J. Microbiol. Biotechnol. (1998), **8**(2), 101 – 110

JOURNAL OF
MICROBIOLOGY AND
BIOTECHNOLOGY

REVIEW

Isolation of Microorganisms for Biotechnological Application

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Received: October 25, 1997

Abstract The extent of biological diversity being revealed by molecular techniques accentuates the need to develop methods to isolate and culture the large numbers of microorganisms that remain to be studied. The discovery and characterisation of novel microorganisms will provide information useful in understanding microbial ecosystems and have the potential to lead to new products for the biotechnology industry. In this review, the use of innovative techniques and exploration of unusual ecosystems, that have begun to address the challenge of isolating the "uncultured" members of the microbial population, are examined.

Key words: Biological diversity, microorganisms, isolation techniques, cultivation, ecological sources, bioprospecting, immunocapture, phage selection

Although advances in molecular biology techniques have revealed the extent of biological diversity that exists in many habitats, the ability to isolate and culture the range of genetically distinct microorganisms has lagged behind.

The biotechnology industry has an increasing appetite for novel microorganisms that can be used for a variety of purposes such as inoculants for bioremediation or biological control, as hosts for heterologous protein expression, as vectors for genetic exchange, as sources of commodity chemicals or enzymes, or for introduction into screening programs for therapeutic lead compounds in the pharmaceutical industry [25].

Molecular phylogenetic techniques based largely on small subunit ribosomal RNA genes have enabled the characterisation of microbial ecosystems without the need for cultivation, and it is now possible to detect and identify a large number of species that have remained uncultivated so far [91]. There are a number of comprehensive reviews covering these developments [1, 27, 34, 77, 80] that include strategies to probe environmental samples to selectively visualise a target species or genus [69, 85]. Phylogenetic analysis has been instrumental in revealing the remarkable diversity of the microbial world [16]. It is estimated that only 0.1% of all microorganisms have been cultivated and that at least 3 million new bacterial species and 1.5 million new fungal species have yet to be described [10, 30]. The main challenge, therefore, lies in the development of conceptually new approaches to enrich for and isolate those microorganisms not in culture, and to transform the "uncultured" into culturable microorganisms.

Biodiversity harnessed via cultivation or through "gene-harvesting" of unculturables has enormous potential for application in modern biotechnology. The modern nucleic acid-based approaches allow this diversity to be accessed directly so that genes and functional proteins can be acquired from uncultivated organisms via high-throughput cloning, screening and expression systems [78]. These techniques can equally be applied to guide cultivation attempts of a phylotype by monitoring its enrichment in culture and, where possible, by using the phylogenetic information to relate the target organism to a biochemical group that has specific nutritional requirements [34]. One example of the isolation of a novel genus was demonstrated with a hyperthermophile archaeum by using a laser 'optical tweezer' that 'traps' and 'moves' a single cell in a focussed beam of light. Huber *et al.* [32] were able to physically isolate a single morphotype from a total of 75 enrichment cultures that they had screened by whole-cell *in situ* hybridisation with a fluorescentally-labelled gene probe to a 16S ribosomal RNA sequence. The trapped cell was moved via a capillary tube into a sterile growth medium where sufficient numbers of the pure culture were obtained for the first time. Ecological information is of immense benefit in the detection and culture of new

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organisms. It can be exploited to isolate known groups that have already shown biotechnological potential from new sources such as vascular plants or insects. On the other hand, it also provides a basis for the isolation of new classes of microorganisms from niches that were neglected such as hyperthermophilic, barophilic [40], psychrophilic [52] and arid [58] environments.

Methodological approaches described in this review are important in providing new avenues for the targeted discovery of novel groups, but through their application also provide a wealth of new ecological data. It is necessary to remember that cultivation methods have not been restricted to pure isolates alone but encompass the role of mixed cultures, or consortia, as exemplified in the selection of agents for bioremediation.

This review will focus on techniques and strategies for the isolation of microorganisms for biotechnological application that have been developed in recent years. It is intended to collate and discuss the recent developments in culture-based microbial ecology in a comprehensive manner and will add to the information presented in a number of excellent books and review articles that deal with isolation of microorganisms [29, 47, 64, 86, 88].

