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Production of cellulase from *Aspergillus niger* and *Trichoderma reesei* mixed culture in carboxymethylcellulose medium as sole carbon

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Abstract. *Septiani D, Suryadi H, Mun'im A, Mangunwardoyo W. 2019. Production of cellulase from* Aspergillus niger *and* Trichoderma reesei *mixed culture in carboxymethylcellulose medium as sole carbon. Biodiversitas 20: 3539-3544.* Cellulase is one of hydrolytic enzymes that breakdown cellulose into glucose. Cellulases are promising to be applied in natural products which may improve the yield of bioactive in plant extract through cellulose depolymerization. Cellulases from mixed culture of *Aspergillus niger* and *Trichoderma reesei* can produce a high cellulase activity because of the synergism activity among endoglucanase, exoglucanase, and also β-glucoside. Cellulase production and partial purification of monoculture and mixed culture (1:1) of these fungi on carboxymethylcellulose media were investigated in this study. Total cellulase activity was measured by filter paper assay followed by protein estimation with Bradford method. The crude extract of *Aspergillus niger* monoculture has the highest cellulase activity (0.131 U/mL, P<005) followed by mixed culture (0.109 U/mL) and *Trichoderma reesei* (0.106 U/mL). The cellulase activity of partially purified cellulase from mixed culture significantly increased (0.335, 0.348, 0.374 U/mL, P<0.05) compared to crude extract along with stepwise addition of ammonium sulfate. Cellulase activity of mixed culture at 80% ammonium sulfate increase up to 2.238-fold and showed highest value (P<0.05) compared to monocultures. In conclusion, combination of *Aspergillus niger* and *Trichoderma reesei* fungi in carboxymethyl cellulose media followed by 80% ammonium sulfate precipitation can be a promising cellulase production with high cellulase activity.

Keyword: *Aspergillus niger*, cellulase, carboxymethyl cellulose, mixed culture, *Trichoderma reesei*

INTRODUCTION

Cellulase is a group of hydrolytic enzymes which abundant in nature (Kaur and Joshi 2015). Cellulase is a trivial name of β-1,4-glucan-4-glucanohydrolase (EC.3. 2. 1. 4). Cellulase enzyme can hydrolyze β-1,4-glucoside chain in cellulose, cellodextrin, cellobiose, and other cellulose derivative (Zhang and Zhang 2013). Cellulose, as a substrate of cellulase, constructed in linear forms. It contains units of anhydride glucose and linked tightly one another at first (C1) and four (C4) carbon of glucose units that contribute to a tight structure called a β-1,4-glucoside chain (Kaur and Joshi 2015). Cellulase breakdown of the cellulose structure by involving synergistic action of cellulolytic enzyme complexes: endo-β-glucanase, exo-β-glucanase, and β-glucosidase in certain quantity (Jamil et al. 2009).

Microorganisms such as bacteria and fungi are cellulase producers (Zhao et al. 2018). Aerobic fungi of the Ascomycetes, *Aspergillus* sp., and *Trichoderma* sp. have received significant attention as cellulose-degrading with high cellulase activity (Zhao et al. 2018). Furthermore, its extracellular cellulase model with high yield made it easy to produce commercially (Jagavati et al. 2012).

Trichoderma reesei more often produces large quantities of endo-β-glucanase and exo-β-glucanase, rather than β-glucoside (Diana et al. 2012). Meanwhile, *Aspergillus niger* found to be the highest producers of β-glucoside and endo-β-glucanase (Kassim 1982). The consortium of *Trichoderma reesei* and *Aspergillus niger* will be promising to produce synergistic action of highly extracellular cellulase activity.

A cellulase activity can be measured by observing the clear zone around colony in solid medium, meanwhile, filter paper assay (FPase) measured the total cellulase activity in broth medium through glucose reduction from Whatman paper (Dashtban et al. 2010). Carboxymethyl-cellulose (CMC) is a soluble cellulose derivate which easily degraded by many organisms. CMC is a useful carbon source of both liquid and solid medium for cellulase production, and the hydrolysis of CMC also easy to determine by chromogenic reagent Congo red (Johnsen and Krause 2014).

The main challenge for abundant cellulase from fungi is to utilize them in plant extraction to degrade cellulose from natural products. Cellulase from fungi has a bright prospect in future natural product extraction that can provide high yield of bioactive, less time extraction, and reduce extraction cost. The purpose of this study is to obtain the higher cellulase activity from mixed culture of *Aspergillus niger* and *Trichoderma reesei* than monocultures using carboxymethyl cellulose as sole carbon medium.

