# MIR Spectroscopic Analysis on Sugar Metabolic and Ethanol Productive Kinetics of Suspension TBY-2 and Rice Cells Pre-Cultured in Various Media

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## Abstract

The influence of sugars in pre-cultivation media suspended plant cells on the kinetics of the sugar uptake and the ethanol production were 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 and *Oryza sativa* L., Japonica, cv. Nipponbare (rice) cells respectively, in pre-culture and culture media, which had various types of glucose, fructose, sucrose or glucose-fructose mixtures. The results confirmed the kinetic differences between the TBY-2 cells and rice cells. These results suggested that the TBY-2 cells consumed sugar before growth and the rice cells consumed sugar after growth, moreover, that the ethanol content increased just after cell growth was activated based on the non-dimensional cultivation time for the cell growth behavior.

*Keywords Mid-Infrared Spectroscopy; Metabolic Kinetics; Sugar; Ethanol; Tabacum cells; Rice cells; Pre-cultivation*

# 1

# Introduction

Recently, plant-cell cultivation technology has been developing and played a wide 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 scientific and engineering fields. Most of the biochemical reactions of the glycolysis within plant cells are understood [1] and there are a number of studies

on sugar transport, sugar sensing and signaling in plants [2, 3]. However, the kinetic transport phenomena of nutrimental sugars from a liquid medium to suspension plant cells are not completely understood.

The application of spectroscopy, especially in the infrared region, to the above measurement has high potential. Incidentally, in parallel developments of Fourier transform infrared (FT-IR) spectrometers and attenuated total reflection (ATR) techniques [4, 5], the spectroscopic method using the FT-IR equipped with an ATR accessory (FT-IR/ATR) provided substantial potential as a quantitative analytical tool for liquids. Therefore, various FT-IR/ATR spectroscopic methods are under development for the analysis of bioproducts [6-10]. We also studied the quantitative analysis of sugars in aqueous solutions using the FT-IR/ATR method [11-13].

The MIR spectrum using the FT-IR/ATR method has shown that the spectral additivity was applicable for sucrose, glucose and fructose in the MS media, experimentally. In addition, we examined the potential of the sugar content determination in culture media 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 to analyze the sugar uptake rate of the suspension TBY-2 cells [14]. 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 [15].

The characteristics of cellular size, cycle, growth, sugar metabolic kinetics and alcohol production vary for each plant cell. In a partial or total anaerobic environment, the TBY-2 and *Oryza sativa* L., Japonica, cv. Nipponbare (rice) cells are known as plant cells that produce ethanol and release it into the culture medium [16]. However, since the TBY-2 cells have a homogeneous distribution in the culture media, almost all of them can respire easily in aerobic conditions. Consequently, in such conditions, alcohol fermentation rarely occurs. The rice cells tend to form a number of small aggregates that have a heterogeneous distribution in the culture media. Therefore, since the intracellular condition of the rice cell aggregate changes from the aerobic environment to an anaerobic

environment, alcohol fermentation occurs easily. Thus, the sugar metabolism in plant cells relates not only to the sugar uptake phenomena, but also to the ethanol production. Therefore, Hashimoto et al. [17] attempted to measure the nutrient and product contents in the culture medium using the FT-IR/ATR method, simultaneously. As a result, evaluation of the sugar contents using the FT-IR/ATR method under assumption of ethanol existence was superior in quantitative analysis to that under assumption of no ethanol in the culture media of not only rice cells (high ethanol content), but also TBY-2 cells (low ethanol content). In this study, we determined the metabolite contents in the MS and the R2S mediums using the FT-IR/ATR method under an assumption of ethanol existence on the basis of the studies developed by Hashimoto et al. [17].

The objective of the present work was to study the influence of sugar spices 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. Furthermore, we aimed to understand and compare the common and varying points of sugar metabolic kinetics and the relationships between cell growth behavior and ethanol production for each plant cell simultaneously.

# 2

# Materials and Methods

## 2.1

## Plant Cell Cultivation

This study was carried out with the TBY-2 and rice cells, which were essentially sub-cultured under the same condition as that described in the previous paper [14, 15, 18, 19].

In the TBY-2 cell cultivation, 1.5 ml of the 7-days-old inoculums in the Murashige-Skoog (MS) [20] medium was inoculated into 95 ml of each fresh medium in a 300 ml flask, and the pre-cultivation was carried out at 298 K on the

rotary shaker (150 rpm) in the dark for 14 days. Those pre-cultured cells were then inoculated and cultured in the above conditions for 14 days.

In the rice cell cultivation, 0.05 g (dry-cell weight) of 14-day-old inoculums in the R2S [21-23] medium was inoculated into 120 ml of each sugar medium in a 300 ml flask and pre-cultivation was carried out at 298 K on the rotary shaker (125 rpm) in the dark for 14 days. The pre-cultured cells were then inoculated and cultured using the method above for 21 days. Before pre-cultivation and cultivation, the suspension-cultured cells were washed in the MS or R2S medium without sugar. At specified time intervals, the metabolite contents and the dry cell weight in the culture medium were measured.

## Culture Media

# Table

The MS and R2S media were used as standard mediums, while sucrose was used as the typical carbon source for the TBY-2 cell and rice cell cultivations. A glucose, fructose or binary mixture was substituted respectively for sucrose in the other media. Three types (sucrose, glucose and fructose) of media were prepared at pre-cultivation, and four types (plus glucose-fructose mixture) of media at cultivation (Table 1). The experimental patterns for each plant cell were 3 x 4 ways. The sugar and the other components in the medium were prepared as separate solutions and each sugar was solved into pure water. Next, 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

## Analysis

The sugars and ethanol contents in the culture medium were determined by the FT-IR/ATR method as described previously [14, 17, 19]. 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 was compared to the turbidity of the culture medium using a UV-VIS-NIR scanning spectrophotometer as previously described [14, 15, 18, 19].

# 3

# Results and discussion

## 3.1

## ATR spectral analysis of culture medium

We applied the MIR spectroscopic method developed by Hashimoto et al. [14, 17] to the metabolite content determination. The outline for this method was shown below.

