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Article

Ethanol Production from Enzymatically Treated Dried Food Waste Using Enzymes Produced On-Site

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Abstract: The environmental crisis and the need to find renewable fuel alternatives have made production of biofuels an important priority. At the same time, the increasing production of food waste is an important environmental issue. For this reason, production of ethanol from food waste is an interesting approach. Volumes of food waste are reduced and ethanol production does not compete with food production. In this work, we evaluated the possibility of using source-separated household food waste for the production of ethanol. To minimize the cost of ethanol production, the hydrolytic enzymes that are necessary for cellulose hydrolysis were produced in-house using the thermophillic fungus *Myceliophthora thermophila*. At the initial stage of the study, production of these thermophilic enzymes was studied and optimized, resulting in an activity of 0.28 FPU/mL in the extracellular broth. These enzymes were used to saccharify household food waste at a high dry material consistency of 30% *w/w*, followed by fermentation. Ethanol production reached 19.27 g/L with a volumetric productivity of 0.92 g/L·h, whereas only 5.98 g/L of ethanol was produced with a volumetric productivity of 0.28 g/L·h when no enzymatic saccharification was used.

Keywords: household food waste; *Myceliophthora thermophila*; thermophilic enzymes; in house enzyme production; enzymatic saccharification; *Saccharomyces cerevisiae*; ethanol

1. Introduction

During the last century, there has been a growing deterioration of the environment due to rapid industrialization of human production. In addition, the dependence of countries on imported fossil fuels has meant that satisfaction of their energy needs is not assured. These considerations made the discovery of renewable fuels—which will be based on national resources—one of the most important priorities for most countries worldwide [1–3]. Ethanol is an excellent candidate to replace gasoline, and it has traditionally been produced mainly from sugars or starch [4,5]. The use of sugars and starch as raw materials for ethanol production had a negative effect on their availability as human food, which raised their prices globally and increased concern about the ethics of biofuel production [5–7]. This resulted in a change of focus to the use of renewable raw materials such as lignocellulosic biomass and waste. The use of wastes as raw materials for the production of biofuels not only prevents the "food *versus* fuel" dilemma; it also counteracts accumulation of these wastes in the environment.

Food waste is an interesting potential substrate, as the amount of it in the EU is growing. It has been estimated to increase from 89.3 Mt in 2006 (which resulted in the emission of 170 million tonnes of CO_2) to 126.2 Mt by 2020, according to a report released by the European Commission in 2010 [8]. Food waste can be derived from homes, manufacturing, food services, and the retail sector; domestic food waste production is the largest part of them and accounts for 42% of the total. The problem of food waste production is higher in Asia, where an increase in the total amount of annual urban food waste is expected—from 278 Mt in 2005 to 416 Mt in 2025 [9].

The disposal of large amounts of such waste can be a challenge, and cause severe environmental issues when used in landfill sites—such as uncontrolled gas emissions that contribute to the greenhouse effect, and contamination of water underground [10,11]. It has been estimated that approximately 125 m^3 of gas is produced from each ton of food waste that is used for landfill, with an average composition of 60%–65% methane and 35%–40% CO_2, which is responsible for 8% of the total anthropogenic methane emissions [12]. In many Asian countries, landfill and open dumping of food waste represent a large proportion of waste treatment methods [13]. Other practices of food waste utilization, such as feeding animals, have raised serious hygiene-related issues and the use of them as fertilizers can cause severe pollution of water [14,15]. Moreover, in recent years regulations about feeding animals with food waste have become stricter. For example, in the EU, the EC law no. 1774/2002 prohibits the use of catering waste for animal feed [9]. Finally, incineration is also a waste treatment method used for food waste. Despite the fact that energy can be produced during incineration, the capital and operating costs are considered to be too high and air pollution can also result [13]. It is obvious that these "traditional" ways of food waste manipulation have proven to be problematic from an environmental point of view and do not help in reducing their total amount.

