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DEVELOPMENT OF SOIL CO-CULTURE SYSTEM FOR FERULIC ACID PRODUCTION

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ABSTRACT

Ferulic acid is covalently linked with variety of ester bond in plant cell wall make it challenging to be released naturally. Therefore, appropriate fermentation technique with co-culture could be an effective way to produce ferulic acid. The aims of this research were to develop the co-culture system and to evaluate their performance in releasing ferulic acid. Highest production of ferulic acid from banana stem waste as their sole carbon source was found in co-culture of *Bacillus cereus* CCM 2010, *Bacillus pumilus* SAFR-032 and *Bacillus thuringiensis* Bt407. The co-culture was successfully increased 2.6-fold production compared to single culture in 24 hours period of fermentation. Production of ferulic acid were being consistent each production simply by maintaining the quality of the co-culture. The result of this study suggests that synergistic work by co-culture represents a significant role in manufacturing of valuable product through fermentation of agricultural waste.

Keywords: ferulic acid, co-culture, banana stem waste, bacillus cereus CCM 2010, Bacillus pumilus SAFR-032, Bacillus thuringiensis Bt407.

INTRODUCTION

Synthesis of ferulic acid (FA) was established when it was used as a precursor in the production of vanillin. Vanillin is the world's most highly prized natural flavor and the most important aromatic flavor compounds used in foods, beverages, perfumes and pharmaceuticals (Sarangi and Sahoo, 2009). In nature, ferulic acid is found predominately esterified to a wide variety of compounds such as saccharides, glycoproteins, lignin, hemicellulose, quinic, carboxylic acids, fatty acids, betacyanins and sterols (Zhao *et al.* 2014). Ferulic acid ((E)-3-(4-hydroxy-3-methoxy-phenyl) prop-2-enoic acid) is belongs to the family of phenolic acids plays role in various biochemical processes (Mancuso and Santangelo, 2014). It grabs all the attention as a chemical with many potential applications. Ferulic acid had been used for several applications especially in the food, health, cosmetic and pharmaceutical industries (Ou and Kwok, 2004).

Fermentative production is highly recommended by using cheap by-products and waste substrates for phenolic acid production (Szwajgier and Jakubczyk, 2011). Hence the utilization of banana stem waste (BSW) as the carbon source in fermentation is a good alternative for this phenolic compound production. BSW is a lignocelluloses waste, which consists of lignin, cellulose and hemicellulose. It consists of 15.42% lignin, 53.45% cellulose and 28.56% hemicelluloses (Silveira *et al.* 2008). Most studies showed that ferulic acid ester-linked to arabinoxylan, xyloglucan and pectin, and ether-linked to lignin (Wong *et al.* 2011). Therefore, it requires a range of enzyme to degrade the polysaccharides by releasing arabinoxylan from lignin and breaking the cross- links between arabinoxylan chains (McAuley *et al.* 2004).

FA released from plant cell wall by the action of ferulic acid esterase (FAEs; E.C. 3.1.1.73) (Xiros *et al.* 2009). Microbial ferulic acid esterase has become important materials with considerable roles in biotechnological processes for many industrial and medicinal applications (Huang *et al.* 2011). Ferulic acid esterase is mainly produce by fungi. However bacteria have also been considered as enzyme producers because of their high growth rate, stability at extreme conditions, and presence of multi enzyme complexes (Balasubramanian and Simoes, 2014). Faulds and Williamson (1995) reported a few micro-organisms such as Streptomyces olivochromogenes, Pseudomonas fluorescens subsp, cellulosa Neocallimastix MC-2, PenicilIium pinophilum and several from Aspergillus have been identified that can hydrolyse ferulic and p-coumaric acids from cell wall material, and recently, these enzymes have been purified and partially characterized. Many researchers had been reported in releasing FA by using only a single culture. Sometimes the process needs more than one culture to release FA efficiently. Co-cultivation of different microorganisms may also help to identify and develop new biotechnological substances. Bertrand et al. (2014) had mentioned in their findings, the co-production of enzyme by microorganisms is very important to increase the production of ferulic acid. Some fermentation process needs more than one enzyme (Lemos *et al.* 2014) to release FA efficiently. FAE acts synergistically with other hemicellulase, such as xylanase, to maximise the microbial degradation of plant cell wall (Huang *et al.* 2011).

