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Stabilization of water/gas oil emulsions by desulfurizing cells of *Gordonia alkanivorans* RIPI90A

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It has been previously reported that resting-cells, non-proliferating cells, of *Gordonia alkanivorans* RIPI90A can convert dibenzothiophene (DBT) to 2-hydroxybiphenyl (2-HBP) via the 4S pathway in a biphasic system. The main goal of the current work was to study the behaviour of resting-cells of this strain in biphasic organic media. Resting-cells showed strong affinity for sulfurous organic substrates and were able to stabilize water/gas oil emulsions by attaching to the interface without decreasing the surface tension of their environment. This was consistent with the behaviour of the whole cells but not the surfactants, suggesting that microbial cell-mediated emulsification occurs. It was found that the emulsion-stabilizing activity of the resting-cells was influenced by the growth stage, but was not directly influenced by the metabolic activity of the resting-cells. This activity may be related to cell-surface hydrophobicity, which results from the unique chemical structure of the cell surface. In some biphasic biodesulfurization (BDS) bioreactors, emulsions are created without addition of any surfactant. Cell surface-mediated stabilization helps prolong the emulsions and therefore overcomes mass-transfer limitations in bioreactors. The simultaneous occurrence of emulsion-stabilizing and desulfurization activities of resting-cells was observed for what is believed to be the first time. The results suggest that this strain may have potential for the BDS of diesel oils.

INTRODUCTION

Combustion of fossil fuels results in the emission of SO_2 and the subsequent production of acid rain. Regulations concerning the sulfur content of diesel fuels have become increasingly stringent. To meet the regulations using technology based on hydrodesulfurization (HDS), ever-higher temperatures and pressures will be required, which will lead to increased costs and atmospheric emissions (Borgne & Quintro, 2003). Biodesulfurization (BDS) is often considered as a potential alternative to the conventional deep HDS processes used in refineries (Arenskotter *et al.*, 2004).

It is advantageous to use whole cells when complex transformations are involved that require the action of several enzymes organized in complex metabolic pathways and the presence of cellular cofactors (Borgne & Quintro, 2003; Chiang, 2004). In the 4S pathway, used as the basis of BDS research, there are two monooxygenases that require $FMNH_2$. This cofactor is regenerated by a flavin reductase whose activity depends on NADH (Oldfield *et al.*, 1997; McFarland, 1999). The requirements of complex enzyme systems prohibit the use of purified enzyme systems, and tend to support the use of whole cells for a practical BDS process (Kilbane & Le Borgne, 2004). Therefore, for BDS research, resting-cells have been widely acknowledged to be the most suitable biocatalysts.

In the BDS bioreactor, a limiting factor is the rate of transport of the sulfur compounds from the oil phase to the bacterial cell membrane. Therefore, access to organic sulfur by resting-cells requires the costly dispersal of the oil fraction in the aqueous phase. One problem which has yet to be resolved is whether the chemical surfactants which would create suitable oil/water emulsions are toxic to the process organisms or act against the characteristic adhesion mechanisms of the bacteria to oil-droplet surfaces (Shennan, 1996).

Many bacteria can produce biosurfactants that decrease the surface tension of biphasic media and therefore increase the

Abbreviations: BATH, bacterial adhesion to hydrocarbons; BDS, biodesulfurization; DBT, dibenzothiophene; DMF, dimethylformamide; E_{72}, emulsification index; EC, emulsifying capacity; 2-HBP, 2-hydroxybiphenyl; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

interface area, which results in more efficient transformation. In biphasic systems, emulsification is not always just the result of the activity of biosurfactants; certain intact bacterial cells are also able to stabilize oil/water emulsions without changing the interfacial tension, by inhibition of droplet coalescence (Bredholt *et al.*, 1998; Dorobantu *et al.*, 2004).

The ability of members of the genus *Gordonia* to transform or degrade toxic compounds has encouraged microbiological attempts to investigate their physiological properties. Recently, *Gordonia alkanivorans* RIPI90A has been reported as a desulfurizing strain; the resting-cells of the strain are reported to be able to convert dibenzothiophene (DBT) to 2-hydroxybiphenyl (2-HBP) via the 4S pathway in a biphasic system (Mohebali *et al.*, 2007). The main objects of this study were (i) to show that the resting-cells of strain RIPI90A have an affinity for organic sulfur substrates and are capable of stabilizing the water/gas oil emulsion, (ii) to confirm that this ability is related to cell-surface hydrophobicity, and (iii) to investigate conditions under which both the BDS process and the emulsion-stabilizing activity occur simultaneously.

METHODS

Chemicals and reagents. All chemicals were of analytical grade. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and Gibb's reagent (2,6-dichloroquinone-4-chloroimide) were obtained from Merck. DBT (>95 %) and sodium azide (>99 %) were purchased from Fluka. Dimethylformamide (DMF; 99 %) was purchased from Riedel-de Haën.

