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

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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 duckweed in shake flasks, the filter-paper activity (FPA) reached 6.5 FPU/mL, a value comparable to that induced by avicel. The enzyme-hydrolysis rate induced by steam-exploded corn stalk 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 the 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 of 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 materials hydrolysis.

[**Key words:** Duckweed; Cellulase; Starch; Fermentation; Hydrolysis]

Lignocellulosic biomass is the most abundant renewable source for the production of biofuels. However, conversion of lignocellulose into soluble sugars by lignocellulolytic enzymes remains a major challenge limiting the widespread use of bioenergy (1). The high cost of lignocellulolytic enzymes, which are produced by filamentous fungi, is the major bottleneck limiting the biorefinery of lignocellulose (2).

Among all species of filamentous fungi producing lignocellulolytic enzymes, *Trichoderma reesei* (teleomorph *Hypocrea jecorina*) is the main producer used for commercial lignocellulolytic enzymes preparation. *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 (3). 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 different aspects of the fermentation process, such as the medium composition, pH, agitation, and extracellular-protein supplementation (4–6). Among these approaches, using lignocellulosic materials as inducers might be an effective and simple method to enhance enzyme production.

Using 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 (7), bran (8), rice straw (9), and bagasse (10), have been studied as lignocellulytic enzymes inducers. Different lignocellulosic materials used as enzyme inducers have different effects on lignocellulytic enzymes production, due to the varying compositions among lignocellulosic materials (11). 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 (12). Therefore, a low-density lignocellulosic material with low lignin content is an ideal enzyme inducer in lignocellulytic enzymes 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 (13,14). Duckweed can provide a valuable source of starch, whose content can reach 49% of its dry weight (15). Duckweed is also composed of cellulose, hemicellulose, and lignin (16). 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 (16) and hence may be more easily utilized than other lignocellulosic materials. Furthermore, cellulases and amylases are abundant in the secretomes of *T. reesei* (17). Starch in duckweed

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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 *T. reesei* RUT C30 (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°C 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. 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°C and 180 rpm.

Preparation of duckweed powder and hydrolyzed residue by enzymes The dry duckweed was pulverized into 60 mesh powder with a pulverizer. Extra amylase (1000 U/g), glucoamylase (1000 U/g), proteinase (1000 U/g), and pectinase (1000 U/g) were added to the hydrolysis system, in acetate buffer solution (0.05 M, pH 5.3) containing 10% (w/v) duckweed powder, and cultured for 24 h at 40°C, 100 rpm. The duckweed hydrolyzed residue was then harvested by centrifugation at 10,000 rpm. The hydrolyzed residue was dried at 60°C 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, $(NH_4)_2SO_4$ 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 cultivated at 26°C, 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 stalk, 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, biomass and amylase activities of *T. reesei* at 72 h were shown. The changes in the FPA, *T. reesei* biomass and amylase activities were shown for treatment with a 50 g/L concentration of the 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 S1.

Batch fermentation in a 5-L fermentor For each culture, 5% (v/v) of seed broth was inoculated into a 5-L fermentor (BIOTECH-5BG, Shanghai Baoxing Bio-Engineering Equipment Co. Ltd, China), which contained 3 L culture medium. The medium was the same as that used in shake flasks. The inducers used are shown in Table S2.

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 control strategy was as follows: 0–20 h, natural pH; 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 pH control reaction was carried out with automatic addition of either 2 M H_2SO_4 or 2 M NaOH solution (18).

Reducing-sugar and soluble-protein content Reducing sugar was measured with the dinitrosalicylic acid method, and the concentration of soluble protein was measured with a Bradford protein assay using bovine serum albumin as a standard.