Focusing on the wavenumbers around 1036, 1065, 1055 and 1045 cm-1, we made calibration curves between the absorbance and the metabolite concentrations in the medium, and the following straight line fits all curves.

$$A_{metabolite,wavenumber} = a_{metabolite,wavenumber} C_{metabolite,wavenumber} + b_{metabolite,wavenumber} \quad (1)$$

Here, $A$ and $C$ are respectively the absorbance and the concentration of the metabolite at the wavenumber. The slope, $a$, of the straight line relates to the kind of metabolite, and the value of $b$ theoretically shows the absorbance of the medium without metabolite. All correlation coefficients were greater than 0.92 [17]. Hence, each metabolic concentration is calculated by the following matrix equation.

$$\begin{pmatrix} A_{1055} \\ A_{1035} \\ A_{1065} \\ A_{1045} \end{pmatrix} = \begin{pmatrix} a_{suc,1055} & a_{glc,1055} & a_{fru,1055} & a_{EtOH,1055} \\ a_{suc,1035} & a_{glc,1035} & a_{fru,1035} & a_{EtOH,1035} \\ a_{suc,1065} & a_{glc,1065} & a_{fru,1065} & a_{EtOH,1065} \\ a_{suc,1045} & a_{glc,1045} & a_{fru,1045} & a_{EtOH,1045} \end{pmatrix} \begin{pmatrix} C_{suc} \\ C_{glc} \\ C_{fru} \\ C_{EtOH} \end{pmatrix} + \begin{pmatrix} b_{1055} \\ b_{1035} \\ b_{1065} \\ b_{1045} \end{pmatrix} \quad (2)$$

Here, the subscript *suc*, *glc*, *fru* and *EtOH* respectively mean sucrose, glucose, fructose and ethanol. Then, each metabolite concentration is calculated by using the parameters of calibration curves at four wavenumbers.

For the validity of the above MIR spectroscopic method, Hashimoto et al. [17] reported that the spectral additivity was experimentally observed for 4 metabolic components in MS and R2S media, respectively. Additionally, the excellent agreement between the actual metabolite contents and those estimated using the MIR spectroscopic method were observed, and their correlation coefficients were all higher than 0.99.

## Figure 1

Figure 1 (a), (b) show the time behavior of the ATR spectra, which were obtained after spectral subtraction of water, during the sucrose (MS and R2S) cultivation of the TBY-2 and rice cells, respectively. At the beginning of the cultivation with each plant cell (0 d), the ATR spectra had the same characteristics as that of sucrose in Fig.1. In the finger print region from 1300 to 900 $cm^{-1}$, many peaks were observed such as the CO and C-OH stretching modes, which complicatedly overlap each other [11, 12, 24], especially in the region from 1200 to 950 $cm^{-1}$. The peaks depend on the sugar structure and on the interaction between the sugar molecules and their environments. In addition, ethanol had a significant sharp peak around 1045 $cm^{-1}$, which did not overlap with the sugar peaks [19]. During the cultivation of each plant cell, the absorbance decreased almost over the finger print region with the changes in the spectral patterns. The percentage of decreasing absorbance in the cultured TBY-2 cells was different from that in the cultured rice cells. The spectrum of the TBY-2 cells at 5 d and that of rice cells at 6 d were quite different from each spectrum at 0 d. Those spectra had qualitatively the same spectral characteristics as those of the glucose and fructose mixture in the medium, respectively. Around 1045$cm^{-1}$ appeared a sharp peak identifying ethanol, the sharp peaks after 10 d in the cultured rice cells were observed as shown in Fig.1b, and the slight peaks after 7 d in the cultured TBY-2 cells as shown in Fig.1a. The ethanol production of the cultured rice cells was different from that of the cultured TBY-2 cells. Finally, the ATR spectrum of the culture

media for each plant cell was almost identical to that of each medium without sugar. In comparison to the period of time when sugar in the medium was consumed completely with each plant cell, one in the TBY-2 cells was shorter than the other in the rice cells.

Thus, the spectral change shown in Fig.1 gives evidences for a biological process in which ethanol is produced after sucrose in the medium is hydrolyzed and consumed by the cells. However, the biological process was different for each plant cell. In particular, the amount of ethanol production greatly differed between the cultured cells of TBY-2 and rice. We recognized that the rice cells produced much more ethanol than the TBY-2 cells.

Here, we describe the data processing procedure for the sucrose cultivation instances. In order to determine the metabolite contents in the culture media, the calibration curves between the absorbance and metabolite contents, developed using the MIR spectroscopic method, were applied to the spectral data shown in Fig.1. The calibration curves used in the absorbance at the spectral peaks around 1036, 1065, 1055 and 1045 $cm^{-1}$ are attributed to the glucose, fructose, sucrose and ethanol spectra, respectively.

## Figure 2

Figure 2 shows the time courses for the sugar and ethanol contents evaluated by the method above and the dry cell weight in the culture media during the sucrose cultivation. In this study, we applied to determine the four metabolic contents in each medium using the parameters of the calibration curves [17, 19]. Figure 2 exhibits that the medium initially contained sucrose alone as the carbon source. Shortly after the start of the cultivation, the sucrose content immediately decreased while glucose and fructose successively increased. The glucose and fructose contents in the cultured TBY-2 cells reached peak values around 5 d and decreased afterward (Fig.2a), while the cultured rice cells peaked around 6 d (Fig.2b). This indicated that sucrose in the medium is hydrolyzed to glucose and fructose, which are consumed by the plant cell. The dry cell weight increased with a decrease in the total sugar content and reached the maximum value when the

sugars were almost completely consumed. In Fig. 2b, the ethanol content with the cultivation of the rice cells was negligible at the initial stage and became successively higher and indicated the highest value at 12 d just after the dry cell weight had reached a maximum value. Next, the ethanol content decreased rapidly with a slight decrease in the dry cell weight and was almost equal to zero at the final stage of cultivation. On the other hand, in Fig. 2a, the ethanol content with the cultivation of the TBY-2 cells was less than that with the cultivation of the rice cells. The difference in the two types of plant cells indicated the characteristics of each plant cell.

## 3.2

## Sugar metabolic kinetics

### *3.2.1*

### *Calculation of specific uptake rates*

Next, we then 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 [18], as follows.

