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Isolation and Selection of Appropriate Cellulolytic Mixed Microbial Cultures for Cellulases Production from Oil Palm Empty Fruit Bunch

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Abstract: In order to construct cellulolytic fungal mixed cultures, screening and isolation of cellulolytic fungi was done using rotten oil palm fruit bunches as microorganism source. Three isolated fungi had shown the ability to degrade cellulose based on decolorization of CMC selective agar using Gram's iodine as color indicator. However, only two strains; KS1 and KS5 were selected for construction fungal mixed culture. Based on fungal interaction evaluation test done on PDA agar, both strains showed contact deadlock inhibition interaction with each other. In correlation to cellulase enzymes production, mixed cultures of strains KS1 and KS5 showed low enzymes activity compared to pure culture system. Although, the cellulase enzymes production is low, total cellulase enzymes composition was better than in pure culture system individually.

Key words: Consortia, oil palm empty fruit bunch, cellulase, submerged fermentation

INTRODUCTION

Cellulase enzymes complex is a multi-domain protein that consists of three major enzymes components which are endo-β-(1-4)-D-glucanase, exo-β-(1-4)-D-glucanase and β-glucosidase that works synergically in complex cellulose degradation (Duff *et al*., 1987). In cellulose degradation process, endo-β-(1-4)-D-glucanase or known as carboxymethyl cellulase act by randomly cleave β-(1-4) linkages of glucose chain in the amorphous region of cellulose to open up cellulose structure for subsequent attack from exo-β-(1-4)-D-glucanase (Esteghlalian *et al*., 2002). On the other hand, exo-β-(1-4)-D-glucanase (cellobiohydrolase) act to release cellobiose moiety from end of glucose chain. Finally, β-glucosidase releases glucose from cellobiose and short chain cellooligosaccharides (Krishna *et al*., 1998; Rajoka *et al*., 2004; Ikram-ul-Haq *et al*., 2005). For more than four decades, many researches have been done on cellulase enzymes either in screening and isolation of new strains, optimization processes involved or application of enzymes in industrially. Yet, by far, application cellulases industrially faced difficulties especially in total operational cost which mostly contributed from the enzymes itself compared to the raw material used. Even though various microorganisms have been reported to have the ability to produced cellulase enzymes extracellularly, most studies suggested fungi have better enzymes production compared to bacteria and yeast (Bakri *et al*., 2003). Most reported cellulase enzymes producer are from *Trichoderma* species and *Aspergillus* species (Bhat, 2000).

Trichoderma sp., was widely studied and used industrially especially in production of β-(1-4) exoglucanase and β-(1-4) endoglucanase. Compared to *Trichoderma* sp., *Aspergillus* sp., suffered low production of β-(1-4) exogulacanase and β-(1-4) endoglucanse but high in β-glucosidase enzymes (Madamwar and Patel, 1992). However, in order to obtain high degradation of cellulose material, synergistic effect of all three component of cellulase enzymes have to be achieved. Due to low production of β-glucosidase by *Trichoderma* sp., many approaches have been suggested to improve degradation of cellulosic material (Kovác *et al*., 2009). Many suggested mixed culturing between two strains and supplementation of β-glucosidase enzyme from *Aspergillus* improved total cellulase enzymes activity of *Trichoderma*. Mixed cultures is a cultivation system where two or more different microorganisms were introduce in the same fermentation condition or environment (Yang *et al*., 2003).

According to Correa *et al*. (1999), utilization of fungi mixed culture resulted in higher product yield and growth rate especially in poor nutritional agriculture residue

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and strengthens the protection of the culture against contamination. Due to unique ability of individual strains of microorganism in production of cellulase enzymes complex, it brings great interest in understanding of its microbial ecology with correlation to cellulase enzymes production. Hence, this study focus on the selection of appropriate mixed microbial culture and microbial ecology interaction in correlation to cellulase enzymes production using Oil Palm Empty Fruit Bunch (OPEFB) as substrate.

MATERIALS AND METHODS

The experiments were done from June 2008 until June 2009.

