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MICROBIOLOGY

A novel collagenolytic protease from *Mucor subtilissimus* UCP 1262: Comparative analysis of production and extraction in submerged and stated-solid fermentation

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Abstract: This work aimed to compare the production of collagenolytic proteases produced by *M. subtilissimus* UCP1262 in submerged fermentation (SF) and solid-state fermentation (SSF) as well as extracting in aqueous two-phase system (ATPS). Collagenolytic protease production was performed in using MS-2 culture medium (SF) and soybean bran as substrate (SSF). Subsequently, the fermented liquid from both fermentations were used for the extraction of enzyme by ATPS, it was verified the influence of different variables from a factorial design 2^3. In SSF the highest protease and collagenolytic activities were achieved with 362.66 U/mL and 179.81 U/mL, respectively. When compared with SF (26.33 and 18.70 U/mL) higher values were obtained in the activities. The protease partitioning from SF and SSF in ATPS showed a similar profile showing higher affinity for the polymer rich phase. The highest value for the response variable purification factor (3.49) was obtained in the system using SSF. Thus, SSF shows promise as a bioprocess for extracellular production of collagenolytic proteases, using of soybean bran as substrate had used sustainable raw material, aiming application this possible enzyme in the treatment of burns and postoperative scarring.

Key words: bioprocess, ATPS, filamentous fungus, sustainability.

INTRODUCTION

Proteases are one of the most economically desirable biomolecules in the world market due to their physicochemical and catalytic properties, highlighting their use in leather processing, in the formulation and production of laundry detergents and soap, in the formulation and preparation of food products and beverages, and in the pharmaceutical segment (Gurumallesh et al. 2019). Naturally, microorganisms can produce large amounts of proteases to meet industry demand through the development of bioprocesses to allow fermentation and hence the increase in the desired protease volume. All processing is performed through easily manipulated bioreactors and low-cost production (Alipour et al. 2016, Belmessikh et al. 2013, Gurumallesh et al. 2019, Viniegra-González & Favela-Torres 2006).

Microbial proteases of fungal origin may be produced in submerged or solid-state fermentation, such fermentations have their own characteristics. In submerged fermentation the microorganism is exposed to hydrodynamic forces whereas in solid fermentation the growth is restricted to the surface of the solid matrix. The growth of the fungus and the production of

metabolites by these microorganisms in such systems depend on the availability of nutrients, geometric configuration of the matrix and the same microorganism in different types of fermentation can produce different amounts of enzymes or even different enzymes (Oda et al. 2006).

Among the proteases of industrial importance is the collagenolytic protease or simply collagenase catalyze chemical processes and the ability to cleave the different types of collagen (Alipour et al. 2016, Amaral et al. 2020, Bhagwat & Dandge 2018, Oliveira et al. 2020, Wanderley et al. 2017). Different genus of filamentous fungi has been described as producers of collagenases, *Penicillium* (Lima et al. 2013) and *Aspergillus* (Silva 2018). Collagen, a collagenase substrate, is a fibrous, structural and insoluble protein that has high tensile strength, and is present mainly in the skin, cartilage, bones, tendons, teeth and blood vessels and the peptides resulting from degradation of collagen, also present several biological activities of industrial interest. This protein has broad application in the leather, cosmetic, biomedical, pharmaceutical and food processing industries (Bhagwat & Dandge 2018, Oliveira et al. 2019).

For production microbial enzymes is so easy, but as limiting factor their purification. Conventional systems for separating biomolecules are often unsatisfactory. The two-phase aqueous system (ATPS) is one of the most economical and feasible processes for extraction and pretreatment of biological compounds. ATPS is commonly composed of polymers such as polyethylene glycol (PEG) and dextran or a polymer and a salt. These ATPS components are mutually incompatible hydrophilic solutes which are dissolved in water above a certain critical concentration so that the biphasic system is formed (Ferreira et al. 2009).

This methodology offers several advantages in the purification of biomolecules such as: ease in increasing the scale; rapid mass transfer; achieved balance with low energy resources in the form of mechanical mixing; possibility of fast and selective operation; possibility of operation at room temperature; environmentally friendly (Nainegali et al. 2020). Therefore, the objective of the present work was to compare the production of collagenolytic protease in ATPS produced by *Mucor subtillissimus* UCP 1262 in different types of fermentations.

MATERIALS AND METHODS

Microorganism

The filamentous fungi *Mucor subtilissimus* UCP 1262 was isolated from the soil of Caatinga (Northeast, Brazil), deposited in the Universidade Católica de Pernambuco (UNICAP). The species used is registered in the Patrimônio Genético Brasileiro n° AA30B0B.

