GROWTH KINETICS OF *Citrus suhuiensis* CELL SUSPENSION

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ABSTRACT: **Citrus is one of the major commodities in many countries including Malaysia. However, production of citrus including *Citrus suhuiensis* (*C. suhuiensis)* is declining due to diseases and inability to withstand low temperatures. Plant cultures such as cell suspension have the potential in propagating disease-free and healthy Citrus fruits with value-added characteristics. However, studies related to *C. suhuiensis* is still scarce. Therefore, the growth kinetics of *C. suhuiensis* cell suspension culture was studied. Friable callus of *C. suhuiensis* which was induced from seeds was inoculated into MS medium with 30 g/L sucrose, 0.5 g/L malt extract and 2.0 mg/L 2, 4-D for the cell suspension initiation. Several batch experiments using a few types of sugars (sucrose, glucose and fructose) were carried out. The cell dry weight (CDW) of *C. suhuiensis* was recorded for 30 days of culture period and residual sugars in the medium were analyzed using HPLC. Cells grown in 30 g/L sucrose achieved the highest CDW (9.559 g/L) with μ_{max} equals to 0.00512/h, compared to glucose and fructose. In addition, sucrose is the preferred carbon source with the highest uptake rate (0.213 g/L·h). Cells completely hydrolyzed sucrose into glucose and fructose after 5 days of inoculation. All sugars were completely utilized by *C. suhuiensis* cells after 25 days. The kinetic growth parameters determined from batch experiments were then used for model simulation and verification in MATHCAD 15. After adjustments and refinement to the selected kinetic parameters, the model has fairly described and predicted the growth and sugars profile of *C. suhuiensis* cells. The proposed model can be used to predict sucrose hydrolysis, glucose and fructose formation from sucrose and their consumption by plant cells and also for larger scale of growth.**

KEYWORDS: *Cell suspension; Citrus suhuiensis; Kinetic growth parameters; Cell dry weight (CDW); Sugar uptake.*

1. INTRODUCTION

Citrus suhuiensis (C. suhuiensis) is a citrus species derived from the sub-family of *Aurantioideae* which comes from the family of *Rutacaeae*. The distribution of citrus fruits can be primarily found in the Southeast Asia region, Australia, eastern India and also China (Elcy *et al.*, 2012). *C. suhuiensis* which is also known as *Limau Madu, Limau Angkat* or *Limau Langkat* is cultivated in most of the states in Malaysia including Pahang, Kedah, Perak, Kelantan, Terengganu, Johor and Sarawak as sub-tropical climate is the country's climate. Citrus fruits have high vitamin C content and fiber. Several bioactive compounds of Citrus are identified as abundant with antioxidant activities (Lagha-Benamrouche & Madani, 2013; Toscano-Garibay *et al.*, 2017 and Zou *et al.*, 2016). Antioxidant acts by preventing the oxidation occurrence in human body and can potentially reduce the risk of suffering critical diseases such as cancer and coronary diseases (Arias & Ramón-Laca, 2005), hypoglycaemic activity and an anticholinesterase effect, (Conforti *et al.*, 2007). However, the production of Citrus especially oranges declined over the past couple of years due to diseases such as green mold, citrus greening (huanglongbing) and Citrus tristeza virus (CTV). These low yields have led to the decrease of fruit for processing along with a slight drop in fruit for consumption and exports in 2014 (Berk, 2016).

Plant cell suspension cultures have been widely used in both research and commercial applications especially in the production of secondary metabolites. This technique is utilized in this study due to several advantages. It is easy to maintain and allows great flexibility of experimental approaches (Mustafa *et al.*, 2011 and Evans *et al.*, 2003). It is a more preferable method if various factors and compounds are to be studied, which can affect the growth and differentiation of plant cells. In recent years, there is a number of studies which reported on tissue and cell culture for Citrus plants. A study was reported to investigate the effects of several factors on the embryogenic cells of *C. suhuiensis* in suspension culture such as the concentration of growth hormone, initial cell density and the types of carbon sources (Dita, 2012). However, no kinetic study and simulation were reported.

