Duckweed (*Lemna minor*) is a novel natural inducer of cellulase production in *Trichoderma reesei*

Chen Li [1] , **Jun Feng** [1] , **Xue Bai** [1,2] , **Shulin Chen** [1] , **Dongyuan Zhang** [1] , **Ronglin He** [1] , **Demao Li** [Corresp. 1]

[1] Tianjin Key Laboratory of Industrial Biology Systems and Processing Engineering, Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin, China
[2] College of Life Sciences, The Northwest Normal University, Lanzhou, China

Corresponding Author: Demao Li
Email address: li_dm@tib.cas.cn

An inducer is crucial for cellulase production. In this study, duckweed was used as an inducer of cellulase production by *Trichoderma reesei* RUT C30. In a reaction induced by 50 g l^{-1} duckweed in shake flasks, the filter-paper activity (FPA) reached 6.5 FPU ml^{-1}, a value comparable to that induced by avicel. The enzyme-hydrolysis rate induced by steam-exploded corn stalks was 54.2%, representing a 28% improvement over that induced by avicel. The duckweed starch was hydrolyzed to glucose, which was subsequently used for biomass accumulation during the fermentation process. Furthermore, to optimize control of the fermentation process, a combined substrate of avicel and duckweed was used to induce cellulase production by *T. reesei* RUT C30. The cellulase production and hydrolysis rates for the combined substrate, compared with avicel alone, were 39.6% and 36.7% higher, respectively. The results of this study suggest that duckweed is a good inducer of cellulase production in *T. reesei*, and it might aid in decreasing the cost of lignocellulosic-material hydrolysis.

Duckweed (*Lemna minor*) is a novel natural inducer of cellulase production in *Trichoderma reesei*

Chen Li[1], Jun Feng[1], Xue Bai[1, 2], Shulin Chen[1], Dongyuan Zhang[1], Ronglin He[1]*, Demao Li[1]*

[1]Tianjin Key Laboratory of Industrial Biology Systems and Processing Engineering, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin, China

[2]College of Life Sciences, Northwest Normal University, Lanzhou, China

*Corresponding authors

Demao Li, Email: li_dm@tib.cas.cn; Tel: +86-22-84861932; Fax: +86-22-84861932;
Ronglin He, Email: he_rl@tib.cas.cn; Tel: +86-22-84861993

Abstract

An inducer is crucial for cellulase production. In this study, duckweed was used as an inducer of cellulase production by *Trichoderma reesei* RUT C30. In a reaction induced by 50 g l^{-1} duckweed in shake flasks, the filter-paper activity (FPA) reached 6.5 FPU ml^{-1}, a value comparable to that induced by avicel. The enzyme-hydrolysis rate induced by steam-exploded corn stalks was 54.2%, representing a 28% improvement over that induced by avicel. The duckweed starch was hydrolyzed to glucose, which was subsequently used for biomass accumulation during the fermentation process. Furthermore, to optimize control of the fermentation process, a combined substrate of avicel and duckweed was used to induce cellulase production by *T. reesei* RUT C30. The cellulase production and hydrolysis rates for the combined substrate, compared with avicel alone, were 39.6% and 36.7% higher, respectively. The results of this study suggest that duckweed is a good inducer of cellulase production in *T. reesei*, and it might aid in decreasing the cost of lignocellulosic-material hydrolysis.

Keywords

Duckweed; Cellulase; Starch; Fermentation; Hydrolysis

Introduction

Lignocellulosic biomass is the most abundant and most renewable source for the production of biofuels. However, conversion of lignocelluloses into soluble sugars by lignocellulolytic enzymes remains a major challenge limiting the widespread use of bioenergy (Iqbal et al., 2013). The high cost of lignocellulolytic enzymes, which are produced by filamentous fungi, is the major bottleneck limiting the biorefinery of lignocellulose (Li et al., 2010b).

Among all species of filamentous fungi producing lignocellulolytic enzymes, *Trichoderma reesei* (teleomorph *Hypocrea jecorina*) is the main producer used for commercial lignocellulolytic-enzyme preparations. *T. reesei* cellulase contains several endo-1,4-β-glucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21). These enzymes synergistically hydrolyze cellulose to monosaccharide (Singhania et al., 2010). For cellulase production in different industries, submerged cultures in fermentors are mainly used with *T. reesei*. To decrease the high cost of cellulase production by *T. reesei*, in the past 40 years, enormous efforts have been made in modifying strains and optimizing aspects of the fermentation process, such as the medium composition, pH, agitation, and extracellular-protein supplementation (Abdullah et al., 2014; Ahamed & Vermette 2009; Lv et al., 2015). Among these approaches, using lignocellulosic materials as inducers might be an effective and simple method to enhance enzyme production.

Use of an inducer is crucial for cellulase production. Most cellulases are inductive enzymes, which reach their full activity only in the presence of an inducer. Cellulose, which is present in many lignocellulosic materials, is a commonly used natural inducer. The functional components of cellulose are the disaccharides generated by its degradation (such as sophorose, cellobiose and gentiobiose) and the derivatives that are transported into cells and trigger the expression of enzyme-encoding genes. Several types of lignocellulosic materials, such as corn stover (Zhang et al., 2012), bran (Vijayaraghavan et al., 2016), rice straw (Kang et al., 2004), and bagasse (Camassola & Dillon 2007), have been studied as lignocellulytic-enzyme inducers. Different lignocellulosic materials used as enzyme inducers have different effects on lignocellulytic-enzyme production, due to the varying composition among lignocellulosic materials (Juhasz et al., 2005). The lignocellulosic materials used as enzyme inducers are mainly composed of cellulose, hemicellulose, and lignin. A high lignin content influences the structure of lignocellulosic materials, thus making them inefficient enzyme inducers in submerged cultures (Kumar et al., 2012). Therefore, a low-density lignocellulosic material with low lignin content is an ideal enzyme inducer in lignocellulytic-enzyme production.

Duckweed is being studied by researchers worldwide as a potential source of biofuels, because it grows rapidly and does not contribute to global warming (Campanella et al., 2012; Muradov et al., 2010). Duckweed can provide a valuable source of starch, whose content can reach 49 % of its dry weight (Zhao et al., 2014). Duckweed is also composed of cellulose, hemicellulose, and lignin (Zhao et al., 2012). Because cellulose and hemicellulose are inducers in cellulase and hemicellulase production, duckweed might be a candidate enzyme inducer. Duckweed has a very low lignin content (Zhao et al., 2012) and hence may be more easily used than other lignocellulosic materials. Furthermore, cellulases and amylases are abundant in the secretomes of *T. reesei* (Adav et al., 2013). Starch in duckweed may be hydrolyzed to glucose and utilized as a carbon source for biomass accumulation by *T. reesei*. However, there are no reports on enzyme production in *T. reesei* with duckweed used as an inducer.

