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Physical methods for characterization of microbial cell surfaces

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Summary. There are different concepts for explaining the adsorption of microorganisms to solid surfaces: the DLVO theory and the surface free energy. Basic aspects of both theories are discussed. Established methods for determining the surface properties of microbial cells are reviewed: Electrophoretic mobility, colloid titration, electrostatic interaction chromatography, bacterial adherence to hydrocarbons, partitioning in an aqueous two-phase system, hydrophobic interaction chromatography, contact angle measurement and X-ray photoelectron spectroscopy. They are discussed and classified according to their potential for the correlation of cell surface characteristics and adsorption behavior.
Key words. Cell surface characterization; surface charge; hydrophobicity; chemical analysis; adsorption.

Introduction

Up to now no uniform theory has been developed to explain the fundamental mechanisms of cell adsorption phenomena. Both physical and chemical theories have been proposed [23]. One can distinguish between reversible adsorption, where the microorganisms still exhibit Brownian motion and can be removed from the surface by shearing forces, and irreversible adsorption where the microorganisms are firmly attached to the surface by extracellular polymer fibrils (polymer bridging) or by specific cell adhesion molecules (adhesines), which are supposed to be responsible for selective interactions between microorganisms and plants or animal or human tissues [49].

The present paper is focussed on the influence of physical interactions on adsorption. Such interactions can be classified as long-range (distances > 150 nm), short-range (distances < 150 nm) and very short-range forces (distances < 50 nm) [87]. The DLVO theory, developed by Derjaguin, Landau, Verwey and Overbeek, is applicable in general to explain long-range interactions [73]. According to this theory the total interaction energy of two particles (V_T) is calculated by the sum of the London-van der Waals attractive (V_A) and the electrostatic-like-charge repulsive energy (V_R). The repulsive energy depends on the thickness of the electrical double layer, which is inversely correlated to the ionic strength of the suspending liquid [49]; on the surface potential; on the distance between the interacting particles, and on the dielectric constant of the liquid phase [73, 87]. If the electrical double layer is small, attractive interactions even between surfaces of the same charge are possible. Figure 1 shows the correlation of the interaction energy (V_T) and the distance (h) between like-charge particles; attraction occurs at the primary and at the secondary minimum (h = 5–10 nm). Though for biological systems the exact determination of the terms V_A and V_R is difficult, the DLVO theory may be useful for the interpretation of adsorption phenomena [73].

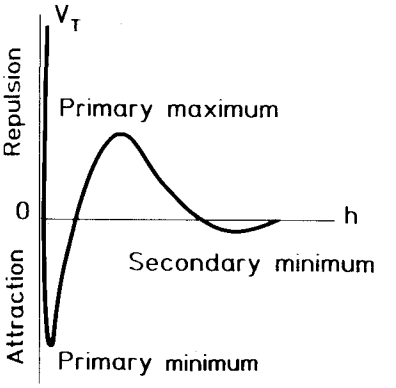

Figure 1. Correlation of the total interaction energy (V_T) between two particles of the same charge and the separation distance (h). (Barclay et al.[10] and Rutter [73], modified).

At short distances the validity of the DLVO theory is doubtful, i.e. it cannot be applied for the accurate prediction of interactions at the primary minimum. Therefore several authors introduced the concept of surface free energy to describe interactions at small distances, where any repulsive forces have already been overcome[87].

The surface charge of microorganisms

Different aspects of electrical phenomena related to particles have been discussed in detail by several authors[5,20,23,40,41,47–49,65]. The main aspects will be summarized in the following.