In this study, the kinetics of *C. suhuiensis* cell growth and sugar uptake were investigated. Kinetics can be simply defined as the rate at which reaction occurs and thus, kinetic models considered the reaction in terms of time. Most mathematical models describing culture growth are restricted to the period of exponential growth. It is a phase where cell division results in the formation of daughter cells which are equally capable of further division. Growth rates as well as other metabolic rates generally depend on the nutritional environment which the cells experienced. It is common that substrate dependence metabolism is approximately described by the mathematical models originally derived from the enzyme kinetics. Rate of growth and nutrient uptake are often described by simple Michaelis-Menten equation (Doran, 2013).

This study involved the initiation of *C. suhuiensis* cell suspension cultures from friable callus culture. The callus culture of *C. suhuiensis* was initiated from its cotyledon under suitable conditions and growth medium (Fathil *et al.*, 2017). Different batch experiments involving three types of carbon sources (sucrose, glucose and fructose) were performed to obtain the values of kinetic parameters for kinetic model. Sampling was done for each batch experiments to monitor *C. suhuiensis* CDW and performing residual sugar analysis. An

unstructured kinetic model was developed based on Monod equation using simple kinetic expressions. MATHCAD 15 software was used for model simulation and verification.

2. MATERIALS AND METHODS

2.1 Media preparation

Murashige and Skoog (MS) media (Duchefa Biochemistry) supplemented with 2.0 mg/L of 2,4-D (PhytoTechnology Laboratories), 30 g/L (w/v) sucrose (Fischer Scientific) and 500 mg/L malt extract (Friendemann Schmidt) were used in the study. The pH of media was adjusted to 5.7 ± 0.1 with either 0.1 M NaOH or HCl. The media was distributed into 100 mL Erlenmayer flasks with a working volume of 50 mL. The flasks containing media were autoclaved for 15 mins at 121°C and 15 psi. The autoclaved flasks were left to cool at room temperature before the cultivation of cells. The procedures were repeated when using glucose and fructose as the carbon source for growth kinetic parameters determination.

2.2 Establishment and maintenance of cell suspension culture

For *C. suhuiensis* cell suspension culture establishment, 2.5 g of friable callus initiated from the seeds of *C. suhuiensis* (Fathil *et al.*, 2017) was inoculated into 50 mL of liquid medium containing 30 g/L sucrose as the carbon source into 100 mL Erlenmayer flasks. The flasks were covered with aluminum foil and sealed with the parafilm. The cultures were maintained on the incubator shaker at 100 rpm and 25°C under continuous illumination and left for 15 days before the next subculture.

Subculturing was carried out by pipetting method adapted from Mustafa *et al.* (2011). Flasks were shaken gently and the culture was refluxed with a pipette (sterile wide-mouth pipette) three times to achieve a more homogenous distribution of cells in the pipette (for inoculum) and to break up the cell aggregates. Then, 10 mL of cell suspension was transferred into a 250 mL Erlenmayer flask containing 90 mL of fresh medium for 10% of inoculum size. The cultures were maintained for 3 months before conducting the experiments.

2.3 Growth kinetics

Growth of *C. suhuiensis* cell suspension culture was determined by measuring the CDW (Mustafa *et al.*, 2011). Sampling was performed every 5 days in 30 days of batch experiment. By using Buchner vacuum filter, cells were collected on a preweighed Whatman filter paper disc (90 mm). The filtered media was collected in centrifuge tubes for residual sugar analysis and flasks were washed three times using distilled water. Total weight of the filter paper and the cells were determined to calculate fresh weight (FW) of the cells. The filter paper was then placed on a glass dish and placed in a 60°C oven for 24 hrs. The filter paper and cells were weighed at intervals after the 24 hrs period until the weight remained constant. The weight of filter paper was subtracted to obtain the CDW reading.

