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# Simultaneous Hydrolysis and Fermentation of Sweet Sorghum Varieties (FS501 and KCS105) into Bioethanol Using *Saccharomyces steineri* – A Kinetics Study

**Hanifrahmawan Sudibyo[1,a,*], Jasman[2,b], Himawan Tri Bayu Murti Petrus[1,c], Agus Prasetya[1,d], Donny Widianto[3,e], Chusnul Hidayat[3,f], Satoshi Harashima[4,g], Irfan Dwidya Prijambada[3,h]**

1 Chemical Engineering Department, Faculty of Engineering, Universitas Gadjah Mada, Yogyakarta 55281 Indonesia
2 Science Education Department, Universitas Nusa Cendana, Kupang 85001 Indonesia
3 Faculty of Agriculture Technology, Universitas Gadjah Mada, Yogyakarta 55281 Indonesia
4 Department of Agricultural Microbiology, Universitas Gadjah Mada, Yogyakarta 55281 Indonesia
5 Department of Biotechnology, Osaka University, Suita, Osaka 565-0871 Japan
Email: [a]hanifrahmawan.s@mail.ugm.ac.id, [b]puskomundana@yahoo.com, [c]bayupetrus@yahoo.co.id, [d]aguspras@ugm.ac.id, [e]donny@faperta.ugm.ac.id, [f]chusnulhidayat@ugm.ac.id, [g]harashima@bio.sojo-u.ac.jp, [h]irfandp@faperta.ugm.ac.id

**Abstract.** In this study, kinetics of bioethanol production by fermentation of three different substrates, which were artificial substrate and the juice of two sweet sorghum varieties (FS501 and KCS105) using *Saccharomyces steineri*, were examined using two proposed models by assuming that simultaneous hydrolysis and fermentation occurred. Fermentation of the substrate of FS501 and KCS105 juices showed better data fitting by using the modified version of the kinetics model while the fermentation of artificial substrate which was free of any other components followed Philippidis's kinetics model. This difference was caused by the change of the yeast behavior in the form of the reduction of both the rate of fructose and/or glucose consumption by the yeast and the rate of fructose and or glucose conversion into ethanol during lag phase. As the consequence, sucrose hydrolysis seems very dominant in the FS501 and KCS105 juices fermentation during the lag phase. The change of behavior of the yeast was estimated being caused by the existence of "impurities" such as acetic acid, glycerol, nitrogen, phosphor, and potassium in the FS501 and KCS105 juices. From statistical analysis using correlation coefficient (between kinetics parameters and "impurities"), acetic acid was the most influential component to change the behavior.

**Keywords:** Bioethanol, hydrolysis, fermentation, *Saccharomyces steineri*, kinetics study.

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**Nomenclature**

| | | | |
|---|---|---|---|
| $k_I$ | specific rate of sucrose hydrolysis ($h^{-1}$) | $[G]$ | concentration of glucose (g/L) |
| $K_m$ | sucrose saturation constant for yeast (g/L) | $[F]$ | concentration of fructose (g/L) |
| $K_{IG}$ | inhibition constant of yeast enzyme by glucose (g/L) | $[E_{th}]$ | concentration of ethanol (g/L) |
| $K_{IF}$ | inhibition constant of yeast enzyme by fructose (g/L) | $[X]$ | concentration of yeast (g/L) |
| $K_G$ | glucose saturation constant for the yeast (g/L) | $m_{SG}$ | specific rate of glucose consumption for maintenance requirements ($h^{-1}$) |
| $K_F$ | fructose saturation constant for the yeast (g/L) | $m_{SF}$ | specific rate of fructose consumption for maintenance requirements ($h^{-1}$) |
| $K_E$ | ethanol saturation constant for the yeast (g/L) | $m_{SS}$ | specific rate of sucrose consumption for maintenance requirements ($h^{-1}$) |
| $Y_{X/G}$ | yield coefficient of yeast mass from glucose (g/g) | $k_d$ | death rate constant ($h^{-1}$) |
| $Y_{X/F}$ | yield coefficient of yeast mass from fructose (g/g) | $\mu_m$ | maximum specific growth rate of the yeast ($h^{-1}$) |
| $Y_{Eth/X}$ | yield coefficient of ethanol from yeast mass (g/g) | $r_X$ | volumetric rate of yeast mass production (g/L/h) |
| $[S]$ | concentration of sucrose (g/L) | $r_H$ | volumetric rate of sucrose hydrolysis (g/L/h) |
| $X$ | value of component concentrations | $n$ | number of data |
| $Y$ | value of kinetics parameters | $[E]$ | enzyme concentration |

**Abbreviation**

| | | | |
|---|---|---|---|
| SHF | Simultaneous Hydrolysis and Fermentation | MEA | Malt Extract Agar |
| SDSF | Simultaneous Delignification, Saccharification, and Fermentation | OD | Optical Density |
| NPK | Total content of $N_2$ + $P_2O_5$ + $K_2O$ | SSE | Sum of Square of Error |
| HPLC | High Performance Liquid Chromatography | | |

## 1. Introduction

Fluctuation of the world crude oil price [1] and environmental concern of greenhouse gas emission have promoted the attraction over biofuel as the alternative renewable energy resources. However, a complete substitution of petroleum-derived fuels by biofuel recently is still impossible from the production capacity and engine compatibility point of views. Yet, marginal replacement by biofuel can remarkably reduce the dependency on the petroleum resources and abate the radical climate change caused by automotive pollutants [2]. Incremental vehicle technology options, such as bioethanol-supported vehicle (with 85 percent bioethanol mixed with 15 percent gasoline), can reduce the greenhouse gas (GHG) emissions to 100 to 300 grams per mile or to about 65 to 74 percent of the emission levels expected from future gasoline-supported vehicles [3]. This signifies the importance of bioethanol to reduce carbon footprint in energy fulfillment distribution throughout the world [4].

Biofuel is undergoing a revolution in terms of technological aspect. As the development of technology continues to rise, the demand of bioethanol-powered fuel keeps proliferating [5]. World production of bioethanol in 2007 was over 46 billion liters, mounting up as many as 167% [6] from 2001 to 2007 [7]. The bioethanol production in 2007 represented about 4% of the 1,300 billion liters of gasoline consumed

worldwide [7]. With all current government programs for renewable energy development in America, Asia, and Europe, the total demand of bioethanol for fuel could exceed 125 billion liters by 2020 [8].

