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FTIR-spectral analysis of two photosynthetic H^2-producing strains and their extracellular polymeric substances

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Abstract The Fourier transform infrared (FTIR) spectra of the cells of two photosynthetic H_2-producing strains, *Rhodoblastus acidophilus* and *Rhodobacter capsulatus*, as well as their extracellular polymeric substances (EPS), were evaluated. The FTIR spectra of *R.capsulatus* and its EPS during its cultivation were also recorded. The main peaks in the spectra, including 1,080 cm^{-1} (carbohydrates), 1,250 cm^{-1} (nucleic acids), 2,830–2,930 cm^{-1} (lipids), 1,660–1,535 cm^{-1} (Amide I and II of proteins), were observed. The relative heights of these peaks in the spectra of the two strains were different, showing the difference in contents of various components in the cells or EPS. The ratios among the main components in the EPS obtained from the FTIR spectra were in good agreement with those from a conventional quantitative chemical analysis. As an easy, rapid, and direct technique, the FTIR spectroscopy could be used to characterize the components and their relative contents of EPS of photosynthetic bacteria.

Introduction

Various photosynthetic bacteria (PSB) can utilize short-chain organic acids as electron donors to produce H_2 at the expense of solar energy (Tsygankov et al. 1998). However, because of the poor flocculation ability, the PSB cells cannot be efficiently separated from supernatant (Watanabe et al. 1998), resulting in a low cell concentration in a H_2-producing reactor. Microorganisms can excrete extracellular polymeric substances (EPS) during their growth. The ratio of proteins/carbohydrates in EPS is closely associated with the bacterial surface characteristics and flocculation ability (Wingender et al. 1999). The EPS of bacteria are also able to complex or precipitate heavy metals in cultivation medium to reduce the toxicity of heavy metals (Sheng et al. 2005a). Analysis on the content and components of EPS would be useful for understanding the functions of EPS of PSB. However, so far, little information is available about the content and components of the EPS of PSB.

In general, the determination of the main macromolecular components in microbial cells or their EPS is based on conventional chemical methods, which include preliminary extraction, purification, and indirect colorimetric analysis. The colorimetric analysis for individual composition is often influenced by other concomitant cellular compositions. For instance, colorimetric determination of protein using the Lowry method is interfered by the presence of lipids, purines, carbohydrates, and other substances (Raunkjær et al. 1994). In some cases, appropriate purification is required. This will require more analytical time and sacrifice for the determination of accuracy. Thus, the conventional chemical analysis on the main macromolecular components in microbial cells or their EPS is fussy and time-consuming. One rapid and sensitive analytical method is needed for characterizing the compositions of the cells and their EPS.

With recent developments in analytical technology, Fourier transform infrared (FTIR) spectroscopy technique has been applied in the field of microbiology. FTIR is able to simultaneously measure the vibrations of functional groups of different cell components. This spectrum contains information about all the IR active functional groups with a permanent dipole moment in cell compounds. In addition to the identification and classification of microorganisms (Tindall et al. 2002), FTIR has been successfully used for the quantitative and qualitative analysis of the molecular compositions of cell parts, e.g., cell wall (Galichet et al. 2001), cell membrane (Kamnev et al. 1999), and bacterial storage products (Kansiz et al. 2000), etc. Methods have also been developed for

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monitoring bioreactors using FTIR spectroscopy (Rhiel et al. 2002). As a rapid and sensitive analytical method, FTIR spectroscopy may also provide information about the relative contents of the main components in EPS (e.g., proteins/carbohydrates). It is essential to analyze the entire or partial microbial cells and allow simultaneous measurement of different compounds in microbial cells and their EPS. Few studies have been pursued on the contents and compositions of EPS by using FTIR, mainly concentrating on identifying the function groups of EPS (Omoike and Chorover 2004; Sheng et al. 2005b). However, quantitative information about the relative contents of different components of EPS obtained with FTIR spectra is not available in literature.

