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Treatment effect on degradation compounds production of microalgal-bacterial biomass grown in photobioreactors for pig manure remediation

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RESUMEN

El desarrollo de metodologías eficientes y baratas para el tratamiento *in situ* de todo tipo de aguas residuales (residuos ganaderos, de ciudad, etc.) es una necesidad creciente. Uno de los métodos más prometedores es el uso fotobiorreactores. La biomasa producida, compuesta por microalgas y bacterias, utiliza los nutrientes presentes en todo tipo de aguas residuales para generar carbohidratos, proteínas y lípidos. Después, estos productos pueden ser procesados y revalorizados. El aprovechamiento de las diferentes fracciones de la biomasa, aplicando el concepto de biorrefinería, mejora la viabilidad económica y ambiental del proceso global.

Este trabajo ha estudiado aspectos concretos de la valorización de la fracción de carbohidratos de biomasa de algas y bacterias crecida en plantas de tratamiento de purín de cerdo en forma de azúcares fermentables. Muestras de biomasa algal, obtenida del tratamiento de purines en una planta piloto, fueron sometidas a varios tratamientos físicos y químicos, seguidos de una etapa de hidrólisis enzimática. En todos los casos, la liberación de azúcares conlleva la co-solubilización de otras fracciones (proteínas y lípidos) y la generación de compuestos de degradación, que reducen el rendimiento del proceso y pueden inhibir las posteriores etapas de fermentación. El objetivo del estudio es identificar la metodología más eficiente para maximizar la producción de azúcares, minimizando simultáneamente su degradación. Para ello se ha investigado el efecto de dichos tratamientos en la generación de subproductos de degradación o inhibidores.

Para ahondar en la comprensión de los procesos de degradación de nutrientes y poder diferenciar la degradación debida al pretratamiento de la degradación biológica, algunos tratamientos (hidrólisis ácida, hidrólisis alcalina y molienda en molino de bolas) se aplicaron a fuentes puras de carbohidratos (celulosa), proteínas (gelatina) y lípidos (aceite vegetal).

Las concentraciones de inhibidores obtenidas se han estudiado con herramientas estadísticas multivariantes (análisis en componentes principales y análisis de conglomerados jerárquicos), evidenciando el efecto de los diferentes tratamientos físicos, químicos y enzimáticos en el tipo y cantidad de productos de degradación liberados.

Los resultados revelaron que, a pesar de la alta producción de inhibidores, los tratamientos con ácido clorhídrico son los más adecuados para maximizar la producción de monosacáridos. Los tratamientos químicos resultaron en mayor degradación, mientras que en los físicos, los inhibidores se produjeron durante la hidrólisis enzimática, debido al metabolismo de las bacterias presentes en la biomasa.

ABSTRACT

The development of efficient and inexpensive methodologies for the treatment of all types of wastewater (manure, municipal wastewaters, etc.) is an increasing need. One of the most promising methods is the usage of photobioreactors. Biomass produced, composed by microalgae and bacteria, use the nutrients present in all types of wastewater to generate carbohydrates, proteins and lipids. These products can be further processed and revalorized. The use of the different fractions of the biomass, applying the concept of biorefinery, improves the economic and environmental viability of the global process.

Certain aspects of the valorization of the carbohydrate fraction of algae and bacteria biomass grown in pig slurry treatment plants in the form of fermentable sugars were studied in this work. Algal biomass samples, obtained from pig manure treatment in a pilot plant, were subjected to several physical and chemical treatments, followed by an enzymatic hydrolysis step. Sugar release entails co-solubilization of other fractions of biomass (proteins and lipids) and generation of degradation byproducts, which can reduce the efficiency and inhibit further processes. The aim of the study is to identify the most efficient method to maximize sugars production while simultaneously minimizing their degradation. The effect of treatments on the generation of degradation byproducts has been investigated.

To deepen the understanding of the processes of nutrient degradation and differentiate degradation due to pretreatment and due to biological action, some treatments (acid hydrolysis, alkaline hydrolysis and bead mill grinding) were applied to pure sources of carbohydrates (cellulose), gelatin (proteins) and lipids (vegetable oil).

The concentrations of inhibitors obtained were studied by using multivariate statistical analysis (principal component analysis and hierarchical cluster analysis), demonstrating the effect of the different physical, chemical and enzymatic treatments in the type and concentration of degradation products released.

Obtained results revealed that even though high byproduct yields, hydrochloric acid treatment is the most suitable method due to the highest monosaccharide recovery. Chemical treatments resulted in higher degradation, while in physical treatments inhibitors were mainly produced during enzymatic hydrolysis as a result of bacteria metabolism.

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2. Introduction

Contamination is one of the major problems that humanity faces nowadays. Emission of greenhouse gasses, water scarcity and energy supply are some of the elements which contribute to its increase. One of the latest and most promising solutions to these problems is the use of biological entities for water purification and bioenergy production [1-3]. Microalgal-based systems have been widely studied in the last decades as a promising solution in the near to medium term. It has been demonstrated that algae perform photosynthesis much more efficiently than superior plants, thus achieving fast growth under the adequate conditions [1, 2, 4]. Algae also present high lipid and carbohydrate productivities, by far exceeding those of conventional energy crops like soybean, sugarcane or corn [2, 5]. Microalgae systems are capable to perform an advanced water treatment by removing nutrients, organic matter and heavy metals from waste waters, and at the same time producing low cost biomass.

Microalgae biomass is mainly composed of carbohydrates, lipids and proteins, which are usable in the production of biofuels (bioethanol and biogas by fermentation; biodiesel by oil esterification), fertilizers or animal feeds. Microalgae systems are also able to recover or generate added value products such as pigments or pharmaceuticals [1, 2, 6-10]. Composition of microalgae can be modulated by adjusting the process variables or the nutritional conditions during biomass grow, and thus compounds of interest can be produced. [11].

Microalgae can behave as autotrophic or heterotrophic systems, and some species show both metabolisms. Autotroph species use sunlight and CO_2 as feedstock to synthetize organic matter, releasing oxygen in the process, whereas heterotroph species use organic matter in the media as source of nourishment. Mixotrophic systems can perform both metabolisms depending on sunlight, CO_2 or organic matter characteristics of the media, making them the most suitable option to biomass production [1, 9-12].

The largest fraction of global biofuel production is *first generation*, which is based on vegetable oils or carbohydrate-rich plants. The employment of these crops changes the usage of arable land, with negative impact on food security, water scarcity and deforestation. Biofuels produced from microalgae biomass are *third generation*, which is a big improvement as feedstock used is no suitable for human or animal consumption. This make algae biomass production independent of arable land availability or crop irrigation water, relieving the scarcity of crops and water for human consumption [2, 4,

5, 12-15]. Biodiesel, bioethanol or biogas can be synthetized efficiently from certain species algae biomass.

A large amount of wastewater is produced every day, including different sources as agricultural activities, industry or urban waste. These are low cost media for microalgae cultivation, as they grow by taking up organic and inorganic nutrients. After that, oxygenated effluent with low organic matter concentration and higher pH is discharged, preventing eutrophication of water bodies. Piggery wastewater is one of the most suitable media for microalgae cultivation, as it is low-cost and widely available. Large amounts of organic matter are present in pig manure, with high concentrations of carbon, nitrogen and phosphorus, the most important nutrients for microalgal growth [9-11].

Microalgae are a potential source of sustainable biomass feedstock, but further investigation is necessary to overcome economical and technical limitations of microalgal mass cultivation. High energy demands and poor recoveries need to be improved. Harvesting is one of the critical steps, due to the size of microalgae organisms (unicellular or slightly pluricellular) [10]. Research in recent years have showed that microalgal-bacterial symbiotic systems could easily solve this problem with the formation of aggregates of appreciable size that can be removed from cultivation tanks. It has been demonstrated that this symbiotic relation has many advantages in biomass production. Aggregates show excellent settling characteristics, making the system more resistant to climate oscillations [8]. A beneficial exchange of macro and micronutrients is stablished within the aggregates. On one hand, bacteria degrade organic matter and generate simple compounds assimilable by microalgae. Algal growth is also promoted by other compounds produced by bacteria (indole-3-acetic acid, vitamin B12). On the other hand, oxygen produced by algal photosynthesis is used by bacterial metabolism, reducing the need of additional oxygen supply. Bacteria also take advantage of fixed carbon compounds synthetized by microalgae. Some polysaccharides and proteins excreted by bacteria trigger the formation of the aggregates, enveloping microalgae and bacteria cells together [7, 16].

After biomass growth and harvesting, some steps are necessary to obtain the desired products. Cell wall disruption is essential to release intracellular compounds as starch, cellulose, lipids or proteins, and to maximize product recovery in downstream processes [17]. A variety of cell disruption methods are currently available, and new technologies are being developed. These methods need to be low cost and energy efficient, resulting in high quality products with excellent yield. Methods are classified in physical, chemical and biological.

Physical treatments can be thermal or mechanical. Thermal treatments are based in temperature changes, sometimes complemented by pressure. *Freeze-fracture* or *steam explosion* are examples of thermal treatments. Mechanical treatments break the components of the cell wall by direct action of a physical force like kinetic force (*bead milling*), waves (*ultrasonic*, *microwaves*) or electric force (*pulse electric field*). Physical methods advantages usually include less contamination of the final product and less dependence of microalgae species. However, more sophisticated equipment is required and needs higher energy inputs. In addition, local heat peaks can damage the released products.

Chemical methods are based on chemical reactions with specific cell wall components. Acid and alkali methods are often used. Methods with concentrated chemicals are faster but have some drawbacks, such as byproduct generation, equipment corrosion, difficult recovery or high operational and preservation costs. Pretreatments with usage of diluted chemicals are more time-consuming but have less inconveniences. Other less common chemical methods for the separation of the compounds of interest use extraction with ionic liquids or supercritical fluids. In general, chemical methods have lower energy consumption, higher efficiency and upscaling is simpler. Cost of chemicals and quality of the final products are the main disadvantages.

Biological methods include enzymatic treatments, where cell disruption is accomplished through the conversion of carbohydrates into monomeric sugars or proteins into amino acids. The use of enzymes has abundant benefits, such as biological specificity, high selectivity, high conversion yield, mild operating conditions, low energy requirements, low capital investment, easy scale-up and the prevention of destructive conditions. Nevertheless, inhibitor production, high enzyme cost, difficult enzyme recovery and long incubation times are intricate problems with no easy solution [18, 19].

Chemical methods are usually reported as the most effective for sugar release, and more specifically, acid treatments. However, these methods contribute to the degradation of released nutrients into byproducts, which could be harmful in further downstream processes. Furfural, hemifurfural (HMF), formic and acetic acids are usual degradation byproducts of cellulose and hemicellulose, originated by these acid pretreatments [4, 15, 17, 20-23]. Inhibitory byproducts can appear as a result of any pretreatment, chemical, physical or enzymatic. Bacteria from microalgae-bacteria aggregates can also generate inhibitory byproducts by metabolism [24].

3. Aims of study

The aim of this work is to investigate the effects of physical, chemical and biological (enzymatic) treatments on the release of inhibitory degradation byproducts during the recovery of nutrients from microalgae biomass grown in pig manure treatment photobioreactors. Since inhibitory products decrease the yields of nutrients and their quality, the objective is to maximize the monosaccharides yields while minimizing the generation of inhibitory byproducts. Lipid and protein co-solubilization are also analyzed as a result of applying the biorefinery concept.

The type and amount of inhibitory byproducts released by the different pretreatments applied have been analyzed using multivariate statistical methods (principal component analysis, PCA, and hierarchical cluster analysis, HCA) in order to find correlations amongst the experimental conditions used in biomass treatment and the degradation products released. This knowledge will allow optimizing the procedure for the obtention of high-quality monosaccharides from biomass grown in pig manure treatment plants.

To better understand the sources of the different degradation byproducts and their dependence on the different biomass treatments, pure materials containing only carbohydrates (cellulose), proteins (edible gelatin sheets) or lipids (sunflower oil) have been also treated using physical (bead mill) and chemical (acid and alkali treatments) methods, combined with enzymatic hydrolysis. These treatments proved to be extreme conditions between monosaccharide solubilization and minimal degradation byproduct release. The results have been analyzed again using PCA and HCA and compared with those obtained from the treatment of microalgal biomass.

Scarce information is found in literature about inhibitory byproducts [20], and there is no previous research using statistical treatment in this field. Therefore, this work can add some relevant knowledge to the subject.

4. Materials and methods

4.1 Biomass assays

Centrifugated biomass was provided by the Cajamar Foundation (Almeria, Spain) and refrigerated at 4 °C for a maximum of 48 h prior to use. Fresh algal-bacterial biomass was cultivated in a thin-layer 1200 L photobioreactor treating pig manure. Two different batches were used. The biomass composition of Batch 1 (on a dry weight basis) was 23.67% carbohydrates, 42.55% proteins, 16.74% lipids, and 16.83% ash, and the main microalgae families in the biomass were *Scenedesmaceae* (71%), *Aphanothecaceae* (11%) and *Chlorellaceae* (12%). The biomass composition of Batch 2 was 38.11% carbohydrates, 24.83% proteins, 12.51% lipids, and 24.50% ash; and the families of microalgae identified within were *Scenedesmaceae* (73%) and *Naviculaceae* (27%) [24].

Bead mill, alkaline (NaOH), steam explosion and alkali-peroxide (H_2O_2) pretreatments were applied to the biomass from Batch 1, and ultrasound and acid (HCl) pretreatments were applied to the biomass from Batch 2. Each pretreatment, from now on abbreviated as PR, was performed at two different levels and in duplicate. Biomass was weighted to achieve concentration of 5% w/w of dry weight. Experimental conditions of each treatment are detailed in Table 1.

Table 1. Experimental conditions of the pretreatment tests of microalgae-bacteria biomass.

Code	Type of pretreatment	Conditions	Duration (min)	Temperature
A	Bead mill	1.25 mm beads	5	Room temp.
B	Bead mill	2.50 mm beads	60	Room temp.
C	Alkaline	NaOH 0.5M	60	120ºC
D	Alkaline	NaOH 2M	60	120ºC
E	Steam explosion	Saturated steam + flash	5	130ºC
F	Steam explosion	Saturated steam + flash	20	170ºC
G	Alkali-peroxide	H_2O_2 0.5% (w/w); pH 11.5	60	50ºC
H	Alkali-peroxide	H_2O_2 7.5% (w/w); pH 11.5	60	50ºC
I	Ultrasound	479 W, 7186 J/g TS	5	Room temp.
J	Ultrasound	115W, 7186 J/g TS	21	Room temp.
K	Acid	HCl 0.5M	60	120ºC
L	Acid	HCl 2M	60	120ºC

After pretreatment and neutralization of pH, enzymatic hydrolysis was performed to an aliquot of the whole pretreated suspension. The enzymatic hydrolysis, henceforth abbreviated as EH, was performed in 100 mL Erlenmeyer flasks containing pretreated biomass suspension, 1.25 mL of 1M citrate buffer and 125 µL of celullase enzyme (Celluclast 1.5L - Cellulase). The assays of biomass were incubated in a rotatory shaker at 50 ºC and 300 rpm for 6 and 12 hours. Results obtained by *Martín-Juárez et al.* showed that 6-hour and 12-hour EH had similar results. The experiments were performed in duplicate for each sample. These experiments were carried out by the group of investigation, and results are submitted for publication [24].

After statistical treatment of the generated inhibitory byproducts, a selection of those pretreatments were performed to pure carbohydrate, protein and lipid samples. Pure carbohydrate material for the study was commercial cotton, composed entirely by cellulose. Commercial edible gelatine sheets (from pork source) were used as pure protein source (86% protein, 0% lipids, 0% carbohydrates). Commercial sunflower oil (100% lipids) was used as lipid pure source. Samples of materials were weighted (12.5 g) to achieve a concentration of 5% w/w by adding distilled water or the corresponding chemical reagent solution to 250 mL. Acid (HCl) and alkali (NaOH) pretreatments were performed at two previous levels (0.5 and 2.0 M; 60 minutes; 120ºC), while bead mill pretreatment was performed only at one level (1.25 mm beads; 5 minutes; room temperature). PR and EH were performed in duplicate. Only 6-hour EH assays were performed on pure materials.

Liquid samples were taken after pretreatment and after enzymatic hydrolysis in duplicate. Solid and liquid fraction were separated by centrifugation (5 min; 10000 rpm) and liquid fraction was filtered (nylon; 0.22 µm) prior to analysis by liquid chromatography.

4.2 HPLC measurements

Monosaccharides and degradation byproducts in the liquid fraction were quantified by HPLC, using a Bio-Rad HPX-87H ion-exclusion column, installed in a Waters e2695 separation module. A 25 mM sulfuric acid solution was used as mobile phase. The flow rate was adjusted to 0.6 mL/min after 2 hours of stabilization. Temperature of the oven was set to 50ºC, while temperature of the detector was 35ºC. A refractive index detector (Waters 2414) was used to quantify the concentration of monosaccharides and degradation byproducts (Table 2) [5]. Cellobiose, glucose, xylose, arabinose and ribose

were the measured monosaccharides. Other degradation byproducts (oxalic, formic, acetic, lactic, butyric, succinic and levulinic acids, furfural and HMF) were also measured with a photodiode detector (Waters 2998) at 210 nm.

External calibration was used to quantify monosaccharides and degradation compounds. Calibration standards were prepared by mixing the adequate amounts of stock solutions of each compound, prepared from reagents with a purity > 95% (Sigma Aldrich, Spain). In following analysis of results, names of inhibitors will be used as appear in figures. In samples, acids are protonated or deprotonated depending the pH of the treatment, but in discussion of results thy will be named as their acid state. All acids were protonated in HPLC analysis, as the mobile phase used was 0.025 M H_2SO_4.

Table 2. Retention times of measured compounds by HPLC.

Nº	Inhibitor	Retention Time	Nº	Inhibitor	Retention Time
1	Cellobiose	7.798	12	Glycerol	13.790
2	Oxalic acid	8.107	13	Formic acid	14.775
3	Citric acid	8.851	14	Acetic acid	15.973
4	Glucose	9.349	15	Levulinic acid	17.93
5	Xylose	10.007	16	Methanol	19.391
6	Malic acid	10.468	17	Ethanol	21.602
7	Arabinose	11.011	18	Butyric acid	23.384
8	Ribose	11.571	19	Acetone	23.814
9	Xylitol	11.700	20	Butanol	37.924
10	Succinic acid	12.833	21	Furfural	38.400
11	Lactic acid	13.451	22	HMF	59.217

4.3 Statistical treatment

Multivariate statistical analysis was carried out using principal component analysis (PCA) and hierarchical agglomerative cluster analysis (HCA). These are non-parametric classification methods, which means that no mean or variance are used in calculations, making them insensitive to data with variety of distributions (no necessary normal distributions). These exploratory data analysis methods allow to uncover hidden relations between the variables and samples and easily visualize these relations [25].

As values of the different measured variables can have different scale and magnitude, these methods are usually performed with standardized values, where all variables have similar weight in final results. In this study, values are transformed into *z-values* (zero mean and unit variance).

Principal component analysis is used to reduce the dimensionality of large data sets of observations of possibly correlated variables into a set of values of linearly uncorrelated variables, called principal components or PCs, in such a way that only a few of the new PCs contain relevant information, thus reducing the dimensionality and facilitating the visualization of the relations between observations. All the original variables have the same amount of associated variance, and are usually correlated. The new set of variables are uncorrelated (orthogonal) and total variance is condensed into the first new PCs, while the last ones have low portions of the total variance. Usually PC1 and PC2 are represented in a plot, as they mean the highest variability, and new associations of values can be observed. Relation between PCs and original variables is expressed by loadings, which will be named as variables or inhibitors in following figures. Individual transformed measurements are called scores, and from now on will be named as values of PCA plots to ease the explanation of the figures. Parallel representation of loadings and scores for the most significant PCs clarifies unseen relations in the raw dataset, as related variables or samples will obtain similar values after PCA [26].
Hierarchical agglomerative cluster analysis is an unsupervised pattern-recognition method which classifies the objects (variables and samples) into groups or clusters, based on similarities between them. Classification is achieved by distance between objects. Groups formed are progressively merged until all objects are in one group. In this work, separation is measured as Euclidean squared distance between objects. Ward method is used as association method, and is obtained as squared sum of deviation of each value to the centroid of the group. Values are hierarchically grouped forming a dendrogram, one for samples and other for variables. Interpretation of dendrograms is helped by heatmaps, which represents whether the value for each sample and variable is above or below the average. Samples with similarities in inhibitors or in amount of them will group together [27].

