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ORIGINAL PAPER

Production of *Trichoderma Reesei* RUT C-30 Lignocellulolytic Enzymes Using Paper Sludge as Fermentation Substrate: An Approach for On-Site Manufacturing of Enzymes for Biorefineries

Thanh Tung Lai[1] · Thi Thanh Ha Pham[1] · Kokou Adjallé[1] · Daniel Montplaisir[1] · François Brouillette[1] · Simon Barnabé[1]

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Abstract Different types of pulp and paper sludge were used as raw materials for the production of a cocktail of lignocellulolytic enzymes of *Trichoderma reesei* RUT C-30 (*T. reesei* RUT C-30). The fungus were grown in pellets to produce cellulases (carbomethylcellulase, β-glucosidase) and hemicellulases (xylanase, β-xylosidase) in three types of sludge: primary (PS), secondary (SS) and mixed sludge (MS). The highest carbomethylcellulase activities obtained after 7 days of fermentation were 7.3, 4.8, and 1.5 IU/ml in MS, SS, and PS, respectively. Sludge modification such as the mixing SS and PS at 1:1 (v/v) ratio, the addition of ammonium sulfate as an inorganic nitrogen source, and the increase of the solids content were shown to improve enzyme production. The crude enzyme mixture obtained from the sludge samples showed a synergistic effect to hydrolyze various biomasses into monosugars. The tested biomasses included highly purified CMC, xylan from birch wood and lignocellulosic materials (corn stover and primary pulp and paper sludge). The addition of a surfactant (polysorbate 20) to the enzyme cocktail enhanced the saccharification efficiency of the sludge, in particular the hydrolysis of the corn stover which contains lignin. The obtained results contribute to the assessment of the feasibility of on-site low cost enzyme production at paper mills generating the sludge, or for neighboring or local biorefineries.

✉ Simon Barnabé
Simon.barnabe@uqtr.ca

[1] Lignocellulosic Materials Research Centre, Université du Québec à Trois-Rivières, 3351, Boul. des Forges, Trois-Rivières G9A 5H7, Canada

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Introduction

Lignocellulosic biomass is a renewable and abundant source of carbohydrates that can be converted into simple sugars to produce biofuels and biochemical through microbial fermentation technologies. Agricultural and industrial residual biomasses are an interesting alternative to crop biomass as secondary generation feedstock for biorefinery that would not contribute to the global food crisis. However, high amounts of various lignocellulolytic enzymes are required to convert complex lignocellulosic materials into fermentable sugars. This particular situation contributes to the high cost of industrial scale biofermentation processes. Several solutions are being investigated to minimize enzyme consumption. Among them, engineering of cellulase enzymes with high hydrolytic efficiency and yield, biomass pretreatment, and alternate raw materials for enzyme production are particularly promising [1–3].

Various lignocellulosic residues have already been successfully used as carbon source and cellulase inducers in the cultivation of cellulolytic bacteria and fungi. Lignocellulolytic enzymes with high specific activities were obtained [4–6]. However, competition for the biomass supply could arise from the fact that the same material, which contains high amounts of cellulose, would be used for the production of enzymes and also as a feedstock for cellulosic ethanol manufacturing. This could result in both enzyme and ethanol yield reductions [7]. Waste streams from agricultural and forestry industries, such as wastewaters and wastewater sludge, contain carbohydrates potentially capable of

inducing cellulosic enzymes production and essential nutrient for microbial culture [8, 9].

Several studies have reported the successful production of enzymes from semi-alternate media containing paper sludge as a carbon source [8, 10, 11]. Paper sludge contains high amounts of water and organic material (such as lignocellulosic derivatives in primary sludge and microbial biomass derivatives in secondary sludge) that are suitable for the submerged culture fermentation of industrial microorganisms. In addition, residual cellulose fibers found in paper sludge can induce the enzyme secretion of cellulolytic bacteria and fungi. In consequence, replacement of the whole synthetic medium with paper sludge would provide a low cost culture medium for enzyme production. Moreover, an on-site concept of enzyme production from paper sludge located near bioethanol plants can be applied to reduce biofuel production cost, since the produced enzymes are directly used for biomass-to-sugar conversion without any need for stabilization, formulation or transportation [12].

The present work focuses on the utilization and modification of paper sludge as a culture medium for *T. reesei* RUT C-30 lignocellulolytic enzyme production. Enzymatic activity and hydrolysis assays were conducted by using the fermented broth on different biomasses to evaluate the saccharification efficiency of the enzyme cocktail.

