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MINIREVIEW

Alpine Microorganisms: Useful Tools for Low-Temperature Bioremediation

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Cold environments, including polar and alpine regions, are colonized by a wide diversity of microorganisms able to thrive at low temperatures. There is evidence of a wide range of metabolic activities in alpine cold ecosystems. Like polar microorganisms, alpine microorganisms play a key ecological role in their natural habitats for nutrient cycling, litter degradation, and many other processes. A number of studies have demonstrated the capacity of alpine microorganisms to degrade efficiently a wide range of hydrocarbons, including phenol, phenol-related compounds and petroleum hydrocarbons, and the feasibility of low-temperature bioremediation of European alpine soils by stimulating the degradation capacity of indigenous microorganisms has also been shown.

Keywords: alpine, bioremediation, petroleum hydrocarbons, phenol

Cold ecosystems: habitats for psychrophiles

The Earth is a cold planet. About 85% of the biosphere is permanently exposed to temperatures below 5°C. Cold habitats span from the Arctic to the Antarctic, from high-mountains to the deep ocean. The major fraction of this low-temperature environment is represented by the the deep sea (nearly 75% of the Earth is covered by oceans and 90% of the ocean volume is below 5°C), followed by permafrost (24% of land surface), sea ice (13% of the earth surface) and glaciers (10% of land surface) (Bakermans, 2007). Other cold environments are cold soils (especially subsoils), deserts, lakes, and caves.

Cold environments are colonized by a wide diversity of microorganisms, including bacteria, archaea, yeasts, filamentous fungi and algae. To thrive successfully in low-temperature environments, these microorganisms have evolved a complex range of structural and functional adaptations. Adaptations include the production of cold-active enzymes with high catalytic efficiency at low temperatures, the incorporation of unsaturated fatty acids in cell membranes to maintain membrane fluidity, the production of cold-shock proteins and cold-acclimation proteins to ensure improved protein synthesis at low temperatures, the synthesis of protective compounds to protect the cell from freezing (e.g. sugars, extracellular polysaccharides, antifreeze proteins), and the production of high amounts of antioxidant enzymes (catalase, superoxide dismutase, dioxygen-consuming lipid desaturases) for the detoxification of reactive-oxygen species (Gerday and Glansdorff, 2007; Margesin *et al.*, 2008).

Microorganisms in cold environments are distinguished from other thermal classes (mesophiles, thermophiles) by their ability to grow and reproduce at temperatures around 0°C and even below. Attempts have been made to classify cold-adapted microorganisms into psychrophiles and psychrotrophs (also termed cold-tolerant) according to growth rates and the upper limit of growth temperature (Morita, 1975). However, this distinction is very questionable since the apparent "optimal" temperature for growth gives no indication about the ability of microorganisms to thrive well at low temperatures prevailing in their natural environment. Temperatures close to the maximum growth temperature induce cellular stress, leading to low cell densities and low activities (e.g. enzyme production) (Gerday *et al.*, 2000).

Can you suggest better criteria or classification to recognize cold-adapted microorganisms, for example, concept of eurypsychrophile and stenopsychrophile (Cavicchioli, 2006, Nature Reviews 4, 331-343)

Alpine climate conditions and microbial activities

It is well-known that polar regions (high-latitude zones) represent an ideal habitat for psychrophiles, whereas comparatively little is known about cold alpine regions. The term "alpine" is generally accepted as a term for a high-altitude belt (about 1,800-2,500 m above sea level) above continuous forests on mountains. The subalpine belt has been defined as covering the forest-tundra ecotone at high altitudes (Löve, 1970).

The change of temperature and other environmental conditions with altitude in mid-latitude mountains has often been compared to their change with latitude: a 1,000 m higher altitude in the Alps may roughly be equivalent to a 1,000 km move northward (Kuhn, 2008). Compared to the Arctic, the European Alpine region is characterized by higher maximum temperatures, similar or lower minimum temperatures,

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large and frequent (diurnal) temperature fluctuations and freeze-thaw events, higher precipitation (2,000-3,000 m per year) and humidity, lower atmospheric pressure, higher intensity of solar radiation.