Screening and isolation of cellulolyic fungi: Sample of rotten oil palm empty fruit bunch was collected for screening and isolation of fungi-producing cellulases purposes. Serial dilution of sample were prepared in sterilized distilled water and 0.1 mL of diluted sample were spread on the surface of Potato Dextrose Agar (PDA) and incubated for 7 days at 30°C. Colonies with different morphological form were picked and sub-cultured to obtain pure culture. Stock cultures were maintained on PDA agar at 4°C for subsequent use as inoculum.

Screening of cellulase-producing fungi was done on CMC selective agar containing 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.2% carboxymethylcellulose (CMC) sodium salt, 0.02% peptone and 1.7% agar. Plates were spot plated with spores suspension of pure culture and incubated at 30°C. After 3 days incubation, plates were flooded with Gram's Iodine solution for 3 to 5 min according to Kasana *et al.* (2008) in order to observed and measured zone of clearance around the colony.

Fungi interaction evaluation: Fungal possible interaction was studied in nutrient-rich medium PDA agar. A loopful of each fungus strain spores suspension was cultured at 4.0 cm apart from each other in the same PDA agar to observe the consequences of different fungal interactions. Control were single cultured strains at one sided of equally divided PDA agar. Interacting fungi were incubated at 30°C for 7 days and observed at third and seventh day of incubation.

Production of cellulase enzymes: Locally isolated fungi of strain KS1 and KS5 were chosen for construction of fungal mixed culture. Each fungal culture were grown on PDA agar and incubated at 30°C for 7 day before harvested with sterile distilled water for subsequent use in inoculum preparation.

The basal medium described by Mandel and Weber (1969) was used in this experiment. Its composition was (g L^{-1}) KH_2PO_4 (2.0), $MgSO_4.7H_2O$ (0.3), $CaCl.2H_2O$ (0.3), $CoCl_2$ (2.0), $MnSO_4.H_2O$ (1.6), $ZnSO_4.H_20$ (5.0), 2 ml L^{-1} Tween 80 and 1 ml L^{-1} trace element. The OPEFB obtained from mill was first ground to fibre with average length of 10 mm. It was delignified by soaking in 2% (w/v) NaOH for 4 h followed by autoclaving at 121°C for 5 min according to Umikalsom *et al.* (1997). The pretreated OPEFB was filtered, washed with distilled water until no traces of alkaline could be detected and dried in an oven at 95°C for 2 day.

In all fermentation, 100 mL medium (pH 5.0) was dispensed in 250 mL shake flask and inoculated with fungi spores suspension containing 1×10^6 spores mL^{-1}. The flasks were incubated at 30°C with agitation speed of 150 rpm on rotary orbital shaker. Each experiment was performed in duplicates. Samples were withdrawn at regular time interval for analysis.

Analytical methods: The cellulase enzymes activity was measured according to Wood and Bhat (1988) and Ariffin *et al.* (2006). One unit activity of CMCase and FPase was defined as 1 μmol reducing sugar released mL^{-1} enzyme/min. Meanwhile, for one unit activity of β-glucosidase was defined as 1 μmol of p-nitrophenol released/mL enzyme/min.

All experiments were duplicates and repeated twice for confirmation.

Samples withdrawn from fermentation broth were assayed for cellulase enzymes activity. Cellulase enzymes activity assay was done in order to determine the cellulase enzyme production for both pure and mixed cultures of locally isolated fungi.

RESULTS

Screening and isolation of celluloytic fungi: During screening process, 7 fungi were obtained from rotten palm oil fruit bunch. Only four fungi were selected for isolation of cellulase producer based on they rapid growth on PDA agar within seven days of incubation. All four selected fungi were subjected to CMC agar (selective agar) for isolation of cellulase producer. Growth of each fungus was observed after 3 days of incubation before each inoculated agar were flooded with Gram's Iodine solution for decolorization zone observation and measurement. From Fig. 1, only three fungi showed decolorization zone around mycelial growth on CMC agar. Decolorization zone made by fungi showed secretion of cellulase enzymes by fungi in order to degrade cellulose structure of CMC. Fungus strain SK3 showed the largest decolorization zones as compared to

Fig. 1: Fungi strain KS1, KS3 and KS5 showed degradation of CMC agar with appearance of decolorization of Gram's Iodine

Table 1: Diameter zone of clearance produced by four isolates on CMC agar stained with Gram's Iodine after 3 day incubation

Isolates	Zone of clearance diameter (cm)
SK1	4.1
SK3	4.5
SK4	Nil
SK5	2.4

strain SK1 and SK5 (Table 1). However, due to its non sporulated fungi morphological form, strain SK1 and SK5 were selected for the construction fungi cellulase mixed cultures.