In this study, we used duckweed as an enzyme inducer of cellulase production during batch fermentation. The effects of duckweed components on cellulase production and hydrolysis were also investigated. Additionally, cellulase production in the presence of duckweed combined with other substrates and the hydrolysis abilities of the obtained enzymes were evaluated.

Materials and methods

Fungus and duckweed

Trichoderma reesei Rut C-30 (ATCC 56765) was purchased from the National Center of Industrial Culture Collection (CICC), Beijing, China. The strains were maintained on potato dextrose agar (PDA) at 4℃ and subcultured once every 3 months.

Dried duckweed, *Lemna minor,* was purchased from Bozhou Jianan Pharmaceutical Co., Ltd., China.

Seed culture

Spores on PDA slants were washed with sterilized water and suspended in sterilized water to a concentration of 10^7–10^8 spores ml^{-1}. For each culture, 1 ml of spore suspension was transferred into a

250-ml Erlenmeyer flask containing 30 ml seed-culture medium (avicel 20 g/L, corn steep liquor powder 10 g/L, glucose 10 g/L, pH 4.5), then cultured for 24 h at 28℃ and 180 rpm.

Preparation of duckweed powder and hydrolyzed residue by enzymes

The dry duckweed was pulverized into 60 mesh powder with a pulverizer.

Excess amylase (1000 U/g), glucoamylase (1000 U/g), proteinase (1000 U/g), and pectinase (1000 U/g) were added to a hydrolysis system, in acetate buffer solution (0.05 M, pH 5.3) containing 10% (w/v) duckweed powder, and cultured at 40℃, 100 rpm, for 24 h. The duckweed hydrolyzed residue was then harvested by centrifugation at 10000 rpm. The hydrolyzed residue was dried at 60℃ for 24 h to achieve a constant weight.

Effects of duckweed on cellulase production

For each culture, a 5% (v/v) inoculum of seed culture was transferred into a 250-ml Erlenmeyer flask that contained 30 ml culture medium. The culture medium comprised corn steep powder 17 g/L, $(NH4)_2SO_4$ 5 g/L, KH_2PO_4 6 g/L, $MgSO_4$ 1 g/L, glycerol 2.5 g/L, and Tween-80 2 ml/L, pH 5.0, with different concentrations of inducers. The Erlenmeyer flasks were cultured at 26℃, 180 rpm for 120 h.

To determine the effects of duckweed powder on cellulase production, duckweed-powder concentrations of 10 g/L, 30 g/L, 50 g/L, and 70 g/L were studied. Then the induction effects of 50 g/L concentrations of corn cob, steam-exploded corn straw, bagasse, avicel, and hydrolyzed residue were tested for comparison with duckweed. The biomass of *T. reesei*, the starch content, and the amylase activity were determined during the cultivation. Among the tests of the effects of different inducers, only the results of FPA, *T. reesei* biomass and amylase activities at 72 h are shown. The changes in the FPA, *T. reesei* biomass and amylase activities are shown for treatment with a 50 g/L concentration of inducer during the 120 h cultivation. Different combinations of duckweed powder, hydrolyzed duckweed residue, and avicel as inducers were studied and are shown in Table 1.

Batch fermentation in a 5-L fermenter

For each culture, 5% (v/v) of seed broth was inoculated into a 5-L stirred fermenter (BIOTECH-5BG, Shanghai Baoxing Bio-Engineering Equipment Co. Ltd, China), which contained 3 L culture medium. The culture-medium composition was the same as that used for batch fermentation in shake flasks. The inducers used are shown in Table 2.

The initial culture conditions were as follows: agitation speed 300 rpm and aeration rate 3 L/min, at 0.05 Mpa and 26°C. The dissolved oxygen (DO) content was kept above 30% by varying the agitation speed and air flow. In batch fermentation, the pH was controlled as follows: 0–20 h, growth without pH control; 20–40 h, pH not less than 4.5; 40–60 h, pH 4.5; 60 h to the end of fermentation, pH 5.0. The control reaction was carried out with automatic addition of either 2 M H_2SO_4 or 2 M NaOH solution (Li et al., 2013).

Analytical methods

Reducing-sugar and soluble-protein content

Reducing sugar was measured with the dinitrosalicylic acid method (Miller et al., 1959), and the concentration of soluble protein was measured with a Bradford protein assay (Bradford, 1976) using bovine serum albumin as a standard.

Determination of enzyme activity

The cellulase activity was determined as filter-paper activity (FPA) with a filter-paper assay, according to the method recommended by the International Union of Pure and Applied Chemistry (IUPAC) (GHOSE 1987). Endoglucanase activity and β-glucosidase activity were also determined according to the method of Ghose (GHOSE 1987). Exoglucanase activity was determined according to the method of Lokapirnasari (Lokapirnasari et al., 2015), and xylanase activity was determined according to the method of Li (Li et al., 2015). The amylase activity was assayed according to the method described by Miller (Miller 1959) with a UV–visible spectrophotometer (Eltek, India). One unit of amylase activity was defined as the amount of enzyme that released 1 μg of reducing sugar as glucose per milliliter per minute under the assay conditions.

Determination of hydrolysis rate

The hydrolysis rate was measured according to the hydrolysis of steam-exploded corn straw (Li et al., 2013). A certain volume of cellulase solution was added into a 100 ml flask containing 10 g/L substrate in citrate buffer (0.05 M, pH 5.0) to ensure that the enzyme loading was 10 FPU per gram substrate. NaN_3 at a concentration of 3/10,000 (w/v) was added to the reaction system to limit the growth of infectious microbes. The total volume of the reaction system was controlled at 30 ml, and the reaction was carried out at 50℃ with a stirring rate of 200 rpm. After 72 h, samples were taken from the reaction mixture and immediately heated at 100℃ to terminate the reaction, cooled and then centrifuged for 10 min at 8000 rpm. The concentrations of reducing sugar in the supernatant were measured. The hydrolysis rate was calculated with the following formula (Selig et al., 2008):

$$hydrolysis\,rate = \frac{reducing\,sugar \in the\,supernatant \times 0.9}{cellulose\,content\,of\,the\,s\,team - exploded\,corn\,straw} \times 100$$

T. reesei RUT C30 biomass determination

T. reesei RUT C30 biomass was determined according to the method of Ma (Ma et al., 2013) by calculating the difference between the total dry weight and the residue in the acid wash.

Analysis of biomass components

The starch analysis method was adapted from Sluiter and Sluiter (Sluiter & Sluiter 2005). The content of cellulose, hemicellulose and lignin in the duckweed was determined though the neutral-detergent fiber (NDF), acid-detergent fiber (ADF), and acid-detergent lignin (ADL) methods, respectively (Vansoest et al., 1991). The pectin content was determined according to Lawrence and Groves (Lawrence & Groves 1954). The lipid content was determined according to Chen (Chen et al., 2017). The protein content was determined according to Li (Li et al., 2010a). The ash content was determined according to Li (Li et al., 2011).

