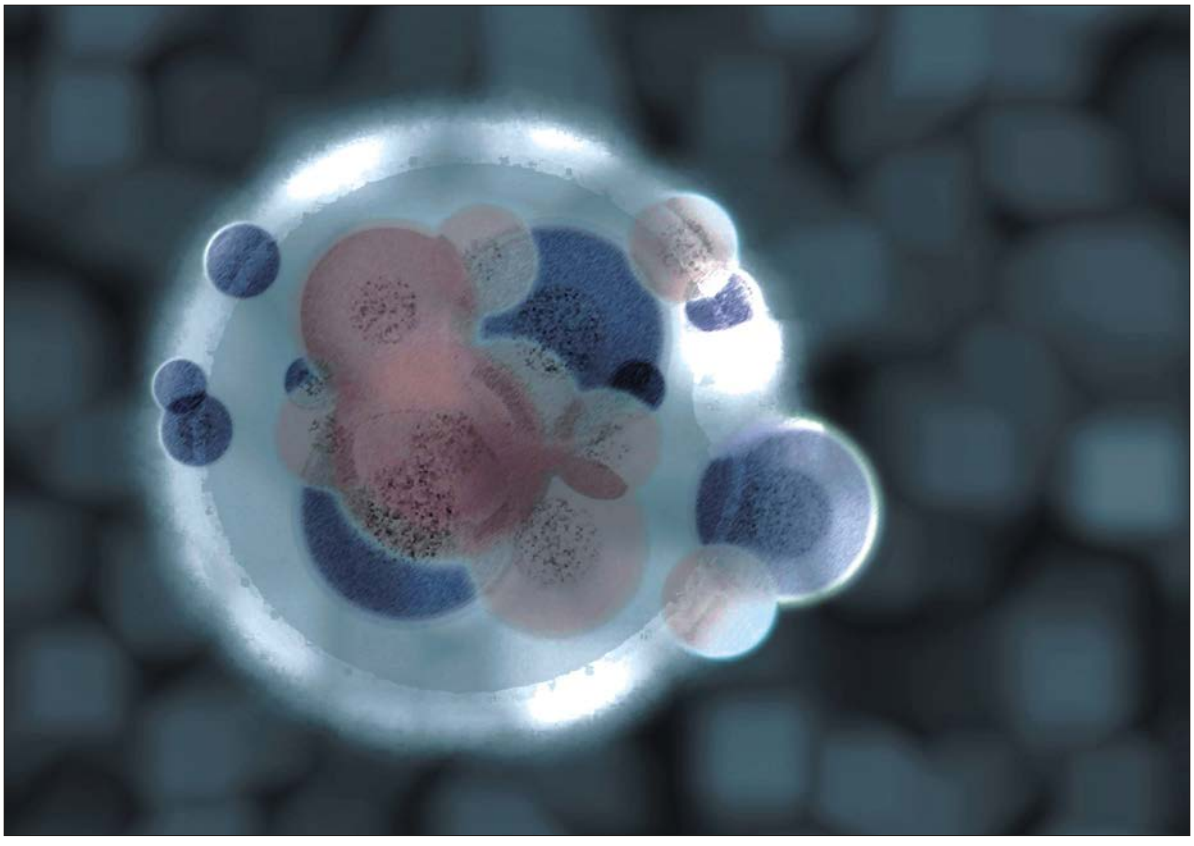

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Cellulolytic Fruits Wastes: A Potential Support for Enzyme Assisted Protein Production

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Abstract: White rot fungus are valuable class of filamentous and spore forming strains capable of use as animal feed supplements when cultivated under submerged state bioconversion. Selected bacidiomycetes; *Phanerochaete chrysosporium, Panus tigrinus* M609RQY (M6) and RO2 were grown solely on liquid and solid substrates of banana peel, pineapple peel and papaya peel. On banana peel (solid and liquid), RO_2 has the highest protein (15.88 and 14.08 mg g^{-1}), followed by *P. chrysosporium* (15.09 and 13.56 mg g^{-1}) and M6 (13.24 and 8.07 mg g^{-1}). On pineapple peel, RO_2 protein is 17.71 and 12.79 mg g^{-1}, M6 12.61 and 12.32 mg g^{-1}. *P. chrysosporium* produced 12.27 and 6.85 mg g^{-1} protein. RO_2 produced 15.64 and 12.94 mg g^{-1} protein on Papaya peel, M6 produced 9.23 and 10.09 mg g^{-1} while *P. chrysosporium* synthesized 8.16 and 10.21 mg g^{-1}. *P. chrysosporium*, M6 and RO_2 produced good α-amylase and cellulase enzyme activities that assisted in substrate degradation for protein synthesis.

