A Consortium of Three Enzymes: Xylanase, Arabinofuranosidase, and Cellulase from *Aspergillus sp.* which liquefied Coffee Pulp Wastes

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A Consortium of Three Enzymes: Xylanase, Arabinofuranosidase, and Cellulase from *Aspergillus sp.* which liquefied Coffee Pulp Wastes

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Abstract. Three enzymes released by *Aspergillus* sp., identified as xylanase, arabinofuranosidase, and cellulase which hydrolyzed coffee pulp during solid state fermentation (SSF) had been investigated. The active fraction of xylanase activity was detected when purification of crude enzymes was performed on DEAE Cellulofine, while cellulase and arabinofuranosidase were fractionated from the previous active fraction using the DEAE Toyopearl 650M and DEAE Mono-Q columns. All enzymes are optimum active at 55°C and stable at temperatures below 50°C and a range of pH 3-6.5. Optimum cellulase activity at pH 5.5 while xylanase and arabinofuranosidase at pH 5. A consortium of these three enzymes can easily hydrolyze the coffee pulp into simple sugar with a degree hydrolysis 79%.
Keywords: coffee pulp, xylanase, arabinofuranosidase, solid state fermentation

1. Introduction

As the world's fourth-largest coffee producer, coffee pulp waste (CPW) is one of the most abundant agro-industrial products available in Indonesia. In contrast, CPW is a very potential model because it contains a lot of polysaccharides, mineral nutrients and the presence of secondary metabolites in bioactive forms such as caffeine and antioxidants such as hydroxyzine acid makes this waste very worthy of its use. [1-4] However, the waste is still much underutilized and is only accumulated without efforts reduced the wastes either to increase the economic value of CPW [5]. Some of the results of research on CPW have been widely reported. However, the utilization in the scale of industrial application is still lacking. It has been reported that CPW can be used as an additive for ceramics and particle board [6-7], as a carbon source in bioethanol production [2,8-9], production of gibberellic acid [10], lactic acid [11], biogas [12], and active carbon sources [1]. Other studies have reported that CPW can be used as a substrate in the production of several enzymes such as endoglucanase [13], xylanase [14], proteases [15], and cellulase [16]. Some of the uses of microorganisms in the degradation process of biomass are one of the most effective strategies because microorganisms are capable of producing various enzymes [17]. In this study, three extracellular enzymes from *Aspergillus* sp. which have an important role in CPW hydrolysis process was reported.

2. Material and Method

Fresh CPW bean was obtained from coffee plantation Durjo village Jember Regency. The fungus *Aspergillus* sp. VTM5 was obtained from previous research [18]. The chromatography media DEAE

Cellulofine, DEAE Toyopearl 650M, DEAE Mono-Q, Sephacryl S-100 gel filtration, and all chemical substances were purchased from Sigma.

2.1. Preparation of coffee pulp alkali substrates

Coffee pulp alkali (CPA) substrate was prepared as follows: 500 g of CPW were suspended in 10% NaOH, shaken overnight, filtered and the filtrate adjusted to pH 5 with acetic acid. The filtrate was made to 50% in ethanol for precipitation by centrifugation at 12000 rpm, for 20 minutes. The precipitates were freeze-dried under reduced pressure at -10^{o}C. The yield as a dry powder of CPA was about 4.6% of the starting material.

2.2. Cultivation and Harvesting of crude enzyme

Sterilized of fresh CPW 500 grams on a 4 L Erlenmeyer flask inoculated with *Aspergillus* sp. was cultivated at 30^{o}C. After day fifth, the enzyme was harvested by shaking at room temperature for 9 hours. In this step, 100 ml H_2O containing 1% NaCl and 0.1% toluene were added. The supernatant as a crude enzyme source was obtained by filtered using glass filter, followed by centrifugation at 8000 rpm for 10 minutes.

