Effects of oxidising atmosphere on brewer's yeast hydrothermal treatment and subsequent biopolymer recovery

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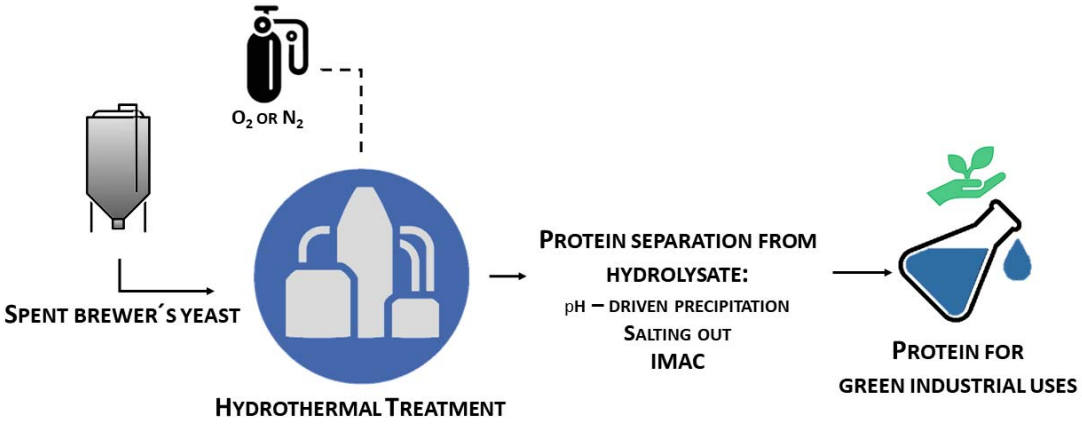
GRAPHICAL ABSTRACT



HIGHLIGHTS

The presence of oxygen enhanced yeast disintegration.

Soluble proteins abated at long times when oxygen was present.

Oxygen caused a convergency in the different protein isoelectric points to 3.

Oxygen decreased protein hydrophobicity, worsening saline precipitation.

Oxygen reduced protein recovery but enhanced selectivity in IMAC.

ABSTRACT

Spent brewer´s yeast is an underused by-product, whose current management offers low returns. This article studies, for the first time ever, the hydrothermal treatment of spent brewer´s yeast, paying special attention to the effect of the presence of oxygen on the physical properties, composition and potential recovery of valuable products from the hydrolysed yeast, as previous step towards the employment of these recovered proteins as renewable chemical feedstock for green industrial uses.

Results showed a higher VSS disintegration employing an oxidising atmosphere, as well as a better solubilisation in terms of solid COD reduction, although it also lowered soluble COD but without significantly reducing soluble TOC. Both treatments led to an almost complete solubilisation of protein and carbohydrates from sludge, which were subsequently degraded in presence of oxygen if reaction times were lengthened. Looking at biopolymer recovery, protein precipitation by pH adjustment worked better in hydrolysates obtained in presence of oxygen, with almost 90% of protein precipitation and good selectivities at pH 2.5 and 3. Salting out was more effective, in terms of selectivity, for samples obtained in absence of oxygen and low concentrations of ammonium sulphate. The application of IMAC showed better results on yeast hydrolysates obtained under an inert atmosphere, reaching maximum sorption capacities of almost 100 mg protein/ g dry resin, three times higher than those observed using the hydrolysate produced in presence of oxygen. A new perspective in yeast management during beer production is open as renewable chemical feedstock for protein, although further studies are required to optimise protein recovery and purification with respect to its applications.

KEYWORDS

Hydrothermal treatments, brewer’s yeast, protein recovery, kinetic model, IMAC

1. Introduction

Beer is one of the most popular drinks all around the globe, particularly in Europe. According to EUROSTAT [1], more than 39 billion litres of beer were produced during 2018 in the old continent. It is well known that the main responsible for beer production from the starch fermentation from cereal are yeasts, mainly those from *Saccharomyces* genus. During the fermentation, the cellular growth involves the production and drainage of around 1.7- 2.3 kg of yeast (expressed as dry weight) per tonne of beer, thus generating a huge amount of wet biomass which must be properly managed [2]. In fact, spent yeast is the second major by-product of the brewing industry [3]. Due to its high nutritional

value [4], brewer´s yeast has been traditionally used as animal feedstock, since it represents an economically friendly source of proteins [5].

Currently, different strategies focused on the transformation of spent brewer´s yeast into other added-value resources are becoming important. The most explored alternative is the use of spent yeast as a nutritional supplement in human diets; for example, as an ingredient to increase the protein content in vegan diet or in functional foods [2,6–8]. However, although this application is extended, it has an important disadvantage due to the high content of nucleic acids in the yeast, that could lead to high values of uric acid and its associated illnesses [8]. In addition, the huge quantities of yeast produced every year provide enough feedstock for a wider variety of applications. Therefore, the research for new applications for spent yeast is providing new innovative uses for this waste in several fields. As it presents high nutritional values for animals, it also represents a source of proteins and minerals for microorganisms. For this reason, yeast extracts have been used as sole media or along with supplements in several interesting fermentation processes such as the growth of lactic acid bacteria, the production of succinic acid or the use of the extracts as fermentation substrates [9–11]. Other applications are based on the fact that yeasts are cells and, as such, produce the enzymes required in their life cycle. These enzymes also have a commercial interest; for instance, the use of pectinase is being studied in the juice industry [12]. Moreover, other constituents of the yeast, such as monosodium glutamate or some nucleotides, have also been employed after being appropriately treated as flavour enhancers in meat, sauces and other foods [2,5,13,14]. Spent yeast, as other biomasses, also shows adsorbent properties and has been employed in the removal of chromium, lead or dyes, hence being useful in bioremediation and treatment of contaminated industrial wastewaters [15,16].

Nevertheless, most of these routes for the reuse of yeasts require their previous disruption in order to release and solubilise their compounds. Different methods of cellular lysis have been reviewed extensively in the bibliography and employed for this purpose, such as homogenization, thermal lysis, sonication, chemical disruption or enzymatic lysis, being categorized mainly as mechanical or chemical ones [17]. During the mechanical lysis, yeast membrane is physically broken down by using shear forces. These methods are the most popular, with high throughputs, although problems such as heating of sample volume, degradation of cellular products, cell debris and higher cost limit the use of this method. On the other hand, chemical lysis methods use reagents such as buffers, detergents or enzymes to disrupt the cell membrane. In this case, their main

drawbacks are that an additional purification step has to be incorporated to remove the corresponding reagent and the difficulty to achieve a completed lysis. Nevertheless, there is another yeast lysis method, the thermal lysis. This one is based on the supply of heat to the cells to denature proteins and lyse the cells. However, the heating for a long period may damage target materials such as proteins and enzymes. It is true that the thermal denaturation has several important consequences, such as a loss of biological activity, the increase in the viscosity of protein solutions, a decrease in the solubility, an increase in the reactivity of side groups and altered surfactant properties and sensitivities to enzymatic proteolysis [18]. These effects may result in problems, but are also recognised as benign and even desirable in non-food applications, such as the use of proteins for the production of coatings, films, adhesives or surfactants [19,20]. It is also well-known that in hydrothermal media under high temperature, carbohydrate and protein produce several chemicals (furfural, 5-hydroxymethyl furfural, and nitrogenous aromatics) which are often inhibitory to microbial growth [21]. In addition, the possibility of treating high volumes with a high percentage of solids, as in the case of the spent brewer´s yeasts, in a short time and with a high yield makes thermal lysis attractive to the supply of proteins from yeast for uses where keeping the protein native structure or the presence of inhibitory compounds to microbial growth are not essential, such as in the production of resins [22], films [23], organic fertilizers [24], adhesives [25] or bioplastics [26].