General Isolation Methods

It is rather obvious that a number of microorganisms are as yet 'unculturable' because their growth requirements have yet to be deciphered and optimised. These requirements are based on a variety of factors such as nutrient composition and concentration, temperature, pH, ionic strength, partial pressure of oxygen, or the presence of light. Further refinements of empirically-derived selective enrichments and isolation methods are still being developed to achieve an incremental increase in isolating new genera and species. This has been achieved more systematically where, for example, stepwise discriminant analysis of the growth response and antibiotic production in a number of physiological and nutritional tests has been applied to identify the most selective substrates to enrich antibiotic-producing soil isolates [28].

Researchers have realised that isolation protocols that attempt to use culture conditions which closely approximate natural environments are likely to be more successful whether they are from marine or terrestrial sources. The introduction of simple strategies such as diffusion gradients and dilution culture [11, 74] that mirror the low nutrient status found in most marine and soil environments has aided in the isolation of uncommon bacteria as well as facultative oligotrophs. On the other hand, abundantly available natural products, such as xylan, pectin, lignin and coal as carbon sources [31, 95], or xylose and lignin as attractants in a simple chemotactic isolation (H. Runmao *et al.* 1997. Abstr. *Proc. 10th Int. Symp. Biol. Actinomycetes*, Beijing, China, p. 7S7) have been used to obtain uncommon genera that are normally exposed to such compounds in their microhabitats.

Direct Recovery of Microorganisms from Soil Environments

Microorganisms present in environmental samples, including both single cells and mycelia, need to be released for isolation purposes as they tend to become tightly bound to the soil matrix. Therefore, to optimise individual cell recovery the soil has to be dispersed to separate cells from the organic matter and clay. Physical disruption with ultrasound or homogenisation are common means of dispersion but are likely to damage cells due to abrasion. Chemical dispersion methods that are less destructive, such as the use of ion-exchange resins in the presence of polyethylene glycol, have been shown to increase the extraction efficiency significantly [82]. Smith and Stribley [75] have added an aqueous two-phase partitioning technique that increases the number of microorganisms released from an ion-exchange dispersed soil by 15 times. The dispersed soil is added to a biphasic solution composed of dextran and polyethylene glycol and inverted repeatedly before it is allowed to settle to a stable interphase. Removal of the top phase following a repetition of the partitioning procedure resulted in the sequential extraction of soil to yield a more representative sample of the indigenous microbial population. Countercurrent extraction is another underutilised procedure currently being developed to increase the speed and scale of the aqueous two-phase partitioning technique [33].

Electrical Conductivity

Differences in electrical conductivity of microbial cells is well established, though this property has not been exploited to a great extent to separate microorganisms in mixtures. When a non-uniform current is applied to cells suspended in an electric medium a dipole moment is induced in each cell resulting in a transductional force. If the effective conductivity of the particle exceeds that of the suspending medium, the translational force will direct the suspended cell towards regions of high electric field strength, and vice versa [66]. Therefore, separation can be achieved by selecting a suspending medium conductivity and electric field frequency so that suspended particles in a mixture experience dielectrophoretic forces of opposite polarity [53]. This technique has been used to separate mixtures of microorganisms in pure culture, but would need to be modified for application to environmental samples, where a number of non-biological, charged particles are also present.

Selective Isolation Using Phage

The continued importance of natural products in industrial screening programmes for new therapeutic compounds

requires new microorganisms to be isolated in high numbers. The requirement is most often for the less-studied genera but there is a continuing need to include new members of highly-screened genera, such as the *Streptomyces*, which are recognised as prolific producers of chemical diversity. Because the commonly isolated genera are ubiquitous and tend to swamp isolation plates due to their high numbers and more rapid growth, they need to be eliminated selectively when attempting to isolate other taxa. For example, common streptomycetes appear even when conditions are made highly selective for non-streptomycetes, thus preventing the establishment of other targeted actinomycetes [89]. To overcome this hurdle, Kurtböke *et al.* [44] introduced polyvalent phage to soil suspensions to selectively reduce the number of *Streptomyces* spp. and *Streptosporangium* spp. that came up on soil dilution plates. The 2~17 times reduction in numbers of these two genera that was achieved clearly showed the effectiveness of this principle. However, it did not result in a significant increase in numbers of other actinomycete genera. These findings highlight the importance that a strategy to selectively eliminate common colonisers is not sufficient by itself unless it is used in combination with pretreatments and selective media developed specifically for a target genus [50]. Phage-resistant streptomycetes that appear after treatment with *Streptomyces*-Specific polyvalent phage are likely to be new members of this genus, provided the host spectrum of the phage is broad enough to lyse most common colonisers. Broadening the host spectrum yet reducing the number of polyvalent phage applied for the pretreatment of soil was accomplished by Long and Amphlett [49] with a superlytic actinophage system (SLA) containing four actinophage only. With the SLA system they achieved a 50% reduction in streptomycete numbers and at least a doubling in the number of other genera isolated, resulting in a concomitant 3~10 fold increase in the number of isolates from those genera.

