Effect of UV and EMS Mutagenesis on Xylanase Production of *Streptomyces thermocarboxydus* ME 742 for Oil Palm Waste Composting

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ABSTRACT

Composting is one of most favored alternative to manage the large quantity of wastes generated from palm oil mill including empty fruit bunches (EFB) and decanter cake (DC) to avoid environmental pollutions. Microorganism is one of factor determinant for the degradation rate of raw material, therefore applying superior microorganism in process is a promising approach to enhance composting efficiency. In this study, the main aim was to improve xylanase production by *Streptomyces thermocarboxydus* ME742, which had been classified as thermotolerant lignocellulolytic bacteria and can be used in compost preparation. Firstly, UV exposure was applied to increase the production of lignocellulolytic enzyme, mainly xylanase. Unfortunately, this method could not induce a significant increase in xylanase production when mixed substrate of EFB and DC was used as nutrient for microbial growth, even they showed clear zone on xylan-containing agar about 10-13 cm bigger than that of wild-type. Mutants with enhanced 0.05-0.23 fold in xylanase production were obtained by EMS treatment for 60 min at concentration of 15 µg/mL. Through cultivation for 5 generations, 8 mutants possessed significantly increase in their xylanase production reached to 1.95-2.06 fold higher than wild type. Among these mutants, MEEMS16 was most genetically stable mutant which produced xylanase at 704.90 ± 21.91 U/g substrate, accounted to 2.06 fold of wild type. This study indicates that EMS was an effective mutagenic agent to generate mutation of *S. thermocarboxydus* ME742 with promising xylanase activity for use in decomposition of EFB and DC.

Keywords: Oil palm waste, Xylanase, UV mutation, Ethyl methanesulfonate mutation, Streptomyces thermocarboxydus

INTRODUCTION

Oil palm (*Elaeis guineensis*) is an important commercial crop vastly cultivated in Southern Thailand. It has made contributions to industrial development and economic growth of Thailand. In line with the increasing of palm oil production,

Article history:
Received 20 June 2021; Received in revised from 30 November 2021;
Accepted 7 December 2021; Available online 8 December 2021.

a vast quantity of oil palm biomass is generated every year. In the extraction process, crude palm oil is gained as a main product by only 10 % of oil palm biomass and 90 % of remaining biomass is released as wastes (Fauzianto, 2014), which need proper managements to reduce environmental pollutions. Over the last decade, wastes from oil palm mill have been utilized as renewable source for many bio-products such biofuel (Duangwang, et al., 2015; Wattanasit, et al., 2013) and biocatalysts (lignocellulolytic enzyme) (Khangkhachit, et al., 2020) as well. However, composting is a simple and most favored option to manage the large quantity of oil palm wastes (Cherypiew, et al., 2014; Nutongkaew, et al., 2014).

Oil palm empty fruit bunche (EFB) is a kind of solid waste extensively recycled into organic fertilizer since EFB contains a high content of organic matter (52.83 %) and mineral elements (Nutongkaew, et al., 2011; Nutongkaew, et al., 2014), which can be used to restore soil fertility (Nutongkaew, et al., 2011). In composting preparation, many formulations have been developed to achieve greater properties of product in shorter maturation time. Co-composting of EFB and decanter cake (DC) has been proposed as one alternative to balance nutrients in compost system. Because DC contains high nitrogen content (2.9 %), it is usually used as a potent nitrogen source to activate microorganisms in the composting process (Adam, et al., 2014; Nutongkaew, et al., 2011; Yahya, et al., 2010). In addition, high moisture content (78 %) in DC can accelerate the rate of compost formation (Nutongkaew, et al., 2011). However, natural composting normally takes long time than 60 days to obtain matured compost (Kananama, et al., 2011). Therefore, the research is still going on to improve composting efficiency.

