CHEMICAL ENGINEERING TRANSACTIONS
VOL. 118, 2025
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.cetjournal.it
Guest Editors: Laura Piazza, Francesco Donsì, Giorgia Spigno, Paolo Ciambelli, Luca di Palma

ISBN 979-12-81206-19-9; **ISSN** 2283-9216
DOI: 10.3303/CET25118001

Enzymatic Hydrolysis of Hemp Seed Cake Flour: Impact on Technological Properties

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This study explored improving the technological properties of hemp seed cake flour through enzymatic hydrolysis. Before hydrolysis, the biomass underwent a delignification pre-treatment using a deep eutectic solvent made of glycerol and choline chloride to remove lignin and enhance treatment yield. Hydrolysis significantly increased the polyphenol content from 1821 mgGAE/kg_{dw} for untreated flour up to 4387 mgGAE/kg_{dw} due to enzymatic action and lignin removal, which made phenolic compounds more accessible within the matrix. The hydrolyzed flour exhibited higher oil adsorption and water-holding capacities, attributed to the increased availability of hydrophobic and polar ionizable groups. Moreover, the process transformed insoluble fibers into a more amorphous form, enhancing water interaction and improving the swelling and water adsorption indices. Regarding foaming properties, enzymatic treatment increased foam capacity up to 8.1 v/v but reduced its stability, leading to its complete disappearance within 45 minutes. Finally, the hydrolyzed flour demonstrated reduced emulsifying capacity but improved emulsion stability.

1. Introduction

Food waste, traditionally considered as an environmental issue, has recently been recognized as a rich source of various valuable compounds. In the oilseed processing industry, a substantial amount of by-products is generated, including peels, seeds, defatted oilseed meals. Among these, oilseed meals stand out due to their high content of proteins, dietary fibers and other bioactive compounds that offer health benefits, making them valuable for use in food or animal feed (Caponio et al., 2022). A particularly yet poorly investigated oilseed meal is the by-product of hemp seed (*Cannabis sativa* L.) processing, that can be easily ground into hemp seed cake flour (HSCF) and could serve as a valuable ingredient for fortifying food products.
Hemp seed cake flour is characterized not only by its high content of polyunsaturated fatty acids and essential amino acids but also by several health-promoting compounds, including phenols, tocopherols, phytosterols, vitamin E, and various minerals. Additionally, it is characterized by a significant level of insoluble dietary fiber, particularly cellulose, hemicellulose, and lignin, which can be further processed and incorporated into food products for their potential health benefits (Barta et al., 2021; Farinon et al., 2020). To date, numerous studies have explored the potential applications of HSCF in the food sector, particularly within the bakery industry (Istrate et al., 2021; Rusu et al., 2021), as well as in the production of fresh and dried pasta (Merlino et al., 2022; Teterycz et al., 2021) and gluten-free products (Jagelaviciute et al., 2021; Korus et al., 2017).However, despite its nutritional value, HSCF exhibits poor technological properties, due to the presence of lignocellulosic compounds which limits its application in food formulations to low percentages. A useful approach used for the increasing technological properties of the lignocellulosic-based food waste is represented by the enzymatic treatments due to their high efficiency, specificity, and mild processing conditions (Wang et al., 2020). Cellulase-catalyzed hydrolysis increases the porosity of the fiber and the surface area, allowing for greater exposure of hydrophilic groups, which improves both water-holding and water-binding capacities (Li et al., 2024). Moreover, enzymatic hydrolysis using proteases leads to the formation of peptide molecules that can self-assemble into

Paper Received: 28 May 2025; Revised: 18 July 2025; Accepted: 4 September 2025
Please cite this article as: Malvano F., Condurso C., Galgano F., Miller A., Condelli N., Albanese D., 2025, Enzymatic Hydrolysis of Hemp Seed Cake Flour: Impact on Technological Properties, Chemical Engineering Transactions, 118, 1-6 DOI:10.3303/CET25118001

smaller particles, promoting their adsorption at the oil/water interface and positioning them as effective emulsifying agents (Bashash et al., 2024).
To our knowledge, there are currently few studies focusing the application of enzymatic treatments to enhance the technological properties of HSCF. Therefore, this study aims to investigate the effects of enzymatic treatment using cellulases, hemicellulases, and proteases on the key technological parameters of HSCF, including bulk density, swelling index, water-holding capacity, solubility, emulsion and foam capacity and stability. Before the enzymatic hydrolysis, a delignification pretreatment of the biomass was conducted using deep eutectic solvents to remove lignin and enhance the yield of the treatment.

