# Analysis of Kinetic Uptake Phenomena of Monosaccharide and Disaccharide by Suspension TBY-2 Cells Using an FT-IR/ATR Method.

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## Abstract

The influence of sugars in culture media on the kinetics of the mono- and disaccharide uptake and cell growth behavior was studied by mid-infrared spectroscopy using a Fourier transform infrared spectrometer (FT-IR) equipped with an attenuate total reflection accessory (ATR). We performed the plant cell cultivation with *Nicotiana tabacum* cv. Bright Yellow No.2 (TBY-2) cells in the culture media, which contained glucose, fructose, mannose, galactose, sucrose, trehalose, maltose or lactose. Consequently, the differences of the kinetics sugar uptake and cell growth behavior among all the cultivations were confirmed. In particular, a very long lag time before the galactose uptake was observed, and the spectral-pattern of the maltose medium presented almost the same as the initial one during the cultivation. Furthermore, base on the non-dimensional cultivation time for cell growth behavior, it was suggested that the TBY-2 cells consumed sugar before cell growth and produced the ethanol just after cell growth.

*Keywords Mid-Infrared Spectroscopy; Sugar Metabolic Kinetics; Plant Cell ; Maltose; Galactose*

# 1

# Introduction

Recently, plant-cell cultivation technology has been developed and plays a significant role in various fields, from plant physiology to bioprocess engineering. The precision control of the cultivation process is one of the most important subjects in both the scientific and engineering fields. Most of the biochemical reactions during glycolysis within plant cells are understood [1] and there are a

number of studies on the sugar transport, sugar sensing and signaling in plants. For example, in disaccharides, Riesmeier et al. [2] discovered and successfully isolated a sucrose transporter (SUT) from spinach. Additionally, they suggested that the SUT had the ability to transport maltose. After this, the SUT was isolated from other plants. A maltose transporter had not been previously identified. Niittyla et al. [3] discovered a maltose transporter which was unrelated to other sugar transporters in Arabidopsis leaves. On the other hand, the monosaccharide transporter (MST) has been significantly reported from various plants. The monosaccharide transport activity was found in various areas such as the spice of plant, tissue in plants and the MST family. However, little is known about the kinetic transport phenomena of nutrimental sugars from a liquid medium to suspension plant cells.

Incidentally, the spectroscopic method using Fourier transform infrared (FT-IR) spectrometers equipped with an attenuated total reflection accessory (FT-IR/ATR) provides significant potential as a quantitative analytical tool for liquids [4, 5]. Therefore, various FT-IR/ATR spectroscopic methods are under development for the analysis of bioproducts [6-9]. We also studied the quantitative analysis of sugars in aqueous solutions using the FT-IR/ATR method [10-12].

The MIR spectrum using the FT-IR/ATR method has experimentally shown that the spectral additivity was applicable for sucrose, glucose and fructose in the MS media. In addition, we examined the potential of the sugar concentration determination in culture media containing suspended *Nicotiana tabacum* cv. Bright Yellow No.2 (TBY-2) cells using the FT-IR/ATR method by a comparison with the high-performance liquid chromatography (HPLC) method in order to analyze the sugar uptake rate of the TBY-2 cell suspension [13]. We then discovered the importance of understanding the influence of sugar in the sub- and pre-culture media on the sugar uptake kinetic phenomena during cultivation [14].

Hashimoto et al. [15], moreover, made it possible to simultaneously measure the nutrient and product concentrations in a culture medium using the FT-IR/ATR method. Using the FT-IR/ATR method developed by Hashimoto et al. [15], we studied the influence of sugar species in the pre-culture and culture media on the

kinetic sugar uptake phenomena and the ethanol production by comparing the characteristics of the TBY-2 cells with the rice cells [16].

Additionally, since water is a major constituent in the bioproducts, the interaction between the saccharide molecules and water is one of the most important factors for monosaccharide and disaccharides [17]. As a major translocatable product of photosynthesis, sucrose (glucose and fructose) is the main soluble component of the phloem sap [18]. Trehalose (a dimer of glucose linked in α1-2) substitutes for water and maintains the life of organisms exposed to arid environments [17]. In leaves, maltose (a dimer of glucose linked in α1-4) with glucose and isomaltose are principal products starch mobilization exported from chloroplasts at night [19, 20]. Mannose is known to be toxic for plant growth, supposedly due to a sequestration of inorganic phosphate as mannose-6-phosphate [21-23]. Similarly, galactose is highly toxic when supplied exogenously to plants in concentration of 1-10 mM [24, 25]. Moreover galactose which is one of the pectic polysaccharide components in the cell wall is related to intercellular contacts [26]. Kanou et al. [17] studied the infrared spectral characteristics of monosaccharides and disaccharides in aqueous solution.

In this study, we defined that glucose and sucrose as the typical carbon source of monosaccharide and disaccharide, respectively. We applied three monosaccharides (fructose, mannose and galactose) which corresponded to glucose epimers and three disaccharides (trehalose, maltose and lactose) which had various glucose linkages in addition to both typical carbon sources to this study. Additionally, those sugars are related to sugar metabolism as described the above paragraph.

The objective of the present work was to get a good grasp of the influence of sugar species, that is, the difference of molecular structure in monosaccharide and kinds of glucose links in disaccharide, in the culture media on the kinetic sugar uptake phenomena. Furthermore, we applied the FT-IR/ATR method developed by Hashimoto et al. to measure not only sugar concentrations but also ethanol concentration which plant cells produce in anaerobic environment, simultaneously.

# 2

# Materials and Methods

## 2.1

## Plant Cell

The experiments reported in this paper were carried out with *Nicotiana tabacum* cv. Bright Yellow No.2 (TBY-2), which were sub-cultured in the Murashige-Skoog (MS) [27] medium for 7days at 298 K on a rotary shaker (150 rpm) in the dark as described in the previous paper [13-16, 28].

## 2.2

## Culture Media

The MS media were used as the standard medium, and sucrose was used as the typical carbon source for the TBY-2 cell cultivation. Four types each of the monosaccharides (glucose, fructose, mannose and galactose) and disaccharides (sucrose, trehalose, maltose and lactose) were prepared as the carbon source in the culture media. The monosaccharide and disaccharide concentrations in the culture media were fixed at 31.58 g/l and 30.00 g/l, respectively; adjusted to the sucrose carbon number. Trehalose, which was used in this study, was of special grade and obtained from Hayashibara Biochemical Laboratories (Okayama, Japan). All of the others chemicals were of special grade and were obtained from Wako Pure Chemical Industries (Osaka, Japan). The sugar and the other components in the medium were prepared as separate solutions, and each sugar was dissolved in pure water. They were mixed after being sterilized. The sugar solution was sterilized by filtration using a 0.2-micrometer membrane filter, and the medium without sugar was autoclaved for 15 min at 394 K.

## 2.3

## Cultivation

During cultivation, 1.5 ml of the 7-day-old inoculums in the MS medium was washed by the MS medium without sugar and inoculated into 95 ml of each fresh medium in a 300 ml flask, and the cultivation was carried out at 298 K on a rotary shaker (150 rpm) in the dark. At specified time intervals, the metabolite concentrations and the dry cell weight in the culture medium were measured.

## 2.4

## Enzyme assay

# Table 1

The enzymes, invertase and maltase in the sucrose and maltose culture media, were assayed under the conditions listed in Table 1, which showed that pH and temperature were optimum conditions of invertase (EC 3.2.1.26) [29] and maltase (EC 3.2.1.20) [30]. Each of culture medium at lag, logarithmic and stationary phases was filtrated through a 0.45μm membrane filter in order to remove the cells from culture media. Furthermore, the sugar concentration, pH, and temperature of the culture media were adjusted to the conditions in Table 1. These culture media were incubated with a shaking. At specified time intervals, the sugar concentrations were measured. In additionally, as a control, each of the culture medium in 0.35 unit/ml invertase and maltase was incubated at the same conditions, respectively.

## 2.5

## Analysis

The sugars and ethanol concentrations in culture medium were determined by FT-IR/ATR method as described previously [13-16, 28, 31]. For analysis by Mid-infrared spectroscopy, an FT-IR spectrometer (Nicolet, Magna 750) was equipped

with a KBr beam splitter and a deuterated triglycine sulfate KBr detector was used to obtain the spectra. The ATR spectra were obtained with a horizontal zinc selenide ATR sampling accessory (GRASEBY SPECAC, SPECACLAMP ATR 11080). The dry cell weight after drying was compared to the turbidity of culture medium by at 600 nm as described previously [13-16, 28].