MATERIALS AND METHODS

Pure isolate of *Aspergillus niger* (UICC 371) and *Trichoderma reesei* (IPBCC) were obtained from IPB Culture Collection, Bogor, and Universitas Indonesia Culture Collection, Depok respectively. The fungi were recultured and maintained on potato dextrose agar (Difco) tube at 28°C. The partially purified enzyme with high cellulase activity from this study deposited in dry powder form to extend its shelf life. The instruments used were autoclave (Tomy, Japan), oven (WTB Binder, Germany), analytical balance (Acculab, USA), hotplate stirrer (Corning, USA), pH meter (Hanna, USA), centrifugation (Kubota, Japan), incubator (Memmert, Germany), microscope (Micromax, USA), air shaking incubator (Heidolph, Germany), laminar airflow (Kubota, Japan), vortex, haemacytometer (Improved Neubauer, Germany), UV-Vis spectrophotometer (Shimadzu UV-1601, Japan), freeze dryer, ose, volumetric pipette and other glasswares commonly used in laboratories. All the chemicals were analytical grade, such as glucose (Merck, Germany), sodium carboxymethyl cellulose (CMC), sodium hydroxide (Bratachem, Indonesia), nitric acid (Bratachem, Indonesia), urea (Merck, Germany), ammonium sulphate (Merck, Germany), potassium dihydrogen phosphate (Merck, Germany), tween 80 (Merck, Germany), sodium potassium tartrate (Merck, Germany), magnesium sulfate heptahydrate (Meck, Germany), iron (II) sulfate heptahydrate (Merck, Germany), yeast extract (Difco, USA), bacteriological agar (Merck, Germany), dinitrosalicylic acid (Sigma, USA), sodium chloride (Merck), congo red (Sigma, USA), bovine serum albumin (Sigma, Germany), Bradford dye reagent concentrate (Bio-Rad, USA), Whatman paper no. 1, aquadest, and aquabidest.

Screening of cellulase activity based on clear zone

Fungi isolates, *A. niger*, and *T. reesei*, as monoculture stock were cultured on potato dextrose agar tube and incubated at 28°C for 3 days. After 3 days incubation, each fungal isolate was subcultured on agar medium plates that contained 1% carboxymethylcellulose, 0.1 g $NaNO_3$, 0.05 g yeast extract, 0,1 g KH_2PO_4, 0.1 g $MgSO_4.7H_2O$, 0.1 g glucose (Murti et al. 2018). The plates were incubated at 28°C for 3 days and screened for cellulase production at least twice. The clear zone was observed by stained the plates with 1% congo red solution for 30 minutes followed by destained with 1 M NaCl solution for 20 minutes. The clear zone was determined by the ability of fungi to produce cellulase. The clear zone measured by ratio of clear zone diameter and colony diameter (cm) (Khokhar et al. 2012).

Cellulase production

Preparation of pre-culture and production medium

The media for cellulase enzyme production, in this research, were preculture and production medium, which added with CMC as sole carbon. The preculture and production medium contain 200 mg yeast extract, 750 mg urea, 280 mg $(NH_4)_2SO4$, 400 mg KH_2PO_4, 150 mg $MgSO_4.7H_2O$, 1 mg $FeSO_4.7H_2O$, diluted in 0.1 M acetic acid buffer 200 mL (Murti et al. 2018). Preculture and production medium contain CMC at 0.5 and 2.5% concentration, respectively. The higher concentration of CMC in production media than preculture media was to enrich media in order to produce high yield of cellulase.

Preparation of inoculum

The inoculum of *A. niger* and *T. reesei* were used at a density 1 x 10^8 cfu/mL and observed by haemacytometer counting chamber under light microscope. Each fungi inoculum was a spore suspension obtained from harvested the isolate in sterile aquabidest and 0.1% tween 80 (Murti et al. 2018).

Production of cellulase enzyme

Two mL of *A. niger* and *T. reesei* inoculums were inoculated into 500 mL Erlenmeyer flasks containing 100 mL pre-culture medium. There were 3 sets of Erlenmeyer flasks, contain monoculture of *A. niger* and *T. reesei* and mixed culture (1:1) fungal inoculum. The flasks were incubated for 48 hours in shaker incubator at 28°C 150 rpm for inoculum development before they were used for cellulase production in production medium (Zhao et al. 2018). After 48 hours incubation, about ten milliliters of preculture media were inoculated into 3 sets of 200 mL production media in 500 mL Erlenmeyer flasks. The production media were stirred in incubator shaker at 150 rpm 28°C for 72 hours.