Despite these different climatic conditions, alpine microorganisms are equally well-adapted to low temperatures as polar microorganisms. The comparison of cold-active enzymes (pectate lyase) from two psychrophilic *Mrakia frigida* strains, isolated from alpine glacier cryoconite and from North Siberian sediment, clearly showed that the enzymes produced by these strains had an almost identical activity and stability pattern. Both enzymes displayed apparent optimal activity at 30°C and pH 8.5-9.0 and were thermolabile, but resistant to repeated freezing and thawing (Margesin *et al.*, 2005a). The two strains had almost identical growth characteristics (high cell densities at 1-15°C, no growth above 20°C), however, the enzyme production pattern was completely different. The Siberain strain produced pectate lyase over the whole growth temperature range, with a maximum at 1°C, wherease enzyme production by the alpine strain was highest at 5°C, very low at 15°C and absent at 20°C. Enzyme production patterns may be related to the natural environmental conditions of the strains. The Siberian strain originated from a sediment sample that might be subjected to more temperature fluctuating conditions, while the natural habitat of the alpine strain is permanently cold with temperatures never exceeding +3°C.

There is evidence of a wide range of metabolic activities in all cold ecosystems. Like polar microorganisms, alpine microorganisms play a key ecological role in their natural habitats for nutrient cycling, litter degradation, and many other processes. They are generalists and utilize a wide range of natural organic compounds, such as protein, lignin, cellulose, and hydrocarbons (Margesin *et al.*, 2002). Hydrocarbon pollution in cold climates is an area of particular importance, since contaminated areas are often remote, and thus the degradation capacity of indigenous microorganisms is required. This review is focused on our studies on the potential of alpine hydrocarbon degraders for low-temperature bioremediation.

Molecular characterization of microorganisms in pristine and contaminated alpine soils

The ubiquity of microorganisms able to degrade petroleum hydrocarbons is well known. Detailed characterization of 12 contaminated soils (0.4-30.0 g TPH/kg soil; sampled near diesel storage tanks, petrol stations or garages from various alpine sites in Tyrol, Austria, at altitudes from 600 to 2,900 m above sea level) and the corresponding pristine soils demonstrated the presence of significant microbial heterotrophic and oil-degrading cold-adapted populations in all of the soils, independent of the contamination level (Margesin *et al.*, 2003a). The numbers of culturable cold-adapted hydrocarbon degraders (diesel oil, hexadecane, 2-10°C) were greater by up to 4 orders of magnitude than those of the corresponding mesophilic populations. This points to the important role of cold-adapted microbial communities in the bioremediation of contaminated alpine soils.

Various methods were used for the molecular characterization of microorganisms in contaminated and pristine alpine soils; before, such analyses were only available for arctic and antarctic soils. Most of the alpine soils had a low organic matter content (<2%), pH values ranged from 4.8 to 9.2. Phylogenetic analyses were applied to evaluate the prevalence of various bacterial classes (Labbé *et al.*, 2007). The relative amount of representatives of the phyla Actinobacteria (18 and 20% in pristine and contaminated soils, respectively) and Proteobacteria (73 and 76%, respectively) was independent of the contamination. However, the distribution pattern of bacterial classes among Proteobacteria was significantly related to the presence or absence of the contamination. The prevalence of Alphaproteobacteria was larger in pristine (46%) than in contaminated soils (24%), while Beta- and Gamma-proteobacteria were only detected in contaminated soils (8 and 24%, respectively). This was confirmed by a significant positive correlation ($P<0.01$) of the soil TPH contents with the amount of Gamma-proteobacteria but not with other bacterial classes.

Studies on the prevalence of catabolic genotypes involved in the degradation of representative fractions of petroleum hydrocarbons (n-alkanes, mono- and polyaromatic hydrocarbons) demonstrated that genotypes with degradative genes from Gram-negative bacteria (*Pseudomonas putida*, *Acinetobacter* sp.) prevailed in contaminated soils (50-75% and 0-12% in contaminated and pristine soils, respectively), while genotypes with degradative genes from Gram-positive bacteria (*Rhodococcus* spp., *Mycobacterium* sp.) were found at a high frequency in all of the soils. Consequently, there was a highly significant ($P<0.001$) positive correlation between the level of contamination and the prevalence of genotypes containig genes from Gram-negative bacteria, whereas no significant correlation was found between the TPH content and the prevalence of genotypes derived from Gram-positive bacteria (Margesin *et al.*, 2003a).

