Simple and Rapid Determination of Metabolite Content in Plant Cell Culture Medium Using an FT-IR/ATR Method

ATSUSHI HASHIMOTO*, ATSUSHI YAMANAKA, MIKIHITO KANOU, KENICHI NAKANISHI, TAKAHARU KAMEOKA

Department of Sustainable Resource Sciences, Faculty of Bioresources, Mie University, 1515 Kamihama-cho, Tsu, Mie 514-8507, Japan

e-mail: hasimoto@bio.mie-u.ac.jp Tel: +81-59-231-9603 Fax: +81-59-231-9603

Abstract

A simple, rapid and accurate method for determining the product (ethanol) content in plant-cell culture media simultaneously with the nutrient (sugars) contents by mid-infrared (MIR) spectroscopy was developed using a Fourier transform infrared (FT-IR) spectrometer equipped with an attenuated total reflectance (ATR) accessory. We examined the potential of this method by comparison with a high-performance liquid chromatography (HPLC) method and applying the developed method to the simultaneous measurement of the ethanol content with the sugar ones in the liquid culture media suspended with the rice and tabaccum cells, respectively. The experimental results suggested that the sugar consumption and ethanol production behavior of the suspension plant cells would be non-destructively and simultaneously monitored by the developed method. Furthermore, the spectroscopic method provided in this study could be developed as the technique to analyze the kinetic overall metabolism of the suspension plant cells.

Keywords *Mid-Infrared Spectroscopic Quantification; FT-IR/ATR Method; Plant Cells; Sugar; Ethanol.*

1

Introduction

A wide range of useful products is produced from plant cells and the precise control of the plant cell cultivation process is one of the most important subjects in both scientific and engineering fields. The cell cultivation control in a biological reactor would depend on the accuracy of the real-time monitoring of nutrients such as sugars supplied and the products. In addition, glycolysis is usually the first step of a series of highly developed and very complicated metabolic pathways and starts simply to consume sugars such as

glucose. Moreover, the plant cells uptake the nutritive sugars in the medium directly and after hydrolyzing those with enzymes secreted from the cells. Thus a good grasp of the kinetic sugar uptake phenomena is very important to control the cultivation process precisely in the both scientific and engineering fields. Furthermore, the suspension cells produce various materials to the medium and most of these products are the final materials through series of highly developed and very complicated metabolic pathways and cycles.

The development and utilization of sophisticated strategies for the bioprocess control are stymied by the lack of suitable on-line sensors for the direct and simultaneous measurement of substrates and products in real time. Therefore, a simple and rapid analysis of the medium in which the cells are is crucial for a good understanding of their kinetic metabolisms for the control. Recently, near-infrared (NIR) spectroscopy in conjunction with chemometric techniques has offered as an alternative to the aforementioned techniques [1-6]. Nevertheless, the mid-infrared (MIR) spectroscopic method has advantages in respect to the spectral information in comparison to the NIR spectroscopy theoretically, although it has been very difficult to obtain the useful MIR spectrum from wet materials due to the strong absorption of MIR radiation by water. The MIR information is based on the fundamental vibrational modes of the objective molecules, but only the combination tones, overtones and the combinations are observed in the NIR region and the intensity is much lower than that in the MIR region [7]. In parallel with the development of the Fourier transform infrared (FT-IR) technology, the attenuated total reflection (ATR) techniques have evolved and provide FT-IR spectroscopy with substantial potential as a quantitative quality control tool for bioprocesses [8, 9]. The ATR provides a simple and reproducible means of handling products in the form of liquids and pastes and the ATR fiber probe may offer even more [10].

Various methods by FT-IR/ATR are under development for the analysis of bioproducts and the cell cultivation process [11-17]. We also studied the quantitative analysis of sugars in aqueous solutions using the FT-IR/ATR method [18, 19] and applied this technique to the quantification of the actual liquid medium suspended plant cells [20]. As a result, each sugar content in the culture medium in which the plant cells were suspended was simultaneously and accurately measured by the MIR spectroscopy.

Additionally, a simple, rapid and accurate method for analyzing the sugar metabolic kinetics was developed by the FT-IR/ATR method [21-23]. Also, the FT-IR/ATR method has a potential to theoretically determine most of the chemical components relating to the metabolism as well as sugars. The application of the MIR quantification results of the metabolite for both the nutriment and productive components during the cultivation process to the kinetic metabolic analysis has been reported [2, 11, 14]. However, the chemometric methods over a wide range of the MIR spectra were carried out in order to analyze the complicated spectra including the information of both the nutriment and productive components, although the MIR information is based on the fundamental vibrational modes of the objective molecules.

The objective of the present study is to develop a simple, rapid and accurate determination method for the nutrient and product contents in the culture medium suspended plant cells using the FT-IR/ATR method. In addition, we examined the potential of the developed method by comparison with a high-performance liquid chromatography (HPLC) method. We then performed a plant cell cultivation with the rice and tabacum cells in the different media in order to study the influence of the cell characteristics and the medium components on the quantification accuracy. Rice cells are one of the typical plant cells producing much ethanol and can easily form aggregates during cultivation. On the other hand, the tabacum cells are almost dispersing in the liquid culture medium and the ethanol production is negligible in comparison to the rice cells.

2

Materials and Methods

2.1

Culture media

All chemicals used in this work were of special grade and were obtained from Wako Pure Chemical Industries (Osaka, Japan). Sucrose was used as the ordinary carbon source for plant cell cultivation. Glucose and fructose, which are used to synthesize

sucrose, were also studied. In addition, ethanol was prepared as the product during plant cell cultivation.

The R2S [24-26] and Murashige-Skoog (MS) [27], which contain 30-g sucrose per liter, were used respectively as the standard media for the rice and tabaccum cell cultivation. In addition, various types of the modified R2S media containing sucrose, glucose, fructose and ethanol were prepared and the number of carbon molecules of sugar per unit volume of each medium was almost the same as that of the standard R2S (sucrose) medium. The sugar and ethanol contents in these media are listed in Table 1.

Table 1

The sugar and the other components in the medium for plant-cell cultivation were prepared as separate solutions and mixed after being sterilized. The sugar solution was sterilized by filtration using a 0.2-μm membrane filter and the medium without sugar was autoclaved for 15 min at 394 K. In addition, each sugar was dissolved in pure water purified by utilizing an AUTO STILL WG221 (Yamato Scientific, Tokyo, Japan) and a Simpli Lab ultrapure water system (Millipore, Bedford, MA).

