Application of Michaelis–Menten in the kinetics of oil palm frond enzymatic hydrolysis for ferulic acid production

Zulsyazwan Ahmad Khushairi^{1,2} · Kamaliah Abdul Samad¹ · Nurul Aliaa Abdul Rahman¹ · Hafizuddin Wan Yussof¹ · Norazwina Zainol³

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Abstract

Ferulic acid (FA) production has become a frequent focus on today's research due to its antioxidant properties. However, there has been little to none studies reported on the usage of mixed culture as inoculum in FA production. This study aims to determine the kinetics FA production in a mixed culture fermentation by applying the Michaelis–Menten kinetic model. In this study, mixed culture fermentation process using oil palm frond bagasse (OPFB) as substrate was applied for FA production improvement. Optimum condition was applied to study ferulic acid esterase (FAE) mechanism for kinetic modelling purposes. The kinetic model used was based on the Michaelis–Menten kinetic model. Runge–Kutta Fourth Order method was used to solve the kinetic model. Maximum FAE activity was achieved at the 28 h of fermentation process at 3.7×10^{-3} mU mL⁻¹. This result proved that enzymatic hydrolysis occurred during fermentation process. Kinetic study was conducted with Michaelis–Menten kinetic model used as a reference kinetic equation. Three kinetic constants, V_{max} , K_m and K_s were determined as 3.725×10^{-3} g L⁻¹ h⁻¹, 28.231 g L⁻¹ and 1.33×10^{-2} h⁻¹ respectively using Runge–Kutta Fourth Order approach. The outcome of this study confirms that the kinetics of the process fit well with the Michaelis–Menten model. This also suggests that the usage of mixed culture has the potential to induce enzymatic hydrolysis hence improving FA production from OPFB during fermentation process.

Keywords Ferulic acid · Oil palm frond · Mixed culture fermentation · Kinetic study · Michaelis–Menten

1 Introduction

Malaysia is a country rich with biodiversity. The agricultural sector stands as one of the largest industries of this country. This sector has been contributing to a majority amount of gross domestic products available [1]. Almost 62.3% of the country's land is covered with agricultural land with the palm oil tree being one of the common sightings. Malaysia and Indonesia leads the global palm oil production which covers up to 87% of the world palm oil production [2]. The leaves of this tree, the oil palm frond (OPF) are

usually cut down and maintained to allow easy access to their fruits. OPF is the largest portion from the palm oil biomass which is up to 70% which is approximately 83 million tonnes (wet weight) annually [3]. The abundance of OPF leads to the imminent research of its utilization for fine products. The OPF itself is rich with feruloyl-polysaccharide content making it a suitable candidate for the base carbon source in biorefineries. The same study stated that OPF was able to be utilized in the production of succinic acid using the strain *Actinobacillus succinogenes*. There were reports that OPF biomass was able to enhance enzymatic

✉ Hafizuddin Wan Yussof, hafizuddin@ump.edu.my | ¹Faculty of Chemical and Process Engineering Technology, Universiti Malaysia Pahang, Lebuhraya Tun Razak, 26300 Gambang, Kuantan, Pahang, Malaysia. ²School of Foundation and Interdisciplinary Studies, DRB-HICOM University of Automotive Malaysia, DRB-HICOM Automotive Complex, Lot 1449, PT 2204, Peramu Jaya Industrial Area, 26607 Pekan, Pahang, Malaysia. ³College of Engineering, Universiti Malaysia Pahang, Lebuhraya Tun Razak, 26300 Gambang, Kuantan, Pahang, Malaysia.

hydrolysis with the proper pre-treatment approach [4]. Other parts of the oil palm waste which were utilized for biorefinery includes empty fruit bunch and oil palm trunk [5, 6]. OPF was discovered to be able to produce renewable sugars simply by pressing the fresh OPF to obtain the juice or sap [7]. The oil palm frond bagasse (OPFB) is the fibre of the OPF after the removal of its sap. These fibres are commonly thrown away as waste or used as animal fodder. Surprisingly, this OPFB is known to contain high content of feruloyl-polysaccharide, which makes it the perfect source for ferulic acid (FA). With this alternative, the use of expensive raw material that cause the rise in production cost can be disregarded. Furthermore, utilization of various food-based raw material including rice bran, soybean, corn fibre and sugar cane could be minimized [8–11].

