Chapter Two

MICROBIAL CELLULASE: PRODUCTION AND APPLICATION IN ENZYMATIC TREATMENT OF BIOSOLIDS

A. A. Al-Gheethi[1,*], A. N. Efaq[2,3], R. M. S. R. Mohamed[1], J. D. Bala[4] and M. K.Amir Hashim[1]

[1]Micro-pollution Research Centre (MPRC), Department of Water and Environmental Engineering, Faculty of Civil & Environmental Engineering, Universiti Tun Hussein Onn Malaysia, Batu Pahat, Johor, Malaysia

[2]Environmental Technology Division, School of Industrial Technology, Universiti Sains Malaysia (USM), Penang, Malaysia

[3]Applied Microbiology Department, Faculty of Applied Sciences, Taiz University, Taiz, Yemen

[4]Department of Microbiology, Federal University of Technology, Minna, Abuja, Minna, Nigeria

ABSTRACT

One of the main objectives in the treatment of biosolids is to reduce the cellulolytic materials which represent 40 to 50% of the total dry weight. Cellulose degradation is initiated primarily by micro-organism (fungi, bacteria and protozoa) with the aid of extracellular enzymes. The cellulase(s) is a multiple enzyme system consisting of endoglucanase, exoglucanase and β-glucosidase which act as a synergistic effect for the

* Email: alisaeed@uthm.edu.my; adelalghithi@gmail.com.

degradation of complex cellulose to glucose. Cellulase enzyme has applied for several industrial activities including production of animal feed, and wine, formulation of detergents, juice clarification, as well as in the treatment of domestic and industrial wastes. The enzymatic treatment of biosolids is named white technology which has raised recently due to the efficiency and absence of toxic by-products. In this chapter the factors affecting production of cellulase enzyme by microorganisms, the potential of indigenous microorganisms for the bio-augmentation process of biosolids and occurrence of catabolite repression phenomenon of cellulase production during the enzymatic degradation of biosolids will be discussed.

Keywords: cellulase, microorganism, biosolids, catabolite repression

INTRODUCTION

Cellulose represent the main component in the biosolids generated from sewage treatment plants (STPs). It represent between 40-50% of the dry weight of biosolids (Wang et al. 1994). Therefore, the reduction for the total mass of biosolids is the major concern of the treatment processes. Currently the biosolids generated from STPs are incinerated or disposed in to the landfill. However, these practice are unacceptable due to high risk associated with the incineration process such as production of secondary by-products which including dioxins and furans (Efaq et al. 2015). The direct disposal of biosolids into the landfill might contribute significantly in the distribution of pathogenic microorganisms to the environment and then transmit via food chain into the human. Biosolids have high concentrations of pathogenic bacteria, viruses, protozoa and fungi (Abdel-Monem et al. 2008a). These organisms have the ability to survive in the environment for several months and sometime for years (Abdel-Monem et al. 2008a). In some developing countries biosolids generated from STPs are used as soil fertilizers, these practices accelerate the distribution and transmission of pathogens into the human and animals (Al-Gheethi et al. 2015a).

Several technologies have been rised during the last decades, which aimed to reduce the health risk and increase the total dry mass of biosolids. These technologies included thickening, stabilization, conditioning and de-watering processes (Abdel-Monem et al. 2008b). However, they could not contribute in the reduction of the biosolids volume. The new strategies are to reuse the biosolids as biofuel resources. Cellulose represent one of the important resources for biofuel production, however, it has to subject for the enzymatic

hydrolysis using cellulase enzyme in which the polysaccharides are degraded into glucose that can be used as substrate for fuel ethanol production (Salmon et al. 2014). The degradation of cellulolytic materials by cellulase(s) enzymes produced from numerous microorganisms is very important in several agricultural and biosolids treatment processes (Hamer, 2003; Angenent et al. 2004; Schloss et al. 2005). Since, the cellulose is very stable with a half-life of 5-8 million years (Wolfenden & Snider, 2001).

The enzymatic treatment process is a technology falls between the biological and physicochemical processes (Karam & Nicell, 1997). The Bio-catalysis technology which is based on utilization of enzymes is known as white biotechnology and fully participates in the "green chemistry" concept (Alcalde et al. 2006). It has introduced in the 90s by Sheldon & van Rantwijk (2004). The enzymatic technology has several advantages including: the high potential to degrade complex compounds, effective under a wide range of temperature, salinity and pH (Karam & Nicell, 1997). It is eco-friendly process which means there is not toxics are produced, conversely it remediate a wide range of compounds unfriendly to the environment.

In this chapter the microbial production of cellulase enzyme, mechanism of action, the application of cellulase for the reduction of biosolids volume and the challenges are discussed.

1. Microbial Production of Cellulase Enzyme

The production of cellulase among different microorganisms have been reported in literature. Several anaerobic bacteria degrade cellulose in anaerobic digestion (Chynoweth & Pratap, 1996). The aerobic microorganisms have high potential to produce copious amounts of free cellulases which act synergistically to degrade cellulose (Blouzard et al. 2007; Mingardon et al. 2007). *Cellulomonas* sp. is the most common bacteria which has the ability to produce high concentrations of cellulase. Other bacteria including *Bacillus* sp. and *Pseudomonas* sp. Among several species of fungi *Aspergillus* spp. have high potential to degrade the cellulolytic materials and produce cellulase enzyme.