Sucrose is synthesized with glucose and fructose, which relates to the hydrolysis of sucrose in the medium by enzymes such as invertase [25, 26]. ~~Additionally, Fig. 1 experimentally and spectroscopically suggests that the suspension cells consumed sucrose after hydrolysis.~~ Consequently, if the suspension cells were cultivated in the sucrose media, we would need to calculate the uptake of the glucose and fructose from the medium to the cells, using the following equations.

$$W_{sugar} = C_{sugar,ini} + \left(C_{suc,ini} - C_{suc}\right)\frac{M_{sugar}}{M_{suc}} - C_{sugar} \quad (3)$$

Here, $C$ [g/l] is the sugar content. $M$ [g/mol] is the molecular weight. $W$ [g/l] is the uptake amount of sugar. The subscript *sugar* indicates glucose or fructose and the subscript *suc* indicates sucrose. Moreover the subscript *ini* indicates the value at the initial stage. Equation (3) is derived from the assumption that the suspension cells consume sucrose after hydrosis. ~~This result is the amount of sugar uptake, namely $U$ [g/l].~~

We applied the logistic functions expressed by Eq. (4) in order to make *Wsugar* fit.

$$W_{sugar} = \frac{W_{sugar,ini} - W_{sugar,fin}}{1 + e^{\left(t - t_{0,sugar} / w_{w,sugar}\right)}} + W_{sugar,fin} \quad (4)$$

In Eq. (4), $t$ [d] is the cultivation time. The parameters *t0* and *ww*, respectively, mean the inflection point of the time course and the time constant ticking its curve. The subscript *fin* indicates the value at the final stage. The calculated result lies beneath the linear plot. The glucose and fructose uptake rates were calculated by differentiating Eq. (4) with the cultivation time.

We quantitatively examined the above time course data and their rates. The logistic function expressed by Eq. (4) was applied to the plots of the dry cell weight against the cultivation time.

$$X = \frac{X_{ini} - X_{fin}}{1 + e^{\left(t - t_{0,x} / w_x\right)}} + X_{fin} \quad (5)$$

This equation is mathematically the same function as Eq. (4) and $X$ [g-dry cell·l] is the dry cell weight. The specific uptake rates were calculated by dividing the uptake rates with the dry cell weight predicted by Eq. (5).

### *3.2.2*

### *The comparison of TBY-2 cell and Rice cell on specific uptake rates in actual cultivation time*

**Figure 3**

## Figure 4

Figure 3 shows the time courses of specific uptake rates of sugar with the TBY-2 cells, and Figure 4 exhibits that with the rice cells. The peak values of the specific uptake rates as shown in Fig. 3 and 4 differed from each other. The values of the TBY-2 cells were higher than that of the rice cells. This was supposed to be the difference in sugar consumption ability for each plant cell.

### *Characteristic of TBY-2 cell*

In Fig. 3, the peak time of the specific uptake rate for the glucose pre-cultivation was earlier than that for the other sugars pre-cultivation (except for fructose cultivation) without being related to the types of sugar in the culture medium. In case glucose and fructose were both present in the medium, glucose was primarily utilized and followed by fructose. However, the peak time of the specific uptake rate for fructose cultivation was earlier than that for the glucose cultivation without being related to the types of sugar in the pre-culture medium.

### *Characteristic of Rice cell*

The kinetic behavior of the sugar uptake in the culture is dependent on the type of sugar used. In particular, the significant differences in the time indicating maximum specific uptake rates among all pre-cultivation conditions were observe for glucose cultivation (Fig.4 (b)).

### 3.2.3

### The comparison of TBY-2 cell and Rice cell on specific uptake rates in non-dimensional cultivation time

## Figure 5

# Figure 6

As shown in Fig. 3 and 4, the influences of sugar in the pre-cultivation medium, in particular, reflected the kinetic sugar uptake behavior of the test organisms. In addition, Fig. 2 also indicates that the cell growth rate was effected by the sugar in the pre-cultivation medium. Thus, Fig. 3 and 4 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 attempted to apply the non-dimensional cultivation time [15, 17] of $t_{non,\,x}$, expressed by Eq. (4), to the data displayed in Fig. 3 and 4.

$$t_{non,x} = (t - t_0) / w_x \quad (4)$$

The above non-dimensional time can be calculated using the parameters, $t_0$ and $w_x$ in Eq. (3), 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 sugar rate and the non-dimensional cultivation time could signify the kinetic sugar uptake characteristics of each plant cell based on the cell growth stage.

Fig. 5 shows the influence of the specific uptake rate on the non-dimensional cultivation time in the TBY-2 cells and Fig. 6 shows that in the rice cells. The horizontal axis $(t - t_0) / w_x$ of Fig. 5 and 6 is the non-dimensional cultivation time standing for the cell growth behavior and was calculated with the parameters of the logistic function expressed by Eq. (3) fitting the time course of the cell density. We could compare the sugar metabolic kinetics with the TBY-2 cells and the rice cells, or with the different cultivation conditions using the non-dimensional the culture time.

## *Characteristics in common between Rice cell and TBY-2 cell*

Each peak time of the specific uptake rate as shown in Fig. 3 or 4 was varied in cultivation time, however as shown in Fig. 5 or 6 this was almost uniform in the non-dimension cultivation time. The time from the start of cultivation to the sugar

uptake phenomena was changed by the influence of sugar spices in the pre-culture media. However, the sugar metabolic process of sugar uptake based on the cell growth stage was not changed. This was experimentally confirmed by the TBY-2 cells and by rice cells.

### *Characteristic of each plant cell*

Figure 5 displays the $(t - t_0) / w_x$ values of the peaks of the specific uptake rates with the TBY-2 cells below zero. Contrarily, Fig. 6 displays that the rice cells were above zero. The trend in Fig. 5 was found in all types of cultivation with the TBY-2 cells. Therefore, this characteristic was supposed to be that of the TBY-2 cells. Moreover the trend in Fig. 6 was found in all types of cultivation with the rice cells. Therefore this characteristic was supposed to be that of the rice cells. These results indicated that the TBY-2 cells consumed sugar before growth and that the rice cells consumed sugar after growth. Since we aim the specific uptake rates of sugars based on the non-dimensional cultivation time for cell growth behavior, we find that each plant cell has the original characteristic of the sugar metabolic kinetics

## 3.3

## Ethanol content

### *The comparison of TBY-2 cell and Rice cell on ethanol content in non-dimensional cultivation time*

**Figure 7**

**Figure 8**

Figure 7 and 8 show non-dimensional courses of ethanol content during cultivation in the TBY-2 cells and rice cells, respectively. Comparing the peak

value of the ethanol content with the TBY-2 cells and the rice cells, those of the rice cells were higher than that of the TBY-2 cells. These results indicated that the ethanol production kinetics of the TBY-2 cell was different from that of the rice cell. In Fig. 8, the peak values of the ethanol content were various. However, the peaks had very similar shapes. Moreover, the $(t - t_0) / w_x$ values of the peaks of the ethanol content were generally above zero. These experimental results found that the rice cells had those trends; the ethanol content increased just after cell growth was activated, without relation to sugar in the pre-culture or culture media. On the other hand, in Fig. 7, the peak values of the ethanol content were very small. As a result, we do not clearly find a trend in the TBY-2 cells. Since we aim the ethanol content for cell growth behavior based on the non-dimensional cultivation time, we find the original characteristics of the ethanol production kinetics of each plant cell.