2.2

Plant cell cultivation

The cultivation reported in this study was carried out with *Oryza sativa* L., Japonica, cv. Nipponbare (rice) and *Nicotiana tabacum* cv. Bright Yellow No.2 (tabaccum; TBY-2), which were sub-cultured respectively in the dark in the R2S medium for 14 d at 298 K on a rotary shaker (125 rpm) and in the MS medium for 7 d at 298 K on a rotary shaker (150 rpm). The 0.5 g-dry cell of the fourteen-day-old inoculums of the rice cells was inoculated into 120 ml of each fresh medium in a 300 mL flask, and cultivation was carried out for 21 d under the same conditions as mentioned above for the rice cell cultivation. One and one half milliliters of the seven-day-old inoculum of the TBY-2 cells was inoculated into 95 mL of the fresh medium in a 300 mL flask and cultivation was carried out under the same conditions as mentioned above for the TBY-2 cell cultivation. At regular time intervals, the metabolite contents and the dry cell weight in the medium were measured as follows.

2.3

Analytical procedure

A Magna 750 FT-IR spectrometer (Nicolet Inst. Corp., WI) equipped with a KBr beamsplitter and a deuterated triglycine sulfate KBr detector were used to obtain the spectra. The ATR spectra were acquired with a horizontal zinc selenide ATR sampling accessory (SPECACLAMP ATR 11080; Graseby Specac, IA). Sixty-four scans of symmetrical interferograms at 4 cm^{-1} resolution were added for each spectrum. The ATR spectra of the culture media passed through a 5-micrometer membrane filter were obtained in 4000 to 800 cm^{-1}.

The dry cell weight after drying at 363 K for 24 h was obtained. For the TBY-2 cell cultivation, the dry cell weight was compared to the turbidity of the culture medium at 600 nm. The turbidity was measured by an UV-VIS-NIR scanning spectrophotometer (UV-3100PC; Shimadzu, Kyoto, Japan) with a cuvette of 1-cm light path.

As the measurements of the reference values, the analysis of the sugars and ethanol in the culture media was made by a Tosoh (Tokyo, Japan) HPLC system, comprised of a model DP-8020 pump and a model RI-8020 differential refracting detector. A Shim-Pack SCR-101N (Shimadzu, Kyoto, Japan) HPLC analytical column (7.9×300 mm) with a guard column was used. 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 flow rate was 0.5 ml/min, and the column temperature was maintained at 313 K with a column oven (CO-8020, Tosoh, Tokyo, Japan). The refractactive index of the elute was monitored. This method has an accuracy of approximately ±0.8 mM (data not shown).

3

Results and Discussion

3.1

Mid-infrared spectral analysis of R2S culture medium

Spectral characteristics of metabolites in R2S media

We subtracted the spectrum of sucrose in the aqueous solution [18] from that of the components in the R2S medium containing sucrose. The subtracted spectrum is shown in Fig.1, in which the spectrum subtracted water from the R2S medium without sugar is also represented. Both indicted almost the same spectral features and the differences between them were negligible over the fingerprint region of the metabolite components. Thus, Fig.1 experimentally suggests the spectral additivity among the components in the R2S culture medium and we may acquire the sucrose information in the R2S culture medium as well as in the aqueous solution.

Figure 1

Figure 2a shows the ATR spectra of sucrose, glucose, fructose and ethanol in the R2S media, which were obtained after spectral subtraction of water, in the finger print region from 1300 to 900 cm^{-1}. For the sugars, there were many peaks such as CO and C-OH stretching modes, which complicatedly overlap each other [18, 19, 28], especially in the region from 1150 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 significant sharp peak around 1045 cm^{-1}, which did not overlap the sugar peaks mentioned above. Thus, we can identify each sugar and ethanol in the medium with its spectral pattern in this region. The peaks around 1055, 1036, 1065 and 1045 cm^{-1} mainly characterize the sucrose, glucose, fructose and ethanol spectra, respectively.

Figure 2

Determination of metabolite content in R2S media

Focusing on the above wavenumbers, we made the calibration curves between the absorbance and the metabolite concentrations in the media and the following straight line fits all curves.

$$A_i(\nu) = a_i(\nu)C_i + b_i(\nu) \qquad (1)$$

Here, $A_i(\nu)$ and C_i are respectively, the absorbance and the concentration of the metabolite (i) at the wavenumber (ν). The slope, $a_i(\nu)$, of the straight line relates to the types of metabolite and the value of $b_i(\nu)$ may theoretically show the absorbance of the medium without metabolite.

The calibration curves for metabolites in the R2S medium are displayed in Fig. 3a and excellent linearity was observed for each metabolite at each wavenumber. Also, the parameters of the calibration curves are listed in Table 2. The correlation coefficient for ethanol at 1045 cm^{-1} where the ethanol spectrum has the sharpest and most stainable peak as shown in Fig. 2a, is 0.999. In addition, those for sugars at that wavenumber are all higher than 0.999. Moreover, the $b_i(\nu)$ values at the four wavenumbers for all metabolites can equal to the experimental values of the absorbance of the medium without metabolite as shown in Fig. 1. These indicate that the b values can be used as only the function of the wavenumber.

Figure 3

Table 2

Next, we examined the spectral additivity for the sugar mixtures in the culture media. Figure 4a shows the spectrum of the glc-fru-suc-EtOH6 mixture in the R2S medium and was calculated by adding the metabolite spectra according to each actual concentration (see Table 1). Very good agreement between the two spectra is observed in the finger print region as shown in Fig. 4a. For all mixtures in the R2S media, the calculation values are compared with the actual ones in Fig. 4b-1 and b-2. The ordinate is the ratio of the calculated absorbance to the actual one which means the error of the spectral additivity. All values of the ordinate in the finger print region are roughly less than ±10%. At the four wavenumbers where the calibration curves were obtained, the

error is less than ±3%. Therefore, the spectral additivity could be experimentally confirmed and the calibration curves obtained in this study are accurately applicable to evaluate each metabolite concentration in the R2S medium. Additionally, it is thinkable, based on our previous studies on the MIR spectroscopic analysis of the sugar aqueous solutions [18, 19], that the spectral additivity would be acceptable over the concentration range for the plant cell cultivation.

Figure 4

Since the above results experimentally suggest that the FT-IR/ATR method may have a potential for the metabolite content determination in the R2S medium, the MIR spectroscopic metabolite content determination in the R2S medium was performed using the Bouguer-Beer's law under the assumption of the spectral additivity as follows:

$$A(\nu) = \sum_i a_i(\nu) C_i + b(\nu) \quad (2)$$

Eq.(2) is rewritten as the matrix expression.

$$\begin{pmatrix} A_{1055} \\ A_{1035} \\ A_{1065} \\ A_{1045} \end{pmatrix} = \begin{pmatrix} a_{suc,1055} & a_{glc,1055} & a_{fru,1055} & a_{EtOH,1055} \\ a_{suc,1035} & a_{glc,1035} & a_{fru,1035} & a_{EtOH,1035} \\ a_{suc,1065} & a_{glc,1065} & a_{fru,1065} & a_{EtOH,1065} \\ a_{suc,1045} & a_{glc,1045} & a_{fru,1045} & a_{EtOH,1045} \end{pmatrix} \begin{pmatrix} C_{suc} \\ C_{glc} \\ C_{fru} \\ C_{EtOH} \end{pmatrix} + \begin{pmatrix} b_{1055} \\ b_{1035} \\ b_{1065} \\ b_{1045} \end{pmatrix} \quad (3)$$