Micro-organism. *G. alkanivorans* RIPI90A was studied in this work. This strain was isolated from an enrichment programme performed in minimal salt (MS) medium containing sodium benzoate (MS-SB medium) and sterile gas oil (250–375 °C) as the sole source of sulfur; the presence of gas oil increased the chance of isolating hydrophobic bacteria. The strain was suspended and maintained in nutrient broth containing 20 % (v/v) glycerol at −70 °C, and also on glass beads under the same conditions.

Media. MS-SB medium, which contains sodium benzoate as the sole carbon source, was used for cultivation of the strain as previously described (Mohebali *et al.*, 2007). DMF was used as co-solvent, in which DBT was dissolved and then added to the medium. Enriched nutrient broth (ENB) medium containing nutrient broth (13.0 g), yeast extract (2.0 g), tryptone (4.0 g) and distilled water (970 ml) was used. This medium was solidified by agar (15 g l^{-1}) to give enriched nutrient agar (ENA), when necessary.

Gibb's assay. Gibb's reagent reacts with aromatic hydroxyl groups such as those of 2-HBP to form a blue-coloured complex (Kayser *et al.*, 1993). The Gibb's assay was used to detect and quantify 2-HBP produced by strain RIPI90A in MS-SB medium as previously described (Mohebali *et al.*, 2007). A standard curve was created using known 2-HBP concentrations, and a linear relationship was observed at 2-HBP concentrations between 0.1 and 12 mg l^{-1}.

Analysis. All samples were centrifuged to remove cellular mass. The clarified supernatants were analysed by HPLC and GC-MS, as previously described (Mohebali *et al.*, 2007).

Preparation of resting-cell suspensions. Cells were grown in liquid MS-SB medium containing DBT as the sole sulfur source, as previously described (Mohebali *et al.*, 2007), and harvested in late-exponential phase by centrifugation (4000 r.p.m., 20 min). The cell suspension was obtained by resuspending the pellet in phosphate buffer (0.1 M, pH 7.08) and adjusting the cell concentration (OD_{660}) to a suitable value.

Resting-cell reaction programme. Resting-cell suspension (5 ml, $OD_{660}=20$) was prepared as described above and mixed with DBT (10 % solution in DMF). The final concentration of DBT was 600 mg l^{-1}. The reaction mixture was incubated at 30 °C on a rotary shaker (120 r.p.m.) for 8 h. Following incubation, the mixture was tested by Gibb's assay and also extracted by ethyl acetate (1 : 1, v/v) three times. Before the extraction, the pH of the mixture was adjusted to 2.0 by 4 M HCl. The total volume of the extract was evaporated at room temperature to a volume of 250 µl, and the sample was analysed by GC-MS and HPLC for the detection and quantification of 2-HBP. The results were used to calculate the specific activity [µmol 2-HBP (g dry cell weight)$^{-1}$ h^{-1}] with respect to dry cell weight and the reaction time of resting-cells with DBT.

Surface-tension measurement. In order to explain the effect of the cells of strain RIPI90A on surface tension, a resting-cell suspension was prepared as described above. The surface tension of phosphate buffer (0.1 M, pH 7.08), resting-cell suspension, MS-SB medium, and the whole culture from which the resting-cells were harvested, was measured. The surface tension of resting-cell suspension filtrate and whole-culture filtrate (both filtered through a 0.22 µm pore-size filter) was also measured.

The cell culture was also prepared following inoculation of the strain in enriched medium (ENB) and incubation at 30 °C until the later stages of the growth phase. The surface tension of the ENB medium, cell culture and culture filtrate (filtered through a 0.45 µm pore-size filter) was then measured using a Krüss tensiometer K9.

Organic phase. Since aromatic hydrocarbons cause lysis of some bacterial species (Pembrey *et al.*, 1999), aliphatic hydrocarbons have been widely used in hydrophobicity assessment as the hydrophobic phase (Dorobantu *et al.*, 2004; Rosenberg *et al.*, 1980); however, gas oil has not previously been used as a hydrophobic phase. In this work, the emulsion-stabilizing activity of resting-cells was measured by various tests using the gas oil fraction (250–360 °C, Tehran Refinery, Iran) as the oil phase. Strain RIPI90A was found to be resistant to gas oil (data not shown).

Bacterial adhesion to hydrocarbons (BATH). The BATH method was used to investigate the cell-surface hydrophobicity of resting-cells. The BATH method was performed as previously elsewhere (Dorobantu *et al.*, 2004; Purdy *et al.*, 1993; Sokolovská *et al.*, 2003). Using this method, the partitioning of cells between the aqueous and hydrophobic phases can be measured. The test was carried out at room temperature as follows. Resting-cell suspensions were prepared with a final OD_{660} of 2.0, 3.0, 4.0, 5.0 and 6.0. Aliquots of each sample (2 ml) were then transferred to individual test tubes (180 × 18 mm), mixed with 0.2 ml gas oil fraction and vortexed at full speed for 30 s. The tubes were allowed to stand for 10 min. When the particle-stabilized emulsions had formed and remained stable, an additional volume (0.2 ml) of gas oil was added to the mixture. The process was repeated three times. The OD_{660} of the aqueous phase was then determined. The partitioning of the resting-cell suspensions was expressed as the percentage of cells absorbed by the hydrocarbon phase (Dahlback *et al.*, 1981; Pembrey *et al.*, 1999); the percentage cell partitioning was calculated using the equation $1-(OD_{660\text{-}1}/OD_{660\text{-}0})\times 100$, where $OD_{660\text{-}0}$ is the initial OD_{660} of the cell suspension and $OD_{660\text{-}1}$ is the OD_{660} of the aqueous phase determined after addition of total gas oil. The mean percentage partitioning of the cells into the hydrocarbon phase was calculated from triplicate samples.

