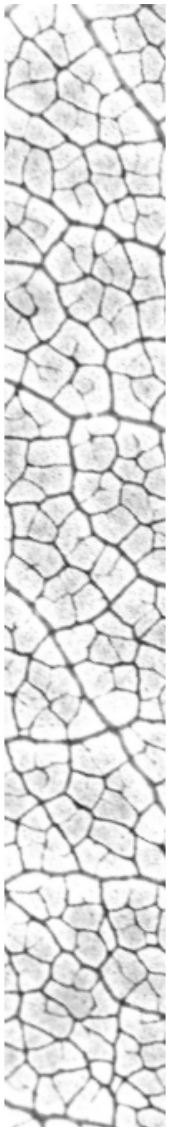

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Biochemical study of the effect of *Nephrolepis biserrata* pretreatment on exo-polygalacturonase production by *Aspergilllus niger* in solid state fermentation

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Abstract
The substrate plays an important role in solid state fermentation (SSF) in the production of exo-polygalacturonase (one type of the pectinase enzymes). The aim of this study was to investigate a pretreatment method on *Nephrolepis biserrata* leaves as a substrate for the production of exo-polygalacturonase. The pretreatment steps improved the decomposition of the lignocellulosic material in the structure of *N. biserrata* leaves as a substrate and allowed *Aspergillus niger* better access of the substrate to the enzymatic reaction. This optimised both exo-polygalacturonase activity and the growth of *A. niger* in SSF. Pretreatment was performed under alkali, acidic, autohydrolytic and control conditions. The highest exo-polygalacturonase activity was 39.16 U/g and biomass of *A. niger* was 0.39 mg/gdsf at 120 h was found for the autohydrolysis pretreatment, as compared to alkali pretreatment, acid pretreatment and control. Scanning electron microscope images showed a marked change in the physical appearance of treated *N. biserrata* leaves as compared to untreated *N. biserrata* leaves, especially following autohydrolysis pretreatment.

Keywords: pretreatment, *Nephrolepis biserrata* leaves, exo-polygalacturonase, *Aspergillus niger,* solid state fermentation, SEM

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INTRODUCTION

Pretreatment is an important step for the process of biochemical conversion of lignocellulosic biomass. It is necessary to alter cellulose biomass structure to enable the enzyme to work more effectively in converting carbohydrate polymers to simple sugars (Göçmen *et al*. 2015). Various pretreatment strategies have been developed to improve cellulose reactions and to improve the fermentation of sugar. All the pretreatment process is to be considered for increase the sugar content in enzyme hydrolysis (Brodeur 2011, Yüksel et al. 2015).

Ceballos *et al*. (2015), stated that cellulose is the main component of plant cell walls and is almost never found in nature, but rather bound to another substance, namely lignin and hemicellulose, in the form of lignocellulosic material. Young plant cell walls are composed of cellulose, hemicellulose, and pectin, and lignin is incorporated into the cell wall as the plant grows. Hemicellulose and lignin bind to phenolic compounds through covalent bonds between cellulose and lignin are not yet fully known the crystalline structure of lignin, hemicellulose and cellulose is a major obstacle concerning the hydrolysis of cellulose. Crystalline cellulose and lignin form fibrils of lignocellulosic compounds that are difficult to degrade by microorganisms.

This problem can be overcome by pretreatment. Pretreatment is a method to open up the structure of lignocellulose, usually via microorganism-mediated degradation (Harmsen *et al*. 2010). Cellulose is a polymer of glucose with α-1,4-glucoside bonds containing with about 50-90% crystallinity; the rest is composed of α-1,4 glucoside bonds in cellulose fibres that can be broken down into glucose monomers by hydrolysis or fermentation (Perruzza 2010). Hemicellulose is more easily hydrolysed by pretreatment and can be degraded into monomers of glucose, mannose, galactose, xylose, and arabinose. Lignin contributes not only to hardening cellulose microfibrils, but is also physically and chemically bonded with hemicellulose in a hydrophobic matrix. Thus, pretreatment can break bonds and lead to the degradation of lignin, which is a method used to increase