Microorganism carrying out the composting process is a key determinant for increase in the degradation rate and decrease in composting time (Dayana Amira, et al., 2012). The capacity of microbe to degrade EFB as well as DC directly relates to its high ability to produce lignocellulolytic enzyme which is an important tool drives chemical change during composting. The studies on composting lignocellulolytic biomass showed that xylanase was normally detected at early stage (Dayana Amira, et al., 2012; Kananama, et al., 2011; Nutongkaew, et al., 2011), indicating the role of the enzyme in decomposition of raw material to release nutrient as mono- and di-saccharides which can be assimilated by microorganisms that do not produce extracellular hydrolytic enzyme. Large quantity of the enzyme can facilitate decomposition rate of lignocellulose. However, its activity was often in decline at later stage (after 2 weeks). It might be due to thermal inactivation of enzyme by heat generated during composting. This indicates that thermotolerance of microbial enzyme is a crucial factor for maintaining enzymatic degradation which might be help in faster production.

Previous studies employed UV exposure and chemical treatment using N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and ethane methane sulfonafe (EMS) to enhance the production of microbial lignocellulolytic enzymes (Jafri, et al., 2017; Kamar, et al., 2009; Kamar, 2015). Induced mutation by UV and chemical is known as a simple and affordable technique for strain improvement. However, the effectiveness of mutagen depends on the operating conditions such as time and concentration as well as microorganism used. In experiment, therefore, it is

necessary to determine optimal treatment condition for high mutation frequency of tested strain.

In previous study, our research group succeeded in isolating *Streptomyces thermocaboxydus* ME742 from natural source (Khangkhachit, et al., 2021). This bacterial strain could utilize EFB as carbon source to produce thermotolerant lignocellulolytic enzyme in which xylanase was a dominant activity in crude enzyme. Therefore, it has the potential to be used as inoculum for composting EFB. Nevertheless, its ability to degrade DC has not been investigated. Thus, the present work aimed to examine its capacity to produce lignocellulolytic enzyme, mainly xylanase from mixed substrate of EFB and DC. In addition, strain improvement was performed using EMS and UV mutation to enhance the production of xylanase by this bacterial strain for further application in composting EFB and DC.

MATERIALS AND METHODS

1. Materials and microorganism

Empty fruit bunch (EFB) and decanter cake (DC) were kindly provided by Larp Tavee Palm Oil Co., Ltd., Satun Province, Thailand. *Streptomyces thermocarboxydus* ME742 was previously isolated by Khangkhachit et al. (2021).

2. Evaluation of the ability of *S. thermocarboxydus* ME742 to produce xylanase used mixed substrate of EFB and DC

S. thermocarboxydus ME742 was grown on the nutrient broth (NB) in shaking incubator at 45 °C, 150 rpm for 24 h to obtain cell turbidity at OD_{660} around 0.6 (10^7 CFU/mL). Afterward, the culture (10 % v/v) was inoculated into 50 mL of Mandel and Andreotti (MA) broth medium (Dashtban, et al., 2011) consisting of 50 g/L carbon source (DC and EFB was mixed at ratio 1:1 by weight). The broth medium was incubated at 45 °C and shaken at 150 rpm for 7 days. The crude supernatant samples were collected at 4-6 days to measure the activity of xylanase and also cellulase. The experiments using individual EEB and DC at 50 g/L were conducted for comparison.

3. Induced mutagenesis of *S. thermocarboxydus* ME742 by UV

S. thermocarboxydus ME742 was grown on NB at 45 °C 150 rpm until OD_{660} reached around 0.6 (10^7 CFU/mL). Cell suspension (0.1 mL) was spread on nutrient agar (NA) and exposed to UV lights (280 nm) at different distances (5, 10 and 15 cm) from the UV source (Bio-Rad 805BR GS Gene Linker Crosslinker UV Chamber, Bio-Rad Laboratories, California) and for varying exposure time (2, 3, 5, 10, 30, 60 and 120 s). Then, plates were incubated at 45 °C in the dark for 48 h to avoid photo-activation. Colonies after UV treatment were recorded to determine survival rate. The colonies on plates showing 0.1-10 % survival rate were screened their capacity to produce xylanase. The survival rate was calculated using equation as follows:

Survival rate (%) = (C1-C2)/C1 x 100

Where C1 are colonies on plate without UV exposure, C2 are colonies on plate after UV exposure.

