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Hydrolysis of carboxymethyl cellulose (CMC) by *Bacillus* isolated from compost

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Abstract
The ability of *Bacillus* sp. to produce cellulase was studied by isolating the bacteria from the oil palm empty fruit bunch compost. Eight isolates were identified as genus *Bacillus* after amplification on universal 16S rRNA primer: Com1 and Com2. Screening of C1, C3, C4, C8, C9, C10, C12 and EB6 on the carboxylmethyl cellulose (CMC) plate resulted in clear zone or halos after staining with Congo red. The biggest clear zone was produced by C4, C12 and EB6 with diameter size of 2.4 cm, 2.6 cm and 3.4 cm respectively. The most suitable pH for the cellulase-producing bacteria growth was in neutral condition with pH between 7 and 8. Later, the above three isolates were analysed for the CMC assay to measure the cellulase activity based on the reduction of glucose released by these isolates. C12 and EB6 showed a higher rate of glucose reduction with 1.29 mg/ml and 1.64 mg/ml respectively after incubation for 24 h at 37 °C as compared to C4 with only 0.503 mg/ml.

Keywords: *Bacillus*, cellulase enzyme, carboxymethyl cellulose (CMC), 16S rRNA

Introduction

Cellulose is the major component of plant biomass (Camassola and Dillon 2007). Its crystalline structure and insoluble nature is a great challenge for enzymatic hydrolysis (Lynd et al. 2002). At the initial stage, the microorganisms responsible for cellulose decomposition bring about an enzymatic hydrolysis of the complex polymer, that is, the enzyme system which make up of a group of different enzymes collectively known as cellulase (Balamurugan et al. 2011).

Cellulases are comprised of independently folding, structurally and functionally discrete units called domains or modules, making cellulases modular (Henrissat et al. 1998). Cellulases are consortium of free enzymes which comprise endoglucanases, exoglucanase and cellobiases (β-D-glucoside glucohydrolase) (Siddiqui et al. 2000). Endoglucanase are active in amorphous regions of cellulose, and thus their activity can be assayed using soluble cellulose substrates, i.e. the CMCase assay. The binding of cellulase components to the accessible cellulose surface is a key step in cellulose enzymatic hydrolysis (Grethlein 1985; Woodward et al. 1988). In practice, the authors found that cellulolytic microorganisms produced a variety of complementary cellulases of different specificities from many different families.

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Hatami et al. (2008) described that cellulose decomposition occurs at a wide range of temperature and different aeration conditions that involved aerobic, microaerobic, facultative and obligate anaerobic microorganisms (which may be psychrophilic, thermophilic or mesophilic). The maximum rate of decomposition can be achieved by mesophilic microorganisms in aerobic condition. The other factors which affect cellulose decomposition are the ambient pH and the level of available nitrogen (Ljungdahl and Eriksson 1985; Kluepfel 1988).

Cellulolytic organism can convert cellulose in various economically important products and monomeric sugars, single cell protein, antibiotics and compost to usable products (Gautam et al. 2011). The cellulolytic potentials in soil bacterial communities were mostly studied based on the screening of isolates using different cellulose substrates such as soluble, colloidal or amorphous, or insoluble microcrystalline cellulose (Hendricks et al. 1995; Ruijssenaars and Hartsmans 2001; Wirth and Ulrich 2002).

This study was conducted to determine the ability of the *Bacillus* spp. isolated from oil palm empty fruit bunch (EFB) compost to produce cellulase. Screening on the CMC plate incurred to choose isolates capable to hydrolyse the cellulose substrate. An analysis of the CMCase activity was performed to assess the quantity of the reducing sugar that can produced by the selected isolates.

Materials and methods

The isolation of bacteria from EFB compost was carried out on the appropriate blended material (1 g compost in 99 ml distilled water). Serially diluted liquor was plated on nutrient agar. The growing colonies were separated based on their morphological differences; they were subsequently purified. The isolates were characterised by observing their morphology and staining method; gram positive and gram negative.

The isolates genomic DNA was extracted using Macherey Nagel NucleoSpin Tissue followed by the manufacturer protocol. The confirmation of their identities was done on the amplification by the 16S rRNA primer set; Com1; 5' CAGCAGCCGCGGTAATAC -3' and Com2; 5' CCGTCAATTCCTTTGAGTTT -3' (Schwieger and Tebbe 1998). The amplification condition was as follows: initial denaturation at 94 °C for 3 min followed by 35 cycles of 94 °C for 45 s, 61 °C for 45 s and 72 °C for 2 min. The final extension was at 72 °C for 10 min. The PCR products were viewed using Bio-Rad gel documentation system. The visible band on the 1.0% (w/v) agarose gel were outsourced to First Base Laboratories Sdn. Bhd., Selangor for sequencing analysis. The sequencing nucleotides results were analysed by using the Basic Local Alignment Search Tool (BLAST) to identify the sequences. The sequencing results were then aligned under Clustal Omega software to determine the phylogenetic relatedness of the different species. The alignment results were opened under the Tree View program to view the phylogeny tree.

