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Enzymatic hydrolysis of the organic fraction of municipal solid waste: Optimization and valorization of the solid fraction for *Bacillus thuringiensis* biopesticide production through solid-state fermentation

Esther Molina-Peñate [a,b], Antoni Sánchez [a], Adriana Artola [a,*]

[a] GICOM Research Group, Department of Chemical, Biological and Environmental Engineering, School of Engineering, Edifici Q, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain
[b] Aeris Tecnologías Ambientales S.L, Carrer Santa Rosa, 38, local, 08290 Cerdanyola del Vallès, Barcelona, Spain

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ABSTRACT

To reach a more sustainable society, the implementation of a circular economy perspective in municipal waste management becomes essential. In this work, the enzymatic hydrolysis of source-separated organic fraction of municipal solid waste (OFMSW) has been optimized as a sugar-releasing step. A liquid sugar concentrate, with a maximum reducing sugar concentration of 50.56 g L^{-1}, and a solid hydrolyzed fraction were obtained. The effect of the harshness of the hydrolysis conditions was evaluated on the performance of the resulting solid fraction as a substrate for *Bacillus thuringiensis* biopesticide production through solid-state fermentation. A production of 3.9 $\times$ 10^8 viable cells g^{-1} dry matter with a 33% sporulation ratio was achieved for milder hydrolysis conditions, highlighting the potential of the solid fraction of hydrolysis as a substrate of SSF processes. The proposed valorization pathway for the OFMSW results in a sugar concentrate with potential for fermentative processes and a fermented solid containing biopesticides from *Bacillus thuringiensis*.

1. Introduction

The organic fraction represents nearly half of the globally generated municipal solid waste (MSW), which is produced at a rate of 2.01 billion tonnes per year (Kaza et al., 2018). This organic fraction of municipal solid waste (OFMSW) comprises green and food waste from parks, households, commerce, and restaurants (Al Seadi et al., 2013). It is an abundant organic waste, with high moisture content and complex composition in polysaccharides, lignocellulose, sugars proteins, lipids, and macro/micronutrients (Campuzano and González-Martínez, 2016). From a circular economy perspective, in which organic waste as a source for new bioproducts must be kept within the production cycles as long as possible (Sánchez et al., 2015), the OFMSW becomes a potential feedstock for biotechnological processes.

To date, the valorization of the OFMSW has been focused mostly on composting and biogas production through anaerobic digestion. Pretreatments have appeared as a tool to reduce the chemical complexity of the OFMSW and to increase its biodegradability by enhancing access to individual components (Romero-Cedillo et al., 2017; Romero-Güiza et al., 2016). In this sense, enzymatic hydrolysis has been applied successfully as a pretreatment for enhancing biogas yield (Mlaik et al., 2019). Recently, the focus has been shifted to the production of higher market value products and enzymatic hydrolysis has emerged as a tool for extracting functionalized compounds, such as sugars, fatty acids, or proteins (Pleissner and Peinemann, 2020). Sugars are of special interest as they can be the platform for the production of bioproducts through fermentation processes (Cabas Candama et al., 2020; Zhang et al., 2020). Cellulases are the most commonly used enzymes for the hydrolysis of OFMSW but, considering its complex composition, the use of complementary enzymatic activities, such as xylanases or amylases, is advisable to increase the rate and yield of fibers conversion (de la Torre et al., 2017; Hu et al., 2013).

Most studies dealing with enzymatic hydrolysis as a step to release sugars from the OFMSW have been focused on the use of the liquid fraction of the hydrolysis. Different valorization scenarios have been considered with relative success, for example, the production of succinic acid (Stylianou et al., 2020), acetic acid (López-Gómez et al., 2019), or lipids (Ghanavati et al., 2015). Conversely, few studies have also taken into consideration the valorization of the solid fraction, which has been either diluted and fermented together with the liquid fraction

* Corresponding author.
E-mail address: adriana.artola@uab.cat (A. Artola).

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(Ebrahimian and Karimi, 2020) or simply directed to anaerobic digestion processes (Mahmoodi et al., 2018). To attain the implementation of enzymatic hydrolysis as an extractive step and provide a zero-waste alternative to the current model, it is necessary to ensure the full exploitation of the OFMSW potential. In this regard, solid-state fermentation (SSF) appears as a potential technology for the utilization of unhydrolyzed solids.

SSF, defined as a process that occurs in the absence or near absence of free water (Pandey, 2003), has been established as an efficient and environmentally friendly tool for the valorization of various solid organic waste (Yazid et al., 2017) to produce different marketable products, such as aroma compounds (Martínez-Avila et al., 2021), biosurfactants (Jiménez-Peñalver et al., 2016), or biopesticides (Mejias et al., 2020; Sala et al., 2020). Among them, biopesticides derived from *Bacillus thuringiensis sp*. (Bt), the most widely used microbial biopesticide, have shown a robust production on SSF using OFMSW or similar wastes because of their content in easily biodegradable organic matter and a great variety of macro and micronutrients (Ballardo et al., 2017; Rodríguez et al., 2019; Zhang et al., 2013; Zou et al., 2016). During the sporulation phase, Bt species produce crystal inclusions containing toxic proteins (Cry or Cyt protein) (Bravo et al., 2011). These proteins are selectively toxic for a wide spectrum of hosts including insects of Coleoptera, Lepidoptera, Diptera, Hymenoptera, Hemiptera, and Orthoptera orders, as well as phytopathogenic nematodes and terrestrial gastropods (Malovichko et al., 2019). The toxicity is produced by ingestion causing gut cell lysis. Bt-derived biopesticides are already available in the market commercialized under different names and forms, such as DiPel®or XenTari® (Kenogard), Agree®(Bioamvac), or Deliver®(Certis), and produced through submerged fermentation employing defined synthetic media.

