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Characterization of Partitioning Behaviors of Microorganisms and Their Homogenates in Aqueous Two-Phase Systems

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A method for the control of the partitioning behavior of microorganisms and their homogenates in aqueous two-phase systems (ATPS) was investigated based on their surface properties, such as isoelectric point (pI) and surface net hydrophobicity (HFS). The pI value of *Saccharomyces cerevisiae* was determined as 1.3±0.2 based on the partitioning behavior of cells in the ATPS with different salts (i.e. the cross-partition method). The amounts of cells partitioned at the interface (IPF values) were also found to be useful for the determination of the pI value, suggesting that IPF was dependent on the electrostatic effect. HFS values of *S.cerevisiae* were determined by the relationship between the hydrophobic factor of the ATPS (HF) and the partition coefficients of cells at their pI. The cell surface of *S.cerevisiae* had a rather hydrophilic nature (-854 kJ/mol) comparing with that of *Escherichia coli* (-524 kJ/mol). The HFS values of these cells were found to affect the IPF values. Following the disruption of the microorganisms, the surface properties of the homogenates were also characterized in the same way. The pI and HFS values were found to increase as cell disruption proceeded. It was quantitatively shown that the partitioning behavior and the amounts of cells partitioned at the interface were dependent on the above surface properties and could be controlled by the use of electrostatic and hydrophobic effects.

1.Introduction

Because of the relative composition of various cell membrane materials, the membrane surface of microorganisms exhibits various properties, such as charge, hydrophobicity, and the other specific properties. The efficiency and the selectivity of the recovery of target products in practical bioprocessing,

including the selection and separation of microorganisms, their cultivation, their disruption and/or lysis, and the removal of their homogenates from the target products, may be directly affected by these surface properties. These properties can also be utilized for the clarification of the mechanisms of the stress-response function of the microorganisms because the membrane surface will, firstly, recognize the environmental stress and respond against it. The development of evaluation methods of these surface properties is, therefore, important and necessary.

Various methods for the characterization of these cell properties have been previously reported by many researchers [1-9]. The aqueous two-phase partitioning method is particularly good for the following two reasons. (1) The surface properties can be analysed under mild conditions because these systems are mainly composed of water. (2) The systematic analysis of the surface properties, such as isoelectric points, surface net hydrophobicity, and other specific properties, can be achieved by varying the partitioning mode. Albertsson [10] and, recently, Kuboi *et al.* [11] investigated the partitioning behavior of macromolecules, such as microorganisms, bacteria, and viruses, micromolecules, such as amino acids, peptides, proteins and enzymes, respectively.

In this study, the surface properties of microorganisms and their homogenates are quantitatively characterized by using the aqueous two-phase partitioning method. Then, the partitioning behaviors of cells at the interface are also investigated in order to clarify the effects of cell surface properties and the partitioning systems on them. Based on the surface properties, such as hydrophobicity and isoelectric point, the possibility of controlling the amounts of cell homogenates partitioned at the interface in order to complete the separation of them thoroughly is discussed.

2. Theoretical

The analytical methods for determination of the surface properties of microorganisms using ATPS containing the polymer, to which charged, hydrophobic, or biospecific ligands are attached, have been typically reported by Miorner *et al.* [12,13]. When such ligands are not incorporated in the polymer, the partition coefficients of biomolecules in ATPS have been found empirically to depend on several effects which act roughly independently. The partitioning coefficient, K, of biomolecules, such as amino acids, peptides, proteins, virus, and cell, may therefore be written as Baskir *et al.* [14].

$$\ln K = \ln K_{hphob.} + \ln K_{el.} + \ln K_{salt} \tag{1}$$

$K_{hphob.}$, $K_{el.}$, and K_{salt}, are, respectively, the contributions to the partitioning of biomolecules from hydrophobic, electrostatic, and salt effects. Based on these effects, the protein surface can be systematically characterized [11,15]. The concept is shown in Fig.1. It is thought that thus characterization method can be

applied to microorganisms. When the isoelectric point (pI) is selected the pI at low ionic strength, $\ln K_{el}$, and $\ln K_{salt}$ can be assumed to be negligible, and thus,

$$\ln K \fallingdotseq \ln K_{hphob.} \quad (2)$$

Nozaki and Tanford [16] evaluated the hydrophobicities of several amino acids in water/ethanol and water/dioxane systems. Kuboi *et al.* [17] have reported the relationship between their evaluated values and the partition coefficients of amino acids and defined a hydrophobicity factor (HF) as the indicator of the hydrophobicity of two-phase systems. The net hydrophobicity of proteins (HFS) were determined from the slope of equation (3) using the ATPS.

$$\ln K = HFS \times HF \quad (3)$$

Following the above scheme, the surface properties of bacterial cells were characterized in the same way as applied to the characterization of protein surfaces.