2.3. Enzyme assays

Enzyme activities were measured by incubating the reaction mixture of enzyme and CPA substrate at 37^{o}C and measuring the released of reducing sugars by the method of Nelson and Somogy. One unit of enzyme activity was defined as the amount of enzyme that produced reducing sugar at a rate of 1 µmol per minute. For pH range at 1-6 and 6-8, 20 mM acetate and phosphate buffer were used, respectively. All assays were performed in 1 ml total volume. For cellulase and xylanase activity, carboxymethyl cellulose 1% and xylan 1% substrates in the same buffer were used. The α-L-arabinofuranosidase activity was also determined by measuring the amount of p-nitrophenol released from the appropriate p-nitrophenyl-arabinofuranoside.

2.4. Enzymatic hydrolysis product analysis

To analysis the enzymatic hydrolysis product, the mixture of dialyzed crude enzyme and 1% of CPA were incubated at 37^{o}C for 2 hours. In this analysis, 1% the CPA and fully hydrolyzed CPA was used as the control. For full hydrolysis of CPA, 2M of HCl containing 1% of CPA was incubated at 100^{o}C for 6 hours, followed by filtration and centrifugation 4000 rpm for 10 minutes to recover the hydrolyzates. The sugars component of hydrolysates analysis were performed as alditol acetate [19], and then 5µL of the sample containing transmethylated D-Glucose as an internal standard was injected to Gas Chromatograph (GC) Thermo Scientific Trace 1310 equipped with TG-225MS 15mx0.25mmx 0.25µm column for sugars component analysis.

2.5. Purification

Purification was carried out at 30^{o}C using 20 mM acetate buffer at pH 5, and the steps were explained under "Result and Discussion."

2.6. Enzymes properties

The effect of pH and temperature on optimum activity and stability of these three enzymes were determined at the pH range of 2 to 10 and a temperature range of 20 to 65^{o}C. Determination the molecular weight of the enzymes, gel filtration Sephacryl S-100 column equilibrated with the same buffer containing 100 mM NaCl was used. The purified enzymes together with standards blue dextran (MW 2,000,000), apoferritin (MW 44,300), and ribonuclease A (MW 13,700) were loaded into Sephacryl S-100 column, then eluted with the same buffer. The molecular weight of the enzymes was calculated from the effluent volume.

3. Results and Discussion

Microbial utilization of CPW has been demonstrated in these investigations that *Aspergillus* sp. could utilize CPW as carbon, nitrogen and mineral sources. The growth of mycelium and spores from *Aspergillus* sp. as long as SFF can be easily detected and seems to have very good growth. As reported from the previous study [20] , *Aspergillus* can grow well in an environment that is relatively acid to alkaline from pH 3.5 to 8 [21-27]. And it has also been reported that *Aspergillus* can use various sources of carbon and nitrogen from the biomass because it is capable of producing enzymes in the broad spectrum [28-29]. In this study, it was proven that *Aspergillus* was readily to hydrolyze CPW substrate, and fifth day of fermentation the liquid form was produced. The analysis liquid form showed that sugars-rich hydrolyzate 140 µg/ml hydrolyzate was produced. The extracellular enzymes which secreted by *Aspergillus* sp. during SSF of CPW could hydrolyze up to 79%.

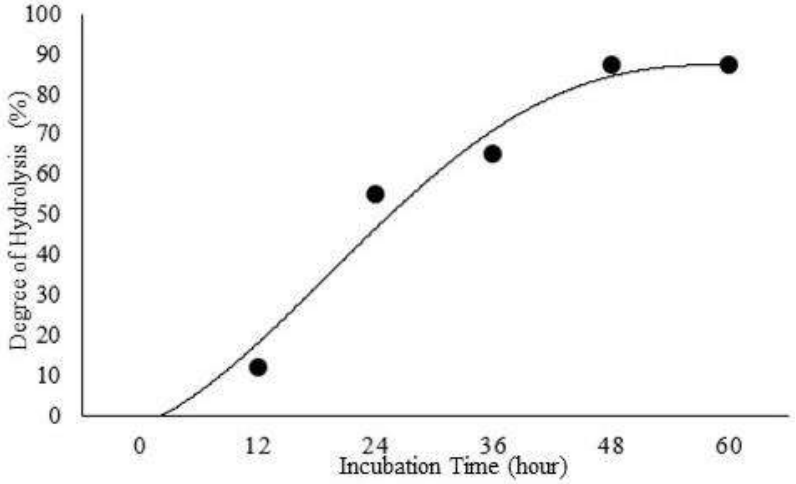

Figure 1. Maximum reducing sugar produced during hydrolysis of 1% CPA substrate in 20 mM acetate buffer at pH 5 was achieved after 48 hours incubation at 37ºC.

