Utilization of *Moringa oleifera* seed waste as substrate in lipase production under different pretreatments

A R Irfansyah1, M P Koentjoro2, Isdiantoni3, I Ekawati3 and E N Prasetyo1

1 Department of Biology, Institut Teknologi Sepuluh Nopember, Surabaya, 60111 Indonesia
2 Faculty of Health, Nahdlatul Ulama University of Surabaya, Surabaya, 60237 Indonesia
3 Faculty of Agriculture, Wiraraja University, Sumenep, 69451 Indonesia

Abstract. Moringa industry in Sumenep Regency experienced growth in recent years along with the increase in export demand. Moringa seed waste, which is the coproduct of oil extraction contains a massive amount of lipid and biomass. As much as 30% of lipid residue from *Moringa oleifera* seeds waste are potentially usable as a substrate for lipase production. However, the phenolic compounds contained in the waste are difficult to degrade and have antimicrobial property which is needed to be removed. This study aims to determine the best pretreatment method in removing phenolic content in Moringa seed waste. The proposed pretreatment methods in this study including enzymatic pretreatment, Laccase Mediator System pretreatment, basic chemical pretreatment, acidic chemical pretreatment, and peroxide pretreatment. Lipase production carried out using *Bacillus* sp. SK II-5 isolates. Total protein content of lipases produced from various pretreatment then measured using the Bradford method. Lipase enzymatic activity determined qualitatively using the Quantofix formaldehyde test. Characterization of lipase based on isoelectric point. The result of this study is that laccase pretreatment is the best pretreatment method which capable of reducing total phenol concentration in Moringa seed waste by 70% to 3.87 mgGAE/g. The results were followed by a high total protein concentration of 0.43 mg/ml and lipase activity in the range of 100-200 ppm formaldehyde.

1. Introduction
The Moringa industry in Sumenep Regency experienced growth in recent years along with the increase in export demand. The main products of the moringa industry are moringa powder and moringa oil [1]. Oil is obtained by extracting oil from the seeds. The extraction process using the cold pressing method leaves oil residue of around 30% of the dry weight of the seed waste [2,3]. *M. oleifera* has an oil content of about 36.7% by weight of seeds, *M. oleifera* seed oil or known as Ben Oil contains high levels of monounsaturated fatty acid (MUFA) with a concentration of around 76.73%. Of the total fatty acids content, there are about 73.57% oleic acid [2,3]. Oleic acid has good oxidative stability compared to polyunsaturated fatty acids (PUFA) so it is often used in the food industry because it has the advantage of longer storage and high-temperature frying processes [4]. Apart from the oil, Moringa seeds have 31.4% of protein content and 18.4% of carbohydrates. Nutrient content and oleic acid content in Moringa seed waste have the potential to be used as a substrate in lipase production. Gupta [5] states that good compounds used as substrates in lipase production are compounds containing triglycerides which are long-chain fatty acids.

Lipase (EC 3.1.1.3) or triacylglycerol acyl hydrolase is a class of hydrolase enzymes that catalyzes the hydrolysis of triglycerides into glycerol and free fatty acids [6,7]. Lipases can be produced from...
microorganisms and are more desirable in industry because of their stability, selectivity, and more specifically to broad substrates [8,9].

Lipases are mostly active in organic solvents that catalyze several reactions including esterification, transesterification, regioselective acylation of glycol and menthol, and peptide synthesis. Lipase application occurs in organic chemical processes, detergent formulations, biosurfactant synthesis, oleochemical industries, pharmaceutical processes [6,10]. Research conducted by Hidayah [11] shows the ability of Bacillus sp. SK II-5 for producing lipases. The obtained activity value reaches 3000 U/ml by using visceral substrate waste of abattoir. M. oleifera seed waste (MSW) can be a good substrate in the production of lipase enzymes but has a phenolic content that has a functional group that is difficult to degrade and is an antimicrobial compound such as gallic acid, ellagic acid, and caffeic acid, also found p-coumaric acid, acid vanilla, protocatechuic acid, ferulic acid, and cinnamic acid in small quantities [12,13].