Means of maintenance and sporulation

It was maintained on Czapek medium at 30ºC for 7 days and stored in mineral oil, sterilization of the medium was performed by autoclaving at 121ºC, 1 atm pressure for 20 minutes. For sporulation the microorganism was incubated in BOD (Body Oxygen Demand) at 30ºC for 7 days.

Preparation of inoculums for submerged and solid-state fermentations

For submerged and solid-state fermentations the spores were collected using nutrient solution comprised of 0.5% yeast extract, 1% glucose and 0.01% Tween 80 diluted in sodium phosphate buffer 245 mM and pH 7.0 previously sterilized: The spores were counted in Neubauer chamber to a final concentration of 10^7 spores/mL for state solid fermentation, but for submerged

fermentation was use final concentrated 10^4 spores/mL.

Production of collagenolytic protease by submerged fermentation (SF)

For production of collagenolytic protease in submerged fermentation was used the MS-2 medium (Porto et al. 1996) composed of: 2.0% soybean meal, $MgSO_4.7H_2O$, 0,1%, NH_4Cl, 0,435%, K_2HPO_4 and 0.1 mL mineral solution (100 mg $FeSO_4$, 100 mg $MnCl_2$, 100 mg of $ZnSO_4$, 100 mg of $CaCl_2$ H_2O in 100 ml of distilled water, initial pH 7.2). Therefore, a solution containing spores of *Mucor subtillissimus* UCP 1262 was inoculated in a 125 mL Erlenmeyer flask containing 50 mL of the autoclaving at 121ºC, 1 atm pressure for 20 min. The fermentation was carried out in an orbital shaker at 120 rpm, 30°C, for 72 hours. After the culture period the samples were centrifuged at 3500 rpm for 10 min to separate the biomass and the supernatant obtained was used to determine the enzymatic activities.

Production of collagenolytic protease by solid-state fermentation (SSF)

For production of collagenolytic protease in solid-state fermentation the soybean bran with a granulometry between 0.6 to 2.0 mm was used with 5g and the solution containing spores of *Mucor subtillissimus* UCP 1262 was inoculated in a 125 ml Erlenmeyer flask and the moisture of 40% at 30˚C for 72 hours for production. Thus, the enzyme extraction was realized with purpose 7.5 mL of 245 mM sodium phosphate buffer, pH 7, were added per g of substrate and the flasks were placed in an orbital shaker at 150 rpm for 90 min at room temperature (Nascimento et al. 2015).

Preparation of aqueous two-phase systems and extraction of collagenolytic protease

For formation of ATPS was utilized concentrations phosphate buffer (PBS, 40% w/w) in pH 6.0 at room temperature (25 ± 1ºC), was addition PEG solutions (60%, w/w) with different molecular weight (200, 500, 1000) determined for 2^3 experimental design de according Table I and transferred 15 ml graduated tubes and were addition 2 g sample. After these addictions were homogenized, stand by until 60 min for separated the phases. The volumes phase was measured, and determination collagenolytic activity and protein concentration were determination of two phases. The extracts of submerged and solid-state fermentations were used for extraction of collagenolytic protease in each 2^3 experimental design.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was determined by Laemmli (1970). Protein staining was done by immersing the gel in a solution containing 0.1% Coomassie Blue R-250 and then staining with silver (Fluka Fine Chemical Co., Ltd., Tokyo, Japan). The molecular mass was calibrated using as a standard a molecular mass marker (GE Healthcare 17044601, São Paulo, SP, Brazil)

Table I. Factor levels of the 2^3-experimental design used for the extraction of collagenolytic protease from *M. subtilissimus* UCP 1262 from by PEG/phosphate ATPS.

VARIABLE		LEVEL	
	Low (-1)	Central (0)	High (+1)
M_{PEG} (g/mol)[a]	200	550	1000
C_{PEG} (% w/w)[b]	17.5	20.0	22.5
C_{SALT} % (w/w)[c]	15.0	17.5	20.0

[a]PEG molar mass; [b]PEG concentration; [c]Citrate concentration.

HMW-SDS maker kit, pageruler unstained broad range protein ladder – Thermo Scientific – 26630.

Analytical determinations

Protein determination

Total protein content of samples was determined using Coomassie Brilliant Blue G-250 as a dye and Bovine Serum Albumin as a standard (Bradford 1976).

