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Full Length Research Paper

Production of aspartic peptidases by *Aspergillus* spp. using tuna cooked wastewater as nitrogen source and further extraction using aqueous two phase system

Darío Spelzini[1]*, Beatriz Farruggia[1], Nelson Perez Guerra[2], Maria Luisa Rua[2] and Lorenzo Pastrana[2]

[1]Laboratorio de Fisicoquímica Aplicada a Bioseparación. Departamento de Química-Fisica, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 571, 2000 Rosario, Argentina.
[2]Laboratorio de Biotecnología, Área de Nutrición y Bromatologia, Facultade de Ciencias de Ourense Universidade de Vigo, As lagoas s/n, 32004 Ourense, España, Spain.

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The production of extracellular aspartic peptidase by the fungi *Aspergillus niger* and *Aspergillus awamori* was carried out in a shake flask and in stirred tank submerged fermentations using tuna cooked wastewater, an industrial effluent, as nitrogen source for culture medium. In stirred tank fermentation, biomass production of 6 g/l and 2 arbitrary units was obtained. In addition, the partitioning of extracellular aspartic peptidases from cell-free. *A. awamori* broth culture produced in a stirred tank reactor was carried out in aqueous-two phase systems formed by polyethylene glycol and potassium phosphate. Previous results showed that aspartic peptidases have a high affinity for the PEG-rich phase. A Kp of 4.5 for ATPS PEG 1450-Pi; in ATPS PEG 8000-Pi, Kp value of the range of 2 to 2.5 was obtained. A purification factor 2 was obtained. The method appears to be suitable as a first step for the purification of these proteins from these complex medium.

Key words: Tuna cooked wastewater, aspartic peptidases, aqueous two-phase system.

INTRODUCTION

Proteolytic enzymes account for nearly 60% of the industrial market in the world. They find application in a number of biotechnological processes, including food, pharmaceutical, leather and detergent industries, bioremediation processes, etc (Rao et al., 1998).

In cheese production, reduced supply and increasingly high prices of calf rennet have led to numerous attempts to find suitable rennet substitutes. Several animal and plant proteases (Lopes et al., 1998) have been identified as possible rennet substitutes. However, rennet- like enzymes from *Rhizomucor, Mucor* (Yegin et al., 2010), *Endothia parasitica* and *Aspergillus niger* (Siala et al., 2009) have received wide acceptability because of their high milk clotting and low proteolytic activities.

Aspergillus spp. produces two acid proteases, aspergillopepsin I and II, which are known to be secreted into the medium. Aspergillopepsin I (394 residues) is a typical aspartic peptidase, inhibited by pepstatin with milk clotting activity, whereas aspergillopepsin II (282 residues) is not inhibited by pepstatin (Schomburg and Schomburg, 2002).

The selection of a substrate for enzyme production by fermentation depends on several factors, including cost and substrate availability. Agro-industrial wastes are generally considered as a good source of low-cost substrates for the production of enzymes by fermentation processes. These wastes are usually supplemented with sufficient amounts of nutrients to satisfy the nutritional requirements of enzyme-producing microorganisms.

*Corresponding author. E-mail: dario.spelzini@conicet.gov.ar
Tel: 54 341 480 45 92/ int 253.

Abbreviations: TCWW, Tuna cooked wastewater; **ATPS,** aqueous two phase system; **PEG,** polyethylene glycol; **Pi,** potassium phosphate.

A medium-size canned tuna processing plant yields more than 15 tons/day of tuna cooked wastewater (TCWW), which contains approximately 4% of valuable proteins are discarded. However, TCWW which contains high biological oxygen demand value is generally rejected (Jao and Ko, 2002). This results in water pollution as well as in a loss of a beneficial protein source. The use of TCWW would allow to industrialists diminish the wastewater treatment cost and to recover high added value molecules. Therefore, a possible way to process this waste is its utilization as a nitrogen source in microbial culture medium for the growth of fungi. Many raw materials have been studied as substrates for the production of microbiological products like ram horn hydrolysate (Kurbanoglu and Algur, 2002) or thin stillage from a rice-spirit distillery (Yang and Lin, 1998).

