International Research Journal of Engineering and Technology (IRJET) e-ISSN: 2395 -0056
Volume: 03 Issue: 08 | Aug-2016 www.irjet.net p-ISSN: 2395-0072

Release of Ferulic Acid from Banana Stem Waste by Ferulic Acid Esterase from Co-culture

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Abstract - *The use of microbial in producing ferulic acid esterase for ferulic acid production from agricultural waste currently becomes important. This study was focus on investigating this capable enzyme from co-culture in releasing ferulic acid from banana stem waste by enzymatic hydrolysis. Submerged fermentation of banana stem waste by co-culture from Bacillus cereus, Bacillus pumilus, and Bacillus thuringiensis was conducted. The analysis found that ferulic acid yield gradually increased with the increasing of ferulic acid esterase activity until the late log phase or early stationary phase of co-culture growth. Ferulic acid esterase activity was increased up to 3.1-fold during the log phase and successfully released 571 mg/kg ferulic acid content. The discovery indicates that co-culture of Bacillus spp. have the capability of producing the desired enzyme in order to produce high-value compound from agricultural waste.*

Key Words: Ferulic acid esterase, Enzymatic hydrolysis, Submerged fermentation, Co-culture, Banana stem waste

1. INTRODUCTION

Ferulic acid (4-hydroxy-3-methoxycinnamic acid, FA) was first time isolated from *Ferula foetida* and an enormously copious and almost ubiquitous phytochemical phenolic derivative of cinnamic acid, present in plant cell wall components as covalent side chains [1]. FA together with dihydroferulic acid is a component of lignocelluloses which conferring cell wall rigidity by cross-linking lignin and polysaccharides [2]. According to Yagi and Ohishi (1979), a group of Japanese researchers was succesfully found steryl esters of FA of FA that extracted from rice oil. Discovery of the antioxidant properties became into notice of researcher on biological usages of FA.

At present, extensive researches have been conducted in producing FA from agricultural waste. Generally, FA obtained from the chemical process cannot be considered as natural, so various attempts have been made for enzymatically release of FA from natural sources. Ferulic acid esterase (FAE) (EC. 3.1.1.73) is a key enzyme for the recovery and purification of ferulic acid and related hydroxycinnamic acids by hydrolyzing the ester bond between hydroxycinnamic acids and sugars present in the plant cell walls [4].

Many studies have been described on FA production by the action of FAE-producer microorganisms. The activity has been reported most in fungi especially in *Aspergillus* spp. such done in *A. niger*, *A. clavatus*, and *A. terreus* [5-7] and a few in bacteria species such reported in *Bacillus* spp. [8-9], *Lactobacillus fermentum* [8], *Streptomyces* spp. [10-12] and, *Staphylococcus aureus* [13]. However, lack of research has been done for co-cultivation through fermentation to produce ferulic acid.

Currently, four types of microbial FAEs namely A, B, C, and D have been classified based on substrate preference to enzyme to form an enzyme-substrate complex [14]. These enzymes have different specificity for hydroxycinnamic acid methyl esters to release FA from the complex structure. As a diverse group of hydrolases, FAEs act in releasing FA by breaking down the linkage of FA to hemicellulose and lignin the plant cell wall [15]. Certain microflora can well survive in the presence of phenolic acids by utilizing them as carbon source [12]. Thus, the utilization of acclimatized microbe to substrate prior conducting an experiment could enhance the performance of an organism over another organism that has not had the opportunity to acclimate to that particular environment [16].

Fermentative production is highly recommended by using cheap by-products and waste substrates for FA production [17]. Hence, the exploitation of banana stem waste (BSW) as carbon source in fermentation is a good alternative for this FA production. Besides, the used co-cultivation could also improve the FA production [18]. Therefore, the purpose of this study was to discover the FAE production by co-culture in hydrolyzing BSW, which results in the release of ferulic acid. The activity of the enzyme release by co-culture was investigated through submerged fermentation process.

2. MATERIAL AND METHODS

2.1 Substrate

Banana stem waste (BSW) was obtained from banana plantation in Pahang, Malaysia. The stem was cleaned to

remove any dirt attached before use as substrate. The stem was chopped up into a cube (1 cm x 1 cm). One part of the chopped stem and two part of distilled water were blended. The mixture was adjusted to a pH 9.5 by an addition of 1 M of NaOH. Substrate was prepared in 250-ml Erlenmeyer flask and autoclaved at 121°C for 15 min for sterilization to kill undesired microorganism.