FA is a phenolic acid which can be found in almost any plant cell wall. It has been previously reported to possess certain properties including anti-oxidative, anti-inflammatory and other health related properties [12]. These properties made FA stood among the high value-added compounds and has been used widely in the medical, food and cosmetic industries [13, 14]. There has been a study on the compilation of food-based plant that may naturally contain a decent amount of FA [15]. This includes commelinid plants such as rice, wheat oats and pineapple. However other type of plants such as grasses, grains, vegetables, flowers, fruits, leaves, beans, peanut and nuts were also reported for their FA content in natural condition. FA is found to be available in the feruloyl compound that is linked with hemicellulose chains with arabinose residues by ester bonds while connected ether bonds with lignin through [16]. In theory, the feruloyl compound is required to be released from the ester bonds link for FA production. Hydrolysis process has been reported to be able to break these bonds and producing FA [17]. There are also reports that enzymatic hydrolysis with the presence of feruloyl esterase (FAE) have increased FA production due to the mechanism of FAE which cleave the hemicellulose in plant structure [9, 18, 19]. These reports indicate that the presence of FAE may induce FA production via enzymatic hydrolysis.

The FAE is an enzyme that specifically catalyses chemical reaction to induce FA production [20]. To produce FA via enzymatic hydrolysis, the presence of FAE is must for the process to be successful. FAE can be found naturally in plants with the presence of another microorganism. Both fungi and bacteria has been reported to be able to release FAE during FA production [21, 22]. Among the fungi kingdom, the *Aspergillus* spp. has been reported to be able to produce FAE consistently [16, 23]. As for bacteria, the *Bacillus* spp. has shown the ability to produce FAE [22, 24, 25]. Since both of these microorganisms can be found in soil culture, it is safe to deduce that soil culture has the ability

to produce FAE. This is where the hypothesis and idea of using a soil-based mixed culture for enzymatic hydrolysis to produce FA was developed. The utilization of mixed culture fermentation in biotechnology is not a new area to be explored. There are studies that indicate the mixed culture fermentation may perform better in biodegradation compared to pure culture system [26]. However, in FA production, there is no study on the mixed culture fermentation yet except from our previous research [27, 28]. Our previous researches have studied on the screening and optimization process of FA production via enzymatic hydrolysis of OPFB using mixed culture. The optimum conditions for FA production are temperature at 26 °C, pH value at 9, agitation speed at 150 rpm, 24 h of response time and with the presence of 2% of mixed culture volume yielding a value of 205.724 mg FA/kg OPFB.

In this manuscript, the kinetics of FA production from enzymatic hydrolysis of OPFB was determined. This kinetics serves as the novelty of this manuscript as this report has never been published yet. This study is also a continuation of our previous work as this part of the study was not included in both of our works [27, 28]. However, to determine the kinetics of FA production from enzymatic hydrolysis of OPFB, an existing and validated model needs to be used as a reference source. In this work, the Michaelis–Menten kinetic model was used as a reference for the kinetics. The kinetic parameters were solved using Runge–Kutta Fourth Order (RK4) method. The mechanism of FAE activity was also studied as an effort to understanding the kinetic model. The kinetic model developed was able to determine the substrate–enzyme binding constant and the maximal velocity of the process. These two constants serve as a crucial tool in any enzymatic hydrolysis process. The kinetic parameters were discussed in this study for its relevancy and the competency of this method.

2 Materials and methods

2.1 OPFB preparation

As for this study, both OPFB and soil used were obtained from a local oil palm plantation located near Gambang, Kuantan, Pahang. The OPF used in this study was first squeezed for at least three times using conventional sugarcane presser machine to remove sap from OPF [7]. The fibres obtained from this process is the OPFB. The OPFB was then dried under the sun for 2–3 days. The fibres were shredded into powder form and sieved into particle size as low as 2 mm. In this study, OPFB was characterized and used as substrate for the fermentation process.