Electrical double-layer concepts

The surface potential of microorganisms, which is generated by dissociated chemical groups and not by ion adsorption[23], is counterbalanced by ions of the opposite charge (counter-ions). In this way an electrical double layer is built up. There are two main concepts describing the structure of the double layer, the Gouy-Chapman and the Stern models[41,48]. Whereas Gouy and Chapman postulated a diffuse structure of the double layer due to the thermal energy of the ions, the Stern model, now widely accepted, is founded on the theory that the electrical double layer is divided into two parts. The ions of the first part, the Stern layer, are attached strongly enough by electrostatic and van der Waals forces to overcome the thermal forces. In the second part the ions are diffuse, and free movement is possible[41]. Figure 2 shows the exponential potential drop in such a double layer.

When an external electric field is applied to charged particles suspended in an electrolyte solution, the particles and firmly attached ions migrate to the appropriate electrode. The remaining counter-ions of the diffuse part of the double layer move in the opposite direction. The boundary between the compact and the diffuse part of the double layer is defined as the plane of shear or slipping plane, the potential at this plane, relative to the bulk medium, is the electrokinetic or zeta potential ζ.

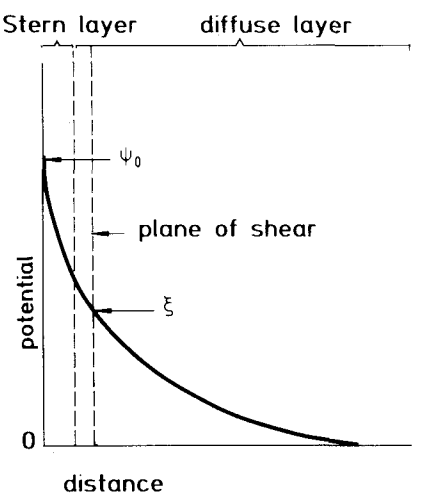

Figure 2. Potential drop in an electric double layer; Ψ_0, surface potential; ζ, zeta potential. (James[41], modified).

From the velocity of the charged particles over a known distance, which is measured, the electrophoretic mobility can be calculated[49]. There are different equations to determine the zeta potential from the electrophoretic mobility[12]. For bacteria, the Helmholtz-Smoluchowski equation is normally used.

On the one hand, it is possible to calculate the surface charge density of microorganisms from the zeta potential[5,12]. On the other hand, most investigators prefer to use only zeta potential or electrophoretic mobility data because of uncertainties concerning the value of several parameters needed for the calculation of the surface charge density.

Measurement of the electrophoretic mobility

Usually the variation with pH of the electrophoretic mobility or zeta potential of microorganisms or isolated cell walls[55] is studied at constant ionic strength. The surface charge can be characterized by the isoelectric point[32,81] or the zeta potential or electrophoretic mobility at pH 4[6,7]. The isoelectric point of most microorganisms that have been studied is in the range of pH 2–3.5. The exact determination is difficult. One has to take into account that at extreme pH values the cell surface may be irreversibly altered[47], i.e. when the pH has been readjusted to pH 7 the original mobility is not retained[55]. A direct comparison of experimental pH values with those of pure organic substances is not possible because the dissociation behavior of ionogenic groups on the outer cell surface is influenced by their surroundings[46]. No fundamental differences between the isoelectric point of gram-positive and gram-negative microorganisms could be observed[32].

pH-mobility curves can be used to characterize the surface ionogenic groups. In this respect, there are two types of microorganisms. The first one only shows zero or constant negative electrophoretic mobility at pH values above ≈ 4. In this case all ionogenic groups are acidic. Dissociation occurs at pH values above 4.0; at pH 2.0 these groups are undissociated and so the microorganisms are uncharged. The other type shows a positive charge at low pH values and a constant net negative surface charge between pH 9.0 and 11.0. These microorganisms are amphoteric; they carry both acidic and basic ionogenic groups. The charge reversal at low pH is due to dissociated basic (amino) groups together with undissociated acidic groups. At pH values up to ≈ 7.0 both the acidic and the amino groups are dissociated. The net negative charge is due to an excess of acidic groups. At higher pH, the amount of dissociated amino groups decreases, which results in an increase of the net negative charge[49]. Variations of the pH-mobility curves after chemical or enzymatic modification of the surface ionogenic groups are also used to identify the main compo-