Aqueous two-phase systems (ATPS) have been widely used in biochemical research for the separation and purification of macromolecules, cells and cell particles. ATPS have also been employed in several fields of the biotechnological industry for full-scale enzyme purification. This technique is considered potentially attractive for to obtain industrial enzymes due to its potential uses in scale-up, its low material cost and minimization of protein denaturation, among other advantages. In order to achieve both high yield recovery and a good purification factor for a target protein, a composition of the ATPS has to be selected to quantitatively extract the desirable protein from one of the phases with minimal concentration of contaminant molecules (Rito-Palomares, 2004).

In ATPS, macromolecules are selectively distributed between the two phases. The partitioning of soluble molecules occurs between the two bulk phases. This is characterised by the partition coefficient, Kp, which is the ratio between the concentration of solute in the top phase and in the bottom phase (Hatti-Kaul, 2000). The partition coefficient has been found to depend on several factors, such as polymer phase-forming, presence of salt, affinity ligands and other factors which act roughly independently (Albertsson, 1971).

In a previous paper, we partitioned chymosin and pepsin in ATPS composed by PEG-phosphate and found that both enzymes have high affinity for the top phase. The results indicated a strong protein-PEG interaction, in which PEG has a stabilizing effect on chymosin and pepsin (Spelzini et al., 2005).

In this study, the production of aspartic peptidase from *Aspergillus* spp. using tuna cooked wastewater as nitrogen source was studied and a procedure to recover the enzyme from the culture broth by aqueous-two phase systems was proposed.

MATERIALS AND METHODS

Chemicals

Malt culture media was purchased from Panreac Co. (Spain). (Hemoglobin and polyethyleneglycol (PEG) of average molecular mass 1450 and 8000 were purchased from Sigma Chem. Co. USA) and used without further purification. Bicinchoninic acid (BCA) assay was purchased from Pierce (Rockford, Ill) and used according to the manufacturer's specifications. Tuna cooked wastewater (TCWW) was kindly supplied by Jealsa Rianxeira (A Coruña, Spain).

Fungi strain

A. niger NRRL 3 and *A. awamori* NRRL 3112 was kindly supplied by CEREMIC (Rosario).

Pretreatment of TCWW, culture media and growth conditions

The TCWW obtained from industrial facilities was filtered through two layers of gauze to remove floating fat and solids, it was then concentrated by ultrafiltration using tangencial flow Prep/Scale-TFF cartridges (Millipore) with a molecular weight cut-off at 10,000 Da in two stages: concentration were followed by diafiltration, in order to remove saline content and the molecular weight components. The process stopped when it reached a salt dilution factor of 2.5.

The strain was maintained at 28°C for 7 days on malt agar slants. For conidia production, mature conidia was harvested by gentle agitation with glass beads and quantified by Turbidimetric methods.

The culture medium contained 25% (v/v) ultrafiltrated TCWW, glucose 20.0 g/l and yeast extract 1.0 g/l. For shake flask fermentation, 2 g/l of casein was added to a medium. The media was autoclaved at 121°C for 15 min in shake flask fermentation and 45 min in batch stirred tank fermentation.

The shake-flask cultures were operated at 300 rpm, 30°C, on a rotary shaking incubator. Batch fermentation in the stirred-tank fermentor was carried out with 4 L of culture medium without pH control at 30°C and 150 rpm and air flow rate was 3.0 vvm. An inoculum suspension containing 0.5×10^6 conidia/ml was used.

Partitioning in aqueous two-phase systems

Cell-free broth harvested from *Aspergillus* spp. culture was centrifuged at 1500 g for 15 min and used to assemble ATPS formed by PEG-potassium phosphate. Aspartic peptidase activity and protein content in the broth were determined by the Anson method (Anson and Mirsky, 1932) and bicinchoninic acid method, respectively (Brown et al., 1989).

The ATPS were obtained at pH 7 at longer tie line were published elsewhere (Lei et al., 1990), the water content was partially replaced for cell-free culture broth; the ATPS composition is shown in Table 1.

