Conesa Domínguez, C.; Seguí Gil, L.; Fito Maupoey, P. (2017). Hydrolytic performance of Aspergillus niger and Trichoderma reesei cellulases on lignocellulosic industrial pineapple waste intended for bioethanol production. Waste and Biomass Valorization. 1-10. doi:10.1007/s12649-017-9887-z

Hydrolytic performance of *Aspergillus niger* and *Trichoderma reesei* cellulases on lignocellulosic industrial pineapple waste intended for bioethanol production.

Claudia Conesa, Lucía Seguí*, Pedro Fito

Institute of Food Engineering for Development (IUIAD) – Universitat Politècnica de València, Camino de Vera s/n, 46022 Valencia, Spain

*Corresponding author. Tel.: +34 96 387 70 00 Ext. 73676

E-mail address: lusegil@upvnet.upv.es

ABSTRACT

Purpose

The hydrolytic action of *Aspergillus niger* and *Trichoderma reesei* commercial cellulases, alone or combined with *A. niger* hemicellulase, against industrial pineapple waste as a previous step to produce bioethanol was investigated.

Methods

Enzymatic hydrolysis experiments were conducted in static conditions in an incubation oven, by adding the corresponding enzyme mixture to the pineapple waste (combinations of 0, 0.1, 0.2 and 0.4 (w/w) of cellulase from *A. niger* or *T. reesei* and hemicellulase from *A. niger*). pH and total soluble solids were examined along the treatments, and the sugar profile in the final hydrolysates was evaluated by High-Performance Anion-Exchange Chromatography.

Results

T. reesei cellulase exhibited a significantly faster initial hydrolysing rate than *A. niger* cellulase (0.258±0.004 *vs.* 0.15±0.07, for the maximum enzyme concentrations assayed), although differences regarding soluble sugars increments were not significant at the end of the treatment (0.349±0.009 *vs.* 0.34±0.05). Glucose, fructose, sucrose, arabinose, xylose and cellobiose were identified in the hydrolysates. Increasing enzyme concentration (cellulase or hemicellulase) produced an increase in total and fermentable sugars released (17 and 11%, respectively, for the maximum enzymatic concentration assayed); besides, a synergistic effect of combining hemicellulase and cellulase was identified. Accumulation of cellobiose (up to 4.4 g/L), which may slow down hydrolysis, evidenced the weaker β-glucosidase activity of *T. reesei* cellulase. Due to its performance and the lower cost of the enzyme, *A. niger* cellulase was chosen as an alternative.

Conclusions

Commercial *A. niger* cellulase represents an efficient alternative to *T. reesei* cellulase for the saccharification of industrial pineapple waste, especially when combined with a hemicellulase. Total sugars present in the final hydrolysates indicated that *A. niger* cellulase performed similarly at a lower cost, with no cellobiose accumulation. However, if processing time is a limiting factor, *Trichoderma reesei* cellulase could be the one preferred.

Keywords: pineapple waste, lignocellulosic biomass, enzymatic hydrolysis, cellulases, bioethanol.

INTRODUCTION

At present, the search for renewable, sustainable and environmentally-friendly energy sources is encouraged so as to face the need for energy supply and in response to climate change [1]. In this context, bioethanol is recognized worldwide as an alternative to petroleum-derived transport fuels [2]. However, competition for food resources has made first generation bioethanol production a controversial issue, for which much of recent research has been focused on second generation bioethanol. Among the raw materials available to produce the so-called second generation of biofuels, those obtained from agricultural, forestry or industrial wastes are characterized for being low-cost raw materials that do not compete with food supplies or threaten biodiversity. The use of residues or waste feedstocks for energy production continues to be an interesting issue due to growing energy demand, depletion of fossil fuels, greenhouse emissions and climate change concern, among other reasons. On the other hand, residual biomass needs to be properly disposed in order to reduce its environmental impact, for which there is a great interest in giving added value to these waste materials. The agro-food industry is particularly characterized by generating significant amounts of organic residues. This is the case of the pineapple industry since industrial pineapple waste (peel, core and crown) generally accounts for at least 50% of the total pineapple weight [3], 20 to 40% in the form of peel and core [4]. Disposal of this waste is of capital importance for the industry due to its high biochemical and chemical oxygen demand [5]; therefore, valorisation of these residues would contribute both to facilitate their disposal and to obtain a benefit from an otherwise non-valuable material.