Some of the degradation products determined in this study were undetected or measured under limits of detection. When a variable was undetected in several samples, inhibitor was excluded from statistical study, or a value near to zero ($<10^{-4}$ $g{\cdot}L^{-1}$) was assigned to each sample to be able to perform multivariate statistical analysis (software is not capable to work with zeros or empty cells). In other cases, where a variable only has a few 'zero' values, a random number between zero and limit of detection was assigned [28, 29].

Multivariate statistical analysis was carried out with Statgraphics Centurion 18. Heatmaps were constructed with MATLAB 2016.

5. Results and discussion

5.1 Microalgal-bacterial biomass

The solubilization yield of principal components of biomass (carbohydrates, proteins and lipids) was measured for each pretreatment in a previous study carried out by *Martín-Juárez et al.* Results are represented in Figure 1. Acid pretreatments achieved the highest solubilization yield for carbohydrates, with remarkable effect of acid concentration. Acid pretreatment also produced high solubilization of proteins and lipids. Alkali pretreatment obtained good solubilization yields for all components. Proteins are preferentially solubilized with alkali pretreatment. Steam explosion and alkali-peroxide also resulted in moderate solubilization for all biomass components but only under severe conditions. Ultrasound pretreatment obtained low solubilization yields, slightly better when pretreatment was applied for long times. The lowest solubilization yields were obtained with bead mill pretreatment. Effect of enzymatic hydrolysis is clear in all samples, especially in those physically pretreated. Only strong acid treatment obtained similar solubilization of carbohydrates after EH, probably explained by total solubilization in PR step due to the severity of the treatment. Values of 6-hour and 12-hour enzymatic hydrolysis are not very different, indicating that EH can be performed for only 6 hours and obtain comparable yields. Results from untreated samples of both biomass batches are also represented (sample *UT1* from batch 1; sample *UT2* from batch 2).

As said above, chemical pretreatments produced higher solubilization yields than physical pretreatments, but also produce greater amounts of inhibitory byproducts. In Figure 2, total concentration of inhibitory byproducts is exposed for each treatment. Monosaccharide recovery is also represented, to give an idea of the goodness of each treatment. As said before, the ideal treatment should obtain good monosaccharide recovery while concentration of inhibitors remains low. For maximum monosaccharide concentration, carbohydrates need to be preferentially solubilized in high yields.

Bead mill pretreatment produce negligible amounts of degradation byproducts, while acid and alkali were the most degrading pretreatments. The amount of these compounds increased with the harshness of the pretreatment. Furfural and HMF were not detected, probably because of further degradation to simpler compounds.

Monosaccharide recovery was moderate for all chemical pretreatments except for acid pretreatment. At the same time, degradation of the released sugars is higher in acid pretreatments. In alkali and alkali-peroxide pretreatments, monosaccharides recovery is much lower compared to the yield of carbohydrate solubilization, due to high degradation of the released products. A good compromise between monosaccharide recovery and degradation need to be achieved to optimize the process.

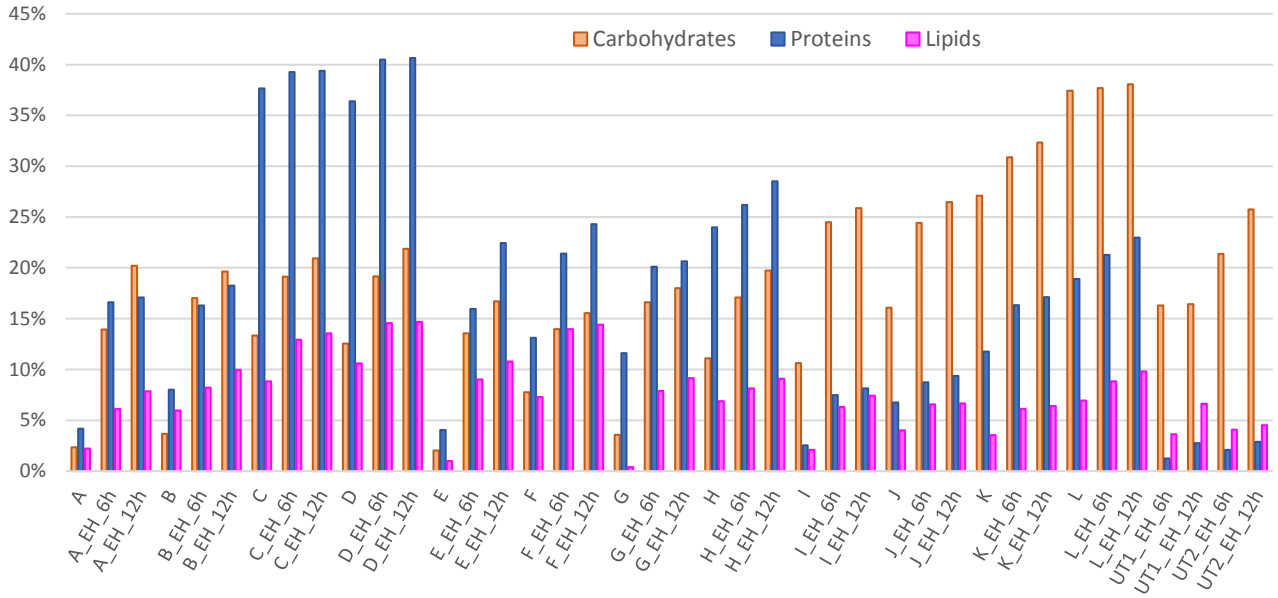


Figure 1. Concentration of components of biomass (carbohydrates, proteins and lipids) released to liquid fraction during PR and EH steps. Concentration in % (w/w). (Data from Martin et al, submitted for publication).

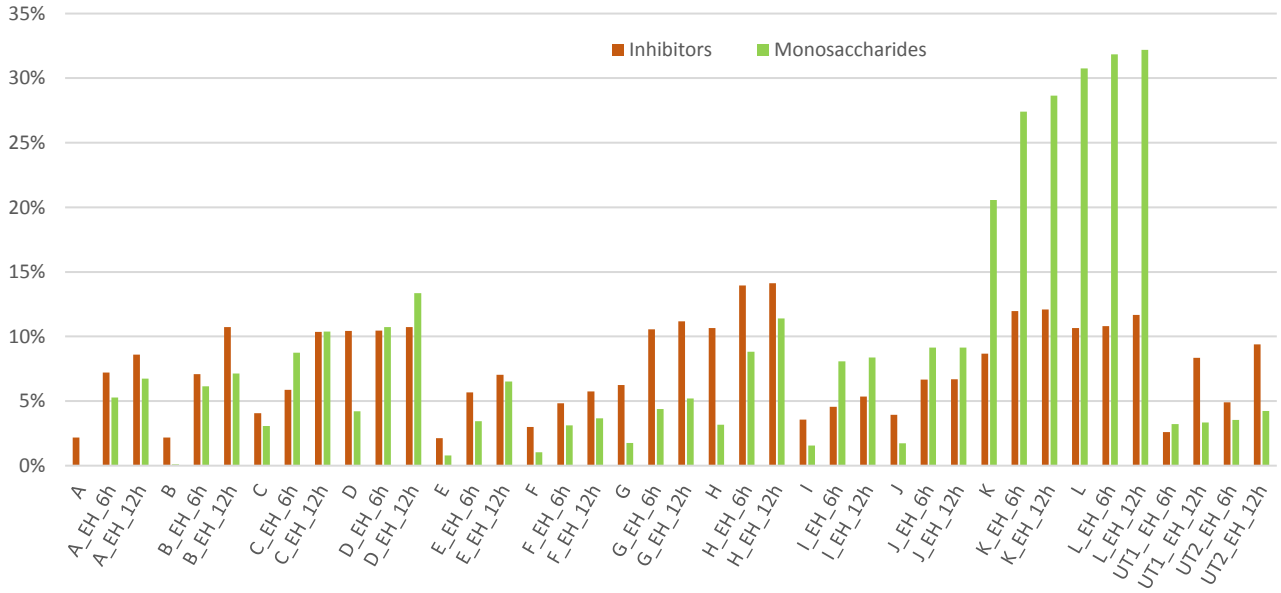


Figure 2. Total concentration of inhibitory byproducts and monosaccharides obtained in PR and EH steps. EH data from 6-hour and 12-hour assays. Concentration in % (w/w). (Data provided by the research group).

Enzymatic hydrolysis step was performed after pretreatment. The type on enzyme used selectively solubilizes carbohydrates, enhancing the recovery. Enzymatic hydrolysis was applied to untreated samples from batch 1 and batch 2 to evaluate the effects of not performing a pretreatment step and compare results of different batches of microalgal biomass. Low monosaccharide recovery was obtained, with high degradation attributed to active bacterial metabolism present in the suspension. Significative concentrations of methanol, ethanol, acetic acid and succinic acid were found after EH of untreated biomass, which are common products of bacteria metabolism mentioned before.

Enzymatic hydrolysis produced high monosaccharide recovery in chemical pretreated samples. This effect is mostly due to pretreatment action before EH and can be observed in samples of 2 M HCl. Monosaccharide recovery achieved in PR, 6-hour EH and 12-hour EH of 2 M HCl treatment was similar. In soft acid samples and alkali samples, enzyme action increases the recovery as chemical PR is not able to fully solubilize carbohydrates. This effect is determining in bead mill pretreated samples, where EH produced a huge increase in carbohydrate solubilization, which was very low after PR. However, the concentration of inhibitory byproducts originated by degradation also increased, caused by the action of living microorganisms in the suspension using solubilized compounds as feedstock. Concentration of inhibitors visibly increased from 6 to 12-hour EH, while recovery yields remained similar. This effect highlights that duration of EH step does not mean an improvement in the process.

Martín-Juárez et al. [24] confirmed the action of bacterial metabolism by analysing DNA integrity in each sample. Viable bacteria can degrade solubilized compounds into undesired inhibitors and compete with other microorganisms in subsequent valorisation steps. Results showed that after enzymatic hydrolysis step, bacterial DNA was clearly degraded. No bacterial DNA was found in samples of acid, alkali and 7.5% H_2O_2 alkali-peroxide pretreatments, suggesting that inhibitors are mostly originated by chemical degradation. Other pretreatments did not degrade bacteria as well as chemical treatments, resulting in higher carbohydrate degradation and lower monosaccharide recovery.

Concentrations of inhibitors released by PR and EH action can be found in *Supplementary Materials* section, in Table I. Solubilization of carbohydrates, proteins and lipids of biomass is summarized in Table II, alongside total monosaccharide recovery and total inhibitor release.

Principal component analysis (PCA) and hierarchical cluster analysis (HCA) were used to analyze data of inhibitors obtained from pretreatments and enzymatic hydrolysis of microalgal-bacterial biomass. First, pretreatment step will be evaluated. After that, 6-hour and 12-hour EH assays will be discussed, and finally both PR and EH will be studied all together, including data of inhibitors from only EH and from PR+EH. The plot for loadings and the plot for scores for the principal components 1 and 2 will be always represented, named as Variables (A) for loadings and Samples (B) for scores of the treatments in new PCs.

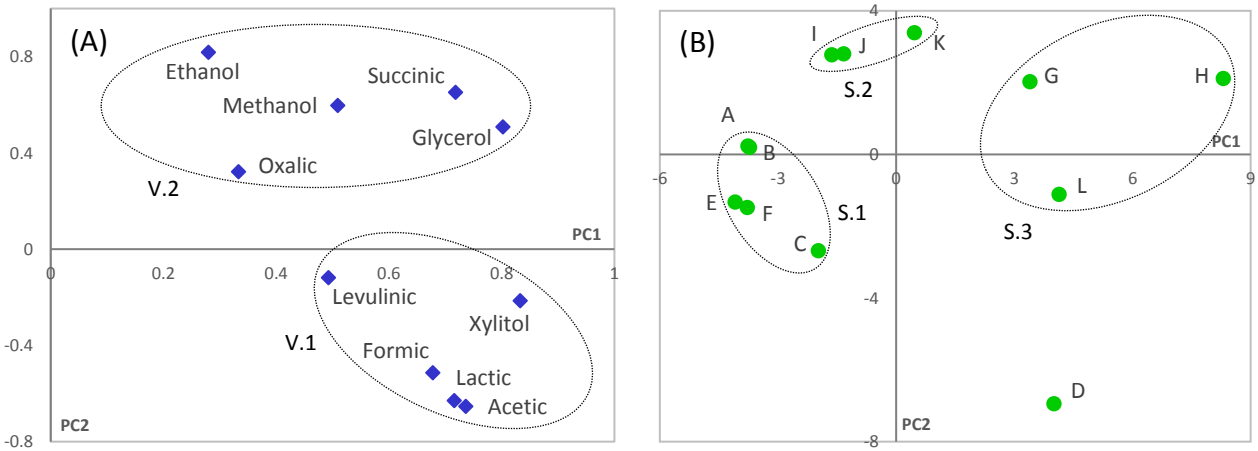
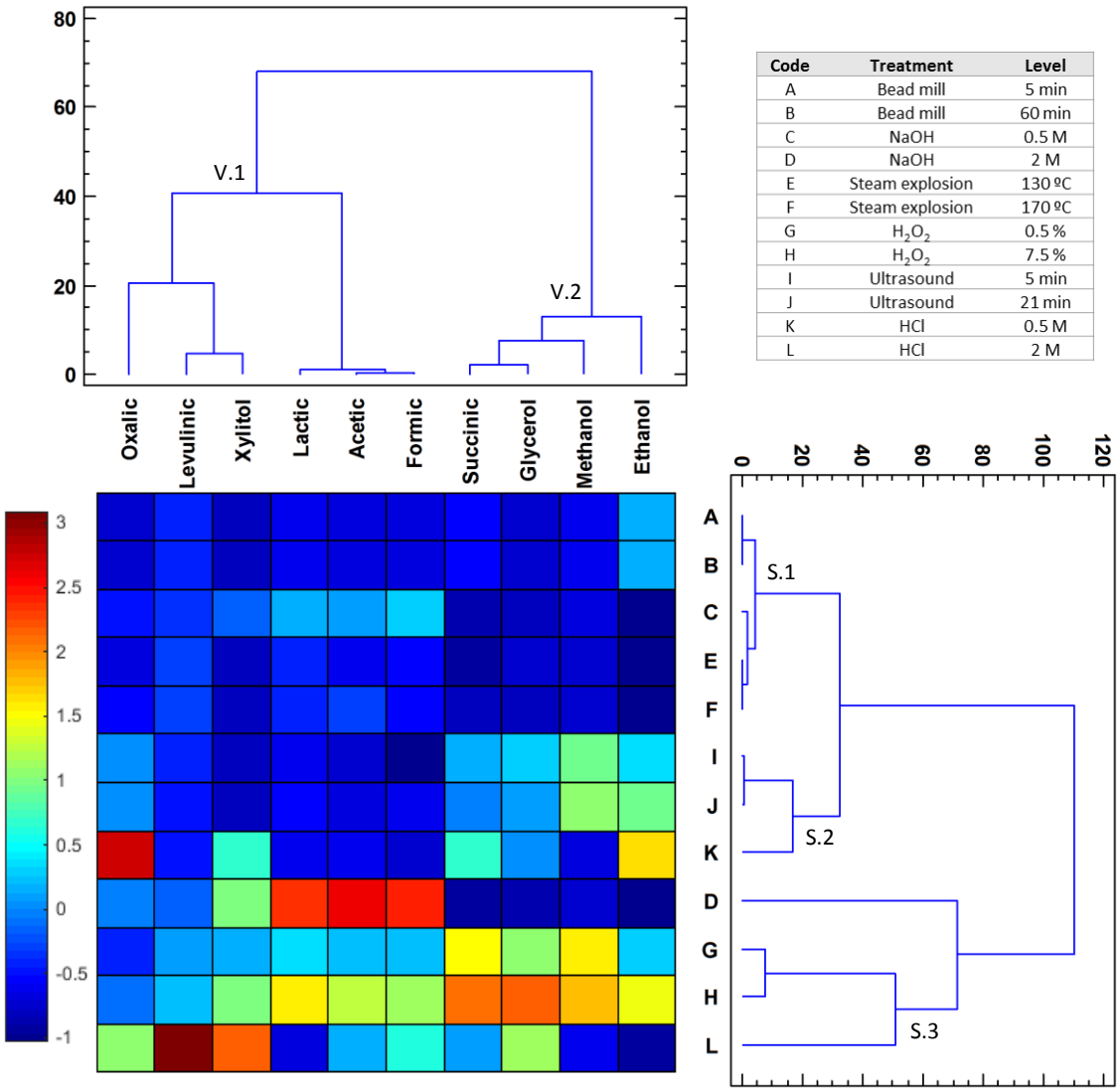


Figure 3. PCA plot of different PR applied to microalgal biomass, with: (A) Variables; (B) Samples.

In Figure 3A, separation of inhibitors is not achieved by PC1, as all of them resulted in positive values. PC2 clearly separate the variables in two groups, with positive (*V.2*) and negative coefficients (*V.1*). Plot B shows that an increase in the intensity of the pretreatment does not produce an increase in degradation byproducts of physically pretreated samples (A, B, E, F, I, J), as values remain together. Separation can be appreciated in chemical pretreatments (C, D, G, H, K, L), where increase in severity of the treatment produce an increase in the production of inhibitors.

In HCA (Figure 4), groups are arranged slightly different. Group *V.2* does not include oxalic acid, which is moved to group *V.1*. Group *V.1* can be divided in two smaller groups: lactic, acetic and formic acids, strongly related to 2 M NaOH treatment; and xylitol, oxalic and levulinic acids, related to acid pretreatments. Heatmaps represent the standardized concentrations of each inhibitor, ranging from dark blue for very low concentrations to dark red for concentrations much higher than the mean. There are three clusters for samples: group *S.1* includes treatments with very low degradation, group *S.2*, not far from first, with medium/low concentrations of inhibitors.

Group *S.3* includes alkali-peroxide and strong acid treatments, with higher concentrations of inhibitory byproducts. Finally, 2 M NaOH treatment, which is included in group *S.3* of samples in dendrogram but is far from the group in PCA. Group *S.1* is not related to any group of inhibitors because of the very low concentrations present in the samples. Groups *S.2* and *V.2* are related, and group *S.3* is related to both groups *V.1* and *V.2*. Alkali peroxide samples released inhibitors from group *V.2*, and strong acid PR obtained also high concentrations of oxalic, levulinic and xylitol, in group *V.1* of inhibitors. As said before, treatment *D* is strongly related to acetic, lactic and formic acids, and that is the reason why group *V.1* can be divided and is placed in the fourth quadrant of PCA.



Code	Treatment	Level
A	Bead mill	5 min
B	Bead mill	60 min
C	NaOH	0.5 M
D	NaOH	2 M
E	Steam explosion	130 ºC
F	Steam explosion	170 ºC
G	H_2O_2	0.5 %
H	H_2O_2	7.5 %
I	Ultrasound	5 min
J	Ultrasound	21 min
K	HCl	0.5 M
L	HCl	2 M

Figure 4. Dendrograms of inhibitory byproducts produced in pretreated samples, obtained by HCA.