The partition of cell-free culture broth between both phases was equilibrated at 8°C. After mixing by inversion for 1 min and leaving it to settle for at least 30 min, the system was centrifuged at low speed for the two-phase separation. Samples were withdrawn from separated phases and aspartic peptidase activity and total protein content in top phase were determined. Partition coefficient (Kp), purification factor (PF) and yield percentage (y%) were calculated in the top phase.

The partition coefficient for total protein was calculated as:

$$Kp = \frac{[P]_{top}}{[P]_{bottom}} \quad (1)$$

Where, $[P]_{top}$ and $[P]_{bottom}$ are the equilibrium protein concentrations in the top and the bottom phase, respectively. The partition coefficient for aspergillopepsin was determined as:

Table 1. PEG-Pi ATPS composition.

PEG MW	Pi 28 % w/w(g)	PEG (g)	Water (g)	Cell-free culture broth (g)
1,450	49	18.6	14	20
1,450	49	18.6	24	30
8,000	41	15.5	23.5	20
8,000	41	15.5	13.5	30

PEG, Polyethylene glycol; Pi, potassium phosphate.

Table 2. Composition of TCWW in each fraction of ultrafiltration process.

Parameter	Protein (g/l)	Carbohydrate (g/l)	NaCl (g/l)
TCWW raw	10	16.55	3.46
Permeate 1	3	6	2.83
TCWW retentate 1	14.84	14.32	2.56
Permeate 2	2.43	0.46	1.48
TCWW retentate 2	11.44	8.69	1.4

1, Concentration stage; 2, diafiltration stage. **TCWW,** Tuna cooked wastewater.

$$Ke = \frac{\bar{A}_{top}}{\bar{A}_{bottom}} \quad (2)$$

Where, $[A]_{top}$ and $[A]_{bottom}$ are the equilibrium enzymatic activity in the top and the bottom phase, respectively.

The purification factor in the top phase was calculated as:

$$PF = \frac{\bar{Act}_{top}}{\bar{Act}_{broth}} \quad (3)$$

Where, $[Act]_{top}$ and $[Act]_{broth}$ are the specific enzymatic activities in the phase top and cell-free broth, respectively.

Analytical methods

The protein content was determined using the Folin phenol reagent (Lowry et al., 1951). Due to the high collagen content in TCWW, for routine determination of protein concentration, a modification of Lowry's method was used (Komsa-Penkova et al., 1996). For protein determination in cell-free culture broth partition in ATPS, a bicinchoninic acid (BCA) assay was used. Aspartic peptidase activity has been described by Anson. There is no international agreement about units obtained by the hemoglobin digestion method. In this study, we used an arbitrary system whereby the amount of enzyme that causes an increase in the absorbance of the hemoglobin filtrate of 0.001 at 280 nm in 1 min is taken as 1 unit (Barret, 1995). The carbohydrate content was estimated with the conventional phenol-sulfuric acid reaction procedure (Dubois, 1956).

RESULTS AND DISCUSSION

Composition of TCWW

The composition of both retentate and permeate in each stage of ultrafiltration process are shown in the Table 2. Asymmetric distributions on protein and carbohydrate contents between both permeate and retentate fractions were observed. The concentration stage achieved adequate protein concentrations to support the fungi growth, although, the carbohydrate level was not enough. The diafiltration stage probed to be useful for TCWW desalting, since the initial NaCl concentration was usually high in these juices.

Shaked flask cultures

Cultivation of conidia was conducted in 250 ml Erlenmeyer flasks containing 50 ml of basal medium. Each Erlenmeyer was placed on a rotary shaker at 300 rpm and 30°C.

In Figures 1 and 2, biomass and extracellular aspartic peptidase activity production for *A. niger* and *A. awamori* are shown. The influence of casein (2 g/l) in the medium was analyzed. The control medium had no TCWW.

The highest biomass yield (9 g/l) was obtained at 48 h incubation time for *A. niger* and 72 h for *A. awamori* independent of the presence of casein. The extracellular aspartic peptidase activity reached a maximum (2 A.U.) at 72 h for *A. niger* without casein; for *A. awamori*, the highest enzymatic activity was obtained at 48 h. Both fungi strain were not developed in the absence of TWCC.

