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Screening of Potential Strains and Co-substrate for Production of Cellulase Enzyme using Sewage Treatment Plant Sludge as Major Substrate

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Abstract: Potential fungal strains belonging to the genera of *Penicillium*, *Trichoderma* and *Aspergillus* were isolated from various waste sources and tested for their capability to produce cellulase enzyme using Sewage Treatment Plant (STP) sludge as a major substrate. The isolation technique was used in the Potato Dextrose Agar (PDA) media incorporated with the sewage sludge (0.5% Total Suspended Solids, TSS). In addition 1 mL of streptomycin was used to control the growth of bacteria. Colonies appeared in the plate were transferred to the fresh PDA plate for purification and identification. Out of 35 strains, five strains were identified as the potential strains to produce cellulase through Filter Paper Activity (FPA) assay. These five promising strains were TH(U), S-105A and P2-STP isolated from STP sludge, P1-EFB isolated from Empty Fruit Bunched (EFB) compost and O-102A isolated from rotten orange. Various carbon sources; wheat flour, cassava flour, commercial sugar and cellulose were evaluated as a co-substrate with and without EFB supplement using these five potential strains. The strain O-102A was identified as the most promising strain that able to produce cellulase enzyme using STP sludge as a major substrate, cellulose as a co-substrate with supplement of oil palm Empty Fruit Bunches (EFB).

Key words: Endo-glucanase, bioconversion, media optimization, filamentous fungi, sewage treatment plant sludge

INTRODUCTION

Sewage is the largest contributor of organic pollution to water resources as well as to surrounding environments all over the world. In Malaysia, its contribution is dominant (47.8%), followed by manufacturing industry (45.0%), animal husbandry wastes (4.6%) and agro based (2.6%) in term of biological oxygen demand (BOD) load (Department of Environment, 2006). Approximately 4.2 million cubic meters of sewage sludge is produced by Indah Water Konsortium (IWK) annually in Malaysia and this sludge volume is expected to rise to 7 million cubic meters by the year 2020 (Alam *et al*., 2003). The management of the ever-increasing volume of domestic and industrial organic wastes has been one of the prime environmental issues in Malaysia. The safe and environmental-friendly disposals of these huge quantities of sludge are the main concern of Indah Water Konsortium (IWK).

In recent years, interest has twisted to the methods or processes based on the resource recovery approach known as recycling and reuse of organic waste produced from different sources of domestics as well as industry. The concept of product recovery from organic residues by applying biological-based treatment is becoming more popular to be applied for various purposes. Previously composting was more acceptable alternative for the sludge treatment due to its potential use for land application as biofertilizer and soil conditioner. Bioconversion of sewage treatment plant sludge by Liquid State Bioconversion (LSB) is being proposed to solve these problems through the recovery of products especially lignocellulolytic enzymes such as cellulase (Alam *et al*., 2008), hemicellulase, xylanase, lignin peroxidase (LiP) (Alam *et al*., 2009) and manganese peroxidase (MnP). This is a new biotechnological approach for the biodegradation and biosolids accumulation of sludge beside the production of industrial enzyme which exhibits the benefit of being very low treatment and production cost and environmentally friendly (Alam *et al*., 2003).

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Cellulase refers to a group of hydrolytic enzymes (endoglucanases, exoglucanases and α-glucosidases) that are capable of hydrolyzing insoluble cellulose to glucose (Wen *et al*., 2005). It is an important commercial enzyme that widely used in food, animal feed, textile, pulp and paper, grain alcohol fermentation; starch processing, pharmaceuticals, malting and brewing industries (Oksanen *et al*., 2000). Fungi are the main cellulase producing microorganisms, though a few bacteria and actinomycetes have also been reported to yield cellulase activity (Chand *et al*., 2005). The cellulolytic fungi *Trichoderma* spp. and *Aspergillus* spp. have been extensively studied for their cellulase production. Many studies involved the *Trichoderma* species such as *Trichoderma harzianum* (Alam *et al*., 2008), *Trichoderma viride* (Liu *et al*., 2006) and the most common cellulase producer *Trichoderma reesei* (Wen *et al*., 2005) have been published. Several *Aspergillus* species also have been proved as a cellulase producer such as *Aspergillus niger* (Kang *et al*., 2004) and *Aspergillus flavus* (Ojumu *et al*., 2003). Cellulase production has been influenced by a number of factors including the type of strain used the culture conditions and substrate types (Lynd *et al*., 2002). The relationship between these variables has a marked effect on the ultimate production of the cellulase enzyme complex. Thus, the aim of this study was to identify the potential strain for cellulase production by liquid state bioconversion of Sewage Treatment Plant (STP) sludge and to identify the best co-substrate to be use in fermentation media. Besides, the potential of domestic Sewage Treatment Plant (STP) sludge used as a basal media for cellulase production was highlighted.