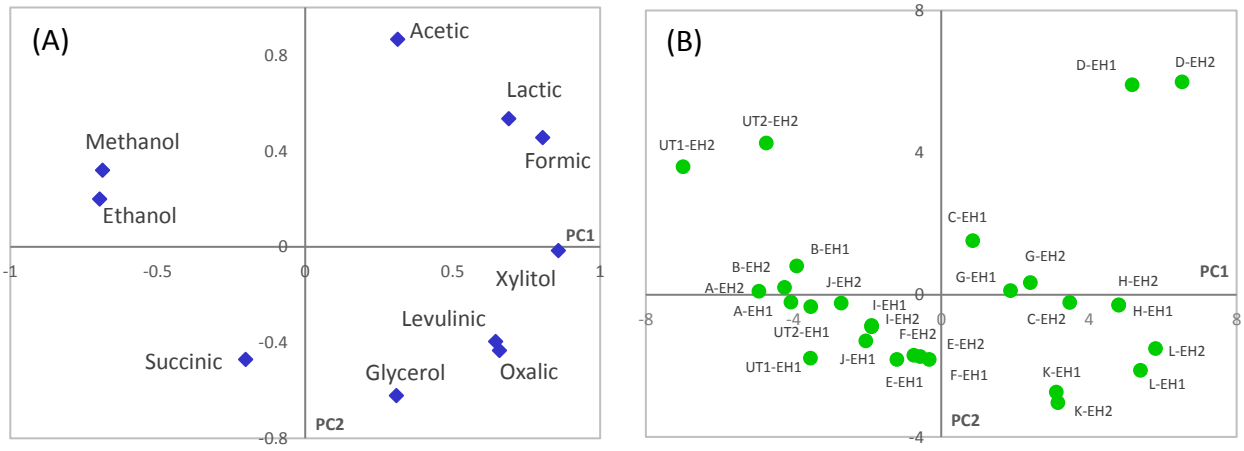


Figure 5. PCA plot of 6-hour and 12-hour (EH1 and EH2, respectively) enzymatic hydrolysis step of previous pretreatments, with: (A) Variables; (B) Samples.

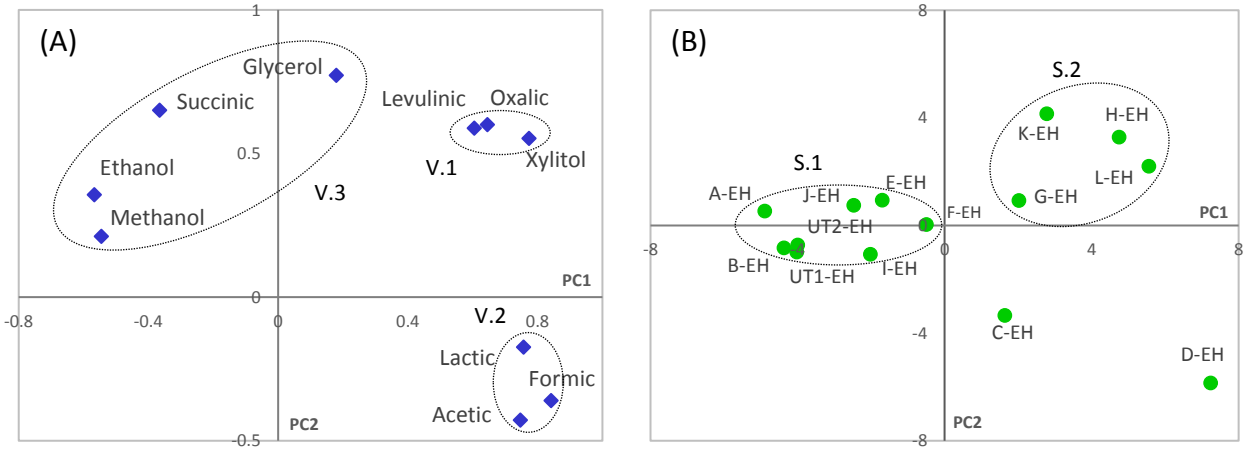
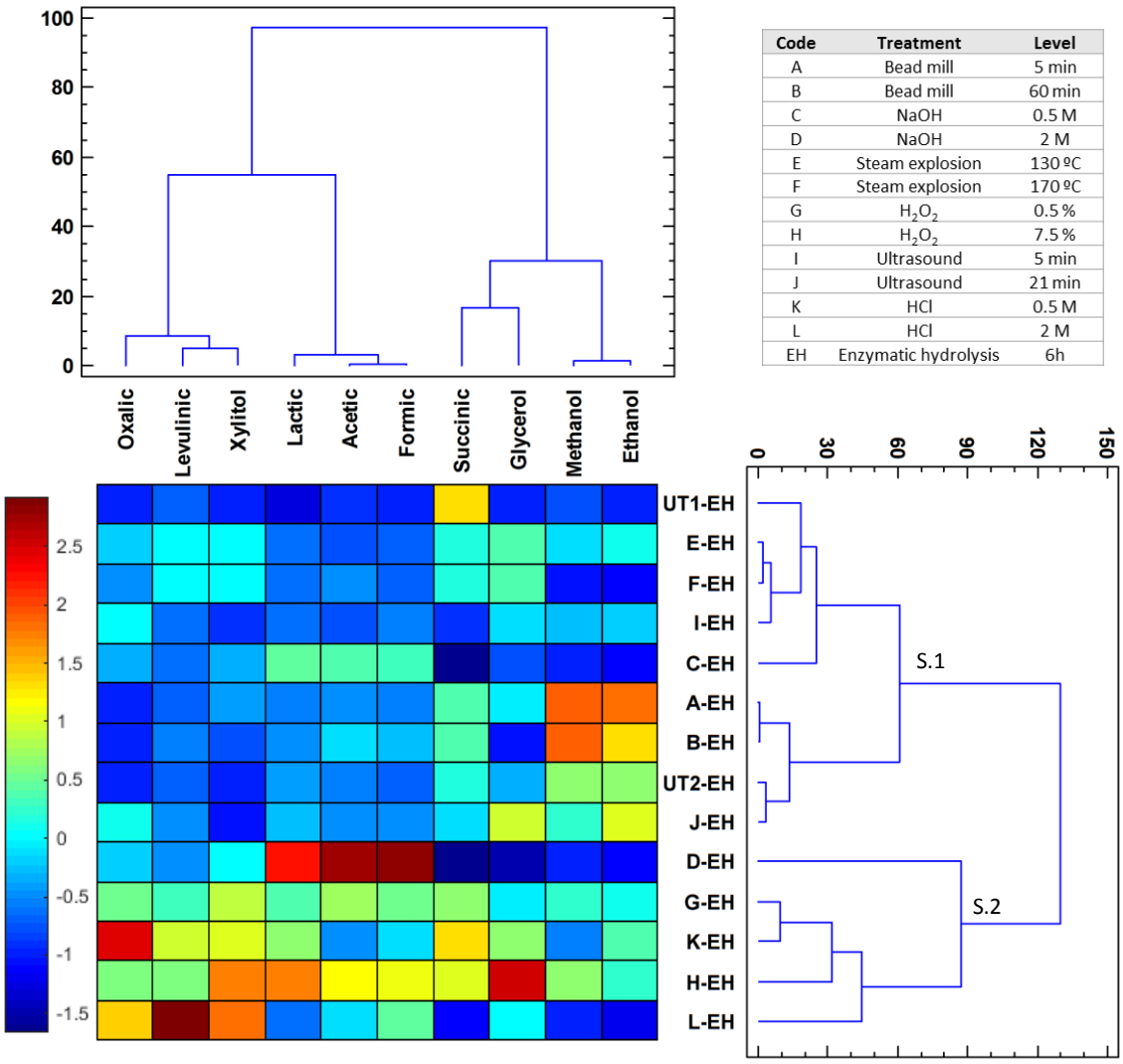


Figure 6. PCA plot of enzymatic 6-hour hydrolysis step of previous pretreatments, with: (A) Variables; (B) Samples.

As said in Figures 1 and 2, an increase in EH duration did not necessarily mean better results. Monosaccharide recovery was shortly better after 12 hours of enzymatic hydrolysis, in strong acid treatment results were even similar. However, concentration of degradation byproducts increased in most of the samples when duration of enzymatic hydrolysis was longer.

Regarding these results, two different PCA were prepared: first one includes data from 6 and 12-hour enzymatic hydrolysis, while second is based in data from 6-hour assays. These PCA are shown in Figures 5 and 6, respectively. PCA plots of variables found similar results. Groups of inhibitors are the same, changes between plots are only in the position of clusters. In PCA of only 6-hour data results are slightly better because clusters of inhibitors are more defined and separation between them is evident.



Code	Treatment	Level
A	Bead mill	5 min
B	Bead mill	60 min
C	NaOH	0.5 M
D	NaOH	2 M
E	Steam explosion	130 ºC
F	Steam explosion	170 ºC
G	H_2O_2	0.5 %
H	H_2O_2	7.5 %
I	Ultrasound	5 min
J	Ultrasound	21 min
K	HCl	0.5 M
L	HCl	2 M
EH	Enzymatic hydrolysis	6h

Figure 7. Dendrograms of inhibitory byproducts produced in 6-hour enzymatic hydrolysis, obtained by HCA.

In PCA of 6 and 12-hour EH, values of samples with same pretreatment are not far from each other. Only untreated and 0.5 M NaOH samples show separation between values for short and long EH, due to big increase in concentration of inhibitors. Separation of groups is not clear. In PC1 two groups can be considered: physically pretreated samples at negative values and chemically pretreated samples at positive values. Severe alkali pretreatment samples are shifted to high positive PC2 values. Identically, samples of 12-hour untreated samples are separated from the big cluster. As results show that EH for 12 hours is not clearly useful, HCA was performed only with 6-hour EH data, and compared with respective PCA plots (Figure 7).

Groups of samples are more difficult to understand. A big cluster appears in negative PC1 values, including samples physically pretreated. In dendrogram, this big cluster could be divided in two smaller groups. In second groups, samples with high contents of

methanol and ethanol would be included (*A-EH, B-EH, UT2-EH, J-EH*). These samples are located in the left part of the cluster in PCA plot, related to the position of group *V.3* of inhibitors. Soft NaOH sample is included in this group in HCA, because concentration of inhibitors obtained is similar to others in the group, but inhibitors obtained are more related to strong NaOH sample. In the same way, 2 M NaOH belongs to group *S.2* in dendrogram because its high concentration of inhibitors. In PCA is far from the group, placed in the fourth quadrant. Samples of alkali treatment are shifted to fourth quadrant as they are strongly correlated with the production of acetic, formic and lactic acids (group *V.2*).

Groups of variables are similar to PCA of pretreatments, but now separation is achieved in both PC1 and PC2. First group (*V.1*) is formed by oxalic, levulinic and xylitol, in first quadrant. Acetic, lactic and formic acids remain grouped in fourth quadrant (group *V.2*). Methanol and ethanol are closer, and with succinic and glycerol constitute group *V.3*. Methanol and ethanol are probably produced by metabolism of bacteria and are related to untreated biomass as they have similar positions in PC plots (Figure 6). This effect is produced by the lack of sterilization of the biomass suspension and can be also appreciated in physical pretreatments such as bead mill (close to untreated biomass in Figure 6B).

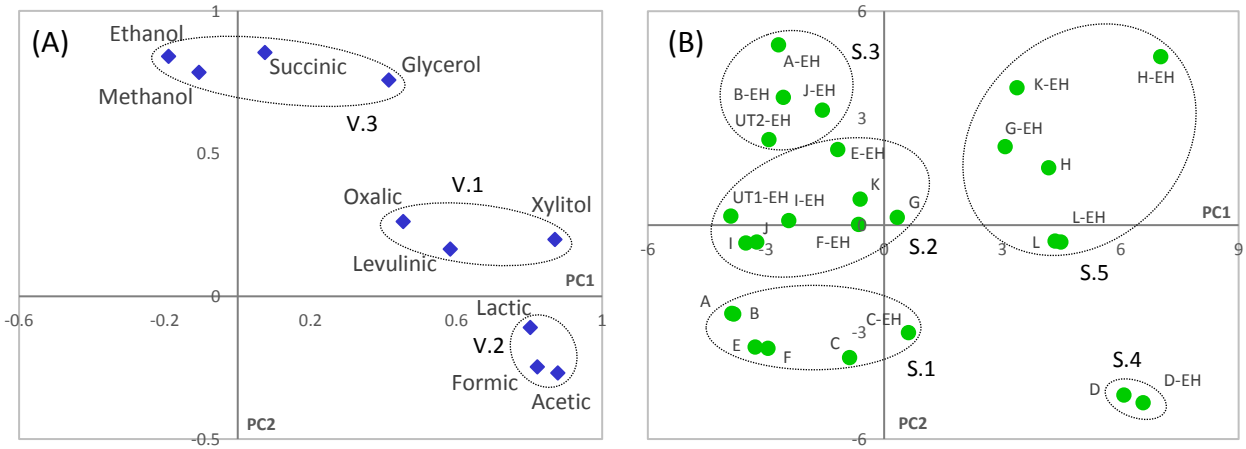
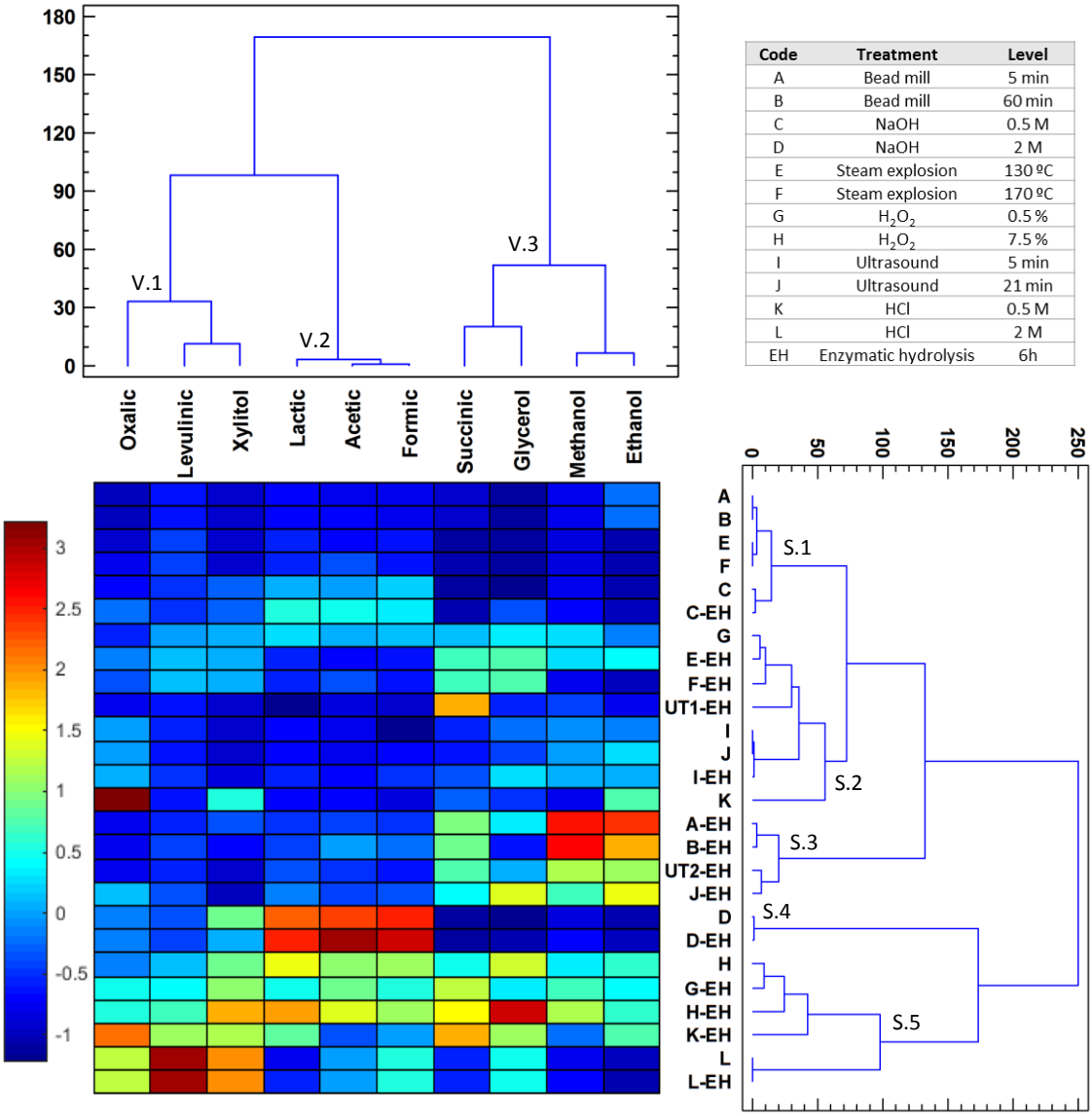


Figure 8. PCAs plot of pretreatments and enzymatic hydrolysis of previous pretreatments, with: (A) Variables; (B) Samples.

Finally, a comparative study including data from both PR and EH was performed. PCA plot of inhibitors (Figure 8A) is more similar to the plot obtained from PR than the plot obtained from EH, indicating the key role of the pretreatment step in the overall process. Groups obtained are even more defined: high correlation for lactic, formic and

acetic acids in fourth quadrant (group *V.2*), not far from other group including xylitol, levulinic and oxalic (group *V.1*). Final cluster, *V.3*, includes glycerol, succinic, ethanol and methanol, these last two very close. Plot of samples (Figure 8B) is not easy to understand, as values are not well clustered. Separation is achieved between physical and soft chemical treatments (negative PC1 values) and severe chemical treatments (positive PC1 values). First group can be divided into three smaller clusters, with the help of the dendrogram of samples. Group *S.1* contains samples with very low degradation, including *C* and *C-EH*. Group *S.2* includes samples with medium/low concentration of inhibitors. Only *K* and *UT1* obtained relatively high concentration of any inhibitor.



Code	Treatment	Level
A	Bead mill	5 min
B	Bead mill	60 min
C	NaOH	0.5 M
D	NaOH	2 M
E	Steam explosion	130 ºC
F	Steam explosion	170 ºC
G	H_2O_2	0.5 %
H	H_2O_2	7.5 %
I	Ultrasound	5 min
J	Ultrasound	21 min
K	HCl	0.5 M
L	HCl	2 M
EH	Enzymatic hydrolysis	6h

Figure 9. Dendrograms of inhibitory byproducts produced in pretreatment step and subsequent enzymatic hydrolysis, obtained by HCA.

In group *S.3* appear samples with low concentration of inhibitors except those from group *V.3*. High concentrations of methanol and ethanol, which are common degradation byproducts of bacteria metabolism, are present in bead mill and untreated samples. Strong chemical PR reaches high sterilization of the biomass, and degradation in EH is low. However, soft chemical PR and particularly physical PR are not strong enough to obtain this effect, and remaining bacteria are able to metabolize monosaccharides solubilized in EH and produce simple alcohols as byproducts. Group *S.4* of samples includes only strong alkali treatment (PR and EH), located far from other samples in fourth quadrant. This group is strongly related to group *V.2* of inhibitors (acetic, lactic and formic acids). Last group of samples,*V.5*, contains other chemical treatments.

Position of groups of variables and samples reveals the interaction between them. As said before, groups *V.2* and *S.4* are closely related. Likewise, inhibitors of group *V.1* are related to strong acid treatments, but also are produced in alkali-peroxide treatments. Inhibitors in group *V.3* are released mostly in samples of group *S.3*, but also in alkali-peroxide treatments, so it is placed between them. There are no inhibitors related to groups *S.1* and *S.2* of samples because release of inhibitors in these samples is low.

PCA plot of samples reveals the effect of the enzymatic hydrolysis in the generation of degradation byproducts. Strong acid and base treatments (L and D) remain close to their EH value. This effect is caused by great solubilization of compounds during PR step, and also degradation of these compounds. The most part of inhibitors present in these samples are a result of PR step, and EH has low impact. On the other hand, soft physical treatments that yielded low degradation in PR step are an optimal media for bacterial metabolic action, and concentration of inhibitors clearly increase. This can be appreciated in plot B, where PR physical pretreatments are grouped in group *S.1* while values of their EH are clearly separated in group *S.2* and *S.3*, so PC2 clearly separates these pretreatments from their enzymatic hydrolysis. At the same time, PC1 separates high-degrading chemical treatments, with positive values, and less degrading chemical treatments and physical treatments, with neutral and negative PC1 values.

