Enhancement of lytic polysaccharide monooxygenase activity derived from *Neurospora* sp. in solid substrate fermentation of oil palm empty fruit bunch

L J Virginia¹, D Wijaya¹ and A Suwanto*²

¹ Research and Development for Biotechnology, PT. Wilmar Benih Indonesia, Cikarang, Indonesia
² Bogor Agricultural University, Bogor, Indonesia

*Corresponding author: asuwanto@indo.net.id

Abstract. Oil Palm Empty Fruit Bunches (OPEFB) are lignocellulose by products which are generated from oil palm fruit pressing. To date, OPEFB were mainly burnt to generate energy and often contribute to air pollutions. Therefore, OPEFB utilization into more valuable products is timely demanded. Component of OPEFB is classified as lignocelluloses family where cellulose is the major component (46%), followed by hemicellulose (34%) and lignin (20%), these complex components must be degraded into smaller molecules before further downstream bioconversion. In this study, OPEFB degradation was conducted biologically employing *Neurospora* sp. due to its ability in producing cellulases and Lytic Polysaccharide Monooxygenase (LPMO). The objectives of this study were to examine OPEFB degradation employing *Neurospora* in solid substrate fermentation and detection of LPMO activity derived from *Neurospora*. *Neurospora intermedia* isolate O3 could produce 11.9 ± 0.4 µmol/g reducing sugars when incubated for three days in OPEFB without delignification, while it increased to 77.4±2.3 µmol/g in OPEFB with delignification treatment. Interestingly, copper ion supplementation into OPEFB medium significantly increased in reducing sugar production up to 172.9±15.0 µmol/g, which might be due to the enhancement of LPMO activity.

1. Introduction

Lignin is one of potential biopolymers that can be obtained from pretreatment of lignocellulosic materials. On top of that Indonesia generates abundance of lignocellulosic waste which mostly comes from estate crops, such as oil palm. Indonesia is well-known as the largest palm oil producer in the world and palm oil is among estate crops that plays important role in Indonesia’s economy. The process from fresh fruit bunches into crude palm oil results in biomass wastes in large amount of oil palm empty fruit bunch fiber (OPEFB). In 2012, production of crude palm oil (CPO) reached 26 million ton which equal to 26.4 million ton OPEFB [1], and Indonesia is predicted to generate more lignocellulosic waste due to high demand on crude palm oil for various application such as in food and pharmaceutical industry. Unfortunately, to date, handling of OPEFB in Indonesia was still depending on non-environmentally treatment such as burning this lignocellulosic waste which generates air pollution, thus OPEFB utilization into more valuable products was required.
As a lignocellulosic biomass OPEFB contains cellulose, hemicellulose and lignin. Cellulose, the major component of lignocelluloses, is a polymer consisting of glucose units that are connected to each other by β-1,4-glycosidic bonds [2], where in lignocellulosic wastes the cellulose fibers are embedded in a matrix of hemicelluloses and lignin, which protect cellulose from being degraded by enzymes under certain condition [3]. Cellulose content of EFBs of palm oil is 37.3 – 46.5%, while hemicellulose and lignin content are 25.3 – 33.8% and 27.6 – 32.5%, respectively. Due to its high cellulose content, EFBs of palm oil is potential to be converted for renewable energy, such as bioethanol, a promising alternative fuel to gasoline. Utilization of OPEFB into value added product involves bioconversion process to reduce the crystalline structure of cellulosic material and lignin degradation, cellulosic hydrolysis, etc. While these processes could be conducted chemically, however biological degradation towards enzymatic is preferable due to less severe effect to environment and more specific activity which produce less by product [4].

In relevance with cellulose as the major component in OPEFB, this study will focus on harnessing cellulase derived from microorganism to break glycosidic bond (β-1,4-D-glukopyranose) in cellulose. Cellulase commonly perform toward hydrolyzing activity, however hydrolysis is still a major cost factor in the production of second-generation biofuels from lignocellulosic biomass considering the costs of currently available enzyme mixtures [5]. Until recently, the finding of enzymes from glycosol hydrolase family 61 (GH61), known as lytic polysaccharide monooxygenase (LPMO) in combination with the flavocytochrome cellulbiose dehydrogenase (CDH) enhance the action of hydrolytic enzymes added a new dimension to the classical concept of cellulose degradation [6].