MATERIALS AND METHODS

Microbial strain and its inoculum preparation: Various fungal species of *Trichoderma* spp., *Penicillium* spp. and *Aspergillus* spp. were isolated from different waste samples (STP Sludge, empty fruit bunches (EFB) compost, rotten orange and rotten lemon). The *Trichoderma harzianum* strain and commercial strain *T. reesei* Rut C-30 (ATCC 56765) which obtained from laboratory stock were tested for their potential of producing cellulase enzyme. The fungal strains were grown on the Potato Dextrose Agar (PDA) plate and incubated at 32°C until the entire plate was covered by fungus. Four PDA plates of fungal culture were washed with 100 mL sterile distilled and the surface of the agar was gently scraped with sterilized glass rod. The spore suspension was then filtered using Whatman filter paper No1 and kept at 4°C until further used. This study has been conducted in year 2010.

Evaluation of fungal strains for cellulase production: Sewage treatment plant (STP) sludge (0.5% w/v of TSS) was used as a major substrate or medium with supplementary of wheat flour (2% w/v) as co-substrate for initial microbial growth and oil palm empty fruit bunch, EFB (0.2% w/v) as cellulosic material in the fermentation process. The initial pH of the media was adjusted to pH 5.0 and sterilized at 121°C for 20 min before inoculation. All potential fungal strains were cultured in 100 mL Erlenmeyer flask with working volume of 50 mL containing the fermentation media. The medium was inoculated with 3% (v/v) inoculums. Fermentation was carried out for 2 days at 30°C and shake at 150 rpm. Supernatants were analyzed for cellulase activity.

Evaluation of co-substrates as supplementary nutrients for cellulase production: Sewage treatment plant (STP) sludge (0.5% w/v of TSS) was used as a major substrate with supplementary of 2% (w/v) different co-substrate: wheat flour, commercial sugar, cassava flour and cellulose powder (Sigma-Aldrich). The effect of lignocellulosic material on cellulase production also have been studied here by varied the fermentation media with and without addition of 0.2% (w/v) of oil palm Empty Fruit Bunch, (EFB). Five strains selected having a potential to produce cellulase enzymes were cultured in media with different co-substrate. Samples were harvested on second, third and fourth days and filtered using filter paper Whatman No.1. Supernatants were analyzed for cellulase activity.

Comparison of *Trichoderma reesei* Rut C-30 and locally isolated strain O-102A: A 3% (v/v) inoculum concentration of O-102A and *T. reesei* Rut C-30 was inoculated to sterilized fermentation medium containing STP sludge (0.5% w/v of TSS) as a major substrate with supplementary of 2% (w/v) of cellulose powder and 0.2% (w/v) EFB for production of cellulase enzyme. All the fermentations for cellulase production were carried out in 250 mL Erlenmeyer flasks containing a total volume 150 mL in three replications. The initial pH was about 5.0 and it was not controlled during the fermentation. Fermentation was carried out for 7 days at 30°C and shake at 150 rpm. Samples were collected everyday starting from third until seventh day of fermentation and filtered through filter paper Whatman No. 1. The filtrates then centrifuged at 10,000 rpm for 5 min and proceed for the cellulase analysis.

Enzyme assay: The activities of total cellulase (filter paper activity, FPA) and endo-α-1,4 glucanase activity (CMCase) were determined using IUPAC recommended procedure (Ghose, 1987). One international Filter Paper

Unit (FPU) and CMCase activity were defined as the amount of enzyme that release 1 μmol glucose/min and activities were reported as FPU mL^{-1} and U mL^{-1} respectively. Glucose equivalents (reducing sugars) generated during the assay were estimated by using the 3,5 dinitrosalicylic acid (DNS) method (Miller, 1959), with glucose as standard. All samples were analyzed in triplicate and the mean value calculated.

