

## Effects on pre-cultivation on sugar metabolic kinetics of suspension plant cells measured by mid-infrared spectroscopy

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### Abstract

In order to study the sugar metabolic kinetics of suspension plant (*Nicotiana tabacum* cv. Bright Yellow No.2: TBY-2) cells by focusing our attention on sugars in the pre-cultivation media, a simple, rapid and accurate method for analyzing the sugar metabolic kinetics was developed by applying a mid-infrared spectroscopic method using a Fourier transform infrared spectrometer equipped with an attenuate total reflection accessory to the sugar content determination and by introducing a non-dimensional cultivation time denoting for the cell growth behavior in the analysis. Glucose could have almost the same characteristics as sucrose as the carbon source in pre-cultivation, and fructose would play a role different from the other sugars during both pre-cultivation and cultivation. In addition, the time differences in the sugar uptake rates among the sucrose, glucose and fructose pre-cultivation conditions would mainly depend on the lag time from the start of the cultivation to the sugar consumption by the suspension cells.

**Key words:** MIR spectroscopy, *Nicotiana tabacum*, sugar uptake, sucrose, glucose, fructose.

### Introduction

In the higher plants, sucrose is one of the most important substrates for carbohydrate catabolism and is very popular as nutritive and stabilized substance. Also, therefore, sucrose is most commonly used as the carbon source for plant cell cultivation. The influences of sucrose in a culture medium on the metabolic characteristics have been widely investigated<sup>1-3</sup>. Sucrose is rapidly hydrolyzed extra-cellularly into glucose and fructose by the invertase activity before being taken up by the cells<sup>4-8</sup>. In addition, the other sugars such as glucose and fructose can be used to support the plant cell cultivation, and most of the biochemical reactions for glycolysis within plant cells are almost totally known<sup>9</sup>.

Recently, plant-cell cultivation technology has been developed, and plays a very important role in many fields from plant physiology to bioprocess engineering. The precision control of the cultivation process is one of the most important subjects in both scientific and engineering interests. Glycolysis is usually the first step of a series of highly developed and very complicated metabolic pathways, and starts by simply consuming sugars such as glucose. Hence, the kinetic understanding of the sugar metabolic phenomena would be very effective in system biological research and in the precise control of cell cultivation processes. However, the kinetic transport phenomena of nutrimental sugars from medium to suspension plant cells are not well understood. Additionally, such studies, even on microorganisms and protist, were not kinetically quantitative and the analytical procedures proposed were complicated<sup>10-12</sup>. Therefore, we need to study the sugar metabolic dynamics for the plant cell system and to construct a model system for the transport phenomena from the outside. For such an experiment, it is very important that not only the contents of the metabolic components but also their molecular structures are nondestructively and simultaneously monitored in real time.

Application of spectroscopy, especially in the infrared region, to the above measurement is desirable as a high potential technique. Incidentally, in parallel developments of Fourier transform infrared (FT-IR) spectrometers and attenuated total reflection (ATR) techniques<sup>13, 14</sup>, the spectroscopic method using the FT-IR equipped with the ATR accessory (FT-IR/ATR) provides substan-

tial potential as a quantitative tool based on the molecular structure and on the interactions between the molecule and its environment. In addition, mid-infrared (MIR) spectroscopy has advantages theoretically with respect to spectral information in comparison with near-infrared (NIR) spectroscopy. Therefore, various FT-IR/ATR spectroscopic methods are now under development for the analysis of bioproducts<sup>15-23</sup>.

We then studied the quantitative analysis of both monosaccharides and disaccharides in aqueous solutions by the FT-IR/ATR method as the fundamental system<sup>14-26</sup> and developed the infrared spectroscopic determination of the sugar content in culture media suspending plant (*Nicotiana tabacum* cv. Bright Yellow No.2: TBY-2) cells by comparison with a high performance liquid chromatography method focusing on the sugar uptake<sup>27</sup>. These studies represented an important step in the development of an analysis method of the cellular metabolism through the sugar contents in culture media. Additionally, we provided a simple, rapid and accurate evaluation of the kinetic sugar uptake phenomena by the suspension TBY-2 cells in culture media, which have various combinations of glucose, fructose and sucrose concentrations during the initial stage of cultivation, using the sugar content detected by the FT-IR/ATR method<sup>28</sup>. As a result, it was found, for the TBY-2 cells sub-cultured in sucrose medium, that the fraction of sucrose in the initial total sugar content of the culture medium might kinetically affect the sugar uptake process and cell growth. These experimental results suggested the importance of understanding the influence of sugar in the sub- and pre-culture media on the sugar uptake kinetic phenomena during cultivation.