Bioethanol can be produced from either various biomasses or various natural resources. A number of agricultural crops, such as wheat, corn, sweet sorghum, sugarcane molasses, sugar beet, and sweet potatoes [9] not only provide foods [10], feeds [11], and fibers [12] but also produce sugars which can be converted to alcohol that can subsequently be used as energy sources. Utilization of sweet sorghum as the source for bioethanol production offered more advantages compared to other resources: (1) Sorghum harvest cycle is shorter compared to cane, (2) The plant requires less water than cane or corn, (3) Operational expenditure for sorghum production is only a third compared to cane production because of lower water and fertilizer requirement [13]. Sorghum production also boosted the utilization of dry lands which is unsuitable for corn or cane production. These make sorghum is highly suitable to be developed in the region with the dry and hot climates such as Southeast Asia, Middle East, and Africa.

As the juice content of the sweet sorghum stalk, where the fermentable sugars (sucrose, glucose, and fructose) are located [14], is between 62-74% by weight [15], it is more feasible to extract the juice first before converted into bioethanol. The remaining weight composition coming from sweet sorghum bagasse actually contains lignocellulosic biomass which is potential to be converted into bioethanol too [16]. However, the pretreatment process required for delignification of the biomass makes the overall process of bioethanol production less feasible than the process using only the sweet sorghum juice as the raw material [9]. Therefore, this study focused on the conversion of the juice into bioethanol rather than both the juice and lignocellulosic biomass simultaneously.

To create an effective method to produce bioethanol from sweet sorghum, both good and comprehensive understanding about reaction mechanism and kinetics model are required. Kinetics modeling of sucrose hydrolysis is an important tool in estimating the rate of hydrolysis by the microorganism's enzymes and the rate of fermentation by the microorganism itself [17]. Consequently, kinetics modeling of simultaneous hydrolysis and fermentation (SHF) process is a powerful step toward industrialization of bioethanol production from sweet sorghum due to the fact that concepts establishment of production process totally based on experimental, such as reactor optimization, can be expensive and time-consuming. Moreover, decent kinetics model and reliable model parameters are necessary to optimize the performance of SHF process. Several kinetics models had been developed since the past years but it was to explain the production mechanism of bioethanol from lignocellulosic or cellulosic biomass through simultaneous delignification, saccharification, and fermentation (SDSF) process [18, 19, 20]. In this study, kinetics model of a batch SHF process was developed to incorporate the variations of substrate composition of the juice of different sweet sorghum varieties. Evaluation of kinetics models and kinetics parameters correlation to the various substrate composition helps to obtain a comprehensive understanding of which model can be applied to a wide range of substrate compositions. The comprehensive understanding was made due to the possibility of sweet sorghum juice to contain minerals which can be the group of required macronutrients or micronutrients by the yeast or the inhibiting component to the yeast growth. The effect of mineral existence in the juice to the growth of the yeast and to the bioethanol production was studied by comparing the hydrolysis and fermentation process of sweet sorghum juice of two varieties (FS501 and KCS105) to the artificial substrate which was comprised of sucrose, glucose, and fructose of certain concentration. Therefore, in this study, the best kinetics model and its parameters were estimated by fitting the models to experimental data using numerical completion method. Afterward, the correlation of the obtained kinetics parameters with the mineral content of the substrate was verified.

## 2. Kinetics Modelling of SHF

Simultaneous hydrolysis and fermentation (SHF) of sweet sorghum juice is a multi-step process and interactions between enzymes inside the yeast and the sophisticated substrate. In addition, SHF also undergoes mechanisms by the product and other components inside the substrate. The process, interactions, and as well as inhibition mechanism of SHF are not fully understood. A modified kinetics model based on those reported by Philippidis [21] and Shadbahr [20] were used in this study to quantify the sucrose hydrolysis and sugars fermentation. However, the original version of kinetics model by Philippidis [21] was also used for the comparison. The kinetics model assumes that the yeast both consume and hydrolyze the sucrose into glucose and fructose. The rate of sucrose hydrolysis is shown on Eq. (1) below. One mole of glucose or fructose (180 g/mol) will be fermented to two moles of ethanol (46 g/mol) and two moles of carbon dioxide

(44 g/mol). The reaction pathway for the biochemical conversion of sucrose is supposed to follow the pathway shown by Fig. 1.

$$r_H = \frac{k_1.[X][S]}{K_m.\left(1+\frac{[G]}{K_{1G}}+\frac{[F]}{K_{1F}}\right)+[S]} \tag{1}$$

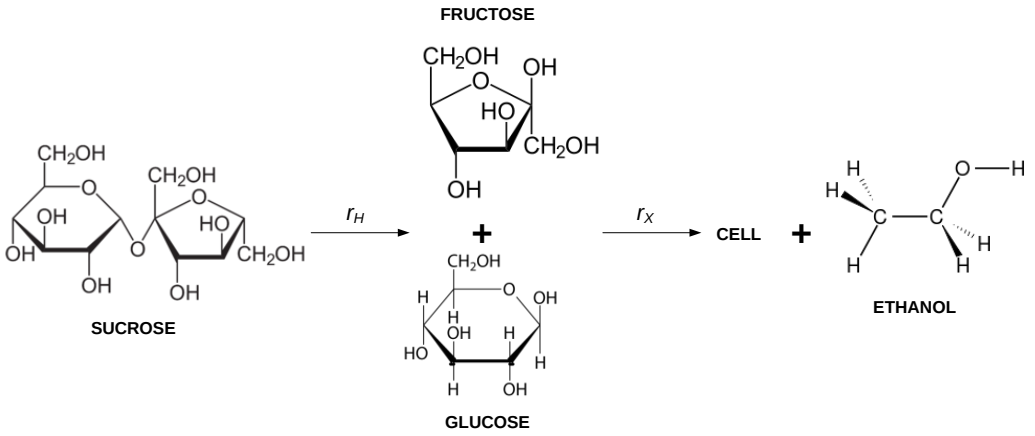


Fig. 1. Pathway of simultaneous hydrolysis and fermentation of sweet sorghum juice.

