Cellulolytic Yeast Isolated From Raja Ampat Indonesia

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Abstract

The objective of this study was to select and characterize three yeast isolates originating from soil of Raja Ampat region of Papua, Indonesia for itspotential to produce cellulase . Selection and characterization of cellulolytic yeast was carried out by measuring cellulolytic Index (IS)with congo red method and measurement of Carboxy Methyl Cellulase (CMC-ase) activity through determination of reducing sugar with dinitrosalycilic methods. Cellulolytic Index (IS) of the isolates *Sporobolomyces poonsookiae* Y08RA07, *Rhodosporidium paludigenum* Y08RA29 and *Cryptococcus flavescens* Y08RA33were 1.40, 2.60 and 1.66 respectively. CMC-ase produced optimum at pH 8 at 37ºC by isolate Y08RA07, whereas for Y08RA29 andY08RA33 were at pH 6, at 28ºC. Paper waste was good substrate for cellulase enzyme production by isolate Y08RA07, while for two other isolates the best substrate was CMC. Isolate Y08RA29 having highest cellulase activities when grown in CMC, while isolates Y08RA07 and Y08RA33 achieved highest enzyme activity when grown in bamboo leaf.

Key words: Cellulolytic yeast, Raja Ampat, waste paper, bamboo leaf

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Introduction

The energy and food crises encourage exploration of untapped potential of tropical microbial resources. Enormous amount of agricultural and forestry waste generated annually. Those are believed to be good sources as raw materials for cellulosic and lignocelluloses based biofuel industry.The Research Center for Biology of the Indonesian Institute of Sciences play vital role in exploration of Indonesian biodiversity, including microorganism manifested by regular explorations to many parts of the Indonesian archipelago. The biodiversity exploration program in 2007 found several important yeasts from soil and litter of Raja Ampat Papua, Indonesia. To date not many intensive works have been focused on the exploration of cellulolytic yeast, and harness their physiological potential (Jimenez *et al.*, 1991).

Yeasts are ecologicallydistributed from marine, terrestrial and fresh water ecosystem (Fell *et al.*, 2000). They have been isolated from numerous biotopes including leaf surface, root, litter, flower, gill of marine fish, and event from dessert soil. The occurance of yeast in diverse biotopes, indicating its strong physiological characters including its roles in deteriorating wood and litter. Exploration of cellulolytic yeast may have triple objectives: to verify yeast diversity, to obtaincelulolytic yeast, and to find potential strain (s) having prominent cellulolytic power for industrial used.

The standard protocol in assesing cellulolytic capacity of yeast is through determining enzymatic properties of extracellular enzymes produced during aerobic growth. Cellulases responsible in hydrolysing cellulose are mainly three types: (1) endoglucanases (EC. 3.2.1.4), (2) cellobiohydrolases (EC. 3.2.1.91) and β-glucosidases (EC.3.2.1.21). The endoglucanases (EG)1 are able to hydrolyze soluble, substituted celluloses such as carboxymethyl cellulose (CMC) by attacking the carbohydrate chain (1-4,β glucosidic bond) internally and randomly but are less active against highly ordered crystalline cellulose such as cotton fibers or Avicel.

Cellobiohydrolases (CBH) are ineffective towards substituted celluloses but are able to degrade crystalline cellulose and produce primarily cellobiose. β-Glucosidases hydrolyze cellobiose and other short cello-oligosaccharides produced by the other enzyme to glucose. With the recent development of biotechnology, there has been vast interest to use cellulose digestive microorganisms to convert cellulosic biomass to glucose that can be used in different applications such as production of fuel ethanol, use in animal feed, use in waste water treatment and in brewing industry.

Although there have been many papers dealing with more efficient cellulose degrading enzyme from various organisms such as *Trichoderma reesei, Trichoderma viride, Trichoderma lignorum*(Fahrurrozi *et al*., 2010), *Chrysosporium lignorum*, *Chrysosporium pruinosum* and *Fusarium solani*, only limited research has identified the yeast as cellulase producer. The cost of production and low yields of this enzyme are the major problems for industrial application. Therefore, investigations on ability of microbial strains to utilize inexpensive substrate and improvement of enzyme productivity have been done. In the present study, we reported isolation and identification of 3 cellulolytic yeasts from Raja Ampat and to characterize their physiological properties. The ability of those strains in utilizing various carbon sources includes CMC, waste paper, and bamboo leaf were also discussed.