# 4

# Conclusion

In conclusion, the consumption rate for each sugar and the behavior of the ethanol production could be measured simultaneously and accurately by the FT-IR/ATR method. In addition, by applying the logistic function to the predicted sugar contents and dry cell weight in the media during cultivation, the specific uptake rates of sugar by the suspension plant cells can be easily and continuously obtained. Accordingly, we suggested the importance of understanding the influence of the sugar in pre-cultivation and cultivation for the sugar metabolic kinetics. Moreover, we were able to determine the common or overlapping characteristic for the two types of plant cells and the specific metabolic features exhibited by each plant cell. When the ethanol production behavior phenomena in each plant cell were compared, the ethanol contents during cultivation were significantly different. The amount of ethanol production with the TBY-2 cells was very small, so this phenomenon of the TBY-2 cell could not be to be measured. On the other hand, when the ethanol production behavior phenomena with the rice cells based on the non-dimensional cultivation time for cell growth behavior was compared with every cultivation of prepared condition, the common

characteristic in all types of cultivation was seen between the cell growth behavior and the ethanol production: the ethanol content increased just after cell growth activation. This indicated the influence of the type of sugar in the culture media on the kinetic sugar uptake phenomena, but was hardly the influence of that on the ethanol production phenomena.

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## Figure Legends

**Fig.1.** Time behavior of the ATR spectra of the medium component during sucrose cultivation (a) in TBY-2 and (b) rice cells

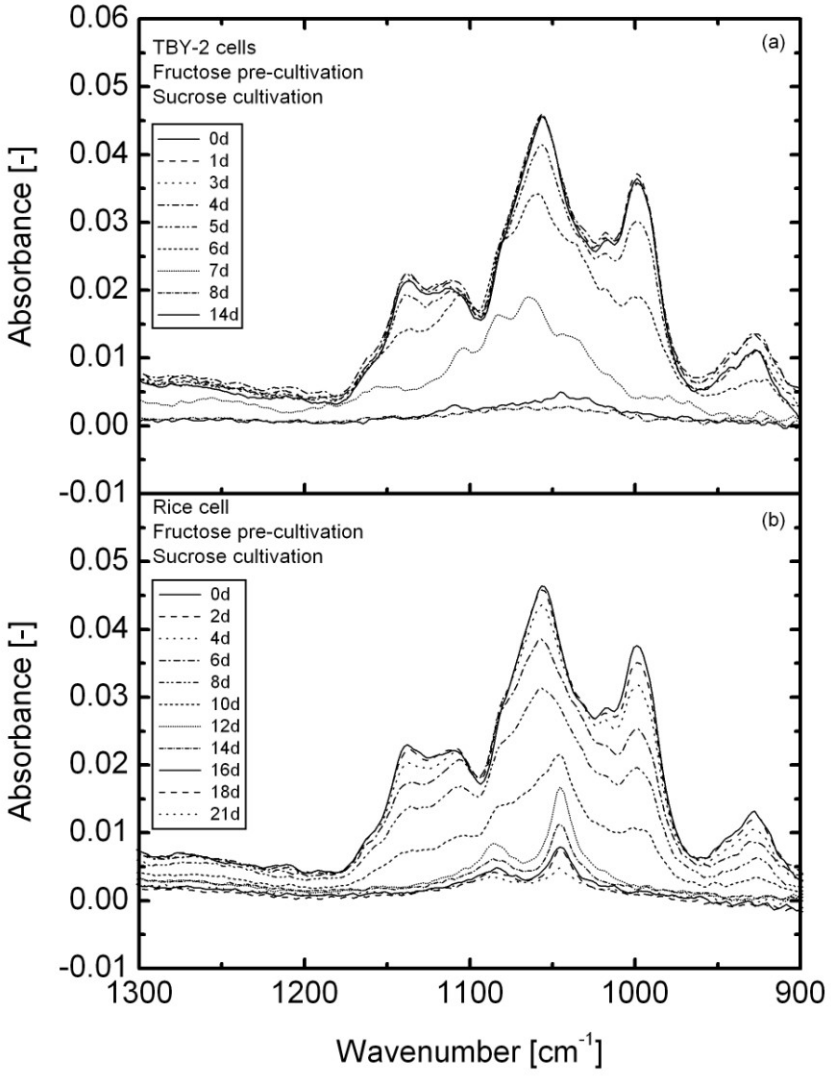


**Fig.2.** Time courses of sugar content, ethanol content, and dry cell weight during sucrose cultivation (a) in TBY-2 and (b) Rice cells