Protease activity

Protease activity was measured using 1% azocasein as the substrate for reaction, with 1 unit of enzymatic activity (U) varying from 0.01 at absorbance to 420 nm per 1 hour (Ginther 1979).

Azocoll assay for collagenolytic enzyme activity determination

The determination of the collagenolytic activity was performed according to the methodology described by Wanderley et al. (2017) using as substrate Azo dye-impregnated collagen (Azocoll; Sigma Chemical Co., St Louis, MO). The reaction occurred at 37°C for one hour. After this time, each assay was centrifuged and 1mL of the supernatant was removed for spectrophotometer reading at wavelength at 520nm. One unit of enzyme activity (U) was defined as the amount of enzyme, per milliliter, necessary to increase the absorbance by 0.1.

Determination of partition coefficient (K), activity yield (Y), purification factor (PF), Mass balances (MB) for ATPS

The partition coefficient (K) of collagenolytic protease was defined as the ratio of collagenolytic activity, expressed in U/mL, in the top phase (Ca_t) to that in the bottom phase (Ca_b):

$$K = \frac{C a_t}{Ca_b}$$

Where: "Ca_t" and "Ca_b" were collagenolytic activities (U/mL) in the top and bottom phases, respectively.

The activity yield (Y) collagenolytic protease in top phase was calculated from the formula below:

$$Y = \frac{Vs.Cat_t}{Vce.Ca_{ce}} X\,100$$

Where: "Vs" and "Cat_t" are volume of the top phase and collagenolytic activity (U/mL), respectively. As "Vce" and "Ca_{ce}" are the initial volume (crude extract) and the initial collagenolytic activity (U/mL) (crude extract) respectively.

The purification factor (PF) was calculated according to this formula:

$$PF = \frac{Cat/Pt}{Cace/Ptce}$$

Where: "Cat" and "Pt" are collagenolytic activity (U/mL) and protein concentration (mg/mL) in top phase, respectively. Thus, "Cace" e "Pt*ce*" are collagenolytic activity (U/mL) and protein concentration (mg/mL) in crude extract.

The mass balance (MB) was certificated according to this formula:

$$MB = \frac{(Pt.Vt)+(Pb.Vb)}{Ptce.Gce} X\,100$$

Where: "P*t*" and "V*t*" are the protein concentration (mg/mL) and volume top phase respectively. Then, "Pb" and "V*b*" are the protein concentration (mg/mL) and volume top phase, respectively. Then, "Pt_{ce}" and "G_{ce}" are the protein concentration (mg/mL) and volume of crude extract, respectively. Ultimately, "Pt" is protein concentration (mg/mL) crude extract and quantity in grams of the sample used, respectively.

Statistical analysis

Statistical analysis of results obtained through the factorial experimental design and central composite rotary design were performed using the software Statistical 8.0. In order to compare the means of the effects of inhibitors on protease activity, we used the Student t-test for independent samples. The results were considered statistically significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Collagenolytic protease production by *Mucor subtilissimus* UCP1262 in Submerged Fermentation (SF) and Solid-State Fermentation (SSF)

The production industrial of enzymes is a process basically carried out by two fermentation system: Submerged fermentation (SF) and Solid-State Fermentation (SSF), whose differences in bioprocess and composition of the medium are determinant in the enzymatic production from microorganism (Silva 2018). In present work the collagenolytic protease production from *Mucor subtilissimus* UCP 1262 was evaluated using these two techniques. The total proteolytic activity of 362.66 U/mL and the collagenolytic activity 179.81 U/mL were higher in the SSF process compared to SF where the production was 26.33 U/mL and 18.70 U/mL for the total proteolytic activity and collagenolytic activity, respectively.

Around the 90% of the industrial enzymes production use the SF technique and most of the microbial collagenases reported have been produced from these process (Pal & Suresh 2016). SF is the mostly utilized because it provides advantages such as used a bioreactor equipped with techniques for monitoring and control (Dasari et al. 2019). However, the byproducts are diluted and enzyme extracts may be less stable when compared to solid-state fermentation (Gimenes et al. 2019). Viniegra-González & Favela-Torres (2006) explain that the low enzymatic production in SF process may be related to important catabolite repression phenomenon. According to these authors, this phenomenon is the inhibition of the microbial synthesis of many enzymes that require a chemical signal, called the inducer, and are repressed when there is abundance of a ready fermentable substrate such as glucose, glycerol or other carbon sources.