The objective of the present study is to better understand the kinetic sugar uptake phenomena of the TBY-2 cells by focusing our attention on sugars in pre-cultivation media. In this study, we also applied the MIR spectroscopic method developed in our previous study<sup>27</sup> to simultaneously determine the sugar content in the culture media. Therefore, we pre-cultured the TBY-2 cells in sucrose, glucose or fructose medium and performed cultivation with the pre-cultured cells in the four kinds of sugar media.

## Experimental

**Materials:** The experiments reported in this paper were carried out using TBY-2 cells. The TBY-2 cells were sub-cultured in a 300-mL flask with the Murashige-Skoog (MS) medium, which contained 30-g sucrose (Wako Pure Chemical Industries, Ltd., special grade) per liter at 298 K on a rotary shaker (150 rpm) in the dark. Sucrose was used as the typical carbon source for the TBY-2 cell cultivation. Glucose and fructose (Wako Pure Chemical Industries, Ltd., special grade), which synthesize sucrose, were also studied. Four kinds of media were prepared as listed in Table 1. The number of carbon molecules per unit volume in each medium was almost the same as that of the MS medium (sucrose medium). The sugar and the other components in the medium were prepared as separate solutions, and they were mixed after being sterilized. The sugar solution was sterilized by filtration using a 0.2- $\mu\text{m}$  membrane filter, and the medium without sugar was autoclaved for 15 min at 394 K.

**Methods:** The seven-day-old inoculum in the sucrose medium was washed with sterilized pure water. One and a half milliliters of the suspension was inoculated into 95 mL of the glucose, fructose or sucrose medium, and the pre-cultivation was carried out under the above cultivating conditions for 14 d. Again, the pre-cultured cells were cultured with the fresh glucose, fructose, sucrose or glucose-fructose mixture medium for 14 d after being washed. At regular time intervals, the sugar content and the cell density were measured by the following methods. An FT-IR spectrometer (Nicolet, Magna 750) was equipped with a KBr beamsplitter and a deuterated triglycine sulfate KBr detector. The FT-IR was also equipped with a horizontal zinc selenide ATR sampling accessory (Graseby Specac, Specacamp ATR 11080) placed within the sample compartment. This instrument was allowed to purge to minimize the spectral contribution due to atmospheric water vapor. Sixty-four scans of symmetrical interferograms at 4  $\text{cm}^{-1}$  resolution were co-added for each spectrum. The ATR spectra of the culture medium, passed through a 5- $\mu\text{m}$  membrane filter, were obtained from 4000 to 800  $\text{cm}^{-1}$ . Also, the ATR spectrum of the pure water was measured under the same conditions. The dry cell weight after drying at 363 K for 24 h was compared to the turbidity of the culture medium at 600 nm. The turbidity was measured by an UV-VIS-NIR scanning spectrophotometer (Shimadzu, Kyoto, Japan; UV-3100PC) with a 1-cm light path cuvette.

## Results and Discussion

**Specific uptake rates of sugars based on MIR spectral information:** We subtracted the water spectrum from the sucrose culture media, and the time variations of the subtracted spectra in the finger print region during cultivation of the TBY-2 cells pre-cultured in sucrose, glucose and fructose media are displayed in Fig. 1 (a), (b) and (c), respectively. These sucrose spectra at 0 d of cultivation time had almost the same characteristics as that of sucrose in its aqueous solution<sup>24</sup> and indicated that sucrose was the only carbon source in the medium. In the finger print region from 1300 to 900  $\text{cm}^{-1}$ , there are many peaks such as the CO and C-OH stretching modes<sup>24, 29-31</sup>, which significantly overlap each other. These peaks depend on the sugar structure and on the relationship between the sugar molecules and their environments. As shown in Fig. 1, the absorbance decreased with the change in the spectral pattern during cultivation, and the pattern spectroscopically indi-