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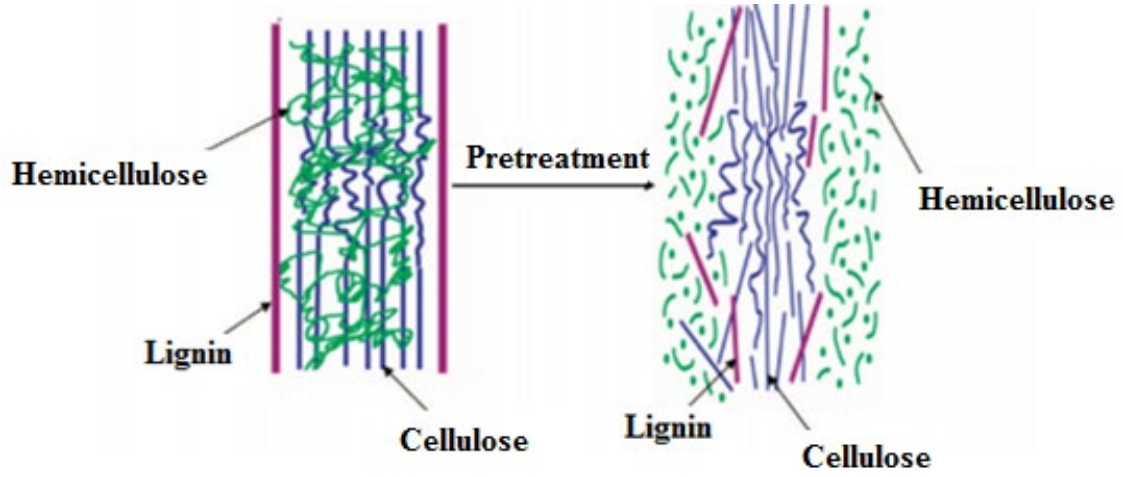


Fig. 1. The effect of pretreatment of lignocellulosic biomass (Kumar *et al*. 2009)

the access and penetration of the enzyme into the substrate (Taherzadeh and Karimi 2008).

The goal of pretreatment also includes the removal of lignin and breakdown of the cellulose crystal structure. **Fig. 1** shows the results of the pretreatment process of lignocellulosic biomass. The pretreatment process leads to an increase in the enzymatic hydrolysis of lignocellulosic materials, increases the surface area and porosity, structurally alters and removes lignin, depolymerises hemicellulose, and reduces the crystallinity of cellulose. Alkali and acid pretreatment, which are commonly used, are convenient, cheap and effective. In addition, steam explosion treatment is often also used as a pretreatment for lignocellulosic material because it does not involve chemicals and is more environmentally friendly; however, it involves the use of energy. This method is carried out at high pressure and saturated steam is injected into the reactor containing the biomass. During the injection of steam, the temperature rises to 160-260°C, and then the pressure is suddenly to allow the biomass to undergo explosive decompression, which degrades hemicellulose and lignin. It is important to note that steam explosion treatment is affected by time, temperature, particle size, and the moisture content (Agbor *et al*. 2011).

The effectiveness of pretreatment to increase the digestibility of lignocellulosic biomass depends on the structure and composition of the substrate in both pretreatments conditions. The pretreatment methods were compared regarding their ability to remove lignin from the lignocellulosic matrix (Ceballos *et al*. 2015). Usually, cellulose is the dominant fraction in the plant cell wall (35–50%), followed by hemicellulose (20–35%) and lignin (10–25%) *(*Mussato *et al.* 2012). The result of fractionation of lignocellulose content of *N. biserrata* leaves most is lignin (43.29%) pectin-oligosaccharide (34.7%), followed by cellulose (20.66%) and at least is hemicellulose (1.78%) (Pagarra *et al*. 2014). According to Essuman *et al.* (2014), found that *N. biserrata* flour contains a high carbohydrate amounting to 43.01% (g/g). In this case, the purpose of this study was to evaluate the effect of pretreatment of autohydrolysis, alkali treatment, and acid. on the structure, composition, and susceptibility to enzymatic hydrolysis on the *N. biserrata* leaves. Then dried leaves with oven was used as the control treatment.

MATERIALS AND METHODS

Pretreatment *N. biserrata* Leaves

N. biserrata leaves were obtained from palm trees at the Universiti Teknologi Malaysia, Johor Bahru, Johor, Malaysia. Three different methods of pretreatment, i.e. acid, alkali, and autohydrolysis, were performed. Oven drying the leaves at 60°C for six days was used as the control treatment in this study to solve and eliminate lignin lignocellulose. A total 10 g of dried *N. biserrata* powder was then treated with acid, alkaline and autohydrolysis treatment methods. The substrate from each pretreatment and control is used in SSF for the production of exo-polygalacturonase and biomass of *A. niger* to obtain the best pretreatment. Then the best results between these three methods are used as a substrate in the fermentation of exo-polygalacturonase and biomass of *A. niger*. Acid pretreatment was performed using hot liquid H_2SO_4 at a concentration of 0.01 M (Yang and Wyman 2009). Alkali pretreatment was performed using 0.2 N NaOH (Yoon *et al*. 2012). Autohydrolysis pretreatment was performed in an autoclave at 121°C, 15 psi (Myoung *et al*. 2008).