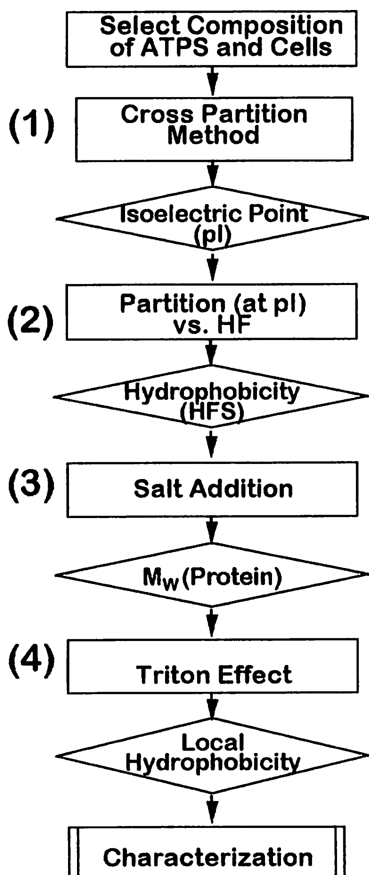


Fig. 1 Scheme for the characterization of the surface properties of biomolecules

3. Experimental

3.1 Materials. Poly(ethylene glycol) (PEG1540, 4k and 6k; M_w =1.5k, 3k and 7k daltons, respectively) and dextran (Dex; M_w=100~200k daltons) were obtained from Wako Pure Chemicals Ltd. (Osaka, Japan). Casamino acid was purchased from Difco Lab. (Michigan, USA). Baker's yeast (*Saccharomyces cerevisiae*) was purchased from the Oriental Yeast Co. Ltd. (Osaka, Japan). The salts and other chemicals were of analytical grade.

3.2 Cultivation of Microorganisms and Preparation of Their Homogenates. Two types of typical microorganisms, such as prokaryotic *Escherichia coli* W3110 and ML308 and eukaryotic *S.cerevisiae*, were used in this study. These *E.coli* cells were grown in a modified M9 media supplemented with 5 g/dm^3 glucose and 50 g/dm^3 glycerol as a carbon source [18]. After the cells were cultivated (100 cm^3, 210 K) to the stationary growth phase in a shaking flask, they were washed once and were suspended in 0.02 mol/dm^3 Tris-HCl buffer.(3 ~4 mg [dry-cell] / cm^3-buffer) *S.cerevisiae* cells were suspended in 0.05 mol/dm^3 potassium phosphate (KPi), 6wt% PEG1540, and 12wt% PEG1540 / 12wt% KPi two-phase

systems. (20 mg [wet-cell] / cm^3) These cell suspensions were disrupted either by an ultrasonic method (Tomy Seiko, UD-201, Japan) or by beads-milling (Bead-Beater, USA). After these homogenates were washed with the respective buffer, they were recovered by centrifugation (15000 rpm, 10 min 277 K) and resuspended in the buffer (*E.coli* and *S. cerevisiae* homogenate suspension). The disrupted fraction of cells, DF_C, and the released fraction of intracellular products, RF_i (i; types of intracellular products) were determined from the concentration of cells and total soluble proteins, respectively, as previously reported [18].

3.3 Partitioning Procedure of Microorganisms and Their Homogenates in Aqueous Two-Phase Systems. The basic composition of the phase systems for the *E.coli* cells were 9~10.8wt% PEG1540, 4000, 6000 / 9wt% Dex systems. The pH of these systems was adjusted by the addition of concentrated HCl and NaOH solutions. For partitioning of yeast cells, 12wt% PEG1540 / 12wt% KPi systems were used. The phase systems were prepared by mixing stock solutions of PEG, Dex, and KPi together with the above cell suspensions and their homogenate suspensions. After mixing in a 10 cm^3 centrifugal tube (inside diameter; 10 mm) by gentle inversion, the systems were carefully centrifuged ($10 \times g$, 5 min, 298 K) in order to enhance the phase separation but not to induce the sedimentation of cell particles. This condition has been selected from the pre-experimental determination of the sedimentation curve of the cells in the buffer. The ATPS were separated into top and bottom phases. The volume and the cell concentrations (C_{Cell}) of these phases were then analysed.