Materials and Methods

Sampling of soil. Multiple soil cores (15-cm depth by 2-cm diameter) were collected and mixed in a plastic bags from Batanta Island Raja Ampat, West Papua Province, Indonesia. At the same places litter were collected and put in paper envelope.

Isolation Methods of Yeasts and Yeast-like Fungi. Yeasts were aseptically isolated from the soil and litter using Rose Bengal Chloramphenicol Agar (RBCA), which prevents growth of bacteria and controls the spread of molds. As 1.0 g leaves-litter wasweighed-in and cut into pieces. The leaf-litter were put into 30 ml sterile distilled water and vortex for 5 min. The soil andlitter were filtered using filter membrane *Millipore* 0.45 µm, and the membrane was put onto the RBCA medium. The soil and litter suspensions were filtered again using filter membrane *Millipore* 0.45 µm, and the membrane was put onto the RBCA medium. Incubation of the plates is done for 5 days at room temperature. As 0.1 g soil is weighed-in. We have successfully used this medium extensively in recent microbial surveys. Plates were incubated aerobically at an appropriate temperature, approximating that of the habitat of origin. One or two representative colony from the primary isolation plates were selected and purified by streaking for single colonies twice on rich media such as Potato Dextrose Agar (PDA) or YM agar.

Morphological and physiological characters. The analyses were done following Nakase, 2000.

DNA Preparation. The cells were harvested during the logarithmic phase of growth, cultivated in YM broth (containing 3 g of yeasts extract, 3 g of malt extract, 5 g of polypeptone and 10 g of glucose per 1,000 mL of distilled water). The cultures are harvested by centrifugation. The DNA wasextracted and purified by using the DNeasy Plant Tissue Kit (QIAGEN).

Amplification of D1/D2 of LSU rDNA and ITS regions by PCR. The D1/D2 region of nuclear large subunit ribosomal DNA and the ITS regions are amplified and sequenced using the primer sets, NL1 (GCATATCAATAAGCGGAGGAAAAG) and NL4 (GGTCCGTGTTCAAGACGG), and ITS4 (TCCTCCGCTTATTGATATGC) and ITS5 (GGAAGTAAAAGTCGTAACAAGG), respectively.

Sequencing of D1/D2 of LSU rDNA and ITS regions. The nucleotide sequences are determined with Big Dye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems) following the manufacturer's instructions. The gel electrophoresis and data collection are performed on ABI Prism Genetic Analyzer (Applied Biosystems). The sequence of D1/D2 region of LSU rDNA and ITS regions of the strains are aligned with other LSU rDNA

sequences on the basis of similarity of the sequences.

Phylogenetic Analysis. The data collections are performed on an Applied Biosystems automated DNA sequencer. Sequences are aligned using CLUSTAL X (Thompson *et al*, 1994) and were adjusted manually. The gaps are not included in our phylogenetic analyses. The distance matrix for the aligned sequences was calculated using the two-parameter method of Kimura (1980). The neighbor-joining (NJ) method (Saitou and Nei, 1987) was used to construct all phylogenetic trees. The robustness for individual branches was estimated by bootstrapping (Felsenstein, 1985) with 1,000 resamplings.

Production of CMCase. Cellulolytic yeast was grown in YM-CMC broth medium (3 g L^{-1} Yeast extract, 5 g L^{-1} peptone and 10 g L^{-1} CMC, pH 6.2 ± 0.2) at 25°C for 48 h as a pre-culture. The culture medium was inoculated with 2% of the pre-culture and incubated at 25°C with shaking at 120 rpm for 72 h. To assess the ability of culture to hydrolyze cellulose from agriculture waste, 2 % grinded printed paper, straw and bamboo leaf were used as carbon sources to medium containing 3 g L^{-1} Yeast extract, 5 g L^{-1} peptone, pH 6.2 ± 0.2 and grown at rotary shaker (125 rpm) at 25 ºC (Kang *et al*, 2004).