A significant result in increasing the activity of crude enzyme against 1% CPA substrate in 20 mM at pH 5 (Figure 1), and it was estimated that the maximum degree hydrolysis up to 87% (52.2 µg/ml) when incubated at 37ºC for 48 hours.

Gas Chromatography analysis as alditol acetates of the hydrolyzates proved that the main component monosaccharides after enzymatic hydrolysis were glucose (78.1%), xylose (18.1%), and arabinose (3.8%), but galactose and rhamnose were less than 0.2% detected, shown in Table 1.

Regarding the results shown in Table 1, it can be understood that the amount of glucose, arabinose, and xylose contained in hydrolysate is due to cellulase, arabinosidase, and xylanase activity during the hydrolysis process. It has been reported from previous studies that several *Aspergillus* species are capable of producing extracellular cellulase, arabinosidase, xylanase, and several groups of hydrolase enzymes [30-32] .

Table 1. Comparison of sugar component after enzymatic hydrolysis, full hydrolysis and without hydrolysis of CPA substrate by the crude enzyme.

Sugar component	Enzymatic hydrolysis		Full hydrolysis		Without hydrolysis	
	%	µg/mL	%	µg/mL	%	µg/mL
Glucose	78.1	40.8	71.0	45.3	ND	ND
Xylose	18.1	9.4	16.3	10.4	ND	ND
Arabinose	3.6	1.9	4.5	2.9	ND	ND
Galactose	0.17	0.09	5.0	3.2	ND	ND
Rhamnose	0.03	0.02	0.1	0.07	ND	ND

ND: Not detected

The enzyme purification was started by dialysis of 100 ml of the filtrated-crude-enzyme using water to remove remaining sugar and then buffered using 20 mM of acetate buffer pH 5. Subsequently, the crude enzyme was put into an on DEAE Cellulofine column which was pre-equilibrated using the same acetic buffer. Purification was carried out by elution using a 500 mL buffer with a gradient concentration of 0 to 0.5M NaCl. Two peaks as active fractions were obtained which hydrolyzed CPA substrate (Figure 2). The fractions for each peak were pooled separately, and re-dialyzed against the buffer to remove NaCl. Also, the first active fraction was identified as xylanase because this fraction easily hydrolyzed xylan substrates. While xylanase activity was not detected in the second active fraction, but this fraction was able to hydrolyze the substrate of carboxymethyl-cellulose and p-nitrophenyl-arabinofuranoside. This evidence means that there are two types of enzymes that cannot be fractionated through the DEAE Cellulofine column, as shown in Figure 2.