Co-culture provides a promising method to improve the substrate conversion and the product yield. To have a stable co-culture, certain requirements must exist. One is that the two strains must be compatible and able to grow together. Compared with single culture, interactions between the different microorganisms play a critical role in co-culture systems (Chen, 2011) Hence, these present studies aims to develop the best co-culture

system by utilizing banana stem waste as their sole carbon source by considering combination up to five potential of soil microbe. Production of ferulic acid was evaluated by performance of each co-culture in releasing ferulic acid from banana stem waste. Developing a new stable soil co-culture was important for a better ferulic acid production and reduces the enzyme cost for hydrolysis, since hydrolysis and fermentation are completed by the same strain combinations.

METHODOLOGY

Microorganism

Strain of Bacillus cereus strain CCM 2010, Bacillus weihenstephanensis KBAB4, Bacillus pumilus SAFR-032, Bacillus thuringiensis Bt407 and Lysinibacillus fusiformis strain NBRC 15717 were isolated from soil. Bacterial strains were acclimatized for a month in banana stem waste prior to isolate. Each colony was identified and confirmed by 16S rRNA sequence analysis identification method. All the strains were streaked on nutrient agar plate and incubated at 37°C for 24 hours for inoculum preparation. All the strains were maintained on nutrient agar plate and kept at 4°C until further use.

Substrate

Banana stem waste was (BSW) obtained from banana plantation near Kuantan, Pahang. Before using as substrate, the agricultural waste was cleaned to remove any dirt attached. The stem was chopped into cube (1cm) and mixed with distilled water. The mixture was blended and autoclave at 121ºC for 15 min for sterilization to kill undesired microorganism.

Growth Pattern

Bacterial strains were streaked on nutrient agar plate to obtain pure colony. A full loop of each pure culture was inoculated in 10 ml of nutrient broth. Cultures were incubated overnight before start the measurement of optical density (OD) for construction of growth pattern. The enrich culture were inoculated into the new 250ml-Erlenmeyer flask containing 100 ml nutrient broth. Sample from the flask was taken for every two hours to measure the concentration of cell by using spectrophotometer at wavelength of 600nm until the growth rate decrease.

Inoculum Preparation

Each five flask of inoculum of single culture were incubated until stationary state at 37°C. Inoculums of co-culture were prepared by mixing the stationary state of single culture at equal volume. In this experiment, 31 sets of inoculum consisted of five types of single culture and 26 types of co-culture were developed as provided in Table-1. Those five types of single culture were used to compare with the production of ferulic acid by co-culture.

Table-1. Sets of inoculum of single and co-culture.