The lytic phage principle was applied to the selective isolation and recovery of thermophilic actinomycetes from composted eucalyptus bark, where a poor recovery and diversity is vitiated by the presence of thermophilic bacteria [45]. Isolation of genus-specific phage is achieved with relative ease provided a suitable source material and the host culture is available [46, 50]. The bacteriophage used for this pretreatment were isolated directly from the source material. This was done by incubating the source material with an inoculum of the host *Pseudomonas* and *Bacillus* sp., followed by spotting this mixture onto a lawn of the host bacteria. The plaques that arise indicate the successful isolation of a phage that can be further propagated on the pure host bacterium and stored for further use. This is known as the Host Enrichment method.

Phage susceptibility properties, therefore, are proving valuable for selective isolation. As phage specificity does not cross wall chemotype [43] or even genus boundaries [46], the phage can also be used for rapid identification purposes (phage-typing). It is speculated that the ease of detecting the presence of a phage in an environmental sample can be exploited to indicate the presence of the host genus in the sample.

Immunocapture Techniques

Immunocapture (IMC) techniques were developed for the food industry where microbiologists require methods to detect specific microorganisms, usually pathogenic or faecal, against a mixed background microflora. A major constraint is that the time factor for isolation and detection has to be shortened considerably. Hence the lengthy selective enrichment stage that would normally be used has to be replaced by a non-growth related procedure [15]. These target-specific bacterial magnetic separation methods are expensive for routine use, but are gaining acceptance due to increasing outbreaks of food poisoning. They have been reviewed by Safarik *et al.* [70] for the food industry and wastewater treatment and by Olsvik *et al.* [63] for clinical microbiology.

This technology employs specific monoclonal and polyclonal antibodies as affinity ligands to recognise three-dimensional structures of surface components of target cells. The antibodies are immobilised onto magnetic polystyrene beads, such as Dynabeads®; to effect an immunomagnetic separation on capture when a magnetic field is applied. In the direct IMC technique, the primary antibody bound to the magnetic particles is added directly to the cell suspension. After an appropriate incubation time to allow for binding, the captured target cells are removed from the suspension with a magnetic particle separator that attracts the magnetic beads to which they are bound. In the alternative indirect method, the free primary antibody is first added to the cell suspension. After binding to the target cells the magnetic beads coated with secondary antibody (against the primary antibody) are introduced. For each system a number of parameters needs to be optimised to increase specificity and reduce non-specific binding. These parameters include the magnetic-bead to target-cell ratio, the incubation time, the use and choice of blocking agent, and the number of washing steps.

For ecological studies, immunological methods were first used to detect and capture genetically modified organisms from water samples [56]. The application of IMC technology to isolate target cells from soil samples, however, is a lot more challenging due to non-specific binding of soil particles and the separation and release of cells from soil matrices. The first reported study of the successful recovery of cells from a soil

sample was of *Bacillus polymyxa* from the soil and rhizosphere of wheat [54]. In this serological method, microtitre plates were coated with an antibody to *B. polymyxa*. Soil suspensions were added to the wells, and after a number of incubation and washing steps the captured bacilli were eluted with 0.1 M KCl. The percentage selective recovery of *B. polymyxa* achieved was as high as 20% as opposed to 0.1% when a classical enrichment was attempted.

Streptomycete spores have since been captured directly using a monoclonal antibody [90], where recovery was improved by the use of blocking agents such as bovine serum albumin, hydrolysed gelatin or skimmed milk, and a nonionic detergent (Nonidet P40) to prevent non-specific binding to the beads. Recovery of the target microorganism from spiked sterile soil was approximately 30%, whereas it was a low 4% from a non-sterile soil indicating that the direct method still needs further optimisation to make it an effective tool.