Materials and methods

Strain

The fungus *T. reesei* RUT C-30 (ATCC 56765) was used in this study. The fungal culture was recovered from freeze-dried spores (2 days at 25 °C and 200 rpm) in a potato dextrose broth. A loop of *T. reesei* RUT C-30 cells inoculum was transferred to potato dextrose agar slants and incubated for 7 days at 25 °C. The greenish conidia, appearing on the slants, were suspended in sterile saline water (0.9 % NaCl) and harvested to determine the spore concentration with a hemocytometer equipped with a microscope Axio scope.A1 (Carl Zeiss company) [13]. The harvested spores were stored in 10 % glycerol at −80 °C [14].

Culture Media

A synthetic Mandel medium was prepared with the following composition: 10 g/L Sodium carboxymethylcellulose (CMC); 0.3 g/L urea; 1.4 g/L $(NH_4)_2SO_4$; 2.0 g/L KH_2PO_4; 0.3 g/L $CaCl_2$; 0.3 g/L $MgSO_4$; 0.25 g/L yeast extract; 0.75 g/L proteose peptone, and trace elements (5 mg/L $FeSO_4.{}_7H_2O$; 20 mg/L $CoCl_2$; 1.6 mg/L $MnSO_4$ and 1.4 mg/L $ZnSO_4$).

Primary (PS) and secondary (SS) paper sludge samples were collected from the wastewater treatment facility of the Kruger Crabtree mill, Quebec, Canada. The total solids concentration of the sludge samples was adjusted to 15 g/L. The SS (initial solids concentration of 8 g/L) was concentrated by gravity clarification for 1 day at 4 °C followed by centrifugation at $3000\times g$ for 5 min at 4 °C (Multifuge X3 FR, Thermo Scientific). The supernatant was used to dilute the precipitate to targeted solids concentrations. The PS (initial solids concentration of 40 g/L) was diluted with demineralized water to 15 g/L. Mixed sludge (MS) was prepared by mixing the 15 g/L PS and SS to different ratios. The pH of both synthetic and sludge-based media was adjusted to 5 with 1 N HCl before sterilization. The chemical characterization of sludge was followed standard methods for examination of water and wastewaters [15] and Sluiter et al. [16]. Sludge properties are presented in Table 1.

Cellulase Production

A starter culture of *T. reesei* RUT C-30 was prepared by transferring 4 ml of a 1.0×10^8 spore/ml stock culture in 200 ml of potato dextrose broth and inoculating in a shaking incubator at 25 °C and 200 rpm for 2 days. A 10 % v/v solution of the actively growing cells (starter culture) was used as inoculum for enzyme production by shake-flash fermentation.

Submerged culture fermentation was carried out in 1 L shake flask with 200 ml of working volume at 25 °C and 200 rpm for 10 days. Samples were taken at 1 day

Table 1 Chemical analysis of pulp and paper sludge sample

Properties	Primary sludge	Secondary sludge
pH	6.5–6.8	5.8–6.2
Total solid (g/L)	40.0 ± 0.5	12.0 ± 0.5
Suspended solid (g/L)	37.0 ± 0.5	8.0 ± 0.5
Dissolved solids (g/L)	0.80 ± 0.02	3.80 ± 0.02
Ashes (g/L)	0.28 ± 0.02	0.080 ± 0.005
Total organics mater (g/L)	3.70 ± 0.02	4.50 ± 0.02
Elemental analysis		
Nitrogen (% w/w)	0.33 ± 0.01	4.34 ± 0.02
Carbon (% w/w)	39.44 ± 0.01	42.50 ± 0.01
Hydrogen (% w/w)	5.47 ± 0.01	6.39 ± 0.01
Sulfur (% w/w)	LOQ	1.23 ± 0.01
Glucan (% w/w)	22.28 ± 0.01	2.10 ± 0.01
Xylan (% w/w)	9.32 ± 0.01	0.61 ± 0.01
Hemicellulose (% w/w)	11.29 ± 0.01	2.72 ± 0.01

intervals. The fermented broth supernatant containing extracellular enzymes was separated from the fungal biomass by centrifugation at 10,000×*g* for 10 min at 4 °C for further enzyme activity assays.

