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Potential use of vetiver grass for cellulolytic enzyme production and bioethanol production

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ABSTRACT

Vetiver grass (VG) high cellulose is an abundant lignocellulosic material used in both cellulolytic enzyme and bioethanol production. Appropriate VG size for fungal growth and enzymes production was 500 μm, while the optimum cellulolytic enzyme production by *Aspergillus tubingensis* HS1–5 under solid state fermentation using response surface methodology (RSM) was the substrate volume of 12.36 g, moisture content of 56.45% and pH at 4.4. Filter Paperase (FPase), Carboxymethylcellulase (CMCase) and β-glucosidase activities of 156.84, 2096.67 and 63.11 U/g substrate, respectively were investigated under these conditions. The highest yield of reducing sugar was found with pretreatment of VG with 1% (w/v) NaOH followed by 0.5% (v/v) H_2SO_4 at 121 °C for 60 min. Maximum fermentable sugar from enzymatic hydrolysis (90 FPU/mL) and bioethanol production were obtained with 21.10 and 5.85 g/L, respectively. The crude cellulase enzyme was suitable for VG hydrolysis and the sugar released can be further utilized for bioethanol production.

1. Introduction

Increases in populations and greater industrialization have led to increasing demand for energy globally, whilst fuels such as petroleum and natural gas are mostly located in a small group of countries. Energy consumption continues to increase, but conversely fossil fuel reserves are rapidly decreasing. The whole world is concerned about future energy shortages and the impact of fossil fuels which release carbon dioxide into the atmosphere causing climate change. Therefore, renewable energy sources are keenly researched and developed. Bioethanol is an alternative energy source which can be produced from any material containing carbohydrates. The three main groups of raw materials for biofuel production are sugar, starch, and non-food (cellulosic materials). This process has proved popular because a variety of raw materials can be used. However, sugar and starch are food crops and so the production process can impact on food and ethanol prices, as well as causing shortages. Therefore, it would be advantageous to identify non-food materials to serve as renewable feedstock for ethanol production (Telebnia, 2015). Currently, development of technology for ethanol production from lignocellulosic materials with agricultural wastes (straws, corncobs, and bagasse), woody feedstock, perennial grass and municipal solid waste (MSW) have been proposed for second-generation bioethanol production (Watanabe, 2013).

Lignocellulose is a major component of biomass which consists of polymers generated from the byproducts of agricultural residues, forest and considered waste (Soccol et al., 2011). Cellulose is the main content of lignocellulose, which consists of a linear polymer (Gnansounou and Dauriat, 2011), and cellulose chain that can be converted into glucose by either chemical or enzymatic digestion. The glucose from cellulose hydrolysis is used for bioethanol fermentation by microbe.

Cellulolytic enzymes are a major group of enzyme which can degrade cellulosic biomass into glucose including endoglucanase, exoglucanase and β-glucosidase. Hydrolysis of cellulose to glucose requires the synergy of these three enzymes (Wood and McCrae, 1986; Wood et al., 1989). Endoglucanase (EC 3.2. 1.4), randomly attacks the β-(1,4) glycosidic bonds of amorphous regions and randomly cleaves the internal bonds of the glycan chains. This reaction results in a rapid decrease of the viscosity of the cellulose water mixed liquor, which provides reducing or non-reducing ends of cello-oligosaccharide (Behera et al., 2017). Exoglucanase (EC 3.2.1.91) releases cellobiose from the non-reducing ends of cello-oligosaccharides. This enzyme can hydrolyze both amorphous and crystalline cellulose, but generally does not hydrolyze substituted cellulose such as carboxymethyl cellulose (Cheng, 2010). β-glucosidase or cellobiase (EC 3.2.1.21) can greatly improve the hydrolysis efficiency of cellulose by degrading cellobiose which hydrolyzes cellobiose to glucose (Wood and McCrae, 1986; Wood et al., 1989).

There are many groups of microorganisms involved in cellulase

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production including bacteria, white rot and soft fungi (Kuhad et al., 2016). Cellulase enzymes are mostly produced from filamentous fungi for industrial application (Srivastava et al., 2018). Submerged fermentation and solid state fermentation processes have potential for industrial scale of cellulase enzyme production (Biswas, 2014). However, solid state fermentation is suitable to support the fungal growth and cellulase enzyme production on lignocellulosic material due to lower energy consumption increased enzyme concentration and an ability to directly hydrolyze (Yoon et al., 2014).

Vetiver grass (VG) or *Chrysopogon zizaniodes*, is a tropical plant which can grow in different environments, possessing deep root systems which spread into the soil directly. The grass has no rhizomes or stolons and depth of up to 3 m (The World Bank, 1992). VG has been successfully used for land protection, such as soil and water conservation (Danh et al., 2010). Vetiver leaves sprout from the bottom of the clump and are long, narrow and rough. The leaf edge and apex are sharp (Nakorn et al., 2000). Vetiver grows rapidly under low nutrient conditions and has the capacity to produce high biomass (Raman and Gnansounou, 2015). In Thailand, 10 types of VG consist of cellulose (31.85–38.51%), hemicellulose (37.87–42.61%) and lignin (3.67–5.06%) were reported (Wongwatanapaiboon et al., 2012).

Vetiver grass (VG) is used in land stabilization, forming hedges to prevent soil erosion and increase water conservation in Thailand. It has been planted throughout the country. The VG leaves are cut off every 3 months to increase root stimulation. The leaves can be utilized as roof thatching, or for making handicrafts. Because the VG leaf consists of high cellulosic content, it is suitable as a raw material for cellulolytic enzyme and bioethanol productions. The pretreatment process for increasing the enzymatic saccharification of lignocellulosic materials has become a key step of the cellulosic ethanol production. This research aimed to produce the cellulolytic enzyme from VG by *Aspergillus tubingensis* HS1–5 and use that enzyme to hydrolyze pretreated VG with ethanol fermentation by simultaneous saccharification and fermentation (SSF).