The evaluation of cellulose hydrolysis was carried out on actively growing pure cultures on a CMC agar plate which was also used for the screening purpose. The reagent used was as follows: $NaNO_3$, 2.0 g; K_2HPO_4, 0.5 g; $MgSO_4.7H_2O$, 0.2 g; $CaCl_2.2H_2O$, 0.02 g; $MnSO_4.H_2O$, 0.02 g; $FeSO_4.7H_2O$, 0.02 g; CMC, 5.0 g and technical agar, 15 g. The initial pH of the medium was 7.0. The bacteria were then allowed to grow overnight. The diameter of each colony and the respective clear zone was measured after staining with Congo red (Hankin and Anagnostakis 1977) for 15 min.

The mineral salt broth consisted of 0.1% (w/v) of CMC was prepared with different initial pH levels; pH 4, 5, 6,

7 and 8. The pH medium was adjusted by pipetting sodium hydroxide (NaOH) and hydrochloric acid (HCl) to alkaline and acidic condition respectively. All mediums were autoclaved prior to inoculation. After autoclaving, the medium was dispensed into the sterilised test tube labelled with pH level. This test was conducted in triplicate. These inoculated tubes with test cultures were incubated at 37 °C for 48 h. The growth of the cultures was spectrophotometrically measured by optical density at 540 nm.

Cellulose degradation activity was expressed as cellulase (CMCase) activity. The cellulase-producing bacteria were grown in a nutrient broth for 24 h at 37 °C. The CMC solution was prepared as follows: KH_2PO_4 1.0 g, $MgSO_4.7H_2O$ 0.5 g, NaCl 0.5 g, $FeSO_4.7H_2O$ 0.01 g, $MnSO_4.H_2O$ 0.01 g, NH_4NO_3 0.3 g, distilled water 1.0 litre. A concentration of 1% (w/v) of CMC was added to the solution. The culture growth medium was centrifuged at 8000 rpm for 20 min and 200 µl of the supernatant was pipettes onto the test tube. A volume of 200 µl of the 1% (w/v) CMC solution was dispersed in the same test tube. The mixture was incubated in a water bath at 60 °C for 30 min. The enzymatic reaction was stopped by adding 600 µl of DNS solution and was then incubated at boiling temperature for 15 min (Singh et al. 2001; Faiez et al. 2008). The amount of simple sugar released was determined by the DNS method (Miller 1959) and measured by UV-VIS spectrophotometer under 540 nm. The standard curve was built using the standard glucose range from 1 mg/ml to 5 mg/ml.

Results and discussion

A total of eight isolates of cellulase-producing bacteria were isolated from the EFB compost. EFB compost was used as the source for isolation because EFB is a lignocellulosic composite and the potential cellulolytic bacteria isolated from the original high cellulose substrate was supposed to have the ability to produce high cellulase enzyme activity (Emmyrafedziawati 2013). They were morphologically characterised to differentiate the isolates. All isolated bacteria were gram positive and most of them exhibited the same characteristics.

The identification of the isolated bacteria was analysed through the PCR amplification using the universal 16S rRNA primer sets: Com1 and Com2 as described in materials and methods. All sequences belong to genus *Bacillus*. The C3 and C8 strains were definitively identified as *B. thuringiensis* and *B. atrophaeus* respectively. The identities were based on 100% homology appeared in BLAST database. The isolated C1, C9 and C10 were 99% homologous to the *B. cereus* while isolated C4, C12 and EB6 were most closely related to *B. subtilis* with 100% homology in nucleotide database. However, C4 and EB6 were identified as similar strains *(Table 1)*.

Based on the 16S rRNA gene sequence analysis results, a dendogram for the eight isolates were constructed. The phylogeny diagram *(Figure 1)* shows the genetic relatedness between the isolates. Two major branches were formed with each grouped by the distance of the percentage identity. The upper branch consisted of clusters belonged to *B. subtilis* while the lower branch consisted of clusters belong to *B. cereus, B. thuringiensis* and *B. atrophaeus*.