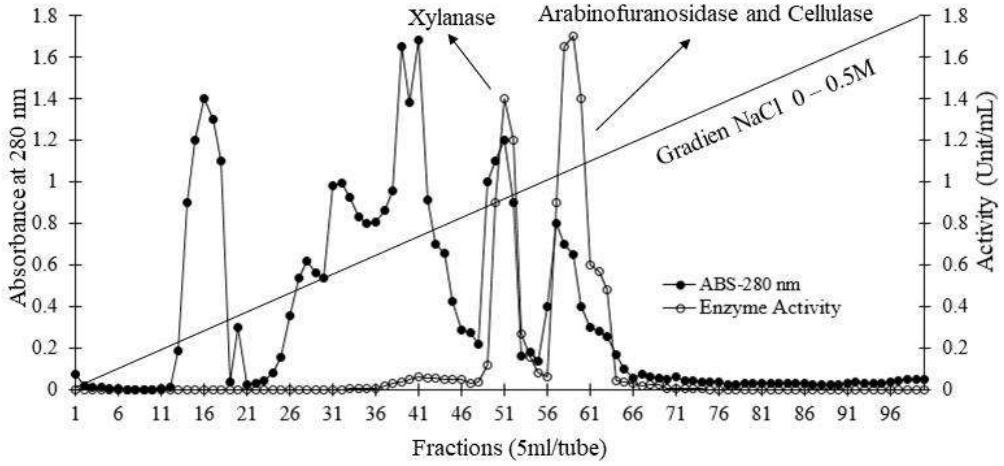

Figure 2. The buffered crude enzyme was loaded an on DEAE Cellulofine column and eluted using the same buffer with gradient 0 - 0.5 M NaCl. The protein concentration (●) was monitored at 280 nm, and the activity (○) was checked after 10 minutes incubation of the mixture (100 µl crude enzyme in 1ml of 1% CPA substrate containing 20 mM acetate buffer, pH 5).

Further step purification of xylanase and arabinofuranosidase and cellulase were done by re-chromatograph of the second active fraction. In this step, the active fraction was diluted four times from the initial volume with the same acetate buffer and loaded to DEAE-Toyopearl 650M column. And then to remove remaining NaCl, the column re-eluted and pre-equilibrated using the same buffer. The purification was run under the same gradient concentration of NaCl. One peak as active fraction was produced, which means that purification through DEAE-Toyopearl 650M column the arabinofuranosidase and cellulase could not be purified. In this step showed that total protein concentration of the active fraction of protein decreases significantly, but there was an increase in specific activity against CPA, p-nitrophenyl-arabinofuranoside, and carboxymethyl cellulose substrate. Even though the arabinofuranosidase and cellulase could not be fractioned, this evidence explained that some non-target proteins were removed when the second active fraction loaded into DEAE-Toyopearl 650M column. Again, an active fraction from the previous step was pooled, diluted five times from initial volume, loaded into DEAE Mono-Q columns, pre-equilibrated with buffer, and fractioned with the gradient 0-0.4 NaCl in the same buffer. Shown in Figure 3, two peaks active fraction were obtained and identified as purified arabinofuranosidase and cellulase as well.

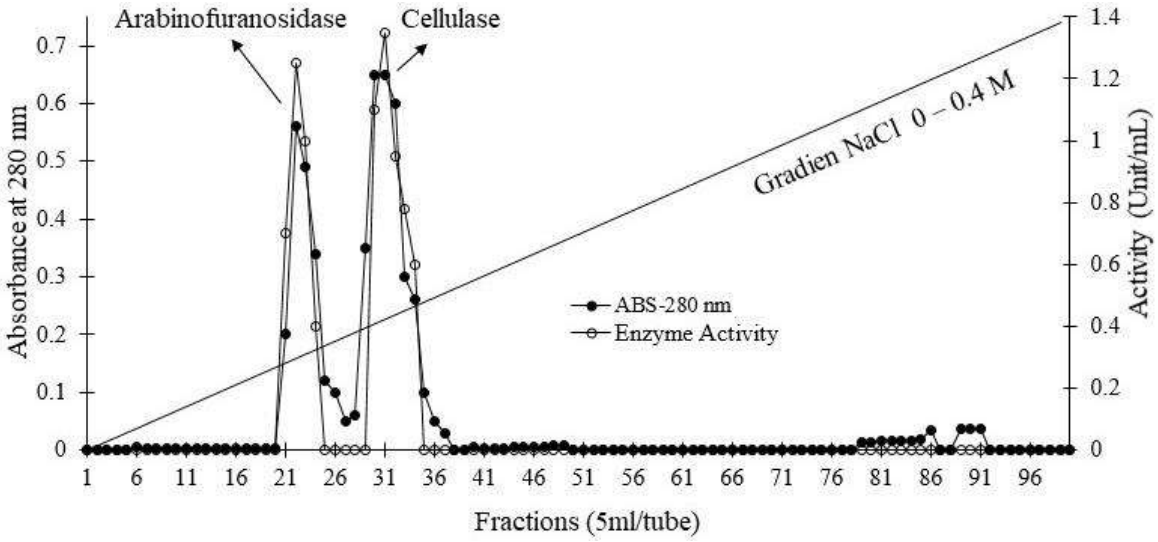

Figure 3. Two active fractions identified as arabinofuranosidase and cellulase when the second active fractions were loaded onto a DEAE Mono-Q column and eluted using the same buffer with gradient 0 - 0.4 M NaCl.