2. Material and methods

2.1. Preparation and compositions analysis of vetiver grass

Fresh vetiver grass (VG) was dried in sunlight and cut into 3–4 cm pieces. These were dried in an oven at 70 °C overnight and milled to small particles. Dry VG was separated by 12, 20, 35, 50 and 100 mesh-size sieves (American Standard Test Series, ASTM). The cellulose, hemicellulose, lignin, ash and moisture content in the solid VG were determined according to the method of Klinpratoom et al. (2015).

2.2. Microorganism and inoculation

Aspergillus tubingensis HS1–5 (Yurayard et al., 2012) was cultured in potato dextrose agar (PDA) slant and incubated at 30 °C over 5 days for spore production. Spore suspension was prepared by adding 0.1% Tween 80 (v/v) solution to the grown culture. The spore concentration was adjusted to 10^8 spores/mL for use as seed starter culture.

2.3. Cellulolytic enzyme production

2.3.1. The effect of substrate size on cellulolytic enzyme production

VG in different particle sizes of 1.7 mm, 850 μm, 500 μm, 300 μm and 150 μm was used as substrate for enzyme production. The solid state culture was comprised of 12 g of VG and 8 g of wheat bran for production media. Media moisture was adjusted to 65% w/w by 40 mL of distilled water at pH 5.0. The cultivation was carried out in a polypropylene bag (12 × 18 in.). The open end of the bag was put on a plastic tube (2 × 4.5 in.) and was closed with a cotton wool plug (Lotong and Suwanarit, 1983; Sotthisawad et al., 2017). The solid state medium was autoclaved at 121 °C for 60 min.

Five milliliters of the spore suspension of *A. tubingensis* HS1–5 was added into the bag. The cultures were incubated at 30 °C and samples were taken on days 1, 3, and 5 for glucosamine and enzyme activity analysis.

2.3.2. Response surface optimization of enzyme production

Response surface methodology was used to optimize the factor of substrate volume, moisture content and initial pH on cellulolytic enzyme production in solid state fermentation. Twenty experiments were designed for the three factors, with and five levels (-α, −1, 0, +1, +α) using central composite design (CCD) with the Design-Expert® Software (Version 11, Stat-Ease, Inc., USA). The variables of experimental design are shown in the Table 2. All experiment results of Filter Paperase (FPases), Carboxymethylcellulase (CMCase) and β-glucosidase activity were used in ANOVA analysis.

2.3.3. Enzyme assay and cell growth determination

The cellulolytic enzymes were extracted using 30 mL of 0.05 M citrate phosphate buffer (CP buffer, pH 5.0) and kept at 4 °C for 24 h before separation. Crude enzyme extract was filtrated with straining cloth and centrifuged at 3220 × g for 10 min. The supernatant was stored at −20 °C before being used to determine enzyme activity.

FPase activity was determined using filter paper (whatman No.1), size 3 × 0.5 cm^2 (0.02 g) as a substrate according to Ghose (1987). CMCase activity was measured using 0.5 mL of 1% (w/v) carboxymethyl cellulose (CMC) as substrate with 0.5 mL of enzyme solution at 45 °C, for 30 min, followed by the addition of 1.0 mL of dinitrosalicylic acid (DNS) reagent (Ekperigin, 2007). One unit (U) of FPase and CMCase were defined as the amount of enzyme that released 1 μmol of glucose per minute under experimental conditions.

β-glucosidase activity was assayed by incubating 0.2 mL of 0.002 M *p*-nitrophenyl-β-D-glucoside (*p*NPG) with 0.1 mL of enzyme solution at 45 °C, for 30 min, followed by the addition of 1 mL of 0.25 M Na_2CO_3 and absorbance at 405 nm was spectrophotometrically measured (Swangkeaw et al., 2008). One unit (U) of β-glucosidase was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenyl per minute.

Fungal biomass was measured according to Morgan-Elson assay method (Van de Loo, 1976). Determination of glucosamine was carried out in a test tube containing 1 mL of sample and 1 mL of acetyl acetone at 100 °C for 20 min. Ten milliliters of 95% ethanol and 1 mL of Erhich's reagent were added into the sample and incubated for 30 min. The samples were measured by spectrophotometer at 530 nm and glucosamine concentration was compared with glucosamine hydrochloride standard.

2.4. Bioethanol production

2.4.1. Optimization of alkaline-acid pretreatment

For the first step, VG sample was pretreated with sodium hydroxide (NaOH), at concentration of 0.5% and 1% w/v with a ratio of 1:10 (solid: liquid) then autoclaved at 121 °C, for 60 min. The biomass samples were washed with tap water until neutral pH was obtained and samples were dried overnight at 60 °C. Next, the dried VG from the first step was mixed with sulfuric acid (H_2SO_4) concentration 0.5%, 1% and 2% (v/v) with a ratio of 1:10, dried VG: liquid, heated in an autoclaved at 121 °C, for 60 min and dried at 60 °C overnight. The dried sample was stored in a plastic box at room temperature.

2.4.2. Cellulolytic enzyme hydrolysis

The amounts of crude enzyme from *A. tubingensis* HS1–5 (30, 50, 70 and 90 FPU/mL) were measured in test tube with a 5% w/v solid loading in 0.05 M CP buffer, pH 5. The reaction was performed in an incubator at 45 °C. Samples were collected at 6, 12, 18, 24, 36 and 48 h and centrifuged at 3220 × *g* for 10 min. Reducing sugar was determined using DNS method (Miller, 1959).

