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MIXED MICROBIAL AND THERMAL DEGRADATION OF AGRICULTURAL DERIVED PLANT WASTES

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Abstract

Agricultural and food industries produce a lot of waste biomass which needs to be disposed of. In recent years it has been understood how these biomasses can be recovered and transformed in order to produce organic derivative products with high added value to be reintroduced on the market. A non-exhaustive list of these biotechnological products includes protein hydrolysates, consisting of bioactive peptides and amino acids, growth-stimulating fertilizers for plants and nutritional additives for animal feed. The recovery and transformation of the protein component into protein hydrolysates gives great added value to the waste biomass and is a process that is generally performed chemically. This approach results however unfavourable because of the possible production of toxic side-products, and the high level of energy required, which makes this process eco-unfriendly. In the light of all these limitations, we have designed and developed a mixed procedure, applicable to plant-derived wastes, based on a microbial preliminary degradation, followed by a mild thermic treatment to produce protein hydrolysates from agriculture waste biomass.

Key words*: Chemical Hydrolysis, Circular Economy, Microbial Hydrolysis, Protein Hydrolysates, Waste Biomasses.*

INTRODUCTION

The production of protein hydrolysates from waste biomass represents one of the main challenges of *Circular Economy,* which sees the transformation of waste into a resource. Protein hydrolysates are bio-based chemicals with high added value. Depending on the biomass used for their production, they can be equipped with high bio stimulating, hormonal and fertilizing capacities and therefore produce significant qualitative-quantitative improvement of agricultural crops, without causing problems concerning environmental pollution. The hydrolysates of plant origin do not have any toxicity for the plant and humans; they are in line with European directives (EU Directive 91/676) which aim at a consistent reduction in the use of chemical products in agriculture, and at the same time promote the reduction in organic matter content of the soils. They are also marketable in areas that do not bear the use of products currently on the market that are generally prepared from animal waste. Protein hydrolysates are made up of polypeptides, oligopeptides, and amino acids and are produced from protein sources through various hydrolysis processes (McCarthy et al., 2013; Schaafsma, 2009). Protein hydrolysates can be obtained through acid, basic, enzymatic or mixed protein digestion. Enzymatic hydrolysis is carried out both through the use of microorganisms and the use of purified enzymes. According to the production method used, hydrolysates with different characteristics can be obtained. On the one hand, chemical hydrolysis is more aggressive and acts irregularly leading to the formation of greater quantities of single amino

acids, many of which are not bioavailable (Celus et al., 2007; McCarthy et al., 2013). On the other hand, enzymatic hydrolysis, conducted at lower temperatures, is less aggressive and leads to the formation of some free amino acids and many dipeptides, polypeptides and bioavailable peptides (Korhonen & Pihlanto, 2006; Schaafsma, 2009).

Protein hydrolysates find various applications in the medical, nutritional and agro-food fields. In the medical field, protein hydrolysates are used for parenteral nutrition in states of serious malnutrition following surgery, in diseases that compromise the digestion and intestinal absorption of food, in animal cell cultures for the production of monoclonal antibodies, therapeutic proteins, therapeutic drugs, and vaccines (Ganglberger et al., 2005; Heidemann et al., 2004; Lee et al., 2008; Mazurkova et al., 2008; Pasupuleti & Braun, 2008; Tripathi et al., 2009). Protein hydrolysates are also used in the treatment of patients with diseases such as Phenylketonuria, a disease affecting the amino acid metabolism, which prevents the conversion of phenylalanine into tyrosine, due to the absence or deficiency of the *phenylalanine hydroxylase* enzyme. This causes the accumulation of phenyl pyruvic acid in the blood, which can lead to serious neurological damage. The use of phenylalanine-free hydrolysates is therefore a viable alternative for infants with this type of enzyme defect, bringing beneficial effects on physical growth and mental development (Acosta et al., 1998; Berry et al., 1976; McCarthy et al., 2013). In the nutritional field, whey proteins, soy proteins, and bovine collagen are used to produce new generation sport products thanks to the hypoallergenic nature, peculiar rheological properties and better digestibility of the hydrolysates obtained (Bequette et al., 1998; Cordoba et al., 2005; Gilbert et al., 2008). In the agro-industrial sector, protein hydrolysates have found widespread application in recent years as biostimulants and fertilizers, as they improve the absorption and assimilation of nutrients (e.g. nitric nitrogen and iron), tolerance to environmental stress (salinity, drought, extreme temperatures) and product quality. In this light, by-products of agriculture-derived waste can be effectively recovered and reused without causing any adverse effect on human and animal health or on the environment (Corte et al., 2014; da Silva, 2018; Luziatelli et al., 2015; Mihalache et al., 2014; Planques et al., 2012).