Key words: *Phanerochaete crysosporium, Panus tigrinus*, animal feed, filamentous, bioconversion

INTRODUCTION

Surging prices of animal feed supplements is one of the challenges facing the livestock industries all over the world. Incidentally, solid unwanted agricultural materials resulting from postharvest activities of farmers and food processors are also growing at a faster rate due to improved farming methods and high fruit and vegetable survival (Jamal *et al*., 2012). Therefore, fruit and vegetables residues remained source of solid agricultural waste. A large quantity of the waste include peels, seeds and pulps depending on the type of fruits (Correia *et al*., 2007). Banana waste (peel), pineapple waste and papaya waste are examples of agricultural wastes found abundantly in several tropical and sub-tropical areas such as India subcontinent and Southeast Asian countries (Roslina, 2008).

White rot fungi constitute a class of filamentous and spore forming molds capable of metabolizing the complex carbon materials found in fruit wastes that contain reducing and non-reducing sugars; others may contain complex sugars such as cellulose, hemicellulose and lignin. Several filamentous fungi are capable of secreting lignocellulotic extracellular enzymes which are used to breakdown the glycosidic bonds of complex sugar molecules (Ramanathan *et al*., 2010). Among these organisms, *P. chrysosporium* is a prominent member well researched for its production of extracellular enzymes that can breakdown lignin and other celluloses with its peroxidase enzymes (Nazareth and Sampy, 2003). Similarly, *P. chrysosporium* is among important bacidiomycete whose genes have been expressed (Singh and Chen, 2008). M6 and RO_2 on the other hand are bacidiomycete belonging to *Lentinus* or *Panus tigrinus* which are edible mushrooms capable of growing on lignocellulolytic agricultural residues to form non-toxic products (Kadimaliev *et al*., 2004). They as well produced laccases, cellulases and certain peroxidases which helps themto breakdown complex lignolytic residues through similar process like *P. chrysosporium* (Tijani *et al*., 2011).

Moreover, *P. chrysosporium* has been investigated deeply at both solid and submerged state bioconversion (SmB) with promising results recorded in many of such bioconversion. In literature, M6 and RO_2 have been grown under Solid State Bioconversion (SSB) but these edible fungi have not been deeply investigated under SmB for their biomass and bio-protein synthesis potential. Therefore, elucidating their performance under SmB will be a good step further in understanding their biochemical reaction pattern leading to product formation through effective breakdown of lignocellulosic materials.

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Bacidiomycetes and other group of edible fungi are microbial strains capable of producing essential amino-acids-rich biomass which could improve nutritional status of non-ruminant animals (Saheed *et al*., 2012). Apart from amino acid content, many of these white rot fungi produce essential vitamins and minerals in the biomass thereby making them a viable substitute for synthetic feed supplement (Kadimaliev *et al*., 2004).

The main objective of this research is to look critically into the possible utilization of three abundant fruit wastes (banana peel, pineapple peel and papaya peel) which are usually generated by food processing industries, hotels and cottage restaurants for sustainable production of value added products. Similarly the paper seeks to investigate the potentiality of high profile filamentous fungi in effective management of solid agricultural waste which could improve revenue for farmers and food processors. To the best of our knowledge, this is the first time M6 and RO_2 will be cultivated under SmB with the three substrates under investigation.

