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EXPLORATION OF METAGENOMES FOR NEW ENZYMES USEFUL IN FOOD BIOTECHNOLOGY – A REVIEW

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Metagenomics is the genomic analysis of the collective genomes of an assemblage of organisms, or the metagenome. Metagenomic analysis has been applied to diverse problems in microbiology and has yielded insight into the physiology of uncultured organisms to access the potentially useful enzymes and secondary metabolites they produce.

DNA isolation methods have to be strictly adapted to the type of isolated biological material; of great importance is also the size of the obtained DNA. Small DNA fragments are usually sufficient for an analysis of individual genes or their small groups, whereas large inserts are required for analysing metabolic pathways, genome structures or sequencing large DNA fragments.

There are two types of methods of extracting genomic DNA. One of them consists of the direct extraction of nucleic acids from an environmental sample after the cell lysis (*in situ*), followed by purification of the obtained DNA. The other method consists of the separation of bacterial cells from an environmental sample, followed by lysis of the cell suspension and DNA extraction.

In the presented review methods of the environmental DNA isolation, cloning and new enzymes discovery are presented.

INTRODUCTION

Metagenomics is the culture-independent genomic analysis of microbial communities. The term combines two words: *meta*-analysis, *i.e.* a method of statistical analysis of the results of two different analyses, and *genomics*, *i.e.* analysis of genetic material [Rondon *et al.*, 2000]. Microorganisms are the product of about 3.8 billion years of evolution and a valuable source of biodiversity. However, most microorganisms present in the environment cannot be cultivated using the available methods (Table 1). How rich is the soil as a source of microbial species? The soil sustains an immense diversity of microbes, and it is estimated that 1 g of forest soil contains 4×10^7, whereas one gram of cultivated soil and grasslands contains about 2×10^9 prokaryotic cells, and thousands of different species, but more than 98%, in general, are unknown [Daniel, 2005; Rosello-Mora & Amann, 2001; Torsvik & Ovreas, 2002].

TABLE 1. Cultivability of microorganisms with the use of traditional methods in various habitats [Amann *et al.*, 1995].

Habitat	Cultivability* (%)
Seawater	0.001 – 0.1
Freshwater	0.25
Mesotrophic lake	0.1 – 1
Activated sludge	1 – 15
Sediments	0.25
Soil	0.3

*Cultivability was determined as the ratio of the number of colony forming units (CFU) to the total microorganism count

In the past, selection and screening was performed in order to isolate a pure culture. It is now known that metagenomic analysis conducted in abundant and biodiverse environments, such as soil, sea and ocean water, have revealed the presence of many new microorganisms. Methods of genomic analysis, independent of methods of microorganism cultivation, are being applied in the examination of: genetic and phylogenetic diversity, population structure, ecological aspects, discovering new metabolic pathways and metabolites, polymers and new enzymes [Handelsman *et al.*, 2002; Riesenfeld *et al.*, 2004].

The new era in microbiology, associated with isolation and examination of non-cultivated microorganisms began with the sequencing of their rRNA (5S rRNA). In 2004, the GenBank contained twice as many sequences of 16S rRNA of non-cultured microorganisms than cultivated prokaryotes. The abundance of microorganisms, hidden and unavailable to the traditional isolation methods (selection and screening), opens up new opportunities for metagenomics. Apart from the methods of directed molecular evolution applied in improving enzyme properties and methods of mutation and rational protein engineering, isolation of metagenomes should enable considerable progress in obtaining enzymes for specific ap-

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plications in industrial biotechnology (white biotechnology) [Drepper *et al.*, 2006; Lorenz *et al.*, 2003].

Metagenomics describes the functional and sequential analysis of the total microbiological genome contained in environmental samples. Frequently, various terms are used, *e.g.* zoolibraries, soil DNA libraries, eDNA, but all of them refer to metagenomes [Robe *et al.*, 2003; Schloss & Handelsman, 2003]. Analysis of metagenome opens up great opportunities for exploring secondary metabolites whose genes frequently form clusters. Microorganisms found in the soil are a rich source of bioactive components applied in medicine, agriculture, *e.g.* antibiotics, antitumors, immunosupressors [Sharma *et al.*, 2005]. Based on the function and sequence, metagenomics describes the total genome of the microorganisms present in environmental samples (Figure 1).

Obtaining metagenomic libraries, which provide complete genetic information from an environment (biotope), enables a subsequent structural and functional analysis of the genomes of a microorganism non-cultured with the use of traditional methods of cultivation. The isolated genomic DNA is further used for cloning with various types of vectors, *e.g.* cosmid, fosmid (accepted insert up to about 45 kb) and bacterial artificial chromosome (BAC; inserts from 100 to 350 kb) [Schloss & Handelsman, 2003]. In addition, the use of various analytic methods, such as gene 16S rRNA analysis, DNA re-association, DGGE (denaturant gradient gel electrophoresis), ARDRA (amplified rDNA restriction analysis), T-RFLP (terminal restriction fragment length polymorphism) and RISA (ribosomal intergenic spacer analysis – analysis of fragments of rRNA-coding DNA; from 16S to 23Ss) provides valuable information about genetic diversity and microorganism evolution [Handelsman, 2004; Steele & Streit, 2005].