Degradation of phenolic compounds on a substrate can be done by oxidation of hydroxyl groups in aromatic benzene rings and deformation of aromatic benzene ring structures [14]. Therefore, in this study several pretreatments were conducted to find out the optimal method in removing phenolic compounds in MSW. Types of pretreatment used including enzymatic oxidation using laccase, chemical enzymatic oxidation using Laccase Mediator System (LMS), chemical oxidation using hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), chemical hydrolysis using sulfuric acid (H\textsubscript{2}SO\textsubscript{4}) and sodium hydroxide (NaOH) [15,16].

2. Methods

2.1. Culture Preparation
Bacteria used in this research are Bacillus sp. SK II-5 collection of Microbiology and Biotechnology Laboratory, Department of Biology ITS. Isolate of Bacillus sp. SK II-5 is subcultures on Nutrient Broth (NB) medium. Acclimatization is carried out on a fermentation medium with 10% cooking oil as a substrate.

2.2 Substrate Preparation and Pretreatment
Types of pretreatment used include: enzymatic oxidation using laccase, chemical enzymatic oxidation using Laccase Mediator System (LMS), chemical oxidation using hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), chemical hydrolysis using sulfuric acid (H\textsubscript{2}SO\textsubscript{4}) and sodium hydroxide (NaOH)

2.2.1 Enzymatic pretreatment
The pretreatment was carried out at 20% (w/v) ratio of MSW to 50mM buffer citrate solution pH 5 containing 20 U/ml crude laccase of Trametes versicolor. The incubation process occurred 24 hours long. The substrate was filtered and the pH was adjusted to 7. Finally, the substrate was dried out using an oven at 40°C [17].

2.2.2 Laccase Mediator System (LMS) pretreatment
The pretreatment was carried out at 20% (w/v) ratio of MSW to 50mM buffer citrate solution pH 5 containing 20 U/ml crude laccase of Trametes versicolor. 10% of 0,1 mM of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) or ABTS were added to the solution as mediators. The incubation process occurred 24 hours long. The substrate was filtered and the pH was adjusted to 7. Finally, the substrate was dried out using an oven at 40°C [17].

2.2.3 Basic chemical pretreatment
The pretreatment was carried out at 20% (w/v) ratio of MSW to 2% of NaOH solution. The suspension was heated at 121°C as long as 20 minutes. The substrate was filtered and the pH was adjusted to 7. Finally, the substrate was dried out using an oven at 40°C [16].
2.2.4 Acidic chemical pretreatment
The pretreatment was carried out at 10% (w/v) ratio of MSW to 2% of H$_2$SO$_4$ solution. The suspension was heated at 121°C as long as 20 minutes. The substrate was filtered and the pH was adjusted to 7. Finally, the substrate was dried out using an oven at 40°C [18].

2.2.5 Oxidative pretreatment
The pretreatment was carried out at 10% (w/v) ratio of MSW to 2% of H$_2$O$_2$ solution. The suspension was heated at 121°C as long as 20 minutes. The substrate was filtered and the pH was adjusted to 7. Finally, the substrate was dried out using an oven at 40°C [19].

2.3 Lipase Production
4% of Bacillus sp. SK II-5 starter cultures which had absorbance values of 0.6 at a wavelength of 600 nm spectrophotometer were inoculated on a fermentation medium with a glucose composition (0.2%); peptone (0.05%); cooking oil (1%); KH$_2$PO$_4$ (0.1%); K$_2$HPO$_4$ (0.3%); Na$_2$SO$_4$ (0.2%); MgSO$_4$·7H$_2$O (0.01%); Arabic gum (0.1%); tween 80 (0.1%); pH 7. Cooking oil can be substituted with M. oleifera waste powder which has an oil content of 30% per dry weight. Production is carried out for 10 hours at a speed of 150 rpm with a temperature of 30 ° C. Every 2 hours sampling and centrifuging at 4 °C and speed of 10000 rpm [20]. Supernatant containing crude lipase was added with glycerol and stored at 0 ° C for further testing [21].