Determination of enzyme activity The cellulase activity was determined as filter-paper activity (FPA) according to the method recommended by the International Union of Pure and Applied Chemistry (IUPAC) (19). Endoglucanase activity and β-glucosidase activity were also determined according to the method of Ghose (19). Exoglucanase activity was determined according to the method of Lokapirnasari et al. (20), and xylanase activity was determined according to the method of Li et al. (21). The amylase activity was assayed according to the method described by Miller (22) 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 using the hydrolysis of steam-exploded corn stalk as the substrate (18). 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 inhibit the growth of infectious microbes. The total volume of the hydrolysis system was controlled at 30 mL, and the reaction was carried out at 50°C, 200 rpm. After 72 h, samples were taken from the reaction mixture and immediately heated at 100°C 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 (23):

$$\text{Hydrosis rate \%} = \frac{\text{Reducing sugar in the supernatant} \times 0.9}{\text{Cellulose content of the steam} - \text{Exploded corn stalk}} \times 100 \quad (1)$$

***T. reesei* RUT C30 biomass determination** *T. reesei* RUT C30 biomass was determined according to the method of Ma et al. (24) 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 (25). The content of cellulose, hemicellulose and lignin in the duckweed was determined through the neutral-detergent fiber (NDF), acid-detergent fiber (ADF), and acid-detergent lignin (ADL) methods, respectively

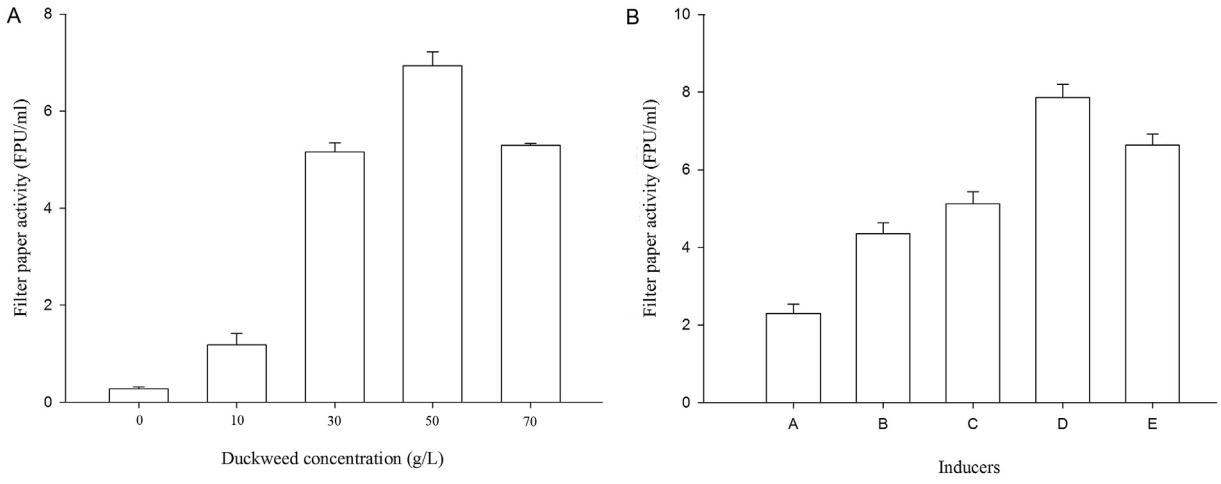

FIG. 1. Effects of different inducers in cellulase production by *T. reesei*. (A) Cellulase production induced by different concentrations of duckweed (10 g/L, 30 g/L, 50 g/L, and 70 g/L). Cellulase production without any inducer is shown as control. (B) Cellulase production induced by 50 g/L of different inducers: A, corn cob; B, bagasse; C, steam-exploded corn stalk; D, avicel; E, duckweed.

(26). The pectin content was determined according to Lawrence and Groves (27). The lipid content was determined according to Chen et al. (28). The protein content was determined according to Li et al. (29). The ash content was determined according to Li et al. (30).