METHODS OF GENOMIC DNA ISOLATION

The methods of genome analysis require DNA free of various types of impurities, commonly found in environmental samples. The DNA must represent the entire microbial diversity and contain the whole genomic DNA [Robe *et al.*, 2003; Schloss & Handelsman, 2003]. Methods of DNA isolation must be strictly adjusted to the type of isolated biological material (Table 2). Additionally, while isolating the DNA, the size of the DNA obtained is very important. Small DNA fragments are sufficient for the analysis of single or small groups of genes, while large inserts are required for the analysis of multigenic pathways, genome organization or large DNA fragment sequence analysis [Gillespie *et al.*, 2005; Lorenz & Schleper, 2002]. Currently, there are two approaches to genomic DNA isolation. The first approach is based on direct extraction of nucleic acids from the environmental sample, after a previous lysis (*in situ*) and subsequent purification of the DNA. The other method involves separation of bacterial cells from the environmental sample, followed by lysis of cell suspension followed by extraction of the DNA (Figure 2) [Lorenz & Schleper, 2002].

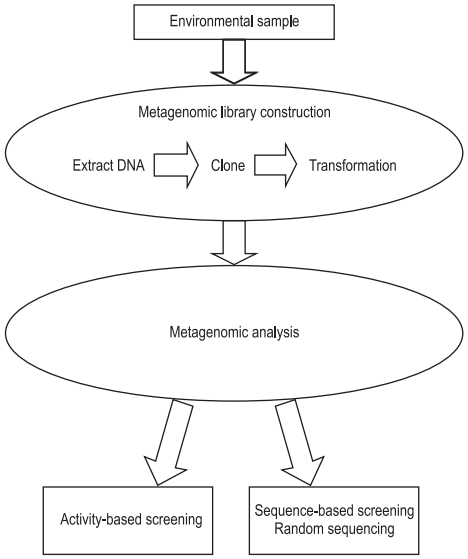

FIGURE 1. The procedure of obtaining DNA libraries from environmental samples.

DIRECT METHODS OF ISOLATING METAGENOMIC DNA

The aim of applying these methods is to achieve a complete lysis of all cells of microorganisms found in the environmental sample, *in situ*. Generally, their application provides a higher quality of DNA compared to indirect methods [Robe *et al*., 2003] (Table 2). The application of direct methods includes two basis steps: cell lysis and DNA extraction and purification. The first step (lysis) is critical as regards the efficiency of the process; and due to the variety of microorganisms present in samples, it is difficult to perform this process properly [Roose-Amsaleg *et al.*, 2001]. The efficiency of the process, besides the susceptibility of the microbe cell wall to lysis, depends on the location of bacterial cells in the microstructures of the material from which DNA is isolated, and the interaction with environmental particles, *e.g.* soil [Robe *et al.*, 2003].

METHODS OF MICROORGANISM CELL LYSIS

Currently, there are three basic methods of microorganism cell lysis applied, *i.e*. physical, chemical and enzymatic. These methods can be used as independent procedures, but most often they are applied in various combinations, thus increasing the efficiency of the process.

The application of physical methods of cell lysis generally facilitates the access to each single cells, which results in a higher efficiency of DNA isolation. However, the use of radical physical treatment results in obtaining DNA of a smaller size. These methods include: freezing-thawing and freezing--boiling cycles, which take advantage of the disintegrating effect of ice crystals and the temperature on the cell wall of microorganisms [More *et al.*, 1994] (Table 2). The thermal shock method is a more gentle method of lysis than the mechanical methods, though equally efficient. The number of cycles, time of incubation in liquid nitrogen or ice and heating at 50°C, 60°C, 100°C can all vary [Picard *et al.*, 1992].

The method modified by Kauffmann *et al.* [2004] consists of conducting three cycles of freezing a soil sample mixed with an extraction buffer in liquid nitrogen and thawing it in

TABLE 2. Characteristics of metagenomic DNA obtained by different methods of isolation.

Material for isolation	Applied method	Amount of DNA (μg/g)	Size of DNA (kb)	Purity of DNA ($A_{260/280}$)	Reference
		Direct methods			
Soil	1. Homogenisation with beads 2. Microwaves 3. Grinding in liquid nitrogen	18.5 0.57 0.09	23	ND*	[Lakay *et al*., 2007]
Soil	1. Homogenisation with beads at 4 m/s 2. Homogenisation with beads at 5 m/s 3. Homogenisation with beads at 6 m/s	30.7** 45.7** 106.3**	12.2 10.2 6.0	ND*	[Burgmann *et al*., 2001
Wet sediment	Isolating kit FastDNA spin Kit (Bio101 Inc.)	17.7**	1-20	ND*	[Wilkinson, *et al*., 2002]
		Indirect methods			
Soil	Separation of cells in a buffer/ mechanical grinding (beads)	0.5-2.25	1-10	1.32	[Kozdrój & Dirk van Elsas, 2001]
Soil	Separation of cells in EDTA, SDS, PVP/heating in a microwave oven	1.7**	20-23	>2.0	[Orsini & Romano-Spica, 2001]
Soil	Separation of cells by the method with Nycodenz / lysis of cells immobilised in agarose gel	ND*	50-500	ND*	[Berry *et al*., 2003]
Soil	Separation of cells by shaking in Chelax - 100 Separation of cells by homogenisation in a buffer with PVPP	0.13 0.54	23	2.0 1.55	[Tien *et al*., 1999]
Soil	Separation of cells by the method with Nycodenz / isolation with the use of a kit manufactured by A&A Biotechnology	13.56	23	1.54	(the authors' study)
Soil	Separation of cells by the method with Nycodenz / lysis of cells immobilised in agarose gel	8.93	200 - 250	ND*	(the authors' study)