The partition coefficients of bacterial cells, K_{Cell}, were defined as the ratio of the cell concentration in both phases, $C_{Cell,Top}$ / $C_{Cell,Bottom}$. The amounts of cells and their homogenates, which were partitioned at the interface, were evaluated in the following two ways, based on their weights and volume. The interfacial partitioning factors of *S.cerevisiae* and *E.coli* cells, $IPF_{W,Cell}$ [%], were determined as the percentage of weight of *E.coli* cells partitioning at the interface against the total amount, ($C_{Cell,Top}$ [mg-cell/cm^3] $\times$ V_{Top} [cm^3]) / ($C_{Cell,Bulk}$ [mg-cell/cm^3] $\times$ V_{Bulk} [cm^3]) from the mass balance. Further, the $IPF_{V,Cell}$ values of *S.cerevisiae* were also determined as the percentage of the volume of the homogenates partitioned at the interface of the systems, $V_{Cell,\ Interface}$ [cm^3] to overall phase volume, V_{Bulk} [cm^3]. The centrifugal clarification process of the homogenates of *S.cerevisiae* was monitored at 1200 rpm by light transmittance measurements using a Shimadzu Centrifugal Particle Size Analyzer CP-50.

3.4 Measurement. The concentration of bacterial cells was determined by optical density (OD). The HF values of ATPS were determined according to Kuboi *et al.* [17]. The size distributions of various particles were measured by the Dynamic Scattering method (DLS-700Ar, Otsuka. Electr. Co. Ltd., Japan). The activity of enzymes (i=1, acid phosphatase (AcP); i=2, glucose-6-phosphate dehydrogenase (G6PDH); i=3, fumarase (Fum)) was determined by the previously described method [18]. The concentration of total soluble proteins (i=T) was measured by the Pyrogaroll Red Method [19].

4. Results and Discussion

4.1 Control of Partitioning of Microorganisms and Their Homogenates in Aqueous Two-Phase Systems. In a practical process using aqueous two-phase systems for the production and separation of useful biomolecules, an important problem is the removal of cell homogenates from the interface of both phases. The partitioning behaviors of cell homogenates at the interface of the PEG/phosphate ATPS, which show favorable economic and operational factors, were investigated using the evaluated values of the amounts of the cell homogenates partitioned at the interface.

4.1.1 Separation of Cell Homogenates by using Aqueous Two-Phase Systems. The observed clarification process of cell homogenates in the ATPS is compared with that in a single phase system (water solution) in Fig.2. *S.cerevisiae* cells were disrupted with Bead-Beater in 12wt% PEG1540 / 12wt% KPi systems and the homogenates were diluted with pure systems of the same composition and with water. The light transmittance was measured at a point above the final liquid-liquid interface. Settling time for the homogenate in the ATPS was extremely short in the range of several seconds at 1200rpm, while that in the homogeneous water solution was over 10 times longer. Although the actual concentration of cell homogenates in an industrial-scale process is much higher than above, it is clear that the solid-liquid separation including the fractionation of the cell homogenates and the following liquid-liquid separation is expected to be much easier in a PEG/phosphate aqueous two-phase system for production of a clear top phase with the target enzyme than in a single-phase system. This is because the time consuming solid-liquid separation process in a homogeneous solution can be converted into a rapid liquid-liquid separation process in PEG/phosphate systems. Therefore, a shorter time and lower centrifugal speed are achieved for the removal of cell homogenates and other insoluble impurities in the ATPS, especially