Enzyme Activity

All enzyme activities were determined using the same experimental conditions: 50 °C and pH 4.8, adjusted with a 0.05 M acetate buffer. Only the reaction time varied. Cellulase activities were determined in terms of carbomethylcellulase (CMCase) and β-glucosidase while hemicellulases were determined in terms of xylanase and β-xylosidase.

CMCase activity was determined by measuring the release of reducing sugars from the enzymatic hydrolysis of 2 % CMC for 30 min according to the dinitrosalicylic acid (DNS) standard method [17]. The reducing sugars released from the enzymatic hydrolysis of 1 % birch wood xylan for 15 min were measured to calculate xylanase activity according to previously described methods [18, 19]. One international unit of activity (IU) of CMCase or xylanase is defined as the amount of enzyme that releases 1 µmol of glucose or xylose, respectively as reducing sugar equivalent per minute.

β-glucosidase and β-xylosidase activities were determined by the enzymatic hydrolysis of 4-nitrophenyl-β-D-glucopyranoside and 4-nitrophenyl-β-D-xylanopyranoside for 10 min. The release of 4-nitrophenol was measured according to method C210-00 of Genecor (now Dupont Industrial Biosciences), which using a modification of the assay described by Hagerdal et al. [20]. A 1 ml portion of a 1 mM substrate was heated to 50 °C. Then, 0.2 ml of the diluted sample was added to the substrate, and the reaction was stopped by adding 0.5 ml of 1 M sodium carbonate. The solution was diluted by 10 ml distilled water and mixed well by vortex. The absorbance was read at 400 nm. One IU of β-glucosidase or β-xylosidase is defined as the amount of enzyme that releases 1 µmol of 4-nitrophenol per minute.

Enzymatic Hydrolysis

Cultivations of *T. reesei* RUT C-30 in Mandel and MS media were carried out in 2 L shake flasks for 7 days under the conditions described above. Extracellular enzymes were separated from the fermented broth biomass by centrifugation (10,000×*g*, 10 min, 4 °C) and used directly as enzyme cocktails for hydrolysis. Saccharification experiments were performed in 125 ml shake flasks with a working volume of 45 ml according to the NREL protocol [21]. Commercial celluloses, namely Avicel pH101 and CMC, were used as substrates for the enzymatic hydrolysis assays. Lignocellulosic residues including filtered PS and corn stover pretreated with 5 % NaOH at 105 °C in a twin-screw extruder were also used. The total sugar content of substrate were 97, 92, 64 and 33.5 % for Avicel, CMC, PCS and PS respectively. The substrate suspended in a 0.05 M sodium citrate buffer to 2 % solid loading (w/v), were sterilized at 121 °C for 20 min and cooled down to ambient temperature before enzyme addition. The enzymatic digestibility tests were carried out at pH 4.8, temperature varied from 50 to 70 °C and surfactant varied 0.5–2 % tween 20. Enzyme loading is 266 CMCase IU per gram of substrate. The commercial enzyme—Accellerase 1500 of Genecor (now Dupont) was used as positive control. The monosaccharide content of hydrolysed samples was determined by anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) with a Dionex ICS-5000 system. C5 and C6 sugars were separated using CarboPAC® PA100 columns at 30 °C with 50 mM NaOH used as eluent at a flow rate of 1 ml/L. The injected volume was 10 µL. Hydrolysate sugar concentrations were quantified using glucose, fructose, xylose, mannose, arabinose, galactose, and cellobiose standards.

Statistical Analysis

The experiments of fermentations and enzymatic hydrolysis have been performed in duplicate in which the results were analysed using software Sigmaplot 13. Enzyme activity data are presented with the standard deviation of triplicates. The significance of variation in sugar concentrations at different enzymatic hydrolysis was determined by considering sample triplicates using paired *t* test at $p < 0.01$.

Results and Discussion

Growth of *T. reesei* RUT C-30 in Paper Sludge

The growth of *T. reesei* RUT C-30 was observed through the formation of pellets in all three sludge media (PS, SS and MS). *T. reesei* RUT C-30 grew mainly in mycelia form in the synthetic medium, meanwhile pellets dominated in fungal morphology in the sludge based media. Loose, fluffy and hollow spherical pellets were formed from the close attachment of fungal mycelia on sludge insoluble matters. This observation is consistent with many other studies reporting that the presence of insoluble substrates stimulates pellet formation at high agitation speed in submerged fermentation [22, 23]. Interestingly, the prevalence of pellets may help to reduce the medium viscosity thus improving mass transfer during fermentation. The pellet

formation may also promote a simple downstream recovery of enzymes.