Fungi Interaction on PDA agar: Visible fungi interaction evaluation was tested on both SK1 and SK5 fungi strain on PDA agar within seven days of incubation according to Iluyemi and Hanafi (2009). For mixed culture interaction observation, PDA agar was divided into equal area and each strain of fungi was inoculated 4 cm apart on PDA agar. As for control, each strain were inoculated on one sided of equally divided agar. After 3 days of incubation, both fungi grow well in the PDA agar yet did not perform any direct contact to each growing mycelium as shown in Fig. 2. Meanwhile, in PDA agar that contains only pure culture strain showed initial growth of mycelium especially for strain KS1. Strain KS1 grown as greenish white mycelium initially after 3 days incubation. Contradictly, growth of KS5 greenish mycelium spread unevenly due to it morphological form that is highly sporulated than KS1 strain. After 7 days incubation, full growth of mycelium from both pure cultures can be observed. In mixed cultures agar, there was deadlock interaction at touching point between two strains of fungus. However, strain KS1 mycelium showed early stage of invasion toward strain KS5 mycelium according to Molla *et al.* (2001). Mixed culture agar also showed slight inhibition where visible demarcation line 1-2 mm between two strain fungi can be observed.

Cellulase enzymes production by single and mixed cultures: Production of cellulase enzymes by single and mixed cultures were carried out in shake flask fermentation using Oil Palm Empty Fruit Bunch (OPEFB) as substrate. Figure 3a-c show the production of cellulase enzymes by single and mixed cultures. Based on the results obtained, single cultures of strain KS1 and KS5 gave highest activities activity in certain component of cellulase enzymes. No mutual synergism was observed between strain KS1 and KS5 in mixed cultures fermentation. However, according to maximum enzymes production shown in Table 2 indicated that even though the production of respective enzymes was low in mixed cultures fermentation compared to single culture fermentation, improved total enzymes composition can be observed in mixed cultures.