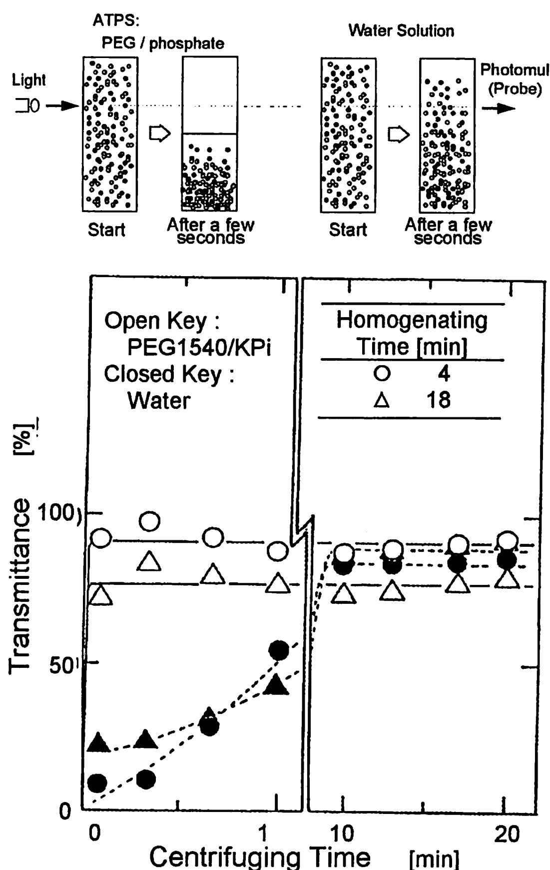
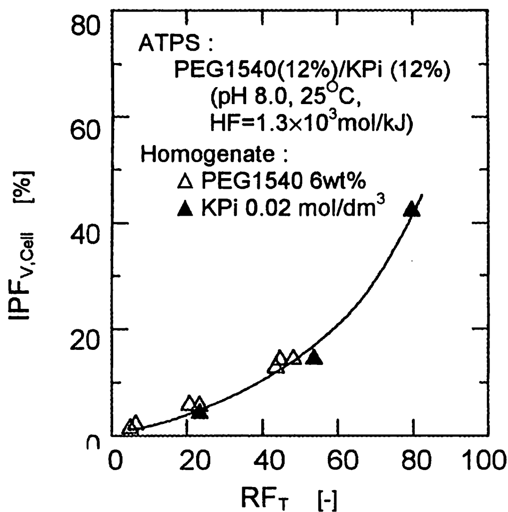


Fig.2 Solid-liquid separation in PEG/phosphate aqueous two-phase systems and in a homogeneous solution (disruption time: O● 4min; Δ▲ 8min)



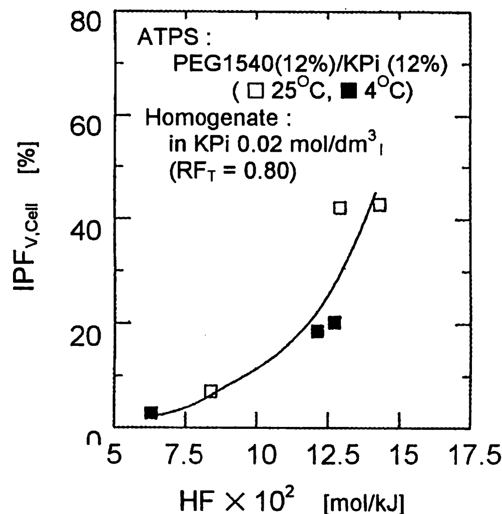


Fig.3 Correlation of the interfacial partitioning factor, $IPF_{V,Cell}$, of cell homogenates with the released fraction, RF_T, of total proteins by cell disruption

Fig.4 Correlation of the interfacial partitioning factor, $IPF_{V,Cell}$, of cell homogenates with the hydrophobic factor, HF, of the phase system

in the PEG/phosphate two-phase systems. In the following experiments, the partitioning behaviors of cell homogenates at the interface were characterized by using the evaluated values, $IPF_{V,cell}$.