Residual biomass mostly consists of lignocellulose, which contains large quantities of sugar polymers such as cellulose and hemicellulose [6]. According to Roda et al. [7], pineapple peel waste is made of 35-50% cellulose, 20-35% hemicellulose, and 5-30% lignin. Manisha et al. [8] have recently reported a content of 35% cellulose, 19% hemicellulose, and 16% lignin on dry basis. Therefore, industrial pineapple waste is a good candidate for biofuels or other metabolites production, since cellulose and hemicellulose are polymers potentially hydrolysable to simple fermentable sugars.

Producing ethanol from lignocellulosic biomass requires the hydrolysis of part of the cellulose and hemicellulose to fermentable sugars [9]. Among the hydrolysis treatments, enzymatic hydrolysis has a lower utility cost than acid or alkaline hydrolysis, since the hydrolysis is conducted at milder conditions [10]. Together with sulphuric acid, cellulolytic enzymes are the major hydrolysers of cellulose and hemicelluloses, and

present fewer disadvantages than the use of chemicals. Filamentous fungi segregate two types of enzymatic complexes that can hydrolyse the lignocellulosic matrix: cellulases that hydrolyse the crystalline cellulose into small oligosaccharides and then into glucose; and hemicellulases, which hydrolyse the hemicellulose into monomeric sugars. Fungal cellulase is made up of three major groups of cellulases: endoglucanases, cellobiohydrolases or exoglucanases and β-glucosidases [11]. Among the enzymes produced by fungi, the enzymes derived from *Trichoderma reesei* represent the best characterized and have been assayed for the enzymatic saccharification of lignocellulosic materials [12]. *T. reesei* cellulase has been said to be the most productive and powerful destroyer of crystalline cellulose; however, it has also demonstrated a relatively weak β-glucosidase activity for which the reaction from cellobiose to glucose has been shown to be slow [13, 14]. *Aspergillus niger* cellulase has also been tested in some lignocellulosic raw materials [14, 15, 16, 17]. Although not such hydrolytic power have been attributed to it, it could represent an efficient alternative to *T. reesei* cellulase. In addition, enzyme costs can be significantly reduced with the use of *A. niger* cellulase, since some of the commercial options available are less expensive. On the other hand, the combined use of a cellulase and hemicellulase could potentially increase the final amount of sugars available for fermentation, since hemicellulase would partially cleave hemicellulose bonds to yield monomeric sugars such as glucose or xylose.

Therefore, the aim of the present study was to analyse and compare the hydrolytic action of both cellulases, *Aspergillus niger* and *Trichoderma reesei*, against industrial pineapple waste as a previous step to produce bioethanol. Additionally, the synergistic effect of combining them with *A. niger* hemicellulase is discussed. Finally, optimum condition of *A. niger* cellulase hydrolysis were determined.

MATERIALS AND METHODS

Raw material and pre-treatment of pineapple waste

Pineapple fruits (*Ananas comosus* [L.] Merr., MD-2 cv.) were selected based on external factors such as the absence of injuries, ripeness and weight. The crown was first removed, and the pulp was separated from the rest of the fruit (peel and core) by using a pineapple cutter. The waste material (crown, peel and core) was cut into smaller pieces and then physically treated by grinding it in a blender in order to decrease particle size and increase cellulose and hemicellulose accessibility. After grinding, pH was adjusted

to 5 by adding $Ca(OH)_2$. The resulting product was then thermally treated in an autoclave at 121 ºC for 20 min.The grinded and sterilized material was finally frozen and kept at -22 °C until the experiments were conducted.

Characterization of the pineapple waste liquid phase

Pineapple waste was characterized in terms of pH, total soluble solids (TSS) and sugars present in the waste liquid phase. The pH was measured with a digital pH meter (Mettler Toledo Inlab) and the TSS (º Brix) were measured by refractrometry (table-top ABBE-Atago refractometer thermostated to 20 °C). The sugars present in the liquid phase of the grounded waste were measured by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detector (HPAEC-PAD), using a Metrohm IC chromatograph system equipped with a 716 Compact module and an ICnet 2.0 software program. A three-step PAD setting was used with the following path intervals (ms) and potentials (V): t_1: 400/E_1 = +0.05 (detection); t_2: 200/E_2 = +0.75 (cleaning); t_3: 400/E_3 = -0.15 (regeneration). The column used was a Metrosep Carb 1 250/4.6 column (250 mmL x 4.6 mmID) coupled to a guard column. Analyses were done at 32 ºC, 8.8 MPa, injection volume: 20 µL and using sodium hydroxide 0.1 M as the mobile phase (1 mL/min). Liquid phase was previous filtered (0.45 µm nylon filter) and diluted (1:2000 v/v in bidistilled water). Glucose, fructose and sucrose standards (Sigma-Aldrich, Co.; purity ≥ 99.5%) were used to prepare standard calibration curves (2.5, 5, 10, 15, 25 and 50 ppm). Determinations were carried out in triplicate.