PLFA (phospholipid fatty acids) patterns of microbial communities further confirmed this trend. PLFA indicative of the Gram-negative population were significantly ($P<0.05$) increased in soil samples containing high amounts of diesel oil, whereas the Gram-positive population was not significantly influenced by the TPH content (Margesin *et al.*, 2007a).

All together, the molecular characterization of pristine and contaminated alpine soils indicated a shift in microbial community composition after a contamination event: Gram-negative bacteria (especially representatives of gammaproteobacteria) are enriched after a contamination event, whereas Gram-positive bacteria (Actinobacteria) are already present in substantial numbers before such an event, and their prevalence is not affected by the presence of petroleum hydrocarbons. This may be explained by the *r-K* scheme (Atlas and Bartha, 1998), according to which *r* strategists (Gram-negative, Gamma-proteobacteria) are adapted to rapid reproduction and thus were predominant in contaminated soils where nutrients were represented by hydrocarbons, while *K* strategists are better adapted to resource-limited conditions and are usually permanent members of the microbial community, such as typical Gram-positive representatives of the Actinobacteria in soil. Similar results have been reported from arctic and antarctic soils (MacNaughton *et al.*, 1999; Juck *et al.*, 2000; Whyte *et al.*, 2001).

Bioremediation of alpine soils contaminated with petroleum hydrocarbons

The capacity of a broad spectrum of microorganisms to utilize hydrocarbons as the sole source of carbon and energy (biodegradation) has been recognized very early. Bioremediation attempts to accelerate the biodegradation rates through the optimization of limiting environmental conditions, such as temperature, nutrients, bioavailability of contaminants.

Low temperatures affect the rates of biodegradation through the modification of the physical nature of the contaminants (Wu *et al.*, 1997). For petroleum hydrocarbons, a decrease in temperature results in increased viscosity, decreased volatilization and increased water solubility (resulting in higher toxicity of short-chain alkanes), and decreased bioavailability of some compounds such as long-chain alkanes (Whyte *et al.*, 1998). Beside of these effects, the environmental temperature influences microbial activity. Due to the Q_{10} effect, reaction rates are reduced in the cold, however, local environmental conditions select for populations with high activities at low temperatures.

Successful bioremediation at low temperatures has been described for arctic and antarctic soils (for a review see Aislabie *et al.*, 2006). We focused our studies on the bioremediation of European alpine soils contaminated with petroleum hydrocarbons.

Bioremediation strategies can involve biostimulation of the indigenous soil population and/or bioaugmentation (inoculation with efficient degraders). A number of studies on low-temperature bioremediation of alpine soils clearly demonstrated that biostimulation by nutrient supplementation (NPK) enhanced biodegradation to a significantly greater extent than inoculation with microorganisms that degraded diesel oil efficiently in liquid cultures (Margesin and Schinner, 1997a, 1997b). In a long-term study (150 days at 10°C) with two alpine soils, a total loss of 95% of the initial experimental contamination (4,000 mg diesel oil/kg soil) was obtained with fertilization. Inoculation only contributed to a short-term, temporary acceleration of the biodegradation process (Margesin and Schinner, 1997b).

Remarkably, about 30% of the decontamination could be attributed to abiotic processes (e.g. adsorption onto soil colloids, transformation and evaporation) which play an important role in freshly contaminated soils but are of minor importance in soils with aged pollution.

Fertilization may not be a general solution to increase natural biodegradation; the investigation of nutrient effects at a specific site is essential for successful bioremediation (Braddock *et al.*, 1997). Natural attenuation (abiotic loss plus natural biodegradation by indigenous soil microorganisms) played a major role in the decontamination of soil samples from a former tank farm (mean contamination of 4,700 mg/kg soil); fertilization had no significant effect ($P<0.05$), despite the nutrient deficiency of the soil. After 5 months at 10°C (this temperature corresponded to the mean annual environmental temperature of the tank farm area and to the groundwater temperature), a TPH loss of 20-49% and 23-66% was found in unfertilized and fertilized soil samples, respectively. The negative effect of fertilization on the number of cold-adapted hydrocarbon degraders and microbial activity (soil respiration) was explained by the oligotrophic nature of the soil microorganisms (Margesin and Schinner, 1999).

