Appl Biochem Biotechnol (2009) 158:552–560
DOI 10.1007/s12010-008-8476-9

Water Hyacinth as Carbon Source for the Production of Cellulase by *Trichoderma reesei*

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Received: 14 June 2008 / Accepted: 3 December 2008 /
Published online: 18 December 2008

Abstract Water hyacinth (*Eichhornia crassipes*), an aquatic weed common to the subtropic/tropical regions, was utilized as an inexpensive lignocellulosic substrate for production of cellulase by *Trichoderma reesei*. The effects of process parameters like substrate pretreatment, substrate concentration, initial medium pH, mode of inoculation, and incubation temperature on cellulase production were investigated. Under optimal conditions, a maximal cellulase activity of 0.22±0.04 IU/ml (approximately 73.3 IU/g cellulose) was recorded at the end of 15-day incubation period. Specific activity of the enzyme was 6.25 IU/mg protein. Hydrolysis of 1% substrate (water hyacinth) using crude enzyme dosage of 1.2 IU/g water hyacinth showed 28.7% saccharification in 1 h. The observations in present study indicate that saccharification of cellulose from water hyacinth was significantly higher by laboratory-produced cellulase than the commercial blend.

Keywords Water hyacinth · *Trichoderma reesei* · Cellulase · Saccharification

Introduction

Cellulases are significant for their potential applications in food, textile, and paper processing [1, 2]. Use of cellulases for enzymatic deinking [3], cellulosic waste management [4], and lactic acid production [5] has been reported. Cellulose is an important renewable resource in the emerging possibilities of biomass utilization. Using cellulase enzymes, cellulose can be hydrolyzed to its constituent sugars and used as feedstock for production of ethanol [6, 7], organic acids [5, 8], and other chemicals [9]. However, the commercial viability of such processes is limited due to the high cost of cellulase production. Cost of carbon source is one of the significant factors contributing to the cost of cellulase production [10]. The search for low-cost substrates has led to investigations in the

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utilization of wheat straw [11, 12], sugar cane bagasse [13, 14], corn cob residues [15], banana stem [16], and dairy manure [17] for cellulase production. Water hyacinth (WH) or *Eichhornia crassipes*, an aquatic weed common to the subtropics and tropics, is often the cause of ecological and socioeconomic problems to contiguous populations [18, 19]. It disrupts water transport and fishing, blocks irrigation canals, depletes aquatic biodiversity, and destroys the overall ecological balance. This has motivated research not only on WH growth control measures but also on utilization of WH in beneficial ways [20, 21]. WH is an abundantly available lignocellulosic biomass source composed of cellulose and hemicellulose polymers and can be a good substrate for economical production of cellulase. This work investigates the utilization of WH as the sole carbon source for production of cellulase by *Trichoderma reesei*. Further, application of the laboratory-produced enzyme for saccharification of WH was tested.

Methods

Microorganisms

The fungal strain *Trichoderma reesei* NCIM 1052 (ATCC 24449), referred here onwards as *T. reesei*, was obtained from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (NCL), Pune, India and maintained on potato dextrose agar (PDA) plate [22]. For cellulose production, the fungal culture was cultivated in Mandel's medium [23]. Pretreated WH, 1–8% (*w*/*v*), was added to the medium as the sole carbon source. The medium containing the pretreated WH was sterilized by autoclaving at 121 °C at 15 lb for 20 min.

Substrate Pretreatment

WH collected from the ponds in Nerul, Navi Mumbai was cleaned by washing with tap water, spread on trays, sun dried, and then oven dried at 60 °C for 24 h. The dried plant material was subjected to alkali treatment by soaking in NaOH solution of varying concentrations (1–5%; *w*/*v*), for 15 h, at ambient temperature. The material was then washed thrice with water. The water for the fourth wash was amended with 1% phosphoric acid. The material was subjected to two more washes with tap water, sun dried, and then baked at 70 °C for 1 h. Alternatively, dried WH was subjected to pretreatment in an autoclave. In brief, WH was suspended in water and heated to 120 °C at 15 lb for time periods of 5, 10, 15, and 20 min. The solid (gram) to liquid (milliliter) ratios for alkali and heating pretreatments were 1:100 and 1–4:100, respectively. The treated material was then dried as described above. A combination of the two treatments consisted of sequential alkali and steam treatments. After pretreatment, the dried plant material was hand-crushed (dried plant material held in gloved hands was broken using pressure of the fingers) to obtain small random sized pieces (1–5 cm long). Alternatively, the weeds were crushed in a blender, sieved to obtain fairly uniform sized particles (0.05–0.1 cm long). The material was wrapped in paper and stored dry in polyethylene bags at room temperature.

