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Synergistic effect of high hydrostatic pressure, hydrogen peroxide, and a *Trametes hirsuta* BM-2 enzymatic extract on polyphenol release from *Sargassum* spp.

Efecto sinérgico de la alta presión hidrostática, el peróxido de hidrógeno y un extracto enzimático de *Trametes hirsuta* BM-2 sobre la liberación de polifenoles a partir de *Sargassum* spp.

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Abstract

Massive influxes of *Sargassum* spp. in the Mexican Caribbean, exacerbated by pollution and climate change, pose severe environmental challenges but also represent a promising resource for biorefineries. This study assessed the effect of high hydrostatic pressure (HHP) combined with chemical and enzymatic pretreatments on polyphenol release from *Sargassum fluitans* and *Sargassum natans*. Biomass was subjected to HHP (0.101–200 MPa) either alone or with hydrogen peroxide (H_2O_2), a crude enzymatic extract from *Trametes hirsuta* BM-2, or their sequential application. HHP alone produced no significant effect, but in combination with pretreatments it markedly enhanced polyphenol extraction. Maximum concentrations reached 8.10 and 7.54 mg GAE eq g^{-1} TS for peroxide and enzymatic treatments at 150 MPa, while the sequential process achieved 16.18 mg GAE eq g^{-1} TS. Comparable yields using individual treatments at 50 and 150 MPa indicate potential energy savings for scale-up. These results demonstrate that coupling pretreatments with HHP significantly improves phenolic recovery, offering a sustainable strategy to valorize invasive sargassum biomass and reduce its coastal impact.

Keywords: *Sargassum* spp., high hydrostatic pressure, polyphenols, hydrogen peroxide, laccase, delignification.

Resumen

Las afluencias masivas de *Sargassum* spp. en el Caribe mexicano, agravadas por la contaminación y el cambio climático, representan graves desafíos ambientales, pero también constituyen un recurso prometedor para el desarrollo de biorrefinerías. Este estudio evaluó el efecto de la alta presión hidrostática (HHP) combinada con pretratamientos químicos y enzimáticos sobre la liberación de polifenoles a partir de *Sargassum fluitans* y *Sargassum natans*. La biomasa se sometió a HHP (0.101–200 MPa), ya sea de manera independiente o junto con peróxido de hidrógeno (H_2O_2), un extracto enzimático crudo de *Trametes hirsuta* BM-2, o su aplicación secuencial. La HHP por sí sola no produjo un efecto significativo, pero en combinación con los pretratamientos mejoró notablemente la extracción de polifenoles. Las concentraciones máximas alcanzaron 8.10 y 7.54 mg GAE eq g^{-1} TS para los tratamientos con peróxido y enzimático a 150 MPa, mientras que el proceso secuencial logró 16.18 mg GAE eq g^{-1} TS. Rendimientos comparables obtenidos con tratamientos individuales a 50 y 150 MPa sugieren un potencial ahorro energético para el escalamiento. Estos resultados demuestran que el acoplamiento de pretratamientos con HHP mejora significativamente la recuperación de compuestos fenólicos, ofreciendo una estrategia sostenible para valorizar la biomasa invasiva de sargazo y reducir su impacto costero.

Palabras clave: *Sargassum* spp., Alta presión hidrostática, Polifenoles, Peróxido de hidrógeno, Lacasa, Deslignificación.