2.4 Total Protein Content Assay
Total protein levels were carried out by the Bradford method using bovine serum albumin (BSA) as a standard. Bradford reagent was prepared by dissolving 0.1 g of Coomassie Brilliant Blue (CBB) G-250 with 50 ml of 95% ethanol, adding 100 ml of 85% phosphoric acid and 850 ml of distilled water. Measurement of the protein content of the sample was conducted by reacting 0.1 ml of the enzyme sample solution with 5 ml of Bradford solution. The solution was homogeneous using vortex and incubated at room temperature for 2 minutes in dark conditions. The solution was measured its absorbance value by UV-Vis spectrophotometer at a wavelength of 595 nm [22].

2.5 Isoelectric Point
The isoelectric point is measured by reacting to each 1ml crude lipase with 1ml acetate buffer with pH 3,4,6,7, and 8. The reaction was carried out in a water bath for 10 to 30 minutes. The fastest precipitate formation in solution is the enzyme isoelectric point [23].

2.6 Lipase Activity Determination
Lipase activity was measured qualitatively using the Quantofix Formaldehyde test strips of the Machener-Nagel (MN) brand. The determination of lipase activity begins by dissolving 1 ml of triglyceride (TG) with 9 ml of pH 8 buffer phosphate solution containing 0.44% tween 80 and 0.11% Arabic gum. Lipase reaction was carried out by adding 0.1 ml of crude lipase with 0.9 ml of substrate solution at 30 °C and then incubated for 10 minutes.

Determination of formaldehyde was carried out by making periodate reagents. Sodium periodate was added to distilled water until it reaches a concentration of 18 mg/ml then 10% (v/v) acetic acid was added until it was homogeneous. Next is 77mg/ml of ammonium acetate was added to the mixture. A total of 200 μl of the sample was reacted with 200 μl of periodate reagent and incubated for 10 minutes then 1 drop of Formaldehyde-1 (Quantofix) solution was added and re-homogenized. The formaldehyde test strips are then dipped in the solution and drained. The strip is left to oxidize for 1 minute then the color formed is adjusted to the available color parameters [21,24].

2.7 Total Phenolic Content Assay
Total phenol levels were measured using the Folin-Ciocalteu method with standard gallic acid curves [25]. A total of 100 mg of substrate was dissolved with distilled water to a volume of 10 ml to obtain a
concentration of 10 mg/ml. 1 ml of suspension is added with distilled water until it reaches a volume of 10 ml and a concentration of 1 mg/ml is obtained. 0.2 ml of suspension was added to 15.8 ml of distilled water and 1 ml of the Folin-Ciocalteu reagent then homogenized and incubated for 8 minutes. Na₂CO₃ was added then incubated for 2 hours at room temperature. The absorbance of each solution was measured by a UV-Vis spectrophotometer at a wavelength of 765 nm. Total phenol levels can be determined from standard gallic acid curves [26,27].

3. Results and Discussions

3.1. Substrate Optimization
This research was initiated by giving pretreatment to MSW with different agents. The results of each pretreatment were determined by quantifying the residue of the total phenolic contained to each substrate compared to positive control as well as the negative control. Effectiveness of the pretreatment process resulting in lower total phenolic content. Table 1. Shows the total phenolic content of the pretreated substrate,

| Pretreatment | Total Phenol (mg GAE/g) |
|--------------|-------------------------|
| K (+)² | 10.08 ± 0.007 |
| K (-)² | 4.84 ± 0.002 |
| NaOH | 3.87 ± 0.002 |
| H₂SO₄ | 7.84 ± 0.004 |
| LMS | 7.67 ± 0.001 |
| Laccase | 3.17 ± 0.001 |

² Positive control, cooking oil as a substrate.
² Negative control, unpretreated substrate.

Figure 1. Visualization of substrate after pretreatment process:
(a) K (-) negative control, (b) NaOH, (c) H₂O₂, (d) H₂SO₄, (e) LMS, (f) Laccase

Pretreatment variations in this study interprets the high rate of delignification process due to a decrease in the total phenolic content in MSW. This is because lignin which the most abundant
component composing the seed are indicated as phenolic compounds. The statement following [28,29] the reduced phenolic compound is a result of the delignification process.