The same laboratory employed polyclonal antibodies with better success to selectively recover *Streptosporangium fragile* from soil by indirect immunocapture [59]. This species was selected as a model organism that is difficult to culture on isolation media due to its slow growth and competition from the faster growing actinomycetes. The polyclonal immunoglobulin G fraction from rabbits challenged with mycelium and spores of *S. fragile* was added to the chemically-mediated dispersed soil extract, after adding partially hydrolysed gelatin to counteract non-specific binding. Indirect capture was carried out with sheep anti-rabbit antibodies attached to the Dynabeads®. Recovery rates from spiked sterile soils increased to 40%, and to between 6.1 and 8.3% for non-sterile soil, but with significantly lower background spore capture [59] as compared to the direct immunocapture method. The advantage of a polyvalent antibody is that a broader specificity is expected and that novel genera or species may be recovered with similar cell wall properties to the target microorganism used to raise the antibody.

Flow Cytometry

Flow cytometry has been applied successfully to the analysis of community structure of bacteria in natural environments, primarily aquatic systems [14, 67], and for the detection of specific microorganisms in environmental samples [84]. For isolation purposes, flow cytometry coupled with cell sorting has been used to separate viable cells stained with non-lethal dyes such as Hoechst 33423 and Rhodamine 123 on the basis of their optical properties. Sorted cells are separated and placed into individual agar plates or microtitre wells. While applicable for gross separation and isolation it has limited use for the selection of viable cells stained with genus- or species-specific (16S rRNA-fluorochrome conjugate) probes as the staining protocol would reduce the viability of the cells. In any case the intensity of the fluorescent signal with these probes is too low to be effective. Nevertheless, this technique is well suited to microorganisms with unique morphological or chemical properties, especially those containing their own fluorescent molecules, such as methanogens which contain F_{420} and cyanobacteria. The unique 'signature' supplied by these molecules provides the basis for identification and cell sorting [18, 19]. Soil samples contain particulate matter which interferes with fluorescence, hence the application of flow cytometry to soil microbial communities still presents a problem. Fluorescent-labelled antibodies have been used together with cell sorting to separate viable *E. coli* from lake water samples [67]. This technique has the species-specificity required for a target-directed approach to siolation and its application does not affect cell viability.

Thermophiles

Hyperthermophiles growing at temperatures between 80~110°C are isolated almost exclusively from environments with *in situ* temperatures between 80~115°C, such as water-containing volcanic areas, terrestrial solfatane fields and hot springs, submarine hydrothermal systems and abyssal hot vent "black smokers", smouldering coal-refuse piles and geothermal power plants and oil wells [79]. Isolation has been performed using aerobic and anaerobic enrichment cultures on various substrates using conditions corresponding to the original environement. Fermentaion of these organisms to high cell densities is achievable [42] illustrating the possibility of their use in bioprocesses.

The hyperthermophiles identified to date belong to 25 genera and 11 orders within the kingdom Bacteria and Archae and are adapted to the extremes of pH, redox potential and salinity. Extreme acidophilic thermophiles that are strict aerobes grow autotrophically by oxidising S^0, S^{2-} and H_2. The microaerobic members, such as *Sulfolobus* isolates can reduce ferric ion and molybdate at low ionic strength, whereas the *Aciadanaus* can grow both aerobically and anaerobically and can withstand up to 4% salt. This group also contains a strict anaerobe, *Stygiolobus* spp., an extreme acidophile that grows chemolithotrophically. Both microscopy and 16S rRNA analysis [5] reveal many other members of the thermophilic community than have been characterised. With a range of physiological and nutritional requirements the challenge remains to improve isolation and cultivation techniques. Such techniques could include the use of an optical laser tweezer, as mentioned earlier, to trap and select a novel hyperthermophilic archaeum previously detected only with a phylogenetic stain [32].