RESULTS AND DISCUSSION

Effects of duckweed on cellulase production The components of duckweed were determined. Duckweed consisted mainly of cellulose (30.4 ± 0.3%), hemicelluloses (23.6 ± 0.2%), starch (19.4 ± 0.5%), protein (10.4 ± 0.3%), ash (8.2 ± 0.3%) pectin (4.3 ± 0.4%), lignin (1.5 ± 0.1%), and lipids (1.1 ± 0.4%). The low content of lignin (1.5 ± 0.1%) indicated that duckweed 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 enzymes increased with increasing duckweed concentrations (Fig. 1A), and the highest FPA (6.5 FPU/mL) 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 stalk, corn

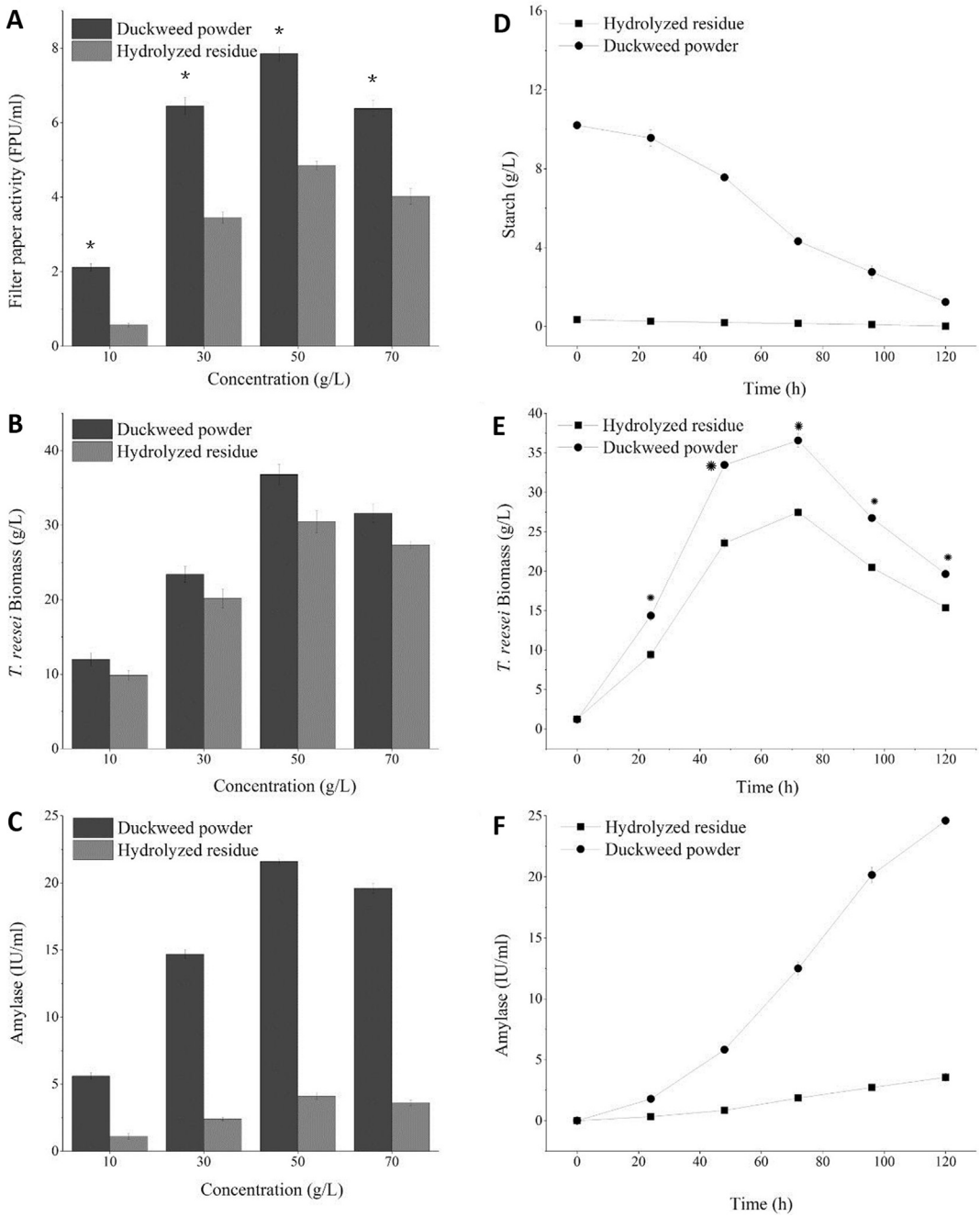

FIG. 2. Effects of duckweed powder and hydrolyzed residue on cellulase production. Effects of different concentrations of inducers on FPA (A), *T. reesei* biomass production (B), and amylase production (C), starch utilization (D), *T. reesei* biomass production (E), and amylase activity (F) during cultivation with 50 g/L duckweed and hydrolyzed residue as inducers. Asterisks indicate statistically significant differences calculated by student's *t*-test ($p < 0.05$).

cob, bagasse, and avicel (Fig. 1B). The results indicated that duckweed had an inducing effect on cellulase production. Different inducers resulted in different levels of cellulase activity in *T. reesei*. The FPA activity of the enzyme induced by duckweed was 195% higher than that induced by steam-exploded corn stalk, 55% higher than that induced by corn cob, and 32% higher than that induced by bagasse. In addition, The FPA activity of the enzyme induced by duckweed was 14% lower than that induced by avicel.

As seen in Table S4, 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 40%, 33% and 43% lower than that in steam-exploded corn stalk, corn cob and bagasse, respectively. 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 (31). Thus, duckweed's low lignin content may be the reason for its high inducing ability of cellulase production.