Here, the left subscripts suc, glc, fru and EtOH respectively mean sucrose, glucose, fructose and ethanol. The right subscripts indicate the wavenumbers. Moreover, Eq.(3) has the other expression (Eq.(4)).

$$\begin{pmatrix} C_{suc} \\ C_{glc} \\ C_{fru} \\ C_{EtOH} \end{pmatrix} = \begin{pmatrix} a_{suc,1055} & a_{glc,1055} & a_{fru,1055} & a_{EtOH,1055} \\ a_{suc,1035} & a_{glc,1035} & a_{fru,1035} & a_{EtOH,1035} \\ a_{suc,1065} & a_{glc,1065} & a_{fru,1065} & a_{EtOH,1065} \\ a_{suc,1045} & a_{glc,1045} & a_{fru,1045} & a_{EtOH,1045} \end{pmatrix}^{-1} \begin{pmatrix} A_{1055} - b_{1055} \\ A_{1035} - b_{1035} \\ A_{1065} - b_{1065} \\ A_{1045} - b_{1045} \end{pmatrix} \quad (4)$$

Each metabolite concentration is then calculated using the parameters of the calibration curves at the four wavenumbers for each metabolite (Table 2).

Figure 5 displays a comparison of the metabolite contents in the R2S medium determined by the above MIR spectroscopic or HPLC method, which is one of the present standard methods, with the actual ones. As shown in Fig. 5, excellent agreement between the actual and estimated metabolite content for both analytical methods is obtained. Their correlation coefficients are all higher than 0.99. This may suggest that

the FT-IR/ATR spectroscopy is applicable for the content determination of not only the sugars but also ethanol (the metabolic product) as an accurate method. In addition, it takes ten or twenty minutes for the HPLC measurement while the ATR spectrum was obtained within a minute. Furthermore, the MIR spectrum gives the information of all components having infrared activity, though the HPLC data provide only the information for each component after the pre-treatment for the HPLC measurement. Hence, the MIR spectroscopy has a higher performance than the other analytical methods for monitoring the cultivation process from the viewpoint of the kinetic metabolic analysis.

Figure 5

Changes in metabolite concentrations during rice cell cultivation

For the R2S media during the sucrose cultivation of the rice cells, we applied the FT-IR/ATR method to the metabolite content determination. Figure 6a shows the time behaviors of the ATR spectra, which were obtained after the spectral subtraction of water, during the rice cell cultivation with the R2S media. At the beginning of cultivation (0 d), the ATR spectrum had the same characteristics as that of sucrose in the R2S medium as shown in Fig. 2a. During cultivation, the absorbance decreased almost over the finger print region with the changes of the spectral patterns. The spectrum at 6 d was quite different from that at 0 d and had qualitatively the same spectral characteristics as that of the glucose-fructose mixture in the aqueous solution [18]. This spectroscopically indicated that there were only glucose and fructose left in the medium after 6 d of cultivation. Around 1045 cm^{-1} where the sharp peak identifying ethanol is indicated in Fig. 2a, the broad peak at 10 d and the sharp peaks at 12 and 14 d were observed. Finally the ATR spectrum was almost equal to that of the R2S medium without sugar as shown in Fig. 1. Thus, the spectral change shown in Fig. 6a gives the evidence for the biological process in which ethanol is produced after sucrose in the medium is hydrolyzed and consumed by the rice cells.

Figure 6

Figure 7a shows the time courses of the sugar and ethanol contents evaluated by the method above and of the dry cell weight in the R2S culture media during the rice cell cultivation. The graph displays that the R2S medium initially contained only sucrose as the carbon source. Just after the start of cultivation, the sucrose content immediately decreased and the glucose and fructose contents slightly increased. The glucose and fructose contents reached peak values at 4 to 6 d and decrease afterward. The dry cell weight increased with a decrease in the total sugar content and reached a maximum value when the sugars were almost consumed. The ethanol content that was negligible in the initial stage became successively higher and indicated the highest value of about 4 g/L at 12 d just after the dry cell weight had reached the maximum value. After that, the ethanol content rapidly decreased and was less than 1 g/L in the final stage of cultivation (21 d).

Figure 7

Figure 8a represents a comparison of the quantification results of the metabolites in the R2S medium during the rice cell cultivation using the MIR spectroscopic method with those using the HPLC method. Very good agreements for the sugar and ethanol contents between both were observed and the validity of the application of the simple and rapid MIR spectroscopic methods to the simultaneous determination of the nutrimental and productive content during the rice cell cultivation were experimentally confirmed. Hence the results above indicated that the sugar consumption and ethanol production behavior during cultivation could be continuously observed through the spectra in the R2S culture medium for the rice cell cultivation using the FT-IR/ATR method.

Figure 8

3.2

Mid-infrared spectral analysis of MS culture media

Mid-infrared Spectroscopic determination of metabolite contents in MS media

In our previous study [20], we developed the sugar content determination in the MS media using the FT-IR/ATR method focusing on sugar uptake by the TBY-2 cells [21]. In this study, we researched the simultaneous quantification of both the sugars as the nutrients and the ethanol as the product.

Figure 2b represents the ATR spectra of ethanol in the MS medium, which were obtained after the spectral subtraction of water, in the finger print region from 1300 to 900 cm^{-1} and those of the sugars reported in our previous work [20]. Ethanol had a significant sharp peak around 1045 cm^{-1}, which did not overlap the sugar peaks mentioned above, in the same MS medium as that in the R2S medium (Fig. 2a). Also, in the MS media, we can identify each sugar and ethanol in the medium with its spectral pattern in this region.

Then we remade the calibration curves using the peaks around 1055, 1036, 1065 and 1045 cm^{-1} mainly characterizing the sucrose, glucose, fructose and ethanol spectra, respectively, because the spectral additivity for the MS medium had been experimentally checked in our previous work [20]. The calibration curves for the metabolites in the MS medium are displayed in Fig. 3b and excellent linearity was also observed for not only the sugars but also the ethanol at each wavenumber. As listed in Table 3, the correlation coefficient for ethanol at 1045 cm^{-1} where the ethanol spectrum has the sharpest and most stainable peak as shown in Fig. 2b is 0.999. In addition, those for sugars at that wavenumber are all higher than 0.999. These correlation coefficients were the same as those for the R2S medium. Therefore, these results experimentally suggest that the FT-IR/ATR method may have a potential for the content determination of both the sugars and ethanol also in the MS medium using the Bouguer-Beer's law under the assumption of the spectral additivity.