This paper aims to evaluate the enzymatic hydrolysis of OFMSW as a sugar-releasing step to obtain a sugar-rich solution, and the subsequent use of the unhydrolyzed solid fraction to produce Bt-derived biopesticide. First, an optimization of the hydrolysis to increase the amount of reducing sugars (RS) is presented, providing two optimum scenarios for the Viscozyme L® enzymatic cocktail. Then, the suitability of the resulting solid fraction to be used as a substrate of a SSF process producing Bt spores is analyzed. To the best of our knowledge, this is the first work using SSF technology to valorize the solid fraction resulting from enzymatic hydrolysis of the OFMSW.

2. Materials and methods

2.1. OFMSW collection and preparation

Source separated OFMSW (ssOFMSW) samples, kindly provided by Mancomunitat La Plana (Malla, Barcelona), were collected upon arrival at the MSW treatment plant. First, samples were screened manually for the presence of inert materials such as glass, plastics, metals, or textiles. Bones, hard shells, hair, and excess paper were also removed. Then, samples were homogenized mechanically using a home composting shredder (Tecoinsaen SL, Spain), packed into 1 kg bags, and stored at −20°C for a maximum of two months. Initial characterization of the two homogenized OFMSW batches collected for this study (September and November 2020) was performed. Before use, samples were defrosted overnight at 5°C and sterilized by autoclaving at 121°C for 30 min.

2.2. Enzymatic hydrolysis

For the enzymatic hydrolysis, the commercial cocktail Viscozyme L® from Novozymes (Denmark) was selected. This cocktail has been gaining interest in the pretreatment of food waste, a major component of OFMSW, (Cabas Candama et al., 2020; Chua et al., 2021; Gabiatti Junior et al., 2020) because of its wide range of carbohydrases, including cellulase, β-glucanase, hemicellulase, xylanase, arabinase, and pectinase. The declared activity by the provider is 100 Fungal Beta Glucanase Units (FBGU) per gram with an operating pH range of 3.5–5.5 and a wide operating temperature range of 25–55°C.

Experiments were conducted in sterile conditions using 500 mL Erlenmeyer flasks containing 100 g of sterile ssOFMSW diluted with 0.05 M sodium citrate buffer to reach a 10% (w v^{-1}) solid to liquid ratio. Experiments were conducted for 24 h at 180 rpm and temperature, initial pH, and enzyme load were adjusted according to the experimental design (Table 2). The adjustment of the pH was done by changing the pH of the sodium citrate buffer. Immediately after the enzymatic hydrolysis, samples were centrifuged at 6000 rpm for 15 min at 4°C, both the supernatant (liquid fraction) and the pellet (solid fraction) were collected. The liquid fraction was centrifuged at 8000 rpm for 10 min, and the supernatant was collected and stored at −20°C for RS determination. The solid fraction was collected for RS determination, characterization, and further use in the SSF process.

2.2.1. Optimization of the enzymatic hydrolysis procedure

Enzymatic hydrolysis conditions were evaluated and optimized by RSM using a Box-Behnken design. Temperature, initial pH, and enzyme load were chosen as the three independent variables of the design and were tested at three different levels (low, medium, and high). For temperature (25°C, 40°C, 55°C) and pH (3.5, 4.5, 5.5), the selected levels were based on the operating ranges specified by the enzyme cocktail supplier (Novozymes, Denmark). The enzyme load levels (0.01, 0.05, 0.1 mL g^{-1} DM) were equivalent to 1.2, 6.6, 12 FBGU g^{-1} dry matter (DM) according to the supplier and were based on the range proposed by Arapoglou et al. (2010) and Cabas Candama et al. (2020) and assessed on preliminary experiments (data not shown). Time was fixed in 24 h, agitation in 180 rpm, and solid load in 10% (w v^{-1}) because it was found as the maximum percentage ensuring a proper mixing under the experimental set-up. The final design consisted of 15 runs with a triplicate in the central point to allow for the estimation of the pure error (Box and Behnken, 1960). Two responses were considered, RS concentration in the liquid fraction (mg g_i^{-1} DM) and RS concentration in the solid fraction per initial gram of DM (mg g_i^{-1} DM). Calculations were done considering the 18 g of initial dry OFMSW in all the experiments and the % of recovered wet solids after centrifugation (around 70%). A second-order polynomial model, as presented in Equation (1), was fitted for the results of each response.

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{3}\beta_{ii} X_i^2 + \sum_{i=1}^{2}\sum_{j=i+1}^{3}\beta_{ij} X_i X_j \quad (1)$$

where Y is the predicted response; β_0 is model constant and β_i, β_{ii}, β_{ij} are regression coefficients of linear, quadratic, and cross-product terms; X_i and X_j are coded independent variables. The quality of fit of the polynomial model equations was expressed by the coefficient of determination (R^2) and their prediction capability by the predicted R^2.

To verify the obtained models, the enzymatic hydrolysis was performed in triplicate under the optimum conditions predicted. The experimental and predicted response values were compared and the predictive capability of the model was assessed. The efficiency of the hydrolysis is reported through a performance index computing the increase of RS in both fractions after the hydrolysis with respect to the RS initially present in the substrate.