Inoculum

The fungus *T. reesei* was cultured on PDA plate for 6–7 days till good spore crop developed. Sterile saline solution (2 ml) was added to the plate and swirled about gently to

release the spores. In general, 1 ml of the spore suspension consisting of 10^4–10^5 spores was used to inoculate 100 ml Mandel's medium. Alternatively, a pre-inoculation culture was prepared by incubating the spore-containing medium at 30 °C in a shaker at 100 rpm for 6–7 days to allow for spore germination and formation of mycelia. The mycelial mass was harvested by centrifugation and unless otherwise indicated, approximately 0.1 mg wet weight of mycelia was used to inoculate 100 ml medium.

Cellulase Production

The pretreated/untreated and dried WH was added to Mandel's medium (100 ml) in a 250-ml Erlenmeyer flask, autoclaved, and cooled to room temperature. The substrate (WH) concentration ranged from 1% to 8% (*w*/*v*). *T. reesei* spores or mycelial mass were inoculated into the medium and the culture flasks incubated in a shaker at 30 °C, 100 rpm. Aliquots were drawn aseptically at regular intervals and analyzed for presence of cellulase activity.

Investigation of Process Parameters

Cellulase production process parameters investigated in this study include substrate pretreatment method (NaOH; 1–5% (*w*/*v*), autoclaving and both), substrate particle size, initial medium pH (range 4.5–8.0), culture incubation temperature (25–50 °C), substrate concentration (1–8%; *w*/*v*), and mode of inoculation. All the experiments were repeated three times.

Enzyme Assay

Cellulase activity was measured as described by Ghose [24] using the filter paper assay method and expressed as international units per milliliter. International unit is defined as the amount of enzyme that produces 1 μM glucose (reducing sugars as glucose) per minute during the hydrolysis reaction. Reducing sugar concentration was determined using the dinitrosalicylic acid (DNS) method [25]. Protein was estimated by the method of Lowry using bovine serum albumin as the standard [26].

WH Hydrolysis

Ability of the laboratory-produced cellulase enzyme to hydrolyze WH was evaluated using the crude enzyme at different dosages [24]. Laboratory-produced cellulase was the *T. reesei* whole culture broth following centrifugation of solids (5,000×*g* at 4 °C for 10 min) and stored at 4 °C. Hydrolysis assay reactions were performed in 20 ml glass tubes that contained 1% substrate (WH pretreated with 1% NaOH) suspended in 5 ml (final volume) pH 4.8, 50 mM citrate buffer, to which was added the crude cellulase solution. Hydrolysis was performed at 50 °C in a water bath, for 1–2 h. Glucose concentration in the hydrolysate was determined by DNS assay. WH hydrolytic capacity of the laboratory-produced enzyme was compared with that of the Cellulase C8546 (Sigma Alrich, Saint Louis, MO, USA). Loading of the two enzymes was adjusted to same international unit activity. Saccharification (%) was calculated as: reducing sugar (mg/ml)×0.9×100/initial cellulose (mg/ml) in WH. Cellulose content in the WH substrate was estimated by the method of Updegraff [27].

Results and Discussion

WH as the sole source of carbon supported growth of *T. reesei* as was indicated by the presence of rich mycelial growth and spore formation at the end of 5–6-day incubation period. Cellulase production was monitored by filter paper assay of the culture broth. Factors like substrate pretreatment method, substrate particle size, initial medium pH, culture incubation temperature, substrate concentration, and mode of inoculation were investigated to obtain optimal conditions for cellulase production. Changes in cellulase production with variation in process parameters in the different experimental setups are expressed as relative cellulase yield.

