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A MODEL FOR *ARABIDOPSIS THALIANA* CELL SUSPENSION GROWTH AND SUGAR UPTAKE KINETICS

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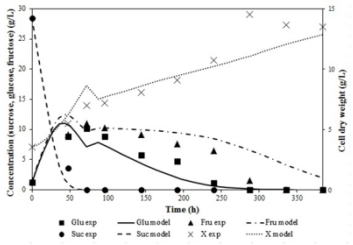
Graphical abstract



Abstract

Arabidopsis thaliana (A. thaliana) is a small weed which is related to the cabbage and mustard family. This flowering plant has been used widely as a model plant in genetics and molecular biology research since it is the first plant the full sequenced genome. Thus, *A. thaliana* literature provides plentiful information from genomics and transcriptomics point of view. However, there is still a lack of physiological information regarding its cell suspension cultures which can be powerful research tools in Plant Biotechnology and especially in Plant Systems and Synthetic Biology. In this study, cell growth and sugar uptake of *A. thaliana* Col ecotype grown in the continuous dark condition were modelled using the modified Monod and Michelis-menten equations. The model included sucrose hydrolysis by the cell-wall invertase enzyme into hexoses (glucose and fructose) and consumption of these hexoses at different rates to support cell growth. All kinetic model parameters were obtained from a control experiment where Col cells were grown on 30 gL^{-1} sucrose as well as other independent experiments where Col cells were supplied with different concentrations and combinations of sugars. The model adequately described and predicted the growth and sugars profile of *A. thaliana* cells. This model can also be applied for larger scale of growth with extended expressions for oxygen uptake rate, carbon dioxide production rate etc.

Keywords: *Arabidopsis thaliana*, Columbia (Col) ecotype, kinetic mode, cell growth, sugar uptake

1.0 INTRODUCTION

Research on plant cell suspension cultures goes back a few decades. Although there was extensive investigations on the use of plant cell cultures for the production of primary and secondary metabolites in the 1980s [1], the lack of understanding of the plant molecular biology, metabolism and physiology resulted in major problems with the non-reproducibility and low product yields at large scale. Potential applications of

plant cell cultures not only include the production of pharmaceutical compounds, flavors, pigments, fragrances and oils but also synthetic seed production via somatic embryogenesis which is very important for some agricultural, forestry and horticultural cases where seed germination is very difficult.

With advances in "omics" science and technology, it is now timely to revisit plant cell biotechnology as solutions to some sustainable production of platform chemicals, biofuels such as biodiesel, germplasm preservation and artificial seed production of endangered plant species, and heterologous protein production may have become feasible [2, 3]. Understanding both cell growth and production of compounds (primary and secondary products) is crucial to increase the formation of secondary metabolites in plants. With renewed interest in plant secondary metabolites and bioactive compounds research, a kinetic model is a powerful complementary tool. Some of the early kinetic models for plant cell suspension cultures include that for apple cells [4] and *Catharanthus roseus* [5, 6].

A. thaliana has been used widely as a model plant for research in genetics, physiology and metabolism because its genome is completely sequenced [7]. In most of these research, the whole plant of *A. thaliana* is used as its complete growth cycle from seed germination to seed setting covers only about three weeks. Research covering biochemical engineering aspects of its cell suspension cultures however, is still comparatively scarce.

To our knowledge, there are no reports yet on the kinetic models for *A. thaliana* cell suspension growth and its sugar uptake. In the literature, kinetic models which were reported for this plant are focus mainly on the metabolism and secondary metabolite production using different part of *A. thaliana* *i.e.* root cultures, chloroplast stroma, and whole leaves [8-11].

Therefore, the aim of this work is to develop a kinetic model that relates sucrose hydrolysis, glucose and fructose consumption as well as the growth of *A. thaliana* Col ecotype batch culture grown in the continuous dark condition.

2.0 METHODOLOGY

2.1 Plant Materials and Maintenance

A. thaliana Col callus cells were initiated from the seeds, obtained from the Faculty of Life Sciences, University of Manchester. Prior to germination, seeds were surface-sterilized by soaking them into 1% (v/v) sodium hypochlorite for 20 minutes. Seeds were rinsed with the sterile distilled water for 3 times. Sterile seeds were then transferred by using sterile forceps on the seed germination medium containing Gamborg's B5 (B5) medium supplemented 0.8% (w/v) agar at pH 5.6. Within 3 days of incubation, seeds started to germinate. After 2 weeks, leaves and shoots were harvested and transferred in to callus induction medium containing B5 medium with 20 gL^{-1} glucose, 0.5 mgL^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.05 mgL^{-1} kinetin, and 8 gL^{-1} agar[12]. Germination and callus initiation were under 16 h photoperiod at 25±1°C. Callus maintenance was performed every 3 weeks by transferring friable calli into the callus maintenance medium (the composition of this medium was similar as the callus initiation medium).