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1 Introduction

Since 2011, recurrent massive arrivals of *S. natans* and *S. fluitans* have affected the Caribbean, The Mexican Caribbean is among the most affected regions, receiving 10,105 to 40,932 m^3 km^{-1} yr^{-1} of beach-cast biomass in 2018, and basin-scale biomass reaching 24.2 million tons in 2022. Decomposition of sargassum biomass releases ~316 L of leachate per ton and contributes 6,150 kg N and 61 kg P km^{-1} yr^{-1} to coastal waters, driving eutrophication, 31-fold reduction in light penetration, and oxygen declines to 0.67–3 mg L^{-1} (Rodríguez-Martínez *et al.*, 2025; Chávez *et al.*, 2020). These conditions cause severe ecosystem degradation, including losses of seagrass biomass and shifts toward carbonate-rich sediments derived from *Sargassum* epibionts; additionally, evidence has indicated that sargassum decomposition led to a significant pH drop in coastal waters (Santana-Martí *et al.*, 2026). Economically, cleanup operations in Quintana Roo have exceeded USD 17 million, while hotels between Cancun and Puerto Morelos spent between USD 128,770–USD 284,830 per year on manual removal during peak seasons (Rodríguez-Martínez *et al.*, 2025; Chávez *et al.*, 2020). Extreme regional landings of up to 10,000 wet tons per day have been documented during major events, underscoring the persistence, scale, and economical and environmental impacts of this phenomenon to date (Santana-Martí *et al.*, 2026; Rodríguez-Martínez et al., 2025; Tapia-Tussell *et al.*, 2018). In addition, the release of toxic gases and contaminated leachates poses risks to human health, while beach closures, reduced water quality, and declines in tourist arrivals further exacerbate the socio-economic burden on coastal communities (Rodríguez-Martínez *et al.*, 2025; Escobar *et al.*, 2024; Resiere *et al.*, 2021; Chávez *et al.*, 2020). Collectively, these impacts highlight the urgent need to develop sustainable valorization pathways for this abundant biomass.

These brown macroalgae are rich in lignocellulose, polysaccharides, minerals, proteins, and polyphenolic compounds (Tapia-Tussell *et al.*, 2018). This biomass is a promising biorefinery feedstock, with potential for high-value products in the food, cosmetic, pharmaceutical, and renewable energy sectors. The seaweed polyphenolic fraction (SPF) includes phlorotannins with inhibitory activity against AChE, TNF-α, NF-κB, tyrosinase, trypsin, collagenase, and elastase, conferring anti-inflammatory, skin-whitening, and anti-aging properties (Jiang et al., 2024; Puspita *et al.*, 2017; Vijayan *et al.*, 2018). Purified SPF also shows strong antioxidant capacity, commonly assessed by ABTS, DPPH, CUPRAC, FRAP, and the Folin–Ciocalteu assay (Gisbert *et al.*, 2023; Lee *et al.*, 2022).

Lignocellulosic material in seaweed is another source of polyphenols and phenolic acids, recoverable by delignification. Removing these before biofuel production can both yield bioactive compounds and reduce microbial inhibition, improving substrate accessibility (Chikani-Cabrera *et al.*, 2022). However, strong binding to lignocellulose hinders release. Conventional solvent extraction with heat may degrade sensitive molecules, lower yields, and require large solvent volumes (Lee *et al.*, 2022). Alternatives include enzyme-assisted extraction (EAE) with proteases, carbohydrate hydrolases, or ligninolytic enzymes such as lignin peroxidase, manganese peroxidase, and laccase (Puspita *et al.*, 2017; Chikani-Cabrera *et al.*, 2022; Tapia-Tussell *et al.*, 2018). Other methods, such as hot-water extraction (HWE) ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), and supercritical fluid extraction, can be effective but are energy-intensive and often challenging to scale (Gisbert *et al.*, 2023).

Hydrogen peroxide (H_2O_2) has emerged as an oxidative delignification agent and potential enhancer of phenolic release. Wan *et al.* (2022) reported a H_2O_2/ascorbic acid method to extract antioxidant polysaccharides, but such pretreatments have not yet been applied to sargassum for phenolic recovery. High hydrostatic pressure (HHP) is a non-thermal, eco-friendly method that disrupts cell structures, increases membrane permeability, and facilitates the release of intracellular compounds such as polyphenols (Navarro-Baez *et al.*, 2022). Moreover, HHP can enhance the catalytic efficiency of lignocellulose degrading enzymes, including laccases and cellulases, by inducing conformational changes that improve enzyme–substrate interactions (Albuquerque *et al.*, 2016; Ma & Mu, 2016). Based on these considerations, this study evaluated the effect of HHP combined with H_2O_2 and a crude enzymatic extract from Trametes hirsuta BM-2 (white rot fungus from Yucatán, Mexico) on polyphenol release from *Sargassum* spp.