Characterisation of Lignocellulolytic Enzymes Produced from Paper Sludge

Paper sludge was able to support the growth of *T. reesei* RUT C-30 and its enzyme secretion. The profile of CMCase activity with time, shown in Fig. 1, demonstrates that the highest CMCase activity for MS, SS, and PS enzymes obtained after 7 days of fermentation are 7.3, 4.8, and 1.5 IU/ml, respectively. Since *T. reesei* RUT C-30 needed time to adapt to the complex composition of the sludge samples, the production of enzyme in the sludge media occurred 3 days later than in the synthetic medium with the highest activity obtained on the fourth day (data not shown). Moreover, MS was the better medium for enzyme production compared to the PS and SS. In pulp and paper wastewater treatment systems, PS is generated from wastewater sedimentation. It contains mainly cellulosic fibers residues and waste that could supply cellulose and xylan for cellulase and xylanase induction. The same observation on enzyme activity was reported by Wang et al. [24] working on cellulase production of *T. reesei* RUT C-30 on Kraft paper mill sludge. SS, generated from activated sludge treatment, contains nutritive elements from endogenous activity of bacteria that activities of cellulolytic and hemicellulolytic enzymes of Mandel, SS and MS were continuously measured and highest results are presented in Table 2. The mixture of enzymes responsible for the complete conversion of biomass into monosugars including

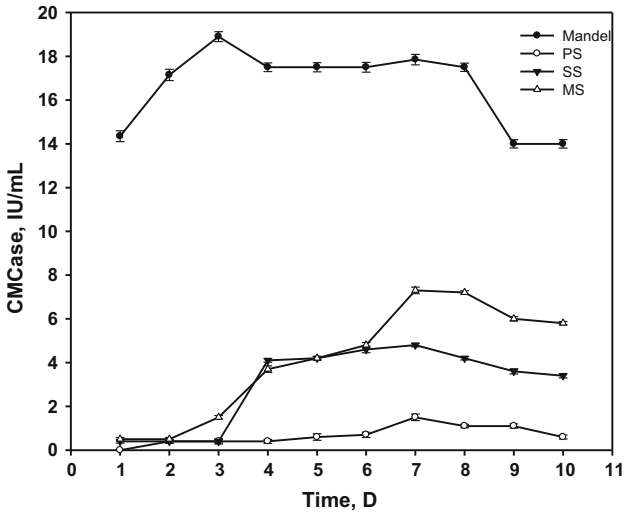

Fig. 1 CMCase production during *T. reesei* RUT C-30 fermentation of pulp and paper sludge

CMCase, Xylanse, β-glucosidase, and β-xylosidase was found in both SS and MS enzymes. The proportion of β-glucosidase per CMCase was very low with 9, 10 and 12 % for SS, Mandel and MS, respectively. The same low level of β-glucosidase and β-xylosidase production in paper sludge was also reported previously [8]. In fact, many studies proved that a low secretion of these enzymes is an intrinsic character of *T. reesei* RUT C-30 strains. In addition, the major part of these enzymes is tightly bound to the cell wall of the fungus during cultivation [2, 25]. The higher enzyme mixture yield was obtained in MS. CMCase and xylanase were 1.7 and 2.1 times higher than in SS. This confirmed the important role of PS in the induction of both cellulases and hemicellulases. For this reason, the utilization of MS as enzyme production medium could be the best choice for the production of a lignocellulolytic enzyme cocktail from paper sludge.

Improvement of Sludge-Based Culture Media for Enzyme Production

Paper sludge composition, which is known to be variable, can influence the performance of microbial fermentation. The primary/secondary mixing ratio, the C/N ratio, and solids concentration are important parameters that might need to be adjusted to stabilize the sludge and make it a proper culture medium to improve enzyme production [26].

Modification of Mixing Ratio

As mentioned above, fermentation using MS reached higher enzyme activity than using only PS or SS. Residual cellulose in PS could be required for *T. reesei* RUT C-30 enzyme secretion. However, the primary sludge contains high amount of ash which could be detrimental to fungal growth and consequently to cellulase production. Optimizing the PS to SS ratio is necessary to determine the best fermentation medium. Therefore, different PS to SS mixing ratios (1:3; 1:1; and 3:1 (v/v)) were prepared for fermentation. The 1:1 mixing ratio was the most suitable for enzyme production with the highest CMCase activity of 7.3 ± 0.2 IU/ml as presented in Fig. 2. Compared to the optimal ratio of 1:1, adding a lower concentration of PS could not meet the demand of substrate inducer of the strain, but adding more substrate could lead to substrate inhibition and lower enzyme production. The presence of more inorganic compounds (ash) could explain this phenomenon. In addition, the high amount of residual cellulosic fibers found in PS increases the viscosity of sludge medium, thus hindering mass transfer of the strain during fermentation [27].