A viable alternative is the SSF technique, since that, several authors related the increase in enzyme production using this bioprocess (Bhavsar et al. 2013, Mazotto et al. 2013). Moreover, this type of fermentation is indicated for filamentous fungi because the cultivation conditions are similar to their natural habitat; the concentration of the products after extraction is much higher when compared to submerged fermentation and generates less liquid residue, being this process economically interesting mainly in regions with abundant agro-industrial waste (Soccol et al. 2017).

The total protein content found in the SSF enzymatic extract was 1800.22 mg/mL, which is higher than that obtained in SF with 344.01 mg/mL. SSF process the microorganism has greater difficulty in degrading the substrate to get nutrients, as compared to the aqueous medium (SF), where the nutrients concentration is more accessible. Thus, the microorganism was induced to increase the production of proteins (enzymes) to degrade the substrate. Several studies show a higher concentration of secreted proteins in the solid medium than in the liquid medium; and the morphology and chemical composition of the substrate used in fermentation process are very important for enzyme production (Mazotto et al. 2013, Shivanna & Venkateswaran 2014).

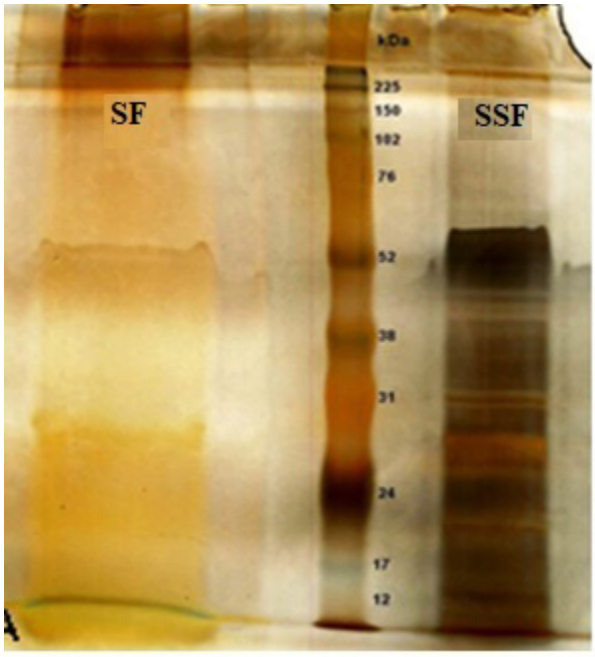

Figure 1. Comparison of the protein profile obtained by *Mucor subtilissimus* UCP 1262 in submerged (SF) and solid state (SSF) fermentation on silver nitrate stained SDS-PAGE (12%) with protein molecular mass marker (High ranger RN756E, GE Healthcare Life Sciences).

Regarding the protein profile in both types of fermentations (Figure 1) the protein profile of SSF is more complex, as it has more differentiated bands, while in SF the protein diversity is lower. Similary with the results of Oda et al. (2006) who compared the amount of protein secreted in the two cultivation forms, using wheat bran as a carbon source, the study reports that solid state growth resulted in four to six times more secreted protein compared to submerged culture, resulting in a more complex protein profile. In present work the proteolytic activity obtained from SSF had a 13.7-fold increase compared to SF. Likewise, the results obtained in the present study, was reported *Aspergillus oryzae* protease production in submerged and solid-state fermentation and observed a 3.5-fold increase in the amount of enzyme produced in solid state using wheat bran as substrate (Sandhya et al. 2006). Corroborating with the results, Belmessikh et al. (2013) in the work realized a comparison of the neutral protease production from the cultivation of *Aspergillus oryzae* in solid-state fermentation (SSF) and submerged fermentation (SF) using tomato pomace based medium, and reported a ratio of 9 between SSF and SF, showing the efficiency of the solid-state fermentation compared to the submerged fermentation, the nature of fermentation, submerged or solid state, influences various aspects of microorganism growth as well as enzyme production.

Though, the use of this fermentation technique presents challenges to be overcome such as insufficient nutrient employment due to heat transfer in substrates and the low quantity of oxygen. Moreover, the challenges of scale-up, purification of the bioproduct and biomass estimation are drawbacks of the technique that have encouraged researchers to search for better solutions (Gimenes et al. 2019).

Comparative extraction of collagenolytic protease from submerged fermentation (SF) and solid-state fermentation (SSF) using aqueous two-phase system

The experimental results of extraction of collagenolytic protease produced for *Mucor subtilissimus* UCP 1262 in SF and SSF was analyzed according to factorial design with two levels and three variables. The statistical influence was determined within 95% of confidence. The results for the response variables, partition coefficient (K), purification factor (PF) and activity yield (Y) are summarized in Table II and Table III for collagenolytic enzyme extraction obtained by SF and SSF, respectively.