2.2 OPFB characterization

Characterization was done in order to determine the exact composition of ashes, cellulose, hemicellulose, lignin, and other extractives. Ash analysis was carried out according to National Renewable Energy Laboratory (NREL) procedure by using ash furnace AAF 11/3 (Carbolite, UK) [29]. The temperature was set at 575 ± 25 °C during the process. Empty porcelain crucibles were heated in the muffle furnace for 4 h. The crucibles were then left to cool in a desiccator for an hour. The mass of the empty crucibles was weighed with analytical balance. Crucibles were then filled with 1.2 ± 0.2 g of OPFB and their mass were taken. The crucibles with OPFB were placed in the furnace at 575 ± 25 °C for 24 h. At the end of the duration, the crucibles were left to cool down again in desiccator for an hour. The crucible with the OPFB mass were weighed and recorded again. Equation 1 was to calculate the ash content of OPFB.

$$\text{Ash, \%} = \frac{A_3 - A_1}{A_2 \times (1 - MC_{TWB})} \times 100\% \quad (1)$$

From Eq. 1, A_1 is the weight of crucibles in gram, A_2 is the initial weight of OPFB samples in gram, A_3 is the final weight of crucibles and ash in gram and lastly, MC_{TWB} stands for the moisture content percentage.

The structural carbohydrate composition in the OPFB was determined by applying the NREL method for acid hydrolysis [30]. Three grams of OPFB sample were weighed and placed in autoclave vial. Another autoclave vial containing sugar recovery standard was placed alongside the OPFB sample vial. The sugar recovery standard consists of 0.2 g of glucose, 0.1 g of xylose and 0.1 g of arabinose for sugar degradation quality control. After the autoclave process, 3 mL of 72% H_2SO_4 was added into the sample and standard. Both of the vials were left at the incubator shaker at 30 °C with 150 rpm of agitation speed for an hour. The step was continued with the addition of 84 mL deionized water into all vials. As for the sugar recovery standard solution, the percentage of recovery was calculated using Eq. 2.

$$\text{SRS, \%} = \frac{V - H_5}{H_2} \times 100\% \quad (2)$$

From Eq. 2, the sugar recovery standard solution (SRS) percentage can be represented with the total volume of acid and deionized water in Litre (V), Mass of monomer sugar after acid hydrolysis in gram (H_2) and the concentration of monomer sugars after acid hydrolysis in g/L (H_5). The vials were then placed in autoclave at 121 °C in the liquid sterilization cycle for an hour. After

the autoclave process, the vials were cooled down using running water. The samples were filtered into HPLC shell vial for sugar content analysis via HPLC. OPFB mass that was added into the hydrolysis vial (HM) was calculated using Eq. 3.

$$HM = \frac{H_1 - MC_{Ext2}}{(1 - ET\%)} \quad (3)$$

For Eq. 3, H_1 is the initial biomass in grams, MC_{Ext2} is the moisture content percentage while ET is the total extractives composition percentage. The composition of the structural carbohydrates for glucan, xylan and arabinan were calculated using Eqs. 4–6 respectively.

$$\text{SC2}_{Glu}\% = \frac{0.9 \times V \times H_{3,Glu}}{\text{SRS}_{Glu} \times HM} \times 100\% \quad (4)$$

$$\text{SC2}_{Xyl}\% = \frac{0.88 \times V \times H_{3,Xyl}}{\text{SRS}_{Xyl} \times HM} \times 100\% \quad (5)$$

$$\text{SC2}_{Ara}\% = \frac{0.88 \times V \times H_{3,Ara}}{\text{SRS}_{Ara} \times HM} \times 100\% \quad (6)$$

As for Eqs. 4–6, V is the total volume of acid and deionized water in Litre, H_3 is the mass of sugar for each respective monomer after hydrolysis process, SRS is the monomer sugar recovery standard solution percentage and ET is the percentage of total extractives in the OPFB.

Lignin composition was determined via acid-insoluble lignin analysis method from NREL procedures [31]. Samples that have gone through acid hydrolysis process were filtered with the vacuum filtration. Filter paper used were weighed first prior to be used for filtration purposes. The residues left on the filter paper were dried overnight in the desiccator. After the drying process, the weight of filter paper with residues was taken. Acid-insoluble lignin can be determined using Eq. 7.

$$\text{AIL\%} = \frac{L_2 - L_1}{HM} \times 100\% \quad (7)$$

In Eq. 7, $\text{AIL\%}$ represents the acid-insoluble lignin percentage, L_1 is the weight of filter paper without its residue, L_2 is the final weight of the filter paper with its residue and HM is the mass of OPFB added to the hydrolysis vial. All of the mass weighed in this calculation were in the gram unit.