Analysis of the enzyme assay was performed using a Tosoh (Tokyo, Japan) HPLC system, consisting of a model DP-8020 pump and a model RI-8020 differential refracting detector. A Shim-Pack SCR-101 N (Shimadzu, Kyoto, Japan) HPLC analytical column (7.9×300 mm) with a guard column was used. The column temperature was maintained at 313 K with a column oven (CO-8020, Tosoh, Tokyo, Japan). The mobile phase was the pure water after filtration through a 0.2 μm membrane filter and degassing with an ERC-3310 degasser (Erma Optical Works, Tokyo, Japan). The refractive index of the elute was monitored as described previously [14].

# 3

# Results and discussion

## 3.1

## ATR spectral analysis of culture medium

**Figure 1a**

**Figure 1b**

Figure 1 shows the ATR spectra of the monosaccharide (glucose, fructose, mannose and galactose), disaccharide (sucrose, trehalose, maltose and lactose) and ethanol from 1300 to 900 $cm^{-1}$ (finger print region) in the MS medium. These spectra were obtained after the spectral subtraction of water. For the sugars, there were many peaks, such as the CO and C-OH stretching modes, which complicatedly overlapped each other [10, 11, 32], especially in the region from

1200 to 950 $cm^{-1}$. These peaks depend on the sugar structure and on the interaction between the sugar molecules and their environments. In addition, ethanol had a significantly sharp peak around 1045 $cm^{-1}$ (Fig. 1a), which did not overlap the sugar peaks. Thus, we can identify each sugar and ethanol in the medium with its spectral pattern in this region. The peaks around 1036, 1065, 1070, 1076, 1055, 993, 1149 and 1076 $cm^{-1}$ characterize the glucose, fructose, mannose, galactose (Fig. 1a), sucrose, trehalose, maltose and lactose (Fig. 1b) spectra, respectively.

**Figure 2a**

**Figure 2b**

**Figure 3a**

**Figure 3b**

Figures 2a, 2b, 3a and 3b show the time behavior of the ATR spectra of the medium during the glucose, galactose, trehalose and maltose cultivation, respectively. At the beginning of the cultivation (0 day), the ATR spectra had the same characteristics as that of each disaccharide shown in Fig. 1. During the monosaccharide cultivation, the absorbance decreased almost over the finger print region without any change in the spectral patterns as shown in Fig. 2. As the mannose and galactose cultivations are known to be toxic, the absorbance decreased with the cultivation time. On the other hand, during the disaccharide cultivation, the ATR spectra had the characteristics of two patterns. In one pattern, during the trehalose cultivations, the absorbance decreased with the changes in the spectral patterns. Especially, a sharp peak identifying the glycosidic linkage around 993 $cm^{-1}$ decreased. These spectra qualitatively had the spectral characteristics as those of the trehalose and glucose mixture in the medium. The spectral patterns had gradually changed from trehalose to glucose (Fig. 3a). During the sucrose cultivation, the ATR spectral patterns had also gradually changed from a sucrose to a glucose and fructose mixture (data not shown). The other pattern, during the maltose cultivation, the absorbance decreased, however, there was little change in the spectral pattern (Fig. 3b). Additionally, during the

lactose cultivation, the ATR spectra hardly decreased within 180 days (data not shown).

Around 1045 $cm^{-1}$ appeared a sharp peak identifying ethanol, this sharp peak appeared after 20 days and was observed as shown in Fig. 3a. This phenomenon was observed with some sugar cultivations. Thus, the spectral change shown in Fig. 3a gives evidences for a biological process in which ethanol is produced after trehalose in the medium is hydrolyzed and consumed by the cells. However, the biological process was different from each carbon source. Finally, the ATR spectrum of the culture media was almost identical to that of the MS medium without sugar.

## Table 2

We applied the MIR spectroscopic method developed by Hashimoto et al. [13, 15] to the metabolite concentration determination. Focusing on the above wavenumbers, we made calibration curves between the absorbance and metabolite concentrations in the media. The fitting parameters and correlations coefficients of the calibration curves are displayed in Table 2. An excellent linearity was observed for each metabolite at each wavenumber [15].

## Figure 4

Figure 4 shows the time courses for the sugar and ethanol concentrations evaluated by the above method and the dry cell weight in the culture media during the trehalose cultivation. In this study, we determined the sugar and ethanol concentrations in the MS medium using the parameters of the calibration curves shown in Table 2. Figure 4 shows that the medium initially contained trehalose as the carbon source. The trehalose concentration decreased while the glucose successively increased. The glucose one reached peak values around 17 days and then decreased. This indicated that a part or most of the trehalose in the medium was hydrolyzed to glucose, which was consumed by the TBY-2 cell. Moreover, in the sucrose medium, the sucrose concentration decreased with an increase in the

glucose and fructose concentrations, which the sucrose in the medium was hydrolyzed similar to the previous studies [13-16, 32]. In contrast to these results, in the maltose medium, we rarely observed any sugar other than maltose by both the FT-IR/ATR and HPLC method (data not shown).

On the other hand, the dry cell weight increased with a decrease in the total sugar concentration and reached the maximum value when the sugars were almost completely consumed. The ethanol concentration, which was negligible during the initial stage, became successively higher and indicated the highest value at 19 days just after the dry cell weight had reached a maximum value. The ethanol concentration then rapidly decreased with a slight decrease in the dry cell weight and was almost equal to zero during the final stage of cultivation. These results suggested that the sugar metabolism and ethanol production during cultivation could be continuously observed on the base of the spectra in the culture medium using the FT-IR/ATR method.

## 3.2

## Sugar metabolic kinetics

We studied the sugar metabolic kinetics in the mono- and disaccharides by comparing with all sugar metabolic kinetics. However, the sugar uptake phenomena on galactose and maltose presented very different behaviors when compared to the other sugars used in this study. We first understood both phenomena and then made a comparison of each sugar's metabolic kinetics.

### *3.2.1*

### *Galactose*

**Figure 5a**

**Figure 5b**

**Figure 6a**

**Figure 6b**

We observed that the TBY-2 cell growth on galactose medium required a longer lag time period from the start of the cultivation to the sugar uptake phenomenon than that on the other carbon source medium. The lag time was about 5 times longer than the second longest lag time (= maltose medium) in Fig. 10. Furthermore, Figs. 5a and 5b show the micrographs of cultured TBY-2 cells in sucrose medium for 0 day and for 7 days, respectively. These cultured cells were homogeneously proliferating. Figures 6a and 6b show the micrographs of those in the galactose medium for 0 day and for 25 days, respectively. The TBY-2 cells in Fig 6a had a homogeneous distribution the same as those in Fig. 5a. However, those in Fig. 6b formed a number of small aggregates.

Galactose inhibits the growth of roots, coleoptiles and pollen, and promotes leaf abscission. It is a poor carbohydrate source for cultured plant cells [24]. It is toxic to sugarcane cells, however, galactose-adapted cells that grow on 100 mM galactose have been propagated [25]. In sugarcane cultures, UDP-galactose 4-epimarase appears to be a key enzyme for the formation of glycolytic intermediates from galactose. The activity of UDP-galactose 4-epimarase in the cell walls of galactose-adapted cells is ten-fold greater than that in the cell walls of the sucrose cultured cells. On the other hand, the strength of the intercellular contacts should depend on the cell wall structures, which include galactose in pectic polysaccharides [33]. The galactose content in the cell walls of the embryogenic callus of a carrot cell culture is lower than that in the cell walls of the non-embryogenic callus [26].

From these results, it was likely that the TBY-2 cells cultured on galactose medium for a long time period were roughly under similar conditions to the galactose-adapted sugarcane cells, and that UDP-galactose in the cell walls of the TBY-2 cells was transformed to glycolysis as the nutrition resource after it was transformed into UDP-glucose. The galactose content in the cell walls of the TBY-2 cells cultured on galactose medium would have decreased with cultivation. Therefore, it seems that a low galactose content causes strong intercellular contacts as shown in Fig. 6b.

We then examined the sugar uptake phenomena of the cultured cells in galactose medium that formed some aggregates. Furthermore, we studied the specific uptake rates of sugar, which is one of the most important indices characterizing the sugar metabolic kinetics of cells. We then determined the specific uptake rates after quantification of the sugar uptake rate and the cell density according to the method provided in our paper [14, 16, 28] as follows.