Table 3

Influences of ethanol peaks on mid-infrared spectroscopic determination of metabolite contents

We also subtracted the water spectrum from the MS medium during the sucrose cultivation of the TBY-2 cells and the time variations of the subtracted spectra in the finger print region during cultivation are displayed in Fig. 6b [20]. At the beginning of cultivation (0 d), the ATR spectrum had the same characteristics as that of sucrose in the MS medium as shown in Fig. 2b. The absorbance spectra of the MS medium shown in Fig. 6b decreased with the change in the spectral pattern during the TBY-2 cell cultivation and the pattern changes spectroscopically indicated that the sucrose in the MS media during the TBY-2 cell cultivation was hydrolyzed the same to glucose and fructose as that in the R2S medium during the rice cell cultivation. However, only the broad peak around 1045 cm^{-1} characterizing ethanol at the final stages of the TBY-2 cell cultivation in the MS medium was observed in Fig. 6b, and was much lower than that of the rice cell cultivation in the R2S medium. In addition, the changing kinetics of the spectral pattern of the MS medium was different from that of the R2S medium.

We also applied the FT-IR/ATR method to the metabolite content determination in the MS medium during the TBY-2 cell cultivation. The metabolite contents were determined by Eq.(4) with the calibrating parameters listed in Table 3. Figure 7b shows the time courses of the sugar and ethanol contents evaluated by the method above and of the dry cell weight in the culture media during the TBY-2 cell cultivation. In Fig. 7b, the time courses of the sugar contents evaluated by the method above and of those evaluated by the HPLC method [20] and the dry cell weight in the culture media during the TBY-2 cell cultivation were displayed. The calculated time behavior in consideration of the ethanol existence in the MS medium in this study vary from those neglecting the ethanol existence in the MS medium in our previous study [20] and are qualitatively and quantitatively similar to those evaluated by the HPLC method. Both results evaluated by the MIR spectroscopic method in consideration of the ethanol existence in the MS medium and by the HPLC method indicate that the sucrose content immediately decreased just after the beginning of the cultivation and that the glucose and fructose contents successively increased. The ethanol content that was negligible at the initial stage reached a concentration of about 1 g/L at 7 d of cultivation when the dry

cell weight had just reached the maximum value. After that, the ethanol content successively decreased and was almost zero at the final stage of cultivation (14 d).

For the metabolite contents in the MS medium during the TBY-2 cell cultivation, Figure 8b represents a comparison of the quantification results obtained by the two MIR spectroscopic methods with those by the HPLC method. The sugar contents calculated by the MIR spectroscopic method in consideration of the ethanol existence agreed with those by the HPLC method more consistently than those by the previous MIR spectroscopic method [20]. Thus the validity of the application of the simple and rapid MIR spectroscopic method to the simultaneous determination of the nutrimental sugar and the productive ethanol content was also experimentally indicated for the TBY-2 cell cultivation in the MS medium, although the ethanol production by the TBY-2 cells was almost negligible. Therefore the results above suggested that the kinetic sugar uptake phenomena of the TBY-2 cells could be analyzed more accurately using the FT-IR/ATR method in consideration of the ethanol peaks of the MS medium spectra than by the previous MIR spectroscopic method [20].

4

Conclusion

The rapid and accurate determination of the ethanol content simultaneous with the sugar ones in the liquid culture media suspending plant cells was developed using the MIR spectroscopic method, which can obtain the information based on the fundamental vibrational modes of the objective molecules and has advantages with respect to spectral information in comparison with the near-infrared spectroscopy, theoretically. So we apply the developed method to the simultaneous measurement of the ethanol content with the sugar ones in the R2S media suspending the rice cells, which are one of the typical plant cells producing much ethanol and can easily form aggregates during liquid cultivation. The experimental results suggested that the sugar consumption and ethanol production behavior of the suspension rice cells would be measured. In addition, for the MS medium suspending the TBY-2 cells which produce little ethanol during cultivation, the sugar content determined by the MIR spectroscopic method in consideration of the ethanol existence in the medium indicated a better accuracy than

that determined by the previous method neglecting the absorption peaks of the ethanol in the medium [20]. Therefore, the spectroscopic method provided in this paper could be developed as the technique to analyze the kinetic overall metabolism of the suspension plant cells because the FT-IR/ATR method has a potential to determine theoretically most of the metabolite including the ionic dissociative materials [29, 30]. This study represents a very important step in such developments.

References

1. Schmidt S, Kircher M, Kasala J, Locaj J (1998) Near infrared spectroscopy in fermentation and quality control for amino acid production. Bioprocess Eng. 19: 67-70

2. Sivakesava S, Irudayaraj J, Ali D (2001) Simultaneous Determination of Multiple Components in Lactic Acid Fermentation Using FT-MIR, NIR, and FT-Raman Spectroscopic Techniques. Process Biochem. 37: 371-378

3. Pillonel L, Luginbühl W, Picque D, Schaller E, Tabacchi R, Boset JO (2002) Analytical Methods for the Determination of the Geographic Origin of Emmental Cheese: Mid- and Near-Infrared Spectroscopy. Eur. Food Res. Technol. 215: 260-267

4. Chung H, Arnold MA, Rhiel M, Murhammer DW (1996) Simultaneous Measurements of Glucose, Glutamine, Ammonia, Lactate, and Glutaminate in Aqueous Solutions by Near-Infrared Spectroscopy. Appl. Spectrosc. 50: 270-276

5. Hall JW, McNeil B, Rollins M J, Draper I, Thompson BG, Macaloney G (1996) Near-Infrared Spectroscopic Determination of Acetate, Ammonium, Biomass, and Glycerol in an Industrial Escherichia coli Fermentation. Appl. Spectrosc. 50: 102-108

6. Yano T, Suehara K, Nakano Y. (1998) Determination of the Content of Water and Rice Bran in Solid Media Used for Mushroom Cultivation Using Near-Infrared Spectroscopy. J. Ferment. Bioeng. 86: 472-476

7. Bernath PF (1995) Spectra of Atoms and Molecules, Oxford University Press, New York

8. Harrick NJ (1967) Internal Reflection Spectroscopy, Harrick Scientific Corporation, New York

9. Mirabella FM, Harrick NJ (1985) Internal Reflection Spectroscopy: Review and Supplement, Harrick Scientific Corporation, New York

10. Heise HM, Küpper L, Pittermann W, Butvina LN (2001) New Tool for Epidermal and Cosmetic Formulation Studies by Attenuated Total-Reflection Spectroscopy Using a Flexible Mid-Infrared Fiber Probe. Fresenius J. Anal. Chem. 371: 753-757

11. Maculey-Patrick S, Arnold SA, McCarthy B, Harvey LM, McNeil B (2003) Attenuated Total Reflectance Fourier Transform Mid-Infrared Spectroscopic Quantification of Sorbitor and Sorbose during a *Gluconobacter* Biotransformation Process. Biotechnol. Lett. 25: 257-260

12. Pollard DJ, Buccino R, Connors NC, Kirschner TF, Olewinski RC, Saini K, Salmon PM (2001) Real-Time Analyte Monitoring of a Fungal Fermentation, at Pilot Scale, Using Situ Mid-Infrared Spectroscopy. Bioprocess Biosyst. Eng. 24: 13-24

