Evaluation of *Myceliopthora thermophila* as an Enzyme Factory for the Production of Thermophilic Cellulolytic Enzymes

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Enzymatic hydrolysis is a key step in bioethanol production. Efficient hydrolysis requires a consortium of different enzymes that are able to hydrolyze cellulose and hemicellulose into fermentable sugars. *Myceliopthora thermophila* is a promising candidate for the production of thermophilic cellulolytic enzymes, the use of which could reduce the cost of ethanol production. The growth conditions of the fungus were optimized in order to achieve increased secretion of extracellular cellulases. Optimal conditions were found to be 7.0% w/v brewer's spent grain as the carbon source and 0.4% w/v ammonium sulfate as the nitrogen source. The cellulases obtained were characterized for their optimum activity. The optimum temperature and pH for cellulase activity are 65 °C and pH 5.5, respectively. Studies on thermal inactivation of the crude extract showed that the cellulases of *M. thermophila* are stable for temperatures up to 60 °C. At this temperature the half-life was found to be as high as 27 h. Enzymatic hydrolysis of cellulose resulted in 31.4% hydrolysis yield at 60 °C after 24 h of incubation. Finally, the recalcitrance constant for cellulose and cellulose pretreated with ionic liquids was calculated to be 5.46 and 2.69, respectively.

Keywords: Enzymatic hydrolysis; Myceliopthora thermophila; Sporotrichum thermophile; Thermophilic enzymes; Lignocellulosic biomass; Biofuels; Bioethanol

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INTRODUCTION

During the past century, human activities have led to a dramatic release of greenhouse gases into the atmosphere, causing deterioration of the environment. Second-generation biofuels, which are produced using plant biomass (mainly lignocellulosic materials), are an attractive alternative to fossil fuels and provide economic, environmental, and energy benefits (Ajanovic and Haas 2010; Reinhardt and von Falkenstein 2011; Ribeiro 2013; van Eijck *et al.* 2014). Lignocellulose-based feedstock consists of recalcitrant materials that require costly processes in order to obtain fermentable sugars from cellulose and hemicellulose. Thus, there are technical difficulties that need to be overcome in order to use lignocellulosic biomass efficiently for the production of biofuels (Naik *et al.* 2010).

During lignocellulose-based ethanol production, four stages are required—namely pretreatment, hydrolysis, fermentation, and distillation (Limayem and Ricke 2012). Efficient enzymatic degradation of lignocellulosic biomass remains a major challenge for biofuel production processes (Sun and Cheng 2002). It requires the availability of a range of enzymes that are able to hydrolyze cellulose, hemicellulose, and other polysaccharides

to fermentable sugars. Efforts have been made towards reduction of enzyme loads and incubation time with the development of new strategies that focus on the properties of improved and low-cost biocatalysts. This could help to reduce the cost of production of fermentable sugars and, therefore, also reduce the cost of ethanol production, opening the way for commercialization.

The use of thermotolerant enzymes in hydrolysis processes allow for the use of high reaction temperatures. High reaction temperatures provide numerous advantages since enzymes derived from thermophilic fungi have better hydrolytic capability than enzymes derived from mesophilic fungi, such as higher hydrolysis rates and reduced incubation time (Berka *et al.* 2011; Singh 2014). The use of high temperatures also reduces the risk of contamination, reduces the substrate viscosity, and improves mass transfer. Also, thermophilic enzymes can be stored for prolonged periods of time at room temperature and tolerate organic solvents; they also lose less of their activity during processing at the elevated temperatures that are often used in pretreatment of raw material (Turner *et al.* 2007).

Myceliopthora thermophila (syn. *Sporotrichum thermophile*) is a thermophilic filamentous fungus that offers a relatively rich inventory of genes encoding enzymes that break down cellulose and hemicellulose (Karnaouri *et al.* 2014a). This fungus occurs naturally in self-heated soil masses of vegetable matter, where it contributes to the decomposition of polysaccharides in the cell wall of plants (Bhat and Maheshwari 1987). *M. thermophila* grows optimally at temperatures between 45 °C and 50 °C, but it can be cultivated at temperatures between 25 and 55 °C. Its exponential growth on cellulose (0.09 to 0.16 h^{-1}) is similar to that on glucose (0.10 h^{-1}) (Maheshwari *et al.* 2000), revealing the remarkable ability of this fungus to use cellulose as efficiently as glucose. This efficient use of the glucose contained within cellulose makes this fungus stand out as a promising candidate for the production of enzymes that can be used during hydrolysis of plant biomass. *M. thermophila* has been increasingly attracting interest in the last few years due to the fact that its genome was successfully sequenced by Berka *et al.* in 2011, revealing a large number of putative genes encoding enzymes of industrial importance (plant cell wall-degrading enzymes, proteases, oxidoreductases, and lipases).

Numerous enzymes from *M. thermophila* that are involved in the degradation of lignocellulose have been studied. In particular, an endoglucanase (Karnaouri *et al.* 2014b), an ethanol-tolerant *β*-glucosidase (Karnaouri *et al.* 2013), the polysaccharide monoxygenase Cel61a (Dimarogona *et al.* 2010), the feruloyl esterase MtFae1a (Topakas *et al.* 2012), and the glucuronoyl esterase StGE2 (Topakas *et al.* 2010) have been cloned from strain ATCC 42464 and have been heterologically expressed in the mesophilic host *Pichia pastoris* before characterization. Moreover, the determination of the crystal structure of StGE2 has given new insights into the importance of this class of enzymes as potential biocatalysts (Charavgi *et al.* 2013).

Cellobiose dehydrogenases from this strain have also been studied (Canevascini *et al.* 1991; Subramariam *et al.* 1999). Derived from strain ATCC 34628, xylanases (Katapodis *et al.* 2003; Vafiadi *et al.* 2010), a thermostable *β*-xylosidase (Katapodis *et al.* 2006), two types of feruloyl esterase, StFAE-A and StFAE-C (Topakas *et al.* 2004; Topakas *et al.* 2005), and the glucuronoyl esterase StGE1 (Vafiadi *et al.* 2009) have been purified and their properties characterized. From the C1 strain, xylanases (Ustinov *et al.* 2008; Van Gool *et al.* 2012; van Gool *et al.* 2013), feruloyl esterases (Kuhnel *et al.* 2012), acetyl xylan esterases (Pouvreau *et al.* 2011; Kool *et al.* 2014), and arabino-hydrolases

(Kuhnel *et al.* 2010; Kuhnel *et al.* 2011) have been studied for their specificity and properties.