Experimentally and spectroscopically, a decrease in absorbance of the culture medium suggests that the suspension cells consumed sugar. In order to determine the sugar and ethanol concentrations in the MS medium using the parameters of the calibration curves shown in Table 2 [15], we showed the time courses for the sugar and ethanol concentrations and the dry cell weight in the culture media during cultivation as shown in Fig. 4. We applied the logistic functions expressed by Eq. (1), which could experimentally represent the time course of $W_{sugar}$ very well [14, 16, 28, 31], ~~in order to make them fit~~ to the fitting ~~of $W_{sugar}$~~ in order to compare ~~with~~ the sugar metabolic kinetics in all the cultivations (Figs. 10 and 11) ~~as previously [14, 16, 28, 31], though it might be improper for only galactose medium that formed some aggregates~~.

$$W_{sugar} = \frac{W_{sugar,\,ini} - W_{sugar,\,fin}}{1 - e^{\left(t - t_{0,\,sugar} \,/\, w_{x,\,sugar}\right)}} + W_{sugar,\,fin} \quad (1)$$

Where, $W$ [g/l] is the uptake amount of the sugar. The subscript sugar indicates the monosaccharide. $t$ [d] is the cultivation time. The parameters $t_0$ and $w_w$, respectively, denote the inflection point of the time course and the time constant ticking its curve. Moreover the subscript *ini* and *fin* indicates the value at the initial and final stage, respectively. The calculated result lies below the linear plot. The sugar uptake rates were calculated by differentiating Eq. (1) with the cultivation time, $t$.

We quantitatively examined the above time course data and their rates. The logistic function expressed by Eq. (1) was applied to the plots of the dry cell weight versus the cultivation time for the same purpose~~reasons~~ as the above-mentioned.

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

This equation is mathematically the same function as Eq. (1) and $X$ [g-dry cell/l] is the dry cell weight. The specific uptake rates were calculated by dividing the uptake rates by the dry cell weight predicted by Eq. (2).

**Figure 7a**

**Figure 7b**

Figure 7a shows the time courses of the specific uptake rates of sugar during the galactose cultivation. We performed the cultivation with galactose 10 times. The peak values of the specific uptake rates widely differed from one another. Every sample required a long lag period from the start of the cultivation to consuming sugar. These lag periods were abnormally long times. The longest lag period in this study was about 80 days.

Next, Fig. 7b 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 while neglecting the cell growth behavior, we attempted to apply the non-dimensional cultivation time [14-16] expressed by $t_{non,x} = (t - t_0)/w_x$ to the data displayed in Fig. 7a. The non-dimensional cultivation time can be calculated using the parameters, $t_0$ and $w_x$, which respectively denote the inflection point of the time course of the cell density and the time constant ticking its curve, so that the relationship between the specific uptake rate and the non-dimensional cultivation time could signify the kinetic sugar uptake characteristics of each sugar cultivation based on the cell growth stage.

Figure 7b shows the influence of the specific uptake rate on the non-dimensional cultivation time. The horizontal axis $(t - t_0) / w_x$ is the non-dimensional cultivation time denoting for the cell growth behavior and was calculated using the

parameters of the logistic function expressed by Eq. (2) fitting the time course of the cell density. We could compare the sugar metabolic kinetics with each of the galactose cultivation sample~~s~~ using the non-dimensional cultivation time.

As shown in Fig. 7b, each peak value was almost uniform regarding the non-dimensional cultivation time, nevertheless, each peak in Fig. 7a varied in cultivation time. Additionally, in Fig. 7b, almost the $(t - t_0) / w_x$ values of the peaks of the specific uptake rate are below zero.

These results indicated the following. Although the time from the start of cultivation to the sugar uptake phenomena was very long and uneven, almost all of the sugar metabolic processes of the sugar uptake based on the cell growth stage did not changed. Moreover, the cultured cells consumed sugar before their growth.

### *3.2.2*

### *Maltose*

No maltose transporter has been identified and the importance of maltose export in vivo is poorly understood [3]. Though the absorbance decreased with little or no change in the spectral pattern during the maltose cultivation, the glucose concentration did not increase at all (Fig. 2b).

Cased on these results, several possibilities were considered for the sugar uptake phenomenon. For example, one possibility is to assume that the glucose uptake rate is significantly faster than the hydrolysis rate of maltose, therefore, the glucose in the medium is not able to be observed. Another possibility is that since the maltose transporter exists in the apoplast, the maltose in the medium is imported from the extracellular to intracellular without hydrolysis.

In contrast, it has been previously reported that sucrose in the medium was hydrolyzed with glucose and fructose by enzymes such as invertase for the plant cells [13-16, 28, 31]. In this study, in order to understand the maltose uptake

phenomenon, the enzymes in the maltose culture medium were assayed as compared with the ones in the sucrose culture medium.

The disaccharide like sucrose and trehalose is hydrolyzed into two monosaccharides in the medium by enzymes such as invertase. This enzyme reaction is expressed by the Michaelis-Menten equation as follows:

$$[E]+[S]\underset{k_2}{\overset{k_1}{\rightleftharpoons}}[ES]\underset{k_4}{\overset{k_3}{\rightleftharpoons}}E+P \quad (3)$$

Here, [*E*], [*S*], [*ES*] and [*P*] are the concentrations of the enzyme, substrate, enzyme-substrate complex and product, respectively. $k_1$, $k_2$, $k_3$ and $k_4$ are the reaction rates.

$$v=\frac{v_{\max}[S]_0}{[S]_0+K_m} \text{ where, } v_{\max}=k_3[E]_0 \quad (4)$$

Where, $v$ is the reaction rate, and $v_{\max}$ is the maximum reaction rate. $K_m$ is the Michaelis constant. $[E]_0$ and $[S]_0$ are the initial enzyme and substrate concentrations, respectively. The rate equation of [*P*] ~~are~~ is given as follows:

$$\frac{d[P]}{dt}=k_3[ES]-k_4[E][P] \quad (5)$$

Where, $t$ is the reaction time. The product is hydrolyzed to a substrate by the enzyme. The relationship between [*P*] and [*S*] is expressed by $[P]=[S]_0-[S]$.

When the reaction is under steady state conditions, the following equation is expressed by Eq. (6).

Where, $X=\frac{k_1-k_4}{(k_1k_3+k_2k_4)[E]_0}$, $Y=\frac{(k_2+k_3)(k_1k_4[S]_0+k_1k_3+k_2k_4)}{(k_1k_3+k_2k_4)^2}$,

$Z=\frac{k_2k_4}{k_1k_3+k_2k_4}[S]_0$

$$t=X([S]_0-[S])-Y\ln\frac{[S]-Z}{[S]_0-Z} \quad (7)$$

Rearranging Eq. (7), we get the following equation.

$$t = X([S]_0 - [S]) - Y\ln\{1 - Z'([S]_0 - [S])\} \quad (8)$$

where $Z' = \dfrac{1}{[S]_0 - Z}$, at $t \to \infty$: $Z' < \dfrac{1}{[S]_0}$

The initial velocity of the reaction, $v_0$ is defined by Eq. (8) as follows;

$$v_0 = \left(\frac{d[S]}{dt}\right)_{t=0} = \frac{1}{X + YZ'} \quad (9)$$

**Figure 8a**

**Figure 8b**

**Figure 9a**

**Figure 9b**

**Table 3**

Figure 8a shows the time courses for the sucrose uptake rate, sucrose concentration and dry cell weight during the sucrose cultivation. Moreover, for the enzyme assay, Fig. 8b displays the time courses of the substrate consumption in the sucrose culture media filtered at 3, 6 and 10 days during incubation. The substrate consumption increased during the incubation in all the three of the filtered culture media. As shown in Fig. 8a, since the sucrose hardly remained in the culture medium at 10 days, the sucrose uptake rate was almost zero. However, this medium had the greatest potential for the hydrolysis of sucrose among of the three culture media in Fig. 8b. These experimental results reconfirmed that the enzyme by which sucrose in the culture medium was hydrolyzed to glucose and fructose was released into the culture medium by the TBY-2 cells.

On the other hand, Fig. 9a shows the time courses for the maltose uptake rate, maltose concentration and dry cell weight. Moreover, in the enzyme assay, Fig. 9b displays the time courses of the substrate consumption in the maltose culture media filtered at 14, 19 and 28 days during incubation. These enzymatic activities

were very low. We had confirmed that invertase (EC 3.2.1.26) and maltase (EC 3.2.1.20) hydrolyzed disaccharide in the culture media to a monosaccharide under the conditions listed in Table 1 (data not shown).