*- ND –no data, ** - μg/g dry matter, PVPP - polivinylpolypyrrolidone

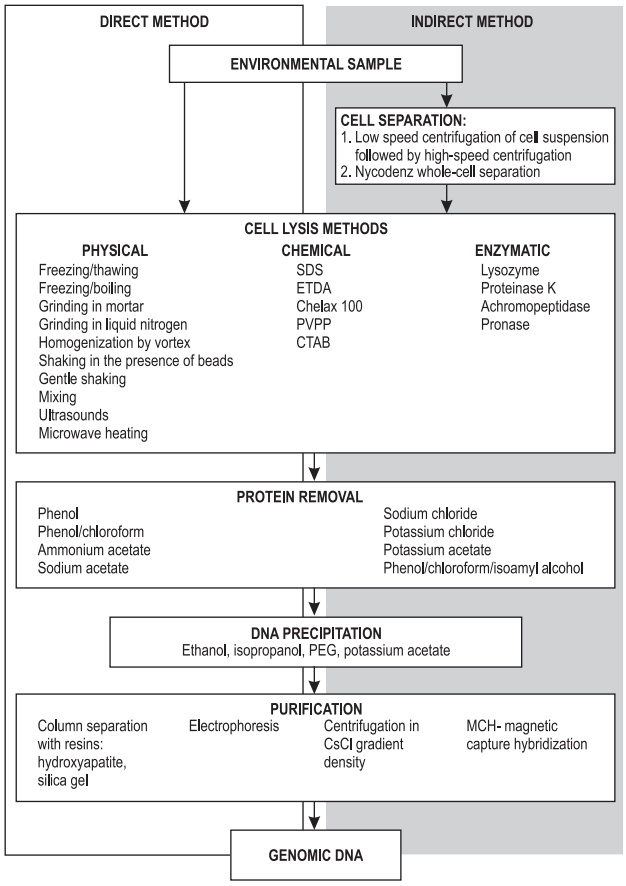

FIGURE 2. Scheme of metagenomic DNA isolation by direct or indirect method.

PEG – polyethylene glycol, CTAB – hexadecyltrimethylammonium bromide, PVPP -polyvinylpolypyrrolidone, SDS – sodium dodecyl sulfate, ETDA – ethylenediaminetetraacetic acid, Chelex® 100 – chelating resin in a sodium form.

water bath at 65°C. This method, due to the relatively gentle conditions of DNA isolation, is not effective for the release of nucleic acids from environmental samples which are biotically, microbiologically poor.

An easier method of cell lysis is bead-beating, which can be used for obtaining inserts of very different sizes, *e.g.* 0.1-0.5 kb [Picard *et al.*, 1992], 2-5 kb [Gillespie *et al*., 2005], and even 20 kb [Yeates *et al.*, 1997]. An originator of this method was Ogram *et al.* [1987], and the commercial kits that are currently available constitute a modified version (time and speed of homogenization, volume and composition of lysing buffer) of the original method [Gabor *et al.*, 2003]. Power Soil™ DNA Isolation Kit (MO BIO), ZR Soil Microbe DNA Kit™ (Zymo Research) are examples of commercially available products that use beads for disintegration. Additionally, there are also certain devices available, such as Beadbater™, allowing very efficient disintegration with the use of beads of various sizes, made of glass, steel, zircon or silica. This type of DNA isolation kit, manufactured by Qbiogene, Inc. (FastDNA® spin kit) has been used for creating a metagenomic library from a sample of geothermal sediment, with the use of pCR-XL-TOPO vector [Wilkinson *et al.*, 2002].

Other physical methods used in isolating genomic DNA include: grinding in mortar, grinding in liquid nitrogen, and applying ultrasounds [Robe *et al*., 2003]. Orsini & Romano-Spica [2001] applied, successfully, microwaves for separating nucleic acids from the soil sample. The DNA obtained with the use of this method featured a higher purity, a size of 20-23 kb. A higher DNA yield in comparison to DNA samples obtained after an enzymatic lysis of cells was obtained. The advantages of this method are also its low cost, rapid completion, and the possibility of using it for mRNA isolation.

Chemical methods of cell lysis used independently or in combination with physical or enzymatic methods are very commonly used for DNA isolation. The most popular detergent used for DNA isolation is sodium dodecyl sulfate (SDS), which washes out lipids found in the cell membrane of microorganisms [Roose-Amsaleg *et al*., 2001]. This detergent can be used in various concentrations (0.1% – 20%), at elevated temperature (65°C), and most often with chelating chemical compounds such as ETDA (ethylenediaminetetraacetic acid) or Chelex® 100 (chelating resin in a sodium form) [Gabor *et al*., 2003; Maarit Niemi *et al.*, 2001; Robe *et al*., 2003] (Table 2). The increase of EDTA concentration had a favourable effect on the intensity of lysis; however, it reduced the purity of DNA. Consequently, EDTA concentration must be selected very carefully in order to reach a compromise between the amount and the purity of DNA [Krsek & Wellington, 1999].

The application of a chemical method of DNA isolation in combination with gentle shaking can significantly reduce DNA damage, consequently leading to obtaining DNA fragments of up to 80 kb, but the size of the obtained DNA depends on the type of microorganisms found in the examined sample [Rondon *et al.*, 2000].

Maarit Niemi *et al.* [2001] have shown that the DNA isolation method consisting of: cell lysis in the SDS solution with the addition of guanidine isothiocyanate, and freezing-thawing or homogenization with beads, did not have any significant effect on the content of DNA but did reduce DNA size, in comparison with a gentle lysis just with SDS. A larger amount of DNA was obtained by using a thermal and mechanical lysis, but the purity of DNA was low [Zhou *et al.*, 1996].