This procedure was able to characterize proximate chemical composition of the OPFB such as ash content [29], lignin content [31], cellulose content and hemicellulose content [30]. All data were recorded for analytical purposes.

2.3 OPFB substrate preparation

As for the substrate preparation, OPFB was mixed with distilled water at a ratio of 1:10 weight to volume (g mL^{-1}). This method is a slight modification from another study on FA production [32]. Substrates were kept in freezer to avoid any contamination. The OPFB substrates were thawed on the same day before use. The total volume of substrate used was determined first before thawing them. Substrate was sterilized by autoclaving for 15 min at 121°C purposes [33].

2.4 Mixed culture preparation

The soil used in this study was collected from the same palm oil plantation where the OPFB was obtained. The soil sample was collected at approximately 5–10 cm layers below the surface [34]. The soil is estimated to be the habitat of millions of microbes. The soil acclimatization with OPFB substrate was performed through continuous fermentation by mixing one part of soil containing mixture of microorganism with three parts of substrate into 7 L bioreactor as depicted in Fig. 1. The first part is filled in with soil and then periodically with OPFB substrate at a controlled rate of 100 mL day^{-1} . Three replications were made to control the mixed culture performance. The soil mixed with substrate were incubated at ambient temperature for 1 month before being used as mixed culture inoculum for the process [32].

2.5 Experimental setup

As for the experimental setup, a batch fermentation system is proposed as it offers more control to the process compared to continuous fermentation system. The

fermentation process was performed by inoculating mixed culture into 250-mL conical flask which contained OPFB substrates. Fermentation process was performed at optimum condition obtained from previous study [28]. The optimum condition is temperature at 26°C , agitation speed at 150 rpm, pH value at pH 9 and inoculum culture percentage at 2% of volume. FA concentration was obtained via High-Performance Liquid Chromatography (HPLC) analysis. Both substrate and biomass concentrations were obtained by controlling the time parameter [35]. The mixed culture growth was also recorded during this process. Samples were collected for 30 h with 2 h interval between each reading. Each sample were analysed to determine the kinetic parameters of the model.

2.6 The Michaelis–Menten kinetic equation

The Michaelis–Menten kinetics mathematical model is one of the oldest yet applicable model for a simple enzyme-catalysed reaction [36, 37]. Michaelis–Menten equation can be expressed as the equation below (Eq. 8).

$$V = \frac{V_{max} \cdot [S]}{K_m + [S]} \quad (8)$$

In Eq. 8, the relation between both initial rate of product formation and substrate concentrations can be expressed. The Michaelis constant (K_m) and maximal velocity (V_{max}) are constants that need to be determined. V_{max} may also vary depending on the quantity of enzyme present in the reactor [38]. The determination of the kinetic parameters was conducted using the RK4 method. The rate of substrate consumed during FA production was studied by applying Eq. 9 to determine substrate constant, K_s . The rate of FA production from Eq. 8 can also be expressed as Eq. 10. From Michaelis–Menten equation, both of Eqs. 9 and 10 can be solved simultaneously. The experimental results for substrate concentration, $[S]$ and FA production $[P]$ were used to determine all of the three kinetic constants: K_s , V_{max} and K_m . The determination of the model is conducted using the RK4 method.

$$\frac{dS}{dt} = -K_s[S] \quad (9)$$

$$\frac{dP}{dt} = \frac{V_{max}[S]}{(K_m + [S])} \quad (10)$$

2.7 Substrate concentration analysis

The substrate concentration was analysed by measuring the optical density (OD) of the sample. OD was determined

Fig. 1 Simple design of the bioreactor composition

using spectrophotometer (Varian Cary 50, USA) at 600 nm wavelength. The blank used in this analysis was the substrate liquid itself. Samples were first centrifuged for the supernatant part. The supernatant was then filtered through a pre-weight nylon membrane filter (47 mm diameter, 0.2 μm pore size) via vacuum pump [39]. The filtrate was used for OD analysis while the membrane was then dried at 105 $^{\circ}\text{C}$ overnight before being dried in desiccator until constant weight. The substrate dry weight and sample volume were used to determine substrate concentration. Samples were collected every 4 h for 48 h. The procedure was conducted in triplicates to avoid disambiguate data recordings. An identical sample of the fermentation broth was used as a control set. A calibration curve of sample OD versus the substrate weight was plotted from the control set. The substrate concentration was able to be determined by referring to the calibration curve.