2.4 Residual sugar analysis

Samples that were originally at -20°C were defrosted and shaken for 30 seconds to ensure homogenous suspension. Samples were then filtered using 0.45µm Nylon (N6) membrane syringe filter and the filtrate were collected in HPLC vials. The residual sugars were analyzed by using

SUPELCOSIL LC-NH_2 column (250 mm x 4.6 mm, 5 micron) (Sigma-Aldrich, USA) with a guard-column at a flowrate of 1 mL/min and the temperature of 30°C controlled by a temperature controller module using a mixture of HPLC grade acetonitrile and HPLC grade water as the mobile phase at the ratio of 75:25 (v/v). Prior to the analysis, the mobile phase was filtered using a 0.45 µm Nylon filter paper. Ten (10) µL of sample was injected twice using the auto injection (Waters 717plus autosampler) and the peak was detected by a refractive index (RI) detector (Waters 2414 RI detector). All chromatograms were viewed using a computerized integrator (Empower software ver. 2).

Prior to the sample analysis, the standard curves for sucrose, glucose and fructose were constructed using five different concentrations of each sugar; *i.e.*: 1.0, 2.5, 5.0 and 10.0 g/L. The concentration of residual sugars in the samples were determined based on the standard curves using the Empower software.

3. RESULTS AND DISCUSSION

3.1 Growth profile of *C. suhuiensis* cells

The growth profile of *C. suhuiensis* cells using sucrose as the carbon source and its sugar uptake are shown in Fig. 1. It can be observed that when sucrose was supplied as the sole carbon source in *C. suhuiensis* cell suspension medium, it was hydrolyzed into almost equal amounts of glucose and fructose by the cell wall invertase enzyme (Tauzin & Giardina, 2014). This phenomenon has been reported previously for other types of plant cells such as *Arabidopsis thaliana*, *Taxus cuspidata*, *Thalictrum rugosum* and carrot (Puad *et al.*, 2017; Kanabus *et al.*, 1986; Son *et al.*, 2000 and Choi *et al.*, 1999). The growth profile of *C. suhuiensis* cells is segmented into three different phases in which Phase 1 is the period where sucrose hydrolysis takes place and cells adjustment to the new environment. Phase 2 is the growth phase of cells with simultaneous uptake of glucose and fructose. Phase 3 is the death phase due to depletion of carbon sources. As the interest of this study is the growth of *C. suhuiensis* cells, only Phase 1 and Phase 2 are taken into account for model simulation. Brief description of each phases are tabulated in Table 1.

It is assumed that sucrose hydrolysis followed Michaelis-Menten kinetic and thus the mass balance for sucrose, glucose fructose and biomass for Phase 1 were established, respectively (Table 2). The amount of cell wall invertase was assumed to be directly proportional to the cell concentration (Combes & Monsan, 1983). There was no significant changes in *C. suhuiensis* cell growth during Phase 1 and no glucose inhibition on fructose uptake by cells was observed in Phase 1 since both hexoses were present in the medium with the same concentration. It was also found that in other plants, fructose uptake is not inhibited by glucose if there is a presence of sucrose in the medium (Stanzel *et al*., 1988 and Oliveira *et al*., 2002). Sucrose was completely hydrolyzed in Phase 1 with the formation of glucose and fructose.

In Phase 2, rapid growth of *C. suhuiensis* cells were observed. During this period, it is assumed that all cells are viable and there is no cell death. From Fig. 1, it can be deduced that glucose uptake occurred at a higher rate compared to fructose. This reflects that both sugars were taken up by the cells at different specific uptake rates. In addition, *C. suhuiensis* cells prefers glucose over fructose as their carbon source which depicts the inhibition of glucose on fructose consumption. The inhibition of glucose on fructose can also be found in other plant