In order to improve the purity of DNA during chemical and/or physical lysis, such compounds as: CTAB (hexadecyltrimethylammonium bromide) or PVPP (polyvinylpolypyrrolidone) are used, which can partially remove humus compounds found in environmental samples [Roose-Amsaleg *et al*., 2001]. CTAB is used more often for DNA isolation, as PVPP causes losses in yield of DNA [Zhou *et al.*, 1996]. However, PVPP application allows for better results while DNA is purified in spin columns [Krsek & Wellington, 1999].

Numerous methods of DNA isolation are based on enzymatic lysis. Most often used enzyme is lysozyme which hydrolyzes polysaccharides found in the cell wall of microorganisms [Maarit Niemi *et al*., 2001; Roose-Amsaleg *et al*., 2001]. Another enzyme equally often used in cell lysis is proteinase K [Gabor *et al*., 2003; Maarit Niemi *et al*., 2001; Roose-Amsaleg *et al.*, 2001]. Proteolytic enzymes that are less frequently used in enzymatic lysis of cells include achromopeptidase [Liu *et al.*, 1997] and pronase [Roose-Amsaleg *et al*., 2001].

INDIRECT METHODS OF DNA ISOLATION

Faegri *et al.* [1977], and later, among others Torsvik & Goksoyr [1978], have proposed a method of genomic DNA isolation from environmental samples after a preliminary separation of microorganism cells. An indirect method of metagenomic DNA isolation consists of the following steps: dispersion of the environmental sample, cell separation, cell lysis, DNA isolation and purification [Robe *et al*., 2003] (Figure 2).

DISPERSION

Procedures of dispersing environmental samples or cells can be divided into physical and chemical methods. Homogenization with the use of a warning blender [Hardeman & Sjoling, 2007; Lindahl & Bakken, 1995] or a rotating rubber pestle treatment [Berry *et al.*, 2003] are frequently used for releasing microorganism cells from the environmental sample. The time of mechanical impact on cells should not exceed 18 min, because after this time is exceeded, some of the microorganisms are subject to disintegration [Lindahl & Bakken, 1995]. This method brings very good results, when microorganisms are strongly adsorbed on soil particles.

More gentle methods, such as shaking or ultrasonic treatment, are also used for dispersing microorganism cells. The application of ultrasound is not recommended, due to difficulties in establishing the conditions of the process, caused by a lack of information concerning microorganisms present in the environmental sample [Hardeman & Sjoling, 2007]. Gentle shaking decreases the efficiency of cell extraction, but has a positive impact on the size of the DNA inserts.

Mechanical methods of dispersion are most often used together with chemical methods. Specific chemical compounds, such as: sodium cholate and deoxycholate (interacting on lipopolysaccharides), polyethylene glycol or SDS (dissolving hydrophobic chemical compounds) assist the process of dispersion [Bertrand *et al.*, 2005; Lindahl & Bakken, 1995; Robe *et al*., 2003] (Table 2).

SEPARATION OF MICROORGANISM CELLS

Separation of microorganism cells from soil impurities is based on two speeds of centrifugation [Robe *et al.*, 2003]. The first stage, *i.e.* centrifugation at low rotational speed of 500 – 1000×g for 5-15 min aimed at separating particles of soil, fungal cells and other compounds [Hardeman & Sjoling, 2007; Krsek & Wellington, 1999]. The obtained supernatant containing bacterial cells is centrifuged at high rotational speed, *e.g.* 10000×g, in order to separate microbe cells [Hardeman & Sjoling, 2007] (Table 2). This method allows the easy removal of a certain part of contamination, however, it causes large losses of biomass, which is not representative and the DNA separated from it later does not contain the whole genomic DNA.

Experiments by Krsek & Wellington [1999] have demonstrated that the yield of DNA isolation after using the *ex situ* method was only about 60% of that acquired by using *in situ* method. Luna *et al.* [2006] using the analysis of terminal restriction fragment length polymorphism of 16S rRNA gene (T-RFLP), established that indirect extraction of DNA from the soil makes it impossible to isolate the abundance and the variety of the examined soil. Although indirect methods of genomic DNA isolation will never enable the collection of the whole genetic diversity, the losses of DNA can be compensated for by repeated separation of cells from the same sample [Robe *et al*., 2003].

Another method of separating bacteria from environmental samples is centrifugation at a high *g*-factor value, with the application of compounds creating a density gradient [Carac-

ciolo *et al.*, 2005], *e.g.* Percoll, metrizamide and Nycodenz® [Robe *et al*., 2003] (Table 2). This technique is based on a high density (1.3 g/cm^3) of the Nycodenz®, whose value is higher than the density of microorganisms [Lasken *et al.*, 2005]. Centrifugation at the speed of 10,000×g of disperged cells added to Nycodenz® results in separating microorganisms from soil particles and biomass gathering in the upper layer of the test tube [Berry *et al*., 2003]. Inorganic compounds of soil, due to their higher density, gather at the bottom of the test tube [Lasken *et al*., 2005]. Caracciolo *et al.* [2005] determined the number of microorganisms in each phase after separation achieved with the application of Nycodenz® using fluorescent *in situ* hybridization (FISH). It was found that depending on the kind of the soil sample, it is possible to separate 76-78% microorganisms living in a given sample. It is probably caused by the fact that although the majority of bacteria demonstrate a density close to 1.12 g/cm^3 and should aggregate in the upper phase of the solution after centrifugation, some of the bacteria are strongly bound to soil particles and sediment along with it [Robe *et al*., 2003]. However, it must be emphasized that the application of Nycodenz® allows a relatively pure solution of bacteria to be obtained in comparison with methods using centrifugation at low rotational speed. Density gradient centrifugation cannot be used for complete removal of humus substances and soil particles.