Table 2 Enzyme activities of *T. reesei* RUT C-30 growth on different medium

Sample	CMCase (IU/ml)	ß-glucosidase (IU/ml)	Xylanase (IU/ml)	ß-xylosidase (IU/ml)
SS	4.2 ± 0.1	0.38 ± 0.003	3.1 ± 0.06	0.02 ± 0.004
MS (1:1)	7.3 ± 0.2	0.9 ± 0.01	6.6 ± 0.1	0.45 ± 0.004
Mandel	18.9 ± 0.2	1.8 ± 0.01	10 ± 0.1	0.6 ± 0.004

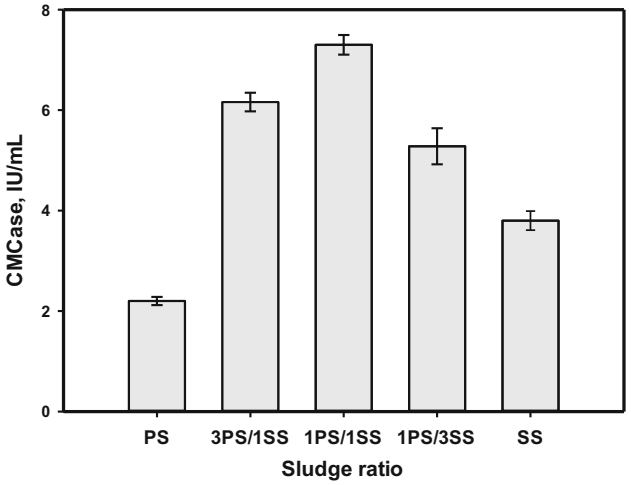

Fig. 2 CMCase production at different PS and SS mixing ratios

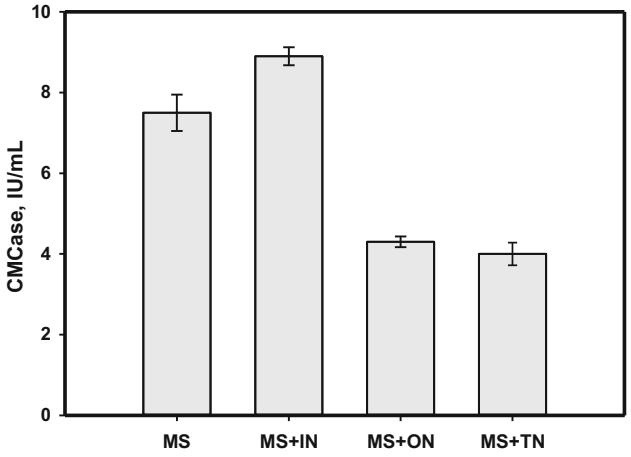

Fig. 3 CMCase production with the addition of different nitrogen sources: *IN* inorganic nitrogen; *ON* organic nitrogen; *TN* inorganic and organic nitrogen

Effect of Nitrogen Supplementation

The MS have a C/N ratio of 18, which is much higher than that of the Mandel synthetic medium (3.7 C/N) as shown in Table 3. It was indicated that MS contains abundant C, but insufficient N for fungal growth and enzyme production. Thus, in order to modify the C/N ratio of MS to meet nitrogen demand, inorganic and organic nitrogen sources (urea, yeast, and peptone) were supplemented in the 1:1 MS. The concentration of nitrogen sources as follows: 1.4 g/L $(NH_4)_2SO_4$ in MS adding inorganic nitrogen (MS + IN); 0.3 g/L urea 0.25 g/L yeast extract and 0.75 g/L proteose peptone in MS adding organic nitrogen (MS + ON). The MS adding total nitrogen (MS + TS) comprised of both inorganic and organic nitrogen at the same concentration as the ones in MS + IN and MS + ON. As shown in Fig. 3, supplementation of organic nitrogen compounds, alone or with ammonium sulfate, affected enzyme production. A significant decrease in enzyme activity was observed. Otherwise, the addition of the inorganic nitrogen enhanced enzyme production. Enzyme activity slightly increased from 7.5 to 8.9 IU/ml. Protein rich nitrogen compounds such as peptone and yeast extract could induce a co-secretion of extracellular protease with cellulases. The proteolysis of cellulase by protease was suggested as the main reason for cellulase reduction [28].