Enzymatic hydrolysis experiments

Cellulase from *Aspergillus niger* (1.08 U/mg solid; Sigma-Aldrich, Co.) and *Trichoderma reesei* ATTC 26921 (6 U/mg solid; Sigma-Aldrich, Co.) were combined with *Aspergillus niger* hemicellulase (5 U/ mg solid; Sigma-Aldrich, Co.) at the following concentrations: 0, 0.1, 0.2 and 0.4% (w/w). The corresponding enzyme mixture was added to 50 g of thawed pineapple waste in a 100 mL beaker, and samples were placed in an incubation oven at 40 °C, where enzymatic hydrolysis was performed in static conditions for 24 h. Conditions of pH and temperature were fixed according to information sheet of supplier, whereas duration was based on preliminary studies. Experiments were conducted in triplicate.

The evolution of the TSS and pH values was registered at hourly intervals for the first seven hours and at the end of the experiment (24 h). Sugars present in the final hydrolysate

(24 h) were determined by HPAEC-PAD as stated previously. Arabinose, glucose, xylose, fructose, sucrose and cellobiose standards (Sigma-Aldrich, Co; purity ≥99.5%) were used in order to identify and quantify the sugars released. Standard curves were prepared from dilutions of these standards (2.5, 5, 10, 15, 25 and 50 ppm). Determinations were performed in duplicate.

Optimum conditions for A. niger cellulase performance against pineapple waste

After the enzymatic hydrolysis experiments, *A. niger* cellulase was selected for further investigations, with the aim of determining the optimum conditions for *A. niger*. An experimental design with two independent variables (pH and temperature) at three levels (pH = 4, 5 and 6; T = 40, 50 and 60 ºC) was performed. Pineapple blended waste was saccharified with 0.4% (w/w) of cellulase and 0.2% (w/w) of hemicellulase from *A. niger* at the above mentioned pH and temperatures. Sugar profile was measured at the end of the saccharification process (24 h).

The data were analyzed by multiple regressions to fit second-order polynomial regression models for total sugars containing the coefficient of linear, quadratic and two factor interaction effects (Equation 1).

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^{\,2} + \sum_{i<j}^{k} \beta_{ij} x_i x_j + e \qquad \text{(Eq. 1)}$$

Where, Y is the response parameter (total sugar yield), β_0 is the intercept value, β_i (i = 1, 2, …, k) is the first order model coefficient, β_{ij} is the interaction effect and β_{ii} represents the quadratic coefficient of x_i. x_i and x_j are the independent variables that influence the response parameter (pH and temperature) and e represents the random error.

Statistical analysis

Statgraphics Centurion XVI (Manugistics Inc.; Rockville, MD, USA) was used for statistical analyses. One-way and multifactor analyses of variance (ANOVA, 95% confidence level) were performed on the data obtained.

RESULTS AND DISCUSSION

Characterization of the raw material (industrial pineapple waste)

Glucose (27 ± 2 g/L), fructose (22 ± 2 g/L), and sucrose (20 ± 3 g/L), were the sugars identified in the liquid phase of pineapple waste. The amount of total simple sugars obtained (69 ± 1 g/L) was similar to the reported by Abdullah and Mat [5], and lower than the reported by Ban-Koffi and Han [16]. Differences were probably due to processing and/or raw material characteristics. Total soluble solids, measured as Brix degrees, were 10.6 ± 0.2. Pineapple waste had an acid pH (3.63 ± 0.01) as a result of the different organic acids present in the liquid phase [7].