nents responsible for the surface charge of microorganisms[26,77] or fungal spores[25], for example.
However, it has been shown that the electrophoretic mobility of microorganisms is not only determined by the chemical composition of the cell wall itself but also by structures associated with the cell surface like flagella and capsular materials[11,12,42].
The correlation of the electrophoretic mobility with growth conditions and growth phase has been studied by several investigators. Contradictory results were obtained; whereas Thonart et al.[79] observed no change of the zeta potential of haploid and diploid cells of *Saccharomyces cerevisiae* during growth, Moyer[52] found a decrease in the electrophoretic mobility of rough and smooth cells of *E. coli* in the lag and early exponential phases. Hubert and Werner[39], who determined the zeta potential of *Saccharomyces cerevisiae* during growth in chemostat cultures, observed an influence of growth rate, substrate and fermentation type on the zeta potential. Therefore they proposed use of the measured zeta potential as a parameter for biotechnological processes.

Colloid titration
The surface charge of particles can also be characterized by colloid or polyelectrolyte titration. In contrast to the zeta potential measurement, this method requires no expensive equipment. The colloid titration method is based on the fact that polyelectrolytes of opposite charge form complexes in a stoichiometric way. The endpoint of the complex formation can be determined colorimetrically by using indicators, for example the metachromatic cationic dye toluidine blue[37].
The charged particles have to be incubated with an excess of polymer of opposite charge. As a cationic polymer, polydiallyldimethylammonium chloride is usually used; as an anionic polymer, potassium polyvinylsulfate. After complex formation between the oppositely-charged groups the remaining amount of polyelectrolyte can be titrated[36–38,74]. If the titer of the polyelectrolyte solutions is known it is possible to calculate the bacterial surface charge, as equivalents per mg dry weight, for example. Noda et al.[63] applied the colloid titration method to determine the surface charge of whole cells, protoplasts and cell wall fractions of *Micrococcus luteus*. The results obtained showed good reproducibility.
Theoretically, colloid titration should be suitable for determining the surface charge of microorganisms. However, our investigations indicated that the values obtained are in the same range as the standard deviation. The sensitivity and the accuracy were not sufficient. Therefore the applicability of the colloid titration method seems to be questionable.

Electrostatic interaction chromatography (ESIC)
Another method for studying the surface charge of microorganisms is electrostatic interaction chromatography, applied for example by Pedersen[66] and Hermansson et al.[34]. The procedure is very similar to that of hydrophobic interaction chromatography (see below). As a charged matrix, anion exchange resins like Dowex 1 × 8 and DEAE-Sepharose CL-6B can be used, or cation exchange resins like Dowex 50 W × 8 and CM-Sepharose CL-6B.
The ESIC is a much simpler technique than zeta potential measurement[66]. The main difference is that by zeta potential measurement the sum of the charges of all surface groups of a cell is determined, whereas by ESIC only the interactions of parts of the cell surface, i.e. localized charges, are measured[34].

Cell surface hydrophobicity

Theoretical background
The theoretical background of the importance of cell surface hydrophobicity for bacterial adhesion to solid surfaces has been excellently discussed by several authors[4,9,59,71,72]. The main aspects can be summarized as follows:
From the thermodynamic point of view the adsorption of microorganisms to solid surfaces is favored when the change in the Gibbs free energy of the process of cell attachment is negative. Neglecting charge effects and specific biochemical interactions the change in the interfacial free energy of adhesion ΔF_{adh} per unit surface area is defined as[16,53]:

$$\Delta F_{adh} = \tau_{CS} - \tau_{CL} - \tau_{SL} \quad (1)$$

τ_{CS} = cell-substratum interfacial free energy (ergs/cm^2)
τ_{CL} = cell-liquid " " "
τ_{SL} = substratum-liquid " " "

The surface free energy term τ is defined as the work necessary to create a unit area of new surface under certain conditions[9]. For liquids this energy can be measured directly, but not for solids. It can be calculated by two different approaches; the equation of state, which is employed for most biological systems, and the geometric mean equation.