2.8 Feruloyl-esterase assay

As for the analysis of FAE activity, the FAE assay was determined using a method proposed by a recent study [39]. In this procedure, the mixed culture capability to produce FAE was determined using a standard substrate rather than the OPFB itself. A 50 mM methyl ferulate stock solution (AbcamBiochemicals, United Kingdom) was prepared in methanol. The FAE assay was performed by diluting methyl ferulate 10 times with 0.1 M citrate buffer (pH 6.0) and preheated at 40 $^{\circ}\text{C}$. Next, 2 mL of inoculum from the sample were added and incubated in water bath at 40 $^{\circ}\text{C}$ for 4 h. Then, the mixture was further heated at 95 $^{\circ}\text{C}$ for 10 min to stop the reaction. Samples were prepared to determine FA content via HPLC by filtering it using 0.45 μm membrane syringe filter. Each unit of FAE activity was represented by the amount of FAE used to release 1 μmol of FA hydrolysed from methyl ferulate per min. FAE assay was run in triplicate to maintain the consistency of the data. Samples were collected at a 4-h interval up to the 48th hour.

2.9 HPLC analysis

The FA concentration was determined via HPLC analysis for samples at each time interval. HPLC analysis method used in this study was a modification of a procedure from a previous study [40]. The study stated that the method was first developed for the estimation of FA from asafoetida and a polyherbal preparation. HPLC (Agilent 110 series) equipped with a C18 column was used for sample analysis. A solution with ratio of 20:80 (v/v) containing acetonitrile and 10% acetic acid was prepared as mobile phase. Injection volume of 20 μL and 1.0 mL min^{-1} of flow rate was set. Column temperature

was controlled during analysis by fixing the temperature at 30 $^{\circ}\text{C}$. Sample was detected at 319 nm of wavelength. The slight modification in this procedure was the maximum retention time of the analysis which is at 10 min. Figure 2 shows the standard curve used to determine FA concentration from HPLC absorbance (mAU). The standard FA was used to plot out this curve.

2.10 Mixed culture dry weight cell analysis

Mixed culture concentration was measured based on optical density (OD). Fermentation samples were first filtered to separate substrate particles from mixed culture liquid before being used for OD analysis. Substrate liquid without mixed culture was used as a blank for reference. Dry weight of fermentation mixed culture was determined by comparing with the standard OD values obtained from a known volume of mixed culture broth. The mixed culture was inoculated into a 250-mL flask containing 100 mL nutrient broth. Every 2 h, 1 mL of the sample was taken from the flask to measure the OD with spectrophotometer (Varian Cary 50, USA) at wavelength of 600 nm until the growth rate decreased. From the OD data and mixed culture dry weight, a standard curve of mixed culture concentration versus time was plotted. This data is available in Fig. 4 at Sect. 3.2 along with the FAE activity trending on a 48-h timeline. The substrate concentration versus time standard curve was plotted by referring it with nutrient broth. This data is available in Fig. 3. With this set of data, the mixed culture (g L^{-1}) and substrate concentration (g L^{-1}) was able to be determined.

Fig. 2 Standard curve of FA concentration at different HPLC absorbance readings

Fig. 4 Mixed culture growth and FAE activity by fermentation time

Fig. 3 Substrate concentration trending over the duration of time

3 Results and discussion

3.1 OPFB proximate chemical analysis

Since FA can be found covalently linked to polysaccharides by ester bonds as a part of lignin the determination of the chemical composition of OPFB is crucial in this study as it gives a good estimation on the availability of FA in the OPFB [41]. Certain types of plant species are able to be characterized by using proximate chemical analysis. These analyses are done to determine quantitatively the major components of the pulp under normal circumstances. The proximate analysis is shown on Table 1. Each of these analyses are performed on the raw OPFB.

Proximate chemical analyses of OPFB were carried out via the NREL's methods. The total carbohydrate structure is found to be at 67% consisting 43.3% of cellulose and 23.7% of hemicellulose. The amount of lignin discovered is approximately at 29.9% while ashes content is found to be at 3.1%. The cellulose content is almost identical with other similar studies which are 41.7% [7] and 42.8% [3].