2 Materials and methods

2.1 Experimental design

The experimental work was structured according to a full factorial design aimed at evaluating the combined influence of high hydrostatic pressure and chemical or enzymatic pretreatments on the release of polyphenols from *Sargassum* spp. The design incorporated two independent factors: the pressure applied during the hydrostatic treatment and the type of pretreatment administered to the biomass. The pressure factor comprised five levels corresponding to atmospheric pressure (0.101 MPa), 50 MPa, 100 MPa, 150 MPa, and 200 MPa. The pretreatment factor consisted of four conditions that included an untreated control, a hydrogen peroxide pretreatment, an enzymatic pretreatment based on the *Trametes hirsuta* BM-2 extract, and a sequential process that combined hydrogen peroxide followed by enzymatic treatment. Each specific combination of these factors was performed with six independent replicates, giving a total of 120 experimental units.

For analytical clarity, the factorial structure may be represented in terms of two indices. The index i denotes the level of hydrostatic pressure and assumes values from 1 to 5. The index j denotes the pretreatment condition and assumes values from 1 to 4. Each experimental unit is therefore associated with a unique pair (i,j), referred to as T_{ij}, which identifies the treatment defined by the i-th pressure level and the j-th pretreatment. The response variable corresponding to each treatment is the concentration of polyphenols released from the biomass and is denoted by Y_{ijk}, where k indicates the replicate and ranges from 1 to 6.

The response data were interpreted within the framework of the standard two-factor factorial model,

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk},$$

where μ represents the overall mean response, α_i describes the effect of the i-th pressure level, β_j describes the effect of the j-th pretreatment condition, and $(\alpha\beta)_\mathrm{ij}$ represents the interaction between both factors. The term ε_ijk denotes the experimental error, which is assumed to be independent and normally distributed.

In addition to the factorial arrangement described above, a complementary experimental set was performed to determine whether high hydrostatic pressure alters the activity of the enzymatic extract. This resulted in two new treatment configurations: (i) non-pressurized biomass treated with pressurized extract, and (ii) pressurized biomass treated with pressurized extract; pressurized biomass treated with non-pressurized extract was used as control. The chemical–enzymatic treatment sequence was also included within these same configurations. This second experimental component followed a one-factor design with six levels, each conducted with six independent replicates (36 experimental units).

All experimental data derived from the factorial combinations were subjected to two-way or one-way ANOVA in order to determine the significance of the main effects and their interaction. When significant differences were detected, Tukey's test with a significance level of $p < 0.05$ was applied to compare individual treatment means. The assumption of normality was evaluated by examining the distribution of residuals in a Q–Q plot prior to statistical testing.

2.2 Sample selection and preparation

Mixed *Sargassum* spp. (*S. fluitans and S. natans*) were manually collected offshore at Puerto Morelos, Quintana Roo, Mexico (20°50.9195' N, 86°52.5743' W) during 2022. The biomass was first rinsed with tap water to remove sand and salts, after which it was wrapped in a permeable cloth and submerged in running water for 30 min with manual agitation. This washing procedure was repeated three times to ensure the removal of residual debris. The material was then sun-dried for three days and subsequently stored at 4 °C until use. Dried samples were shredded in a commercial blender (Waring 51BL30) and sieved to retain particles larger than 1 mm.

2.3 High hydrostatic pressure (HHP) preconditioning

Portions of dried sargassum were placed in Teflon tubes filled with water, sealed with beryllium–copper clamps, and pressurized at 50, 100, 150, or 200 MPa for 30 min at 25°C in a manually operated hydraulic press (Eureka Ltda., MG, Brazil). Pressure was monitored with a calibrated Woler Ind. manometer. Compression/decompression times were < 1 min (Albuquerque *et al*., 2016). Immediately after decompression, samples underwent chemical, enzymatic, or sequential pretreatments.

2.4 Enzymatic extract production

The crude extracellular enzymatic extract was produced as described by Tapia-Tussell *et al*. (2018) using *Trametes hirsuta* Bm-2 (GQ280373), isolated in Yucatán, Mexico. The fungus was maintained on 2% (w/v) malt extract agar at 35 °C for 4–5 days. Four 1 cm mycelial plugs were inoculated into 250 mL Erlenmeyer flasks containing sterilized medium (g L^{-1}: malt extract 10, peptone 2, yeast extract 2, KH_2PO_4 2, $MgSO_4{\cdot}7H_2O$ 1, thiamine 1) and 2% (w/v) wheat bran. Cultures were incubated at 35 °C, 150 rpm for 7 d, and filtered to obtain the crude extract. Laccase activity was determined using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate at 420 nm (ε = 36,000 L moL^{-1} cm^{-1}). One unit was defined as 1 μmol of product min^{-1}.