Results and discussion

Effects of duckweed on cellulase production

The components of *L. minor* duckweed were determined and are shown in Table 3. *L. minor* consisted mainly of starch, cellulose, hemicelluloses, and pectin. The small quantity of lignin indicated that *L. minor* may be a good inducer for cellulase production.

Duckweed was chosen as the inducer for cellulase production and was tested at different concentrations (10 g/L, 30 g/L, 50 g/L, and 70 g/L). The FPA of the obtained enzyme increased with increasing duckweed concentrations (Fig. 1a), and the highest FPA, 6.5 FPU ml^{-1}, was obtained at a duckweed concentration of 50 g/L. This result indicated the potential of duckweed as a cellulase inducer.

The inducing effect of duckweed was compared with those of the following inducers at 50 g/L: steam-exploded corn straw, corn cob, bagasse, and avicel (Fig. 1b). The results indicated that duckweed had an induction effect that was favorable for cellulase production. Different inducers resulted in different levels of cellulase activity in *T. reesei*. The FPA activity of the enzyme induced by duckweed was higher than that induced by steam-exploded corn stalk, corn cob, and bagasse, and was lower than that induced by avicel.

As seen in Table 4, the content of cellulose, hemicellulose, and lignin in steam-exploded corn stalk, corn cob, duckweed, bagasse, and avicel differed. Except for avicel, the other substrates had approximately the same content of cellulose and hemicellulose. The content of lignin in duckweed was lower than that in the other tested inducers. Lignin content affects the hydrolysis of cellulose material: a low lignin content notably promotes enzymatic hydrolysis, whereas non-specific combinations of lignin cause irreversible cellulase inactivation (Zhang et al., 2016). Thus, duckweed's low lignin content may be the reason for its high induction of cellulase production.

Duckweed contains (30.4 ± 0.3%) cellulose, which is the main substance responsible for cellulase induction (Table 3). Duckweed exhibited the highest induction ability (measured in FPA per unit cellulose) among the inducers studied. The hydrolyzed residue was mainly composed of cellulose and hemicellulose (Table 1). The ability of hydrolyzed residue and non-pretreated duckweed powder to induce cellulase activity was compared (Fig. 2). The FPA values induced by the duckweed powder were all higher than those induced by the hydrolyzed residue at different concentrations (Fig. 2a). The starch and pectin content were the primary differences in composition between the duckweed powder and the hydrolyzed residue (Table 3). Thus, further studies on the differences in the induction ability of these two materials are clearly needed.

Starch is a carbohydrate consisting of many glucose units joined by glycosidic bonds. It can be hydrolyzed to glucose in the presence of amylase. The resultant glucose is then transported into the cell for metabolism. *T. reesei* produces both cellulase and amylase (Adav et al., 2013). Hence, we speculated that duckweed starch could be used in the cellulase-production process. To analyze the cellulase-production process by duckweed powder and hydrolyzed residue, the biomass growth of *T. reesei*, amylase production, and starch content were determined (Fig. 2b-f). The starch in the culture medium decreased gradually along with the induction of cellulase by duckweed powder and the increased amylase production (Fig. 2d and f). The biomass accumulation after treatment with duckweed powder was faster than that after treatment with the hydrolyzed residue. The maximum biomass production after treatment with duckweed powder was also higher than that after treatment with the hydrolyzed residue at a 50 g/L inducer concentration, which was optimal for induction (Fig. 2e). The results indicated that the starch of the duckweed powder was hydrolyzed to glucose, absorbed into the cells, and used for growth and respiratory metabolism by the amylase in *T. reesei*. Thus, in the cellulase-production process, glucose from starch hydrolysis was used for biomass growth and maintaining respiratory metabolism, which is beneficial for growth of biomass.

To study the characteristics of cellulase induced by avicel, hydrolyzed residue and duckweed powder, the activity and hydrolysis rates of endoglucanase, exo-glucanase, β-glucosidase, and xylanase were determined (Fig. 3a). The endoglucanase, exo-glucanase, and β-glucosidase activity induced by avicel was higher than that induced by duckweed powder and hydrolyzed residue, as was evident in the changes in FPA. However, the xylanase activity induced by avicel was lower than that induced by the duckweed powder and hydrolyzed residue. The differences among these enzymes activities may be correlated with the compositions of the inducers studied (Table 3). Compared with avicel, duckweed powder and hydrolyzed residue contained more hemicellulose, which has been shown to induce more hemicellulase and cellulase activity (Liao et al., 2014).

The enzyme-hydrolysis rate in the presence of steam-exploded corn stalk was 54.2% when 10 FPU per gram substrate was loaded, thus representing a 28% improvement over that induced by avicel only (Fig. 3b). The balance of cellulase and hemicellulase is important for lignocellulose hydrolysis (Dondelinger et al., 2016). The present results indicated that duckweed induction produces a balance of cellulase and hemicellulase that is favorable for biomass hydrolysis.

Effects of combinations of avicel, duckweed powder, and hydrolyzed residue on cellulase production

Duckweed powder and hydrolyzed residue were combined with avicel and used to induce cellulase production (Table 1). The FPA values improved with increasing concentrations of duckweed powder or hydrolyzed residue and were maximal when 22.5 g/L duckweed powder or hydrolyzed residue was used as an inducer (Fig. 4a). Moreover, the FPA induced by the combination of avicel and duckweed powder was higher than that induced by the combination of avicel and hydrolyzed residue. The enzyme-hydrolysis rates in the presence of different inducers were higher than those induced by avicel alone and were similar to the FPA results (Fig. 4b). The enzyme-hydrolysis rates induced by the hydrolyzed residue were higher than those induced by non-pretreated duckweed powder at concentrations lower or higher than 22.5 g/L. Thus, the maximal hydrolysis rate was obtained at the concentration of 22.5 g/L, at which the duckweed powder and hydrolyzed residue elicited nearly identical rates.

The reason for this result may be that the balanced ratio of cellulose and hemicellulose (for avicel and hydrolyzed-residue complex) favored balanced enzyme-system production, thereby increasing cellulase production.

Batch fermentation in a 5-L fermenter

Duckweed was also used as an inducer in batch fermentation in a 5 L fermenter (Table 2). Different levels of cellulase production by *T. reesei* were observed in the presence of various inducers (Fig. 5). Similarly to the results of the flask experiments, the highest FPA was produced by the enzymes induced by avicel and duckweed powder. However, the FPA values obtained from batch fermentations in the 5 L fermenter were higher than those obtained in shake flasks in the presence of the same inducers.

Batch fermentation in a 5 L fermenter at the laboratory level may serve as a basis for scaling up to industrial production. The results of the present study showed that cellulase production induced by duckweed and composite inducers is feasible at the laboratory level.