2.4.3. Bioethanol fermentation

Saccharomyces cerevisiae TISTR 5339 (Sotthisawad et al., 2017; Salakkam et al., 2017) was pre-cultured in yeast extract peptone dextrose (YPD) agar plate at 30 °C for 48 h. A single colony of the yeast was cultured in yeast extract peptone dextrose (YPD) broth on a rotary shaker at 30 °C, 150 rpm, for 18 h. Yeast cells were centrifuged at 3220 × *g* for 10 min and washed with distilled water. Inoculum containing 10% yeast cell (10^9 cells/mL) was used for ethanol fermentation.

Enzymatic hydrolysis was performed in a 250 mL flask containing 5 g of alkaline-acid pretreated VG and 100 mL of crude enzyme (90 FPU/mL). and enzyme saccharification reaction measured in an incubator at 45 °C for 18 h.

After the VG pre-hydrolysis, ethanol production medium (20 g/L KH_2PO_4, 10 g/L $(NH_4)_2SO_4$, 10 g/L $MgSO_4{\cdot}7H_2O$, 5 g/L yeast extract and 1 g/L $MnSO_4$) and 10% v/v of *S. cerevisiae* (10^9 cells/mL) were added. The fermentation flask was sealed with air lock and incubated at 30 °C for 36 h in static condition. The experiment compared simultaneous saccharification and fermentation condition (SSF), and non-ethanol production supplement culture under the same condition. Samples were taken at 0, 6, 12, 18, 24 and 36 h with reducing sugar determined by DNS method and ethanol production measured by Gas Chromatography (GC). Ethanol was analyzed by a Perkin-Elmer Clarus® 680 with a FID detector and Perkin-Elmer capillary column (0.32 mm i.d. × 30 m L; d.f. 0.25 mm). The GC program ran with a 50 °C (5 min), 30 °C min^{-1}, 250 °C (1 min), 200 °C injection temperature, 250 °C detector temperature and Helium was the carrier gas. Yeast growth was determined by viable plate counting technique in YPD agar.

2.4.4. Scanning electron microscopy (SEM)

The untreated, alkaline-pretreated, alkaline-acid pretreated and crude enzyme hydrolysis of VG were oven-dried at 60 °C, 24 h for SEM analysis. All samples were coated with sputtering of gold under argon gas by electrical conductivity and images taken by the LEO 1450VP scanning electron microscope (SEM).

3. Results and discussion

3.1. Chemical compositions analysis of vetiver grass

The chemical compositions of VG consisted of 45.22% (w/w) cellulose, 32.07% (w/w) hemicellulose, 14.45% (w/w) lignin, 5.9% (w/w) moisture and 2.37% (w/w) ash. Restiawaty and Dewi (2017) reported that vetiver leaves had a high content of hemicellulose (34.55% w/w), cellulose (31.39% w/w), and lignin (17.58%w/w). In another report, hemicellulose comprised 40% w/w with cellulose and lignin as 30–35% w/w and 10% w/w, respectively (Methacanona et al., 2003) The cellulose composition of VG in this research was higher than other reports, whilst hemicellulose content was similar (Table 1).

3.2. Effect of particle size on cellulolytic enzyme production by solid state fermentation

The material particle size has an effect on the void space of the fermentation process which results in mass and oxygen transfer. The transfer of oxygen affected the growth and metabolism (Melikoglu et al., 2013). Small particles, or particles with large flat surfaces, pack together closely, making aeration and mass transfer difficult. In contrast, the larger particles provide better chance for aeration (Ruiz et al., 2012). Therefore, appropriate substrate size is very important for fungus growth and enzyme production. The effects of substrate in particle size between 150 μm and 1.7 mm on FPase, CMCase, β-glucosidase production and cellular growth were observed. After 5 days of cultivation in particle size between 150 μm and 1.7 mm of VG, both FPase and CMCase production were not significantly different (Fig. 1a.-b). A particle size 500 μm of VG (Fig. 1c.) yielded the highest β-glucosidase production (40.81 U/g substrate). Variations of cellulolytic enzyme production in various sizes of substrate particle have been reported. The highest cellulase (CMCase) production from *A. tubingensis* IMMIS2 under solid state fermentation was found when cultured in the particle size 425 μm of corn stover (Imrana et al., 2017), which closely correlated to particle size in this study. However, the maximum CMCase and FPase activities of *Pleurotus ostreatus* IE-8 were observed when grown in particle size 920 μm of sugar cane bagasse (Membrillo et al., 2008). Ang et al. (2013) reported cellulase production from different sizes of oil palm trunk (125, 250, 500, 600, 710 and 800 μm) by *A. fumigatus* SK1 under solid state fermentation. The maximum of FPase, CMCase and β-glucosidase was 3.36, 54.27 and 4.54 U/g substrate, respectively when cultured in 125 μm of substrate size.

N-acetylglucosamine content in the fungal cell wall (Fig. 1d.) determined fungal biomass. The highest glucosamine concentration of *A. tubingensis* HS1–5 was 2.18 mg/g substrate when cultured in the particle size 500 μm of VG within 5 days of cultivation. Suitable substrate particle size promoted enzyme production and fungi growth. Fungal cultivation with large-sized substrate could reduce preparation time and saved energy required to make the substrate smaller. A particle size 500 μm of VG was chosen for optimum cellulolytic enzyme production.

Table 1
Comparison of main composition of VG from another research.

Reference	Component (%)		
	Cellulose	Hemicellulose	Lignin
This study	45.22	32.07	14.45
Restiawaty and Dewi (2017)	31.39	34.55	17.58
Raman and Gnansounou (2015)	32.6	31.5	17.3
Wongwatanapaiboon et al. (2012)	31.85 – 38.51	37.87–42.61	3.67–5.06
Methacanona et al. (2003)	30–35	40	10

3.3. Response surface analysis

The optimization of cellulolytic enzyme production by *A. tubingensis* HS1–5 was investigated under solid state fermentation at 5 days of cultivation. RSM with CCD technique was used to select the condition and influence for FPase, CMCase and β-glucosidase productions. The variables on 3 factors including substrate volume, initial moisture content and initial pH were analyzed. The experiment response summary, observation and predicted values are shown in Table 2.