To totally understand PR and EH steps and the inhibitors generated, one last study was performed including data from PR and EH. Concentrations from PR step are the same as before. Concentrations from EH are now the result of subtracting the amount of inhibitors obtained after PR to the total obtained after EH. This way, the value obtained represents the amount of each inhibitor released only during EH, while in previous figures the value represented the total concentration of inhibitor obtained after both PR and EH steps.

PCA plot of variables (Figure 10A) is identical to previous. Three groups are visibly defined, also in the dendrogram: group *V.1*, with oxalic, levulinic and xylitol; group *V.2* with lactic, acetic and formic; and group *V.3*, including succinic, glycerol, ethanol and methanol. The most important change is the arrangement of the samples. Values of physically pretreated samples continue approximately in the same positions, but some values from chemical EH are now mixed in these clusters. Group *S.1* includes samples *A*, *B*, *E* and *F* as before, but now *D-EH* and *L-EH* are also included in this group. As said before, severe chemical PR have sterilization effects in biomass. Inhibitors are released only during PR step, as a result of chemical reactions. During EH there are no remaining bacteria and very low amount of inhibitors is released. Other chemical treatments, such as *K*, *G* of *H*, have medium sterilization effects, and some amounts of inhibitors are generated in EH step. The total amount of inhibitors of these samples is produced in both PR and EH , with no predominant step. These samples appear now in group *S.2*, which contains all samples with moderate concentrations of inhibitors. As before, physical PR with medium/low generation of inhibitors are contained within this cluster. The borders of groups *S.1* and *S.2* are not well defined comparing dendrogram and PCA plot. Some samples can be moved to other group, as concentration of degradation byproducts are similar.

Group *S.3* is the same as previous figures. Samples with high content of methanol and ethanol (group *V.3*) are included in this cluster. Samples from strong chemical pretreatments are placed at positive values of PC1. These samples appear at the end of the dendrogram. Two groups are formed: *D* and *H*, more related to group *V.2*, and *K* and *L*, with relation to group *V.1* of inhibitors. In PCA these groups are not well-defined; distance between *D* and *H* is high, and *K* is closer to group *S.2*. This figure highlights the effect of enzymatic hydrolysis in the release of degradation byproducts. Pretreatments with sterilization effects did not increase the concentration of inhibitors during EH, confirming that degradation is caused by metabolism of bacteria present in biomass. Relation between alkali pretreatment and release of acetic, lactic and formic acids is also confirmed. Alike, levulinic, oxalic and xylitol are related with acid treatments.

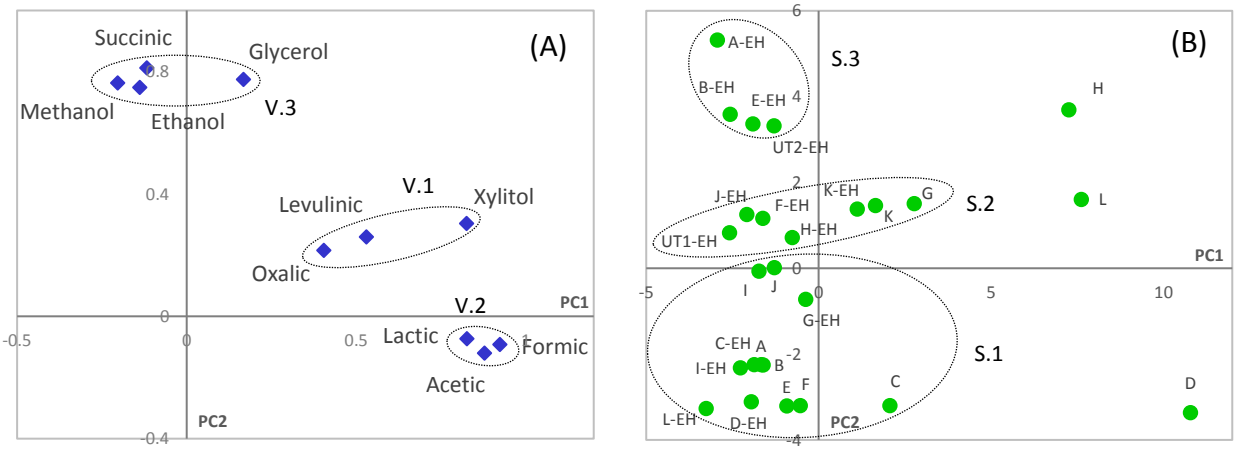
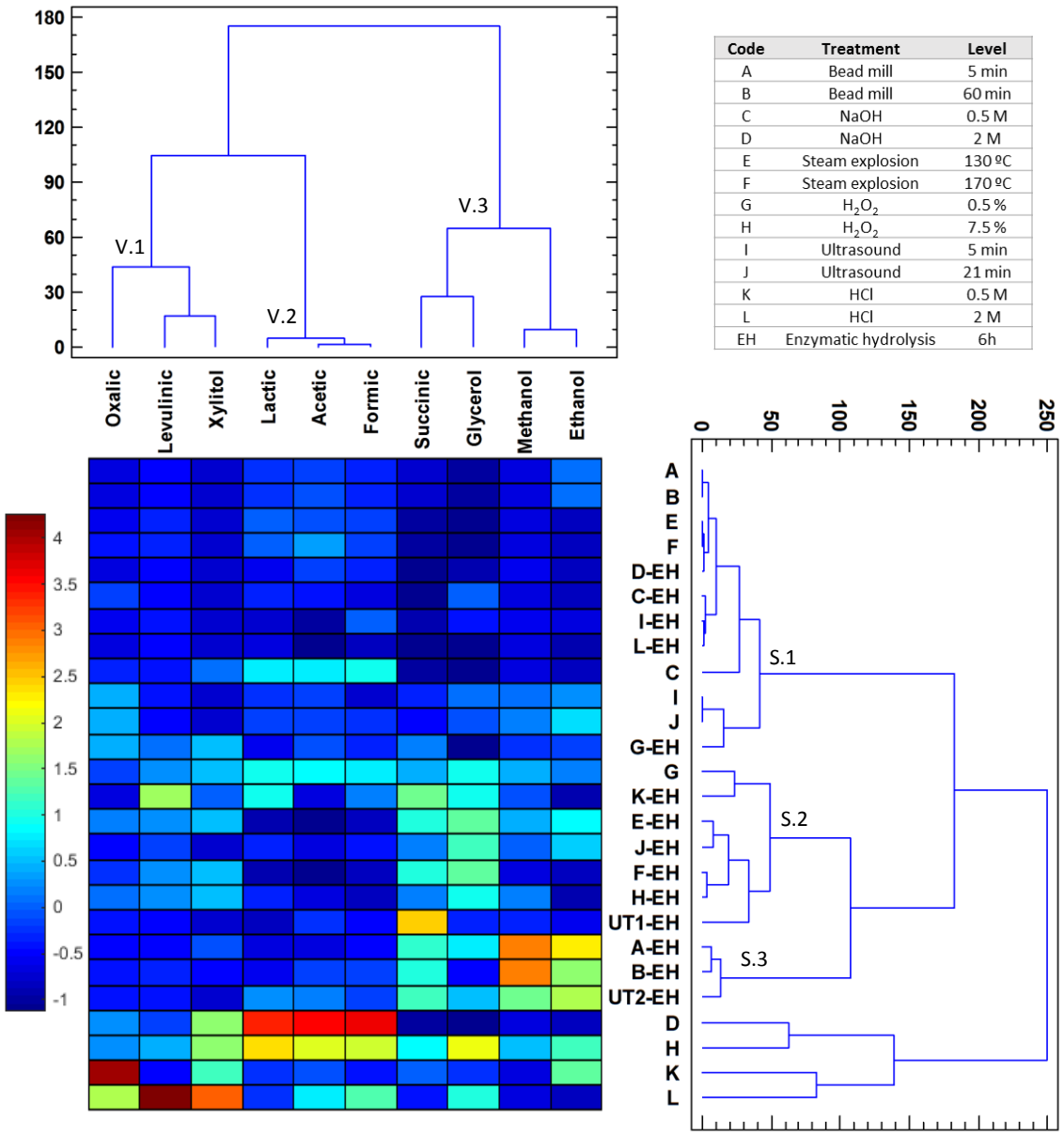


Figure 10. PCA plot of PR and EH, when inhibitors from PR are subtracted to the total concentration of PR+EH, with: (A) Variables; (B) Samples.



Code	Treatment	Level
A	Bead mill	5 min
B	Bead mill	60 min
C	NaOH	0.5 M
D	NaOH	2 M
E	Steam explosion	130 ºC
F	Steam explosion	170 ºC
G	H_2O_2	0.5 %
H	H_2O_2	7.5 %
I	Ultrasound	5 min
J	Ultrasound	21 min
K	HCl	0.5 M
L	HCl	2 M
EH	Enzymatic hydrolysis	6h

Figure 11. Dendrograms of inhibitory byproducts produced in PR and EH separately, obtained by HCA.

5.2 Pure materials

After statistical treatment of data of inhibitors obtained from different PR and the posterior EH step, experiments with pure materials of carbohydrates, proteins and lipids were carried out. These materials were chosen as they are the main components of microalgae biomass, and can serve as reference to elucidate which material is the source of each inhibitor, and the influence of different pretreatments on each material. Only few pretreatments were used for these experiments. Acid, alkali and bead mill were selected as they represent the maximum solubilization and minimum inhibitory byproduct generation. After pretreatment, enzymatic hydrolysis step was performed for 6 hours as before. In the following figures, data for pure materials will be labeled as A, B and C, standing for pure lipids (sunflower oil), pure proteins (gelatin) and pure carbohydrates (cellulose), respectively. Pretreatments will be named as M for bead mill, 0.5NaOH and 2NaOH for 0.5 M and 2 M sodium hydroxide treatments, and 0.5HCl and 2HCl for 0.5 M and 2 M hydrochloric acid treatments.

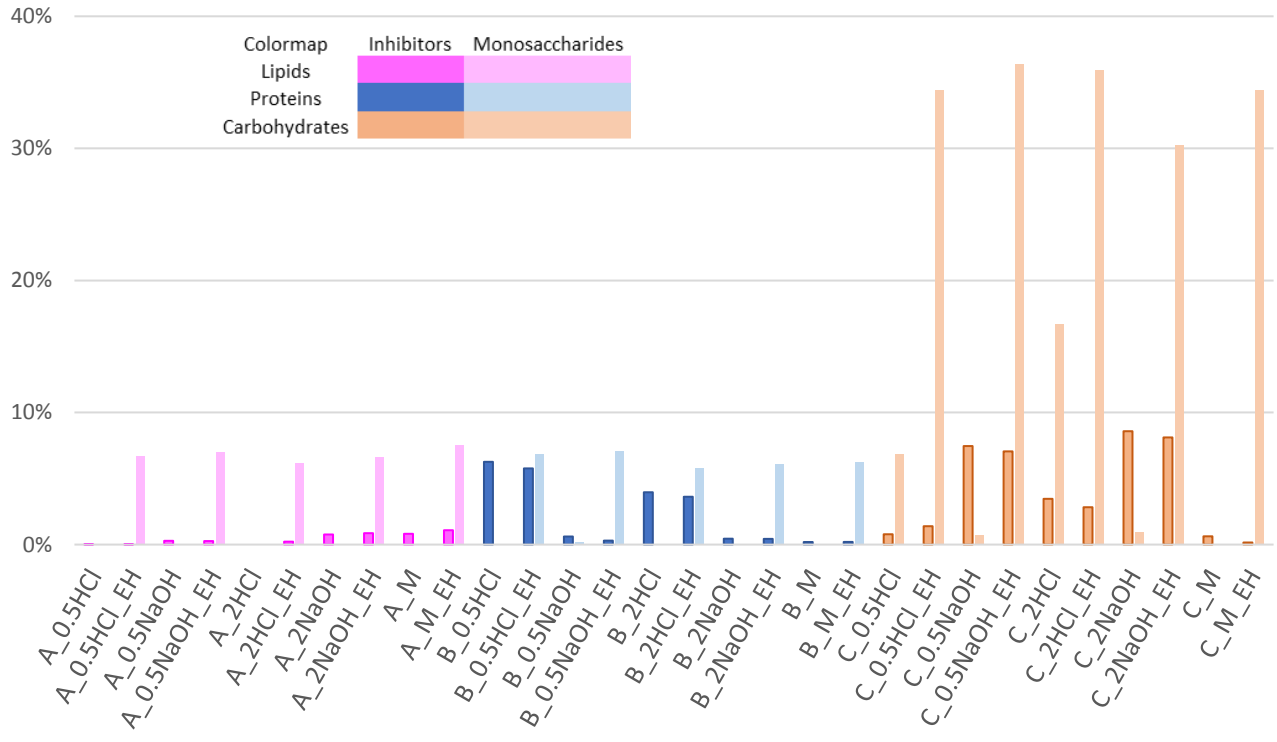


Figure 12. Total concentration of inhibitory byproducts and monosaccharides (% w/w) produced in PR and EH of: (A) sunflower oil, (B) edible gelatin sheets and (C) commercial cotton.

Figure 12 reveals that, for carbohydrates, inhibitory byproducts are mostly produced by alkali treatments, which released high concentrations of formic and lactic acids and xylitol. In the same way, proteins are degraded by acid treatments, producing methanol and acetone. Acid treatment for carbohydrates resulted in low-medium degradation, while solubilization was very good. Similarly, proteins were well solubilized by alkali, obtaining low degradation. This is in concordance with data of microalgae biomass, where it was concluded that acid pretreatments solubilize preferentially carbohydrates, alkali pretreatments were selective for proteins and lipids have low to medium solubilization for both agents. Inhibitors obtained in enzymatic hydrolysis differ from those released from biomass, likely because pure materials do not have bacteria or microorganisms which could contribute to further degradation as biomass. Concentrations obtained from enzymatic hydrolysis of pure materials are similar or even lower than those obtained from pretreatment steps. Concentrations after EH treatment from microalgal biomass were significantly higher, suggesting that a degradative action of bacteria metabolism during EH step takes place, especially in physically pretreated samples.

Figure 12 also represents the amount of monosaccharides recovered from each experiment. Sunflower oil and gelatin yielded insignificant amounts of monosaccharides after PR step, but increased to nearly 8% in EH step. Cellulose obtained moderate concentration of monosaccharides after acid PR, and lower amounts after alkali and bead mill PR. After applying EH, the amount of monosaccharides clearly increases for all samples, indicating the well performance of the enzyme. The increase is especially important after bead mill PR. Using microalgal biomass the amount of inhibitors after EH was much higher. This effect visibly confirms the degradation action of bacteria metabolism present in biomass.

Inhibitors and pretreatments will be evaluated for each material by PCA and HCA. First, PCA plot for inhibitors (variables) will be analyzed. Then, PCA plot for samples will be studied, divided in three categories: classified by type of pretreatment, by intensity of pretreatment and by step. Type of pretreatment includes acid, alkali and bead mill, as mentioned before. Intensity of pretreatment consists on soft pretreatments (0.5 M), severe pretreatments (2 M) and bead mill, which was performed only at one level. Classification by step refers to pretreatment or enzymatic hydrolysis data. This classification will help to deeper understand the behavior of pure materials in each experiment.

Concentrations of inhibitors released in PR and EH assays of each material are shown in Table III of *Supplementary Materials* section. Solubilization of total monosaccharides and total inhibitors is summarized in Table IV.

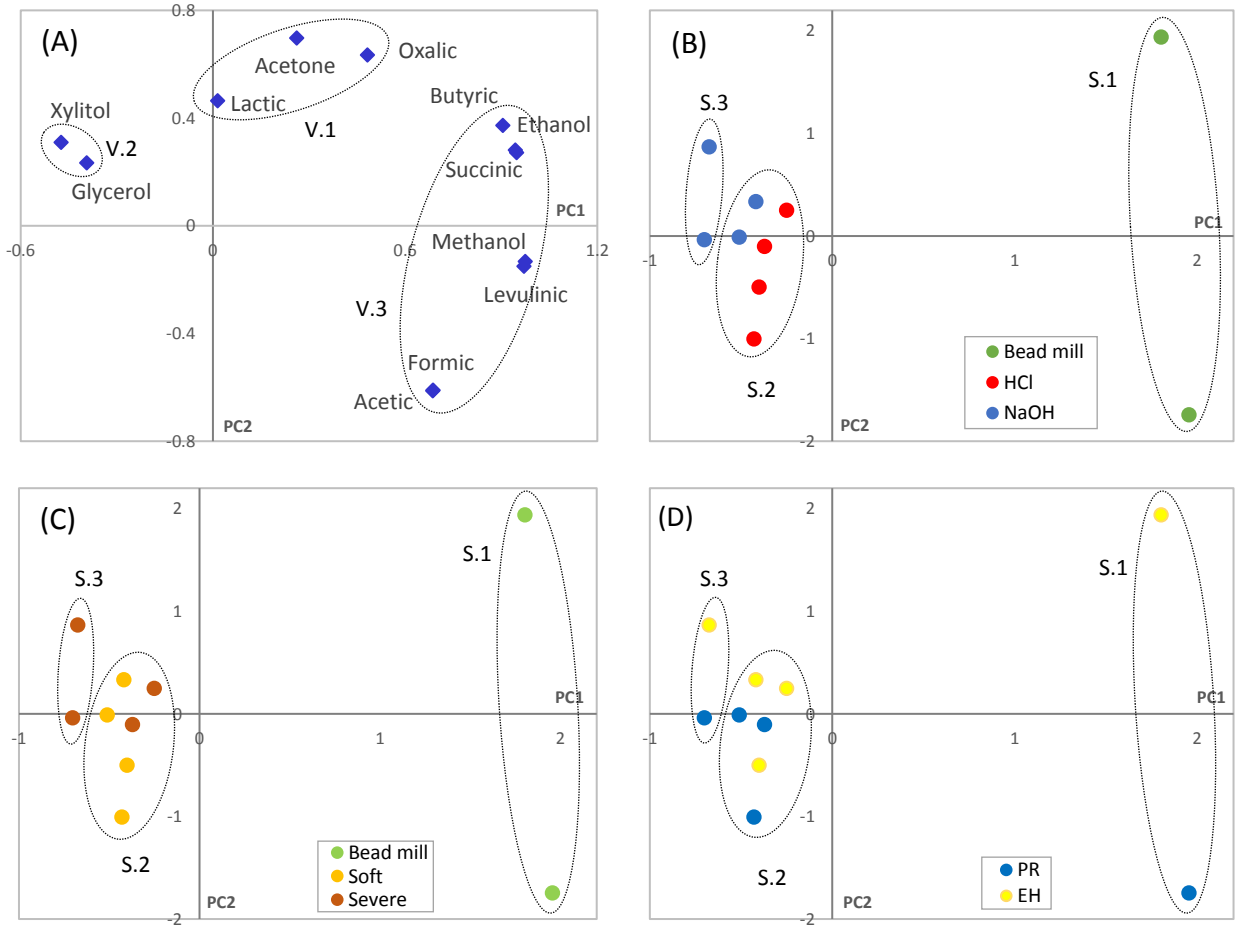
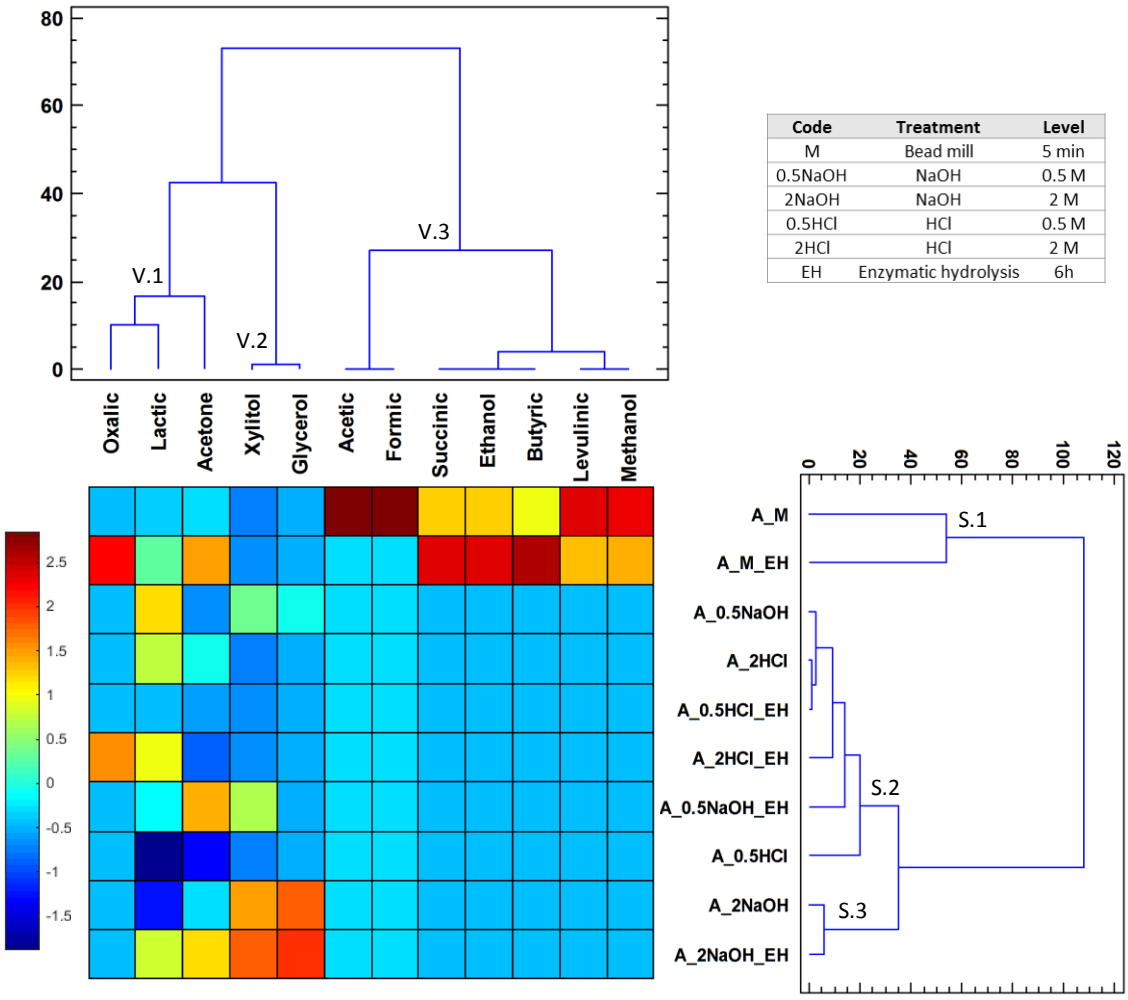


Figure 13. PCA plot of inhibitors produced in PR and EH of sunflower oil, with: (A) Variables; (B) Samples classified by type of PR; (C) Samples classified by intensity of PR; (D) Samples classified by PR or EH.