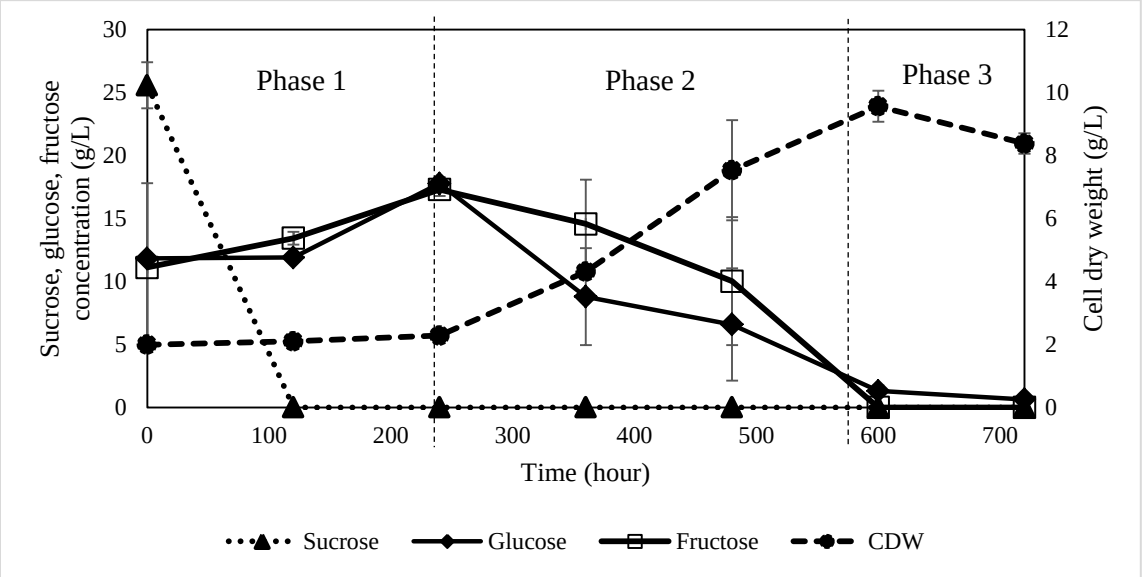

Figure 1: Growth profile of *C. suhuiensis* cell suspension culture and the residual sugar concentrations grown in continuous illumination with 10% (v/v) inoculum.

Table 1: Growth profile characteristics of the three different phases of *C. suhuiensis* growth as in Fig. 1.

Phase	**Description**
Phase 1	0 to 240 h. Sucrose hydrolysis into glucose and fructose, no significance growth of cells during the period. At the end of the phase, sucrose was completely hydrolyzed.
Phase 2	240 to 600 h. Growth of *C. suhuiensis* cells on glucose and fructose, fructose depleted first at 600 hours while glucose concentration is low which almost equals to zero at the same time.
Phase 3	600 h onwards. Death phase with no carbon sources in the medium.

culture with studies on the sugar uptakes such as soybean (*Glycine max*) (Klerk-Kiebert *et al*., 1983), *Phaseolus vulgaris* (Botha & O'Kennedy, 1998), *Daucus carota (*Kanabus *et al.*, 1986) and *Lilium* bulblets (Lian *et al.*, 2002). Table 3 summarizes the expressions for the volumetric rate of sugar uptake (glucose and fructose) as well as the biomass formation rate which incorporate glucose inhibition on fructose uptake constant.

3.2 Initial rate experiment

Initial rate experiment is the technique used to study the enzyme-catalyzed reactions and also the relationship between an enzyme and a substrate (Combes & Monsan, 1983 and Waley, 1981). The

outcome of this technique is also essential in understanding the functions of a particular enzyme and can serve as a starting point in designing enzyme reactors for the industrial application such as the sugar-related industry. Hydrolysis involves the cleaving of sugar polymers into their monomers. Hydrolysis can be carried out chemically or enzymatically.

For *C. suhuiensis* suspended cells, it was thought to be beneficial to study the

Table 2: Volumetric rate expression sucrose hydrolysis and uptake of hexoses in the Phase 1.

Rate of sucrose depletion due to hydrolysis without consumption by the cells, where V_{max} and K_S are the saturation parameters	$\frac{dS}{dt} = -r_S = q_S x_v = -V_{max} \cdot \frac{S}{K_S+S} \cdot x_v$ (1)
Rate of glucose formation from sucrose in the medium without consumption by the cells	$\frac{dG}{dt} = r_G = Y_{G/S} \cdot r_S$ (2)
Rate of fructose formation from sucrose in the medium without consumption by the cells	$\frac{dF}{dt} = r_F = Y_{F/S} \cdot r_S$ (3)

Table 3: Kinetic expressions corresponding to growth profile and uptake of hexoses in Phase 2.