Duckweed contained (30.4 ± 0.3%) cellulose, which is the main substance responsible for cellulase induction (Table S3). Duckweed exhibited the highest cellulase induction ability (measured in FPA per unit cellulose) among the inducers in this study. The hydrolyzed residue was mainly composed of cellulose (58.0 ± 0.5%) and hemicellulose (36.0 ± 0.4%). The cellulase induction ability of hydrolyzed residue and non-pretreated duckweed powder was compared (Fig. 2). The FPA values induced by the duckweed powder were significant higher than those induced by the hydrolyzed residue at different concentrations (Fig. 2A). The starch and pectin contents were higher in duckweed powder than in the hydrolyzed residue (Table S3). Thus, further studies on the differences in the cellulase induction ability of these two lignocellulosic materials were 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 further metabolism. *T. reesei* produces both cellulase and amylase (17). Hence, we speculated that duckweed starch could be used in the cellulase production process. To analyze the cellulase production process using duckweed powder and the hydrolyzed residue as inducers, the biomass accumulation 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 significant faster than that treated with the hydrolyzed residue. The maximum biomass accumulation 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 cellulase 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 was beneficial for biomass accumulation.

To study the characteristics of cellulase induced by avicel, hydrolyzed residue and duckweed powder, the activity of endoglucanase, exoglucanase, β-glucosidase, and xylanase and hydrolysis rates were determined (Fig. 3A). The endoglucanase, exoglucanase, and β-glucosidase activity induced by avicel were significant 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 significant lower than that induced by the duckweed powder and hydrolyzed residue (Fig. 3B). The different compositions of endoglucanase, exoglucanase, and β-glucosidase activity may be led by the different compositions of the inducers used in this study (Table S3). Compared with avicel, duckweed powder and hydrolyzed residue contained more hemicellulose, which had been shown to induce more hemicellulase activity (32).