Determination of emulsification index (E_{72}). The E_{72} was used to help explain the emulsion-stabilizing activity of cells and cultures.

The E_{72} was determined at room temperature using a modification of the method described elsewhere (Bodour *et al.*, 2004; Cooper & Goldenberg, 1987). The resting-cell suspension (2 ml, $OD_{660}=2.0$) was transferred to a test tube (180 × 18 mm) and 0.2 ml gas oil was added, followed by vigorous mixing for 30 s. The tube was allowed to stand for 10 min; when the particle-stabilized emulsion remained stable, a second volume (0.2 ml) of gas oil was added. The cycle was repeated 10 times. The creamy emulsion was transferred to a graduated cylinder and left for 72 h. The height of the emulsified zone was divided by the height of the aqueous phase and multiplied by 100. The resulting number, the E_{72}, was used as a measure of the stability of emulsions after 72 h. The index was calculated from triplicate samples.

Determination of emulsifying capacity (EC). The EC was used to explain the emulsion-stabilizing activity of cells and cultures. The EC was determined at room temperature using a modification of a method previously elsewhere (Cassidy & Hudak, 2001). The strain was grown in MS-SB medium as described above. Whole culture at the late-exponential phase ($OD_{660}=1.725$) was used. Whole culture (2 ml, $OD_{660}=1.725$), whole-culture filtrate (2 ml, filtered through a 0.22 μm pore-size filter), resting-cell suspension (2 ml, $OD_{660}=1.725$) and resting-cell suspension filtrate (2 ml, filtered through a 0.22 μm pore-size filter) were transferred into test tubes (180 × 18 mm) and then gas oil (0.2 ml) was added, with the contents being mixed vigorously for 30 s. The tubes were allowed to stand for 10 min. When the particle-stabilized emulsion remained stable, a second volume (0.2 ml) of gas oil was added. The cycle was repeated until a distinct and clear oil fraction was observed on the top of the liquid. The total volume of gas oil used was divided by the primary volume of the aqueous phase and multiplied by 100. The resulting figure represented the EC (%). In order to study the effect of resting-cell concentration on the EC value, four increasing cell concentrations ($OD_{660}=1.0$, 3.0, 5.0 and 7.0) were used. The EC of the whole culture and of the resting-cell suspension was determined at different growth stages. The tests were carried out using triplicate samples.

Affinity of resting-cells for DBT crystals. The adherence of the active cells to DBT crystals was assessed using an MTT-based assay. MTT was used as an indicator for metabolically active cells. The method is based on the respiratory reduction of MTT, a water-soluble and yellowish tetrazolium salt; this compound can be reduced intracellularly to a water-insoluble purple formazan by micro-organisms (Freimoser *et al.*, 1999; Johnson *et al.*, 2002).

The method was adapted from that described by Johnson *et al.* (2002). DBT solution (400 μl, 5 mg ml^{-1} in n-hexane) was added to a sterile vial (4 ml, 15 × 45 mm, Waters). The solvent was allowed to evaporate in a sterile laminar flow cabinet, resulting in a coating of DBT crystals on the bottom and wall of the vial. In addition, a vial containing hexane alone was evaporated under the same conditions and used as a control. The resting-cell suspension was prepared as described above. One drop of the suspension was streaked on ENA medium to check for purity. Resting-cell suspension (400 μl, $OD_{660}=2$) was added to both vials (group A). The vials were plugged using sterile cotton wool and incubated at 30 °C on a rotary shaker (100 r.p.m.) for 7 h. Following incubation, the contents of the vials were transferred into two sterile and uncoated vials (group B). Phosphate buffer (400 μl, 0.1 M, pH 7.08) was added to each of the empty vials (group A). MS medium (400 μl) containing ethanol (2 g l^{-1}) as the sole carbon source and electron donor was then added to each of the four vials (groups A and B). To start the colour reaction, 80 μl MTT stock solution was added to the cultures (final concentration 0.5 mg ml^{-1}). The vials were incubated at 30 °C for 9 h. The cells were pelleted by centrifugation (4000 r.p.m., 20 min), and 1 ml 1-propanol was then added to the pellets and mixed thoroughly; lysed cells and debris were pelleted under the same conditions. The A_{570} of the supernatants was measured with a spectrophotometer (Shimadzu UV-Mini 1240). The A_{570} of a control containing 1-propanol alone was subtracted from all values. The A_{570} of the group A vials was divided by the A_{570} of groups A and B and multiplied by 100. The total A_{570} of groups A and B was used as the value for the initial cell concentration. The resulting values represent the adherence of the resting-cells to DBT (%). The tests were carried out in quadruplicate.