3.3.1. Statistical analysis and optimization of enzyme production

The CCD design of RSM was applied to determine the optimal levels of 3 factors which influenced enzyme cocktail (FPase, CMCase and β-glucosidase) productions. The significant effect of the 3 factors on enzyme production was analyzed by ANOVA. The values of multiple correlation coefficients (R^2) of FPase, CMCase and β-glucosidase productions were 0.9569, 0.9722 and 0.8854, respectively. The adjusted R^2 values of FPase, CMCase and β-glucosidase productions were 0.9182, 0.9472 and 0.7822, respectively. The results indicated that the model was significant at p-value < 0.001. The lack of fit for FPase was not significant (p-value $= 0.233$), which means that the model is fit for data experimentation. However, the lack of fit for CMCase and β-glucosidase was significant (p-value $= 0.0222$ and 0.0053), which means that making it an unsuitable data experiment model. Initial pH and moisture content are the main factors of CMCase and β-glucosidase production, which results to not fit to experiment data. FPase referred as total cellulase activity which mainly lignocellulose hydrolysis was manipulated for optimization and validation.

The predicted result of FPase activity CMCase and β-glucosidase are presented in Eq. (1), Eq. (2) and Eq. (3), respectively. The contour plots showing interaction between substrate volume (A), initial moisture (B) and initial pH (C) on enzyme activity are presented in Fig. 2. The results

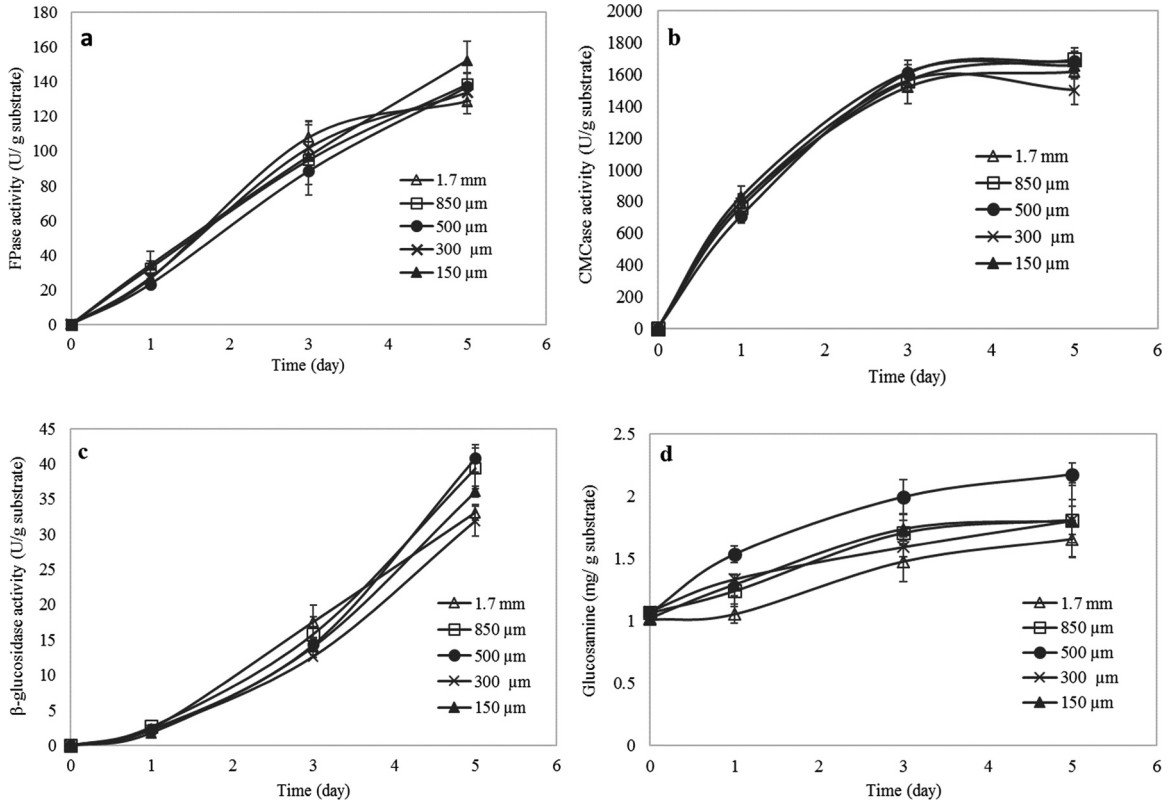

Fig. 1. Effect of substrate size on FPase activity (a), CMCase activity (b), β-glucosidase (c), fungal biomass (d) observed from solid state fermentation.

showed interaction between substrate volume and initial moisture, indicating an optimal zone in the center of the plot. FPase activity increased with lower pH, while CMCase activity increased with lower substrate, moisture content and pH. However, minimum activity was found near the center point of plot. The β-glucosidase activity increased with lower substrate, moisture content and pH. As with CMCase activity, a minimum point of activity was found at the center of the plot.

$$\text{FPase activity} = +179.52 - 13.45A - 17.44B - 69.57C + 0.5628AB - 21.23AC + 14.13BC - 23.35A^2 - 19.84B^2 - 23.91C^2 \quad (1)$$

$$\text{CMCase activity} = +1801.96 - 222.10A - 150.67B - 436.14C + 6.42AB - 307.81AC + 8.51BC - 82.41A^2 - 82.90B^2 - 233.48C^2 \quad (2)$$

Table 2
Experimental design and result of the central composite design.

