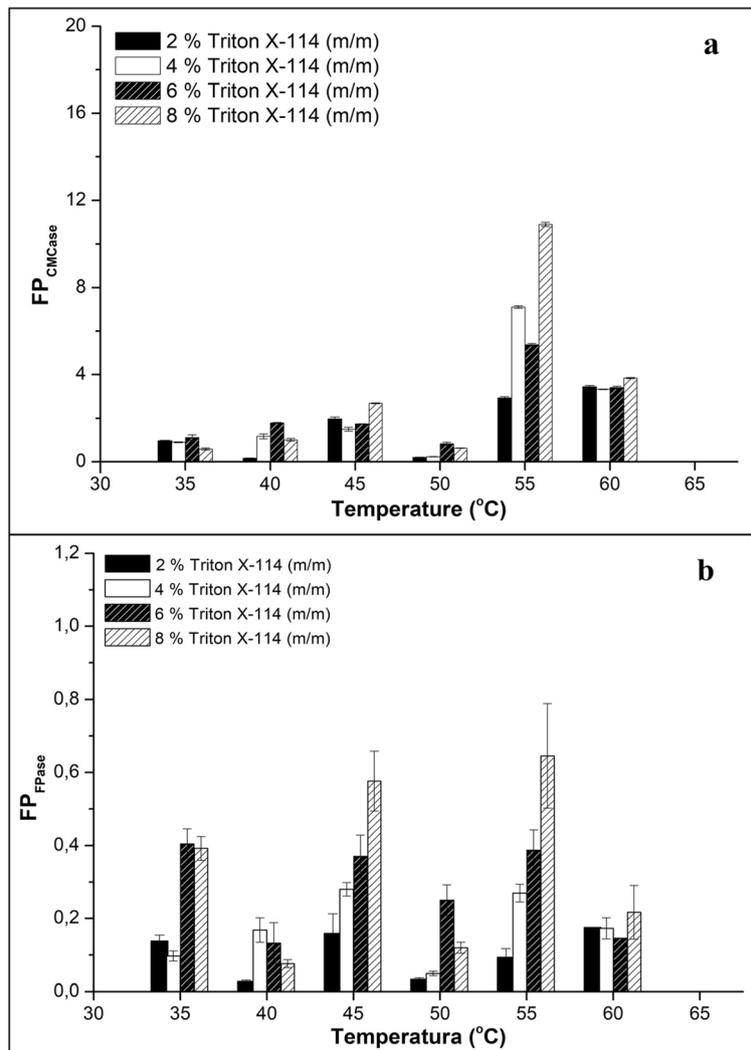


Fig. 3 Purification factor for CMCCase (a) and FPase (b) obtained during partitioning in the ATPMS using different Triton X-114 concentrations and changing the temperature from 35 to 65 °C at 40% enzymatic extract concentration

washed with 1.0 M NaCl until the bands appeared, confirming the cellulolytic activity.

Results

Triton X-114 coexistence curves

The quantification of coexistence curves is crucial for understanding the ATPMS. The coexistence curve delimits the single phase from the biphasic region in the diagram. Figure 1 shows the coexistence curves formed at different temperatures, changing both the enzymatic extract content as well as the Triton X-114 concentration.

Our results clearly demonstrate that the coexistence curve is influenced by the extract content. A higher extract concentration led to a lower critical temperature (T_c) for the ATPMS.

Influence of Triton X-114 concentration and temperature on cellulolytic enzyme partitioning

The influence of Triton X-114 on the cellulase yield (CMCase and FPase) was assayed by keeping the enzymatic extract at 40% (w/w) and changing the Triton X-114 concentration and the temperature, as shown in Fig. 2. For the crude extract, the initial conditions were 0.70 IU/mL, 0.11 IU/mL, and 0.64 mg/mL for CMCase, FPase, and total protein, respectively. It was observed that for enzyme CMCase, the highest yields were obtained at 60 °C, regardless of the surfactant concentration in the system (Fig. 2a). The highest value for the partition coefficient (K) for CMCase was 9.33 (see supplemental Table S1), obtained at 60 °C when the Triton X-114 concentration was 8% (w/w).