4.1.2 Effects of Cell Disruption on the Partitioning of Homogenates at the Interface. The effects of disruptive conditions, such as the disruptive media and the disruption time, on the partitioning of cell homogenates were investigated. The homogenates of *S. cerevisiae*, which were disrupted with the Bead-Beater (1~16 minute, 11600rpm, 0.5mm beads) in 6wt% PEG1540 or 0.02 mol/dm^3 KPi solution, were partitioned in a PEG1540 (12wt%) / KPi (12wt%) system at pH 7.1 to 9.2, and the systems were centrifuged at 1200 rpm x 10 min. As previously reported [18,20], the presence of PEG and KPi has an influence on the released fraction of total proteins (RF_T) in different ways. As the interfacial partitioning factor of cell homogenate, $IPF_{V, Cell}$, and the released fraction of total proteins, RF_T, increase with disruption time and change with the surrounding medium in a similar fashion, fine cell homogenates, produced by longer disruption times and consequent larger release of intracellular protein are likely to be partitioned to the PEG-rich phase. This is thought to be because of the increase in the hydrophobicity of the surface of cell homogenates during the disruption process. Figure 3 shows the variation of $IPF_{V, Cell}$ with RF_T obtained under various disruption conditions. For a given phase system, $IPF_{V, Cell}$ can be correlated with a single parameter, RF_T. The $IPF_{V, Cell}$ value is therefore found to depend on the value of RF_T.

4.1.3 Partitioning Behaviors based on Hydrophobic Effect. The hydrophobic effect on the $IPF_{V,Cell}$ was investigated. $IPF_{V,Cell}$ values of cell homogenates were determined for various conditions (271~298 K, pH

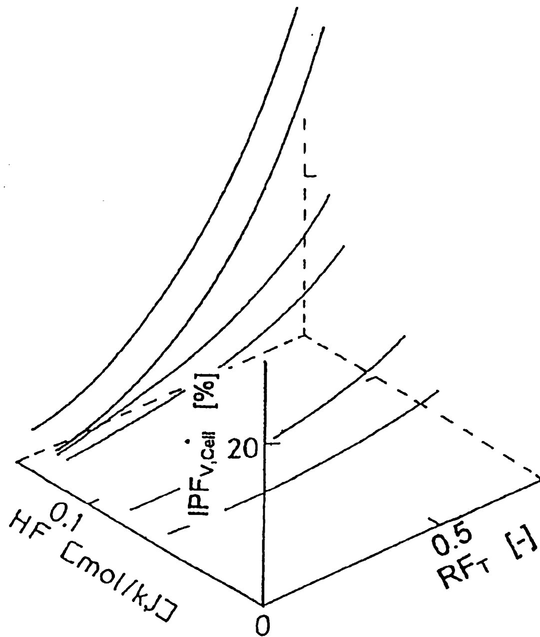


Fig.5 Summary of the dependence of the interfacial partition factor, $IPF_{V,Cell}$, of cell homogenate on the released fraction of total proteins, RF_T, and on the hydrophobic factor of the phase systems, HF.

7.8~9.2). Increasing both pH and temperature raises the hydrophobic factor of the PEG/phosphate system, HF. The dependence of $IPF_{V,Cell}$ on the hydrophobic factor of the system, HF, is shown in Fig. 4. For a given disruption time and thus a given PR value, $IPF_{V,Cell}$ can be correlated with the single parameter, HF, regardless of individual pH and temperature. $IPF_{V,Cell}$ increases steadily with HF. The undisrupted cells have larger densities and they are usually partitioned to the bottom phase. However, for smaller cell homogenates obtained after more protein is released, the hydrophobic interaction between the system and cell homogenates may play an important role in its partitioning and hence, more cell homogenates tends to be partitioned to the top phase.

Dependencies of the $IPF_{V,Cell}$ on the released fraction, RF_T and the HF values of the ATPS are summarized in the Fig. 5. The $IPF_{V,Cell}$ values are thought to be controlled by the selection of the released fraction of total proteins (RF_T) and the hydrophobicity of two-phases (HF).

4.2 Characterization of Surface Properties of Microorganisms and Their Homogenates. The partitioning behaviors of the microorganisms and their homogenates at the interface of the ATPS, which were described in the above section, are thought to depend on their surface properties such as isoelectric point and surface net hydrophobicity. These properties were analysed by using the aqueous two-phase partitioning method, following the scheme for the characterization of the surface properties (Fig.1). The methods for control of their partitioning behaviors are then discussed on the basis of the evaluated values.