Hydrothermal treatments, that is to say, the thermal lysis at high temperatures and pressures in absence (thermal hydrolysis) or presence (wet oxidation) of oxygen, have already been successfully applied to different waste biomasses with high protein content, mainly waste activated sludge from wastewater treatment plants and food industry wastes [27,28]. Nevertheless, the literature dealing with the thermal hydrolysis of spent brewer's yeast in order to obtain valuable biomolecules is extremely scarce. In this regard, Espinosa et *al*. [29] evaluated the hydrothermal treatment of oleaginous yeast biomass at 280 ºC and 500 psi as an alternative bioprocessing strategy for hydrolysis and lipid extraction resulting in fatty acids used for biofuel production. During the treatment, the original biomass lipids, mainly triacylglycerides, were converted into fatty acids and recovered by hexane extraction, thus obtaining a stream of these compounds free of sulphur and low in salts and nitrogen. In fact, only one paper deals specifically with the hydrothermal treatment of Baker´s yeast cells for the production of proteins and amino acids [11]. The purpose of that research was to determine the effect of temperature and hydrolysis time during the thermal hydrolysis under an inert atmosphere on the amount

of residual yeast, TOC, and the amount of protein and amino acids in the soluble products, not paying attention to other biopolymers or to the subsequent potential separation of compounds from the hydrolysate. They found that the amount of solubilised protein increased with an increase in temperature, while that of amino acids decreased with increasing temperature. The highest yield of protein and amino acids obtained were 0.16 and 0.063 mg/mg of dry yeast, respectively.

Most of the available works involve thermal hydrolysis at severe conditions to achieve a total liquefaction of the yeast in order to obtain biofuels [30–32] and not proteins, so they will not be here discussed. Only Miao et *al*. [31] studied a sequential hydrothermal liquefaction process to firstly obtain sugar and protein at a lower temperature, then converting the remaining biomass to bio-oil at a higher temperature. They reported that high temperatures (>180 ºC) in the first step increased the production of inhibitory compounds. The authors proposed the use of isolated polysaccharides and protein at low temperature as potential carbon and nitrogen sources for repeated culture of yeasts. Regarding the application of wet oxidation treatments to brewer's yeast, to the best of our knowledge, the literature in this field is inexistent, even when wet oxidation has been proved to be a suitable technique during the treatment of other biomasses with a high protein content [33].

The potential applications of the proteins previously described are in the line of the context of circular economy and green industrial uses, by taking advantage of a waste. However, the first step that has to be taken is the recovery of the proteins. Although the number of protein recovery methods is high, from an industrial point of view, it would be desirable the use of safe, cheap and/or reusable materials. In this basis, some interesting methods for protein recovery from the hydrolysates are pH driven precipitation, salting out or IMAC (Immobilized Metal Affinity Chromatography). These techniques have been used in other protein recovery strategies from effluents of food industry [34]. The adjustment of pH and the use of salting out have been widely studied and require specific testing in each case to find the isoelectrical point or the salting out point. IMAC is based on the bind of the specific groups of protein to the metal [35]. The most interesting fact regarding IMAC is the cheapness of the technique, since the resin could be reused by eluting protein with a saline solution. Although other methods match the premises of reusability and non-toxicity, as can be the case of membrane filtration, their application to recover protein from hydrolysates is extremely complex. Previous studies in sewage sludge hydrolysate shed light on the size distribution of its components, showing that the

solubilized molecules had different sizes that make the separation almost impossible by membrane technologies [36].

Due to the aforementioned, the aim of this article is to study the hydrothermal treatment of spent brewer´s yeast, paying special attention to the effect of the presence of oxygen on the physical properties, composition and potential recovery of valuable products from the hydrolysed yeast, as well as proposing and validating a kinetic model based on the results obtained. These knowledge may well constitute a first step towards the employment of spent brewer´s yeast as renewable chemical feedstock for green industrial uses.

2. Material and methods

2.1. Spent brewer´s yeast

The yeast samples were withdrawn from the bottom of a conical-cylindrical fermenter from an artisan brewery (Cerveza Caleya), situated in Asturias (Spain). The yeast species was identified as *Saccharomyces pastorianus*. It is important to point out that yeast samples did not contain hop or other flavouring compounds. Once collected, the yeast samples were stored at 4 ºC until ready to use. An initial analysis of the yeast showed a high concentration of TSS (more than 150 g/L) and carbohydrates, as expected due to the origin of the sample. It has to be reminded that yeast samples were withdrawn from a beer fermenter containing barley wort as fermentation medium, thus explaining the high initial concentration of carbohydrates. The initial analysis also showed that more than 98% of TSS were VSS, so suspended solids will be treated as VSS throughout the text. Therefore, before being hydrolysed, spent yeast was washed 3 times with distilled water, in order to remove the remaining liquid corresponding to the fermentation medium. The characteristics of the washed yeast that was charged into the reactor are shown in Table 1.

Table 1. *Initial parameters after washing the yeast samples employed in the experiment.*

Parameter	***Units***	***Value***
pH		5.6 ± 0.5
TSS	*g/L*	26 ± 6
VSS	*g/L*	26 ± 6
SVI	*mL/g*	18 ± 3
TCOD	*g O_2 /g VSS_o*	1.6 ± 0.3
SCOD	*g O_2 /g VSS_o*	0.22 ± 0.07
TOC	*g C /g VSS_o*	0.07 ± 0.03
Soluble proteins	*g /g VSS_o*	0.07 ± 0.04
Soluble carbohydrates	*g /g VSS_o*	0.02 ± 0.02

2.2. Hydrothermal treatments

Hydrothermal treatments were carried out in a 1 litre capacity reactor (PARR series 4520 reactor) equipped with a six-bladed turbine stirrer. In order to study the effect of an inert or oxidizing atmosphere on the process, nitrogen or oxygen, respectively, were added at a flowrate of 1200 mL min^{-1}, after both being previously conditioned by means of an upstream humidifier of 2 litre capacity. Both reactor and humidifier were filled at a 70% of their maximum capacity aiming to safety purposes. The pressure was controlled using a back-pressure valve at the end of the gas line, whereas the temperature of both reactor and humidifier, the flowrate and the stirrer speed were regulated by a PID controller.

2.3. Biopolymers recovery

With this purpose, hydrolysates obtained after 80 minutes of treatment were used. This time was selected attending to the biopolymers concentration that will be show in figure 2 (section 3.2). In this figure, it can be observed that protein did not increase in high amounts from minute 80 and carbohydrates were also high at that time. In addition, treatment time was not too long, thus avoiding excessive damage in the biopolymers.

The recovery methods selection strategy was set on the basis of four main premises: one step, low cost, the avoiding of harmful chemicals and feasibility of industrial implementation. Therefore, the methods selected were pH driven precipitation, ammonium sulphate and IMAC sorption.

2.3.1. pH-driven precipitation

Different pH values (2, 2.5, 3, 3.5, 4 and 9) were tested by adding HCl 1 M or NaOH 1 M to the hydrolysates obtained either in presence or absence of oxygen to find out which value yields the higher precipitation of one of the biopolymers with the less precipitation of the other. Precipitate was separated by centrifugation at 10000 g for 10 min. This method has been successfully applied to protein separation strategies and its mechanism involves the shifting of pH near the isoelectric point of the proteins to achieve protein to protein hydrophobic interactions, thus making them precipitate [34].

2.3.2. Saline precipitation

The addition of salts to precipitate proteins is known as salting out. Different percentages of the saturation concentration of ammonium sulphate (0.764 g/mL) were employed: 100%, 90%, 80% and 50%. Precipitate was separated by centrifugation at 10000 g for 10 min.

2.3.3. IMAC sorption

Immobilized Metal Affinity Cromatography (IMAC) was employed, using the Lewatit TP-207 resin (Sigma). Copper was bound to the resin by soaking into a $CuCl_2$ 1M solution for at least four hours. This metal was chosen owing to its high affinity to peptide bonds [37]. Then, different L/S relations between resin and hydrolysates were tested in order to elucidate the resin capacity for protein retention.

The main advantage of IMAC technology is the reutilisation of the resin once protein has been eluted, thus sharply decreasing the recovery step cost.

