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RESEARCH ON BIOSURFACTANTS – TREHALOSE LIPIDS IN RHODOCOCCI

Рассматриваются вырабатываемые микроорганизмами биологические поверхностно-активные вещества, которые благодаря своим физическим и химическим свойствам значительно снижают поверхностное и интерфациальное натяжение как водных растворов, так и углеводородных смесей.

The article studies biosurfactants synthesised by microorganisms and which are due to their physical and chemical properties considerably reduce surface and interfacial tensions in both aqueous solutions and hydrocarbon mixtures.

Biosurfactants are surface-active, amphiphilic compounds characterised by a hydrophilic and a hydrophobic molecule part. Due to their physical and chemical properties biosurfactants considerably reduce surface and interfacial tensions in both aqueous solutions and hydrocarbon mixtures. (Bio-) surfactants are supposed to act in three principal ways: they accelerate the dissolution of the substrate with increasing mass transfer rate; they support the formation of fine substrate droplets (process of micro-emulsification or pseudosolubilisation); and they can facilitate the contact between cell and substrate (Hommel, 1990). Biosurfactants are synthesised by various microorganisms as a kind of adaptive mechanism in order to increase the bio-availability of low water-soluble substances, which enables them to utilise aliphatic and aromatic hydrocarbons as carbon and energy sources.

Bio-surfactants represent a structurally very heterogeneous group. According to their size they can be divided into high- and low-molecular bio-surfactants. Glycolipids, e.g. rhamnolipids or trehalose lipids, as one major group of bio-surfactants are by far the most common and best-investigated type. They basically consist of low-molecular, water-insoluble sugars that are acylated with long-chain fatty acids or hydroxy fatty acids. Their emulsifying and emulsion-stabilising properties which are not remarkable changed by shifts in temperature or salinity, and also their low toxicity favour these biosurfactants in a number of environmental applications, namely in bioremediation purposes (Banat et al., 2000; Christofi & Ivshina, 2002; Desai & Banat, 1997). Certain trehalose lipids produced by rhodococci have also been shown to possess immunostimulating and cell-differentiating features (Isoda et al., 1997; Ryll et al., 2001). Therefore they may even have the potential for future medical application, particularly in cancer research.

Due to their metabolic potential, their high tolerance against various forms of stress, and their remarkable adaptiveness, rhodococci presumably do play an important role in the bioremediation of xenobiotic compounds under natural conditions (Warhurst & Fewson, 1994). During growth on *n*-alkanes rhodococci often synthesise a complex mixture of trehalose-containing glycolipids. Typical structures evidenced within such mixtures are non-ionic trehalose monomycolates (Kretschmer & Wagner, 1983), trehalose dimycolates (Philp et al., 2002; Rapp et al., 1979), trehalose trimycolates (Tomiyasu et al., 1986), as well as various acylated derivatives of trehalose (Kretschmer & Wagner, 1983; Philp et al., 2002; Singer et al., 1990). But also the formation of anionic trehalose lipids, such as trehalose tetraesters could be observed (Rapp & Gabriel-Jürgens, 2003;

Ristau & Wagner, 1983). Formation of the latter was shown to be affected by shifts in pH, temperature and/or nitrogen supply (Kim et al., 1990; Ristau & Wagner, 1983). The synthesis of surface-active compounds by rhodococci and other nocardioform actinobacteria, their physicochemical characterisation and their possible application in bioremediation have been subject to many studies (Banat et al., 2000; Christofi & Ivshina, 2002; Khan & Forster, 1988; Lang & Philp, 1998; Pirog et al., 2004; Ramsay et al., 1988). Still, compared to the well understood biochemical and molecular basics of rhamnolipid formation by *Pseudomonas aeruginosa* (Maier & Soberon-Chavez, 2000), less information is available for the genetic and biochemical background of trehalose lipid biosynthesis.

In order to bridge this gap in knowledge it was of interest to know to which extent rhodococci are able to grow on water insoluble compounds and whether they form trehalose lipids during this process. Since formation of trehalose lipids is supposed to be an *n*-alkane-induced process, several strains of *Rhodococcus* were grown with *n*-alkanes of distinctive chain-length (*n*-decane, *n*-dodecane, *n*-tetradecane, *n*-hexadecane) as sole sources of carbon and energy. The hydrocarbons were supplied at concentrations of 0,5 or 1 % (w/v) in mineral medium. In case of nitrogen-limiting conditions the above mineral medium did not contain ammonium sulphate and thus had a high carbon surplus. Evidence of produced biosurfactants was achieved by extracting whole cultures with polar organic solvents. Organic phase extracts were then separated by thin-layer chromatography using anisaldehyde/ sulphuric acid reagent for derivatisation. Trehalose lipids were identified by comparing obtained spots with co-chromatographed authentic reference compounds for trehalose monocorynomycolate, trehalose dicorynomycolate and trehalose tetraester. Most of the investigated strains were indeed found to synthesise trehalose lipids. Some produced non-ionic trehalose dicorynomycolates, others produced the anionic trehalose tetraesters, preferably under nitrogen limited conditions. Further purification some of the trehalose lipids for structural characterisation was achieved by column chromatography. The mere products were structure analysed using various analytical techniques including NMR spectroscopy, analytical thin-layer chromatography, GC-MS analytics, and IR spectroscopy, respectively (Niescher, 2002; Pfeiffer, 2004; Zimmermann, 2003).