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 (33). The present results indicated that duckweed induction produced a balance of cellulase and hemicellulase that was favorable for lignocellulosic 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 S1). The FPA values improved with increasing concentrations of duckweed powder or hydrolyzed residue and reached maximal when 22.5 g/L duckweed powder or hydrolyzed residue was used as the 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 hydrolysis rates in the presence of different inducers were higher than those induced by avicel alone and were similar to as was evident in the changes in FPA (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

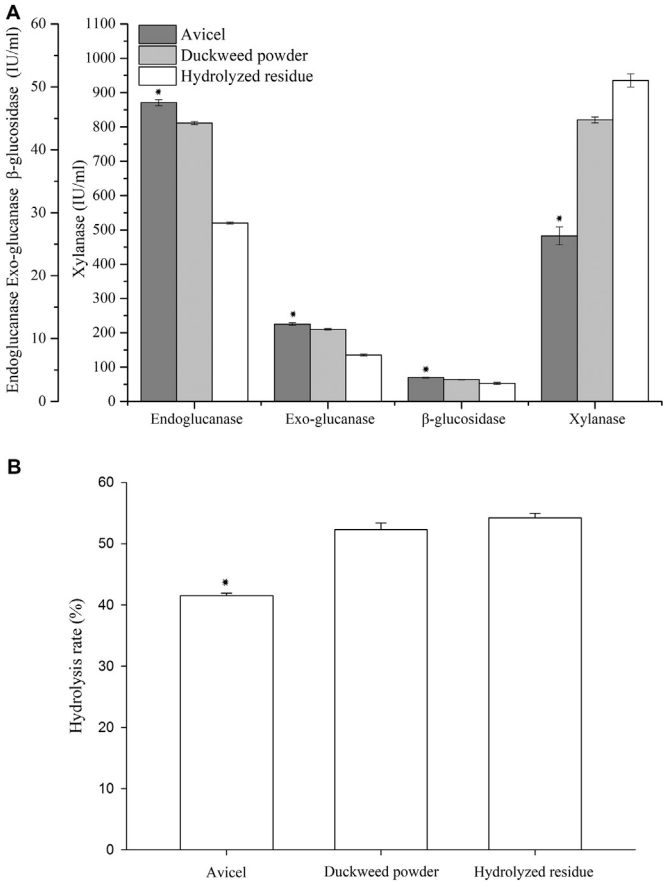

FIG. 3. Production of cellulase components induced by avicel, duckweed powder, and hydrolyzed residue. (A) Exoglucanase, endoglucanase, β-glucosidase and xylanase activity, (B) hydrolysis rate. Asterisks indicate statistically significant differences calculated by student's *t*-test ($p < 0.05$).

