Research Article

Isolation, identification and characterization of soil bacteria for the production of ferulic acid through co-culture fermentation using banana stem waste

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Abstract
Exploitation of soil bacteria for production of ferulic acid (FA) is extensively performed since bacteria are the largest soil community that have the potential in producing degrading enzymes. This study aims to isolate, identify and characterize the most efficient soil bacteria for high FA yield via co-culture fermentation of banana stem waste (BSW). Bacteria were isolated and screened from acclimatized mixture of soil culture and BSW. ARB programme package and biolog system were employed for identification and characterization. The results reveal that four isolates closely related to *Bacillus* spp. and one *Lysinibacillus* sp. had greater potential to produce FA in very large amounts. Specifically, the maximum FA yield of 394.76 mg/kg was achieved using co-culture of *Bacillus* sp. MB2, *Bacillus* sp. WB8A and B. *pumilus* strain WB1A, which was 2.5-fold higher than FA produced by single culture. The profiles of substrate utilization exposed strong hydrolyzation of pectin in those three potential cultures, while one showed strong hydrolyzation of glucuronic acid. The ability to efficiently hydrolyze the components proves that the chosen co-cultures are good sources of hydrolytic enzymes. The results suggest that the co-culture has contributed cooperative actions among the cultures to synergistically breakdown the FA linkage in BSW to produce high FA yield.

Keywords Co-culture · Banana stem waste · Ferulic acid · ARB software · Biolog system

1 Introduction

The possible use of ferulic acid (FA) as medicinal, food preservative and in several other potential applications has drawn considerable industrial and commercial interest towards this phenolic compound. Some of the notable industries that make use of this compound include pharmaceutical, food and cosmetic industries. FA is copiously present in the plant cell wall as covalent side chain components [1]. Different researchers have investigated various agriculture wastes such as sugar beet pulp, wheat bran, corncobs, paddy straw, switchgrass and triticale bran for their FA content. In this study, banana stem waste is used as substrate for FA production. Notably, banana stem contains about 15.42% lignin, 53.45% cellulose and 28.56% hemicellulose [2]. The use of this second largest cultivated tropical fruit, that generates several tons of underused by-product and waste for ferulic acid production presents

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immense benefits. More interestingly, it is abundantly found and cheap. FA is ether-linked to lignin, and ester-linked to arabinoxylan, xyloglucan and pectin which often makes its recovery from these natural sources very challenging [1, 3].

Different methods have been explored to produce FA from various types of lignocellulosic biomass such as mechanical extraction, chemical extraction and enzymatic hydrolysis [4–6]. Chemical and enzymatic hydrolysis are the most widely-used methods for FA production that have been reported. However, the use of chemical processes such as alkaline hydrolysis, has certain drawbacks that the method is considered unsafe and unacceptable in the food and medical industries compared to a more conventional enzymatic approach. Hence, enzymatic hydrolysis is preferred as an alternative for FA production. The use of purified enzyme and fermentation by microorganism are currently used to produce FA from biomass by breaking down the linkage. Feruloyl esterase or ferulic acid esterase (FAE) is known as the most important enzyme to cleave the feruloyl-polysaccharide. On the other hand, several enzymes have been observed to facilitate the production of FA [7, 8].

FAE acts synergistically with other hemicellulases such as xylanase to maximize the microbial degradation of plant cell wall [9]. This has been proven by Uraji et al. [10] in their finding where combinational enzymes have significantly enhanced FA production in rice bran, wheat bran and corncob. Notwithstanding, it is worthy to note that despite the reported beneficial use of co-enzymes simultaneously, the process might give different results with the utilization of different types of substrates. Therefore, co-culture fermentation is being considered as a useful method that could effectively support FA production from BSW, by simultaneous act of enzymes induced naturally by co-culture during fermentation.

Besides high growth rate and resistance to extreme condition, the ability to produce multi enzyme complexes indicates that bacteria have the potential to produce several hydrolytic enzymes for FA production [11]. Soil contains the highest number of microbial communities which actively takes part in releasing chemical nutrients from the decaying of organic matter processes in soil through enzymatic hydrolysis. Bartolomé et al. [12] and Sarangi and Sahoo [13] are among the researchers that have utilized bacteria in their research to produce FA from brewer's spent grain and wheat bran through fermentation. While, fermentation using mixture of unknown cultures on oil palm frond (OPF) and BSW have been explored by Khushairi et al. [14] and Mohd Sharif et al. [15]. However, the mixed culture fermentation technique may sometimes be followed by limitations to obtain higher FA production since biotransformation of FA into other phenolic compounds, such as vanillin and vanillic acid, may occur during fermentation [16, 17]. Therefore, FA production through co-culture (combination of several known cultures) fermentation is recommended in the production of high FA yield. Wei et al. [18] has observed FA production in the range of 0.2–1.8 µg/mL on chopped wheat straw, corn stalk, rice straw, Chinese wildrye and medicago sativa using co-cultures of *Piromyces* and *Methanobrevibacter ruminantium* where wheat straw was identified as the most potential source of production. While, Razak et al. [19] found that FA production from rice bran was four times greater than *Aspergillus oryzae* through fermentation of *A. oryzae* and *Rhizopus oryzae* co-culture. Therefore, the selection of the most useful microbe to produce co-culture may offer many advantages in the production of FA. Specifically, the synergistic action would help to release FA from plant cell wall more efficiently, thereby minimizing the risk of possible conversion into other products. Till this moment, there has not been a report on isolation of soil bacteria for co-culture fermentation to improve FA production from BSW. This makes it necessary to investigate some other bacterial strains which have ability to release ferulic acid effectively through co-culture fermentation. Co-culture therefore promotes the production of lignocellulolytic mixtures or complexes that improve the efficiency of biodegradation. Significant polysaccharide hydrolase production during fermentation will degrade various lignocellulose substrates with the ability to release FA from lignocellulosic biomass [18].