2.2 Microorganism

Soil bacteria were acclimatized for a month in BSW before isolation. Several colonies were isolated, and only three types of strains were chosen in this experiment. They were identified by 16S rRNA sequence analysis as *Bacillus cereus* strain CCM 2010 (A), *Bacillus pumilus* SAFR-032 (C) and *Bacillus thuringiensis* Bt407 (D). Isolated strains were maintained on nutrient agar plate and kept at 4°C until further use.

2.3 Preparation of co-culture

Before starting the experiment, the strains were revived by plating onto nutrient agar plate and incubated at 37°C for 24 hours. A single colony of each strain was scraped and propagated in universal bottle containing 10 ml of nutrient broth at 37°C for 22 hours. The cultures were aseptically dispensed into 100-ml Erlenmeyer flask containing 50 ml of sterile nutrient and incubated further 22 hours at 37°C. Co-culture AD and ACD were prepared as co-culture inoculum by adding a total of 50 ml stationary phase inoculum of each single culture at equal volume into 1000-ml Erlenmeyer flask containing 500 ml nutrient broth. Before used in fermentation, the inoculum was incubated for another 22 hours at 37°C.

2.4 Experimental set-up

In this study, fermentation was performed at the optimum condition by using the best type of co-culture that has good performance in releasing FA. For that reason, optimum condition was prior determined by studying the effect of agitation (0 rpm and 150 rpm) and volume of inoculum (5% and 15%) using co-culture AD and ACD. Fermentation of BSW as their sole carbon source by co-culture ACD and AD was performed for 24 hours. The selected optimum condition and co-culture was used in evaluating the enzyme production from that capable co-culture. The stationary phase of co-culture inoculum was inoculated into 250-ml Erlenmeyer flask containing substrate and incubated at 26°C for up to 48 hours. The culture supernatant was collected at certain interval time to check the growth profile, FA production and for enzyme assay preparation. Bacterial growth was checked using a spectrophotometer, while FA content was analyzed by HPLC.

2.5 Growth pattern

The culture supernatant was collected at certain interval time, and the cell concentration was monitored by using a spectrophotometer. Absorbance was measured at a wavelength of 600 nm using a UV/Visible spectrophotometer. Sterile liquid of substrate without co-culture was used as a blank for zeroing purpose.

2.6 Analytical methods

2.6.1 Preparation of crude enzyme and enzyme assay

Fermentation sample was centrifuged for 20 minutes at 4000 x g at 4°C to separate the liquid from the bacterial biomass and suspended material. The clear supernatant was collected and used as the crude enzyme for enzyme assay. A modified method of Xie et al. (2010) was applied to determine the activity of ferulic acid esterase. A 50 mM methyl ferulate was prepared in methanol as stock solution. The activity of ferulic acid esterase was assayed by diluting 1 ml of methyl ferulate solution with 9 ml of 0.1 M citrate buffer (pH 6.0). Then 2 ml crude enzyme was added to 2 ml of preheated (at 40°C) diluted methyl ferulate solution. The reaction mixture was incubated for 4 hours at 40°C. The reaction was stopped by heating the mixture to 95°C for 10 min. Then it was cooled to room temperature and filtered using a 0.45µm membrane. HPLC was used to analyze the content of ferulic acid in each sample. The activity of one unit ferulic acid esterase was referred as the amount of enzyme used to release 1 µmol ferulic acid that hydrolyzed from the methyl ferulate per minute at pH 6.0 and 40°C.

2.6.2 Ferulic acid determination by HPLC

Sample was centrifuged for 20 minutes at 4000 x g and supernatant was used for analysis. Samples were analyzed by using HPLC (Agilent 1100 system) equipped with Agilent Zorbaq SB-AQ C18 analytical column. The modified method of Chamkha et al., (2001) was applied using an isocratic mobile phase consisted of acetonitrile, distilled water and acetic acid (30:69.9:0.1, v/v). The flow rate was set at 0.6 ml/min with 25 ml volume of the injection loop. Ferulic acid was quantified using a diode array detector (DAD) at 240 nm wavelength. Before analyzing, samples were filtered into vial by using 0.45 µm nylon syringe filter. The vials of sample were placed on HPLC plate to analyze the amount of ferulic acid content.