cated that the sucrose in the media was hydrolyzed to glucose and fructose for all the pre-cultivation media. However, the changing kinetics was different from each other among the three pre-cultivation conditions. The spectral characteristics of the glucose-fructose mixture<sup>27</sup> were observed at 5 d of cultivation time for the sucrose and glucose pre-cultivation conditions (Fig. 1 (a) and (b)) and at 7 d of cultivation time for the fructose pre-cultivation conditions (Fig. 1 (c)), respectively. For the glucose, fructose or glucose-fructose mixture cultivation, the spectral changes in the culture media during cultivation were kinetically different among the three pre-cultivation conditions. These results spectroscopically suggested that the effects of the pre-cultivation conditions could kinetically reflect on the sugar uptake phenomena of the TBY-2 cells and not on the qualitative mechanisms. We describe the data processing procedure for the sucrose cultivation instances. In order to determine the sugar content in the culture media, calibration curves between the absorbance and sugar content<sup>27</sup>, developed using the MIR spectroscopic method, were applied to the spectral data shown in Fig. 1. The calibration curves used the absorbance at the spectral peaks around 1036, 1065 and 1055  $\text{cm}^{-1}$ , which are attributed to the glucose, fructose and sucrose spectra, respectively<sup>24, 25</sup>. Figure 2 shows the time courses of the sugar content and the cell density during sucrose cultivation after sucrose, glucose or fructose pre-cultivation. For all the pre-cultivation conditions, the cell density increased with a decrease in the total sugar content, and reached the plateau stage when the sugars were almost completely consumed. For the sucrose and glucose pre-cultivation conditions, the sucrose content immediately decreased just after the start of cultivation, and the glucose and fructose content successively increased. The glucose and fructose content reached the peak values at about 5 d, and decreased afterward. For the fructose pre-cultivation conditions, the sucrose content decreased after the lag time longer than that for the sucrose or glucose pre-cultivation conditions, and the peaks of the glucose and fructose content were observed at 6 to 7 d. The time variation of the sugar content changes in the glucose, fructose and glucose-fructose mixture cultivation media was also affected by the sugar in the cultivation medium. These results suggested that the difference of the sugar in the pre-cultivation medium reflected the kinetics of the sugar metabolic process of the TBY-2 cells. We then studied the specific uptake rates of the sugars, which is one of the important indices characterizing the sugar metabolic kinetics of cells. We calculated the specific uptake rates after quantification of the sugar uptake rate and the cell density according to the method provided in our previous paper<sup>28</sup> as follows.

Since glucose and fructose in the sucrose culture medium are produced by the hydrolysis of sucrose in the medium by enzymes such as invertase<sup>4-8</sup> during cultivation, we calculated the sugar uptake rates,  $R_{c,s}$ , using the following logistic functions with the uptake amounts of glucose ( $W_{\text{glc}}$  [ $\text{g}\cdot\text{L}^{-1}$ ]) and fructose ( $W_{\text{fru}}$  [ $\text{g}\cdot\text{L}^{-1}$ ]) that were evaluated based on the assumption that the suspension cells consume sucrose after hydrolysis.

$$R_{c,\text{glc}} = \frac{dW_{\text{glc}}}{dt} = k_{w,\text{glc}} (W_{\text{glc}} - W_{\text{glc},\text{fin}}) (W_{\text{glc}} - W_{\text{glc},\text{ini}}) \quad (1a)$$

$$R_{c,\text{fru}} = \frac{dW_{\text{fru}}}{dt} = k_{w,\text{fru}} (W_{\text{fru}} - W_{\text{fru},\text{fin}}) (W_{\text{fru}} - W_{\text{fru},\text{ini}}) \quad (1b)$$

Here, the parameters,  $k_{w,\text{glc}}$  and  $k_{w,\text{fru}}$ , are the rate constants for the sugar uptake. The subscripts ini and fin indicate the values at the initial and final stages, respectively. On the other hand, we quanti-

tatively examined the time course data of the cell density and applied the logistic function, expressed by Eq.(2), to the data. This equation is mathematically the same function as Eq.(1), and  $x$  [g-dry cell·L<sup>-1</sup>] is the cell density. The parameters  $t_0$  and  $w_x$ , respectively, denotes the inflection point of the time course of the cell density and the time constant ticking its curve. The lines for the cell density shown in Fig.2 fit the calculated results. The specific uptake rates were then calculated by dividing the uptake rates evaluated by Eq.(1) with the cell density predicted by Eq.(2).