Therefore, this study was performed to isolate, identify and characterize an isolated co-culture from soil that has potential as FA producer using BSW as their sole carbon source. Isolates were obtained from acclimatized soil bacteria and their performances were evaluated for FA production via single and co-culture fermentation. Identification of the most efficient bacteria was achieved through 16S rRNA gene sequencing. In addition, phylogenetic analysis was performed by ARB software package and calculated by Bayesian analysis to obtain a phylogenetic tree. The ARB software used in this study provides a comprehensive interaction tool to analyze ribosomal RNA data including import and export data in different format. In addition, the use of Bayesian analysis could provide a satisfactory and reliable phylogenetic at statistical consistency.

2 Materials and methods

2.1 Raw material

Collection of banana stem waste (BSW) from banana plantation near Kuantan, Pahang was done once the banana was harvested. The stem was cleaned to eliminate any

dirt attached and the damaged stem layer was discarded before using as substrate. The stem was then sliced into 1 cm cube size and blended with distilled water at ratio of 1:1 (g/mL) using a Panasonic blender. The BSW slurry mixture was sterelized in an autoclave (Hirayama HVE-50, Japan) at 121 °C for 15 min to kill undesired microorganisms.

2.2 Acclimatization and isolation of soil microbe

Soil sample was also collected from a depth of about 5–10 cm below ground surface at banana plantation in Kuantan, Pahang. Acclimatization of soil microbe in BSW was prepared in triplicate by mixing a part of soil containing mixture of microorganism, with four parts of substrate containing BSW and distilled water into 5 L-container. The containers were closed with cotton plug to avoid environmental contamination. The mixtures were incubated for a month at ambient temperature prior to isolation.

After a month, 1 mL of acclimatized soil microbe was pipetted out and suspended in 9 mL of 0.85% (w/v) NaCl buffer (Merck KGaA, Germany). The mixture was shaken vigorously using mechanical vortex. One milliliter of the first dilution was added to the next 9 mL of NaCl buffer to create a serial dilution up to 10^{-6}. Then, 100 µL of each dilution was spread evenly on a petri dish containing nutrient agar (Merck KGaA, Germany) and incubated in an incubator (Memmert, USA) at 37 °C for 24 h. Pure colonies observed was further cultured in a parallel agar plate to ensure the purity of each colony. Pure colonies were kept at 4 °C for further use.

2.3 Preparation of inoculum

Pure culture inoculum was prepared for the use in screening of five potential strains with maximum production of FA from 46 isolates. Inoculum was prepared from a fresh bacterial colony. Quadrant streaking method was performed to obtain the colony. Streaked plate was incubated in an incubator for 24 h at 37 °C. Each pure colony of the 46 strains was scraped and inoculated into a different 28 mL-Universal bottle containing 10 mL of nutrient broth. For use in preliminary studies involving single culture fermentation, inoculum was then incubated at 37 °C for 24 h.

Similar steps were followed in order to prepare co-culture inoculum as prepared in the preliminary study. However, the pure culture inoculum of potential strains were incubated at 22 h instead on the basis of our results in previous work on the bacterial growth profile to obtain high density cell culture [20]. Further stage was accomplished by combining several combinations of the selected potential soil bacteria which showed high production of FA into a new 28 mL-Universal bottles. The number of possible combinations can be calculated as in Eq. 1 [21].

$$C(n,r) = \frac{n!}{r!(n-r)!} \quad (1)$$

where n represents the total number of strains, and r represents the number of strains being chosen in a group. From the calculation, the number of possible combinations of two, three, four or five strains generated are 10, 10, 5 and 1 types of co-culture without considering the order of the strain and without any repetition. In this study, a combination generator tool (https://www.statisticshowto.datasciencecentral.com/calculators/permutation-calculator-and-combination-calculator/) was used to obtain the possible combination cultures. A total of 26 types of co-culture could be developed from five selected strains which then being used for co-culture inoculum preparation. Identical volume of each pure culture was equally distributed with a total of 10% into another 100 mL-Erlenmeyer flask containing 50 mL fresh nutrient broth. Co-culture inoculum was further incubated for 22 h at 37 °C before use.

