

Finding all the bugs

Electrophoresis technique can identify damaging bacteria more accurately

***Corresponding author:**

Monika Lamoratta
Lanxess Deutschland
GmbH
Leverkusen, Germany
monika.lamoratta@
lanxess.com

Monika Lamoratta*
Frank Sauer

All current procedures for identifying bacteria that degrade paints use culturing and this procedure may bias the results. Attempts were therefore made to identify bacterial species directly from their DNA. Detailed identification was obtained from a contaminated and degraded paint sample, but components of undegraded paint may interfere with the test procedure.

There has been a trend for some time now in the paint and coatings industry for the supply of environmentally friendly product solutions and for sustainability in product development as well as in manufacture of modern coating systems.

A publication by the "Wissenschaftliche Dienste des Deutschen Bundestages" [scientific services of the German Bundestag] from 2004 [1] describes what is termed the "three-pillar model" of sustainable development.

This is based on the concept that sustainable development is only achievable if environmental, social and economic aims are pursued at the same time and are given equal importance, so as to ensure the ecological, economic and social effectiveness of a society. The three aspects are mutually interdependent.

If this concept is applied to a wall paint, for example, appropriate preservation firstly prevents the coating in the wet state from contamination with bacteria, fungi or yeasts and subsequently protects the cured paint film after application from

growth of micro-organisms. These are for example moulds and algae, which possibly might be harmful to health, which negatively influence the decorative aspect of a facade. With an optimum preservation, the substrate remains presentable on long term and the integrity of the film matrix is retained for longer, compared to unpreserved coatings. This means that expensive and resource-consuming redecoration does not have to be carried out so often.

Apart from avoiding risks to health, appropriate preservation therefore makes a substantial contribution to prolonging the service life of a coating, thereby saving natural resources. In line with the above definition, high-performance protection against micro-organisms can therefore be seen as sustainable product optimisation.

How micro-organisms are conventionally identified

It is usually complex microbial communities (consisting, for example, of bacteria, fungi and yeasts) that are responsible for the structural degradation of emulsion paints in wet state, so that the selection of the right microbicide must be based on the type and prevalence of the destructive micro-organisms in the product.

To effectively control the spectrum of these micro-organisms, in an ideal situation the composition of the communities must be precisely known. Until now, conventional methods of identification have been used which require cultivation of the affected product (Figure 1).

However, with this cultivation step, there is always the danger that it will also support the growth of those micro-organisms which may be insignificant in terms of paint destruction. This means that the relevance of these strains in the microbial destruction of paint would be substantially over-rated.

In particular, there are two classical methods of bacterial identification. The first is based on phenotyping, in which the grown colony is examined for size, shape and colour, for example (Figure 2). This examination is normally followed by biochemical methods such as Gram staining and oxidase reaction, which allow the species and genus to be identified more precisely.

Bacteria can also be identified from their DNA

Another method in which micro-organisms are characterised at genetic level was recently established in routine practice. This characterisation is based on evolutionary changes at DNA level in the information-carrying regions, the genome. During the course of development, e.g. because of adaptations to changed environmental conditions, different species have developed from just a few antecedents, for example. Molecular biological identification is based on differences in the genetic code.



Figure 1: Classical laboratory cultivation of micro-organisms



Figure 2: Bacteria seen in a coloured electron microscope image

An area of the DNA that is used in practice for bacterial identification is, for example, the 16S rRNA gene [2, 3, 4, 5]. The corresponding sequence of the base pairs from a specific segment of this gene (*Figure 3*) can be assigned precisely to a specific species of bacteria – in a similar way to comparing human fingerprints.

With this method, too, however, the bacteria have first of all to be cultured in the products for identification purposes, and the grown colonies have to be isolated. Genomic DNA is isolated from each colony and the 16S rRNA gene segment is amplified by polymerase chain reaction (PCR) (*Figure 4*).

The PCR technique is a process for the amplification of DNA in vitro with the use of a special enzyme for this purpose, known as DNA polymerase. The products from previous cycles are used as starting materials for the next cycle, thereby allowing exponential amplification.

By sequencing this gene segment and making a comparison with published sequences in a database, the individual organisms can be identified by family, genus and ultimately species.

Cultivation step may introduce unwanted bias

The disadvantage of the methods described above is that they need a cultivation step. With this process, however, because of the chosen experimental parameters such as nutrient supply, temperature and oxygen level, the result can be an unintended pre-selection of certain micro-organisms. Organisms that are unable to reproduce under the selected culture conditions cannot be detected and so cannot be identified by the methods described above.