The first material analyzed was sunflower oil. PCA plot of variables (Figure 13A) divides inhibitors in PC1 and PC2. Xylitol and glycerol (group *V.2*) are clustered in negative PC1 values, as they are mostly produced by severe NaOH pretreatment (group *S.3*), while other inhibitors are placed in positive PC1 value. These inhibitors can be separated in two groups: *V.1* and *V.3*. Group *V.1* is formed by acetone, oxalic and lactic acids, with medium-low concentrations in all samples. Because of this, it is not related specifically with any group of samples. The other inhibitors constitute group *V.3*, and are strongly related with bead mill treatments, grouped as *S.1*, which can be found at positive PC1 values in PCA plots of samples, quite far from groups *S.2* and *S.3* (Figure 13B, C and D). Relative concentrations of inhibitors from group *S.1* are visibly over the average in bead

mill treatments. Last cluster is formed by the rest of inhibitors (group *V.3*). Ethanol, butyric and levulinic acids are more related to bead mill EH, while acetic and formic are found essentially in bead mill PR. Group S.2 includes the rest of the samples (acid and soft alkali pretreatments), which released low total concentration of inhibitors, though some of them appear in medium concentration (e.g. lactic acid in soft alkali PR).



Code	Treatment	Level
M	Bead mill	5 min
0.5NaOH	NaOH	0.5 M
2NaOH	NaOH	2 M
0.5HCl	HCl	0.5 M
2HCl	HCl	2 M
EH	Enzymatic hydrolysis	6h

Figure 14. Dendrograms of inhibitory byproducts produced in PR and EH of sunflower oil, obtained by HCA.

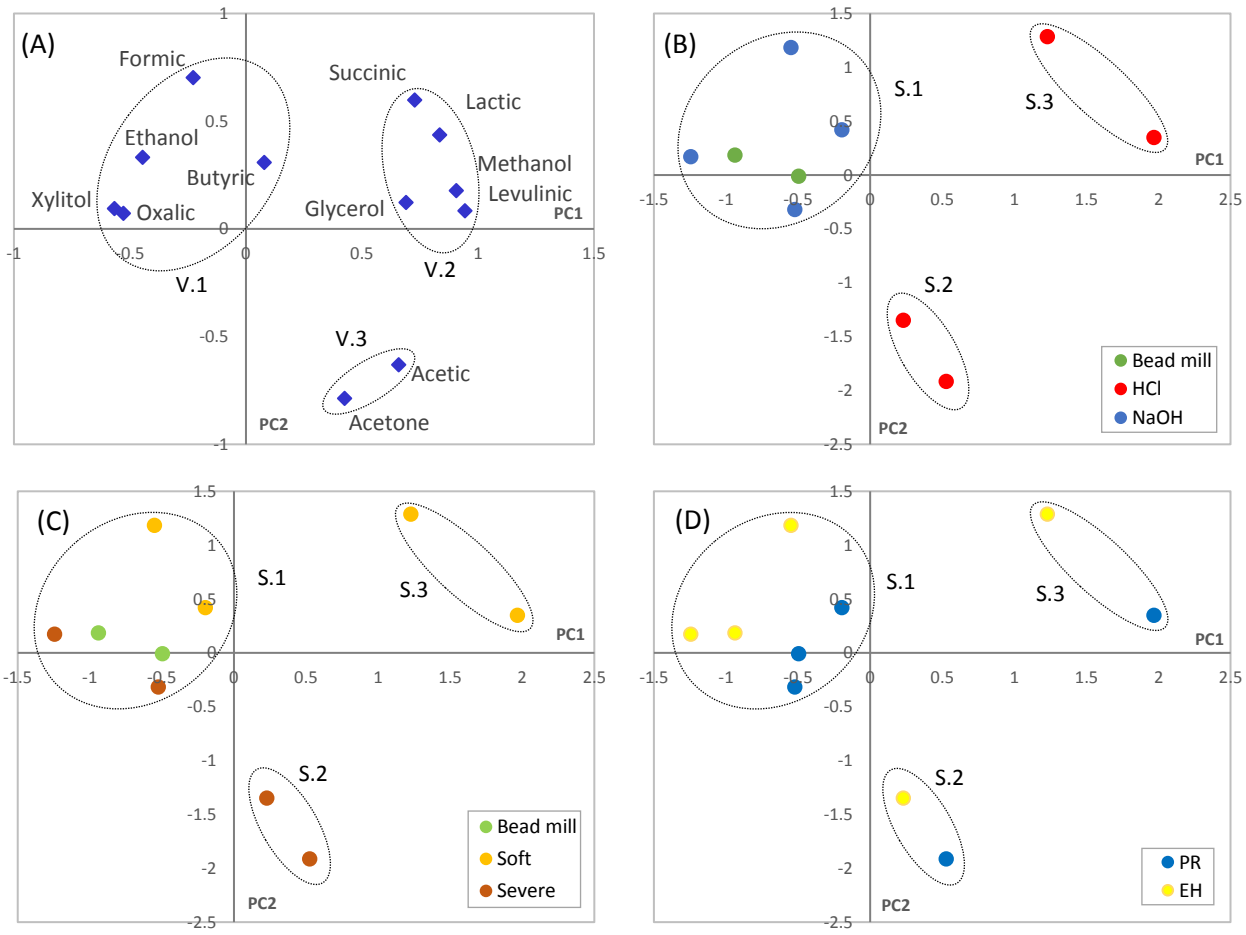
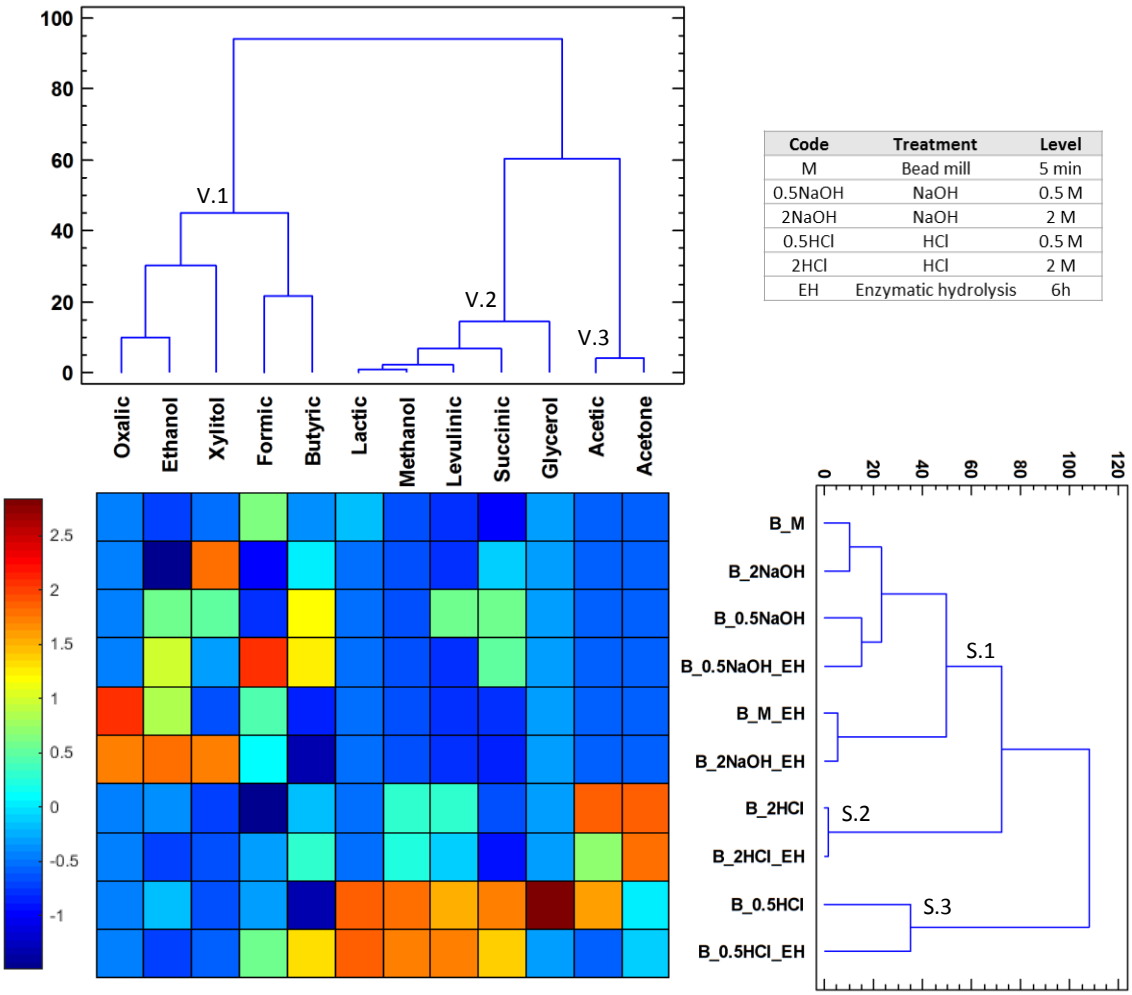


Figure 15. PCA plot of inhibitors produced in PR and EH of edible gelatin sheets, with: (A) Variables; (B) Samples classified by type of PR; (C) Samples classified by intensity of PR; (D) Samples classified by PR or EH.

The second material analyzed was edible gelatin sheets obtained from pork, with nearly 90% of protein content. As seen in Figure 12, proteins are degraded by acids, while solubilization is achieved by alkali treatment. Heatmap shows high relative concentration of inhibitors for acid treatments, while only EH of 2 M NaOH treatment obtained high concentration of inhibitors.

PCA plot for inhibitors (Figure 15A) shows good separation in both PC1 and PC2. Group *V.2* is placed in the first quadrant, and includes inhibitors formed preferentially in 0.5 M HCl PR (*S.3*). Group *V.3*, in the fourth quadrant, is composed by only acetone and acetic acid, related with 2 M HCl treatments (*S.2*). Other treatments are included in group *S.1*, with negative PC2 values in PCA plots. This big aggregate is related to group *V.1* of inhibitors, which are produced essentially in alkali and bead mill treatments and appear in relatively low concentrations in acid treatments.



Code	Treatment	Level
M	Bead mill	5 min
0.5NaOH	NaOH	0.5 M
2NaOH	NaOH	2 M
0.5HCl	HCl	0.5 M
2HCl	HCl	2 M
EH	Enzymatic hydrolysis	6h

Figure 16. Dendrograms of inhibitory byproducts produced in PR and EH of edible gelatin sheets, obtained by HCA.

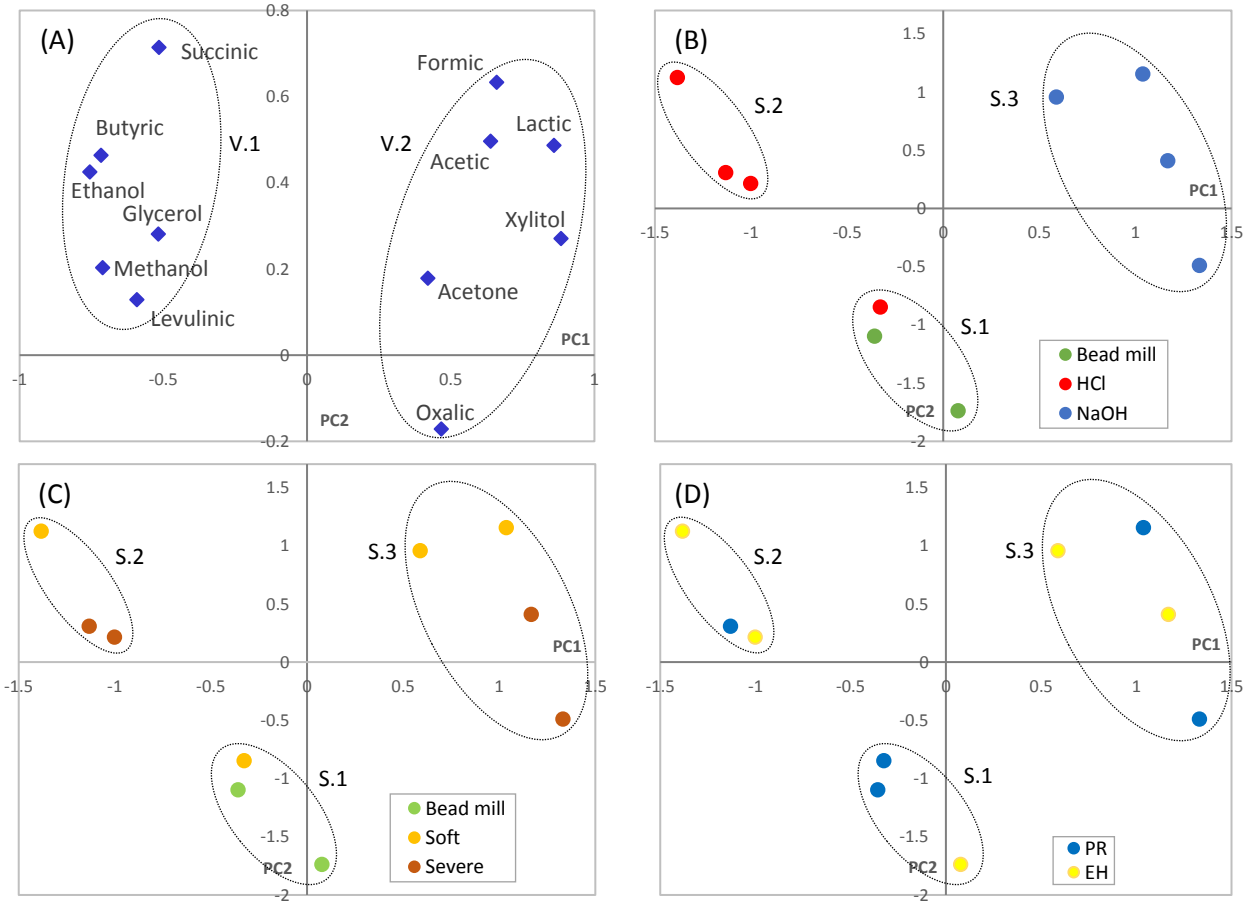
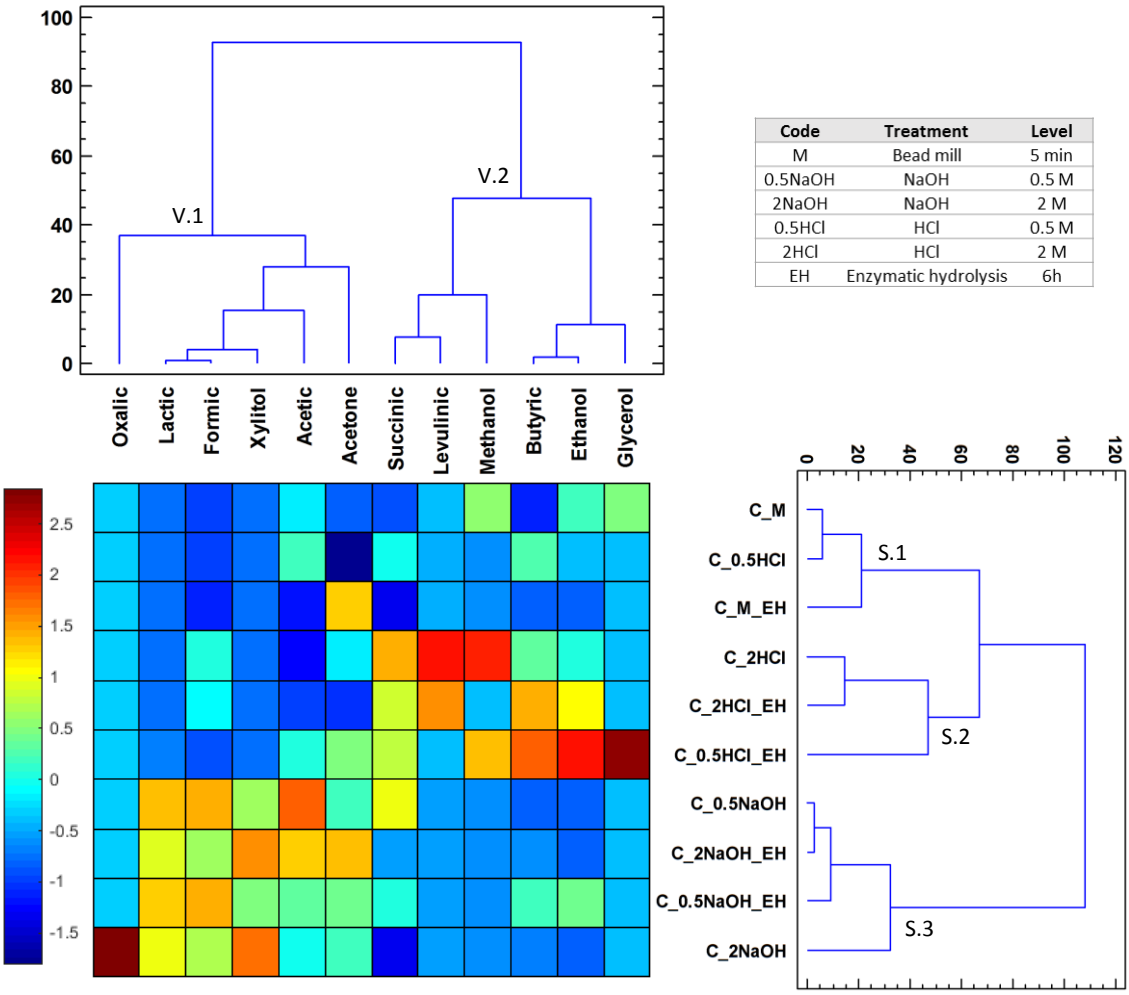


Figure 17. PCA plot of inhibitors produced in PR and EH of commercial cotton, with: (A) Variables; (B) Samples classified by type of PR; (C) Samples classified by intensity of PR; (D) Samples classified by PR or EH.