Kinetics of the hydrolysis

Kinetics of saccharification were studied by measuring the increase in total soluble solids present in the liquid phase (Δz_s) during the treatments. Results are given in figure 1. Each graph (A, B, C, D) corresponds to a different concentration of hemicellulase, while different concentrations and origin of cellulase are plotted within a graph. As it is deduced from the plots, the rate of enzymatic hydrolysis was faster during the first two hours of saccharification, fell in the following hours and was negligible at 24 h of enzymatic treatment, indicating the end of the process. Increasing enzyme concentration (cellulase and hemicellulase) increased the hydrolysis rate as well as the final result of hydrolysis.

As can be deduced from the plots, *T. reesei* cellulase implied a faster initial hydrolysis rate, although significant differences between *T. reesei* and *A. niger* cellulases were not as manifested at the end of the treatment. Variation in the soluble solids content of the liquid phase followed a hyperbolic type pattern (figure 1). In order to determine the effect of the different enzyme combinations and particularly the cellulase source in the initial rate of hydrolysis, an adaptation of the empirical model proposed by Peleg [19] was used. This model has been used to describe different processes that respond to this type of curves and it has not been deduced from any law or physical principle [20]. Thus, the evolution of the increment in total soluble solids in the liquid phase (Δz_{ss}) along the enzymatic process was fitted to equation 2, in which the inverse of the constant K_1 corresponds to the initial rate of the reaction (R_0), and the inverse of the constant K_2 represents the asymptotic value of the curve ($\Delta z_{ss\ assymptote}$). Data were fitted to the model by non-linear regression using Statgraphics Centurion XVI. Results (R_0, $\Delta z_{ss\ Pred.}$, R^2) as well as the experimental value at 24 h ($\Delta z_{ss\ Exp.24\ h}$) are shown in table 1.

$$\Delta z_{ss} = \frac{t}{K_1 + K_2 \times t} \quad (2)$$

Where $R_0 = 1/K_1$ and $\Delta z_{ss\ predicted} = 1/K_2$

The determination coefficients (table 1) indicate a good agreement between experimental ($\Delta z_{ss\ experimental}$) and predicted ($\Delta z_{ss\ predicted}$) values. The coefficients were generally greater when cellulase from *T. reesei* was employed, for which it was deduced that the model fitted better these data. Multifactor ANOVA indicated that *T. reesei* and *A. niger* cellulases have a statistical significant effect on the initial rate of the hydrolysis (p-value < 0.05). R_0 values were higher when cellulase from *T. reesei* was used, indicating a faster initial reaction rate of this enzyme, which is in line with the greater hydrolytic power attributed to this enzyme in the literature [21]. However, differences between both cellulase types were not maintained at the end of the experiments according to the experimental values after 24 h of hydrolysis ($\Delta z_{ss\ Exp.24h}$). Therefore, the present results suggest that both enzyme combinations may lead to a similar increase in the TSS content after 24 h of hydrolisis, despite the lower initial saccharification rate of *A. niger* cellulase.

As for pH, it decreased as the enzymatic hydrolysis proceeded, this reduction being faster in the first two hours of saccharification, concurrently to sugar release. The action of both cellulases, *A. niger* and *T. reesei*, had a statistically significant effect on pH (p-value < 0.00); nevertheless, lower pH values were obtained when *A. niger* cellulase was used (Figure 2).

Sugar profile of the hydrolysates

Total soluble solids is an indirect measure of the amount of sugars present in the hydrolysate; however, in order to obtain more accurate results, the specific sugars present in the final hydrolysate (24 h) were identified and quantified by ion exchange chromatography. Results of this analysis are summarized in table 2.

Lignocellulosic hydrolysates are characterized by presenting a variety of sugars. Among them, six-carbon sugars (hexoses) such as glucose or fructose and five-carbon sugars (pentoses) such as xylose and arabinose are usually present if both cellulose and hemicellulose have been hydrolysed [13]. In this particular case, and apart from the sugars identified in the pineapple waste (glucose, fructose and sucrose), arabinose, xylose and cellobiose were present in the hydrolysates.

The yeast *Sacharomyces cerevisiae*, which is naturally able to ferment sucrose, fructose and glucose, is one of the most effective and well-known ethanol producing microorganisms. It is successfully employed at industrial scale, allowing for high ethanol

productivity, since it has high tolerance to ethanol and to inhibitors normally present in lignocellulosic residues [13, 22, 23]. Since sucrose, fructose and glucose are naturally fermented by *S. cerevisiae* and other ethanologenic microorganisms such as *Zymmomonas mobilis*, they are frequently grouped under the concept of fermentable sugars. Fermentable sugars significantly increased when increasing both enzymes concentration (cellullase and hemicellulase) to the pineapple waste (Table 2). In particular, *T. reesei* cellulase exhibited a higher hydrolytic power at lower enzyme concentrations. In contrast, enzyme concentration effect was more significant for *A. niger* cellulase so that increasing the enzyme concentration up to 0.4 resulted in a similar fermentable sugars content in both hydrolysates (*T. reesei* or *A. niger*). In all cases, the addition of hemicellulase had a positive impact in fermentable sugars release.