On the other hand, the rich composition of food waste—in carbohydrates, proteins, and minerals—makes it an excellent raw material for the production of biofuels and bio-based chemicals through microbial conversion [16,17]. The advantage of this "alternative" use of food waste is not only to solve the "food *versus* fuel" dilemma and exploitation of low-cost raw materials for the production of biofuels and chemicals; it would also lead to a decrease in the use of landfill areas, minimizing greenhouse gas emissions and improving land use. Biogas production is a well-established biological process regarding production of biofuels from food wastes, and high yields of methane have already

been reported [18–20]. These good yields have resulted in the construction and operation of biogas plants, which make use of food waste as raw material, either alone or in combination with other wastes such as municipal wastewater. During the last years, there has also been increased interest in exploitation of food waste for the production of ethanol [10,15,21,22].

Another interesting use of food waste is as a raw material for the production of different kinds of enzymes. The rich and diverse composition of food wastes—which includes proteins, starch, insoluble carbohydrates, and lipids—can facilitate the production of a diverse range of enzymes. Some characteristic categories of enzymes that have already been produced from food wastes are amylases [23,24], proteases [25,26], ligninocellulolytic enzymes [27,28], lipases [29,30], and pectinolytic enzymes [31,32].

On the other hand, use of food wastes presents some challenges, such as the difficulty in separating them from the whole waste mass and their easy degradability. The rich composition of food wastes makes them easily contaminated by various microorganisms, which makes their storage and handling a considerable challenge. Moreover, the high water content of food wastes results in high volumes—which must be stored and transferred. These properties lead to difficulties in storage and a requirement for large cooling units, which in turn have a negative impact on the cost of the raw material and its availability. Separation of food waste could be improved by using a source separation system in houses. Moreover, drying of food waste *in situ* could be one possible solution to improve their stability in storage and to reduce the total volume.

Except for the readily available soluble carbohydrates, food waste also contains insoluble carbohydrates such as cellulose, which makes the inclusion of an enzymatic saccharification step important in order to liberate more glucose. Generally a pretreatment step before saccharification could improve the saccharification yields, but the presence of soluble sugars makes it unsuitable for application to food waste as the sugars can undergo degradation reactions, with formation of inhibitors [21]. Also, incorporation of a saccharification step not only facilitates an increase in soluble sugars but also leads to a decrease in the viscosity of the slurry, which in turn enables better mixing of the microorganism [33]. Thermostability of the cellulolytic enzyme(s) is an important consideration, as cellulose hydrolysis takes place at high temperatures and the enzyme should be able to maintain high levels of activity for the time required for the saccharification. For this reason, enzymes from thermophilic microorganisms are considered to be more favorable for industrial applications, due to their increased activity and stability at high temperatures [34]. Furthermore, when thermostable enzymes are used, less of them is needed—as they have higher specific activity at elevated temperatures [35], thus reducing the cost of the process. Finally, higher temperatures result in reduced viscosity of the slurry, which in turn enables the application of higher substrate consistencies—with a positive impact on process economics [36].

The filamentous fungus *Myceliophthora thermophila* is an excellent candidate for cellulolytic enzyme production, as it has a powerful "portfolio" of enzymes and presents similar growth rates to insoluble cellulose and glucose [37,38]. Moreover, despite the fact that *M. thermophila* has lower activities of extracellular cellulolytic enzymes than, for example, the hyper-producing strain *Hypocrea jecorina* (syn. *Trichoderma reesei*), which is widely used for preparation of commercial cellulolytic enzyme solutions, it has more rapid growth and is able to degrade cellulose faster [37,38]. This observation made researchers conclude that the cellulolytic system of *M. thermophila* contains unknown catalytic mechanisms that result in more efficient cellulose degradation than with *H. jecorina* [39]. For this reason, the aim of this work was to evaluate enzyme production by *M. thermophila* using source-separated

household food waste (HFW) as raw material and subsequent use of thermophilic enzymes to saccharify the HFW at high dry material consistencies in order to produce ethanol.