Figure 3 shows the purification factor for the two classes of enzymes assayed. The results revealed that the purification process for CMCase is favorable when the

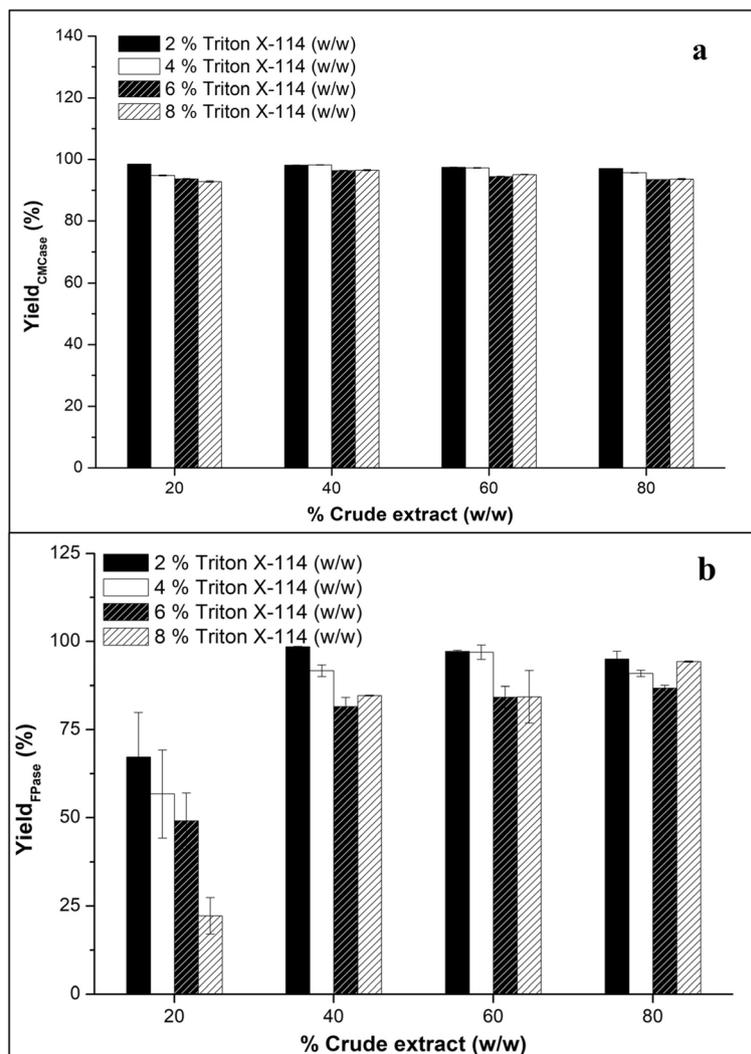


Fig. 4 CMCase (a) and FPase (b) yields obtained during partitioning in the ATPMS using different Triton X-114 concentrations and changing the crude extract concentration at 55 °C.

ATPMS is operated at 5 °C with a Triton X-114 concentration of 8% (w/w). In this case, a PF of 10.89 was reached for CMC_{Case}.

For FPase, even though a temperature of 55 °C using a Triton X-114 concentration of 8% (w/w) showed a higher PF, the PF value was still lower than 1.0. This revealed that these enzymes prefer the micelle-rich phase. Similar to the CMC_{Case}, the FPase also showed an increase on the PF due to the increase of the temperature of the system with the same Triton X-114 concentration (8% (w/w)). In this case, the increase on the PF was an approximately 8.1-fold increase, as shown in Fig. 3.

Influence of the Triton X-114 concentration and crude extract on cellulolytic enzyme partitioning

The influence of Triton X-114 on the cellulase yield (CMC_{Case} and FPase) was assayed by changing the Triton

X-114 concentration as well as the crude extract concentration while keeping the temperature at 55 °C, since this temperature produced the best results for *K* and PF for both enzymes. Figure 4a shows a yield value of 92% for the CMC_{Case} regardless of the crude extract concentration used in the system. However, the use of the 20% (w/w) crude extract strongly reduced the yield for the FPase.