2.4 Selection of the most potent co-culture

The most potent co-culture was established through their achievement in the production of high amounts of FA. Prior to co-culture fermentation, a preliminary study was performed to determine the effectiveness of each isolate in the production of FA using single culture fermentation. The study was conducted by submerged fermentation using the BSW medium as its primary carbon source. The overnight pure culture inoculum previously prepared was used freshly for the fermentation process. Each of the inoculum culture was inoculated about 10 mL into 250-mL Erlenmeyer flask, containing the BSW slurry substrate at ratio of 1:1 (g/mL). The working volume of the mixture was up to 100 mL. The flask was closed using cotton plug and incubated for 24 h at 35 °C and 150 rpm using incubator shaker (Infors HT Ecotron, Switzerland) aseptically. Then the culture supernatant was collected after centrifugation process for 15 min at 5800 rpm by refrigerated centrifuge (Eppendorf 5810R, Germany). The sample was analysed using high performance liquid chromatography (HPLC) to check the ferulic acid content. Co-culture fermentation was further conducted by inoculating 10% of co-culture inoculum instead, following same steps as the single culture fermentation method.

2.5 Quantification of ferulic acid

The FA content was determined using Agilent 1100 HPLC equipment (USA) equipped with diod array detector (DAD)

according to Chamkha et al. [22]. Chromatographic analysis was performed on Agilent Zorbaq SB-AQ C18 analytical column (USA) with an isocratic mobile phase made up of acetonitrile (Fisher Scientific, UK), Milli-Q ultrapure water (Millipore, USA) and acetic acid glacial (Fisher Scientific, UK) at a ratio of 30:69.5:0.5 (v/v). A 25 µL injection volume at 0.6 mL/min was set at wavelength of 280 nm. Samples were filtered using 0.2 µm nylon syringe filter prior to analyzing and the FA content was measured by comparing the result with the calibration curve of standard ferulic acid (99%, Sigma Aldrich, USA).

2.6 Biochemical test

Biochemical test for the selected five bacterial strains from the preliminary study which showed best performance in releasing FA was performed using Biolog GEN III Micro-Plate System by referring the OmniLog ID System User Guide instructed by the manufacturer (Biolog, Hayward, CA). A pure colony was picked using a sterile cotton swab and transferred into the inoculating fluid B (IF-B). The IF-A was tilted gently upside-down to obtain a uniform suspension. Then, the target cell density was obtained at range 93–98% by using a turbidimeter. About 100 µL of cell suspension was inoculated into 96-well of the GEN III Microplate which consist of 71 types of carbon source and 23 chemical sensitivity assay tests using a multichannel micropipette. The mixture plate was then incubated at 37 °C into Omnilog Unit for 24 h. The result was interpreted by an identification system's software (Biolog, Hayward, CA).

2.7 Identification by 16S rRNA gene sequencing

Genomic DNA was extracted from exponentially grown culture by using a GF-1 Bacterial DNA extraction kit (Vivantis Technologies, USA). Those five selected strains based on the preliminary study which showed higher contribution towards FA production were grown on nutrient agar plate overnight at 37 °C to obtain pure culture colonies. Each type of bacteria was suspended in sterile distilled water and centrifuged at 12,138 rpm for 10 min in a microcentrifuge (Heraeus Instrument, Germany). The cell pellet was re-suspended to wash the cell and re-centrifuged twice prior to DNA extraction according to the manufacturer's instructions (Vivantis Technologist, USA). DNA purity was checked using Eppendorf BioPhotometer Plus (Eppendorf AG, Germany) with absorbance at A230, A260, A280 and A340. The sample and 1 kb ladder were examined through gel electrophoresis at 70 V for 30 min on 1% agarose gel in a sub-cell GT agarose gel electrophoresis system (Bio-Rad Laboratories, USA) [23].

PCR was performed using bacterial ID kit according to the manufacturer's instruction (Profound Kestral Laboratories, Malaysia). Amplification of PCR product was performed using the primer 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) and primer 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) (First Base Laboratories, Malaysia). The PCR conditions used for initial denaturation were 95 °C for 4 min, followed by 30 s denaturation cycles at 95 °C, an annealing at 57.5 °C for 25 s, an initial chain extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min. PCR products were loaded into the well of 1% agarose gel with the control and ladder in Sub-Cell GT agarose gel electrophoresis system (Bio-Rad Laboratories, USA). Gel electrophoresis was run at 90 V for 1 h 50 min [23]. The agarose gel then was visualized with an UV transilluminator using Bio-imaging machine (Alpha Innotech, Germany) after stained. The gel was cut and purified for sequencing. The PCR products were sequenced at First Base Laboratories (Selangor, Malaysia). Briefly, the nearest neighbor of each isolate was searched and compared from the National Centre for Biotechnology Information (NCBI) database, using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/). All the sequences were deposited to NCBI GenBank with accession numbers between MH443316–MH443320.