Finally, an exoglucanase (Frachboud and Canevascini 1989) and *β*-glucosidases (Canevascini and Meyer 1979; Meyer and Canevascini 1981; Gaikwad and Maheshwari 1994) have also been isolated from strain IIS220. The *β*-glucosidases isolated differ in their molecular weight, optimum temperature and pH, thermostability, and specificity for different substrates. They are found either in the cytoplasm or bound in the cell walls. It is clear that there is a high degree of research interest in the cellulolytic enzymes of *M. thermophila*, as they have interesting characteristics. For this reason, this study is focused on the evaluation of the biotechnological potential of *M. thermophila* for the efficient production of cellulolytic enzymes and subsequent cellulose hydrolysis.

EXPERIMENTAL

Materials

Raw materials, chemicals, and microorganisms

Brewer's spent grain (BSG) was supplied by Athenian Brewery S.A. and wheat bran was supplied by Agiou Georgiou Flour Mills S.A. Rice straw, corn cob, and sweet sorghum were supplied by the Agricultural University of Athens. Sweet sorghum bagasse was prepared as previously described in the work of Matsakas and Christakopoulos (2013a). BSG was stored at −18 °C and, prior to its use, was dried at 65 °C until it reached constant weight. Wheat bran was stored at 4 °C. Rice straw, corn cobs, and sweet sorghum bagasse were kept at room temperature. Before use, all raw materials were milled to a particle size less than 3 mm using a laboratory mill. All chemicals and reagents used were of analytical grade.

The thermophilic fungus *Myceliopthora thermophila* (syn. *Sporotrichum thermophile*) ATCC 42464, which originally was isolated from soil, was used for the production of extracellular cellulolytic enzymes.

Media and culture

Stock cultures were maintained at 4 °C in slants containing a medium consisting of 3.9% w/w potato-dextrose-agar (PDA) and 0.2% w/w yeast extract. Slants inoculated with the fungus were incubated at 47 °C for 5 to 6 days. Before the experiments, the fungus was cultivated in 250 mL Erlenmeyer flasks containing 50 mL of pre-culture medium with the following composition: corn cobs, 30 g/L; $NH_4H_2PO_4$, 7 g/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; $FeSO_4 \cdot 7H_2O$, 5 mg/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 (Bhat and Maheshwari 1987; Katapodis *et al.* 2003). The pH was adjusted to 5.0 prior to sterilization (121 °C, 20 min). After being inoculated from the slants, the culture flasks were incubated in an orbital shaker for 48 h at 47 °C, at an agitation rate of 200 rpm. One slant was used for the inoculation of two pre-culture flasks.

During the trials for the optimization of cellulolytic enzyme production, *M. thermophila* was cultured in 250 mL Erlenmeyer flasks containing 100 mL of medium of the same composition as the pre-cultures, except for the carbon and nitrogen source. Inoculation was done with 5% v/v of the pre-culture broth. Initially, the effect of different carbon sources (brewer's spent grain, wheat bran, rice straw, corn cob, or sweet sorghum bagasse) on cellulase production was evaluated at a concentration of 3.0% w/v, using 0.7%

w/v $NH_4H_2PO_4$ as nitrogen source. Subsequently, the effect of organic nitrogen sources (meat peptone, urea, or yeast extract) and inorganic nitrogen sources ($NH_4H_2PO_4$, $(NH_4)_2SO_4$, NH_4NO_3, or $NaNO_3$) was evaluated at a concentration of 1.0% w/v in the presence of the carbon source that was previously found to be optimal. When the optimal carbon and nitrogen sources had been determined, their concentrations were optimized further in order to increase cellulolytic activity. In all the experiments, the flasks were incubated for 5 to 6 days at 47 °C at an agitation rate of 200 rpm. At certain time intervals, samples were withdrawn under sterile conditions and centrifuged (5,000 rpm, 20 min) to remove solids and fungal biomass from the broth. Finally, the clear supernatant was collected and analyzed for cellulase activity.

Production of crude enzyme extract for characterization experiments

Culturing was performed under the conditions that were found to be optimal for increased cellulase activity. At the end of cultivation, the culture broth was collected and centrifuged (12,000 rpm, 45 min) at 4 °C. The clarified supernatant was concentrated using ultrafiltration membranes (cutoff 10 kDa; Amicon) until the desired cellulase activity had been reached. The concentrated crude enzyme extract was used for the enzyme characterization experiments.

Methods

Analytical methods

Cellulase (filter paper activity), endoglucanase (EC 3.2.14), exoglucanase (EC 3.2.1.91), *β*-glucosidase (EC 3.2.1.21), and *β*-xylosidase (EC 3.2.1.37) activity were determined according to standard assays (Ghose 1987; Ghose and Bisaria 1987). Xylanase activity (EC 3.2.1.8) was assayed according to Bailey *et al.* (1992) on 1.0% w/v birch-wood xylan (Sigma). Unless otherwise stated, all assays were carried out at 50 °C in 100 mM citrate-phosphate buffer, pH 5.0, in a thermomixer with 1,000-rpm agitation. One unit (U) of activity was defined as the amount of enzyme that released 1 μmol of product per minute under assay conditions. Total protein concentration was measured using the Lowry method (Lowry *et al.* 1951). The concentration of reducing sugars was determined according to the dinitro-3,5-salicilic acid (DNS) method (Miller 1959).