Regarding the purification factor for both CMC_{Case} and FPase, a higher crude extract of 40% (w/w) was favorable, mainly for a Triton X-114 concentration of 8% (w/w), as shown in Fig. 5 and in the Supplemental Table S2. The PF obtained for CMC_{Case} was again higher than that for FPase.

Influence of the Triton X-114 concentration and inorganic salts on cellulolytic enzyme partitioning

The salt type and content played a key role in the partitioning of biomolecules using the ATPMS. Therefore, in

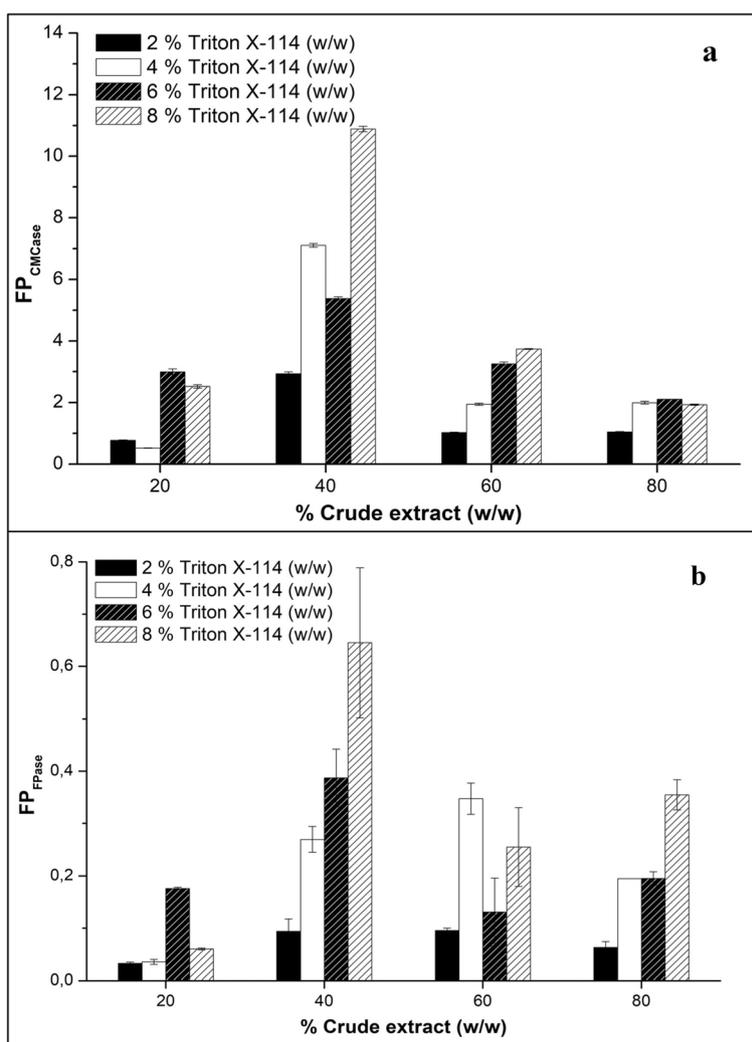


Fig. 5 Purification factor for CMC_{Case} (a) and FPase (b) obtained during partitioning in the ATPMS using different Triton X-114 concentrations and changing the crude extract concentration at 55 °C

this study, we investigated the influence of the inorganic salts on the partitioning of cellulolytic enzymes produced by the filamentous fungus *A. fumigatus* CCT 7873 using ATPMS. For these experiments, the temperature and crude extract were 55 °C and 40% (w/w), respectively. These values were chosen since they led to the best results overall, for yield and PF, for both enzymes, as can be seen in Figs. 3, 4, and 5. Thus, the enzyme partitioning was carried out in the presence of the salts CaCl_2 , MgSO_4 , MnSO_4 , and NaCl , changing the Triton X-114 concentration from 2% (w/w) up to 8% (w/w), as shown in Figs. 6 and 7. The addition of CaCl_2 favored the partition coefficient for both enzymes, as seen in Supplemental Table S3. For instance, a K value of 50 was obtained for ATPMS containing 2% (w/w) Triton X114 operating at 55 °C with 40% (w/w) crude extract, while the K value for FPase was 13.5.