The isolated bacteria were then grown on specific media containing the CMC, which allowed only the cellulase-producing bacteria to grow. The activity of cellulose degradation was measured as the ratio between the diameters of the clearing zone over that of the colony size. The clear zone diameter was related to bacterial activity in cellulose decomposition (Pavlostathis et al. 1998; Valjamae 2002). The cell colonies hydrolysed CMC and easily recognised by a clear zone after staining with congo red. The differences in the clear zone size presumably due to the individual hydrolytic

Table 1. Clear zone for sample isolated from compost

Isolated no.	Isolate identification	Genbank accession no.	Homology (%)	Clear zone (cm)
C1	*Bacillus cereus* strain HLB-5	GU451184.1	99	1.3
C3	*Bacillus thuringiensis*	JQ922258.1	100	1.5
C4	*Bacillus subtilis* strain BAB-2742	KF053069.1	100	2.4
C8	*Bacillus atrophaeus* strain LY7	JQ246905.1	100	2.5
C9	*Bacillus cereus* strain SZAN-2	GU222440.1	99	1.4
C10	*Bacillus cereus* strain LY10	JQ246908.1	99	1.6
C12	*Bacillus subtilis* strain SBRh5	KF053071.1	100	2.6
EB6	*Bacillus subtilis* strain BAB-2742	KF053069.1	100	3.4

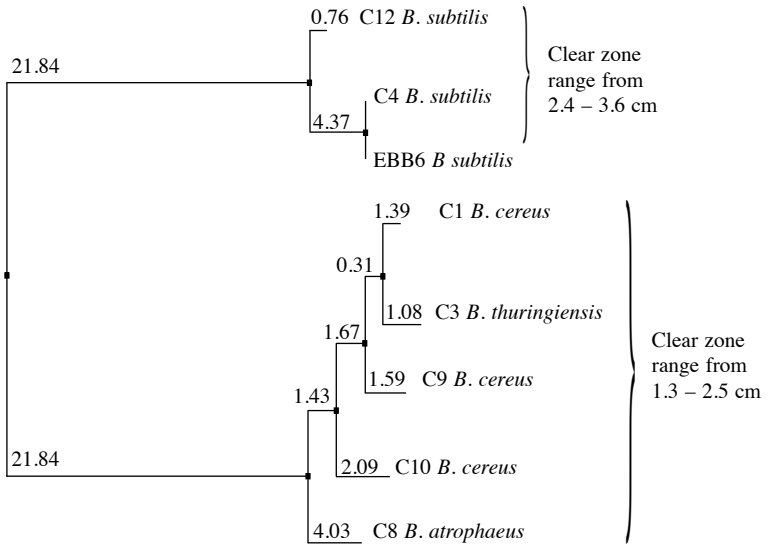

Figure 1. The phylogeny of the neighbour joining tree using % identity with distance produced from the alignments of 16S rRNA fragments presented in TreeView

activity on CMC. The results indicated that these bacteria could utilise CMC as a sole carbon source.

The interesting results were determined when the screening analysis can be related to the bacteria position in the phylogenetic tree (*Figure 1*). The upper branch of the phylogenetic tree showed that all strains had greater clear zone than strain from the lower branch. Substantially, the difference of these two branches were in terms of the species in which the upper branch was *B. subtilis* while the lowest branch was other groups of *Bacillus*. The *B. subtilis* strains consisted of C4, C12 and EB6 which produced larger clear zone than other *Bacillus* which consisted of C1, C3, C9 and C10 (*Table 1*). The individual cluster on each group although of similar species (refer to *B. cereus* and *B. subtilis*); they are from the different strains.

The screening of the cellulolytic activity on the CMC plate was used to narrow down isolates for further cellulose

Table 2. The effect of initial medium pH on the growth of cellulase-producing bacteria

Strain no./ID	Optical density (OD)				
	pH 4	pH 5	pH 6	pH 7	pH 8
C4 – *Bacillus amyloliquefaciens*	0.013	0.014	0.033	0.081	0.090
C12 – *Bacillus subtilis*	0.013	0.011	0.034	0.098	0.088
EB6 – *Bacillus subtilis*	0.009	0.011	0.039	0.085	0.100

study such as effect on pH and CMCase activity. The growth of the selected cellulase-producing bacteria on different pH was measured by optical density (OD) at 540 nm. Results showed that the suitable initial medium pH was 7 – 8 for all strains (*Table 2*). As the pH of the culture medium increased from 4 – 8, the OD increased from 0.009 to 0.1 for EB6. The maximum OD was recorded on EB 6 at pH 8 and C12 at pH 7 after incubation at 37 °C for 48 h. The acidic condition, which is below pH 7, seems not suitable for cellulase-producing bacteria growth. The medium growth that contained CMC indicated the ability of the isolates to grow in the cellulose substrate under suitable pH. This result was supported by claims made by Hatami et al. (2008) that cellulose decomposition will progress more rapidly in neutral pH and abundance of nitrogen. This is because under the aerobic condition, the mesophilic microorganisms will decompose the cellulose at maximum rate. The ambient pH and the level of available nitrogen are among other affecting factors (Ljungdahl and Eriksson 1985; Kluepfel 1988).