2. Results

2.1. Evaluation of the Production of Thermophilic Enzymes Using HFW as Raw Material

Source-separated HFW was used as raw material for the production of extracellular cellulolytic enzymes by *M. thermophila*. The HFWs used during this work contained both soluble sugars (sucrose, fructose, and glucose) and insoluble carbohydrates (cellulose, hemicellulose, *etc*.), but, surprisingly, they do not contain any starch. The presence of cellulose can trigger the expression of cellulolytic enzymes by the fungus in the culture broth. Moreover, the HFW contained soluble and insoluble protein [21] which could be used as a nitrogen source during growth and cellulase secretion. Two factors were evaluated and optimized concerning enzyme production by *M. thermophila*, namely the effect of the addition of an external nitrogen source and the concentration of the HFW.

The ability of the HFW (at a concentration of 4% *w/v*) to support the growth of the fungus and promote the secretion of cellulolytic enzymes without the addition of an external nitrogen source was evaluated by supplementing the HFW with 0.7% *w/v* ammonium sulphate. We selected an inorganic source of nitrogen, as it has already been demonstrated that inorganic nitrogen stimulates the secretion of enzymes better than organic nitrogen in *M. thermophila*, with ammonium sulphate and ammonium phosphate promoting the highest activities of extracellular xylanases [40]. As can be seen in Figure 1, extracellular cellulolytic activity was enhanced from 0.16 FPU/mL to 0.21 FPU/mL when ammonium sulphate was added, which corresponds to an increase of 31.3%. The yield obtained (activity per gram) from HFW when an external nitrogen source was added reached 5.25 FPU/g.

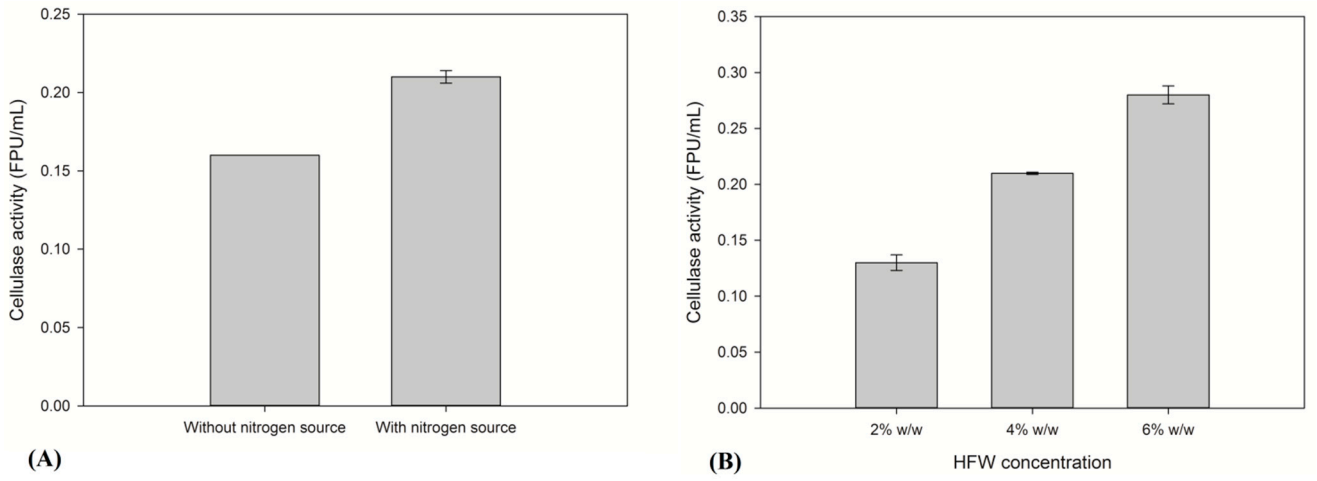

Figure 1. Effect of addition of external nitrogen source (**A**) and initial concentration of HFW (**B**) on the produced enzyme activity.

Then, the effect of the initial solids' concentration on the ability of the fungus to secrete cellulolytic enzymes was evaluated at a range of 2%–6% *w/w*. Cellulolytic activity increased with increasing concentration of HFW, and it reached 0.28 FPU/mL when 6% *w/v* HFW was used as raw material

(Figure 1). On the other hand, the yield (cellulolytic activity per gram) from HFW decreased from 6.50 FPU/g to 4.67 FPU/g when the HFW concentration was increased from 2% *w/v* to 6% *w/v*.