MATERIALS AND METHODS

Substrate collection, preparation for analysis and bioconversion: Banana peel (BP), Pineapple Peel (PP) and papaya peel (Pw) were collected from fruit processors at Gombak area in Kuala Lumpur. The peels were washed, drained and blended with distilled water at 1:1 volume; each peel slurry was sieved with 002 sieve size excess substrates were kept at -20°C for further use. Powdered (solid) substrates were produced by drying at 60°C for 48 h immediately after collection and washing to prevent inherent chemical reactions within the peels. Same sieve size was used to screen the powdered peels and was stored in air tight container for further use.

Proximate composition of substrates: In order to ascertain the potentiality of the intended substrates regarding provision of support for selected microorganisms, they were subjected to initial chemical analysis.

Protein content analysis: The initial protein content of the peels was determined using the Lowry *et al.* (1951). Protein was extracted from 100 mg of the sample with 1N NaOH for 24 h and centrifuged at 6000 RPM for 15 min. 0.5 mL of the supernatant was used for the analysis. Folin Phenol reagent was added to the supernatant and incubated at room temperature for 30 min before absorbance was read at 660 nm. Bovine serum albumin was used for standard curve generation from which the protein value was deduced and triplicate samples were prepared.

Total soluble sugar (TOS) and Total carbohydrate (TC): Carbohydrate content of the peels was determined using phenol-sulphuric acid reagent. 100 mg of sample was hydrolysed with 2.5 N HCl for 3 h, the solution was neutralized with sodium carbonate and resulting mixture was filtered with whatman No. 1 filter paper. 5 mililiter Phenol-sulphuric acid reagent were added to 1 mL of filterate and absorbance was read at 490 nm. For total soluble sugar, 1 mL of filterate from the slurry was used while distilled water was used to elute TOS from the powdered sample before other steps were carried out (DuBois *et al*., 1956).

Total reducing sugar (TRS): Aqueous extraction of reducing sugar from BP, PP and Pw was done in 50 mL stoppered conical ask containing air dried peels for solid sample and slurry for liquid sample. 10 mL of 0.2 (mol L^{-1}) of disodium hydrogen phosphate/0.1 (mol L^{-1}) of citrate buffer (pH 4.8) was added before centrifugation was performed. Total reducing sugar of the supernatant was determined by Miller method using dinitrosalicylic acid reagent (DNS).

Ash content: Ash content represents the amount of trace and major minerals in the biomass. To determine ash content of the peels, 1 g of pre-dried sample and 1 g equivalent of slurry sample were weighed in the crucible. They were ignited at 550°C for 6 h and cooled in the dessicator before being reweighed. The difference in mass was recorded in percentage as %ash (Poorter and Bergkotte, 1992).

Moisture content: The moisture content of the peels was determined by weighing 1 g of powdered sample and its equivalent for slurry sample and subjected to drying in hot air oven at 105°C for 16 h. The loss in weight of the samples was taken as the moisture and recorded as %moisture content (AOAC Official Method 950.46).

Organic matter: Organic matter was calculated from the difference between the mass of dry substrate and its final mass after ashing.

Inoculums preparation

Fungal inoculums: Innoculum was prepared by using 25 mL of sterilized distilled water assisted with L-shaped rod to wash a petri dish of 7 days old fungi mycelium. For spore forming fungi, whatman No. 1 filter paper was used to filter out suspended spores (Saheed *et al*., 2012).

Submerged state bioconversion: Submerged state bioconversion was carried out in 250 mL Erlenmeyer flasks

with about 2% substrate, 2% innoculum. The volume was adjusted to 50 mL mineral solutions. Inoculums were added after sterilization of media at 121°C for 15 min in an autoclave. The flasks were kept at ambient temperature and 150 RPM for 7 days.