Al-Gheethi (2015) found that *Sporosarcina pasteurii*, *Bacillus megaterium*, *Staphylococcus xylosus*, *B. subtilis* and Burkholderia *cepacia* obtained from wastewater and biosolids have exhibited high productivity of cellulase enzymes. Bala et al. (2015) isolated *Micrococcus luteus*, *S. maltophilia*, *B. cereus*, *K. pneumoniae*, *B. subtilis*, *Aspergillus fumigatus*, *A. nomius*, *A. niger*, *Meyerozyma guilliermondii* from POME and revealed that these species

exhibited high activity for the production of cellulase. Efaq et al. (2016a) found that *A. niger*, *A. fumigatus*, *A. tubingensis*, *A. terreus var. terreus*, *P. simplicissimum* and *P. waksmanii* obtained from clinical wastes have a cellulase activity on the production medium.

The first screening for the production of cellulase enzyme among the microorganisms is conducted by using the pate technique which has introduced by Hankin & Anagnostakis (1975). The technique is depend on the subculture of the microorganism on the culture medium containing cellulase substrate. The most common substrate used for the detection of cellulase enzyme is Carboxymethyl cellulose (CMC). A pure culture of the microorganism is streaked on the CMC medium and then incubate at 28ºC for fungi and 35-37ºC for bacteria. After the incubation period for 3-5 days, the CMC plates are flooded with 1% (w/v) congo red for 20 min and then the dye is decanted and the plates are again flooded with one molar (1 M) NaCl for another 20-30 min (Singh et al. 2013; Bala et al. 2015; Efaq et al. 2016b). The cellulase production is detected by the presence of a pale orange to clear zone around the microorganism colony which indicate to cellulose hydrolysis (Sadhu & Maiti, 2013). The occurrence of clearing zone around the cellulolytic bacteria or fungi colony is due to colour change of Congo red which take place as a result of degradation of cellulose into glucose and routinely metabolize glucose to organics acids. This process lead to decrease the pH to be in acidic condition and then Congo red change from red to pale orange (Yoon et al. 2007). Another reagent for the detection of cellulase enzyme might be conducted by using iodine $ZnCl_2$ solution (3% $ZnCl_2$ is added to gram's iodine solution), where the appearance of clearing zone around the colony mean the production of enzyme is positive (Al-Gheethi, 2015).

In order to confirm the presence of extracellular cellulase, the microorganism is cultured in a CMC-broth medium for 5-7 days. The cells are separated by the centrifugation 4000-6000 rpm for 10 min to obtain the supernatant. The detection of cellulase in the supernatant is carried out by using CMC cup-plate clearing zone (C.C.Z) assay. In this assay 2% (w/v) CMC is dissolved in citrate buffers (pH 7) for bacteria or phosphate buffer for fungi and supplement with 1.5% agar for solidification. After sterilization, equal amounts of assay medium (20 ml) is poured in sterilized petri dishes (12 cm in diameter). Cups (10 mm in diameter) is made in each plate using a sterile cork borer. Equal amounts of the enzyme solution (cultural supernatant) are put into each cup. Plates with cups containing enzyme solutions are incubated at 37°C for 24 h, then the surface of the plates were floated with the reagent (Congo red or $ZnCl_2$ solution. Diameters of blue zones are measured (Al-Gheethi, 2015).

The concentration of cellulase enzyme is determined by using spectrophotometer by using the dinitrosalicylic (DNS) method according to Miller (1959). In this method glucose is used as the standard. Measurements are made in a spectrophotometer at 540 nm wavelength in the presence of the blank which is containing heat-inactivated post-culture liquids (boiled for 5 min).

Cellulases enzymes are complex enzyme consist of endoglucanases (EC3.2.1.4), exoglucanases, including D-cellodextrinases (EC3.2.1.74) and cellobiohydrolases (EC 3.2.1.91) and (3) b-glucosidases (EC 3.2.1.21) (Lynd et al. 2002; Zhang & Lynd, 2004). Endoglucanase hydrolyze accessible intra-molecular β-1-4 glucosidic bonds of cellulose chain ends, exoglucanases processively cleave cellulose soluble cellobiose or glucose and β-glucosidase hydrolyze cellobiose to glucose in order to cellobiose inhibition (Krishna, 1999; Zhang et al. 2006).

2. Enzymatic Treatment of Biosolids

The main objective of wastewater treatment plants is to degrade the total suspended solids (TSS) which represent the cellulolytic materials in wastewaters (Wu et al. 2010). The primary and secondary process contribute significantly in the reduction of TSS in the effluents. However, these concentrations still high in the biosolids which remains a problem to be resolved further (AbdulKarim et al., 2011). The use of microbial enzymes as an advance technology for biosolids might offers an alternative solution to reduce the chemical oxygen demand (COD), biological oxygen demand (BOD) and total suspended solids (TSS) parameters (Alam et al., 2009).