2.5 Chemical, enzymatic and sequential treatments

For chemical pretreatment, 10 g of biomass was treated with 200 mL of 2.5% (v/v) H_2O_2 (Chikani-Cabrera *et al*., 2022). Enzymatic pretreatment involved preparing a 10% (w/v) suspension in 0.05 M citric acid/0.05 M sodium citrate buffer (pH 5), adding the enzymatic extract to reach 7000 U laccase g^{-1} dry sargassum (10 mL of enzymatic extract with an activity of 7000 U ml^{-1}), and incubating at 40°C, 150 rpm for 48 h (Tapia-Tussell *et al.*, 2018). Sequential pretreatments applied H_2O_2 followed by enzymatic treatment under the same conditions.

2.6 *Total phenolic content*

Phenolics were quantified following Box (1983). Briefly, 50 μL of extract were mixed with 1.55 mL distilled water and 100 μL Folin–Ciocalteu reagent. After 5 min in the dark, 300 μL of 7.5% (w/v) Na_2CO_3 were added. The mixture was incubated at 40°C for 30 min, and absorbance was measured at 760 nm (Varian Cary 3 UV–Vis). Results were expressed as mg gallic acid equivalents (GAE eq) g^{-1} total solids (TS).

3 Results and discussion

3.1 *Effect of hydrostatic pressure on the polyphenol release from Sargassum spp. biomass*

As shown in Table 1 and Figure 1a, HHP alone slightly increased phenolic content relative to atmospheric pressure, although differences were not statistically significant. This aligns with reports that HHP mainly disrupts cell walls without extensive lignocellulose depolymerization (Albuquerque *et al.*, 2016; Navarro-Baez *et al.*, 2022). The concentrations were comparable to those reported for brown macroalgae, e.g., *U. pinnatifida* (1.2 mg GAE eq g^{-1} TS), *S. latissima* (1.2 mg GAE eq g^{-1} TS), *S. polyschides* (1.7 mg GAE eq g^{-1} TS), *H. elongata* (7.3 mg GAE eq g^{-1} TS), and *H. muticum* (19 mg GAE eq g^{-1} TS) (Jard *et al.*, 2013), and to values from *S. muticum* HWE (2.6 mg GAE eq g^{-1} dry mass) or EAE (3.8–6.4 mg GAE eq g^{-1} dry mass) (Puspita *et al.*, 2017). Higher yields from cellulase, hemicellulase, and amylase (4.2–6.4 mg GAE eq g^{-1} dry mass) compared to protease (3.8 mg GAE eq g^{-1} dry mass) are consistent with the greater phenolic release observed here using *T. hirsuta* BM-2 extract at 0.101 MPa (6.31 ± 0.46 mg GAE eq g^{-1} TS), likely due to enhanced lignocellulose degradation.

In contrast to HHP alone, combining hydrostatic pressure with H_2O_2 or the laccase-rich fungal extract significantly increased phenolic release, with concentrations peaking at 150 MPa. At 0.101 MPa, H_2O_2 and laccase treatments yielded 6.30 ± 0.20^{b} mg GAE eq g^{-1} TS and 6.31 ± 0.46^{b} mg GAE eq g^{-1} TS, respectively, both substantially higher than the untreated control (2.63 ± 0.60^{a} mg GAE eq g^{-1} TS) (Figure 1a). This confirms the effectiveness of both chemical and enzymatic pretreatments in liberating polyphenols from the lignocellulosic matrix, and the capacity of HHP to enhance this effect. Rodrigues *et al.* (2017) also reported that HHP improved extraction of antioxidant polysaccharides from *Sargassum muticum*, with yields of 32.0–40.4 g 100 g^{-1} dry mass at 300, 450, and 600 MPa 36–72% higher than those obtained by HWE (23.5%) or UAE (24.0%). The latter methods also produced extracts with comparatively lower antioxidant activity.