Enzyme assays

α-amylase assay: Amylase enzyme activity was carried out by first preparing the substrate which consists of 0.2% soluble starch (Sigma) dissolved in boiling 0.05 M KH_2PO_4-NaOH buffer (pH 6.0) and cooled to 40°C. The iodine reagent was prepared fresh by diluting 1 mL of stock solution (0.5% I_2 in 5.0% KI) into 500 mL of deionized water containing 5 mL of 5 N HCl. For the assay, 1.0 mL of enzyme solution was placed in a test tube and warmed to 40°C in a water bath for 10 min. The reaction was stopped by taking a 0.2 mL sample and adding it to 5.0 mL of iodine reagent. Absorbance was measured at 620 nm against blank (0.2 mL of water in 5 mL of iodine reagent). 1.0 mL of buffer was used in place of the enzyme to produce enzyme blank. Amylase activity was calculated from the absorbance by using the equation:

$$\alpha\text{-amylase units per milliliter} = \frac{\text{Control-test}}{\text{Control}} \times 4D$$

where, D is the enzyme dilution factor and 40 represents the 4.0 mg of starch present in the reaction tube times 10. One unit of α-amylase is defined as the amount of enzyme that will hydrolyze 0.1 mg of starch in 10 min at 40°C when 4.0 mg of starch is present. Dilution is required for activities resulting in absorbance of <0.125 after 10 min required dilution to give linear reactions.

Cellulase assay: The cellulase activity assay was conducted as outlined by the NREL LAP-006 (Adney and Baker, 1996), with a few modifications. Cellulase was diluted with 0.05 M Na-citrate buffer at a pH of 4.8 so that the final volume was 1.0 mL. Three replications of dilutions were used. An additional 2.0 mL of 0.05 M Na-citrate buffer was added to each of the sample test tubes, as well as to the blank test tubes. Two filter paper strips (1×6 cm; approximately 50 mg) were added to all sample and blank test tubes. All tubes were incubated in a water bath for 1.0 h at 50°C. After incubation, the tubes were immediately placed in an ice bath to stop the hydrolysis reaction. 1 mL diluted enzyme solution was added to the respective blank tubes. The soluble sugar content was then determined using the phenol-sulfuric acid assay (DuBois *et al*., 1956). Cellulase activity was calculated by comparing the sugar concentrations to a standard curve.

RESULTS AND DISCUSSION

Chemical analysis of the substrates (liquid and solid) showed that they contain appreciable level of reducing sugar, soluble sugar and acid soluble carbohydrates (Table 1 and 2) which are carbon sources necessary to support growth of selected microorganisms. Chemical properties of selected substrate were also documented by several investigators with results showing presence of same types of sugars and carbohydrate (Dhanasekaran *et al*., 2011; Jamal *et al*., 2012; Krishna *et al*., 2008) this qualified them for use as substrates for bioconversion processes.

Performance of each selected strain on the substrates: The performance of each selected filamentous fungus on both liquid and solid substrates under submerged phase bioconversion was carried out. Figure 1-3 showed that the microbes are capable of using the substrates as carbon source. Result obtained after 7 days of submerged bioconversion showed that more protein was produced by solid substrates comprising of BP, PP and Pw solely as carbon source. When selected microorganisms were tested on BP (solid and liquid), more protein was synthesized on solid substrate (Fig. 1). RO_2 synthesized highest amount of protein (15.88 mg g^{-1}) on solid matrix, followed by *P. crysosporium* (15.09 mg g^{-1}) while M6 produced the least (13.24 mg g^{-1}). The difference in

Table 1: Proximate composition of liquid substrates

Sample	Soluble sugar (mg g^{-1})	Carbohydrate (mg g^{-1})	Reducing sugar (mg g^{-1})	Protein content (mg g^{-1})	Ash content (%)	Organic matter (%)	Moisture content (%)
Banana peel	36.71	22.83	1.30	0.54	0.86	99.14	96.16
Pineapple peel	75.45	40.22	1.80	0.50	0.32	99.68	96.30
Papaya peel	52.35	47.51	4.54	0.61	0.60	99.40	95.77

Table 2: Proximate composition of solid substrates

Sample	Soluble sugar (mg g^{-1})	Carbohydrate (mg g^{-1})	Reducing sugar (mg g^{-1})	Protein content (mg g^{-1})	Ash content (%)	Organic matter (%)	Moisture content (%)
Banana peel	32.84	20.23	1.29	0.83	18.07	81.93	39.23
Pineapple peel	40.74	36.84	1.70	0.80	5.33	94.67	40.50
Papaya peel	24.94	18.92	0.86	0.73	12.21	87.79	41.07