The main objective of this study was to characterize two photosynthetic H_2-producing strains: *Rhodoblastus acidophilus* and *Rhodobacter capsulatus*, and their EPS by using FTIR spectroscopy. The ratio of proteins/carbohydrates of cells and their EPS were also calculated from FTIR spectra, and the results were compared with those obtained from the conventional chemical methods.

Materials and methods

PSB strains

Rhodoblastus acidophilus, obtained from the East Sea Fisheries Research Institute, China, was used as one target PSB, whereas *Rhodobacter capsulatus*, provided by Chenxin Microbial, Yizheng, China, was used as another target strain. Both strains could utilize acetate, propionate, and butyrate as a substrate to produce H_2 (Sheng et al. 2005b; Shi and Yu 2005). They were anaerobically grown in a medium at pH 7.0. The composition of this medium could be found in our previous paper (Sheng et al. 2005b). The strains were grown in 300-ml rubber-stopper vials at 30 °C and 3,000 μE m^{-2} s^{-1}. Anaerobic conditions were created by purged with pure argon.

EPS extraction

The EPS of the two PSB were extracted using EDTA method according to Sheng et al. (2005b) and cation exchange resin technique (CER, Dowex, Fluka 91973) adopted from Frolund et al. (1996). For the CER extraction, both bacteria were harvested by centrifugation at 9,000×*g* for 10 min to remove the medium, and then the pellets were washed twice with 0.9 % NaCl solutions. After that, the cell pellets were resuspended to a pre-determined volume in 0.9 % NaCl solution, and CER with a dosage of 60 g/g dry cell was then added. These suspensions were stirred for 12 h at 200 rpm and 4 °C. Later, the CER/cell suspension was settled for 3 min to remove CER, and the extracted EPS were harvested by centrifugation at 9000×*g* and 4 °C for 30 min to remove remaining cell components. The supernatant was then filtrated through 0.45-μm acetate cellulose membranes and was used as the EPS fraction for analyses.

Cellular components extraction

The cellular carbohydrates and proteins in the bacteria were collected using alkaline extraction with boiling (Grube et al. 1999). Cells of both bacterial suspensions of 20 ml were harvested by centrifugation at 9,000×*g* for 10 min, and the pellets were then washed twice with 0.9 % NaCl solutions. After that, the cell pellets were resuspended in 10 ml of double distilled water, and 10 ml of 1-mol l^{-1} NaOH solution was then added, and later the mixture was boiling for 10 min. This solution was cooled to ambient temperature. Thereafter, the solution was adjusted to pH 7.0 with 1 mol l^{-1} HCl solution. The sample solution was then diluted to a predetermined volume. The extracted cell components were harvested by centrifugation at 9,000×*g* for 30 min. The supernatant was then filtrated through 0.45-μm acetate cellulose membranes and was used to determine the cellular proteins and carbohydrates.

Chemical analysis

All chemicals used in this work were of analytical grade. Cell growth was measured from the dry cell weight. The contents of carbohydrates and proteins were determined using the anthrone method with glucose as a standard and the Lowry method with egg albumin as a standard (Frolund et al. 1996), respectively. The content of nucleic acids was determined according to Boonaert et al. (2001), using a UV spectrophotometer (UV751GD, Analytical Instrument, China).

FTIR spectrum

All spectra were recorded between 4,000 and 500 cm^{-1} with a FTIR spectrometer (Magna-IR 750, Nicolet Instrument, USA). The absorption spectra of the two strains were recorded in the FTIR technique as dried films on Thallium Bromo-Iodide KRS-5 crystal (Schuster et al. 1999). The cells for FTIR spectrophotometry were separated from cultivation medium by centrifugation at 9,000×*g* for 10 min, and then washed twice with double distilled water. After that, the cell pellets were spread on a KRS-5 crystal and dried thereafter. The spectrum of the crystal plate coated with cells was then recorded with the spectrometer. After subtracting the background spectrum, the FTIR spectrum of bacterium could be obtained. The EPS solution was transferred to a quartz mortar and was mixed and milled with potassium bromide crystal. After that, the solution was dried, and the mixture was then pressed to a tablet and used for FTIR measurement.