Table 3 shows the initial velocity, $v_{0,}$ of the enzyme assays and the disaccharide uptake rate during cultivation using the sucrose and maltose culture media. In the sucrose culture media, the values of $v_0$ were higher than the sucrose uptake rate. In particular, although the sucrose uptake rate at 10 days was about zero, the value of $v_0$ was the highest at that time. In the maltose culture media, the values of $v_0$ were lower than the maltose uptake rate. Furthermore, these values of $v_0$ were about zero. This result suggested that almost all of the maltose in the culture medium was not hydrolyzed to glucose, but probably imported to the intracellular by the maltose transporter, directly. Therefore, based on the assumption that the suspension cells consume maltose without hydrolysis, we calculated the specific uptake rates of maltose in this study.

### *3.2.3*

### *Comparison of each sugar metabolic kinetics*

## Figure 10

Figure 10 shows the time courses of the specific uptake rate of sugar during the cultivation. The kinetic behavior of the sugar uptake in the culture is dependent on the type of sugar used. Each peak time of the specific uptake rates as shown in Fig. 10 differed from each carbon source. However, these cultivation conditions in this study could be classified into the early and later groups. The early peak time group with the specific uptake rate consisted of the sucrose (5 days), fructose (6 days) and glucose (9 days) cultivation. These sugars are usually used as the carbon source by plant cells. The later group consisted of mannose (13 days), trehalose (15 days), maltose (16 days) and galactose (50 days, Fig. 7a, sample No. 7) cultivation. In particular, the peak time of the specific uptake rate for the galactose cultivation was about 3 times later than that for the maltose cultivation.

Sucrose and trehalose as disaccharides were consumed by the TBY-2 cells after hydrolysis of the monosaccharide. On the one hand, the peak time of the specific uptake rate for the sucrose cultivation was earlier than that for both the glucose and fructose cultivations. On the other hand, the peak time of the specific uptake rate for the trehalose cultivation was later than that for the glucose cultivation. Additionally, in the case when glucose and fructose were both present in the culture medium, namely, the sucrose cultivation, glucose was primarily utilized then followed by fructose. However, the peak time of the specific uptake rate for the fructose cultivation was earlier than that for the glucose cultivation.

## Figure 11

Figure 11 shows the influence of the specific uptake rate on the non-dimensional cultivation time. The horizontal axis $(t - t_0) / w_x$ in Fig. 11 is the non-dimensional cultivation time as well as that in Fig. 7b. We compared the sugar metabolic kinetics with each of the sugar cultivation conditions using the non-dimensional cultivation time. Each $(t - t_0) / w_x$ value for the peak was below zero. This indicated the cultured cells consumed sugar before their growth without being related to the types of sugars in the medium. We reported that this characteristic in the sucrose, glucose and fructose cultivation was supposed to be that of the TBY-2 cells [15]. In particular, these values for the maltose and galactose cultivation were more negative than that of the other sugar cultivations. These results indicated that the time from the start of sugar uptake phenomenon to cell growth on the maltose and galactose cultivation was longer than that on the other sugar cultivations.

# 4

# Conclusions

The influences of the difference in the molecular structures of the monosaccharides and kinds of glucose links in the disaccharides on the kinetic sugar uptake phenomenon were studied by the FT-IR/ATR method and enzyme assay.

In the monosaccharide, mannose [21-23] and galactose [24, 25] which are known to be toxic to plant growth, were consumed by the TBY-2 cells in this study. In general, TBY-2 cells are known to homogeneously proliferate. However, for only the galactose cultivation, each cell formed a number of small aggregates with proliferation. Furthermore, we observed that the cell growth on the galactose medium required a longer lag time period from the start of the cultivation to the sugar uptake phenomenon than that on the other carbon source media.

In the disaccharide, sucrose and trehalose were consumed with hydrolysis of the monosaccharide, such as glucose and fructose, by TBY-2 cells. However, maltose would be consumed without the hydrolysis of glucose, but imported to the intracellular by a maltose transporter. Therefore, we calculated the specific uptake rates based on the assumption that the suspended cells consume maltose without hydrolysis. Additionally, the TBY-2 cells cannot consume lactose.

Base on these results, the specific uptake rates of sugar by the suspended plant cells can easily and continuously be obtained by applying the logistic function to the predicted sugar concentrations and dry cell weight in the media during cultivation. Accordingly, we suggested the importance of understanding the influence of the monosaccharide and disaccharide species in the culture media on the kinetic sugar uptake phenomena. The order of the peak time of the specific uptake rate was sucrose (5 days), fructose (6 days), glucose (9 days), mannose (13 days), trehalose (15 days), maltose (16 days) and galactose (50 days) cultivation. As a result, the lag time from the start of the cultivation to consuming sugar was different from that for one other cultivation. In particular, the peak time for the galactose cultivation was about 3 times longer than that for the maltose cultivation. However, based on the non-dimensional cultivation time for the cell growth behavior, we found common characteristics in all the sugar types of cultivation; the TBY-2 cells consumed sugar before cell growth and then the ethanol concentration in the culture media increased just after cell growth. Additionally, for the maltose and galactose cultivation, it was suggested that the time from the start of the sugar uptake phenomena to cell growth was longer than that for the other sugar cultivation.

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## Figure Legends

Fig. 1. ATR spectra of (a) monosaccharide (glucose, fructose, mannose and galactose), (b) disaccharide (sucrose, trehalose, maltose and lactose) and ethanol in MS medium

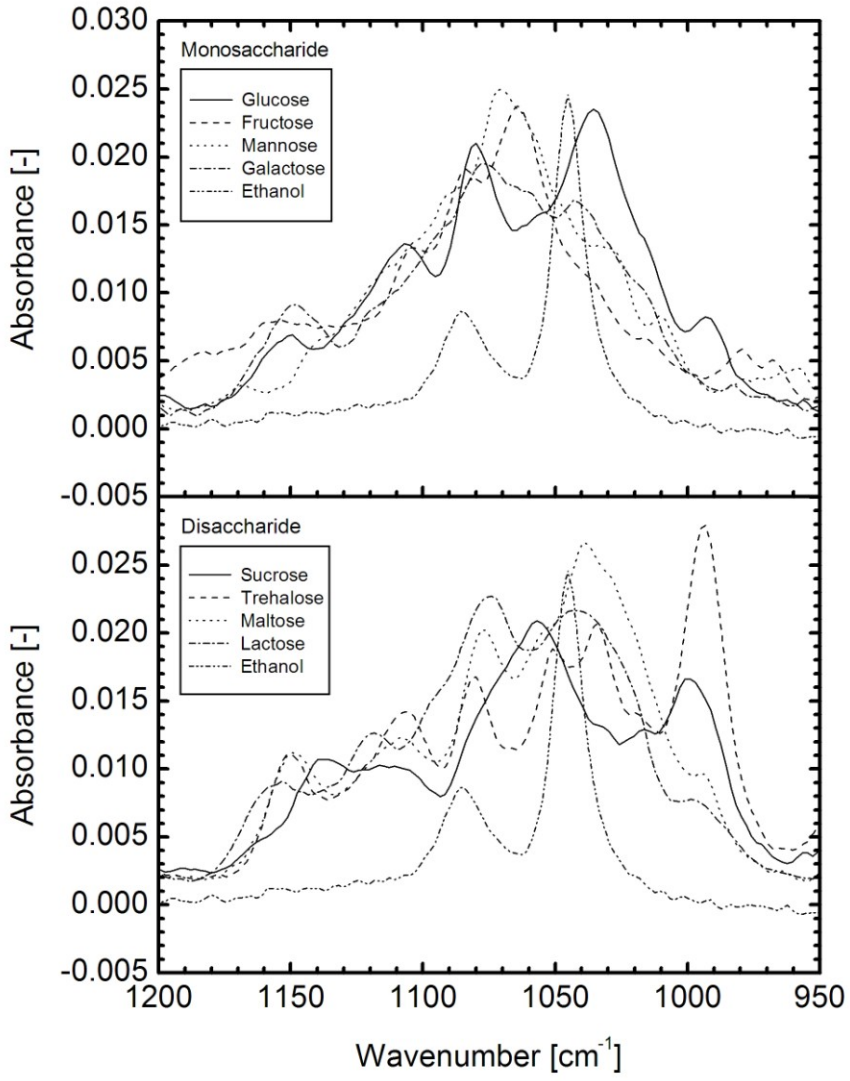


Fig. 2. Time behavior of the ATR spectra of the medium during (a) glucose and (b) galactose cultivation.