PCA plot of inhibitors from commercial cotton (Figure 17A) shows two clear groups of variables, separated in positive and negative values of PC1. Comparing plots of variables and samples (Figure 17A and B) it is possible to see a clear relation between the pretreatment applied and the inhibitors detected. Alkali treatments, in group *S.3* of samples and at positive PC1 values, produced essentially oxalic, acetic, lactic and formic acids, acetone and xylitol (group *V.2* of inhibitors). Oxalic acid could be excluded from this cluster, as it only appears in 2 M NaOH PR. On the other side, acid treatments (group *S.2*) produced more succinic, levulinic and butyric acids, methanol, ethanol and glycerol, which constitute group *V.1* of inhibitors, and are placed at negative PC1 values. This two big clusters can be clearly seen in dendrogram of variables. Bead mill pretreated samples resulted in low degradation of the material, so they appear in the middle of both groups, slightly more related with group of acid pretreatments. Soft acid PR is clustered with bead mill samples in group *S.1*, due to low degradation. Value of bead mill EH is shifted near to values of alkali treatments as acetone concentration is above the average.

In plot C, classified by intensity of PR, strong acid pretreatments obtained similar values (value for PR and value for EH), indicating that inhibitors are mostly produced in PR step and EH action does not contribute. However, in plot D, values for soft acid pretreatments are separated. The distance between PR and EH values is caused by an increase in concentration of degradation byproducts. Soft acid pretreatment is not able to totally solubilize the sample, so enzyme action is necessary. Some amounts of cellulose could be wrongly transformed into inhibitors during EH, explaining this increase. These conclusions are in close agreement with PCA plots analyzed for microalgal biomass. Alike algal biomass, alkali treatments released preferentially lactic, acetic and formic acids, among other compounds.



Code	Treatment	Level
M	Bead mill	5 min
0.5NaOH	NaOH	0.5 M
2NaOH	NaOH	2 M
0.5HCl	HCl	0.5 M
2HCl	HCl	2 M
EH	Enzymatic hydrolysis	6h

Figure 18. Dendrograms of inhibitory byproducts produced in PR and EH of commercial cotton, obtained by HCA.

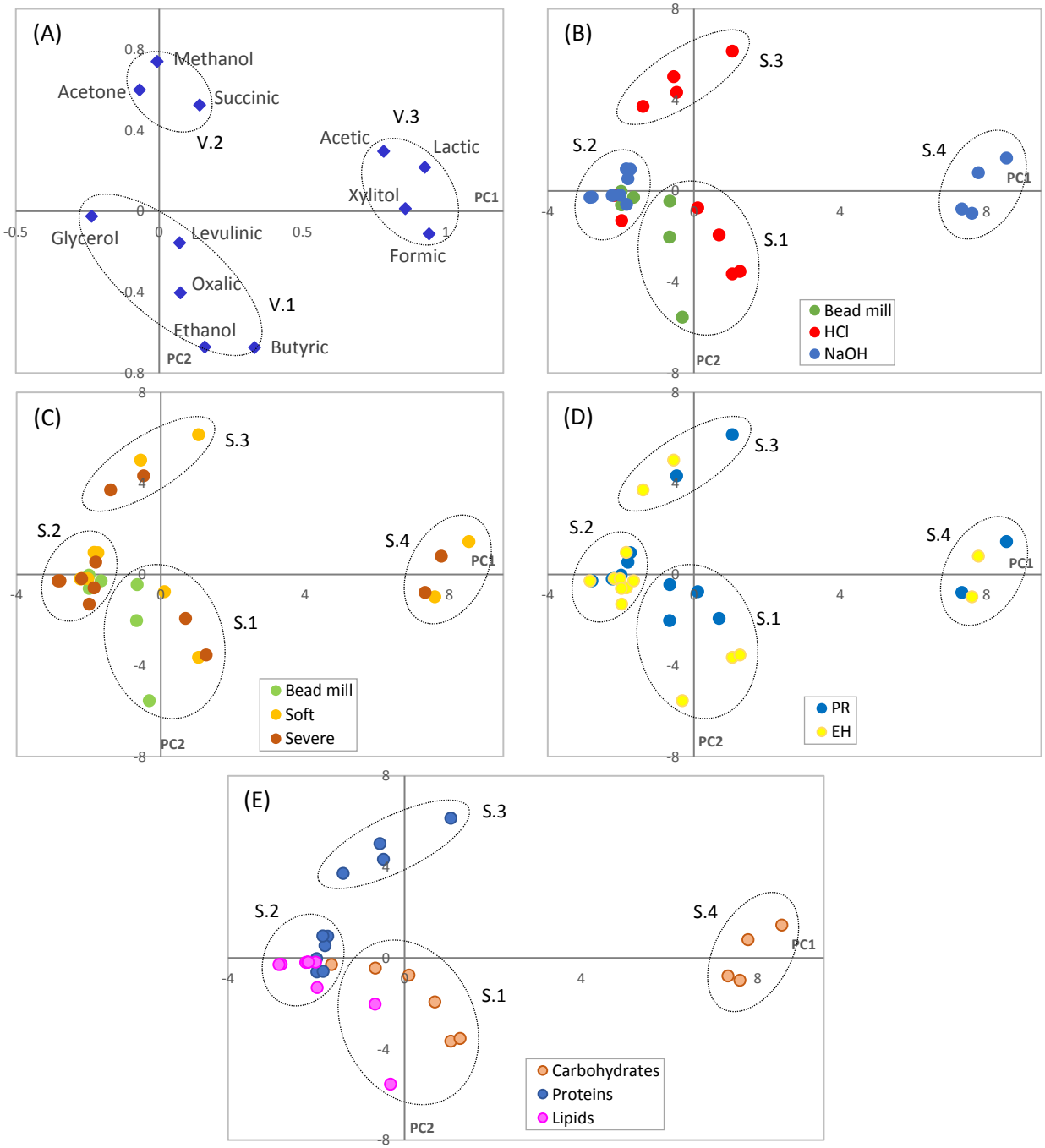
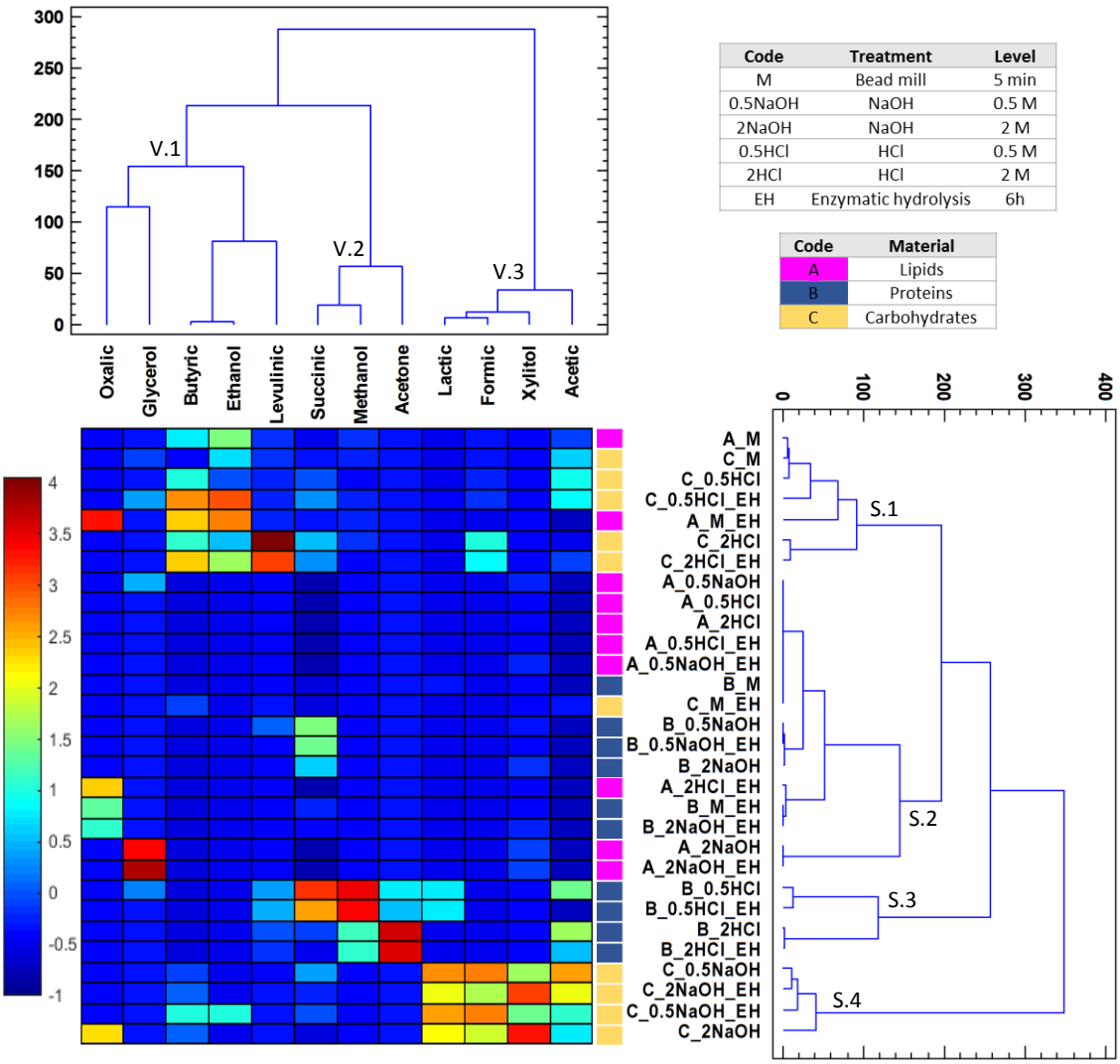


Figure 19. PCA plot of inhibitors produced in PR and EH of pure materials of lipids, proteins and carbohydrates, with: (A) Variables; (B) Samples classified by type of PR; (C) Samples classified by intensity of PR; (D) Samples classified by PR or EH; (E) Samples classified by material.

After analysis of each material separately, data of inhibitors from lipids, proteins and carbohydrates were combined to perform PCA and HCA. Results and relations between inhibitors produced by each material were studied, again using classification by type of PR, by intensity of PR, by PR or EH and by material. Amount of data is much higher now, so relations are easier to understand using HCA combined with heatmap representation.



Code	Treatment	Level
M	Bead mill	5 min
0.5NaOH	NaOH	0.5 M
2NaOH	NaOH	2 M
0.5HCl	HCl	0.5 M
2HCl	HCl	2 M
EH	Enzymatic hydrolysis	6h

Code	Material
A	Lipids
B	Proteins
C	Carbohydrates

Figure 20. Dendrograms of inhibitory byproducts produced in PR and EH of commercial cotton, obtained by HCA.

Clusters obtained are labeled in PCA plots and HCA, using *V* for variables (inhibitors) and *S* for samples (treatments). PCA plot of variables (Figure 19A) divides inhibitors in three groups. This division can be understood by observing the dendrograms of inhibitors and samples (Figure 20). Group *V.3*, formed by xylitol and acetic, lactic and formic acids, is related to NaOH pretreatments of carbohydrates. As said before, these are the most degrading treatments for cellulose, and acetic, formic and lactic acids are released in relatively high concentrations. This effect was also appreciated in microalgal biomass. In PCA these values are grouped far from the rest (group *S.4*), with high positive values in PC1, in the same zone than the cluster formed by inhibitors of last group in HCA.

Other inhibitors have similar PC1 values, as seen in Figure 19A, but separation is achieved along PC2. At positive values of PC2 a group (*V.2*) is formed by acetone, methanol and succinic acid. This cluster is related to HCl treatments of proteins (group *S.3*), which resulted in the greatest release of inhibitors for this material (Figure 12). In PCA, these samples are located at high PC2 positive values near PC2 axe (Figures 19B, C, D and E), according with position of the group of inhibitors in plot A.

Last group of inhibitors, *V.1*, is less defined than previous, formed by the rest of inhibitors at negative values of PC2 in plot A. There is a big group of values in the center of PCA plots of samples. This big cluster can be divided in two parts: one narrow cluster with more negative PC1 values (group *S.2*), while the second cluster is shifted to more positive PC1 values and negative PC2 values (group *S.1*). Samples corresponding to group *S.1* obtained moderate amounts of inhibitory byproducts, and are associated with inhibitors of group *V.1*. It is formed by five carbohydrate samples and two lipid samples (those of bead mill) as seen in plots B, C, D and E.

Finally, last cluster contains all samples with low concentration of inhibitors. In the dendrogram of samples, this big cluster appears in the center, and includes samples from all the materials (group *S.2*). Two samples can be differentiated from the group, shifted to negative PC2 values. They correspond to 2 M NaOH treatments (PR and EH) of lipids, which obtained relatively high concentrations of glycerol, related with the position of this inhibitor in plot A. These two samples are located last in their group of the dendrogram, with clear separation from other samples in the group.

5.3 Comparison of biomass and pure materials

Joint interpretation by PCA and HCA was performed to samples from biomass and from pure materials to elucidate the origin and relation between the released inhibitors and the treatments carried out. Some changes were made to be able to perform the statistical analysis and to simplify the study. Some inhibitors (butyric acid and acetone) were not detected in microalgal biomass and were eliminated before statistical treatment. As before, citric acid was eliminated from the study because buffer containing citric acid is added before EH. Only samples of 5-minutes bead mill, acid and alkali treatments from biomass have been used for the study, as these are the treatments performed in assays with pure materials. Results from 6-hour EH of untreated microalgal biomass have been included.

A first comparative study includes samples from PR of pure materials and algal biomass, and results of PCA and HCA are presented in Figures 21 and 22. Microalgal biomass samples differ notably from the rest, as higher concentration of inhibitors was usually obtained. Cellulose is the material behaving more similarly to biomass, as values usually appear in the same groups. In PCA plots for samples, three groups can be separated: group *S.1* with all samples from lipids and proteins, and also some from carbohydrates; group *S.2* with samples from carbohydrates and biomass; and group *S.3* with only acid treatments of microalgal biomass. Groups of samples achieved well-defined separation in PC1 and PC2 axes.

Group *S.1* included some samples slightly separated from the rest. These values can be easily identified in the dendrogram of samples; they are the last five samples of group *S.1*. This effect is explained by concentrations of some inhibitors above the average, but not high enough to belong to other cluster. Value of *Alg_0.5NaOH* is shifted towards group *S.2*, as it contains same inhibitors but in lower concentrations. Group *S.2* is related to group *V.2* of inhibitors, which appears in the fourth quadrant of PCA plot of variables. The samples in group *S.3* are located in the first quadrant, and correspond to acid treatments of biomass. These samples are related to group *V.1* of inhibitors.

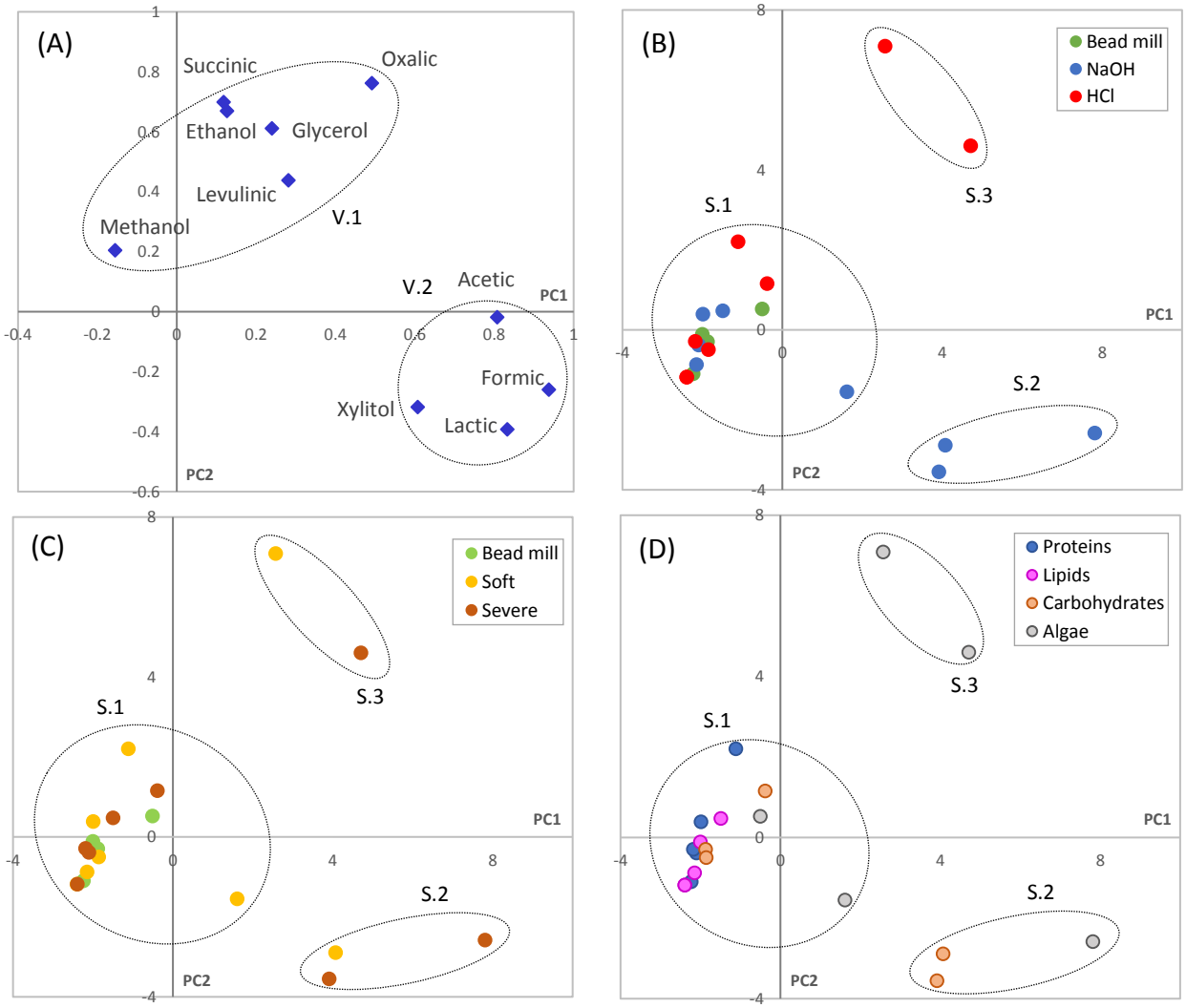
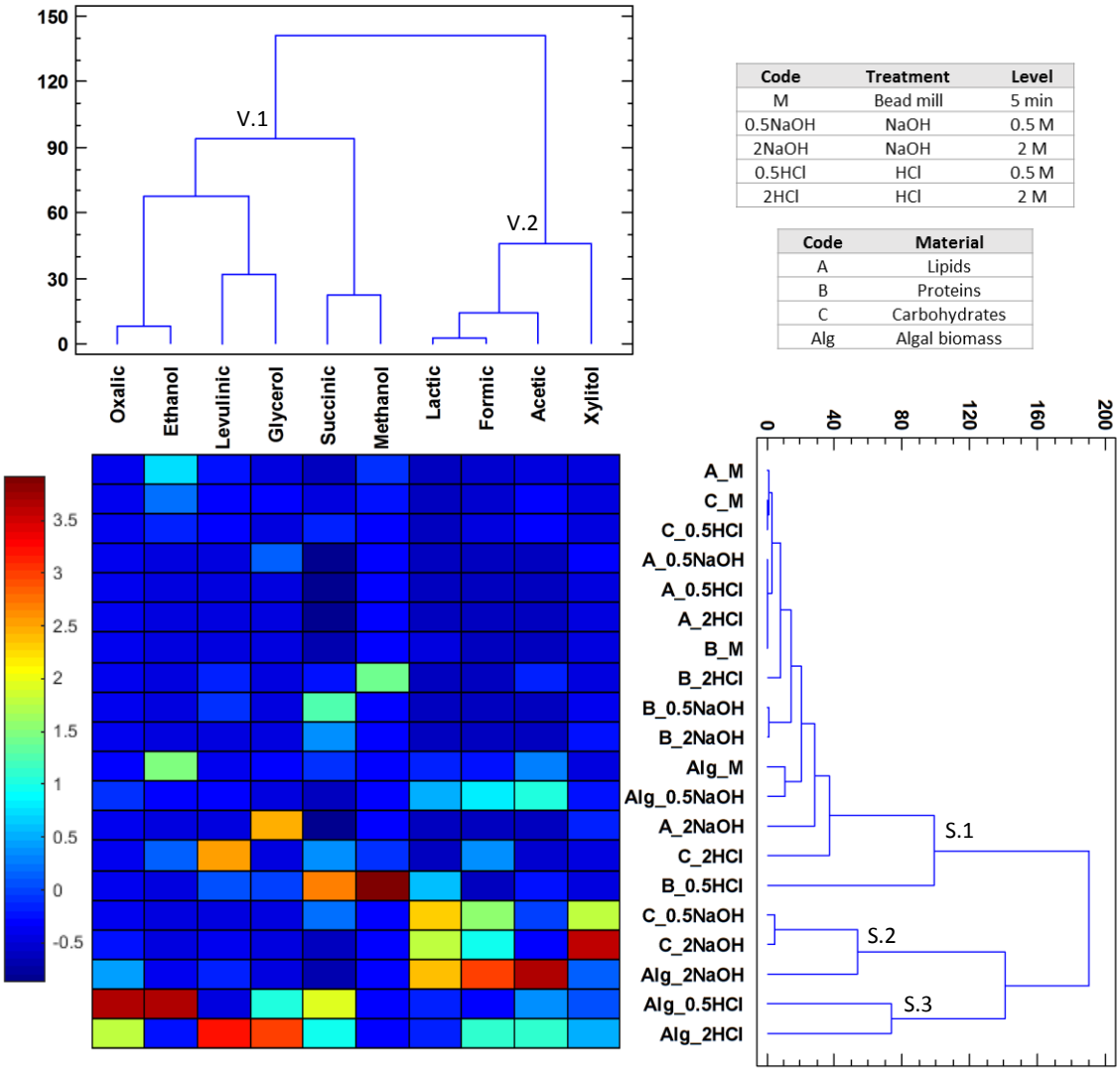


Figure 21. PCA plot of inhibitors produced in PR of algal biomass and pure materials of lipids, proteins and carbohydrates, with: (A) Variables; (B) Samples classified by type of PR; (C) Samples classified by intensity of PR; (D) Samples classified by intensity of PR; (D) Samples classified by material.