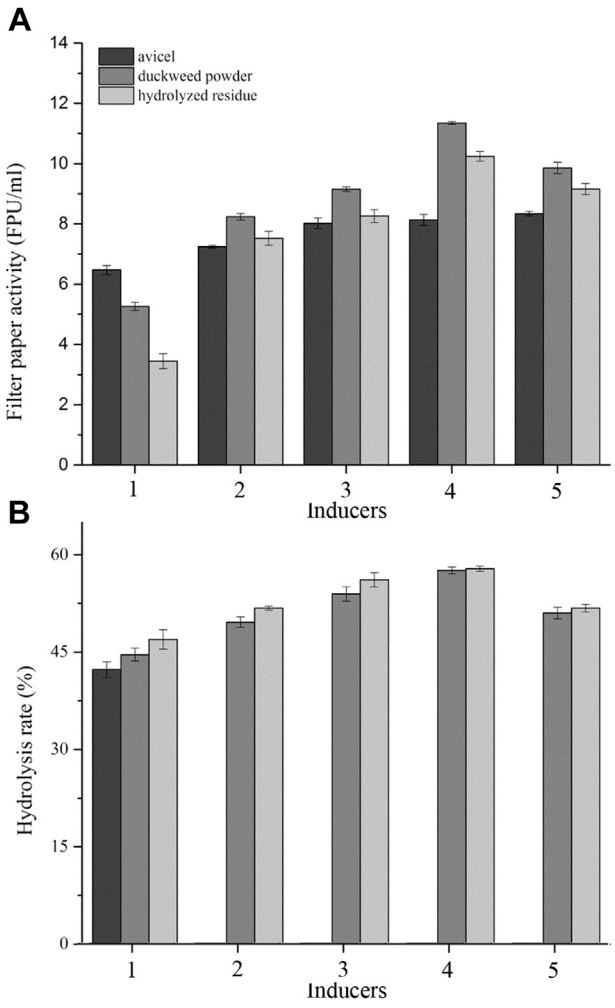

FIG. 4. Cellulase production by *T. reesei* in the presence of different combinations of inducers. (A) Filter paper activity, (B) hydrolysis rate. 1–5 of the x axis indicated the different combined inducers as follows: 1, avicel 30 g/L, hydrolyzed residue 30 g/L, duckweed power 30 g/L; 2, avicel 37.5 g/L, avicel 30 g/L + hydrolyzed residue 7.5 g/L, avicel 30 g/L + duckweed power 7.5 g/L; 3, avicel 45 g/L, avicel 30 g/L + hydrolyzed residue 15 g/L, avicel 30 g/L + duckweed power 15 g/L; 4, avicel 52.5 g/L, avicel 30 g/L + hydrolyzed residue 22.5 g/L, avicel 30 g/L + duckweed power 22.5 g/L; 5, avicel 60 g/L, avicel 30 g/L + hydrolyzed residue 30 g/L, avicel 30 g/L + duckweed power 30 g/L.

than 22.5 g/L. Thus, the highest hydrolysis rate was obtained at the concentration of 22.5 g/L, at which the duckweed powder and hydrolyzed residue elicited nearly identical hydrolysis 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 fermentor Duckweed was also used as an inducer in batch fermentation in a 5 L fermentor (Table S2). Different levels of cellulase production by *T. reesei* were observed in the presence of various inducers (Fig. 5). Similar 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 fermentor were higher than those obtained in shake flasks in the presence of the same inducers. Batch fermentation in a 5 L fermentor 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.

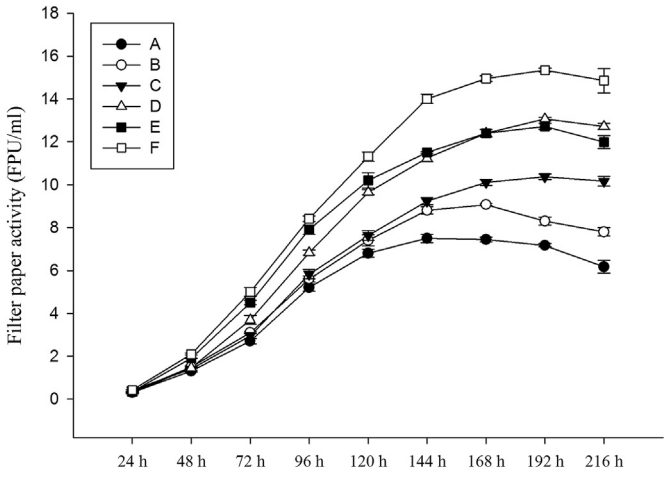

FIG. 5. Cellulase production by different inducers in a 5-L fermentor. A–F indicate the different combined inducers: A, duckweed power 30 g/L; B, avicel 30 g/L; C, duckweed power 52.5 g/L; D, avicel 52.5 g/L; E, avicel 30 g/L + hydrolyzed residue 22.5 g/L; F, avicel 30 g/L + duckweed power 22.5 g/L.

In this study, duckweed was used as an inducer of cellulase production by *T. reesei* RUT C30. A combined substrate of avicel and duckweed was used to induce cellulase production by *T. reesei* RUT C30. The cellulase production and hydrolysis rates of the combined substrate, compared with avicel alone, were both improved more than 30%. Results from scaling-up studies indicated that duckweed may be a potential candidate lignocellulosic material for the industrial production of cellulase.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2018.09.017.

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