Estimation of the molecular weight of the three purified enzymes by using gel filtration Sephacryl S-100 column as described in the method revealed that xylanase, arabinofuranosidase, and cellulase were 67,000, 45000, and 29,000 Da. Figure 4 shows that purified enzymes are optimum active at 55°C and stable at temperatures below 50°C and a range of pH 3-6.5. Optimum cellulase activity at pH 5.5 while xylanase and arabinofuranosidase at pH 5.

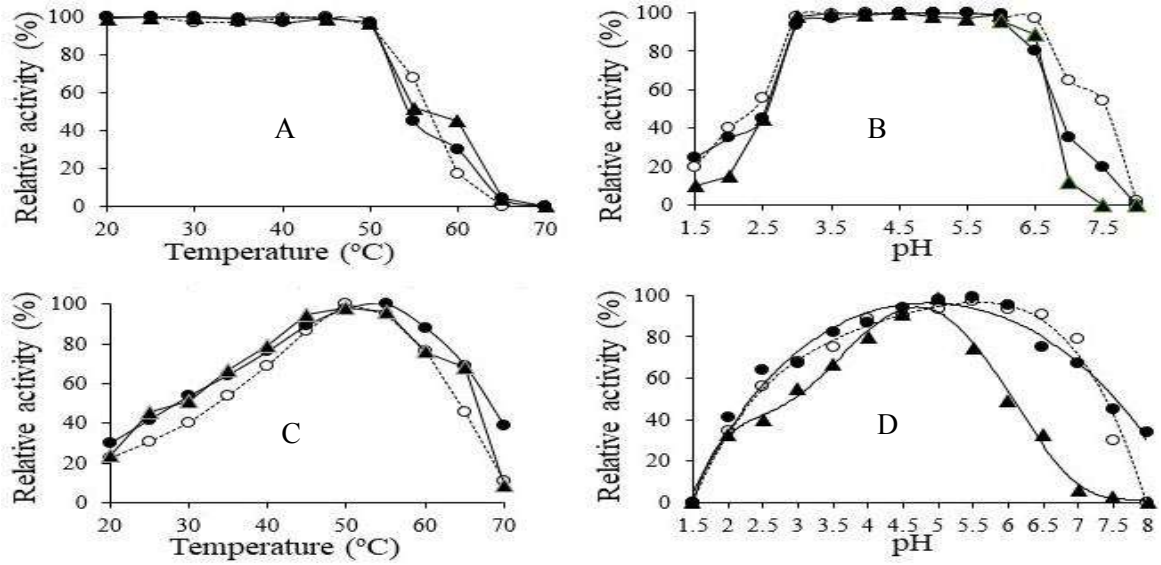

Figure 4. Stability (A, B) and optimum (C, D) on temperature and pH on the activity of purified xylanase (●), arabinofuranosidase (○), and cellulase (▲).

Based on the results, all purified xylanase, arabinofuranosidase, and cellulase tend to work at acid pH. Facts, in previous studies that some hydrolases can work in acidic conditions . Also, it has been reported that under acidic conditions *Aspergillus* species are capable of producing xylanase, cellulase, amylase, and some hydrolases [23, 30-32].

4. Conclusion

Xylanase, arabinofuranosidase, and cellulase from *Aspergillus* sp. which liquefied coffee pulp during SSF had been elucidated. A consortium of these three extracellular enzymes easily hydrolyzed the CPW into simple sugar with a degree hydrolysis up to 79%. All enzymes are optimum work at 55°C and stable at temperatures below 50°C and a range of pH 3-6.5. Optimum cellulase activity at pH 5.5 while xylanase and arabinofuranosidase at pH 5. Suggested that by using this isolate and CPW as a cheap material, the production of xylanase, arabinofuranosidase, and cellulase can be done. However, the ideal process for industrial purposes, improvement research to increase yield still needed.