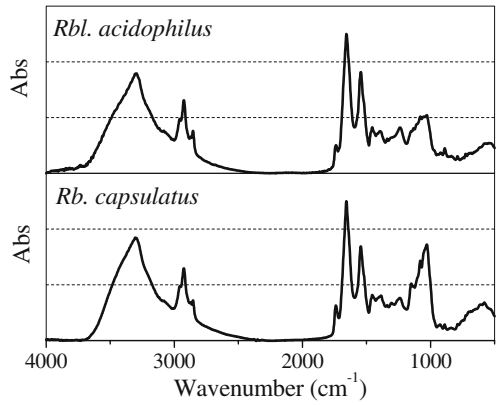

Fig. 1 FTIR spectra of *Rbl. acidophilus* and *Rb. capsulatus*

Calculation of relative contents of major components of bacterial cells and EPS from FTIR spectra

According to the Lambert–Beer Law, the optical density of a mixture is equal to the sum of the partial optical densities, corresponding to the absorbance of each component. At wave number ν, the absorbance of cell or EPS can be expressed as follows:

$$A(\nu) = \varepsilon_1(\nu)C_P d + \varepsilon_2(\nu)C_{Ca} d + \varepsilon_3(\nu)C_{NA} d + \varepsilon_4(\nu)C_L d$$

where $A(\nu)$ is absorbance at wave number ν, $\varepsilon_1(\nu)$, $\varepsilon_2(\nu)$, $\varepsilon_3(\nu)$, $\varepsilon_4(\nu)$ are extinction coefficients of proteins, carbohydrates, nucleic acids, and lipids respectively, at wave number ν; C_P, C_{Ca}, C_{NA}, C_L are the concentrations of proteins, carbohydrates, nucleic acids, and lipids, respectively; d is the path length.

In this study, to perform the spectrophotometric analysis on the cells or EPS, the following characteristic absorption bands were used: 2,930 cm^{-1} for lipids, 1,080 cm^{-1} for carbohydrates, 1,250 cm^{-1} for nucleic acids, and 1,660 cm^{-1} for proteins. Their extinction coefficients at these wave numbers can be found in Grube et al. (1999). The spectra were normalized to the Amide I peaks at 1,660 cm^{-1}. The absorbance was obtained with the baseline technique. From the four equations of absorbance at 2,930; 1,660; 1,250; and 1,080 cm^{-1}, the ratios of proteins/carbohydrates in cells and their EPS can be calculated.

Results

FTIR spectra of *Rbl. acidophilus* and *Rb. capsulatus*

Figure 1 shows the typical FTIR spectra of *Rbl. acidophilus* and *Rb. capsulatus* at their stationary phase. The region between 4,000 and 500 cm^{-1} holds characteristic bands and is suitable for the characterization of microorganisms (Schmitt and Flemming 1998). The functional groups of proteins, carbohydrates, phospholipids, and nucleic acids could be observed (Table 1). As illustrated in Fig. 1, wide and intensive absorption bands at frequencies of 1,660 and 1,540 cm^{-1} could be identified, corresponding to the characteristic vibrations of the –CONH– group of Amide I and Amide II in proteins (Schmitt and Flemming 1998). In the 1,250 cm^{-1} region, a typical band of phosphate group absorption was observed, and this band was mainly attributed to nucleic acids (Schuster et al. 1999). At wave numbers 980–1,200 cm^{-1}, a wide and intensive carbohydrate bands were found, which were attributed to –COC– group vibrations in the cyclic structures (Gomez et al. 2003). Cellular lipids showed intensive duplicate absorption bands at 2,860 and 2,930 cm^{-1}, corresponding to the respective symmetrical and asymmetrical –CH– vibrations (Schmitt and Flemming 1998).

As illustrated in Fig. 1, the peak positions of proteins, carbohydrates, nucleic acids, and lipids were almost the same for the two stains. However, the relative intensities of their peaks were different. The absorption ratio between the peak intensities at 1,660–1,535 and 1,080 cm^{-1} bands (the bands of proteins and carbohydrates) for *Rbl. acidophilus* were higher than those for *Rb. capsulatus* (Fig. 1), suggesting that the relative contents of proteins and carbohydrates in the two strains were different.