Visualization of cells on oil droplets. The affinity of resting-cells for gas oil as the organic phase was observed microscopically. To make the active resting-cells more visible during microscopy, filter-sterilized MTT solution (5 mg ml^{-1} in the aqueous phase) was added to the emulsified mixture of the resting-cells ($OD_{660}=2.0$, in 0.1 M phosphate buffer, pH. 7.08) and hexadecane (1 : 5, v/v). Hexadecane is the basic component of the gas oil fraction. The mixture was stirred and incubated at 30 °C for 60 min before being studied by wet mount microscopy (× 400 magnification). Purple cells could be seen on the surface of the oil droplets. The affinity of growing cells of strain RIPI90A for gas oil droplets was also measured. To make the growing cells more visible during microscopy, filter-sterilized MTT solution (5 mg ml^{-1} in the aqueous phase) was added to the emulsified mixture of cells and gas oil that was obtained from the growing culture–gas oil interface. The mixture was stirred and incubated at 30 °C for 60 min before being studied by wet mount microscopy (× 1000 magnification). The cells could be seen on the surface of the oil droplets.

Effect of metabolic activity of resting-cells on their emulsion-stabilizing activity. The method used to explain the effect of the metabolic activity of resting-cells on their emulsion-stabilizing activity was adapted from that described by Botsford *et al.* (1996). The test was performed as follows. Resting-cell suspension ($OD_{660}=2$) was prepared as described above. Using the resting-cell suspension, the minimum effective toxic concentration and contact time for sodium azide were determined. The resting-cell suspension (2 ml) was incubated with sodium azide (0.5 %) at room temperature for 60 min; another resting-cell suspension (2 ml) was incubated as a control without sodium azide. Following incubation, MTT (0.5 mg ml^{-1}) was added to the suspensions. The metabolic activity of resting-cell suspensions was measured after 15 min, as described above. The resting-cell suspension treated by sodium azide was used to determine EC (%), as described above. In parallel, the EC test was performed using a non-azide-treated resting-cell suspension under the same conditions.

Effect of harvest time on desulfurizing activity of resting-cells. The desulfurization activity of resting-cells harvested at various stages of cell growth was studied. Culture samples were taken at defined time intervals during growth on liquid MS-SB medium containing DBT as the sole sulfur source. Using each sample, a resting-cell suspension was prepared and the resting-cell specific activity determined as described above. Following incubation, the sample was analysed for 2-HBP. All the reaction mixtures were prepared in duplicate.

Effect of harvest time on emulsion-stabilizing activity of resting-cells. The emulsion-stabilizing activity of resting-cells harvested at various growth stages was investigated using the EC test. Culture samples were taken at defined time intervals during growth on liquid MS-SB medium containing DBT as the sole sulfur source. Using each sample, aliquots (2 ml) of the resting-cell suspension ($OD_{660}=2$) were prepared and tested to measure EC (%), as described above. All the reaction mixtures were prepared in duplicate.

Emulsion-stabilizing activity during various growth stages. The EC test was performed to compare the emulsion-stabilizing

activity of whole-culture and resting-cell suspensions at various growth stages. Whole-culture samples were taken at defined time intervals during growth on liquid MS-SB medium containing DBT as the sole sulfur source. Whole-culture samples were used directly, and the resting-cells were harvested from the whole culture and then used in the EC test as the resting-cell suspension.

RESULTS

Dry-cell-weight measurement of *G. alkanivorans* RIPI90A

A correlation between OD_{660} and the dry cell weight of *G. alkanovorans* RIPI90A was established (data not shown). It was found that the relationship between OD_{660} and dry cell weight was linear in the OD_{660} range 0.39–1.24, according to the equation:

$$\text{Dry cell weight } (\text{g l}^{-1}) = (0.0763 \times OD_{660}) + 0.2475$$

It was found that 0.323 gram dry cell weight of culture was equivalent to 1.0 OD_{660} unit.

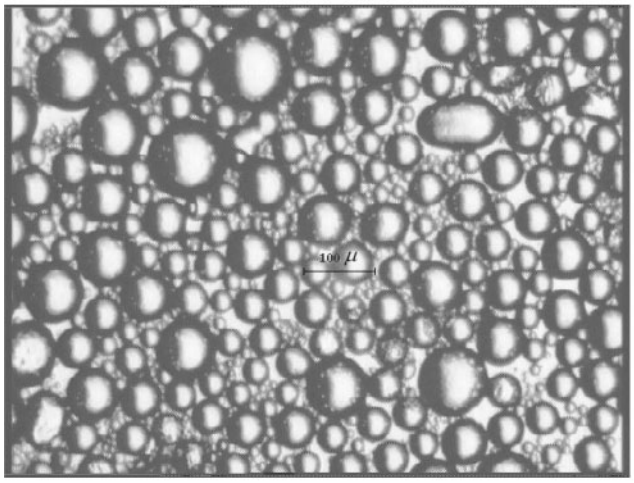
Affinity of resting-cells for DBT and oil phase