Initiation of suspension culture was done using the callus that has been maintained on solid media after fourth subculture. Approximately, 1 g of soft friable callus was transferred into 50 mL of Murashige and Skoog (MS) medium containing 30 gL^{-1} sucrose, 0.05 mgL^{-1} 1-naphthaleneacetic acid (NAA) and 0.5 mgL^{-1} kinetin and 50 mgL^{-1} in a 125 mL sterilized Erlenmeyer flask. Suspension culture was maintained in the cycled light condition (16 h photoperiod/8 h dark) at 110 rpm. In the first 6 months, suspension cultures were subcultured every 3 weeks. After that period, cell suspension was maintained by transferring every 10 days 10 mL of cells into 90 mL of fresh medium in 250 mL Erlenmeyer flask.

2.2 Growth Measurements in Suspension Cultures

In order to determine the growth characteristics of *A. thaliana* Col cell suspension cultures, the fresh and dry weight measurements were taken during the batch culture experiments. About 25 mL of cell suspension cultures were vacuum filtered onto a pre-weighed Whatman No. 1 filter paper. Then, the filter paper containing biomass was again weighed and the fresh weight of cells was determined by subtracting the weight of filter paper with biomass and the empty filter paper. The filtrate was filtered again and pH measurement was taken before the samples were stored at -20°C for further analysis. For dry weight measurements, filter paper with the cells was dried in the oven at 80°C until constant weight was achieved, and the difference in weight was recorded.[13]

2.3 Analysis of Sugars

The residual sugar contents in *A. thaliana* Col cell-free suspension medium were analyzed by the High Performance Liquid Chromatography equipment (HPLC) (Varian, Inc. (USA)) using PL Hi-Plex Ca column (300 x 7.7 mm) (Polymer labs) with a guard column (Polymer labs) at working flow rate of 0.6 mL min^{-1} at 85°C (Shimadzu CTO-6A) using HPLC grade water as the mobile phase. The defrosted filtrate was first filtered through a 0.45 μm cellulose acetate membrane filter (Sartorius). Each sample was injected twice using the auto injection (Pro Star 410) and the peak was detected by evaporative light scattering detector (ELSD) (PLS-2000). The evaporation temperature of ELSD was set to 90°C while 35°C for nebulization. The nitrogen flow rate was 1.60 L min^{-1}. All chromatograms from the analysis were visualized using a computerized integrator (Prostar/Dynamax System). 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^{-1} [14]. The remaining

concentrations of sucrose, glucose and fructose in the samples were identified by comparing their counted peak area with the standard curves. The retention times for sucrose, glucose and fructose were 11.132, 13.209, and 15.846 minutes, respectively.

3.0 RESULTS AND DISCUSSION

In the development of our model, the experimental results depicted in Figure 1 were used. When sucrose was supplied as the sole carbon source in the growth medium of *A. thaliana* Col suspension cells, it was hydrolyzed into stoichiometrically equal amounts of glucose and fructose by the cell wall invertase (EC 3.2.1.26). Both hexoses were consumed by the cells at different uptake rates. As all carbon sources were depleted, cells eventually reached their stationary phase (Figure 1). The overall growth of these cells could be summarized in three distinct phases as shown in Figure 1 and Table 1. Since the main focus in this study was the modeling of the growth of *A. thaliana* Col cells, the death phase (Phase 3 in Figure 1 and Table 1) was omitted from the model.