4. Induced mutagenesis of *S. thermocarboxydus* ME742 by EMS

Cell culture was performed as described above. After that, cells were collected by centrifugation at 10,000 x g for 10 min at 4°C and re-suspended in 0.1 M phosphate buffer solution (pH 6.0). Ethyl methanesulfonate (EMS) dissolved in same buffer at concentrations (10, 15, 20, 25, 30, 50, 70, 90 and 100 µg/mL) were then added into cell suspension. Each cell mixture was incubated in shaking incubator at a speed of 150 rpm at 45°C for 60 min. After given time, cells were centrifuged at 10,000 x g for 10 min at 4°C and washed twice with 0.85 % (w/v) sterile sodium chloride solution before grown on NA by spreading method. The cultivation was done in a dark incubator at 45°C for 48 h. The colonies on plates showing 0.1-10 % survival rate were screened their capacity to produce xylanase. The survival rate after EMS treatment was calculated using equation as follows:

Survival rate (%) = (C1-C2)/C1 x 100

Where C1 are colonies on plate without EMS treatment, C2 are colonies on plate after EMS treatment.

5. Selection of desirable mutant improved the production of xylanase

Initially, the survivors after mutagenesis were screened their improvement in xylanase production by a spot test on agar medium. A single colony was grown on MA agar containing 0.25 % (w/v) xylan as carbon source for 48 h to allow sufficient clear zone production. The mutants produced the clear zone around colony larger than that of wild type, *S. thermocarboxydus* ME742 (WTME742) were selected for screened their ability to secrete xylanase on MA broth contained 50 g/L EEB and DC (1:1 by weight). MA broth inoculated with 10 % (v/v) mutant cell suspension was incubated at 45 °C with shaking speed of 150 rpm for 5 days. The enzyme activities in crude supernatant were measured as described below. Mutants exhibited xylanase production higher than wild type were selected for determination of their genetic stability for enzyme production for 5 successive generations. The interval for each generation was 10 days. The ability of each mutant after interval cultivation on fresh NA slant to produce xylanase on broth medium contained EEB and DC was evaluated. The mutant maintained stable xylanase production was chosen for further experiment.

Enzyme activity assay

Xylanase activity of each culture was measured according to the method described by Miller (Miller, 1959, pp. 426-428). The reaction mixture containing 0.5 mL of crude supernatant and 0.5 ml of 1 % (w/v) xylan (Oats spelt xylan; Sigma Chemical Ltd.) solubilized in 50 mM sodium citrate buffer (pH 4.8) was incubated at 50 °C for 10 min. Then, 2.0 mL of 3,5 dinitrosalicylic acid (DNS) reagent was added into the mixture. After boiling the mixture at 100 °C for 10 min, it was put in

ice box for 5 min before measurement of absorbance at 520 nm using spectrophotometer (Spectroquant ® Pharo 300, Thomas Fisher Scientific Co., Ltd). One unit of xylanase was determined as amount of enzyme releases 1 µmol of reducing sugar equivalent to xylose per min under the assay condition.

Cellulase activity was also determined. The activity of cellulase was measured using the method similar to assay of xylanase. However, the reaction mixture used 1 % (w/v) carboxymethylcellulose (CMC) (BHD Chemicals Ltd.) as substrate and incubated at 50 °C for 30 min. One unit of cellulase activity was determined as amount of enzyme releases 1 µmol of reducing sugar equivalent to glucose per min.

Statistical analysis

All experiments were conducted in triplicate. The obtained results were statistically analyzed by one-way analysis of variance (ANOVA). P value of ≤ 0.05 was regarded as significant.

RESULTS AND DISCUSSION

1. Xylanase production by *S. thermocarboxydus* ME742 using mixed substrate of EFB and DC

This experiment was conducted to evaluate the ability of *S. thermocarboxydus* ME742 to produce extracellular xylanase in broth medium contained EFB and DC as nutrient source in order to ensure its potential application in preparing co-compost of EFB and DC. Xylanase production was compared with that using individual EFB or DC as carbon source. The activity was observed at 4-6 days incubation. Figure 1A showed the highest activity of xylanase (327.5±1.89 U/g) at 6 days in medium contained mixed substrate. Compared with EFB, broth medium containing EFB + DC gave higher xylanase active, indicating supplemented DC might be more favored for xylanase production by *S. thermocarboxydus* ME742. Cellulase production was also investigated. The considerable cellulase production (47.37±1.40 U/g) was found in medium mixed EFB and DC (Figure 1B).