The collagenolytic protease produced by SF and SSF partitioned preferentially for the polymeric phase (partition coefficient $K > 1$). According to Table IV the independents variables C_{PEG} and M_{PEG} showed significant influence under partition of collagenolytic protease obtained

Table II. Results of 2^3-experimental design for collagenolytic protease extraction from *M. subtilissimus* UCP 1262 in submerged fermentation (SF) using PEG/phosphate ATPS.

Runs	M_{PEG}	C_{PEG} (%)	C_{SALT} (%)	CA_{PEG} (U/mL)	CA_{SALT} (U/mL)	K	Y (%)	PF	MB
1	200	17.5	15	12.0	4.13	2.903	175.14	1.106	106.432
2	1000	17.5	15	1.3	4.53	3.000	194.11	1.323	91.938
3	200	22	15	13.3	4.43	0.287	123.00	0.818	99.032
4	1000	22	15	10.9	4.33	2.515	147.3	1.415	90.978
5	200	17.5	20	14.0	4.67	3.000	174.05	2.174	135.365
6	1000	17.5	20	8.4	4.57	3.000	198.65	2.389	104.727
7	200	22	20	14.7	4.90	1.839	81.73	1.333	127.697
8	1000	22	20	13.7	4.83	2.834	162.92	1.441	101.601
9_{cp}	500	20	17.5	13.4	7.00	1.914	159.35	1.544	92.171
10_{cp}	500	20	17.5	13.2	6.97	1.895	156.97	1.508	112.664
11_{cp}	500	20	17.5	8.8	6.77	1.300	109.41	1.280	89.708
12_{cp}	500	20	17.5	11.0	6.93	1.587	124.86	1.551	85.405

C_{PEG} - PEG concentration; C_{SALT} - Phosphate concentration; M_{PEG} - PEG molar mass; CA_{PEG} - Collagenolytic activity off PEG; CA_{SALT} - Collagenolytic activity of salt; K - Partition coefficient; Y - Activity yield; PF - Purification factor; MB - Mass balances; $_{cp}$ - Central points.

by SF and SSF too. The influence of C_{PEG} had the negative effect, the polymer concentration decrease favored the partition of the enzyme for this phase. Because the volume occupied by the polymer increases with the polymer concentration and which results in reduced space for biomolecules in the top phase (Porto et al. 2008). On the contrary, M_{PEG} was positively significant for K, runs composed with higher molar mass of the polymer (1.000 g/mol) favored the enzyme partition to the upper phase. Is commonly reported that in biphasic systems formed by polymers and salts, when the polymer has a high molar mass, which is caused by the molecule size, can lead to the volume exclusion effect, when the biomolecule tends to migrate to a salt-rich phase where there is more space (Siqueira et al. 2020). In the present work or opposite behavior was observed once as microbial collagenases are generally small, with molecular weight ranging from 28 kDa to 116 kDa (Jucá et al. 2008), and indicating high affinity enzymatic for the PEG-rich phase.

The interaction of independents variables C_{PEG}-C_{SALT} (2*3) exerted significant linear positive effect (Table IV) on partition of collagenolytic protease from SSF, meaning that the simultaneous increase or decrease of PEG concentration (C_{PEG}) values and phosphate concentrations (C_{SALT}), the higher the values K values. This effect was observed in the present study, where the lowest concentration of PEG 17,5% in combination with the lowest phosphate concentration 15% (w/w) were the conditions that favored the partition of collagenolytic protease to the PEG rich phase. Low PEG concentration values associated with low phosphate concentrations favored the partition of the molecule to the upper phase, increasing the partition coefficient.

This effect was observed in the present study, where the run compound whit lowest concentration of PEG 17,5% (w/w) in combination with the lowest phosphate concentration 15% (w/w) was the conditions that favored the partition of collagenolytic protease to the upper phase, increasing the partition coefficient (K=3.45).

ATPS has great versatility in separating complex mixtures due to the large number of variables that interfere with the target molecule

Table III. Results of 2^3-experimental design collagenolytic protease extraction from *Mucor subtilissimus* UCP 1262 in solid-state fermentation (SFF) using PEG/phosphate ATPS.