2. Material and Methods

2.1 Materials

Monovarietal hemp seed (*Cannabis sativa* var. Futura 75) cake flour (HSCF) was purchased from *Molino Crisafulli*, a Sicilian company. Choline chloride, glycerol, sodium citrate, citric acid and hydrochloric acid were obtained from Merck (Darmstadt, Germany).

2.2 Deep Eutectic Solvent – pretreatment and Enzymatic Hydrolysis

The enzymatic hydrolysis was performed on raw and pretreated HSCF. For the pretreatment, a deep solvent (DES) made of choline chloride and glycerol in a molar ratio 1:2 was employed. The DES was prepared by continuously stirring the mixture at 80°C until a homogeneous colorless liquid was formed. The biomass was then mixed with the DES with a biomass/DES ratio 1:8. Pretreatment was carried out at 120°C for 3h.
After the reaction time, the mixture was washed with water and the flour was dried at 38 °C until a constant weight was achieved. The enzymatic hydrolysis of raw and DES-pretreated HSCF was carried out according to the procedure proposed by Procentese et al. (2018). Cellulase, hemicellulase and protease enzymes were provided by Biocatalyst Ltd. (Wales, UK). 10 g of biomass was mixed with 100 mL of 0.1 M Sodium Citrate Buffer (pH 5.0). The enzymatic hydrolysis was carried out with the addition to the mixture of a cocktail solution made of cellulase and hemicellulose (10 $mg_{enzyme}/100g_{biomass}$) and protease (12 $mg_{enzyme}/100g_{biomass}$). The hydrolysis was carried out under gentle mixing on a rotary shaker at 50 °C for 40 h.

2.3 Chemical and physical characterization of raw and hydrolyzed hemp seed cake flour

2.3.1 Total Polyphenols Content

The total polyphenol content (TPC) of raw and hydrolyzed HSCF was determined through the Folin-Ciocalteu method (Singleton et al., 1965). For the polyphenols extraction, 2.5 g of sample was mixed with 50 mL of a 80:20 methanol:water (v/v) solution. The solution was left to stir for 30 min and then centrifuged at 4000 g for 5 min. The supernatant was collected and analyzed. The absorbance at λ = 760 nm was determined at room temperature using a UV – 6300PC Double Beam Spectrophotometer (VWR International S.r.l., Milano, Italy). Quantification was based on a standard curve generated using gallic acid. The results were expressed as mg of gallic acid equivalent (GAE)/kg of dried flour weight (dw).

2.3.2 DPPH Radical Scavenging Activity

Antioxidant activity (AA) of raw and hydrolyzed HSCF was determined by 1,1-diphenil-2-picrylhydrazyl (DPPH) radical scavenging assay (Brand-Williams et al., 1995). Methanolic extracts were prepared by dissolving 0.5 g of each sample in 5 mL of methanol under mixing for 30 min. 3.9 mL of freshly prepared DPPH radical solution ($6*10^{-5}$ M) was added to 134 µL of methanolic extract (0.1 g/ml) and incubated in the dark for 40 min at room temperature. The bleaching of DPPH was recorded at 515 nm by a UV – 6300PC Double Beam Spectrophotometer (VWR International S.r.l., Milano, Italy) at room temperature (A_{sample}). A blank experiment was also carried out by applying the same procedure to a solution without sample (A_{blank}). The percentage inhibition was calculated as follows:

$$\%\ \text{inhibition of DPPH} = \frac{(A_{blank} - A_{sample})}{A_{blank}} * 100 \qquad (1)$$

AA was expressed as µmol of Trolox equivalent per gram of sample. Values were determined by a calibration curve of Trolox standard solutions in the range 60-600 µM, performed according to sample preparation.

2.3.3 Color Evaluation

The color of raw and hydrolyzed HSCF was evaluated with a Minolta CR-400 colorimeter (Konica Minolta Sensing, Osaka, Japan) for the detection of L*, a*, and b* (CIE L*a*b* color space). The overall color difference (ΔE), Chroma (C*) and Hue angle (h°) were calculated as follows:

$$\Delta E^* = \sqrt{(L_1{}^* - L_2^*)^2 + (a_1^* - a_2^*)^2 + (b_1^* - b_2^*)^2} \quad (2)$$

$$C^* = \sqrt{a^{*2} + b^{*2}} \quad (3)$$

$$h^\circ = \tan^{-1}\left(\frac{b^*}{a^*}\right) \quad (4)$$

where L_1*, a_1* and b_1* are the color values of hydrolyzed samples, while L_2*, a_2* and b_2* are the color values of raw flour.