2.3.4. Recovery and selectivity

The percentage of biopolymer precipitation, or recovery, was calculated according to equation 1:

$$\%_{precipitated} = \left(1 - \frac{\text{supernatant concentration after precipitation}}{\text{supernatant concentration before precipitation}}\right) x100 \quad (1)$$

A selectivity factor was calculated according to the following equation [38]:

$$\alpha_{p/c} = \frac{\left(\frac{(C_p)_{\text{before precipitation}} - (C_p)_{\text{supernatant after precipitation}}}{(C_c)_{\text{before precipitation}} - (C_c)_{\text{supernatant after precipitation}}}\right)}{\frac{(C_p)_{\text{supernatant after precipitation}}}{(C_c)_{\text{supernatant after precipitation}}}} \quad (2)$$

2.4. Analytical methods

pH, total and volatile suspended solids (TSS and VSS), sludge volumetric index (SVI), total and soluble chemical oxygen demands (TCOD and SCOD, respectively) were measured according to the Standard Methods [39]. Total soluble organic carbon (TOC) was determined by means of a TOC-VCSH Analyzer (Shimadzu, Japan) and colour number (CN) was calculated according to the equation proposed by Tizaoui et *al*. [40]:

$$CN = \frac{SAC_{436}^2 + SAC_{525}^2 + SAC_{620}^2}{SAC_{436} + SAC_{525} + SAC_{620}} \quad (3)$$

In equation 1, SAC_i are the spectral absorption coefficients at a wavelength of *i* nanometers, which were determined using an AnalitykJena Spectrophotometer. At this point, it should be pointed out that sCOD, TOC and CN were measured in the supernatant obtained after centrifuging the yeast samples at 10000 g for 10 minutes.

The average oxidation state of carbon (AOSC) is calculated by means of SCOD and TOC values, following the next equation [41]:

$$AOSC = 4 - 1.5 \cdot \frac{SCOD}{TOC} \quad (4)$$

AOSC values can oscillate from -4 to 4, being negative for reduced carbon (-4 is associated to the most reduced carbon molecule, CH_4) and positive when carbon is

oxidized (4 is the value given to CO_2, the most oxidized form of carbon). For the sake of a better understanding, it has to be pointed out that AOSC refers to the oxidation state of a mixture, whereas the term employed to indicate the oxidation state of a specific compound is MOC (Mean Oxidation state of Carbon).

The concentration of soluble biopolymers (proteins and carbohydrates) were also determined in the supernatant. The concentration of soluble proteins was measured by the Lowry method, using BSA (Sigma) as standard [42]. Regarding the soluble carbohydrates, its concentration was determined by the phenol-sulphuric method, with D-+-glucose (Sigma) as reference [43].

3. Results and discussion

3.1. Effect of the atmosphere on the physical properties

The evolutions of the pH, VSS, SVI, TCOD, SCOD, TOC, AOSC and CN during the hydrothermal treatment of spent yeast under an inert or oxidising atmosphere are shown in figure 1.

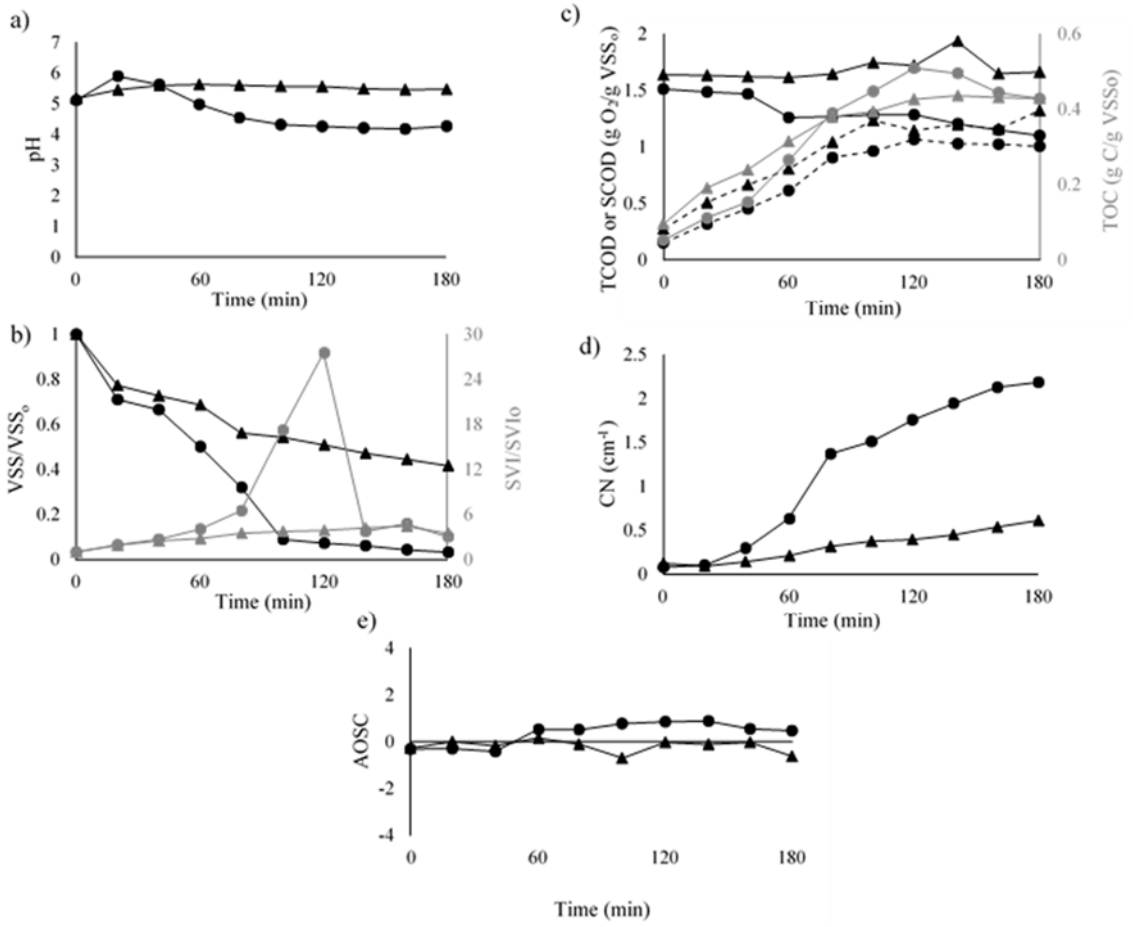


Figure 1. *Evolution of a) pH, b) VSS and SVI (grey), c) TCOD, SCOD (dashed lines) and TOC (grey), d) CN, e) AOSC during wet oxidation (●) or thermal hydrolysis (▲) of brewer´s spent yeast. In all cases: 160 °C and 40 bar, 1200 mL min^{-1} of either N_2 or O_2 and 125 rpm of agitation.*

Before starting the discussion of the results, it is important to take into account that, under an inert atmosphere, the yeast solubilisation via VSS disintegration is supposed to be only based on hydrolysis reactions, accelerated by the use of high temperatures and pressures, these latter to avoid the vaporization of the liquid phase. On the other hand, when an oxidizing atmosphere is selected, oxidation reactions are present as well. These oxidation reactions are more aggressive than the hydrolysis ones, being able to modify to a greater extent the structure of the molecules by generating oxidized functional groups, such as carboxyl, carbonyl, ketone, hydroxyl… [44,45]. Results here obtained were in line with these premises. So, the changes in the pH (fig. 1a) under an inert atmosphere were almost negligible, with only a slight increase in the alkalinity for the first 60 minutes (from 5.17 to 5.63), probably related to the solubilisation of proteins and amino acids. When an oxidising atmosphere was selected, in contrast, the initial increase in the pH attributed to the release of cytoplasmic materials was followed by an acidification due to the oxidation reactions from 5.45 to 4.26 after 120 minutes, with this value remaining constant until the end of the treatment. Focusing on VSS behaviour (fig. 1b), in both thermal hydrolysis and wet oxidation, the reduction was noticeable after only 20 minutes even at low temperatures, probably due to the solubilisation of soluble microbial products (SMP) and lightly bound extracellular polymeric substances (LB-EPS) [36]. From this point, the effect of the oxidizing reactions was evident. The disintegration of the VSS was 10% higher in presence of oxygen than in its absence after 60 minutes. From this point until the end of the process, the behaviours totally differed. Thermal hydrolysis only provoked a slight VSS decrease from this time to the end of the treatment (180 min), whereas wet oxidation led to an almost complete abatement of the VSS. To illustrate these differences, at minute 180, thermal hydrolysis disintegrated a 59% of the initial VSS, whereas wet oxidation reduced a 97% the initial VSS. To put some light on the mechanism that makes wet oxidation more efficient in VSS destruction, it has to be known the characteristics of the reactions. Hydrolysis reactions require a lower activation energy than oxidation reactions, so the last only occurred when temperature is high, after the initial heating-up of the reactor (around 40 min). This effect was also observed in other works [33]. However, VSS diminution can be related to solubilisation or their complete destruction to carbon dioxide or volatile compounds that could leave the reactor. The SVI can be used to discern between these two options. In thermal hydrolysis, SVI increased during all treatment time, being more than three times higher at the end, due to the biopolymers release. This phenomenon will be discussed later, but briefly, the more the

protein concentration, the worse the settleability [46]. However, during wet oxidation, the SVI astonishingly rose from minute 80 to minute 120 (multiplying by 27 the initial SVI value), indicating an intense solubilisation (corresponding with the high decrease in VSS observed) during those minutes. During the final minutes, SVI dramatically dropped, suggesting the oxidative degradation of those biopolymers that hindered the sedimentation, ending with a final SVI value 3 times higher than the initial value.