2.2. Saccharification of HFW and Subsequent Ethanol Production

The conditions that were found to be favorable for the production of cellulolytic enzymes by *M. thermophila* were applied in order to produce adequate amounts of extracellular broth, which would be used for the saccharification of HFW. After concentration of the broth in order to increase the activity per mL (from 0.28 FPU/mL initial activity to 3.98 FPU/mL after concentration), it was used to saccharify HFW at an initial solids concentration of 30% *w/v*. In order to evaluate the effect of the addition of the cellulolytic enzymes on ethanol production, an extra fermentation was run at the same solids' concentration without the addition of enzymes. Ethanol production when enzymes were added reached 19.26 g/L after 21 h of fermentation, which corresponds to a volumetric productivity of 0.92 g/L·h (Figure 2). Ethanol production reached 38.6% of the maximum theoretically possible based on both soluble and insoluble carbohydrates, whereas the same value based only on soluble carbohydrates was 102.7%.

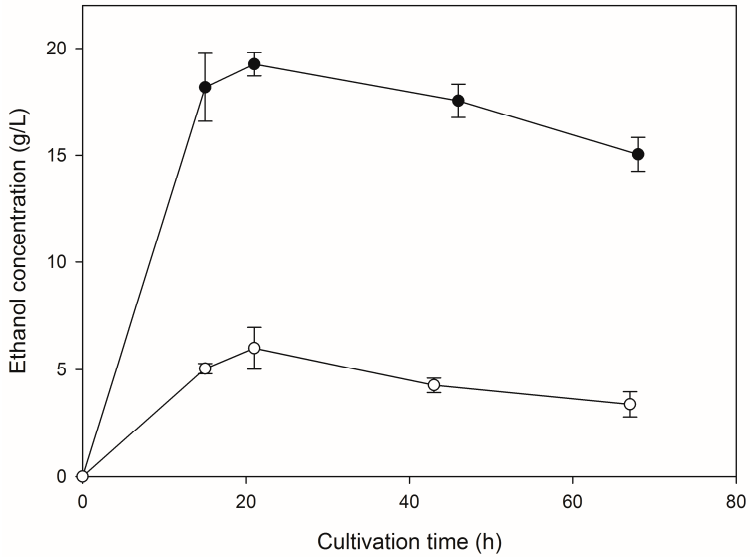

Figure 2. Ethanol production with (●) and without (○) inclusion of saccharification with the thermophilic enzyme solution.

During the control experiment without the addition of enzymes, the ethanol concentration was rather low, reaching only 5.98 g/L (which was more than three times less than when thermophillic enzymes were added) after 21 h of fermentation, which corresponds to 0.28 g/L·h (Figure 2). This ethanol concentration was equal to 11.98% of the maximum theoretically possible based on both soluble and insoluble carbohydrates, and 31.88% of the maximum theoretically possible based on soluble carbohydrates only.

3. Discussion

Hydrolysis of insoluble carbohydrates to monomeric sugars is an important step during second-generation ethanol production. This can be achieved by either acid hydrolysis or enzymatic

hydrolysis, with the latter being more preferable—as acid hydrolysis can cause degradation of sugars and is less environmentally friendly. For this reason, the use of cellulolytic enzymes is considered to be crucial during production of second-generation ethanol. On the other hand, the cost of the enzymes plays an important role in the viability of the process of second-generation ethanol production [36]. One strategy to reduce the cost of enzymes is producing them in the same place as they will be used for the saccharification of biomass. This strategy could prove to be economically beneficial as the enzymes do not need to be transferred long distances, which also minimizes the need for large cooling units to store them and concentrate them; finally, low-cost raw materials can be used [4]. Moreover, production of enzymes based on the same material as that used for ethanol production could also prove to be beneficial, as the microorganism will express and secrete the specific enzymatic activities that are necessary for better exploitation of this raw material.