Determination of optimum pH, optimum temperature, and thermal stability

The optimum temperature was determined by assessing the cellulase activity of the crude enzyme extract over a range of temperatures (40 to 80 °C) at pH 5.0. The optimum pH was determined by measuring the cellulase activity at the optimum temperature over a pH range of 4.0 to 7.0. Thermal stability was determined by measuring the residual activity (at 50 °C and optimum pH) after incubation of the extract at several temperatures (40 to 80 °C) for 24 h in the absence of substrate and without agitation. In all trials, the pH value was adjusted by using 100 mM citrate-phosphate buffer. The initial cellulase activity of the enzyme extract used was 1.43 ± 0.04 FPU/mL (measured at 50 °C and optimum pH). All reactions were carried out in duplicate.

Effect of temperature on hydrolysis yield

Hydrolytic reactions were performed over a range of temperatures (between 40 °C and 65 °C) at optimum pH and with an agitation rate of 1,000 rpm. The initial concentration of cellulose was 3.3% w/v (Whatman filter paper No. 1, Sigma) with an enzyme load of 10 FPU/g of substrate. Reaction mixtures were incubated for 24 h. At certain time intervals,

samples were withdrawn for measurement of the concentration of reducing sugars. All reactions were carried out in duplicate.

Effect of substrate conversion on reaction rate

Enzymatic hydrolysis reactions were performed on untreated cellulose (filter paper, Whatman No. 1) and cellulose pretreated with ionic liquids (IL-treated cellulose; CELLIONIC BCW 1100, Sigma) under optimum conditions and with an agitation rate of 1,000 rpm. Substrate (20 mg) was added to an adequate amount of enzyme and 100 mM citrate-phosphate buffer in order to achieve 38 FPU/g cellulose. Every 30 min, the hydrolysate was removed by centrifugation (3200 rpm, 2 min). The partially hydrolyzed cellulose was then washed thoroughly with fresh buffer under vigorous agitation and re-incubated after the addition of fresh buffer and fresh enzyme. The concentration of reducing sugars was measured just before and just after the hydrolysate replacement. All reactions were carried out in duplicate.

RESULTS AND DISCUSSION

Optimization of Cellulase Production

Effect of carbon source

Carbon source is an important factor affecting extracellular enzyme production (Petitdemange *et al.* 1992; Juhász *et al.* 2005; Niranjane *et al.* 2007). In order to reduce the cost of enzyme production, low-cost renewable raw materials should be used as carbon source. Agro-industrial by-products are an interesting category of lignocellulosic biomass that could be used as raw materials for the production of cellulolytic enzymes. For this reason different kinds of these by-products were evaluated for their ability to support cellulolytic enzyme production by *M. thermophila.* The different carbon sources were tested at a fixed concentration (3% w/v) during the induction of extracellular cellulase secretion, and the cultures were incubated for 6 days. Brewer's spent grain (BSG) gave the highest activity in the culture broth during the fifth and sixth day of cultivation, 0.11 ± 0.01 FPU/mL, as illustrated in Fig. 1. The yield of cellulase reached 3.67 FPU/g of substrate.

Effect of nitrogen source

The presence of a nitrogen source is vital for the production of enzymes, as nitrogen supplies the microorganisms with the building blocks of organic molecules, such as proteins. When a number of nitrogen sources were tested at a fixed concentration (1.0% w/v), there was a difference in the enzyme production pattern between inorganic and organic sources, as illustrated in Fig. 2. Growth on inorganic sources resulted in an exponential increase in cellulase production until the fifth day of incubation. On the other hand, using organic sources resulted in high cellulase production followed by very low activity after the fourth day of incubation. The highest activity, 0.13 ± 0.00 FPU/mL, was obtained during the fifth day of cultivation when ammonium sulfate was used. In a previous study by Katapodis *et al.* (2003), it was found that inorganic sources were more favorable for the secretion of enzymes by *M. thermophila*, with ammonium phosphate resulting in the highest xylanase activity. These findings are very important because inorganic nitrogen sources are much cheaper than using complex organic sources, such as yeast extract, which would have a positive effect on the economics of the process.

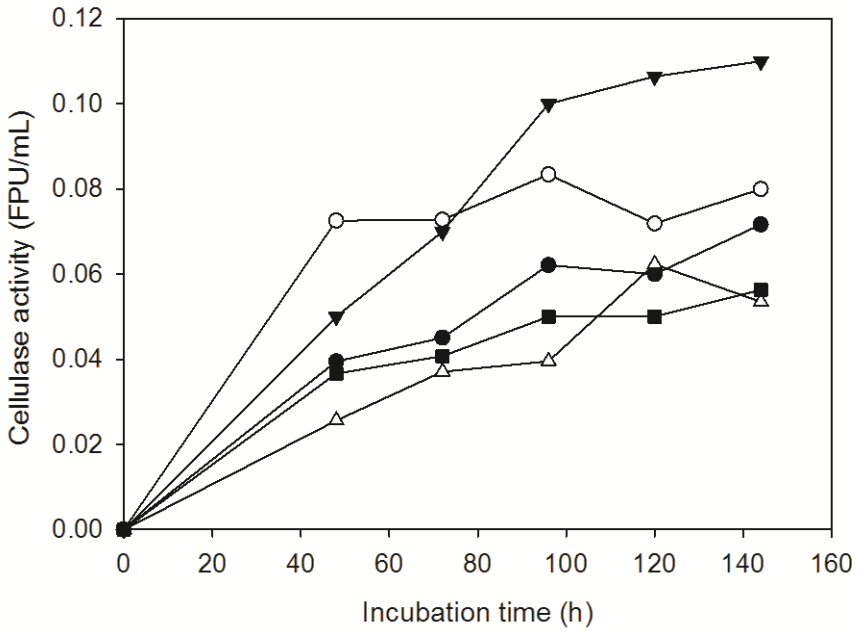

Fig. 1. Effect of carbon source (3% w/v) on the production of cellulases. The carbon sources used were: brewer's spent grain (▼), wheat bran (○), corn cob (●), sweet sorghum bagasse (Δ), and rice straw (■). $NH_4H_2PO_4$ (0.7% w/v) was used as nitrogen source