13. Toraño JS, van Hattum SHC (2001) Quantitative Analysis of Active Compounds in Pharmaceutical Preparations by Use of Attenuated Total-Reflection Fourier Transform Mid-Infrared Spectrophotometry and the Internal Standard Method. Fresenius J. Anal. Chem. 371: 532-535

14. Bellon V, Vallat C, Goffinet D (1995) Quantitative Analysis of Individual Sugars during Starch Hydrolysis by FT-IR/ATR Spectroscopy. Part I: Multivariate Calibration Study -Repeatability and Reproducibility. Appl. Spectrosc. 49: 556-562
15. Dupuy N, Huvenne JP, Sombret B, Legrand P (1993) Quantitative Analysis by Mid-Infrared Spectrometry in Food and Agro-Industrial Fields. J. Mol. Struc. 294: 223-226
16. Kemsley EK, Holland JK, Defernez M, Wilson RH (1996) Detection of Adulteration of Raspberry Purees Using Infrared Spectroscopy and Chemometrics. J. Agric. Food Chem. 44: 3864-3870
17. Schindler R, Lendl B (1999) Simultaneous Determination of Enzyme Activities by FTIR-Spectroscopy in an One-Step Assay. Anal. Chim. Acta 391: 19-28
18. Kameoka T, Okuda T, Hashimoto A, Noro A, Shiinoki Y, Ito K (1998) FT-IR Analysis of Sugars in Aqueous Solution Using ATR Method. J. Jpn. Soc. Food Sci. Technol. 45: 192-198 (in Japanese)
19. Kameoka T, Okuda T, Hashimoto A, Noro A, Shiinoki Y, Ito K (1998) A Rapid FT-IR/ATR Method for Sugar Content Determination in Food. J. Jpn. Soc. Food Sci. Technol. 45: 199-204 (1998) (in Japanese)
20. Hashimoto A, Kameoka T (2000) Mid Infrared Spectroscopic Determination of Sugar Content in Plant-Cell Culture Media Using an FT-IR/ATR Method. Appl. Spectrosc. 54: 1005-1011
21. Hashimoto A, Nakanishi K, Motonaga Y, Kameoka T (2001) Sugar Metabolic Analysis of Suspensions of Plant Cells Using an FT-IR/ATR Method. Biotechnol. Prog. 17: 560-564
22. Hashimoto A, Yamanaka A, Kanou M, Nahar F, Kameoka T (2003) Effects on Pre-Cultivation on Sugar Metabolic Kinetics of Suspension Plant Cells measured by Mid-Infrared Spectroscopy. Food Agric. Environ. 1: 168-175
23. Hashimoto A, Nakanishi K, Yamanaka A, Nahar F, Motonaga Y, Kameoka T (2002) Mid-Infrared Spectroscopic Analysis of Sugar Metabolism by Rice Cells Suspending in Culture Medium. In: Dochain D, Perrier M (eds) Computer Applications in Biotechnology 2001, Elsevier Science, Oxford, pp. 379-384
24. Ohara K, Ojima K, Saigusa M, Fujiwara A (1975) Studies on the nutrition of rice cell culture II. Microelement requirement and the effects of deficiency Plant cell physiol. 16: 73-81.
25. Ohara K, Ojima K, Fujiwara A (1973) Studies on the nutrition of rice cell culture I. A simple, defined medium for rapid growth in suspension culture Plant cell physiol. 14: 1113-1121
26. Kyozuka J, Shimamoto K (1991) Transformation and regeneration of rice protoplasts In: Lindsey K (ed) Plant Tissue Culture Manual, B1, Kluwer Academic Publishers, Dordrecht, pp. 1-16
27. Murashige T, Skoog F (1962) A Revised Medium for Rapid Growth and Bioassay with Tabocco tissue Culture Physiol. Plant 15: 473-497
28. Cael J, Koening J, Blackwell J (1974) Infrared and Raman Spectroscopy of Carbohydrates PartIV. Carbohydrate Res. 32: 79-91
29. Nakanishi K, Hashimoto A, Kanou M, Pan T, Kameoka T (2003) Mid-Infrared Spectroscopic Measurement of Ionic Dissociative Materials in Metabolic Pathway. Appl. Spectrosc. 57: 1510-1516
30. Pan T, Hashimoto A, Kanou M, Nakanishi K, Kameoka T (2003) Development of a Quantification System of Ionic Dissociative Materials in the Glycolytic Pathway Using an FT-IR/ATR Method. Bioprocess Biosyst. Eng. 26: 133-139

Figure Legends

Fig.1. Subtracted ATR spectra of the components in R2S medium other than sugar.

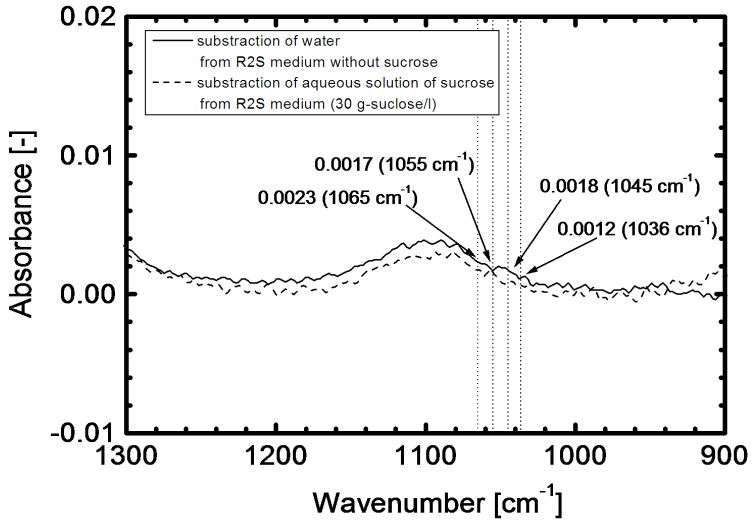


Fig.2. ATR spectra of glucose, fructose, sucrose and ethanol. (a) in R2S medium. (b) in MS medium.