2.3.4 Particle Size Distribution and Bulk Density

The particle size distribution (PSD) of raw and hydrolyzed HSCF was estimated through sieve analysis by using a set of ten round sieves with a rectangular mesh made of metal fibres with mesh sizes of 630, 560, 450, 400, 355, 280, 180, 140, 125, 80 μm placed on top of each other. A sample of 50 g was sieved was sieved for 10 min and the masses of individual fractions retained on sieves screens were determined.
For the determination of bulk density, 10 g of sample was quantitatively transferred to a 25 mL measuring cylinder. The cylinder's bottom was gently tapped on the laboratory bench, allowing vibrations to aid particle settling and minimise excess air gaps until a stable volume was achieved. The bulk density was expressed as g/cm^3.The measurement was carried out in triplicate.

2.3.5 Technological Properties

The technological properties of raw and hydrolyzed HSCF were assessed through the determination of water holding capacity (WHC), oil absorption capacity (OAC), swelling index (SI), water absorption index (WAI), water solubility index (WSI), emulsion capacity (EC) and stability (ES), and foam capacity (FC) and stability (FS). WHC and SI were determined by referring to the methods reported by Merlino et al. (2022), whereas the OAC was assessed according to Girotto et al (2024). The WAI and WSI were determined on the same sample after the SI determination. In particular, the supernatant was separated from the solid residue and dried at 105°C to constant weight. The weight of the dried sediment was used to calculate the solubility index, whereas the weight of the solid residue was used for calculating the WAI. The EC and ES were determined by using a 7.0 % aqueous suspension of raw or hydrolyzed HSCF and sunflower oil following the procedures reported by Du et al (2014). FC was estimated using 2.5% aqueous flour suspensions that were whipped into graduated cylinders using a Ultra-Turrax T18 homogeniser (Janke & Kunkel, IKA Instruments, Staufen, Germany); the volume of foam formed was recorded after 0.5 for FC assessment and at various intervals up to 120 min for FS. All the determinations were performed in triplicate and the results were expressed as below reported.

$$\text{SI}\ (\%) = \frac{Final\ sediment\ volume}{Initial\ powder\ volume * initial\ powder\ weight} * 100 \quad (5)$$

$$\text{WAI}\ (\%) = \frac{\text{Final sediment weight}}{\text{Initial powder weight}} * 100 \quad (6)$$

$$\text{WSI}\ (\%) = \frac{\text{Weight of sediment from dried supernatant}}{\text{Initial powder weight}} * 100 \quad (7)$$

$$\text{WHC}\ (\%) = \frac{Wheight\ of\ hydrated\ powder - weight\ of\ dry\ powder}{Weight\ of\ dry\ powder} * 100 \quad (8)$$

$$\text{OAC}\ (\%) = \frac{\text{Weight of oiled powder} - \text{weight of dry powder}}{\text{Weight of dry powder}} * 100 \quad (9)$$

$$EC\ (\%) = \frac{Volume\ of\ the\ emulsified\ layer}{Volume\ of\ emulsion\ before\ centrifugation} * 100 \quad (10)$$

$$ES\ (\%) = \frac{Volume\ of\ the\ emulsified\ layer\ after\ heating}{Volume\ of\ emulsion\ before\ centrifugation} * 100 \quad (11)$$

$$FC\ (\%) = \frac{Foam\ volume}{Volume\ before\ whipping} * 100 \quad (12)$$

$$FS\ (\%) = \frac{Foam\ volume\ after\ time\ t}{initial\ foam\ volume} * 100 \quad (13)$$

2.4 Statistical analysis

All the trials were conducted in triplicate. Experimental data were reported as mean and standard deviation and subjected to one-way analysis of variance (one-way ANOVA). The significant differences ($p<0.05$) among

samples were determined via Duncan's test through JMP statistical software (SAS Institute. Inc. Cary, NC, USA).

3. Results and Discussion

3.1 Total polyphenols content and antioxidant activity

Raw and hydrolyzed HSCF have been characterized by their polyphenol content and antioxidant activity (Table 1). A significant increase in the polyphenol amount was observed in the hydrolyzed flour compared to the raw one. This increase may be due to the enzyme's activity which, along with the removal of lignin through DES pretreatment, further improves the accessibility of phenolic compounds in the matrix, as confirmed in the literature (Pontonio et al., 2020). AA of hydrolyzed flour, assessed through DPPH assays, was found to be higher than that of the raw flour. However, the slight increase in the AA observed in hydrolyzed HSCF is not directly linked to the significant increase in polyphenol compound amount. This finding highlighted that the increased bioavailability of the released phenolic compounds doesn't necessarily lead to improved antioxidant activity. Different factors, such as their chemical stability, interaction with other compounds, and metabolic transformations, could influence their overall effectiveness as antioxidants, as reported by Pontonio et al. (2020).