The FTIR spectra of the cells of *Rbl. acidophilus* and *Rb. capsulatus* after EPS extraction are illustrated in Fig. 2. The typical absorption bands (1,660; 1,540; 1,250; and 1,080 cm^{-1}) could also be seen in these spectra. However, the relative intensities of the peaks in their spectra changed after EPS extraction. The ratio of absorbance at 1,660 cm^{-1} to absorbance at 1,080 cm^{-1} of *Rbl. acidophilus* after EPS extraction was higher than the corresponding ratio before EPS extraction, and it is the same case for the ratio of absorbance at 1,660 cm^{-1} to the absorbance at 1,250 cm^{-1}. For *Rb. capsulatus*, before and after EPS extraction, the same peaks were observed in the FTIR spectra. However, after EPS extraction, the height of the carbohydrate bands reduced, corresponding to an increase in the ratio of $A_{1,660}$/

Table 1 FTIR peaks of function groups of the two strains and their EPS

Wave number (cm^{-1})	Group	Reference
1660 1540	Amide I (–CO–)and Amide II (–NH–) in proteins	Schmitt and Flemming 1998
1250	Phosphate group in nucleic acids	Schuster et al. 1999
1080 1160	–COC– group vibrations in the cyclic structures of carbohydrates	Gomez et al. 2003
2860 2930	Symmetrical and asymmetrical –CH– vibrations in lipids	Schmitt and Flemming 1998

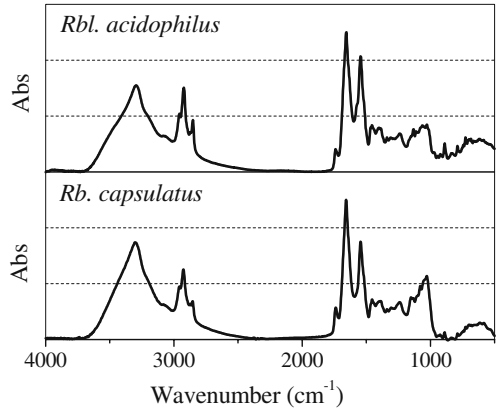

Fig. 2 FTIR spectra of *Rbl. acidophilus* and *Rb. capsulatus* after EPS extraction

$A_{1,080}$. However, the value of the ratio of absorbance at 1,660 cm^{-1} to absorbance at 1,250 cm^{-1} of *Rb. capsulatus* after extraction was higher than the corresponding value before extraction, while the ratio of absorbance at 1,080 cm^{-1} to the absorbance at 1,250 cm^{-1} after extraction was lower than that before extraction.

The difference in the main components of *Rbl. acidophilus* and *Rb. capsulatus* could be found from the FTIR spectra according to the measurement of the relevant intensities of these characteristic adsorption bands. The ratios of proteins/carbohydrates in the two strains were calculated from their FTIR spectra, and the results are listed in Table 2. The proteins/carbohydrates ratio of *Rbl. acidohpilus* was higher than that of *Rb. capsulatus*. For the two bacteria, the ratios of proteins/carbohydrates increased after EPS extraction. The ratios of proteins/carbohydrates of the two bacterial cells were greater than 1, indicating that proteins were the major components for the two bacteria.

FTIR spectra of EPS

Figure 3 shows the spectra of the EPS, extracted using the CER and EDTA methods, of *Rbl. acidophilus* and *Rb. capsulatus* at their stationary phase. The EPS spectra were not the same as those of the bacterial spectra. Although the typical absorption bands of proteins and carbohydrates could be observed in the EPS spectra, the relative intensities of various bands and the shapes of these bands were different from those of cells. The peaks at 2,930 and 2,860 cm^{-1} of the EPS spectra were lower than those of cell spectra. There was a big peak between 1,500 and 1,700 cm^{-1}, while there were two peaks in bacterial spectra. Such a difference suggests that the contents of intracellular and extracellular substances were substantially different. The difference could be readily found from the FTIR spectra. As listed in Table 2, for the EPS extracted using the EDTA or CER methods, the ratios of proteins/carbohydrates of EPS were lower than those of the two strains.