Rate of glucose consumption for cell growth	$\frac{dG}{dt} = -r_{G_{x_v}} = -\left(\mu_{max} \cdot \frac{G}{K_G + G} \cdot x_v\right) \cdot \frac{1}{Y'_{x_v/G}}$ (1)
Rate of fructose consumption for cell growth. Inhibition of glucose on fructose uptake	$\frac{dF}{dt} = -r_{F_{x_v}} = -\left(\mu_{max} \cdot \frac{F}{K_F \cdot \left(1 + \frac{G}{K_{ig}}\right) + F} \cdot x_v\right) \cdot \frac{1}{Y'_{x_v/F}}$ (2)
The biomass formation rate is directly proportional to the cell concentration. Cells consumed glucose and fructose to support their growth.	$\frac{dx_v}{dt} = r_x = \mu \cdot x_v$ $= \mu_m \cdot \left[\frac{G}{K_G + G} + \frac{F}{K_F \cdot \left(1 + \frac{G}{K_{ig}}\right) + F}\right] x_v$ (3)

hydrolysis of sucrose by cell wall invertase using initial rate experiment as the cell growth takes a longer time than most of plant cells. Normally, the initial rate is performed in a short period of time *i.e.* within an hour or more with sampling every 5 or 10 mins (Waley, 1981).

In the study, sucrose hydrolysis was investigated by performing initial rate experiments with different initial sucrose concentrations to determine the value of kinetic parameters. Different sucrose concentrations of 30 g/L, 20 g/L 10 g/L and 5 g/L were prepared with 10% (v/v) inoculum

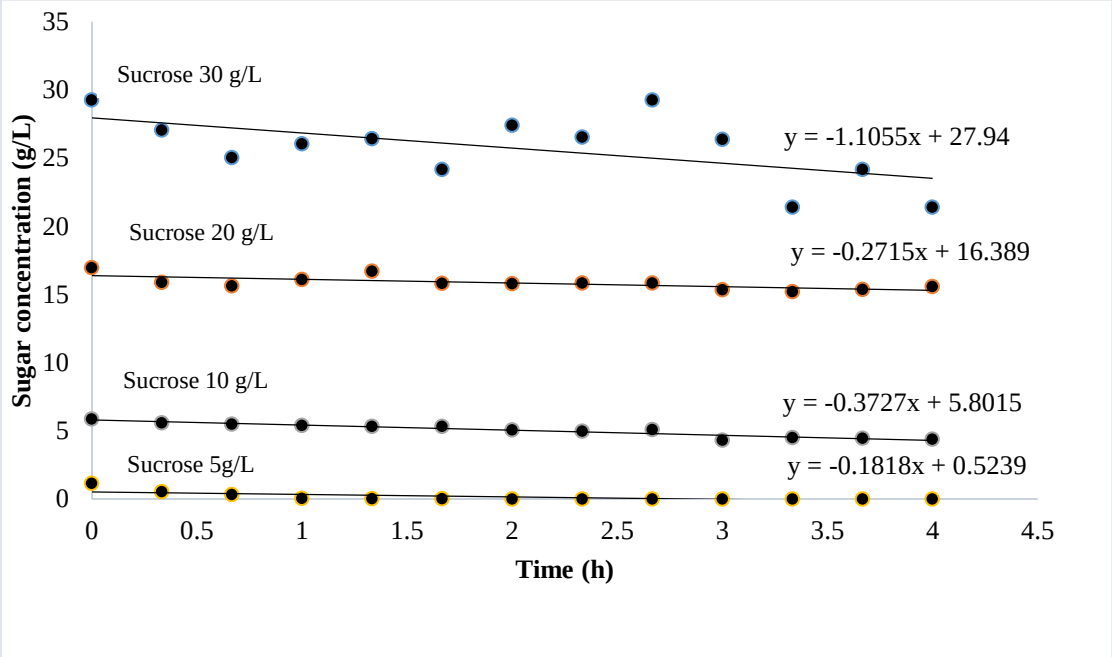

Figure 2: Initial rate experiment results using different initial sucrose concentrations.