Runs	M_{PEG}	C_{PEG} (%)	C_{SALT} (%)	CA_{PEG} (U/mL)	CA_{SALT} (U/mL)	K	Y (%)	PF	MB
1	200	17,5	15	27.6	16.6	1.66	125.64	0.557	96.49
2	1000	17,5	15	32.4	25.8	1.25	116.06	0.406	139.77
3	200	22	15	65.2	18.9	3.45	291.94	1.925	83.84
4	1000	22	15	34.8	25.7	1.35	96.09	3.495	103.10
5	200	17,5	20	37.5	25.2	1.48	123.13	1.409	92.06
6	1000	17,5	20	29.6	16.6	1.78	97.19	0.500	96.16
7	200	22	20	25.1	16.9	1.48	93.66	0.775	80.38
8	1000	22	20	29.1	16.2	1.79	69.49	1.516	86.57
9_{cp}	500	20	17,5	40.3	25.1	1.60	132.33	1.860	85.65
10_{cp}	500	20	17,5	50.1	26.2	1.91	134.60	2.348	110.61
11_{cp}	500	20	17,5	47.5	26.7	1.77	127.61	2.176	103.24
12_{cp}	500	20	17,5	40.0	25.5	1.56	131.34	1.779	90.45

C_{PEG} - PEG concentration; C_{SALT} - Phosphate concentration; M_{PEG} - PEG molar mass; CA_{PEG} - Collagenolytic activity off PEG; CA_{SALT} - Collagenolytic activity of salt; K - Partition coefficient; Y - Activity yield; PF - Purification factor; MB - Mass balances; $_{cp}$ - Central points.

partition. Through the systematic manipulation of extrinsic variables, the behavior of the protein of interest and its contaminants can be modified (Tubío et al. 2007). However, with this wide range of variables, and most of them being interdependent, the theoretical understanding of the behavior of the molecule of interest in ATPS becomes exceedingly difficult. As a result, the conclusions regarding collagenolytic protease partition presented in this study were based on the physicochemical characteristics of the system components and the chemical structure of the target molecule.

The partition of the enzyme produced in SF and SSF to the PEG rich phase may be justified by the hydrophobic interaction between PEG and collagenolytic protease. In this respect, it is possible to infer that such interaction occurs due to the presence of hydrophobic domains in collagenase, as well as in chitinases, cellulases and xylanases (Bockle et al. 1995). The hydrophobic domains present in microbial collagenases, as well as in all metalloproteases, are formed by the methionine residues contained in these enzymes that are responsible for such hydrophobic bases (Bode et al. 1993). In addition, PEG has high hydrophobicity due to its long carbon chains and is a neutral molecule (Pereira & Coutinho 2019). Thus, the hydrophobic domains of collagenase interacted with the hydrophobic PEG chains allowing, through hydrophobic forces, the migration of the enzyme of interest to the upper part of the biphasic system.

The activity yield (Y) values of collagenolytic protease from SF and SSF obtained were: 198.65% and 291.94% respectively (Table II and III). Other authors using biphasic system for enzyme extraction also reported yield values above 100%, this fact can be explained by the possible decrease of the enzyme inhibitors of interest during the extraction process (Oliveira et al. 2020) or the PEG phase may have promoted greater stability to the target enzyme. This behavior was also observed extracting tannase from *Aspergillus tamarii* where the highest

Table IV. Statistical effects calculated for the responses of collagenolytic protease from *Mucor subtilissimus* UCP 1262 in submerged and solid-state fermentation using PEG/phosphate ATPS performed according to the 2^3 -experimental design.

Variable or Interaction	Submerged fermentation			Solid-state fermentation		
	K^a	Y^b_{PEG}	PF^c	K^a	Y^b_{PEG}	PF^c
$M_{PEG}{}^d$	-5.3833*	-4.8668*	3.1270*	4.2018*	10.8222	6.4074*
$C_{PEG}{}^e$	4.0375*	3.7435*	-5.4592*	4.2002*	-31.0174*	1.6564
$C_{SALT}{}^f$	3.8022*	1.2756	7.3568*	-2.5914	-29.8910*	-2.8905
M_{PEG}*C_{PEG}	2.3936	2.4877	0.7508	-3.7063*	-22.3946*	4.4636*
M_{PEG}*C_{SALT}	2.1583	1.1759	-1.3504	6.8878*	-24.7008*	-5.3958*
C_{PEG}*C_{SALT}	-1.6176	-0.6944	-4.3811*	4.1546*	18.8527*	-2.8905
M_{PEG}*C_{PEG}*C_{SALT}	-1.3822	-0.8565	-1.3394	3.7775*	22.8242	-0.0940