The affinity of the resting-cells for sulfurous organic substrates was determined by bacterial adhesion to DBT crystals followed by visualization of bacteria on the oil droplets. This test was intended to study the affinity of metabolically active resting-cells for DBT crystals. The adherence of resting-cells to DBT crystals did not result in any inhibition of their metabolic activity. Under the test conditions, the adherence of cells to the glass surface (wall and bottom of control vial) was 6.62 % and the value for vials coated with DBT crystals was 12.42 %, which showed that the affinity of resting-cells of strain RIPI90A for DBT crystals was higher than that for glass; no further adhesion took place because of the likely saturation of the wall surface with resting-cells. As a statistical measure of the dispersion of distribution, the coefficient of variation (CV) was calculated for all the test groups. The CVs of group A and its control were 7.211 and 8.127 %, respectively. The CVs of group B and its control were 2.163 and 2.771 %, respectively. The affinity for and adherence of the cells to the liquid organic phase (gas oil fraction) was observed microscopically (Fig. 1b). The presence of cells on the surface of oil droplets confirmed the high affinity of the cells for the oil phase.

Effect of growing- and resting-cells on surface tension

The resting-cell suspension and its filtrate were found to have surface tensions of 58 and 66.9 mN m^{-1}, respectively, while whole-culture filtrate and whole culture from which the resting-cells were harvested had surface tensions of 56.2 and 52 mN m^{-1}, respectively; these results together with those presented in Fig. 5 indicate that the emulsion-stabilizing activity of the resting-cell suspension was a particle-related phenomenon.

(a)



(b)

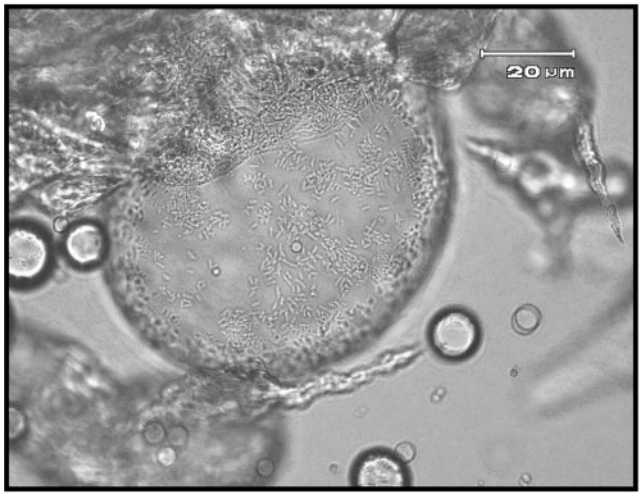


Fig. 1. (a) Photomicrograph of a creamy gel emulsion sample (×100). Bar, 100 μm. The sample was studied microscopically during 72 h incubation at room temperature. (b) Photomicrograph of the growing cells of *G. alkanivorans* strain RIPI90A on a gas oil droplet (×1000). Bar, 20 μm.

The original ENB medium was found to have a surface tension of 55.3 mN m^{-1}. After growth, the surface tension of the whole culture and its filtrate increased (64.2 and 60.3 mN m^{-1}, respectively). It can therefore be suggested that under the test conditions no biosurfactant was secreted into the medium.

Emulsion-stabilizing activity of resting-cells

The E_{72} of resting-cell suspensions (OD_{660} = 2.0 and 4.0) was 150 and 207 %, respectively; the cell suspensions yielded creamy emulsion gels (Fig. 1a), which were stable for at least 22 days. The creamy emulsion gel did not flow in the sample

tubes unless shaken vigorously, indicating high emulsion stability. The long-lasting emulsions confirmed that the strain acts as an efficient emulsion stabilizer.

In order to understand the effect of the metabolic activity of resting-cells on their emulsion-stabilizing activity, the minimum effective concentration and contact time for sodium azide were found to be 0.5 % and 60 min, respectively; under these conditions the resting-cell suspension was metabolically inactivated (62 %), as assessed using the MTT assay. The emulsion-stabilizing activity of both resting-cell suspensions, treated and untreated with sodium azide, was determined as EC (%). Both suspensions showed the same activity, suggesting that the emulsion-stabilizing activity of the resting-cells is not directly related to their metabolic activity.

EC values of the whole culture, the whole-culture filtrate, the resting-cell suspension and the resting-cell suspension filtrate were calculated as 240, 120, 150 and 65 %, respectively. The whole culture showed greater emulsification capacity; with the whole culture, the whole-culture filtrate and the resting-cell suspension, a creamy and stable emulsion gel was created, but with the resting-cell suspension filtrate, the emulsion was almost clear and was unstable. These results suggest that there is an unknown extracellular component(s) that acts partially as an emulsion stabilizer.

The effect of resting-cell concentration on EC was investigated (Fig. 2). Concomitant with increasing cell concentration, the EC value increased; that is, a clear correlation was observed between cell concentration and EC value. Emulsion stabilization was therefore found to be a cell density-dependent phenomenon.

