

Cell Surface Hydrophobicity and Assimilation of Hexadecane by *Pseudomonas frederiksbergensis*

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A study was undertaken to investigate the mechanisms for biosurfactant-enhanced hexadecane uptake into *P. frederiksbergensis*. Biosurfactants production was observed within 4-6 days from the incubation. The biosurfactant(s) were able to cause a drop in the surface tension from 72 to 25 mNm⁻¹ after 10 days of incubation. The stability of hexadecane emulsion in the supernatant of the culture was observed after 24 h, where 15 % of the hexadecane was converted to an emulsion. After 8 days, the conversion reached maximum value of 73.3 % when the adhesion of the cells to hexadecane was 88%. This study supports the hypothesis of alkane transport into microbial cells by direct contact with larger alkane droplets and by pseudosolubilization. Also, it appears that both mechanisms occur simultaneously.

Key words: *P. frederiksbergensis*, Biosurfactants, Hexadecane, Assimilation.

Although the actual uptake of alkanes by bacteria is thought to be a passive transport process, microorganisms possess a number of adaptive mechanisms for accumulating and transporting hydrocarbons into the cell for initial enzymatic catabolism (Sikkema *et al.*, 1995). Bacteria can transport and assimilate soluble alkanes that are dissolved in the aqueous phase. Indeed, it was initially thought that bacteria could utilize only solubilized hydrocarbons (Britton, 1984). However, alkanes are degraded at rates which exceed the rates of dissolution of hydrocarbons in the aqueous phase, indicating

that other uptake mechanisms are also utilized by hydrocarbon degrading microorganisms (Leahy *et al.*, 1990). Different mechanisms for the uptake of aliphatic hydrocarbons have been proposed. Due to the low solubility of long chain alkanes, a transport through the water phase in a dissolved state was ruled out (Cuno, 1996). Commonly discussed hydrocarbon uptake mechanisms by bacteria are: a) very small hydrocarbon droplets (micelles) are enclosed into the cells; b) the direct contact of cells to the bigger hydrocarbon phase enables the cells to enclose hydrocarbons into their cells

Ron and Rosenberg, (2002) reported that hydrocarbon degrading microorganisms adapted to grow and thrive in oil containing environments and have an important role in the biological treatment of the pollution. One of the limiting factors in this process especially at low temperature

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is the bioavailability of many fractions of the oil. The hydrocarbon degrading microorganisms produce biosurfactants of diverse chemical nature and molecular size.

These surface active materials increase the surface area of hydrophobic substrates and increase their bioavailability, thereby enhancing the growth of bacteria and the rate of bioremediation. Desai, (1997) confirmed that these amphiphatic compounds reduce the surface tension by accumulating at the interface of immiscible fluids and a solid and increase the surface areas of insoluble compounds, which leads to increased bioavailability and subsequent biodegradation of the hydrocarbons (Eastcott et al., 1988). Microorganisms may also take up insoluble hydrocarbons by adhering to hydrocarbons at the water hydrocarbon liquid or solid interface (Volkerling et al., 1998). To facilitate adhesion to hydrophobic substrates, hydrocarbon degrading bacteria may increase cell surface hydrophobicity by modifying cell surface components (Morgan and Watkinson, 1993). In addition, microbial cells may produce extracellular polymeric substances (EPS) in the form of capsules or mucoid secretions that may interact with hydrophobic substrates, such as hydrocarbons. Bouchez-Naitali et al., (1999), explained the uptake of hydrocarbons used by bacteria for the degradation of long chain alkanes. The first mode of uptake, direct interfacial accession, involves the contact of cells with hydrocarbon droplets. In the second mode, biosurfactant mediated transfer, the cell contact takes place with hydrocarbons emulsified or solubilized by biosurfactants. Psychrophilic strains with high oil oxidizing and bioemulsifying activities were also described by Chugunov et al., (2000).

The aim of this study was study cell surface hydrophobicity and assimilation of Hexadecane *Pseudomonas frederiksbergensis* with the physiological characterization with respect to such hydrophobic uptake of the insoluble substrates.