Conclusion

Duckweed is a good inducer of cellulase production by *T. reesei*, and its components (starch, cellulose, and hemicellulose) can be used for improving biomass growth, cellulase production, and enzyme-hydrolysis rates of *T. reesei*. Starch from duckweed can be hydrolyzed to glucose, which in turn serves as a carbon source for biomass growth. Cellulose and hemicellulose from duckweed can be efficiently utilized because of duckweed's low lignin content. The hydrolysis rate can be further improved through induction with both substrates. Moreover, duckweed combined with avicel can further increase cellulase

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26529v1 | CC BY 4.0 Open Access | rec: 16 Feb 2018, publ: 16 Feb 2018

production and hydrolysis rates. Results from scaling-up studies indicated that duckweed may be a potential candidate material for the industrial production of cellulase.

Acknowledgments

This work was supported by the Chinese National Natural Science Foundation for the Youth (21406259) and the Jilin City Science and Technology Innovation and Development Program (2015313008).

Conflicts of interests

The authors have declared that no conflicts of interests exist.

Ethical statement

This study was focused on analyzing a novel natural inducer of cellulase production in *Trichoderma reesei*. Every parts of the research did not involve human participants and other animals. Our manuscript complies to the Ethical Rules applicable for Journal of Industrial Microbiology and Biotechnology.

References

Abdullah R, Zafar W, Nadeem M, Iqtedar M, Naz S, Syed Q, and Kaleem A. 2014. Process optimisation for the biosynthesis of cellulase by Bacillus PC-BC6 and its mutant derivative Bacillus N3 using submerged fermentation. *Nat Prod Res*:1-6 DOI 10.1080/14786419.2014.981186.

Adav SS, Chao LT, and Sze SK. 2013. Protein abundance in multiplexed samples (PAMUS) for quantitation of Trichoderma reesei secretome. *J Proteomics* 83:180-196 DOI 10.1016/j.jprot.2013.03.023.

Ahamed A, and Vermette P. 2009. Effect of culture medium composition on Trichoderma reesei's morphology and cellulase production. *Bioresour Technol* 100:5979-5987 DOI 10.1016/j.biortech.2009.02.070.

Camassola M, and Dillon AJ. 2007. Production of cellulases and hemicellulases by Penicillium echinulatum grown on pretreated sugar cane bagasse and wheat bran in solid-state fermentation. *J Appl Microbiol* 103:2196-2204 DOI 10.1111/j.1365-2672.2007.03458.x.

Campanella A, Muncrief R, Harold MP, Griffith DC, Whitton NM, and Weber RS. 2012. Thermolysis of microalgae and duckweed in a CO 2-swept fixed-bed reactor: bio-oil yield and compositional effects. *Bioresource Technology* 109:154-162 DOI 10.1016/j.biortech.2011.12.115.

Chen L, Zhu T, Fernandez JSM, Chen S, and Li D. 2017. Recycling nutrients from a sequential hydrothermal liquefaction process for microalgae culture. *ALGAL RESEARCH-BIOMASS BIOFUELS AND BIOPRODUCTS* 27:311-317 DOI 10.1016/j.algal.2017.09.023.

Dondelinger E, Aubry N, Ben Chaabane F, Cohen C, Tayeb J, and Remond C. 2016. Contrasted enzymatic cocktails reveal the importance of cellulases and hemicellulases activity ratios for the hydrolysis of cellulose in presence of xylans. *Amb Express* 6:24 DOI 10.1186/s13568-016-0196-x.

GHOSE TK. 1987. MEASUREMENT OF CELLULASE ACTIVITIES. *Pure & Appl Chem,* 59:257-268

Iqbal HMN, Kyazze G, and Keshavarz T. 2013. Advances in the Valorization of Lignocellulosic Materials by Biotechnology: An Overview. *Bioresources* 8:3157-3176 DOI 10.15376/biores.8.2.3157-3176.

Juhasz T, Szengyel Z, Reczey K, Siika-Aho M, and Viikari L. 2005. Characterization of cellulases and hemicellulases produced by Trichodermareesei on various carbon sources. *Process Biochemistry* 40:3519-3525 DOI 10.1016/j.procbio.2005.03.057.

Kang SW, Park YS, Lee JS, Hong SI, and Kim SW. 2004. Production of cellulases and hemicellulases by Aspergillus niger KK2 from lignocellulosic biomass. *Bioresour Technol* 91:153-156 DOI 10.1016/s0960-8524(03)00172-x.

Kumar L, Arantes V, Chandra R, and Saddler J. 2012. The lignin present in steam pretreated softwood binds enzymes and limits cellulose accessibility. *Bioresource Technology* 103:201-208 DOI 10.1016/j.biortech.2011.09.091.

Lawrence JM, and Groves K. 1954. Fruit Pectin, Determination of a Soluble Pectin in Apples. *Journal of Agricultural and Food Chemistry* 2:882-885

Li C, Yang ZH, Zhang RLHC, Zhang DY, Chen SL, and Ma LJ. 2013. Effect of pH on cellulase production and morphology of Trichoderma reesei and the application in cellulosic material hydrolysis. *Journal of Biotechnology* 168:470-477 DOI 10.1016/j.jbiotec.2013.10.003.

Li D, Chen L, Zhang X, Ye N, and Xing F. 2011. Pyrolytic characteristics and kinetic studies of three kinds of red algae. *Biomass and Bioenergy* 35:1765-1772 DOI 10.1016/j.biombioe.2011.01.011.

Li D, Chen L, Zhao J, Zhang X, Wang Q, Wang H, and Ye N. 2010a. Evaluation of the pyrolytic and kinetic characteristics of Enteromorpha prolifera as a source of renewable bio-fuel from the Yellow Sea of China. *Chemical Engineering Research and Design* 88:647-652 DOI 10.1016/j.cherd.2009.10.011.

Li XH, Yang HJ, Roy B, Park EY, Jiang LJ, Wang D, and Miao YG. 2010b. Enhanced cellulase production of the Trichoderma viride mutated by microwave and ultraviolet. *Microbiol Res* 165:190-198 DOI 10.1016/j.micres.2009.04.001.

Li YY, Zhong KX, Hu AH, Liu DN, Chen LZ, and Xu SD. 2015. High-level expression and characterization of a thermostable xylanase mutant from Trichoderma reesei in Pichia pastoris. *Protein Expr Purif* 108:90-96 DOI 10.1016/j.pep.2014.11.014.

Liao H, Li S, Wei Z, Shen Q, and Xu Y. 2014. Insights into high-efficiency lignocellulolytic enzyme production by Penicillium oxalicum GZ-2 induced by a complex substrate. *Biotechnology for Biofuels* 7:162 DOI 10.1186/s13068-014-0162-2.

Lokapirnasari WP, Nazar DS, Nurhajati T, Supranianondo K, and Yulianto AB. 2015. Production and assay of cellulolytic enzyme activity of Enterobacter cloacae WPL 214 isolated from bovine rumen fluid waste of Surabaya abbatoir, Indonesia. *Vet World* 8:367-371 DOI 10.14202/vetworld.2015.367-371.