Determination of total Yeast biomass. During cultivation, two samples of the broth from each cultivation flask were centrifuged (5min, 4000 rpm) and the supernatant discarded. The pellet was washed three times with distilled water in order to remove loosely associated substances from yeast cell surface. Washed yeast biomass was dried at 105°C to constant mass.

Enzyme assay. CMCase activity was determined by measuring the amount of glucose released from carboxymethyl cellulose sodium salt (CMC-Na salt) by the Dinitrosalicylic method with glucose as the standard (Haggett *et al*, 1979). Reaction mixtures contained 0.45 ml of 1% CMC-Na salt in pH 4-8 and 0.45 ml of bulk each enzyme fraction from the culture grown in CMC, filter paper, paddy straw and bamboo leaf. Distilled water was used as control. After incubation at 28-37°C for 1 hour, the reaction was terminated by adding 1.0 ml of DNS reagent. The mixture was vortex, placed in a boiling-water bath for 10 min, and cooled to room temperature.The mixture was centrifuged to remove any precipitate, and the absorbance of the supernatant was measured at 540 nm. One international unit (IU) of enzyme activity was defined as the amount of enzyme producing 1 µmol of reducing sugars in glucose equivalents per min.

Results and Discussion

Isolation and Identification of Cellulolytic Yeast from Raja Ampat.

About 50 colonies were obtained from litter and soil samples. Based on the morphological characteristics, 10 isolates were selected for cellulolytic test (Table 1). Out of 10 strains selected 6 strains were able to form clear zone surrounding growing colony. The formation of clear zone is due to congo red cannot bind to the reducing sugar form during the hydrolyses of CMC, while orange color form CMC hydrolyzes.

Three isolates (*Sporobolomyces poonsookiae* Y08RA07, *Rhodosporidium paludigenum* Y08RA29 and *Cryptococcus flavescens* Y08RA33) that produced highest clear zone, and have highest cellulolytic-index were selected for further study. The morphological character of growing colonies grown in PDA shown in Figure 1.

S. poonsookiae was reported by Takashima & Nakase (2000), having reddish orange and shiny to semi-shiny colonies. This species produce pseudohyphae and true hypae during they grow on corn meal agar. Ballistoconidia are allantoids to ellipsoidal. *R. paludigenum* was reported by Fell & Statzell-Tallman (1980). Morphological character indicated by polar budding, colony surface smooth and has an entire margin, having salmon – orange colour. This physiological character of this species was indicated by their ability to assimilate D-glucose, sucrose, raffinose, galactose, trehalose and maltose. They did not ferment D-glucose, D-galactose and maltose. *C. flavescens* glucose fermentation was negative and they assimilate D-glucose, D galactose, D-xylose but they could not assimilate L-sorbose. *C. flavescens* is common soil yeast, some members of this genus has cellulolytic capacity. Phylogenetic

affiliation of those cellulolytic yeasts isolated from Raja Ampat is shown in Figure 2.

The phylogeny of these isolates (*S. poonsookiae* Y08RA07, *R. paludigenum* Y08RA 29 and *C. flavescens* Y08RA33) were distributed at different cluster, implying that cellulolytic yeast distributed in wide taxa.

Tabel 1. Celulolytic Indexs of Yeast

Isolate	Species Identification by sequencing of D1/D2 of LSU rDNA and ITS regions	Mean Diameter of Clear zone (mm)	Mean diameter of colony (mm)	Cellulolytic Indexs
Y08RA04	*Barnettozyma californica*	3.0	2.5	1.20
Y08RA07	*Sporobolomyces poonsookiae*	7.0	5.0	1.40
Y08RA13	*Cryptococcus bestiolae*	4.0	3.0	1.33
Y08RA15	*Wickerhamomyces sp*	-	2.0	No clear zone formed
Y08RA23	*Candida sonorensis*	6.0	5.0	1.2
Y08RA24	*Rhodosporidium paludigenum*	-	1.8	No clear zone formed
Y08RA26	*Cryptococcus flavescens*	-	2.1	No clear zone formed
Y08RA29	*Rhodosporidium paludigenum*	13.0	5.0	2.0
Y08RA31	*Candida intermedia*	-	2.1	No clear zone formed
Y08RA33	*Cryptococcus flavescens*	8.3	5.0	1.66

Figure 1. Morphological characters of growing colonies in PDA media after 5 days incubation

Confirmation of cellulolytic capacity of isolated yeast

To have better understanding on hydrolyses rate of CMC by the tested isolates then each strain was grown in media with CMC as the main carbon sources.The ability of 10 isolates to hydrolyze CMC is shown by Figure 3.