2.8 Sequence processing and analysis of phylogeny

All the gene sequences of isolates and the nearest neighbor were analyzed using ARB software package to generate phylogenetic tree. The sequences were aligned using SILVA online SINA alignment to get the compatible sequence prior to being imported. They were then analyzed using an ARB 6.0.6 software [24, 25]. Bayesian phylogenetic tree was constructed through several steps. All the sequences were subjected to removal of ambiguous sequences using Gblocks [26]. Bayesian analysis was performed using MrBayes v3.0b4. Markov chains were run for 10,000,000 generations with sampling frequency of 100. The calculated tree obtained was imported into the ARB, and the tree was constructed via an ARB parsimony method without changing the tree topology.

3 Results and discussion

3.1 Isolation and selection of ferulic acid-producing strains

The purpose of isolation in this study was to evaluate and identify the performance of various bacteria with the highest capacity to produce FA from BSW through submerged fermentation. FA is one of the most common

phenolic compound that can be produced in high amounts in fermented product which is synthesized by microorganisms during fermentation [27]. This early stage of this research has to do with acclimatization of soil microbe for adaptation to utilize BSW as their sole carbon source prior to isolation. Acclimatization is a process where continuous exposure of a microbial population to a substrate results in a more rapid biodegradation of a compound to which it is exposed than initially observed [28, 29]. Use of acclimatized microbe to substrates before performing a research is believed to be a way of improving its ability to achieve high performance compared to other microbes that were not acclimatized to such environment [30]. In this study, 46 acclimatized soil microbes were successfully isolated from the BSW mixture after a month of acclimatization. The result suggested that the obtained bacteria might make effective use of BSW to survive and tolerate the provided environment. The ability of these bacteria utilize BSW effectively might be explained by mutation in one or more genes caused by change of the environmental condition exposed to allow the cell to degrade provided substrate [29, 31].

A number of research studies have used acclimated organism in obtaining adapted strains with improved biodegradable characteristic to enhance productivity [31–36]. A study conducted by Mate and Pathade [37] found that isolated microbes which earlier subjected to acclimatization with azo dye were usefully treated wastewater containing reactive dyes without giving toxicity of the biodegradation product. Similarly, Saha et al. [38] discovered a promising strategy using a microbiome acclimatized to lipidic-waste such as fat, oil, and grease (FOG) to improve hydrogen production and simultaneous production of C4–C7 fatty acids through hydrogenogenic acidogenic fermentation. Both studies showed similarities to this study which productivity have improved using acclimatized microbes with high tolerant and great degradation capabilities on the specified substrate compared to the unacclimated one.

The efficiency of each isolated bacterium to produce FA from BSW was further evaluated for 24 h fermentation. The result of preliminary study on FA production via single culture fermentation is presented in Fig. 1a. Among the 46 bacteria, only 37 were observed to be significant in producing FA. From the observation, six bacteria were