During the initial stage of this work, the secretion of cellulolytic enzymes was optimized regarding the addition of an external nitrogen source and the initial solids' concentration. High enzymatic activities are necessary in order to reduce the cost of the subsequent concentration of the enzyme solution, which will be used for the saccharification of lignocellulosic biomass. It was previously found that an inorganic nitrogen source stimulates the secretion of extracellular xylanase [40], and for this reason the low-cost inorganic salt ammonium sulphate was used to supplement HFW. HFW contains a considerable amount of protein, both soluble and insoluble, which reaches a total concentration of 11.05% *w/w* [21]. On the other hand, the presence of nitrogen in organic form might not be beneficial for the production of cellulolytic enzymes. Furthermore, proteins may not be easily accessible to the fungus, as microorganisms do not have the ability to utilize all forms of proteins. Addition of ammonium phosphate enhanced the production of cellulolytic enzymes by 31.3% compared to the raw HFW. The exploitation of the raw material also improved, as the enzyme yield increased from 4 FPU/g to 5.25 FPU/g.

Despite the fact that the enzymatic activity obtained was higher than previously reported for the same fungus (strain IIS 220), which was around 0.12 FPU/mL [37], we made an attempt to further increase the activity by evaluating the effect of the concentration of HFW. By increasing the substrate consistency, higher enzymatic activities would be expected to be produced. Special care should be taken in order to not under-exploit the raw material, as the yield of enzymes per gram of substrate would be expected to fall to some extent. Moreover, a higher solids concentration could result in semi-solid or solid cultivation, which would in turn increase the difficulty in enzyme recovery. For this reason, during this work a range between 2% *w/w* and 6% *w/w* was chosen for evaluation. Enzyme activity increased with increasing substrate consistency, and reached a maximum of 0.28 FPU/g when the HFW concentration was 6% *w/v*. At this HFW concentration, the activity increased by 33.3% compared to the 4% *w/v* concentration. On the other hand, enzyme production (yield per gram of HFW) declined by approximately 11% when the HFW concentration was increased from 4% *w/v* to 6% *w/v*. This decrease in the yield of enzyme was low compared to the increase in enzyme activity, which indicates that using an HFW concentration of 6% w/v is the most favorable for efficient enzyme production.

The cellulolytic activity produced during this work is comparable to that described by other authors (Table 1) despite the fact that *M. thermophila* is considered to achieve lower extracellular activities than other microorganisms. Moreover, the cellulose concentration in HFW is lower (18.3% *w/w*) than in most lignocellulosic materials used for enzyme production, which could also result in lower yields of enzyme.

Table 1. Comparison of the enzyme activity achieved during this work with other works.

Microorganism	Raw Material	Enzyme Activity (FPU/mL)	Enzyme Yield (FPU/g)	Reference
Trichoderma asperellum	Wheat bran	n.a.	2.2	[41]
Neurospora sitophila	Steam exploded wheat straw	n.a.	6.4	[42]
Trichoderma reesei	Alkali treated sugarcane bagasse	0.09	n.a.	[43]
Trichoderma reesei	Avicel	0.16	n.a.	[43]
Fomitopsis sp.	Wheat bran	n.a.	6.8	[44]
Bacillus subtilis	Banana wastes	n.a.	2.8	[27]
Trichoderma viride	Banana peel	n.a.	5.6	[28]
M. thermophila	Household food wastes	0.28	4.7	Present work

n.a. = not available.