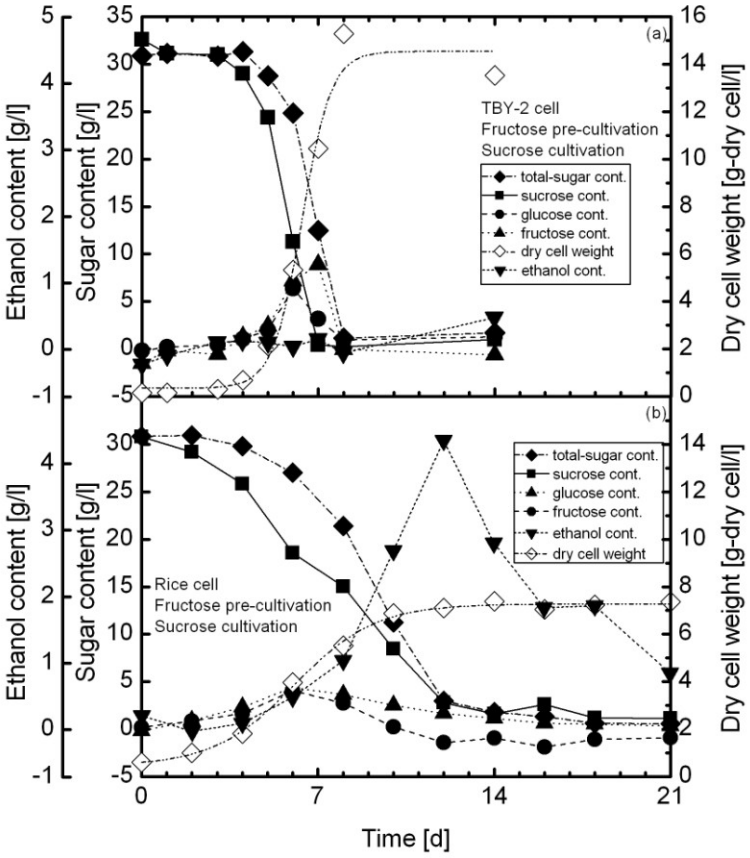


**Fig.3.** Time courses of specific uptake rates of sugar (a) during sucrose cultivation, (b) glucose cultivation, (c) fructose cultivation, and (d) glucose - fructose mixture cultivation in TBY-2 cells

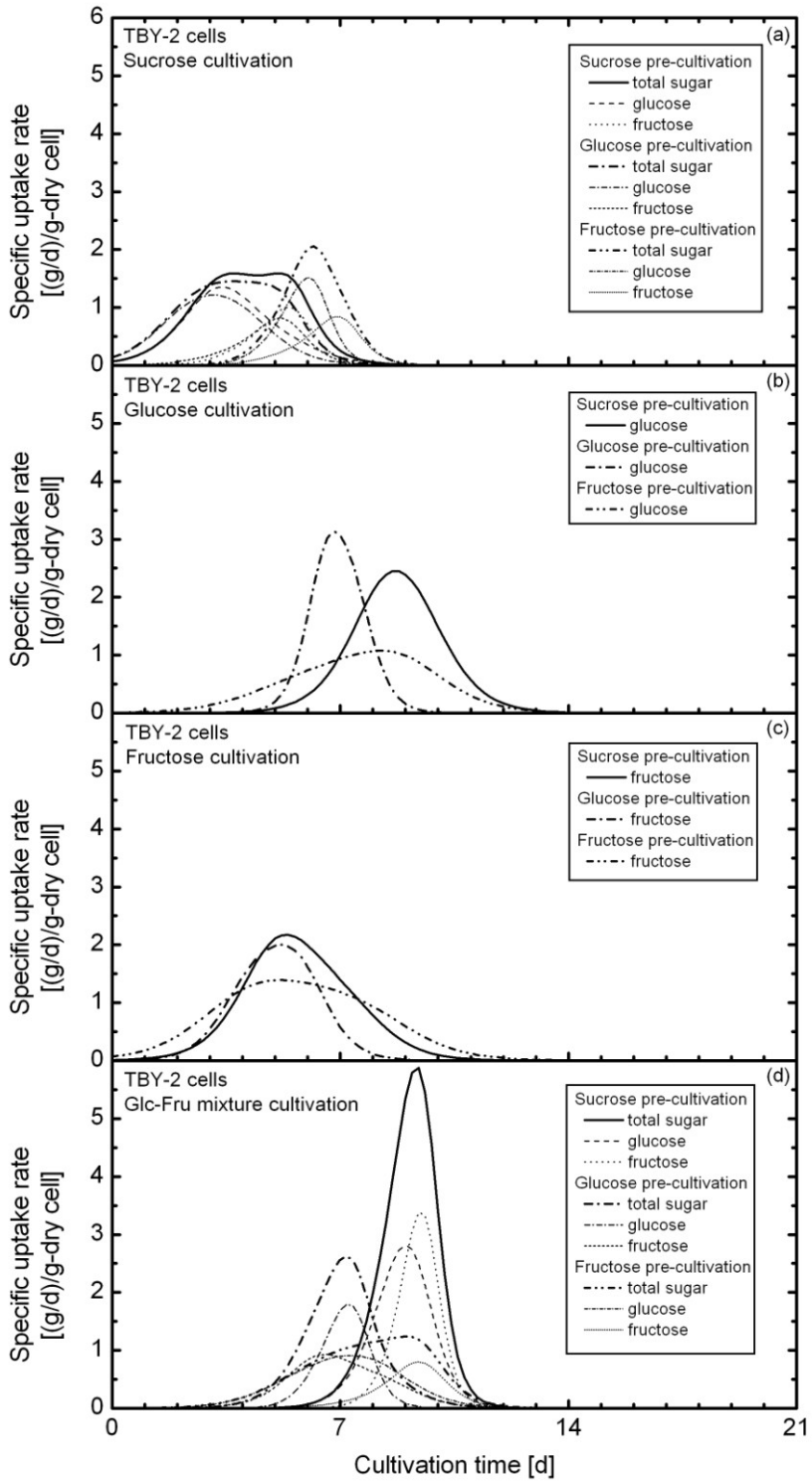


**Fig.4.** Time courses for specific uptake rates of sugar (a) during sucrose cultivation, (b) glucose cultivation, (c) fructose cultivation and (d) glucose - fructose mixture cultivation in rice cells