4.2.1 Isoelectric Point (pI) of Microorganisms. The isoelectric point (pI) of microorganisms was determined by the cross partition method [10,12,13]. The cells were partitioned in the two ATPS containing two different salts (0.4 mol/dm^3 NaCl and 0.2 mol/dm^3 Na_2SO_4) at almost the same ionic strength of anion, the contribution of which is important to cell partitioning. Figure 6 shows the dependence of the partition coefficient, K_{Cell}, and interfacial partitioning factor, $IPF_{W,Cell}$, of the *S. cerevisiae* on the corresponding pH

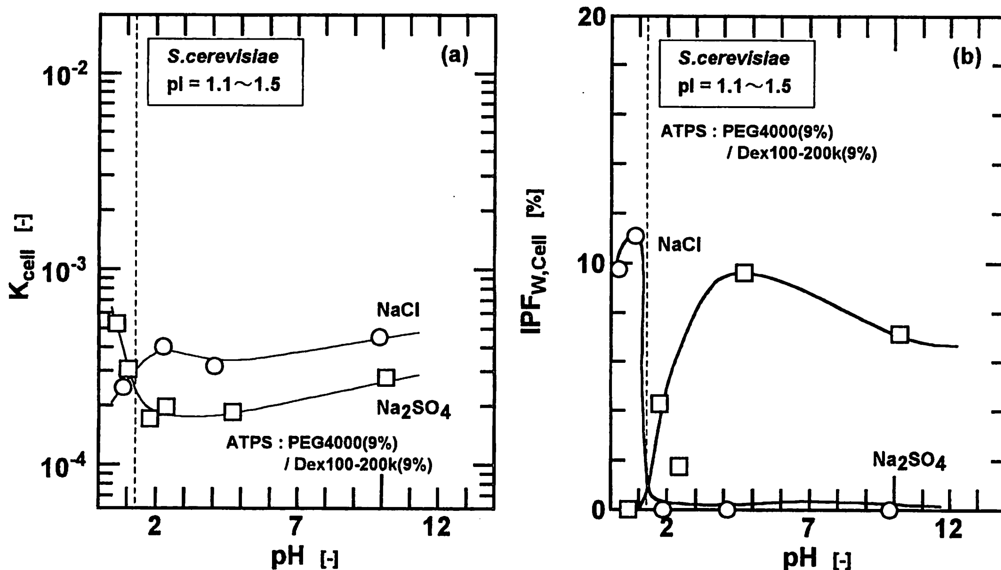


Fig. 6 Dependence of K_{Cell} and $IPF_{W,Cell}$ values of *S.cerevisiae* on the pH values in PEG4000/Dex ATPS in the presence of 0.4 mol/dm^3 NaCl and 0.2 mol/dm^3 Na_2SO_4 by the cross partition method [10].

values in the presence of the above salts. When the pH values decrease, the K_{Cell} values increase in the presence of Na_2SO_4 (Fig. 6 (a)). On the other hand, they gradually increase with decreasing pH values in the presence of NaCl. These two curves cross at a pH value of 1.3 ± 0.2 and this value can, therefore, be determined to be the isoelectric point. Similarly, the values of $IPF_{W,Cell}$ in the ATPS increase and decrease with decreasing pH values, respectively, in the presence of NaCl and Na_2SO_4 (Fig. 6 (b)). The two curves cross at the pH value of pI. It is found that the isoelectric points of microorganisms can be determined by using the values of K_{Cell}, and $IPF_{W,Cell}$ and that the partitioning behaviors of microorganisms in the top and bottom phases and at the interface can be controlled by the above electrostatic effect .

4.2.2 Surface Net Hydrophobicity of Microorganisms. The partitioning of cells is mainly dependent on the hydrophobic effect in the ATPS at the isoelectric point. The partitioning behaviors of cells were investigated in various ATPS which differed in hydrophobicity between the two-phases. Figure 7 shows the relationship between the K_{Cell} and $IPF_{W,Cell}$ values of two different cells and the corresponding hydrophobicity factor (HF) at the isoelectric point. As shown in Fig.7(a), the K_{Cell} values of *S. cerevisiae* decreased linearly with increasing HF values within a HF value range of less than 0.015 mol/kJ. The value of the slope of this line is -854 kJ/mol. The K_{Cell} values of *E.coli* ML308 cells also decreased with increasing HF values and the value of the slope of this line is -369 kJ/mol (Fig.7(b)). The slope of these lines, HFS, (Eq.(3)) can be determined as the surface net hydrophobicity of cells. The cell surface of *S. cerevisiae* is found to have a rather hydrophilic nature comparing with that of *E coli*. The $IPF_{W,Cell}$ values of cells are,