Enzyme Extraction

Fermented samples were collected every 24 h. At each sampling, 1 g of the sample was drawn out and mixed with 25 mL of citrate phosphate buffer (pH 4.0). The mixture was then placed on a vortex for 1 min to ensure that the enzyme or sugars on the surface were well-mixed with the buffer. Next, the suspension was centrifuged at 4000 rpm at 4°C for 20 min to separate

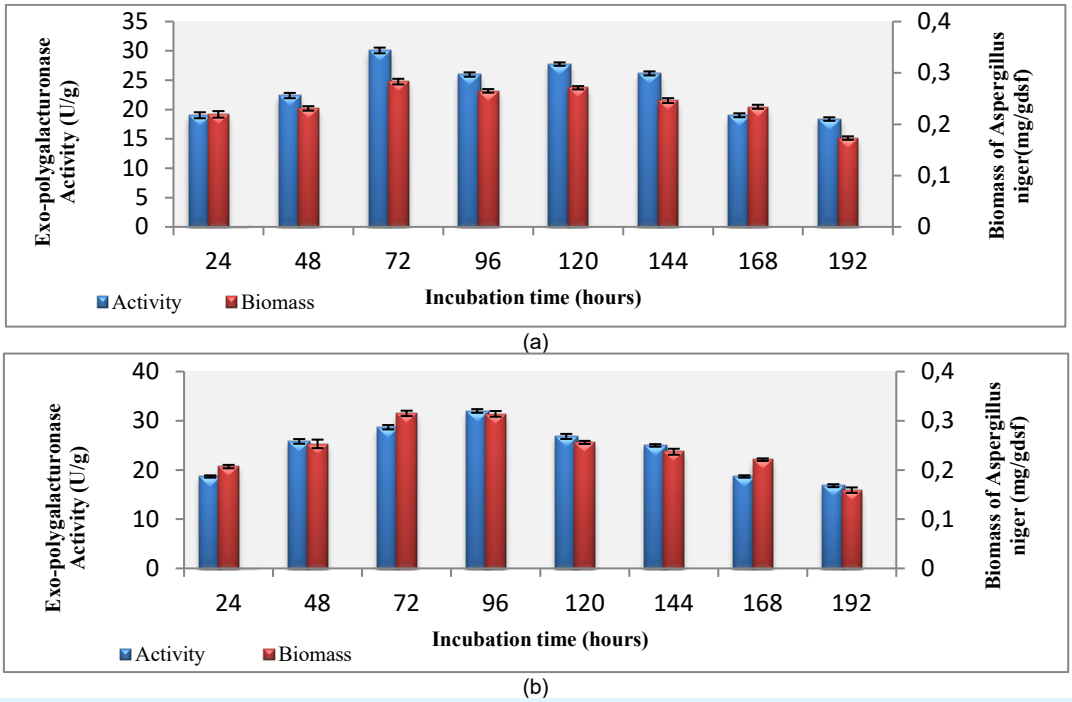


Fig. 2. Effects of pretreatment on *N. biserrata* leaves with exo-polygalacturonase activity and biomass of *A. niger* in the incubation time of 24 to 192 h: (a) NaOH pretreatment and (b) H_2SO4 pretreatment

the solid and liquid phases. The supernatant was used as the crude enzyme for various assays.

Analytical Procedures

The determination of enzyme activity was based on the DNS (dinitro salicylic acid reagent) method with some modifications. This method provides information on the carbonyl groups of reducing sugars. Exo-polygalacturonase activity is identified by a change in colour intensity using a spectrophotometer at a wavelength of 540 nm. One unit of exo-polygalacturonase is amount per µM of reducing sugars, which is measured in terms of glucose, produced as a result of the action of enzymes in the extract (1.0 mL for 1 min at 35°C ± 1°C) (Ali *et al*. 2010).

Determination of *A. niger* Biomass

The determination of biomass was carried out using the indirect biomass estimation method proposed by Ramachandran and Kurup (2013). Fungal biomass estimates were performed to determine the amount of N-acetyl-glucosamine produced by acid hydrolysis of chitin contained in the fungal cell wall. A total of 0.5 g of fermentation material was mixed with 2 mL of concentrated sulphuric acid and put in a 500 mL conical flask, the mixture was held at 30°C for 24 h. The mixture was then diluted with distilled water to make a 1 N solution and autoclave (15 psi for 1 h at 121°C) and then filtered through filter paper. This solution was then neutralised with 4 N NaOH and made up to 100 mL with distilled water. Then, 1 mL of this solution was mixed with 1 mL of acetyl acetone and incubated in a boiling water for 20 min. After cooling, 6 mL of ethanol was added, followed by the addition of 1 mL Ehrlich reagent, then incubated at 65°C for 10 min. After cooling, the optical density of the reaction mixture was read on a spectrophotometer at 530 nm. The results are expressed as milligrams of glucosamine per gram of dried substrate fermentation (mg/gdsf).