Effect of Substrate Pretreatment and Particle Size

Use of 1% NaOH pretreated WH showed at least twofold increase in cellulase yield than that obtained with untreated (sun dried and powdered) WH as substrate, at the end of 4-day incubation period. During the same period, cellulase production using steam-pretreated WH was up to 1.5-fold less than that obtained with alkali-pretreated WH. Use of WH subjected to both steam and alkali pretreatment showed no significant difference in cellulase yield in comparison to that obtained with WH pretreated with alkali alone (Fig. 1). Cellulase production was minimal with fresh pieces/paste of WH as substrate. In comparison, a 12% increase in cellulase yield was obtained using dried WH as substrate. Alkali pretreatment of the dried WH with 1% NaOH enhanced the yield by 28%. It was observed that pretreatment of WH with higher concentrations, 2% to 5% NaOH, showed no significant variation in the cellulase yield from that obtained with 1% NaOH pretreated WH (data not shown). These observations indicate that pretreatment of WH with 1% NaOH is optimal for its use as substrate for cellulase production by *T. reesei*. Pretreatment is carried out to reduce lignocellulosic biomass crystallinity, render cellulose accessible, and remove lignin [28]. Saponification of intermolecular ester bonds cross-linking hemicelluloses and lignin, and

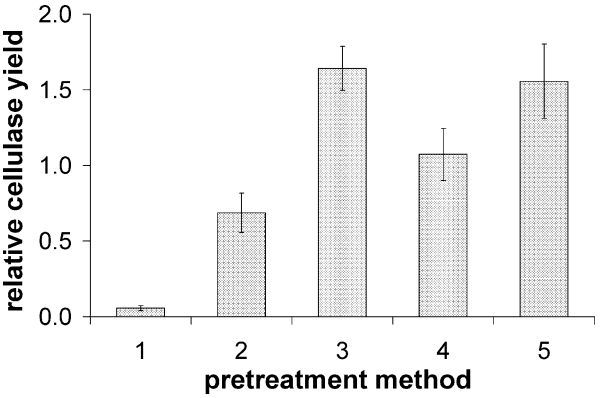

Fig. 1 Effect of WH pretreatment on cellulase production by *T. reesei*. Relative cellulase yield in media with *1* green, *2* dried, *3* alkali-pretreated, *4* steam-pretreated, and *5* alkali- and steam-pretreated WH as the sole carbon substrate, at the end of 4-day incubation period are shown. Relative yield is the cellulase yield obtained using substrate subjected to a particular pretreatment method divided by the average of the cellulase yield in all cultures with the different pretreated substrates in that experimental set. The results shown are representative of three experiments. *Error bars* represent standard error

disruption of the lignin structure is reported to occur on NaOH treatment of lignocellulosic materials [28]. Palonen et al. [29] report the adsorption of cellulases on lignin. Lignin is known to inhibit cellulase activity [30].

Investigation on the effect of substrate particle size on cellulase production showed that in the early stages of incubation, cellulase yield with powdered WH was up to 1.6-fold more than that obtained with unpowdered WH incubated under identical culture conditions (data not shown). However, the apparent lag observed with unpowdered WH was transient. Towards the end of 14-day incubation period, cellulase yield was found to be similar with powdered and unpowdered WH indicating that the substrate particle size had no effect on the cellulase production by *T. reesei*.

Effect of Incubation Temperature and Initial Medium pH

Cellulase production with *T. reesei* cultivated in medium with 1% alkali-pretreated WH as substrate showed cellulase production at 25 °C, 30 °C, 37 °C, 40 °C, and 50 °C. Among these cellulase activity was consistent and maximal in the culture incubated at 30 °C. On an average, the relative enzyme yield at 30 °C was up to five times and two times more than the yield at 25 °C and 50 °C, respectively.

Evaluation of the effect of initial pH of *T. reesei* culture medium on cellulase production showed that cellulase yield was comparable in the pH range of 6 to 8. Cellulase yield in medium with initial pH value of 6 was twofold of that obtained in medium with initial pH 4.5 (Fig. 2). Usually, *T. reesei* is cultured in Mandel's medium which has a pH of 4.8–5.0. However, our observations indicate that cellulase yield is better at pH higher than 5. Juhász et al. [31] report the significance of pH-controlling strategies for improving cellulase yields.