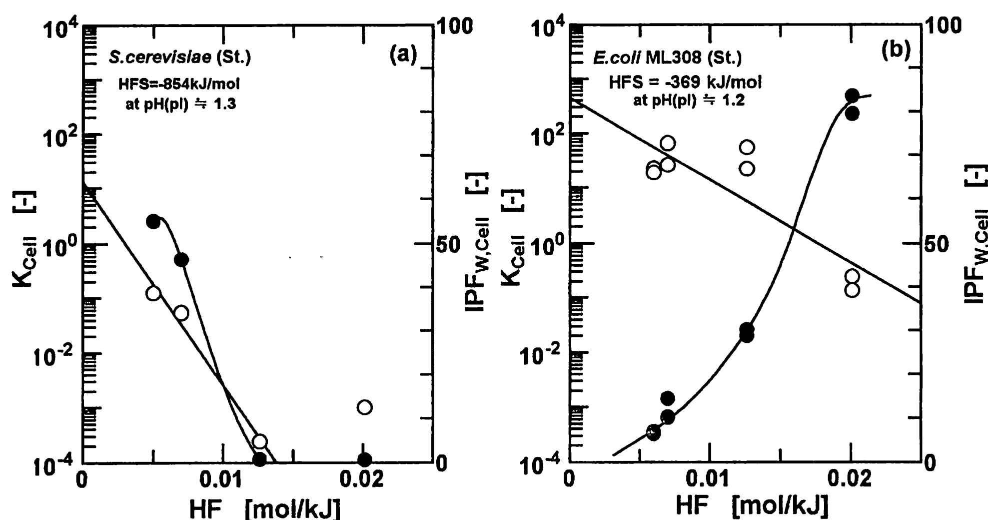


Fig. 7 Relationship between the HF values and the corresponding K_{Cell} and $IPF_{W,Cell}$ values in some aqueous two-phase systems at the isoelectric point

therefore, affected by the surface net hydrophobicity of these cells. The $IPF_{W,Cell}$ values of the hydrophilic *S. cerevisiae* cells decreased with increasing HF values (Fig.7 (a)). On the other hand, that of the rather hydrophobic *E.coli* cells increased with increasing HF value (Fig.7(b)). In this way, the partitioning behavior of cells and their amounts partitioned at the interface were found to be dependent on the above surface hydrophobicity and could be controlled by the use of hydrophobic effects.

4.2.3 Comparison of Surface Characteristics between Different Microorganisms. By the use of the above methods, the surface properties of microorganisms, such as *S.cerevisiae* and *E.coli* (W3110 and ML308) are characterized. These values are summarized in Table 1. All cells are shown to have isoelectric points at acidic pH values. Typically, it has been reported that cell surfaces have isoelectric points at low pH values [21]. The above isoelectric point results agree with the previous findings. This suggests that i) the pI values indicate the average charge of the cell surfaces owing to the relative number of anionic and cationic lipids and lipoproteins which are stacked out from the cell surface and that ii) some cell surfaces have similar compositions of ionic groups on their cell membrane.

Table 1 Isoelectric points and surface net hydrophobicity of three different microorganisms

Types of Cells	pI [-]	HFS [kJ/mol]
S.cerevisiae (Baker's yeast: st.phase)	1.3	-854
E.coli W3110 (st.phase)	2.8	-524
E.coli ML308 (st.phase)	1.2	-369

The cell surface hydrophobicity is also shown in this table. All cells are found to have a rather hydrophilic nature compared with peptides, proteins, and enzymes [11]. These results correspond to the typical findings for the surface properties of microorganisms. The hydrophobicity of *S.cerevisiae* is found to be smaller than that of *E.coli* cells. This is because of the contribution of hydrophobic groups (hydrophobic amino acid, and/or, mainly lipid materials or other hydrophobic domains), which exist in the membrane surface of microorganisms.

It is found that the surface properties of microorganisms, such as the isoelectric point and surface net hydrophobicity, are dependent on their types and their partitioning behaviors in the ATPS can be controlled on the basis of these properties.