Figure 8 shows the SDS-PAGE and the zymogram obtained using an ATPMS consisting of 40% (w/w) crude extract operating at 55 °C using different concentrations of Triton X-114 (2, 4, 6, and 8% w/w). The crude extract showed at least four bands with molecular masses of 25, 33, 39, 52, and 70 kDa.

Discussion

The coexistence curve showed that a higher extract concentration led to a lower critical temperature (T_c) for the ATPMS. Additionally, the lowest T_c occurred at 20.4 °C at a Triton X-114 concentration of 0.5 (w/w). An aqueous solution containing the nonionic surfactant Triton X-114 can suffer from macroscopic changes in the phase separation with an increase in temperature, thus forming a micelle-rich phase (bottom phase) and a micelle-poor phase (top phase) (Ramelmeier et al. 1991).

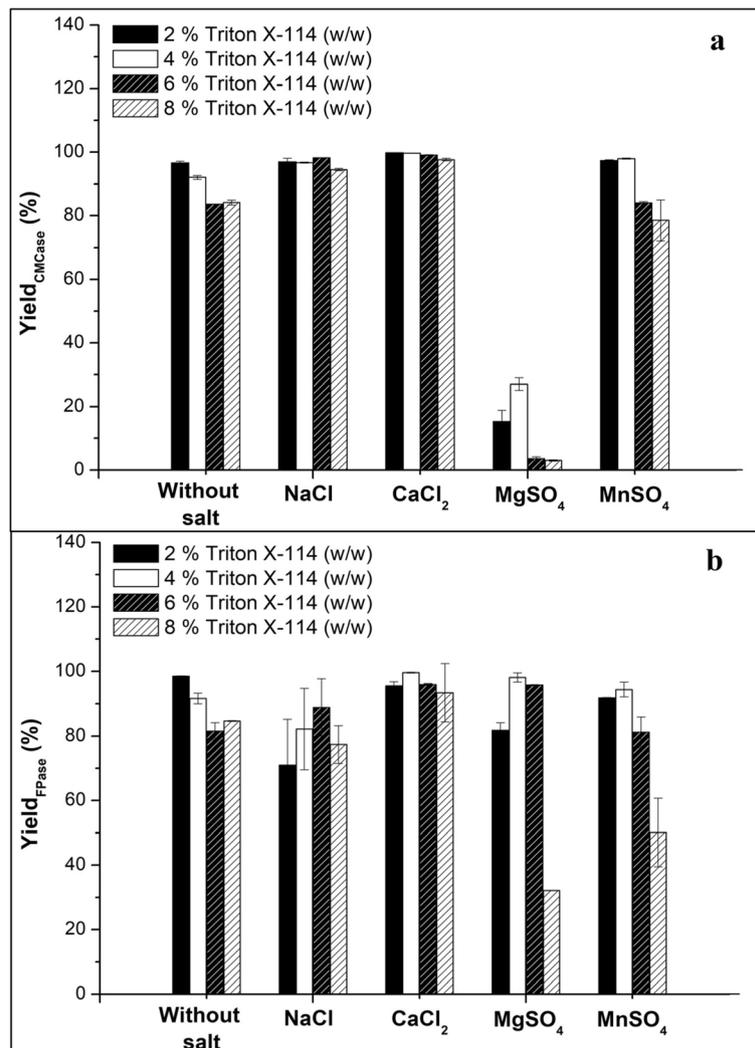


Fig. 6 CMCCase (a) and FPase (b) yields obtained during partitioning in the ATPMS using different Triton X-114 concentrations and inorganic salts at 55 °C and 40% (w/w) crude extract concentration