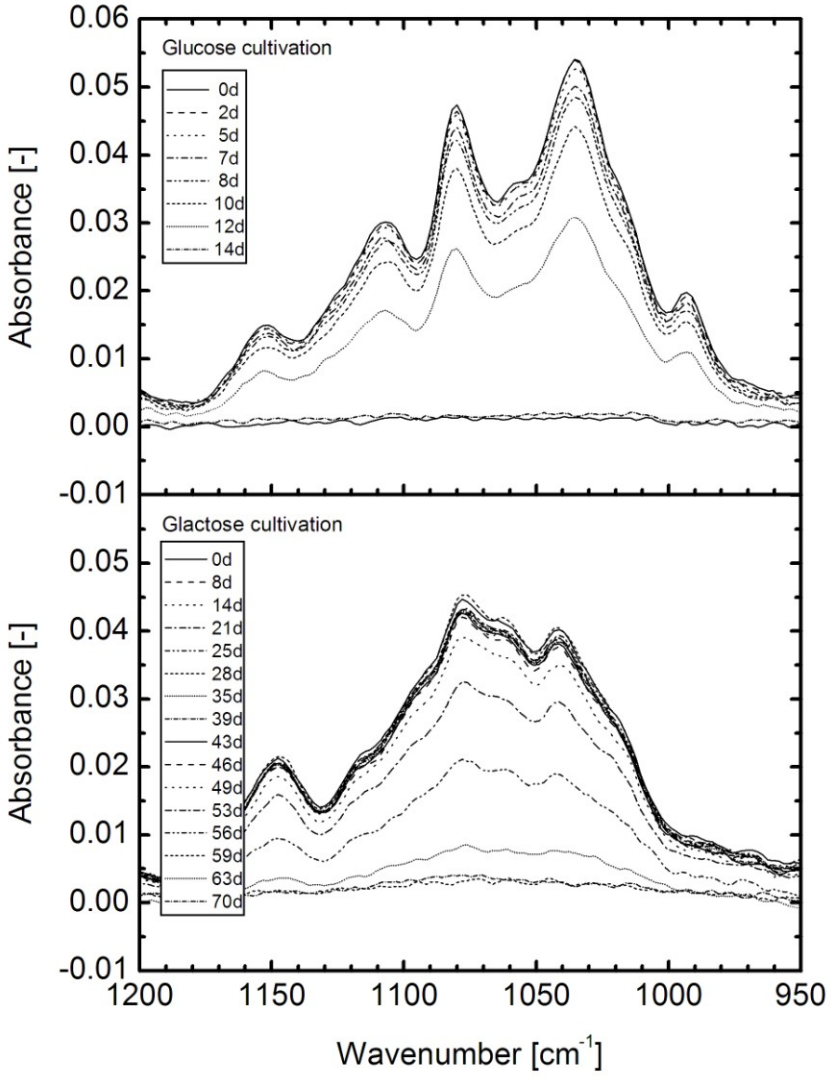


Fig. 3. Time behavior of the ATR spectra of the medium during (a) trehalose and (b) maltose cultivation.

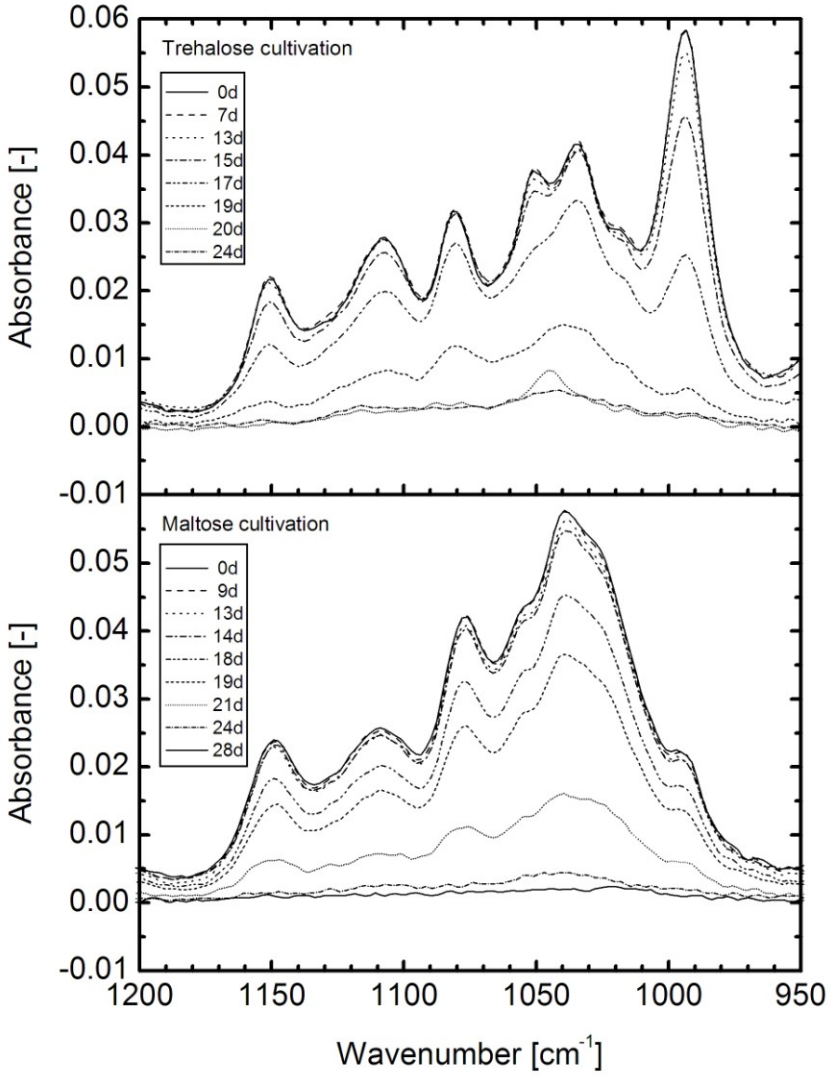


Fig. 4. Time courses of sugar concentration, ethanol concentration, and dry cell weight during trehalose cultivation.