Table 3 Estimated C/N ratio of synthetic and pulp and paper sludge media

	Synthetic	PS	SS	1:1 MS	N added MS
C, % (w/w)	66.7	39.4	42.5	12.5	12.5
N, % (w/w)	18.0	0.3	4.3	0.7	3.4
C/N	3.7	120	10	18	3.7

Effect of Total Solids Concentration

Total solids concentration is one of the most critical parameters of fermentation bioprocesses, especially when residues and waste media are used. High solids concentrations suggest more nutrients for microbial use, but it can lead to a higher viscosity that affects mass transfer during fermentation. Thus the effect of MS solids concentration on enzyme production was evaluated in the 5–40 g/L range. As shown in Fig. 4, enzyme activity shows an increasing profile with solids concentration. The highest enzyme activity (12.6 IU/ml) was obtained at 30 g/L solids concentration. The enzyme activity decreased slightly of 12.2 and 11.8 IU/ml at higher solids concentration of 35 and 40 g/L, respectively. It was reported that the viscosity of PS increased at higher solids concentration [29] that may limit the mass transfer during fermentation and thus inhibiting the growth and enzyme production of the strain.

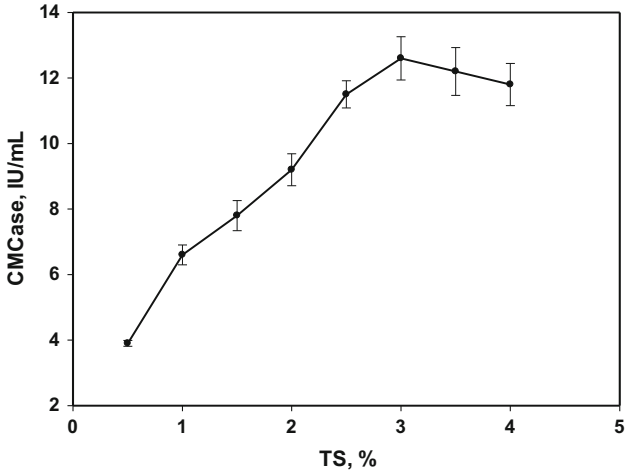

Fig. 4 Effect of sludge solids concentration on CMCase production

Effect of Temperature and Surfactant on Enzymatic Hydrolysis

Effect of Temperature

To investigate the effect of temperature on CMCase activity of fermented broth, an enzymatic essay was carried on using 2 % of substrate concentration at temperature range from 50 to 90 °C in 30 min. The results presented in Fig. 5 show that CMCase activity in sludge medium did not change, while in Mandel medium, the CMCase activity decreased significantly (26 %) in temperature range of 50–70 °C. The enzymes produced in complex medium of sludge may have better properties than convenient medium that could be other advantage of using sludge as fermentation medium. Therefore, the enzymatic hydrolysis was carried out at different temperatures from 50 to 70° C to investigate the effect of temperature on performing of enzyme during conversion process. Unfortunately, the sugar yield decreased significantly for all substrates at high temperature of 60 and 70 °C (Fig. 6,).thus the cellulases of *T. reesei* RUT C-30 produced from the sludge are not stable over 50 °C.

Effect of Surfactant Concentration

Non-ionic surfactants such as polysorbate 20 and 80 are known to have multiple functions in enhancing hydrolysis efficiency: biomass disruptor, enzyme stabilizer, and enzyme effectors [30]. Figure 7 shows the improvement of hydrolysis with the addition of 0.5–2 % polysorbate 20 for all substrates. CMC to sugar conversion yield was improved slightly (7 % of sugar released) with the addition of 0.5 % polysorbate 20, but further increases did not have any effect on the hydrolysis. A linear relationship between polysorbate 20 concentration (below 1 %) and sugar yield was observed in the hydrolysis of Avicel, PCS, and PS with an increase in sugar amount of 61, 95, and 21 %, respectively. The effect of the surfactant addition was more important on the enzyme hydrolysis of insoluble and complex structure substrates, particularly PCS, which was assumed to have high lignin content. The positive effect of the surfactant addition might be explained by a strong affinity of polysorbate 20 for this substrate, leading to a hydrophobic interaction with lignin on substrate surface that prevented unspecific adsorption of the enzymes on lignin [31, 32].