Table 1 Chemical composition in OPFB

Proximate analysis	Composition (%)
Cellulose	43.3
Hemicellulose	23.7
Lignin	29.9
Ashes	3.1

However, the hemicellulose and lignin content from both of the studies is quite different compared to the value obtained in this study. This difference is mainly due to the different pre-treatment methods applied. Other factors that may affect different chemical composition between lignocellulosic material includes different geographic location, ages, climate and soil conditions [42]. In FA production, the hemicellulose structure is broken down by FAE via hydrolysis. FAE cleave the arabinose hydroxycinnamate ester in hemicellulose, releasing the FA bonded to the arabinose [43, 44]. FA production is affected by the hemicellulose content present in the OPFB.

3.2 Mixed culture growth profile and FAE activity

The growth of mixed culture and FAE activity were plotted in a time course graph for 48 h. The findings showed that FAE activity had a proportional increase to the log phase of mixed culture growth. The mixed culture density rapidly increasing reaching its peak at the 28 h during the log phase. The following hours showed that there was a slight increase in the mixed culture density. This data suggested that the mixed culture density has become stable at the 28 h.

From the FAE activity plot in Fig. 4, the graph curve had a stable increasing trend right off after the 4 h. The trend continued up to the 28 h, whereas after that point, the graph showed a declining trend. The maximum FAE activity was recorded at 3.7×10^{-3} mU mL⁻¹ at the 28-h compared to the lowest FAE activity, 1.4×10^{-3} mU mL⁻¹ at the 4 h. The FAE activity was quite low compared to other studies such as 34.8 mU mL⁻¹ using *Bacillus pumilus* [24], 2.0 mU mL⁻¹ using *Streptomyces* S10 [45] and 82 mU mL⁻¹ using *S. avermitilis* CECT 3339 [46]. One of the reasons behind this low FAE activity was due to the nature of the FAE source used. The three studies cited used pure culture system to determine the FAE activity. In comparison with pure culture system, the mixed culture system consists of other microbes that may affect FAE activity that may bring disadvantages to the system. However, the simplicity of the study stands as one of the advantages in using mixed culture as a source of FAE. Pure culture system requires specific isolation technique for the culture to be used for

fermentation purposes. As for mixed culture used in this study, the mixed culture can be obtained from the soil of the oil palm tree for fermentation purposes.

The comparison between the mixed culture growth and FAE activity implied that FAE activity was relatively affected by the mixed culture growth. Enzyme reaction by *Bacillus* sp. is heavily affected by the growth of microorganism [47]. The growth would eventually reach a limitation stage as the nutrients become less available. The same paper also discovered that beyond 72 h of incubation period, the declining of enzyme activity occurred as a result of enzyme denaturation from the interaction with other components in the medium.

3.3 Kinetic parameters

The first estimation of the kinetic parameters was determined by Lineweaver–Burk plot. The Lineweaver–Burk plot was simplified to linear form from the Michaelis–Menten equation. It is also known as the double-reciprocal [48]. The Lineweaver–Burk plot is expressed in Eq. 11.

$$\frac{1}{V_0} = \frac{K_m}{V_{max} \cdot [S]} + \frac{1}{V_{max}} \quad (11)$$

where V_0 stood as the rate of product formation, V_{max} as the maximum product formation rate, K_m as the Michaelis–Menten constant and $[S]$ as the substrate concentration. Figure 5 shows the FA production from OPFB on Lineweaver–Burk plot.

From Fig. 5, the values from Lineweaver–Burk equation was able to be determined. The V_{max} was calculated by inverting the y-axis interception point of the straight line and the value of K_m was determined from the negative reciprocal of the x-intercept of the graph [49]. Since the x-intercept is not visible in the plot, the graph is plotted with an extended line across the y-axis to look

Fig. 5 Lineweaver–Burk plot for K_m and K_s estimation

for the x-intercept. From Lineweaver–Burk plot, the value obtained for V_{max} and K_m were $0.004 \text{ g L}^{-1} \text{ h}^{-1}$ and 0.193 g L^{-1} respectively. These values were used as estimations for RK4 method in Microsoft Excel solver.