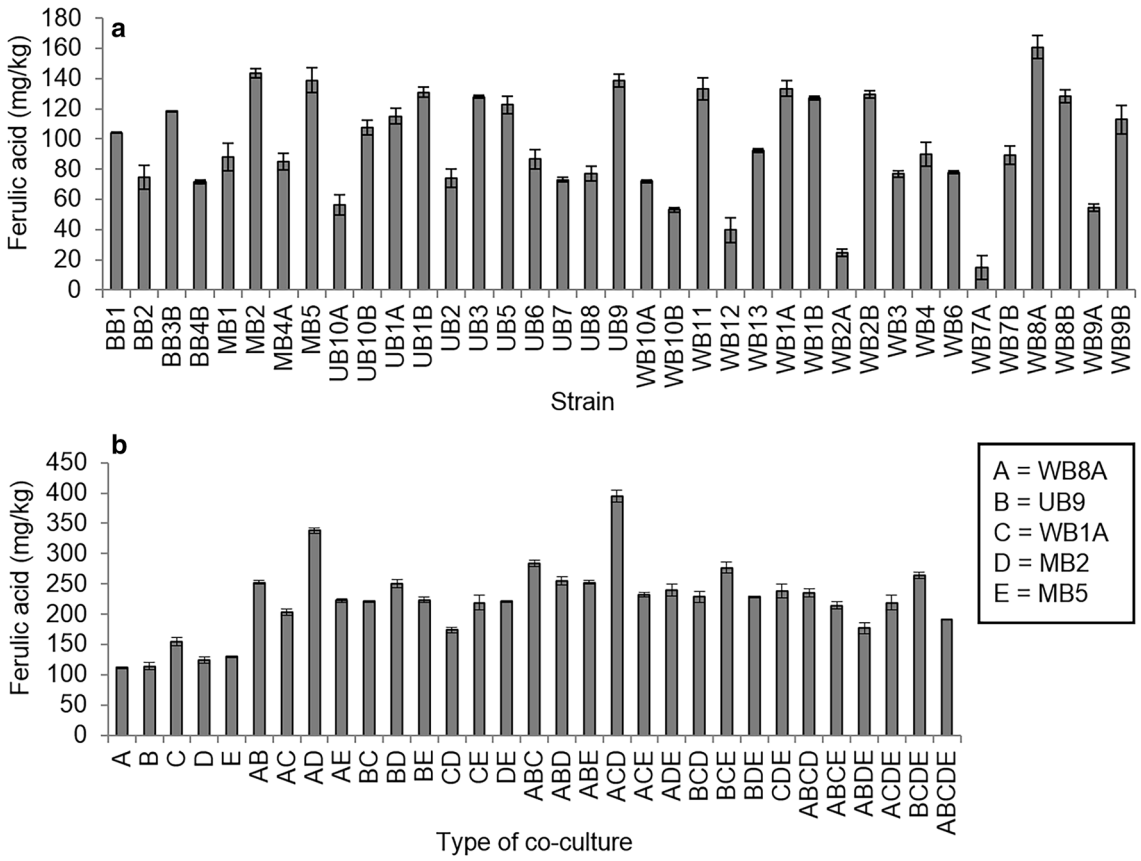


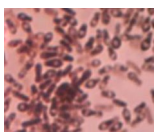
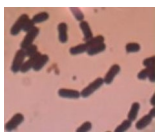
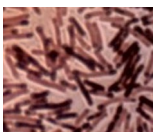
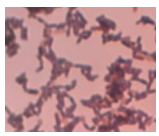
Fig. 1 Production of ferulic acid released by **a** single culture and **b** co-culture. Fermentation was performed under the same conditions for 24 h

unable to show the presence of FA. A possible explanation to this might be due to insignificant amount of FA produced during the fermentation, or perhaps the bacteria was not capable to express an appropriate enzyme to hydrolyze feruloylated polysaccharide to release FA from BSW. Strain WB8A was observed to be the highest FA producer with a total production of about 160.78 mg/kg. On the other hand, strain WB7A was found to produce the lowest amount of FA producer with a total production of about 14.81 mg/kg. These values present a significant difference between the highest and the lowest FA producer. Specifically, the quantity of FA produced by WB8A is 10.86-fold higher than WB7A. Strain MB2 was the second highest producer of FA followed by MB5, UB9 and WB1A with range values between 133.53 and 143.81 mg/kg. From the result, the FA production achieved in this study was found to be lower than previously reported by Sarangi and Sahoo [13] where as much as 275 mg/kg of FA was recorded after 7 days fermentation with wheat bran using *Staphylococcus aureus*. However, at 24 h incubation, only 48 mg/kg of FA was observed in their study which showed that FA produced by strain WB8A in this study was 3.35-fold higher and more efficient in utilizing BSW in shorter time.

Figure 1b shows the FA production by 26 types of co-culture after 24 h fermentation. Notably, the result revealed significant improvement in FA production as shown by the co-cultures in comparison with single culture. Specifically, co-culture ACD presents an increase in FA production of up to about 394.76 mg/kg compared to just about 160.78 mg/kg of FA which was initially obtained through fermentation by WB8A (A) alone. On the other hand, the FA produced by AD which is the second highest producer of FA is about 338.08 mg/kg. This is a difference of about 56.68 mg/kg when compared with the quantity produced by ACD. The presence of strain C in ACD might be the reason for increasing of FA production. FA could be effectively released from lignocellulosic biomass due to the synergistic interaction between strains during fermentation. This significant difference in FA production indicates that co-cultures have better capability to increase FA production over the single culture. Co-culture could produce several polysaccharide hydrolases including FAE and xylanase with high biodegrading capabilities which useful for lignocellulosic bioconversion to FA [18]. This is well demonstrated in the study done by Wu et al. [39] which has recorded 70% of FA production from wheat bran under AnXyn11A (xylanase) and AnFaeA (feruloyl esterase) synergistic activity compared with 16.8% only produced by AnFaeA alone. Combination of both xylan-degrading enzymes portrayed a great synergistic effect towards the production of high amount of FA. It is also encouraging to compare this result with that found by Valdez-Vazquez et al. [40] who also demonstrated that co-culture of *Clostridium cellulovorans* and *Clostridium acetobutylicum* showed positive interaction by improving 2- to 3-fold productivity compared to single culture.

However, it was observed herein that the additions of strain may not always present good synergistic effect towards increased FA production. For example, combination of five strains (ABCDE) was found to be the third lowest producer of FA followed by ABDE (second lowest) and CD (lowest). Notably, a difference of about 203.90 mg/kg was observed between ACD and ABCDE. This result suggest that poor synergistic effect of co-enzyme might occur due to inappropriate type of enzyme expressed by co-culture which might ineffectively degrade the substrate [39]. Therefore, it is important to ensure accurate combination through proper evaluation, in order to identify the most suitable combination to produce FA naturally.