Run	Variables			Observed response			Predicted response		
	A	B	C	FPase activity (U/g)	CMCase activity (U/g)	β-glucosidase (U/g)	FPase activity (U/g)	CMCase activity (U/g)	β-glucosidase (U/g)
1	12.00	70.00	6.00	183.58	1718.15	21.73	179.52	1801.96	23.99
2	8.00	80.00	5.00	116.68	1647.78	9.83	142.09	1588.00	15.51
3	16.00	60.00	7.00	25.24	463.06	2.73	10.92	572.86	3.39
4	18.73	70.00	6.00	77.67	1389.44	7.73	90.85	1195.36	9.05
5	5.27	70.00	6.00	164.94	1819.07	40.52	136.09	1942.41	30.23
6	16.00	60.00	5.00	213.67	1989.44	24.02	220.78	2077.79	28.12
7	16.00	80.00	5.00	170.15	1615.37	7.66	158.76	1772.27	4.46
8	8.00	60.00	5.00	200.71	1881.11	44.68	206.36	1919.21	49.65
9	12.00	53.18	6.00	159.85	1873.70	42.82	152.75	1820.90	34.42
10	12.00	70.00	6.00	165.17	1825.56	26.46	179.52	1801.96	23.99
11	8.00	80.00	7.00	69.69	1386.67	3.84	73.66	1348.35	6.09
12	12.00	70.00	7.68	0.00	370.00	0.58	0.00	408.06	0.00
13	8.00	60.00	7.00	58.92	1752.41	7.99	81.40	1645.53	17.54
14	12.00	70.00	6.00	178.48	1837.59	21.26	179.52	1801.96	23.99
15	12.00	86.82	6.00	102.67	1332.04	5.47	94.09	1314.09	4.90
16	12.00	70.00	6.00	164.02	1775.56	22.20	179.52	1801.96	23.99
17	12.00	70.00	4.32	239.48	1983.89	27.94	228.90	1875.07	24.13
18	16.00	80.00	7.00	0.00	289.44	1.06	5.44	301.37	2.43
19	12.00	70.00	6.00	173.04	1761.67	24.33	179.52	1801.96	23.99
20	12.00	70.00	6.00	210.16	1881.11	26.45	179.52	1801.96	23.99

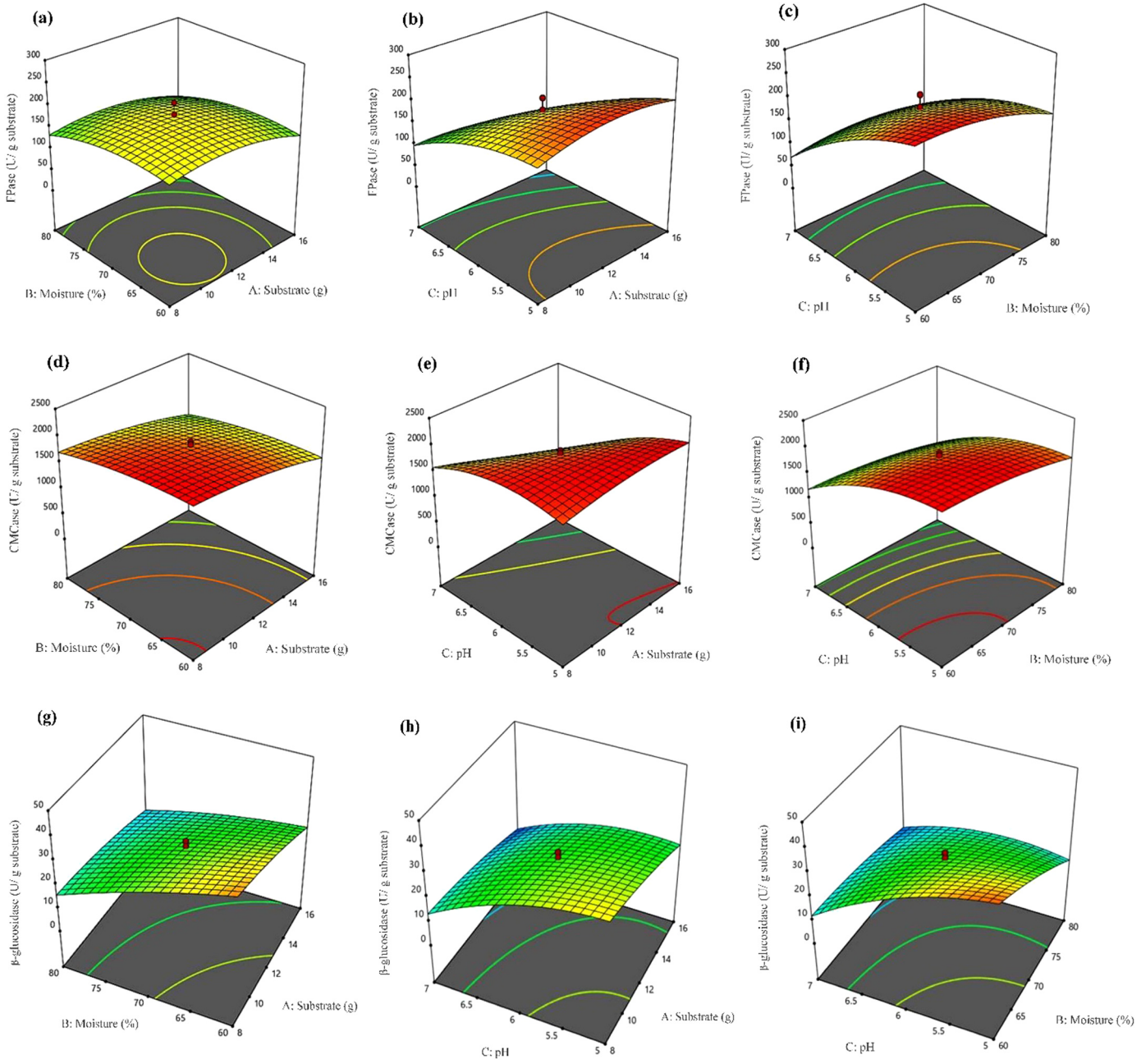

Fig. 2. Response surface plots for FPase activity (U/g substrate); moisture content and substrate loading (a), initial pH and substrate loading (b), initial pH and moisture content (c). CMCase activity (U/g substrate); moisture content and substrate loading (d), initial pH and substrate loading (e), initial pH and moisture content (f). β-glucosidase activity (U/g substrate); moisture content and substrate loading (g), initial pH and substrate loading (h), initial pH and moisture content (i).