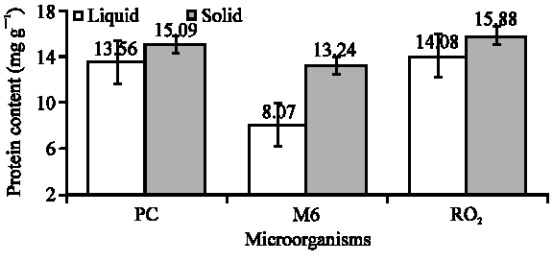

Fig. 1: Protein synthesis of *P. chrysosporium*, M6 and RO_2 on banana peel

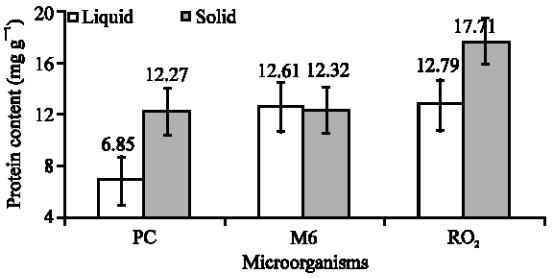

Fig. 2: Protein synthesis of *P. chrysosporium,* M6 and RO_2 on pineapple peel

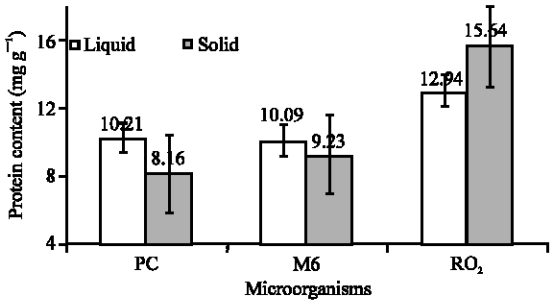

Fig. 3: Protein synthesis of *P. chrysosporium,* M6 and RO_2 on papaya peel

protein production between solid and liquid substrate can be attributed to the presence of lignocellulosic materials in the solid matrix which triggers production of degrading enzymes by the fungus. This result was consistent with that of other researchers irrespective of whether fungi or yeast were cultivated on the substrate but majority favoured either heavy supplementation with other carbon source or nitrogenous minerals to produce high biomass and enhancement of protein (Essien *et al*., 2005; Yabaya and Ado, 2008; Tijani *et al*., 2011).

On PP, same trend was recorded with selected microbes producing more protein on solid substrate. RO_2 leads the park with 17.71 mg g^{-1} protein secretion while *P. crysosporium* and M6 followed with 12.27 and 12.32 mg g^{-1}, respectively. RO_2 produced more on PP compared to BP; this was caused by the presence of more soluble and insoluble sugar in PP which supports growth and product synthesis over the bioconversion period. However, the margin between *P. crysosporium* and M6 on both substrates was insignificant signaling similarities in their biochemical behavior over same substrate. Similarly, *P. crysosporium* and RO_2 produced 13.56 and 14.08 mg L^{-1} on liquid BP showing similarities in their protein production efficiency over the bioconversion period. Furthermore, M6 and RO_2 behaviour on liquid PP was similar signaling a possible similarity in their metabolic pathway for sugar under SmB. On Pw, RO_2 produced highest protein (17.71 mg g^{-1}) on solid Pw while liquid substrate supports more protein production between *P. crysosporium* and M6 with no significance in their production. The difference in performance of selected strains on Pw showed how fungi strains could behave on same substrate during bioconversion (Jamal *et al*., 2009; Kadimaliev *et al*., 2004; Dhanasekaran *et al*., 2011).