The physico-chemical properties of EFB showed that it can be used as alternative carbon source for microbial growth owning to the richness in organic carbon (45 %, w/w) but low in nitrogen content (0.55-0.90 % w/w) (Nutongkaew, et al., 2011, pp. 1-5; Kavitha, et al., 2013, pp. 930-937), while DC contains high nitrogen content (2.9 %) (Nutongkaew, et al., 2014, pp. 275-281). Mixing two raw materials might provide better C/N balance contributing to high xylanase production by *S. thermocarboxydus* ME742. This result indicates the feasibility of applying *S. thermocarboxydus* ME742 in composting process using co-substrate of EFB and DC.

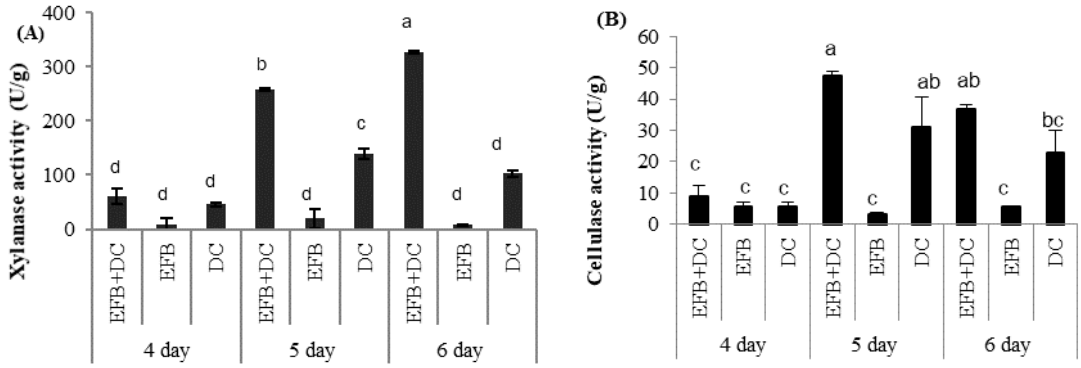

Figure 1 The average amount of xylanase (A) and cellulase (B) production by *S. thermocarboxydus* ME742 in broth media containing 50 g/L oil palm waste as carbon source. EFB indicates broth medium containing 50 g/L EFB, DC indicates broth medium containing 50 g/L DC, and EFB+ DC indicates broth medium containing 25 g/L EFB and 25 g/L DC as carbon source.

2. Improvement of xylanase production by UV mutagenesis

As mentioned above, *S. thermocarboxydus* ME742 would be used as inoculum in co-composting EFB and DC in further research. In order to achieve the goal, induced mutation by physical and chemical mutagens were employed as tool to improve xylanase production by this bacterial strain as high as possible**.**

Mutagenesis of *S. thermocarboxydus* ME742 was initiated by UV exposure for varying time (2, 3, 4, 5, 10, 30, 60 and 120 s) and distance (5, 10 15 cm). The survival percentage was calculated and showed in Figure 2. The lethality about 99 % was seen with increase in exposure time. Exposure longer than 4 s killed almost cells in all tested distances. The survival rate in range 1-10 % was observed by exposure at 5 cm for 2-3 s.

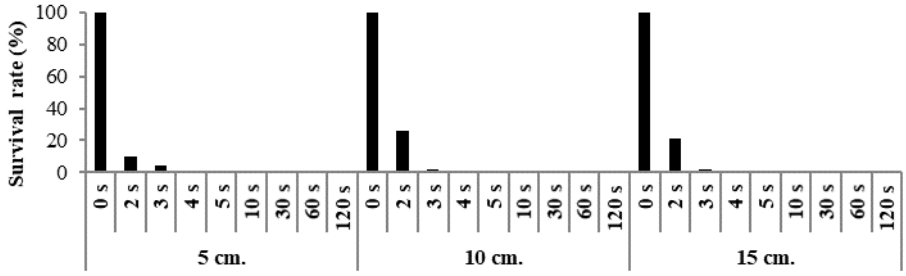

Figure 2 The survival rates of *S. thermocarboxydus* ME742 after exposure to UV at varying time and distance.