MATERIALS AND METHODS

Source of microorganisms and enrichment and cultivation of organisms

Microorganisms used in the present study were isolated and enriched according to the method described by Abdel-Megeed et al., (2014).

Methylene Blue Method for Active Substance (MBAS)

The purpose of the MBAS assay is to determine the presence of biosurfactants in the culture medium (Lide, 1991). 1 ml from the Hexadecane mineral salt medium culture was vigorously shaken for 30 s with 0.003 % methylene blue, then an equal amount of chloroform was added to the sample. The mixture was left for 20 min to extract the methylene blue anionic surfactant ion pair into chloroform layer. At this point, it is necessary to note that all blue dye has migrated into the chloroform layer. The tube was centrifuged at 3,000 rpm for 5 min (Jones and Esposito, 2000). After the extraction with chloroform, the absorbance of each sample was measured at 625 nm against a reference of pure grade chloroform.

Detection and quantification of the biosurfactants

10 ml of the culture supernatants (pH 6.5) were concentrated by the addition of ZnCl_2 to a final concentration of 75 mM. The precipitated material was dissolved in 10 ml sodium phosphate buffer (pH 6.5) and extracted twice with equal volumes of diethyl ether. The pooled organic phases were evaporated to dryness and the pellets dissolved in 100 ml of methanol. The concentrated culture supernatants were spotted on paper filter discs and then put onto agar plates containing 5 % sheep blood. The blood agar plates were incubated at room temperature for 2 days. In addition, blue agar plates containing 0.2 mg ml^{-1} cetyltrimethylammonium bromide (CTAB) and 5 mg ml^{-1} methylene blue were used to detect extracellular biosurfactants production according to the method described by Kästner et al., (2000). Biosurfactants were observed by the formation of halos around the colonies.

Interface and hydrophobicity analyses of *P. frederiksbergensis*

In order to detect the formation of a biosurfactant(s) produced by *P. frederiksbergensis*, the cells were grown in a water bath shaker at 15°C with 200 rpm in 500 ml screw cap sterilized flasks containing 200 ml of MSM supplemented with 1 mM Hexadecane. In a Pyrex beaker, 20 ml was transferred from the culture flask. The surface tension (ST) of the biosurfactant containing medium was measured during the growth by the Ring method described by Magnitis, (1979) using Du Nouy Tensiometer at RT. In different

intervals of time (0, 1, 2, 3, 4, 5 days), 15 ml from the bacterial growth on MSM were subjected to the Du Nouy Tensiometer.

Detection of biosurfactant activity

Samples of the culture media were centrifuged at 8,000 rpm for 20 min. The emulsifying activity and stability of the culture supernatant was measured by adding 0.5 ml of the supernatant and 0.5 ml of hexadecane to 4.0 ml of distilled water. The tube was vortexed for 10 s, held stationary for 1 min, and then visually examined for turbidity of a stable emulsion. Emulsification power was measured by vortexing equal volumes of the centrifuged culture with hexadecane for 1 min and determining the percentage of volume occupied by the emulsion. The mixture was allowed to settle for 24 h and the height of the emulsion was measured (Noordman and Janssen (2002)). The surface tension was determined as a function of the bacterial growth and the relative hydrophobicity. To determine if *P. frederiksbergensis* biosurfactant was released into the medium or remained associated with the cells, 25 ml of culture medium were centrifuged at 12,000 rpm for 10 min at 4°C, and the cell free supernatant was separated from the cell pellet. The latter was resuspended in 25 ml of fresh MSM, and the surface tension was determined. All surface tension measurements were performed at RT.