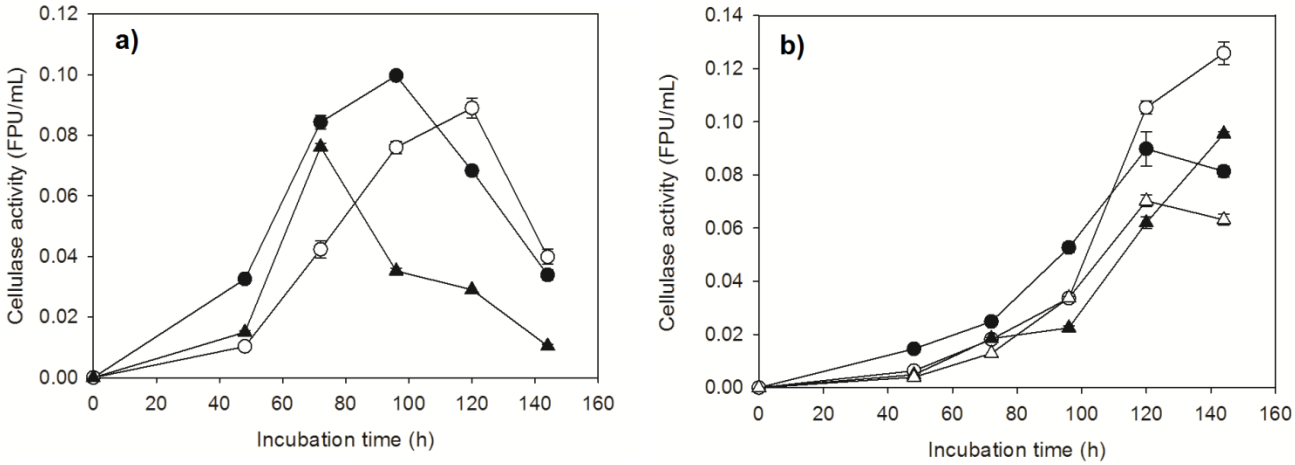

Fig. 2. Effect of a) organic nitrogen sources (yeast extract (●), meat peptone (○), and urea (▲)) and b) inorganic nitrogen sources ($NH_4H_2PO_4$ (●), $(NH_4)_2SO_4$ (○), NH_4NO_3 (▲), and $NaNO_3$ (Δ)) (B) (all 1.0% w/v) on the production of cellulases. BSG (3.0% w/v) was used as a carbon source

Combined effect of BSG and ammonium sulfate on cellulase secretion

For further optimization of the growth conditions for the enhanced production of extracellular cellulases, the combined effect of the concentration of carbon and nitrogen sources was evaluated. Twenty-four different combinations of the previously determined as optimum carbon and nitrogen sources were used. The BSG concentration was varied in six levels (1, 3, 5, 7, 9, and 11% w/v), while the ammonium sulfate concentration was varied in four levels (0.1, 0.4, 0.7, and 1.0% w/v). The highest activity (0.37 ± 0.01 FPU/mL) was detected under growth conditions consisting of 7.0% w/v BSG and 0.4% ammonium sulfate on the fifth day of culture (Fig. 3). On the sixth day of culture, the optimum growth conditions were found to be the same but the activity was reduced to 0.24 ± 0.00 FPU/mL (a 35.1% reduction).