RESULTS AND DISCUSSION

Screening of potential fungal strains for cellulase production: In this present investigation, 35 strains were tested for production of total cellulase enzyme using filter paper as a substrate based on activity screening approach. The result presented in Table 1 indicated the production of cellulase enzyme expressed in Filter Paper Unit (FPU) activity for all strains on the second day of fermentation.

Out of the 35 strains screened, five strains isolated from STP sludge showed a negative result where the cellulase activity could not be detected. While *Trichoderma* spp. isolated from STP sludge noted as TH(U) showing a good result which produced 0.155 FPU mL^{-1}. Other strains noticed having a potential to produce cellulase enzyme are strain P2-STP (0.131 FPU mL^{-1}) and S-105A (0.055 FPU mL^{-1}) isolated from STP sludge, *Penicillium* spp. (P1-EFB) with 0.074 FPU mL^{-1} isolated from EFB compost and strain O-102A with 0.067 FPU mL^{-1} isolated from rotten orange. Fungi mainly from the *Aspergillus* spp. and *Trichoderma* spp. are reported by many authors as the cellulase producer microorganisms (Liming and Xueliang, 2004), although a few bacteria and actinomycetes have also been reported to yield cellulase activity (Chand *et al*., 2005). The production of cellulases by the fungal strains using other substrates such as pure cellulose, corn cob, etc with optimum fermentation conditions cited in the literatures are higher (2-5 IU (cellulose-based)/mL) than the present study. From the screening experiment, these five strains were identified as a good cellulase producer using STP sludge as a substrate and further used for screening of co-substrate which is expecting to increase with optimum fermentation study.

Screening of co-substrate for cellulase production: Cellulase enzymes were produced by fermentation of five potential strains selected from screening mentioned before using wheat flour, commercial sugar, cassava flour and microgranular cellulose (Sigma-Aldrich) as the carbon sources. The fermentation medium used in this study was noted as SW (fermentation media with sludge and wheat flour), SS (fermentation media with sludge and commercial sugar), ST (fermentation media with sludge and cassava flour) and SC (fermentation media with sludge and cellulose powder) as well as with and without supplement of EFB. Cellulase enzyme activity was measured using most known test: filter paper assay for total cellulase activity and results are shown in Table 2.

In SW media, strain TH(U) and P2-STP were showing the ability to produce the cellulase enzyme with supplement of EFB. The effect of commercial sugar (sucrose) as a carbon source was observed in SS media. Contrast from observation in SW media, total cellulase activity for strain P1-EFB (0.080 FPU mL^{-1}), P2-STP (0.073 FPU mL^{-1}) and O-102A (0.064 FPU mL^{-1}) without supplement of EFB observed were higher. The activity of cellulase with supplement of EFB found were 0.036 FPU mL^{-1} (P1-EFB), 0.047 FPU mL^{-1} (P2-STP) and 0.016 FPU mL^{-1} (O-102A). No activity was detected for these three strains on second day fermentation. Whereas, strain TH(U) and S-105A begin to produce cellulase enzyme on second day of fermentation but with very low activity and start increased on the fourth day of fermentation supplemented with EFB with 0.054 and 0.070 FPU mL^{-1} cellulase activity respectively compared only 0.023 and 0.024 FPU mL^{-1} on second day. Production of cellulase enzyme in media contained cassava flour as a co-substrate (ST media) was similar to SW media, whereby the cellulase activity was higher in media with supplement of EFB than media contained no EFB. Strain O-102A was showing the potential in producing cellulase enzyme where 0.111 FPU mL^{-1} of cellulase activity was recorded in fourth day which is the highest activity recorded in ST media followed by TH(U) with 0.064 FPU mL^{-1} of cellulase activity. Other strains in ST media not showing the ability to produce higher cellulase enzyme compared to O-102A and TH(U). Wayman and Chen (1992) have studied on the wheat flour effects on the cellulase production using *T. reesei*. Some other studies showed various carbon source effects on the production of cellulases using different microbial treatments (Hideno *et al*., 2010; Petitdemange *et al*., 1992).