4.2.4 Alternation of Surface Properties during the Cell Disruption Process. As a case study of the application of the evaluated surface property values such as pI and HFS, these values for disrupted *E.coli* W3110 cells were determined by using the above method. Figure 8 shows the relationship between the disrupted fraction of cell, DF_C, and the various corresponding parameters (the size distribution of cell (f(w)), the released fraction (RF_i), and the surface hydrophobicity (HFS), and isoelectric point (pI)). When the DF_C values are increased, the size distribution of cells is decreased (Fig.8(a)) and the intracellular enzymes are then released to the surrounding media, depending on their intracellular locations (Fig.8(b)). As the cell disruption proceeds, the hydrophobicities of *E.coli* cells gradually increased and the pI values increased toward mid-range pH values (Fig.8(c)). Further, the $IPF_{W,Cell}$ values increased with the disrupted fractions in most experiments using aqueous two-phase partitioning systems. In the cell disruption process, the microorganisms are disintegrated into many pieces and then the intracellular products, which have ionic and or hydrophobic

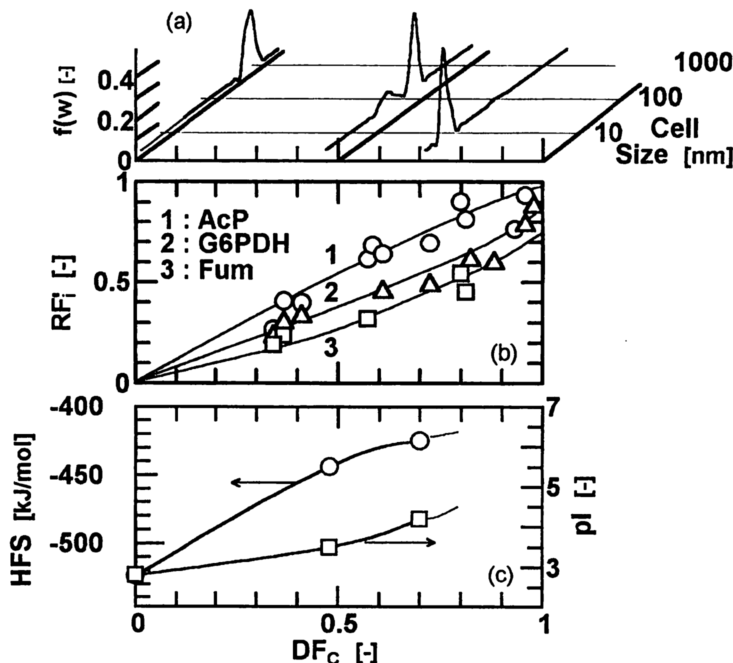


Fig. 8 Change in the corresponding distribution of cell size, released fraction, RF_i, and the surface hydrophobicity, HFS, and isoelectric point, pI as the cell disruption process proceeds. Strain: *E.coli* W3110

groups, appear on the surface of the homogenates. The surface properties of cells, such as HFS and pI, and their partitioning behaviors at the interface are affected by these phenomena.

It is found that the partitioning behavior of cell homogenates at the interface can be controlled not only by the above electrostatic and hydrophobic effects but also by the control of the cell disruption process, based on the relationship between the disrupted fraction of cells and their evaluated properties.

5. Conclusion

The partitioning behavior of microorganisms and their homogenates can be explained on the basis of the evaluated values of their surface properties. The surface properties of microorganisms and their homogenates, such as isoelectric points and net hydrophobicities, can be evaluated by using aqueous two-phase partitioning methods based on similar methods for the characterization of protein surfaces. The partitioning behavior of microorganisms between the phases and at the interface of the aqueous two-phase systems were found to be affected by hydrophobic and electrostatic effects. The values of the hydrophobicity and isoelectric points of microorganisms were shown to increase when the cells were disrupted. It is suggested that the partitioning of the cell homogenates can be controlled through these properties.

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Nomenclature

ATPS ; aqueous two-phase systems

C_{Cell} ; cell concentration determined from the optical density [mg-(dry)cell/cm^3]

DF_C ; the disrupted fraction of cell [-]

HF ; hydrophobic differences between two-phases [mol/kJ]

HFS ; surface net hydrophobicity of biomolecules [kJ/mol]

$IPF_{V.Cell}$; interfacial partitioning factor on the basis of the volume of cell homogenates at the interface [%]

$IPF_{W.Cell}$; interfacial partitioning factor on the basis of the cell weight at the interface [%]

OD ; optical density

pI ; isoelectric point [-]

RF_i ; the released fraction determined from the concentration of total proteins (i=T) and the activity of enzymes (i=1; AcP, 2; G6PDH, 3; Fum) [-]

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