Polyphenol concentrations were consistently higher in peroxide-treated samples than in those treated with the enzymatic extract alone, and this trend was observed across all pressure levels tested (Table 1, Figure 1a). These results indicate that HHP can enhance chemical and enzymatic access to the lignocellulosic polymer network by altering cell wall structure and increasing surface area, thereby improving delignification efficiency (Navarro-Baez *et al.*, 2022). The data also suggest that less pressure is required to potentiate H_2O_2 action than to enhance enzymatic action, likely due to the smaller molecular size of H_2O_2. This is supported by the absence of statistical difference between the peroxide treatment at 50 MPa (7.54 ± 0.12^{cde} mg GAE eq g^{-1} TS) and the enzymatic treatment at 150 MPa (7.54 ± 0.94^{cde} mg GAE eq g^{-1} TS).

The sequential combination of H_2O_2 and the fungal extract significantly enhanced polyphenol recovery, reaching 13.47 ± 1.13^{f} mg GAE eq g^{-1} TS at 0.101 MPa, more than twice the values obtained with individual treatments.

Table 1. Effect of high hydrostatic pressure on polyphenol release (mg GAE eq g^{-1} TS) from *Sargassum* spp. treated with hydrogen peroxide (H_2O_2) and a fungal enzymatic extract with laccase activity.

Pressure (MPa)	No treatment	H_2O_2	EE	H_2O_2 and EE
0.101	2,63±0,60^{a}	6,30±0,20^{b}	6,31±0,46^{b}	13,47±1,13^{f}
50	2,91±0,44^{a}	7,54±0,12^{cde}	6,70±0,34^{bc}	15,32±0,58^{g}
100	3,03±0,28^{a}	7,98±0,59^{de}	6,82±0,29^{bcd}	15,66±1,37^{g}
150	3,23±0,32^{a}	8,10±0,27^{e}	7,54±0,94^{cde}	16,19±0,47^{g}
200	3,12±0,21^{a}	8,03±0,38^{de}	6,68±0,64^{bc}	15,94±0,24^{g}

Different letters indicate statistically significant differences (Tukey's test, $p < 0.05$). EE: Enzymatic Extract, H_2O_2: Hydrogen Peroxide. Total solids = 0.9237 ± 0.0284 g TS g^{-1} dry mass.

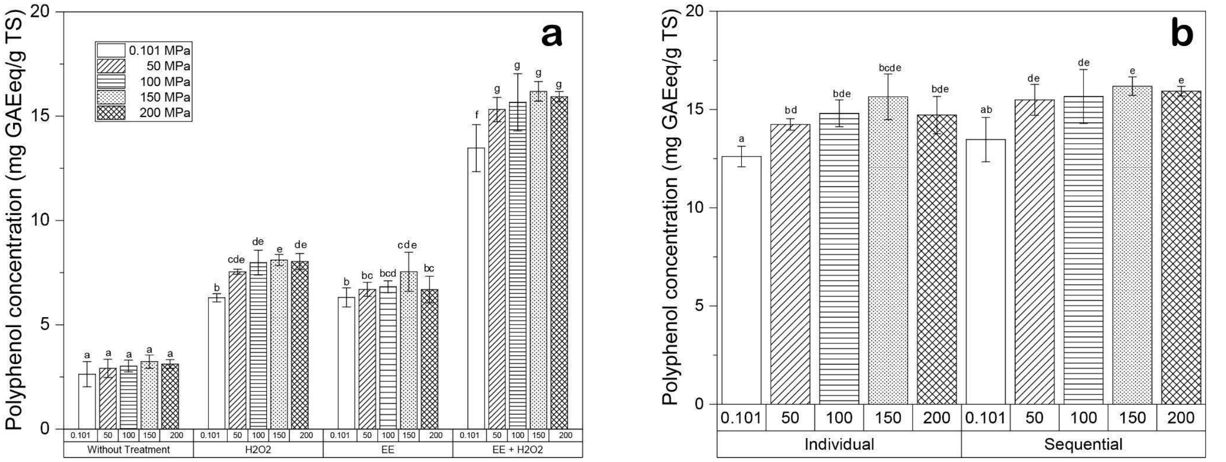

Figure 1. Polyphenol concentration (mg GAE eq g^{-1} TS) released from *Sargassum* spp. biomass preconditioned with high hydrostatic pressure (0.101, 50, 100, 150, and 200 MPa). (a) Effect of hydrogen peroxide (H_2O_2), a fungal enzymatic extract from *T. hirsuta* with laccase activity, or their sequential application. (b) Sum of individual treatments (chemical and enzymatic) versus sequential treatment, with and without HHP preconditioning. Different letters indicate significant differences (Tukey's test, $p < 0.05$).

