

**Microbes in oil industry –
Isolation and characterization of a novel *n*-alkane-
degrading strain, *Acinetobacter haemolyticus* AR-46**

PhD THESIS

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In the course of the production of crude oil, paraffinic-asphaltenic deposition occurs on the inner surface of the tubing and in the near-wellbore zone. Oil companies have to face this costly problem, which can necessitate constant intervention to eliminate mechanical blocks, otherwise paraffin precipitation restricts production to uneconomical levels, causing premature abandonment of well and leaving the main part of the oil resource in the reservoir unrecovered.

1. The Department of Bioremediation, Bay Zoltán Foundation for Applied Research, Institute for Biotechnology and the MOL Hungarian Oil and Gas Plc. have been developed and patented a microbial deposition-inhibiting technology (Hlatki et al., 2003b).
2. Our preliminary analysis on production stream of Dorozsma-55 wellbore zone detected 2 ppm of dissolved oxygen, thus the presence of aerobic degradation processes were presumed. However, suitable microbial treatment could only been performed under closed-well-state only, which resulted in anaerobic conditions. Consequently, during the development of the technology, the consortia of facultatively anaerobic *Pseudomonas* and *Bacillus* isolates with the degrading ability of different heavy oil fractions (paraffin, asphaltene, maltene) or with biosurfactant producing ability were chosen and applied.

Other publications:

Kesserű P., Kiss I., **Bihari Z.**, Polyák B., (2002), The effects of NaCl and some heavy metals on the denitrification activity of *Ochrobactrum anthropi*. J. Basic Microbiol. **42**, 268-276. Impact factor: 0.512

Kiss I., **Bihari Z.**, Kesserű P., Fehér B., Polyák B. (2002), Co-immobilization of symbiotic green algae and *Saccharomyces unispora*. Symbiosis **32**, 247-256 Impact factor: 0.795

Summa impact factors: 6.765

Publications referred in Dissertation:

Bihari Z., Pettkó-Szandtner A., Csanádi Gy., Balázs M., Bartos P., Kesserű P., Kiss I., Mécs I., (2007), Isolation and characterization of a novel *n*-alkane-degrading strain, *Acinetobacter haemolyticus* AR-46. Z. Naturforsch. C – accepted manuscript. Impact factor: 0.602

Kesserű P., Kiss I., **Bihari Z.**, Polyák B. (2002), Investigation of the denitrification activity of immobilized *Pseudomonas butanovora* cells in the presence of different organic substrates. Water Res. **36**, 1565-1571. Impact factor: 1.611

Kesserű P., Kiss I., **Bihari Z.**, Polyák B. (2003), Biological denitrification in a continuous-flow pilot bioreactor containing immobilized *Pseudomonas butanovora* cells. Bioresour. Technol. **87**, 75-80. Impact factor: 1.382

Kesserű P., Kiss I., **Bihari Z.**, Polyák B. (2005), Nitrate-dependent salicylate degradation by *Pseudomonas butanovora* under anaerobic conditions. Bioresour. Technol. **96**, 779-784. Impact factor: 1.863

Kovács T., **Bihari Z.**, Hargitai A., Mécs I., Kovács K. L. (2002), Stress related changes of cell surface hydrophilicity in *Bacillus subtilis*. Acta Microbiol. Immunol. Hung. **49**, 21-35. Impact factor: -

3. As nitrate or nitrite electronacceptors were not detected in formation water samples, technology fluid was supplemented with nitrate. Inorganic N, P and S sources were found to be either in limiting concentration or they were not present at all, so these nutrients were externally amended.
4. During the closed intervals, the oxygene tension decreased in the wells, which had positive effect on the denitrification processes. The *Pseudomonas aeruginosa* strains were known to be able to decompose *n*-hexadecane (Chayabutra and Ju, 2000) under these conditions; and we had proved previously that *Pseudomonas butanovora* not only has denitrification ability (Kesserű *et al.*, 2002, 2003), but it cometabolizes aromatic substrates as well (Kesserű *et al.*, 2005).
5. During oil production, the added electronacceptor disappears, and the waterflow regenerates the microaerophilic condition in the tubing. This phenomenon was observed in the case of well Dorozsma-55. The bacterial treatment took place five years ago and since then the elevated oxygene tension has evoked the paraffin deposition inhibition effect.
6. Taken together, the technology was succesfull both scientifically and economically in the two-third of the microbially injected wells. Mechanical tube clearing was either not needed at all, or the

- intervention intervals were elongated drastically. In line with it, the oil production increased by 30-100%. The durability of the microbial treatment was at least 3 months, and the average value was 6 months.
7. The effect of the applied method was evident on the increasing total cell numbers in oil and deposit samples recorded after the treatment; and the injected strains could be reisolated. The increase of cell concentration by 0.5-4.5 order of magnitude correlated well with the positive technological observations, the decrease in total cell number coexisted with the falling of the inhibition effect.
 8. Consortia were able to brake oil/water emulsions both in laboratory batch experiments and in industrial treatments of surface tank oil (Hlatki *et al.*, 2003a). Similar effect occurred during oil production, the previously uniform production stream was separated to oil and water phases. It yielded to a decrease in viscosity, which could lead to the appearance of extra oil production.
 9. In line with the phenomena mentioned above, changes in the alkane-composition of depositions were also measured. Due to the most successful treatments, the quantity of deposits decreased, and the longer *n*-alkanes (>*n*C₃₀) concentrated in it. According to literature data, this phenomenon is an unambiguous consequence of the biodegradation limit of *Pseudomonas* and *Bacillus* isolates.
 22. Similarly to *A. sp.* ADP1, in the upstream regions of *alkM* gene, *in silico* analysis revealed the same types of putative promoter elements and inverted repeat sequences serving binding targets for the AraC-XylS-like AlkR transcription regulator in AR-46 too.
 23. Unfortunately, deeper genetic analyses are hampered by the very low efficiency of site-specific recombination. Similarly as for *A. haemolyticus* AR-46, deriving gene disruptants failed for strain M-1 too.
 24. Based on our results, the strain *A. haemolyticus* AR-46 can be involved in different technological applications, which are under preparation.