An interesting thing happened in the pretreatment using LMS which showed that the total phenol levels were much higher compared to the laccase pretreatment. ABTS was used as a mediator in this research which provides more accessibility of laccase to its particular substrate. LMS enables oxidizing not only phenolic compounds but also some lipids with aromatic groups such as oleic acid and linoleic acid according to the statement [30], unique ability did not occur in the oxidation of laccases in the absence of a mediator.

Figure 1 shows that pretreatment using laccase oxidation produced a darker color than other pretreatment groups. This shows that the action of laccase on MSW targeting aromatic phenolic molecules that produce radicals. Hydroxy radicals in phenolics will produce darker pigmentation color, due to the delocalization of electrons in the aromatic ring [17]. Otherwise, pretreatment by the hydrolysis method, both acidic and basic, produces brighter color characters and relatively lighter masses. This is due to the breaking of the glyceryl-β-aryl (β-O-4) ether bond and the diaryl ether bond (4-O-5) in the lignin structure [31,32].

3.2. Lipase Production
Production of lipase made through fermentation techniques by isolates of Bacillus sp. SK-5 on the production medium containing pretreated MSW and cooking oil as positive control. Lipase harvest time is based on the growth profile of Bacillus sp. SKII-5 in cooking oil medium consisting of lag phase, logarithmic phase, and stationary phase.

![Figure 2. The graph demonstrates the growth curve (OD600nm) of Bacillus sp. SK II-5 to protein concentration (mg/ml) under several substrates](image)
Figure 2 shows that pretreatment using acids and alkaline produces a growth profile that is in harmony with total protein production. Increased protein concentrations in the logarithmic phase until the beginning of the stationary phase indicate the use of carbon sources other than lipids which is contained in the substrate. This is inversely proportional to the profile generated at K (+) which shows an increase in the stationary phase.

As stated by Bora and Bora [33] that the increase in enzyme production is due to lipids as a substrate resulting in the breakdown of lipids into fatty acids which are used as the main carbon source. In contrast, the growth profile and protein production in the experimental group using laccase and LMS promoting protein production in the stationary phase of growth. Thus, it can be predicted that the proteins in the laccase and LMS experimental groups are secondary metabolites, one of which is lipase protein.

Based on studies from [34,35,36], it shows that acid and alkaline hydrolysis will produce precursors and some dissolved reducing sugars which are predominant for primary metabolite production, whereas oxidation by the enzymatic laccase system only reduces the total amount of phenols which had antimicrobial property so that the content protein increases occurred in the stationary phase.

Figure 2

![Graph showing protein concentration](image)

**Figure 3.** Bar graph demonstrates the total protein content of the pretreated substrates

Figure 3 shows that the highest total protein concentration was obtained through a NaOH pretreatment agent with a concentration of 0.50 mg/ml then followed by a Laccase and H₂O₂ pretreatment agent with concentrations of 0.43 mg/ml and 0.40 mg/ml.

When referring to Table 1, the lower total phenol in the pretreatment group should be able to increase the production of enzyme proteins because the rate of antimicrobial inhibition due to phenol is lower [37]. This is not observed in Figure 2, considering the value of the protein content of the alkaline pretreatment production is not directly proportional to the total residual phenol. Pretreatment using alkaline compounds and strong acids can damage the structure of lignin and hemicellulose thereby increase the accessibility of microbes to the particular substrate. Therefore, the measured protein cannot be ascertained to contain the target protein in this case lipase protein. In the pretreatment of laccase, H₂O₂, and LMS, shown in Figure 3, the oxidation target molecule is a phenolic compound such as lignin. Decreasing the number of phenolic compounds that can act as antimicrobials triggers an increase in the production of target lipase proteins during fermentation [38].