Microbial cell degrade the organic compounds by using two types of enzymes which are endo-enzymes and exo-enzymes. Endo-enzymes are produced within the cell and used for the metabolic reactions of soluble substrates. In this case the substrates with low molecules weight are transported across the cell membrane via the transport systems of the microorganism into the cytoplasm. Exo-enzymes are synthesized inside the microbial cell and then release to the surrounding medium to breakdown the high molecular compounds which could not transported across the cell membrane of the cell (Gerardi, 2003). The cellulose has high molecular weight with about 570,000 units of glucose. Therefore, the microbial cell produce cellulase enzyme as a hydrolysis enzyme to degrade it into small units with low molecular weight like glucose (180 MW) to facility the transportation process through the cell membrane of the microorganism.

The simplest method for the enzymatic treatment of biosolids is by introducing of the microbial cell that produce cellulase into the biosolids reactor. However, it has to consider several factors which affecting microbial cellulase production in biosolids such as initial concentrations of the microorganisms, temperature, pH value, carbon and nitrogen sources as well as heavy metals concentrations in biosolids. Besides, the potential of the introduced microorganism has to survive and compete with the indigenous organisms in the biosolids. In terms of initial concentrations of inoculated microorganism, it has to mention that the concentrations rely on the age of microorganism for example for the bacterial cells it supposed to be between 24 to 48 hrs, while for the fungi it has to be between 72 and 96 hrs. At this age the microbial cells still have high activity for the production of the enzymes, because it will be in the stationary phase of the growth curve. Krishna (1999) revealed that 15% (v/w) of *B. subtilis* (24 hrs) produced maximum concentrations of the enzymes in the culture medium. Most of the researchers used the bacteria such as *B. pumilus*, *Streptomyces omiyaensis*, *Psudomonas fluorescens* within 24 hrs of the age as inoculum for the production of cellulase in the medium with initial concentrations ranged from a loop of the pure culture to 15% of the production medium without need to detect the numbers of the bacterial cells concentrations (Kotchoni et al. 2003; Bakare et al. 2005; Al-Gheethi 2015). Moreover, Al-Gheethi & Norli (2014) indicated that 10^6 cell/mL is the best initial concentrations for the enzymatic degradation of pharmaceutical restudies in the wastewater. It has to indicate that the initial concentrations of inoculated microbial cells would not effect directly on the amount of the cellulase production, since the microorganism will grow and multiply during the treatment process. Therefore, the microbial concentrations during the enzymatic treatment process of biosolids can be calculated according to the following equations (Davis and Cornwell 2013).

$$P = P_0 + 2P_0 + 2 \times 2P_0 + 2 \times 2 \times 2P_0 + \cdots \quad (1)$$

Where P is the population, P_0 is the initial concentrations, and due to the multiplication of bacterial cells by Logatherm numbers the total population is calculated as following;

$$P = P_0(2)^n$$

Where n is the generation number which is depend on the generation time and different form one microorganism to others and can calculated as following:

$$n = \frac{treatment\ period}{generation\ time} \quad (2)$$

The cellulolytic microbial growth during the treatment process is depend on the concentrations of cellulose in the biosolids as substrate. Therefore, in order to determine the microbial growth the Monod Equation can be used.

$$\mu = \mu_{max}[\frac{S}{S+Ks}] \quad (3)$$

Where, μ_{max} = maximum growth rate, t^{-1}, S = concentration of cellulose in the biosolids (in term of BOD), mg/L, K_s = half saturation constant, mg/L which can be calculated from the growth curve slop of substrate concentrations and microbial concentrations.

The growth rate of microorganisms can be determined by using the following question

$$\frac{dX}{dt} = \frac{\mu_m SX}{K_s + S} - k_d X \quad (4)$$

Where, dX/dt is growth rate of biomass, X is concentration of biomass, k_d is endogenous decay (natural die-off of microorganism during the treatment process).

The degradation of cellulolytic materials in the biosolids (in term of BOD) is calculated based on the microbial growth rate and decimal fraction of cellulolytic material mass utilized by the microorganism (Y) as following:

$$-\frac{dS}{dt} = \frac{1}{Y}\frac{\mu_m SX}{K_s + S} \quad (5)$$

…

$$Y\ (yield\ coefficient) = \frac{microorgasim\ concentration}{BOD\ reduced} \quad (6)$$

The effect of temperature on the production of cellulase enzyme depend on the microorganism species (bacteria/fungi). Most environmental microorganisms grow with the ambient temperature (25-30ºC). However, the

microbial cells have a wide range temperature for the production of cellulase enzyme. Bacillus spp. and Aspergillus spp. exhibited high potential to produce the enzyme within the range between 30ºC to 60 ºC (Mawadza & Zvauya, 1996; Krishna, 1999; Immanuel et al., 2006; Ikram-ul-Haq et al., 2006; Al-Gheethi 2015). Moreover, the selection of inoculated microorganism species represent the main point in the degradation of cellulolytic materials in biosolids. The utilization an environmental microbe strain might exhibit high effective in the production of the enzyme and thus the treatment process can be conducted at the mesophilic temperature. In most studies the microorganism used are obtained from the biosolids itself because it has already acclimatized naturally to grow and multiply in this medium. This point is very important in the enzymatic treatment of the biosolids as will be discussed below.