No.	Symbol	Type of co-culture
1.	A	B. cereus
2.	B	B. weihenstephanensis
3.	C	B. pumilus
4.	D	B. thuringiensis
5.	E	L. fusimorfis
6.	AB	B. cereu and B. weihenstephanensis
7.	AC	B. cereus and B. pumilus
8.	AD	B. cereus and B. thuringiensis
9.	AE	B. cereus and L. fusimorfis
10.	BC	B. weihenstephanensi and B. pumilus
11.	BD	B. weihenstephanensis and B. thuringiensis
12.	BE	B. weihenstephanensis and L. fusimorfis
13.	CD	B. pumilus and B. thuringiensis
14.	CE	B. pumilus and L. fusimorfis
15.	DE	B. thuringiensis and L. fusimorfis
16.	ABC	B. cereus, B. weihenstephanensis and B. pumilus
17.	ABD	B. cereus, B. weihenstephanensis and B. thuringiensis
18.	ABE	B. cereus, B. weihenstephanensis and L. fusimorfis
19.	ACD	B. cereus, B. pumilus and B. thuringiensis
20.	ACE	B. cereus, B. pumilus and L. fusimorfis
21.	ADE	B. cereus, B. thuringiensis and L. fusimorfis
22.	BCD	B. weihenstephanensis, B. pumilus and B. thuringiensis
23.	BCE	B. weihenstephanensis, B. pumilus and L. fusimorfis
24.	BDE	B. weihenstephanensis, B. thuringiensis and L. fusimorfis
25.	CDE	B. pumilus, B. thuringiensis and L. fusimorfis
26.	ABCD	B. cereus, B. weihenstephanensis, B. pumilus and B. thuringiensis
27.	ABCE	B. cereus, B. weihenstephanensis, B. pumilus and L. fusimorfis
28.	ABDE	B. cereus, B. weihenstephanensis, B. thuringiensis and L. fusimorfis
29.	ACDE	B. cereus, B. pumilus, B. thuringiensis and L. fusimorfis
30.	BCDE	B. weihenstephanensis, B. pumilus, B. thuringiensis and L. fusimorfis
31.	ABCDE	B. cereus, B. weihenstephanensis, B. pumilus, B. thuringiensis and L. fusimorfis

Inoculum sets were incubated at 37°C until stationary state before used in fermentation.

Experimental Set-up

Experiment was performed through fermentation process with BSW medium as their sole carbon source. Inoculum of 31 sets of single and co-culture were prepared freshly. Each 10% of stationary phase inoculum was inoculated into 250 ml Erlenmeyer flask containing 100ml of substrate. Each type of co-culture were prepared triplicate. The samples were incubated in the incubator shaker at 35°C and 150 rpm. After 24 hours incubation, samples were took out, filtered and centrifuged. The supernatant were analyzed by using High Performance Liquid Chromatography (HPLC) to check the ferulic acid content.

Analytical Method

Samples were analysed by using HPLC (Agilent 1100 system) equipped with Agilent Zorbaq SB-AQ C18 analytical column according to method of Chamkha et al. (2001) with modification. An isocratic mobile phase consist of acetonitrile, distilled water and acetic acid (30:69.5:0.5, v/v) was used at a flow rate of 0.6 ml/min. Volume of the injection loop was 25 ml and ferulic acid were quantified using a diod array detector (DAD) at 280nm wavelength. Prior to analyse, fermented samples were prepared by centrifuging at 5800 rpm for 15 minutes and the supernatant were filtered. The samples of supernatant were injected in the vial by using syringe and 0.45 µm of nylon syringe filter. The vials of samples were put on HPLC plate to analyse the amount of ferulic acid content.

RESULTS AND DISCUSSIONS

Determination of Growth Rate of Single Culture

Growths of bacterial strain were constructed in this research to find the maximum number of bacterial cell grown in the inoculum. It was performed to ensure the inoculum culture contain sufficient amount of bacterial cell. Figure-1 shows the growth curve of five bacterial strains for 28 hours based on optical density at wavelength of 600nm. B. cereus, B. pumilus, B. thuringiensis and L. fusimorf is were displayed almost similar growth pattern and grow rapidly without showing lag phase state compared to B. weihenstephanensis. There were no cell division and therefore no increasement of cell number in B. weihenstephanens is at the first four hours. During this phase cellular metabolism is accelerated and prepared for the next phase of cycle.

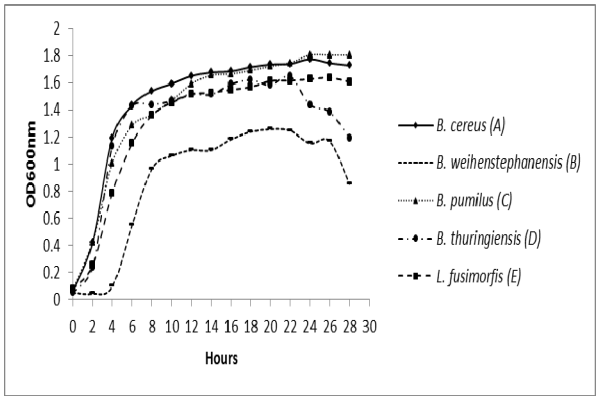

Figure-1. Growth curve of single culture at wavelength of 600nm.