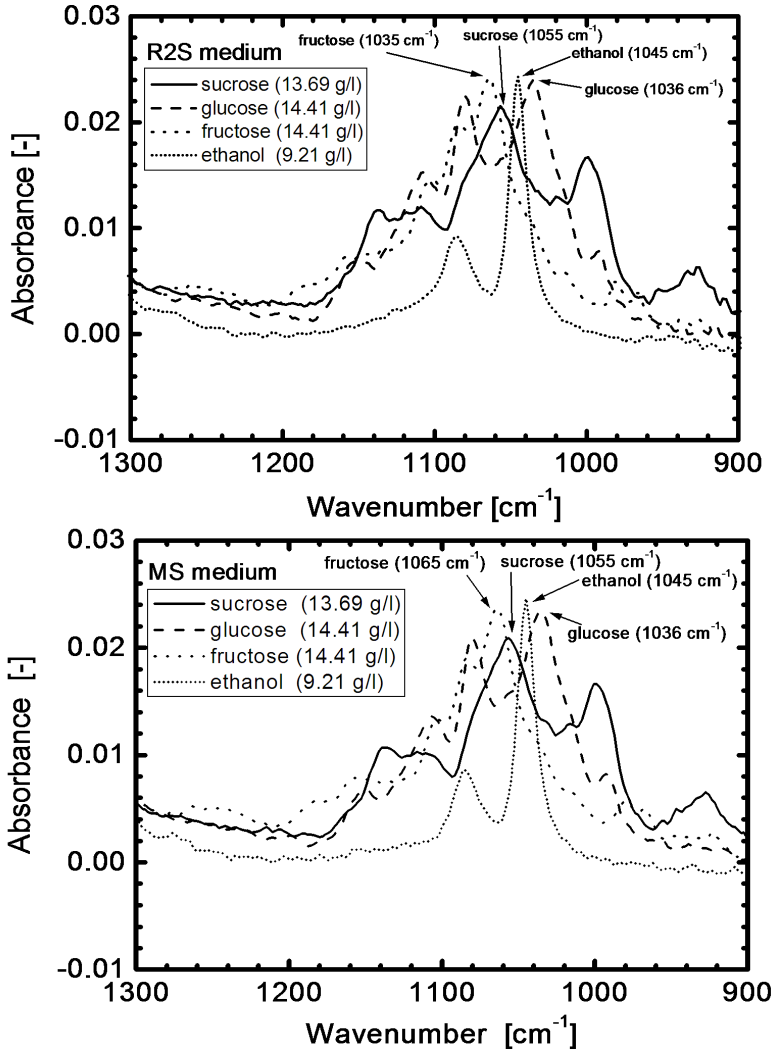


Fig.3. Calibration curves between the absorbance of sugar and ethanol spectra and their concentrations. (a) in R2S medium. (b) in MS medium.

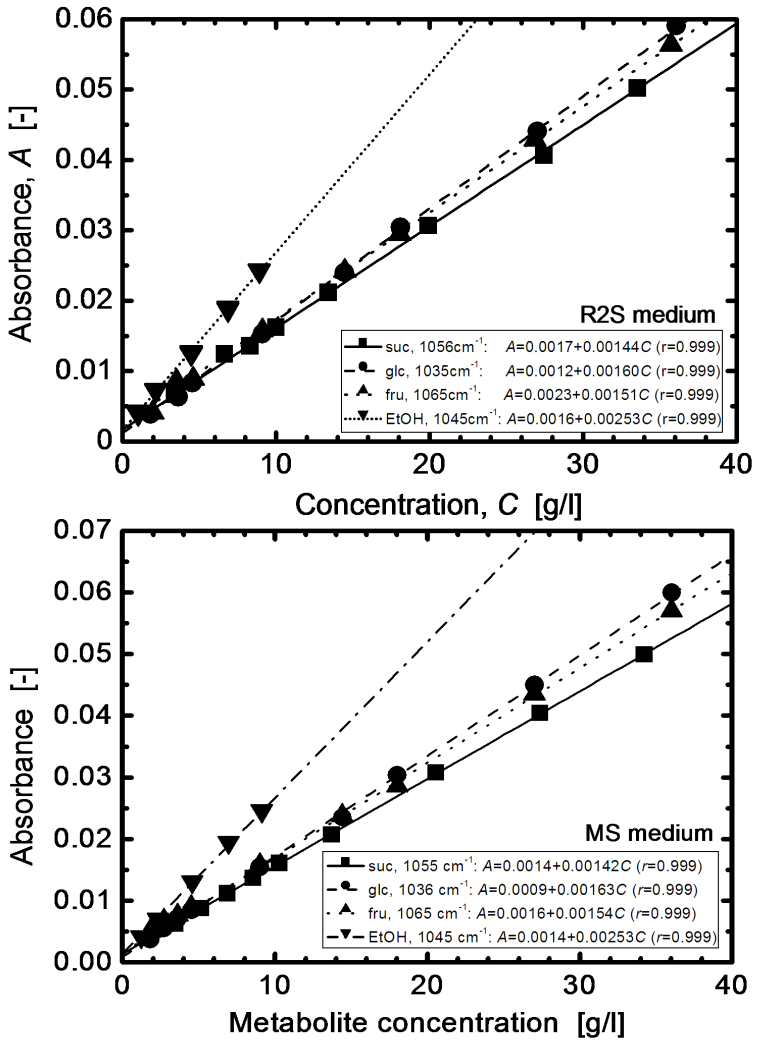


Fig.4. Comparison of calculated spectra with actual ones for R2S medium. (a) Spectral comparison for glucose-fructose-sucrose-ethanol (glc-fru-suc-EtOH6) mixture medium. (b-1) Absorbance ratio of calculated spectra to actual ones (about 3% of ethanol concentration). (b-2) Absorbance ratio of calculated spectra to actual ones (about 6% of ethanol concentration).