Addition of both cellulases, *A. niger* or *T. reesei*, had a statistically significant effect on the release of glucose (p-value < 0.05), although glucose content in the hydrolysates was significantly higher when *A. niger* cellulase was used for saccharification. This is evidenced in figure 3, were main differences between *A. niger* and *T. reesei* performance have been plotted. During cellulose hydrolysis glucose is released due to a synergistic action of three fungal cellulases [12, 13]: 1) the endoglucanases, which act by randomly hydrolysing the internal glycosidic linkages of the cellulose chain; 2) the cellobiohydrolases, also known as exoglucanases, which act on the ends of the chains, releasing glucose monomers, cellobiose and oligosaccharides with a low molecular weight; and 3) the β-glucosidases, which convert cellobiose into glucose [6]. In addition, enzymatic hydrolysis of hemicellulose also releases glucose, among other sugars.

Apart from the hydrolysis phenomenon, a decrease in sucrose concentration together with an increase in fructose and glucose contents was also observed, which suggested the inversion of this dimmer. As shown in figure 3, sucrose decrease was more evident for *A. niger* cellulase as compared to *T. reesei* cellulase, this contributing to a more significant increase in the glucose content. Since the enzymes used in the present work are not expected to promote sucrose inversion, it is postulated that acid pH was responsible for this inversion [5, 18]. This is reinforced by the fact that lower pH values were registered when *A. niger* cellulase was used (Figure 2).

T. reesei cellulase has been claimed to be the most productive and powerful destroyers of crystalline cellulose [13]. However, *T. reesei* cellulases present a relatively weak β-glucosidase activity and the reaction from cellobiose to glucose has been shown to be slow [10, 24, 25]. In addition, cellobiose has a strong inhibition towards endo and

exoglucanases so that the accumulation of this dimmer significantly slows down the hydrolysis process, leading to a non-desirable result. In fact, supplementation of β-glucosidase is sometimes recommended due to its insufficient amount in *T. reesei*, in order to avoid cellulases inhibition due to cellobiose accumulation [13]. Therefore, cellobiose accumulation is an important phenomenon to evaluate. The enzymes assayed in the present work had a statistically significant effect on cellobiose yield (p-value<0.05). It was observed that if low cellulose concentrations were used, (0.1% for both cellulases), cellobiose started to accumulate in the hydrolysates (figure 3). Then, including hemicellulase in the mixture or increasing the amount of cellulase added to the medium promoted cellobiose depletion. The latter phenomenon was more noticeable in the case of *A. niger* cellulase, cellobiose completely dissapearing at the higher cellulase concentration assayed. In line with the literature, the amount of β-glucosidase in *T. reesei* cellulase was lower than the needed for the efficient hydrolysis of cellobiose into glucose, as compared to *A. niger* cellulase. The fact that *T. reesei* cellulase tends to accumulate more cellobiose could be responsible for a slowdown of the hydrolytic process, due to inhibition of endo and exoglucanases, despite the faster initial hydrolysis rate of this cellulase.

As said, xylose and arabinose are pentoses that may appear in the hydrolysates as a result of hemicellulose hydrolysis. Although pentoses are not fermentable by *S. cerevisiae*, some yeast have been reported to be efficient in xylose conversion into ethanol, such as *Pichia stipitis*, *Candida shehatae, Candida parapsilosis* and *Pachysolen tannophilus* [13, 26]. In addition, several genetic engineered strains of *S. cerevisiae* capable of metabolizing pentoses have already been developed [22, 27]. Among bacteria, *Klebsiella oxytoca* is able to grow either on hexoses or pentoses, as well as on cellobiose and cellotriose [28]. Likewise, *Escherichia coli* is naturally able to use a variety of sugars, for which work has been focused on selectively produce ethanol and increase ethanol tolerance [29]. As for *Zymomonas mobilis*, it is naturally able to ferment glucose, fructose and sucrose, producing ethanol at high yields. It has also been engineered to successfully co-ferment xylose and arabinose [29, 30, 31].