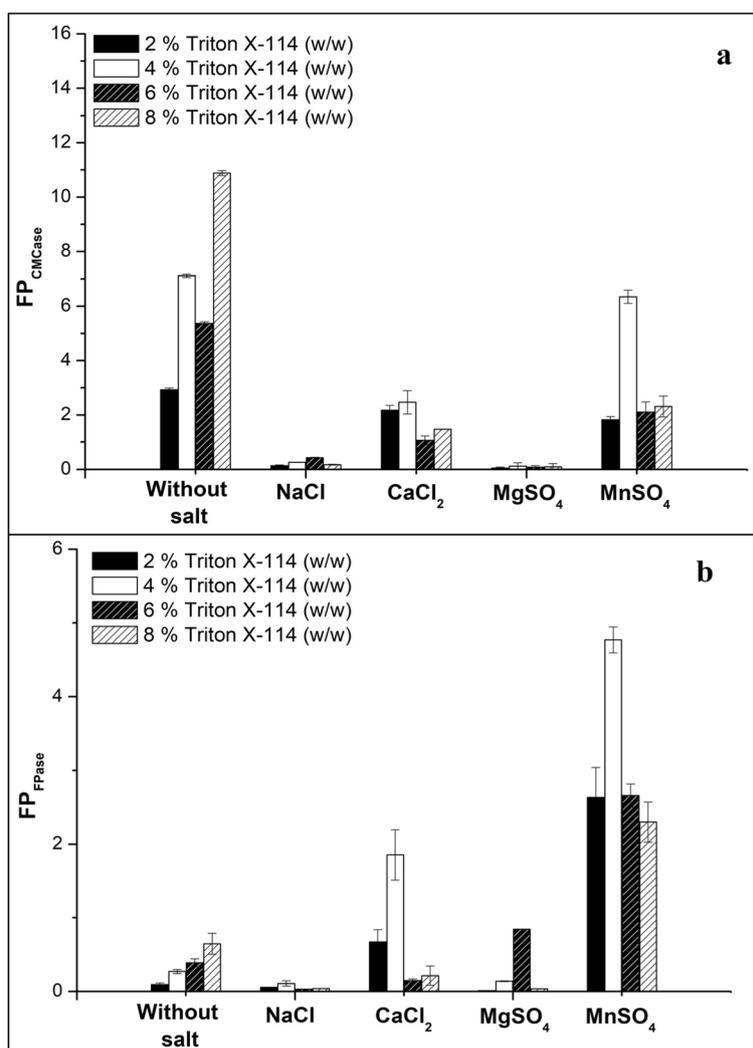


Fig. 7 Purification factor for CMCCase (a) and FPase (b) obtained during partitioning in the ATPMS using different Triton X-114 concentrations and inorganic salts at 55 °C and 40% (w/w) crude extract concentration

The structures, shapes, and sizes of the micelles can be altered by changing the system temperature or the surfactant concentration, or by adding salt to the system (Liu et al. 1996). An increase in temperature causes dehydration of the oxyethylene group, which is responsible for the higher polarity region at the surfactant chain, thus promoting phase separation due to the molecule solubility. The cloud point of the system occurs due to monomer surfactant aggregation, causing phase separation (Quina and Hinze 1999).

With regard to the influence of the Triton X-114 concentration and temperature on cellulytic enzyme partitioning for FPase, the highest value for K was 5.0 and was obtained at 60 °C with Triton X-114 concentrations of 6 and 8% (w/w). Thus, both enzymes preferred the micelle-poor phase. Also, for both enzymes, the yield relied on both the temperature and the surfactant

concentration. This effect is more pronounced for FPase; for instance, we observed that when the Triton X-114 concentration was increased from 2 to 8% (w/w), the yield for this enzyme increased from about 67% at 35 °C to 97.88% at 60 °C, an increase of approximately 46.09%. A higher K value is important, as in this case the enzyme was being concentrated in the surfactant-poor phase.