The maximum production of cellulase enzymes might take place after 4 hrs to three days of the inoculation period for bacteria and might extend to seven days for fungi depend on the microorganism source (Kawai et al., 1988; Ekperigin, 2007). Microorganism needs time to adapt in the new media, the period of the adaption process depend on the microorganism source. Therefore, if the microorganism has obtained from the biosolids, the adaption period will take short time, then the enzymatic degradation of cellulose in biosolids will be fast.

pH value of biosolids is around pH 7, in contrast, microorganism have a wide pH range for the production of cellulase enzymes. Fungi are acidophilic, *Trichoderma reesi* produced the maximum enzyme between pH 2.8 and pH 3.5 (Sternberg & Mandels, 1979), while bacteria such as *Cellulomonas* spp., *Bacillus* spp. and *Micrococcus* spp. produce the enzyme at natural pH (Mawadza & Zvauya, 1996; Immanuel et al. 2006). However, some species of bacteria and fungi have the potential to produce the enzyme at alkaline conditions more than pH 9 (Hakamada et al., 1997). The wide range of pH required for the maximum production of cellulase enzyme facility the enzymatic treatment of biosolids where no adjustment of pH value by using chemical additives are required.

The type and concentration of cellulosic material and carbon source is critical for the production of cellulase enzyme by introduced microorganism in the biosolids treatments. Biosolids contains 40-50% cellulose, 12% hemi-cellulose and 10-15% lignin by dry weight (Wang et al., 1994). These substrates can be used as a carbon source for the cellulolytic organisms. Al-Gheethi (2015) examined CMC, cellulose powder, cotton, filter paper, tobacco leaves, sawdust and bagasse as a carbon sources for *B. megaterium* which is used for the production of cellulase enzymes in the production medium. The study revealed

that CMC was the best for the maximum production of cellulase followed by cellulose powder. The lowest cellulase production was recorded with cotton and sawdust. CMC was also reported as the best carbon source for the production of cellulase enzyme among fungi such as *A. niger, T. viride*, *Paenibacillus* sp. (Ikram-ul-Haq et al. 2006; Emtiazi et al., 2007). Efaq et al. (2016a) used CMC as a substrate for the bioassay of cellulase enzymes among *A. niger, A. fumigatus, A. tubingensis, A. terreus var. terreus, P. simplicissimum* and *P. waksmanii*. In the investigation for the effect of carbohydrate on the cellulase production, Al-Gheethi (2015) found that the mannose, ribose, fructose and xylose have induced the production of cellulase by *B. megaterium*.

The nitrogen source in the medium is one of the most important factors, which greatly affect cellulase production by microorganisms. Biosolids are rich with organic nitrogen sources such as ammonium and urea which play an important role in the enhancement of cellulase production by the introduced microorganism (Chan & Au 1987). Jin & Toda (1989) demonstrated that the increase of urea concentration from 2 to 6 g L^{-1} and reduction of yeast extract from 6 to 4 g L^{-1} in the medium improved endocellulase production by Clostridium thermocopiae. Al-Gheethi & Abdul-Monem (2014) revealed that the organic nitrogen source has improved the production of cellulase enzyme by *B. megaterium* more than the inorganic source because the organic nitrogen compounds stimulated higher growth and cellulase production than inorganic compounds.

The composition of biosolids is the main challenges in the enzymatic treatment process, since the biosolids have many of heavy metals elements which might inhibit the microbial growth and then the production of cellulase enzymes. There are few studies which conducted on the production of cellulase enzyme in the biosolids media. Al-Gheethi (2015) examined the efficiency of bacteria to produce cellulase enzyme in the biosolids medium which contains different concentrations of heavy metals such as zinc (Zn), copper (Cu) and nickel (Ni). The study examined four types of biosolids collected from four different STPs and inoculated with *B. megaterium*. The efficiency of bacteria to produce of cellulase was conducted without chemical additives or pH adjustments. The study found that production of cellulase enzyme was high in the biosolids with low concentrations of Ni^{2+} even when the concentration of Cu^{2+} and Zn^{2+} ions were high. It was due to the toxicity of Ni^{2+} ions in comparison to Cu^{2+} and Zn^{2+} at the high concentration. Some of heavy metals such as Zn, Cu and Ni at the low concentration play role as cofactor for many of enzymes and improve the enzymatic reaction (Nies, 1999). However, at high concentrations some of these metals such as Ni^{2+} becomes more toxicity than

Cu^{2+} and Zn^{2+}. The toxicity of heavy metals is depend on the transport system of microbial cells. For example, the microbial cell take up of $Zn^{2+}$and Cu^{2+} is by specific transport system (Fath & Kolter 1993; Fagan & Saier 1994), while Ni^{2+} is accumulating by the fast and unspecific system (Tao et al. 1995).

On the other hand, the ability of microbial cell to resist heavy metals might improve the over production of cellulase. For example, Al-Gheethi (2015) revealed that *B. megaterium* has the potential to produce detectable amount of cellulase in biosolids with Ni^{2+} ions was due to that *B. megaterium* was acclimatized to tolerate nickel ions before the inoculation to the biosolids medium. Therefore, one of the consideration for the utilization of microbial cells for the enzymatic treatment of biosolids is the selection process for the most potent microorganism.

The adding selected indigenous microbial strains into the biosolids which called bioaugmentation process, might be efficient in degrading cellulolytic materials and can improve the catabolism of specific compounds. It is indeed a promising technique to solve practical problems in wastewater treatment plants, and to enhance removal efficiency (Herrero & Stuckey 2015).