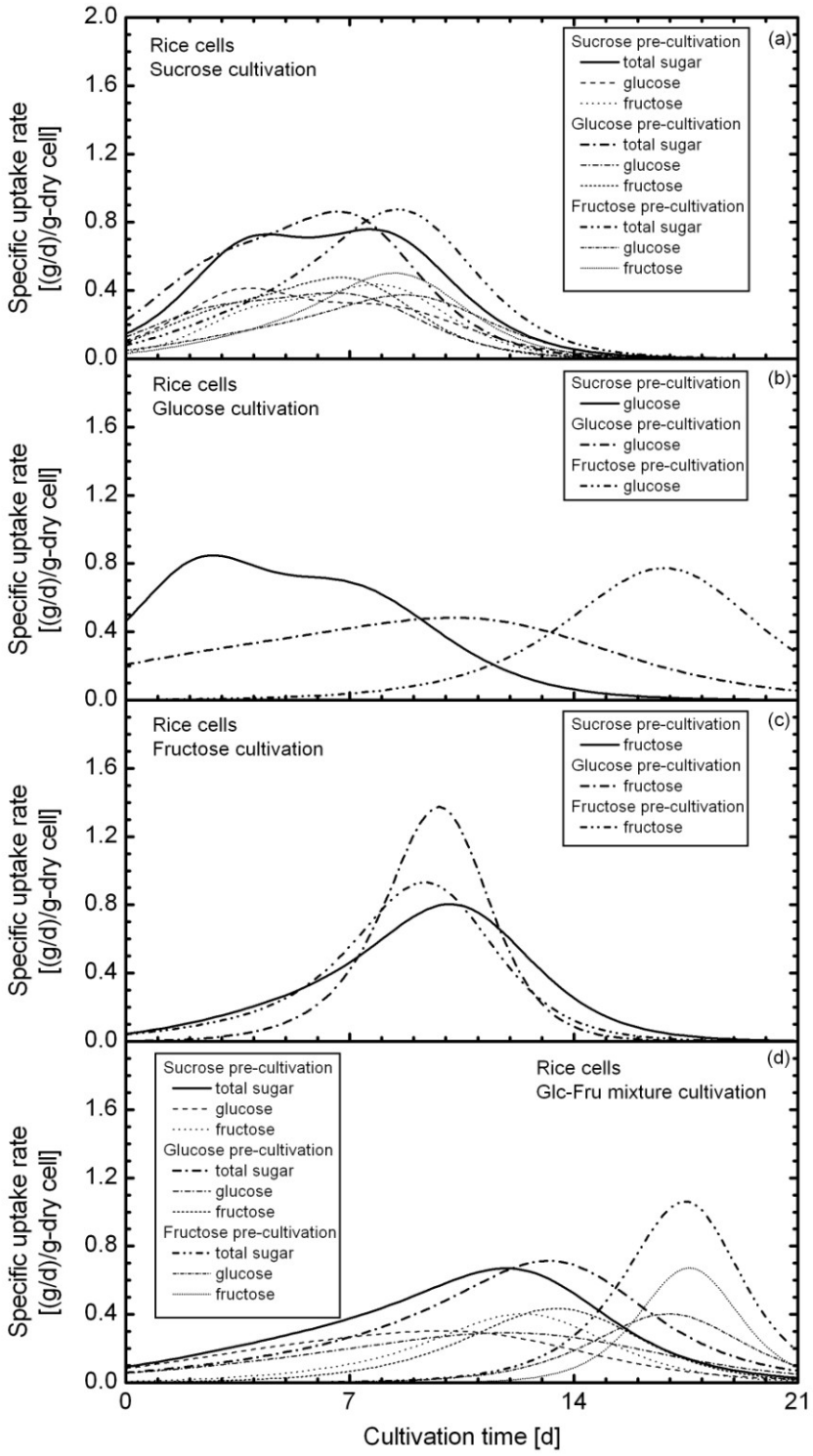


**Fig.5.** Non-dimensional time courses for specific uptake rates of sugar (a) during sucrose cultivation, (b) glucose cultivation, (c) fructose cultivation and (d) glucose - fructose mixture cultivation in TBY-2 cells

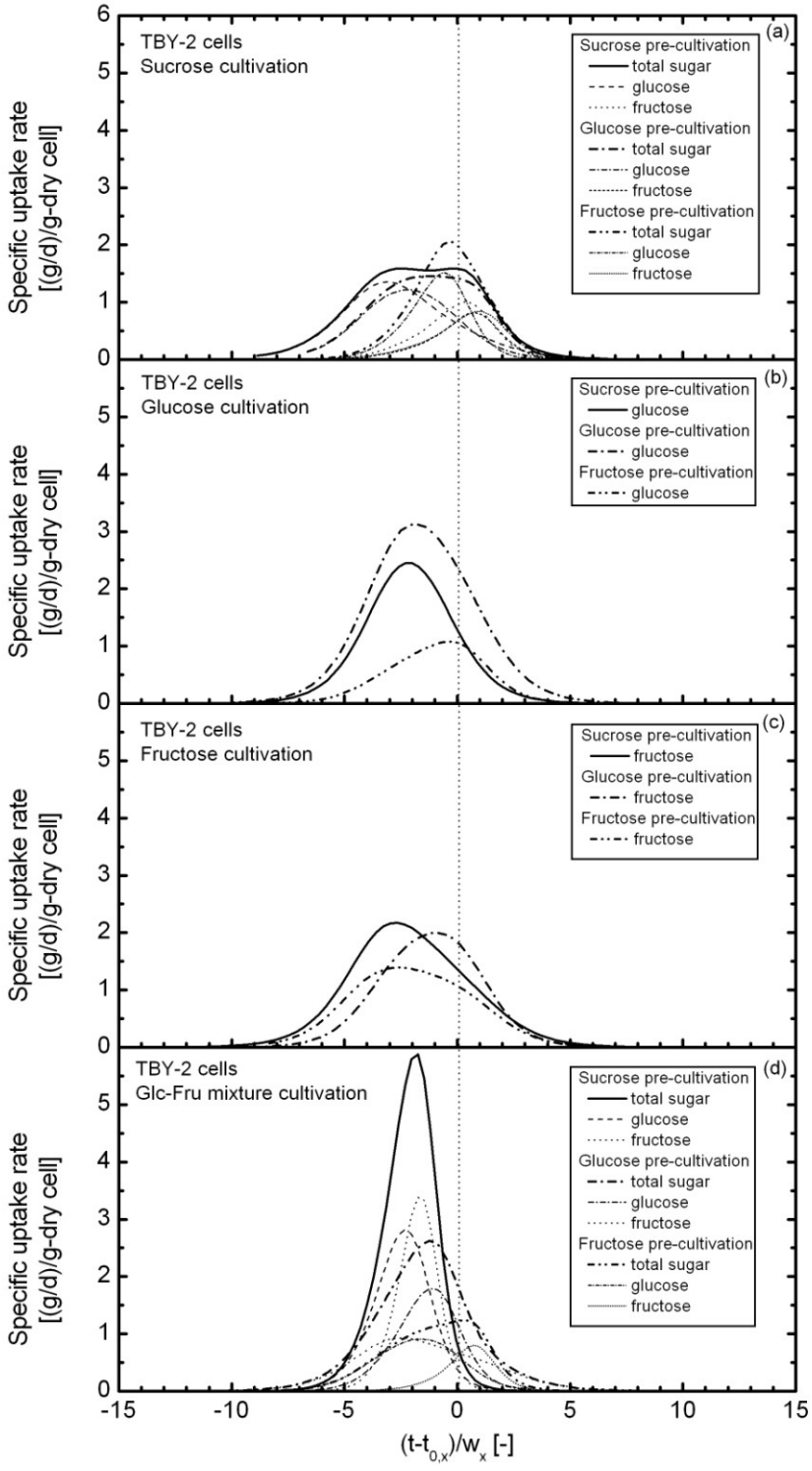


**Fig.6.** Non-dimensional time courses of specific uptake rates of sugar (a) during sucrose cultivation, (b) glucose cultivation, (c) fructose cultivation and (d) glucose - fructose mixture cultivation in rice cells