For production of cellulase in Sludge-Cellulose (SC) media, no cellulase activity was detected for all strains on second day of fermentation. Without supplement of EFB, no enzyme could be detected on the third day of fermentation for all strain except 0-102A. The cellulase activity of O-102A increased up to 0.151 and 0.211 FPU mL^{-1} on third and fourth day of fermentation respectively which is the highest activity recorded in this study when cellulose was used as a co-substrate. In fact, cellulose was proved to be the best carbon source for production of cellulase enzyme (Chand *et al*., 2005;

Table 1: Sources of fungal isolated and their filter paper activity (FPA)

Species	Sources	Strain	Filter paper unit (FPU) mL^{-1}	Sources	Strain	FPU mL^{-1}
Trichoderma spp.	Commercial	TH	0.048	STP Sludge	T07-10	0.007
	STP Sludge	TH(U)	0.155		T07-11	0.009
		T07-01	0.018		T07-12	0.024
		T07-02	0.010		T07-13	0.006
		T07-03	0.021		T07-14	0.010
		T07-04	0.013		T07-15	0.028
		T07-05	ND		T07-16	ND
		T07-06	0.009		T07-17	0.006
		T07-07	0.010		T07-18	0.002
		T07-08	ND		T07-19	0.015
		T07-09	0.010		T07-20	0.010
Aspergillus spp.	STP Sludge	STP-A1	ND	Rotten orange	O-102A	0.067
		S-105A	0.055		O-103A	0.041
		S-106A	ND		O-109A	0.045
	Rotten lemon	L-102A	ND		O-111A	0.052
		L-106A	ND		O-114A	0.040
Penicillium spp.	Rotten EFB	P1- EFB	0.074	STP Sludge	P2-STP	0.131
		P-K	0.045			

ND: The cellulase enzyme not detected; STP: Sewage treatment plant; EFB: Empty fruit bunches

Table 2: Cellulase enzyme (FPU mL^{-1}) of five potential strains according to the fermentation media

	TH(U)		S-105A		O-102A		P1-EFB		P2-STP	
Strain media	W/O	W	W/O	W	W/O	W	W/O	W	W/O	W
Sludge-wheat										
D2	0.084	0.145	0.027	0.054	0.055	0.060	0.082	0.067	0.073	0.117
D3	0.094	0.144	0.033	0.057	0.061	0.072	0.088	0.079	0.087	0.133
D4	0.099	0.154	0.066	0.084	0.082	0.089	0.066	0.087	0.097	0.141
Sludge-sugar										
D2	0.001	0.023	0.014	0.024	ND	ND	ND	ND	ND	ND
D3	0.007	0.040	0.017	0.028	0.003	0.008	0.005	0.013	0.015	ND
D4	0.015	0.054	0.039	0.070	0.064	0.016	0.080	0.036	0.073	0.047
Sludge-cellulose										
D2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
D3	ND	ND	ND	0.022	0.010	0.151	ND	ND	ND	0.080
D4	ND	0.041	0.021	0.026	0.053	0.211	ND	0.058	0.037	0.136
Sludge-cassava										
D2	0.020	0.025	ND	0.008	ND	0.008	0.023	0.026	0.022	0.029
D3	0.024	0.028	ND	0.018	0.010	0.028	0.022	0.034	0.025	0.033
D4	0.065	0.064	0.019	0.032	0.046	0.111	0.038	0.036	0.031	0.043

W/O: Media without supplement of EFB, W: Media with EFB, ND: No cellulase enzyme detected, D: Day

Niranjane *et al*., 2007). Niranjane *et al*. (2007) reported that no cellulase activity recorded in the presence of glucose and xylose in fermentation medium, while substrates such as CMC and microcrystalline cellulose (avicel) proved to be the strongest inducers of the cellulase enzyme by *Phlebia gigantea*. Low cellulase activity was obtained when glucose, corn-steep and low concentrations of soluble and insoluble cellulose were added to the fermentation media, suggesting some synergetic effect as the enzyme activity was lower than that obtained with the media using higher concentration of cellulose. Besides, from overall observation, the EFB can act as inducer for cellulase production for all strains in all media except in SS media, where cellulase activity recorded was higher in media without EFB supplement. EFB has cellulosic components that can induce the production of cellulase when used as carbon source for fungi growth (Umikalsom *et al*., 1997).