Scanning Electron Microscopy (SEM)

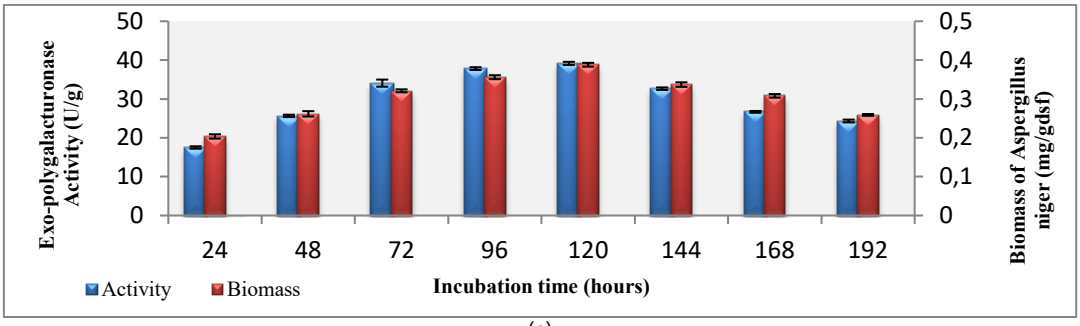
SEM analysis was conducted using VEGA TESCAN microscope. For sample preparation, the fibres were dried and mounted onto a stub. A gold coating was applied to the surface of the stub by a sputter coater.

RESULTS AND DISCUSSION

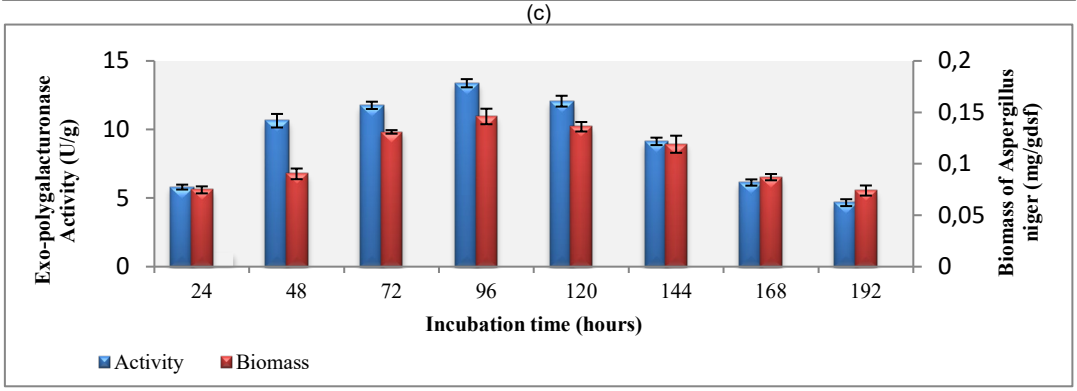
Effects of the Pretreatment of *N. biserrata* Leaves on Exo-polygalacturonase Activity and *A. niger* Biomass

The production of exo-polygalacturonase activity and *A. niger* biomass with the different pretreatment methods are shown in **Fig. 2**. Generally, the three pretreatment methods used to treat *N. biserrata* leaves led to significantly increased enzyme activity and *A. niger* biomass compared to the control treatment using a drying oven. This occurred because oven drying the leaves did not result in the destruction of structures that would allow the fungus to use the leaves as a substrate. These findings clearly show the importance of pretreatment to increase the growth rate of microorganisms and thus increase the production of enzymes.

Fig. 2 (**(a)** to **(d)**) shows the production of exo-polygalacturonase with an incubation time from 24 to



(c)



(d)

Fig. 2 (continued). Effects of pretreatment on *N. biserrata* leaves with exo-polygalacturonase activity and biomass of *A. niger* in the incubation time of 24 to 192 h: (c) Autohydrolysis pretreatment and (d) Control by using *N. biserrata* leaves pectin (oven drying)

192 h. The effects of the pretreatment of *N. biserrata* leaves at the 24-h incubation time point shows increased exo-polygalacturonase activity and *A. niger* biomass, which was almost the same for all three pretreatments, taking into account the experimental error. In this case, the cell mass increases, while the cell density remains constant. In this study, *N. biserrata* leaves treated with alkali (NaOH) (**Fig. 2(a)**) and sulphuric acid (H_2SO_4) (**Fig. 2(b)**) showed an increase in activity and total biomass of *A. niger* after 72 h of incubation (30.07 U/g; 0.28 mg/gdsf) (**Fig. 2(a)**) and 96 h of incubation (31.99 U/g; 0.31 mg/gdsf) (**Fig. 2(b)**). **Fig. 2(c)**.