Thus, with regard to the influence of the Triton X-114 concentration on the purification factor for CMCCase, an increase of almost 19-fold was seen in a system with the same Triton X-114 concentration (8% (w/w)), but operating at 55 °C instead of 35 °C, as shown in Fig. 5a and Supplemental Table S1. Therefore, for CMCCase, there was a phase change due to the increase in temperature when the system contained Triton X-114 at 8% (w/w). This phase change probably occurred due to a preference for the micelle-rich phase (bottom phase) caused

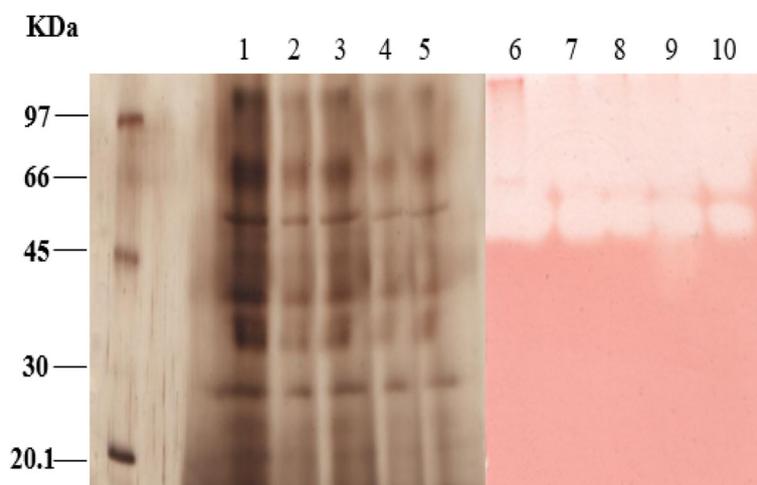


Fig. 8 SDS-PAGE (lanes 1–5) and zymogram (lanes 6–10). 1,6: crude extract; 2,6: 2% Triton X-114 (w/w); 3,8: 4% Triton X-114 (w/w); 4,9: 6% Triton X-114 (w/w); and 5,10: 8% Triton X-114 (w/w)

by the temperature increase. Thus, it reduced the volume occupied by the hydrophilic enzymes, causing the migration of these enzymes to the top phase. Additionally, as can be seen in Fig. 2, the enzymes were denatured at temperatures above 65 °C. It should be noted that there has not previously been a report using the ATPMS approach to recover and purify cellulases existing in the broth, as we have done in the current study. Vicente et al. (2019) previously combined an aqueous two-phase system (ATPS) and ATPMS based on Pluronic L-35, a thermo-responsive copolymer, in order to selectively separate three model proteins: cytochrome c, ovalbumin, and azocasein.

Considering the influence of the Triton X-114 concentration and crude extract on the FPase yield at a constant temperature of 55 °C (Fig. 4b), the use of the 20% (w/w) crude extract strongly reduced the yield. In this case, a decrease from 65 to 20% was observed. Compared to CMCCase, this reduction was quite small, from 98.5 to 93%. In addition, the use of 40% (w/w) crude extract was more favorable for CMCCase. For FPase, an increase in the crude extract above 40% (w/w) did not significantly alter the yield. Thus, greatly increasing the concentration of the crude extract was not effective at improving biomolecule extraction. In this case, it can precipitate the target biomolecule and also influence enzyme partitioning (Malpiedi et al. 2011). Regarding the purification factors for both CMCCase and FPase, the former showed a PF value of 10.89, while the latter showed a higher PF value of 0.65. For FPases at a temperature of 55 °C, regardless of the Triton X-114 concentration and crude extract used in this study, these enzymes showed a preference for the micelle-rich phase (bottom phase), which could be due to the more hydrophobic features of these enzymes. The results of this

study are in accordance with those shown by Jaramillo et al. (2013), which reported 8% (w/w) and 20% concentrated crude extract (w/w) when extracting pectinases using ATPMS.