EXTRACTION AND PURIFICATION OF GENOMIC DNA

After lysis, for both direct and indirect methods of genomic DNA isolation, the next stages include separation, purification and precipitation of DNA. DNA extraction can be carried out, among others, by using classical deproteinisation in organic solvents, such as phenol or mixtures:phenol: chloroform (50:50, v:v) [Ranjan *et al.*, 2005], phenol:chloroform:isoamyl alcohol (25:24:1, v:v:v) [Zhou *et al.*, 1996], which are most often used in chemical and physical methods of DNA isolation, as well as for removing proteins from the mix containing DNA. The application of chloroform brings very good results, while phenol is rather avoided due to its toxicity and the ability to separate DNA together with other compounds [Porteous & Armstrong, 1991].

The following compounds are also used for the precipitation of proteins: sodium chloride, potassium chloride, ammonium acetate, potassium acetate or sodium acetate [Krsek & Wellington, 1999; Porteous & Armstrong, 1991; Roose-Amsaleg *et al.*, 2001] (Figure 2). Protein precipitation takes place during centrifugation and nucleic acids remain in the supernatant. The use of non-toxic sodium chloride allows for the precipitation of soil particles with proteins and fragments of cells and provides relatively pure DNA [Roose-Amsaleg *et al.*, 2001].

The extraction of DNA of high purity and satisfactory size can be carried out after lysis of immobilized cells in agarose plugs. This is a very gentle method of obtaining large DNA fragments (50-100 and more kb) after utilizing standard pulse field gel electrophoresis (PFGE) [Berry *et al*., 2003; Gillespie *et al*., 2005]. The application of PFGE with the addition of PVP (polyvinylpyridine) allows for additional separation of humus compounds from DNA [Robe *et al.*, 2003].

Isolated nucleic acids are condensed and at the same time purified by precipitation with ethanol, isopropanol, sodium acetate or polyethylene glycol (PEG) [Porteous & Armstrong, 1991; Roose-Amsaleg *et al*., 2001]. PEG frequently replaces isopropanol, since it has been observed that alcohol has the ability to precipitate DNA with humus substances of soil [Porteous & Armstrong, 1991]. The addition of sodium acetate during precipitation improves the purity of the DNA.

The oldest methods of DNA purification include centrifugation in a density gradient with caesium chloride (cesium chloride, CsCl) [Roose-Amsaleg *et al*., 2001]. Caesium chloride and ethidium bromide are added to the sample before the centrifugation and various lysate components (proteins, polysaccharides, chromosomal or plasmid DNA and RNA) are separated according to their density [Robe *et al.*, 2003]. Unfortunately, this method is extremely time-consuming and does not completely remove impurities.

Microcolumn chromatography on resins, hydroxyapatite or silica gel is another method of DNA purification [Roose-Amsaleg *et al.*, 2001] (Figure 2). Miller [2001] evaluated a variety of gel filtration resins (Sephadex G200 and G150, Sepharose 6B, 4B, 2B, Bio-Gel P100, P200 and Toyoperal HW 55, HW 65, HW 75) for their ability to remove PCR reaction inhibitors co-extracted from environmental samples. The Sepharoses demonstrated a higher DNA purification ability, and the column with Sepharose 2B proved to be the best. Jacobsen *et al*. [1995] established that it is not possible to purify DNA isolated from forest soils or samples contaminated with carbohydrate on the Sephadex column, whereas the column with Sepharose has larger pores than Sephadex and therefore eliminates small RNA fragments, which affects the improvement of the separated DNA.

Most frequently, a combination of precipitating and purifying substances is used while isolating DNA from lysate. Krsek & Wellington [1999] compared various methods of isolation and purification of DNA from soil. Purification of DNA with potassium acetate had to precede precipitation with PEG, since the simultaneous application of these chemical compounds did not produce any positive effects. Purification with PEG provided cleaner DNA in comparison to precipitation with ethanol. Likewise, purification of DNA with a phenol/chloroform mix proved more efficient than centrifugation on a Sephadex G50 column and Chelex 100 column.

Maarit Niemi *et al*. [2001] have compared the quality of DNA purification with the use of columns filled with: Elutip, Sepharose 4B and Wizard DNA Clean-up System. A commercially available purifying kit has proved to be the most efficient, and resulted in a high yield of DNA, which had an effect on the good result of the PCR reaction. Additionally, the results of the PCR-DGGE analysis demonstrated that the type of microorganisms isolated from the soil is not only dependent on the method of isolation, but also of the method of DNA purification.

An alternative method of purifying genomic DNA is magnetic capture hybridization (MCH). DNA isolation by MCH consists of the hybridisation of non-purified nucleic acids with specified, single-strand, marked DNA, and the separation of impurities and other DNA fragments by using a magnetic field [Jacobsen, 1995].

CHOOSING THE RIGHT METHODS OF GENOMIC DNA ISOLATION

The main problem related to the isolation of metagenomic DNA is to ensure the representatives of a separated DNA sample which can be used to describe the richness of complex microbial communities. This approach requires high purity (free from inhibiting impurities) and a proper size of DNA [Luna *et al*., 2006].

Courtois *et al.* [2001] found that the direct method makes it possible to extract 28-42% DNA from *Actionomyces* and twice as much DNA from *Proteobacterium*. A disadvantage of direct DNA isolation is the relatively low purity and small size of DNA (1-50 kb), which results from applying mechanical lysis of cells. Isolated DNA can be used for creating a gene library with the use of a plasmid vector, which is favourable in the case of a functional analysis of clones [Lorenz *et al.*, 2002].