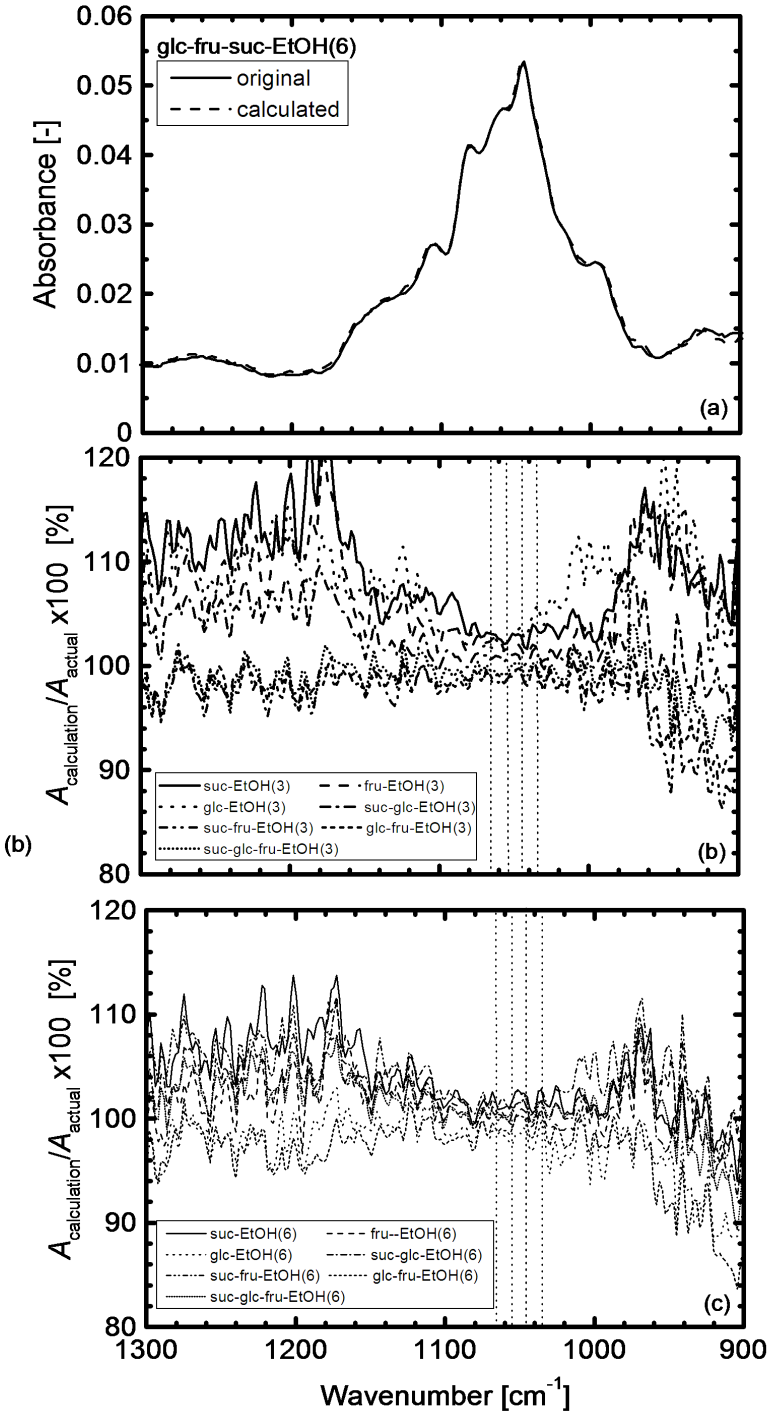


Fig.5. Comparison of evaluated sugar and ethanol contents in R2S media with actual ones. (a) MIR spectroscopy. (b) HPLC method.

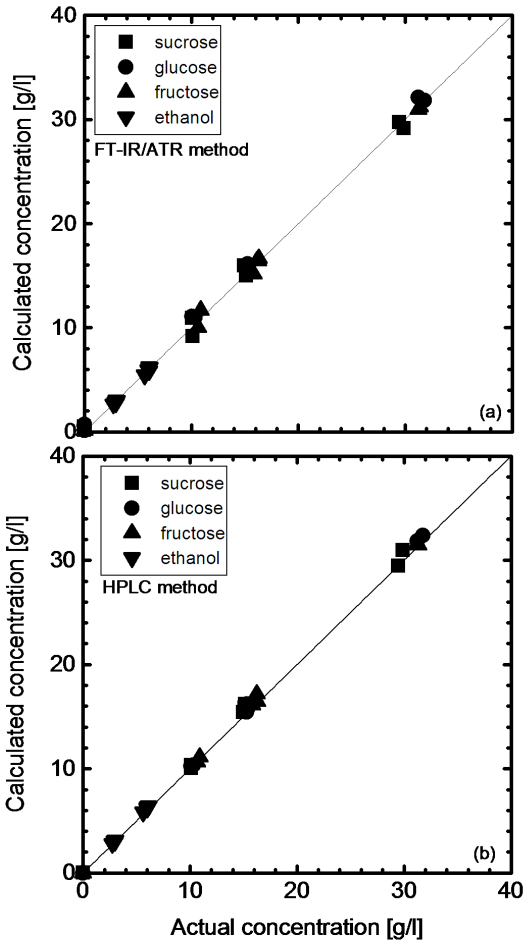


Fig.6. Time behavior spectra of culture medium during sucrose cultivation. (a) Rice cell cultivation with R2S medium. (b) TBY-2 cell cultivation with MS medium.

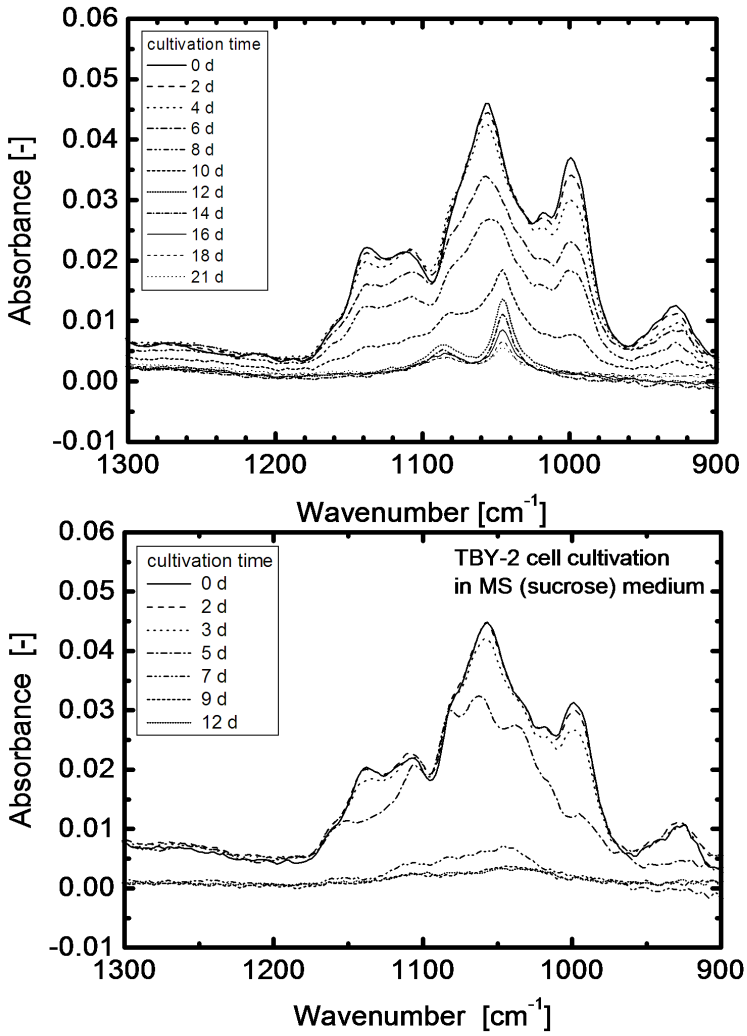


Fig.7. Time courses of sugar contents, ethanol content and cell density during sucrose cultivation. (a) Rice cell cultivation with R2S medium. (b) TBY-2 cell cultivation with MS medium.