Table 1 Characteristics of strain MB5, WB8A, MB2, UB9 and WB1A

Characteristic	Strains				
	MB5	WB8A	MB2	UB9	WB1A
					
Pigmentation	Light brown	White	White	Clear	Buff
Optical property	Opaque	Opaque	Opaque	Transparent	Translucent
Shape	Irregular	Circular	Circular	Irregular	Circular
Surface	Smooth	Rough and shiny	Rough and dull	Smooth	Shining
Elevation	Flat	Umbonate	Raised	Flat	Convex
Margin	Entire	Irregular	Irregular	Entire	Entire

3.2 Identification and characterization of ferulic acid-producing strains

Bacterial morphologies of the five strains were observed in terms of their macroscopic as well as microscopic characteristics as roughly summarized in Table 1. The results revealed that all the strains were confirmed as gram-positive and rod-shape bacterium. Gram-positive bacteria are often differentiated based on chemical and physical properties of the bacteria. Generally, they contain thicker peptidoglycan properties in their cell wall compared to gram negative bacteria and this protect and enables the gram-positive bacteria to withstand extreme environments [41]. Previous studies have established that FAEs take a wide range of temperature and pH stabilities, with more than 60% of optimal activities between temperature of 25 and 50 °C and between pH 5.0 and 7.5 [42, 43]. Nevertheless, there are also some of the FAEs that are categorized into four groups: A, B, C and D depending on their specificity of substrates, may have strong stability at extreme pH between 9.0 and 10.0 and temperature between 55 and 60 °C [44–47]. Gram-positive bacteria are known to have great potential as lignocellulose-degrading bacteria especially for lignin degradation which is important in this study to facilitate the production of desired phenolic compound from lignocellulosic waste [48].

Identification of selected bacteria through 16S rRNA sequence showed that the bacterial strains were classified as the genus of *Bacillus* spp. and *Lysinibacillus* sp. by comparing with BLAST result. The result reflects that of Donaghy et al. [49] who found a number of bacteria from *Bacillus* sp. possess the capabilities in producing FAE activity. On the other hand, *Lysinibacillus* sp. is the first genus that has been reported as having potential for FA production. A phylogenetic tree obtained clearly demonstrates the relatedness between the strains and nearest neighbor of each strain as portrayed in Fig. 2. The tree obviously demonstrated that WB8A, MB2 and UB9 could only be resolved to the genus. They were grouped in the same clade which is closely related to *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus anthracis* with a slight difference in term of sequence compared to the database. *B. cereus* and *B. thuringiensis* are genetically distinguishable. To date, the genetic relationships between *B. cereus* and *B. thuringiensis* have been comprehensively investigated. However, there are no sufficiently convincing results, and this makes it a bit difficult to classify both species. According to Peruca et al. [50] sometimes both species are classified together, whereas at other times *B. thuringiensis* is categorized as a subspecies of *B. cereus* which really makes the classification so complicated.

In contrast, strain WB1A and MB5 were 100% closely related to *Bacillus pumilus* and *Lysinibacillus fusiformis*, respectively. *Bacillus* spp. and related genera are the most attractive group of bacteria in industrial biotechnology due to its unique enzyme which shows resistance to high pH and temperature [51]. Therefore, it is advantageous to enhance FA production using these strains through co-culture fermentation for natural production.

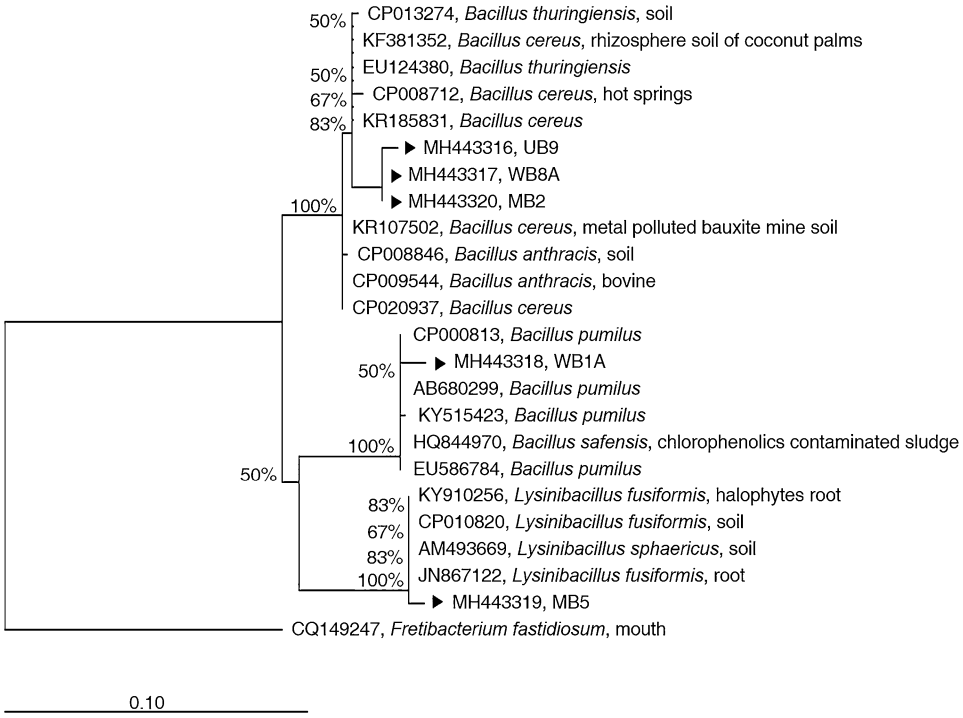


Fig. 2 Phylogenetic tree based on amplified 16S rRNA gene sequence including nearest neighbor of known species. The scale bar for nucleotide sequence difference was recorded as 0.1. *Fretibacterium fastidiosum* is an outgroup