Hydrolysis of Lignocellulosic Biomass

Cultivations of *T. reesei* RUT C-30 in Mandel and 30 g/L MS + IN media were carried out in 2 L shake flasks for 7 days. The supernatant of sludge fermented broth of *T.*

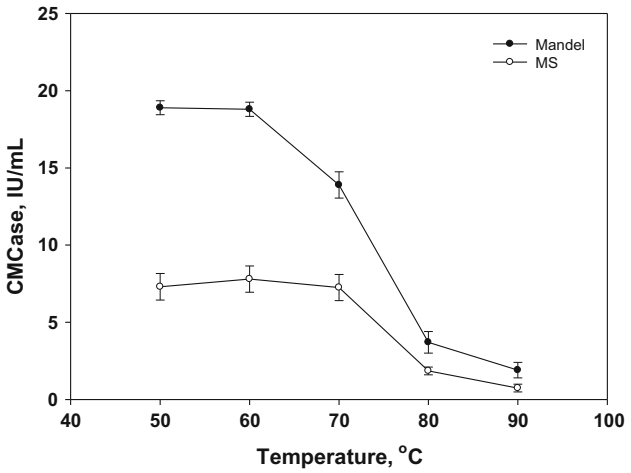

Fig. 5 Effect of temperature on CMCase activity (2 % substrate in 30 min)

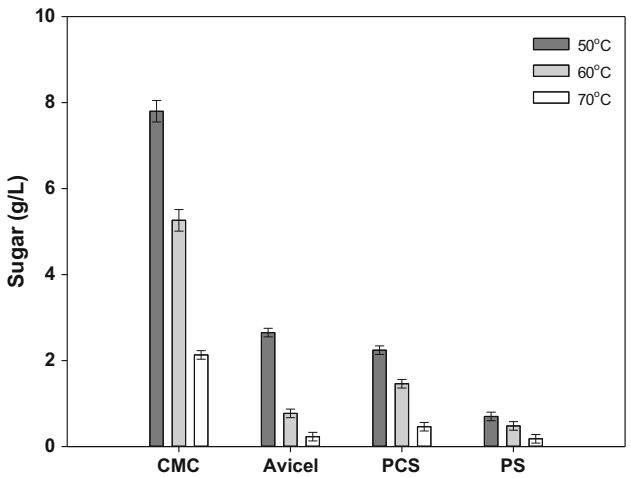

Fig. 6 Effect of temperature on enzymatic hydrolysis using sludge fermented broth (2 % substrate in 72 h)

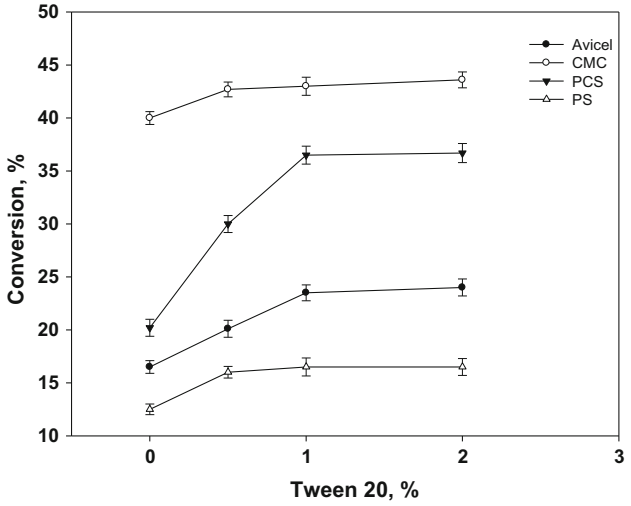

Fig. 7 Effect of polysorbate 20 addition on the enzymatic hydrolysis of different substrates (*CMC* carboxymethyl cellulose; *PCS* pretreated corn stover; *Avicel* bacterial microcrystalline cellulose; *PS* primary sludge)

reesei RUT C-30 was used directly for enzymatic hydrolysis. To evaluate the performance of the enzyme cocktail, different cellulosic biomass were used as substrates. They consisted in Avicel, CMC, lignocellulosic residues (pretreated corn stover (PCS) and primary sludge (PS)). Specific sugar concentrations obtained by hydrolysis were calculated based on total detected sugars. Perform of hydrolysis are presented in Fig. 8. The main reducing sugars found in all substrate hydrolysates are glucose and xylose, as determined by ion chromatography. In addition, small amounts of arabinose and cellobiose were detected in PCS and Avicel. The presence of monosaccharides in the hydrolysates of all four substrates indicates that there is a good functional and synergistic integration of the sludge-produced enzyme mixture