2.2.2. Statistical analysis

Results were analyzed using the Design-Expert® statistical software (Stat-Ease, Inc, United States). With the aid of the program, multiple regression analysis for the construction of the model, and statistical analysis of variance (ANOVA) for evaluating its significance, were conducted. Linear and quadratic effects of the variables and their interactions on the release of RS were calculated and plotted into three-dimensional and contour plots. Maximum RS concentration in each fraction was estimated using numerical optimization.

2.3. Solid-state fermentation of the solid fraction of hydrolysis

2.3.1. Microorganism, inoculum preparation, and growth assessment

Bacillus thuringiensis var *israelensis* (Bti) (CECT 5904) was obtained from *Colección Española de Cultivos Tipo* (Valencia, Spain) and preserved at −80°C using a seed lot system in cryo-pearls (DeltaLab, Barcelona, Spain).

Inoculum preparation was performed according to the methodology presented by Mejias et al. (2020). Shortly, one cryo-pearl was inoculated in 100 mL of sterilized Nutrient Broth N°2 (Oxoid CM0067B, England) and incubated for 20 h, at 130 rpm and 30°C until it reached an optical density of 2.5–3. Afterward, the culture was centrifuged for 10 min at 3500 rpm and 4°C. The obtained pellet was resuspended in 3 mL of supernatant. To reach an inoculum concentration on the solid substrate of approximately 10^7 CFU g^{-1} DM, the resuspended inoculum was diluted at 1:10 (v v^{-1}) with supernatant. The final inoculum contained around 10^8 CFU mL^{-1} and no spores were detected.

Bti growth was assessed in terms of viable cells and spores. The procedure used is as described by Mejias et al., 2020. First, a solid–liquid extraction was performed using Ringer solution in a 1:10 (w v^{-1}) ratio at 150 rpm for 20 min. For spore determination, 20 mL of the previous extract were submitted to a thermal shock by incubating them at 80°C for 10 min and then placing them into ice. Serial dilution banks of both extracts were prepared using Ringer and plated in triplicates onto Petri dishes containing Nutrient agar medium (Oxoid CM0003B, England). The plates were incubated for 20 h at 30°C and viable cells or spores were estimated in terms of colony-forming units (CFUs). The sporulation ratio at a certain time was calculated considering that the viable cell count includes vegetative cells and spores.

2.3.2. Substrate preparation

The solid fractions resulting from the selected as optimal conditions of the enzymatic hydrolysis were collected sterile and mixed with sterile wood chips of particle size between 0.5 and 5 cm (Acalora, Palets Pla d'Urgell). Wood chips act as a bulking agent and are necessary to provide porosity to the solid matrix. The resulting SSF substrate for each reactor consisted of 95 g of solid fraction and 15 g of wood chips manually mixed and inoculated with 2.6 mL of diluted Bti inoculum. Triplicates for each condition studied were performed.

2.3.3. Experimental SSF set up

SSF experiments were conducted in 0.5 L packed bed reactors under aseptic conditions. Filled reactors were placed in a temperature-controlled water bath at 30°C and connected to a mass flow meter (Bronkhorst, Netherlands), which continuously supplied a specific airflow saturated through a humidifier to prevent drying in the solid matrix. Airflow was set to 37 mL h^{-1} g^{-1} DM in all the experiments for ensuring aerobic conditions (Mejias et al., 2017). Exhaust gases exited from the top of each reactor, went through a water trap, and reached an oxygen sensor (Alphasense, UK) connected to a custom-built acquisition system (Arduino® based). With the recorded oxygen concentration, the specific oxygen uptake rate (sOUR) was calculated as an indicator of the biological activity as stated by Ponsá et al. (2010). Experiments lasted 72 h, which has been established as the maximum spore count time for Bt (Cerda et al., 2019). At this time, the final pH, viable cells, and spores were determined.

A summary of the experimental process described can be seen in Fig. 1.

2.4. Analytical methods

RS were measured using the DNS method (Miller, 1959) both in the liquid and solid fractions of the enzymatic hydrolysis. For the solid fraction analysis, a solid–liquid extraction was performed using distilled water in a 1:10 (w v^{-1}) ratio for 30 min at 50°C and 150 rpm. The liquid phase from the extraction, together with the supernatant obtained from the centrifugation of the liquid hydrolysate were filtered through a 0.45 μm membrane filter and diluted with water to obtain a concentration in the range of the calibration curve (glucose, 0.2–3.3 g L^{-1}). RS in the solid fraction is always expressed per initial gram of DM before the hydrolysis was performed.

Solid fractions were characterized physiochemically in terms of moisture content (MC), dry matter (DM), organic matter (OM), ashes, and pH according to standard procedures (Thompson et al., 2001). Cellulose, hemicellulose, and lignin content were determined using an Ankom200 Fiber Analyzer incubator (Ankom Technology, Macedon, NY). C/N ratio was determined using a CHNS elemental analyzer Flash 2000 (Thermo Scientific). Biodegradability was assessed through the dynamic respiration index (DRI), which represents the average oxygen uptake rate during the 24 h of maximum activity observed, as described elsewhere (Ponsá et al., 2010; Sala et al., 2020). DRI is expressed in mg of oxygen consumed per g of dry matter per hour. All measurements were conducted in triplicates.