The introduced microorganism into the enzymatic treatment of biosolids should be subjected for several adaptation process in order to get high degradation process of cellulose. Several authors have applied the adaption strategy for the microorganism used in the bioagumentaion process to achieve high efficiency in the biodegradation of organic pollutant in the wastewater. Among bacterial species *P. aeruginosa, B. flexus, B. subtilis, E. homiense* and *S. aureus* (Sivaprakasam et al. 2008; Li et al. 2010; Al-Gheethi & Norli 2014; Adel et al. 2015; Al-Gheethi et al. 2015; Al-Gheethi et al. 2016a).

The considerations in the bioagumentaion process of enzymatic treatment of biosolids included the insufficiency of substrate, competition with indigenous organisms, pH, moisture, nutrient, osmotic factor. Hence, the choice of organisms is particularly important because of differences in their ability for growth and survival under extreme conditions and affinity for pollutants (Morikawa, 2006). The adaption process depend on the natural selection process which rely on the acclimatization and the competition process for resources (Davies & Davies, 2010; Radwan & Babik, 2012; Al-Gheethi et al. 2016b).

Recently, some researchers trended to use hybrid technology for the biotechnology, such as using the genetic engineering technology to improve the efficiency of introduced microorganisms (Wang & Chen, 2009). However, the limitation in the using genetically modified organisms (GMOs) are their ability to generate persistent strains which might give adverse effect for the natural ecosystems. Therefore, they have been considered a threat to the environment

and human health (Gilsdorf & Zilinskas, 2005; Prescott et al., 2005; Prakash et al., 2011). Hence, the natural adaption process is the best to get more effective organism in the bioaugmentation process. This process has been used to the biodegradation of cellulose in biosolids and pharmaceutical residues in the wastewater and exhibited a good efficiency (Al-Gheethi 2015; Al-Gheethi et al. 2016a).

3. CATABOLITE REPRESSION OF ENZYME PRODUCTION

One of the main challenges in the enzymatic treatment of biosolids lie in the Carbon catabolite repression (CCR) for the production of cellulase by the inoculated microorganisms. This process lead to inhibit the production of cellulase due to high concentrations of glucose in the medium. CCR is a key regulatory system available in the microorganism cell to ensure preferential utilization of energy-efficient carbon sources (Vinuselvi et al. 2012). Cellulase enzyme are produced by the microorganism as genetically or inducible. In the microorganism which produce the enzyme genetically the high level of glucose would not lead to inhibit the enzyme production. In contrast, in the inducible microorganism the presence of glucose in the surrounding medium might reduce the production of cellulase enzyme. Therefore, in the selection process of microorganism the nature of cellulase enzyme production as genetic or inducible should be tested. The test can be conducted by the sub-culturing of microbe in two production medium, one of the medium containing cellulase substrate such as CMC, while the other containing glucose and then determine the amount of the cellulase enzymes in both culture medium. The inducible organism produce less cellulase enzyme in the presence of glucose in comparison to that in the presence of inducer substrate. Al- Gheethi, (2015) has reported that *S. pasteurii*, *B. subtilis* and *S. Xylosus* lose the ability to produce cellulase in the presence of glucose, while B. megaterium has produced the enzyme genetically.

Glucose inhibit the production of cellulase enzyme in the inducible organism because it easily across through cell membrane and stop the gene expression of enzyme production. However, it has to mention that the CCR occur after several days of the enzymatic treatment process. Bala (2016) found that the CCR exhibited after 50 days of enzymatic treatment of POME by bacteria and fungi. Allcock & Woods (1981) and Al-Gheeth (2015) has reported that microbial strains can continually produce hydrolysis enzymes if it does not undergo catabolite repression to degrade its substrate (cellulose) and obtain the

end products (glucose) which can be detectable as long as the degrading microbe produce the enzymes. Even though microbes can utilize glucose for their survival (energy) it will be very less as compare to the production of the glucose in the medium.

Catabolite repression inhibit or repress the production of hydrolytic enzymes of an organisms in the presence of glucose which will cause decrease in the reduction of the parameters since the enzymes involve in the degradation process is inhibited. The CCR can also occur with others enzymes such as lipase, Boekema et al. (2007) has reported that microbial cell does not produce lipase in the medium containing glucose suggesting that lipase gene expression in the microbial cell was prone to catabolite repression. The presence of glucose inhibited or repressed the production of lipase in the medium and hence the degradative activity of the enzymes was inhibited.

CONCLUSION

Cellulase enzyme is one of the most important hydrolysis enzymes which might play an important role in the enzymatic treatment of biosolids. However, the considerations with the application of the enzymatic technology lie in the selection process for the most potent microorganism which is high effective in different types of biosolids compositions.

ACKNOWLEDGEMENT

The authors gratefully acknowledge Ministry of Higher Education of Malaysia for the research project financial support under fundamental research grant scheme (FRGS) vot No. 1453 and prototype research grant scheme (PRGS) vot G004.

REFERENCES

Abdel-Monem, M. O., Al-Zubeiry, A. H. & Al-Gheethi A.A. (2008). Elimination of *Salmonella* and faecal indicator bacteria in sewage sludge by quick lime stabilization. *J Environ Sci, Al- Mansoura Univ. Egypt, 36,* 281-297.

