Production of Cellulase and Xylanase from *Eupenicillium Javanicum* by Solid-State Fermentation Utilizing Pineapple Crown Leaves Waste as the Substrate

Evelyn¹, S Z Amrains¹, E D Pratiwi¹, U N Ismaila¹

¹Department of Chemical Engineering, Faculty of Engineering Universitas Riau, Pekanbaru 28293, Riau, Indonesia

E-mail: evelyn@eng.unri.ac.id

**Abstract.** Vast applications of cellulases and xylanases in many fields over the last few decades and high production cost demand extensive research in improving its quality and finding low cost substrates. These include pulp and paper, food and feed, brewing, agriculture and biofuel industries. The main objective of this study was to investigate the use of pineapple crown leaves waste as the substrate to produce cellulases and xylanases in solid-state cultures (SSF) of *Eupenicillium javanicum* InaCC F154. Three SSF temperatures (25°C, 30°C and 35°C) and pH (4.0, 6.0 and 8.0) were tested for 96 h in solid media containing defatted soybean, (NH₄)₂SO₄, NaNO₃, CaCl₂, and small amount of H₂O. Then, its comparison with submerged fermentation (SmF) was also carried out. The SSF results showed that increasing temperature from 25°C to 35°C, increased the production of these enzymes. The pH also affected its activities, except at pH 4.0. The optimum pH and temperature for cellulase and xylanase production were found to be 6.0 and 35°C, respectively. The maximum cellulase obtained was 0.261 U/mL while much higher was obtained for xylanase with activity of 1.683 U/mL. SSF was better than SmF for xylanases (0.501 U/mL vs. 1.683 U/mL). The outcome of this study exhibits the potential of pineapple crown leaves as the substrate for xylanase production from *E. javanicum* by solid-state fermentation.

1. Introduction
Enzymes are biological catalysts that accelerate the rate of a reaction in biological system. The enzymes itself is not being change by the reaction. Enzymes are primarily made out of protein that are highly substrate specific molecules. After the reaction, substrates are then converted into different molecules known as products. Enzymes have grown applications in recent years such as food and beverages, detergent and textiles, biofuel, animal feed, cosmetics, medication and pharmaceuticals, and many others. According to Oliviera et al. [1], it was worth to a value about $4 billion in 2011 and $6 billion in 2016. Industrial enzyme production from microorganisms has received much attention due to better yields and lower cost and labor [2]. Fermentation is one of good methods to obtain enzymes from microorganisms.

Solid-state fermentation (SSF) is defined as the fermentation method that uses solid medium in absence or near absence of free water to culture microorganism [3]. Industrial enzyme production by this method has attracted more and more researcher’s attention since SSF could provide several benefits compare to submerged or liquid state fermentation (SmF) i.e. more oxygen circulation for the microbial growth, lower energy consumption, simulating natural habitats and simplicity of the subsequent processes, thus is suitable for enzyme production [4, 5]. Filamentous fungi are the most promising and exploiting ones for enzyme production by SSF method due to a better adaptation to the...
substrates and its biochemical characteristics such as tolerance at low water activity [2, 5]. As opposed to SSF, the microorganisms and nutrients are usually submerged in water thus making it suitable for bacterial fermentation [6].

Cellulase and xylanase are two important enzymes that catalyze the hydrolysis of cellulose and xylan in some hemicelluloses, respectively. Both enzymes can be produced from filamentous fungi through SSF. Among the most common fungi that have been used in literature belongs to genera Aspergillus sp., Rhizopus sp., Penicillium sp, Trichoderma sp., and Pseudomonas sp. Examples of species for cellulase production are Trichoderma reesei, Aspergillus niger, Pseudomonas fluorescens, and Penicillium sp. [2, 7]. Examples of species for xylanase production are A. niger, Aspergillus fumigatus, Trichoderma viride, and Rhizopus oryzae [5].

Inexpensive substrates like agro-industrial byproducts and forest residue can be used as the substrate in SSF. They are abundant in the environment and low value however they still have a high content of molecules required i.e. cellulose, hemicellulose, starch, lignin, pectins, and polyphenols for microbial growth [8]. Pineapple fruits make up one of the largest sectors of the world’s fruit production, with global exports of estimated increase to 3.2 million tonnes in 2019 [9]. After the fruits are harvested and processed as juice or other products, the crown is produced in great quantities and normally regarded mainly as waste, creating environmental issue. Little has known in production of cellulase and xylanase enzymes from Eupenicillium javanicum by solid-state fermentation utilizing pineapple crown leaves waste, therefore the objective of this study were: (i) to investigate the effect of temperature and pH on cellulase production, (ii) to investigate the effect of temperature and pH on xylanase production; (ii) to compare cellulase and xylanase production by SSF and SmF. It is believed that such a study will provide information on the use of pineapple crown leaves waste as the substrate used for cellulase and xylanase production by E. javanicum.