18. Besides long and thick fimbriae as primary hydrophobic sites, the products of clustered *ompH-lpxD-fabZ-lpxA* genes are responsible for membrane formation. Therefore, these genes located adjacently to the *alkR-alkM* gene may also play a crucial role in *n*-alkane solubilization.
19. The enzymes which catalyse the oxidation of *n*-hexadecane substrate taken up by AR-46 cells were examined in detail, but neither alkane dioxygenase nor cytochrome P450 activities were detected in cells. These results of enzyme assays and the GC-MS analysis afforded evidence of the involvement of the monoterminial oxidation pathway in AR-46.
20. In support of this, the chromosomal *alkM* gene was identified. The gene has no paralogue in the genom as revealed by Southern hybridization and DGGE analysis.
21. Comparison of the results of Northern hybridization experiments on *A. spp.* demonstrated significant differences in alkane hydroxylase gene induction and regulation. The transcription of *alkM* in *A. haemolyticus* AR-46 is evoked by the broad range of long-chain *n*-alkanes (C₁₆-C₃₀).

These strains were unable to mineralize the long-chain alkanes, so the relative amounts of these compounds could increase in this way.

The isolation of microbes with long chain paraffin-degrading ability was aimed. The effective strain was necessary not only for upgrading the oil well treatment technology, but for bioremediation processess of oil contaminated soil (Hlatki *et al.*, 2002) and water phases (GVOP-3.1.1 grant), and also for developing of a new MEOR technology.

10. Strain AR-46 was sampled from the production water of an oil reservoir in Hungary and was isolated after enrichment on solid paraffin carbon source. Both 16S rDNA analysis and biochemical characterization indicated the highest degree of similarity between AR-46 and the *A. haemolyticus* type strain. We performed the first detailed description (Bihari *et al.*, 2007) of this novel *n*-alkane-degrading isolate belonging in this species from the environment.
11. Under microaerophilic conditions, AR-46 was able to assimilate *n*-alkanes with carbon chain lengths of from C₁₂ to C₃₅. Among the tested compounds, *n*-hexadecane was found to be the best substrate for growth and biodegradation ($\mu_m = 0.253 \text{ h}^{-1}$, $Y'_{X/S} = 0.576 \text{ kg of cells (kg of } n\text{-hexadecane)}^{-1}$ at the optimal temperature of 37 °C).

12. A systematic comparison of AR-46 with related *Acinetobacter* spp. concerning the temperature dependence of *n*-alkane degradation was performed. Although *Acinetobacter* species are routinely maintained at 30 °C, the present results suggest a cultivation temperature of 37 °C for *A. calcoaceticus* EB104 and *A. venetianus* 6A2. The experimental data reported here indicate that this latter strain and our isolate appear to be well suited for relevant biotechnological applications in consequence of their excellent long-chain *n*-alkane-degrading ability at both temperatures.
13. The high rate of *n*-alkane biodegradation correlates with an improved substrate uptake mechanism. Intimate interaction between the cell and *n*-alkane droplet surfaces was observed microscopically, and this adherence is involved in the solubilization and uptake process. Based on the TEM data, the thick fimbriae (11-14 nm in diameter) of the *n*-hexadecane-adhered cells are significantly (2-3 times) longer than the fimbriae of the non-adhered and control cells.
14. The phenomenon that these long structures serve as primary hydrophobic sites is also manifested in their physiological properties. The interactions between the *n*-alkane droplets and the adhered hydrophobic cells are so strong that cells can not be separated from the substrate even by intensive centrifugation. However, the detached *n*-alkane-grown AR-46 cells in the bulk phase, similarly to cells grown in rich media, are predominantly unable to adhere to *n*-hexadecane because of their constant surface hydrophilicity. Fewer than 10% of these free AR-46 cells can attach to *n*-alkane, whereas the analogous values for *A. venetianus* RAG-1 and VE-C3 are approximately 90% (Baldi *et al.*, 1999).
15. Cell surface hydrophilicity values -measured by MATH tests - are generally increased (Kovács *et al.*, 2002) by different stress effects (heat, salt or pH shocks, oxygene-, nutrient and carbon source-limitation), but in the case of AR-46, it is rather the result of the special dynamic balance between the two cell types. After alkane droplet solubilization and uptake, the hydrophobic cells possessing long and thick fimbriae become hydrophilic and migrate to the nutrient-rich bulk phase. The alkane adherence is not further necessary, thus the reduction of the length of the fimbriae can be observed.
16. This theory was strengthened by the fact that the intermediates *n*-hexadecan-1-ol, *n*-hexadecan-1-oic acid and the wax ester *n*-hexadecyl-*n*-hexadecanoate could be measured from free cells, but not from the HNPS media.
17. The accumulation of this latter substance is a unique phenomenon in the genus, as wax ester has only been detected under nutrient-limiting conditions until now.