An increase in the production of proteases in presence of casein was reported (Cardoza et al., 2003; Yegin et al., 2010). In this study conditions of growth, an influence of casein on the production of extracellular peptidic activity was not observed. Due to the fact that casein did not produce a significant increase in the biomass and aspartic peptidase activity, the medium without casein is

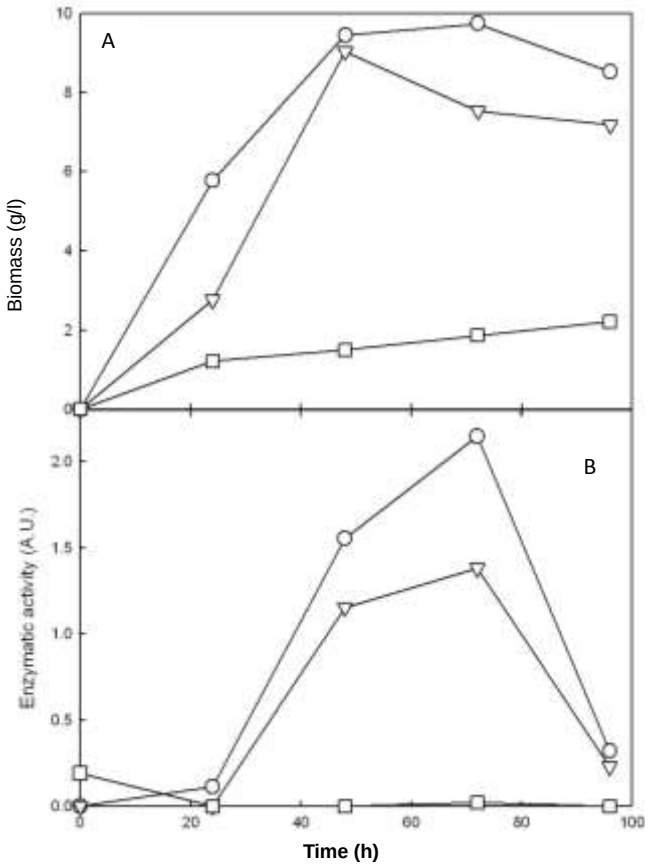

Figure 1. *A. niger* NRRL 3 time course biomass production (A) and extracelular enzymatic activity production (B). Shake flask fermentation: medium without casein, circle; medium with casein (2 g/l), triangle and control medium (without TCWW), square.

to be selected for further assays.

Bioreactor cultures

A. niger and *A. awamori* culture profiles obtained are shown in Figures 3 and 4, respectively. Culture profile for *A. niger* showed a maximum biomass at 96 h incubation time, 5.6 g/l; extracellular aspartic peptidase activity reached a maximum of 0.6 A.U. at 24 h and another peak of 0.3 A.U., at 100 h. Time course biomass production for *A. awamori* was higher, 5.5 g/l, from 48 to 72 h of incubation time. This maximum value coincides with a higher enzymatic activity yield, approximately 2 A.U. Another peak of enzymatic activity during the first 24 h of culture was observed. This fact coincides with reports for other researchers, where two peaks of enzymatic activity of aspartic peptidase was observed, the first without milk clotting activity, possibly aspergillopepsin I and the second peak, with milk clotting aspartic peptidase is aspergillopepsin II (Channe and Shewale, 1998).

Production of extracellular aspartic peptidase by *A. awamori* in stirred tank fermentation was selected for further extraction in PEG-phosphate aqueous two-phase system.

Culture broth partitioning in ATPS

After fermentation, culture broth from *A. awamori* with enzymatic activity was pooled and centrifuged. Cells and insoluble components were discarded. The supernatant was used as a crude enzyme solution.

The ATPS was constructed with PEG, potassium phosphate and cell-free culture broth being used instead of water. After mixing, the systems were centrifuged at 3000 rpm for 3 min and to stand for 2 h at a bath at 8°C. Samples of top and bottom phases were taken in order to quantify the enzyme activity and total protein concentration.