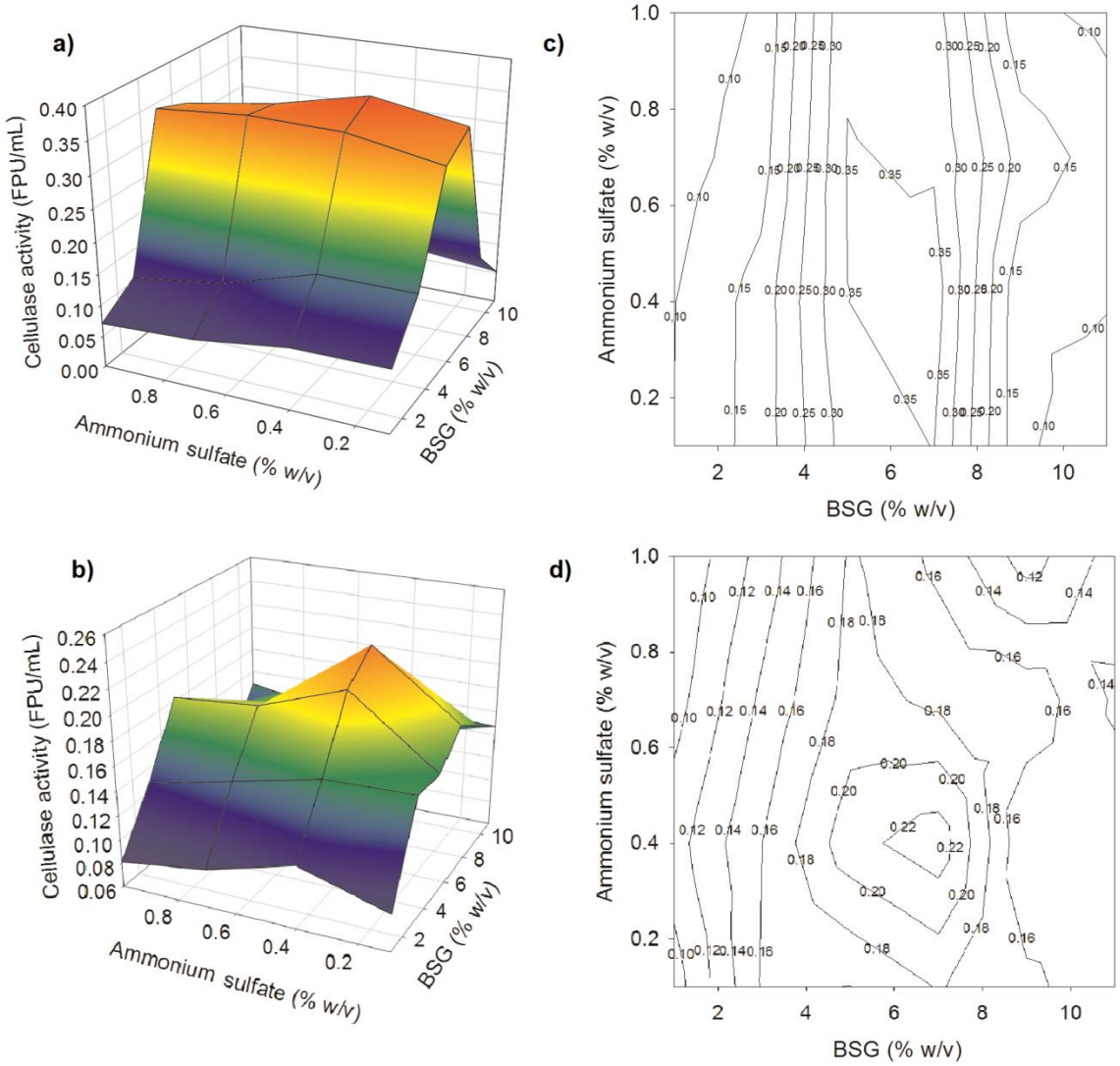

Fig. 3. Combined effect of BSG and ammonium sulfate concentrations (% w/v) on cellulase activity (FPU/mL) a) at the fifth day of cultivation and b) the sixth day of cultivation. Contour plots are also provided for c) the fifth day and d) sixth day of cultivation.

The enzymatic activity obtained on the fifth day of culture was 5.29 times higher than previously reported for the same strain, as demonstrated in Table 1. The crude enzyme extract produced showed high endoglucanase activity. The extracellular *β*-glucosidase activity was very low despite the fact that many sequences on the genome of the fungus are predicted to code putative enzymes with this particular activity (Karnaouri *et al.* 2014a). This leads to the assumption that the optimum conditions of this study did not induce the secretion of extracellular *β*-glucosidase.

The optimum growth conditions also induced high endoxylanase expression. This is an important characteristic, as even small amounts of residual xylan could negatively affect the hydrolysis of cellulose. Removal of xylan is necessary in order to expose cellulose microfibrils (Zhang *et al.* 2011; Matsakas and Christakopoulos 2013b). The cellulolytic activity achieved during this work (0.37 FPU/mL or 5.29 FPU/ g of substrate) is comparable to that described by other authors, despite the fact that *M. thermophila* is considered to produce lower extracellular activities than other microorganisms.

Table 1. Activities on Primary Cellulase and Xylan-degrading Hemicellulase Components of *M. thermophila*

Protein conc. (mg/mL)	Activity (U/mL)						Source
	β-gluco-sidase	*β*-xylo-sidase	Endo-glucanase	Exo-glucanase	Total cellu-lase	Xyla-nase	
4.02 ±0.23	0.03 ±0.002	0.005 ±0.0003	18.07 ±0.13	0.17 ±0.02	0.37 ±0.013	20.9 ±0.14	Current work
0.13 ±0.003	0.26 ±0.01	n/a	1.05 ±0.08	0.10 ±0.03	0.07 ±0.006	n/a	Bhat and Mahe-shwari 1987

n/a: not available

Interestingly, the culture of *M. thermophila* on dried household food waste (HFW) resulted to a cellulase yield equal to 4.7 FPU/ g of substrate (Matsakas and Christakopoulos 2015). In similar studies on cellulase production, the growth of *Trichoderma viride* on banana peels and *Neurospora sitophila* on steam exploded wheat straw resulted in a cellulase activity equal to 5.6 and 6.4 FPU/ g of substrate, respectively (Sun *et al.* 2011; Li *et al*. 2013).

Enzyme Characterization

Effect of temperature and pH on cellulase activity

The effect of temperature on cellulase activity was evaluated over a range of 40 °C to 80 °C. The enzymes showed the highest cellulase activity at 65 °C, which was 2.31 times higher than the activity at 50 °C (reference value) (Fig. 4A). These results align with a similar study concerning the thermal characteristics of *M. thermophila* cellulases produced under different conditions, where the optimal temperature was found to be 68 °C (Margaritis and Creese 1981). Next, the effect of pH on enzyme activity was evaluated at 65 °C over a pH range from 4 to 7. The crude enzyme extract had optimum activity at pH 5.5, which was 1.18 times higher than the activity measured at pH 5.0 (reference value) (Fig. 4B). On the other hand, an increase in pH led to a rapid decrease in cellulolytic activity.

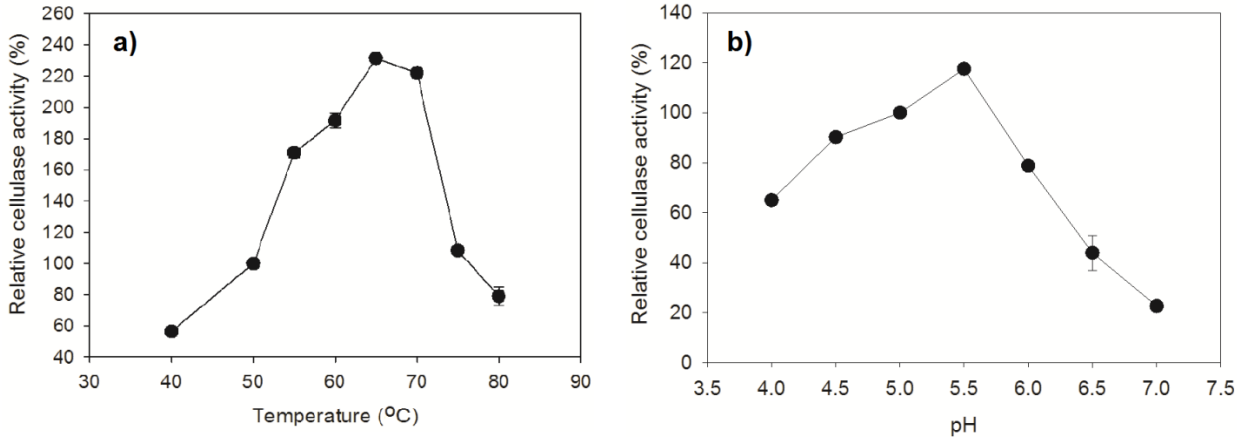

Fig. 4. Effect of a) temperature and b) pH on cellulase activity

The highest temperature at which the crude extract shows optimal activity is a beneficial characteristic, as it may allow for the hydrolysis of cellulose at elevated temperatures. The use of higher temperatures for cellulose hydrolysis brings several advantages, such as higher hydrolysis rates, lower incubation times, prevention of contamination, lower viscosity of the slurry, and improved mass transfer.