Comparison of enzyme activity leading to protein synthesis: White rot fungus is known to produce extracellular enzymes such as amylases and cellulases during metabolism of complex sugar sources. Therefore, amylase and cellulase activities of selected filamentous fungi were determined to further elucidate the mechanism behind the bioconversion process in relation to protein production. Figure 4a presents the α-amylase activities of all the microbes on BP (solid and liquid) over the bioconversion period. As expected, RO_2 produced highest amount of amylase enzyme (5.65 units mL^{-1}) leading all others microbes on solid matrix. Equally, on the liquid substrate, it produced highest amount of extracellular amylases (36.96 units mL^{-1}). This showed that RO_2 converted a lot of inherent starch in the BP to produce bio-protein. *P. chrysosporium* and M6 on the other hand were able to produce amylase activities of 3.26 and 2.47 units mL^{-1} on solid substrate to synthesize their protein. On the liquid substrate, *P. chrysosporium,* amylase activity was 16.98 units mL^{-1} while M6 amylases have 11.83 units mL^{-1}. Results presented here further buttress other researchers opinion that the amount of protein produced by microbial strains is related to their hydrolytic enzyme activity over the bioconversion period (Nazareth and Sampy, 2003; Ohkuma *et al*., 2001).

For cellulase activity (Fig. 4b), *P. chrysosporium* is having the best (0.32 units mL^{-1}) on solid media followed by RO_2 (0.24 units mL^{-1}) and M6 (0.18 units mL^{-1}),

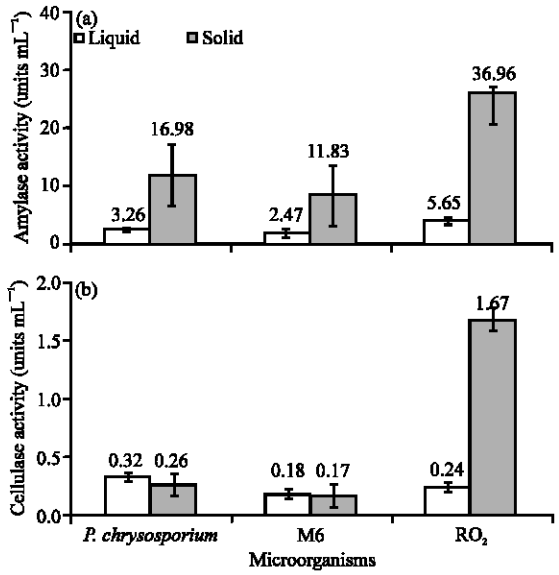

Fig. 4(a-b): (a) Amylase and (b) Cellulase enzyme production by *P. chrysosporium,* M6 and RO_2 on banana peel

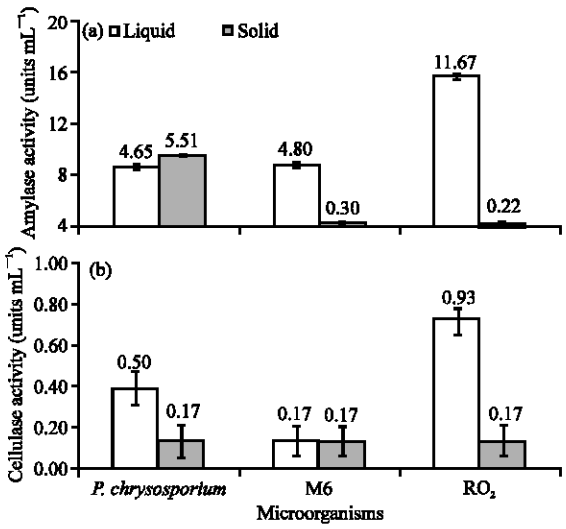

Fig. 5(a-b): (a) Amylase and (b) Cellulase production by *P. chrysosporium,* M6 and RO_2 on pineapple peel

respectively. RO_2 led the park on liquid substrate with 1.67 units mL^{-1} followed by 0.26 units mL^{-1} from *P. chrysosporium* and 0.17 units mL^{-1} by M6. The level of amylases and cellulases activity on BP showed presence of cellulose, hemicellulose and starch. The cellulase activities of all the selected strain are were more than those reported by Alam *et al.* (2005) when *Trichorderma harzianium* was cultivated on oil palm biomass. For *P. chrysosporium* scanty reports opined its production of cellulases but such literatures do agree that the strains contain numerous lignolytic hydrolytic enzymes (Singh and Chen, 2008). Therefore, this paper brings forward the presence of cellulases as part of such enzymes.