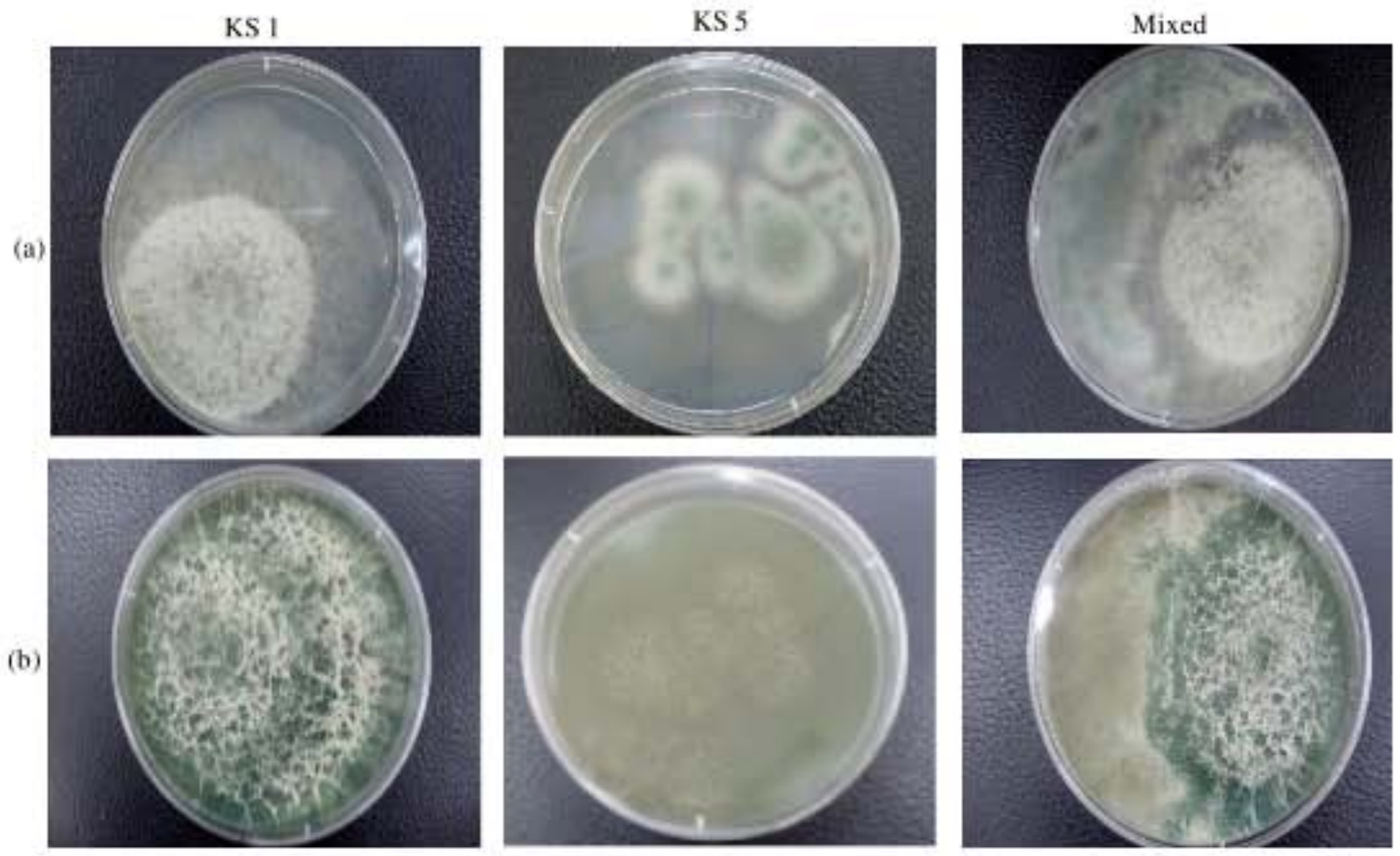

Fig. 2: Observation of fungi single culture (KS1 and KS 5) and mixed culture fungi (KS1 and KS 5) during 7 day cultivation on PDA agar. (a) 3rd day of observation and (b) 7th day of observation