The lipids in the EPS could not be accurately determined due to its very low content, evidenced by the very low peak intensity at 2,930 cm^{-1}. This peak was attributed to the lipids of cells (Grube et al. 1999). Based on the conventional chemical analysis, a relatively higher nucleic acids content would lead to a higher peak intensity at 1,250 cm^{-1}. Furthermore, with the addition of 5 mg/l DNA into the two EPS samples, the absorbance at 1,250 cm^{-1} increased, as shown in Fig. 4. These results suggest that the wavelength of 1,250 cm^{-1} was attributed to nucleic acids (Schuster et al. 1999). However, the low peak intensities indicated that the contents of nucleic acids in the EPS of *Rbl. acidophilus* and *Rb. capsulatus* were of a low level (Figs. 3 and 4). Therefore, in this work the analysis on the EPS was focused on their main components, i.e., carbohydrates and proteins.

The spectra of EPS extracted using the EDTA method were also different from those extracted using the CER method. The band between 1,000 and 1,200 cm^{-1} in the spectra of EPS extracted using the CER methods was a big peak, while in the spectra of EPS extracted using the EDTA method, the band was bimodal. As illustrated in Fig. 3, for the CER extraction, the peaks of proteins and carbohydrates were at 1,655 and 1,078 cm^{-1} for *Rbl. acidophilus*,

Table 2 Ratios of proteins/carbohydrates in the two strains before and after extraction as well as their EPS

Bacterium			FTIR analysis	Conventional chemical analysis
Rhodoblastus acidophilus	Before extraction		2.11	1.69±0.04
	After extraction		3.06	2.39±0.11
	EPS	CER method	1.10	0.99±0.03
		EDTA method	1.52	1.38±0.02
Rhodobacter capsulatus	Before extraction		1.36	1.23±0.07
	After extraction		2.34	2.23±0.33
	EPS	CER method	0.38	0.47±0.04
		EDTA method	0.72	0.83±0.01

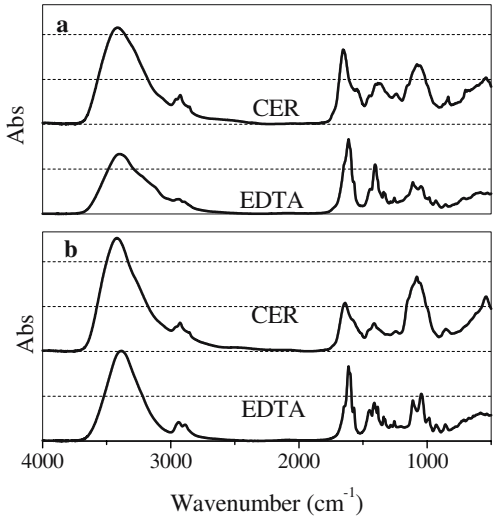

Fig. 3 FTIR spectra of EPS of **a** *Rbl. acidophilus* and **b** *Rb. capsulatus* extracted using the EDTA and CER methods

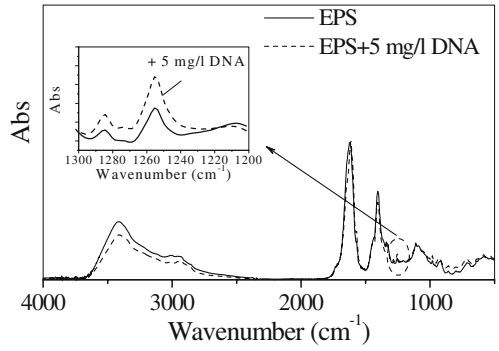

Fig. 4 FTIR spectra of EPS of *Rb. capsulatus* and the sample added with 5 mg/l DNA

and at 1,639 and 1,079 cm^{-1} for *Rb. capsulatus*, respectively. For the EDTA extraction, the peaks were at about 1,612; 1,111; and 1,047 cm^{-1} for *Rbl. acidophilus*, and at about 1,612; 1,111; and 1,043 cm^{-1} for *Rb. capsulatus*, respectively.