Cell surface hydrophobicity

Cell surface hydrophobicity was assessed using the BATH test (Dorobantu *et al.*, 2004; Pembrey *et al.*, 1999; Rosenberg *et al.*, 1980). The assay measures the partitioning of cells between aqueous and hydrophobic phases. Depending on their hydrophobicity, cells may remain in the aqueous phase, or partition into the water–hydrocarbon interface or the hydrocarbon phase (Pembrey *et al.*, 1999). The BATH values for the resting-cell concentrations $OD_{660} = 2.0$, 3.0, 4.0, 5.0 and 6.0 were calculated as 31.25, 44.33, 56.175, 62.2 and 65.62 %, respectively (Fig. 3). A clear correlation was observed between cell concentration and BATH value; bacterial adhesion to the oil phase was found to be a cell density-dependent phenomenon.

Simultaneous occurrence of emulsion-stabilizing and desulfurizing activities

Our hypothesis was that both the biodesulfurization and the emulsion-stabilizing activities of resting-cells could take place simultaneously. EC (%) was used as a measure of emulsion-stabilizing activity. The biodesulfurization and emulsion-stabilizing activities of the resting-cells harvested from various growth stages were investigated simultaneously. The resting-cells harvested at the late growth phase (Fig. 4) showed the highest specific activity. The desulfurization activity of resting-cells harvested at stationary phase was about 28 % of the highest activity. During the exponential-growth phase, the EC value increased, and at the time of transition from the late-exponential to the stationary phase the value reached its highest level, before dropping to 8.3 % of its highest level. With respect to the time-course of cell growth, the resting-cells with the highest desulfurization and emulsion-stabilizing activities can be harvested in late-exponential phase.

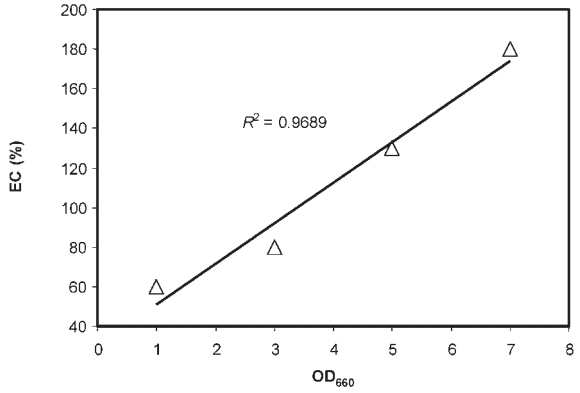


Fig. 2. Effect of the concentration (OD_{660}) of resting-cells on their emulsion-stabilizing activity as EC (%).

Comparison of the emulsion-stabilizing activity of whole culture and resting-cells during various growth stages

In order to investigate the effect of various growth conditions on the emulsion-stabilizing activity of resting-cells, the EC test and whole culture were employed. The EC of resting-cell suspensions and the whole cultures from which the resting-cells were harvested was measured during various growth stages of strain RIPI90A (Fig. 5). The

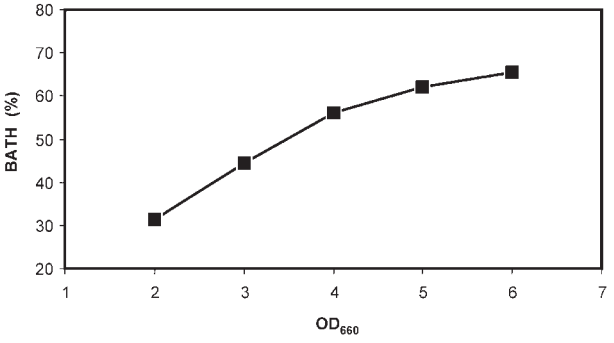


Fig. 3. Effect of resting-cell concentration (OD_{660}) on partitioning of cells between the aqueous and oil phase, shown by the BATH test.

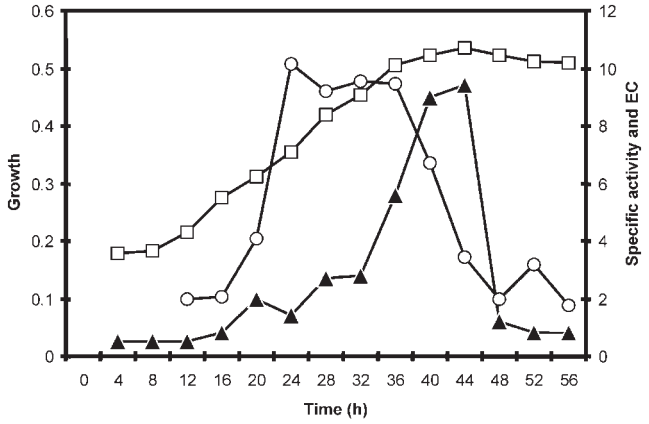


Fig. 4. Emulsion-stabilizing and desulfurization activities of *G. alkanivorans* RIPI90A during the growth phase. □, Growth (grams dry cell weight per litre); ▲, 50 × EC (%, resting-cell suspension); ○, specific activity [mg 2-HBP (g dry cell weight)$^{-1}$ h^{-1}].

similarities in the profile of the EC of whole cultures and resting-cells during various growth stages suggest that only cell-mediated stabilization occurred. Under these conditions, concomitant with the increasing turbidity, the EC of whole-culture and resting-cell suspensions increased, so that at the time of the transition from late-exponential to stationary phase, the maximum values were achieved. A clear correlation was observed between cell harvest times and emulsion-stabilizing activity as estimated by EC. The influence of optimal cell harvest time on the emulsion-stabilizing activity of strain RIPI90A was evident in these results.