This effect may arise from complementary chemical targets and possible enzymatic synergy. H_2O_2 acts as an oxidative delignification agent, generating hydroxyl radicals (·OH) via Fenton-like reactions that cleave aromatic structures and disrupt ether linkages in lignin and phenolic esters (Teong *et al*., 2019), while laccases oxidize phenolic hydroxyl groups in lignin-like compounds through one-electron transfer, forming phenoxy radicals that promote depolymerization or rearrangement (Cañas & Camarero, 2010). In addition, white-rot fungi secrete peroxidases such as manganese peroxidase, lignin peroxidase, and versatile peroxidase, enzymes with H_2O_2-dependent activity (Hofrichter *et al*., 2010). Although the *T. hirsuta* Bm-2 extract was not purified, it may contain such peroxidases, as co-expression with laccases has been reported in *Trametes* spp. under ligninolytic conditions (Moiseenko *et al*., 2023). Oxidation during peroxide pretreatment may also disrupt cell walls, improving enzymatic access and potentially activating peroxide-dependent enzymes, thereby reinforcing the advantage of combining biochemical and chemical pretreatments under hydrostatic pressure.

The sequential chemical and enzymatic treatment was enhanced by HHP preconditioning, showing a significant increase in polyphenol release from sargassum starting at 50 MPa (15.32 ± 0.58^g mg GAE eq g^{-1} TS) compared to 0.101 MPa (13.47 ± 1.13^f mg GAE eq g^{-1} TS). Concentrations increased slightly with pressure, reaching 15.66 ± 1.37^g mg GAEeq/g TS at 100 MPa, 16.18 ± 0.47^g mg GAE eq g^{-1} TS at 150 MPa, and 15.94 ± 0.24^g mg GAE eq g^{-1} TS at 200 MPa, though differences among these pressure levels were not statistically significant. Notably, the 150 MPa maximum was statistically significant only in peroxide (8.10 ± 0.27^e mg GAE eq g^{-1} TS) and enzymatic (7.54 ± 0.94^{cde} mg GAE eq g^{-1} TS) treatments applied individually, suggesting that pressure responsiveness is more pronounced when chemical and enzymatic actions operate separately, whereas their combination under HHP may cause a plateau effect due to early and efficient substrate oxidation. When compared with literature values, the concentrations obtained here appear modest; for example, Prasedya *et al*. (2021) reported 14.19 ± 2.08% total polyphenol content (TPC) in ethanol evaporated extracts (141.9 mg TAN eq g^{-1} dry extract yield 1.03 ± 0.15%), with a threefold increase upon reducing particle size from >4 mm to 45–125 μm. In this study, particle size was >1 mm, suggesting future exploration of size reduction in combination with HHP, H_2O_2, and enzymatic treatment. Other studies have reported higher values, e.g., *Sargassum tenerrimum* at 69.12 ± 0.24% TPC (12 g 100 g^{-1} dry mass yield) using HWE plus Soxhlet extraction (Shunmugiah Mahendran *et al*., 2024), or 4.2–9.8 mg TAN eq g^{-1} dry extract (0.45–1.31 mg GAE eq g^{-1} dry mass) after purification (Lee *et al*., 2022). Vijayan *et al*. (2028) obtained 4.88 mg GAE eq g^{-1} dry mass from *Sargassum wightii* via ethyl acetate extraction. These comparisons highlight that the combined process proposed here achieves phenolic concentrations comparable to those from established food and pharmaceutical extraction methods.