The ratios of proteins/carbohydrates of EPS calculated from FTIR spectra are listed in Table 2. The proteins/carbohydrates ratio values of EPS suggest that proteins were the major components in the EPS of *Rbl. acidophilus*. However, for the EPS of *Rb. capsulatus*, this value was lower than 1, indicating that the carbohydrates were the major component in its EPS. The ratios of proteins/carbohydrates for the EPS extracted using the EDTA method from the two strains were larger than those using the CER method, implying that the extraction efficiencies for proteins, carbohydrates, and other components in EPS were different with various methods. The FTIR spectrum of EPS clearly reflects the difference of various EPS samples, and is, thus, a rapid technique in analyzing the EPS components.

FTIR spectra of *Rb. capsulatus* and its EPS during cultivation

Figure 5 shows the FTIR spectra of *Rb. capsulatus* at various cultivation phases. During the bacterial cultivation, the locations of the typical absorption bands at 1,660; 1,540; 1,250; and 1,080 cm^{-1} did not significantly change. However, as the relative content of various cellular components varied during cultivation, the relative intensities of their peaks changed as well. The ratios of proteins/carbohydrates of cells calculated from the FTIR spectra are listed in Table 3. The ratios of proteins/carbohydrates of *Rb. capsulatus* initially decreased, but did not significantly change at the stationary growth phase.

Figure 6 shows the FTIR spectra of EPS extracted using EDTA method from *Rb. capsulatus* at various cultivation phases. Similarly, the locations of the proteins, carbohydrates, and nucleic acids absorption bands did not change. However, the ratios of these peaks varied during the bacterial cultivation. The ratios of proteins/carbohydrates of EPS during bacterial cultivation calculated from their FTIR spectra are also listed in Table 3. The ratios of

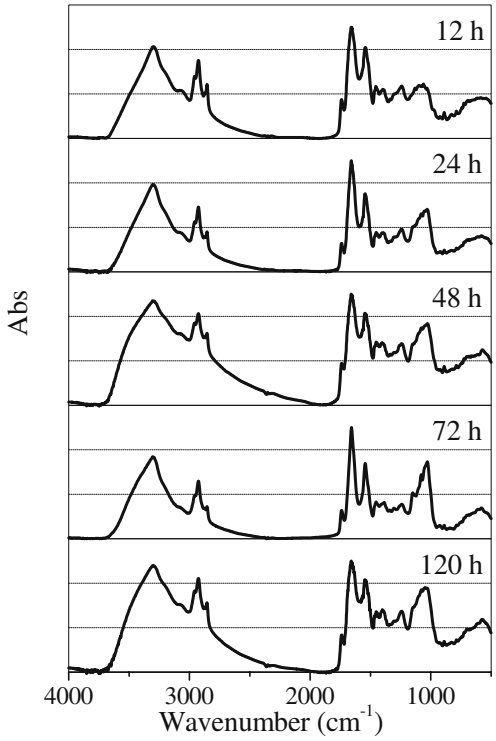

Fig. 5 FTIR spectra of *Rb. capsulatus* at its cultivation phases

proteins/carbohydrates decreased at initial stage, and slightly fluctuated at the stationary growth phase.

Comparison between the FTIR method and conventional chemical analytical method

For comparison, the ratios of proteins/carbohydrates of the two strains and their EPS measured using conventional chemical analysis are also listed in Tables 2 and 3. The two methods had a good agreement. For the bacterial cells and their EPS, the ratios of proteins/carbohydrates were of a similar level with the two analytical methods. This result implies that the determination of the components of

Table 3 Ratios of proteins/carbohydrates of *Rb. capsulatus* during its cultivation and its EPS

	Cultivation time (h)	FTIR analysis	Conventional chemical analysis
Rhodoblastus capsulatus	12	6.20	5.67±0.18
	24	2.38	2.81±0.07
	48	2.41	2.48±0.13
	72	1.36	1.23±0.07
	120	1.89	2.11±0.11
EPS	12	2.00	1.92±0.17
	24	1.28	1.32±0.02
	48	1.10	0.91±0.02
	72	0.72	0.83±0.01
	120	0.94	0.97±0.10