Effect of temperature on cellulase inactivation

Cellulases can be inactivated by various factors including the shear force developed during agitation, temperature, ionic strength, product inhibition, and ineffective adsorption to the substrate (Zhang *et al.* 2010). Temperature is considered to play an important role during inactivation because it affects both enzyme activity and protein chemical stability. Knowledge of the thermal stability of a crude enzyme extract is crucial for efficient hydrolysis during simultaneous hydrolysis and fermentation (SHF) processes because the retention time at high temperatures is long (8 to 24 h). During this time enzymes can undergo thermal inactivation, resulting in reduced hydrolytic activity. Knowledge of thermal inactivation can offer guidance on enzyme dosage and retention time for enzymatic hydrolysis processes. These two factors are critical for reduction of the cost of ethanol production, as cellulases are considered to contribute significantly to this process. It has been calculated that the contribution of cellulolytic enzymes to the cost of cellulosic ethanol production can be up to $1.47/gal of ethanol (Klein-Marcuschamer *et al.* 2012).

Generally, enzyme thermal inactivation is described by first-order kinetics (Shuler and Kargi 2001) (Eqs. 1 and 2),

$$dC_E/dt = -k_d \cdot C_E \quad (1)$$

$$C_E = C_{E0} \cdot e^{-k_d \cdot t} \quad (2)$$

where C_E is the residual enzyme activity (FPU/mL) or relative activity (%), C_{E0} is the initial enzyme activity (FPU/mL), k_d is the thermal de-activation constant (h^{-1}), and t is the incubation time (h).

The crude enzyme extract from *M. thermophila* exhibited high stability when incubated at 40 °C, as no decrease in activity was observed even after 24 h of incubation. High stability was also observed at 50 °C and 60 °C, where after 24 h of incubation 81.2 ± 3.8% and 52.0 ± 1.5% of the initial activity was maintained, respectively. At 65 °C the cellulases retained only 38.1 ± 2.0% of their initial activity after only 2 h of incubation, despite the fact that at this temperature, the crude enzyme extract showed the highest cellulase activity. After 24 h, the residual activity was only 23.4 ± 0.4% of the initial activity. The data on thermal inactivation shown in Fig. 5 fitted well with Eq. (2). The half-lives ($t_{1/2}$) of the enzymes at each temperature were calculated after regression (Table 2). It can be seen that, despite the fact that the half-lives at 65 °C and 70 °C were almost the same, after 24 h of incubation the residual activity at 65 °C was 18.9% higher than at 70 °C.

Table 2. Half-Lives of the Extracellular Cellulases from *M. thermophila* at Various Temperatures

T (°C)	50	60	65	70	80
$t_{1/2}$ (h)	65.00	27.00	0.98	0.96	0.33

The dependence of the inactivation constant on temperature can be described by an Arrhenius-type relationship (Eq. 3),

$$k_d = A_d \cdot e^{-Ed/RT} \quad (3)$$

where E_d is the inactivation energy (kcal/mol), and A_d (h^{-1}) is a constant. The linear relationship between ln(k_d) and 1/T, which can be derived from Eq. 3, is confirmed by the data shown in Fig. 6.

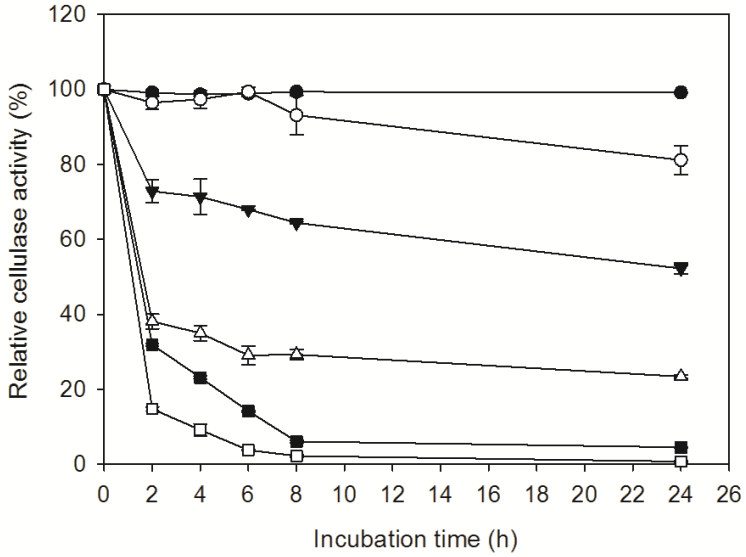

Fig. 5. Thermal stability of the extracellular cellulases from *M. thermophila* at pH 5.5 (40 ºC (●), 50 ºC (○), 60 ºC (▼), 65 ºC (Δ), 70 ºC (■), 80ºC (□))

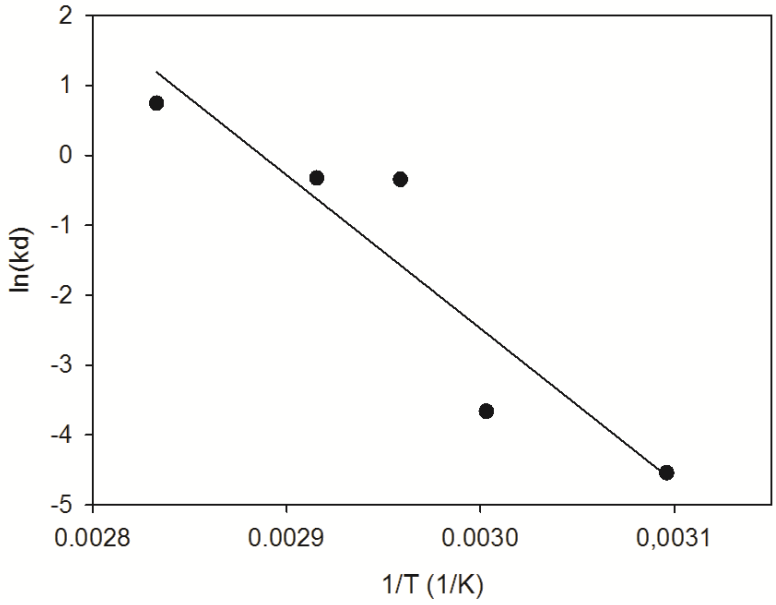

Fig. 6. Logarithmic plot of the inactivation constant of the extracellular cellulases of *M. thermophila* as a function of the reciprocal of temperature