The effect of the sequential application was evaluated by comparing the polyphenol concentration obtained with the sum of the concentrations from the individual treatments (Figure 1b). At 0.101 MPa, the sequential treatment (13.47 ± 1.13^{ab} mg GAE eq g^{-1} TS) was significantly higher than the sum of phenolic phases yielded individual treatments (12.61 ± 0.52^a mg GAE eq g^{-1} TS). The same trend was observed with HHP preconditioning: at 50 MPa,

Table 2. Percent increase in polyphenol release relative to the untreated atmospheric-pressure control.

Pressure (MPa)	No treatment	H_2O_2	EE	H_2O_2 and EE
0.101	0.00±32,44%[a]	139.14±55.37%[b]	139.69±57.68%[b]	411.68±124.94%[f]
50	10.59±30.35%[a]	186.48±65.88%[cde]	154.48±59.78%[bc]	481.96±135.30%[g]
100	14.96±28.43%[a]	203.24±73.08%[de]	159.17±60.45%[bcd]	494.99±146.13%[g]
150	22.79±30.67%[a]	207.87±71.35%[e]	186.55±74.76%[cde]	514.79±142.16%[g]
200	18.47±28.29%[a]	205.14±71.51%[de]	153.87±63.10%[bc]	505.31±139.17%[g]

Different letters indicate statistically significant differences (Tukey's test, $p < 0.05$). EE: Enzymatic Extract, H_2O_2: Hydrogen Peroxide. Total solids = 0.9237 ± 0.0284 g TS g^{-1} dry mass.

Table 3. Effect of High Hydrostatic Pressure (150 MPa) on polyphenol concentration, expressed as gallic acid equivalents (mg GAE/g TS), as an indicator of laccase activity in extracts obtained from *Sargassum* spp.

Treatment	Pressurized Sargassum	Pressurized Sargassum and enzymatic extract	Pressurized enzymatic extract
Enzymatic extract	7.54±0.94[b]	6.63±0.32[a]	6.13±0.26[a]
Hydrogen peroxide (H_2O_2) and enzymatic extract	16.19±0.47[d]	15.97±0.39[d]	14.24±0.29[c]

Different letters indicate statistically significant differences (Tukey's test, $p < 0.05$). Total solids = 0.9237 ± 0.0284 g TS/g dry mass.

14.24 ± 0.29^{bd} vs. 15.49 ± 0.78^{de} mg GAE eq g^{-1} TS; at 100 MPa, 14.81 ± 0.68^{bde} vs. 15.66 ± 1.37^{de} mg GAE eq g^{-1} TS; at 150 MPa, 15.65 ± 1.16^{bcde} vs. 16.18 ± 0.47^{e} mg GAE eq g^{-1} TS; and at 200 MPa, 14.72 ± 0.95^{bde} vs. 15.94 ± 0.24^{e} mg GAE eq g^{-1} TS for the sum of individual and sequential treatments, respectively. These results confirm that H_2O_2 pretreatment enhances enzymatic efficiency through one or more of the mechanisms discussed above, as evidenced by the consistently higher yields obtained when treatments were applied sequentially to sargassum biomass. Similar synergistic behavior has been reported in ultrasound-assisted enzymatic extraction systems, where the combination of physical disruption and enzymatic hydrolysis markedly increases polyphenol release (Loan *et al.*, 2024).

The percent increases relative to the untreated control revealed consistent and substantial enhancements across all pretreatments. Hydrogen peroxide and the enzymatic extract each produced increases of approximately 140–200%, while the sequential process achieved the highest gains, reaching 400–515% depending on pressure. Although HHP contributed modest additional improvements, especially at 150 and 200 MPa, the data indicate that the synergistic chemical–enzymatic interaction exerts the strongest effect on polyphenol extraction. The highest yield was obtained when the biomass was treated sequentially with hydrogen peroxide and *T. hirsuta* extract at 150 MPa, reaching 514.79±142.16%, although this value was not statistically different from those obtained at lower pressure levels.