size in different 250 mL Erlenmeyer flasks. Sampling was done by taking 2 mL of culture media from each flask for every 20 minutes in 4 hours. Based on the results, the rate of sucrose hydrolysis was increased as the initial sucrose concentration increased with just a slight decrease for 20 g/L sucrose (Fig 2). Slightly different pattern for 30 g/L sucrose might be due to a diffusional limitation of sucrose to the active site of the enzyme (Bowski, 1971). The rate was determined by calculating the slope of the plot of sugar concentration against time for each concentration of sucrose.

3.3 Estimation of model parameters

The calculation of $\frac{dS}{dt}$, $\frac{dG}{dt}$, $\frac{dF}{dt}$ and $\frac{dx}{dt}$ at different times were done by using the mid-point slope method for the experimental batch culture profile in Fig. 1.

Lineweaver-Burk type of linear plot was used to plot some of the expressions to obtain the estimates for the kinetic parameters and these parameters are listed in Table 4. The value of the maximum specific growth rate, μ_{max} was estimated from the linear regression of semi-logarithmic CDW against the time during the

exponential phase based on the data in Fig. 1. Since there was no cell growth in Phase 1, only one value of μ_{max} was used in the model.

3.4 Model verification and refinement

Ideally, the model should be verified using a completely different set of experimental data with different operating conditions to test the robustness of the model. The experimental data in Fig. 1 was used for model verification and improvement. The kinetic parameters listed in Table 4 were substituted into the mass balance equations described in Tables 2 and 3. The initial values for sucrose, glucose, fructose and biomass concentration at t = 0 were taken from the control experiment (Fig. 1). The differential equations for the time rate of change of concentrations of sucrose, glucose and fructose and viable cells were solved in MATHCAD 15 software using *rkfixed* function, given by Equation 1:

$$Z(t) = rkfixed\ (y0, x1, x2, npoints, D) \quad (1)$$

where:

y0: a vector of initial values, whose length depends on the order of the differential equation (DE) or the size of the system of DEs,

x1, x2: endpoints of the interval on which the solution to differential equations will be evaluated,

npoints: the number of points beyond the initial point at which the solution is to be approximated. This controls the number of rows (1 + npoints) in the matrix returned by rkfixed,

D = (x, y): real vector-valued function containing derivatives of the unknown functions. This vector will be of the same length as y, and follow similar rules.

The *rkfixed* function uses the fourth order Runge-Kutta method to solve first order differential equations in which the software will return a two-column matrix (Puad *et al.*, 2017). The first column represents the points at which the solution to the differential equations is evaluated at specific time range, *Z(t)* whereas the second column entries are the corresponding values of the solution. The comparison between simulated and experimental data is shown in Fig. 3 using the values of kinetic parameters listed in Table 4. In Phase 1, it was found that sucrose hydrolysis was almost similar with the experimental data with slightly underestimated rate of sucrose hydrolysis. Sucrose hydrolysis was completed after 120 hrs of incubation in which both kinetic model and experimental data showed similar pattern. However, glucose and fructose formation due to sucrose hydrolysis was found to be deviated in the simulated data than the experimental one which was likely caused by the deficiency in sampling points that affected the determination of kinetic parameters (Puad *et al.*, 2017).

In Phase 2, the simulated data predicted that glucose and fructose consumption by the cells take a longer time in comparison with experimental data. Cell growth rate was found to be higher in the simulated data with almost similar behavior where after 600 hrs, *C. suhuiensis* cells were predicted to be in the stationary phase and subsequently entered their death phase.

In order to find the best fitted model, numerical values for V_{max}, K_F, K_G, K_S and K_{ig} were altered accordingly until a slightly better fit was obtained. The comparison between simulated and experimental results is illustrated in Fig. 4. Cell growth and

Table 4: Model parameters and their respective values when sucrose was used as the sole carbon source.