3. Results and discussion

Table 1 summarizes the characteristics of the two batches of ssOFMSW used in this study. The majority of the physicochemical values were in close agreement with the average values from 43 cities in 22 countries presented by Campuzano and González-Martínez (2016). Except for the reducing sugars content in batch 1 ($p = 0.008$), the lignin ($p = 0.049$), and nitrogen ($p = 0.041$) content in batch 2, which showed significant differences (Table S.1). These parameters were reported as highly variable by the same authors. Sugars are a very easily degradable compound and their amount fluctuates depending on the time from waste generation to analysis (Hansen et al., 2007). This higher RS value can also explain the higher biodegradability of batch 1, measured by the DRI, which is a measure of biological activity in terms of oxygen consumption. It is important to highlight that the OFMSW is an inherently variable material that does not only variates among countries or cities,

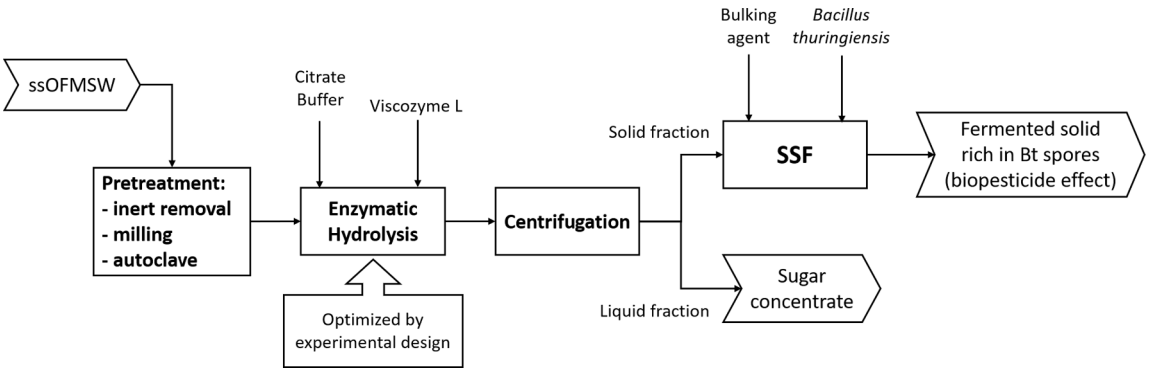

Fig. 1. Scheme of the experimental process conducted for the valorization of source-separated OFMSW (ssOFMSW).

Table 1
Characterization of the two batches of source-separated OFMSW used in this study together with average values from 43 cities in 22 countries reported by Campuzano and González-Martínez, 2016.

Parameter	Batch 1	Batch 2	(Campuzano and González-Martínez, 2016)
Moisture content (%)	81.0 ± 0.1	72.9 ± 1.0	72.8 ± 7.6
DM (%)	19.1 ± 0.1	27.1 ± 1.0	27.2 ± 7.6
OM (%*)	90.1 ± 1.0	89.7 ± 1.7	84.6 ± 9.9
RS (%*)	24.1 ± 0.5[a]	15.7 ± 1.3	10.5 ± 6
C (%*)	45.4 ± 0.3	45.5 ± 1.5	46.6 ± 4.4
H (%*)	6.5 ± 0.03	6.4 ± 0.2	6.6 ± 0.62
N (%*)	2.1 ± 0.7	1.9 ± 0.3[a]	2.9 ± 0.6
S (%*)	0.1 ± 0.04	<0.1	0.3 ± 0.26
C/N ratio	17	24	16
Cellulose (%*)	15.9 ± 0.2	7.4 ± 0.1	18.6 ± 15
Hemicellulose (%*)	10.0 ± 0.1	6.8 ± 0.1	8.6 ± 4.6
Lignin (%*)	7.8 ± 0.3	2.1 ± 0.2[a]	9.7 ± 5.3
pH	5.25 ± 0.05	5.31 ± 0.04	5.02 ± 0.95
DRI (mg O_2 g^{-1} DM h^{-1})	7.5 ± 0.2	4.2 ± 0.3	NA

* Dry basis. NA, not available. Data presented as mean values ± standard deviation (n = 3). [a] significantly different parameter ($p < 0.05$) based on the Tukey test analysis.

but also within the same region by other factors such as weather conditions, seasonal changes, nutritional habits, or recollection system (Campuzano and González-Martínez, 2016; Hansen et al., 2007).

3.1. Enzymatic hydrolysis as sugar releasing step

The evaluation of the enzymatic hydrolysis as a sugar-releasing step was performed using batch 1 of ssOFMSW and based on three operational parameters for two response variables. The operational parameters studied were temperature, pH, and enzyme load, previously identified as significant factors affecting the enzymatic hydrolysis of organic wastes (Guan and Yao, 2008; Yan et al., 2011). These parameters were evaluated based on their effect on the release of RS in the liquid fraction and the solid fraction (Table 2).