Cellulase extraction

Tween 80 (0.1%) was added to production media, then were aliquoted into 15 mL centrifuge tubes. The tubes were centrifuged at 4°C 3000 rpm for 10 minutes (Murti et al. 2018). Supernatant was collected as crude extract of cellulase enzyme and used for cellulase assay (Ghose 1987).

Purification of cellulase enzyme

Purification of cellulase enzymes was carried out by salting out process with ammonium sulfate (Ikeda et al. 2014). Proteins of enzymes were sequentially precipitated from 30 mL of the crude extract by stepwise addition of solid ammonium sulfate from 40, 60, to 80 percent saturation and followed by overnight incubation at 4°C. On the next day, the solution was centrifuged at 4°C 4500 rpm for 20 minutes. The pellet obtained after centrifugation was resuspended in 0.05 M acetate buffer pH 5.5 up to 1/5 filtrate volume. Aliquots of precipitated fractions, as partially purified cellulase enzymes, were analysed for its cellulase activity by FPase (Ghose 1987) and its protein contents by Bradford method (Bradford 1976). The total cellulase activity of partially purified cellulase was compared to crude extract.

Total cellulase activity of filter paper assay (FPase)

Cellulase activity was measured by filter paper assay used Whatman filter paper no.1 in a size of 1 x 6 cm strip as standard substrate (Dashtban et al. 2010; Ghose 1987). FPase assay was performed by add 0.5 mL cellulase enzymes into tube filled with one filter paper (50 mg) and 1

mL 0.05 M sodium citrate. The concentration of glucose was estimated by dinitrosalysilic acid (DNS) reagent (Ghose 1987). About 2 mg glucose equivalents added to the tube and boiled for 60 minutes at 50℃. After that, 1% DNS solution was added and boiled for 5 minutes to stop the enzymatic reaction. The absorbance of solution was measured by UV-Vis spectrophotometer at 540 nm wavelength. Total fungal cellulase activity was estimated by a standard equation compared to the glucose calibration curve. The total cellulase activity of FPase was determined in the standard IUPAC procedure and expressed as an international unit (IU) in U/mL as one micromole of glucose equivalents to per minute of culture filtrate under assay conditions (Ghose 1987).

Total protein content of enzyme

The protein content in crude extract and partially purified cellulase enzyme were determined by Bradford method (Bradford 1976). Protein assay was prepared by diluted one part of concentrate dye reagent with 4 parts aquabidest and filtered with Whatman #1 filter paper to remove particulates. Bovine serum albumin (BSA) was used as standard protein and diluted to 5 different concentrations (0.01, 0.02, 0.04, 0.06, 0.08 mg/mL). About two mL of dye reagent mixed with different concentrations of standard protein, and incubated for 5 minutes at room temperature. The absorbance of solutions was measured under UV-Vis spectrophotometer at 595 nm (Bradford 1976). The absorbance of the standard protein with the concentration was plotted to construct a calibration curve. The total protein content in cellulase crude extract and partially purified cellulase were determined by mixed 1 mL of each sample with 2 mL dye reagent in tube then incubated for 5 minutes at room temperature. The absorbance of samples was measured using UV-Vis spectrophotometer at 595 nm. The protein content in samples was measured by substituted the absorbance data into a calibration curve.

Data analysis

Total cellulase activity of mixed culture compares to monoculture of *A. niger* and *T. reesei* was done by oneway-ANOVA followed by Least Significance Different (LSD) to assess the significant differences (P<0,05). Meanwhile, the total cellulase activity between crude extract and partially purified in each group was analyzed by a paired t-test. All data were analyzed by Statistical Package for Social Sciences (SPSS) software ver. 24.

RESULTS AND DISCUSSION

Screening of cellulase activity based on clear zone

The ability of *A. niger* and *T. reesei* grew in CMC solid medium form a clear zone around colony that indicated the cellulolytic activity (Figure 2). The growth of colony was observed at the end of 5 days incubation. After day 5, the plates were stained with 1% congo red to observe clear zone of hydrolysis. The clear zone of CMC hydrolysis was 0.45 cm on *A. niger* and 0.1 on *T. reesei*. The result indicates that *A. niger* (UICC 371) and *T. reesei* (IPBCC) were capable of utilized CMC and produce cellulase. Their cellulolytic activity was further evaluated by inoculating its spore suspension to cellulase production broth medium supplemented by CMC as sole carbon.