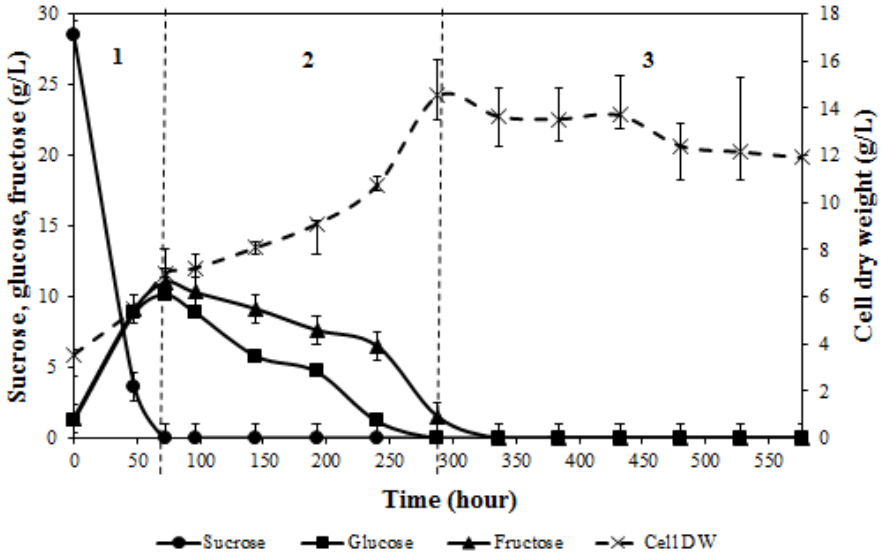


Figure 1 Residual sugar concentrations and growth profile of Col suspension cells under continuous dark condition (10% (v/v) inoculum). Phases 1, 2, and 3 of the experiment are described in Table 1

Table 1 Characteristics of the three different phases of *A. thaliana* Col cell suspension growth as illustrated in Figure 1.

Phase	Description
1	0 to 72 hours. Sucrose hydrolysis into glucose and fructose, growth of *A. thaliana* cells on glucose and fructose. At the end of this phase, sucrose was completely hydrolyzed
2	72 to 288 hours. Growth of *A. thaliana* cells on glucose and fructose, glucose and fructose are assumed to finish at the same time (after 288 hours), glucose inhibition of fructose uptake
3	Death phase. No nutrients in the medium

The unsteady state mass balances in phase 1 of the batch culture are represented by Equations (1) to (4). Mass balance for sucrose gives the rate of sucrose depletion due to hydrolysis as Equation (1):

$$\frac{dS}{dt} = -r_s = q_s x_v = -V_{\max} \cdot \frac{S}{K_S + S} \cdot x_v \quad (1)$$

The mass balance for fructose gives the rate of change of fructose concentration in the medium as a result of formation from sucrose and simultaneous consumption by the cells according to Equation (3):

The mass balance for glucose gives the rate of change of glucose concentration in the medium as a result of formation from sucrose and simultaneous consumption by the cells according to Equation (2):

$$\frac{dG}{dt} = r_G - r_{G_{x_v}} = Y_{G/S} \cdot r_S - \left(\mu_{\max} \cdot \frac{G}{K_G + G} \cdot x_v \right) \cdot \frac{1}{Y'_{x_v/G}} \quad (2)$$

$$\frac{dF}{dt} = r_F - r_{F_{x_v}} = Y_{F/S} \cdot r_S - \left(\mu_{\max} \cdot \frac{F}{K_F + F} \cdot x_v \right) \cdot \frac{1}{Y'_{x_v/F}} \quad (3)$$

The mass balance for biomass gives the rate of biomass production from glucose and fructose consumption according to Equation (4):

$$\frac{dx_v}{dt} = r_x = \mu \cdot x_v = \mu_m \cdot \left[\frac{G}{K_G + G} + \frac{F}{K_F + F} \right] x_v \quad (4)$$

Phase 2 corresponded to the growth phase of *A. thaliana* Col cells on the remaining glucose and fructose after the exhaustion of sucrose. During this period, it was assumed that there was no cell death. From Figure 1, it could be said that glucose consumption occurred at a higher rate compared to that of fructose reflecting that both of them were taken up by the cells at different specific uptake rates. *A. thaliana* Col cells exhibited preference for glucose over fructose as have been reported as well for other plant cells i.e. apple fruit [4], carrot [15] and *Taxus cuspidata* [16]. All this may imply that there was glucose inhibition on fructose consumption. In fact, the inhibition of fructose uptake by glucose was reported for *Glycine max* [17], *D. carota* [18, 19] and *Phaseolus vulgaris* [20]. Using this evidence from the literature, the volumetric fructose uptake rate in the presence of glucose was now expressed to incorporate the effect of inhibition by glucose on fructose uptake.