In terms of enzyme activity, both pretreatments were less effective than *N. biserrata* leaves treated by autohydrolysis. Moreover, in terms of the total weight of the leaves that were treated, the chemical treatment processes were in efficient, as the total mass loss of the leaves around was 70-80% of the total leaf mass before being treated. However, the leaves that were treated by autohydrolysis only lost around 20-30% of the total leaf mass before treatment. Oven drying the leaves did not result in the destruction of structures that would allow the fungus to use the leaves as substrate.

The chemical treatments (acid and alkali) destroyed most of the leaves and required the use of water in the washing process after treatment. In addition, the chemical pretreatments had some negative impact on the operating efficiency, energy, and material costs due to erosion of the chemical pretreatment equipment, although these methods were very effective at breaking lignin and hemicellulose and cellulose outline, leading to improved enzymatic hydrolysis (Yang *et al.* 2011). The removal of lignin increased the surface area and porosity of the leaves and further facilitated the growth and reproduction of microorganisms, thus improving enzymatic hydrolysis (Canilha *et al.* 2012).

Alkali treatment of *N. biserrata* leaves produced the optimum exo-polygalacturonase level within 72 h, while sulphuric acid pretreatment and the drying oven methods were slower, requiring 96 h. Pretreatment is necessary because the treated leaf structure (substrate) are more suitable to be used for the growth of *A. niger* and the secretion of enzymes. Pretreatment in the oven drying the leaves (control) led to lower activity compared to the other methods, with exo-polygalacturonase activity of 13.374 U/g; the autohydrolysis pretreatment generated the highest exo-polygalacturonase activity at 39.16 U/g and biomass of *A. niger* at 0.39 mg/gdsf in 120 h. A reduction in exo-polygalacturonase activity was observed with all pretreatment methods after 120 h. The reduction in exo-polygalacturonase activity and biomass of *A. niger* after the optimal incubation time. Increased and decreased production of enzyme-polygalactonase enzymes closely related to the amount of *A. niger* biomass. Increased activity of the enzyme-polygalacraconase enzyme is followed by the rapid and large growth of *A. niger*. The rapid growth of *A. niger* and the number of cells is increasing as this period is called the exponential phase and subsequently the decreased production of exo-polygalacturonase is followed by the