It was therefore the declared aim of the work discussed below to develop another process that, by combining modern molecular biological methods, would allow the direct iden-



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Figure 3: Schematic representation of a segment of DNA

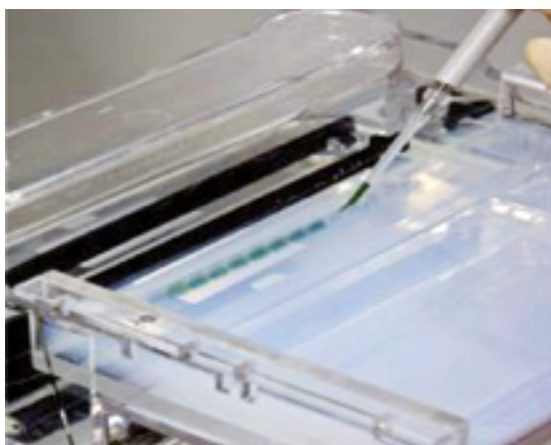


Figure 4: Electrophoresis with DNA fragments obtained by PCR

Results at a glance

» The preservation of paints against microbiological attack prolongs their lifetime and is thus both environmentally and economically desirable. However, all current bacterial identification procedures use intentional culturing to increase their numbers, and this may bias the results.

» Attempts were therefore made to identify bacterial species directly from their DNA using denaturing gradient gel electrophoresis (DGGE). The method appeared satisfactory with a paint that was already naturally contaminated with bacteria, but identification of species added directly to fresh paint was not yet possible, suggesting that components of the paint may interfere with the separation step.

» Classical identification methods and DGGE identified different bacterial species in the contaminated paint, suggesting that DGGE may be developed to give more accurate information on degrading species and so assist in optimising biocidal systems.

tification of bacteria from a contaminated aqueous product such as an emulsion paint or a sample of process water.

Thus a cultivation step, with its potentially adverse effect on the information obtained about the composition of the microbial community, would no longer be necessary. This newly developed method is based on the sequence-dependent separation of gene segments of the same size by denaturing gradient gel electrophoresis (DGGE) [6].

In this case, the DNA of all the bacteria contained in the product is isolated directly from it and the respective 16S rRNA gene segments are then amplified (the process is outlined in *Figure 5*). If there is infestation by a bacterial community, a mixture is obtained of 16S rRNA gene segments of the same size, which differ from one another only in their sequence.

How different gene sequences are isolated

The mixture of 16S rRNA gene segments is then separated by DGGE according to sequence. This separation is possible because gene segments with different DNA sequences have different melting points, i.e. the type and frequency of the building blocks of a DNA sequence determines the temperature at which the DNA double strands separate from one another. This is also called denaturation and cannot only be induced by temperature but also by chemical reagents such as urea and formamide. This characteristic is utilised in electrophoresis as a (partly) separated strand of DNA is less mobile in the gel than a

Table 1: Bacteria identified from a contaminated emulsion paint using test methods with and without initial culturing of the bacteria

With culturing
Comamonas testosteroni
Flavobacterium mizutaii
Pseudomonas aeruginosa
Delftia tsuruhatensis
Pseudomonas spp.
Microbacterium keratanolyticum
Culture-independent
Pseudomonas fluorescens
Pseudomonas putida

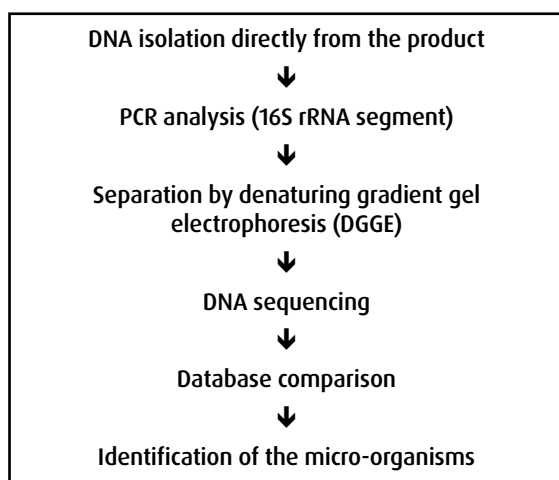


Figure 5: Summary of procedure for bacterial identification without initial culturing

double strand. A mixture of 16S rRNA gene segments can therefore be separated using this method. The fragments which are separated from each other can now be sequenced and compared with known DNA segments in a database. Ideally, using this method, a specific number of identification results is obtained which should reflect the number of contaminating bacterial strains in the product. To be able to use the culture-independent method of bacterial identification by DGGE in practice, however, it is essential that the different DNA of a bacterial community can be isolated directly from the final products to be tested, such as emulsion paints.