During the final stage of this work, we evaluated the possibility of using the *in house*-produced thermophillic enzymes for efficient HFW saccharification and then ethanol production. In order to increase the concentration of carbohydrates and subsequently increase the production of ethanol, the initial concentration of HFW was set at 30% *w/v*. At the same time, a control experiment without enzymatic saccharificaiton was also included in order to evaluate the effect of saccharification. When the thermophillic enzymes were used, ethanol production reached 19.24 g/L after 21 h of fermentation. The yield of ethanol based on soluble sugars only was 102.7% of the maximum theoretically possible. This high yield indicates that part of the cellulose was hydrolyzed and used by the yeast. The same yield based on both soluble and insoluble carbohydrates reached 38.6%. On the other hand, when no enzyme treatment was applied, the ethanol concentration reached only 5.98 g/L after 21 h of fermentation. The same yields (percentage of maximum theoretically possible) were 11.98% for both soluble and insoluble carbohydrates and 31.88% for soluble carbohydrates. The low yields obtained when no enzymatic saccharification was used underscore the importance of the enzyme treatment, as the yield even for the soluble sugars was very low. This indicates that the enzymatic saccharification not only improves ethanol production by increasing the concentration of glucose, but also—by reducing the viscosity of the slurry—facilitates the better growth of the yeast, which is a result of the collapse of the crystal structure of cellulose, giving reduced water-binding capacity [45]. Also, it has been demonstrated that during liquefaction of model municipal solid waste among the different enzymes that were tested, it was cellulases that were responsible for viscosity reduction [46]. It was also observed that ethanol concentration in both trials that ethanol concentration gradually decreased after 21 h of fermentation. This is evidence of a 'diauxic growth' of the yeast where the initially produced ethanol is observed to be re-consumed when glucose in the media is depleted [47]. Moreover this fact indicates the presence of oxygen in the medium, as the accumulated ethanol is converted back to acetaldehyde if oxygen is available [48]. Ethanol production in the presence of oxygen occurs due to the Crabtree effect. According to this phenomenon, the enzymes involved in the oxidative pathway undergo catabolite repression by the high glucose present in the media [49]. Finally, it is worth mentioning that during this work ethanol

fermentation proceeded without any form of sterilization, addition of nutrients and detoxification. All these process can contribute to the increase of the total cost and the absence of them is beneficial.

The ethanol production achieved during this work was higher than that reported using in-house cellulases from *Aspergillus* sp. to hydrolyze sugarcane bagasse, where ethanol production reached 6.98 mL/L [50]. On the other hand, Sukumaran *et al.*, also used in-house produced enzymes and reported ethanol production of 12.34 g/L and 25.56 g/L when alkali-pretreated rice straw hydrolysates where concentrated at a final reducing sugar concentration of 60 g/L and 120 g/L, respectively. However, the actual concentration of reducing sugars before concentration was lower (26.30 g/L), which would result in a lower ethanol concentration [51]. On the other hand, to the best of our knowledge, there is no report where food wastes have been saccharified with cellulolytic enzymes that have been produced in-house. Despite the fact that in other works commercial enzyme solutions were used, ethanol production achieved during this work was higher than the one reported by Walker *et al.* [52] which was 8 g/L. On the other hand, similar results (23.3 g/L) were obtained by Cekmecelioglu and Uncu [22] with the application of a mixture of commercial cellulases and amylases. Some other research works reported higher ethanol concentration, like the one of Moon *et al.* [10] where 29.1 g/L was produced. On the other hand, in many works, the food waste used is rich in starch which is more hydrolysable compared to cellulose (which was the main insoluble carbohydrate of the food wastes used during this work).

One of the advantages of the process proposed during this work is the use of in-house produced enzymes in order to reduce the cost of enzyme application during ethanol production. Enzyme cost still remains high and, as was previously reported by Klein-Marcuschamer *et al.* [53], cellulolytic enzymes can contribute up to $1.47 /gal of cellulosic ethanol. If the necessary enzymes could be efficiently produced in-house, this could have a positive impact on the process cost.

4. Materials and Methods

4.1. Raw Materials and Microorganisms

The HFWs used during this work were kindly donated by Prof. Maria Loizidou, Unit of Environmental Science and Technology (UEST), National Technical University of Athens, Greece. They were separated at source and collected from houses in the Papagos-Cholargos Municipality in Athens, Greece, and were dried *in situ* in a prototype drier, which was developed by UEST [54]. After drying, they were milled to particles of less than 3 mm using a small laboratory mill. The composition of the dried HFW was as follow (% *w/w*): Glucose, 4.39; fructose, 3.47; sucrose, 4.38; soluble protein, 0.54; fats, 11.91; insoluble protein, 10.51; pectin, 3.92; cellulose, 18.30; hemicellulose, 7.55; klason lignin, 2.16; ash, 11.03 [21].

Myceliopthora thermophila (syn *Sporotrichum thermophile*; ATCC 42464) was used for production of the thermophilic enzymes.