The unsteady state mass balances in phase 2 of the batch culture are represented by Equations (5) to (7). The mass balance on glucose gives the rate of change of glucose concentration in the medium according to Equation (5):

$$\frac{dG}{dt} = r_{G_{x_v}} = -\left(\mu_{\max} \cdot \frac{G}{K_G + G} \cdot x_v \right) \cdot \frac{1}{Y'_{x_v/G}} \quad (5)$$

The mass balance on fructose gives the rate of change of fructose concentration in the medium according to Equation (6):

$$\frac{dF}{dt} = -r_{F_{x_v}} = -\left(\mu_{\max} \cdot \frac{F}{K_F \cdot \left(1 + \frac{G}{K_{ig}}\right) + F} \cdot x_v \right) \cdot \frac{1}{Y'_{x_v/F}} \quad (6)$$

The mass balance on biomass gave the rate of change of cell concentration as a result of growth consuming glucose and fructose according to Equation (7):

$$\frac{dx_v}{dt} = r_x = \mu \cdot x_v = \mu_m \cdot \left[\frac{G}{K_G + G} + \frac{F}{K_F \cdot \left(1 + \frac{G}{K_{ig}}\right) + F} \right] x_v \quad (7)$$

3.1 Estimation of Model Parameters

The numerical values of $\frac{dS}{dt}$, $\frac{dG}{dt}$, $\frac{dF}{dt}$ and $\frac{dx_v}{dt}$ at different times during the batch experiment were calculated using the mid-point slope method of the experimental batch culture profiles of Figure 1. Lineweaver-Burk type of linear plot (double reciprocal) was used to plot some of the rate expressions to obtain the estimates for the kinetic parameters from independent batch experiments using different concentrations and combinations of sucrose, glucose and fructose. The respective plots used in the estimation of the numerical values of various kinetic parameters for the model are listed in Table 2.

The value of the maximum specific growth rate, μ_{max} was estimated from the linear regression of the semi-logarithmic cell dry weight against time during cell growth using the data of Figure 1. Since cell growth rate was different during Phase 1 and 2, two values of μ_{max} were used in the model corresponding to these 2 phases (Table 2). The values for V_{max} and K_S were obtained from the Lineweaver-Burk plot of the experimental data from the initial rate experiment using different initial sucrose concentrations.

The biomass yield on glucose, $Y'_{x/G}$ was determined as 0.281 g cells (g glucose)$^{-1}$ from the growth experiment on glucose only while the biomass yield on fructose, $Y'_{x/F}$ was determined as 0.379 g cells (g fructose)$^{-1}$ from the growth experiment on fructose only. The value of the glucose inhibition coefficient, K_{ig} was obtained from [20]. $Y_{G/S}$ and $Y_{F/S}$ are the stoichiometric yields (g hexose (g sucrose)$^{-1}$) from sucrose which are obtained by dividing the molecular weight (MW) of glucose (or fructose) by MW of sucrose. In order to determine the values of K_G and K_F, different independent experiments were conducted in which cells were grown separately in glucose and fructose only media. The growth of cells on glucose only were plotted in the form of a Lineweaver-Burk plot which gave the value of K_G by the division of slope and y-intercept values. Similarly, K_F value was calculated from the Lineweaver-Burk plot of data of a similar set of data from cell growth on fructose only.

3.2 Model Verification

As described in Table 2, the values of most of the kinetic parameters were obtained from the individual plots of different batch experiments independently of the results of Figure 1. The values of the parameters listed in Table 2 were then substituted into the mass balance

differential equations which are described in Equations (1) to (7). The initial values for sucrose, glucose, fructose and biomass concentration at t = 0 were those of the control experiment (Figure 1). The differential equations for the time rate of change of concentrations of sucrose, glucose, fructose and viable cells were solved in Mathcad version 14 using the *rkfixed* function.

The *rkfixed* function uses the fourth order Runge-Kutta method to solve first order differential equations in which Mathcad returns a two-column matrix. The first column (in Mathcad) 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 Figure 2 using the values of kinetic parameters given in Table 2. In Phase 1, it was observed that sucrose hydrolysis and formation of hexoses were adequately described by the model although the model slightly underestimated the rate of sucrose hydrolysis. Sucrose hydrolysis was completed after 72 hours of incubation in which both kinetic model and experimental data were in good agreement with each other. However by the end of Phase 1, the simulated results achieved a slightly higher biomass concentration.

In Phase 2, there was fair agreement between the simulated and experimental values for fructose consumption although the model predicted that fructose was not completely consumed by the cells. Meanwhile lower glucose consumption was predicted by the model. As a result, cells continued to grow after 288 h in the kinetic model although according to the experimental data, cells had already reached the stationary phase and subsequently entered their death phase.