$$x = \frac{x_{ini} - x_{fin}}{1 - e^{-(t-t_{0,x})/w_x}} + x_{fin} \quad (2)$$

Figure 3 shows the influences of the sugar in the pre-cultivation medium on the specific uptake rates during sucrose cultivation, and the specific uptake rates for the total sugars are the sum of each value. The specific uptake rates at 0 d of the cultivation time in Fig.3 (b) are not equal to zero, because their initial values mathematically mean those not at the minus infinity of the cultivation time but at 0 d. For all the pre-cultivation conditions tested in this study, similar to the previous studies<sup>28</sup>, the cells consumed mainly glucose and fructose, respectively, during the initial and final stages of cultivation. Hence, these characteristics of the TB Y-2 cells were maintained after glucose or fructose pre-cultivation. The kinetic behavior of the specific sugar uptake during sucrose cultivation after glucose pre-cultivation (Fig.3 (b)) was almost same as that after sucrose pre-cultivation (Fig.3 (a)), which could be the standard conditions. Additionally, the lag time before the start of the sugar uptake by the TB Y-2 cells pre-cultivated in the fructose medium was the longest among the three pre-cultivation conditions (Fig.3 (c)). Furthermore, the significant differences in the times indicating the maximum specific uptake rates among the three pre-cultivation conditions were not observed for the fructose cultivation (data not shown).

**Nondimensional time behavior of specific uptake rates of sugars:** As shown in Fig.3, the influences of the sugar in the pre-cultivation medium experimentally reflected the kinetic sugar uptake behavior of the test organisms. In addition, Fig.2 also indicates that the cell growth rate was affected by the sugar in the pre-cultivation medium. Thus Fig.3 might be expressed as the kinetic sugar uptake behavior denoting the variations in the cell growth process during cultivation. In order to discuss the kinetic sugar uptake phenomena and neglecting the cell growth behavior, we tried to apply the non-dimensional cultivation time of  $t_{non,x}$ , expressed by Eq. (3), to the data displayed in Fig.3.

Because the above non-dimensional time can be calculated using the kinetic parameters,  $t_0$  and  $w_x$  in Eq. (2), which respectively denote the inflection point of the time course of the cell density and the time constant ticking its curve, the relationship between the specific sugar uptake rate and the non-dimensional cultivation time could signify the kinetic sugar uptake characteristics of the TB Y-2 cells based on the cell growth stage.

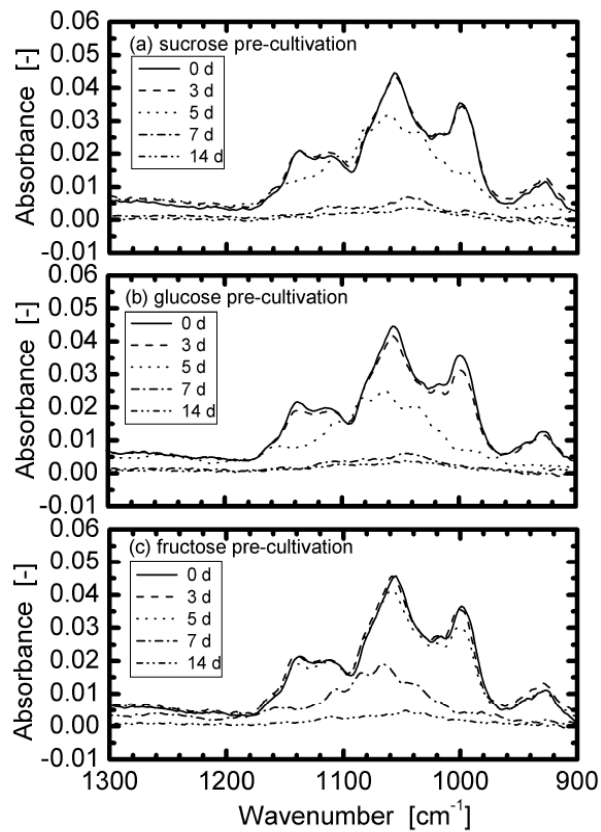
$$t_{non,x} = (t - t_{0,x}) / w_x \quad (3)$$