$$\beta - \text{glucosidase activity} = +23.99 - 6.30A - 8.78B - 8.54C + 2.62AB + 1.85AC + 5.67BC - 1.54A^2 - 1.53B^2 - 5.03C^2 \ldots\ldots \quad (3)$$

3.3.2. Optimization and validation

Design Expert software was used for numerical optimization of enzyme cocktail (FPase, CMCase and β-glucosidase) at substrate volume, initial moisture content and initial pH. The condition with higher enzyme activity gave a substrate volume of 12.36 g, moisture content of 56.45% and pH of 4.4. Confirmation experiments were performed in three replicates under all conditions. The optimized variables were investigated by solution desirability. Maximum FPase, CMCase and β-glucosidase productions were 156.84, 2096.67 and 63.11 U/g substrate, respectively. The FPase, CMCase and β-glucosidase activities increased to 14.18%, 24.36% and 54.64%, respectively when compared with the conventional methods of enzyme production.

There are many reports using RSM to analyze cellulolytic enzyme production from *Aspergillus* spp. Das et al. (2013) reported the production of cellulase by *A. fumigatatus* ABK9, using RSM to optimize mixed substrate under solid state fermentation. The substrate amount of 10.0–10.05, substrate ratio of wheat bran: rice straw, fermentation time of 86–88 h and pH of 6.1–6.2 had a significant influence on enzyme production. These condition yielded FPase and β-glucosidase activities of 102.5 and 255.16 U/g, respectively. The maximum condition of CMCase activity by *A. nidulans* MTCC344 under SSF was 28.84 U/g when cultured in 16.8 mm of bagasse, 60% of moisture content, pH 4.25 and 40 °C (Jabasingh and Nachiyar, 2011). The production of

cellulase by *A. terreus* from rice straw volume, initial pH, inoculum size and moisture ratio were optimized using RSM design by Box-Behnken. The validated FPase activity was 10.96 U/g with 5 g of substrate concentration, initial pH of 5, 1×10^5 spores/ mL and moisture ratio of 1:6.79 (w/v) at 45 °C (Narra et al., 2012).

Substrate type and optimum conditions resulted in efficient enzyme cocktail production for our fungal strain. This method enables cellulase enzyme production from VG and this enzyme will be used in the ethanol production process.

3.4. Alkaline-acid pretreatment and enzyme hydrolysis of VG

3.4.1. Effect of alkaline-acid pretreatment

Alkaline-acid pretreatment can be modified, reducing hemicellulose or lignin in fiber to promote enzyme hydrolysis. Sodium hydroxide pretreatment removes lignin and sulfuric acid reduces hemicellulose in lignocellulosic materials (Kim and Kim, 2013). The optimum condition for total sugar released from VG after hydrolysis was pretreatment with 1% (w/v) of NaOH, followed by 0.5% (v/v) of H_2SO_4 and hydrolysation with crude enzyme. After being pretreated with 1% NaOH, cellulose content was increased to 66.62% w/w. In addition, under treatment with 1%NaOH followed by 0.5% H_2SO_4, the cellulose obtained 78.08% w/w which is higher than only 1% NaOH pretreatment (Fig. 3). The pretreatment condition changed the structure and removed lignin from fibers. As reported by Martin (2012), NaOH pretreatment removed lignin from the cellulose structure and led to unpacked fibers with an open biomass structure. Acid pretreatment of the biomass resulted in broken down grass fibers, while opening and increasing the surface area to promote enzymes binding (Sahoo et al., 2018). Cardona et al. (2016) studied the effects of physiochemical pretreatment of King Grass. NaOH pretreatment gave the highest concentration of reducing sugar. Sahoo et al. (2018) observed the effect of acid or alkaline pretreatment of wild rice grass (*Zizania latifolia*). The pretreatment conditions was performed with different concentrations of dilute H_2SO_4 (0.4–2% w/v), NaOH (0.25–1.5% w/v) with 10% substrate loading. The maximum sugar yield (457 mg/g) was performed with 2% w/v of acids and 10% substrate loading. Shimizu et al. (2018) investigated banana pseudostem pretreatment with different concentrations of acid, alkaline and peroxide. H_2SO_4 pretreatment completely removed hemicellulose from biomass and NaOH and H_2O_2 pretreatments reduced the lignin content to 7.65% and 7.17%, respectively. After hemicellulose and lignin removal, alkaline and peroxide pretreatments increased cellulose content from 60.84% to 75.48% and 74.37%, respectively.

3.4.2. Effect of crude enzyme loading

Enzyme loading quantity is important for enzyme digestibility of cellulosic substrates (Zhu et al., 2015). Different crude enzyme loading

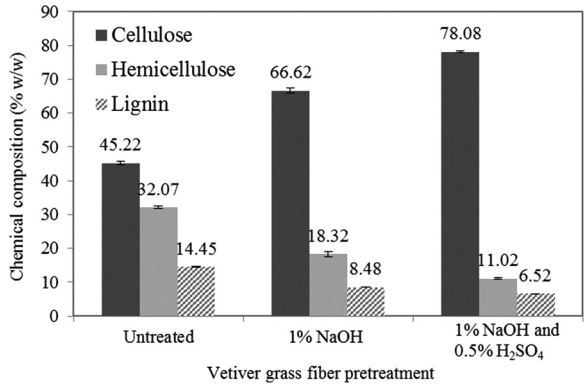

Fig. 3. The composition of untreated, alkaline pretreated and alkaline-acid pretreated VG.