4.2. Cultivation of Myceliophthora Thermophila

M. thermophila was maintained in petri dishes containing medium consisting of 3.9% *w/w* PDA (potato dextrose agar) and 0.2 *w/w* yeast extract. Before cultivation for enzyme production, the fungus was sub-cultured in an 250 mL Erlenmeyer flask containing 50 mL of pre-culture medium with the

following composition: Corn cobs, 30 g/L; $NH_4H_2PO_4$, 7g/L; KH_2PO_4, 3 g/L; K_2HPO_4, 2 g/L; $MgSO_4 \cdot 7H_2O$, 0.5 g/L; $CaCl_2 \cdot 2H_2O$, 0.1 g/L; $FeSO_4 \cdot 7H_2O$, 5mg/L; $MnSO_4 \cdot 4H_2O$, 1.6 mg/L; $ZnSO_4 \cdot 7H_2O$, 1.4 mg/L; and $CoCl_2 \cdot 6H_2O$, 0.2 mg/L. The pH of the medium was 5 and the incubation took place for 48 h at 47 °C with agitation at 200 rpm.

4.3. Enzyme Production and Concentration

Growth of *M. thermophila* for optimization of enzyme production took place in 250 mL Erlenmeyer flasks containing 100 mL medium with the same composition as pre-culture medium, except for the carbon and nitrogen source. As carbon source, different concentrations of HFW were used, and (when used) the nitrogen source was ammonium sulphate. Cultivation took place for 6 days at 47 °C and 200 rpm, after inoculation with 5% *v/v* pre-culture broth. Samples were taken at different time intervals and centrifuged to remove solids. The supernatant was collected and analyzed for cellulase activity. All the trials were performed in duplicates.

In order to produce sufficient quantities of enzyme solution, the optimum conditions were used during growth of the fungus in a 20 L MBR cylindrical bioreactor with a working volume of 16 L. The aeration was adjusted so that the dissolved oxygen was maintained above 20% of saturation and the agitation was 150 rpm. At the end of cultivation, the culture supernatant was collected and centrifuged to remove the solids and the fungal biomass. Finally, the clarified supernatant was concentrated with ultrafiltration membranes (cut-off 10 KDa; Amicon).

4.4. Saccharification and Ethanol Fermentation of HFW

Saccharification took place in 100 mL Erlenmeyer flasks containing 25 g of 30% *w/v* HFW slurry, at 60 °C and 200 rpm for 8 h. The pH of the slurry was adjusted to 5.5 using 50 mM citrate-phosphate buffer. The enzyme loading was 10 FPU/g HFW. At the end of the saccharification, the slurry was left to cool down at room temperature and inoculated with dry baker's yeast (Jotis, Athens, Greece) at a concentration corresponding to 15 mg/g solids. Ethanol fermentation took place in 100 mL erlenmeyer flasks containing 25 g of hydrolyzed food wastes, at 30 °C and 100 rpm. Microanaerobic conditions were maintained by adjusting a plastic cap on the flasks. At certain time intervals, samples were withdrawn and the solids were removed by centrifugation. Then, the supernatant was used for ethanol determination.

When no enzymatic saccharification was included, inoculation with yeast took place directly in the slurry which was previously kept at 60 °C for 8 h. It is worth mentioning that the fermentations took place at non-sterilized conditions. All the trials were performed in duplicates.

4.5. Analytical Methods

Total cellulase activity was measured according to the standard filter paper assay [55]. Prior to ethanol quantification, samples were filtered through 0.45 μm filters. Ethanol concentration was determined using an HPLC apparatus equipped with an RI (refractive index) detector. This was done using an Aminex HPX-87H column (300 mm × 7.8 mm, particle size 9 μm; Bio-Rad, Hercules, CA, USA) working at 40 °C with 5 mM H_2SO_4 as the mobile phase, at a flow rate of 0.6 mL/min.

5. Conclusions

We have demonstrated the possibility of producing thermophilic enzyme solution from source-separated HFW. Use of thermophilic enzymes during cellulose hydrolysis provides several benefits, whereas the in-house production of cellulases could improve the economics of the process. During fermentation of a high dry material concentration of HFW, the use of thermophilic enzymes enhanced ethanol production by a factor of more than 3.

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Author Contributions

Both authors contributed jointly to all aspects of the work reported in the manuscript. Both authors have read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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