As can be seen in figure 1c, TCOD did not show significant changes during the hydrothermal treatment of yeast in absence of oxygen, because no oxidation reactions occurred. Meanwhile, the organic load decreased in presence of oxygen and oxidation reactions caused the mineralization of a 30% of the initial TCOD from minute 40, when the high temperature made these reactions prevail over the hydrolysis ones. Moving on to the soluble organic load, it has to be pointed out that the SCOD was higher in absence than in presence of oxygen, even when the VSS disintegration was significantly higher under an oxidizing atmosphere, as previously explained. This can be easily explained taking into account that oxidation reactions not only caused a faster disintegration of solids but also the mineralization of a fraction of this dissolved organic matter to carbon dioxide. Maximum SCOD concentrations were achieved after 100 minutes of treatment under an inert atmosphere (75% of the initial COD) and after 120 minutes when oxygen was present (70% of the initial COD). These results differ from the initially expectable behaviour, in which wet oxidation should produce a higher SCOD than thermal hydrolysis because the VSS disintegration was also higher under an oxidising atmosphere. The higher SCOD observed under an inert atmosphere can be easily explained taking into account that oxygen not only accelerated the lysis of the yeast, but also partially oxidised the soluble organic load, thus reducing the SCOD.

Regarding TOC, both treatments caused an increase in this parameter during the first minutes of reaction. After this time, TOC concentration remained constant when an inert atmosphere was employed, but decreased in presence of oxygen, due to the formation of carbon dioxide by means of oxidation reactions. Comparative research in sewage sludge between wet oxidation and thermal hydrolysis showed similar results [47]. Unlike the case of SCOD, it should be stressed that an oxidizing atmosphere did led to higher concentrations of soluble TOC than an inert one: 0.51 g C/g VSS_o and 0.44 g C/g VSS_o, respectively (Figure 1c). These differences in the evolutions of SCOD and TOC with or without oxygen suggest that the solubilised compounds were partially oxidized, but not completely mineralized to carbon dioxide, in presence of oxygen. This would

involve the accumulation of partially oxidised intermediates in the medium under an oxidising atmosphere and a faster decrease in the SCOD concentrations than in the TOC ones, as observed experimentally.

These partially oxidised compounds would also be responsible for the colour evolution showed in figure 1d. Although either inert or oxidising atmospheres caused an increase in the CN, this was substantially higher in presence of oxygen. In fact, this difference in colour, which was already noticeable after 40 minutes, progressively increased with the time of treatment, with a final CN in presence oxygen 3.6 times higher than in its absence. The increase in colour in absence of oxygen is probably due to the formation of Maillard and Amadori products at temperatures from 140 to 165 °C, which also show a brownish colour [48]. These molecules are reported to appear in materials with high concentration of sugars and proteins, molecules that our sample showed to be rich in [49]. As time advanced and oxidation reactions began to take place in the wet oxidation experiment, a modification in the nature of the molecules occurred, generating other coloured compounds such as phenolic derivates [50,51].

Finally, and closely related to COD and TOC, AOSC showed in figure 1e represents the oxidation state of the liquid mixture. As can be expected in absence of oxidation reactions, AOSC for the hydrothermal treatment of the yeast under an inert atmosphere did not show significant changes, with a negative value of around -0.1 during all the treatment, thus corroborating the non-oxidative nature of this process. In contrast, a slight increase in AOSC values was observed when the hydrothermal treatment was carried out in presence of oxygen, beginning from an initial reduced mixture (AOSC = -0.3) to a final pool of partially oxidised compounds after 180 minutes (AOSC = 0.47). Due to the oxidation reactions, the molecules released from the cells were enriched in carboxyl, carbonyl, hydroxyl groups… raising their MOC value, as well as reducing the pH of the hydrolysed yeast. This can be checked by comparing figs. 1a and 1e, where AOSC values raised when pH dropped in presence of oxygen, effect not observed for thermal hydrolysis experiments. This phenomenon was attributed to the formation of organic acids, a typical intermediate partially oxidised in wet oxidation treatments [52–54].

3.2. Effect of the atmosphere on the composition

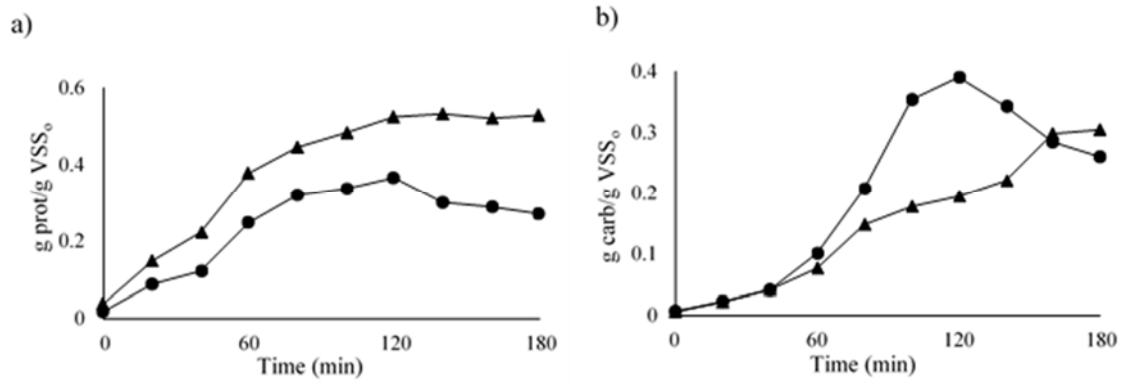


Figure 2. *Evolution of a) proteins and b) carbohydrates during wet oxidation (●) or thermal hydrolysis (▲) of brewer´s spent yeast. In all cases: 160 °C and 40 bar, 1200 mL min^{-1} of either N_2 or O_2 and 125 rpm of agitation.*

Putting now the light in the solubilisation of the biopolymers, both treatments showed a high potential for the yeast lysis and the release of its intracellular content. Proteins were the main biopolymer solubilised from the yeast (fig. 2a), with both wet oxidation and thermal hydrolysis yielding high concentrations of these compounds in the liquid phases with maximum values of 0.36 g protein/g VSS_o and 0.53 g protein/g VSS_o, respectively. It is interesting to note that, according to the bibliography, yeasts are approximately composed by a percentage of proteins comprised between 47% and 60% [4,13,55]. This involves that, according to the data, almost all the protein in the yeast was solubilised by means of thermal hydrolysis. In the case of wet oxidation, the maximum concentration of solubilised protein was only around a 50% of the total protein in the yeast, even when the oxidising atmospheres caused a higher solubilisation than the inert ones, as was previously proved. Therefore, this lower percentage of soluble protein in presence of oxygen was not due to a lower solubilisation, but to a higher degradation of this biopolymer once solubilised due to the oxidation reactions. The maximum soluble protein concentrations were reached after 120 minutes of hydrothermal treatment, independently of the atmosphere used. Although the protein concentration remained constant after this time until the end of the treatment using an inert atmosphere, the presence of oxygen caused a final decrease in the soluble protein to a final value of 0.27 g protein/g VSS_o, that is a 25% less than the highest concentration achieved under an oxidising atmosphere, thus demonstrating the degradation of these biopolymers by oxidation reactions, but not by hydrolysis ones.