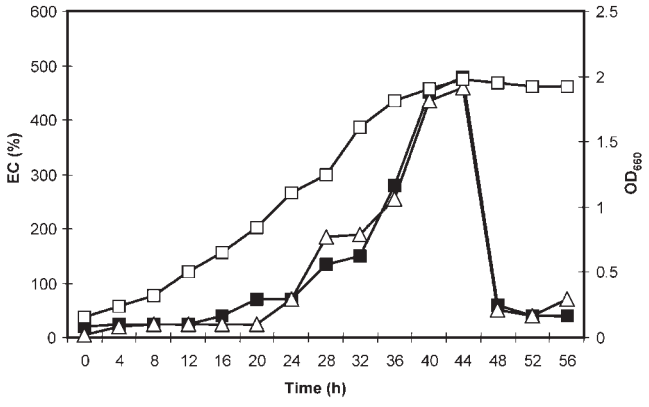


Fig. 5. Emulsion-stabilizing activity of whole-culture and resting-cell suspensions of *G. alkanivorans* RIPI90A during the growth phase. △, EC (whole culture); ■, EC (resting-cell suspension); □, growth (OD_{660}).

DISCUSSION

Gordoniae are potentially useful for environmental and industrial biotechnology. There is continuing industrial interest, as indicated by the increasing number of patents relating to the genus *Gordonia* (Arenskotter *et al.*, 2004). Previously, we have reported *G. alkanivorans* RIPI90A to be capable of desulfurizing DBT via a carbon–sulfur (C–S) bond-targeted pathway (Mohebali *et al.*, 2007). We attempted to learn more about how resting-cells of strain RIPI90A deal with gas oil. It has been reported that in BDS bioreactors at higher cell densities, the emulsion is composed of smaller droplets (Pacheco, 1999). In some bioreactors, such as mechanically mixed reactors and electro-spray reactors, the emulsions are created by physical means. Under these conditions, emulsion stabilization can help to prolong the longevity of the created emulsion. Therefore, we presented the hypothesis that resting-cells of strain RIPI90A could stabilize water/oil emulsions.

Some bacteria produce polymers that act primarily as emulsion stabilizers but do not usually lower the surface tension. The polymers can be expressed on the cell surface and so react with the organic phase and disperse it (Bredholt *et al.*, 1998). It has been reported that some intact and washed bacteria can stabilize oil/water emulsions by adhering to the oil–water interface; these bacteria hinder the coalescence of oil droplets and interact to form emulsion gels. In addition to strong adhesion to the oil–water interface, hydrophobic bacteria possess an affinity for each other, leading to the self-assembly of bacteria at the oil–water interface, which resists coalescence and deformation (Dorobantu *et al.*, 2004). Herein we report that strain RIPI90A could stabilize a water/oil emulsion by attaching to the interface. No biosurfactant was secreted into the medium. The emulsion-stabilizing activity was largely associated with the bacterial cells and was a cell density-dependent phenomenon. The high stability of the emulsion, together with the supporting data presented, confirmed that the strain acts as an efficient emulsion stabilizer by the inhibition of droplet coalescence.

Bacteria that can stabilize oil/water emulsions are often hydrophobic by nature (Dorobantu *et al.*, 2004). The results of this work showed that the affinity of resting-cells of strain RIPI90A for liquid and solid organic phases is high. In the BATH assay there was significant withdrawal of cells from the aqueous phase, indicating that the resting-cells are hydrophobic. Bacterial adhesion to the oil phase was found to be a cell density-dependent phenomenon. The cell-surface hydrophobicity of members of the genus *Gordonia* is related to the presence of mycolic acids in the cell wall (Bendinger *et al.*, 1993; Linos *et al.*, 2000). It has been shown that strain RIPI90A synthesizes a homologous series of mycolic acids ranging from C_{52} to C_{58} (Mohebali *et al.*, 2007). It can be concluded that the cell-surface hydrophobicity of *G. alkanivorans* RIPI90A may be related to its cell-surface long mycolic acids.