With regard to the influence of the Triton X-114 concentration and inorganic salts on cellulolytic enzyme partitioning, it was observed that the addition of CaCl_2 favored the partition coefficient for both enzymes, as seen in the Supplemental Table S3. For instance, a K value of 50 was obtained for ATPMS containing 2% (w/w) Triton X-114 operating at 55 °C with 40% (w/w) crude extract, while the K value for FPase was 13.5. Also, not only was the K favored but a higher yield was also obtained when this salt was added to the system. Overall, in the presence of MgSO_4 , a yield of 70% was obtained for both CMCCase and FPase. A lower yield (less than 30% regardless of the Triton X-114 concentration) was observed for the CMCCase. The effect of the ion type on this enzyme partitioning becomes evident when comparing the yields obtained in the presence of MgSO_4 with those obtained when MnSO_4 is used, as shown in Fig. 6a. Therefore, the use of the latter is better than the former for CMCCase yield. Indeed, the use of MnSO_4 was more favorable for the yield of CMCCase in the micelle-poor phase. An inverse relationship was observed for FPase. Thus, the anion size plays a key role in the salting out: the lower the anion the higher its hydration capacity, and since the radii of the Mn^{+2} is less than that of Mg^{+2} , the former induces the micelle-micelle interaction, thus pushing the CMCCase to the upper phase and improving K , Y , and FP . Additionally, in presence of NaCl and MgSO_4 , CMCCase showed a PF less than 1.0, regardless of the Triton X-114 concentration used, as seen in Fig. 7a. The salt presence was not at all beneficial for the PF of CMCCase, mainly due to the salting-out effect, since

the use of an ATPMS composed by Triton X-114 at 8% (w/w) operating at 55 °C with 40% (w/w) crude extract but without salt addition resulted in a PF of 10.89. For the FPase, the addition of 5% (w/w) MnSO₄ resulted in a PF value of approximately 5.0, as can be seen in Fig. 7b.

The differences in biomolecule partitioning, mainly of enzymes and proteins, were that the ions could exhibit very different behaviors when partitioning between the two phases (Costa et al. 1998, Harris et al. 1998, Umakoshi et al. 1996). The addition of salt, even at millimolar concentrations, can influence the partitioning of the charged biomolecules. Even though the salts can be partitioned almost equally between the phases, it is possible for differences to arise in the partition coefficients of different ion species. This can create an electric potential difference between the phases, subsequently influencing the partitioning of charged biomolecules (Sarubbo et al. 2000). As shown in the zymogram (Fig. 8), the crude extract showed at least four bands with molecular masses of 25, 33, 39, 52, and 70 kDa. The range of the molecular masses of the proteins in the present study is similar to the range reported by Morozova et al. (2010). Thus, based on the results of the present study, an integrative technique (Araújo et al. 2016; Araújo Padilha et al. 2017; Glyk et al. 2015; Wanderley et al. 2017) such as ATPMS (Amid et al. 2013) can be successful at partitioning cellulases. Overall, the highest values obtained for the yield and PF show that ATPMS is an interesting technique for recovering and purifying cellulolytic enzymes.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s13213-020-01573-w>.

Additional file 1: Table S1. Partition coefficient, yield (%) and purification factor for the CMCase and FPase using ATPMS composed by Triton X-114 (2, 4, 6 and 8% (w/w)) at different temperatures at 40% (w/w) crude extract. **Table S2.** Partition coefficient, yield (%) and purification factor for the CMCase and FPase using ATPMS composed by Triton X-114 (2, 4, 6 and 8% (w/w)) at different crude extract, keeping the temperature at 55 °C. **Table S3.** Partition coefficient, yield (%) and purification factor for the CMCase and FPase using ATPMS composed by Triton X-114 (2, 4, 6 and 8% (w/w)) at different inorganic salts. The crude extract concentration was 40% (w/w) and the temperature was 55 °C.

Authors' contributions

The author(s) read and approved the final manuscript.

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Ethics approval and consent to participate

This article does not contain studies with human participants or animals performed by any of the authors. Informed consent was obtained from all individual participants included in the study.

Competing interests

The authors declare that they have no conflicts of interest.

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