Lv X, Zhang W, Chen G, and Liu W. 2015. Trichoderma reesei Sch9 and Yak1 regulate vegetative growth, conidiation, and stress response and induced cellulase production. *J Microbiol* 53:236-242 DOI 10.1007/s12275-015-4639-x.

Ma L, Li C, Yang Z, Jia W, Zhang D, and Chen S. 2013. Kinetic studies on batch cultivation of Trichoderma reesei and application to enhance cellulase production by fed-batch fermentation. *J Biotechnol* 166:192-197 DOI 10.1016/j.jbiotec.2013.04.023.

Miller GL. 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Analytical Chemistry* 31:426-428.

Muradov N, Fidalgo B, Gujar AC, and Ali T. 2010. Pyrolysis of fast-growing aquatic biomass–Lemna minor (duckweed): Characterization of pyrolysis products. *Bioresource Technology* 101:8424-8428 DOI 10.1016/j.biortech.2010.05.089.

Selig M, Weiss N, and Ji Y. 2008. *Enzymatic Saccharification of Lignocellulosic Biomass: Laboratory Analytical Procedure (LAP): Issue Date, 3/21/2008*: National Renewable Energy Laboratory.

Singhania RR, Sukumaran RK, Patel AK, Larroche C, and Pandey A. 2010. Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases. *Enzyme and Microbial Technology* 46:541-549 DOI 10.1016/j.enzmictec.2010.03.010.

Sluiter A, and Sluiter J. 2005. Determination of Starch in Solid Biomass Samples by HPLC. Laboratory Analytical Procedure. *National Renewable Energy Laboratory, Golden, Colorado*

Vansoest PJ, Robertson JB, and Lewis BA. 1991. Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. *Journal of Dairy Science* 74:3583-3597

Vijayaraghavan P, Prakash Vincent SG, and Dhillon GS. 2016. Solid-substrate bioprocessing of cow dung for the production of carboxymethyl cellulase by Bacillus halodurans IND18. *Waste Manag* 48:513-520 DOI 10.1016/j.wasman.2015.10.004.

Zhang L, Liu Y, Niu X, Liu Y, and Liao W. 2012. Effects of acid and alkali treated lignocellulosic materials on cellulase/xylanase production by Trichoderma reesei Rut C-30 and corresponding enzymatic hydrolysis. *Biomass and Bioenergy* 37:16-24 DOI 10.1016/j.biombioe.2011.12.044.

Zhang L, Zhang L, Zhou T, Wu Y, and Xu F. 2016. The dual effects of lignin content on enzymatic hydrolysis using film composed of cellulose and lignin as a structure model. *Bioresource Technology* 200:761-769 DOI 10.1016/j.biortech.2015.10.048.

Zhao X, Elliston A, Collins SRA, Moates GK, Coleman MJ, and Waldron KW. 2012. Enzymatic saccharification of duckweed (Lemna minor) biomass without thermophysical pretreatment. *Biomass and Bioenergy* 47:354-

361 DOI 10.1016/j.biombioe.2012.09.025.

Zhao X, Moates GK, Wellner N, Collins SR, Coleman MJ, and Waldron KW. 2014. Chemical characterisation and analysis of the cell wall polysaccharides of duckweed (Lemna minor). *Carbohydr Polym* 111:410-418 DOI 10.1016/j.carbpol.2014.04.079.

Figure captions

Fig. 1. Effects of different inducers in cellulase production by *T. reesei*

a Cellulase production induced by different concentrations of duckweed (10 g/L, 30 g/L, 50 g/L, and 70 g/L). b Cellulase production induced by 50 g/L of different inducers. A. corn cob, B. bagasse, C. steam exploded corn straw, D. avicel.

Fig. 2. Effects of duckweed powder and hydrolyzed residue on cellulase production. Effects of different concentrations of inducers on FPA (a) *T. reesei* biomass production (b), and amylase production (c). Starch utilization (d) and *T. reesei* biomass production (e) and amylase activity during cultivation with 50g/L duckweed and hydrolyzed residue as inducers.

Fig. 3. Production of cellulase components induced by avicel, duckweed powder, and hydrolyzed residue. (a) exo-glucanase, endoglucanase, β-glucosidase and xylanase activity. (b) hydrolysis rate.

Fig. 4. Cellulase production by *T. reesei* in the presence of different combinations of inducers. a, b, c, d, e show the different combined inducers indicated in Table 1.

Fig. 5. Cellulase production by different inducers in a 5-L fermenter. a, b, c, d, e, f show the different inducers indicated in Table 2.