K^a - Partition coefficient, Y^b_{PEG} - Activity yield, PF^c - Purification factor, $M_{PEG}{}^d$ - PEG molar mass, $C_{PEG}{}^e$ - PEG concentration, $C_{SALT}{}^f$ -Phosphate concentration, *Statistically significant values (at $p < 0.05$).

activity yield, they observed was 160.4% Sena et al. (2017) Oliveira et al. (2020) and Wanderley et al. (2017) using PEG/phosphate ATPS to extract collagenolytic enzyme from peacock bass (*Cichla ocellaris)* and *Chlorella vulgaris*, reached yields of 116% and 244.0%, respectively. Values of activity yield that match those obtained in the present work.

The independent variables C_{PEG} and M_{PEG} were statistically significant on the activity yield (Y) of collagenolytic protease from SF. C_{PEG} showed a negative effect and M_{PEG} was a positive effect under for this response, in the runs 2 and 6 formed with lower concentrations and higher molar mass of the polymer, higher yield activities were obtained (Table II). Considering the results of activity yield obtained for the extraction of collagenolytic protease from SSF (Table III), all independent variables and their interactions were statistically significant on this variable response, the negative effect of the polymer concentration (C_{PEG}) stands out (Table IV). According to Grilo et al. (2016) the relationship between concentration and molecular weight of polymers is an important parameter in extract enzyme ATPS. This author reports that in biphasic system using PEG polymers, as the molecular weight decrease at a constant concentration of polymer, there will be less ethylene oxide groups per PEG molecule, and consequently the PEG-rich phase will be less hydrophobic. In contrast, obviously an increase in the molecular weight of polymer increases hydrophobicity by reducing the hydrophilic groups/hydrophobic area (Iqbal et al. 2016). As well as the previously discussed, in present study a hydrophobic interaction between PEG and collagenolytic protease occurred.

The highest purification factor (PF) values for collagenolytic protease produced in SSF (PF =3.495) when compared with PF values obtained in extraction of enzyme produced by in SF (PF =2.389). However, the production of collagenolytic proteases in SSF was 9.6 x greater than the production of the same enzyme using SF (described in section "Collagenolytic protease production by *Mucor subtilissimus* UCP1262 in Submerged Fermentation (SF) and Solid-State Fermentation (SSF)"). In solid-state fermentation at a concentration of amino acids, proteins and even larger pigments when compared to submerged fermentation and this can make the purification step more costly and, in many cases, not interesting in industrial terms (Hölker

et al. 2004). Because this, considered that the enzymatic purification of the crude extract from the SSF was more effective.

Relative low purification factor obtained in the extraction of the enzyme produced by SSF may be related to the complex protein pattern (Figure 1) present in the crude extract obtained in this bioprocess. Result that corroborates with the obtained by Silva et al. (2017) and Amaral et al. (2020) when evaluated extraction the protease produced in SSF by *Aspergillus tamarii* URM 4634 and *Aspergillus tamarii* Kita UCP 1279 using PEG-citrate aqueous two-phase system and reported purification factor values that 3.95 and 1.6, respectively. For the collagenolytic protease from SSF the most significant effect on PF was the positive effect one of M_{PEG} and was also negatively influenced by the interaction between M_{PEG} and C_{SALT} (1*3). The optimal conditions for this response were those adopted in run 4 (Figure 2) composed by 22% (w/w) PEG 1000 g/mol and 15% phosphate salts (w/w).

Already in SF, biomolecules are produced in smaller quantities and the extremely aqueous culture medium favors the dilution of enzymes and consequently on its inhibitors. Stoykov et al. (2015) observed that chitinase purification in SF is generally more complex because purification steps require large volumes of metabolic liquids due to the low enzymatic concentration. That said, ATPS techniques is an attractive for the steps downstream of enzymes SSF produced. Since is recovery tool that integrates clarification, concentration and partial purification of the target molecule in a single operation (Nadar et al. 2017, Phong et al. 2018, Siqueira et al. 2020). The ATPS showed the highest value for PF in the conditions of the run 6 (Figure 3) M_{PEG} 1000 g/mol, C_{PEG} 17.5% (w/w), C_{SALT} 20% (w/w) from the collagenolytic protease produced by SF. After the results statistical analysis, it was observed that the main effects of variables were the C_{SALT} positive effect and C_{PEG} negative effect, as well as, an interaction of these variables has negatively