Soluble protein concentrations in the hydrolysed yeast were higher than those obtained in other works also dealing with hydrothermal treatments of yeasts, probably

due to the different kinds of sample (baker's yeast was employed instead of brewer's yeast) and different conditions [11]. However, when comparing the data obtained to other hydrothermal treatments of proteinaceous materials, such as deoiled soybean, results were pretty similar, with 50% of protein solubilized after being treated 30 minutes at 200 °C and almost 4 MPa, with these conditions being comparable to those employed in this work [56]. Similar protein solubilization percentages were achieved when treating algae (*Chlorella*) at 150 °C and 0.01 atm [57].

Regarding carbohydrates, the evolutions of their soluble concentrations during the hydrothermal treatments of yeast with nitrogen and oxygen can be observed in figure 2b. It can be noted that an oxidising atmosphere produced a higher, faster solubilisation of carbohydrates than an inert one, which is the opposite to what happened with proteins. A maximum concentration of soluble carbohydrates of 0.39 g carbohydrate/g VSS_o was reached after 120 minutes of reaction in presence of oxygen, the same time at which maximum soluble protein concentrations were observed. As in the case of proteins, soluble carbohydrate concentration also decreased after this time if oxygen was used, ending with a final value of 0.26 g carbohydrate/g VSS_o after 180 minutes of reaction. On the other hand, soluble carbohydrate concentrations during the treatment under an inert atmosphere increased progressively during all the time of reaction, although at a rate lower than the observed for the first 120 minutes of treatment with oxygen, reaching a final value of 0.30 g carbohydrate/g VSS_o at the end of the treatment (180 minutes). It emerges very clearly from these facts that the presence of oxygen accelerated the solubilisation of carbohydrates, especially at short times of reaction, where the temperature was still low. Nevertheless, oxygen also had effect on the stability of the solubilised carbohydrates, causing their degradation by oxidation if the reaction time was longer. Taking into account that around a 30% of the yeast weight corresponds to carbohydrates, it is also interesting to note that hydrothermal treatments caused a high solubilisation of the inner carbohydrates [58]. It is important to bear in mind that the yeast employed also contained some carbohydrates that came from the fermentation media. Considering the previous statement and the referenced percentage, almost all the intracellular carbohydrates were released employing an oxidising atmosphere, whereas a lower percentage, 90%, was solubilised when thermal hydrolysis was selected as hydrothermal treatment. In studies dealing with hydrothermal treatments in other biomasses, such as sewage sludge, almost complete solubilisations of carbohydrates were achieved [38]. Carbohydrates solubilisation of algal biomass at milder conditions

(autoclaving at 120 °C for 40 minutes) yielded a release of the 64% of total carbohydrates, which was lower that the percentages reached in this work with or without and oxidising atmosphere, probably owed to the lower temperature and shorter time of treatment [59]. In the case of wet oxidation for the algae *Ulva*, the carbohydrate solubilization efficiency was 95% after a treatment carried out at 130 °C, 78 minutes, 1.38 bar and pH 1, corroborating the potential of wet oxidation to effectively solubilise carbohydrates from biomasses [60].

Finally, comparing carbohydrate and protein concentration data, it can be deduced that the solubilisation of proteins just by hydrolysis reactions is faster than the carbohydrates one, but the effect of oxidation reactions on solubilisations is more marked in carbohydrates than in proteins. Regarding the stability of the solubilised biopolymers, results suggest that although either proteins or carbohydrates are stable under inert atmospheres, soluble proteins are more susceptible to be degraded by oxidation reactions than the carbohydrates.

3.3. Kinetic modelling

On the basis of the experimental data afore-discussed, a kinetic model involving the concentrations of VSS, TCOD, SCOD, soluble proteins and carbohydrates was proposed and successfully fitted. To this purpose, the fitting parameters were obtained by using Micromath Scientist software, with a least square method for the error minimization.

The kinetic model was deduced from the mechanism proposed in figure 3. To model the changes in the COD, this was divided into three fractions: TCOD, SCOD and CODsf (COD of the solid fraction. The mechanism is based on the direct disintegration of the VSS (or CODsf) into soluble matter, measured as SCOD. This SCOD included the solubilised proteins and carbohydrates from the yeast which, in turn, could be subsequently degraded to other compounds and carbon dioxide, especially in presence of oxygen. In order to tie together COD with biopolymer concentrations, the corresponding yields (grams of biopolymer per gram of CODsf solubilised) were also introduced as fitting parameters in the kinetic model. All these reactions are schematized in figure 3:

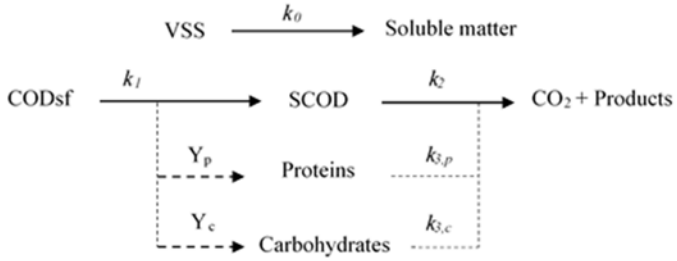


Figure 3. *Proposed mechanism for the hydrothermal treatment of spent yeasts.*

The following kinetic equations can be deduced from the proposed mechanism:

$$r_{VSS} = -k_0 \cdot [\text{VSS}] \quad (5)$$

$$[\text{TCOD}] = [\text{SCOD}] + [\text{CODsf}] \quad (6)$$

$$r_{\text{CODsf}} = -k_1 \cdot [\text{CODsf}] \quad (7)$$

$$r_{\text{SCOD}} = k_1 \cdot [\text{CODsf}] - k_2 \cdot [\text{SCOD}] \quad (8)$$

$$r_{\text{CODsf}} = -k_1 \cdot [\text{CODsf}] \quad (9)$$

$$r_i = Y_i \cdot k_1 \cdot [\text{CODsf}] - k_{3i} \cdot [i] \quad (10)$$

The kinetic model was successfully fitted to the experimental results and the fitting parameters are shown in table 2:

Table 2. *Most relevant fitting parameters for the kinetic model.*

	Thermal hydrolysis	***Wet oxidation***
$\bar{k}_0$ (s^{-1})	0.016 ± 0.001	0.0056 ± 0.0004
r^2	0.975	0.996
$\bar{k}_1$ (s^{-1})	0.0085 ± 0.0005	0.0121 ± 0.0007
r^2	0.978	0.992
$\bar{k}_2$ (s^{-1})	~0	0.0028 ± 0.0003
r^2	0.983	0.994
$\bar{Y}_{\text{protein}}$	0.66 ± 0.17	0.43 ± 0.12
$\bar{k}_{3\text{protein}}$ (s^{-1})	0.0028 ± 0.0003	0.005 ± 0.004
r^2	0.997	0.98
$\bar{Y}_{\text{carb.}}$	0.5 ± 0.2	0.23 ± 0.04
$\bar{k}_{3\text{carb.}}$ (s^{-1})	0.006 ± 0.005	~0
r^2	0.97	0.92

The fitting of the model to the experimental data is shown in figure A1.

The results for the kinetic constants showed in table 2 corroborate the previous discussion about a high efficiency of the hydrothermal treatment in VSS degradation, especially under an oxidising atmosphere, fact that was reflected in the high k_0 values, being this parameter three times higher for wet oxidation than for thermal hydrolysis. These facts are also in accordance with the k_1 values obtained for the CODsf conversion to SCOD under inert and oxidising atmospheres.

It is also noticeable that k_{2,N_2} values were negligible for experiments under inert atmosphere; this was expected because k_2 corresponds to the oxidation of SCOD towards to carbon dioxide, which is negligible under an inert atmosphere.