Parameter	**Value**	**Estimated from**
V_{max}	0.435 g sucrose/(g biomass·h)	Sucrose hydrolysis (initial rate experiments) (Fig. 2)
K_S	18.405 g sucrose/L	
μ_{max}	0.004/h (Phase 2)	Cell growth on 30 g/L sucrose (Fig. 1)
K_G	1.126 g glucose/L	Cell growth on glucose only
K_F	22.79 g fructose/L	Cell growth on fructose only
$Y_{G/S}$=$Y_{F/S}$	0.526 g glucose (or fructose) / (g sucrose)	MW of glucose or fructose divided by MW of sucrose
$Y'_{x/G}$	0.405 g cells/g glucose	Cell growth on glucose only
$Y'_{x/F}$	0.2507 g cells/g fructose	Cell growth on fructose only
K_{ig}	0.2 g glucose/L	Botha and O'Kennedy (1998)

residual sugar profiles in Phases 1 and 2 showed minor adjustment. In Phase 1, rate of sucrose depletion due to hydrolysis was higher in the experimental values compared to simulated result. However, the rate of hexoses formed due to hydrolysis is lower compared to Fig. 3. Phase 2 in contrast predicted that *C. suhuiensis* cell growth is higher than experimental data while the residual sugar profiles exhibit depletion at 600 hrs which is in agreement with the experimental results.

Simulation results from the unstructured model was able to fit the experimental data and represent the growth profile and sugars uptake for *C. suhuiensis* suspension cells with a few deviations in glucose and fructose formation during the lag phase. The parameters can be adjusted with more trials to obtain a more accurate model similar to the experimental data. The proposed kinetic model can be used to predict sucrose hydrolysis, glucose and fructose formation from sucrose and their consumption by *C. suhuiensis* as well as other types of plant cells that exhibit similar growth behavior. This model can also be applied for larger scale of growth with extended expressions for oxygen uptake rate, carbon dioxide production rate etc.

4. CONCLUSION

The proposed unstructured kinetic model can be used to predict sucrose hydrolysis, glucose and fructose formation from sucrose as well as their consumption by plant cells upon adjustments and refinement of selected kinetic parameters. Based on the simulated data, the values of several kinetic parameters such as V_{max}, K_F, K_G, K_S, K_{ig} and μ_{max} were determined as 0.23 g sucrose/g biomass·h