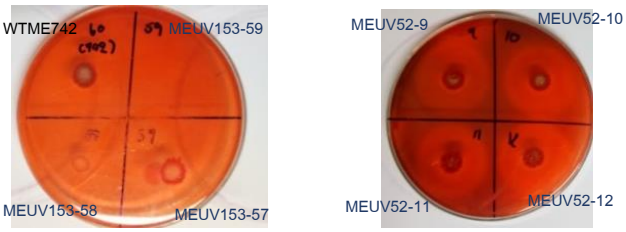

Figure 3 Example of xylan-degrading zone of mutants (code MEUV52-9, MEUV52-10, MEUV52-11, MEUV52-12, MEUV153-57, MEUV153-58, MEUV153-59) and wild type *S. thermocarboxydus* ME742 (designed as WTME742) after 48 h of incubation.

Survivor colonies (68 colonies) were screened for xylanase production as shown in Figure 3. In primary screening on xylan-containing agar medium, 24 conies exhibited clear zone larger than wild type (WTME742) that showed zone of clearance of 0.1 cm. However, this study selected only 12 mutants showing the biggest clear zone in the range from 1.0 cm to 1.3 cm for secondary screening on broth medium supplemented with EFB and DC (1:1 by weight). Of which 12 mutants, only 5 mutants, MEUV52-2, MEUV52-4, MEUV52-9, MEUV52-10, MEUV52-55 showed xylanase production in broth medium. Slightly increase in xylanase production than WTME742 (335.49 U/g) was found by MEUV52-9 (341.93±20.10 U/g) and MEUV52-55 (344.81±24.08 U/g) (Figure 4). The stability of these mutants for xylanase production was studied for 5 generations. Figure 5A shows the average of xylanase activity obtained from 5 generations. The result showed only mutant MEUV52-9 (478.59 ± 81.26 U/g) maintained the stable xylanase production. However, enzyme production level was increased by only 0.03 fold of WTME742 (464.22 ± 69.24 U/g). Considering cellulase production of this mutant, no significant increment was observed (Figure 5B).

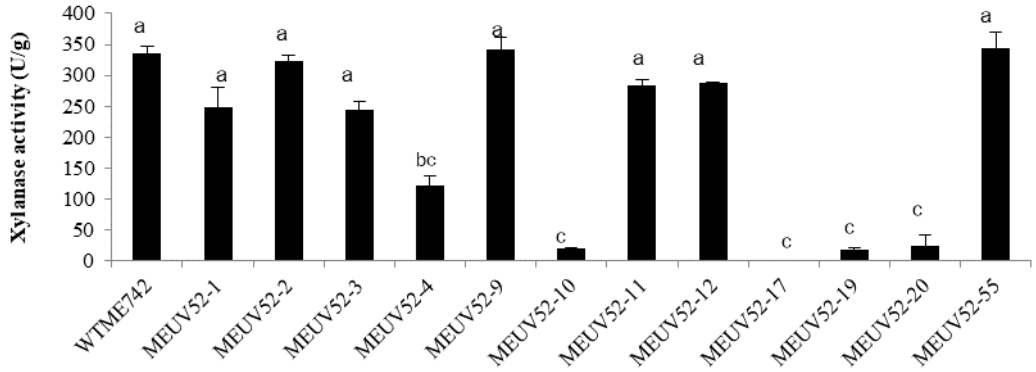

Figure 4 Xylanase production in broth medium contained EFB and DC (1:1 by weight) by WTME742 and mutants generated by UV mutagenesis.