As compared to fermentable sugars, xylose and arabinose were present at significant lower concentrations in the hydrolysates. On the other hand, although it is hemicellulase that hydrolyses hemicellulose into monomeric sugars, results indicated that the addition of cellulases from *A. niger* or *T. reesei* (p-value < 0.05) had a statistically significant effect on pentose release. Moreover, when cellulase was not added to the ground material

neither arabinose nor xylose were detected, suggesting that cellulose hydrolysis promoted hemicellulase action. This was also confirmed the other way around, since hemicelluloses hydrolysis also enhanced cellulose accessibility. This synergistic effect of both enzymes, was probably a consequence of the increased accessibility of the structure thanks to the combined action of both enzymes. Some differences in the amount of xylose and arabinose released to the medium were also found depending on cellulase origin. On the other hand, xylose beings the main constituent of the xylan linear chain, more xylose than arabinose was obtained in all cases.

As for total sugars, which included the six sugars identified, both cellulases and hemicellulase had a significant positive effect on total sugars release (p-value < 0.05). Again, substantial differences between both cellulases are found at low enzyme concentrations, whereas increasing the amount of cellulase up to 0.4 (w/w) and combining it with hemicellulase, led to not significant differences.

Optimum conditions for A. niger cellulase performance against pineapple waste

According to the present results, no significant differences are obtained after 24 h of hydrolysis when *A. niger* or *T. reesei* cellulases are used, mainly if combined with hemicellulase. In addition, cellobiose accumulation was more evident when using *T. reesei* cellulase. This, together with the fact that the commercial *T. reesei* cellulase used in the experiments was significantly more expensive than the *A. niger* cellulase assayed, *A. niger* cellulase was chosen as the best option to perform the enzymatic hydrolysis step of pineapple waste for bioethanol production. Therefore, the optimum conditions (pH and T) for its performance were assayed as explained in the materials and methods section.

Individual sugars were identified and quantified at the different conditions assayed in order to obtain the total sugars values. Total sugars released to the medium were fitted to equation 1 which yielded the following equation (eq. 3). Statistical analysis of the fitting (table 3) indicated that both factors (T and pH), as well as their interactions were significant (p-value < 0.05). The determination coefficient indicates that the model obtained explains 83.6% of the variability in total sugars yield. All coefficients had a positive effect on total sugar release (figure 4). Enzyme action was significantly more affected by pH than temperature, since pH x pH and pH were the most important effects, followed by pH, Temperature, Temperature x pH and Temperature x Temperature. In order to estimate the optimum conditions for the pineapple waste saccharification by *A. niger* cellulase, three dimensional response surface curves (Figure 4) were used. Results

indicated that enzyme performance is enhanced at the 4-5 pH interval, as compared to pH 5-6, where a significant reduction in total sugar's release is observed. Increasing temperature up to 50 ºC also yielded better results. According to the surface curves analysis, 50 ºC and pH = 4.9 were the optimum condition for *A. niger* cellulase, which is in the range of most fungal cellulases [32, 33, 34].

$$\text{Total sugars release (g/L)} = -168.351 - 5.42571 \cdot \text{Temperature (℃)} + 139.256 \cdot \text{pH} + 0.0371494 \cdot \text{Temperature (℃)}^2 + 0.656445 \cdot \text{Temperature (℃)} \cdot \text{pH} - 17.5523 \cdot \text{pH}^2 \quad \text{(Eq. 3)}$$

CONCLUSIONS

Pineapple processing generate significant amounts of residues that need to be properly disposed in order to meet environmental requirements. The use of this waste material for the production of bioethanol is a good opportunity to give added value to this residue of the pineapple industry, in the context of the second generation of biofuels. In order to obtain fermentable sugars from this residual biomass, two different commercial cellulases (*Trichoderma reesei* and *Aspergillus niger*) have been assayed, either alone or combined with hemicellulase from *A. niger*. The increase in the total soluble solids present in the hydrolysate indicate that, in spite of exhibiting a significant slower initial hydrolytic rate than *Trichoderma reesei* cellulase, *Aspergillus niger* cellulase may lead to a similar amount of soluble solids present in the final hydrolysate. This trend has been also confirmed when analysing the specific sugars released to the medium, especially when combined with the enzyme hemicellulase. The fact that *T. reesei* cellulase tend to accumulate more cellobiose, could be responsible for a slowdown of the hydrolytic process, despite the faster initial rate. On the other hand, a synergistic effect of combining both cellulase and hemicellulase enzymes has been proven, since the addition of one enzyme conditioned the action of the other one.