The entire microbe took about four to eight hours to double regularly to reach the maximum number of cell at exponential phase. The process happened rapidly for all those microbes under the same nutritional and physical condition. Maximum growth rate achieved by B. cereus, B. weihenstephanensis, B. pumilus, B. thuringiensis and L. fusimorfis achieved were 1.20, 0.95, 1.12, 1.28, and 1.15 respectively. The deceleration phase began as early hour four in B. thuringiensis and growth cease after 22 hours. The growths of culture were ceasing due to the exhaustion of resource and waste accumulation during the saturation phase (Hall *et al.* 2014). At hour six B. thuringiensis and B. pumilus enter early stationary phase followed by B. cereus, B. thuringiensis and L. fusimorfis. Stationary phase state is the important stage where the bacteria cell maintained its maximum level for a period of time. At this state bacteria cell was obtained sufficiently to be used for preparation of inoculum of co-culture. Stationary phase for B. cereus, B. weihenstephanensis, B. pumilus, B. thuringiensis and L. fusimorfis was achieved at the OD of 1.78, 1.26, 1.80, 1.65 and 1.63 respectively. At this stage there was no further increase in cell number and suitable for preparation of co-culture.

Ferulic Acid Production by Interaction of Co-Culture

Fermentation is one of biological pretreatment process has been used by using microorganism to degrade lignin and hemicellulose in waste material to release vanillin, vanillic acid, ferulic acid, coniferyl aldehyde, guiacyglycerol (Noor Hasyierah *et al.* 2008). Balasubramanian and Simoes (2014) have been reported that Bacillus sp. is the most potent extracellular enzyme producer. The present of enzyme may increase rates of enzymatic hydrolysis, fermentation, and product recovery. Therefore, productions of ferulic acid by co-culture consist of Bacillus sp. and Lysinibacillus sp. were performed through fermentation processes in BSW as their sole carbon source. Figure-2 shows the production of ferulic acid from BSW in 24 hours period of incubation using both single and co-culture. The production of ferulic acid culture Single culture only contributed about 111.48 to154.55 mg/kg of ferulic acid with B. pumilus was

released the highest ferulic acid content. Whilst, all the co-culture was proven to increase the release of ferulic acid production by contributing up to 394.76 mg/kg amount of ferulic acid. The content of ferulic acid were increased up to 2.6 and 2.2-fold respectively by using co-culture ACD (B. cereus, B. pumilus and B. thuringiensis) and AD (B. cereus and B. thuringiensis) compared to the content of the highest ferulic acid of single culture. The result probably due to both co-cultures of AD and ACD produced different type of enzymes to release ferulic acid from cell wall. Previous researches have been reported that, ferulic acid acid can be release efficiently in the present of ferulic acid esterase together with xylanase. Enzymes acts synergistically to break down feruloylated plant cell wall polysaccharides and enhance hydrolysis of lignocellulolytic (Faulds and Williamson, 1995; Huang *et al.* 2011; Xiros *et al*, 2009).