The highest value of RS concentration in the solid fraction was obtained in run 13, 133.2 mg g_i^{-1} DM, corresponding to temperature 40°C, initial pH 4.5, and enzyme load 0.05 mL g^{-1} DM. By contrast, the minimum value was obtained in run 9, 60.8 mg g_i^{-1} DM, corresponding to temperature 40°C, initial pH 3.5, and enzyme load 0.01 mL g^{-1} DM. Comparison with literature values is difficult because, among the few publications dealing with the solid fraction of enzymatic hydrolysis, none of them measured the RS concentration in the solid (Ghanavati et al., 2015; Zhang et al., 2020). As detailed in Table 2, the highest value of RS concentration in the liquid fraction was obtained in run 4, 365.1 mg g_i^{-1} DM, corresponding to temperature 25°C, initial pH 4.5, and enzyme load 0.1 mL g^{-1} DM. By contrast, the minimum value was obtained in run 3, 167.4 mg$_i$ g^{-1} DM, corresponding to temperature 25°C, initial pH 4.5, and enzyme load 0.01 mL g^{-1} DM. Comparing both of them, the effect of enzyme concentration is evident as at equal conditions the amount of liberated RS in the liquid fraction doubled. In contrast to the solid fraction, the maximum release of RS occurred at the maximum enzyme concentration. The maximum obtained RS concentration in the liquid fraction, 50.56 mg mL^{-1} is comparable to other studies performing enzymatic hydrolysis of OFMSW. For example, López-Gómez et al. (2019) obtained 55.41 mg mL^{-1} of glucose from a source selected waste, and Ebrahimian and Karimi (2020) a maximum of 35 mg mL^{-1} of glucose. Generally, the achieved RS concentration is lower than those experiments that employed selected food waste, which reached between 130 and 170 mg mL^{-1} (Yan et al., 2011; Zhang et al., 2020), because this type of waste is lesser in complexity and amount of impurities (Alibardi and Cossu, 2015). An exception was found in Cabas Candama et al. (2020), who reached 25 mg mL^{-1} of glucose using waste from fruit and vegetables and the same enzymatic cocktail as the employed in this paper. In every run the concentration of RS obtained in the solid fraction was lower than that initially present in batch 1 (241 mg g^{-1} DM), indicating the solubilization of the sugars towards the liquid fraction. The total amount of released RS increased almost two-fold in runs 4 (467.2 mg g_i^{-1} DM) and 8 (466.9 mg g_i^{-1} DM) suggesting gradual hydrolysis of the fibers present in the OFMSW. Those runs (3, 7, 9, 12) without enzyme addition did not show an increase in RS after the enzymatic hydrolysis as displayed by the performance index of 1, proving the hydrolysis effect of the cocktail to a greater or lesser extent depending on the conditions of the hydrolysis.

In general, low enzymatic load and high pH lead to lower RS concentrations in both fractions whereas medium and high enzyme loads and medium and low pHs lead to larger amounts of RS released. Reaching an agreement between the sugar release in both fractions, as seen in runs 1, 5, and 8 in Table 2, would maximize the potential of

Table 2
Design matrix of the Box-Behnken model and observed responses: reducing sugars in the solid and liquid fractions. The performance of the hydrolysis is expressed as fold increase of RS.

Run	Temp (°C)	pH	Enzymatic load (ml g^1 DM)	RS solid fraction (mg g_i^{-1} DM)	RS liquid fraction		Hydrolysis performance*
					(mg mL^{-1})	(mg g_i^{-1} DM)	
1	25	3.5	0.05	121.8	43.5	314.3	1.8
2	25	5.5	0.05	121.6	38.0	274.8	1.6
3	25	4.5	0.01	80.2	23.2	167.4	1.0
4	25	4.5	0.1	102.1	50.6	365.1	1.9
5	55	3.5	0.05	130.8	45.2	326.1	1.8
6	55	5.5	0.05	94.4	31.5	227.3	1.3
7	55	4.5	0.01	66.8	25.2	182.0	1.0
8	55	4.5	0.1	128.5	46.9	338.5	1.9
9	40	3.5	0.01	60.8	23.7	171.5	1.0
10	40	3.5	0.1	124.5	40.8	295.0	1.7
11	40	5.5	0.01	69.2	25.1	181.2	1.0
12	40	5.5	0.1	82.2	41.6	300.8	1.6
13	40	4.5	0.05	133.2	33.2	239.5	1.3
14	40	4.5	0.05	119.7	35.4	256.0	1.6
15	40	4.5	0.05	122.3	30.7	221.4	1.4

* Calculated as the total amount of RS (liquid and solid fraction) with respect to the initially measured in batch 1 (241 mg g^{-1} DM).

OFMSW hydrolysis as a pretreatment of fermentative steps.

3.2. Process optimization and verification

The RSM Box-Behnken design was implemented to optimize the sugar release from OFMSW, both in the liquid and solid fractions of hydrolysis. The variables were analyzed by a multiple regression analysis to obtain a regression equation that could predict the response within the specified range (Guan and Yao, 2008). Analysis of variance (ANOVA) was used to investigate the significance of fit for the model equations. As the goal of this paper is the valorization of the solid fraction of hydrolysis the following discussion is focused on it but the model and ANOVA for the liquid fraction can be found in the supplementary material (Table S.2).