We then represent the data in Fig.3 using the non-dimensional cultivation time standing for the cell growth behavior. Figure 4 shows the influences of sugar in the pre-cultivation medium on the non-dimensional time expression of the specific sugar uptake rates during sucrose cultivation. The kinetic behavior of the specific sugar

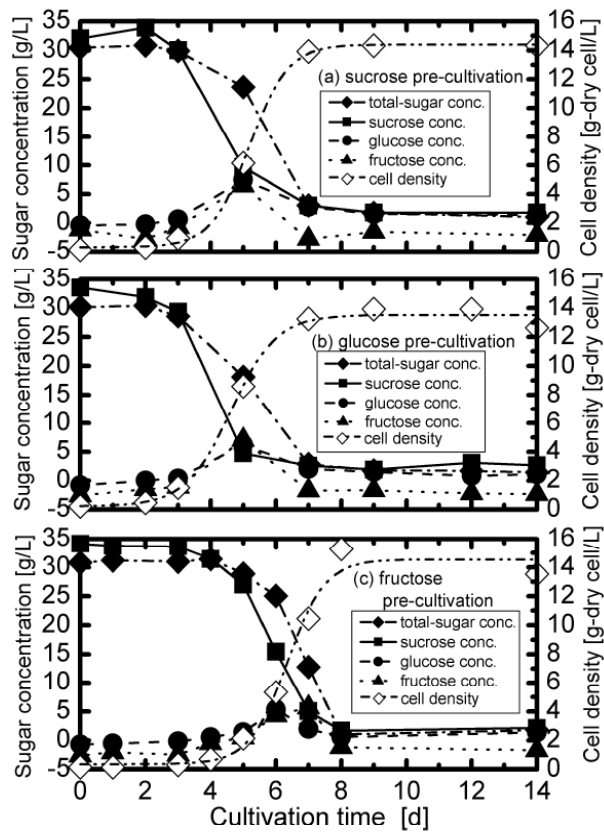
uptake rates of the TB Y-2 cell pre-cultured in the glucose medium was almost same as that pre-cultured in the sucrose medium also in the non-dimensional expression of the cultivation time (Fig.4 (a) and (b)). The tendency, very similar to the results in Fig.4 (a) and (b), were experimentally observed in the real time expression of the cultivation time (Figs.3 (a) and (b)). Thus the sugar metabolic characteristics of the TB Y-2 cells pre-cultured in the glucose medium would be just like those pre-cultured in the sucrose medium, which is the typical medium, both qualitatively and quantitatively. For the fructose pre-cultivation conditions, both the lag time before the start of sugar uptake and the time indicating the maximum specific uptake rate in the non-dimensional time expression were roughly same as those for the sucrose and glucose pre-cultivation conditions, while the former and latter were, respectively, the longest and the last among the three pre-cultivation conditions in the real time expression of the cultivation time. These results experimentally indicated that it was delayable for the TB Y-2 cells pre-cultured in the fructose medium to recognize sucrose in the medium and to metabolize the sugars. Additionally, just after the start of the sugar uptake action, these cells would have sugar metabolic characteristics almost similar to the cells pre-cultured in the sucrose or glucose medium. Furthermore, it could be possible to carry out the analysis and discussion described above by introducing the non-dimensional expression of the cultivation time with the fitting parameters of the logistic function for the cell growth and by comparing the specific sugar uptake rates in both the real and non-dimensional expressions of the cultivation time. Figures 5, 6 and 7 also display the influences of the sugar in the pre-cultivation medium on the non-dimensional time expression of the specific sugar uptake rates during glucose, fructose and glucose-fructose mixture cultivation, respectively. For each pre-cultivation condition, the non-dimensional time indicating the maximum specific uptake rate was roughly similar to each other, while the differences in the time course curves for the real time expression were observed (data not shown). For the glucose and fructose cultivations (Figs. 5 and 6), the time course curves for the glucose pre-cultivation condition were almost the same as those for the sucrose pre-cultivation condition as the standard one, and those for the fructose pre-cultivation condition had different shapes from the others. In addition, the significant differences in the times indicating the maximum specific uptake rates among the three pre-cultivation conditions for the fructose cultivation were not observed in the non-dimensional expression (Fig.6), the same as in the real-time expression (data not shown). This suggested that the influences of sugar in the pre-cultivation medium on the kinetic process of both the sugar uptake and cell growth during fructose cultivation could almost be negligible. For the glucose-fructose mixture cultivation (Fig.7), although no significant influence of the sugar in the pre-cultivation medium was found, the specific uptake rates during cul-