The emulsion-stabilizing activity, as given by the EC value, of whole culture and resting-cells was measured during the growth phase of strain RIPI90A (Fig. 5). It was evident that the emulsion-stabilizing activity of the resting-cells was influenced by the growth stage; the profiles of EC values of whole-culture and resting-cell suspensions during the growth phase were similar, indicating that only cell-mediated emulsion stabilization occurred. Concomitant with increasing turbidity, the EC increased, so that at the time of the transition from late-exponential to stationary phase, the maximum EC value was achieved. The emulsion-stabilizing activity was not directly influenced by the metabolic activity of resting-cells, and therefore the large and sudden reduction in the EC value may be related to changes in the cell-surface properties that occurred during stationary phase. This phenomenon has not been consistently described among hydrophobic bacteria (Dorobantu *et al.*, 2004; Rosenberg *et al.*, 1980). For example, the influence of culture age on the mycolic acid compositions of three *Rhodococcus* isolates has been examined; culture age affects both the lengths and the proportions of saturated mycolic acids detected in cell extracts, but not in the same manner for each isolate (Stratton *et al.*, 2003). However, the explanation for this sudden reduction in the EC value remains unknown and is worthy of further investigation.

Herein for what is believed to be the first time we report the simultaneous occurrence of emulsion-stabilizing and desulfurizing activities of a bacterium. The optimal time for harvesting resting-cells having high emulsion-stabilizing and desulfurizing activities was determined. The EC (%) for a defined resting-cell concentration was calculated to be 450 % (Fig. 5). This high emulsifying capacity suggests a potential property by which the strain can sequester organic substrates directly from the hydrophobic phase with minimum limitation from mass transfer.

The water/oil volume ratio is among the most important technical bottlenecks in the development of petroleum biotechnological processes, and therefore in order to reduce the operational costs associated with water handling, separation and disposal, ideally the water/oil volume ratio should be minimized (Foght, 2004). In this study, the results of the EC test showed that low water/oil volume ratios are physically achievable while the stabilized emulsions provide increased bioavailability of the sulfurous oil fraction. The effect of water/oil volume ratios on the specific activity of desulfurizing resting-cells of strain RIPI90A remains to be investigated. Recently, a water/oil volume ratio of 1 : 1 (v/v) was chosen to investigate the desulfurization activity of strain RIPI90A (Mohebali *et al.*, 2007). Patel *et al.* (1997) have reported that desulfurization increases with increasing oil concentrations up to 50 % (w/w) oil; no activity has been detected at high oil ratios (90 and 100 %, w/w, oil).

The complexity of the biodesulfurization pathway seems to make the use of whole cells the only choice. Nearly all described microbial desulfurization processes take place by mixing a resting-cell suspension with oil. After three decades of efforts focused on the use of metabolically active bacterial cells for fuel desulfurization, and even pilot-plant trials, the process is shown to be limited by several factors, including mass transfer in the three-phase systems. One of the solutions to overcome the mass-transfer limitations is the formation of a stable water/oil emulsion. It has been reported that with increasing catalyst concentrations, particle-stabilized emulsions are formed (Pacheco, 1999). As stabilized emulsions are formed, the difficulty of separation increases. It has been suggested that the formation of a stable water/oil emulsion should be avoided in order to facilitate oil recovery (Ayala & Vazquez-Duhalt, 2004). A BDS process using immobilized cells has been also described (Naito *et al.*, 2001; Lee *et al.*, 2005); by this method it is possible to devise a two-phase system (immobilized cells and oil) to desulfurize oil without any particle-stabilized emulsion formation. It has been reported that the desulfurizing activity in the two-phase system is lower than that of the three-phase system, probably due to the interference of the support with the diffusion of substrates and products (Alaya & Vazquez-Duhalt, 2004).

In order to overcome the mass-transfer and separation problems, an additional step is needed. As shown in Fig. 4, after the transition from the late-exponential to the stationary phase, the emulsion-stabilizing activity of resting-cells decreases to an obvious extent. The reason(s) for the phenomenon remains to be investigated, but when they have been determined they will undoubtedly be helpful to attempts to enhance the demulsification of the treated oil product (desulfurized oil fraction) by changing the bioreactor conditions. Therefore, our intention is to prepare a stable emulsion providing more efficient mass transfer, and in the next step to facilitate oil and cell recovery we can change the reactor conditions to provide stationary-phase conditions and so break down the particle-stabilized emulsions.

Conclusions

G. alkanivorans RIPI90A is capable of converting DBT to 2-HBP, and has been selected to desulfurize the gas oil fraction. We attempted to learn more about how strain RIPI90A deals with the gas oil fraction. Various tests showed a good affinity of resting-cells of the strain for organic substrates including gas oil, suggesting that the mass-transfer limitation in BDS bioreactors can be reduced.

Strain RIPI90A could stabilize water/gas oil emulsions efficiently by adhering to the oil–water interface without any secretion of biosurfactant, and so without decreasing the surface tension of their environment. Attached cells hindered the coalescence of droplets and interacted to form a stable and long-lasting creamy emulsion gel. The emulsification was found to be a cell density-dependent phenomenon. The high stability of the emulsion together with other characteristics confirms that the strain acts as an efficient emulsion stabilizer. It was evident that the emulsion-stabilizing activity of the resting-cells was

influenced by the growth stage, but was not directly influenced by the metabolic activity of resting-cells.

The correlation observed between resting-cell harvest time and emulsion-stabilizing activity enables the harvest of biocatalysts with simultaneously high desulfurizing and emulsion-stabilizing activities, leading to the formation of a stable emulsion without the addition of surfactant to the BDS reaction mixture.

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