C4 as *B. amyloliquefaciens,* C12 and EB6 as *B. subtilis* were selected for CMCase activity as these isolates displayed greater halos on carboxymethyl cellulose (CMC) agar. Cellulase activity was measured by the DNS method (Miller 1959). Measurement of cellulase activity was considered to be a rather difficult task. Most of the enzyme activity measurements were based on reactions using soluble substrates or non-soluble substrate. There are two basic approaches to the assay of cellulose activity.

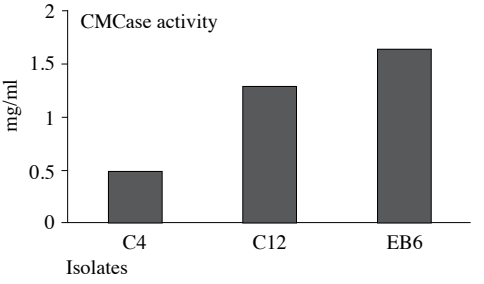

Figure 2. Glucose released from the CMC activity by Bacillus isolates

The first was based on analysis of individual enzymes, while the second was based on the measurement of the whole complex of cellulases (Maki et al. 2009). Assay of the secreted cellulase enzyme was carried out to determine the glucose reduction released from the individual isolates. *Bacillus subtilis* which consisted of the C12 and EB6 exhibited higher rate of glucose reduction with 1.290 and 1.640 mg/ml respectively after 24 h incubation at 37 °C in 1% CMC *(Figure 2)*. Previously, the filter paper assay (FPase) demonstrated on selected isolate showed greater halos. Among the isolates, C12 had highest FPase activity at 1.733 ± 0.023 FPU/ml (Emmyrafedziawati 2013).

The variable structural complexity of pure cellulose and the difficulty of working with insoluble substrates have led to the wide use of the highly soluble cellulose, such as CMC, as a substrate for studies of endoglucanase production (Lynd et al. 2002). CMC as the substrate gave the highest yield of an enzyme. It is assumed that this is due to the less complexity and hence easy assimilation of it by the isolated microbe (Wood and Bha 1988).

Conclusion

Genus *Bacillus* has tremendous potential as cellulose degrader as it was able to produce cellulase enzymes. From this study, the screening and enzymatic assay on the *Bacillus* isolates allowed us to identify the potential cellulase-producing bacteria. Different strains of *B. subtilis* C12 and EB6 had potential in the individual enzyme activities. Although both isolates were from the same species, the different strain may produce variant hydrolytic characteristic. Furthermore, a closer investigation needs to be done so that the isolated bacteria could act as a cellulose degrader. Analysis performed in this study is based on individual analysis. Usually, the microorganisms are more effective if acts as a consortium rather than individually. Future work will be the discovery of the cellulase activity produced by the microbial consortia in large scale production.

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Abstrak

Keupayaan *Basillus* sp. untuk menghasilkan selulase telah dikaji dengan memencilkan bakteria daripada kompos tandan kelapa sawit kosong. Sebanyak lapan pencilan telah dikenal pasti sebagai *Bacillus* selepas amplifikasi dengan menggunakan pencetus universal 16S rRNA: Com1 dan Com2. Saringan ke atas C1, C3, C4, C8, C9, C10, C12 dan EB6 di atas piring CMC telah menghasilkan zon terang atau gelungan apabila diwarnakan dengan Congo red. Didapati bahawa pencilan C4, C12 dan EB6 menghasilkan zon terang yang paling besar di atas piring CMC dengan bacaan diameter masing-masing 2.4, 2.6 dan 3.4 cm. Kesan pH ke atas pertumbuhan pencilan di atas piring CMC menunjukkan pH neutral iaitu pH 7.0 – 8.0 sangat sesuai untuk pertumbuhan bakteria penghasil enzim selulase ini. Kemudian, tiga daripada lapan pencilan yang mempunyai diameter paling besar dipilih untuk ujian esei CMCase bagi mengukur aktiviti enzim selulase masing-masing melalui jumlah penurunan gula yang dihasilkan. C12 dan EB6 masing-masing menunjukkan kadar gula penurunan yang lebih tinggi pada 1.290 mg/ml dan 1.640 mg/ml selepas inkubasi selama 24 jam pada 37 °C berbanding dengan C4 hanya pada 0.503 mg/ml.