**Table 1.** Setup sugar conditions of culture media.

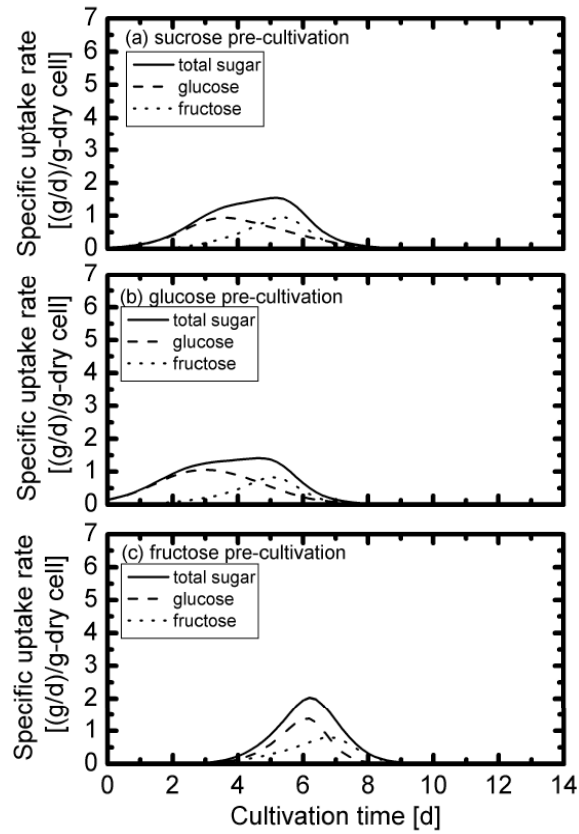
Culture media	Sugar concentrations [g/L]		
	glucose	fructose	sucrose
glucose	31.58	0.00	0.00
fructose	0.00	31.58	0.00
sucrose	0.00	0.00	30.00
glucose-fructose	15.79	15.79	0.00



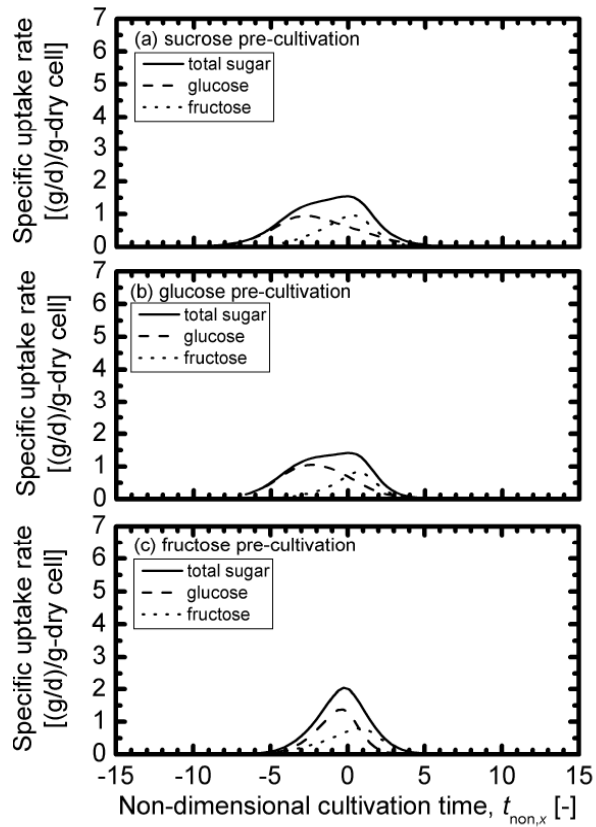
**Figure 1.** Time behavior of ATR spectra of sugars in sucrose culture media: (a) for sucrose precultivation, (b) for glucose pre-cultivation, and (c) fructose pre-cultivation.