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References

[1] Gonçalves, M., Guerreiro, M. C., Oliveira, L. C. A., Solar, C., Nazarro, M. and Sapag, K. (2013). Micro mesoporous activated carbon from coffee husk as biomass waste for environmental applications. *Waste and Biomass Valorization*, **4**(2), 395–400.

[2] Gouvea, B. M., Torres, C., Franca, A. S., Oliveira, L. S.and Oliveira, E. S. (2009). Feasibility of ethanol production from coffee husks. *Biotechnology Letters*, **31**(9), 1315–1319.

[3] Mussatto, S. I., Machado, E. M. S., Martins, S. and Teixeira, J. A. (2011). Production, Composition, and Application of Coffee and Its Industrial Residues. *Food and Bioprocess Technology*, **4**(5), 661–672.

[4] Vijayalaxmi, S., Jayalakshmi, S. K. and Sreeramulu, K. (2014). Polyphenols from different agricultural residues: extraction, identification and their antioxidant properties. *Journal of Food Science and Technology*, **52**(May), 1–9.

[5] Rattan, S., Parande, A. K., Nagaraju, V. D. and Ghiwari, G. K. (2015). A comprehensive review on utilization of wastewater from coffee processing. *Environmental Science and Pollution Research*, **22**(9), 6461–6472.

[6] Acchar, W. J. V. Dultra, E. (2015). Ceramic Materials from Coffee Bagasse Ash Waste, (Oliveira 1998), 23–30.

[7] Bekalo, S. and Reinhardt, H. W. (2010). Fibers of coffee husk and hulls for the production of particleboard. *Materials and Structures*, **43**, 1049–1060.

[8] Menezes, E. G. T., Do Carmo, J. R., Menezes, A. G. T., Alves, J. G. L. F., Pimenta, C. J.and Queiroz, F. (2013). Use of different extracts of coffee pulp for the production of bioethanol. *Applied Biochemistry and Biotechnology*, **169**(2), 673–687.

[9] Woldesenbet, A. G., Woldeyes, B. and Chandravanshi, B. S. (2016). Bio-ethanol production from wet coffee processing waste in Ethiopia. *Springer Plus*, **5**(1), 0–6.

[10] Machado, C. M. M., Soccol, C. R., de Oliveira, B. H. and Pandey, A. (2002). Gibberellic acid production by solid-state fermentation in coffee husk. *Applied Biochemistry and Biotechnology*, **102–103**(1–6), 179–191.

[11] Pleissner, D., Neu, A. K., Mehlmann, K., Schneider, R., Puerta-Quintero, G. I. and Venus, J. (2016). Fermentative lactic acid production from coffee pulp hydrolysate using Bacillus coagulans at laboratory and pilot scales. *Bioresource Technology*, **218**, 167–173.

[12] Battista, F., Fino, D., & Mancini, G. (2016). Optimization of biogas production from coffee production waste. *Bioresource Technology*, **200**, 884–890.

[13] Navya, P. N. and Pushpa, S. M. (2013). Production, statistical optimization and application of endoglucanase from Rhizopus stolonifer utilizing coffee husk. *Bioprocess and Biosystems Engineering*, **36**(8), 1115–1123.

[14] Murthy, P. S. and Naidu, M. M. (2012). Production and Application of Xylanase from *Penicillium* sp. Utilizing Coffee By-products. *Food and Bioprocess Technology*, **5**(2), 657–664.

[15] Kandasamy, S., Govarthanan Muthusamy, B., Senthilkumar Balakrishnan, B., Senbagam

Duraisamy, B., Selvankumar Thangasamy, B., Kamala-Kannan Seralathan, B. and Sudhakar Chinnappan, B. (2016). Optimization of protease production from surface-modified coffee pulp waste and corncobs using *Bacillus* sp. by SSF. **3** *Biotech*, *6*(2), 1–11.

[16] Bui, H. B. (2014). Isolation of cellulolytic bacteria , including actinomycetes , from coffee exocarps in coffee-producing areas in Vietnam. *Int. J. Recycl .Org. Waste Agricult.*, **3**(48), 1–8.