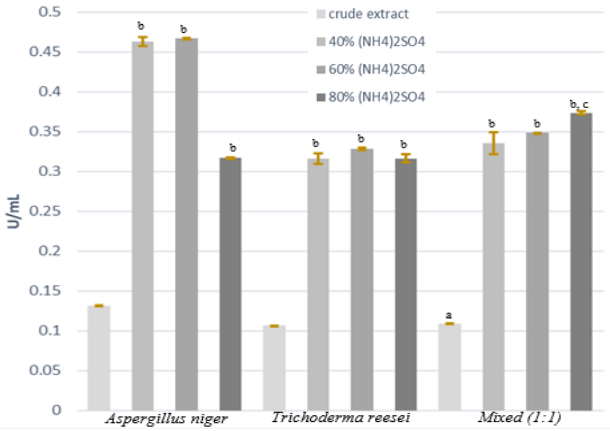

Figure 3. Total FPase cellulase activity in crude extract and partially purified cellulase. Note: [a,c] significant difference compared to monocultures (P<0,05) by ANOVA, [b] significant difference compared to crude extract (P<0,05) by paired t-test

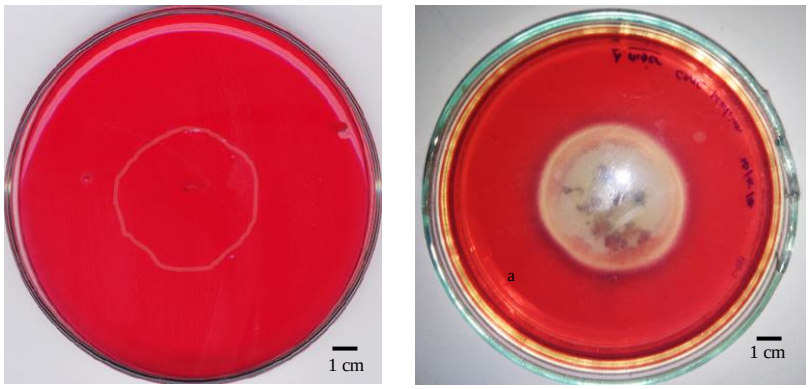

Figure 2. The clear zone of *Aspergillus niger* (*right*) and *Trichoderma reesei* (*left*) in CMC medium agar, 7 days incubation at 28℃

Total cellulase activity of Filter paper assay (FPase)

Total cellulase activity in crude extract and partially purified cellulase at different concentration of ammonium sulfate ($(NH_4)_2SO_4$) presented in Table 1 and Figure 3. Total cellulase activity of crude extract from mixed culture (1:1) significantly different ($P < 0,05$) to monocultures (Table 1). The result showed significant difference between and within groups of monocultures and mixed culture by oneway-ANOVA test followed by LSD test (Table 2). The positive mean difference values in A-T and A-M (Table 2) indicated that cellulase activity produced by *A. niger*, *T. reesei*, and mixed culture was significantly different. A crude extract of *A. niger* produced highest glucose concentration with the highest FPase enzymatic activity (0,131 U/mL) followed by mixed culture (1:1) (0,109 U/mL) and monoculture of *T. reesei* (0,106 U/mL) (Table 1) at 72 h of incubation. We conclude that there was significant difference ($P<0,05$) in total FPase activity between crude extract and partially purified enzyme along with ammonium sulfate addition. Enzyme from monoculture of *A. niger* showed the highest cellulase activity compared to others.

Purification of cellulase enzyme

FPase cellulase activity of partially purified enzymes was significantly difference ($P<0.05$) compared to crude extract in all groups (Table 1 and 3). The partially purified enzymes of *A. niger*, *T. reesei*, and mixed culture were higher than crude extract. Total cellulase activity increase along with step addition of ammonium sulfate saturation up to 60%. The test showed positive *t* values for all pair groups indicated the cellulase activities after precipitated were highly different compared with the crude extract (Table 3). An addition of 80% ammonium sulfate saturation in mixed culture was improved the cellulase activity significantly compared to monocultures (Table 4).

Cellulase purification by 80% ammonium sulfate saturation improved cellulase activity up to 2.238 folds compare to initial activity of crude enzyme (Table 5). The increase in total cellulase activity and specific activity were observed at 0.378 U/mL and 15.768 U/mg respectively. The specific activity of enzyme was measured by dividing the cellulase activity with protein content in enzyme (Nema et al. 2016). The protein contents were determined by Bradford method which compare to the calibration curve of BSA as standard (Bradford 1976). The less protein content at 80% ammonium sulfate saturation was inversely proportional to the total cellulase activity (Table 5).