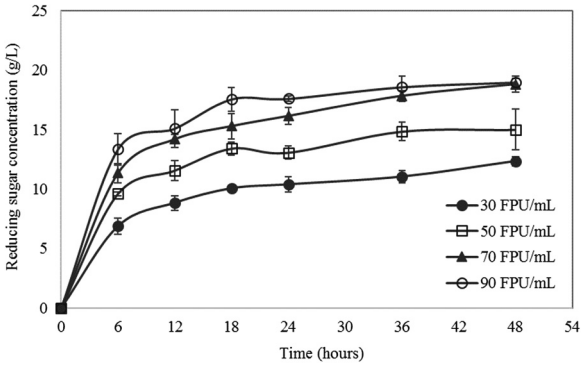

Fig. 4. Reducing sugar concentrations obtained under various Initial of crude enzyme loading. Enzymatic hydrolysis was performed in triplicate with 5% (w/v) solid using 30–90 FPU/mL of crud enzyme at 45 °C, for 48 h.

was determined for hydrolysis of pretreated VG, from 30 to 90 PFU/mL (Fig. 4). Reducing sugar improved as enzyme loading increased. FPase loading increased from 30 FPU/mL to 70 FPU/mL and greatly improved enzyme saccharification. The maximum reducing sugar concentration was 17.55 g/L, in 18 h and 18.97 g/L in 48 h when the hydrolysis was performed with 90 FPU/mL.

3.5. Bioethanol production

Bioethanol production using *S. cerevisiae* TISTR 5339, was carried out under simultaneous saccharification and fermentation condition (SSF) for 36 h. The pretreated VG with 1% (w/v) NaOH, 0.5% (v/v) H_2SO_4 and 90 FPU/mL of crude enzyme was prepared for 18 h and the ethanol was fermented by SSF. Ethanol production levels using ethanol production medium and the non-supplement medium were compared. Time course of sugar consumption, yeast cell growth and ethanol production are presented in Fig. 5. Yeast cell growth with the addition of a production medium was dramatically increased compared to non-supplement medium. Ethanol production increased exponentially at 6 h of fermentation and entered a stationary phase between 12 and 36 h of fermentation. Reducing sugar concentration was decreased for 6 h of cultivation. The maximum ethanol concentration of 5.85 g/L at 18 h of cultivation was obtained from ethanol production medium. However, the non-supplement condition observed a level of 4.93 g/L at 12 h of cultivation. The theoretical efficiency yield of ethanol conversion from the fermentable sugar was 73.82%. The efficiency of ethanol production using sugar from lignocellulosic hydrolysis by *S. cerevisiae* TISTR

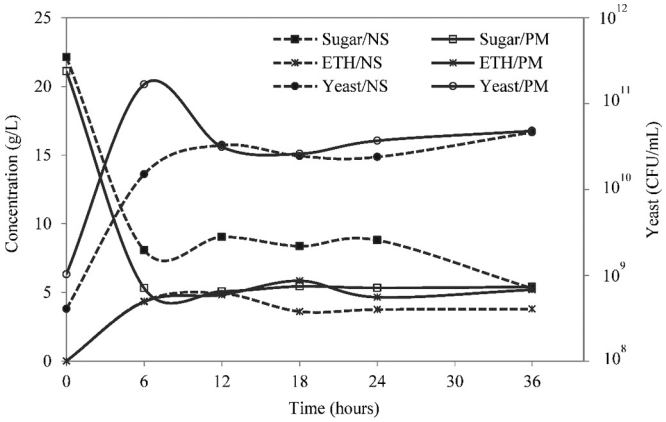

Fig. 5. Simultaneous saccharification and fermentation result of reducing sugar, bioethanol (ETH) and yeast growth (NS: non-supplement, PM: production medium). The fermentation was performed of alkaline-acid pretreated VG by *S. cerevisiae* TISTR 5339. The 5% (w/v) of VG pretreated was hydrolysis by crude enzyme and yeast fermentation in static condition at 30 °C for 36 h.