From Fig. 6, the substrate concentration showed a trending of gradually descending throughout the process. This happened due to substrate utilization for the growth of mixed culture throughout the process. As time passed, more substrates were utilized hence the substrate concentration descending trend over time. The rate of the substrate utilization was directly related to the ability of organisms to consume on the substrate for growth [50]. Low value of K_s indicates that the biomass digestibility on the substrate as food is high. In this study, K_s value for RK4 is $1.33 \times 10^{-2} \text{ h}^{-1}$. This value showed a better rate than to other studies. A recent study indicated that the K_s value obtained is $3.00 \times 10^{-4} \text{ h}^{-1}$ using autotrophic sulphur bacteria [51]. In relation to the current study, the K_s value showed a higher number. This was a clear indication that the substrate used in current study gives better access to microbe for substrate consumption. The structure of OPFB itself was one of the reasons behind this high K_s value. Low lignin content means that mixed culture had much more access to hemicellulose, allowing easier substrate consuming process for FA production since a majority of the feruloyl compound was discovered to be attached to the hemicellulose compound. Another explanation behind this high K_s value was the mixed culture used in this current study. The usage of mixed culture influenced the value of K_s as there were various types and numbers of microbes present in the mixed culture. Through isolation and DNA identification procedure, it was discovered that the mixed culture consists of multiple microbes with bacteria *Bacillus cereus* CCM2010, *B. thuringiensis* AATC 10792 and the fungus *Pseudallescheria boydii* being the dominating microbes in the mixed culture. The presence

Fig. 6 Substrate consumption rate and FA production rate using RK4 method

of these microbes contributed to the high value of K_s as the time goes on.

Figure 6 also shows the FA production rate using RK4 method. The FA concentration showed an increase in FA production at a steady rate of $1.0982 \times 10^{-4} \text{ mg L}^{-1} \text{ h}^{-1}$. This was an indication that the data had a good agreement with the data from mechanism study which also shows a steady increase in FA production. The FA production can be expressed in Eq. 10 to derive the other kinetic constants: V_{max} and K_m . The V_{max} is the rate of reaction at maximum value for total enzyme concentration [52]. The value of V_{max} will vary depending on the number of enzymes added into the reaction while not affected by the substrate concentration. On the other hand, K_m stood as the measurement on the affinity of enzyme on its substrate. From Michaelis–Menten equation, substrate concentration will heavily affect both of K_m and V_{max} . Both kinetic parameters need to be determined as they are clearly crucial in enzyme kinetics [20]. In a more direct meaning, high value of V_{max} is much preferred as it shows that the reaction rate is high. High reaction rate means that the process can obtain high value of FA production rate. Low K_m value is more preferred as it shows that the process would only require a low amount of substrate to achieve a certain value of rate of reaction (half of V_{max}). This would also mean that the process would require low substrate amount for it to reach high FA production rate. The K_s value on the other hand, is quite different for both K_m and V_{max} . A high value of K_s indicates that the substrate is easily accessible by microbes and enzymes, increasing the possibility of other reactions to occur. This would mean that at high K_s value substrate consumption is happening at a fast rate but it does not mean that all of the substrate is consumed for FA production. There is a possibility that it was also used for other reaction process. In this case, high K_s value would affect the substrate cost of the process as more amount of substrate is required. The K_s high or low value preferences can be determined by the value of K_m itself. In this particular study, due to the presence of other bacteria in the mixed culture, a high value of V_{max} , low value of K_m and K_s is much more preferred.

In this kinetic study, the kinetic constant was compared with other studies which emphasize on FA production. The comparison of these values is compiled in Table 2. From Table 2, this study shows a value of V_{max} at $3.730 \times 10^{-3} \text{ g L}^{-1} \text{ h}^{-1}$ with a K_m value of 28.231 g L^{-1} which is quite different with other cited studies. One of the findings from Table 2 reported that using wheat straw as substrate, V_{max} and K_m value were at $0.1142 \text{ g L}^{-1} \text{ h}^{-1}$ and 1.2233 g L^{-1} respectively [20]. A low V_{max} value indicated that the reaction rate of the process was low. A process with low reaction rate would require a longer reaction time than to a process with high reaction rate. As for the