Equation of state approach. Ward and Neumann[86] have shown that for ideal solids the following relation (equation of state) is valid:

$$\tau_{SL} = f(\tau_{SV}, \tau_{LV}) \quad (2)$$

Combining it with Young's equation

$$\tau_{SV} - \tau_{SL} = \tau_{LV} \cos \Theta \quad (3)$$

τ_{SV} = solid-vapor interfacial free energy
τ_{SL} = solid-liquid " " "
τ_{LV} = liquid-vapor " " "
Θ = contact angle solid-liquid

there are two equations with four variables. Two of them, τ_{LV} and Θ, can be determined easily. Experimental data and a computer program have been used to create conversion tables for obtaining values for τ_{SV} and τ_{SL} from the contact angle Θ by using a liquid of known surface tension τ_{LV} [58, 62]. The free energy of the cell-liquid interface can be determined in the same manner. Neuman et al.[61] also described how to obtain the cell-substratum interfacial free energy. In this way the change in the free energy of adhesion can be calculated via equation (1), followed by the possibility of predicting whether adsorption is favored or not[3]. But the question of whether such calculations are justified is still under discussion[60]. Van Loosdrecht et al.[82], for example, prefer to use contact angle data only as a relative measure for the hydrophobicity of cells and solid surfaces, which in most cases is correlated to the surface free energy.

Geometric mean approach. The geometric mean approach, supported by Busscher et al.[15, 16] and van Pelt et al.[84], is based on the assumption that the surface free energy consists of polar and dispersive, non-polar components; London dispersion forces, dipole-dipole (Keesom) forces, induction (Debeye) forces and H-bonds. Interactions between two different surfaces are only due to components of the same type of force.
The geometric mean approach has been used for example to calculate the interfacial free energy of oral microorganisms[16, 84]. In spite of several completions and modifications of this theory[15, 88], the equation of state approach seems to be preferable. This has been shown by Spelt et al.[78], who compared the results obtained by the two approaches.

Methods for measuring cell surface hydrophobicity
A good survey of the development of methods for the determination of the surface free energy of solids is given by Good[29]. Rosenberg and Kjelleberg[71] reported various techniques for measuring the surface hydrophobicity of microbial cells.

Bacterial adherence to hydrocarbons. The adherence of bacteria to hydrocarbons has been used by several investigators as a simple and rapid method for determining the hydrophobicity of microorganisms[24, 45, 56, 64, 67–70]. By this method the percentage of cells which are excluded from the aqueous phase in a water/hydrocarbon two-phase system is measured. The lower the percentage of cells in the water phase, the higher the hydrophobicity. The partition of microorganisms between the two phases is determined by measuring the change in optical density or by counting radiolabelled cells[45]. Variants of this method have been established by Kaeppeli and Fiechter[43], who quantified the amount of hexadecane attached to the cell surface using gas-liquid chromatography, and by Kjelleberg et al.[44] who determined the amount of [^{14}C] dodecanoic acid fixed at the cell surface. As the hydrocarbon phase n-hexadecane is most often used, but n-octane, p-xylene[68] and toluene[54] have also been tested.
Van Loosdrecht et al.[82] compared the results obtained by bacterial adhesion to hydrocarbons with those obtained by contact angle measurement. He found a direct correlation for bacteria with a contact angle above 30°.
Although bacterial adherence to hydrocarbons is widely used to characterize the hydrophobic surface properties of microorganisms there are several uncertainties in the interpretation of the results[54, 70]. One main aspect is the difficulty of standardizing the method[82]. However, we obtained good reproducibility, and the results could be correlated with those obtained by other techniques (HIC, contact angle measurement). Therefore bacterial adherence to hydrocarbons seems to be a suitable method for grouping microorganisms and for differentiating between hydrophobic and hydrophilic ones.