Figure 5 shows Kp for aspartic peptidase activity and total protein value for partition of filtrate culture broth in ATPS formed by PEG-potassium phosphate. It should be noted that after thermodynamic equilibrium was reached,

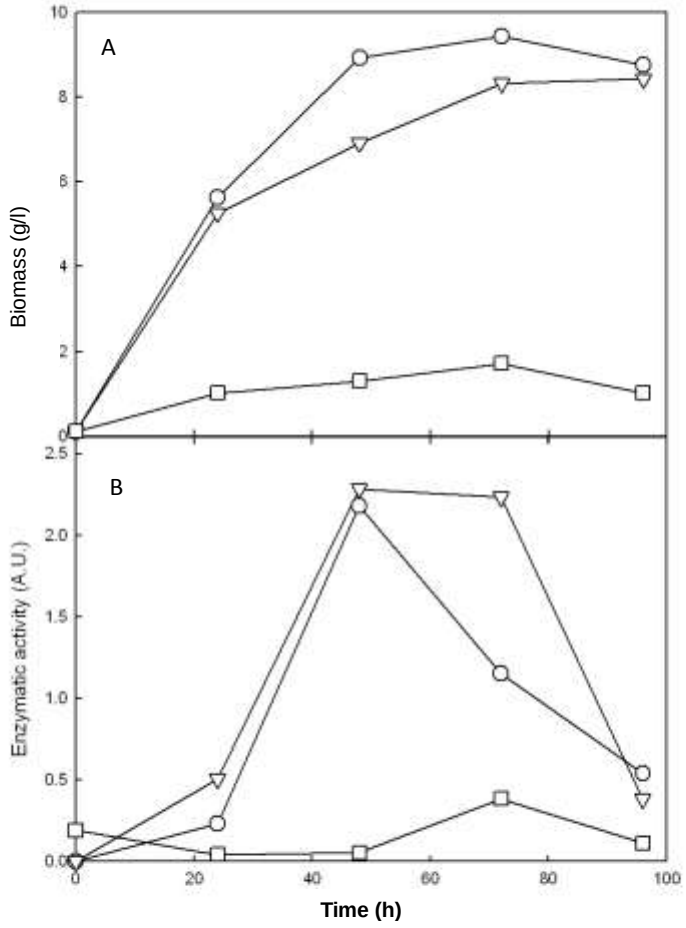

Figure 2. *A. awamori* NRRL 3112 time course biomass production (A) and extracelular enzymatic activity production (B). Shake flask fermentation: medium without casein, circle; medium with casein (2 g/l), triangle and control medium (without TCWW), square.

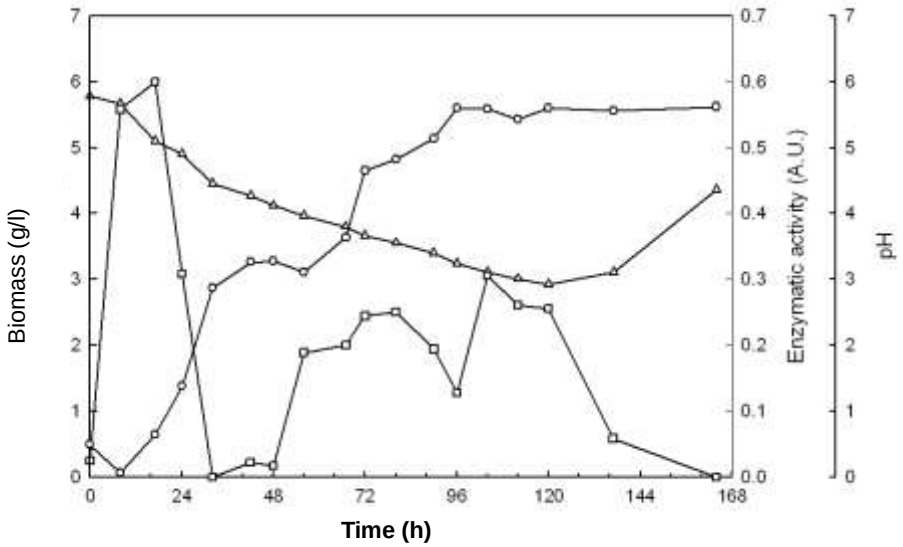

Figure 3. Growth and extracellular aspartic peptidase production in *A. niger* batch fermentation, biomass production (circle), extracellular aspartic peptidase activity (square) and pH (triangle).