Lytic polysaccharide monooxygenase is copper-dependent enzymes which carry out oxidative cleavage of the β-1,4-glycosidic bonds in polysaccharides, using molecular oxygen and an electron donor [6]. Electrons may be supplied by small molecule reductants such as ascorbic acid and gallic acid [7,8] or by enzymes such as cellulbiose dehydrogenase (CDH), which are co-expressed with LPMOs [9,10,11]. Lytic polysaccharide monooxygenase plays a pivotal role in macromolecule degradation, such as crystalline cellulose and chitin in plant cell wall, where its oxidative mechanism considered more effective compare to other hydrolyzing cellulose to disrupt crystalline structure in cellulose and resulted in enhancement of cellulose activity [12].

Biological degradation harnessing microorganisms producing cellulase is preferred to be conducted in solid substrate fermentation technique due to several advantages such as simple process and less instrumentation, more concentrated enzyme production, and utilizing solid waste directly as substrate [13]. In addition Neurospora crassa is well-known to produce LPMO [9] and when compared with Trichoderma reesei, commercial strain producing cellulase, N. crassa S1 showed 2 fold higher activity which make Neurospora a good candidate as OPEFB degrader [14]. Neurospora is a mold used to produce traditional food in Indonesia, called oncom and common to find in OPEFB stack. Reproduction of Neurospora sp. is divided into two ways, asexual and sexual, where asexual reproduction was utilizing shoot, fragmentation, and conidia. Conidia contain carotenoid which has orange color and sexual reproduction was conducted through ascospore [15]. Light induction is known to render disruption on miselium which make Neurospora produces more carotene, as protector agent, inside conidia [16].

In this study, Neurospora was cultured on OPEFB where it was expected to produce LPMO which could destroy cellulose-crystalline structure thus enhance degradation of complex cellulose in OPEFB. The objective of this research was to convert OPEFB using Neurospora through solid substrate fermentation and to detect LPMO activity derived from Neurospora.

2. Materials and Method
Oil palm empty fruit bunch was collected from PT. Agro Masang Perkasa, Padang, Indonesia, Vogel medium, glucose (Sigma-aldrich), Carboxymethyl cellulose or CMC (Sigma-aldrich), BCA assay (Thermo-Fischer), Potato Dextrose Agar or PDA (Sigma-aldrich), Amplex red kit (Invitrogen), CuSO₄ (Sigma-aldrich), Sodium-L-Ascorbic (Sigma-aldrich), DNeasy® Plant Mini Kit (Qiagen), dan 7 pairs
of primer designed with Genious® software. Hemasitometer improved Neubauer (Marienfeld), Microplate Reader (VersaMax), dan Thermal-cycler (Bio-Rad).

The research was divided into 5 major steps; screening of potential Neurospora, delignification of OPEFB, supplementation of CuSO₄ and sodium-L-ascorbic, confirmation of LPMO genes, and detection of LPMO.

2.1. Screening
Isolates were collected from both Oncom and OPEFB which have been overgrown with Neurospora culture. Miselium was sampled then inoculated into PDA medium with 50 µg/mL spectinomycin and 50 µg/mL streptomycin before further incubated in 30°C for 1-2 days. Refreshment into new medium for 3 times was conducted in order to gain a pure culture prior to cryo-preserved in 1 mL PDB (Potato Dextrose Broth) with 20% glycerol at -80°C.

2.2. Oil palm empty fruit bunch medium
Oil palm empty fruit bunch was chopped and filtered until reached 425 µm – 1000 µm. As much as 5 g chopped OPEFB was put into 250 mL Erlenmeyer and gauze lid was applied. Sterilization was conducted with autoclave at 121°C, 15 lbs for 15 minutes.