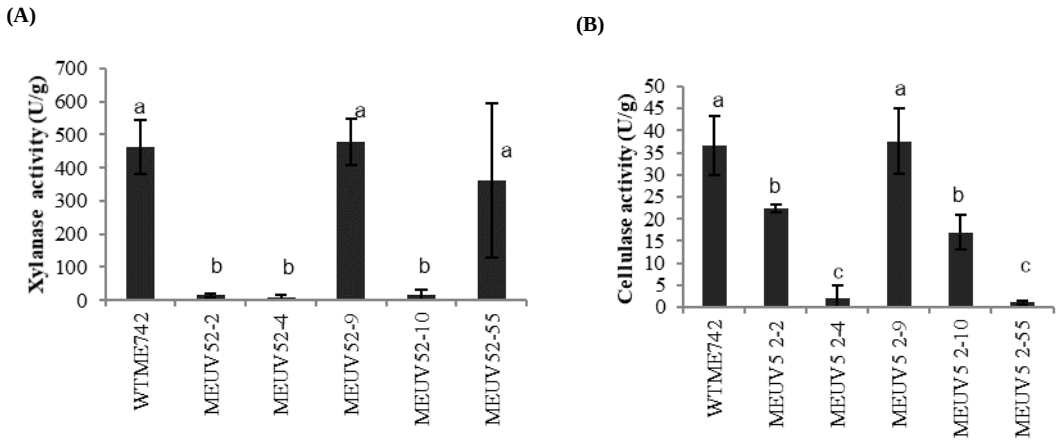

Figure 5 The average amount of xylanase (A) and cellulase (B) produced during 5 generations by WTME742 and mutants generated by UV mutagenesis.

Previous studies reported UV exposure as an effective tool to improve xylanase production by microorganisms such as *Streptomyces pseudogriselus* (Abdel-Aziz, et al., 2011, pp. 1045-1050), and *Streptomyces* sp. CA24 (Porsuk, et al., 2013, pp. 370-375). However, xylanase production by *S. thermocarboxydus* ME742 was not enhanced through this mutagenesis. In present study, we found out an interested phenomenon that xylan-degrading ability of mutant observed on agar plate did not correlate to its ability to produce extracellular in broth medium containing lignocellulosic biomass. This might be due to the occurrence of mutation on other enzymes important in breaking down cell wall of this material, for instance, enzymes belong to ligninase group such as laccase, and lignin peroxidase. It should be noted that the change in cellulase production by mutants obtained in this study had close relation to their xylanase production.

3. Improvement of xylanase production by EMS mutagenesis

EMS treatment was further applied as an alternative method to enhance xylanase production by WTME742. UV mutant MEUV52-9 was also used for EMS treatment to investigate the effect of sequential mutation by UV and EMS. Regarding survival rate, treatment by 15 µg/mL EMS provided the preferable survival rate for WTME742 (8.37 %) and MEUV52-9 (3.72 %). Through primary screening by a spot test, 14 mutants from parent strain WTME742 exhibited xylan-degrading zone (> 0.2 cm) bigger than WTME742 (0.1 cm). Of note, all survivor mutants from MEUV52-9 did not show clear zone on xylan-containing agar plate. Therefore, 14 mutants developed from WTME742 were selected for next screening.

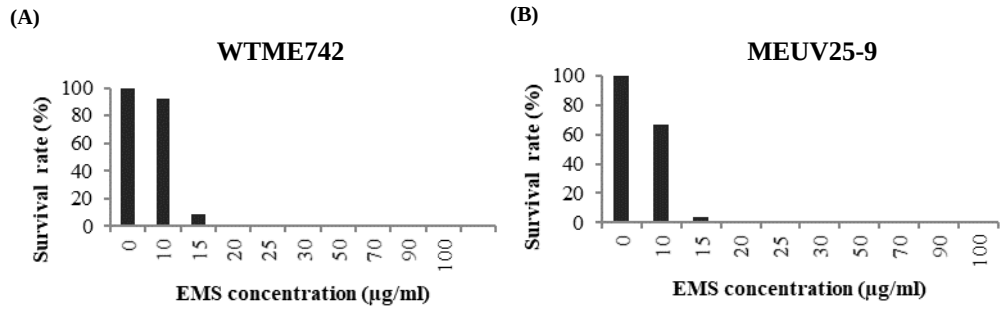

Figure 6 Survival rates after EMS treatment of *S. thermocarboxydus* ME742, WTME742 (A) and UV mutant MEUV52-9 (B).

For secondary screening, broth medium contained EFB and DC was used. It was found that 9 mutants (MEEMS1, MEEMS2, MEEMS4, MEEMS6, MEEMS7, MEEMS9, MEEMS12, MEEMS16, MEEMS19) could produce xylanase in amount higher than wild-type that the xylanase production was increased by 1.05-1.3 fold (Figure 7).