Groups obtained in PCA and HCA from values of EH are slightly different. The effect of untreated samples divides the biggest group in two: group *S.1*, with negative PC1 values; and group *S.2*, shifted to more positive PC1 values and including untreated samples of biomass. This group is related to group *V.2* of inhibitors. Methanol is near PC2 axe due to its anomalous high concentration in sample *B_0.5HCl_EH*, which is located in the left extreme of the group. In the dendrogram, it is possible to see that this sample is a little disconnected from biomass samples. Group *S.1* contains all samples with low concentration of inhibitor byproducts, clustering samples from pure materials. The position of this group of samples does not correspond with any group of variables because of the low concentration of inhibitors obtained.



Code	Treatment	Level
M	Bead mill	5 min
0.5NaOH	NaOH	0.5 M
2NaOH	NaOH	2 M
0.5HCl	HCl	0.5 M
2HCl	HCl	2 M

Code	Material
A	Lipids
B	Proteins
C	Carbohydrates
Alg	Algal biomass

Figure 22. Dendrograms of inhibitory byproducts produced in PR of algal biomass and pure materials of lipids, proteins and carbohydrates, obtained by HCA.

Group *S.3* of samples is placed in the fourth quadrant, as it is composed by alkali treatments of biomass and carbohydrates These treatments released mostly lactic, acetic and formic acids, and xylitol in the case of carbohydrates (group *V.3* of inhibitors). Value of 2 M NaOH treatment of biomass is far from the others because the concentration of inhibitors released was significantly higher. Group *S.4* of samples contains only acid treatments of algal biomass, and is placed in the first quadrant at high PC1 values.

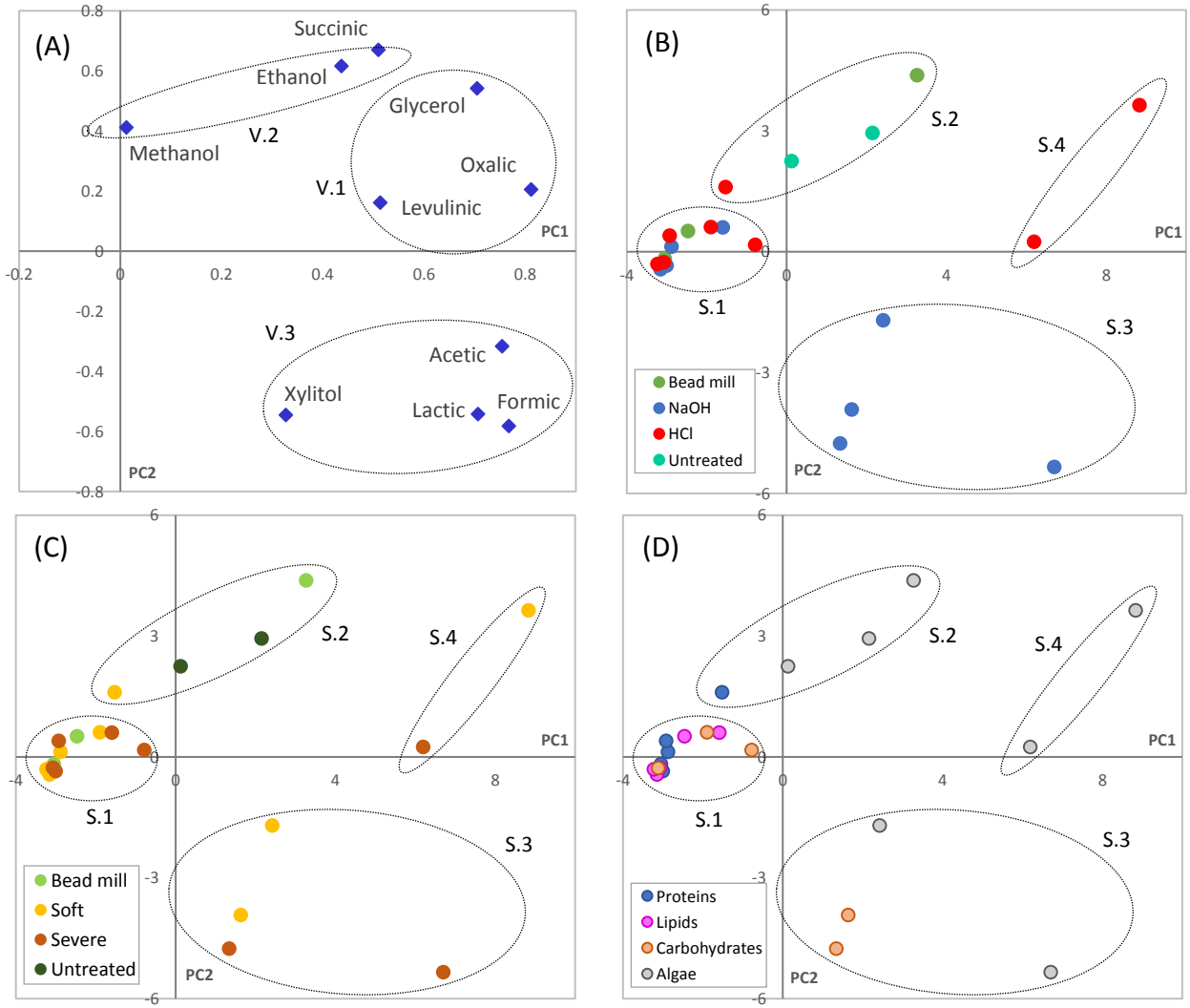
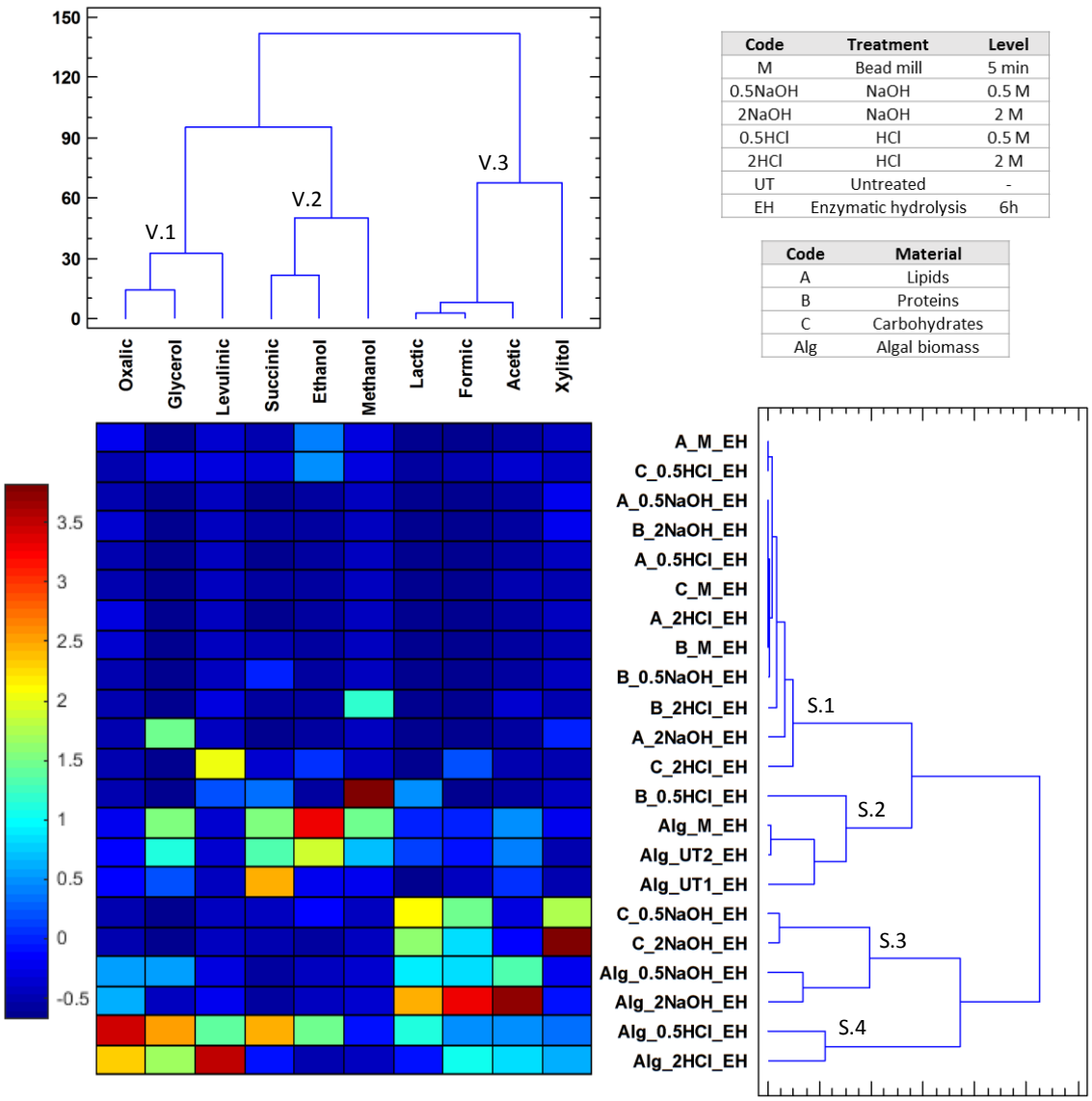


Figure 23. PCA plot of inhibitors produced in EH of algal biomass and pure materials of lipids, proteins and carbohydrates, with: (A) Variables; (B) Samples classified by type of PR; (C) Samples classified by intensity of PR; (D) Samples classified by material.

Inhibitors of group *S.4* (glycerol, oxalic and levulinic acids) are related to acid biomass treatments, and have similar positions in PCA plots (Figure 23). Soft acid treatment also released succinic acid and ethanol, inhibitors that belong to group *V.2*. This is the reason why group *V.2* is placed between *S.2* and *S.4*, in the same way that groups *S.1* and *S.3* in PCA plots of PR (Figure 23).



Code	Treatment	Level
M	Bead mill	5 min
0.5NaOH	NaOH	0.5 M
2NaOH	NaOH	2 M
0.5HCl	HCl	0.5 M
2HCl	HCl	2 M
UT	Untreated	-
EH	Enzymatic hydrolysis	6h

Code	Material
A	Lipids
B	Proteins
C	Carbohydrates
Alg	Algal biomass

Figure 24. Dendrograms of inhibitory byproducts produced in PR of algal biomass and pure materials of lipids, proteins and carbohydrates, obtained by HCA.

Separation of groups can be clearly seen in dendrograms. Shape of dendrograms is very similar to that of PR (Figure 22). The group *S.1* contains half of the samples, all with relatively low concentrations of inhibitors (dark blue). The group *S.2* includes untreated and bead mill samples of biomass. This confirms again that bead mill PR is not degrading but also is not able to sterilize the biomass, so degradation is produced by bacteria metabolism during EH step. Groups *S.3* and *S.4* are separated in the same way as in PCA of PR (Figure 21). Carbohydrate samples in group *S.3* confirm that degradation byproducts in microalgal biomass are generally a result of carbohydrate degradation, especially by alkali treatments.

Figures 25 and 26 shows PCA and HCA results from both PR and EH treatments of pure materials and biomass. A great number of samples are now analyzed, so classification by colors has been included in Figure 26 to ease the understanding of the dendrograms. Color legend is included, and colors are similar to those used before.

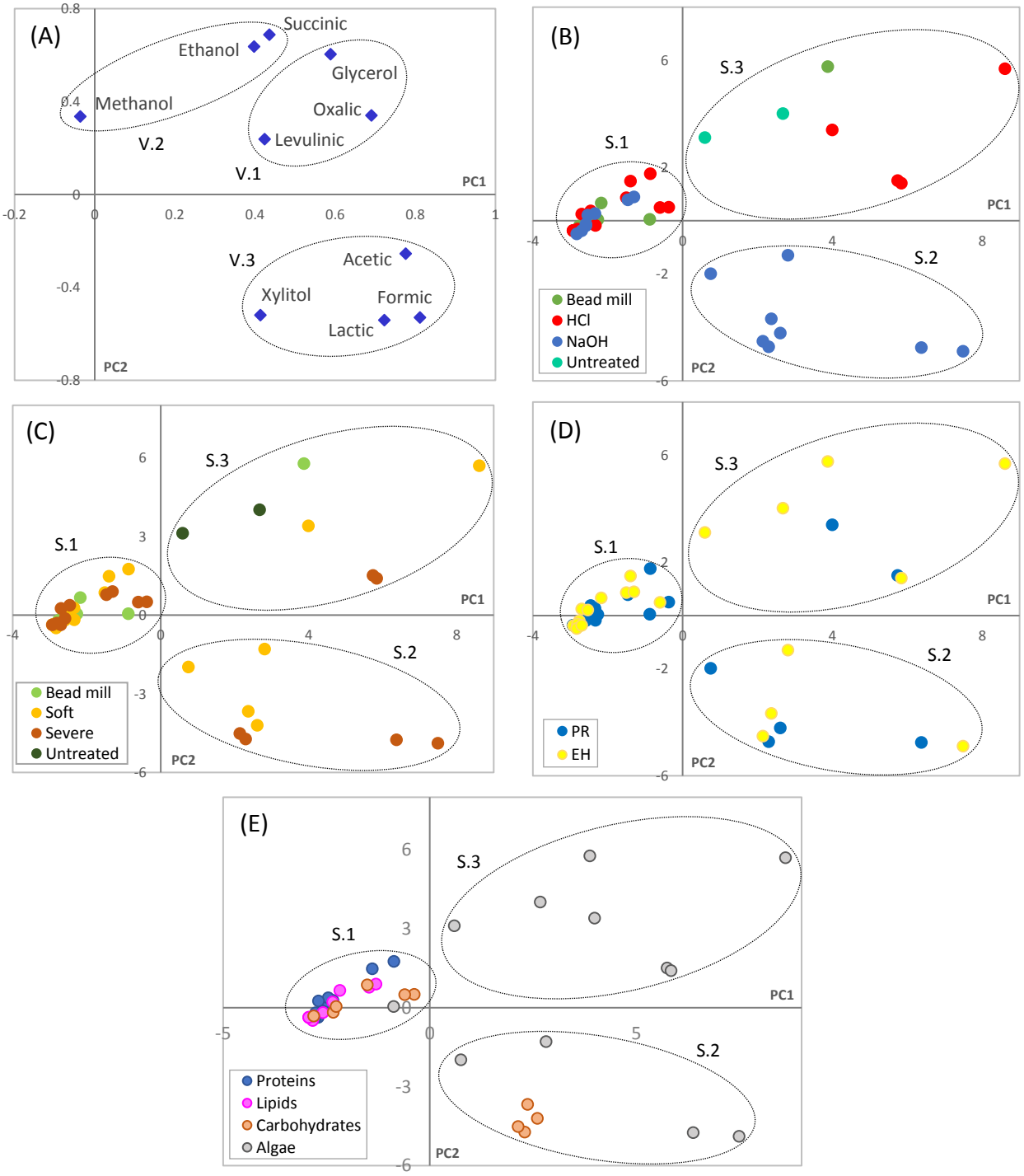


Figure 25. PCA plot of inhibitors produced in PR and EH of algal biomass and pure materials of lipids, proteins and carbohydrates, with: (A) Variables; (B) Samples classified by type of PR; (C) Samples classified by intensity of PR; (D) Samples classified by PR or EH; (E) Samples classified by material.