2.3. Spore suspension
Pure isolate was grown at 30°C for 7 days (5 days dark condition and 2 days light induction) then as much as 10 mL 0.85% sodium chloride solution was used to collect the spores from petri dish. Calculation of spores was conducted through Haemacytometer improved Neubauer (Marienfeld) method. Counting chamber was filled with 20 µL spore suspension and total spore was calculated by using formula:

\[
\text{Spores/ml} = \text{total spores} \times \text{dilution factor} \times \text{conversion factor} \times (10^4)
\]

2.4. Inoculation and OPEFB incubation
Oil palm empty fruit bunch was inoculated with 10⁶ spores of Neurospora, where the final ratio between OPEFB with vogel medium (pH 5.5) : spore suspension = 1:3. Incubation was conducted for 3 days at 30°C.

2.5. Sampling
As much as 50 mL acetate buffer 0.1 M (pH 4.8) was poured into 250 mL erlenmeyer, filled with fermented OPEFB with Neurospora, to conduct sampling. Thorough shaking at 100 rpm for 30 minutes was applied and followed with centrifugation at 12,000 rpm for 5 minutes in order to collect produced enzyme and reducing sugar.

2.6. DNS method
Reducing sugar measurement was conducted by reacting 320 µL sample with 680 µL dinitrosalicylic acid (DNS) reagent for 10 minutes and followed with boiling the solution for 10 minutes and immediate cooling for 5 minutes to stop the reaction. Absorbance was measured at 540 nm [17].

2.7. Delignification
As much as 30 g chopped and filtered OPEFB was soaked into 700 mL technical NaOH 2% (b/v) and autoclaved. Washing was conducted in the following step for 4-5 times to deprive the NaOH residue prior to drying in oven at 70°C for overnight [18].

2.8. CMCase (endoglucanase) activity analysis
CMCase activity is measured by determining reducing sugars released after 10 minutes of enzyme reaction with 1% carboxymethyl cellulose (CMC) at pH 4.8 and 50°C [19]. Reducing sugar measurement was conducted using DNS method, and one unit of enzyme was defined as the amount
of enzyme that liberates 1 µmol of glucose per minute under assay condition. Protein concentration was measured using Bicinchoninic assay (BCA) and specific activity was measured by comparing CMCase activity with its protein concentration. Partial purification was conducted through Amicon® 30 kDa.

2.9. SDS-PAGE
Protein sample was prepared by mixing 30 µL sample and 7 µL buffer then boiled for 5 minutes. As much as 20 µL mixed solution was put into well and electrophoresis was conducted at 80 V for 150 minutes.

2.10. Supplementation of CuSO₄ and Na-L-Ascorbic
Sterilization of 0.12 M CuSO₄ and 0.045 M Na-L-Ascorbic were conducted through milipore 0.22 µm. As much as 1mL CuSO₄ and 0.1 mL Na-L-Ascorbic were added together at spore inoculation where final concentration of CuSO₄ and Na-L-Ascorbic were 2 mg/mL and 300 µM, respectively.

2.11. Identification
DNA extraction was conducted using DNeasy® Plant Mini Kit (Qiagen) where ITS 1 (5’-TCC GTA GGT GAA CCT TGC GG-3’) and ITS 4 (5’-TCC TCC GCT TAT TGA TAT GC-3’) primers were used to amplify ITS gene of isolates at PCR reaction.

2.12. LPMO gene detection
Primers design was conducted using Genious® software with reference gene from NCBI [20]. As many as 7 pairs primers were used to amplify the LPMO gene of Neurospora as shown in table 1.