3.2 *Effect of High Hydrostatic Pressure on the enzymatic and sequential treatments*

The effect of HHP at 150 MPa on laccase activity was evaluated using phenolic release from Sargassum biomass as an indicator (Table 3). Under enzymatic treatment, pressurizing both the biomass and the fungal extract yielded 6.63 ± 0.32^{b} mg GAE eq g^{-1} TS, slightly lower than pressurizing only the biomass (7.54 ± 0.94^{a} mg GAE eq g^{-1} TS), indicating that HHP can reduce laccase activity. This contrasts with Albuquerque *et al.* (2016), where HHP at 3000 MPa was reported to enhance cellulase activity. Pressurizing only the enzyme gave 6.13 ± 0.26^{a} mg GAE eq g^{-1} TS, not significantly different from biomass plus enzyme pressurization, but still higher than HHP alone at 150 MPa (3.23 ± 0.32^{a} mg GAE eq g^{-1} TS; Table 1). These results suggest that HHP at 150 MPa does not fully inactivate laccase, and phenol release from intact sargassum was still feasible after pressurization.

In sequential chemical and enzymatic treatments, the highest release occurred when only the biomass was pressurized (16.19 ± 0.47^{d} mg GAE eq g^{-1} TS), followed by pressurizing both biomass and enzyme (15.97 ± 0.39^{d} mg GAE eq g^{-1} TS) and pressurizing only the enzyme (14.24 ± 0.29^{c} mg GAE eq g^{-1} TS). Significant differences were found only when the enzyme extract was pressurized alone (Figure 2). This indicates that while pressurization can reduce laccase performance, the loss may be mitigated by integrating pressurization and enzymatic steps; moreover, this difference was negligible in sequential treatments, which maintain superior yields. The absence of

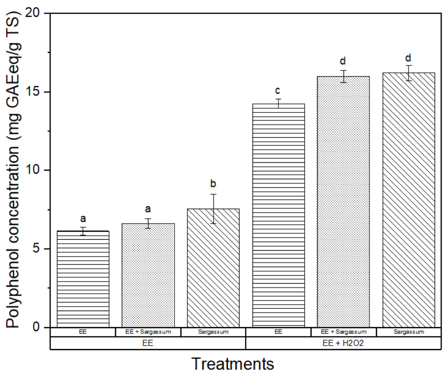

Figure 2. Effect of high hydrostatic pressure (150 MPa) on polyphenol concentration (mg GAE eq g^{-1} TS) as an indicator of laccase activity in extracts from *Sargassum* spp. Different letters indicate significant differences (Tukey's test, $p < 0.05$).

adverse effects on phenolic release is particularly relevant given that offshore sargassum harvesting often involves mechanical compaction systems that apply substantial pressure to dewater and reduce biomass volume (Gray *et al*., 2021).

Conclusions

This study showed that high hydrostatic pressure (HHP) alone does not significantly enhance polyphenol release from the sargassum matrix without prior chemical or enzymatic treatment (no statistical differences across all pressure levels were detected). However, when such pretreatments were applied individually or sequentially, HHP significantly improved extraction, with 150 MPa consistently yielding the highest results. When a 150 MPa pressure was applied to the *T. hirsuta* extracts, it partially reduced its laccase activity, although polyphenol release remained largely unaffected under sequential treatment, likely due to the compensatory effect of H_2O_2. Overall, the combination of chemical and enzymatic pretreatments demonstrates a synergistic effect, further amplified by HHP.

Notably, in the sequential chemical–enzymatic approach, yields at 50, 100, 150 and 200 MPa were similar as no statistical difference was found across all levels of pressure except for atmospheric pressure (0.101 MPa), suggesting potential cost savings for industrial applications because no high pressure is needed to achieve desirable results. In contrast, applied pressure by the HHP method seems to have a significant effect on individual treatments.

Future research should aim to optimize combined pretreatments such as particle size reduction, diluted H_2O_2, and enzymatic extracts from *T. hirsuta* BM-2, under HHP conditions, and further characterize the polyphenolic fraction from *S. fluitans* and *S. natans*. These strategies could enhance polyphenol recovery and support sustainable biorefinery approaches to valorize sargassum, helping mitigate its environmental impact on coastal areas.

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Nomenclature

HHP	High Hydrostatic Pressure
GAE	Galic Acid Equivalent
TS	Total Solids
SPF	Seaweed Polyphenolic Fraction
EAE	Enzyme-Assisted Extraction
HWE	Hot-Water Extraction
UAE	Ultrasound-Assisted Extraction
MAE	Microwave-Assisted Extraction
TPC	Total Polyphenol Content
TAN	Tannic Acid

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