The experimental results were fitted with a quadratic equation. The ANOVA analysis presented in Table 3 resulted in a significant regression model ($p < 0.05$) and a not-significant lack of adjustment ($p > 0.05$). Consequently, the model presents a good adjustment with the experimental data reported. A logarithmic transformation of the response for analyzing the data was performed to achieve a better adjustment in the quadratic model (Joglekar and May, 1987). The parameters enzyme load and pH showed significant effects, specifically, the linear effect of the enzyme load was the most significant as the smaller the p-value, the more significant the corresponding coefficient (Haber and Runyon, 1973). According to the R^2, which is a measure of the degree of fit (Haber and Runyon, 1973), the model could explain 98.76% of the variability in the response. The model also has the capacity to explain 88.40% of the variations in new observations according to the predicted R^2, which is in reasonable agreement (<0.2) with the adjusted R^2 (96.51%) (Haber and Runyon, 1973; Joglekar and May 1987). In addition, it shows a low coefficient of variation (1.11%), being indicative of the reliability of the experimental design. The resulting regression equation for the response of RS concentration in the solid fraction for the conditions studied is presented in Equation (2).

$$\log_{10}RS(mgg^{-1}DM) = +2.10 - 0.007X_1 - 0.033X_2 + 0.10X_3 - 0.035X_1X_2 + 0.045X_1X_3 - 0.059X_2X_3 + 0.011X_1^2 - 0.042X_2^2 - 0.146X_3^2 \quad (2)$$

where X_1, X_2 and X_3 represent the temperature (°C), initial pH, and enzyme load (mL g^{-1} DM), respectively.

The interaction effects of the three variables were corroborated in three-dimensional response surface plots (Fig. 2). Based on the ANOVA analysis (Table 3), the temperature was not a significant parameter as illustrated in plots showing the interaction of temperature with enzyme load and pH (Fig. 2.B and Fig. 2.C, respectively). Therefore, the Viscozyme L cocktail was suitable for the hydrolysis of OFMSW through the whole temperature range described by the provider. This observation has also been recently reported by Cabas Candama et al. (2020) for waste of fruits and vegetables. The effect of the pH, reported as a parameter with significant effects in Table 3, can be seen in Fig. 2.A and Fig. 2.C. The RS concentration increases when pH decreases, reaching the maximum at pH 3.5. However, as shown in Fig. 2.C, for low temperature (25°C) the maximum RS concentration is reached at pH around 4.5. The adequate performance on milder conditions, i.e. lower temperature and not so acidic pH, makes Viscozyme L a notable cocktail for OFMSW hydrolysis as a reduction in energy and chemical requirements highly influences the process economic cost (Alvira et al., 2010). Lastly, enzyme load was found the most significant parameter (Table 3) as evident in the steeper surfaces observed in Fig. 2.A and Fig. 2.B compared with Fig. 2.C. It can be seen that RS concentration increases as enzyme load does until a certain value after which decreases. This observation suggests that the enzymes had limited access to the solid fraction so all attachment sites in the solid were occupied reaching an enzyme saturation effect, which has also been previously reported by Cabas Candama et al. (2020) for waste of fruits and vegetables. At the same time, it is important to highlight that the hydrolysis of the complex composition of OFMSW requires several enzymatic activities. For food waste, the major contributor to OFMSW, it has been shown that polysaccharides such as xylan and pectin, can interfere in the hydrolysis of cellulose and hemicellulose by masking them (de la Torre et al., 2017; Van Dyk et al., 2013). Viscozyme L contains a wide range of carbohydrases addressing these fractions, however, the design of tailor-made cocktails with ratios of enzymatic activities specific for OFMSW or with increased substrate specificity by protein engineering, might bring further the release of sugars from OFMSW (Chapman et al., 2018).

Finally, optimization of the RS concentration in the solid fraction was conducted by a numerical optimization method using the Design-Expert® software. The target was to maximize the RS concentration while keeping temperature, initial pH, and enzyme load within the study range. The optimum operating conditions were close to temperature 55°C, initial pH 3.5, and enzyme load 0.08 mL g^{-1} DM, and correspond to 157.0 mg g_i^{-1} DM. Considering that milder conditions would benefit the economy and energy balance of the process, another optimum that minimizes temperature and enzyme load was also selected. Mild conditions were temperature 25°C, enzyme load 0.06 mL g^{-1} DM, and initial pH 4.5, which correspond to a prediction of 131.9 mg g_i^{-1} DM. Both conditions, extreme and mild, were verified experimentally. Considering the inherent variability of the OFMSW, it was decided to verify the optimum conditions using batch 2 of ssOFMSW to also assess the reproducibility of the process. The experimental results obtained were 122.6 ± 13.4 mg g_i^{-1} DM for extreme conditions and 105.2 ± 9 mg g_i^{-1} DM for mild conditions. The experimental RS concentrations observed using conditions predicted by the model were lower than expected but within the 95% confidence interval for the extreme and the 99% for the mild. It should be noted that batch 2 contained considerably fewer sugars (157.7 mg g^{-1} DM) than batch 1, which might explain the lower RS concentrations achieved. These results are quite satisfactory considering the complexity of working with such a variable substrate as OFMSW (Hansen et al., 2007). This lower initial amount of sugars resulted in greater hydrolysis performance, 2.7 for mild conditions and 2.9 for the extreme.

These conditions were selected for maximizing the RS concentration in the solid fraction, however, they also led to high values in the liquid fraction (304.2 ± 9.5 mg g_i^{-1} DM for mild conditions and 339.4 ± 7.2 mg g_i^{-1} DM for extreme conditions, equivalent to concentrations of 42 and 47 g L^{-1}respectively). This can be explained because the hydrolysis of the solid fibers is a gradual process, and partially hydrolyzed fibers in the solid fraction might be solubilized during the extraction required for RS analysis. The partial hydrolyzation of fibers results in easily accessible sugars during the SSF.

Table 3
ANOVA for the response surface quadratic model when RS concentration in the solid fraction was used as a response.