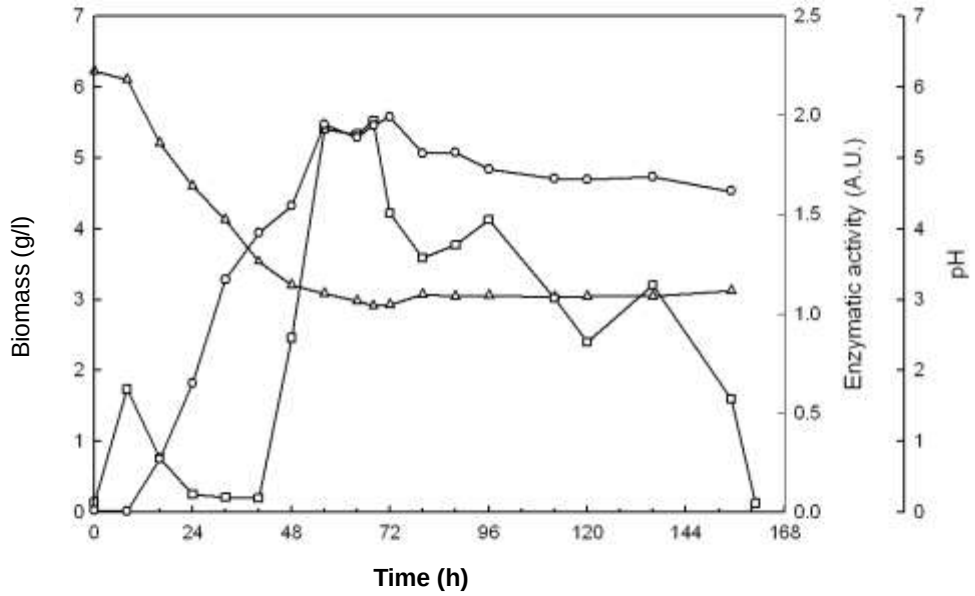

Figure 4. Growth and extracellular aspartic peptidase production in *A. awamori* batch fermentation, biomass production (circle), extracellular aspartic peptidase activity (square) and pH (triangle).

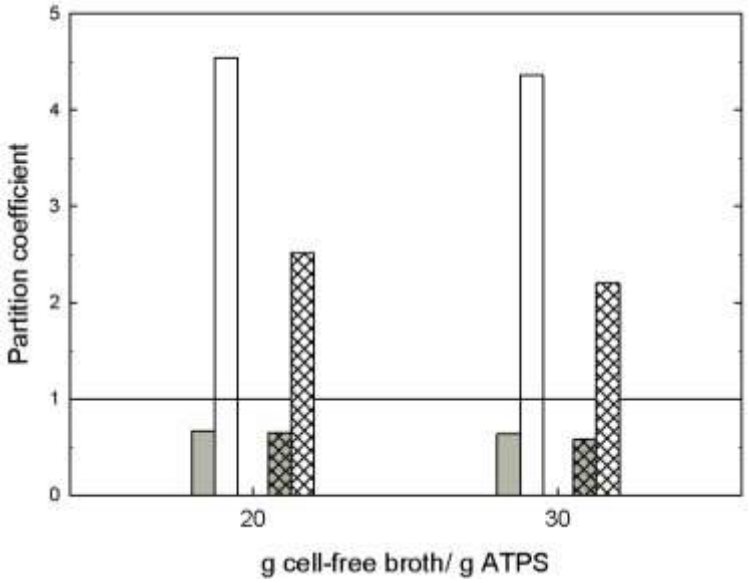

Figure 5. Kp values for total protein (grey bars) and Ke values for aspergillopepsin (white bars) of cell-free broth in ATPS PEG 1450-Pi (flat bars) or ATPS PEG 8000-Pi (rhombuses bars).

an amorphous precipitate was observed in interface. In all cases assayed, contaminant proteins were spread to interface or bottom phase, Kp values for total protein resulted lower than 1, while the extracellular aspartic peptidase was partitioned to the top phase, Ke values for aspergillopepsin were higher than 1. The Ke values for enzymatic activity were greater for ATPS formed with PEG 1450 than those obtained with PEG 8000.