Discussion

There was few previous research that had been carried out using combination of *A. niger* and *T. reesei* as cellulase producers in different strain, substrates, and cellulase assay. The results of this research showed that FPase cellulase activity in mixed culture (1:1) of *A. niger* and *T. reesei* was 0.109 U/mL. The cellulase activity in mixed culture was closed compare to previous research by Jagavati et al. (2012) with FPase of 0.24 U/mL in mixed culture of *Aspergillus* sp. And *Trichoderma* sp. isolated from degrading wood (with ratio 2:1). Lower FPase activity in this research due to differences in substrates, ratio of fungi, and incubation time during cellulase production (fermentation). All these factors must be at optimum conditions (Jagavati et al. 2012).

Table 1. Total FPase cellulase activity of monocultures and mixed culture (1:1) in crude extract and partially purified cellulase with a varying saturation level of ammonium sulfate

Description	Total cellulase activity (U/mL) Means ± SD		
	Aspergillus niger	***Trichoderma reesei***	**Mixed culture (1:1)**
Crude extract	0.131 ± 0.00025^{a}	0.106 ± 0.00025	0.109 ± 0.0004
$(NH_4)_2SO_4$ 40%	0.463 ± 0.00806^{b}	0.317 ± 0.00933^{b}	0.335 ± 0.01958^{b}
$(NH_4)_2SO_4$ 60%	0.467 ± 0.00127^{b}	0.328 ± 0.00247^{b}	0.348 ± 0.00049^{b}
$(NH_4)_2SO_4$ 80%	0.317 ± 0.00134^{b}	0.316 ± 0.00749^{b}	$0.374 \pm 0.00289^{b,c}$

Note: $^{a, c}$ significant difference compared to crude extract in other group ($P<0.05$) by ANOVA, ^{b} significant difference compared to crude extract ($P<0.05$) by paired t-test

Table 2. Statistical evaluation (Oneway-ANOVA and LSD test) of cellulase activity in monoculture and mixed culture crude extract of *Trichoderma reesei* and *Aspergillus niger* at 95% confidence

	Sum of squares	**Df**	**Mean square**	**F**	**Sig.**
Between groups	0.001	2	0.001	5745.799	0.000*
Within groups	0.000	6	0.000		
Total	0.001	8			

	Mean difference	**Standard error**	**Sig.**	**Lower bound**	**Upper bound**
A-T	0.0246	0.0002	0.000*	0.0240	0.0252
A-M	0.0219	0.0002	0.000*	0.02133	0.0225
T-A	-0.0246	0.0002	0.000*	-0.0252	-0.0240
T-M	-0.0026	0.0002	0.000*	-0.0032	-0.0020
M-A	-0.0219	0.0002	0.000*	-0.0225	-0.0213
M-T	-0.0026	0.0002	0.000*	0.0020	-0.0032

Note: *Sig. < 0,05, significant difference; A: *A. niger* crude enzyme; T: *T. reesei* crude enzyme; M: Mixed culture (1:1) crude enzyme

Table 3. Statistical evaluation (paired t-test) of cellulase production and purification by mixed culture of *T. reesei* and *A. niger* with monocultures at 95% confidence

	Mean	Standard deviation	Standard error means	Lower	Upper	t	df	Sig. (2-tailed)
Pair 1	0.3322	0.0054	0.0031	0.3187	0.3457	105.592	2	0.000*
Pair 2	0.3356	0.0011	0.0006	0.3327	0.3385	505.533	2	0.000*
Pair 3	0.1855	0.0007	0.0004	0.1837	0.1872	458.645	2	0.000*
Pair 4	0.2100	0.0063	0.0036	0.1942	0.2258	57.294	2	0.000*
Pair 5	0.2217	0.0020	0.0011	0.2167	0.2266	191.485	2	0.000*
Pair 6	0.2099	0.0055	0.0032	0.1961	0.2237	65.521	2	0.000*
Pair 7	0.2260	0.0134	0.0077	0.1926	0.2594	29.112	2	0.001*
Pair 8	0.2394	0.0007	0.0004	0.2375	0.2413	543.059	2	0.000*
Pair 9	0.2644	0.0016	0.0009	0.2603	0.2685	277.576	2	0.000*