| Name | Sequence | Tₐ (°C) | Reference /Accession No. |
|------|----------|---------|--------------------------|
| C1   | F= 5’-GGCGTCAAGACCTCAACTC-3’, R= 5’-CCACGTCTCAAGTGTCAT-3’ | 58.9, 59.5 | EAA26873, EAA26656, EAA34466 |
| C4   | F= 5’-CTCACAGGCTACCAATCTTC-3’, R= 5’-TACCAGTGTACGTCAAGG-3’ | 57.8, 53.0 | EAA32426, EAA36362, EAA30263 |
| C1,4 | F= 5’-CCACGTCTCAAGTGTCAT-3’, R= 5’-TGTCAGGCGGATGATCT-3’ | 56.2, 58.7 | EAA33178, EAA29018 |
| 9C   | F= 5’-ATGAAGACCGGAAGCATCC-3’, R= 5’-CTAAAGAGGCGACTGGCTG-3’ | 57.9, 57.2 | EAA36362 |
| 9D   | F= 5’-ATGAAGGTCTCGGGCCCTC-3’, R= 5’-TTCAGCAAAGTACACACTGGGACC-3’ | 61 | EAA32426 |
| 9E   | F= 5’-ATGCGGTCCACTCTTGTCA-3’, R= 5’-TTAGACACACTGGGAGTAAATAAGGA-3’ | 58.9, 58.7 | EAA26873 |
| 9F   | F= 5’-ATGCTTCCTCGGACTGCTTCT-3’, R= 5’-TTAGCAGATGCAAGGAGACTGG-3’ | 57.6, 57.4 | EAA26656 |

2.13. Detection of LPMO
Amplex Red Hydrogen Peroxidase was used to detect LPMO [5]. Partial purified enzyme using Amicon® 30 kDa for 50 µL was added with 50 µL sodium phosphate buffer pH 6.0 which contain 30 µM Na-L-Ascorbic, 100 µM amplex red, and 0.2 U/mL horse radish peroxidase (HRP). Incubation of mixtures was conducted in room temperature for 30 minutes with minimum of air contact. Absorbance was measured at 580 nm where 1 unit LPMO was determined as 1 µmol H₂O₂ released per enzyme volume per minutes in reaction condition.

3. Result and Discussion
Selection of isolates was conducted by culturing *Neurospora* for 7 days (5 days dark condition and 2 days light induction, respectively) on PDA. Spore suspension was subsequently inoculated OPEFB for 3 days at 30°C. Reducing sugar and growth rate were pointed as indicator of potential candidate isolate where each isolates were conducted in 3 replicates. Isolates were collected from *Oncom* and OPEFB which most of isolates originated from *Oncom* did not show any growth on OPEFB (table 2). Although 5 isolates gave the same amount of reducing sugar and growth rate, LPMO 3, LPMO 7, LPMO 10, NK, and O3 but isolate O3 and NK were selected for further investigation due to their ability to produce spore.

**Table 2. Reducing sugar and growth rate comparison of *Neurospora* in solid state fermentation of OPEFB for 3 days.**

| Code  | Origin                      | Growth rate | Reducing sugar (µmol/g) |
|-------|-----------------------------|-------------|-------------------------|
| LPMO3 | OPEFB                       | ++          | 12.68±0.45              |
| LPMO5 | OPEFB                       | ++          | 10.74±0.23              |
| LPMO7 | OPEFB                       | ++          | 12.45±0.32              |
| LPMO10| OPEFB                       | ++          | 12.39±0.23              |
| *NK*  | OPEFB                       | ++          | 12.61±0.3               |
| NSA1  | OPEFB                       | ++          | 8.33 ± 0.4              |
| NSB2  | OPEFB                       | +           | 7.35 ± 0.34             |
| NSC3  | OPEFB                       | +           | 7.11 ± 0.21             |
| NSD4  | OPEFB                       | ++          | 10.01±0.16              |
| NSBPK | OPEFB                       | +           | 7.93 ± 0.38             |
| O1    | *Oncom* from supermarket    | -           | 6.11 ± 0.21             |
| O2    | *Oncom* from supermarket    | -           | 5.01 ± 0.22             |
| *O3*  | *Oncom* from traditional market | ++          | 11.98±0.43          |
| O4    | *Oncom* from traditional market | -          | 5.45 ± 0.24          |
| O5    | *Oncom* from traditional market | -          | 5.98 ± 0.23          |
| bControl | -                           | -           | 5.27 ± 0.24          |

OPEFB = oil palm empty fruit bunch

* Isolate NK and O3 were chosen due to their ability to produce more spores when cultured on PDA compare to others which generate the same rate of reducing sugar and growth

b Negative control (OPEFB without any inoculation)

In order to increase reducing sugar, a pretreatment was conducted through delignification of OPEFB. Delignification resulted in a significant visual alteration of OPEFB where OPEFB color became paler and softer in texture. In addition to visual changes, O3 growth rate was faster in pretreated OPEFB. Other than that, delignification process reduced OPEFB mass to 46.1% where reducing sugar also underwent a significant increase after 3 days fermentation (table 3). Therefore, delignification plays a critical role in OPEFB degradation through increment of cellulase access to OPEFB covered by lignin prior to pretreatment.