Focusing on the modelling and fitting of the evolution of the two considered biopolymers, proteins and carbohydrates, it can be easily deduced that k_1 value has to be the same obtained for the fitting of CODsf solubilisation data (eq. 7). The first order kinetic models proposed showed a successful fitting to the data collected during the experimentation. Regarding the fitting parameters for the soluble protein evolution, it can be deduced that thermal hydrolysis led to a higher protein production due to the fact that wet oxidation degraded the solubilized proteins more intensely, since its k_{3,O_2} value was twice the corresponding to thermal hydrolysis value (k_{3,N_2}). Moreover, $Y_{prot.,N_2}$ for thermal hydrolysis was sensitively higher (0.66 for thermal hydrolysis and 0.43 for thermal hydrolysis), supporting the higher soluble protein concentration reported before. Regarding the evolution of soluble carbohydrates, the results were in accordance with the previous evidences. It has to be highlighted that k_{3,N_2} did not reach a noticeable value, thus indicating that thermal hydrolysis did not seem to degrade carbohydrates in a significant amount. In contrast, the value obtained for k_{3,O_2} showed the degradation of the solubilised carbohydrates during the wet oxidation and supported the sharp decrease observed in the experimental points corresponding to the last minutes of treatment (minute 120 to minute 180). The conversion ratio (Y) of CODsf into carbohydrates also showed the better carbohydrate solubilisation under an oxidising atmosphere than under an inert one (0.47 and 0.23, respectively).

3.4. Effect of the atmosphere on the downstream processing

Once yeast has been hydrolysed, many of the applications previously described in the introduction require a subsequent step of separation and purification of either proteins or carbohydrates. As commented, the mechanisms and reactions that yeast suffered during thermal hydrolysis and wet oxidation were different. These differences play an important role not only in the physicochemical properties and composition of the hydrolysate, but also in the subsequent downstream processes, which are focused on the separation of components from the hydrolysate. Due to this, the effect of the kind of atmosphere during the hydrothermal treatment on the separation of proteins from carbohydrates in the hydrolysate obtained was studied in this section. To this end, three different separation techniques were selected: saline precipitation, pH-driven

precipitation and IMAC sorption. This selection was based on that these techniques are profusely used at industrial level.

3.4.1. pH-driven precipitation

The pH of yeast hydrolysates from either thermal hydrolysis or wet oxidation were adjusted to values of 2, 2.5, 3, 3.5, 4 and 9 in order to know the amounts of proteins and carbohydrates precipitated at each pH. These results (as percentages of biopolymer precipitated) are shown in figure 4.

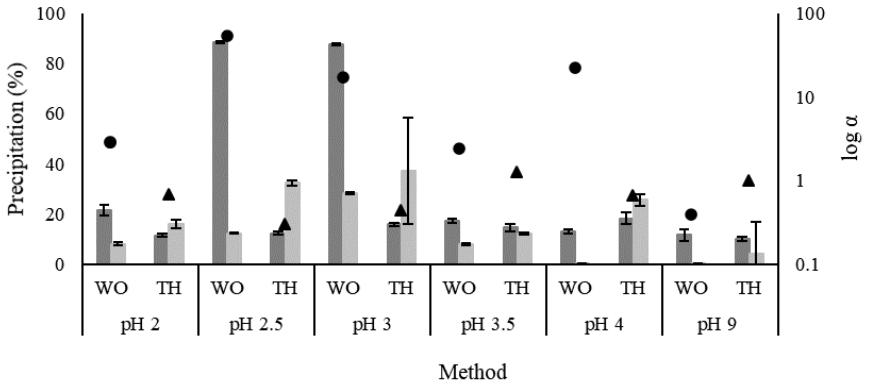


Figure 4. *Percentages of precipitation for protein (■) and carbohydrates (■) precipitation for each pH value and hydrolysate (WO is wet oxidation; TH is thermal hydrolysis). Black points represent the selectivity factor (α) for each method and hydrolysate (● wet oxidation and ▲ thermal hydrolysis).*

As can be easily observed in figure 4, the higher protein precipitations were obtained at acidic pH values, especially when this was from 2.5 to 3. Nevertheless, pH values lower than 2.5 or higher than three involved a poor protein separation. These findings suggest that the mean isoelectric point of the protein pool ranged from 2.5 to 3. This is why low protein recoveries were obtained for pH values outside of this interval. Other authors had reported that more than 80% of protein can be precipitated by pH adjustment at 3.3 of a sewage sludge hydrolysed by a coupled alkaline-ultrasonic treatment [61]. This result was consistent with other studies where pH adjustment was the strategy to recover protein from wastewater, with similar isoelectric points (3.8 – 3.9) and yields (up to 78% for protein recovery) [62]. The pH adjustment to a value of 3 also led to 90% of protein precipitation in papermill sludge hydrolysates [63].

The effect of the atmosphere during the hydrothermal treatment on the protein recovery was also clear. It was observed a higher precipitation of proteins when sludge was hydrolysed under an oxidising atmosphere. It is interesting to notice the excellent precipitation of proteins from wet oxidation hydrolysate at pH 2.5 and 3, with recoveries of 88% and 87%, in comparison to 12% and 16% obtained with the thermal hydrolysis hydrolysate. The better precipitation at acidic pH when an oxidising atmosphere was

selected was probably due to the generation of new carboxyl, carbonyl and hydroxyl groups in the structure of the different proteins by the oxidation reactions, thus modifying the initial isoelectric point of each protein towards a converging value 2.5 - 3. In a similar way, the absence of oxidising reactions is the reason why the pH effect on the recovery of proteins is less marked during the precipitation using the hydrolysate from thermal hydrolysis.

Regarding the carbohydrates, it was also observed a preferential precipitation of these at pH values between 2.5 and 3, particularly when the hydrolysate from thermal hydrolysis was used. The higher carbohydrate recoveries at these pH values were due to the presence of glycoproteins, as well as the afore-mentioned Maillard and Amadori products [49]. Anyway, the percentages of carbohydrates precipitated at different pH values was less marked than for the case of proteins, probably due the former were less susceptible to oxidation reactions.

To elucidate at which pH value the separation was more selective, $\alpha_{p/c}$ parameter was calculated for each pH value and each hydrolysate. As can be easily deduced from figure 4, selectivities were significantly higher if the hydrolysate from wet oxidation instead of the thermal hydrolysis one was used. It is also interesting to note that selectivities for the hydrolysate from wet oxidation were always higher than 1, with the exception of the corresponding one to pH 9, whereas the values for the hydrolysate from thermal hydrolysis were lower or slightly higher than 1. This means that the presence of an oxidising atmosphere during the hydrothermal treatment of the spent yeast highly improved the selective precipitation of proteins by pH adjustment, thus obtaining protein precipitates with a lower percentage of impurities due to carbohydrates, particularly for pH values of 2.5, 3 and 4. It has to be reminded that these pH values were also the corresponding ones to a highest recovery of protein. On the other hand, protein precipitates from hydrolysed yeast under inert atmosphere were lower in amount (lower recovery) and decidedly less pure, containing an appreciable proportion of carbohydrates (low selectivity). In fact, carbohydrates were the main component in the precipitates from thermal hydrolysis at these pH values were selectivities were lower than 1. Previous research in sewage sludge hydrolysates, avoiding the differences with brewer's yeast hydrolysates, showed a worse selectivity in acidic pH for wet oxidation samples, although separation in thermal hydrolysis samples was not good, as in the present study [38].

3.4.2. Saline precipitation

The effect of the atmosphere during the hydrothermal treatment of yeast on the subsequent separation of proteins in the hydrolysate obtained was also studied using saline precipitation as purification technique. The figure 5 shows either the protein or carbohydrates recoveries using different concentrations of ammonium sulphate.