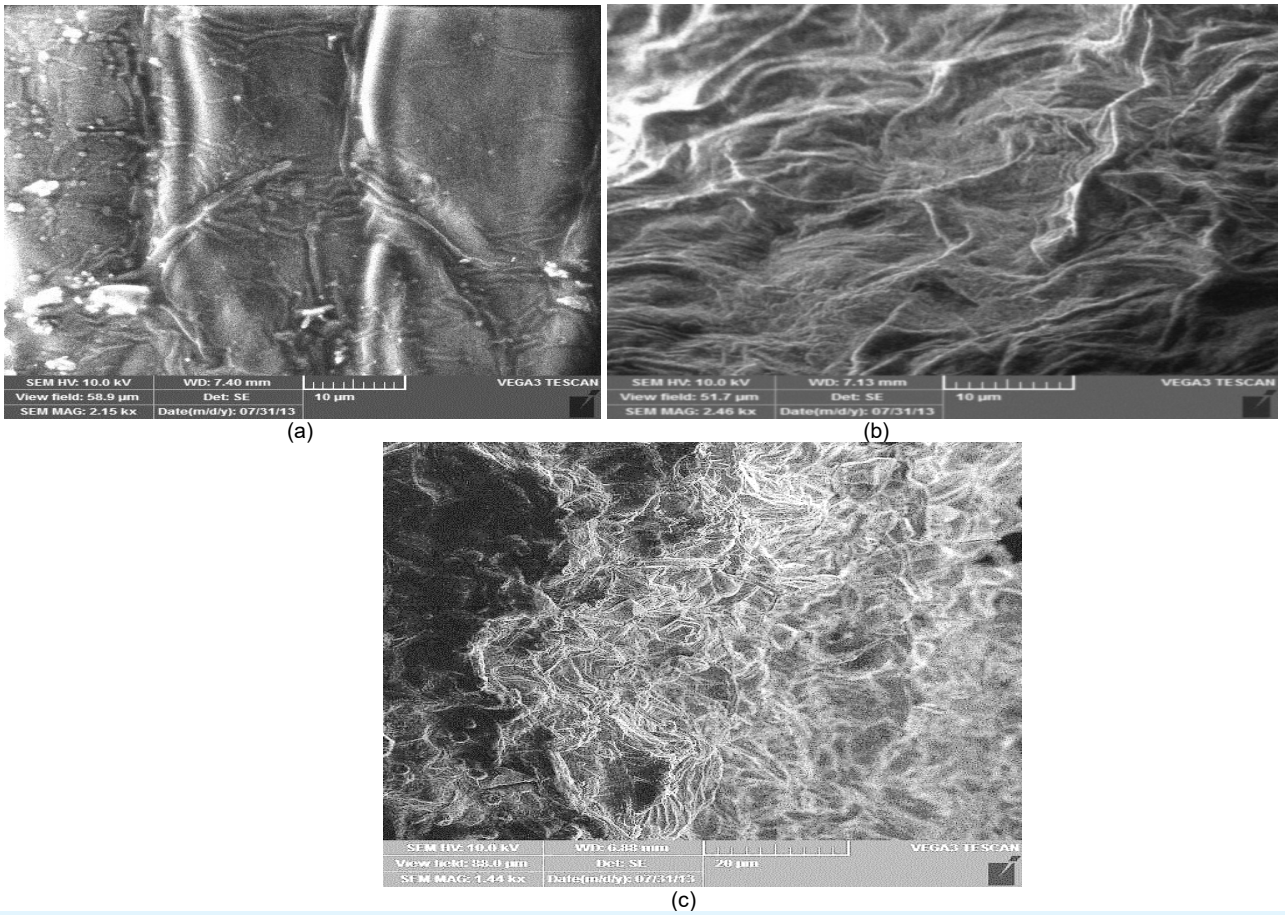


Fig. 3. SEM micrograph of the leaf *N. biserrata*, (a) fresh leaves, (b) pretreatment of auto hydrolysis, and (c) leaves fermented in the solid state

reduced amount of *A. niger* biomass. According to Vaughan (2005), that after the lag phase, where cells adapt to the new environment will begin to multiply rapidly and so the cell count will increase rapidly with time. Thus, this period is called the exponent or logarithm of the growth phase. The next phase is the gel phase in which cell division begins to decline and progressively gradually due to a deficiency of one or more important nutrients or also to the collection of toxic by-products of the growth. The next is the death phase in which the dead cells are released into the medium.

Effects of Pretreatment on the Structure of *N. biserrata* Leaves

A comparison of the structure of fresh *N. biserrata* leaves and the leaves after pretreatment, after autohydrolysis, and after fermentation was performed using SEM micrographs. Significant differences were seen, as the fresh leaf structure had a cohesive and unbroken surface. This is because the fresh leaves contained intact lignin in a natural polymer complex that provided a bond between the walls of the leaf cells.

Pretreatment was required for the production of exo-polygalacturonase in this study. The autohydrolysis treatment, both before and after the fermentation process (**Fig. 3**), led to a modified, broken structure that was split and hollowed. This effect was caused by the removal of lignin, which opened spaces and made the substrate accessible to microorganisms for use as a substrate in SSF. Pretreatment broke the connections between the external surface and the internal structure of *N. biserrata* leaves and shows the structure of microfibrils were clearer than untreated *N. biserrata* leaves. Microfibrils of hemicellulose and cellulose were separated from the initial connecting structure and were completely exposed and ready for the enzyme reaction. The pretreatment results of autohydrolysis showed a decrease in lignin content and increased cellulose and hemicellulose content. Results before pretreatment by Pagarra et al., 2014, ie lignin 43.29% decreased to 16.5%, cellulose 20.66% to 37.3% and hemicellulose from 1.78% to 26.7%. Hemicellulose and cellulose were then degraded and converted into fermentable sugars by *A. niger*, because *Aspergillus sp*. is one of the main agents of decomposition and decay of lignoselulose residues and has the ability to produce various types of enzymes and at high glucosidase levels to complete the conversion of lignosellulose biomass to simple sugars (Mostafaei *et al*. 2014). One typical index used to evaluate the performances of the cellulase and

hemicellulose preparations during fermentation is the conversion rate to say the obtained glucose concentration per time required to achieve it. As Ali *et al*. (2000) stated before, that one enzyme unit rather than ekso-polygalacturase is a µM quantity of reducing sugar measured in terms of glucose, produced as a result of the action of 1.0 ml of enzyme extract in 1 min.

Various pretreatment processes have been reported for the treatment of lignocellulosic materials. Autohydrolysis pretreatment of wheat straw waste is best to obtain fermentable sugars as a raw material for bioethanol production (Tutt *et al*. 2014). In addition, the autohydrolysis pretreatment process was also found to improve the enzymatic hydrolysis of bulrush stems and sweet grass (Bohorquez *et al*. 2014, Tao *et al*. 2011). Autohydrolysis pretreatment can also dissolve most of the hemicellulose and cellulose and improve the digestibility for fermentation to produce ethanol from wheat straw. Autohydrolysis is considered as one of the best pretreatment technologies and has been used for the pretreatment of various forms of lignocellulosic material (Özel *et al*. 2017).