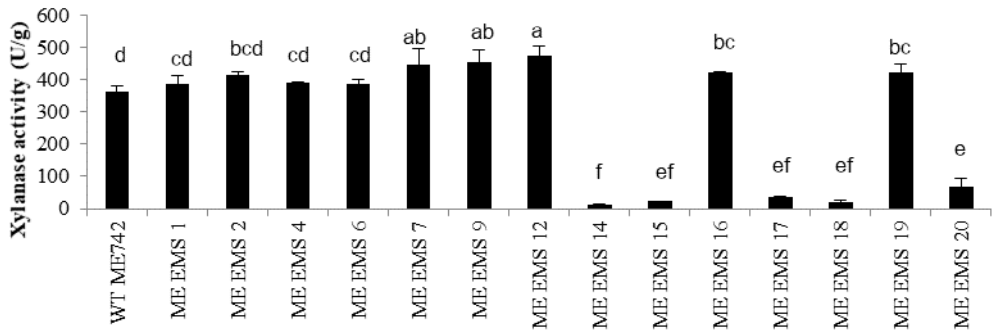

Figure 7 Xylanase production in broth medium contained EFB and DC (1:1 by weight) by WTME742 and mutants generated by EMS mutagenesis.

After cultivation for 5 generations, the increase in xylanase production by MEEMS6, MEEMS9, MEEMS12, and MEEMS16 reached 1.95 - 2.06 fold higher than that of WTME742 (Figure 8A.). It is well-known that the stability of microorganism is most important property for industrial application. According to the amount of xylanase observed in each generation by wild-type and mutants, it was found that MEEMS16 was a more genetically stable mutant which produced xylanase of 704.90 ± 21.91 U/g, accounted to 2.06 fold of wild-type or 206 % increasement. Concerning cellulase, the enzyme production was slightly increased by EMS mutagenesis (Figure 8B). This is comparable to the result reported by previous works studied on UV mutagenesis for *Streptomyces pseudogriseolus* (161% increase) (Abdel-Aziz , et al., 2011) and N-methyl N- nitro N-nitroso guanidine for *Aspergillus niger* GCBT-35 (1.7 fold increase) (Tasneem, et al., 2003).

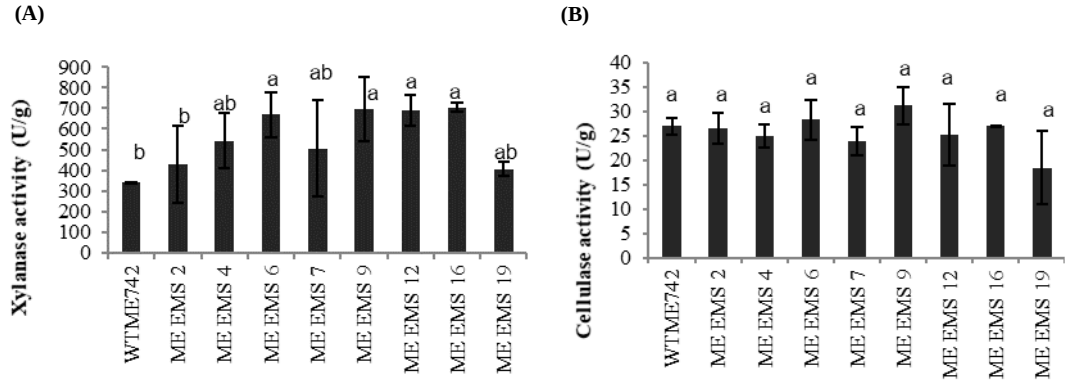

Figure 8 The average amount of xylanase (A) and cellulase (B) produced during 5 generations by wild type (ME742) and mutants obtained by EMS mutagenesis.

CONCLUSIONS

This study indicated the capacity of *S. thermocarboxydus* ME742 to produce xylanase using mixed EFB and DC that can serve as co-substrate for compost production. UV and EMS mutagenesis were applied for development of this bacterial strain capable of increased xylanase production. In this investigation, EMS mutagenesis was more effective to enhance xylanase production by *S. thermocarboxydus* ME742. Among mutants generated, MEEMS16 was selected as most stable mutant that increased 2.06 fold of xylanase production. This shows the promising of mutant MEEMS16 to be used as inoculum in oil palm wastes composting. High xylanase production by this mutant may facilitate degradation rate of raw material and consequently provide good properties of product in shorter maturation time.

ACKNOWLEDGMENTS

This research was financially supported by the Agro-Industry Practice School of Prince of Songkla University, and Graduate School of Prince of Songkla University and Thailand.

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