The CMC-ase activities changed as time of cultivation, but the Figure 3 clearly shows that *S. poonsookiae* Y08RA07, *R. paludigenum* Y08RA29 and *C. flavescens* Y08RA33 have highest CMC-ase activity. Exocellulase or endocellulace activities are not commonly observed in yeast (Federici, 1983), however xylan-degrading activities are more widespread in yeast (Jimenez *et al*., 1991).They observed *Aurebasidium microstictum* and *Rhodotorula grinbergsii* showed the strongest growth on CMC.The highest endocellulase was found in *Trichosporon pullulans*, endocellulase was also found in *Aurebasidium microstictum* (Jimenez *et al*., 1991).

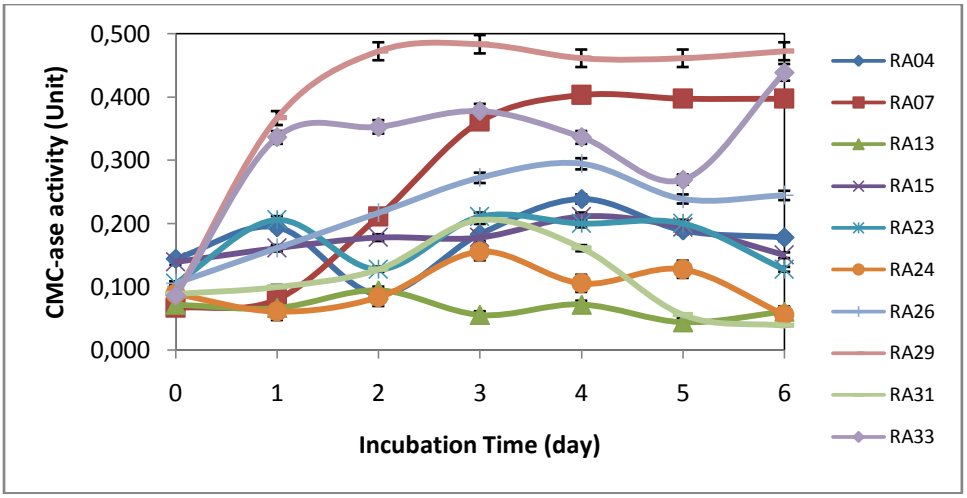

Figure 3. Profile of CMC-ase activity of tested isolated grown for 7 days in media with CMC was the sole carbon sources.