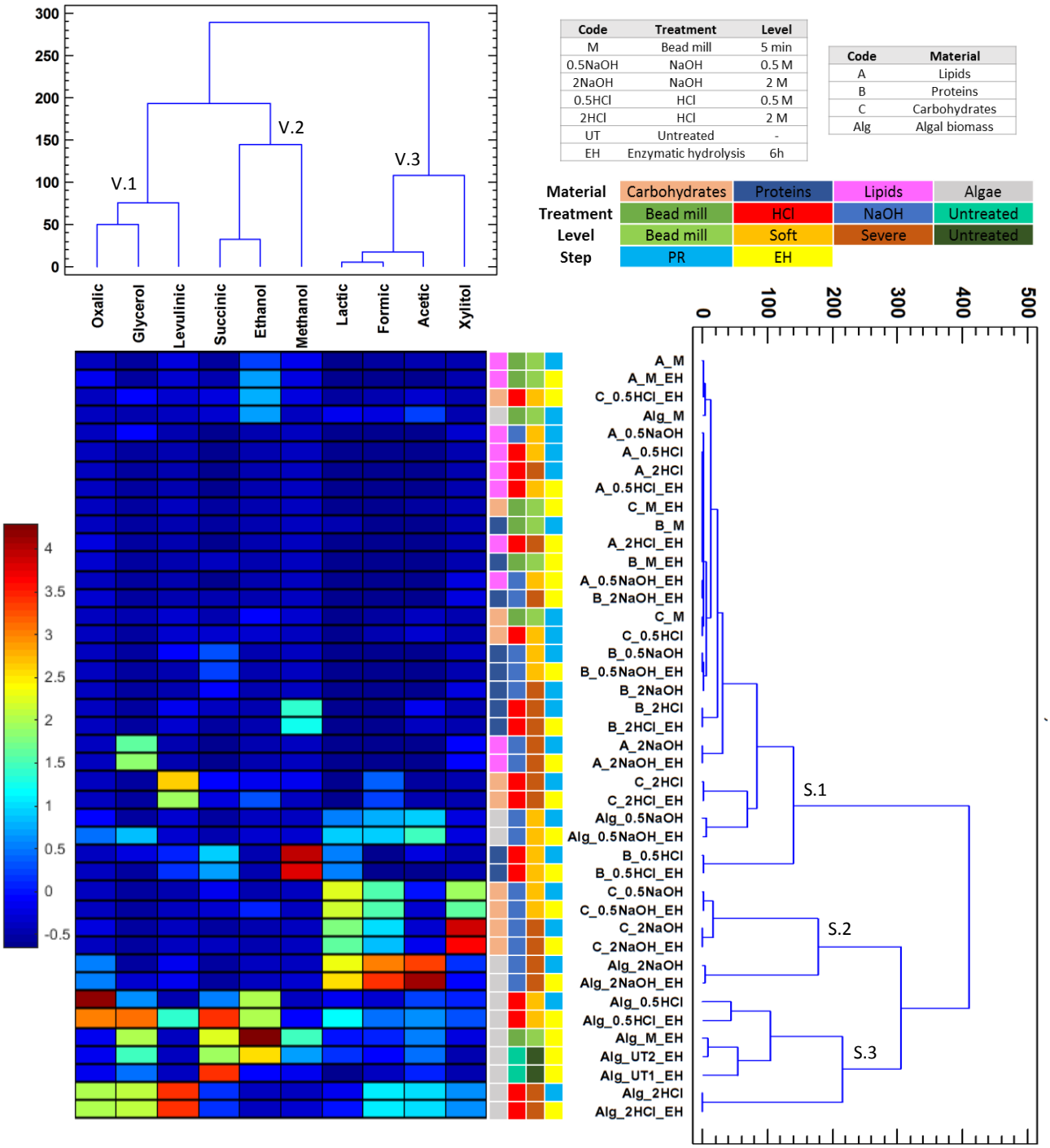


Figure 26. Dendrograms of inhibitory byproducts produced in PR and EH of algal biomass and pure materials of lipids, proteins and carbohydrates, obtained by HCA.

Groups obtained follow the same pattern as before. First, a big compact cluster is formed mainly by samples of pure materials, with very low degradation (group *S.1*). Concentrations in this group are relatively low compared with microalgal biomass. This group is placed at negative PC1 values in PCA plot of samples. In general, samples from PR and from EH are clustered together. Some samples can be highlighted within this

group, as they are less compacted (Figures 26 and 27, respectively). This separation is caused by high concentration of certain inhibitors, but not high enough to be clustered within other group. These inhibitors are located in groups *V.1* and *V.2*, such as glycerol, levulinic and methanol, respectively. These samples are: 2 M NaOH PR and EH of lipids, 2 HCl PR and EH of carbohydrates, 0.5 M HCl PR and EH of proteins and bead mill PR of biomass. Shift of these samples inside group *S.1* is explained by the location of *V.1* and *V.2* at positive PC2 values. Also, samples from 0.5 NaOH PR and EH of biomass are included in this group in dendrogram, but in PCA plot they are assembled in group *S.2* as inhibitors are similar to other alkali treatments.

Group *S.2* contains alkali treated samples of carbohydrates and biomass, and is placed in the fourth quadrant. Group *S.2* is related to group *V.3* of inhibitors, confirming that acetic, formic and lactic acids are obtained from alkali carbohydrate degradation, and also that degradation of carbohydrates has the most important impact in inhibitory byproducts extracted from microalgal biomass. Xylitol is included in this group as is massively released in alkali treatments of carbohydrates.

Group *S.3* is placed in the first quadrant, and contains only biomass samples, including acid and bead mill treatments and also untreated samples. This group of samples has relation with groups *V.1* and *V.2* of inhibitors. Group *V.1* is placed at higher PC1 values, as its inhibitors are produced by acid treatments. Group *V.2* is shifted to lower PC1 values because is more related with untreated and bead mill samples of biomass, which obtained higher concentrations of methanol and ethanol due to bacteria metabolism.

6. Conclusions

Microalgal-bacterial biomass has been proved as a viable source of monosaccharides to improve the economic viability of pig manure treatment by photobioremediation.

Results showed that carbohydrate solubilization is enhanced by acid treatments, while proteins are preferentially released by alkali treatments. On the other hand, acid treatments are more degrading to proteins and alkali treatments damage specifically carbohydrates. Lipids are lowly solubilized and damaged by all treatments. Although high amounts of inhibitory byproducts are released by chemical treatments, acid treatments are preferred because its improved monosaccharide recovery, far from those obtained with other treatments.

After multivariate statistical analysis of the results by PCA and HCA, it is possible to conclude that alkali treatments released essentially lactic, acetic and formic acids in biomass assays. Acid treatments released more inhibitors, oxalic and levulinic acids being the most abundant. Concentration of inhibitors increased with severity of the treatment. Acid and alkali treatments achieved partial or total sterilization of biomass, depending on the severity of the treatment, whereas physical treatments, especially bead mill, obtained low degradation during pretreatment step but also negligible sterilization of microorganisms. Low sterilization resulted in high degradation of released compounds during enzymatic hydrolysis, due to action of bacterial metabolism. Usual byproducts of this metabolism are methanol and ethanol. These compounds are released at much lower concentrations from pure materials as bacteria are not present. Enzymatic hydrolysis was found to affect significantly monosaccharide recovery in all treatments. Only severe acid pretreatment of cellulose achieved monosaccharide recoveries similar to those of EH treatments.

However, when sugar recovery increased, degradation yields also did. No significant differences in monosaccharide recovery were found between 6-hour and 12-hour enzymatic hydrolysis.

Statistical analysis suggested that inhibitory byproducts depend more on the treatment used (pretreatment and enzymatic hydrolysis contributions) than on the composition of the materials treated. Therefore, a careful design of the acidic treatment of biomass must be carried out to assess optimal recovery of monosaccharides.

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8. Supplementary material

Table I. Concentration of inhibitors obtained in pretreatment and enzymatic hydrolysis assays of microalgal-bacterial biomass. Values in % (w/w).

PR	Oxalic	Lactic	Acetic	Succinic	Formic	Levulinic	Xylitol	Glycerol	Methanol	Ethanol
A	0.060	0.440	0.480	0.100	0.320	0.020	0.020	0.020	0.040	0.280
B	0.060	0.460	0.500	0.100	0.320	0.020	0.020	0.020	0.040	0.280
C	0.440	1.060	0.940	0.028	1.060	0.080	0.280	0.002	0.024	0.018
D	1.140	2.720	2.400	0.024	2.720	0.200	0.740	0.000	0.012	0.016
E	0.160	0.600	0.520	0.020	0.420	0.120	0.020	0.016	0.010	0.022
F	0.360	0.600	0.720	0.038	0.420	0.120	0.020	0.008	0.008	0.018
G	0.600	1.200	1.000	0.500	1.000	0.400	0.400	0.400	0.500	0.300
H	1.100	2.120	1.600	0.620	1.720	0.500	0.740	0.640	0.540	0.560
I	1.280	0.460	0.460	0.240	0.020	0.040	0.014	0.240	0.360	0.320
J	1.300	0.480	0.480	0.200	0.360	0.006	0.014	0.200	0.400	0.440
K	5.460	0.440	0.520	0.340	0.260	0.004	0.600	0.180	0.026	0.600
L	2.880	0.420	0.960	0.220	1.280	2.460	1.180	0.420	0.040	0.030
EH	Oxalic	Lactic	Acetic	Succinic	Formic	Levulinic	Xylitol	Glycerol	Methanol	Ethanol
A_EH6h	0.300	0.620	0.700	0.800	0.520	0.040	0.240	0.400	1.580	1.140
A_EH12h	0.640	0.720	0.840	1.100	0.520	0.040	0.240	0.300	1.940	1.620
B_EH6h	0.340	0.700	0.960	0.780	0.720	0.120	0.100	0.140	1.600	0.960
B_EH12h	0.440	0.760	1.720	1.640	0.640	0.080	0.160	0.500	3.360	0.560
C_EH6h	1.040	1.460	1.260	0.046	1.160	0.080	0.280	0.220	0.060	0.040
C_EH12h	3.900	1.860	1.400	0.460	1.460	0.280	0.280	0.540	0.042	0.036
D_EH6h	1.160	2.940	2.860	0.034	3.000	0.160	0.420	0.040	0.058	0.032
D_EH12h	1.140	3.280	3.180	0.100	2.280	0.160	1.380	0.006	0.030	0.056
E_EH6h	1.160	0.600	0.520	0.700	0.420	0.520	0.420	0.500	0.500	0.500
E_ EH12h	1.360	0.800	0.720	0.900	0.620	0.560	0.460	0.540	0.540	0.500
F_EH6h	0.860	0.600	0.720	0.700	0.420	0.520	0.420	0.500	0.030	0.048
F_ EH12h	1.160	0.600	0.720	0.700	0.420	0.520	0.420	0.500	0.500	0.220
G_EH6h	1.920	1.400	1.500	0.900	1.300	0.700	0.800	0.400	0.700	0.500
G_ EH12h	1.920	1.400	1.700	0.900	1.500	0.920	0.800	0.400	0.700	0.500
H_EH6h	2.000	2.520	1.800	1.020	1.720	0.900	1.140	1.040	0.940	0.560
H_ EH12h	2.000	2.520	1.800	1.020	1.720	0.900	1.140	1.040	0.940	0.560
I_EH6h	1.380	0.560	0.500	0.300	0.540	0.100	0.034	0.380	0.420	0.380
I_EH12h	1.460	0.640	0.580	0.360	0.660	0.160	0.006	0.440	0.460	0.600
J_EH6h	1.500	0.880	0.680	0.600	0.600	0.180	0.000	0.660	0.720	0.840
J_ EH12h	1.600	0.680	0.880	0.300	0.560	0.026	0.050	0.500	1.100	1.000
K_EH6h	4.060	1.640	0.720	1.140	0.860	1.160	0.840	0.580	0.300	0.600
K_EH12h	5.460	1.440	0.920	0.740	0.660	0.620	0.600	0.780	0.008	0.600
L_EH6h	2.880	0.560	0.960	0.220	1.280	2.460	1.180	0.420	0.048	0.022
L_EH12h	2.880	0.820	1.360	0.220	1.280	2.460	1.180	0.420	0.014	0.024
UTFeb_EH6h	0.320	0.060	0.420	1.140	0.200	0.020	0.018	0.160	0.180	0.100
UTFeb_EH12h	0.016	0.160	2.420	0.160	0.260	0.014	0.012	0.160	3.080	2.080
UTMar _EH6h	0.360	0.760	0.660	0.720	0.420	0.038	0.016	0.320	0.940	0.720
UTMar_EH12h	0.460	1.700	2.600	0.260	0.044	0.008	0.040	0.080	2.860	1.440

Table II. Solubilized components of biomass and total concentration of inhibitors and recovered monosaccharides, in pretreatment and enzymatic hydrolysis assays. Values in % (w/w).

PR	Carbohydrate release	Protein release	Lipid release	Total inhibitors	Total monosaccharides
A	2.343	4.167	2.225	2.180	0.052
B	3.668	8.004	5.965	2.180	0.096
C	13.352	37.663	8.845	4.060	3.061
D	12.543	36.388	10.606	10.440	4.216
E	2.028	4.035	0.987	2.140	0.783
F	7.769	13.113	7.293	3.000	1.050
G	3.567	11.611	0.382	6.240	1.761
H	11.100	23.977	6.868	10.660	3.169
I	10.617	2.521	2.078	3.560	1.548
J	16.093	6.768	4.000	3.940	1.724
K	27.094	11.757	3.534	8.660	20.575
L	37.444	18.904	6.944	10.660	30.748
EH	**Carbohydrate release**	**Protein release**	**Lipid release**	**Total inhibitors**	**Total monosaccharides**
B_EH6h	17.038	16.301	8.217	7.080	6.155
B_EH12h	19.646	18.237	9.977	10.740	7.134
C_EH6h	19.148	39.276	12.918	5.860	8.739
C_EH12h	20.928	39.391	13.570	10.360	10.380
D_EH6h	19.160	40.494	14.567	10.460	10.729
D_EH12h	21.869	40.659	14.685	10.740	13.353
E_EH6h	13.551	15.948	9.021	5.680	3.454
E_EH12h	16.721	22.442	10.794	7.040	6.513
A_EH6h	13.943	16.615	6.138	7.220	5.280
A_EH12h	20.191	17.087	7.861	8.600	6.737
F_EH6h	13.982	21.404	13.967	4.840	3.116
F_EH12h	15.557	24.291	14.410	5.760	3.665
G_EH6h	16.616	20.124	7.889	10.560	4.388
G_EH12h	17.989	20.636	9.138	11.180	5.191
H_EH6h	17.078	26.185	8.154	13.940	8.810
H_EH12h	19.736	28.528	9.073	14.120	11.404
I_EH6h	24.489	7.492	6.300	4.560	8.066
I_EH12h	25.889	8.145	7.418	5.360	8.366
J_EH6h	24.418	8.736	6.577	6.660	9.141
J_EH12h	26.471	9.380	6.646	6.700	9.146
K_EH6h	30.879	16.324	6.137	11.960	27.402
K_EH12h	32.333	17.108	6.412	12.080	28.650
L_EH6h	37.674	21.263	8.846	10.800	31.850
L_EH12h	38.069	22.969	9.807	11.680	32.201
UTFeb_ EH6h	16.291	1.251	3.624	2.600	3.229
UTFeb_ EH12h	16.411	2.768	6.631	8.340	3.334
UTMar_EH6h	21.354	2.080	4.072	4.900	3.544
UTMar_EH12h	25.751	2.886	4.511	9.400	4.246

Table III. Concentration of inhibitors obtained in pretreatment and enzymatic hydrolysis assays of sunflower oil, edible gelatin sheets and cotton, used as pure sources of lipids, proteins and carbohydrates, respectively. Values in % (w/w).

	Samples	Oxalic	Lactic	Acetic	Succinic	Citric	Formic	Butyric	Levulinic	Xylitol	Glycerol	Methanol	Ethanol	Acetone
Lipids	A_M	0.000	0.000	0.067	0.030	0.000	0.087	0.108	0.117	0.010	0.000	0.223	0.173	0.000
	A_0.5NaOH	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.212	0.072	0.000	0.000	0.000
	A_2NaOH	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.414	0.350	0.000	0.000	0.000
	A_0.5HCl	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.008	0.000	0.000	0.000	0.000
	A_2HCl	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.012	0.000	0.000	0.000	0.000
	A_M_EH	0.270	0.000	0.000	0.050	38.934	0.000	0.230	0.076	0.029	0.000	0.151	0.280	0.000
	A_0.5NaOH_EH	0.000	0.000	0.000	0.000	38.583	0.000	0.000	0.000	0.265	0.000	0.000	0.000	0.000
	A_2NaOH_EH	0.000	0.000	0.000	0.000	33.502	0.000	0.000	0.000	0.475	0.387	0.000	0.000	0.000
	A_0.5HCl_EH	0.000	0.000	0.000	0.000	35.584	0.000	0.000	0.000	0.021	0.000	0.000	0.000	0.000
	A_2HCl_EH	0.201	0.000	0.000	0.000	32.308	0.000	0.000	0.000	0.019	0.000	0.000	0.000	0.000
Proteins	B_M	0.000	0.137	0.000	0.026	0.000	0.000	0.000	0.000	0.030	0.000	0.000	0.000	0.000
	B_0.5NaOH	0.000	0.000	0.000	0.256	0.000	0.000	0.000	0.212	0.142	0.000	0.000	0.000	0.000
	B_2NaOH	0.000	0.000	0.000	0.153	0.000	0.000	0.000	0.000	0.292	0.000	0.000	0.000	0.000
	B_0.5HCl	0.000	1.064	0.219	0.433	0.000	0.000	0.000	0.353	0.013	0.052	3.586	0.000	0.546
	B_2HCl	0.000	0.000	0.242	0.076	0.000	0.000	0.000	0.165	0.010	0.000	1.462	0.000	2.006
	B_M_EH	0.126	0.000	0.000	0.057	31.233	0.000	0.000	0.000	0.016	0.000	0.000	0.000	0.000
	B_0.5NaOH_EH	0.000	0.000	0.000	0.243	37.195	0.000	0.000	0.000	0.055	0.000	0.000	0.000	0.000
	B_2NaOH_EH	0.108	0.000	0.000	0.040	33.134	0.000	0.000	0.000	0.283	0.000	0.000	0.000	0.000
	B_0.5HCl_EH	0.000	1.065	0.000	0.378	35.744	0.000	0.000	0.386	0.020	0.000	3.487	0.000	0.430
	B_2HCl_EH	0.000	0.000	0.130	0.035	29.993	0.000	0.000	0.110	0.016	0.000	1.354	0.000	1.976
Carbohydrates	C_M	0.000	0.000	0.138	0.048	0.000	0.099	0.015	0.097	0.012	0.021	0.095	0.100	0.000
	C_0.5NaOH	0.000	2.672	0.337	0.129	0.000	1.640	0.037	0.001	2.643	0.000	0.000	0.000	0.000
	C_2NaOH	0.199	2.202	0.150	0.029	0.000	1.211	0.049	0.014	4.729	0.000	0.000	0.000	0.000
	C_0.5HCl	0.000	0.000	0.172	0.086	0.163	0.122	0.126	0.068	0.003	0.000	0.000	0.042	0.000
	C_2HCl	0.000	0.000	0.027	0.148	0.072	0.761	0.133	1.991	0.020	0.000	0.223	0.088	0.000
	C_M_EH	0.000	0.000	0.039	0.029	37.609	0.013	0.040	0.019	0.017	0.000	0.000	0.000	0.000
	C_0.5NaOH_EH	0.000	2.567	0.186	0.089	35.753	1.641	0.125	0.014	2.307	0.000	0.000	0.127	0.000
	C_2NaOH_EH	0.000	2.110	0.282	0.063	34.594	1.134	0.054	0.014	4.455	0.000	0.000	0.000	0.000
	C_0.5HCl_EH	0.000	0.080	0.160	0.119	35.888	0.146	0.257	0.081	0.018	0.071	0.162	0.297	0.000
	C_2HCl_EH	0.000	0.000	0.062	0.121	32.444	0.668	0.227	1.536	0.012	0.000	0.015	0.186	0.000

Table IV. Total concentration of inhibitors and recovered monosaccharides in PR and EH assays. of sunflower oil, edible gelatin sheets and cotton, used as pure sources of lipids, proteins and carbohydrates, respectively. Values in % (w/w).

	Samples	Total inhibitors	Total monosaccharides
Lipids	A_0.5HCl	0.009	0.000
	A_0.5HCl_EH	0.022	6.717
	A_0.5NaOH	0.285	0.006
	A_0.5NaOH_EH	0.266	6.969
	A_2HCl	0.014	0.000
	A_2HCl_EH	0.222	6.146
	A_2NaOH	0.764	0.003
	A_2NaOH_EH	0.863	6.640
	A_M	0.816	0.000
	A_M_EH	1.087	7.549
Proteins	B_0.5HCl	6.266	0.024
	B_0.5HCl_EH	5.767	6.851
	B_0.5NaOH	0.611	0.175
	B_0.5NaOH_EH	0.300	7.089
	B_2HCl	3.961	0.069
	B_2HCl_EH	3.622	5.781
	B_2NaOH	0.446	0.028
	B_2NaOH_EH	0.432	6.133
	B_M	0.194	0.000
	B_M_EH	0.201	6.225
Carbohydrates	C_0.5HCl	0.781	6.843
	C_0.5HCl_EH	1.390	34.384
	C_0.5NaOH	7.459	0.729
	C_0.5NaOH_EH	7.056	36.407
	C_2HCl	3.464	16.668
	C_2HCl_EH	2.828	35.915
	C_2NaOH	8.583	0.924
	C_2NaOH_EH	8.112	30.261
	C_M	0.625	0.000
	C_M_EH	0.157	34.400