Comparison of *Trichoderma reesei* Rut C-30 and locally isolated *Aspergillus* spp. (strain O-102A): Based on the screening result, strain O-102A was selected among the locally isolated strain and cellulose was selected as the co-substrate in the media for further studies. The activities of the two cellulases (FPA and CMCase) were measured against the model substrates Whatman No. 1 filter paper and low viscosity carboxymethylcellulose (CMC) (Calbiochem). Total cellulase activity (FPA) and endoglucanase activity (CMCase) of O-102A and commercial strain *T. reesei* Rut C-30 were presented in Fig. 1 and 2, respectively. Obviously from the graph, the commercial strain *T. reesei* Rut C-30 gave highest total cellulase activity on the fifth day of fermentation (0.384 FPU mL^{-1}) before decreased to 0.275 and 0.260 FPU mL^{-1} on sixth and seventh day of fermentation. Whereas, cellulase enzyme produced by strain O-102A was around 0.186 FPU mL^{-1} on third day and increased up to 0.193 FPU mL^{-1} on day five and slightly constant

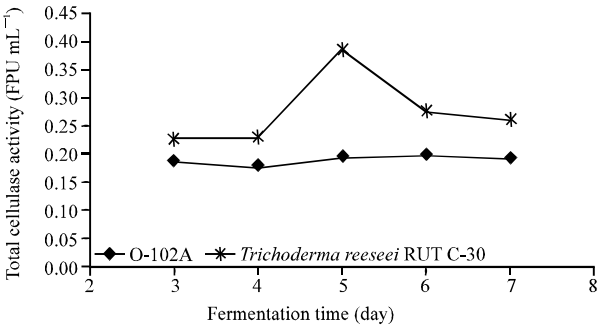

Fig. 1: Total cellulase enzyme production by commercial strain *Trichoderma reesei* Rut C-30 and locally isolated *Aspergillus* spp. O-102A using domestic sewage sludge as a substrate

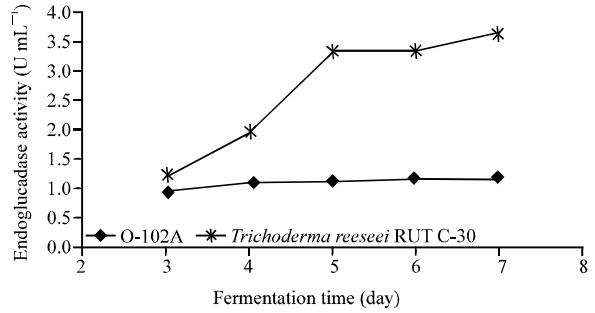

Fig. 2: Endo-β-1,4 glucanase activity of *Trichoderma reesei* Rut C-30 and *Aspergillus* spp. O-102A using domestic sewage sludge as a substrate

after that. The result in Fig. 1 shows that *T. reesei* Rut C-30 produced two times higher cellulase activity than strain O-102A.

Figure 2 shows that the highest CMCase enzyme detected for *T. reesei* Rut C-30 was 3.631 U mL^{-1} which is on the last day of fermentation (day 7). CMCase production on third day was 1.170 U mL^{-1} before suddenly increased to 3.318 U mL^{-1} on day five and slightly increased until seventh day of fermentation (maximum production). Meanwhile CMCase production for strain O-102A was between 1.004 to 1.158 U mL^{-1} from third to seventh day of fermentation which is three times lower than CMCase activity produced by *T. reesei* Rut C-30. Both graphs showed that strain *T. reesei* Rut C-30 was manage to generate maximum cellulase production (FPA and CMCase) on fifth day of fermentation. Similar trend of enzyme activity was observed in previous study, in which Olsson *et al.* (2003) have reported the highest cellulase activity on the fifth day of fermentation using *T. reesei* Rut C-30.

CONCLUSION

Out of 35 strains isolated from various sources and one lab stock strain tested, five strains were identified as promising fungi that producing cellulase enzyme through filter paper activity (FPA) assay. Five selected strains were TH(U), S-105A and P2-STP isolated from STP sludge, P1-EFB isolated from EFB compost and O-102A isolated from rotten orange. These five strains then were further screened for the best co-substrate and cellulase activity produced from O-102A was found to be higher in fermentation media contained cellulose powder as a co-substrate and EFB act as inducer to produce cellulase enzyme. Commercial strain *Trichoderma reesei* Rut C-30 (ATCC 56765) was proved to produced two times higher total cellulase activity and three times higher CMCase activity than locally isolated *Aspergillus* spp. O-102A. This study may show a route of potential and alternative solution for sludge management through bioconversion into value added product (cellulases).

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