**Table 3. Comparison of fermented OPEFB with delignification process.**

| Code  | Spores Inoculum | OPEFB without delignification | OPEFB with delignification | Ratio Y/X |
|-------|-----------------|------------------------------|---------------------------|-----------|
|       | Growth Rate     | Reducing Sugar (µmol/g) (X)  | Growth Rate               | Reducing Sugar (µmol/g) (X) |          |
| *Control | -                | -                            | -                         | 4.68±0.14 | 0×        |
| O3    | 10⁶             | ++                           | 11.98±0.43 (X)            | ++        | 77.43±2.31 | 6.46×     |
| NK    | 10⁶             | ++                           | 12.61±0.3 (X)             | ++        | 71.59±2.37 | 5.68×     |

OPEFB = oil palm empty fruit bunch

* Isolates O3 was selected for further investigation due to its higher reducing sugar production compared to NK. Production of CMCase was conducted for 8 days where sampling was conducted every 24 hours and reducing sugar was measured to defined CMCase activity (figure 1). Production of reducing sugar and CMCase activity was increased significantly from day 0 up to day 3 then reduce in
the following day before increase again in day 6. This occurrence in line with study conducted in previous studies [21, 22] for *Botryosphaeria* sp. and *Trichoderma harzianum*, respectively, which underwent reduction in the available reducing sugar as the mycelia growing since it was used as energy source for fungi.

![Graph](image)

**Figure 1.** Production pattern of O3 towards its reducing sugar and CMCase activity in solid substrate fermentation of OPEGB for 8 days.

Analysis of CMCase activity was conducted on three days after inoculation where CMCase was suggested to play an important role on releasing reducing sugar from OPEFB fiber. Specific activity of CMCase reached 1.64 U/mg where SDS-PAGE profile showed 2 fractions of protein, 36.87 kDa and 45.42 kDa (figure 2).

![SDS Profile](image)

**Figure 2.** SDS profile of partial purified CMCase. M = Marker, S = Sample

Effect of CuSO₄ and Na-L-Ascorbic supplementation during cultivation of *Neurospora* in OPEFB for 3 days was also observed in this study. Production of reducing sugar increase significantly in fermented OPEFB with CuSO₄ supplementation (165.87 µmol/g) and combination of both CuSO₄ and
ascorbic (172.88 µmol/g). More than 3 times higher when compared to positive control (59.07 µmol/g) (figure 3).

Increment of reducing sugar also in line with mycelium growth rate of O3 in OPEFB (figure 4) where both events might be due to the enhancement of LPMO activities which required metalloenzyme or copper dependent enzyme. Thus, supplementation of 2 mg/mL CuSO₄ fulfilled the Cu²⁺ requirement in active site of LPMO as electron recipient from reducing agent [9].

Previous study [12], proved that LPMO application derived from N. crassa able to enhance cellulase activity derived from T. reesei towards crystalline cellulose, which was detected through increment of glucose concentration, and interestingly similar occurrence also seen in this study. Additional of Na-L-ascorbic as reducing agent is predicted to facilitate LPMO activity, however ascorbic is not the sole reducing agent for LPMO. Other natural reducing agents such as lignin and cellobiose dehydrogenase were also competent to assist LPMO activity which made additional of ascorbic did not give a significant improvement of reducing sugar and mycelium growth rate as expected [5, 23]. Study on effect of copper addition towards Neurospora growth rate has been
conducted where it induced copper-methallothionein gene (MTs) which is a metal binding protein and act as protector towards toxic metal contamination [24]. Nevertheless, its effect on Neurospora growth in cellulose rich medium such as OPEFB was still unclear and due to minimum study which showed increment of cellulose derived from Neurospora in relevance with copper presence leads to assumption that LPMO is strongly suggested as the reason of increment towards growth rate and reducing sugar production in OPEFB.