Table 1: Total polyphenol content (TPC) and antioxidant activity (AA) of raw and hydrolyzed HSCF

	Raw HSCF	Hydrolyzed HSCF
TPC [mgGAE/kg_{dw}]	1821.02±20.11^{a}	4386.90±16.56^{b}
AA [mmolTE/100g_{dw}]	0.32±0.04^{a}	0.41±0.02^{b}

Different letters (a, b) in the same row indicate statistical differences (*p<0.05*) between samples.
HSCF: Hemp Seed Cake Flour

3.2 Color evaluation

Table 2 shows L*, a* and b* values evaluated for raw and hydrolyzed HSCF. The results highlight a significant reduction in lightness (L*), confirming that raw flour appears relatively lighter due to its higher L* value, whereas the hydrolyzed sample becomes noticeably darker with a lower L value. Additionally, the hydrolyzed flour shows an increase in a and a* decrease in b* value.
The value of Hue angle and Chroma highlighted the yellowish hue of HSCF, which shifts toward a more brownish hue after hydrolysis treatment. In particular, the raw flour showed a hue angle (88.54) close to a purely yellow hue, along with a higher chroma reflecting a more vivid color. In contrast, hydrolyzed samples displayed a significantly lower hue angle (55.14), shifting toward a reddish color and a reduced chroma. The change in the color of the HSCF is likely due to the thermal impact during the pretreatment with DES, as reported elsewhere (Martinez et al., 2015). Finally, since the total color difference was greater than 3 between raw and hydrolyzed HSCF, an indication of the perception of a colour difference by the human eye was confirmed (Pojic et al., 2014).

Table 2: Colour evaluation of raw and hydrolyzed HSCF

	L*	a*	b*	h°	C*	ΔE*
Raw HSCF	57.13±1.21^{a}	0.36±0.02^{b}	14.30±1.11^{a}	88.54±2.12^{a}	14.31±0.78^{a}	12.65±0.78
Hydrolyzed HSCF	47.34±2.45^{b}	5.97±0.12^{a}	8.57±0.98^{b}	55.14±3.43^{b}	10.45±1.05^{b}	

Different letters (a, b) in the same column indicate statistical differences (*p<0.05*) between samples
HSCF: Hemp Seed Cake Flour

3.3 Particle Size Distribution and Bulk Density

Flour particle size distribution is one of the main factors governing the technological properties of flour and its performance in food applications. It can influence its behavior during processing in terms of mixing characteristics, water adsorption and retention and moisture of the final products (Ahmed et al., 2019). As reported in Figure 1A, both raw and hydrolyzed HSCF displayed bimodal particle size distribution. The raw HSCF showed its highest mass fraction (approximately 37%) at 280 µm, followed by 25% of the mass characterized by a 450 µm dimension. After hydrolysis, the most notable shift appeared at 355 µm, where about 40% of the material was retained, suggesting that the enzymatic activity, along with the removal of lignin, promoted the agglomeration into slightly bigger and more cohesive particles. According to its larger particle size, the hydrolyzed flour showed a significantly lower ($p<0.05$) bulk density than raw flour, i.e. 0.5052 ±0.003 g/cm3 vs. 0.6029 ±0.004 g/cm3.

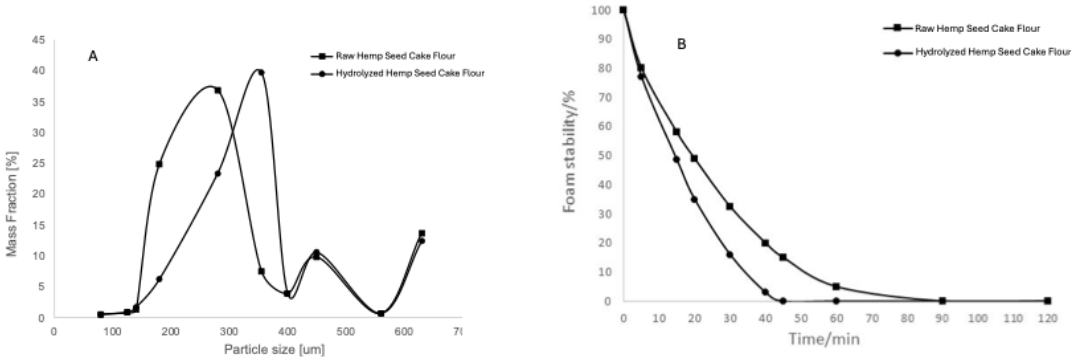

Figure 1: Particle Size Distribution (A) and Foam Stability (B) of raw and hydrolyzed Hemp Seed Cake Flour