Abdel-Monem, M. O., Al-Zubeiry, A. H. & Al-Ghithi A.A. (2008). Survival of enteric indicators and pathogenic bacteria in sewage sludge after thermal treatment. *J Bot, Assut Univ. Egypt, 27 (1),* 171-183.

AbdulKarim, M.I., Daud, N.A. & Alam, M.D.Z. (2011). Treatment of palm oil mill effluent using microorganisms. In: M.D.Z, Alam, A.T, Jameel and A, Amid, (eds). Current research and development in biotechnology engineering at International Islamic University Malaysia (IIUM) Vol. III. IIUM Press, Kuala Lumpur, p. 269-275. ISBN 9789674181444.

Adel, A.S., Lalung, J., Efaq, A.N. & Ismail, N. (2015). Removal of cephalexin antibiotic and heavy metals from pharmaceutical effluents using *Bacillus subtilis* strain. *Expert Opin Environ Biol, 4,* 2.

Alam, M.Z., Rashid, S.S., Karim, M.I.A. & Salleh, M.H. (2009). Management of palm oil mill effluent through production of cellulases by filamentous fungi. *World J Microb Biotechnol* DOI 10.1007/s11274-009-0129-9.

Alcalde, M., Ferrer, M., Plou, F. J. & Ballesteros, A. (2006). Environmental biocatalysis: from remediation with enzymes to novel green processes. *Trends Biotechnol, 24 (6),* 281-287.

Al-Gheethi A.A., Norli, I., Efaq, A.N., Bala, J. D. & Al-Amery R. M. (2015) Solar Disinfection and lime treatment processes for reduction of pathogenic bacteria in sewage treated effluents and biosolids before reuse for agriculture in Yemen. *Water Reuse Des* 5(3): 419-429.

Al-Gheethi, A.A. (2015). Recycling of sewage sludge as production medium for cellulase enzyme by a *Bacillus megaterium* strain. *Int. J Rec Org Waste Agri, 4 (2),* 105-119.

Al-Gheethi, A.A., Mohamed, R.M.S., Efaq, A.N., Norli, I., Amir, H. & Ab. Kadir, M. O. (2016). Bioaugmentation Process of Sewage Effluents for the Reduction of Pathogens, Heavy Metals and Antibiotics. *J Water and Health* (Accepted).

Al-Gheethi, A.A.S & Abdul-Monem, M. O. (2014). Effect of nitrogen source on production of cmcase by *Bacillus megaterium* 1295s isolated from sewage treatment plants. *World Acad Sci Eng., Technol Environ Ecol Eng* 1 (6).

Al-Gheethi, AA, Efaq AN, Radin Maya SRM, Norli Ismail and M.O. Ab. Kadir. Potential of Pre-treated Biomass of Bacillus subtilis for Heavy Metal Ions Removal from Aqueous Solution. CLEAN – Soil, Air, Water (Revised submitted)

Al-Gheethi, A.A., Lalung, J., Efaq, A.N., Bala, J.D., & Norli, I. (2015b). Removal of heavy metals and β-lactam antibiotics from sewage treated effluent by bacteria. *Clean Technol. Environ Policy, 17 (8),* 2101-2123.

Al-Gheethi, A.A.S., & Norli, I. (2014). Biodegradation of pharmaceutical wastes in treated sewage effluents by *Bacillus subtilis* 1556WTNC. *Environ Process 1*, 459-489.

Allcock, E.R. & Woods, D.R. (1981). Carboxymethyl cellulase and cellobiase production by *Clostridium acetobutylicum* in an industrial fermentation medium. *Appl Environ Microbiol 41 (2)*, 539-541.

Angenent, L.T., Karim, K., Al-Dahhan, M.H., Wrenn, B.A. & Domíguez-Espinosa, R. (2004). Production of bioenergy and biochemicals from industrial and agricultural wastewater. *Trends Biotechnol 22*, 477-485.

Bakare, M.K., Adewale, I.O., Ajayi, A.O. & Shonukan, O.O. (2005). Purification and characterization of cellulase from the wild-type and two improved mutants of *P. fluorescens*. *Afr J Biotechnol 4 (9)*, 898-904.

Bala, J. D. (2016). Bioremediation and biodegradation potential of microorganisms isolated from raw palm oil mill effluent Ph.D Thesis, Environmental technology Division, School of Industrial Technology, Universiyi Sains Malaysia (USM).

Bala, J. D., Lalung, J. & Norli, I. (2015). Studies on the reduction of organic load from palm oil mill effluent (POME) by bacterial strains. *Int J Recy Org Waste Agr, 4(1)*, 1-10.

Blouzard, J.C., Bourgeois, C., Philip, P., Valette, O., Bélaïch, A., Tardif, C., Bélaïch, J. P. & Pagès, S. (2007). Enzyme Diversity of the Cellulolytic System Produced by *Clostridium cellulolyticum* Explored by Two-Dimensional Analysis: Identification of Seven Genes Encoding New Dockerin-Containing Proteins. *J Bacteriol 189(6)*, 2300–2309.

Boekema, B.K.H.L., Beselin, A., Breuer, M., Hauer, B., Koster, M., Rosenau, F., Jaeger, K.E. & Tommassen, J. (2007) Exadecane and tween 80 stimulate lipase production in *Burkholderia glumae* by different mechanisms. *Appl Environ Microb. 73*, 3838–3844.