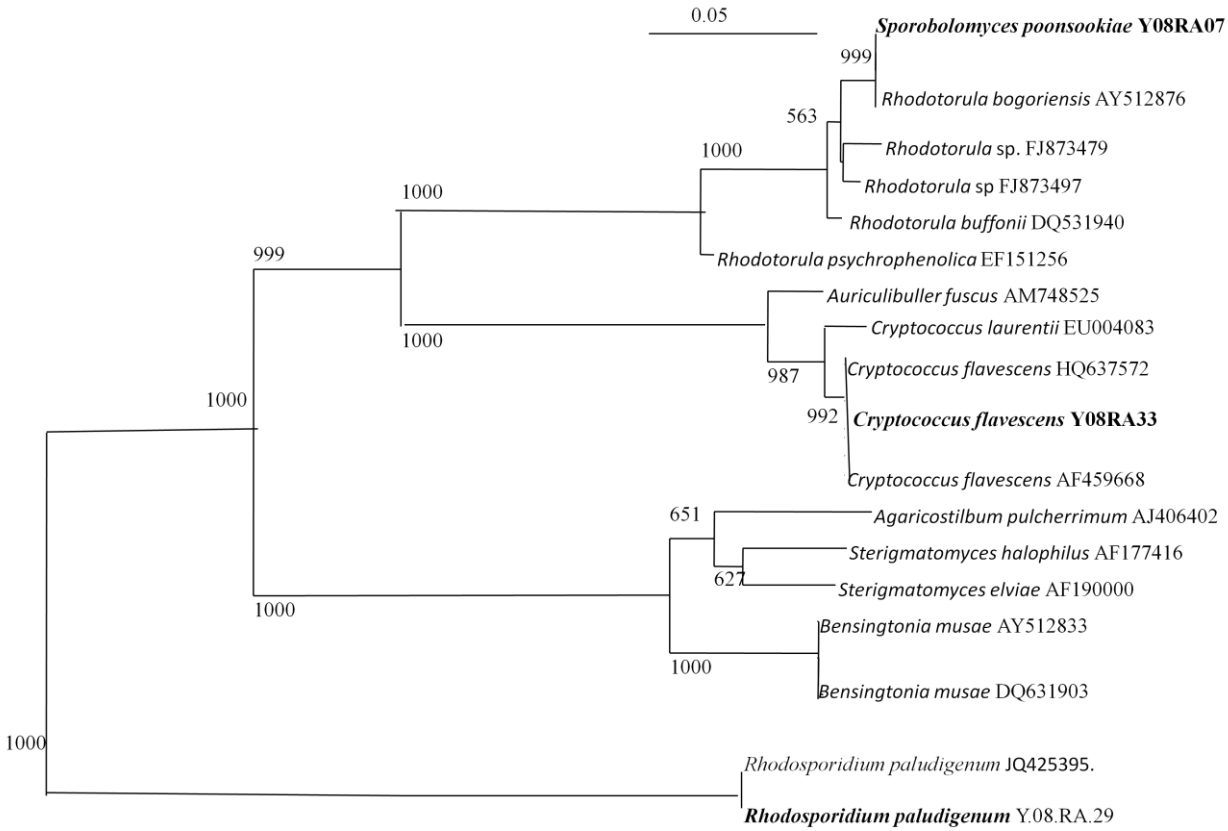

Figure 2. The phylogenetic placement of cellulolytic yeast *Sporobolomyces poonsookiae* Y08RA07, *Rhodosporidium paludigenum* Y08RA 29 and *Cryptococcus flavescens* Y08RA33 based on D1/D2 of LSU rDNA sequences.

Effect of temperature and pH on CMC-ase activity.

Effect of temperature and pH on CMC-ase activity by CMC-ase profile of *S. poonsookiae* Y08RA07 was shown in Figure 4A. Maximum CMC-ase activity of the strain was achieved at pH 8, incubated in 37°C after 4 days cultivation.

Strain *R. paludigenum* Y08RA29 appear to have different CMC-ase activity with other strains. CMC-ase activity of this strain was higher than that of *S. poonsookiae* Y08RA07, maximum enzyme activities was achieved after 7 days incubated at 28°C under pH 7. The CMC-ase activity at 28°C and pH 6 of *R.*

paludigenum Y08RA29 was higher than that of at 37°C and pH 8.

While *C. flavescens* Y08RA33 appeared to perform little different CMC-ase activity profile. Highest CMC-ase activity was achieved after 7 days cultivation at 28°C and pH 6, at. Figure 4 suggest that cellulolytic activity was affected by pH, temperature and species dependent. Those physiological characters are good discriminator for taxonomy studies.