Deep Sea Environments

Deep seas are characterised by high pressures and low temperatures. Barophiles from greater than 2500 metres depth can only grow under high pressure [40] and would be difficult to exploit directly for biotechnology. Their potential utility lies in providing insights into membrane lipids and proteins that function under extreme pressure and temperature [94]. However, another group from the deep oceans (<2000 metres), which grow at atmospheric pressure, are the solvent-tolerant bacteria which have potential for bioremediation applications. These microorganisms combine the properties of barophilicity and organic solvent tolerance that have potential in high-pressure bioreactors and two-phase fermentation systems.

These microorganisms grow under a range of hydrostatic pressures from 0.1~6.0 MPa and optimum temperature of 4~15°C. Organic solvent tolerant (OST) microorganisms have been found to tolerate benzene at 10%; with the first report of such an organism being isolated from mud samples from Kyushu island in Japan [35]. Both OST and barophiles are found in deep sea mud which has a population 100 times higher than terrestrial soil though there is no correlation between barophilicity and OST and these groups of organisms have not been isolated from the same sample. In order to isolate OST bacteria benzene was added at a concentration of 50% to artificial sea water and sediment containing samples of deep sea water. Cultures were incubated for 7 days on a shaker. Then the benzene layers were separated carefully and a portion of each layer spread onto a marine agar [57]. Bacterial strains isolated that degrade crude oil, sulfur compounds, polycyclic aromatic hydrocarbons, and cholesterol have been identified as *Flavobacterium* sp., *Bacillus* sp., and *Arthrobacter* sp., respectively [40]. Microorganisms that can grow in high concentrations of organic solvents are very useful for processing products that cannot dissolve in water or for use in two-phase fermenter systems, such as in the transformation of sterols from cholesterol. Other sources in the marine environment that are yielding new microorganisms are algae [13, 37] and the invertebrates [20, 71]. Psychrophiles from these environments have been cultivated and are being screened for the production of novel enzymes active at low temperatures, and as sources of stable proteins and lipids [21, 52].

Endophytes and Symbionts

Endophytes are microorganisms that have formed associations with healthy plants and are found within living plant tissues. This close association can be exploited for biotechnological application as it may protect the endophyte giving it a growth advantage within the plant. Endophytes that are active against plant pathogens or which produce plant growth promotion activity are likely candidates for further development.

Protocols for the isolation, identification and culture of grass-endophytes, the most studied group of fungal endophytes, have been reviewed recently [3, 68]. These are host-specific, and a number of unique strains have been obtained from the variety of grasses and plants collected. The procedures for isolating endophytes are relatively simple and involve surface sterilisation steps to remove any external contaminant. This is followed by exposing the cut plant tissue to a nutrient agar medium to allow the endophyte to outgrow onto the agar surface. Protocols that have been used for fungi were applied to the isolation of actinomycete and bacterial endophytes with great success [72, 87]. Actinomycetes were found in at least half the plant roots studied with up to 29 isolates recovered from one plant species. As in all isolation procedures, there is a bias introduced by the selective action of the sterilisation process and the growth medium used.

In a similar manner, insects and termites contain microorganisms within their digestive systems [92] or in mutualistic or pathogenic associations and are likely to provide a rich source of new isolates [36, 38].

Isolation of Biological Control Agents

The problems inherent in conventional isolation procedures are exemplified by the relatively low number of successful biocontrol agents which are in commercial use.

One of the greatest problems in the selection of promising agents is that while strains may be effective at controlling specific pests or pathogens in the laboratory using *in vitro* tests, inconsistent levels of control are often observed under field conditions. In general, screens should select for organisms adapted to the environment in which they will operate [12]. The interaction between a biocontrol strain and target pest/pathogen is often complex and a number of factors may contribute to successful control. The successful biocontrol strain *Agrobacterium rhizogenes* K84, and its genetically-engineered derivative marketed as NoGall, produce a number of inhibitory agents towards pathogenic *Agrobacterium* strains. Strain K84 was the first strain obtained from an extensive selection program investigating non-pathogenic strains from soil in an almond orchard affected by crown gall disease [62]. The discovery of strain K84 was very fortuitous as it has proved the most effective control agent for crown gall of stone fruit. The choice of selection method used can be important as the inhibitory effects of strains may vary depending on the assay used [65, 73, 76]. Studies on the multiple factors involved in biological control by the strain K84 have revealed that *in vitro* assay tests may not correlate well with the activity of control strains under more realistic test conditions (Ahmadi and McClure, unpublished). For example, the role of competition in the interaction

between pathogen and biocontrol strain needs to be assessed. Multiple factors have also been shown to play a role in the inhibition of *Phytophthora parasitica* by *Pseudomonas fluorescens* and *P. putida in vitro* [93]. Selective isolation strategies that incorporate the range of factors involved need to be developed to obtain new improved biocontrol agents.