The microbial growth is a very sophisticated phenomenon, nonetheless, the overall microbial growth can often be considered as one single chemical reaction with a simple rate expression. The general idea is that the growth accumulation is the subtraction of bacteria growth and bacteria death. There are two different kinetics models proposed to explain the growth mechanism of the yeast as shown by Eq. (2) and Eq. (4). Equation (3) considered that either glucose or fructose had its own saturation constant due to the fondness of most yeast species to the glucose [22] which led to the different rate of glucose and fructose utilization by the yeast.

a. Growth rate equation of yeast adopted from Philippidis [21]:

$$r_X = \mu_m.[X].\left(\frac{[G]+[F]}{K_G+[G]+[F]}\right).\frac{K_{Eth}}{K_{Eth}+[E_{th}]} \tag{2}$$

The above equation was based on the assumption that the substrate consisting of glucose and fructose could be considered as the single substrate so that their conversion into bioethanol follow the below reaction scheme shown by Eq. (3).

$$[E] + [G + F] \rightleftharpoons [E.(G+F)] \longrightarrow [E] + [P] \tag{3}$$

b. Growth rate equation of yeast adopted from Philippidis [21] with modification:

$$r_X = \mu_m.[X].\left(\frac{[G]}{K_G+[G]}+\frac{[F]}{K_F+[F]}\right).\frac{K_{Eth}}{K_{Eth}+[E_{th}]} \tag{4}$$

The above equation was based on the assumption that different substrate resulted in different rate of yeast growth. The related reaction scheme was shown by Eq. (5) and Eq. (6). A simplification was then made on the maximum specific growth rate $\mu_m$ by making it an overall maximum specific growth rate of both schemes as shown by Eq. (4) above.

$$[E] + [G] \rightleftharpoons [E.G] \longrightarrow [E] + [P] \tag{5}$$

$$[E] + [F] \rightleftharpoons [E.F] \longrightarrow [E] + [P] \tag{6}$$

Another modification was made on the simultaneous differential equations of substrates and product concentration change versus time. In this study, the concentration change over the time of both substrates and products were described using specific yield coefficient and maintenance constant for each component as shown in Eq. (7) – Eq. (11).

$$\frac{d[X]}{dt} = r_X - k_d.X \tag{7}$$

$$\frac{d[S]}{dt} = -r_H \tag{8}$$

$$\frac{d[G]}{dt} = r_H - \frac{1}{Y_{X/G}}.\frac{d[X]}{dt} - m_{SG}.[X] \tag{9}$$

$$\frac{d[F]}{dt} = r_H - \frac{1}{Y_{X/F}}.\frac{d[X]}{dt} - m_{SF}.[X] \tag{10}$$

$$\frac{d[E_{th}]}{dt} = Y_{Eth/X}.\frac{d[X]}{dt} \tag{11}$$

The above differential equations (Eq. (7) – Eq. (11)) were solved numerically and the corresponding kinetics parameters could be determined by minimizing the Sum of Square of Error (SSE) between the calculated and experimental data of the concentration of substrate (sucrose, glucose, and fructose), yeast, and product (ethanol) as shown by Eq. (12). The equation for SSE was formulated using weighting factor as shown by Eq. (13). The weighting factor for each component aims to equalize the order of the experimental data by dividing the average of experimental data of the component whose order is the highest by the average of experimental data of the related component [23].

$$SSE = \sum \left( w_{C,i}.\sum \left( [C_i]_{\exp} - [C_i]_{calc} \right) \right)^2 \tag{12}$$

$$w_{C,i} = \frac{\overline{[C_i]_{\max,\exp}}}{\overline{[C_i]_{\exp}}} \tag{13}$$

Kinetics parameters derived from each substrate was then investigated to find the correlation between the content of other influencing components inside the substrate to the kinetics parameters. Statistical approach was used to identify the correlation. The Pearson correlation coefficient (*r*) (Eq. (14)) was selected as the tool [24, 25]. The judgement whether there is correlation or not between the content of certain components in the substrate to the kinetics parameters used the critical values of Pearson correlation coefficient [26]. This study used three different substrates meaning that the degree of freedom is one. By determining the level of significance of 0.1, the critical value is 0.988.

$$r = \frac{n.\sum x.y - \sum x.\sum y}{\left\{ \left( n.\sum x^2 - \left( \sum x \right)^2 \right) \left( n.\sum y^2 - \left( \sum y \right)^2 \right) \right\}^{0,5}} \tag{14}$$

The absolute value of the calculated Pearson correlation coefficient must be greater than the critical value determined based on the degree of freedom and level of significance (0.988). After the correlation is identified, the negative or positive correlation judgement was based on only the calculated Pearson correlation coefficient value (not the absolute one).

## 3. Materials and Methods

### 3.1. Feedstocks

Feedstocks for batch SHF process in this study included the juice of two different varieties of sweet sorghum (KCS105 and FS501), malt extract agar (Merck), high purity sucrose (Difco), glucose (Merck), and fructose (Merck), $MgSO_4.7H_2O$ (Merck), $K_2HPO_4$ (Merck), yeast extract (Himedia Laboratories, India) and peptone (Himedia Laboratories, India). Both two sweet sorghums seed were obtained from Crop Science Laboratory, College of Agriculture, Ibaraki University and cultivated in Yogyakarta municipal government plantation area in Kricak Village, Yogyakarta, Indonesia.

The estimated influencing mineral contained in the sweet sorghum juices were nitrogen, phosphor, and potassium (NPK). The required feedstocks for nitrogen analysis were $H_2SO_4$ 98%, NaOH, $K_2SO_4$, $CuSO_4$ anhydride, alumina, red methyl indicator, and HCl 37%. The required feedstocks for phosphor and potassium analysis were $HNO_3$, $HClO_4$, $(NH_4)_6Mo_7O_{24}.4H_2O$, $K(SbO)C_4H_4O_6.0.5H_2O$, ascorbic acid, and a standard solution of K 1,000 ppm (Titrisol). All of the chemicals for NPK analysis were supplied from Merck.

### 3.2. Yeast Preparation

There are three recommended strains of yeast from the previous study by Jasman [27] where all of which were *Saccharomyces steineri* but from different sources and were mixed in the same ratio. All yeast isolates were maintained on Malt Extract Agar (MEA) at 4°C in the refrigerator and were sub-cultured every 2 months. Inoculum were prepared by culturing each strain in a medium containing 2.5% glucose, 2.5% fructose, 5% sucrose, 0.15% $MgSO_4.7H_2O$, 0.15% $K_2HPO_4$, 0.5% yeast extract, and 0.5% peptone in 250 mL Erlenmeyer flask with a working volume of 100 mL. Incubation was carried out on an orbital shaker at 100 rpm and temperature of 30°C, for 12-24 hours or until the cell density reached $10^8$ cell/mL.