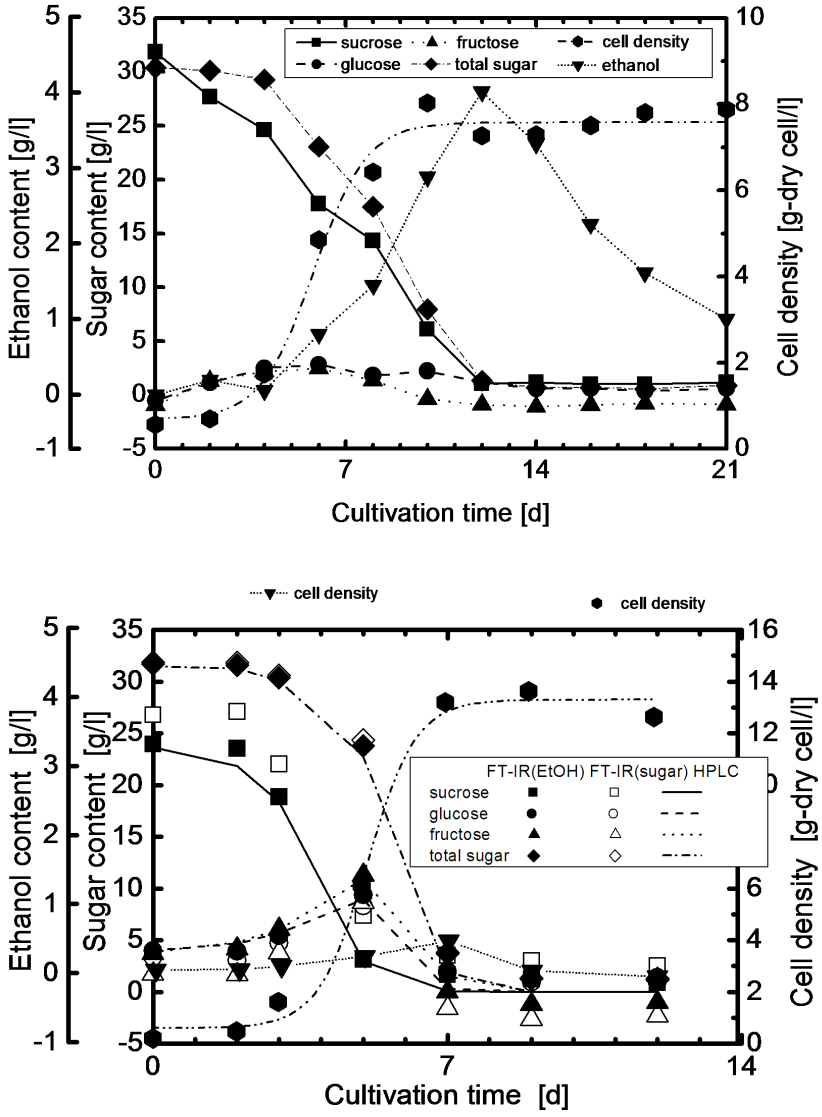
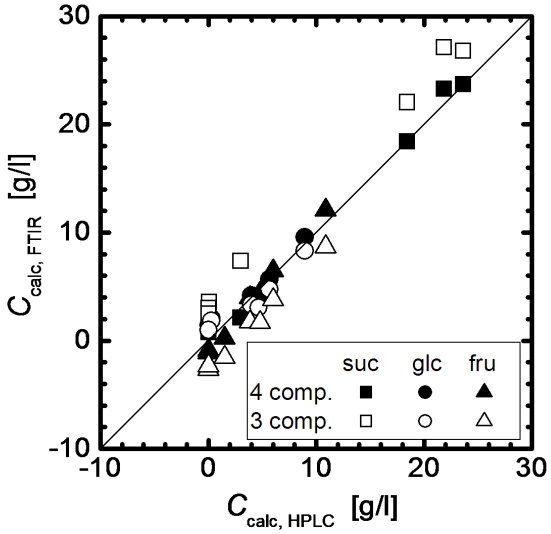


Fig.8. Comparison of sugar and ethanol contents in culture medium during sucrose cultivation evaluated by FT-IR/ATR method with those by HPLC method. (a) Rice cell cultivation with R2S medium. (b) TBY-2 cell cultivation with MS medium. The notations, “4 comp.” and “3 comp.”, mean the sugar contents determined by the MIR spectroscopic method in consideration of the ethanol existence and the absence [20], respectively.



Tables

Table 1. Sugar and ethanol contents in R2S culture media for infrared spectroscopic measurement.

Culture media	concentration [g/L]			
	glucose	fructose	sucrose	ethanol
glc-EtOH3	31.78	---	---	2.90
frc-EtOH3	---	31.23	---	2.99
suc-EtOH3	---	---	29.47	2.72
glc-fru-EtOH3	15.36	16.11	---	3.03
glc-suc-EtOH3	15.32	---	15.13	3.00
suc-fru-EtOH3	---	15.83	14.96	2.90
glc-fru-suc-EtOH3	10.34	10.65	10.11	2.96
glc-EtOH6	31.25	---	---	6.07
frc-EtOH6	---	31.35	---	6.05
suc-EtOH6	---	---	29.90	5.61
glc-fru-EtOH6	15.27	16.28	---	6.00
glc-suc-EtOH6	15.27	---	15.16	6.03
suc-fru-EtOH6	---	16.25	15.16	6.11
glc-fru-suc-EtOH6	10.08	10.90	10.13	5.97

Table 2. Fitting parameters and correlation coefficients of calibration curves for R2S culture medium.

sugars	Wavenumber [cm^{-1}]	Fitting parameter, a [$(g/L)^{-1}$]	Fitting parameter, b [-]	Correlation coefficient, r [-]
glucose	1036	0.00160	0.0012	0.999
	1065	0.00095	0.0023	0.999
	1055	0.00105	0.0017	0.999
	1045	0.00128	0.0016	0.999
fructose	1036	0.00066	0.0012	0.998
	1065	0.00151	0.0023	0.999
	1055	0.00117	0.0017	0.999
	1045	0.00078	0.0016	0.999
sucrose	1036	0.00093	0.0012	0.999
	1065	0.00125	0.0023	0.999
	1055	0.00144	0.0017	0.999
	1045	0.00116	0.0016	0.999
ethanol	1036	0.00082	0.0012	0.994
	1065	0.00015	0.0023	0.995
	1055	0.00066	0.0017	0.922
	1045	0.00253	0.0016	0.999

Table 3. Fitting parameters and correlation coefficients of calibration curves for MS culture medium.

sugars	Wavenumber [cm^{-1}]	Fitting parameter, a [$(g/L)^{-1}$]	Fitting parameter, b [-]	Correlation coefficient, r [-]
glucose	1036	0.00163	0.0009	0.999
	1065	0.00099	0.0016	0.999
	1055	0.00110	0.0010	0.999
	1045	0.00131	0.0014	0.999
fructose	1036	0.00069	0.0009	0.998
	1065	0.00154	0.0016	0.999
	1055	0.00118	0.0010	0.999
	1045	0.00080	0.0014	0.999
sucrose	1036	0.00092	0.0009	0.999
	1065	0.00125	0.0016	0.999
	1055	0.00142	0.0010	0.999
	1045	0.00114	0.0014	0.999
ethanol	1036	0.00088	0.0009	0.985
	1065	0.00024	0.0016	0.939
	1055	0.00069	0.0010	0.992
	1045	0.00253	0.0014	0.999