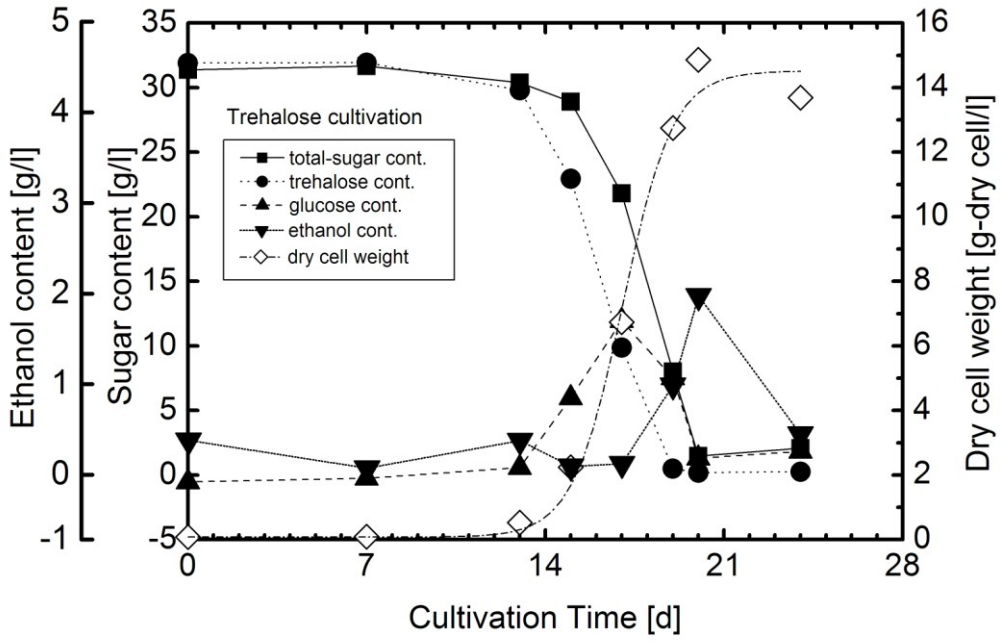
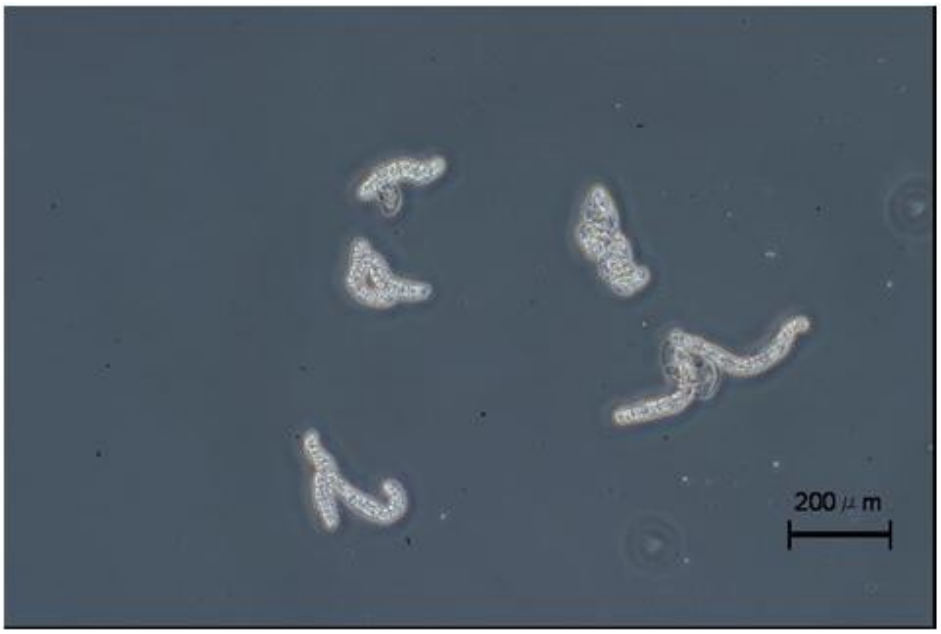
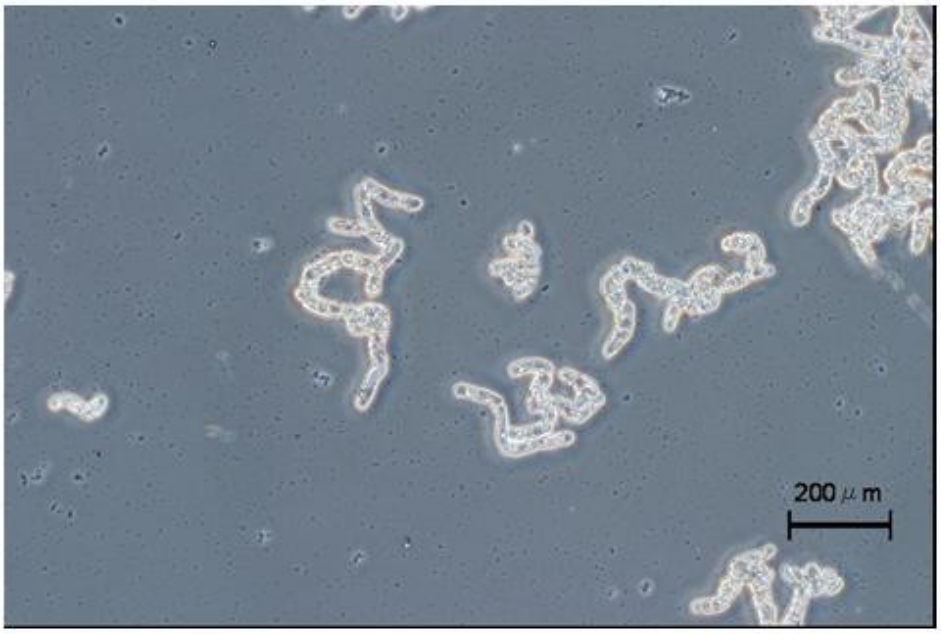


Fig. 5. Photograph of the TBY-2 cells in sucrose cultivation (a) for 0 day and (b) for 7 days.

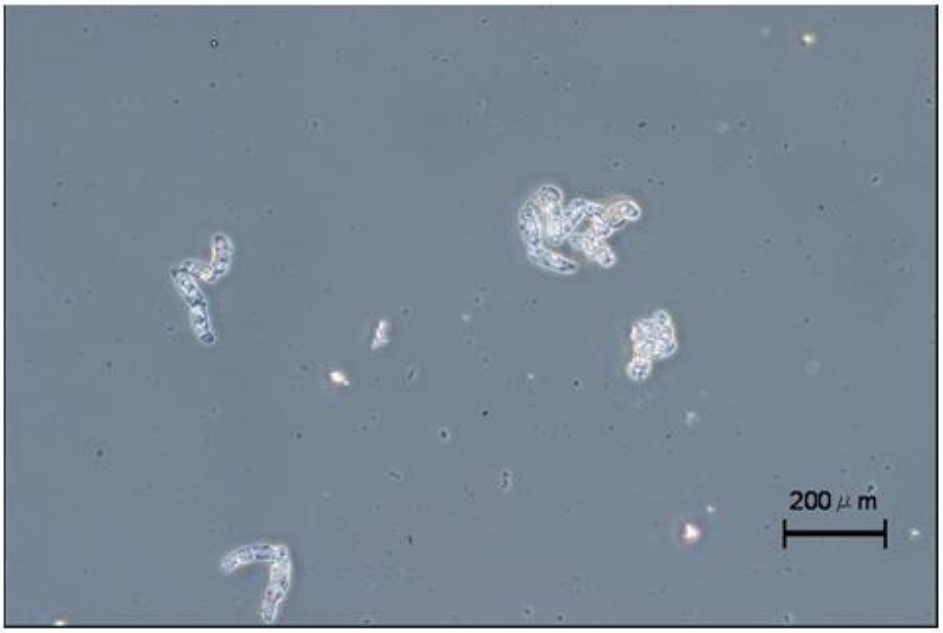



(a)

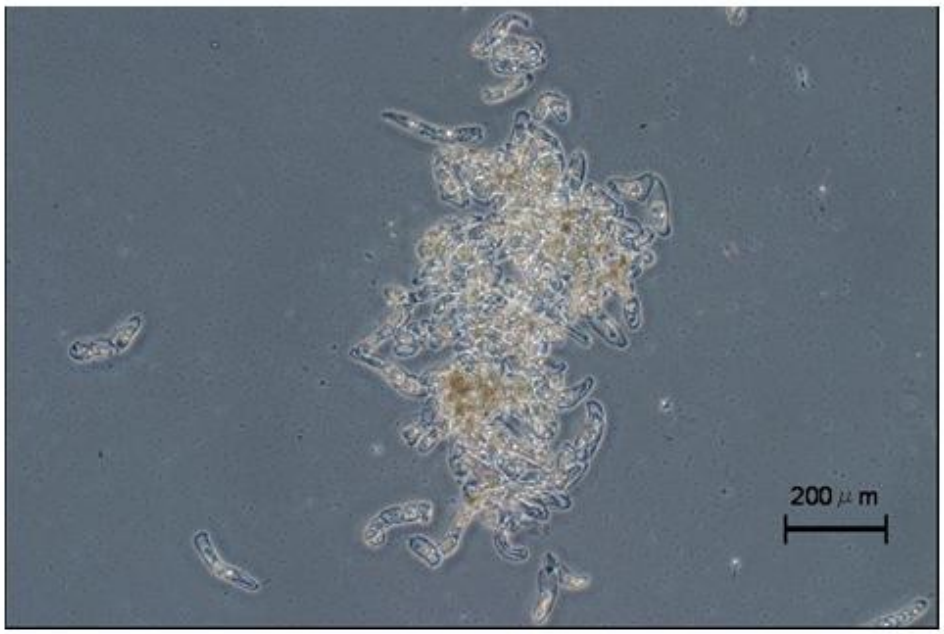



(b)

Fig. 6. Photograph of the TBY-2 cells in galactose cultivation (a) for 0 day and (b) for 25 days.




(a)




(b)

Fig. 7. Specific uptake rates. (a) Time courses of specific uptake rates of sugar during galactose cultivation; (b) Non-dimensional time courses of specific uptake rates of sugar during galactose cultivation.