For closer examination of growth behaviour with respect to the chain length of the *n*-alkane provided, *Rhodococcus opacus* strain 1CP was chosen as a model organism. The strain has originally been isolated from soil by an enrichment on 2,4-dichlorophenol, and was shown to degrade also 4-chlorophenol (Gorlatov *et al.*, 1989), 2-chloro- and 3-chlorophenol, 3-chlorobenzoate (Moiseeva *et al.*, 1999), phenol, benzoate, styrene (Eulberg, unpublished) as well as *m*- and *p*-cresol (Moiseeva et al., 1999; Gröning, unpublished). Due to the insolubility of hydrocarbon substrates, the hydrophobicity of the microbial cells and their high tendency to adhere on glass growth control by ordinary turbidity measurement was not accomplishable. Instead, respirometric control of growth and hence substrate consumption was performed with an OxiTopC system. For all of the tested *n*-alkanes an increase in oxygen demand was observed being highest for *n*-hexadecane and *n*-tetradecane. The utilisation of *n*-dodecane and especially *n*-decane appeared to be significantly slower presumably indicating a toxic effect of the short-chained *n*-alkane on the microbial cells. *R. opacus* 1CP was found to exclusively produce trehalose dimycolates during growth on *n*-alkanes, even under nitrogen-limited conditions (Niescher, 2002; Niescher, 2005). According to the structural elucidation of the purified compound it could be characterised as a trehalose dinocardiomycolate (Hommel, 1990). Quantification of both, growth and trehalose lipid content of *n*-tetradecane-grown cells of the strain suggested a growth-associated formation of the glycolipds. Their concentration sig-

nificantly decreased after the *n*-alkane was exhausted (Niescher, 2002; Niescher, 2005).

Former studies about the biosynthetic pathways of trehalose mycolates clearly indicated that the hydrophobic and the hydrophilic molecule part are synthesised independently (Kretschmer & Wagner, 1983). The identification of key enzymes, corresponding genes and gene products involved in trehalose lipid formation of *R. opacus* 1CP was thought to further contribute to the elucidation of both, the enzymatic and the genetic background of trehalose lipid biosynthesis. The trehalose-6-phosphate synthase OtsA which catalyses the formation of the sugar moiety trehalose-6-phosphate from UDP-glucose and glucose-6-phosphate as part of the OtsA/OtsB trehalose biosynthesis pathway was chosen for closer examination. Using established molecular and genetic approaches two distinctive *otsA* genes were so far identified in *R. opacus* 1CP (Niescher et al., 2004; Pilop, 2002). Their deduced amino acid sequences show high similarities to the respective enzymes of several members of the high-GC Gram-positive group (coryno- and mycobacteria). The existence of more than one *otsA* gene could also been shown for several other rhodococci, it might reflect the various physiological roles OtsA is involved in, including the synthesis of trehalose lipids, cell wall constituents, and together with OtsB the formation of trehalose itself. This is why our present research is particularly focussed on the clarification of the distinct function of each of the two genes mainly by enzymatic approaches. Furthermore an enoyl-acylcarrierprotein reductase InhA was subject of interest taking into account the extensive knowledge about mycolic acid synthesis in mycobacteria. From which it is known that a complex system of enzymes (FASII) is responsible for the elongation of medium sized fatty acids aiming in the long-chained hydrophobic moiety, which is found in trehalose (coryno-) mycolates (Asselineau et al., 2002). InhA as a part of that fatty acid synthase-II system (FASII) is involved in that process of elongation and hence in the formation of trehalose (coryno-) mycolates. PCR and hybridisation approaches applied to total DNA of *R. opacus* 1CP using *inhA*-specific database-derived primers indicated the presence of a putative gene *inhA*. Database comparison with its deduced amino acid sequence resulted in high homology to enoyl-ACP reductases of the high-GC Gram-positive group (Niescher, 2002; Niescher et al., 2004).

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