Since LPMO is estimated to play a pivotal role in crystalline cellulose degradation of OPEFB, thus it should be proven by its presence. Prior to detect existence of LPMO as protein, confirmation on gene encoding LPMO might be simpler to do. In order to design primers to amplify targeted sequence, identification of O3 isolates was conducted through ITS region analysis. Our analysis showed that O3 was identified (99%) as N. intermedia (accession number KT844664.1). Design of 7 pairs of primers was conducted to amplify LPMO gene at its coding sequence (CDS) region in the O3 genome [25, 20]. Both actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers were also designed for positive control in PCR reaction. Amplification on CDS region of LPMO from O3 gave positive results on all primers combination (figure 5) where sequencing analysis was subsequently conducted (table 4).

![Figure 5](image)

**Figure 5.** Amplified CDS region of LPMO gene in O3 genome with various pairs of primers. Both actin and GAPDH act as positive control.

| Code | Band (bp) | Description | Nucleic acid Identity (%) | Amino acid Identity (%) | Accession number (NCBI) | Chromosome |
|------|----------|-------------|---------------------------|------------------------|------------------------|------------|
| C1   | 225      | N.crassa gh61-5, mRNA | 99.5                     | 98.7                   | EAA26873              | III        |
| C4   | 282      | N.crassa gh61-1, mRNA | 96.5                     | 97.9                   | EAA30263              | VII        |
| C1,4 | 448      | N.crassa gh61-2, mRNA | 96.3                     | 98.8                   | EAA29018              | V          |
| 9C   | 1170     | N.crassa gh61-3, mRNA | 97.1                     | 96.7                   | EAA36362              | I          |
| 9D   | 817      | N.crassa gh61-4, mRNA | 85                       | 100                    | EAA32426              | V          |
| 9E   | 1280     | N.crassa gh61-5, mRNA | 96.7                     | 98.5                   | EAA26873              | III        |
| 9F   | 793      | N.crassa gh61-6, mRNA | 89.3                     | 86.8                   | EAA26656              | II         |

**Table 4.** Sequencing result in CDS region of LPMO gene originated from O3 genome.

Primers design could not be conducted in the conserved region from gh61 gene of N. crassa in NCBI due to high diversity in this gene which was explained by Dimarogona *et al.* 2014, where different enzymes activities were required to accommodate different types of substrates. Our study
showed that O3 also possessed various LPMO genes which could be used to produce such enzymes when cultured in OPEFB.

Detection of LPMO was conducted through amplex red kit assay. Trace amount of LPMO activities were able to be detected due to their higher absorbance (0.27 ± 0.04 U/L) compared to negative control i.e. OPEFB without O3 inoculation. Interestingly, in the presence of 0.12 M CuSO₄, O3 could generate higher activity for 2.5 fold (0.67 ± 0.05 U/L) which is shown in figure 6.

Figure 6. LPMO activity measured with amplex red kit. NC = non-inoculated oil palm empty fruit bunch, C = inoculated oil palm empty fruit bunch, Cu = inoculated oil palm empty fruit bunch with CuSO₄ supplementation.

LPMO is classified in auxiliary activity class [25] and produced at only small amount, although in *Neurospora crassa* 14 *pmo* genes are present [5]. Due to its small expression, detailed characterization toward each *pmo* genes were difficult to be conducted. Nevertheless, data gained in this research showed that LPMO is important to be explored, especially in their function to degrade lignocellulosic waste, such as OPEFB.

4. Conclusion
Among 15 *Neurospora* isolates collected from both OPEFB and Oncom, isolate O3 derived from Oncom which is identified as *N. intermedia* was selected as the most potential candidate to study OPEFB biodegradation. Delignification of OPEFB showed better performance in cellulose hydrolysis. In this study, additional of 0.12 M CuSO₄ enhance production of reducing sugar and growth rate of O3 significantly. It might be due to the increase of LPMO activity as auxiliary enzyme of cellulases. The presence of LPMO was confirmed based on molecular and protein data employing PCR and amplex red kit, respectively.