3. RESULTS AND DISCUSSION

3.1 Determination of optimum condition

From our previous research, two (co-culture AD and ACD) out of 26 co-cultures was exhibited good performance in FA production [21]. Fig -1 shows comparison performance of co-culture AD and ACD in producing FA using BSW at different agitation and volume of inoculum. The analyzed result obviously demonstrated that co-culture AD was produced higher FA concentration than co-culture ACD at all conditions. Agitation of 150 rpm and 5% of volume of inoculum increased FA production 1.8-fold and 1.5-fold, respectively using co-culture AD. Volume of inoculum of 5% was optimum to ensure sustainability of bacteria until it reached the stationary phase, while at high agitation (150 rpm) could promote bacteria cell growth and indirectly increased enzyme production. The similar condition was used in fermentation conducted by Zywicka et al. (2015) and Santos et al. (2007) for higher product recovery. Therefore, on the basis of FA production, agitation at 150 rpm and 5% of volume of inoculum were applied for further studies. Meanwhile, co-culture AD was selected to examine the presence of FAE activity during submerged fermentation because of its FA production was better compared to co-culture ACD.

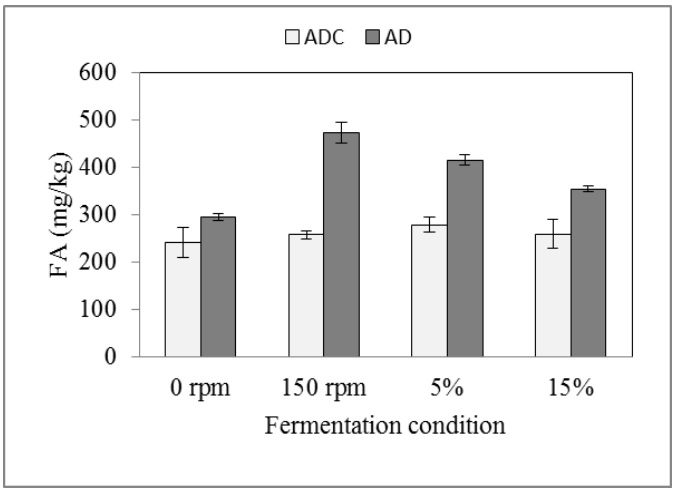

Fig -1: Comparison of ferulic acid production by co-culture ACD (*B. cereus*, *B. pumilus*, and *B. thuringiensis*) and AD (*B. cereus* and *B. thuringiensis*) at different agitation (rpm) and volume of inoculum (%, v/v) from banana stem waste through submerged fermentation.

3.2 Production of ferulic acid esterase by co-culture

The time course of bacterial growth and FAE activity production are shown in Fig -2. The result showed that during the log phase of bacterial growth, FAE activity was increased proportionally to the increase of bacteria cell. The cell density increased rapidly during the log phase, reaching a peak of 30 hours and became almost stable at this point. The results implying that the synthesis of FAE activity was closely associated with the co-culture cell growth and decreased into stabilization state of growth. Mohanasrinivasan et al. (2011) mentioned in their research that enzyme production by *Bacillus* sp. is related to the growth of microorganism. The growth would have reached a stage of limitation due to insufficient nutrients. They also found that by extending the incubation beyond 72 hours resulted in decreasing of enzyme activity might have been due to the denaturation of the enzyme caused by the interaction with other components in the medium.

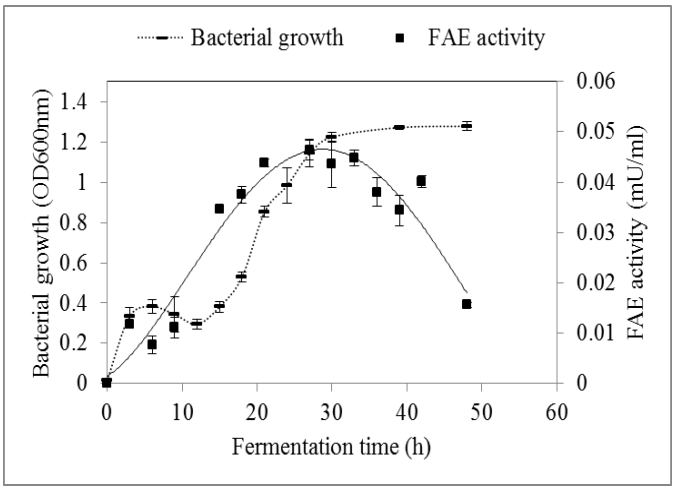

Fig -2: Time course of co-culture AD (*B. cereus* and *B. thuringiensis*) growth pattern and ferulic acid esterase (FAE) production by co-culture AD grown on banana stem waste.