In alpine ski resorts, pollution with hydrocarbons is caused by the use of motor vehicles for the preparation of ski runs and also by leaks and storage tank ruptures. Bioremediation may be the logistically and economically most favorable solution as the use of conventional techniques requires costly excavation. Therefore we evaluated the feasibility of bioremediation as a treatment option in a Tyrolean glacier area (2,900 m above sea level) during a 3 years field study (Margesin and Schinner, 2001). Environmental conditions could be considered as extreme (mean annual air temperature 1.3°C, summer temperatures varying from freezing to above 20°C, annual thaw season from June/July to September). The soil collected from the contaminated zone of the motor pool area (2,600 mg TPH/kg soil) was a mixture of carbonaceous gravel and sand and contained low levels of nutrients. Natural attenuation (untreated soil) was compared with biostimulation. In all three seasons, the decontamination was significantly ($P<0.05$) higher in the fertilized soil, however, the stimulating effect of nutrients slowed down with time, despite repeated nutrient amendment. At the end of the third summer season, a residual contamination of still 30% (770 mg TPH/kg soil) and 50% (1,300 mg TPH/kg soil) was measured in the fertilized and untreated soil, respectively. Nonetheless, a significant reduction of hydrocarbon was obtained in an unfavorable environment and fertilization treatment was effective in terms of accelerated hydrocarbon biodegradation. This was also proved by increased microbial numbers and activities (soil respiration, soil enzyme activities) and significant positive correlations ($P<0.05$-0.001) of all biological parameters with the contents of available nutrients (N, P) and TPH. On the other hand, microbial activities in the unfertilized soil fluctuated around background levels and did not correlate with the residual contamination, which led to the conclusion that a considerable part of the decontamination had to be attributed to nonbiological processes.

This study clearly showed the limits of biological decontamination methods: hydrocarbon contamination, especially aged pollution, cannot always be reduced to zero. Possible reasons are the pollution history, very limited bioavailability of hydrocarbons and the presence of recalcitrant compounds (Allard and Neilson, 1997).

Low-temperature biodegradation of phenol and phenol-related compounds

The use of cold-adapted microorganisms for low-energy wastewater treatment leads to a significant decrease in operational costs. In cold climates, industrial wastewater temperature often decreases to 10°C and below, which requires the activity of cold-adapted degraders for an efficient treatment. Microorganisms that degrade high amounts of organic compounds within a short time at temperatures down to 1°C represent a promising source for accelerated wastewater treatment.

Phenol and phenolic compounds are widely distributed as environmental pollutants. They are common constituents of many industrial wastewaters, such as those produced from crude oil refineries and coal gasification plants. Due to their high toxicity to microorganisms, even low concentrations

of phenolic compounds can often cause the breakdown of wastewater treatment plants by inhibition of microbial growth, which can lead to decreased effluent quality (Ren and Frymier, 2003).

Phenol degradation is well known from mesophiles and thermophiles, but comparatively little is known on phenol degradation at low temperatures (Kotturi *et al.*, 1991). Therefore we examined the potential of alpine microorganisms to degrade phenol. We detected that the susceptibility of cold-adapted and mesophilic bacteria towards toxic compounds is influenced by their growth temperature (Margesin *et al.*, 2004). A mesophilic *Pseudomonas putida* strain showed a significantly ($P<0.05$) lower susceptibility to high concentrations of phenol and related compounds when grown at 25°C compared to 10°C, whereas toxicity for the cold-adapted *Arthrobacter psychrophenolicus* strain was significantly ($P<0.001$) lower when grown at 10°C compared to 25°C. This reflects the adaptation of cold-adapted and mesophilic microorganisms to their respective environmental temperature conditions.

The comparison of the phenol-degrading efficiencies of bacterial and yeast strains (isolated from contaminated alpine soils) at low temperatures demonstrated that yeasts were able to degrade higher amounts of phenol. Using fed-batch cultivation in mineral medium with increasing phenol concentrations as the sole carbon source, bacterial representatives (*Rhodococcus* spp., *Arthrobacter psychrophenolicus*, *Pseudomonas* sp.) degraded up to 10 or 12.5 mM phenol at 10°C, yeast strains (*Rhodotorula psychrophenolica*, *Trichosporon dulcitum*, *Leucosporidium watsonii*) utilized up to 15 mM phenol (Margesin *et al.*, 2003b, 2005b, 2007b). A concentration of 10 mM phenol was fully degraded after 11-14 days by the rhodococci and already after 3 days by *L. watsonii*. This strain was more effective to degrade 10 mM phenol at 10°C than mesophilic bacteria at 30°C (Hinteregger *et al.*, 1992). 12.5 mM phenol was fully degraded after 7 and 10 days by *L. watsonii* and *T. dulcitum*, whereas the rhodococci needed 25 days.