3.4 Technological properties

The hydrolyzed HSCF exhibited higher OAC, WHC, SI, WAI and WSI when compared to raw flour (Table 3). These findings are consistent with literature data (Wouters et al., 2016). OAC is strictly dependent on hydrophobic proteins and, as highlighted by previous studies (Wouters et al., 2016; Bhetwal et al., 2024) protein hydrolysis significantly increases this parameter. This is due to the higher availability of hydrophobic groups resulting from hydrolysis. Likewise, there is substantial evidence that protein hydrolysis leads to a higher availability of polar, ionizable groups with a beneficial impact on WHC. SI and WAI reflect the volume and weight occupied by the polysaccharides after they swell in excess water; the hydrolysis process transforms insoluble fibers into a more amorphous form, making them more accessible to water, thereby improving SI and WAI. Additionally, the higher value of WSI suggests higher levels of water-soluble molecules such as sugars, small peptides, and amino acids, which are also derived from hydrolysis. The hydrolysis treatment increased the foaming capacity and decreased the foaming stability of HSCF. As a result, the foam height was higher, but its volume diminished faster, leading to the foam disappearing within 45 minutes (Figure 1B). These findings are consistent with previous research which shows that hydrolyzed proteins contribute to foam expansion, while larger proteins are necessary for stabilizing the foam (Wouters et al., 2016). Regarding emulsifying properties, the enzymatically treated HSCF exhibited a reduced emulsifying capacity but a higher emulsifying stability. Typically, protein hydrolysis enhances the exposure of hydrophobic groups, which can improve the emulsifying properties of proteins but, at the same time, this can lead to aggregation impairing emulsifying ability. The emulsifying properties of protein hydrolysates can vary considerably according to the degree of hydrolysis; the observed increase in emulsifying stability suggests that the hydrolysis process is not excessive (Vogelsang-O'Dwyer et al., 2022).

Table 3: Technological properties of raw and hydrolyzed HSCF

	OAC (% w/w)	WHC (% w/w)	SI (% v/w)	WAI (% w/w)	WSI (% w/w)	EC (% v/v)	ES (% v/v)	FC (% v/v)
Raw HSCF	95.9±0.0^{b}	128.6±1.3^{b}	103.0±1.1^{b}	181.7±1.2^{b}	1.0 ±0.0^{b}	57.1±0.0^{a}	25.9±0.0^{b}	6.6±0.4^{b}
Hydrolyzed HSCF	211.7±0.9^{a}	257.0±0.0^{a}	234.4±1.2^{a}	292.2±6.6^{a}	2.2±0.1^{a}	42.5±0.1^{b}	35.0±0.1^{a}	8.1±0.4^{a}

WHC: Water Holding Capacity; OAC: Oil Absorption Capacity; SI: Swelling Index; WAI: Water Absorption Index; WSI: Water Solubility Index; EC: Emulsion Capacity, ES: Emulsion Stability; FC: Foam Capacity; FS: Foam Stability. Different letters (a, b) in the same column indicate statistical differences ($p<0.05$) between samples.

4. Conclusions

This study demonstrated the positive impact of enzymatic hydrolysis on the key technological properties of hemp seed cake flour. Through the hydrolysis of the polysaccharide matrix, hemp flour exhibited higher polyphenol content and improved oil adsorption capacity, water-holding capacity, and swelling properties. These improvements are likely due to the increased exposure of hydrophilic and hydrophobic groups in hemp proteins. Changes in particle size distribution and bulk density were also observed, indicating structural modifications that may influence processing and the final characteristics of the product. Furthermore, enzymatic hydrolysis enhanced foam capacity and emulsion stability. In addition to the improvement of technological properties, it is important to underline that the hydrolysis of proteins offers significant nutritional advantages, as it promotes the release of peptides and amino acids, which contribute to improving the digestibility of the flour. In conclusion,

enzymatic hydrolysis represents a promising approach for enhancing the technological properties of hemp seed cake flour, increasing its potential as a sustainable and valuable ingredient for food applications.

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