E_d was calculated to be 43.62 kcal/mol and A_d = $3.34 \cdot 10^{27}$ h^{-1} (R^2= 0.859). The thermal inactivation energy of the crude enzyme extract was slightly lower than the average range of most enzymatically catalyzed reactions (47 to 96 kcal/mol) (Bassetti *et al.* 2000). This means that the thermophilic cellulases of *M. thermophila* are easily inactivated at higher temperatures (above 65 °C).

Effect of temperature on the hydrolysis of cellulose

Efficient cellulose hydrolysis is strongly related to the temperature. The temperature affects both enzyme activity and enzyme stability. Despite the fact that the enzymes can show high activity at a specific temperature, the efficiency of cellulose hydrolysis after some hours of incubation can be reduced as a result of thermal inactivation. For this reason, during this part of the work the effect of temperature on cellulose hydrolysis was evaluated.

The efficiency of enzymatic hydrolysis was calculated by applying the following equation (Vásquez *et* al. 2007) (Eq. 4),

$$Eh = \frac{C_s}{F \cdot (CPn_o / WSR \cdot \rho)} \quad (4)$$

where Eh is the % hydrolysis of cellulose, C_s is the concentration of sugars released during the hydrolysis (g/L), F is the stoichiometric factor due to the hydration of molecules during the hydrolysis ($F_{hexoses}$=1.111), CPn_o is the composition of the raw material for the polysaccharide (% w/w; in this case 100 g cellulose/100 g material), WSR is the water-to-solid ratio (in this case 1,000 g water/30 g raw material, dry basis), and ρ is the density of the hydrolysate (1,025 g/L).

The effect of temperature on the hydrolysis of cellulose is presented in Fig. 7. After the first 2 h of incubation, 8.5 ± 0.4% and 7.7 ± 0.2% of the initial amount of cellulose was converted to sugars at 65 °C and 60 °C, respectively. This agrees with previous results from this work, which found that the crude enzyme extract has the highest cellulase activity at 65 °C. However, after 2 h and until the end of incubation, it was observed that the hydrolysis yield was higher at 60 °C. More specifically, at 60 °C the hydrolysis yield reached 18.0 ± 0.0% after 8 h of incubation and 31.4 ± 1.9% after 24 h. At 65 °C, 15.5 ± 0.3% and 28.3 ± 1.3% of cellulose was converted to sugars after 8 h and 24 h of incubation, respectively. The higher hydrolysis yield at 60 °C compared to 65 °C could be explained by the fact that, despite the higher activity shown at 65 °C, the stability of the enzymes at this temperature was much lower than at 60 °C.

From all the results given above, it can be concluded that, although the temperature at which highest enzyme activity was shown as 65 °C, enzymes were inactivated at a faster rate at this temperature than at 60 °C. It is important to emphasize that assays for the determination of enzyme activity normally last for a short period of time, during which the enzymes do not become inactivated to any great extent. Most of the processes that involve cellulose hydrolysis for the production of fermentable sugars normally last for more than 8 h, and sometimes up to 48 to 72 h. For this reason, it is important to take into account not only the activity of the enzyme solution but also the thermal stability at this specific temperature. These two factors (activity and stability) interact with each other, and their combined effect determines the extent of hydrolysis after a specific time of incubation. On this basis, the best temperature for the enzymatic hydrolysis of cellulose using the crude enzyme extract from *M. thermophila* was judged to be 60 °C at pH 5.5.

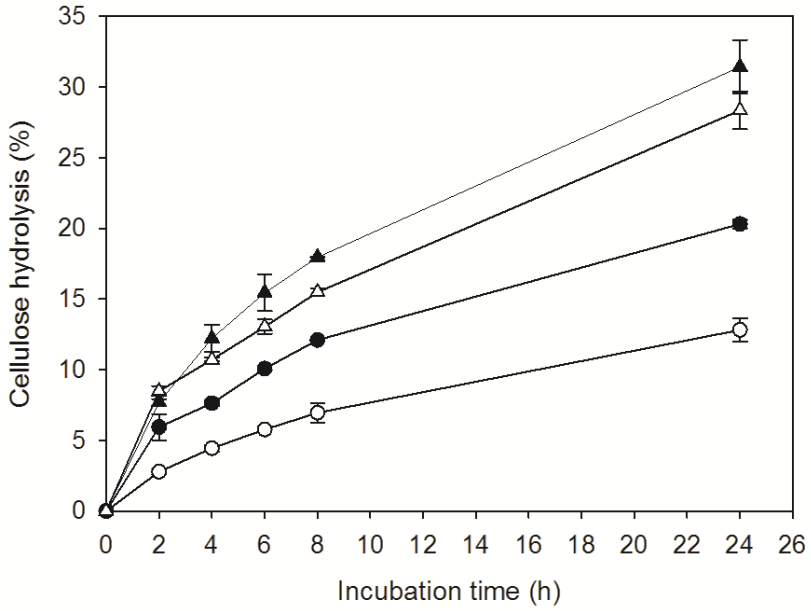

Fig. 7. Cellulose hydrolysis reactions at pH 5.5 (40ºC (○), 50ºC (●), 60ºC (▲), 65ºC (Δ))