[17] Muzakhar, K., Sutoyo, S. and Saragih, A. B. (2015). Phosphate Solubilizing Bacteria Adaptive to Vinasse. *Journal of Mathematical and Fundamental Sciences*, **47**(2), 219–225.

[18] Yuniar, W. (2013). *Skrining dan Identifikasi Kapang Selulolitik pada Proses Vermikomposting Tandan Kosong Kelapa Sawit (TKKS)*.

[19] Muzakhar, K., Sutoyo and Siswoyo. (2014). Sugar Production By Digesting of Oil Palm Empty Fruit. *International Journal of Biosciences and Biotechnology*, **II**(1), 32–38.

[20] Ali, S. (2002). Production of citric acid by *Aspergillus niger* using cane molasses, *5*(3).

[21] Muzakhar, K., Masruroh, Siswoyo, Winarsa, R. and Sutoyo. (2017). Sugar-rich hydrolysates of palm oil empty fruit bunch production through two step solid state fermentations and its conversion to ethanol. *Advanced Science Letters*, **23**(3), 2533–2535.

[22] Guimarães, L. H. S., Jorge, J. A., Terenzi, H. F., Jamur, M. C., Oliver, C. and Polizeli, M. D. L. T. D. M. (2003). Effect of carbon source on alkaline phosphatase production and excretion in *Aspergillus caespitosus*. *Journal of Basic Microbiology*, **43**(3), 210–217.

[23] Hajji, M., Hmidet, N., Jellouli, K., Vallaeys, T., Nasri, M. and Sellami-Kamoun, A. (2010). Gene cloning and expression of a detergent stable alkaline protease from *Aspergillus clavatus* ES1. *Process Biochemistry*, **45**(10), 1746–1752.

[24] Kumar, S. and Ram., M. R. (2017). Optimization Studies on Alkaline Protease From *Aspergillus terreus* Isolated From Marine Soil. *International Journal of Advanced Research*, **5**(4), 300–306.

[25] Prakasham, R. S., Subba Rao, C., Sreenivas Rao, R. and Sarma, P. N. (2007). Enhancement of acid amylase production by an isolated *Aspergillus awamori*. *Journal of Applied Microbiology*, **102**(1), 204–211.

[26] Siala, R., Kamoun, A., Hajji, M., Abid, L., Gharsallah, N. and Nasri, M. (2010). Extracellular acid protease from *Aspergillus niger* I1: purification and characterization. *Journal of Biotechnology*, **8**(18), 4582–4589.

[27] Van Der Gaag, M., Debets, A. J. M., Hoekstra, R. F., Varga, J., Van Diepeningen, A. D. and Swart, K. (2004). Efficient degradation of tannic acid by black *Aspergillus* species. *Mycological Research*, **108**(8), 919–925.

[28] Hartemink, B. A. E., Science, S. and Hartemink, A. (2003). Broad-Spectrum Peptide Inhibitors of Aminoglycoside Antibiotic Resistance Enzymes, **114**, 139–140.

[29] Hrmova, M., Biely, P. and Vr, M. (1989). Cellulose- and xylan-degrading enzymes of *Aspergillus terreus* and *Aspergillus niger*. *Enzyme*, **11**.

[30] Meijer, M., Varga, J., Ehrlich, K., Perrone, G., Noonim, P., Susca, A. and Cozzi, G. (2010). Biodiversity of *Aspergillus* species in some important agricultural products. *Studies in Mycology*, **59**, 53–66.

[31] Okafor, U., Okochi, V., Onyegeme-okerenta, B. and Nwodo-Chinedu, S. (2007). Xylanase Production by *Aspergillus niger* ANL 301 Using Agro - Wastes. *Afican Journal of Biotechnology*, **6**(July), 1710–1714.

[32] Ottenheim, C., Verdejo, C., Zimmermann, W. and Wu, J. C. (2014). Hemicellulase production by *Aspergillus niger* DSM 26641 in hydrothermal palm oil empty fruit bunch hydrolysate and transcriptome analysis. *Journal of Bioscience and Bioengineering*, ***xx***(xx), 1–6.