Source	DF	Mean Square	*p*-value
Model	9	0.1154	0.0003*
X_1 – Temperature (°C)	1	0.0023	0.3908
X_2 – pH	1	0.0474	0.0081*
X_3 – Enzyme load (mL mg^{-1} DM)	1	0.3978	0.0001*
$X_1 \cdot X_2$	1	0.0264	0.0247*
$X_1 \cdot X_3$	1	0.0429	0.0099*
$X_2 \cdot X_3$	1	0.0739	0.0032*
X_1^2	1	0.0024	0.3867
X_2^2	1	0.0347	0.0149*
X_3^2	1	0.4175	0.0001*
Residual	5	0.0026	
Lack of Fit	3	0.0022	0.6315
Pure Error	2	0.0033	

R^2 = 98.76%, R^2 (adj) = 96.51%, R^2 (pred) = 88.40%, and C.V. = 1.11%.
*Significant parameters ($p < 0.05$).

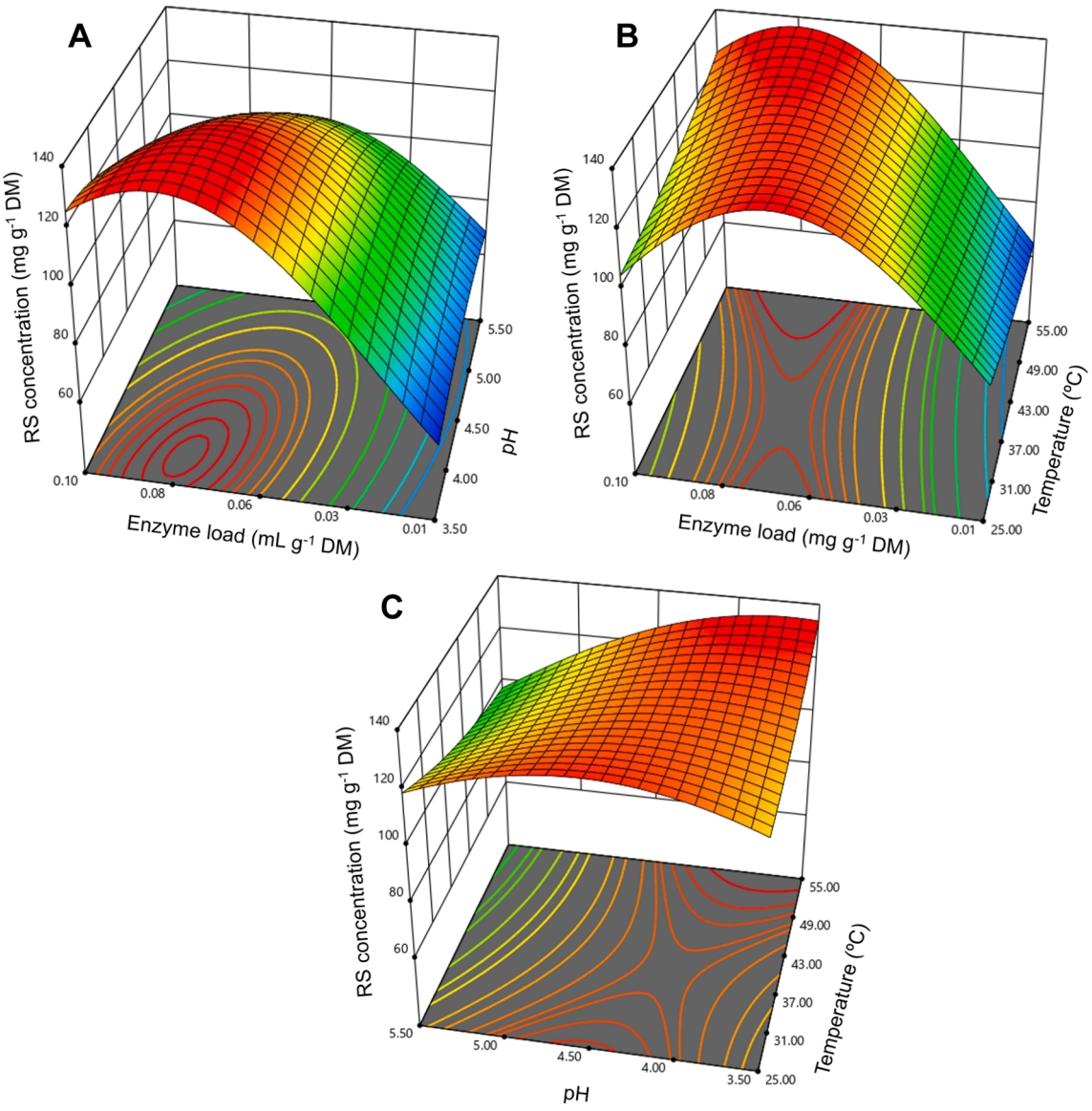

Fig. 2. Combined effect of (A) enzyme load and pH; (B) enzyme load and temperature; and (C) pH and temperature in RS concentration in the solid fraction. Other factors were at medium levels.