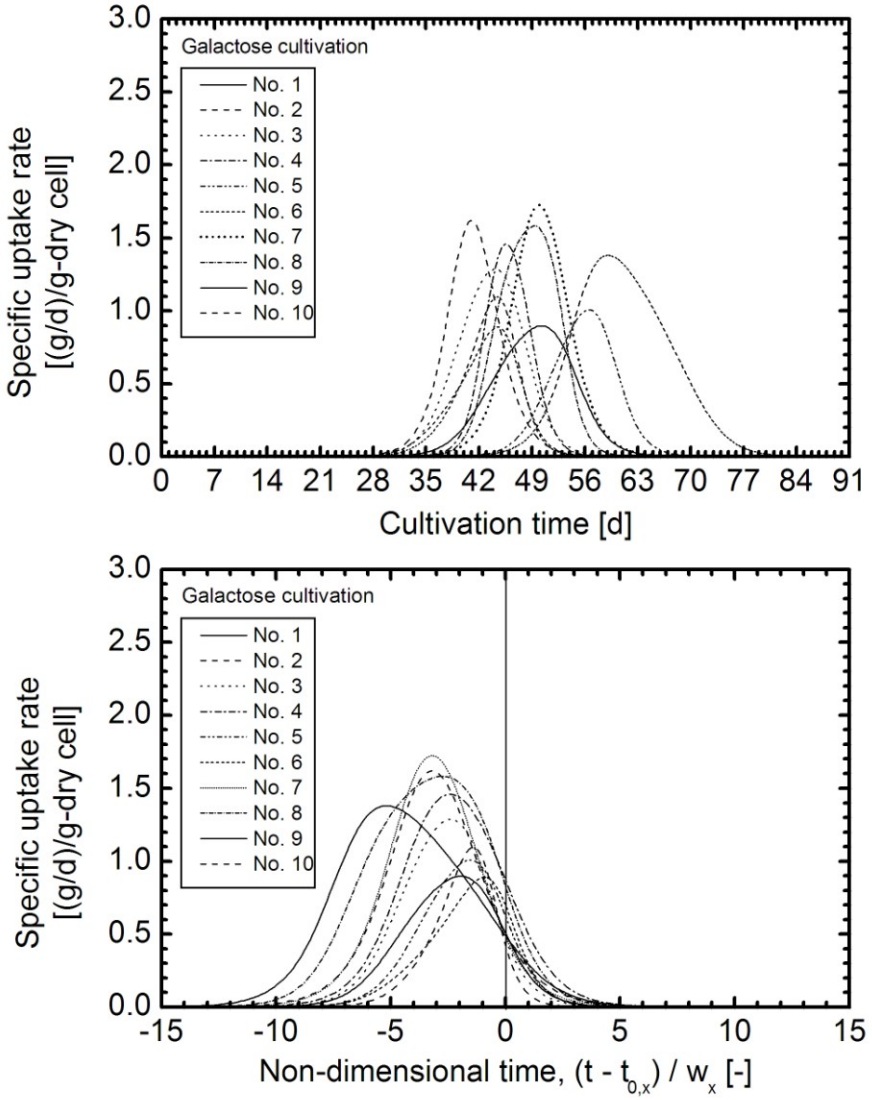


Fig. 8. Enzyme assay (Sucrose). (a) Time courses of sucrose uptake rate, sucrose concentration and dry cell weight during sucrose cultivation; (b) Time courses of substrate consumption in the sucrose culture medium filtered during incubation.

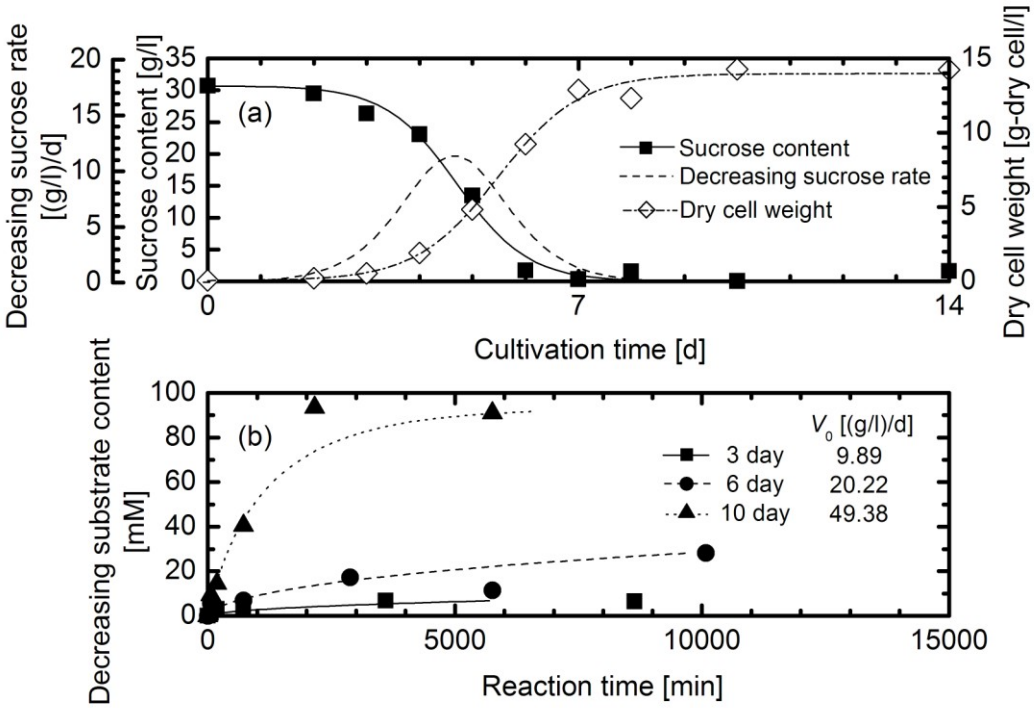


Fig. 9. Enzyme assay (Maltose). (a) Time courses of maltose uptake rate, maltose concentration and dry cell weight during maltose cultivation; (b) Time courses of substrate consumption in the maltose culture medium filtered during incubation.

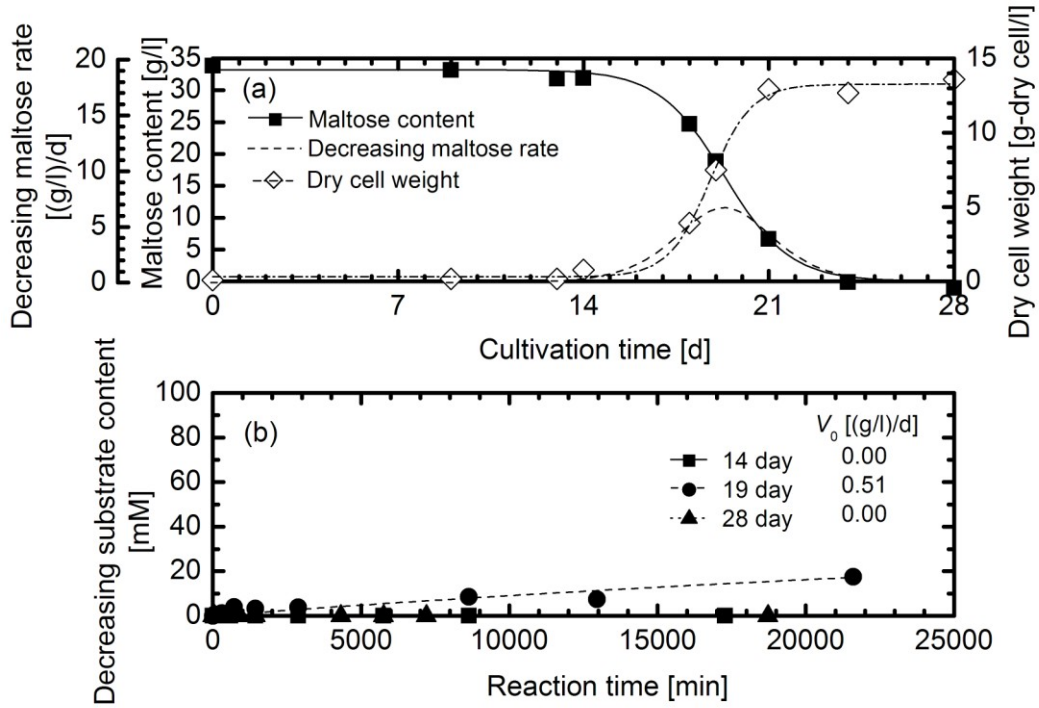


Fig. 10. Time courses of specific uptake rates of sugar during monosaccharide and disaccharide cultivation.