### 3.3. Simultaneous Hydrolysis and Fermentation

The simultaneous hydrolysis and fermentation was conducted in the specific fermentation media. Fermentation media for artificial substrate consisted of glucose, fructose, sucrose, yeast extract, peptone, $MgSO_4.7H_2O$, and $K_2HPO_4$. Fermentation media were prepared by mixing the materials with the composition of 2.5% glucose, 2.5% fructose, 5% sucrose, 0.5% yeast extract, 0.5% peptone, 0.15% $MgSO_4.7H_2O$, and 0.15% $K_2HPO_4$. While for the others, the fermentation media consisted of the sweet sorghum juice, 0.5% yeast extract, 0.5% peptone, 0.1% $MgSO_4.7H_2O$, and 0.1% $K_2HPO_4$.

Simultaneous hydrolysis and fermentation was begun by adding a certain volume of inoculum to the one liter sterilized fermentation jars to achieve the cell density of 3.5 x $10^7$ cell/mL. Afterward, 800 mL of fermentation media were also added and were mixed aseptically. Incubation was carried out at 30°C & pH 5.0 and was conducted statically and anaerobically for certain hours until the concentration of the yeast started to enter the death phase. During SHF experiment, solution pH and temperature were monitored. The pH level was adjusted by NaOH solution. For analytical routines, samples taken at a certain time were refrigerated in the refrigerator with a temperature of -20°C before being analyzed.

### 3.4. Analytical Method

The initial concentration of NPK in sweet sorghum juice was determined to see the effect of initial NPK mineral concentration to the rate of substrate consumption and to the bioethanol productivity.

The nitrogen content in the sweet sorghum juice was determined using Kjeldahl method as explained by AOCA [28]. The phosphor content in the sweet sorghum juice was determined using spectrophotometer (Shimadzu UV-mini 1240, Japan) and flame photometer (Jenway Model PFP7, UK). This method was adapted according to the original works of Hoft [29] and Kirkbright [30]. It can be applied for determination of phosphates, phosphoric acid or total phosphorus in water samples, biological materials or food and beverages. The potassium analytical method using atomic absorption spectroscopy was adapted from the literature [31].

Analysis of sugars and ethanol was performed using an HPLC system (Knauer smart line RI detector 2300, Germany) with a column of Aminex HPX-87C 300 x 7.8 mm (Bio-Rad, USA) at 85°C. Injection volume

is 20 μL and mobile phase was deionized water at flow rate of 0.6 mL/min. The recorded concentration of sugars and ethanol over the time would be fitted by the proposed kinetics models.

Yeast cell concentration was determined indirectly by measuring the optical density (absorbance) of a culture sample [32]. A sample of the culture medium from the fermenter was taken and read its absorbance using spectrophotometer. Up to a certain cell density, the concentration of yeast cells in the sample is proportional to the absorbance reading on the spectrophotometer. The calibration curve correlating cell concentration with absorbance deviates from a linear correlation at high cell densities. Thus, the high optical density (OD) samples (that may be on the non-linear portion of the curve) are usually diluted by a known dilution factor to confirm that the measured OD values fall on the linear portion.

## 4. Results and Discussion

### 4.1. Component Analysis of Sweet Sorghum Juice

Analysis using HPLC to determine the content of initial sugars concentration not only detected the peak resulted by the sugars but there were also visible detected peaks from other components. From two sweet sorghum juices of two different varieties, there were similarities of other component's peaks detected. The components were glycerol and acetic acid. The concentration of acetic acid from FS501 juice was much higher than that of KCS105 while the concentration of glycerol just had tight difference as shown in Table 1. The presence of acetic acid and glycerol was estimated to be potential inhibitors for the yeast during its growth and during bioethanol production.

Table 1. Component analysis of sweet sorghum juice using HPLC.

| Component | Varieties | |
|---|---|---|
| | FS501 | KCS105 |
| Sucrose, g/L | 11.213 | 15.611 |
| Glucose, g/L | 2.502 | 4.890 |
| Fructose, g/L | 2.413 | 3.821 |
| Glycerol, g/L | 0.012 | 0.022 |
| Acetic Acid, g/L | 0.026 | 0.004 |

The explanation of previous studies by Chen et al. [33] and Palmqvist & Hahn-Hagerdal [34] mentioned that under acidic conditions (pH 5), undissociated weak acid might diffuse through the plasma membrane to penetrate into the cellular cytoplasm. Inside the cytoplasm, it might cause acidification of cytoplasm. As the consequence, cells exported the surplus protons across the plasma membrane in order to regulate the intracellular acidity level in an optimum range. This implied on the greater ATP consumption during the proton transportation across the membrane and inhibited the microorganism growth [33]. Another previous study by [35] mentioned that the inhibition of fermentation by acetic acid could probably be explained by decreased activity of fructokinase, hexokinase, phosphofructokinase, and enolase. The study by Pampulha [35] showed that increasing acetic acid concentration from 0 to 0.09 g/L could reduce the maximum specific growth rate and yield coefficient of *Saccharomyces cerevisiae* from substrate as many as 12% and 8% respectively. Although glycerol had minimum concentration in the order of hundreds g/L to start inhibiting the growth of microorganism, such as yeast, by providing high osmotic pressure in the fermentation medium to the cell, its presence must still be considered to study the kinetics of bioethanol production comprehensively [36]

On the other hand, analysis of nitrogen, phosphor, and potassium (NPK) of the two sweet sorghum juices did not show a significant difference of concentration between each other (see Fig. 2). The trend was also similar of which the $K_2O$ content was the highest followed by $P_2O_5$ content and N content. The existence of NPK in the fermentation media was useful to fulfill the nutrient need of the yeast during the growth phase [37, 38, 39]. The previous work by Batista [40] explained that the increasing nitrogen supply using extruded bean could improve the *S. cerevisiae's* growth by 43–79%. However, the increase of nitrogen supply had its limit since the *P. pastoris'* growth was only improved by 20% [40]. Therefore, it is suggested that there was a maximum nitrogen concentration to enhance the yeast growth [41].

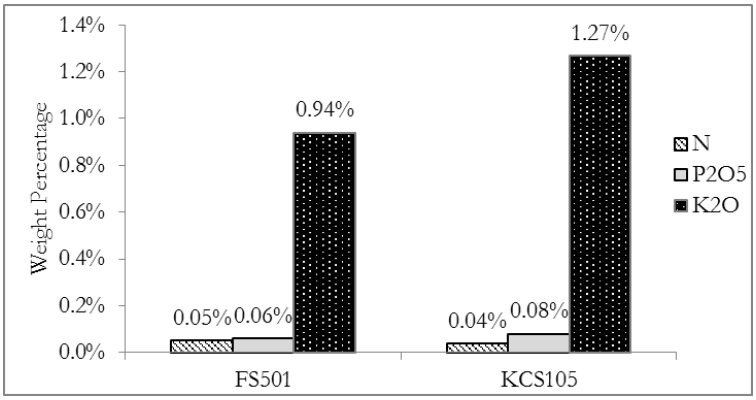


Fig. 2. NPK content in the sweet sorghum juices.