### 3.3 Lipase Activity

Confirmation of lipase activity can be observed qualitatively using the *QuantoFix* Formaldehyde test. Lipase works hydrolyzing lipids into fatty acids and glycerol. Glycerol that yielded during the reaction oxidized by periodate to produce formaldehyde. Accumulation of formaldehyde reacts to the strips...
forming purple color. Figure 4 shows that laccase oxidation gives the Quantofix strip color with the highest intensity in the range of 100-200 ppm formaldehyde. The results of this qualitative measurement prove that higher protein concentrations in NaOH pretreatment are not entirely desirable target proteins, lipase because the color formed on the Quantofix strip is not darker than the pretreatment of laccase. This is an early indication that protein lipase has a high-performance value.

![Figure 4](image)

**Figure 4.** Visualization of qualitative lipase activity assay using the Quantofix Formaldehyde Test Kit. (a) K(+), (b) K (-), (c) NaOH, (d) H2O2, (e) H2SO4, (f) LMS, (g) Laccase, (h) Marker of formaldehyde concentration (ppm)

### 3.4 Lipase Characterization
Characteristics of lipase were analyzed through the isoelectric point (pI). In general, amino acids in protein have a range of different isoelectric values. Based on the test results, the precipitated lipases precipitate at pH 4, 5, and 6. The highest and fastest deposits are at pH 5 shown in Figure 6. This is because the amount of positive and negative protein loads is the same, resulting in the deposition of proteins at that pH. The isoelectric point of lipase produced by the genus Bacillus is reported to occur at pH 4.66; 5.15; and 6 [17, 39, 40, 41].

### 4. Conclusions
This study concludes that laccase is the best pretreatment agent that can reduce the total phenol in *M. oleifera* seed waste by 70% to 3.8 mgGAE/g. These results were followed by a total concentration of lipase protein of 0.43 m/ml. The high total protein concentration was confirmed qualitatively by the presence of lipase activity in the range of 100-200 ppm formaldehyde.

Based on research that has been done, it can be submitted a suggestion, which is carried out a quantitative test of lipase activity using para-nitrophenyl palmitate (pNPP) and measurement of protein molecular weight using SDS-PAGE.