References
[1] Ministry of Agriculture 2013 *Tree Crop Estate Statistics of Indonesia 2012-2014*, ed Juga BS and Sukriya LL (Jakarta: Directorate General of Estate Crop) p 3
[2] Keegstra K 2010 *Plant Physiol.* 154(2):483–6
[3] Chundawat SP, Beckham GT, Himmel ME and Dale BE 2011 Deconstruction of lignocellulosic biomass to fuels and chemicals *Annu. Rev. Chem. Biomol. Eng.* 2:121–45
[4] Isroi, Ishola MM, Milluti R, Syamsiah S, Cahyanto MN, Niklason C and Taherzadeh MJ 2012 *Molecules* 17:14995-15012
[5] Kittl R, Kracher D, Burgstaller D, Haltrich D and Ludwig R 2012 *Biotechnol. Biofuels.* 5(79): 1-13
[6] Horn SJ, Vaaje-Kolstad G, Westereng B and Eijsink VG 2012 Biotechnol. Biofuels. 5(1):45
[7] Vaaje-Kolstad G, Westereng B, Horn SJ, Liu ZL, Zhai H, Sorlie M and Eijsink VGH 2010 Science 330:219-22
[8] Quinlan RJ, Sweeney MD, Lo LL, Otten H, Poulsen JCN, Johansen KS, Krogh K, Jorgensen CI, Tovborg M, Anthonsen A, Tryfona T, Walter CP, Dupree P, Xu F, Davies G J and Walton PH 2011 Proc. Natl. Acad. Sci. USA. 108:15079-15084
[9] Phillips CM, Beeson WT, Cate JH and Marletta MA 2011 ACS Chemical Biology. 6:1399–1406
[10] Langston JA, Shaghasi T, Abbate E, Xu F, Vlasenko E and Sweeney MD 2011 Appl. Environ. Microbiol. 77:7007-15
[11] Sygmund C, Kracher D, Scheiblbrandner S, Zahma K, Felice AKG, Harreither W, Kittl R and Ludwig R 2012 Appl. Environ. Microbiol. 78:6161-71
[12] Eibinger M, Ganner T, Buhner P, Rosker S, Kracher D, Haltrich D, Ludwig R, Plank H and Nidetzky B 2014 J. Biol. Chem. 289(52):35929-38
[13] Sukumaran RK, Singhania RR, Mathew G and Pandey A 2009 Renew Energ. 34: 421-24
[14] Li Q, Ng WT and Wu JC 2014 Microbial Cell Factories 13: 157-65
[15] Nurfaizin and Matitaputty PR 2015 Wartazoa 25(4):189-96
[16] Novianti T, Wignyanto and Nurika I 2012 J. Tek. Pert. 5(2): 64-75
[17] Gusakov AV, Kondratyeva EG and Sinitsyn AP 2011 Int. J. Anal. Chem. https://doi.org/10.1155/2011/283658
[18] Akhtar J, Idris A, Teo CL, Lai LW, Hassan N and Khan MI 2014 IJACES 1(2): 155-7
[19] Dashtban M, Maki M, Leung KT, Mao C and Qin W 2010 Crit. Rev. Biotechnol. 30(4): 302-9
[20] Morgernsten I, Powlowski J and Tsang A 2014 Brief Funct. Genomics 13(6): 471-81
[21] Bahrin EK, Seng PY and Abd-Aziz S 2011 Aust. J. Basic Appl. Sci. 5(3): 276-80
[22] Alam MZ, Muhammad N and Mahmat ME 2005 American J. Appl. Sci. 2(2): 569-72
[23] Dimarogona M, Topakas E and Christakopoulos P 2012 J. Comput. Struct. Biotechnol. 2(3): 1-8
[24] Kumar KS, Dayananda S and Subramanym C 2005 FEMS Microbiol. Lett. 24: 45-50
[25] Busk PK and Lange L 2015 BMC Genomics 16(368):1-13