3.3 Biochemical properties of the selected ferulic acid-producing strains

Biolog Gen III Microplate system is beneficial to evaluate the characteristics of bacteria to react with different type of substrate within a short time. Positive results have been shown by many studies in estimating metabolic activities of microbe using the system [52–56]. Substrate utilization test using the system interprets the potential of bacteria to ferment and hydrolyze various substrates simultaneously at the same plate. Reaction for 24 h in this study was enough for each strain to be evaluated for carbon source utilization by strains or resistance to inhibit the chemicals. Species from the same family could react with the same carbon sources which may display similar substrate utilization profile. Performance of each strain in utilizing 71 types of substrate (column 1–9) and 23 types of chemical sensitivity (column 10-12) are shown in Fig. 3 and the details are described in Supplementary Table S1. From the figures, MB2 shows its ability in utilizing 44 types of substrates and sensitive to 15 types of chemicals which presented by purple well for strong hydrolyzation and half-filled blue for weak hydrolyzation. Meanwhile, MB5, WB1A, WB8A and UB9 are able to utilize 47, 49, 52 and 62 types of substrate and sensitive to 15, 15, 20, 16 and 11 types of chemical, respectively.

The ability of bacteria to utilize several monomeric compounds reflects the ability of the microbes to degrade lignocellulosic biomass during fermentation. Selected substrate utilization from Fig. 3 that reveals hydrolytic activities on lignocellulosic biomass are summarized in Table 2. The results of 11 types of substrate including D-cellobiose, α-D-glucose, D-mannose, D-fructose, D-galactose, D-fucose, L-fucose, L-rhamnose, pectin, D-galacturonic acid and D-glucuronic acid were selected for further evaluation since the profiles could represent hydrolytic activity of lignocellulosic biomass during fermentation process. Utilization of the components could demonstrate the potential of bacterial strains in breaking the ester and ether linkage that link hemicellulose and lignin to FA and other cellulose components to enhance the production of FA from

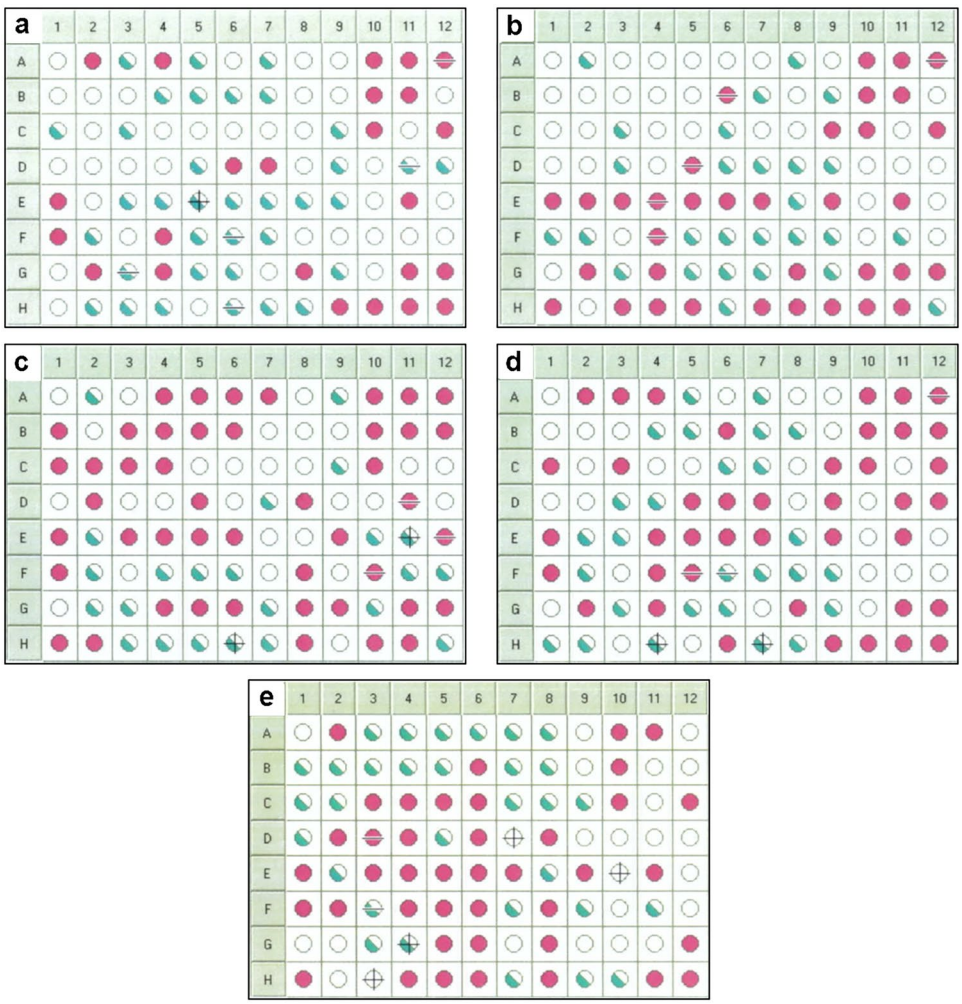


Fig. 3 Profile of different substrate utilization of **a** *Bacillus* sp. MB2 **b** *Lysinibacillus fusiformis* MB5 **c** *Bacillus pumilus* WB1A **d** *Bacillus* sp. WB8A and **e** *Bacillus* sp. UB9. The purple, half-filled blue and blank color well indicate strong reaction, weak reaction and no reaction, respectively during incubation. A1 and A10 are positive and negative control well