3.3. Assessment of the solid fraction of hydrolysis as a substrate for biopesticide production through SSF

A preliminary assessment has been performed to evaluate the capability of the obtained solid hydrolysate to support Bti growth and sporulation. Even though spore count and endotoxicity are not always proportional, spore count can be considered an indirect estimation method because during sporulation each Bt cell produces a spore that might contain toxic proteins (De Lourdes Tirado Montiel et al., 2001). The SSF operation parameters were based on the optimization performed by Mejias et al. (2020) using biowaste mixed with digestate as substrate. The performance of the solid hydrolysate fractions from extreme and mild pretreatment conditions of batch 2 was evaluated on a triplicate after 72 h. The solid fractions were characterized before the SSF in terms of DM and pH, resulting in 23.2 ± 0.9 and pH 6.3 for the mild hydrolysis and 23.9 ± 1.1 and pH 5.2 for the extreme hydrolysis conditions.

The fermentation started with no spore presence in the solid matrix and, as seen in Fig. 3, the solid hydrolysate derived from the mild conditions reached an average concentration of 1.3×10^8 CFU g^{-1} DM for spore count and 3.9×10^8 CFU g^{-1} DM for viable cell count. Viable cell value was in close agreement with those obtained by Mejias et al. (2020), but there still was potential for improvement in the sporulation ratio (33%). Conversely, the solid hydrolysate derived from the extreme conditions did not support Bti growth but resulted in a 12-fold decrease. This could be related to the low initial pH which was 5.2 compared with the 6.3 of the mild treatment. This pH value was lower than the optimum reported for Bti sporulation (7.0) and close to inhibitory (5.5) (De Lourdes Tirado Montiel et al., 2001; Özkan et al., 2003). Therefore, an additional step of pH adjustment using 1 M NaOH to neutral values after the enzymatic hydrolysis was evaluated in the SSF. Results from this SSF experiment can also be seen in Fig. 3. It is illustrated how the growth of Bti was favored by the change of pH, reaching values of viable cells of 2.5×10^7 CFU g^{-1} DM and spores of 1.6×10^6 CFU g^{-1} DM, with a 6% of sporulation ratio. Therefore, after a pH adjustment, the solid hydrolysate derived from extreme conditions also appeared as a suitable substrate for Bti growth but not as promising as the one derived from mild pretreatment conditions. Regarding the RS concentration, values at 72 h were below the detection levels of the method, which indicates consumption of over 90% of the RS. Compared to a previous work using

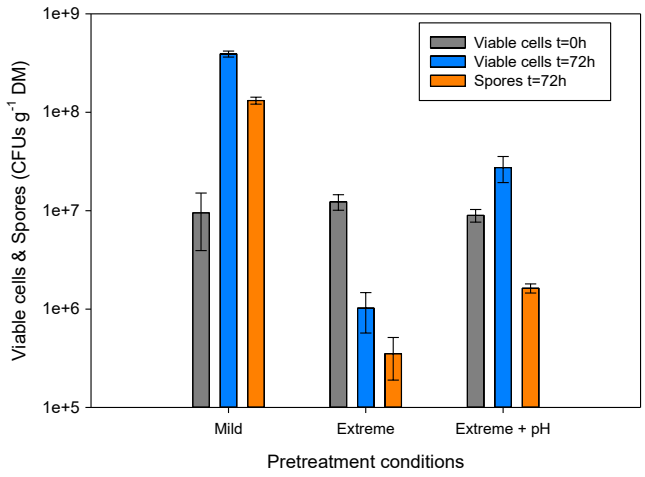

Fig. 3. Bti initial viable cells, final viable cells, and final spore concentration depending on the enzymatic treatment conditions of the solid hydrolysate. The initial spore count was 0.

non-hydrolyzed sterile OFMSW under different operation strategies, which resulted in spore counts between $3.5 \times 10^6 - 2.1 \times 10^7$ CFU g^{-1} (Ballardo et al., 2017), the spore count achieved in this first approach was one order of magnitude higher for mild conditions. Taking this into account and the fact that milder conditions are also related to less energy consumption, the economy of the process favors the hydrolysate derived from mild treatment conditions. A comprehensive material and economic balance of the process, including the utilization of the liquid fraction, would be necessary for achieving maximum profitability from all fractions derived from the enzymatic hydrolysis of OFMSW.

Overall, the OFMSW has been valorized to a sugar concentrate with great potential for fermentative processes and a solid rich in Bt spores, hence providing an alternative pathway for closing the organic matter cycle. This work contributes to the ongoing paradigm shift in waste management, fostered by the Circular Economy Action Plan of the European Commission, which aims to reduce landfilled waste to a maximum of 10% (Union, 2014). Future studies have to validate these results at larger scales and field test the pesticide action of the final product so it can be introduced in the growing global market of biopesticides, projected to reach USD 11,438.1 million in 2026 (Mordor Intelligence, 2018).

4. Conclusions

Enzymatic hydrolysis of source-separated OFMSW has been optimized reaching reducing sugars values of 365.1 mg g_i^{-1} DM in the liquid fraction and 184.11 mg g_i^{-1} DM in the solid fraction, and almost a 2-fold increase in total reducing sugars. Two optimum operational conditions were selected to evaluate the effect of the harshness of the enzymatic hydrolysis on the SSF of the resulting solid fractions. *Bacillus thuringiensis var israelensis* has been grown successfully on the solid fraction deriving from milder conditions. This finding is relevant looking towards process development and its economy. This work provides an alternative scenario for the valorization of organic municipal solid waste, producing a sugar-rich liquid with a concentration of reducing sugars of 50.56 mg mL^{-1} and a solid containing biopesticide from *Bacillus thuringiensis*.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wasman.2021.11.014.

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