This work aims at developing a mixed hydrolysis method designed for soy-derived vegetable biomass, based on a first phase of microbial fermentation and a second phase of heat treatment under alkaline conditions, in order to produce protein hydrolysates with high-added value. More specifically, an attempt was made to understand whether the first hydrolysis step due to fermentative processes by microorganisms known for their proteolytic activity could increase the success of the subsequent phase of thermal hydrolysis carried out in alkaline conditions, thus reducing exposure times at high temperatures and consequently the preparation costs.

MATERIALS AND METHODS

Sample preparation

The biomass consists of soy waste resulting from its processing.

The soy waste was mechanically shredded to form a homogeneous powder. The soy powder was suspended in deionized water and incubated for 1 hour at 80°C. During the incubation, the sample was repeatedly shaken to favour the extraction of proteins. At the end of the incubation time, the sample was centrifuged at 16000 x g at 4°C for 15 minutes and the soluble part collected. This solution was further centrifuged at 16000 x g for 15 minutes and the supernatant containing the total extract of solubilized proteins was quantified by the Bradford method (Bradford, 1976) using the Coomassie Brilliant Blue dye and measuring the absorbance at 595 nm.

1st phase: Microbial fermentation

The biomass extract of interest was subject to microbial fermentation in a mixed consortium culture or with pure cultures known for their proteolytic activity. Five different types of inocula were used: self-digestion with soy microbiota, sourdough, bacterial and yeast mix, *Aureobasidium pullulans* and *Debaryomyces hansenii*.

Each inoculum was used to set up two hydrolysis conditions, one lasting 1 day (Group A) and the other lasting 3 days (Group B).

Determination of the degree of hydrolysis after fermentation

The degree of hydrolysis achieved was assessed primarily by proteomic analysis based on polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE). The samples were diluted in the sample buffer (0.5 M Tris-HCl pH 6.8, 10% SDS (w / v), 25 mM DTT) according to Laemmli protocol (Laemmli, 1970). The samples were incubated at 95°C for 5 minutes, loaded into the gel and subject to a constant electrophoretic run at 40 mA. The run was carried out using molecular weight standard as a reference. The running buffer used was Tris 0.025 M/0.192 M glycine containing 0.1% SDS (w/v). The degree of hydrolysis was also assessed by estimating the concentration of free amino acids in solution through the Ninhydrin assay (Rosen, 1957) and by making a comparison with the concentration of free amino acids found in the starting extract. The test is carried out using 2,2-dihydroxy-1,3-dioxyhydrindene (ninhydrin) supplied by Sigma-Aldrich which, added to the protein hydrolysate, interacts with the primary amines giving a blue-violet colour with absorption at 570 nm.

2nd phase: Thermal hydrolysis under alkaline conditions

The samples corresponding to each fermentation period were subject to alkaline hydrolysis with KOH 1 N and then incubated at 80°C for 15 hours. Subsequently, each sample was centrifuged at 4500 x g for 15 minutes at a temperature of 4°C and the phase corresponding to the hydrolysate was recovered and neutralized with H_2SO_4 18 M. Again, for these samples, the degree of hydrolysis was evaluated by SDS-PAGE and the concentration of free amino acids assessed by Ninhydrin assay, as previously described.

RESULTS AND DISCUSSIONS

In the first phase of microbial fermentation, which was carried out for 1 and 3 days respectively, five different inocula, consisting of single bacterial species or consortia of bacteria and yeasts (Table 1), were used in order to understand which one could give the best degree of hydrolysis based on the type of inoculum and fermentation time.

Table 1. Different types of inocula used in this study

Sample	Composition
1	Self-digestion with Vegetable Biomass Microbiota
2	Vegetable Biomass + Sourdough
3	Vegetable Biomass + Bacteria and Yeast mix
4	Vegetable Biomass + *Aureobasidium pullulans*
5	Vegetable Biomass + *Debaryomyces hansenii*
6	Ctrl (Vegetable Biomass)

At the end of the fermentation process, the degree of hydrolysis of the biomass was assessed for each inoculum and for each time used and compared with that of the control (sample **6**). Table 2 shows the percentage values of the protein concentration obtained by the Bradford assay compared to the protein concentration of the starting biomass extract.

Table 2. Percentage values of the Protein Concentration compared to the Ctrl, after 1 day (Group A) and 3 days (Group B) of fermentation

Sample	Group A	Group B
1	30.95%	25.00%
2	51.24%	16.81%
3	33.0%	10.62%
4	34.62%	20.24%
5	87.14%	15.09%
6	87.28%	

The results show how the fermentation significantly reduces the total protein concentration with the formation of free amino-acids. This is more apparent after 72 hours of treatment (Group B).

In particular, the protein concentration of the control is about 87% of that in the starting biomass extract, while in all of the samples undergone fermentation, the protein content is reduced to 10-25% of the initial value after 3 days. Sample 3 (bacteria and yeast mix) results the best inoculum, with its protein content decreased to 33% after a 24 h incubation and becoming almost 10% after 72 hours. Conversely, sample 5 does not provide a significant degradation in one day (protein content similar to the control), but it later performs a good hydrolysis, in line with that of the other samples.