Meanwhile, FAE activity increased progressively up to 27 hours period of incubation and begin decreased at 36 hours until 48 hours of incubation time. Maximum FAE activity achieved was about 0.046 mU/ml. It increased 3.1-fold during the log phase of co-culture growth. The highest production earlier than previously reported as 48 hours in *Bacillus* spp. [9] and in other species such as *Streptomyces* spp., was reported as 48 hours [11], 72 hours [25] and 96 hours [12]. The activities of other FAE-producing microorganisms from the previous study were also reported at different assay condition as much as 34.8 mU/ml, 2.0 mU/ml and 82 mU/ml using *Bacillus pumilus* [9], *Streptomyces* S10 [12] and *Streptomyces avermitilis* CECT 3339 [10], respectively. However, the yield of FAE activity from co-culture using an inexpensive medium in this study is not possible to compare because the assay was varied in term of condition and enzyme substrate used.

3.3 Release of ferulic acid by co-culture

In this study, the ability of co-culture in releasing FA was also investigated. Release of FA from BSW was examined for 48 hours incubation with co-culture of *B. cereus* and *B.*

thuringiensis. Fig -3 shows that FA production was increased gradually and reached 571 mg/kg at 33 hours incubation. The yield was 2.1-fold higher compared to ferulic acid reported by Sarangi and Sahoo (2010). They obtained 275 mg/kg ferulic acid after six days incubation with Staphylococcus aureus in wheat bran. Besides, through enzymatic hydrolysis by using *Aspergillus* ferulic acid esterase also could only release 260 mg/kg FA content from oat hull [26]. The result showed that the use of co-cultivation could improve the FA production better.

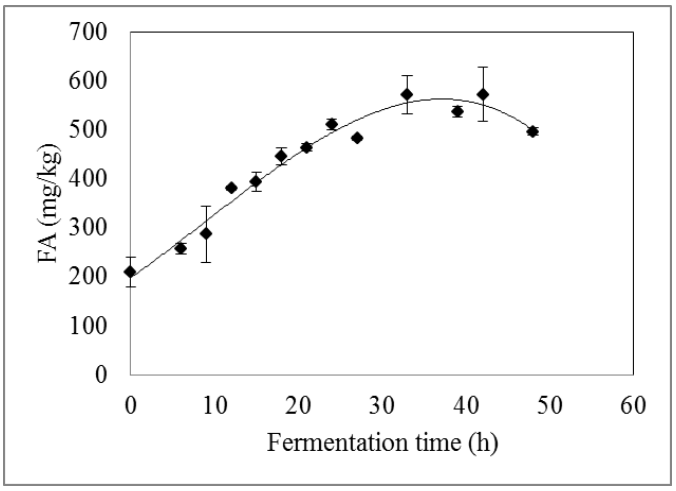

Fig -3: Time course of ferulic acid production by co-culture AD (*B. cereus* and *B. thuringiensis*) from banana stem waste.

Besides, the use of substrate also influenced the release of product. In some case, significant changes in the release of FA from Brewer spent grain was detected although, the same amount of FAE activity was used in all the assays. At the time that the bacterial FAE-producer is grown in a particular carbon source, production of various FAE would be generated depending on diverse substrate specificity. It might be an explanation for the reason of the dissimilarity. That was the case of the fungi of *Aspergillus niger* able to produce several types of FAEs when either sugar beet pulp or oat-spelt xylan are used as carbon sources [10].

However, prolonged incubation up to 48 hours was not resulted in improvement of FA recovery. In fact, the accumulation of FA slightly reduced in the lessened of enzyme activity simultaneously with the stabilization of bacteria growth which entering the stationary phase. Production of FAE activity in this study proved that release of FA from BSW was under hydrolytic reaction. The result suggested that FA production from BSW was in relation to the FAE activity released by co-culture. From the evaluation, it could be claimed that FA production from BSW was part of hydrolytic reaction of FAE produced by co-culture.

4. CONCLUSIONS

Production of FA from BSW through enzymatic hydrolysis was investigated using co-culture. The results demonstrated that co-culture of *B. cereus* and *B. thuringiensis* successfully produced FAE during submerged fermentation of BSW. The highest activity found was 0.046 mU/ml after 27 hours fermentation. FA production increased in parallel with the increasing of FAE activity during the log phase of co-culture growth. The correlation observed suggested that enzymatic hydrolysis occurred in releasing FA from BSW by FAE produced by co-culture. The presence of enzyme during fermentation is paramount in enhancing release of a high-value product rapidly from plant cell wall.

ACKNOWLEDGEMENT

The authors are thankful to Faculty of Chemical and Natural Resources Engineering, Universiti Malaysia Pahang for providing the infrastructure facilities for this study.

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