Table 2 Model parameters and their values obtained independently from various experiments

Parameter	Numerical value	Estimated from
V_{max}	0.323 (g sucrose) (g biomass)$^{-1}$h^{-1}	Sucrose hydrolysis (initial rate experiment)
K_S	12.9 g sucrose L^{-1}	
μ_{max}	0.0094 h^{-1} (Phase 1) 0.0035 h^{-1} (Phase 2)	Cell growth on 30 gL^{-1} sucrose (control experiment)
K_G	3.85 g glucose L^{-1} (estimated in the model as 2.4 g glucose L^{-1})	Cell growth on glucose only
K_F	4.51 g fructose L^{-1} (estimated in the model as 2.74 g fructose L^{-1})	Cell growth on fructose only
$Y_{G/S} = Y_{F/S}$	0.526 g glucose (or fructose) (g sucrose)$^{-1}$	MW of glucose or fructose divided by MW of sucrose
$Y'_{x/G}$	0.281 g cells (g glucose)$^{-1}$ (estimated in the model as 0.30 g cells (g glucose)$^{-1}$)	Cell growth on glucose only
$Y'_{x/F}$	0.379 g cells (g fructose)$^{-1}$	Cell growth on fructose only
K_{ig}	0.210 g glucose L^{-1} (estimated in the model as 0.800 g glucose L^{-1})	[20]

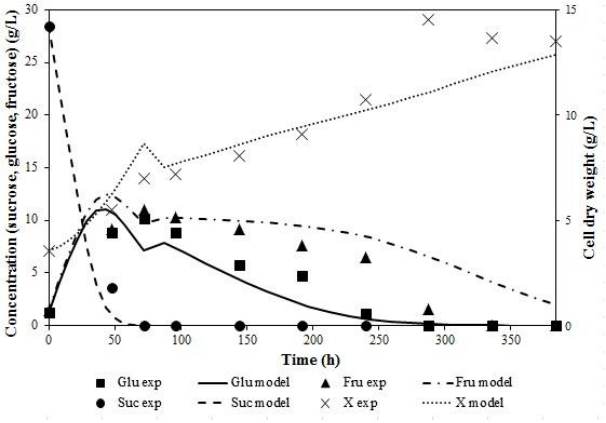


Figure 2 Kinetic model and experimental data of Col suspension cells grown in 30 gL^{-1} sucrose. The symbols represent the experimental data and the lines are the simulated results

4.0 CONCLUSION

Simulation results from the unstructured model could describe the experimental data of the growth and sugars uptake by *A. thaliana* Col cell suspensions reasonably well considering that most of the parameter values were calculated from other independent experiments of these cultures. The proposed kinetic model can therefore be used to predict sucrose hydrolysis, glucose and fructose formation from sucrose and their consumption by plant cells. This model should be able to predict larger scale of growth with extended expressions for oxygen uptake rate, carbon dioxide and product formation rate if there is a secreted product. Such a model can be used generically since it follows the commonly used rate expressions. It can therefore be tested with different plant cell cultures in order to characterize them by obtaining the numerical values of the stoichiometric and kinetic parameters so that experiments and process equipment can be designed more easily.

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Nomenclature

F	fructose concentration (gL^{-1})
G	glucose concentration (gL^{-1})
K_F	Monod constant for fructose uptake (gL^{-1})
K_G	Monod constant for glucose uptake (gL^{-1})
K_M	Michaelis-Menten (or saturation) constant
K_{ig}	inhibition by glucose of fructose uptake rate (g glucose $(L)^{-1}$)
K_S	Michaelis constant for sucrose hydrolysis (gL^{-1})
K_s	Monod saturation constant (kg substrate L^{-1})
r_F	rate of fructose formation from sucrose hydrolysis ($gL^{-1}h^{-1}$)
r_G	rate of glucose formation from sucrose hydrolysis ($gL^{-1}h^{-1}$)
r_S	rate of sucrose hydrolysis ($gL^{-1}h^{-1}$)
r_X	rate of biomass (dry weight) growth ($gL^{-1}h^{-1}$)
$[S]$	limiting substrate concentration (kg subs L^{-1})
S	sucrose concentration (gL^{-1})
V_{max}	maximum rate of sucrose hydrolysis ($gL^{-1}h^{-1}$)
v_{max}	the maximum rate of reaction
x_v	viable biomass concentration (gL^{-1})
$Y_{F/S}$	stoichiometric yield of fructose from sucrose hydrolysis (g fructose(g sucrose)$^{-1}$)
$Y_{G/S}$	stoichiometric yield of glucose from sucrose hydrolysis (g glucose (g sucrose)$^{-1}$)
$Y'_{x/F}$	yield of biomass formation from fructose (g cells (g fructose)$^{-1}$)
$Y'_{x/G}$	yield of biomass formation from glucose (g cells (g glucose)$^{-1}$)
μ	specific growth rate (h^{-1})
μ_{max}	maximum specific growth rate (h^{-1})

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