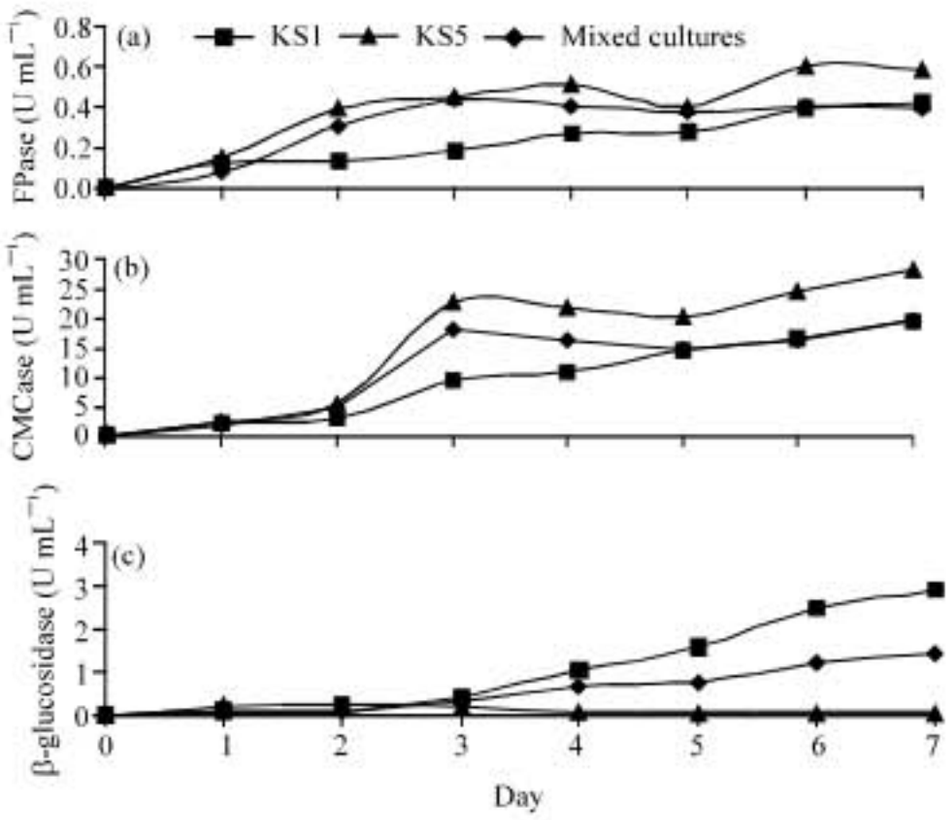

Fig. 3: Cellulase enzymes profiling by strain KS1, KS 5 and mixed cultures

Table 2: Cellulase enzymes production by locally isolated fungi

	Cellulase enzymes activity (U mL^{-1})		
Fungi	FPase	CMCase	β-glucosidase
SK1	0.42	19.61	2.90
SK5	0.60	28.19	0.26
Mixed cultures	0.44	19.68	1.43

DISCUSSION

Production of cellulase enzymes can be done either as pure culture fermentation or mixed culture fermentation. However, lack of deep understanding especially in fungi interaction during mixed culture hindered the advantages of utilization mixed culture for production of industrially important enzymes. Based on results obtained from the experiments, 2 potential cellulase producer fungi had been selected for the construction of appropriate cellulolytic mixed microbial for cellulase enzymes production (Fig. 1). Visible interaction evaluation between opposition colonies revealed that KS1 and KS5 fungi strain possessed a deadlock with mutual inhibition interaction between each other (Fig. 2). The appearance of demarcation line of 1-2 mm according to Stahl and Christensen (1992) confirmed the deadlock inhibition interaction between two strains. Many researches also found that deadlock interaction is the most common fungi interaction when grown on nutrient-rich medium (Iluyemi and Hanafi, 2009; Stahl and Christensen, 1992; Myvan and Shearer, 1988). Rapid growth rate of strain KS1 may caused invasion of mycelium to strain KS5 territory after seven day incubation. As for correlation of cellulase enzymes secretion to fungal interaction, the production of cellulase enzymes was done in submerged fermentation for both culture systems (Fig. 3). In mixed cultures system, low activity of cellulase enzymes especially for FPase and CMCase can be observed compared to strain KS5 pure culture fermentation. In comparison to strain KS1, mixed culture resulted higher FPase and CMCase activities up to 0.44 and 19.68 U mL^{-1}, respectively. Similar results also reported by Castillo *et al.* (1994), where mixed culture of *T. reesei* and *A. niger* produced 4 FPA/g substrates compared to single culture

of *T. reesei* 5 FPA/g substrates. However, if comparison were drawn between both fungi strains, KS1 strain showed better β-glucosidase composition compared to KS5 strain which 0.26 U mL^{-1}. In contrast, KS5 strain produced higher activity of FPase and CMCase enzymes, 0.6 and 28.19 U mL^{-1}, respectively. Deadlock inhibition interaction observed in mixed culture of strains KS1 and KS5 on PDA agar might explain low cellulase enzymes activities obtained in mixed culture system compared to pure culture system. Other than that, production of protease from either fungus strains as defensive mechanism resulted in low enzyme activity. Competition for available carbon resource among the strains may also contribute to low cellulase enzymes activity in mixed culture system. Even though mixed culture of both strain suffered low enzymes activity, in term of total enzymes composition it showed better enzymes composition compared to single strain cellulase enzymes production same as agreed by Castillo *et al.* (1994) and Yusoff *et al.* (2000). It appeared that the results showed no synergistic effect between two fungi and doesn't support for cellulase enzymes production using fungi mixed culture system using both isolated fungi strains. However, further investigation on saccharification of cellulosic materials using cellulase enzymes produced by both pure and mixed cultures system need to be done in order to verify the effectiveness of using fungi mixed cultures for the production of cellulase enzymes.

CONCLUSION

Fungal interaction during mixed culture is essential for construction of fungal mixed culture for the production of cellulase enzymes. Synergistic interaction between each fungus cultures need to be achieved in order to obtain higher enzymes production. The need for investigation especially in saccharification of cellulosic material using crude cellulase enzymes might further explain effectiveness of fungal mixed culturing for cellulase enzymes production.

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