Mode of Inoculation

Cellulase yield in medium inoculated with *T. reesei* mycelia was observed to be up to tenfold more than that obtained in the medium inoculated with *T. reesei* spores. The lag phase in cellulase production in medium inoculated with spores could be attributed to time required for viable spores to germinate, grow, and then commence cellulase production. Thus, use of mycelia (pregrown) as seed may be preferred over spores as inoculum for higher cellulase yield with reduction in incubation period for small and large scale cellulase

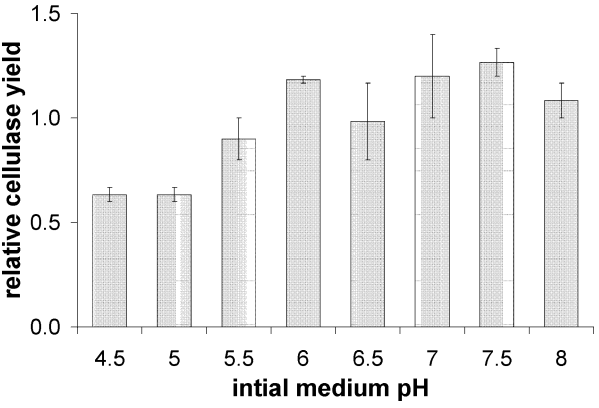

Fig. 2 Effect of initial medium pH on cellulase production. Relative yield of cellulase in culture media with initial pH value ranging from 4.5 to 8.0 at the end of 5-day incubation period are shown. Relative yield is the cellulase yield obtained with a particular initial medium pH divided by the average of cellulase yield in all cultures with the different initial medium pH in that experimental set. The results shown are representative of three experiments. *Error bars* represent standard error

production. Varying the initial inoculum size (mycelial mass) from 0.05 to 0.1 mg and 1.0 mg per 100 ml medium did not show significant variation in the enzyme yield over a 2-week incubation period.

Substrate Concentration

Cellulase production was comparable in culture media with 1–4% (*w*/*v*) substrate concentration, with a given initial inoculum. A marked decrease in cellulase activity was observed in the media containing substrate concentration of 6% (*w*/*v*) and above. The lowering of soluble cellulase yield with increase in substrate concentration may be due to the adsorption of cellulase on WH (cellulose) biomass and its resistance to mass transfer [32, 33].

From the above experiments, 1% (*w*/*v*) substrate concentration, growth temperature of 30 °C, initial medium pH of 6.0–7.5, and the use of mycelial mass as inoculum were found to be the optimized medium and culture conditions for cellulase production using WH as the sole carbon source by *T. reesei*. Under these conditions, 0.1 IU/ml cellulase activity was obtained on day 7 of culture incubation (Fig. 3). The cellulase yield increased to 0.22± 0.04 IU/ml (approximately 73.3 IU/g cellulose) at the end of 15-day incubation period. Specific activity of the enzyme was 6.25 IU/mg protein. A decline in cellulase activity was observed between the days 7 and 13 (Fig. 3). Cellobiose produced during cellulose hydrolysis [34] and aromatic water-soluble substrates produced during delignification [35] may have contributed to repression of cellulolytic action of the enzyme.

Hydrolysis of WH Using the Laboratory-Produced Enzyme

Using the Updegraff method, cellulose content in the sun-dried WH (pretreated with 1% NaOH) was estimated to be around 30% [27]. Hydrolysis of 1% substrate (water hyacinth) using crude enzyme dosage of 1.2 IU/g WH yielded 5.3 mM glucose in 1 h. Increasing hydrolysis reaction time, from 1 to 2 h, enhanced saccharification yield from 27.8% to 48%. An increase in cellulase dosage, from 1.2 to 6.0 IU/g WH, improved saccharification yield by 20% in 1 h (Fig. 4). Interestingly, it was observed that WH saccharification yield using the laboratory-produced crude cellulase solution was up to fourfold of that obtained using similar dosage of the commercial cellulase enzyme. This indicates that the enzyme

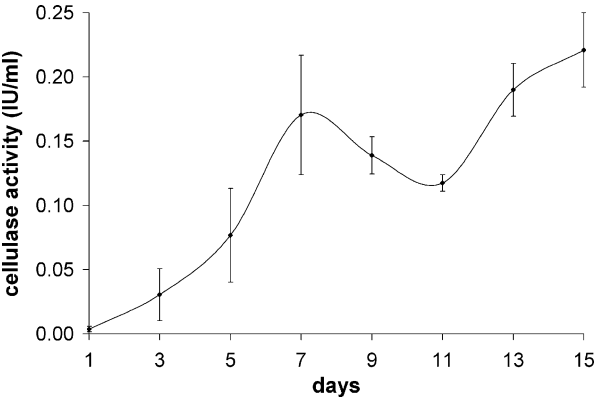

Fig. 3 Time course of cellulase production by *T. reesei* cultivated on 1% WH (*w*/*v*) as the sole source of carbon. Culture conditions: Mandel's medium pH 6.0 containing powdered WH priorly pretreated with 1% NaOH (*w*/*v*) was inoculated with *T. reesei* mycelial mass (1 mg wet weight/l) and incubated at 30 °C. Results shown represent three repeated fermentations. *Error bars* represent standard error