The advantages of autohydrolysis in industrial research and technology include the ability to hydrolyse more hemicellulose and the reduction in the complexity of cell wall components by degradation. During the autohydrolysis process, water vapour rapidly escapes from inside and between cells, thus destroying cell walls and tissue connections (Chen and Qiu 2010).

As a result, a porous or hollow structure is formed after this process and the surface area of the lignocellulosic material is increased, thus increasing the mass transfer rate (Zhang and Chen 2012). Furthermore, the purity of the product can be enhanced after separating the hydrolysate of hemicellulose and lignin from other water-soluble components (Peng and Chen 2012). Therefore, it is clear that autohydrolysis pretreatment is effective and efficient without the need for additional chemicals; moreover, the process is easy to run (Chen and Qiu 2010). This study shows that autohydrolysis pretreatment increases the efficiency of the production of exo-polygalacturonase by *A. niger*, while a thermal pretreatment, such as using a oven drying, is less suitable for enzyme production, which was three-fold lower than with the autohydrolysis method. Based on these results, it is clear that the pretreatment step can change the structure of *N. biserrata* leaves and provide better access to the enzymatic reaction of the substrate and thus improve the process of decomposition of lignocellulosic materials. According to Darah *et al*. (2016), that lignocellulose containing large amount of celluloses, hemicelluloses and pectin, that could serve as a carbon and inducer source for the production enzymee.

CONCLUSION

Autohydrolysis is the best way to alter the physical structure of *N. biserrata* leaves, and leads to higher exo-polygalacturonase activity by *A. niger*. Exo-polygalacturonase activity with autohydrolysis was 39.16 U/g and the biomass of *A. niger* was 0.39 mg/gdsf after 120 h. Pretreatment of *N. biserrata* leaves is important to enhance and accelerate the exo-polygalacturonase substrate reaction to obtain products.

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REFERENCES

Agbor V, Cicek N, Sparling R, Berlin A, Levin DB (2011) Biomass Pretreatment: Fundamentals toward Application. Biotechnology Advances, 29: 675-85. https://doi.org/10.1016/j.biotechadv.2011.05.005

Ali J, Ahmed JS, Assad Q, Hussain A, Abid H, Gul F (2010) Optimization of pectinase enzyme production using sour oranges peel (Citrus aurantium L) as substrate. Pak. Journal Biochemistry Mololeculer Biology, 43(3): 126-30.

Bohórquez C, Amado-González E, Martínez-Reina M (2014) Effect of Pretreatment Dilute Acid - Peroxide on Napier Grass (pennisetum purpureum schumach) to Enhance Reducing Sugar Yield by Enzymatic Hydrolysis. Avances Investigación en Ingeniería, 11(2): 48-55. https://doi.org/10.18041/1794-4953/avances.2.226

Brodeur G, Yau E, Badal K, Collier J, Ramachandran KB, Ramakrishnan S (2011) Chemical and physicochemical pretreatment of lignocellulosic biomass: A review. Enzyme Res: 787532. https://doi.org/10.4061/2011/787532

Canilha L, Kumar AC, Suzane TSM, Antonio FFA, Luiz WCF, Gracas MAF, Silverio SS (2012) Bioconversion of sugarcane biomass into ethanol: an overview about composition, pretreatment methods, detoxification of hydrolysates, enzymatic saccharification, and ethanol fermentation. Journal of Biomedicine and Biotechnology: 1-15. https://doi.org/10.1155/2012/989572

Ceballos RM, Batchenkova NA, Chan MK, Duffing-Romero A, Nelson AE, Man S (2015) Bioethanol: feedstock alternatives, pretreatments, lignin chemistry, and the potential for green value-added lignin co-products. Journal Environmental Analytic Chemical, 2(5): 1-25.

Chen HZ, Qiu WH (2010) Key technologies for bioethanol production from lignocellulose. Biotechnology Advances, 28(5): 556-62. https://doi.org/10.1016/j.biotechadv.2010.05.005

Darah I, Taufiq MMJ, Hong Lim S (2016) Pectinase production by Aspergillus niger LFP-I using pomelo peels as substrate. Indian Journal of Biotechnology, (14): 552-8.

Essuman EK, Ankar-Brewoo GM, Barimah J, Ofosu IW (2014) Functional properties of protein isolate from fern fronds. International Food Research Journal, 21(5): 2085-90.

Göçmen JS, Bozdoğan B, Bozdoğan ME, Çolak Ş, Kılıç A, Sungur E (2015) In vitro investigation of effects of commercially available food and fabric paints in different colors, on Methicillin-resistant Staphylococcus aureus and Pseudomonas aeruginosa. Journal of Clinical and Experimental Investigations, 6(3): 274-278. https://doi.org/10.5799/ahinjs.01.2015.03.0532

Hamed M, Mojtaba H, Hossein J (2014) The Investigation of the Relationship between the Aggression and Addiction Potential High-school Male Students. UCT Journal of Management and Accounting Studies, (1): 01-4.