Figure 1

Effects of different inducers in cellulase production by *T. reesei*

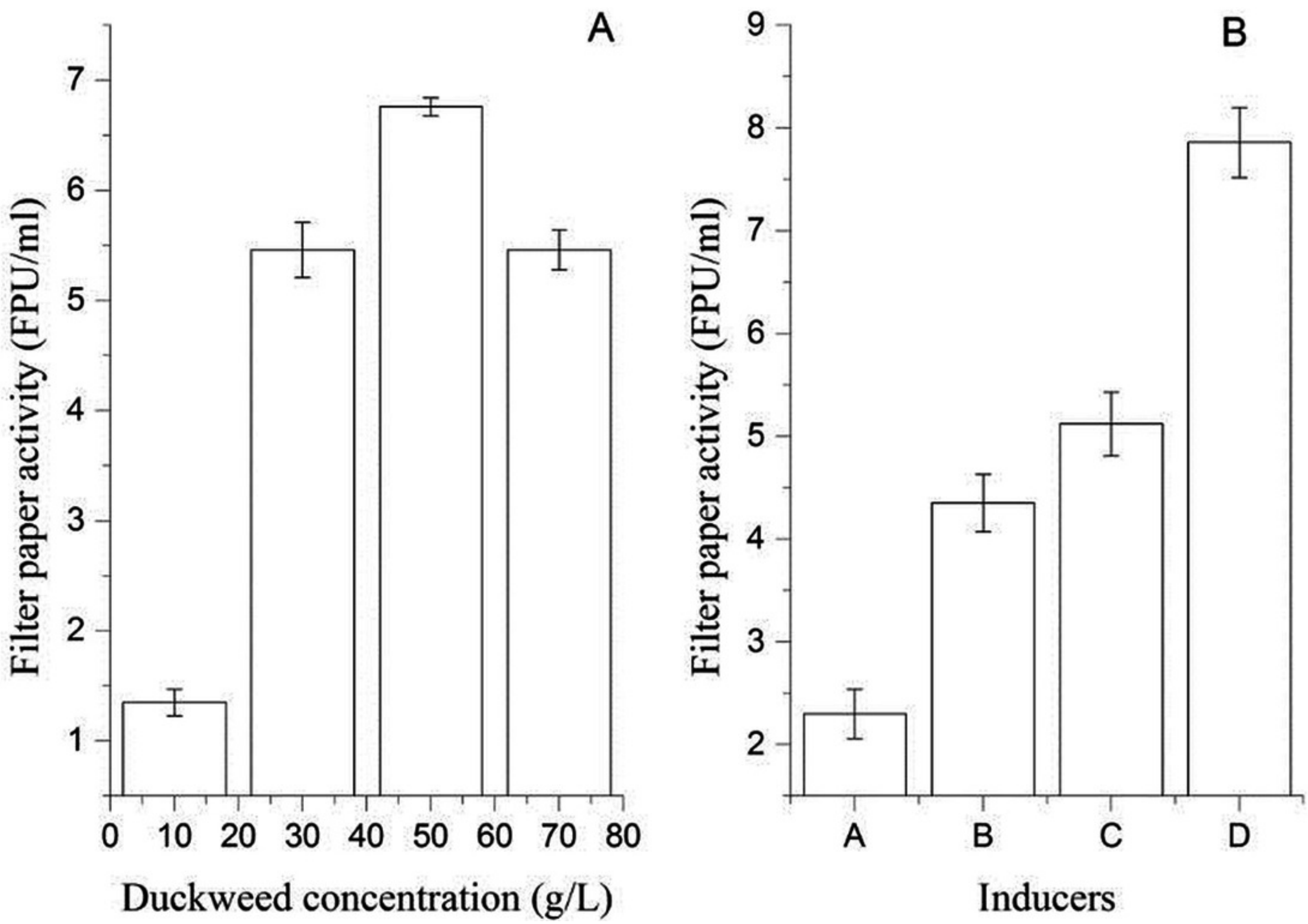


Figure 2

Effects of duckweed powder and hydrolyzed residue on cellulase production

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26529v1 | CC BY 4.0 Open Access | rec: 16 Feb 2018, publ: 16 Feb 2018

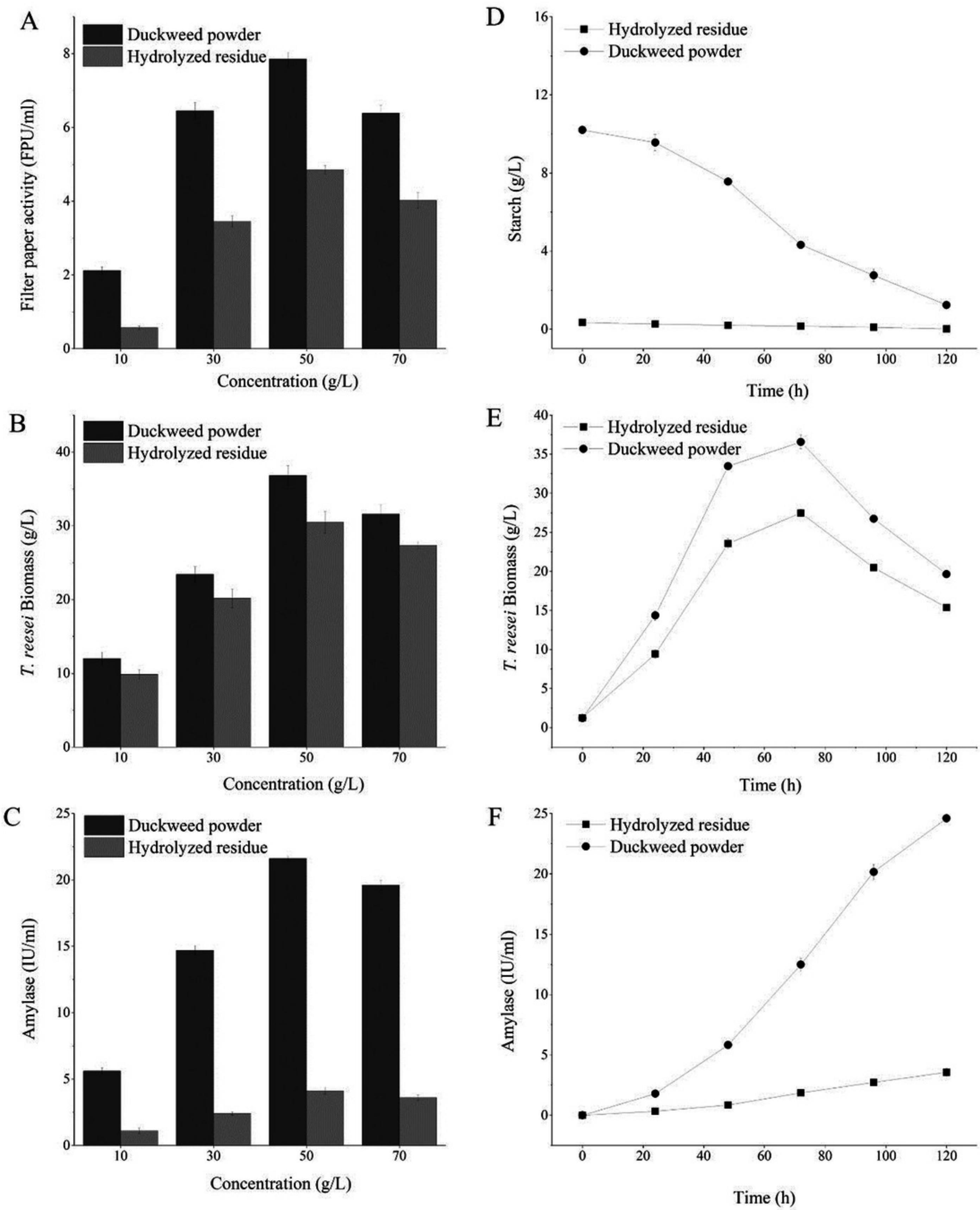

A
Duckweed powder
Hydrolyzed residue
Filter paper activity (FPU/ml)
Concentration (g/L)
B
Duckweed powder
Hydrolyzed residue
T. reesei Biomass (g/L)
Concentration (g/L)
C
Duckweed powder
Hydrolyzed residue
Amylase (IU/ml)
Concentration (g/L)
D
Hydrolyzed residue
Duckweed powder
Starch (g/L)
Time (h)
E
Hydrolyzed residue
Duckweed powder
T. reesei Biomass (g/L)
Time (h)
F
Hydrolyzed residue
Duckweed powder
Amylase (IU/ml)
Time (h)

Figure 3

Production of cellulase components induced by avicel, duckweed powder, and hydrolyzed residue