Chan, K.Y. & Au, K.S. (1987). Studies on cellulase production by *Bacillus subtilis. Antonie Van Leeuwenhoek 53 (2)*, 125-136.

Chynoweth, D.P. & Pratap, P. (1996). Anaerobic digestion of municipal solid wastes: In Palmisano, A. C. and Barlaz, M. A. Microbiology of solid waste. CRC press. Inc. Florida. USA. pp. 71-104.

Davies, J. & Davies, D. (2010) Origins and evolution of antibiotic resistance. *Microbiol. Mol. Biol. Rev. 74 (3)*, 417–433.

Davis M.L. & Cornwell D.A. (2013) Introduction to environmental engineering. 15th edition, McGRAW, HILL, International Edition. New York, USA.

Efaq A.N., Nik Norulaini Nik Ab Rahman, Nagao H., Al-Gheethi AA, Md Shahadat & Ab. Kadir M.O. (2015). Supercritical carbon dioxide as non-

thermal alternative technology for safe handling of clinical wastes. *J Environ Process 2 (4),* 797–822.

Efaq A.N., Nik Norulaini Nik Ab. Rahman, Nagao, H., Al-Gheethi A.A. & Ab. Kadir M.O. (2016a) Inactivation of *Aspergillus* Spores in Clinical Wastes by Supercritical Carbon Dioxide. *Arab J Sci Eng* (AJSE). Online

Efaq, A. N., Nik, N. Nik Abd. Rahman, Nagao, H., Alkarkhi, A. M., Al-Gheethi, A.A., Tengku, N.T.L. & Ab. Kadir M.O. (2016) Treatment of solid clinical wastes using supercritical fluid CO_2 as an advanced green technique for destruction of pathogenic fungal spores. *CLEAN – Soil, Air, Water* (Revised submitted).

Ekperigin, M. M. (2007). Preliminary studies of cellulase production by *Acinetobacter anitratus* and *Branhamella* sp. *Afr. J. Biotechnol. 6 (1),* 28-33.

Emtiazi, G., Pooyan, M. & Shamalnasab, M. (2007). Cellulase activities in nitrogen fixing *Paenibacillus* sp. isolated from soil in N-free media. *World J Agri Sci 3 (5),* 602-608.

Fagan, M.J. & Saier, M.H.J. (1994). P-type ATPases of eukaryotes and bacteria: sequence comparisons and construction of phylogenetic trees. *J Mol Evol 38 (1),* 57-99.

Fath, M.J. & Kolter, R, (1993). ABC-transporters: Bacterial exporters. *Microbiol Rev 57(4),* 995-1017.

Gerardi, M. H. (2003). The microbiology of anaerobic digesters. John Wiley and Sons Inc., New Jersey, pp. 91-118.

Gilsdorf, J. R. & Zilinskas, R. A. (2005). New Considerations in Infectious Disease Outbreaks: The Threat of Genetically Modified Microbes. *Clin. Infect. Dis., 40 (8),* 1160-1165.

Hakamada, Y., Koike, K., Yoshimatsu, T., Mori, H., Kobayashi, T., Ito, S. (1997). Thermostable alkaline cellulase from an alkaliphilic isolate *Bacillus* sp. KSM-S237. *Extremophiles 1(3),* 151-156.

Hamer, G. (2003). Solid waste treatment and disposal: Effect on public health and environmental safety. *Biotechnol Adv 22,* 71-79.

Hankin, L. & Anagnostakis, S.L. (1975). Use of solid media for detection of enzymes production by fungi. *Mycologia 67,* 597–607

Herrero, M. & Stuckey, D. C. (2015). Bioaugmentation and its application in wastewater treatment: A review. *Chemosphere 140,* 119–128.

Ikram-ul-Haq, Javed, M. M. & Khan, T. S. (2006). An innovative approach for hyperproduction of cellulolytic and hemicellulolytic enzymes by consortium of *A. niger* MSK-7 and *Trichoderma viride* MSK-10. *Afr J. Biotechnol. 5 (8),* 609-614.

Immanuel, G., Dhanusha, R., Prema, P. & Palavesam, A. (2006). Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment. *Int J Environ Sci Technol 3 (1),* 25-34.

Jin, F. & Toda, K. (1989). Nutrients effects on cellulase production by the new species, *Clostridium thermocopriae*. *Appl. Microbiol. Biotechnol. 31*, 597-600.

Karam, J. & Nicell, J. A. (1997). Potential applications of enzymes in waste treatment. *J. Chem. Tech. Biotechnol*. *69 (2),* 141-153.

Kawai, S., Okoshi, H., Ozaki, K., Shikata, S., Ara, K., Ito, S. (1988). Neutrophilic *Bacillus* strain KSM.522 that products an alkaline carboxy methyl cellulase. *Agri Biotechnol Chem 52*, 1425-1431.

Kotchoni, O. S., Shonukan, O. O. & Gachomo, W. E. (2003). *Bacillus pumilus* BpCRI 6, a promising candidate for cellulase production under conditions of catabolite repression. *Afr J. Biotechnol*. *2 (6),* 140-146.

Krishna, C. (1999). Production of bacterial cellulases by solid state bioprocessing of banana wastes. *Biores Technol J 69,* 231-239.