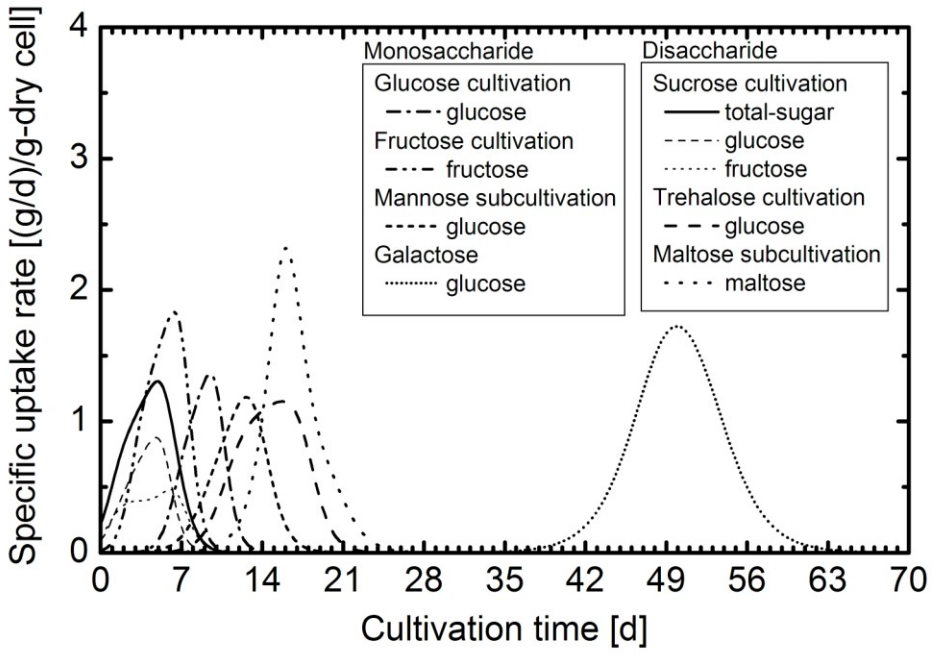
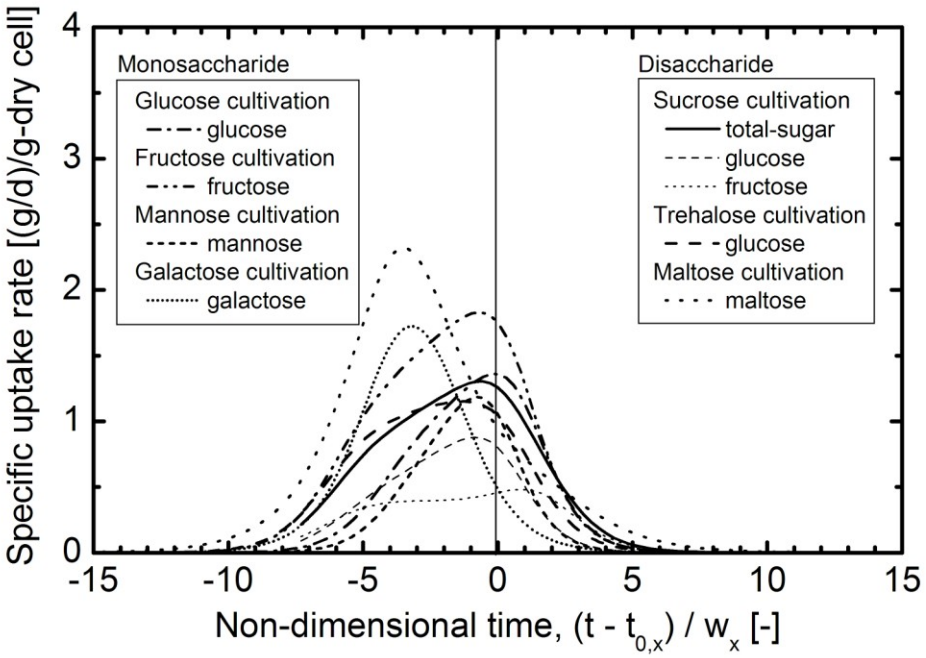


Fig. 11. Non-dimensional time courses of specific uptake rates of sugar during monosaccharide and disaccharide cultivation.



## Tables

**Table 1.** Enzyme assay setup conditions

| | Sucrose culture medium | Maltose culture medium |
|---|---|---|
| Day | 3, 6, 10 | 14, 19, 28 |
| Sugar concentration | 30.00 g/l | 30.00 g/l |
| pH | 5.0 | 6.9 |
| Temperature | 303 K | 313 K |

**Table 2.** Fitting parameters and correlations coefficients of calibration curves.

| Metabolite | Wavenumber [$cm^{-1}$] | Fitting parameter, *a* [M] | Fitting parameter, *b* [-] | Correlation coefficient, *r* [-] |
|---|---|---|---|---|
| Glucose | 1036 (Glc) | 0.2938 | 0.0009 | 0.999 |
| | 1065 (Fru) | 0.1783 | 0.0016 | 0.999 |
| | 993 (Tre) | 0.1032 | 0.0005 | 0.999 |
| | 1055 (Suc) | 0.1985 | 0.0014 | 0.999 |
| | 1080 (Glc) | 0.2433 | 0.0022 | 0.998 |
| | 1149 (Mal) | 0.0737 | 0.0017 | 0.999 |
| | 1045 (EtOH) | 0.2360 | 0.0014 | 0.999 |
| Fructose | 1036 (Glc) | 0.1247 | 0.0009 | 0.998 |
| | 1065 (Fru) | 0.2781 | 0.0016 | 0.999 |
| | 1055 (Suc) | 0.2134 | 0.0014 | 0.999 |
| | 1045 (EtOH) | 0.1439 | 0.0014 | 0.999 |
| Mannose | 1070 (Man) | 0.2836 | 0.0021 | 0.999 |
| | 1045 (EtOH) | 0.1800 | 0.0014 | 0.999 |
| Galactose | 1076 (Gal) | 0.2237 | 0.0027 | 0.999 |
| | 1045 (EtOH) | 0.2011 | 0.0014 | 0.999 |
| Sucrose | 1036 (Glc) | 0.3144 | 0.0009 | 0.999 |
| | 1065 (Fru) | 0.4276 | 0.0016 | 0.999 |
| | 1055 (Suc) | 0.4873 | 0.0014 | 0.999 |
| | 1045 (EtOH) | 0.3917 | 0.0014 | 0.999 |

| | | | | |
|---|---|---|---|---|
| Trehalose | 1036 (Glc) | 0.4413 | 0.0009 | 0.999 |
| | 993 (Tre) | 0.6235 | 0.0005 | 0.999 |
| | 1045 (EtOH) | 0.3663 | 0.0014 | 0.999 |
| Maltose | 1080 (Glc) | 0.4002 | 0.0022 | 0.999 |
| | 1149 (Mal) | 0.2298 | 0.0017 | 0.999 |
| | 1045 (EtOH) | 0.5226 | 0.0014 | 0.999 |
| Lactose | 1076 (Lac) | 0.4790 | 0.0027 | 0.999 |
| | 1045 (EtOH) | 0.4614 | 0.0014 | 0.998 |
| Ethanol | 1036 (Glc) | 0.0417 | 0.0009 | 0.994 |
| | 1065 (Fru) | 0.0118 | 0.0016 | 0.982 |
| | 1070 (Man) | 0.0131 | 0.0021 | 0.942 |
| | 1076 (Gal) | 0.0166 | 0.0027 | 0.968 |
| | 1055 (Suc) | 0.0327 | 0.0014 | 0.996 |
| | 993 (Tre) | - | 0.0005 | - |
| | 1080 (Glc) | 0.0270 | 0.0022 | 0.997 |
| | 1149 (Mal) | - | 0.0017 | - |
| | 1045 (EtOH) | 0.1177 | 0.0014 | 0.999 |

**Table 3.** Initial velocity ($v_0$) of the enzyme assays and disaccharide uptake rate during cultivation using the sucrose and maltose culture media.

| **Kind of cultivation** | **Cultivation period** | **Initial velocity $V_0$ [(g/l)/d]** | **Disaccharide uptake rate (during cultivation) [(g/d)/d]** |
|---|---|---|---|
| Sucrose cultivation | 3 days | 9.89 | 3.10 |
| | 6 days | 20.22 | 5.06 |
| | 10 days | 49.38 | 0.02 |
| Maltose cultivation | 14 days | 0.00 | 0.50 |
| | 19 days | 0.51 | 5.12 |
| | 28 days | 0.00 | 0.06 |