### 4.2. Kinetics Parameters

Determination of kinetics parameters was the output of the experimental data fitting using proposed kinetics models. As previously explained, the difference between the two models proposed was at the growth rate equation of the yeast.

#### 4.2.1. Artificial substrate fermentation

Fermentation of artificial substrate created by mixing pure sucrose, glucose, and fructose showed the lag phase of between 0-10 hours of fermentation, the log phase of between 10-40 hours of fermentation, and the stationary phase of afterward (see Fig. 3 (d)). From Fig. 3 (a), the sucrose concentration decreased over the time. It was surprisingly followed by the consistently decreasing trend of glucose and fructose concentration change over time (see Fig. 3 (b) and Fig. 3(c)). By referring the reaction pathway as shown by Fig. 1, it could be concluded that the rate of hydrolysis of sucrose was relatively equal to or slower than the sum of the rate of glucose (or fructose) consumption by the yeast and the rate of glucose (or fructose) conversion into bioethanol.

Another interpretation could be made from the experimental data was the characteristic of the yeast. From the slope of the peak shown by Fig. 3 (b) and Fig. 3(c), during log phase (between 10-40 hours of fermentation), the glucose concentration decreased faster than the fructose concentration. This indicated that *Saccharomyces steineri* has glucophilic characteristic, which is similar to the previous work of Tronchoni [22] which mentioned that in general, the strain of yeast was glucophilic. From the bioethanol productivity aspect, the ethanol production profile over the time followed the profile of the yeast growth. The productive period was certainly during the log phase of the yeast growth period.

Experimental data fitting using two proposed kinetics models visually or qualitatively showed relatively similar capability to fit with the experimental data as shown in Fig. 3. However, the quantitative approach using SSE showed that kinetics models used by Philippidis [21] in the previous work could fit the experimental data better in the fermentation of artificial substrate into bioethanol using *Saccharomyces steineri.* This is proven by lower SSE value for each component (see Table 2).

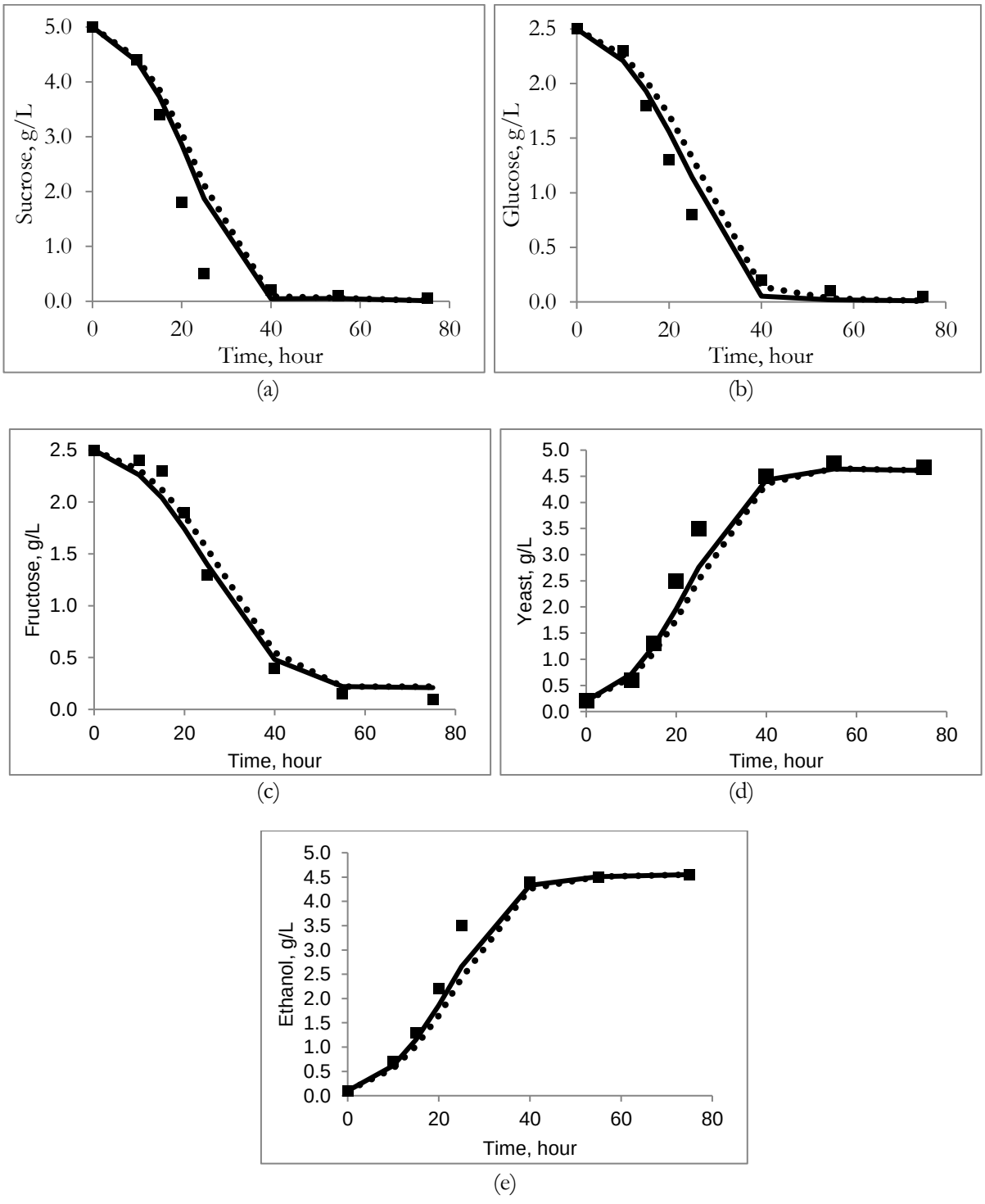


Fig. 3. Experimental data fitting using two proposed kinetics models of artificial substrate fermentation for: (a) Sucrose (b) Glucose (c) Fructose (d) Yeast (e) Ethanol (■: experimental data; ▬: Philippidis; ●●●: Philippidis – Modified).