Partitioning of bacteria in an aqueous two-phase system. The partitioning of microorganisms in an aqueous two-phase system has been used as an alternative to measuring the bacterial adherence to hydrocarbons[27, 28, 82].
Polyethyleneglycol/dextran systems are most often used. The interfacial tension between these two phases is very low (ca 1×10^{-4} erg/cm^2, water/hydrocarbon 50 erg/cm^2) and therefore this method is 3–4 times more sensitive in detecting small differences in the hydrophobicity of hydrophilic particles than the method using bacterial adherence to hydrocarbons[27]. Even for small differences in the cell surface free energy the partition coefficient reaches high values, so that the microorganisms stay definitely in one of the two phases. Only microorganisms with a specific surface free energy will be at the interface. It is possible to measure at the same time both the hydrophobic (dispersive) and hydrophilic (polar) components of the cell surface. Furthermore the procedure is non-toxic for the cells[75]. By this method the validity of contact angle measurements could be checked[82].

Hydrophobic interaction chromatography (HIC). Hydrophobic interaction chromatography is widely used to characterize the hydrophobic cell surface properties of microorganisms[21, 22, 34, 54, 76, 80]. The quantity of cells which are retained in a hydrophobic gel (phenyl- or octyl-sepharose) is determined under various conditions, i.e. different pH and ionic strength. The higher the percentage of microorganisms retained, the higher the hydrophobicity. The cells are detected by measurements of optical density of by radiolabelling[21, 34].
By hydrophobic interaction chromatography we only measured slight differences for most tested microorganisms, even for those with different contact angles. Only the extreme values could be detected. Probably the phenyl-sepharose gel used is not sensitive enough to detect small differences in cell surface hydrophobicity. Hjertén et al.[35] and Smyth et al.[76] were able to enhance

the sensitivity by varying the hydrophobic characteristics of agarose gels by coupling side chains of different polarity to the matrix.

Contact angle measurement. The determination of the surface free energy of solid surfaces by contact angle measurement is based on Young's hypothesis that the cohesive forces between the liquid particles and the adhesive forces between a liquid and a solid surface result in an equilibrium contact angle which is constant and specific for a certain system[57].

A good survey of different techniques for measuring contact angles is given by Andrade et al.[9] and Neumann and Good[60]. Most commonly used are methods which make it possible to determine the contact angle directly from the drop profile (sessile drop and adhering bubble technique). Most investigators employ the sessile drop technique[16, 54, 80]. The microorganisms are collected on filter membranes. So that they will reach a constant moisture content they are put on a layer of 1% (w/v) agar and 10% (v/v) glycerol for about 30 min to 4 h. The filter membranes are fixed on to microscope slides by double-sided adhesive tape. A number of different probe liquids are used for contact angle measurement (water[7, 51, 54, 57, 80, 82], water-n-propanol mixtures[15], α-bromonaphthaline[15,80], 0.1 M NaCl[82]). Different times for the drying of the filter membranes have been reported; they vary between 90 min[16] and 3 h[82]. Because of liquid penetration into the cell layer the contact angle has to be determined immediately after drop positioning.

It is also possible to determine the contact angles of cell layers immersed in a liquid phase[2, 31, 75]. The advantage of this method is that the cells do not have to be dried and therefore no denaturation of macromolecules on the cell surface can occur. By measuring the hydrophobicity of living cells in an aqueous environment the in vivo situation can be approximated. Water/hydrocarbon systems[57] can be used, or the aqueous two-phase system polyethyleneglycol/dextran[27, 31]. Owing to the extremely low interfacial tension of this system more exact measurements are possible, especially for cell layers of low surface tension. If the concentration and the molecular weight of the polymers are varied a series of interfacial tensions between the two phases can be obtained, so that it is also possible to determine the critical surface tension, i.e. the surface tension of a liquid which would just spread on the surface[75].