On PP (Fig. 5a) lesser amount of amylase activity was recorded overall for all cultivated fungi when compared to BP for both solid and liquid substrates. This could be attributed to lesser amount of starch in the substrate since the peel contains more soluble sugar and reducing sugars unlike other substrates. RO_2 produced highest amount of amylase activity (11.67 units mL^{-1}) on solid matrix, followed by M6 (4.8 units mL^{-1}) and *P. chrysosporium* (4.65 units mL^{-1}). The liquid substrate on the other hand did not efficiently support amylase enzyme activity with *P. chrysosporium* producing 5.51 units mL^{-1} while M6 and RO_2 produced a paltry 0.30 and 0.22 units mL^{-1}, respectively. The cellulase activities of the filamentous fungus on PP (Fig. 5b) showed a decline when compared to production on BP. RO_2 produced 0.93 units mL^{-1} activity as highest on PP solid matrix while 0.5 and 0.17 units mL^{-1} were produced by *P. chrysosporium* and M6, respectively. On liquid PP, all the strains showed same cellulase activity of 0.17 units mL^{-1}. Enzyme activities (amylase and cellulase) presented in this research signals a synergistic process regarding catalytic efficacy of extracellular hydrolytic enzymes in converting cellulolytic fruits wastes to intracellular protein by bacidiomycetes. The result was in agreement with previous studies on individual substrates (Jamal *et al.*, 2009; Vijayaraghavan *et al.*, 2011).

Amylase enzyme production by Pw was the highest among all the solid substrates with 102.63 units mL^{-1} activity by RO_2 (Fig. 6a). M6 produced 24.98 units mL^{-1} while *P. chrysosporium* produced 17.03 units mL^{-1} on same substrate. Similarly, 13.39 and 12.24 units mL^{-1} were produced by RO_2, M6 and *P. chrysosporium* on liquid substrate. Furthermore, RO_2 cellulase production was highest on solid Pw matrix with 0.87, 0.71 units mL^{-1} for *P. chrysosporium* while 0.41 units mL^{-1} was produced by M6 (Fig. 6b). On liquid Pw, *P. chrysosporium* produced 0.39 units mL^{-1}, followed by 0.26 units mL^{-1} by RO_2 whereas 0.17 units mL^{-1} was produced by M6. The results showed that Pw effectively supported growth and development of selected strains for producing their extracellular enzymes which breaks down available complex sugars to simple molecules easier to assimilate by the strains to produce protein. Similarly, production of cellulases showed that Pw contained cellulose as part of its components. According to Khan *et al.* (2009), amount

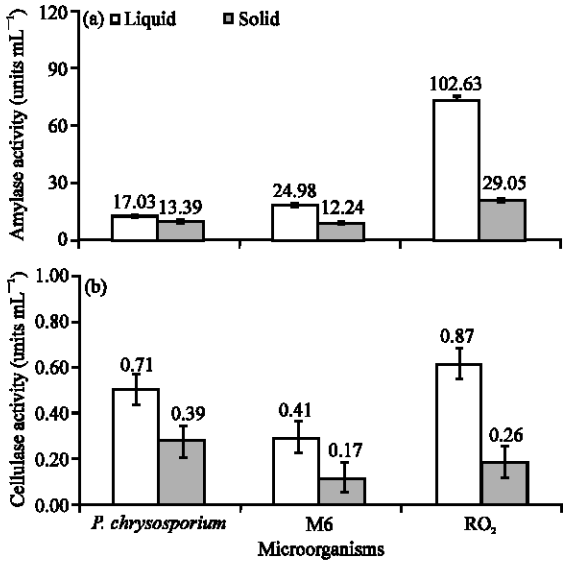

Fig. 6(a-b): (a) Amylase and (b) Cellulase production by *P. chrysosporium,* M6 and RO_2 on papaya peel

of protein synthesized by *Rhizopous oligosporous* on Pw is related to how efficient the enzymes are in converting microbial growth to protein accumulation.

CONCLUSION

Results presented above have demonstrated that selected substrates supported microbial growth without heavy supplementation. Similarly, the synergism between enzyme production and protein synthesis by fungi biomass was further elucidated. Finally, solid substrates selected in the research provided more support for fungal growth, development and product synthesis.

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