Note: *Sig. (2 tailed) < 0,05, significant difference.
Pair 1: *Aspergillus niger* 40% $(NH_4)_2SO_4$ precipitation: *Aspergillus niger* crude enzyme
Pair 2: *Aspergillus niger* 60% $(NH_4)_2SO_4$ precipitation: *Aspergillus niger* crude enzyme
Pair 3: *Aspergillus niger* 80% $(NH_4)_2SO_4$ precipitation: *Aspergillus niger* crude enzyme
Pair 4: *Trichoderma reesei* 40% $(NH_4)_2SO_4$ precipitation: *Trichoderma reesei* crude enzyme
Pair 5: *Trichoderma reesei* 60% $(NH_4)_2SO_4$ precipitation: *Trichoderma reesei* crude enzyme
Pair 6: *Trichoderma reesei* 80% $(NH_4)_2SO_4$ precipitation: *Trichoderma reesei* crude enzyme
Pair 7: *Mixed culture* 40% $(NH_4)_2SO_4$ precipitation: *Mixed culture* crude enzyme
Pair 8: *Mixed culture* 60% $(NH_4)_2SO_4$ precipitation: *Mixed culture* crude enzyme
Pair 9: *Mixed culture* 80% $(NH_4)_2SO_4$ precipitation: *Mixed culture* crude enzyme

Table 4. Statistical evaluation (Oneway-ANOVA and LSD test) of partially purified cellulase at 80% saturation of ammonium sulfate

	Sum of squares	df	Mean square	F	Sig.
Between groups	0.007	2	0.003	294.475	0.000*
Within groups	0.000	6	0.000		
Total	0.007	8			

	Mean difference	Standard error	Sig.	Lower bound	Upper bound
A-T	0.0001	0.0027	0.953	-0.0064	0.0068
A-M	-0.0570	0.0027	0.000*	-0.0636	-0.0503
T-A	-0.0001	0.0027	0.953	-0.0068	0.0064
T-M	-0.0571	0.0027	0.000*	-0.0638	-0.0505
M-A	0.0570	0.0027	0.000*	0.0503	0.0636
M-T	0.0571	0.0027	0.000*	0.0505	0.0638

Note: *Sig. < 0,05, significant difference; A: *Aspergillus niger* partially purified enzyme; T: *Trichoderma reesei* partially purified enzyme; M: Mixed culture (1:1) partially purified enzyme

Table 5. Purification of cellulase in mixed culture of *A. niger* and *T. reesei* at 40, 60, and 80% saturation of ammonium sulphate

Steps	X glucose (mg/mL)	Total cellulase activity (U/mL)	Protein content (mg/mL)	Specific activity (U/mg)	Purification (fold)
Crude extract	0.590	0.109	0.015	7.045	1
40%	1.815	0.335	0.047	7.059	1.002
60%	1.883	0.348	0.048	7.276	1.033
80%	2.007	0.374	0.024	15.768	2.238

Cellulase activity in monoculture *A. niger* showed the highest enzyme activity (0.131 U/mL); therefore, *A. niger* may act as a donor of cellulase enzyme within mixed culture in specific quantities. *A. niger* produced more extracellular cellulase contain exo-β-glucanase and β-glucosidase that significantly degrade filter paper (Kassim 1982). On the other hand, *T. reesei* produced more endo-β-glucanase, exo-β-glucanase, and lack of β-glucosidase (Jamil et al. 2009). Hence, cellulase produced by mixed culture of *A. niger* and *T. reesei* were able to convert cellulose substrates into simple sugars through synergy effect of cellulase complexes produced by these two fungi (Jamil et al. 2009).

Addition of ammonium sulfate increased cellulase activity because of *salting-out* process. The process of *salting out* decreases protein solubility since salt ions were

given in high concentrations. The exact concentration of salt allowed the separation of different proteins and precipitate them (Jamil et al. 2009). Hence, the addition of 80% ammonium sulfate in mixed culture increased the cellulase activity significantly ($P<0.05$) because its hydrophilic cellulase enzyme needs high concentration of ammonium sulfate to precipitate (Jamil et al. 2009). The less activity of cellulase in crude extract showed many undesired contaminants in crude extracts compared to partially purified enzyme (Nema et al. 2016). The specific activity of enzyme in mixed culture was improved along with the addition of the ammonium sulfate concentration that causes an increase in activity up to 2.238 folds. Hence, the combination of *Aspergillus niger* and *Trichoderma reesei* in carboxymethyl cellulose media production followed by ammonium sulfate precipitation at 80% saturation can be a promising cellulase production for highly cellulase activity

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