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References

[1] GIZ. (2013). Moringa Products: Opportunities and Challenges for Mozambique.
[2] Leone, A., Spada, A., Battezzati, A., Schiraldi A., Aristil, J. and Bertoli, S. (2016). “Moringa oleifera seeds and oil: characteristics and uses for human health”. Int. J. Molecular Sciences. 17(12):1–14.
[3] Ogunsina, B.S., Indira, T.N., Bhatnagar, A.S., Radha, C., Debnath, S., Krishna, A.G. (2014). “Quality characteristics and stability of Moringa oleifera seed oil of Indian origin”. J. Food. Science and Techno. 51(3):503–510.
[4] Palafox, J.O., Navarrete, A., Sacramento-Rivero, J.C., Rubio-Atoche, C., Escoffie, P.A., Rocha-Uribe, J.A. (2012). “Extraction and characterization of oil from Moringa oleifera using supercritical CO2 and traditional solvents”. Am J. Anal Chem 03:946–949.
[5] Mekala, N.K., Potumarthi, R., Baadhe, R.R. and Gupta, V.K. (2014). “Current bioenergy researches: strengths and future challenges”. Bioenergy Research: Advances and Applications (pp.1-21).Elsevier.
[6] Sharma, R., Chisti, Y. and Chand, U. (2001) “Production, purification, characterization, and applications of lipases”. Biotechno. Advances 19(8):627–662.
[7] Treichel, H., de Oliveira, D., Mazutti, M.A., Di Luccio, M., Oliveira, J.V. (2010) “A review on microbial lipases production”. Food Bioprocess Techno. 3:182–196.
[8] Dutra, J.C., Terzi, S.D.C., Beviluca, J.V., Damaso, M.C., Couri, S., Langone, M.A. and Senna, L.F. (2008). “Lipase production in solid-state fermentation monitoring biomass growth of Aspergillus niger using digital image processing”. Applied Biochemistry and Biotechno. 147(1-3):63–75.
[9] Griebeler, N., Polloni, A.E., Remonatto, D., Arbter, F., Vardenega, R., Cechet, J.L. et al. (2011). “Isolation and screening of lipase-producing fungi with hydrolytic activity”. Food and Bioprocess Techno. 4(4):578-586.
[10] Guerrand, D. (2017). “Lipases industrial applications: focus on food and agroindustries”. OCL Oilsseeds and Fats Crops and Lipids 24(4):D403.
[11] Hidayah, K.N., Koentjoro, M.P., Nurhayati, A.P.D. and Prasetyo, E.N. (2016). “Visceral organ waste as a substrate for lipase production by Bacillus sp. SKII-5”. Proceeding of ICMSE 3(1).
[12] Singh, B.N., Singh, B.R., Singh, R.L., Prakash, D., Dhakarey, R., Upadhyay, G. et al.(2009). “Oxidative DNA damage protective activity, antioxidant and anti-quorum sensing potentials of Moringa oleifera”. Food and Chemical Toxicology 47(6):1109–1116.
[13] Singh, Y. and Prasad, K. (2013). “Moringa Oleifera leaf as functional food powder: characterization and uses”. Int. J. of Agriculture and Food Science Techno. 4(4):317–324.
[14] Cragg, S.M., Beckham, G.T., Bruce, N.C., Bugg, T.D., Distel, D.L., Dupree, P. et al (2015). “Lignocellulose degradation mechanisms across the tree of life”. Current Opinion in Chemical Biology 29:108–119.
[15] Rencoret, J., Pereira, A., del Río, J.C., Martínez, Á.T. and Gutiérrez, A. (2017). “Delignification and saccharification enhancement of sugarcane byproducts by a laccase-based pretreatment”. ACS Sustainable Chemistry and Eng. 5(8):7145–7154.
[16] Fernandes, M.C., Torrado, I., Carvalheiro, F., Dorés, V., Guerra, V., Lourenço, P. M. et al. (2016). “Bioethanol production from extracted olive pomace: dilute acid hydrolysis”. Bioethanol 1 (open-issue).
[17] Nyanhongo, G.S., Gomes, J., Gübitz G.M., Zvanyu, R., Read, J. and Steiner, W. (2002). “Decolorization of textile dyes by laccases from a newly isolated strain of Trametes modesta”. Water Research 36(6):1449–1456.
[18] Michalska, K., Bizukojć, M. and Ledakowicz, S. (2015). “Pretreatment of energy crops with sodium hydroxide and cellulolytic enzymes to increase biogas production”. Biomass and Bioenergy 80:213–221.
[19] Sharma, D., Kumbhar, B.K., Verma, A.K. and Tewari, L. (2014). “Optimization of critical growth parameters for enhancing extracellular lipase production by alkalophilic Bacillus sp.”
Biocatalysis Agricultural Biotechno. 3(4):205–211.

[20] Afati, F., Setiyoningrum, F. and Priadi, G. (2018). “Characterization of curd kefir milk with the addition of beetroot (Beta vulgaris)”. Pros. Sem. Nasional Masyarakat Biodiversitas Indonesia 4(2):270-273.

[21] Ertuğrul, S., Dönmez, G. and Takaç, S. (2007). “Isolation of lipase producing Bacillus sp. from olive mill wastewater and improving its enzyme activity”. J. of Hazardous Materials 149(3):720–724.

[22] Carvalho, N.B., Barbosa, J.M.P., Oliveira, M.V.S., Fricks, A.T., Lima, Á.S. and Soares, C.M.F. (2013). “Biochemical properties of Bacillus sp. ITP-001 lipase immobilized with a sol-gel process”. Quimica Nova 36(1):52–58.

[23] Kuhn, J., Müller, H., Salzig, D. and Czermak, P. (2015). “A rapid method for an offline glycerol determination during microbial fermentation”. Electronic J. of Biotechno. 18(3):252–255.

[24] Blainski, A., Lopes, G.C. and De, Mello, J.C.P. (2013). “Application and analysis of the folinic acid method for the determination of the total phenolic content from Limonium brasiliense L.”. Molecules 18(6):6852–6865.

[25] Orak, H.H. (2007). “Total antioxidant activities, phenolics, anthocyanins, polyphenol oxidase activities of selected red grape cultivars and their correlations”. Scientia Horticulturae 111(3):235–241.