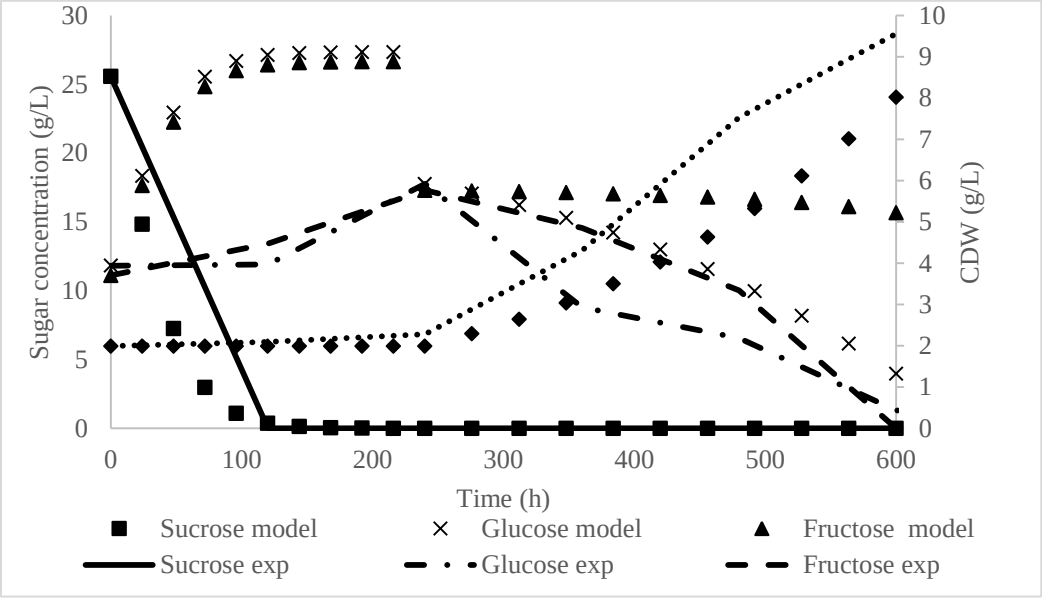

Figure 3: Kinetic model and experimental data of *C. suhuiensis* cells grown in 30 g/L of sucrose. The lines represent the experimental data and symbols represent the simulated results.

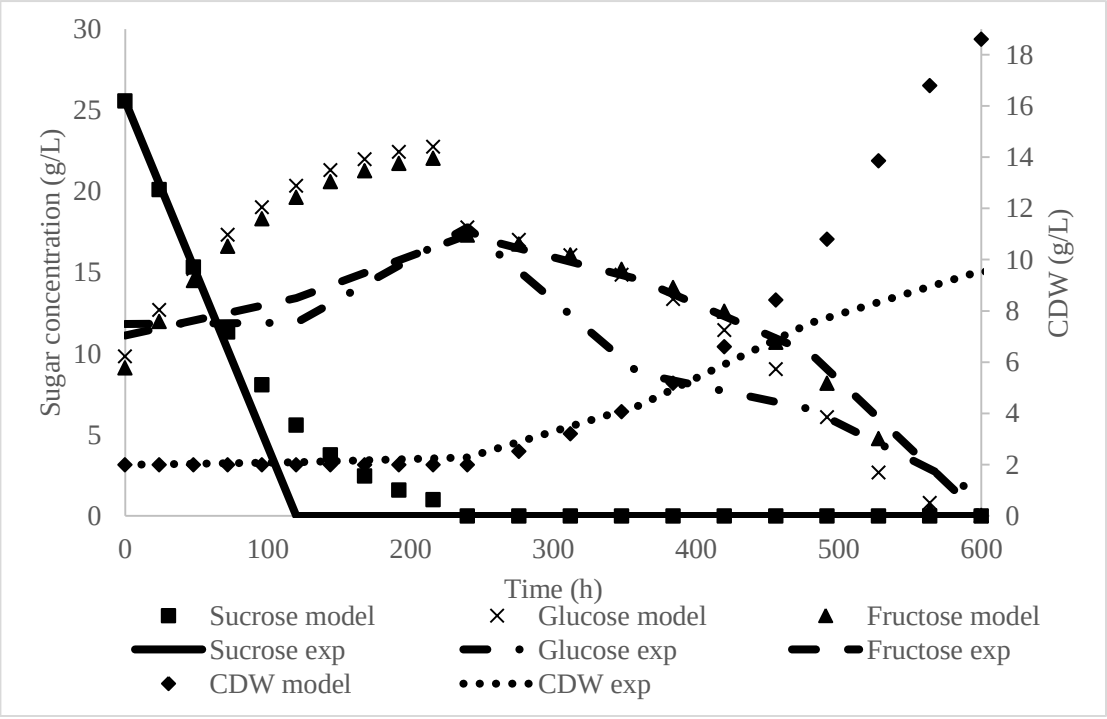

Figure 4: Comparison of the calculated values and the experimental data of Fig. 1 for *C. suhuiensis* cells, with minor adjustment of the values of V_{max}, K_F, K_G, K_S and K_{ig}. V_{max} = 0.23 g sucrose/g biomass·h, K_F = 1.3 g/L fructose, K_G = 1.126 g/L glucose, K_S = 23 g/L sucrose and K_{ig} = 0.45 g/L glucose.

1.3 g/L fructose, 1.126 g/L glucose, 23 g/L sucrose and 0.45 g/L glucose and 0.004/h, respectively. The growth kinetics of *C. suhuiensis* studied throughout this project can be a good start and platform for further studies of *C. suhuiensis* and other citrus species in order to understand and overcome the problems related to this species.

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