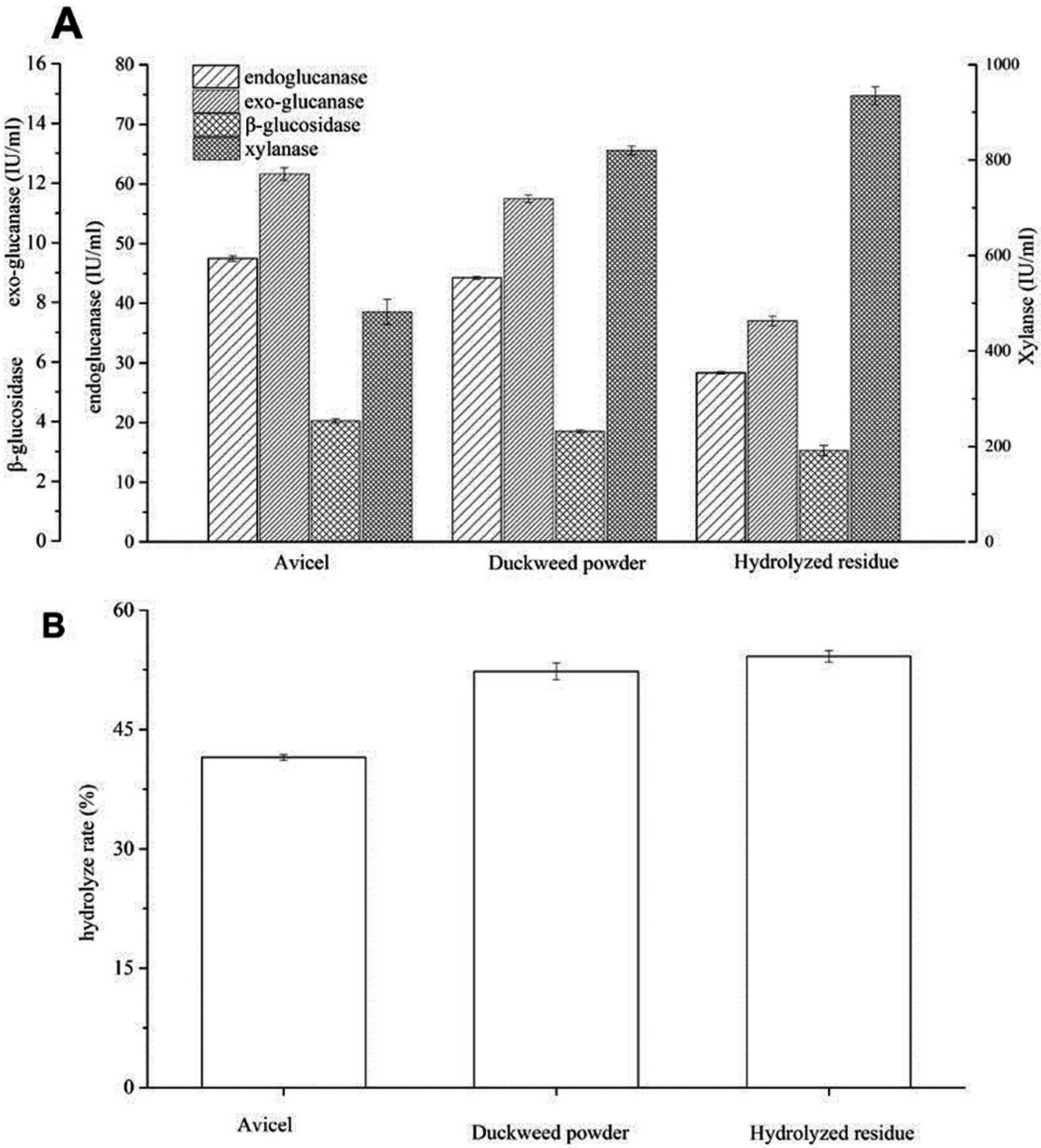
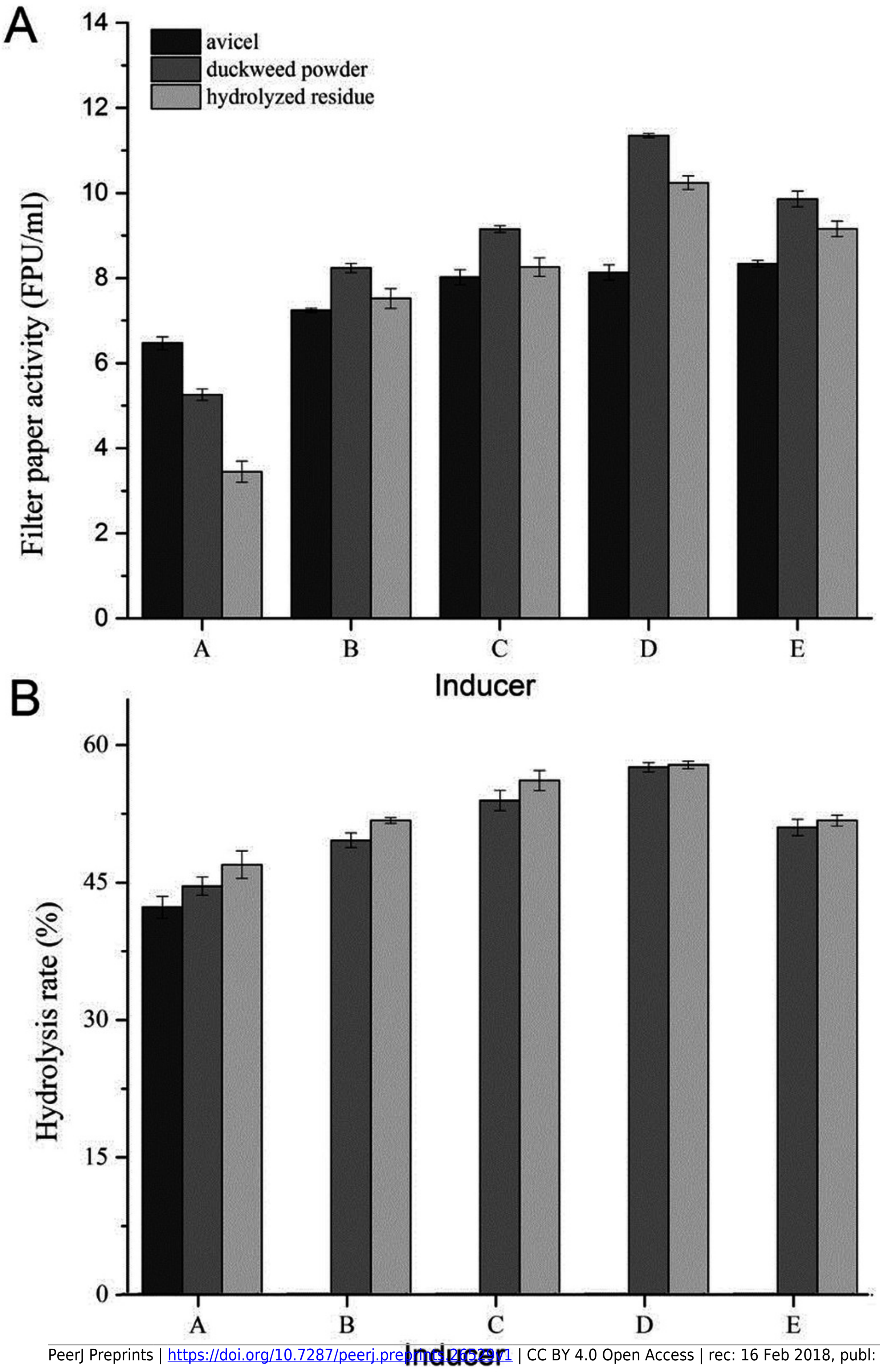