Determination of cell surface hydrophobicity

The determination of cell surface hydrophobicity of *P. frederiksbergensis* grown on hexadecane at 15°C was carried out by bacterial adhesion to hydrocarbons (BATH) test according to Rosenberg *et al.*, (1980). This assay was used also to determine changes in cell surface hydrophobicity during growth on minimal salt medium with hexadecane. Bacteria were harvested from grown cultures by centrifugation at 8,000 rpm for 10 min at 4°C, washed twice, and suspended in PUM buffer. The OD was adjusted to an initial absorbance of 1.0 at 400 nm. 0.5 ml hexadecane sample and 2.0 ml cell suspensions were vortexed in a test tube and equilibrated for 15 min. The bottom aqueous phase was carefully removed with Pasteur pipette and the absorbance at 400 nm was measured. The adherence was expressed as the percentage decrease in optical absorbance of the lower aqueous phase following the mixing procedure, compared to that of the cell suspension

prior mixing. The cell surface hydrophobicity was expressed as percent cells transferred to hydrocarbon phase by measuring the OD of the aqueous phase before and after mixing the substrate

RESULTS AND DISCUSSION

Detection of the biosurfactants activity produced by *P. frederiksbergensis*

During the growth on alkanes *P. frederiksbergensis* released biosurfactants which had the ability to form light blue halos around the colonies on blue agar. The formation of this blue halo was due to the formation of dye-ionic surfactant complex (Siegmund and Wagner, 1991). Biosurfactants production was observed within 4-6 days from the incubation at RT. Another test using the fact that glycolipids possess hemolytic properties was used.

Effect of *P. frederiksbergensis* biosurfactant(s) on the medium surface tension and Hexadecane uptake

Physiological adaptations for alkane uptake at low temperature by *P. frederiksbergensis* were examined in order to get a better understanding of the utilization of insoluble hydrocarbons (e.g., hexadecane). *P. frederiksbergensis* produced biosurfactant(s) capable of lowering the surface tension (ST) of the medium. It formed a substrate water emulsion during growth on hexadecane at both 4 and 15 °C. The biosurfactant(s) were able to cause a drop in the surface tension from 72 to 25 mNm⁻¹ after 10 days of incubation (Fig. 1).

It can be concluded that the surface tension decreased rapidly with the transition to the exponential growth phase. The shorter *Lag* phase and the rapid drop of ST may be partially explained by the fact that hexadecane availability for the cells was enhanced by the biosurfactants. In contrast, there were no significant changes in the surface tension when the suspension of the washed pellet was used.

Cell surface hydrophobicity and adhesion to the insoluble substrate

One of the most common tests for cell surface hydrophobicity is Bacterial Adhesion to Hydrocarbon (BATH) assay. At the beginning of the growth of *P. frederiksbergensis* on hexadecane, the adhesion was 10 % (Fig. 2). Affinity of the cells

towards hexadecane was measured by the method of Rosenberg *et al.*, (1980).

After 240 hours of incubation on rotary water bath shaker at 200 rpm, the value of the adhesion increased to 88 %. On the other hand, the stability of hexadecane emulsion in the supernatant of the culture was observed after 24 h, where 15 % of the hexadecane was converted to an emulsion. After 8 days, the conversion reached maximum value of 73.3 % when the adhesion of the cells to hexadecane was 88 %.

So far all data concerning the uptake of the strongly hydrophobic substances like aliphatic hydrocarbons are only hypothetical. In addition, the molecular mechanisms of the uptake process of these substrates (e.g. alkanes) have still been unclear. Initially, it was estimated that bacteria

could utilized only solubilized hydrocarbons (Britton, 1984). However, diverse authors considered that some bacteria may adapt to the low bioavailability of hydrophobic substrates. Indeed, the bioavailability of such hydrophobic compounds was suggested to be controlled by other factors than solubility (Jobson *et al.*, 1972). *Pseudomonas* are increasingly recognized as ideal candidates for the biodegradation of hydrocarbons because of their hydrophobic cell surface, their production of biosurfactants and their ubiquity and robustness in the environment. In case of *P. frederiksbergensis*, the presence of glycolipids enhanced the degradation of decane, eicosane and hexadecane by decreasing not only the adhesion process, developing the cell surface hydrophobicity, but also reducing the surface