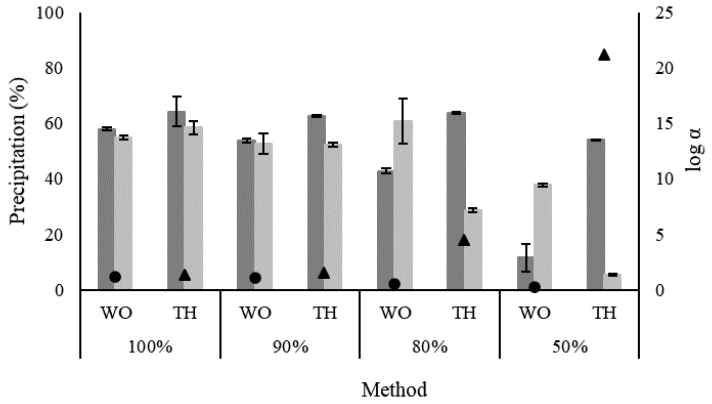


Figure 5. *Percentages of precipitation for protein (■) and carbohydrates (■) precipitation for each $(NH_4)_2SO_4$ concentration and hydrolysate (WO is wet oxidation; TH is thermal hydrolysis). Black points represent the selectivity factor (α) for each method and hydrolysate (● wet oxidation and ▲ thermal hydrolysis).*

As can be seen in the figure 5, the results revealed there was no significant differences in the proteins and carbohydrates recoveries at the highest concentrations of ammonium sulphate (> 90%), regardless the atmosphere used during the hydrothermal treatment used. Thus, the values for 100% and 90% were 57% and 64%, 54% and 62%; for hydrolysates obtained in presence or absence of oxygen, respectively. Nevertheless, for ammonium sulphate saturations lower than 90%, some discrepancies in protein and carbohydrate behaviour became evident, particularly if the hydrolysate was obtained under oxidising atmosphere. Thus, although protein recoveries for hydrolysates from thermal hydrolysis remained approximately constant at an average value of 60% for all the saline concentrations, this parameter gradually increased from 12 for 50% $(NH_4)_2SO_4$ saturation to 58% for 100% saturation, when the hydrolysate from wet oxidation was employed. The reason for these discrepancies was again related to the formation of new carboxyl, carbonyl and hydroxyl groups in the protein structure due to the oxidising atmosphere. These polar groups reduced the hydrophobic interactions and increased the hydrophilic ones of the proteins with the cellular environment, leading to the protein precipitation at higher ammonium sulphate concentrations.

Regarding carbohydrate recovery, this achieved a maximum stable value of around 55% for ammonium sulphates concentrations above 90% saturation, regardless the atmosphere used during the hydrothermal treatment. For salt concentrations lower than

90% saturation, an increase in the ammonium sulphate concentration did involve an increase in the precipitation, being the carbohydrate recovery always higher for the wet oxidation hydrolysate than for the thermal hydrolysis one for the same concentration of salt tested.

Moving on to selectivity, the corresponding values obtained for each hydrolysate and salt concentration tested are showed in figure 5 as well. As can be seen, the selectivities obtained for saline precipitation were clearly lower than the corresponding to the pH-driven one. The selectivity values obtained for ammonium sulphate concentrations equal or higher than 90% saturation were always around one for both hydrothermal treatments, indicating a non-selective precipitation of proteins and the obtaining of precipitates highly impurified by carbohydrates. In fact, carbohydrates turned out to be the main biopolymer precipitated for ammonium sulphate concentrations lower than 80% saturation when the yeast hydrolysed under an oxidising atmosphere was employed. The highest selectivity was observed at the lowest ammonium sulphate concentration tested (50%) and with the hydrolysate from thermal hydrolysis. Nevertheless, these conditions also coincided with the lowest recovery observed. Although salting out is a very common technique to purify proteins, to the best of our knowledge, there are no studies dealing with the separation of proteins and other biopolymers in hydrolysates from hydrothermal treatments but previous works in sewage sludge. In this case, the precipitation percentages of proteins were higher in both thermal hydrolysis and wet oxidation, although selectivity was worse, probably due to the different feedstock employed [38].

3.4.3. IMAC sorption

Eventually, the effect of the atmosphere during the hydrothermal treatment of spent yeast on the protein recovery and selectivity during the sorption of these on a copper-functionalized resin using the hydrolysates obtained was also discussed (figure 6).

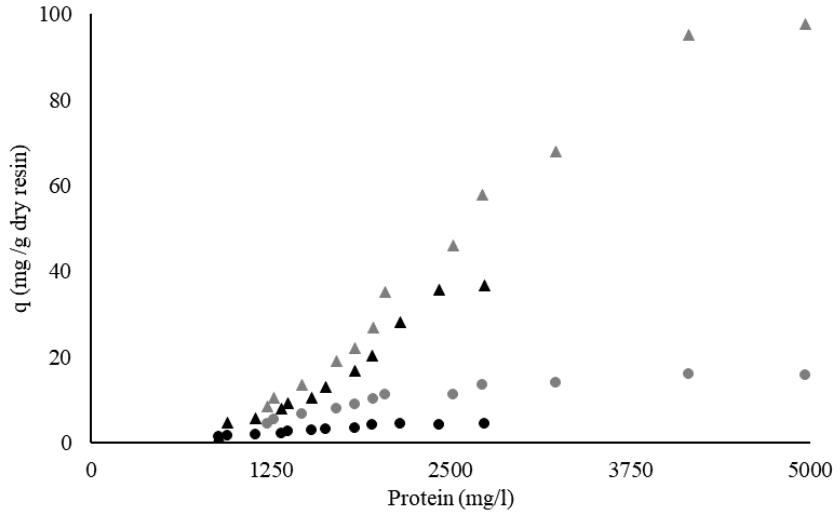


Figure 6. *Adsorption isotherms. In all cases: grey for thermal hydrolysis samples, black for wet oxidation samples, ▲ indicates protein data and ● is carbohydrates data. Hydrolysates were obtained after 80 minutes at 160 °C and 40 bar.*

Sorption isotherms showed a preferential adsorption of proteins by IMAC resins for both kind of hydrolysates, although carbohydrates were also adsorbed in small amounts, probably due to the presence of glycoproteins and the formation of Maillard and Amadori products [49]. In this sense, other authors reported that during the use of IMAC resins for protein sorption, these were also able to bind impurities such as aromatic rings similar to the amino acid side chains [64].

The atmosphere selected during the previous hydrothermal treatment also played a crucial role. As can be seen in figure 6, either proteins or carbohydrates obtained by a hydrothermal treatment under an inert atmosphere shower greater affinity for the IMAC resin than those obtained from the treatment in presence of oxygen. Thus, the maximum sorption capacities for proteins or carbohydrates when hydrolysate from thermal hydrolysis was used were 98 and 16 mg/g dry resin, respectively. On the other hand, these parameters were reduced to 37 mg proteins /g dry resin and to 4 for mg carbohydrates /g dry resin if the previous hydrothermal treatment of the spent yeast was carried out in presence of oxygen. The reason for these behaviours is again related to alterations in the molecular structures of proteins and carbohydrates during the hydrothermal treatments. In this line, wet oxidation was known to be more aggressive than thermal hydrolysis, thus leading to greater changes of proteins and reducing their affinity for the IMAC resin in a higher degree. In any case, the theoretical maximum sorption capacity, 429 mg protein per gram of resin was not achieved, due to the thermal denaturation caused during the hydrothermal treatment [65].

Regarding selectivity, this parameter was calculated by dividing the $q_{prot.}$ by $q_{carb.}$ (figure 7).

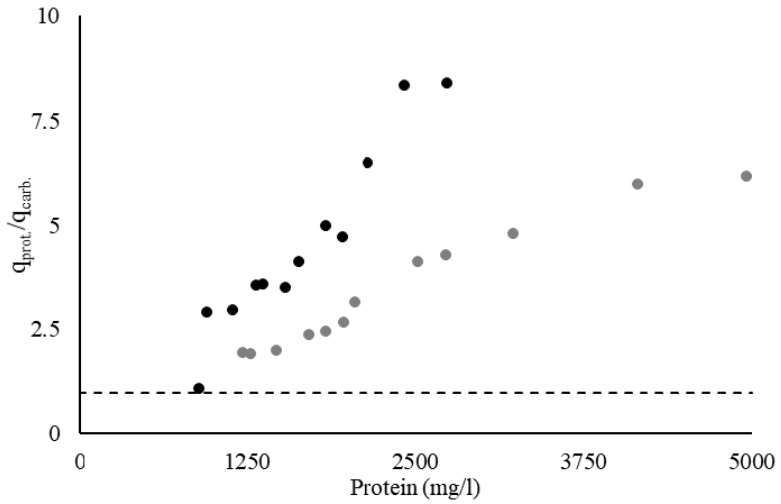


Figure 7. *Selectivity for the IMAC sorption. Grey represents thermal hydrolysis data and black wet oxidation data .*

As can be observed, the IMAC resin selectivity (as $q_{prot.}/q_{carb.}$) was higher for hydrolysates obtained in presence of oxygen, which means a lower retention of carbohydrates per gram of protein adsorbed, that is to say, a lower amount of impurities (as carbohydrates) attached to the resin. This higher selectivity for proteins from the wet oxidation hydrolysates can be explained in two points. Firstly, attending to wet oxidation ability to degrade complex molecules like glycoproteins or Maillard products, thereby releasing the carbohydrate monomers or oligomers from protein and avoiding their indirect binding to the resin. Secondly, Klinke et *al.* [66] reported the formation of phenols, furans and carboxylic acids during the wet oxidation of wheat straw, a waste also rich in carbohydrates. Therefore, if carbohydrates were transformed into the mentioned molecules, less carbohydrates were able to bond to the resin. It is important to point out that this is not an absolute concept, only a few percentage of the carbohydrates suffered the above-mentioned reactions.