Figure 4

Cellulase production by *T. reesei* in the presence of different combinations of inducers

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26529v1 | CC BY 4.0 Open Access | rec: 16 Feb 2018, publ: 16 Feb 2018



PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26537v1 | CC BY 4.0 Open Access | rec: 16 Feb 2018, publ: 16 Feb 2018

Figure 5

Cellulase production by different inducers in a 5-L fermenter

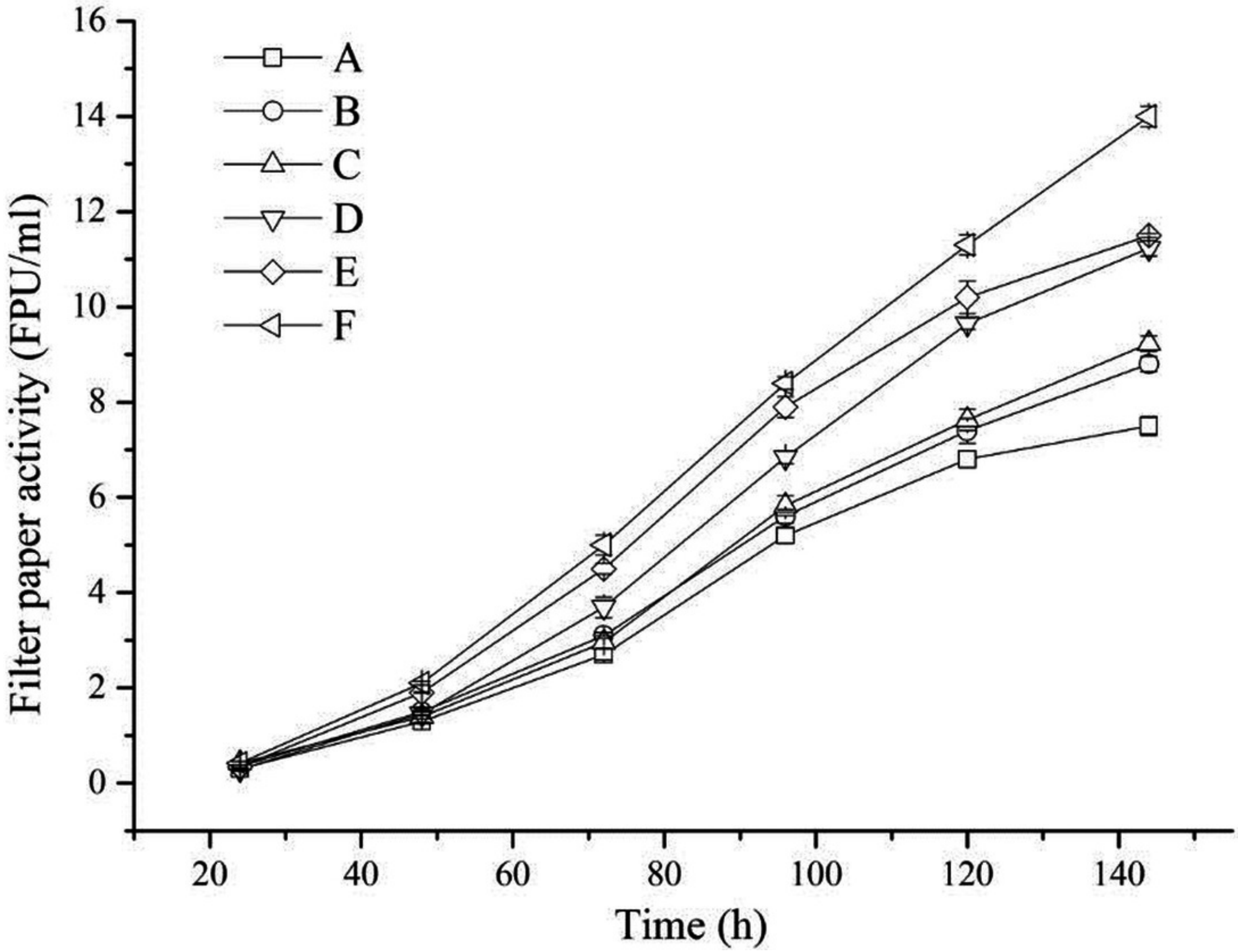


Table 1 (on next page)

Combinations of inducers

Table 1 Combinations of inducers

Treatment	Avicel (g/L)	Avicel (g/L) + hydrolyzed residue (g/L)	Avicel (g/L) + duckweed powder (g/L)
A	30	0 + 30	0 + 30
B	37.5	30 + 7.5	30 + 7.5
C	45	30 + 15	30 + 15
D	52.5	30 + 22.5	30 + 22.5
E	60	30 + 30	30 + 30

Table 2(on next page)

inducers used in a 5-L fermenter

Table 2 Inducers used in a 5-L fermenter

Treatment	Inducer	Concentration (g/L)
A	Duckweed powder	30
B	Avicel	30
C	Duckweed powder	52.5
D	Avicel	52.5
E	Avicel	30
	Hydrolyzed residue	22.5
F	Avicel	30
	Duckweed powder	22.5

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26529v1 | CC BY 4.0 Open Access | rec: 16 Feb 2018, publ: 16 Feb 2018

Table 3(on next page)

Component analysis of *Lemna minor* and hydrolyzed residue

1 Table 3 Component analysis of *Lemna minor* and hydrolyzed residue

Composition	Content (%, w/w of dry matter)	
	Duckweed (*L. minor*)	Hydrolyzed residue
Cellulose	30.4 ± 0.3	58.0 ± 0.5
Hemicellulose	23.6 ± 0.2	36 ± 0.4
Lignin	1.5 ± 0.1	3.3 ± 0.2
Pectin	4.3 ± 0.4	0
Starch	19.4 ± 0.5	0
Protein	10.4 ± 0.3	0
Lipids	1.1 ± 0.4	0.5 ± 0.1
Ash	8.2 ± 0.3	0

2

Table 4 (on next page)

Cellulose, hemicellulose, and lignin content of different inducers

Table 4 Cellulose, hemicellulose, and lignin content of different inducers

	Cellulose (%)	**Hemicellulose (%)**	**Lignin (%)**
Steam-exploded corn stalk	38	22	20
Corn cob	30	40	18
Bagasse	36	32	21
Avicel	85	2	12

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26529v1 | CC BY 4.0 Open Access | rec: 16 Feb 2018, publ: 16 Feb 2018