2. Materials and Methods

2.1 Microorganism
Eupenicillium javanicum InaCC F154 obtained from Indonesian Culture Collection (InaCC) Research Center for Biology, Indonesian Institute of Sciences or LIPI, was used in this study. The strain was maintained and cultured according to Tao et al. [10]. Briefly, stock cultures were maintained on potato dextrose agar slants at 4°C. Spore production was carried out from the stock cultures for period of 4 days at 28°C, which then suspended in sterile distilled water. The final concentration of fungal suspension of 10^6-10^7 spores mL^-1 was used for fermentation.

2.2. Substrate preparation
Pineapple was obtained from local market in Pekanbaru, Indonesia. After pineapple crown leaves were cut and separated from the fruits, they were washed with water. Then, it was dried in an oven at 50°C until a constant weight was obtained and further milled with a grinder [10]. Finally, it was stored in a polyethylene bag at room temperature until used.

2.3. Fermentation
The solid-state fermentations (SSF) were performed in 100 mL erlenmeyer flasks containing pretreated and dried pineapple crown leaves 2 g, defatted soybean 0.5 g, (NH₄)₂SO₄ 0.05 g, NaNO₃ 0.1 g, CaCl₂ 0.1 g, and H₂O 5 mL [10]. These flasks were sterilized at 121°C for 20 min prior to fermentation. Processes were carried out after inoculating the medium with 1 mL of the spore suspensions followed by incubation for 96 h at different temperatures (25, 30 and 35°C) and pH (4.0, 6.0 and 8.0). The optimal operating conditions obtained at SSF was then employed to submerged method (SmF) and compared. The total volume of medium used for SmF was 100 mL. All experiments were performed in triplicate.
2.4. Enzyme extraction
For SSF, enzyme extraction was started by adding 10 volumes of distilled water to the fermentation flasks. Then, the flasks contents were mixed in a rotary shaker at 30°C, 250 rpm for 1 h, from which the crude enzymes were extracted. Next, the slurries were squeezed through double cheesecloth and the resulting extracts were purified by centrifugation (3200 g, 4°C for 25 min). The clear supernatants were used for further enzyme activity analysis. Similar centrifugation process as for SSF was also performed to obtain the supernatants in SmF method.

2.5. Enzyme assays
The cellulase activity was measured by the method of Ghose et al. [11] using carboxymethylcellulose, whereas the xylanase activity was assayed by the method of Bailey et al. [12] using oat spelt xylan. The amount of reducing sugars release was determined using dinitrosalicylic (DNS) acid reagent and photometric method [13]. One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 μmol of reducing sugar from the appropriate substrate per minute under the assay conditions. The final enzyme activities were expressed as Units/mL (U/mL) according to the below equation.

\[ EA = \frac{c}{v \times t \times D_f} \]  

(1)

where \( EA \) is enzyme activity (U/mL), \( c \) is mol of glucose release (μmol), \( V \) is enzyme volume (mL), \( t \) is reaction time (min) and \( D_f \) is dilution factor.

2.6. Statistical Analysis
Differences among means were analyzed for statistical significance by Student’s t test (with significance assigned at \( p<0.05 \)).

3. Results and Discussion
3.1. Effect of temperature and pH on cellulase production
Cellulase activities at three fermentation temperatures (25°C, 30°C and 35°C) and pH (4.0, 6.0 and 8.0) are presented in Figure 1. Increasing the fermentation temperature led to an increase in the total cellulase activities produced by \( E. javanicum \) InaCC F154. When the temperature was increased from 25°C to 35°C the total enzyme activities were increased by 0.087-0.132 U/mL (\( p<0.05 \)). Maximum activities were obtained at 35°C with 0.133 U/mL, 0.261 U/mL and 0.172 U/mL at pH 4.0, 6.0 and 8.0, respectively. It has been known that temperature is a critical parameter affecting growth and enzyme production by microorganisms and this is usually varied from one organism to another. Although there still seems an increasing trend, the maximal cellulase activities observed at 35°C are in agreement with several past results. For example, Alfiah and Kuswytasari [14] showed that the optimum temperature for cellulases production from \( P. sp \), utilizing corn cob was 35°C, with activity of 0.595 U/mL. Pham et al. [15] obtained the maximal activities of cellulases i.e. 2.09 U/mL at slightly higher temperature (37°C) with \( A. niger \) VTCC-F021 using mixed agricultural residues as the substrates. Tao et al. [9] used temperatures between 25°C and 34°C to produce four feed enzymes (endoglucanase, b-glucosidase, pectinase and xylanase) by SSF and reported lower optimal temperature (30°C) from citrus processing waste and \( E. javanicum \) as the microorganism. Increasing further the fermentation temperature in this study could possibly increase the amount cellulases produced by \( E. javanicum \) InaCC F154.