Table 2 Biochemical test for substrate utilization by different strains

Type of carbon source	MB2	WB8A	WB1A	MB5	UB9
D-Cellobiose	W	W	+	–	W
α-D-Glucose	W	+	+	–	W
D-Mannose	–	–	+	–	W
D-Fructose	W	+	+	W	+
D-Galactose	–	–	+	–	+
D-Fucose	–	W	–	W	+
L-Fucose	–	W	–	–	W
L-Rhamnose	–	–	–	–	W
Pectin	+	+	+	W	+
D-Galacturonic acid	W	W	W	W	+
D-Glucuronic acid	W	+	W	W	+

Symbol of +, W and – represent the positive, weak, and negative reaction of strains on substrate, respectively

lignocellulosic biomass. Glucose is a basic unit of cellulose which cellobiose is the intermediate product [57]. The finding showed that all strains excluding strain MB5 may hydrolyze cellulose during fermentation since they could utilize both D-cellobiose and D-glucose. Although strain MB5 was unable to utilize the component, the finding however revealed that all the bacterial strains have capabilities to utilize D-fructose, pectin, D-galacturonic acid and D-glucuronic acid. Hydrolyzation of those substrates are important because hemicellulose is made up of different combination of monomeric hexose and pentose sugar including D-glucose, D-mannose and D-galactose and organic acid such as D-galacturonic acid and D-glucuronic [58]. FA residues and diferulic acid esterified to arabinofuranosyl residue of glucuronoarabinoxylan which consisted of arabinose and glucuronic acid that branched to core chain of xylan [59]. While, galacturonic acid is the main structure of pectin in primary cell wall which may also contain rhamnose, xylose, arabinose, and galactose [60, 61]. Interestingly, all strains could also hydrolyze pectin with specifically strong hydrolyzation by strain MB2, WB8A, WB1A and UB9, and weak hydrolyzation by MB5. The hydrolyzation of pectin is an important step in fermentation of BSW to produce FA as product since FA is a side chain of pectin which could be completed by type B feruloyl esterase [62]. These results corroborate the findings of Kühnel et al. [42] who reported 60% of FA production using type B feruloyl esterase from sugar beet pectin oligomers as a substrate.

In this research, only five microbes were used to evaluate the biodegradable ability of the strains utilizing substrate utilization profile since the co-culture used in this study was developed from these strains. Substrate degradation profiles suggest that the presence of hydrolytic enzymes produced by bacterial strains could use the lignocellulosic biomass component as their carbon source. The results explain why higher FA production could be observed in these five strains from *Bacillus* spp. and *Lysinibacillus* sp. as where the microbes could produce mixtures of enzymes to degrade lignocellulosic biomass during fermentation. Although these strains have potential as a good enzyme producer, the substrate utilization profile provided does not include lignin or lignin-related aromatic compounds as a substrate to clarify the activity of lignin degradation, which is the major limitation of this study to determine its effectiveness as lignin degraders. However, it is predicted that these strains could attack lignin structure parts to gain access to cellulose and hemicellulose rich in energy since BSW is the only carbon source provided for the fermentation [63]. Therefore, rationally, the probability of these strains producing lignin degrading enzymes is higher. Indeed, the findings of this study are aligned with other studies where it has been reported that *Bacillus* spp. and *Lysinibacillus* spp. could exhibit ligninolytic, cellulolytic and/or hemicellulolytic activities [64–66]. From the experimental point of view, more research is necessary to explore the ability of these strains as degrading bacteria to expand our understanding since several questions still remain to be answered.

4 Conclusions

The present study was performed to isolate the most promising soil co-culture for ferulic acid production through submerged fermentation. This study identified five out of thirty-seven strains as the highest FA producer which belongs to *Bacillus* spp. and *Lysinibacillus* sp. The study found that most of the strains could degrade D-cellobiose, α-D-glucose, D-mannose, D-fructose, D-galactose, D-fucose, L-fucose, L-rhamnose, pectin, D-galacturonic acid and D-glucuronic acid which proved their capabilities to manifest hydrolytic activities to produce FA effectively. From the investigation, co-culture of *Bacillus* sp. strain MB2, *Bacillus* sp. strain WB8A and B. *pumilus* strain WB1A was found to efficiently contribute 394.76 mg/kg of FA production from BSW in comparison with single culture (only 160.78 mg/kg of FA produced by *Bacillus* sp. strain WB8A). The findings suggest that co-culture fermentation may enhance FA production as a result of synergistic degrading activity.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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