Table 2. Comparison of SSE resulted from experimental data fitting of artificial substrate fermentation.

| Components | Kinetics Models | |
|---|---|---|
| | Philippidis-Modified | Philippidis |
| Yeast | 1.59 | 0.88 |
| Sucrose | 4.47 | 3.14 |
| Glucose | 0.50 | 0.24 |
| Fructose | 0.15 | 0.15 |
| Ethanol | 1.59 | 0.86 |

4.2.2. KCS105 juice fermentation

Once the sucrose was hydrolysed, there would be glucose and fructose produced together. The fermentation of KCS105 juice showed different result from the fermentation of artificial substrate. The difference existed on the profile of fructose concentration over the time which was firstly increasing before it decreased afterward (see Fig. 4 (c)). This indicated that the sum of the rate of fructose consumption and the rate of fructose conversion into bioethanol was slower than that of glucose. Therefore, the yeast behaved differently when it grew in a different substrate but nevertheless, the result of KCS105 juice fermentation proved that *Saccharomyces steineri* was glucophilic.

The interesting result of the KCS105 juice fermentation was the productivity of the bioethanol (see Fig. 4 (e)). The ethanol concentration reached 14.20 g/L after 72 hours fermentation, much higher than that of artificial substrate fermentation after 75 hours fermentation showing 4.55 g/L. The presence of relatively low NPK and glycerol concentration made the ethanol productivity better than the artificial substrate. Low glycerol concentration could be used as the carbon source for the yeast to grow and be productive.

Qualitative evaluation by visual observation of the experimental data fitting as shown in Fig. 4 showed that both kinetics models had difficulty in fitting the glucose concentration data. However, both models could accommodate the profile of fructose concentration which was firstly increasing. Quantitative evaluation based on the lower SSE result, as shown in Table 3, showed that KCS105 juice fermentation mechanism was described better using the modified kinetics model of Philippidis [21] which was proposed in this study.

Table 3. Comparison of SSE resulted from experimental data fitting of KCS105 juice fermentation.

| Components | Kinetics Models | |
|---|---|---|
| | Philippidis-Modified | Philippidis |
| Yeast | 3.64 | 10.67 |
| Sucrose | 12.91 | 17.23 |
| Glucose | 7.13 | 12.67 |
| Fructose | 1.18 | 2.54 |
| Ethanol | 3.06 | 5.73 |

4.2.3. FS501 juice fermentation

The presence of hydrolysis in the SHF process might be clearly seen from the FS501 juice fermentation experimental data. Both glucose and fructose concentration firstly increased (see Fig. 5 (b) and (c)). This indicated that during the lag phase of the yeast growth (4-9 hours of fermentation), the rate of sucrose hydrolysis was relatively higher than the sum of the rate of glucose (or fructose) consumption by the yeast and the rate of glucose (or fructose) conversion into bioethanol. After the lag phase ended, the glucose and fructose concentration started to decrease.

The productivity of bioethanol production was also higher than that of artificial substrate fermentation but lower than KCS105 juice fermentation. The FS501 juice fermentation reached 7.72 g/L of ethanol concentration after 52 days of fermentation (see Fig. 5(e)).

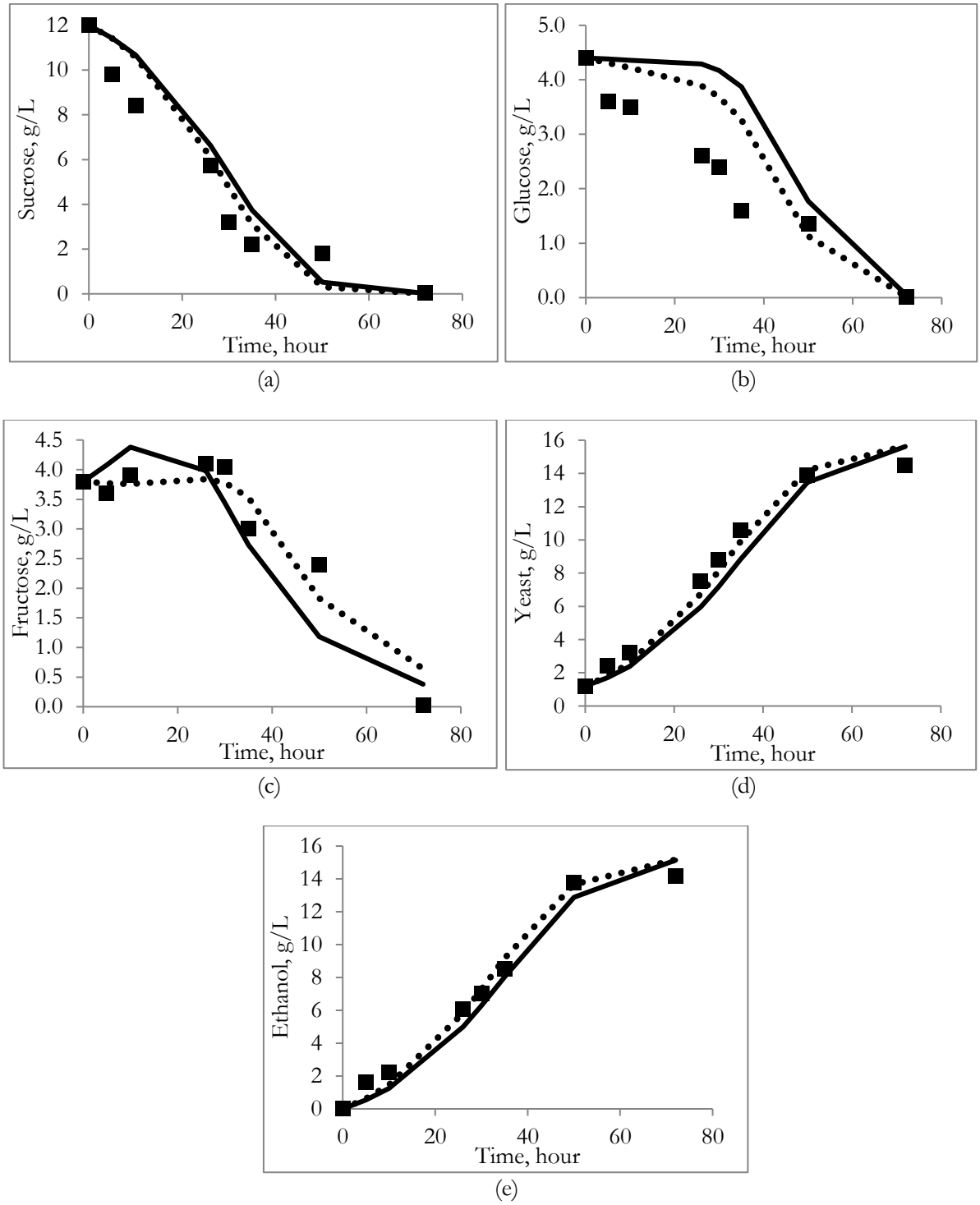


Fig. 4. Experimental data fitting using two proposed kinetics models of KCS105 juice fermentation for: (a) Sucrose (b) Glucose (c) Fructose (d) Yeast (e) Ethanol (■: experimental data; ▬: Philippidis; ●●●: Philippidis – Modified).