How DNA is isolated from contaminated products

In order to isolate DNA from different materials, their polarity or negative charge is used. Two widely used isolation techniques are based on this. In phenol/chloroform extraction, a product containing DNA is mixed in a phenol/chloroform mixture, shaken and the phenolic phase is then separated from the chloroform phase. In this process, the DNA dissolves in the phenolic phase, while most contaminants such as proteins or other organic compounds are found in the chloroform phase. Another method of isolating DNA is purification by anion exchange resins. Here, at low salt concentrations in a de-

finer pH range, the DNA binds to the anion exchange resin and could be purified of any impurities. By subsequently increasing the salt content, the DNA in the anion exchange resin can be displaced again and thus eluted from the stationary phase.

A purified and concentrated DNA product is obtained in every case. However, these methods were originally devised for the isolation of nucleic acids from tissues, blood or other biological sources. DNA purification from a relatively complex mixture such as that of an emulsion paint can theoretically lead to problems with existing experimental protocols.

DNA identification tested in emulsion paints

The method of culture-independent bacterial identification described above was therefore developed in practice in a test system with three strains of bacteria, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, in the same bacterial concentration in three different paint systems. Typical exterior emulsion paints based on pure acrylic, styrene acrylic and polyvinyl acetate binders were used.

It was possible to isolate DNA by purification using anion exchange resins from bacteria in a polyvinyl acetate based emulsion paint which had been naturally infested for some time. It was also possible to amplify this DNA by PCR and to use it in DGGE. However, it has not been possible to date to isolate DNA from a freshly contaminated paint using this method of purification. Nor did phenol/chloroform extraction provide any better results in this respect.

Thus, experiment shows that DNA can be isolated from an emulsion paint which is severely infested with bacteria and already degraded, while isolation of DNA from a freshly produced emulsion to which bacteria have been artificially added needs further optimisation. This appears to suggest that at least some components of the paint impede the use of the two methods of purification used.

One reason for this could be, for example, that components of the paint are bound to the surface of the anion exchange resins so that they are no longer available for the DNA to bind to. If the paint is already decomposed by bacterial infestation, a substantial amount of the organic components in the paint are degraded so that they cannot block the anion exchange resin. This may now allow the DNA to bind to the matrix and effective purification becomes possible.

Isolated DNA can be identified by DGGE

As expected, all the organisms from an artificial mixture of bacteria were identified with a high degree of conformity. Somewhat surprisingly, from above named contaminated emulsion paint, only the *Pseudomonas aeruginosa* species was prominently represented (Figure 6). A comparison of the methods shows that culturing gives a varying number of identification results (see Table 1), the non-culture-dependent DGGE method allows a rather more accurate identification.

The fact that relatively fewer bacterial species were found culture-independently from a paint product compared to culture-dependent methods could be an indication of the



“Encyclopedia of analytical science, Ten-Volume Set”
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fact that not all bacterial strains which are present are equally involved in the destruction of the product. Separation using DGGE correlates to some degree with the concentration of bacterial DNA in the paint. To this extent, DGGE can be regarded as a semi-quantitative method. Thus, the bacteria detected by DGGE could be the dominant species in the paint product that are mainly responsible for destruction of the product in the wet state. In contrast, with the conventional method, the growth of those bacteria which may not be relevant in terms of destruction of the paint is also promoted, so that the detection of these strains of bacteria can be wrongly interpreted.

Detailed information on bacteria may be obtained

These experimental results show that it is possible in principle for a bacterial community to be identified by DGGE without culturing. As regards contaminated products such as emulsion paints, at present fewer strains are identified than with the conventional method. At the moment it is to clarify if this is conditioned on the method or the relevance of the bacteria with regard to the paint destruction. It seems possible, however, that optimisation of the DGGE method would answer this question, thus providing new insights into the processes involved in the destruction of paint in the container which would allow users to protect products not only effectively but also in a sustainable way in line with the motto "As much as necessary, as little as possible".