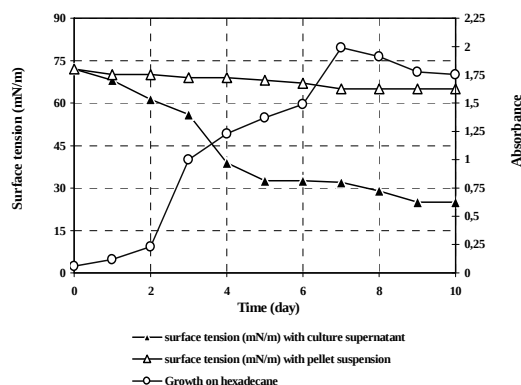


Fig. 1. Surface tension reduction during growth of *P. frederiksbergensis* on hexadecane

tension between the substrate and the bacterial surface membrane as well. Moreover, the transport of the substrate by this psychrophilic organism could be achieved by the cell interaction with the pseudosolubilized or micellar phase hydrocarbon. During the growth at low temperature, *P. frederiksbergensis* might modulate the viscosity of the membrane lipids to maintain or increase the membrane fluidity by decreasing the degree of saturation, by shortening the chain length, by increasing the cis/trans fatty acids ratio and by increasing the relative amount of branched fatty acids (Herbert, 1986).

The ability of *P. frederiksbergensis* to adhere to both solid (eicosane) and liquid (hexadecane) alkanes was especially intriguing and may be the key mechanism by which this organism

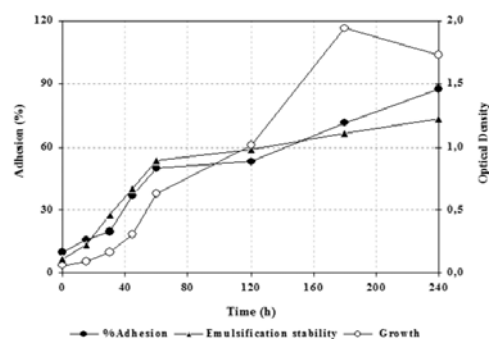


Fig. 2. Emulsion stability and *P. frederiksbergensis* cell adhesion of Hexadecane

took in and assimilated alkane substrates at low temperatures. Attachment of the cells to the crystals on the one hand might be due to membrane bound oxygenases (Rosenberg *et al.*, 1992) that catalyze initial degradation steps. Explanation and understanding of the hydrophobic effect remained difficult. The adhesion of *P. frederiksbergensis* was observed during the growth on these hydrophobic substrates, as determined by the BATH test performed with the supernatant not the cell pellet suspension.

Therefore, glycolipids produced by this psychrophilic organism may promote alkane uptake through solubilization of the eicosane or hexadecane in micelles allowing for bulk phase transport of the micellar hydrocarbon to the cell. According to Witholt *et al.*, (1990), the growth of

Pseudomonas on alkanes accompanied with emulsifiers excreted as small membrane vesicles which interacted with alkane droplets. These biosurfactants interacted and coated the eicosane crystals with an outer layer of hydrophilic chains and thus became water soluble.

Simultaneous development of the ability to oxidize alkanes and emulsify eicosane crystals

During the growth on hydrophobic compounds, *P. frederiksborgensis* produced glycolipids to solubilize and take up these compounds. These modifications include formation of a hydrophobic cell surface, and emulsification of the hexadecane. All the inducers of hydrophobic substrate emulsification from their structure and biochemistry seem to demand an alkane oxidation system. It therefore seemed possible that all the inducers of hexadecane emulsification may also be inducers of the alkane oxidation system and of cell surface hydrophobicity. The alkane oxidation system is needed for growth on hydrophobic compounds utilized by *P. frederiksborgensis*, and a hydrophobic cell surface was necessary for the effective transport of these substances into cells. These two phenomena seem to operate together, suggesting that the genes coding for enzymes involved in these two processes could be under the same repressor control or part of the same operon. The emulsification was a consequence of the release of the hydrophobic cell surface components and, therefore was observed only if the cells had been able to form hydrophobic cell surface (Bredholt *et al.*, 2002).

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