A previous report showed that Kp values of other aspartic peptidase, like chymosin and pepsin, diminish when PEG molecular weights increase in ATPS. The excluded volume theory shows that PEG concentration or its increase in molecular mass induces a diminution of the protein solubility in the phase where the protein is

Table 3. Purification table of partition of filtrate broth in ATPS PEG 1450-potassium phosphate and PEG 8000- potassium phosphate, pH 7, 8°C.

ATP	Culture broth (g)	Total protein (g/l)	Total activity (A.U./ml)	Specific activity (A.U./mg)	Purification factor	Yield (%)
PEG 1450-Pi	Fraction centrifugated	3.52 ± 0.02	1.93 ± 0.05	0.55 ± 0.02	1.0	100
	30*	1.03 ± 0.04	1.32 ± 0.07	1.28 ± 0.06	2.3 ± 0.4	68
	20*	0.70 ± 0.02	0.85 ± 0.04	1.22 ± 0.09	2.2 ± 0.1	44
PEG 8000-Pi	30*	0.97 ± 0.09	1.12 ± 0.03	1.16 ± 0.04	2.1 ± 0.3	58
	20*	0.62 ± 0.03	0.68 ± 0.02	1.10 ± 0.06	2.0 ± 0.2	35

*Cell-free culture broth added to ATPS (%w/w).

Table 4. Purification table of partition of filtrate broth in ATPS PEG 8000-potassium phosphate pH 7, temperature 8°C.

Culture broth (g)	Total protein (g/l)	Total activity (A.U./ml)	Specific activity (A.U./mg)	Purification factor	Yield (%)
Cell free culture broth	3.52	1.93	0.55	1.0	100
30*	0.97	1.12	1.16	2.1	58
20*	0.62	0.68	1.10	2.0	35

*Cell-free culture broth added (%w/w).

situated (Spelzini et al., 2005).

Kp values for chymosin and pepsin observed were higher than those obtained in this work; this fact may be due to several interactions between the target protein and another protein present in natural products. The phase equilibrium was modified due to interfacial precipitate formed. It should be noted that the binodial diagram of an ATPS may change when a complex mix is partitioned modifying the PEG and salt concentration at the equilibrium, thus, modifying the protein partition coefficient.

Tables 3 and 4 show the purification factor and yield percentage in the top phase for extracellular aspartic peptidase from cell-free broth in ATPS formed with PEG 1450 and PEG 8000, respectively and potassium phosphate.

There were no substantial differences in PF obtained when different amounts of culture broth were added to ATPS; the PF obtained was independent of the PEG molecular weight used. The yield was higher for PEG 1450-containing ATPS. The percentage yield increased when the amount of broth added was greater. Decrease in the yield observed would occur by the formation of the interfacial precipitate, but the precipitate would comprise for contaminant protein.

The ATPS was used to extract the target protein from a complex mixture, since this molecule is partitioned mainly in the upper phase, rich in PEG and its impurities are driven towards the interface or the bottom phase. Purification factors higher than those presented in this study were reported but with similar yields even after a precipitation with ethanol followed by an ion exchange chromatography, and size-exclusion chromatography was carried out for the isolation of an aspartic peptidase from *Streblus asper* (Senthilkumar et al., 2006).

Conclusions

The non-conventional media derivate from ultrafiltrate TCWW, supplemented with glucose and vitamin were adequate to *A. awamori* growth and production of extracellular aspartic peptidase activity in submerged culture. These media which were prepared with a waste product could also be used in other fungi cultures to obtain high valuable compounds under other growing conditions.

The extraction of aspartic peptidases from broth culture with ATPS formed with PEG 1450 and potassium phosphate could be a method to apply as first extraction step with an adequate yield.

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