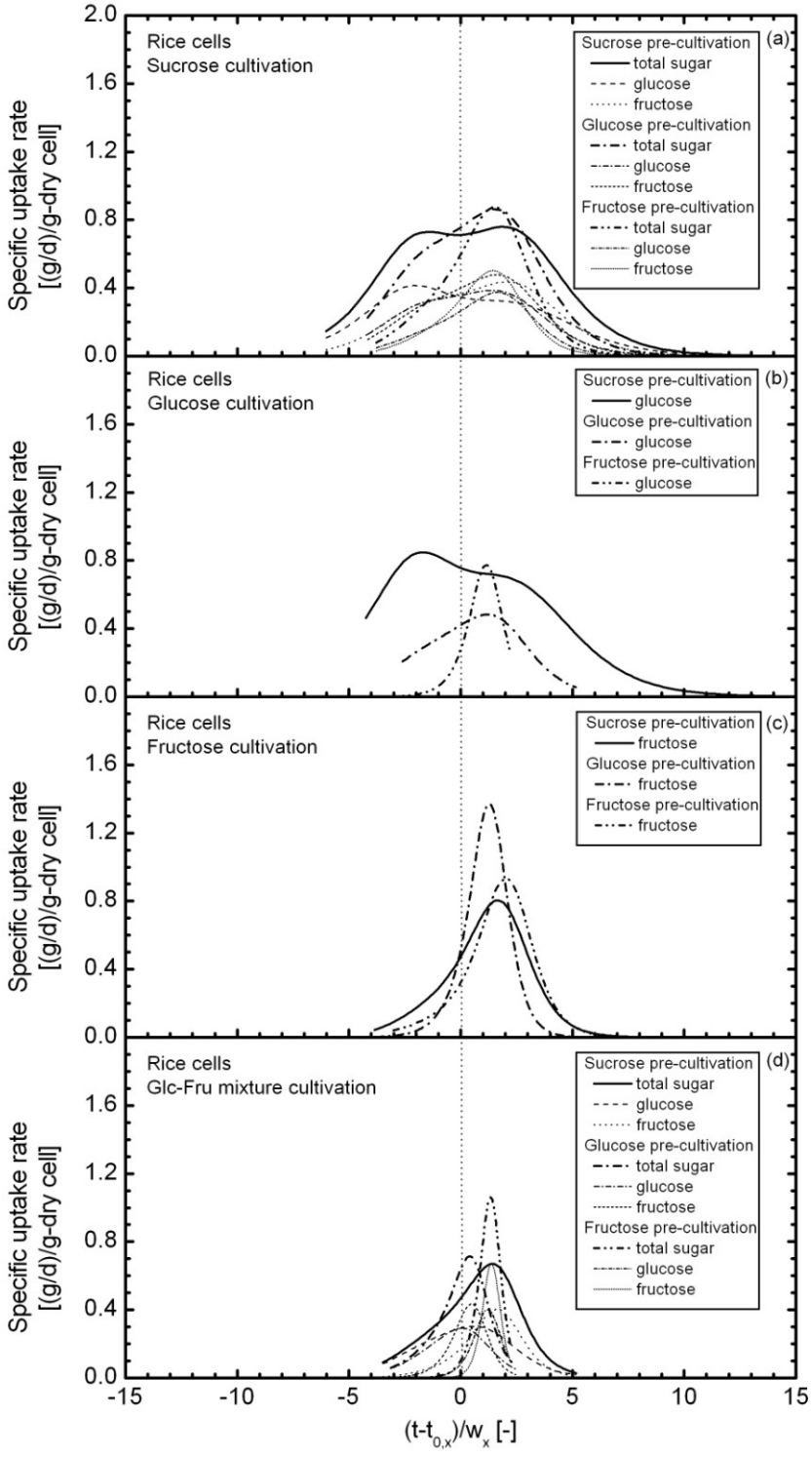


**Fig.7.** Non-dimensional time courses of ethanol content (a) during sucrose cultivation, (b) glucose cultivation, (c) fructose cultivation and (d) glucose - fructose mixture cultivation in TBY-2 cells