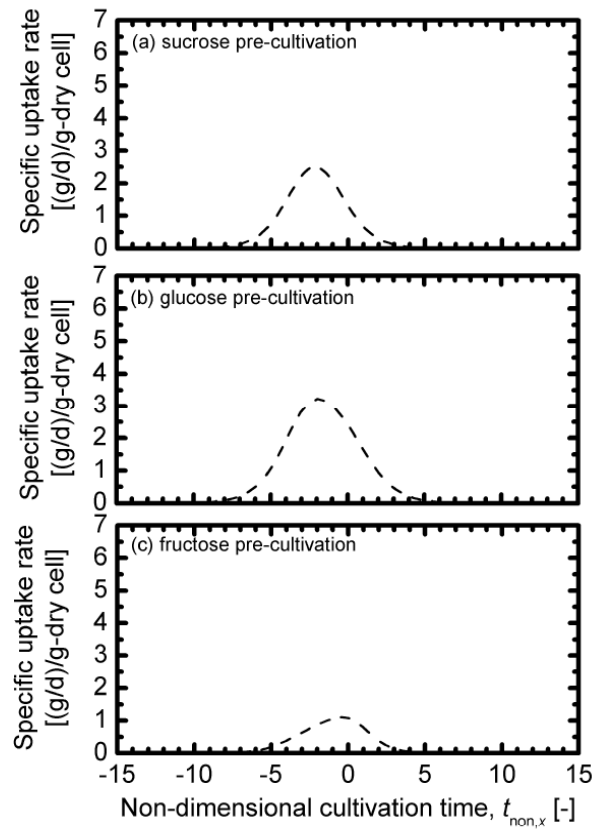
**Figure 2.** Time courses of sugar contents and cell density in sucrose culture media: (a) for sucrose pre-cultivation, (b) for glucose pre-cultivation, and (c) fructose pre-cultivation.



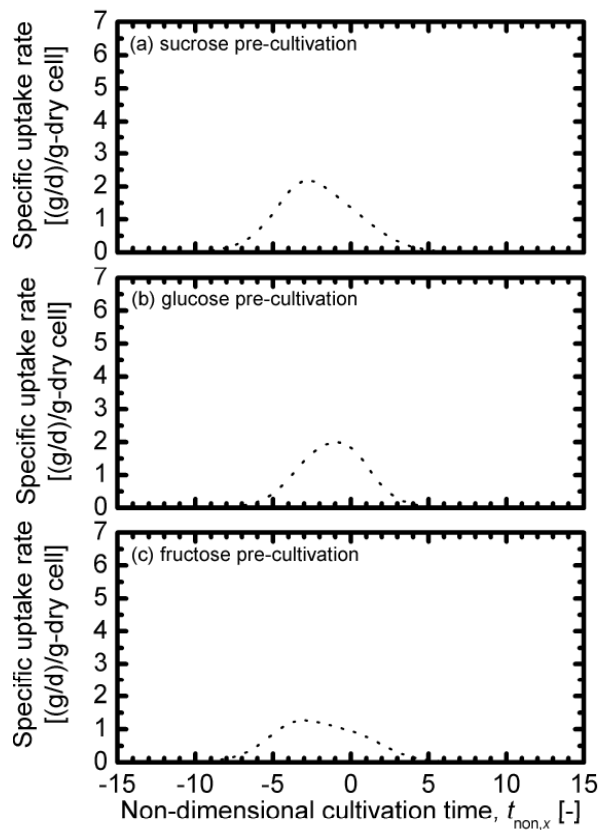
**Figure 3.** Time courses of specific uptake rates during sucrose cultivation: (a) for sucrose pre-cultivation, (b) for glucose pre-cultivation, and (c) fructose pre-cultivation.



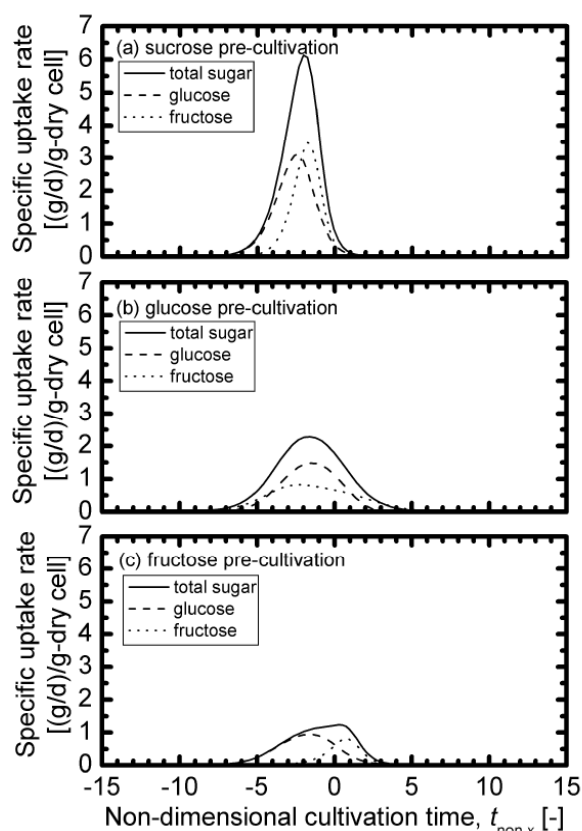
**Figure 4.** Non-dimensional time courses of specific uptake rates during sucrose cultivation: (a) for sucrose pre-cultivation, (b) for glucose pre-cultivation, and (c) fructose pre-cultivation.