On the other hand, a disadvantage of the indirect method is the fact than only 25-50% of microorganisms can be obtained with the use of this method [Robe *et al*., 2003]. An analysis of soil microbiological diversity by PCR has shown that the use of the density gradient cell separation (Nycodenz®) method gave similar results as in the case of DNA isolated by the direct method. Consequently, the Nycodenz® cell separation procedure can be applied as an alternative method of obtaining DNA from the microbiological biodiversity of environmental samples [Courtois *et al.*, 2001].

However, indirect methods of DNA isolation make it possible to reduce impurities found in soil, just by a preliminary extraction of cells and generally gentle lysis conditions, *e.g.* lysing immobilized cells. Bertrand *et al.* [2005] demonstrated that indirect DNA isolation from soil is preferred as a method for obtaining larger DNA inserts. Larger DNA fragments can be obtained by enrichment of the soil sample; unfortunately, such an operation modifies the original microflora composition of the soil sample.

The size of the collected environmental sample can also determine the quality and the representative character of the DNA obtained. Ranjard *et al*. [2003] used an automatic analysis of DNA fragments, coding rRNA from 16S to 23S (A- RISA – automatic ribosomal intergenic spacer analysis). The research found that a soil sample <1g is sufficient for collecting the majority of bacterial DNA, while in the case of isolating fungal DNA, it is necessary to use samples of at least 1 g. In another experiment, Kang & Mills [2006] demonstrated that a sample of meadow soil of 0.1 to 1 g is sufficient to provide comprehensive and representative information about the microorganism population. However, the best results were obtained with 0.25 g soil samples.

Isolation of genomic DNA from the environment, and particularly from the soil, is not an easy task, since it is not possible to develop a single, universal, efficient procedure for DNA isolation from such a complex samples. Therefore, methods of DNA isolation must be strictly selected according to the type of isolated biological material and the aim of DNA use. The proper selection of the method, the choice of lysis conditions, purification and the precipitation process all determine the success of this task.

CLONING METAGENOMIC DNA AND NEW ENZYME EXPRESSION

Culture-independent methods for biocatalyst discovery usually requires creating a library of DNA inserts smaller than 10 kb. It is also required that a relatively large number of clones should be obtained in order to make up for a small number of clones which are active on selective substrates [Henne *et al.*, 2000]. Such small DNA fragments do not have to be obtained in mild conditions of DNA isolation, so it is acceptable to apply such methods as bead-beating.

As it has been reported earlier, isolating large DNA fragments requires very mild conditions and consists mainly in lysis of microorganism cells immobilised in plugs of agarose gel. A trade-off should be made between the need to obtain large DNA fragments and the sample representativeness and the amount of obtained DNA. According to some reports, cell lysis in agarose plugs is ineffective, which stands in contrast to the authors' own observations (unpublished data). In other variants, large DNA fragments are isolated from the soil after repeated freezing and thawing samples, followed by extraction with warm phenol [Rondon *et al*., 2000].

Cloning large DNA fragments provides a better chance of finding the sought phylogenetic marker or a gene which encodes the specific function or enzyme. Large inserts are also required in order to analyse large fragments of genomes or operons which encode the biosynthesis of secondary metabolites [Handelsman *et al.*, 1998].

Bacterial artificial chromosomes (BAC) are vectors which are able to accept large inserts of up to 350 kb. Medium-sized DNA fragments are accepted by fosmids and cosmids (up to 45 kb). The vectors are found as individual copies in a host's cells. This is an advantageous feature of the methods if the product of a cloned insert is toxic to the cell. However, if cloning aims at isolating a vector or analysing the expression products, the small amount of the analysed material becomes a problem.

The CopyControl® system, based on the method developed at the Szybalski laboratory, employing fosmid vectors pCC1FOS™ and pCC2FOS™ or BAC (pCC1BAC), offered by the Epicentre company, is an example of a system of expression which allows for controlling a vector copy [Wild *et al.*, 2001]. The vectors contains the *E. coli* F-factor single-copy origin of replication and *oriV,* a second origin of replication, which can be selectively induced to a high copy number. An "on-demand" number of clone copies can be induced to 10-20 copies per cell (fosmid vector) or 10-200 (BAC).

There are many methods available for cloning small fragments of metagenomic DNA (<10 kb) after amplification with the PCR method (if a specific gene is sought) or after preparing the blunt ends of all the DNA fragments. Applying blunt-end ligation is an alternative to preparing sticky-end DNA fragments and allows for preserving the properties of small DNA fragments [Wilkinson *et al.*, 2002]. Vectors used in cloning small DNA fragments can be found in small, medium or large number of copies. Example vectors used in metagenomic DNA cloning are, for example, pBluescript SK (+/-) (Startagene), pETBlue or pTZBlue, pT7Blue (Novagen), pCR-TOPO vectors (Invitrogen). The pros and con of

small- and large-insert soil libraries are presented in Table 3 [Daniel, 2005].

Cells of *E. coli* are mainly used as host strains, although recently a strain of *Streptomyces lividans* has been applied in the biosynthesis of new antibiotics [Wang *et al.*, 2000], and then perfected for obtaining the *E. coli-Streptomyces lividans* cosmid shuttle vector for drug discovery from the soil [Courtois *et al.*, 2003]. Reports have also emerged of the application of an eukaryotic, heterologous fungal host, *Pichia pastoris* KM71 [Jiang *et al.*, 2006] and *Pseudomonas putida,* or *Rhizobium leguminosarum* [Gabor *et al*., 2007].