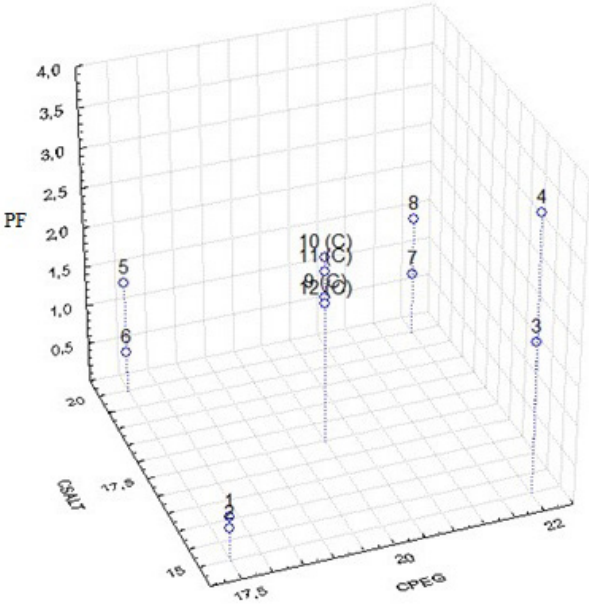

Figure 2. Simultaneous effects of concentrations PEG (C_{PEG}, %) and phosphate concentration (C_{SALT}, % w/w) on the purification factor (PF) the collagenolytic protease of solid-state fermentation in the top phase (PEG) ATPS.

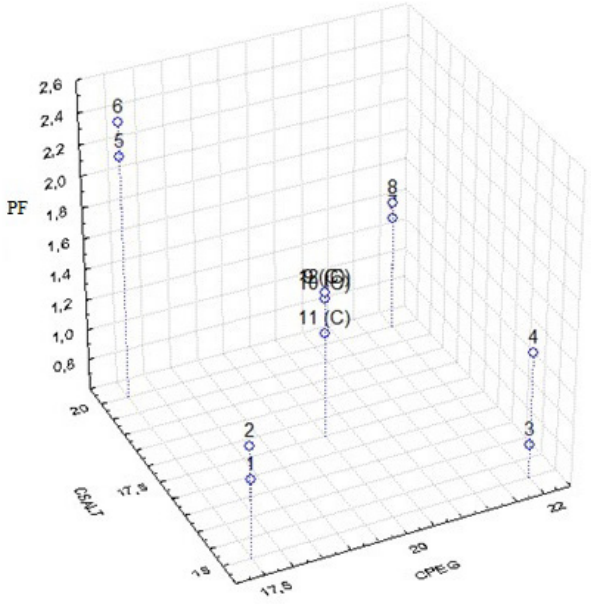

Figure 3. Simultaneous effects of PEG (C_{PEG}, %) and phosphate concentration (C_{SALT}, % w/w) on the purification factor (PF) the collagenolytic protease of submerged fermentation in the top phase (PEG) ATPS.

significant effect under PF of the collagenolytic protease produced by SF. This means that the variables exerted an antagonist effect, whereby of the PF increase was observed in the tests formed by a higher phosphate concentration and lower PEG concentration. Wanderley et al. (2017) reported the effect of salt on the partitioning coefficient of collagenolytic enzyme produced by microalgae *Chlorella vulgaris* was positive meaning that high phosphate concentration makes collagenolytic enzyme migrate to the PEG rich phase. Several authors explain that a higher concentration of salt leads to a decrease in protein solubility in the salt-rich bottom phase, resulting in the migration of the protein and, hence, partition directly to polymer-rich upper phase, the interaction occurs between salt ions and oppositely charged protein groups, creating a double layer of ionic groups, this phenomenon is the salting out effect (Gimenes et al. 2019, Goja et al. 2013, Grilo et al. 2016).

CONCLUSIONS

A collagenolytic protease with potential pharmaceutical application was successfully produced by *Mucor subtilissimus* UCP 1262. The results obtained in this study showed that SSF is a promising method of cultivation in obtaining of this type of biomolecule. The collagenase produced in both fermentative processes (SSF and SF) were partial purified by aqueous two phases systems PEG/phosphate. In ATPS were obtained recovery values higher than 100% and high purification factors, thus ATPS was a viable tool for the extraction of the enzyme, in addition, it is a process that can be used in large scale, constituted by components of low cost, and the conditions used in the PEG/phosphate favored the extraction of these enzymes, becoming an alternative raw material source for the global protease market.

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