Selection for Bioremediation

The use of selection programs for isolating organisms which will be useful in practical bioremediation processes has had limited application to date. To some extent this may be due to inconsistent results obtained when using microbial inoculants in laboratory, pilot or large-scale bioremediation projects, a process termed bioaugmentation. A large number of studies have shown enhancement of pollutant degradation or removal following inoculation of laboratory test systems with specific microorganisms [17, 39, 51, 55]. However, other studies have shown that bioaugmentation has been unsuccessful in laboratory and field studies [61, 83].

Conventional methods for isolating microorganisms with specific catabolic capabilities often involve the use of enrichment techniques using the contaminated matrix as a source of acclimatised organisms [6]. Novel enrichment techniques have been devised for pollutants which have low water solubility and high toxicity [2]. Those which mimic the process to be utilised for application are more likely to result in the isolation of microorganisms which are well adapted to survive and enhance degradation rates in full scale processes. In most cases, isolation of a consortium is necessary as this provides the suite of catabolic enzymes for complete mineralization that a single species is unlikely to possess.

Alternatively, microorganisms with specific catabolic activities can be engineered using molecular techniques [55, 60, 81], although there are limitations and difficulties associated with the release of such organisms into the environment.

The essential features of a microbial selection strategy have been discussed by Bull *et al.* [10]. Examples of the use of dedicated selection programs to identify potentially useful bioremediation inoculants include the selection of white rot fungi capable of degrading polycylic aromatic hydrocarbons (PAH). The lack of specificity associated with PAH and lignin degradation has enabled the use of polymeric dyes such as Poly R for isolating white rot fungi which may be useful for PAH degradation [22, 23, 24, 48].

Other rapid selection tests have also been used to identify and characterise microorganisms which could degrade polychlorinated biphenyls [7], crude oil [9] and phenanthrene [8], and could easily be adapted for use in selection programmes. The major limitation to success is likely to involve extrapolating activities in the laboratory to the target environment where conditions are often sub-optimal for microbial growth and activity.

Isolation, Bioprospecting and the Convention on Biological Diversity

A number of countries have signed the UN Convention on Biological Diversity (UNCBD) with the realisation that conservation of global genetic resources and preservation of indigenous knowledge associated with their use is extremely important, especially if their potential is to be developed in a proper manner. The articles of the UNCBD dictate codes of conduct for the study and sustainable use of biological diversity. The first 19 articles are relevant to biodiversity prospecting and deal with sovereign rights of source countries, responsibilities of governments and contracting parties, the search and development of natural products that have commercial potential, information exchange and technology transfer, deposition of specimens, and the equitable sharing of benefits [4, 41]. It is therefore expected that biotechnologists engaged in bioprospecting, including the corporate managements of pharmaceutical companies, will recognise the need to obtain authorisation from the source country and deal with issues of compensation and intellectual property rights via formalised agreements [26]. Kelley [41] also suggested that *ex situ* culture collections as well as editors of publications should ask for a full isolation history for all microorganisms deposited or reported on. Indeed, scientists appear ready to meet these requirements and one finds that there is a trend to report on the source of the producing organisms. For example, in the 1996 issues of the Journal of Antibiotics at least half the new entries on novel secondary metabolites indicated the country of origin of the environmental samples from which the producing strain was isolated. Source countries mentioned are Korea, Egypt, India, Indonesia, Italy, Kenya, Japan, Nepal, Nigeria, South Africa, Portugal, Thailand, U.S.A., and Peru.

Future Challenges

The major advances in molecular techniques for assessing microbial diversity have underscored the slow progress being made in our ability to isolate and characterise environmental microorganisms. In this review we have summarised the achievements that are providing new isolates that can be exploited for biotechnological application. The real challenge for the immediate future will be the development of improved isolation and culture techniques to realise the extensive metabolic potential offered by this vast biological diversity.

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