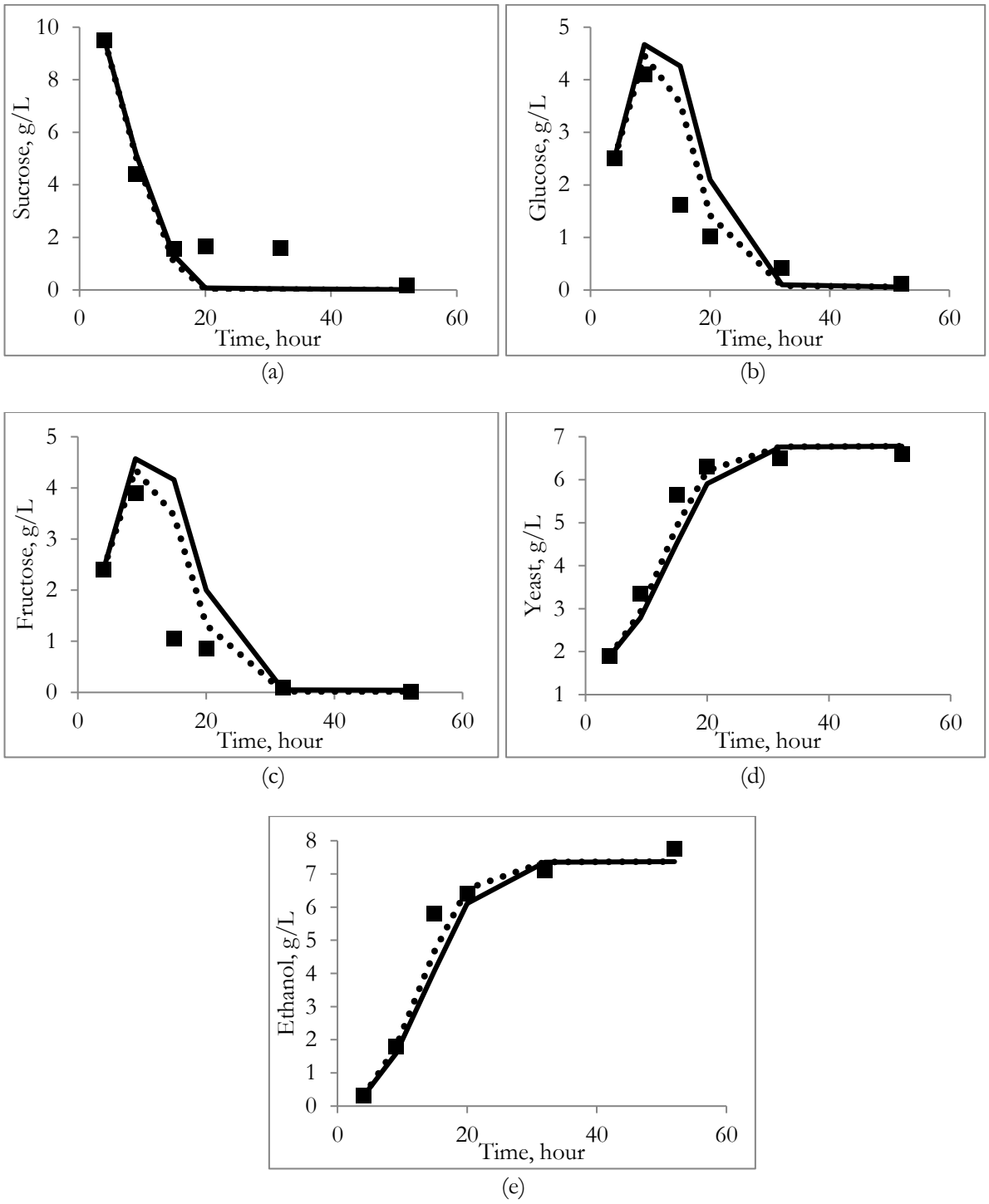


Fig. 5. Experimental data fitting using two proposed kinetics models of FS501 juice fermentation for: (a) Sucrose (b) Glucose (c) Fructose (d) Yeast (e) Ethanol (■: experimental data; ▬: Philippidis; ●●●: Philippidis – Modified).

Due to the unique phenomenon where the glucose and fructose concentration had an opportunity to increase before it decreased, the determination of the suitable kinetics model must be strictly in accordance with the SSE result. Similar to the KCS105 juice fermentation, the better kinetics model was the modified kinetics model of Philippidis [21] because of the lower SSE value (see Table 4). The compilation of all kinetics parameters of the best kinetics model for each substrate was shown in Table 5.

Table 4. Comparison of SSE resulted from experimental data fitting of FS501 juice fermentation.

| Components | Kinetics Models | |
|---|---|---|
| | Philippidis-Modified | Philippidis |
| Yeast | 0.82 | 1.83 |
| Sucrose | 5.88 | 5.55 |
| Glucose | 4.18 | 8.59 |
| Fructose | 6.24 | 11.42 |
| Ethanol | 1.49 | 3.23 |

Table 5. Kinetics parameters of all substrates using the best kinetics model based on the lower SSE result.

| Parameters | Substrate | | | Unit |
|---|---|---|---|---|
| | Artificial (Philippidis) | KCS105 (Philippidis-Modified) | FS501 (Philippidis-Modified) | |
| $\mu_m$ | 0.76 | 0.30 | 0.30 | $h^{-1}$ |
| $k_1$ | 0.28 | 0.18 | 1.50 | $h^{-1}$ |
| $K_m$ | 17.00 | 17.33 | 15.27 | g/L |
| $K_{IG}$ | 55.00 | 55.00 | 15.04 | g/L |
| $K_{IF}$ | 55.00 | 55.00 | 15.00 | g/L |
| $K_G$ | 25.00 | 25.00 | 20.31 | g/L |
| $K_F$ | - | 25.05 | 20.26 | g/L |
| $K_{Eth}$ | 10.00 | 10.00 | 15.00 | g/L |
| $Y_{X/G}$ | 1.00 | 1.00 | 0.45 | g/g |
| $Y_{X/F}$ | 1.11 | 1.10 | 0.45 | g/g |
| $Y_{Eth/X}$ | 1.00 | 1.05 | 1.45 | g/g |
| $m_{SG}$ | 0.00 | 0.00 | 0.00 | $h^{-1}$ |
| $m_{SF}$ | 0.00 | 0.00 | 0.00 | $h^{-1}$ |
| $k_d$ | 0.0001 | 0.0015 | 0.0010 | $h^{-1}$ |