[26] Marjoni, M.R., Afrinaldi, A. and Novita, A.D. (2015). “Kandungan total fenol dan aktivitas antioksidan ekstrak air daun kersen (Muntingia calabura L.)”. YARSI Medical J. 23(3):187-196.

[27] Modenbach, A.A. and Nokes, S.E. (2013). “Enzymatic hydrolysis of biomass at high-solids loadings - A review”. Biomass and Bioenergy 56:526-544.

[28] Sitarz, A.K., Mikkelsen, J.D., Højrup, P. and Meyer, A.S. (2013). “Identification of a laccase from Ganoderma lucidum CBS 229.93 having potential for enhancing cellulase catalyzed lignocellulose degradation”. Enzyme and Microbial Techno. 53(6-7):378-385.

[29] Molina, S., Rencore, J., José, C., Lomascolo, A., Record, E., Martínez, A.T. et al. (2008). “Oxidative degradation of model lipids representative for main paper pulp lipophilic extractives by the laccase-mediator system”. Applied Microbiology Biotechno. 80(2):211-222.

[30] Mukherjee, A., Mandal, T., Ganguly, A. and Chatterjee, P.K. (2016). “Lignin degradation in the production of bioethanol - a review”, ChemBioEng Reviews 3(2):86–96.

[31] Röbiger, B., Unkelbach, G. and Pufty-Heinrich, D. (2018). “Base-catalyzed depolymerization of lignin: history, challenges and perspectives”. Lignin - Trends and Applications 99:120.

[32] Bora, L. and Bora, M. (2012). “Optimization of extracellular thermophilic highly alkaline lipase from thermophilic Bacillus sp isolated from hotspring of Arunachal Pradesh, India”. Brazilian J. of Microbiology 43(1):30–42.

[33] Jönsson, L.J. and Martín, C. (2016). “Pretreatment of lignocellulose: formation of inhibitory byproducts and strategies for minimizing their effects”. Bioresource Techno. 199:103–112.

[34] Amin, F.R., Khalid, H., Zhang, H., Rahman, S., Zhang, R., Liu, G. et al. (2017). “Pretreatment methods of lignocellulosic biomass for anaerobic digestion”. AMB Express 7(1):72.

[35] Kumar, A.K. and Sharma, S. (2017). “Recent updates on different methods of pretreatment of lignocellulosic feedstocks: a review”. Bioresources and Bioprocess 4(1):7.

[36] Ouerghemmi, I., Rebey, I.B., Rahali, F.Z., Bourgu, S., Pistelli, L., Ksouri, R. et al. (2017). “Antioxidant and antimicrobial phenolic compounds from extracts of cultivated and wild-grown Tunisian Ruta chalepensis”. J. of Food and Drug Analysis 25(2):350–359.

[37] Maddox, C.E., Laur, L.M. and Tian, L. (2010). “Antibacterial activity of phenolic compounds against the phytopathogenic Xylella fastidiosa”. Current Microbiology 60(1):53–58.

[38] Imamura, S. and Kitaura, S. (2000). “Purification and characterization of a monoacylglycerol lipase from the moderately thermophilic Bacillus sp. H-257”. The J. of Biochemistry 127(3):419-425.
[39] Castro-Ochoa, L.D., Rodríguez-Gómez, C., Valerio-Alfaro, G. and Ros, R. O. (2005). “Screening, purification and characterization of the thermoalkalophilic lipase produced by Bacillus thermoleovorans CCR11”. Enzyme and Microbial Techno. 37(6):648–654.

[40] Paulsson, M. and Parkás, J. (2012). “Review: light-induced yellowing of lignocellulosic pulps - mechanisms and preventive methods”. BioResources 7(4):5995–6040.

[41] Prasetyo, E.N., Rodríguez, R.D., Lukesch, B., Weiss, S., Murkovic, M., Katsoyannos, E. et al. (2015). “Laccase–cellobiose dehydrogenase-catalyzed detoxification of phenolic-rich olive processing residues”. Int. J. of Environmental Science and Techno. 12(4):1343–1352.