Effect of substrate conversion on reaction rate—Substrate recalcitrance

The abrupt fall-off in the release of reducing sugars as the enzymatic hydrolysis progresses can only partly be attributed to product inhibition or enzyme inactivation. According to Drissen *et al.* (2007), substrate recalcitrance describes the decrease in susceptibility of the substrate to enzymatic degradation due to the fact that the easily hydrolysable cellulose is digested first, whereas the increasingly difficult and more recalcitrant cellulose remains in the solid phase. This phenomenon can be ascribed to two mechanisms: the lower specific turnover rate from the amount of enzyme adsorbed onto the cellulose and the decrease in the amount of enzyme that is adsorbed (Desai and Converse 1997). To account for the recalcitrance of cellulose towards the cellulolytic system of *M. thermophila*, the following equation was used (Drissen *et al.* 2007) (Eq. 5),

$$v = v(0) \cdot e^{-K_{rec} \cdot (1 - C_c(t)/C_c(0))} \quad (5)$$

where v is the cellulase activity (g/L · h), $v(0)$ is the initial reaction rate (g/L · h), and K_{rec} is the recalcitrance constant—a measure of the decrease in initial reaction rate at increasing conversion factors. Equation 5 shows a good fit with the experimental data on relative hydrolysis rate plotted against the cellulose conversion factor, $1 - C_c(t)/C_c(0)$, as illustrated in Fig. 8. The values for the recalcitrance constant obtained from the regression were K_{rec1} = 2.69 (R^2 = 0.886, p < 0.0001) for the pretreated cellulose and K_{rec2} = 5.46 (R^2 = 0.985, p < 0.0001) for the untreated cellulose. In the case of the pretreated cellulose, the initial reaction rate was $v(0)_1$ = 23.5 ± 0.1 g/L · h. The substrate conversion to glucose reached and remained stable at 73.2%. The initial reaction rate of untreated cellulose was $v(0)_2$ = 9.4 ± 0.6 g/L · h, and 33.5% of the substrate was converted to glucose.

Previous studies on substrate recalcitrance have found that the recalcitrance constant of hydrothermally pretreated wheat straw (PWS) towards the enzymatic system of the fungus *Fusarium oxysporum* was 9.0 (Xiros *et al.* 2009), while that for

microcrystalline cellulose (Avicel) towards the commercial enzyme mixture Cellubrix was 2.8 (Drissen *et al.* 2007).

Substrate recalcitrance expresses the difficulty that is encountered by a particular enzyme system during substrate hydrolysis. Another approach is to examine the constant K_{rec} as a measure of the heterogeneity of the substrate, since a relatively large value of K_{rec} for a given substrate means that it consists of parts that are easily hydrolysable and parts that are very resistant to the cellulase system. A K_{rec} value of zero indicates that every fraction of the substrate would be turned into glucose at the same rate, so it would effectively be a very homogeneous substrate. Comparing it to the aforementioned studies, it can be concluded that the cellulolytic system of *M. thermophila* is a highly promising candidate for the efficient hydrolysis of cellulose.

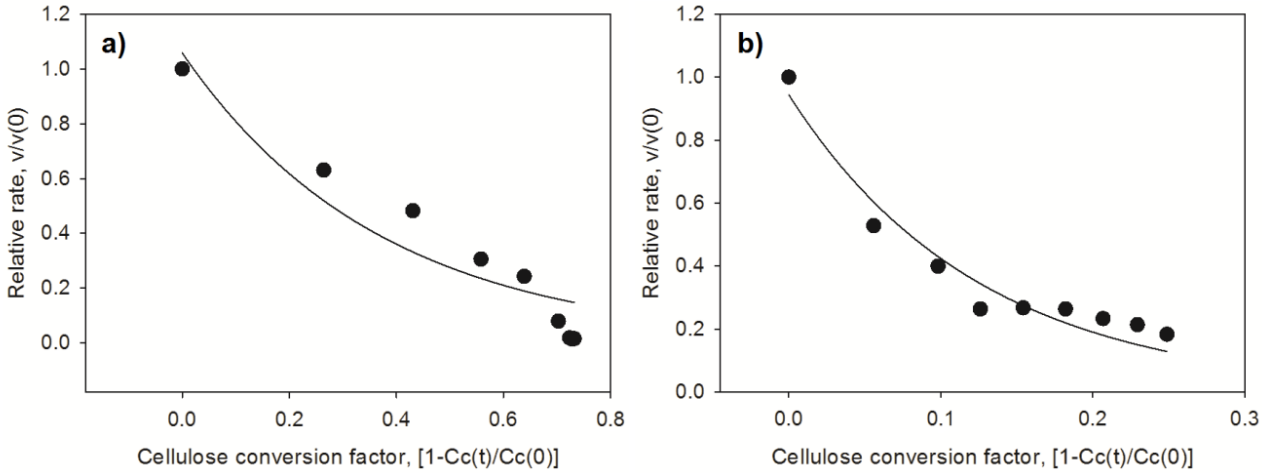

Fig. 8. Relative hydrolysis rate plotted against the cellulose conversion factor for a) cellulose pretreated with ionic liquids and b) for untreated cellulose

CONCLUSIONS

1. It was found that it is possible to use crop by-products and an inorganic nitrogen source, both low-cost raw materials, for the production of a high-activity crude enzyme solution from *M. thermophila*. The cellulase activity obtained after culture under optimal conditions was the highest that has been reported for this strain (5.29 FPU/ g of substrate).
2. The obtained enzymes showed higher activity at elevated temperatures, potentially allowing their use in simultaneous hydrolysis and fermentation processes. The optimum temperature and pH for cellulase activity were found to be 65 ºC and 5.5, respectively. However, the obtained enzymes have higher thermal stability at temperatures up to 60 °C. The highest hydrolysis rate for cellulose was obtained at 60 °C, giving a conversion yield of 31.4%. The recalcitrant constant for cellulose and cellulose pretreated with ionic liquids was calculated equal to 5.46 and 2.69, respectively, showing that the recalcitrance of cellulose towards the cellulolytic system of *M. thermophila* is relatively low. *M. thermophila* is a promising candidate for the efficient degradation of cellulose.

3. The obtained crude enzyme solution presented high activity, and it could act optimally at higher temperatures, which is a beneficial characteristic to be used during second-generation ethanol production. Moreover, the use of low cost materials for the production of the solution can result in lower process cost during ethanol production.

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