In conclusion, this study shows that commercial *A. niger* cellulase represents an efficient alternative to *T. reesei* cellulase for the saccharification of industrial pineapple waste, especially when combined with a hemicellulase. Total sugars present in the final hydrolysates indicated that *A. niger* cellulase performed similarly at a lower cost, with no cellobiose accumulation. However, if processing time is a limiting factor, *Trichoderma reesei* cellulase could be the one preferred.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the financial support of the Universitat Politècnica de València FPI grant programme.

REFERENCES

1. EC of European Parliament and of the Council, Directive 2009/28/ of 23 April 2009 on the promotion of the use of energy from renewable sources, Official Journal of the European Union L 140/64, 5.6 (2009).
2. Demirbas, A. Production of Fuels from Crops. In: The Biofuels Handbook. J. G. Speight (Ed.), RSC Publishing, Cambridge, UK pp. 201-227. (2011)
3. Ketnawa, S., Chaiwut, P. and Rawdkuen, S. Pineapple wastes: A potential source for bromelain extraction, Food Bioprod. Process. 90, 385-391 (2012)
4. Nga, N.T. and Trang, N.T. Influence of the fermentation of pineapple wastes with the use of methanobacterium strains separated in Vietnam on the production of biogas from them. J. eng. phys. thermophys. 88 (2), 1204-1208. (2015).
5. Abdullah, A. and Mat, H. Characterisation of solid and liquid pineapple waste. Reaktor. 12, 48-52. (2008)
6. Fujii, T., Fang, X., Inoue, H. and Murakami, K., and Sawayama, S. Enzymatic hydrolyzing performance of *Acremonium cellulolyticus* and *Trichoderma reesei* against three lignocellulosic materials. Biotechnol. Biofuels. 24, 1-8. (2009).
7. Roda, A., Faveri, D.M., Dordoni, R., Lambri, M. Vinegar production from pineapple wastes - preliminary saccharification trials. Chem. Eng. Trans. 37, 607- 612. (2014).
8. Khedkar, M.A., Nimbalkar, P.R., Gaikwad, S.G., Chavan, P. V. and Bankar, S.B. Sustainable biobutanol production from pineapple waste by using *Clostridium acetobutylicum* B 527: drying kinetics study. Bioresource Technol. doi: http://dx.doi.org/10.1016/j.biortech.2016.11.058. (2016).
9. Galbe, M., Zacchi, G. A review of the production of ethanol from softwood. Appl. Microbiol. Biotechnol, 59: 618-628. (2002).
10. Sun, Y. and Cheng, J. Hydrolysis of lignocellulosic materials for ethanol production: A review, Bioresource Technol. 83, 1-11. (2002).
11. Fujii, T., Fang, X., Inoue, H., Murakami, K., Sawayama, S. Enzymatic hydrolyzing performance of *Acremonium cellulolyticus* and *Trichoderma reesei* against three lignocellulosic materials. Biotechnol. Biofuels. 2:24, 1-8. (2009).