The reproducibility of the techniques mentioned above is about $\pm 2°$[30]. It can be enhanced, for example by always using liquid droplets of the same size[60]. Furthermore, contact angles on non-ideal solid surfaces show hysteresis effects due to surface roughness and heterogeneity[57].

An important point to bear in mind is that the process of sample preparation (drying the filters or putting them in a polymer liquid) might cause changes in the physiological properties of the cell surface.

Influence of growth conditions on the hydrophobicity of microorganisms

The influence of growth conditions and growth phase on the hydrophobic cell surface properties has been studied by several investigators. Ofek et al.[64] tested *Streptococcus pyogenes,* and Hazen et al.[33] *Candida albicans* and *Candida glabrata.* Van Loosdrecht et al.[83] found out that during the exponential growth phase or at high growth rates the hydrophobicity of various bacteria increased. It was also shown that starvation could change the cell surface properties[45].

Büchs et al.[13] modified the surface characteristics of *Corynebacterium glutamicum* changing the culture conditions, i.e. the phosphate concentration of the medium. Cells grown under conditions of phosphate saturation were more hydrophobic (contact angle, HIC) than those grown under phosphate limitation. It was suggested that when phosphate is limited the synthesis of the hydrophobic compound lipoteichonic acid is stopped, and this leads to reduced hydrophobicity.

Hermansson et al.[34] and Cunningham et al.[19] observed that rough strains of *Salmonella typhimurium* (reduced O-side-chains and core oligosaccharides) were more hydrophobic than smooth ones. But the cell surface hydrophobicity is also influenced by structures associated with the cell wall; Hermansson et al.[34] showed that fimbriated cells of *Serratia marcescens* were less hydrophobic than non-fimbriated ones.

Chemical analysis of cell surfaces by X-ray photoelectron spectroscopy (XPS)

The method of XPS or ESCA (electron spectroscopy for chemical analysis) has been used by several investigators to analyze the chemical composition of the cell surface of microorganisms. The basic principles of this method are well described by Andrade[8]. It is possible to carry out a total elemental analysis, except for hydrogen and helium, and to obtain information about the nature of the chemical bonding of the elements involved.

An X-ray electron, usually from the aluminium or magnesium $k\alpha_{1,2}$ line, interacts with an atomic orbital electron in such a way that there is a complete transfer of the photon energy to the electron. The electron is emitted with a kinetic energy approximately equal to the difference between the photon energy and the binding energy. From the kinetic energy of the electron the electron binding energy can be calculated, which makes it possible to identify the atom. The advantage of XPS is the fact that the X-rays emitted are of low energy and cannot penetrate deeper than 2–20 nm into the sample[8]. Therefore in microorganisms, the elemental composition of only the outer layers of the cell surface is analyzed.

XPS has been applied by several investigators for analyzing the chemical composition of the cell surface of bacteria and yeasts[6, 7, 81]. By the use of pure silica powder as external standard it was possible to calculate not only

concentration ratios but also the actual concentrations[81].

XPS data were used to explain the electrophoretic mobility behavior of microorganisms. From the fact that the surface phosphate concentration was directly correlated to the zeta potential at pH 4, Amory and Rouxhet[7] concluded that dissociated phosphate groups are the main components responsible for the net negative surface potential at pH 4. Carboxylic groups, which could not be detected because of their low numbers compared to the total carbon and oxygen content, seemed to have less influence. An inverse correlation was detected between the N/P ratio and the electrophoretic mobility at pH 4[6]. Protonated nitrogen, a fraction of the total nitrogen detected, seemed to partly neutralize the negatively charged phosphorous groups. This was also shown by Van Haecht et al.[81], who found a direct correlation between the N/P surface concentration ratio and the isoelectric point of various yeasts. By analyzing the individual values an inverse correlation was observed for the P content and a direct one for the N content.