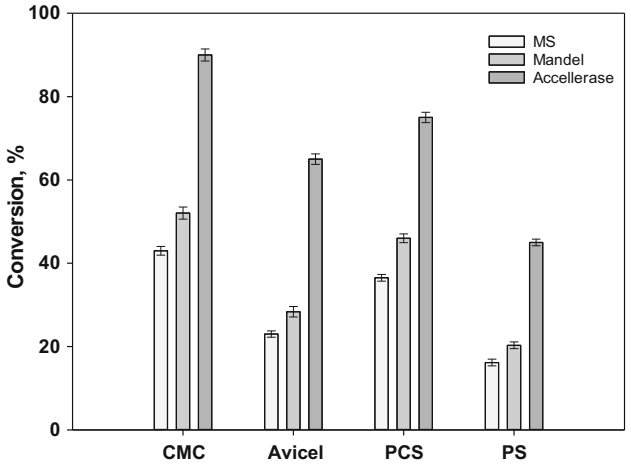

Fig. 8 Enzymatic hydrolysis of different substrates at pH 4.8, 50 °C, 1 % Tween 20 and 72 h (*CMC* carboxymethyl cellulose; *Avicel* bacterial microcrystalline cellulose; *PCS* pretreated corn stover; *PS* primary sludge)

responsible for degrading these lignocellulosic substrates into fermentable sugars. However, the biomass to sugar conversion efficiency of the enzymes was affected by the complexity of the composition and structure of the biomass. For the hydrolyse using the enzyme from sludge, maximum conversion was obtained from CMC (43 %), containing mainly soluble and amorphous cellulose, followed by PCS (36.5 %). The low rate conversion of Avicel (23 %), can be explained by the substrate containing mainly microcrystalline cellulose. Also, the paper sludge-based enzyme cocktail is capable of degrading PS, resulting in 16.2 % sugar yield, In the case of the enzymes from Mandel medium, the conversion rate was obtained higher slightly (52 % for CMC, 28.3 % for Avicel, 46 % for PCS and 20.3 % for PS). The significantly higher conversion was observed on the use of Accellerase 1500 (90 % for CMC, 65 % for Avicel, 75 % for PCs and 45 % for PS). It was noted that the proportion of β-glucosidase per CMCase (20–25 %) of this commercial enzyme cocktails is two-fold higher compared to the one of the enzymes from sludge and Mandel medium. Thus, a supplementation of β-glucosidase to enzyme cocktail from sludge is recommended to improve the conversion. The twin-screw extruder treatment was effective at removing lignin and extractives present in corn stover. These products inhibit enzyme accessibility, resulting in significant sugar amounts in the PCS hydrolysate, the remaining lignin (8 %) could be minimized by help of supplemented surfactant 1 % [33, 34]. PS contains mainly small cellulose fibers and other materials extracted during the deinking process such as ink pigments, clay and adhesives that could hinder the catalyst activity of the enzymes. Previous study suggested that, pretreatment process could improve the sugar content in PS as well improve the digestibility [24].

Conclusions

The activity of various lignocellulolytic enzymes, including CMCase, β-glucosidase, xylanase, and β-xyloxidase, was determined in the fermented broth of *T. reesei* obtained with pulp and paper sludge. It was necessary to modify the sludge to obtain a stable and efficient culture medium for *T. reesei* and enhance enzyme production. The optimal PS and SS mixing ratio for enzyme production was found to be 1:1 (v/v) in this particular case. In addition, enzyme production was enhanced with an inorganic nitrogen supplementation and an increase in sludge solids concentration. The enzyme mixture obtained with paper sludge was efficient to hydrolyse pretreated corn stover and the pulp and paper primary sludge into fermentable sugars. Moreover, the sugar yield was improved by adding polysorbate 20 during the hydrolysis of the lignocellulosic substrates. Overall, paper sludge can be used to produce

lignocellulolytic enzymes with minimal chemical supplementation. When such paper sludge are available near to a biorefinery using enzymes to convert biomass components into valuable products such as second generation sugars, on-site production could be considered to decrease the manufacturing cost of enzymes.

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