It can be also observed that the higher the resin to hydrolysate mass ratio, the lower the selectivity, regardless of the hydrolysate employed. This fact is related to a higher availability of sorption sites per molecule of biopolymer.

In summary, it is possible to obtain a stream of 0.373 g prot/g VSS_o from spent brewer's yeast, with an 86% of purity, by means of IMAC technique application after a thermal hydrolysis treatment. When pH-driven precipitation was the selected method, only 0.281 g prot/g VSS_o could be recovered at pH 3, being its purity of 88%, however this results where only achieved in wet oxidation hydrolysates. Salting out yielded a

stream of 0.240 g prot/g VSS_o with a purity of 97%. This allows spent brewer's yeast to become a renewable and cheap source of biopolymers. Proteins can be used in the fabrication of resins, bioplastics or fertilizers. Meanwhile, the remaining carbohydrates could also be employed as a fermentation media. Therefore, the application of hydrothermal treatments followed by biopolymers recovery by IMAC allows a complete revalorization of the spent brewer's yeast, making it competitive against its traditional uses as animal food or food additive. However, as a first approach, this study only dealt with one treatment condition. The evaluation of other temperatures and pressures could provide useful information in order to find the optimal treatment conditions and balancing the waste minimization and products recovery. Regarding the possibilities of scale-up of the process, this should not be very difficult, taking into account either the hydrothermal treatment or the separation methods here tested have already been successfully implemented at industrial scale for other substrates [44,67–72]. In this sense, it is interesting to note that it was previously found out that batch laboratory degradation rates during hydrothermal treatments were usually significantly lower than those found in industrial continuous stirred operation, due to the degree of backmixing and the synergistic effects [73]. Obviously, the final operation conditions of both the yeast hydrothermal treatment and the hydrolysate downstream processing should be adapted to the product specifications, which will depend on the proposed green industrial use for the recovered protein.

4. Conclusions

In this work, the application of hydrothermal treatments to spent brewer's yeast was assayed as potential source of protein for green industrial uses. The presence of an oxidising atmosphere was found to be a key factor since it strongly enhanced the VSS disintegration and the solubilisation in terms of solid COD reduction, but also lowered soluble COD without significantly reducing soluble TOC for long treatment times.

Both thermal hydrolysis and wet oxidation effectively solubilized proteins and carbohydrates, although if time was lengthened, the presence of oxidation reactions was able to degrade both biopolymers. In the basis of the collected data, a first-order kinetic model including the conversion of volatile suspended solids into soluble matter and the transformation of the solid fraction into SCOD and, particularly, into proteins and carbohydrates, was proposed and successfully fitted.

The potential recovery processes for these biomolecules were also highly influenced by the presence of oxygen in the previous treatment. It was found that

oxidation reactions led to a convergency in the different protein isoelectric points to a value near three, which allowed an excellent and selective precipitation of proteins at pH values between 2.5 and 3. In contrast, the higher diversity of functional groups provided by thermal hydrolysis favoured a selective protein precipitation by salting out, especially at low ammonium sulphate concentrations. This variety of functional groups also made a difference in subsequent IMAC processes, favouring the recovery, although selectivity was higher when the hydrothermal treatment was carried out in presence of oxygen.

5. Acknowledgements

The authors are grateful for the financial support from the Employment, Industry and Tourism Office of Principality of Asturias (Spain) through project GRUPIN IDI/2018/000127. Authors also acknowledge the financial support from the Spanish Ministry of Economy and Competitiveness (MINECO) through Project MCIU-19-RTI2018-094218-B-I00 and FEDER funds from European Union. The authors would like to especially thank Caleya Beers for the yeast samples provided during the experimentation.

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APPENDIX A

Effects of oxidising atmosphere on brewer's yeast hydrothermal treatment and subsequent biopolymer recovery

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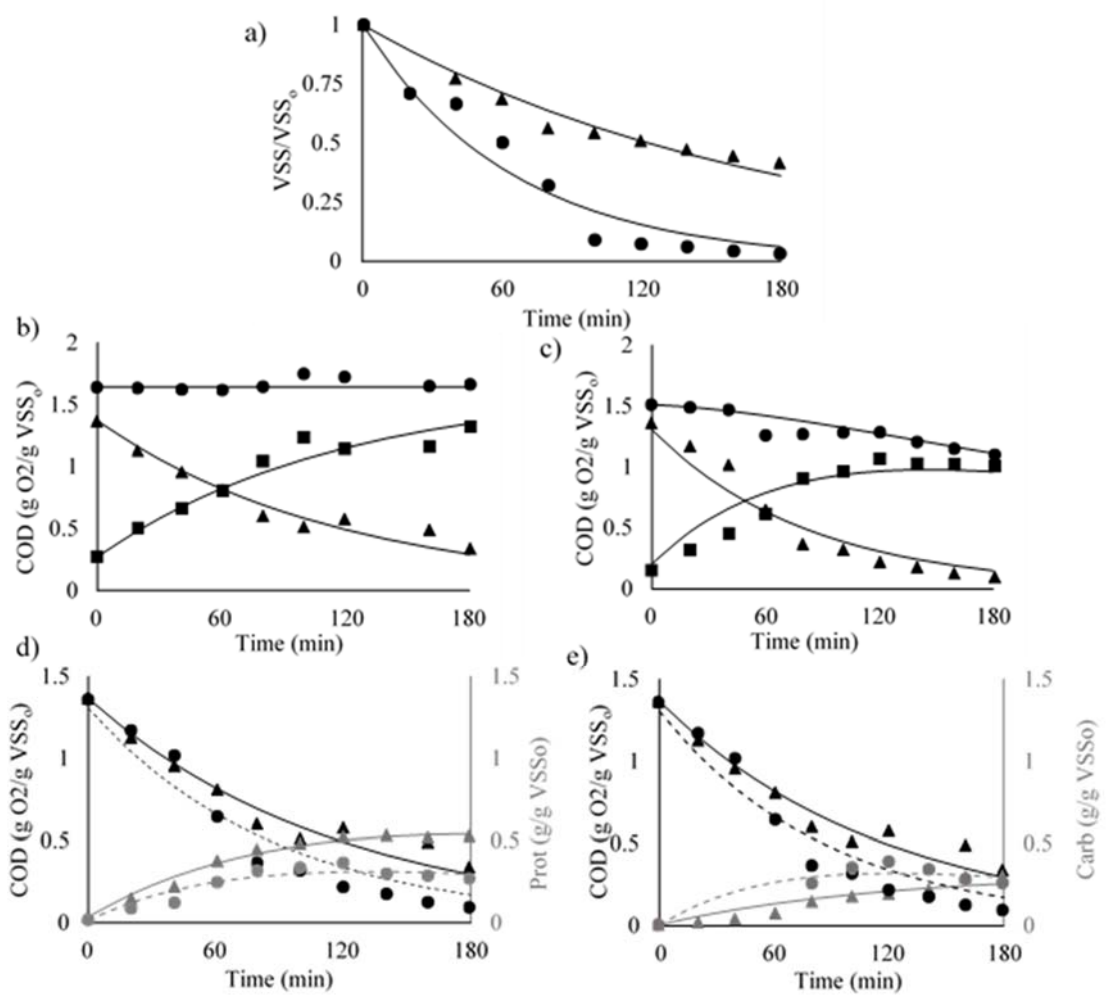

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