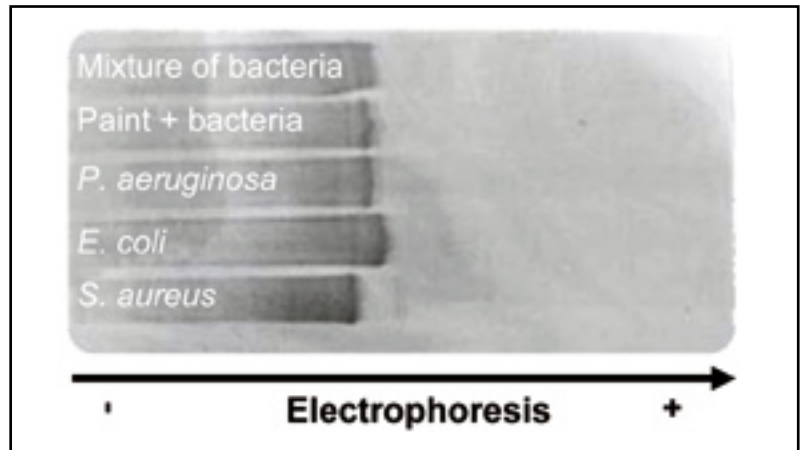
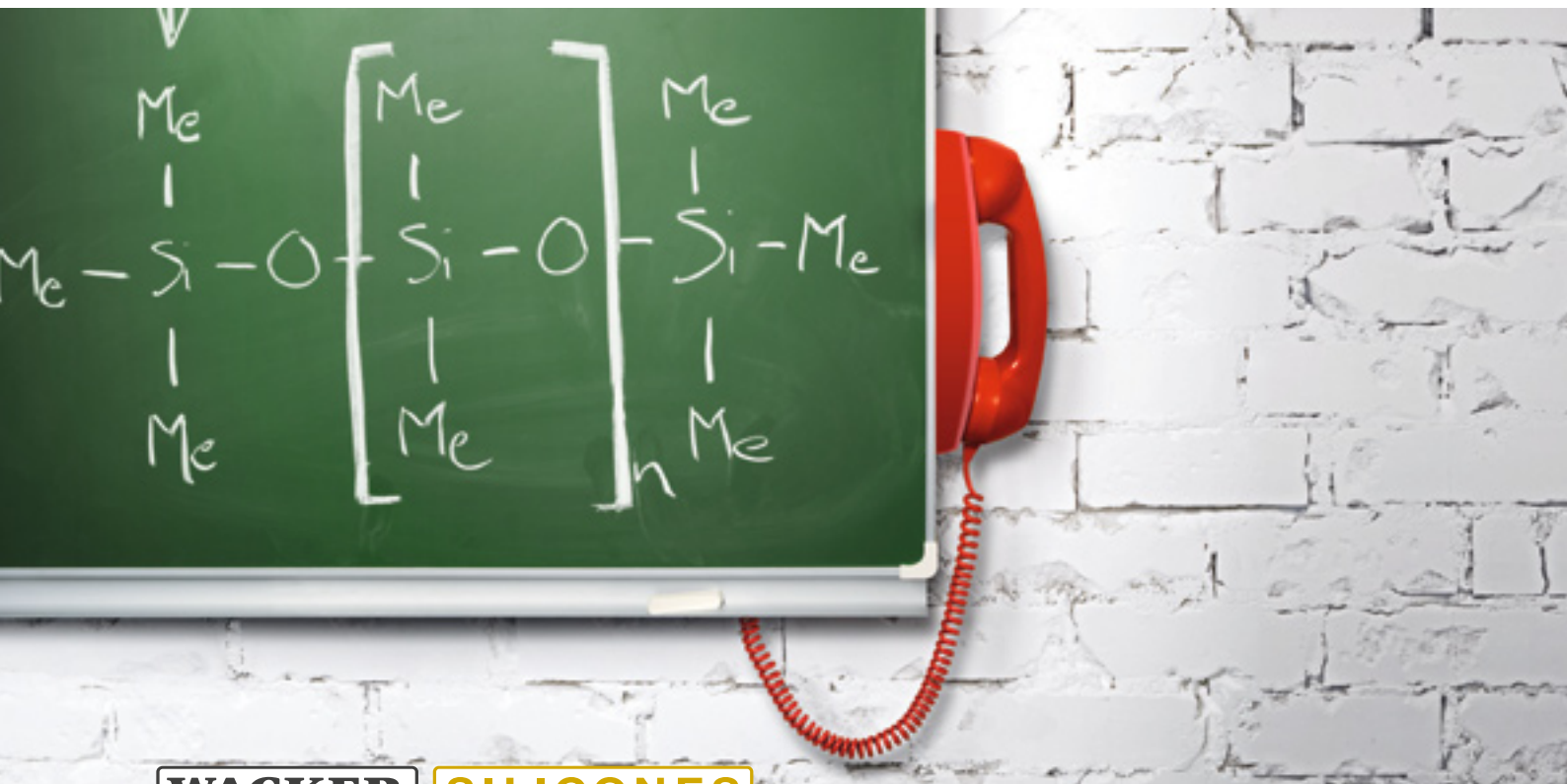


Figure 6: Electrophoretic separation of DNA from the three strains of bacteria, the mixture of bacteria and the contaminated emulsion paint by using DGGE

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