### 4.3. Explanation about Model Validity for FS501 and KCS105

In general, the use of the natural substrate (FS501 and KCS105) as the raw material for bioethanol production pushed the reaction mechanism to follow the modified kinetics model of the previous work by Philippidis [21] (see Table 3 and Table 4). The reason was *Saccharomyces steineri* being more sensitive to the substrate difference when there were other components inside the substrate. Assumption made by Philippidis [21] was no longer capable of accommodating the hindrance from other components such as acetic acid which led to the intolerance to the different substrate composition. As explained on section 4.1, the presence of acetic acid possibly affected enzymes involved in glycolysis which were fructokinase, hexokinase, phosphofructokinase, and enolase. The glycolytic activity would run slowly initially so that the glucose or fructose consumption also ran slowly too. This made the sucrose hydrolysis step during the lag phase be visible as the concentration of glucose or sucrose increased initially during the lag phase (see Fig. 4 and Fig. 5 on point b and c). However, relatively low NPK and glycerol concentration had no significant impact to the yeast growth and bioethanol production rate. The section 4.4 below analysed statistically to find the correlation of the acetic acid, NPK, and glycerol presence to the kinetics parameters.

### 4.4. Statistical Approach

To enable the identification of the effect of the other components presented in the KCS105 and FS501 juice, the correlation coefficient was calculated for each parameter to see if there was any correlation of the kinetics

parameters with the acetic acid concentration, NPK concentration (represented the sum of N, $P_2O_5$, and $K_2O$), and glycerol concentration. The result was shown in Table 6 below.

Generally, the existence of acetic acid had dominant effect to the kinetics parameters compared with NPK and glycerol. From Table 6 below, acetic acid existence in the substrate showed significant correlation by influencing all kinetics parameters value except $\mu_m$, $k_1$, $K_m$, $K_{Eth}$, and $k_d$. Acetic acid presence was seemed to enhance the sucrose hydrolysis rate. According to the kinetics parameters, there were $K_{IG}$ and $K_{IF}$ as the inhibition constant of hydrolysis product (glucose and fructose) to the yeast enzyme which were decreasing. Therefore, the rate of sucrose hydrolysis increased. The increasing rate later caused the glucose and or fructose concentration profile increased during the lag phase of the yeast growth in the batch SHF process (see Fig. 4 and Fig. 5 on point b and c). As the consequence of high enough concentration of glucose and fructose resulted from faster rate of sucrose hydrolysis, the ethanol productivity increased due to enough supply of reactants to be converted. That is why the value of yield coefficient of ethanol from yeast mass ($Y_{Eth/X}$) increased.

Table 6. Statistical approach to identify the effect of other components initial concentration in the substrate to the simultaneous hydrolysis and fermentation (SHF) process.

| Parameters | Correlation coefficient with acetic acid | Correlation coefficient with $N+P_2O_5+K_2O$ | Correlation coefficient with glycerol |
|---|---|---|---|
| | Artificial: 0 g/L<br>KCS105: 0.004 g/L<br>FS501: 0.026 g/L | Artificial: $N+P_2O_5+K_2O$= 0%<br>KCS105: $N+P_2O_5+K_2O$= 1.39%<br>FS501: $N+P_2O_5+K_2O$= 1.05% | Artificial: 0 g/L<br>KCS105: 0.022 g/L<br>FS501: 0.012 g/L |
| $\mu_m$ | -0.619 | -0.972 | -0.891 |
| $k_1$ | 0.978 | 0.217 | -0.016 |
| $K_m$ | -0.957 | -0.137 | 0.097 |
| $K_{IG}$ | **-0.990** | -0.283 | -0.052 |
| $K_{IF}$ | **-0.990** | -0.283 | -0.052 |
| $K_G$ | **-0.990** | -0.283 | -0.052 |
| $K_F$ | **-1.000** | **1.000** | **1.000** |
| $K_{Eth}$ | -0.169 | 0.825 | 0.934 |
| $Y_{X/G}$ | **0.990** | 0.283 | 0.052 |
| $Y_{X/F}$ | **-0.990** | -0.283 | -0.052 |
| $Y_{Eth/X}$ | **-0.992** | -0.296 | -0.066 |
| $k_d$ | 0.302 | **0.992** | **0.994** |

On the other hand, acetic acid presence also gave disadvantages in the form of yeast growth inhibition proven by the reduction of the value of $Y_{X/G}$, $K_G$, $K_F$, and $Y_{X/F}$ as the acetic acid content increased. Fortunately, the net growth rate of the yeast could still be higher than that which grew in the artificial substrate. It was due to the abundant supply of glucose and fructose to be consumed which were resulted from faster sucrose hydrolysis reaction.

Specifically for fructose saturation constant for the yeast ($K_F$), the kinetics parameter only appeared on the fermentation of KCS105 and FS501 juices (see Table 5). The correlation coefficient of $K_F$ was calculated using only two values from KCS105 and FS501 juice fermentation. The negative effect of acetic acid to $K_F$ was overcome by the positive effect of NPK and glycerol since the correlation opposed to each other. Thus, lower $K_F$ value caused by higher acetic acid concentration could be said had no impact on the yeast growth.

The last parameter which is only affected by the NPK and glycerol existence was $k_d$. The value reduction of $k_d$ might describe that the NPK additional supply from the sweet sorghum juice exceeded the maximum limit of the tolerable concentration by the yeast. While for glycerol, the inhibition might be caused by the nature of it as the chemical from alcoholic group which partially had antibiotic characteristics, similar to ethanol.

## 5. Conclusions

The fermentation of artificial substrate (mixture of sucrose, glucose, and fructose) followed kinetics models proposed by Philippidis [19]. However, when the natural substrate was used (in this case the sweet sorghum juice of KCS105 and FS501 varieties), the kinetics model followed the modified one as proposed by this study. It was found that acetic acid, nitrogen, phosphor, potassium, and glycerol existence in the sweet sorghum juice incurred the change of yeast behaviour during its growth phase with the acetic acid as the most influencing component. The existence of those other components, on the other hand, enhanced the yeast growth and increased the bioethanol productivity of the SHF process. Therefore, elemental analysis to identify all components existed in the substrate for bioethanol production was important to be conducted firstly.

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