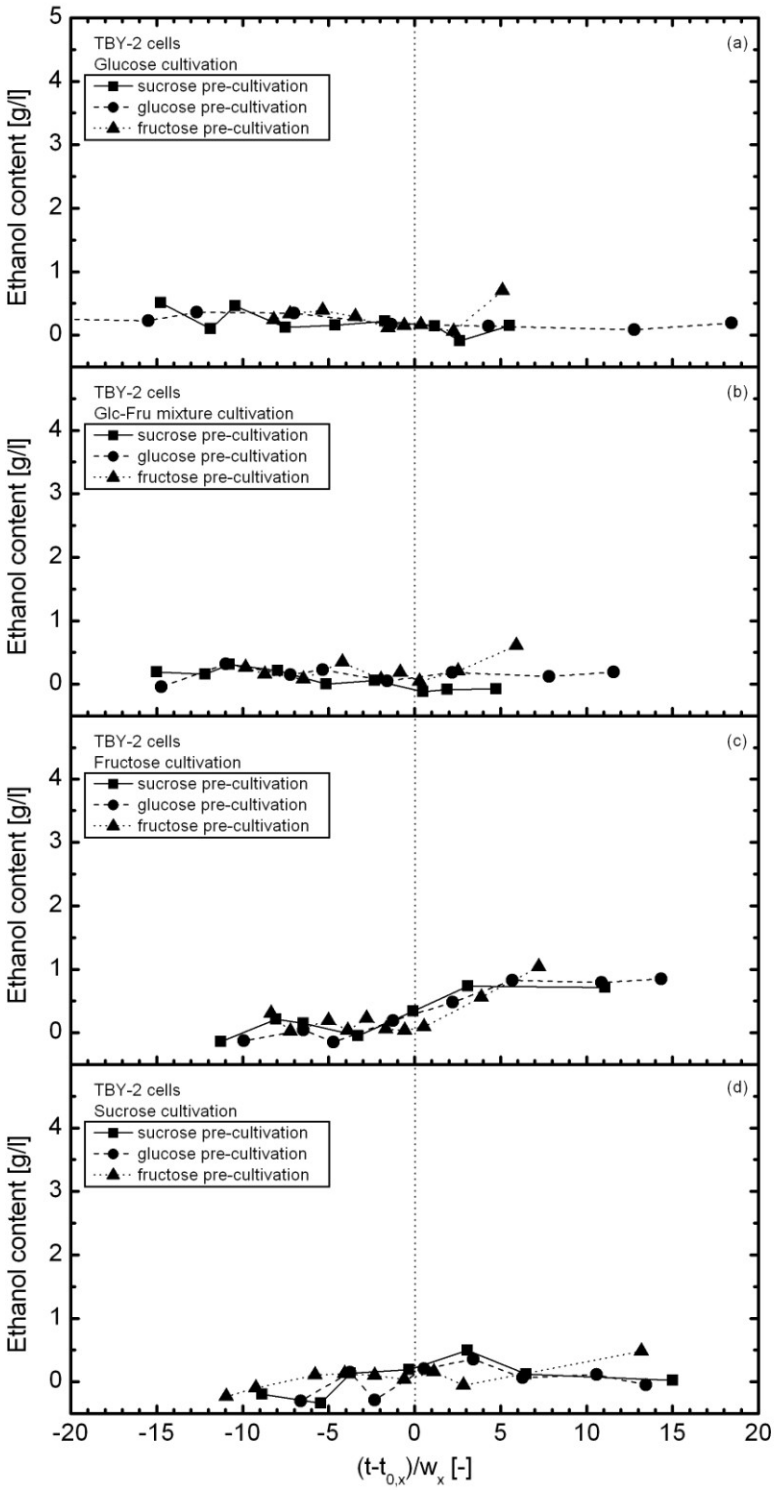
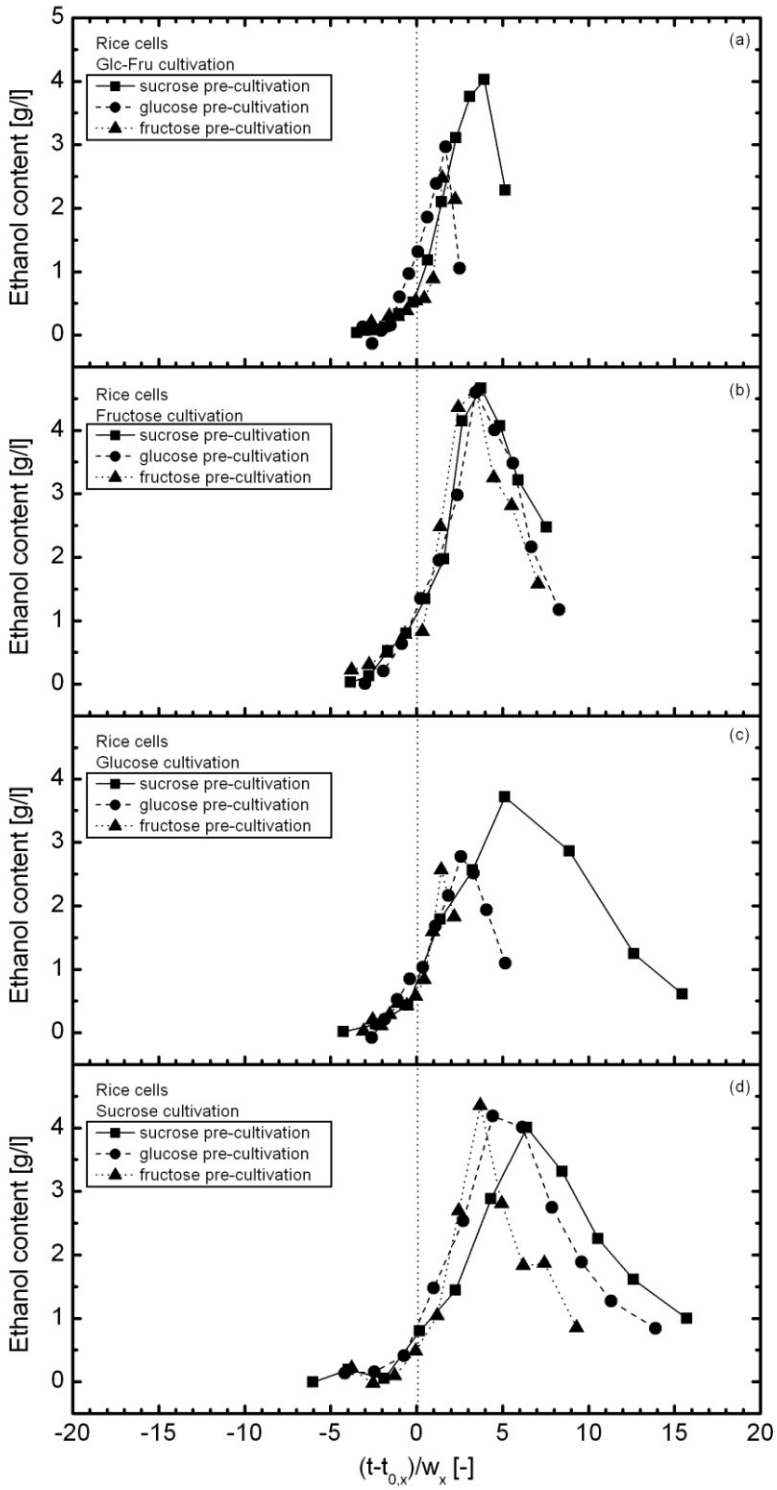


**Fig.8.** Non-dimensional time courses of ethanol content (a) during sucrose cultivation, (b) glucose cultivation, (c) fructose cultivation and (d) glucose - fructose mixture cultivation in rice cells




## Tables

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

| Culture media | Sugar concentration [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 |