The search for an ideal biocatalyst which meets the biochemical requirements *(e.g.* high values of k_{cat} and k_{cat}/K_M, and bioprocess engineering, simple and efficient synthesis, immobilisation) has become possible recently by applying methods of reaction environment engineering, protein engineering, directed molecular evolution, metagenomics, *etc*. [Adamczak & Krishna, 2004; Bornscheuer & Kazlauskas, 2004]. Metagenomic studies, including obtaining new enzymes from the metagenome, have recently revolutionised the earlier paradigm (Figure 3). The classic procedure of isolating pure bacterial strains has been replaced by cultivation-free approaches.

There are two strategies which allow for identification from metagenomic DNA of genes which encode new biocatalysts or genes related to the synthesis of bioactive chemical compounds: (1) screening based on functional analysis, (2) screening based on DNA sequence. According to an interesting discussion of the problem by Beja [2004], both strategies will be followed simultaneously, as in the Human Genome Project. In addition, the possibilities of shotgun sequencing of entire communities without the need to construct large-insert libraries has been borrowed from the Human Genome Project and is now being applied in the study of the Sargasso Sea metagenome. As expected, the two different approaches, shotgun sequencing and the large-insert BAC and the fosmid approach, gave a great deal of concordance with respect to the obtained results [DeLong, 2000].

TABLE 3. Pros and cons of small- and large-insert soil libraries [Daniel, 2005].

Advantages	Disadvantages
Small-insert library, *e.g.* plasmids	
High-copy number allows detection of weakly-expressed foreign genes Expression of foreign genes from vector promoters is feasible Technically simple Cloning of sheared DNA or soil DNA contaminated with matrix substances is possible	Small insert size Large numbers of clones must be screened to obtain positives Not suitable for cloning of activities and pathways that are encoded by large gene clusters
Large insert library, *e.g.* cosmids, fosmids, BAC	
Large insert size Small number of clones can be screened to obtain positives Suitable for cloning enzyme activities and pathways that are encoded by large gene clusters Suitable for partial genomic characterization of uncultured soil microorganisms	Low copy-number might prevent detection of weakly-expressed foreign genes Limited expression of foreign genes by vector promoters Required high-molecular soil DNA of high purity for library construction Technically difficult

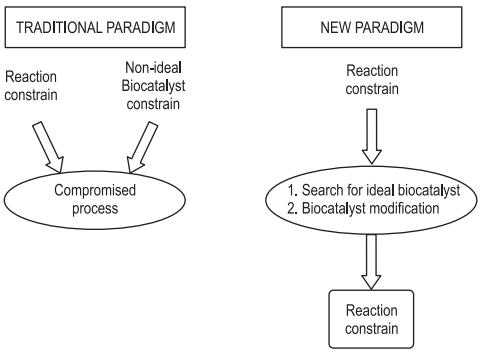

FIGURE 3. Modified paradigm shift [Burton *et al.*, 2002]. The new paradigm enables new enzymes (metagenome) or modified enzymes (directed evolution, mutagenesis, *etc*.) to be obtained that fit the ideal process.

The metagenome search has mainly focused on the isolation of rather small genes encoding enzymes and antibiotics [Streit *et al.*, 2004]. Due to their great catalytic abilities and selectivity, considerable interest has been focused on lipases and esterases (Table 4) – enzymes which are active in the media of controlled, low water activity coefficient, a_w. Lipases are probably the most frequently applied enzymes in research laboratories around the world. In the food processing industry they are used for flavour intensification of food products and in the manufacturing of flavour additives, removing contaminants and lipid waste, synthesis of structured triacylglycerols, improving bread characteristics, *etc*. [Bornscheuer *et al.*, 2003; Bornscheuer & Kazlauskas, 2006].

Ferrer *et al*. [2005a] described the discovery of five esterases from a distinct deep sea hypersaline biotope. It is in the analysis of DNA of such extreme environments that metagenomics seems to play its unique role. There is no technology of microorganism culturing which can imitate extreme conditions, such as high pressure, high temperature or extreme pH values. Two out of the five isolated esterases had no significant sequence homology to known esterases. One has an unusual structural signature incorporating three catalytic active centers mediating distinct hydrolytic activities and an adaptive tertiary-quaternary structure that alters between three molecular states, according to the environmental changes. Additionally, esterase (named O.16) efficiently resolves solketal acetate (E>100), a chiral building block.

In another publication, Ferrer *et al*. [2005b] presented the results of an analysis of a DNA library, created from the isolated total DNA from the rumen content of one New Zealand dairy cow. A metagenom expression library was created in the bacteriophage lambda ZAP phagemid vector (Startagene). Initially, 2×10^5 phage particles were obtained, with an average size insert of 5.5 kb (environmental genome 1.1. GB). The selection procedure provided the characteristics of 12 esterases, 9 endo-β-1,4-glucanases and 1 cyclodextrinase. The sequence analysis showed that 8 enzymes were entirely new.

Another group of desirable enzymes in the metagenome which are attractive to the food processing industry are carbohydrate hydrolases, *e.g.* amylases, glucoamylases, cellulases, chitinases (Table 4). Brennan *et al*. [2004] constructed genomic libraries from microbial DNA isolated from insect intestinal tracts from the orders Isoptera (termites) and lepi-

TABLE 4. Example of the enzymes identified from metagenome-derived genes (from the first data to the recent).