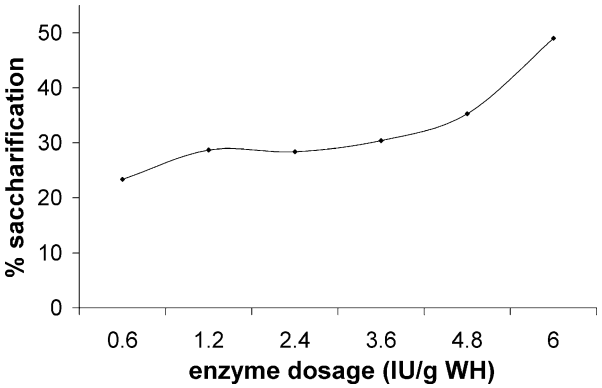

Fig. 4 Percentage (WH cellulose) saccharification yield obtained using varying dosage of the crude cellulase enzyme. For calculation of % saccharification, see "Methods"

produced using WH as carbon source hydrolyzes WH more effectively. The possibility of extending the use of this enzyme produced using WH as the carbon source for improved saccharification of other lignocellulosic substrates needs to be investigated.

Cellulase production using WH as the sole carbon source by *Humicola* spp. [36], a *Trichoderma* isolate [37], *T. reesei* [38], *Aspergillus flavus* S-7 [39], *Aspergillus niger* 1 [40], bacteria [41], and cocultivation of *A. niger* RK3 and *T. reesei* MTCC164 [42] is reported. Culture conditions, namely, nutritional (especially, nitrogen sources) and environmental parameters (pH, incubation temperature, and period), for maximal cellulase production were investigated [36–39]. In this work, maximal cellulase (FPase/exoglucanase) production observed by *T. reesei* 1052 cultivated on 1% WH (pretreated with 1% NaOH) was approximately 0.22±0.04 IU/ml (or 73.3 IU/g cellulose or 21.9 IU/g dry WH) at the end of 15-day incubation period. In another work, application of stimulators (sodium citrate, sodium phytate, Tween-80, asparagine, potassium dihydrogen phosphate, and wheat bran) yielded an enzyme preparation with high cellobiase/exoglucanase ratio of 63.8:1 (18.5 U/ml cellobiase, 0.29 U/ml exoglucanase, and 2.21 U/ml endoglucanase) by *A. niger* 1 [40]. The basal cellulase production of 25.2 filter paper units per g dry substrate with native isolate WHB3 improved to 216 U/g dry substrate on optimization of its culture conditions (added moisture, pH, temperature, incubation time, inoculum concentration, addition of urea, and sucrose in the medium). The exoglucanase activity (22.89 U/g substrate) obtained by cocultivation of *A. niger* RK3 and *T. reesei* MTCC164 on alkali-treated WH was comparable to that of *T. reesei* 1052. The cocultivation was advantageous with about 13% increase in the β-glucosidase activity over the maximum activities observed under single culture conditions of *A. niger* RK3 and *T. reesei* MTCC164 [42].

The cellulolytic fungus *T. reesei* was chosen in this work because it has been extensively studied for cellulase production [43]. This work reports the optimization of process parameters for cellulase production by *T. reesei* NCIM 1052, utilizing WH from the local water bodies as the carbon source. Further, the ability of the laboratory-produced crude enzyme to saccharify WH cellulose has been established. Hronich et al. [44] report the potential of WH as a competitive feedstock. The enzyme produced in this work can be effectively used to hydrolyze WH cellulose to its constituent sugars which are potential feedstock for production of valuable chemicals. In addition, the utilization of WH as a renewable resource will contribute to reduction of the socioeconomic problems associated with the extensive growth of this proliferative weed.

Acknowledgment This work was supported by a grant from the Department of Biotechnology, Ministry of Science and Technology, Government of India.

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