**Figure 5.** Non-dimensional time courses of specific uptake rates during glucose cultivation: (a) for sucrose pre-cultivation, (b) for glucose pre-cultivation, and (c) fructose pre-cultivation.



**Figure 6.** Non-dimensional time courses of specific uptake rates during fructose cultivation: (a) for sucrose pre-cultivation, (b) for glucose pre-cultivation, and (c) fructose pre-cultivation.



**Figure 7.** Non-dimensional time courses of specific uptake rates during glucose-fructose cultivation: (a) for sucrose pre-cultivation, (b) for glucose pre-cultivation, and (c) fructose pre-cultivation.

cultivation after fructose pre-cultivation were lower than those after sucrose or glucose pre-cultivation. Based on these results, glucose could have almost the same characteristics as sucrose as the carbon source in pre-cultivation, and fructose would play a different role from the other sugars during both pre-cultivation and cultivation. Consequently, the experimental results suggested the importance of understanding the influences of sugar in the sub- and pre-culture medium on the sugar uptake kinetic phenomena during cultivation. The above analyzing method as shown in Figs.3 to 7 could be performed by adopting the kinetic parameters of the logistic function, which were accurately and rapidly determined based on the MIR spectroscopic information of the culture medium. The probe or fiber accessories for the MIR spectroscopic measurement are easily available for the continuous, non-destructive and real-time monitoring of the cultivation process. Additionally, the analyzing method of the kinetic sugar uptake phenomena is very unique for determining the cell growth behavior. Therefore, the efforts obtained in this study could provide only an initial indication of the kinetic sugar uptake phenomena of the TBY-2 cells pre-cultured in the three kinds of sugar medium but also a new methodology to analyze the overall metabolic dynamics of the suspension cells. Furthermore, the MIR spectroscopy would be applied to the metabolic analysis in a broad scientific area because the FT-IR/ATR method has a potential to theoretically determine most of the chemical components related with the metabolism as well as sugars. Thus the present study represents the first step in the development of an analysis method for the overall metabolic dynamics of the suspension plant cells based on the MIR spectroscopic information and of non-destructive on-line monitoring devices.

## Conclusions

The influences of sugars as carbon sources during pre-cultivation on the kinetic sugar uptake phenomena during sucrose, glucose, fructose and glucose-fructose mixture cultivation were studied using the time variations of the sugar contents in the media simultaneously and accurately determined by the MIR spectroscopic method developed in our previous study<sup>27</sup>. By applying the logistic functions (Eq.(1) and (2)) to the predicted sugar contents and cell density, the specific uptake rates of the suspension's TBY-2 cells were easily and continuously obtained. In addition, the sugar uptake rates were discussed in the non-dimensional expression of the cultivation time denoting the cell growth behavior, which was calculated with the kinetic parameters of the logistic function (Eq.(3)) for the cell growth and was an original expression in the present study. The kinetic sugar uptake characteristics of the suspension TBY-2 cells were then well confirmed as follows. For the TBY-2 cells after glucose or fructose pre-cultivation similar to those after sucrose pre-cultivation, the glucose uptake led the fructose one in the sucrose and glucose-fructose mixture cultivation. Moreover, glucose could have the almost same characteristics as sucrose as the carbon source during pre-cultivation, and fructose would play a different role from the other sugars during both pre-cultivation and cultivation. Furthermore, the time differences in the sugar uptake rates among the three pre-cultivation conditions would mainly depend on the lag time from the start of cultivation to the sugar consumption by the suspension cells.

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