Harmsen P, Huijgen W, Bermudez L, Bakker R (2010) Literature review of physical and chemical pretreatment processes for lignocellulosic biomass. Wageningen UR Food and Biobased Research.

Kumar P, Barrett DM, Delwiche MJ, Stroeve P (2009) Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Industrial and Engineering Chemistry Research: 3713–3729. https://doi.org/10.1021/ie801542g

Mussato SI, Ballesteros LF, Martins S, Teixeira JA (2012) Use of Agro-Industrial Wastes in Solid State Fermentation Processes. In: Kuan-Yeow Show and Xinxin Guo. (Ed). Industrial Waste. InTech., Croatia: 126-140.

Myoung JL, Shi J, Venditi RA, Jameel H (2008) Autohydrolysis pretreatment of coastal Bermuda grass for increased enzyme hydrolysis. BioResources Tecnology, 100(24): 6434-41. https://doi.org/10.1016/j.biortech.2008.12.068

Özel BA, Aksel G, Kılıçlı E, Muratoğlu M, Kavalcı C, Gülalp B, Kayipmaz AE (2017) Metoclopramide-induced acute dystonic reaction misinterpreted as conversion disorder and seizure. European Journal of General Medicine, 14(4): 122–4. https://doi.org/10.29333/ejgm/81746

Pagarra H, Rahman RA, Jusoh M (2014) Isolation of pectin from Nephrolepis biserrata leaves at different extraction time. Jurnal Teknologi (Sciences & Engineering), UTM Malaysia, 69(5): 17–9. https://doi.org/10.11113/jt.v69.3196

Peng XW, Chen HZ (2012) Hemicellulose sugar recovery from steam exploded wheat straw for microbial oil production. Process Biochemistry, 47(2): 209-15. https://doi.org/10.1016/j.procbio.2011.10.035

Perruzza LA (2010) Exploring Pretreatment Methods and Enzymatic Hydrolysis of Oat Hulls. Master of Applied Science Graduate Department of Chemical Engineering and Applied Chemistry University of Toronto.

Ramachandran S, Kurup G (2013) Screening and isolation of pectinase from fruit and vegetable wastes and the use of orange waste as a substrate for pectinase production. International Research Journal of Biological Sciences, 2(9): 34- 39.

Taherzadeh MJ, Karimi K (2008) Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review. International Journal of Molecular Sciences, 9: 1621-51. https://doi.org/10.3390/ijms9091621

Tao L, Aden A, Elander RT, Pallapolu VR, Lee YY, Garlock RJ, Balan V, Dale BE, Kim Y, Mosier NS, Ladisch MR, Falls M, Holtzapple MT, Sierra R, Shi J, Ebrik MA, Redmond T, Yang B, Wyman CE, Hames B, Thomas S, Warner RE (2011) Process and technoeconomic analysis of leading pretreatment technologies for lignocellulosic ethanol production using switchgrass. Bioresources Technology, 102: 11105–14. https://doi.org/10.1016/j.biortech.2011.07.051

Tutt M, Kikas T, Olt J (2012) Influence of different pretreatment methods on bioethanol production from wheat straw. Agronomy Research. Biosystem Engineering Special Issue, 1: 269-76.

Vaughan KT (2005) Microtubule plus ends, motors, traffic of Golgi membranes. Biochimica et Biophysica Acta 1744(3): 316-24. https://doi.org/10.1016/j.bbamcr.2005.05.001

Yang B, Wyman CE (2009) Dilute acid andautohydrolysis pretreatment. Methods in Molecular Biology, 581: 103-14. https://doi.org/10.1007/978-1-60761-214-8_8

Yang B, Ziyu Dai, Shi-You D, Wyman CE (2011) Enzymatic hydrolysis of cellulosic biomass. Biofuels, 2(4): 421-50. https://doi.org/10.4155/bfs.11.116

Yoon LW, Cheng GN, Seak AMC (2012) Simultaneous production of cellulase and reducing sugar from alkali-pretreated sugarcane bagasse via solid state fermentation. BioResources, 7(4): 5319-32. https://doi.org/10.15376/biores.7.4.5319-5332

Yüksel R, Gürol G, Akkılık ZS, Sarı S, Arabacı S, Güneri D, et al. (2015) The expression of Fetuin-A in brain tissues of WAG/Rij Rats, genetic rat model of absence epilepsy. J Clin Exp Invest., 6(4): 387-90. https://doi.org/10.5799/ahinjs.01.2015.04.0554

Zhang YZ, Chen HZ (2012) Multiscale modeling of biomass pretreatment for optimization of steam explosion conditions. Chemical Engineering Science, 75: 177-82. https://doi.org/10.1016/j.ces.2012.02.052