12. Rosgaard, L., Pedersen, S., Langston, J., Akerhiem, D., Cherry, J.R., Meyer, A.S. Evaluation of Minimal *Trichoderma reesei* Cellulase Mixtures on Differently Pretreated Barley Straw Substrates. Biotech. Progress. 23(6), 1270-1276. (2007).
13. Balat, M. Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review. Energy Convers. Manage, 52(2), 858-875. (2011)
14. Sternberg, D., Vijayakumar, P. and Reese, E.T. β-glucosidase: microbial production and effect on enzymatic hydrolysis of cellulose, Can J Microbiol, 23: 139-147. (1997)
15. Chootnut, A., Saejong, M. and Angkharak, K. The Production of Ethanol and Hydrogen from Pineapple Peel by *Saccharomyces Cerevisiae* and *Enterobacter Aerogenes*. Energy Procedia, 52, 242-249. (2014).
16. Aguiar, C.L. Biodegradation of the cellulose from sugarcane bagasse by fungal cellulase, Cienc. Tecnol. Aliment. 3, 117-121. (2001).
17. Park, Y.S., Kang, S.W., Lee, J.S., Hong, S.I. anc Kim, S.W. Xylanase production in solid state fermentation by *Aspergillus niger* mutant using statistical experimental designs, Appl. Microbiol. Biot. 58, 762-766. (2002).
18. Ban-Koffi, L. and Han, Y.W. Alcohol production from pineapple waste, World J. Microb. Biot. 6, 281-284. (1990).
19. Peleg, M. An empirical-model for the description of moisture sorption curves, J Food Sci. 53,1216-12. (1988).
20. Cárcel, J.A., García-Pérez, J.V. Sanjuán, N., Mulet, A. Influence of pre-treatment and storage temperature on the evolution of the colour of dried persimmon, LWT-Food Sci. Technol. 43, 1191-1196. (2010)
21. Bayer, E.A., Chanzy, H., Lamed, R. and Shoham, Y. Cellulose, cellulases and cellulosomes, Curr. Opin. Struc. Biol. 8, 548-557. (1998).
22. Hahn-Hagerdal, B., Karhumaa, K., Fonseca, C., Spencer-Martins, I. and Gorwa-Grauslund, M.F. Towards industrial pentose-fermenting yeast strains, Appl. Biochem. Biotech. 74, 1207-1209. (2007).
23. Matsushika, A., Inoue, H., Kodaki, T. and Sawayama, S. Ethanol production from xylose in engineered *Saccharomyces cerevisiae* strains: current state and perspectives, Appl. Microbiol. Biot. 84, 37-53. (2009).
24. Medve, J., Karlsson, J., Lee, D. and Tjerneld, F. Hydrolysis of microcrystalline cellulose by cellobiohydrolase I and endoglucanase II from *Trichoderma reesei*:

adsorption, sugar production pattern, and synergism of the enzymes, Biotechnol. Bioeng. 59, 621-634. (1998)

25. Verardi, I. De Bari, E. Ricca, V. Calabro: Hydrolysis of Lignocellulosic Biomass: Current Status of Processes and technologies and Future perspectives. In: Bioethanol, M. A. Pinheiro Lima, A. Pardo Policastro Natalense (Eds.), InTech, Rijeka, Croatia pp: 95-122. (2012).
26. Huang, C.F., Lin, T.H., Guo, G.L. and Hwang, W.S. Enhanced ethanol production by fermentation of rice straw hydrolysate without detoxification using a newly adapted strain of Pichia stipitis, Bioresource Technol. 100, 3914-3920. (2009).
27. Mussatto, S.I., Dragone, G., Guimaraes, P.M.R., Silva, J.P.A., Carneiro, L.M., Roberto, I.C., Vicente, A. Domingues, L. and Teixeira, J.A. Technological trends, global market, and challenges of bio-ethanol production, Biotechnol. Adv. 28, 1873-1899. (2010).
28. Cardona, C.A. and Sánchez, O.J. Fuel ethanol production: Process designs trends and integration opportunities, Bioresource Technol. 98, 2415-2457. (2007).
29. Dien, B.S., Cotta, M.A. & Jeffries, T.W. Bacteria engineered for fuel ethanol production: current status. Appl. Microb. Biotec., 63: 258. (2003).
30. Zhang, M., Eddy, C., Deanda, K., Finkelstein, M. and Picataggio, S. Metabolic Engineering of a Pentose Metabolism Pathway in Ethanologenic Zymomonas mobilis. Science. 267(5195): 240–3. (1995).
31. Deanda, K., Zhang, M., Eddy, C. and Picataggio, S. Development of an arabinose-fermenting Zymomonas mobilis strain by metabolic pathway engineering. Appl. Environ. Microbiol. 62 (12): 4465–70. (1996).
32. Taherzadeh, M.J. and Karimi, K. Process for ethanol from lignocellulosic materials I: Acid-based hydrolysis processes, Bioresources, 2(3), 472-499. (2007).
33. Qin, Y. Wei, W., Song, X., Qu, Y. Engineering endoglucanase II from *Trichoderma reesei* to improve the catalytic efficiency at a higher pH optimum. J. Biotechnol, 135(2), 190-195 (2008).
34. Jahangeer, S., Khan, N., Jahangeer, S., Soail, M., Shahzab, S., Ahmad A., Khan, S.A. Screening and characterization of fungal cellulases isolated from the native environmental source. Pak. J. Bot., 37(3):739-748. (2005).