The N/P ratios were also correlated to the cell surface hydrophobicity, determined by contact angle measurement. The higher the N/P ratio the more hydrophobic the cells were[7].

Influence of microbial cell surface properties on adsorption behavior

Correlations between cell surface hydrophobicity, surface potential and adsorption capacity of various supports gave different results. Van Loosdrecht et al.[83] have shown that the adsorption of hydrophobic microorganisms on negatively charged sulfated polystyrene was directly correlated to the hydrophobicity detected by contact angle measurement, whereas the electrophoretic mobility had no influence. On the other side the adsorption of hydrophilic microorganisms was inversely correlated with the electrophoretic mobility. It was concluded that in this case the surface free energy of the microorganisms was the dominant factor for cell adsorption, whereby the repulsive forces between the like charges on the surfaces of cells and support had been overcome.

By studying the influence of the surface free energy of the support it could be shown that hydrophobic microorganisms are preferentially adsorbed on hydrophobic supports[17,50,51,53,84]. This was in good agreement with our results. By studying the adsorption of different microorganisms on porous glass we found an inverse correlation between cell surface hydrophobicity and cell loading.

Busscher et al.[17] observed a reversible adhesion of microorganisms with a high surface free energy (hydrophilic) to supports with a low surface free energy (hydrophobic) and vice versa, due to a positive ΔF_{adh} value, but there was an irreversible adhesion of cells to supports with a similar surface free energy.

There are also investigations which indicate the importance of the surface potential for the adsorption. For example, it was possible to enhance the immobilization yield resulting from adsorption onto negatively charged supports by decreasing the zeta potential of the microorganisms. The cells were treated with $CaCl_2$, $Al_2(SO_4)_3$ and cationic starch[79] or chitosan[18] to bridge the negatively charged surface groups. Abbott et al.[1] changed the zeta potential of *Streptococcus mutans* by altering the electrolyte concentration of the suspending liquid. Carrier modification was also successful. Büchs and Wandrey[14] modified the surface of porous glass by adding NH_3- and CH_3-groups, Champluvier et al.[18] used chitosan to cover various supports.

Conclusions

Different techniques have been established to characterize the cell surface properties of microorganisms. A clas-

Classification of various methods to characterize the cell surface properties of microorganisms

Theoretical background	Method	Applicability	Detection of cell surface variability	Correlation to adsorption	Analytical potential
Cell surface free energy	Hydrophobic interaction chromatography	**	*	*	*
	Bacterial adherence to hydrocarbons	**	**	**	*
	Partitioning in an aqueous two-phase system	*	*	*	*
	Contact angle measurement	***	***	***	***
Surface potential	Electrostatic interaction chromatography	**	**	*	*
	Zeta potential measurement	***	***	**	***
	Colloid titration	*	*	*	*
Elemental analysis	XPS	*	***	***	***

* poor; ** good; *** very good.

sification scheme is shown in the table. The most suitable method for characterizing the cell surface free energy seems to be the contact angle measurement. The cell surface potential can best be determined by the zeta potential. Mostly, different aspects of the same property (hydrophobicity and surface charge) are measured. Therefore a combination of the results obtained by various methods can provide useful information about the surface characteristics. However, it should be taken into account that the surface properties might be changed by cell preparation. XPS is a suitable method for analyzing the chemical composition of the cell surface, and the data obtained can be used to explain the zeta potential behavior. However, very expensive equipment and experienced operators are necessary to carry out this kind of investigations.

It has been shown that the adsorption of microorganisms to solid surfaces is influenced by both the surface potential and the surface free energy of the cells. To check whether the adsorption of microorganisms to supports with different surface characteristics is favored or not, the theoretical principles of the DLVO theory and the concept of the change in the interfacial free energy of adhesion might be useful. But one has to take into account that bacterial adhesion is not only due to physical interactions but also to surface polymers which may favor attachment under conditions where physical measurements alone suggest it would not be possible [73].