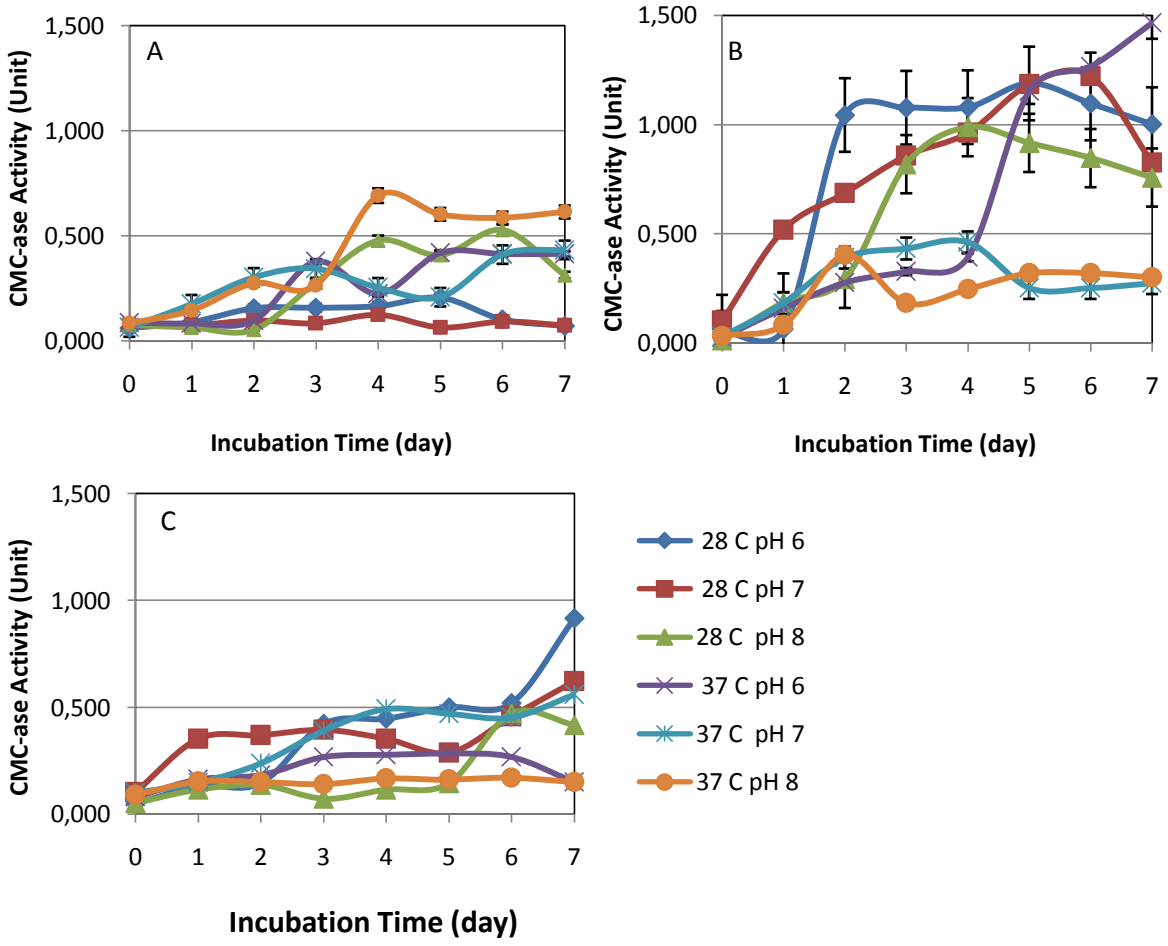

Figure 4. Profile of CMC-ase activity of *Sporobolomyces poonsookiae* Y08RA07 (A), *Rhodosporidium paludigenum* Y08RA29 (B), and *Cryptococcus flavescens* Y08RA33 (C) as affected by temperature and pH

CMC-ase activities at various substrates

It is important to obtain yeast that can grow at various lignocelluloses containing material. We were very keen to see whether the three cellulolytic yeast (*S. poonsookiae* Y08RA07, *R. paludigenum* Y08RA29 and *C. flavescens* Y08RA33) were able to hydrolyze cellulose of waste paper, paddy strawand bamboo leaf, using CMC as a positive control. The results are presented in Figure 5. Figure 5A clearly shows that strain Y08RA07 was able to hydrolyze all substrates, the CMC-ase activities however highest when the strain grown in autoclaved bamboo leaf. Much reducing sugar formed when bamboo leaf was used as the main carbon sources. Paddy straw appeared bit difficult to be hydrolyzed by Y08RA07. The highest enzyme activitywas achieved after 3 day incubation in bamboo leaf medium.

Similarly to *S. poonsookiae* Y08RA07, hydrolyses activity of *C. flavescens* was much higher in bamboo leaf medium (Figure 5C).