Li, J. X., Gao, J., Luo, X. & Guo, Y. (2010). Functions of *Bacillus subtilis* BS7.29 in wastewater treatment. International conference on digital manufacturing and automation (ICDMA) 18-20 Dec. 2010, 1: 753-756.

Lynd, L.R., Weimer, P.J., Van Zyl W.H. & Pretorius, I.S. (2002). Microbial cellulose utilization: Fundamentals and Biotechnology Microbiology. Microbiol *Mol Biol Rev* 66, 506-577.

Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem 31,* 426-428.

Mingardon, F., Chanal, A., López-Contreras, A.M., Dray, C., Bayer, E.A. & Fierobe, H.P. (2007). Incorporation of fungal cellulases in bacterial minicellulosomes yields viable, synergistically acting cellulolytic complexes. *Appl Environ Microbiol 73 (12),* 3822-3832.

Morikawa, M. (2006) Beneficial biofilm formation by industrial bacteria *Bacillus subtilis* and related species. *J. Biosci. Bioeng*. *101 (1),* 1–8.

Nies, D.H. (1999). Microbial heavy metals resistance. *Appl Microbiol Biotechnol 51 (6),* 730-750.

Prakash, D., Verma, S., Bhatia, R. & Tiwary, B. N. (2011). Risks and precautions of genetically modified organisms. Review Article. ISRN Ecology. 2011: 1-13.

Prescott, V. E., Campbell, P. M., Moore, A., Mattes, J., Rothenberg, M. E., Foster, P. S., Higgins, T. J. V. & Hogan, S. P. (2005). Transgenic expression

of bean α-amylase inhibitor in peas results in altered structure and immunogenicity. *J. Agric. Food Chem. 53 (23),* 9023–9030.

Radwan, J. & Babik, W. (2012) The genomics of adaptation. *Proc. R. Soc. B.* p 1-5. (online).

Sadhu, S., Saha, P., Sen, S.K., Mayilraj, S. & Maiti, T.K. (2013). Production, purification and characterization of a novel thermotolerant endoglucanase (CMCase) from strain isolated from cow dung. Springer plus, 2:1-10.

Salmon, D. N. X., Spier, M. R., Soccol, C. R., de Souza Vandenberghe, L. P., Montibeller, V. W., Bier, M. C. J., & Faraco, V. (2014). Analysis of inducers of xylanase and cellulase activities production by *Ganoderma applanatum* LPB MR-56. *Fungal biology*, 118(8), 655-662.

Schloss, P.D., Hay, A.G., Wilson, D.B., Gossett, J.M. & Walker, L.P. (2005). Quantifying bacterial population dynamics in compost using 16S rRNA gene probes. *Appl Microbiol Biotechnol 66,* 457-463.

Sheldon, R. A. & van Rantwijk, F. (2004). Biocatalysis for sustainable organic synthesis. *Austr J Chem 57:* 281-289.

Singh, S., Moholkar, V.S. & Goyal, A. (2013). Isolation, Identification, and characterization of a Cellulolytic *Bacillus amyloliquefaciens* Strain SS35 from Rhinoceros Dung. *Int Scholarly Res Net(ISRN)Microbiol 2013,* 1-7.

Sivaprakasam, S., Mahadevan, S., Sekar, S. & Rajakumar, S. (2008). Biological treatment of tannery wastewater by using salt-tolerant bacterial strains. *Microbial Cell Fact.* 7 (15), 1-7.

Sternberg, D & Mandels, G.R. (1979). Induction of cellulolytic enzymes in *T. reesei* by sophorose. *J Bacteriol 139,* 761-769.

Tao, T., Snavely, M.D., Farr, S.G., Maguire, M.E. (1995). Magnesium transport in *Salmonella typhimurium*: mgtA encodes a P-type ATPase and is regulated by Mg^{2+} in a manner similar of the mgt B P-type ATPase. *J Bacteriol 177 (10),* 2654-2662.

Vinuselvi P., Kim, M. N., Lee, S. K. & Ghim, C. (2012) Rewiring carbon catabolite repression for microbial cell factory. *BMB reports,* 59-70.

Wang, Y.S., Byrd, C.S., Barlaz, M.A. (1994). Anaerobic biodegradability of cellulose and hemicellulose in excavated refuse samples. *J Indus Microbiol 13,* 147-153.

Wang, J. & Chen, C. (2009). Biosorbents for heavy metals removal and their future, Research review paper. *Biotechnol. Adv. 27 (2),* 195–226.

Wu, T.Y., Mohammad, A.W., Jahim, J.M., Anuar, N. (2010). Pollution control technologies for the treatment of palm oil mill effluent (POME) through end-of-pipe processes. *J Environ Manage 91,* 1467–1490.

Yoon, J.H., Park, J.E., Suh, D.Y., Hong, S.B., Ko, J.S. & Kim SH (2007). Comparison of dyes for easy detection of extracellular cellulases in Fungi. *Mycobiol 35(1),* 21-24.

Zhang, Y.H. & Lynd, L.R. (2004). Toward an aggregated understanding of enzymatic hydrolysis of cellulose. Non complexed cellulose systems. *Biotechnol Bioeng 88*: 797-824.

Zhang, Y.H.P., Himmel, M.E. & Mielenz J.R. (2006). Outlook for cellulase improvement: Screening and selection strategies. *Biotechnol Adv 24*, 452-481.

RR