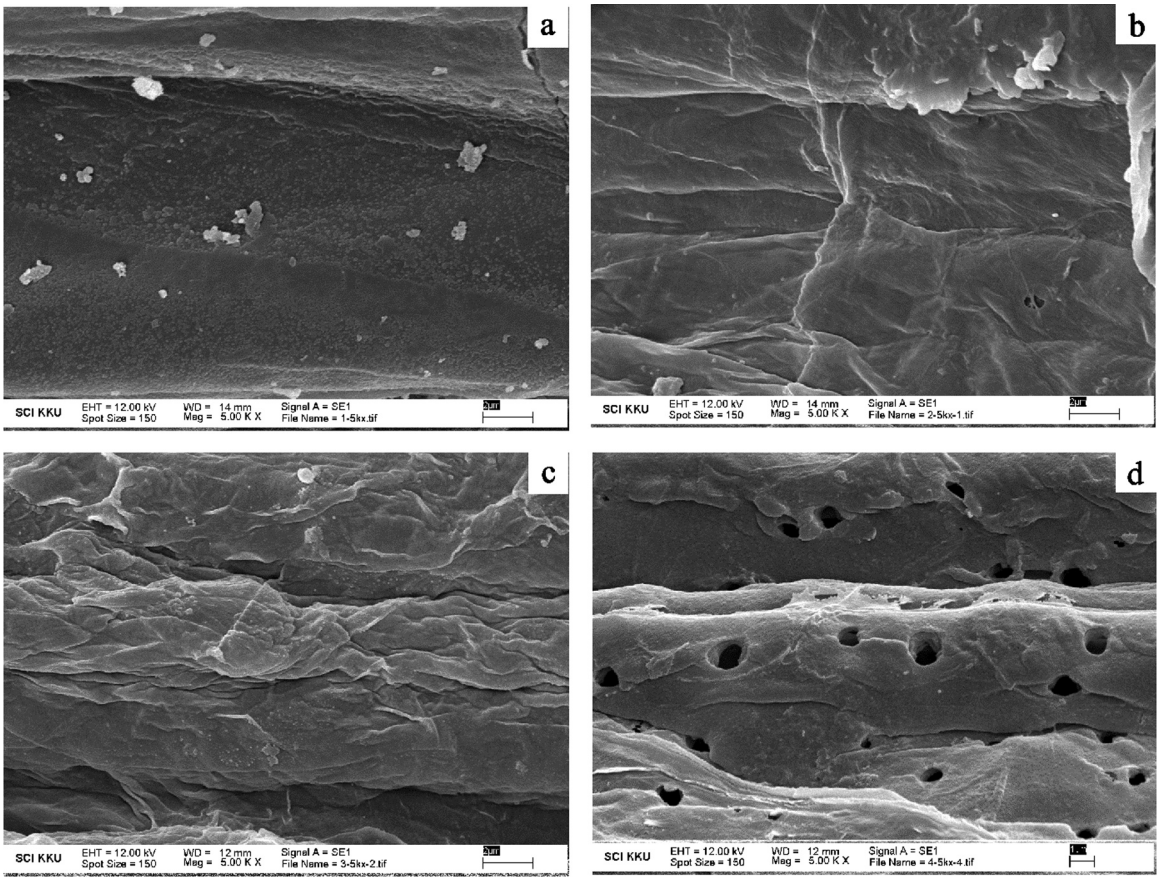

Fig. 6. Electron microscopic images of untreated VG (a), 1% (v/v) NaOH pretreated VG (b), 1% (v/v) NaOH pretreated VG and following pretreated by 0.5% (v/v) H_2SO_4 (c), alkaline-acid pretreated VG and crude enzyme hydrolysis (d) at magnification of 5000×.

5339 was reported as 30–80% theoretical yield by Wongwatanapaiboon et al. (2012), Sotthisawad et al. (2017) and Salakkam et al. (2017).

Soares et al. (2011) observed ethanol production from hot water pretreated purple elephant grass (*Pennisetum purpureum Schum.*) in SSF process. The pretreatment condition was performed at 100 °C for 25 min and ethanol fermentation performed using dried *S. cerevisiae* with 0.2 g (NH_4) H_2PO_4 and 0.1 g $MgSO_4$ at 30 °C for 10 h. The maximum ethanol yield was 1.8 g/L after 4 h fermentation. Dahnum et al. (2015) studied ethanol production from empty fruit bunches pretreated with 10% NaOH at 150 °C for 30 min, comparing hydrolysis fermentation (SHF) and SSF processes. The substrate with 1% (g/mL) of *S. cerevisiae* and 40 FPU/g dry biomass was fermented for ethanol production at 32 °C, 150 rpm for 72 h. The ethanol productions from SHF and SSF processes were 4.74% (72 h) and 6.05% (24 h), respectively.

The production of bioethanol from sugarcane bagasse pith with dilute acid pretreatment was performed in SHF and SSF process using *Pichia stipitis* JCM 10742 with fermentation medium (1.50 g/L yeast extract, 3 g/L peptone, 2 g/L KH_2PO_4,1 g/L $(NH_4)_2SO_4$ and 0.5 g/L $MgSO_4$,$7H_2O$) at 30 °C. The ethanol concentration in SSF was higher than SHF process which were 3.70 g/L (24 h) and 2.58 g/L (30 h), respectively (Sritrakul et al., 2017). Ethanol production from Napier grass under simultaneous saccharification and co-fermentation (SSCF) with Bushnell-Haas selection E medium (BHSD) was performed in a modified bioreactor for cellulose hydrolysis by *A. niger* and *Trichoderma reesei*, and for ethanol fermentation by *Zymomonas mobilis*. The highest ethanol concentration production was 0.51 g/L by using 15 g/L of substrate loading (Liu et al., 2017). Ethanol production levels in this study are comparable to the other reports. These results indicate that VG could be a candidate biomass for bioethanol production. In addition, ethanol concentration produced from ethanol production medium and non-supplement medium exhibited no significant difference. It is not necessary to add any supplement medium for ethanol production in this process. Therefore, ethanol production costs would be reduced.

3.6. Scanning electron microscopy

The surfaces area and morphology of untreated VG, pretreated VG and enzyme hydrolyzed VG were analyzed by SEM. The images of the morphological observation of untreated VG and pretreated VG with 1% NaOH, 0.5% H_2SO_4, crude enzyme hydrolysis are shown in Fig. 6a-d. Alkaline-acid pretreatment of the VG surface loosened its compact structure, increased surface area and also removed lignin and hemicelluloses from the structure (Toquero and Bolado, 2014). In addition, the change of structure expands the accessible surface area for enzyme attack. After enzymatic hydrolysis the cellulose structure had open fibers, and increased porosity for VG hydrolysis (Saini et al., 2013).

4. Conclusion

This study confirmed that VG containing cellulose of 45.22% w/w is a candidate of lignocellulosic biomass for cellulolytic enzyme production and bioethanol fermentation. The optimization of cellulolytic enzymes from *A. tubingensis* HS1–5 was successfully carried out in the Central composite design (CCD) with Response surface methodology (RSM) technique which was higher than conventional methods. Enzyme and ethanol production can be performed without added supplement. Use of VG as substrate would lower costs and cheaper for promote to sustain renewable energy feedstock a more attractive alternative energy source.

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