These results were confirmed by the protein profile obtained by SDS-PAGE and Coomassie Blue Staining. The proteomic analysis carried out through SDS-PAGE has provided

information on the degradation profile of the proteins constituting the biomass.
After 1 day of fermentation (Figure 1A), there is no high degree of hydrolysis, while after 3 days (Figure 1B) a satisfactory level of hydrolysis is obtained.

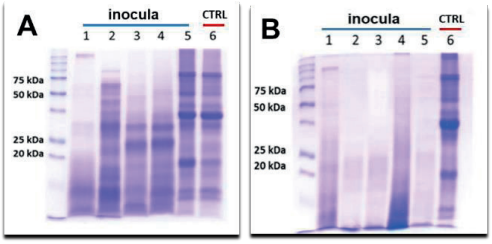

Figure 1. SDS-PAGE of fermented samples after 1 day (A) and 3 days (B)

Each of the microbial hydrolysis described above was then subject to thermal hydrolysis under alkaline conditions. In particular, thermal hydrolysis was carried out at 80°C overnight in the presence of KOH 1N. Also, in this case, the degree of hydrolysis was assessed by SDS-PAGE followed by Coomassie Blue Staining. The results in Figure 2 show that while the one-day fermentation alone does not produce a high level of hydrolysis (Figure 1A), the double passage produces a satisfactory level of hydrolysis with the formation of low molecular weight polypeptides (Figure 2A).
At the same time, the prolonged fermentation for three days produces by itself a satisfactory degree of hydrolysis (Figure 1B), which is however completed by the basic hydrolysis under mild working conditions (Figure 2B).

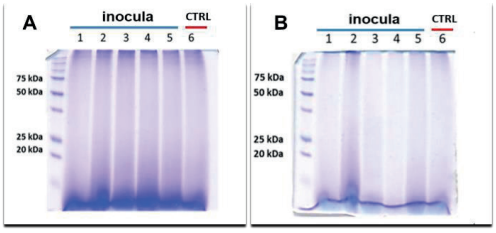

Figure 2. SDS-PAGE of 1 day (A) and 3 days (B) fermented samples treated with thermal hydrolysis under alkaline conditions

The same samples were also analysed employing the Ninhydrin assay, to obtain an estimate of the free amino acids in solution before and after the basic hydrolysis. In fact, ninhydrin is a reagent that interacts with the primary amines of amino acids leading to the formation of a complex that absorbs at 570 nm. The graph shown in Figure 3 shows how, following the microbial degradation, the concentration of free amino acids is higher compared to the control, especially after 3 days of fermentation and in particular for sample 3 composed of a mixture of bacteria and yeasts (left side of the graph).
Following thermal hydrolysis, however, after 1 day of fermentation the concentration of free amino acids is similar to that of the control; conversely, after 3 days, there is an increase in the concentration of free amino acids and again in this case sample 3 delivers the best performance (right side of the graph).

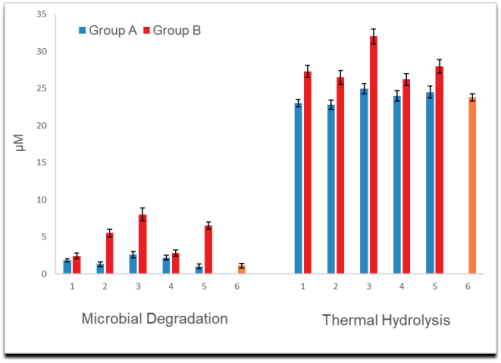

Figure 3. The amino acid concentration obtained using the Ninhydrin assay after microbial hydrolysis (left side of the graph) and mixed hydrolysis (right side of the graph)

CONCLUSIONS

The present work demonstrates the effectiveness of a mixed method of hydrolysis of the protein component of agro-food waste biomass, where soy was chosen as proof of principle.
In general, most of the protein hydrolysates are produced through thermal hydrolysis under acidic or alkaline conditions at high temperatures, usually above 100°C.
This approach is not only extremely aggressive, but it also leads to the formation of a high quantity of free amino acids which are mostly dextrorotatory and therefore not assimilable. Moreover, thermal hydrolysis is extremely disadvantageous from an economic point of view as a result of the high energy required.
Therefore, this study shows how the pre-

treatment of waste biomass with microorganisms, naturally known for their hydrolytic capacities, performs a crucial step in the process that leads to the formation of protein hydrolysates.

The action of microorganisms and in particular of the mixture consisting of consortia of bacteria and yeasts has proven effective in initiating the degradation process of the protein component of the concerned biomass promoting the subsequent passage of thermal hydrolysis, which was conducted at lower temperatures compared to the canonical one.

In this way, it would be possible to obtain not only savings in terms of costs and energy but also a higher quality product.

In fact, the hydrolysates obtained through this process will be subject to follow-up studies to evaluate their effectiveness as bio-fertilizers and bio-stimulants to be reintroduced in agriculture.

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