Studies on the effect of temperature on growth and biodegradation of 5 mM phenol showed that *L. watsonii* degraded 5 mM phenol at 1°C (11 days) faster than two *Rhodococcus* strains at 10°C (16 days), but no growth occurred at 20°C in the presence of phenol. Biodegradation by rhodococci was fastest at 20 and 30°C (full degradation after 4 days), considerably delayed at 10°C and almost negligible at 1°C. A different pattern was observed with *A. psychrophenolicus*, with full degradation after 2, 3, and 7 days at 20, 10 and 1°C (Margesin *et al.*, 2003b, 2005b). The restricted growth temperature range of yeasts indicate their potential for low temperature bioremediation processes in permanently cold environments. The application of degraders that are active over a wide temperature range might be advantageous in environments that undergo large temperature fluctuations.

Low-temperature biodegradation of phenol-related monoaromatic compounds was evaluated using 32 basidiomycetous yeast strains isolated from alpine soils and glacier cryoconite (Bergauer *et al.*, 2005). All strains could grow up to 15°C, but 12 of the strains could not grow above 20°C, and 4 of them were even not able to grow at 20°C. This confirmed results from our previous studies, according to which yeasts have a more restricted growith temperature range than bacteria (Margesin *et al.*, 2003b). The strains investigated were representatives of the Hymenomycetes (*Cryptococcus) or Urediniomycetes (Rhodosporidium*, *Rhodotorula*, *Mastigobasidium*, *Sporobolomyces*). Among the genus Rhodotorula, three novel species (*Rh. psychrophila*, *Rh. psychrophenolica*, *Rh. glacialis*) could be described (Margesin *et al.*, 2007b).

The toxicity of the tested 19 monoaromatic compounds was influenced by the chemical structure (functional groups) of the compounds. Methylated compounds were highly toxic, followed by methoxylated and hydroxylated compounds; carboyxlated compounds had the lowest toxicity. Biodegradability of phenol-related compounds was influenced by volatility and water solubility of the compounds. Interestingly, we could show that the taxonomic affiliation of the strains also influences toxicity and biodegradability. *Rhodotorula creatinivora* strains were characterized by higher IC_{50} values (50% growth inhibition in the presence of nutrients) than all other yeast species, whereas *Sprorobolomyces roseus* was the most sensitive species. In addition, representatives of *Rh. creatinivora* were characterized by a higher metabolic versatility (i.e. ability to utilize a wide spectrum of compounds) than representatives of other species (Bergauer *et al.*, 2005). Immobilized cells of *Rh. creatinivora* strains also exhibited a high ability to produce biofilm and degrade phenol on carriers of zeolite or filter sand under normal and under high osmotic conditions (Krallish *et al.*, 2006).

Conclusion

Alpine microorganisms are subjected to low temperatures, large temperature fluctuations and frequent freeze-thaw events. Beside of the key ecological role for nutrient cycling and litter degradation in their natural habitat, alpine bacteria and yeasts represent a considerable potential for low-temperature bioremediation, including low-energy bioremediation of wastewaters contaminated with phenol and related compounds and of soils contaminated with petroleum hydrocarbons. A number of studies demonstrated the important role of indigenous soil microorganisms for the treatment of European alpine soils contaminated with petroleum hydrocarbons. The stimulation of indigenous soil microorganisms proved to be an appropriate method to accelerate natural biodegradation; however, the investigation of nutrient effects at a specific site is essential for successful bioremediation. Moreover, bioremediation success may be limited due to reduced hydrocarbon bioavailability and recalcitrant compounds, especially in soils with aged pollution. Hydrocarbon contamination results in a shift of the composition of soil microbial communities with an enrichment of Gram-negative bacteria, especially members of Gammaproteobacteria.

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