The crude enzyme samples were also tested for the effect of pH on cellulase production (Figure 1). Though maximum cellulase activities were obtained at the highest temperature tested (0.261 U/mL at 35°C), it was recorded at intermediate pH i.e 6.0 instead of 8.0. Increasing pH from 4.0 to 6.0, increased cellulase activities obtained at all temperatures. However, increasing pH to 8.0 decreased the maximum activity by 0.089 U/mL. Similar to temperature, pH is an important parameter for the growth of microorganism thus fermentation or metabolic reactions leading to the production of
enzymes. It was clear from the above results that the fungal isolate was able to grow and produce cellulase over a selected pH range (4.0-8.0) and pH 6.0 was found to be the optimum pH value for the production in case of *E. javanicum* InaCC F154. Previous studies also showed that cellulase production by fungi was capable to bear the pH change of 4.0-7.0 and optimum at this range [10, 14-16]. According to Tao et al. [9] that alkaline pH could have inhibitory effect on the growth and enzyme production of an acidophilic fungi which could be the reason for the decrease in the enzyme production at pH 8.0.

![Figure 1. Effect of temperature and pH on the cellulase production by *Eupenicillium javanicum* InaCC F154.](image)

### 3.2. Effect of temperature and pH on xylanase production

Xylanase activities at three fermentation temperatures (25°C, 30°C and 35°C) and pH (4.0, 6.0 and 8.0) are illustrated in Figure 2. Similar to cellulase production, the total cellulase activities produced by *E. javanicum* InaCC F154 also increased significantly (*p*<0.05) with increasing temperature, except at pH 4.0. Maximum activities were obtained at 35°C with 1.683 U/mL and 0.915 U/mL at pH 6.0 and 8.0, respectively. These results were 5.3-6.5 times higher than cellulases, suggesting the potential of pineapple crown leaves as the substrates for xylanase production. Several past investigators reported that cellulose can induce xylanase production in *P. echinulatum* and *T. reesei* [18, 19]. Xylanases obtained from microorganism have many useful biotechnological applications such as pre-bleaching of kraft pulp, juice clarification and degumming of vegetal fibers, and improving the digestibility of animal feed [17]. The maximum xylanases obtained in this study (1.683 U/mL) were comparable to the studies performed by Savanth and Patel i.e. 1.85 U/mL using isolated *A. niger* and corncob [20], and much higher than that was reported by Jovanovic et al. [21] i.e. 0.14 U/mL with *T. reesei* QM 9414 and wheat chaff. Other authors achieved 6.47 U/mL, 1.35 U/mL and 0.39 U/mL with wheat bran, sawdust and xylan after fermentation of *Penicillium chrysogenum* PCL501 for 96-120 h [22].
Figure 2. Effect of temperature and pH on the xylanase production by *Eupenicillium javanicum* InaCC F154.

3.3. Comparison of solid-state and submerged fermentation

Figure 3 shows a comparison on the total cellulase and xylanase activities achieved by solid-state and submerged fermentation of *E. javanicum* InaCC F154 at 35°C and pH 6. It can be seen that almost no cellulases were detected after SmF in compared to SSF with 0.261 U/mL. Likewise, xylanases produced in SSF were higher than SmF by 3.4 times. These results confirmed the well adaptation of this fungi to its growth and metabolite production environments.

![Cellulases and xylanases comparison](image)

**Figure 3.** Comparison of cellulase and xylanase activities by solid-state (SSF) and submerged (SmF) fermentation at 35°C and pH 6.

4. Conclusion

The present work demonstrated that the cellulases and xylanases can be produced utilizing pineapple crown leaves waste as the substrate in solid-state fermentation. Temperatures and pH clearly affected its total activities over the selected range, in which increasing temperature increased the production of both enzymes while pH showed its maximum at near neutral pH. Solid-state is a better method than
submerged for producing cellulases and xylanases from *E. javanicum*. The results sheds lights on obtaining cheap source from agro-residues for enzyme production particularly xylanase and their application in industries which may be economically feasible.

5. Acknowledgement
The support from laboratory and administrative staff from the Chemical Engineering Department, University of Riau is appreciated.