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Application of living microbial cells entrapped with synthetic resin prepolymers

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Summary. Living and growing microbial cells were immobilized by entrapping in synthetic resin gels prepared from their prepolymers, and used in the production of various useful substances. The production of the desired metabolites and also both the activity and the stability of the catalytic systems were seriously affected by the physico-chemical properties of the prepolymers, and those of the resin gels subsequently formed, such as gel network, hydrophilicity-hydrophobicity balance and ionic nature, as well as by the type of bioreactors. Hydroxylation of steroids and production of antibiotics, polypeptides and other biologically active substances, and the effects of gel properties on them are discussed as examples.
Key words. Immobilization; entrapment; immobilized cells; microbial cells; synthetic resin prepolymers; bioprocesses; bioconversion.

Introduction

With the development of enzyme engineering, immobilization of multienzyme systems has been attracting worldwide attention, because these systems can mediate complicated reactions for synthesis and conversion of various compounds, with regeneration of ATP and/or pyridine nucleotide coenzymes which participate in oxidation-reduction reactions. Microbial cells, especially living ones, contain metabolic systems that catalyze such complicated reactions. Therefore, immobilization of living cells means in effect the immobilization of multi-step and cooperative enzyme systems with coenzyme regeneration.

Immobilized living cells have several disadvantages, such as undesirable metabolic activities which reduce product yields and form by-products. The existence of a permeability barrier for various substrates and metabolites is another disadvantageous feature of the system. Nevertheless, immobilized living cells, especially immobilized growing cells, serve as renewable and self-proliferating biocatalysts, maintaining specific enzyme activities for a very long period. The application of immobilized living or growing cells was first demonstrated by Slowinski and Charm[22] in 1973 for the production of glutamic acid and, thereafter, developed by Larsson et al.[10] for the hydroxylation of hydrocortisone to yield prednisolone. Some aspects of immobilized cells, dead or alive, were reviewed by Chibata and Tosa[4] and by the present authors[6].

This article deals with the application of living microbial cells entrapped with synthetic resin prepolymers, mainly carried out in the authors' laboratory.

Entrapment of microbial cells with synthetic resin prepolymers

Microbial cells, dead or alive, can be immobilized by means of physical adsorption, ionic binding, entrapment etc. on or in inorganic materials, natural or modified polysaccharides, proteins and synthetic resins. We have developed novel immobilization methods, which are applicable to the entrapment of enzymes, cellular organelles, microbial cells, plant cells and animal cells, with synthetic prepolymers of photo-crosslinkable resins and urethane resins[5,7]. Specific features of these prepolymer

Application of microbial cells entrapped with synthetic resin prepolymers

Microorganism (condition)	Application	Ref.
Corynebacterium sp. (living)	9α-Hydroxylation of steroid	26
Rhizopus stolonifer (living)	11α-Hydroxylation of steroid	25
Curvularia lunata (living)	11β-Hydroxylation of steroid	23, 24
Sepedonium ampullosporum (living)	16α-Hydroxylation of steroid	9
Candida albicans etc. (living)	Asymmetric reduction of β-keto esters	1
Arthrobacter sp. (growing)	Production of muconic acid	19, 21
Corynebacterium glycinophilum (growing)	Production of L-serine	31
Streptomyces carbophilus (growing)	Production of pravastatin	3
Streptomyces rimosus (growing)	Production of oxytetracycline	15
Streptomyces peucetius (growing)	Production of daunorubicin	28
Agaricus campestris (growing)	Production of pyruvic acid	27
Methanogenic bacterium (growing)	Production of methane	2
Saccharomyces sp. (growing)	Production of ethanol	11, 13
Saccharomyces cerevisiae (growing)	Production of α-mating factor and a specific peptidase	16, 17