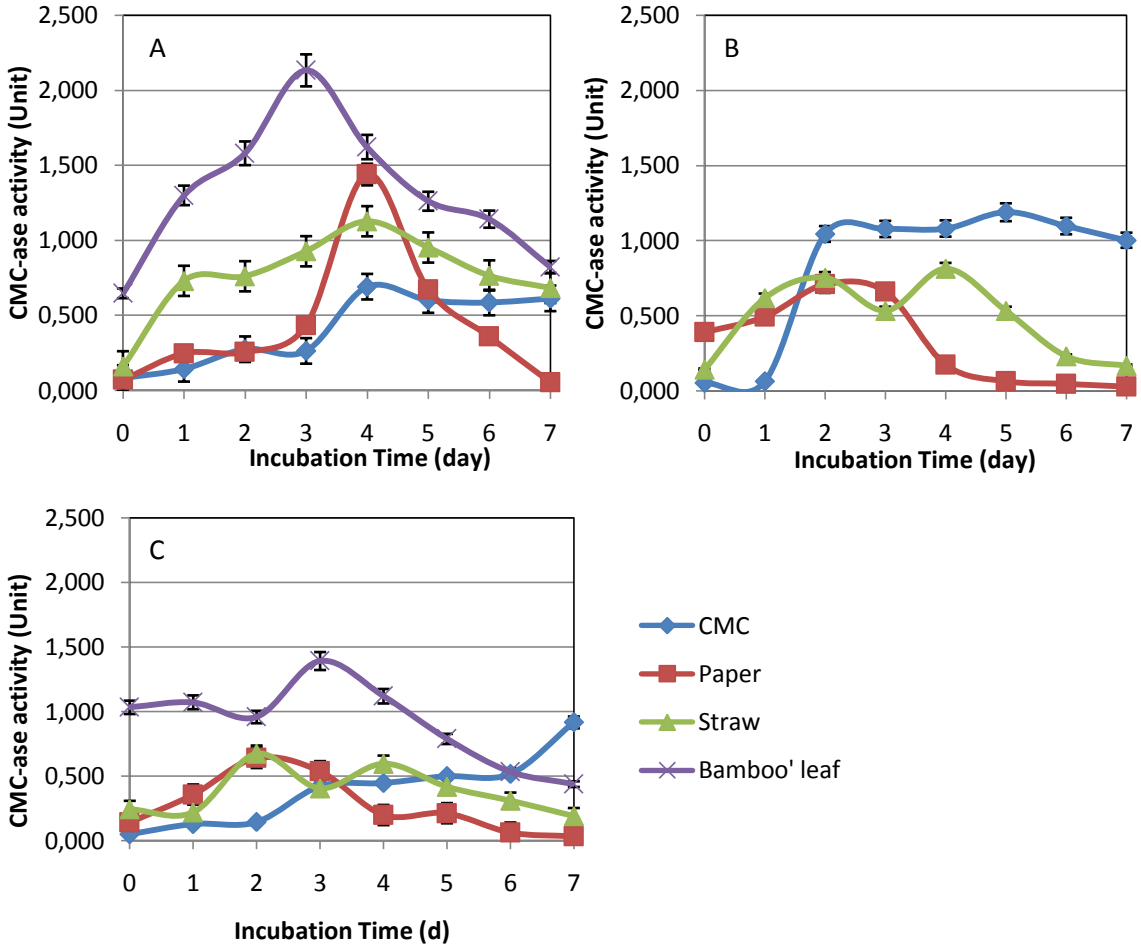

Figure 5. Cellulase activity of *Sporobolomyces poonsookiae* Y08RA07 (A), *Rhodosporidium paludigenum* Y08RA29 (B), and *Cryptococcus flavescens* Y08RA33 (C) grown in bamboo leaf, paper waste, and paddy straw

Conclusion

S. poonsookiae Y08RA07, *R. paludigenum* Y08RA29 and *C. flavescens* Y08RA33 are cellulolytic yeast of Raja Ampat. Physiological characters of those strains in hydrolyzing cellulose were pH, temperature and strain dependent. These strains were able to hydrolyze cellulose of waste paper, bamboo leaf and paddy straw implying that those strains can be exploited for bioconversion of agricultural waste for biofuel production.

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