Table 2 Comparison of kinetic constants with other studies

Substrate	V_{max} ($\text{g L}^{-1} \text{ h}^{-1}$)	K_m (g L^{-1})	Source
OPFB	3.730×10^{-3}	28.231	This study
Wheat straw	0.1142	1.2233	[20]
Rice	1.6×10^{-3}	29.127	[53]
Grape	1.119	0.237	[54]

K_m value, it represented the substrate-enzyme binding capability. Lower value of K_m would indicate that the substrate-enzyme binding occurred more than to the reaction with high K_m value. The binding of substrate-enzyme was reported to be affecting the reaction rate at negative way. The value of K_m obtained in this study is considered quite high compared to other studies from Table 2. Since K_m itself is a measurement of the substrate needed to reach a half of V_{max} , the value of K_m is directly related to substrate consumption rate, K_s . The mixed culture used in this current study possess numerous microbes that consumed the OPFB substrate during this process which contributed to the high value of K_s , as been discussed in the previous paragraph. High value of K_s affected the value of K_m as both of them are a measurement of substrate consumption. This led to the high value of K_m which also affected the substrate-enzyme binding. In other terms, the various microbe present in the mixed culture consumed OPFB substrate alongside FAE, making the substrate needed to reach a half of V_{max} , which is K_m high. This also proved that the substrate-enzyme binding occurred less with the presence of other microbes consuming the substrate. For the reaction rate to be high, the K_m value needed to be low as this will increase the affinity between them. High substrate-enzyme affinity would result in a high value of reaction rate.

However, the V_{max} and K_m value of this study is almost identical with another research using rice as substrate [53]. The usage of a food-based substrate proved to be a crucial point for the research. The cited work utilized a food source as a substrate for its FA and p-coumaric acid production. Rice as a food source is a very valuable resource. It is also labelled as a luxury in certain parts of the world. The utilization of food-based source in FA production is not an applicable approach as it disrupts the food source of a community. This seemed to be the same factor for another study [54]. The cited study utilized grape as a substrate for FA production. The usage of OPFB is far more applicable as it is abundant and widely available than to the utilization of other food sources.

In this current study, there are several factors that may affect the values of K_m and V_{max} in a fermentation process for FA production. Two of these factors that stood out compared to others are the difference in substrates type

and the mixed culture used. Each unit of enzyme activity stands for the amount of enzyme that able to deliberates 1 μmol of FA per minute. The enzyme activity (U) changed when different substrates were used due the different amount of enzyme released during substrate degradation. The use of difference substrate could affect the relative enzyme activity by up to tenfold [55]. The value of kinetic constants is heavily influenced by the substrate used in the process. The presence of other microbes in the mixed culture also contributed to the value of K_m and V_{max} value. As for K_m , the microbes in mixed culture factors has been discussed in the previous page. As for V_{max} , the value was also affected by the microbes present in mixed culture. The presence of other microbes in the mixed culture created a system which the microbes need to compete with each other for substrate consumption in order to survive. In this system, with the competition for substrate for survival, it may induce a situation where there is insufficient substrate for FAE to use for FA production. Insufficient substrate may lead to the low value of FA production hence, the low V_{max} value. The lack of substrate to be consumed by FAE reduced both enzyme activity and enzyme affinity. In a way, this also shows that the usage of mixed culture plays a critical role in determining the kinetic constants value.

4 Conclusions

In this study, the FAE mechanism in mixed culture was studied. FAE activity reached its highest value at the 28 h during incubation which was $3.7 \times 10^{-3} \text{ mU mL}^{-1}$. The FAE activity showed an increasing trend during the log phase of the mixed culture growth. FA production was also discovered to be following the FAE trending throughout the study. This correlation indicated that enzymatic hydrolysis occurred in FA production from OPFB using mixed culture. In the kinetic study, the Michaelis–Menten was able to fit with the proposed model with RK4 method as numerical solutions. From RK4 method, the values of the kinetic constant of V_{max} , K_m and K_s were $3.730 \times 10^{-3} \text{ g L}^{-1} \text{ h}^{-1}$, 28.231 g L^{-1} and 0.013 h^{-1} respectively. The usage of mixed culture affected the values of kinetic parameters as there were various microbes present in the process. These microbes were crucial in determining the kinetic parameters. Kinetic parameters play an important role in determining the reaction rate of the FA production. This is crucial in this study as it paves the path to other future works such as scale-up process and pilot plant.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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