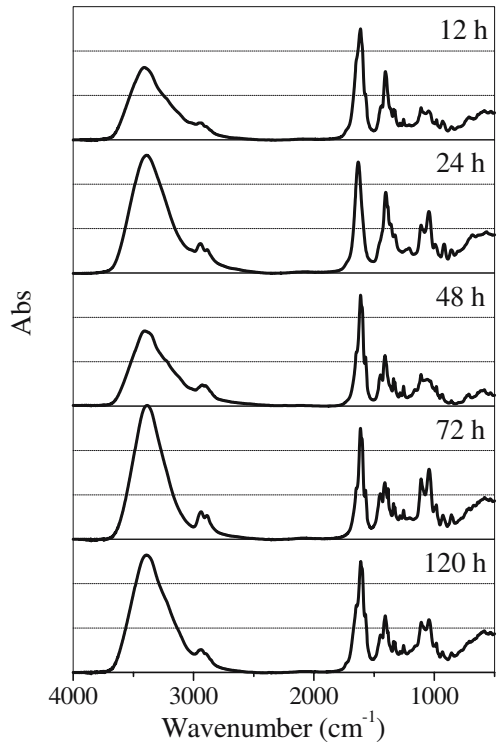

Fig. 6 FTIR spectra of EPS extracted from *Rb. capsulatus* using the EDTA method at its cultivation phases

microbial cell and EPS using the FTIR spectroscopy was feasible.

Discussion

The utilization of FTIR-spectroscopy in the field of microbiology has been proven to be a promising technique (Kamnev et al. 1999; Tindall et al. 2002; Galichet et al. 2001). FTIR spectroscopy allows a direct and quantitative analysis of specific components in multi-component mixtures without separation into individual components. The major components in microorganisms have a large number of sharp and characteristic absorption bands in the fundamental infrared region, allowing quantitative evaluation of the composition of cell part. For EPS, the main components were proteins and carbohydrates. The proteins/carbohydrates ratio significantly influences the bacterial surface characteristics (Watanabe et al. 1998). However, the conventional chemical analysis of EPS components is time-consuming. The samples would suffer from a partial loss in each purification step, resulting in a measurement deviation. On the other hand, FTIR spectroscopic analysis could be rapidly performed, and information about multi-components could be simultaneously obtained. Therefore, it is a rapid technique for characterizing the components of bacteria and their EPS.

In the present study, the absorption bands of the major components in the two PSB strains and their EPS were clearly observed in the FTIR spectra. For *Rbl. acidophilus* and *Rb. capsulatus*, the FTIR spectra were different in the relative intensity and the shape of absorption bands (Fig. 1), suggesting the difference in the contents of cell components. The components of bacterium and its EPS could be readily obtained from the FTIR spectra without any purification. The differences in the EPS of various strains or extracted using various methods could be found from the FTIR spectra. The shifts of the peak locations of EPS extracted using the EDTA method might be associated with the interaction between EDTA and EPS (Comte et al. 2006). The value of the proteins/carbohydrates ratio obtained by the FTIR spectral method was generally consistent with that by the conventional chemical one. Results in Tables 2 and 3 show that the values obtained with the two methods were of a very similar level. This also confirms that for cells and their EPS samples, the analytical results using both FTIR and conventional chemical colorimetric methods were consistent.

FTIR-spectroscopy is a good means for studying microbial EPS. However, typical characteristic adsorption bands are not readily obtained for quantitative analysis because of the overlap of spectral bands (Huffman et al. 2003). Therefore, an improved FTIR-spectroscopy analysis is needed. For instance, a coupled high-performance liquid chromatogram-FTIR spectrometer may be a better option for analyzing microbial components (Huffman et al. 2003), and other mathematical tools, e.g., stoichiometric methods, could be used to separate the signals of different components and overcome the drawbacks of FTIR. Nevertheless, the FTIR spectroscopy analysis is easy and rapid to perform, and could simultaneously obtain much information without purification. Thus, it is a promising technique for analyzing the function group characteristics and components of bacterial EPS.

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