References
[1] Oliveira F, Salgado J M, Abrunhosa L, Pérez-Rodriguez N, Domínguez J M, Venâncio A and Belo I 2017 Optimization of lipase production by solid-state fermentation of olive pomace: from flask to laboratory-scale packed-bed bioreactor *Bioproc. Biosyst. Eng* 40(7) 1123–1132
[2] Niyonzima F N, Veena S M and More S S 2020 *Microbial Enzymes: Roles and Applications in Industries* (Singapore: Springer Nature)
[3] Pandey A 1994 *Solid State Fermentation* (New Delhi: Wiley)
[4] Chen H and Wang L 2016 *Technologies for Biochemical Conversion of Biomass* (Academic Press)
[5] Londoño-Hernandez L, Ruiz H A, Toro C R, Ascacio-Valdes A, Rodriguez-Herrera R, Aguilera-Carbo A, Tubio G, Pico G, Prado-Barragan A, Gutierrez-Sanchez G and Aguilar C N 2020 *Microbial Enzymes: Roles and Applications in Industries* (Singapore: Springer Nature)
[6] Graminha E B N, Goncalves A Z L, Pirotta R D P B, Balsalobre M A A, de Silva R and Gomes E 2008. Enzyme production by solid-state fermentation: application to animal nutrition *Anim. Feed Sci. Technol* 144 1–22
[7] Singhania R R 2011 *Biofuels: Alternative Feedstocks and Conversion Processes* (Academic Press)
[8] Soccol C R, da Costa E S F, Letti L A J, Karp S G, Wociejchowski A L, Vandenberghie L P d S 2017 Recent developments and innovations in solid state fermentation *Biotechnol. Res. Innov* 1(1) 52–71
[9] Food and Agriculture http://www.fao.org/3/ca9213en/ca9213en.pdf Accessed 18th August 2020
[10] Tao N, Shi W, Liu Y and Huang S 2011 Production of feed enzymes from citrus processing waste by solid-state fermentation with *Eupenicillium javanicum* 46 1073–1079
[11] Ghose T K 1987 Measurement of cellulase activities *Pure Appl. Chem* 59 257–268
[12] Bailey M J, Biely P and Poutanen K 1992 Interlaboratory testing of methods for assay of xylanase activity J. Biotech 23(3) 257–270
[13] Miller G L 1959 Use of dinitro salicylic acid reagent for determination of reducing sugar *Anal. Chem* 31(3) 426–428
[14] Alfiah I and Kuswytasari N D 2012 Production of cellulase by *Penicillium sp.* at varied temperature, pH, and agricultural residues *Final year Report FMIPA ITS*
[15] Pham T H, Quyen D T and Nghiem N M 2010 Optimization of endoglucanase production by *Aspergillus niger* VTCC-F021 *Austr. J. Basic Appl. Sci.* 6 4151–5157
[16] Liang X, Huang Y, Hua D, Zhang J, Xu H, Li Y and Zhang X 2012 Cellulase production by *Aspergillus sp.* on rice grass (*Spartina spp.*) under solid-state fermentation *African J. Microbiol. Res* 6 6785-6792
[17] Bandikari R, Poondla V and Obulam V S R 2014 Enhanced production of xylanase by solid state fermentation using *Trichoderma koningi* isolate: effect of pretreated agro-residues *Biotech* 4(6) 655–664
[18] Aro N, Saloheimo A, Ilmén M and Penttilä M 2001 ACEII, a novel transcriptional activator involved in regulation of cellulase and xylanase genes of *Trichoderma reesei* *J. Biol. Chem* **276**(26) 24309–24314

[19] Ritter C E T, Camassola M, Zampieri D, Silveira M M and Dillon A J P 2013 Cellulase and xylanase production by *Penicillium echinulatum* in submerged media containing cellulose amended with sorbitol *Enzyme Res* 240219

[20] Savanth V D and Patel S J 2010 Enhanced production of xylanase from local fungal isolates and effectiveness in pulp treatment *Int. J Innov Res Sci Eng Technol* **2**(12) 7670-7676

[21] Jovanovic M, Vucurovic D, Vucurovic, Bajić B, Dodic S, Vlajkov V and Jevtic-Mucibabic R 2020 Optimization of the simultaneous production of cellulase and xylanase by submerged and solid-state fermentation of wheat chaff *J Serbian Chem Soc* **85**(2) 177-189

[22] Okafor U A, Emezue T N, Okochi V I, Onyegeme-Okerenta B M and NwodoChinedu S 2007 Xylanase production by *Penicillium chrysogenum* (PCL501) fermented on cellulosic wastes *African J Microbiol Res* **1**(4) 048-053