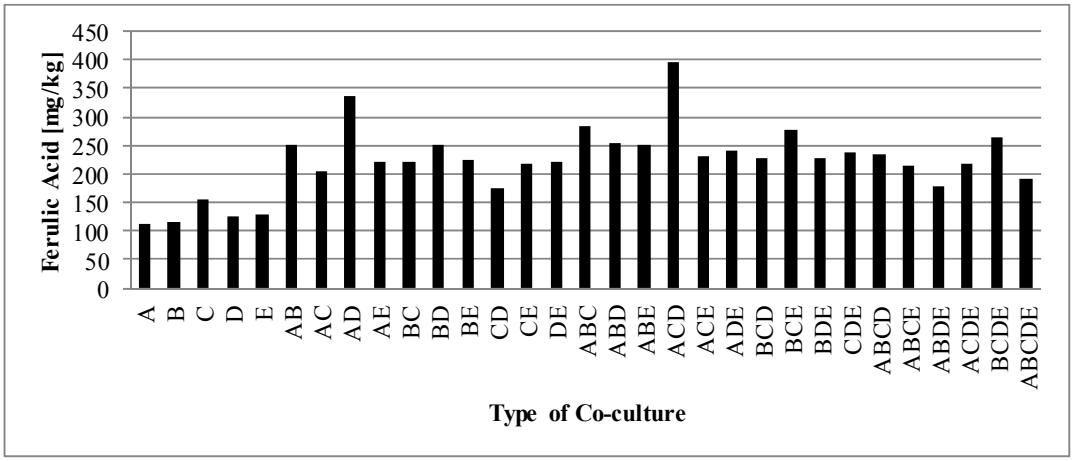

Figure-2. Production of ferulic acid from five single culture and 26 types of co-culture.

Development of Co-culture

Inoculum of co-culture was developed based on their capabilities to release ferulic acid from BSW. Only two type of co-culture were carried out for inoculum development. Both co-culture were performed the best performance to release ferulic acid in BSW. Figure-3 shows the growth curve of both co-culture AD (B. cereus and B. thuringiensis) and ACD (B. cereus, B. pumilus and B. thuringiensis). The graph pattern for both co-culture almost similar at every phase state without lag phase state were shown. The graph showed the maximum OD of co-culture AD and ACD at stationary phase was at 1.72 and 1.74 respectively showed only slight different. At this state each co-culture contained sufficient amount of bacterial cell to be inoculated for fermentation process in the further research.

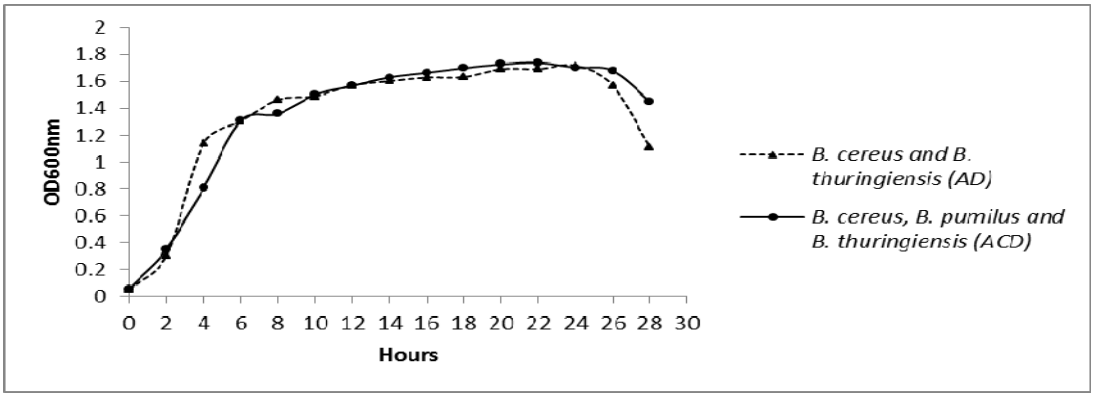

Figure-3. Growth curve of co-culture at wavelength of 600nm.

CONCLUSIONS

Two types of co-culture were successfully developed and observed their accumulation significant amount of ferulic acid. The co-culture was cultivated using a stationary phase of inoculum by indication of OD to enhance the fermentation process. Stable combination of B.cereus, B. pumilus and B. thuringiensis was showed the highest releasing of ferulic acid from BSW. The co-culture

was contributed about 394.76 mg/kg of ferulic acid content in 24 hours period of incubation. The development of co- culture may become important part in maintaining the sufficient amount of bacterial cell for production consistency. Likewise, a stable co-culture is a critical aspect to be emphasized to ensure the ability of each microbe working synergistically to improve the production of ferulic acid.

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