Origin	Vector type	Number of clones	Average insert size (kb)	Total DNA (Gb)	Enzyme	Reference
Meadow	Plasmid	~1,500,000	5-8	7.8	Lipolytic enzyme	[Henne *et al*., 1999, 2000]
Alkaline loessian soil	Plasmid	100,000	8-12	1.0	Protease	[Gupta *et al*., 2002]
Forest topsoil	Plasmid	33,700	35	ND	Lipolytic enzymes	[Lee *et al*., 2004]
Soil samples	Plasmid (*Pichia pastoris*)	ND	~1.4 bp	ND	Lipase	[Jiang *et al*., 2006]
Baltic Sea sediment	Fosmid	>7000	24-39	ND	Low-temperature-active lipase	[Hardeman & Sjoling, 2007]
Soil, compost	Plasmid	~21,000	3.3	ND	Different hydrolytic activity	[Lammle *et al*., 2007]
Deep-sea sediment	Plasmid	ND	804 bp	ND	Alkaline esterase	[Park *et al*., 2007]
Soil	Plasmid	ND	~1.0	ND	Halotolerant cellulase	[Voget *et al*., 2006]
Mud	Plasmid	ND	~1.0	ND	Fibrinolytic metalloprotease	[Lee *et al*., 2007]

ND-no data

doptera (moths). Xylanases were isolated which catalyzed the hydrolysis of a variety of substituted β-1,4-linked xylose oligomeric and polymeric substrates and produce unique hydrolysis products,

Oxidoreductases are highly enantioselective and are applied in the synthesis of carbonyl compounds, hydroxyl acids, amino acids and chiral alcohols. New genes encoding alcohol oxidoreductases were also isolated from metagenome isolated from soil [Eschenfeldt *et al.*, 2001; Knietsch *et al.*, 2003].

Genomic DNA can be isolated directly from environmental samples or from enrichment cultures. Amides, either singly or as a mixture of aromatic and non-aromatic forms, were supplied to soil samples and sediment as a nitrogen source. The DNA obtained from such samples has proven to be a carrier of new and highly active forms of amidases, including penicillin amidase [Gabor *et al.*, 2004].

DeSantis *et al.* [2002] used the metagenome approach to discover over 130 novel nitrilases, compared to fewer than 20 nitrilases previously isolated by classical cultivation methods.

Of key importance for efficient selection and screening of new enzymes is the development of high-throughput methods employing robotics and a microtiter plate [Kuznetsova *et al.*, 2005; Reymond & Babiak, 2007], which enables functional screening.

The drawback of the all sequence-based assays (the most popular PCR and PCR-generated hybridization) is that the novel genes to be detected should resemble previously known genes. This largely restricts the scope of search for new genes. It is possible to apply degenerate PCR primers for the isolation of genes encoding different enzymes [Bell *et al.*, 2002].

TABLE 5. The examples of commercialization of metagenomic technologies for enzyme discovery.

Company	Obtained enzyme	Application
BASF	Amylase	Food industry, aiding with the digestion of starch
B.R.A.I.N.	Nitrile hydrolase, cellulase	Bioactive peptides, pharmaceuticals
Diversa	Nitrilase, glycosidase, phytase	Drugs, digestion of animal feed compounds
Genencor	Lipase, protease	Cleaning industry
Prokaria	Rhamnosidase, β-1,4-gluconase	Food and agricultural industry

The application of microarray technology for searching a metagenome is a promising new approach [Sebat *et al.*, 2003] and is an effective method for analysis of many clones.

Other methods which enable high-productivity screening include the application of a phage- or bacterial-display expression library. Phage-display expression libraries provide a means for isolating DNA sequences by affinity selection of the surface-displayed expression. This method has many advantages, but the size of proteins on a phage surface is restricted to 50 kDa [Paschke, 2006].

Uchiyama *et al.* [2004] proposed substrate-induced gene-expression screening (SIGEX). The approach enables the selection of catabolic genes by trapping operons in vectors from which the expression can be detected by the expression of green fluorescent protein in the presence of the relevant substrate. Fluorescence-activated cell sorting (FACS) is used to capture the GFP-expressing clones [Yun & Ryu, 2005]. The system is limited to the orientation of cloning and is not effective for constitutively expressed genes [Ward, 2006].

A new "–omic" technology was introduced by Wilmes & Bond [2004]. The metaproteomics, *i.e.* "the large-scale characterization of the entire protein complement of environmental microbiota at a given point of time". The procedure uses standard proteomic methods, such as a 2D-PAGE for characterization protein profile, time of flight mass spectrometry and protein sequencing. Proteins that may originate from uncultured microorganisms are identified.

Commercialisation of metagenomic technologies has become a fact and such companies as Diversa [www.diversa.com], BASF [www.corporate.basf.com], B.R.A.I.N. [www.brain-biotech.de] and Genencor (www.genencor.com) are conducting intensive research in the field (Table 5).

SUMMARY

One of the most important international metagenomic projects is the global ocean survey (GOS), a research programme aimed at describing picoplankton smaller than

0.8 μm in all the oceans; the study was started in 2004 with a metagenomic analysis of the Sargasso Sea water [Piganeau & Moreau, 2007].

Recent developments indicate the huge potential of metagenome exploration. It seems that discovering the potential of metagenomic DNA libraries has only just started. Efficient and effective mining will require high-throughput functional screens and selection and also rapid methods for identifying sequences of interest. It has been suggested that it is too early to claim that metagenomic gene discovery is a technology that has "come of age" [Cowan *et al.*, 2005], as new methods and techniques have been emerging, including efficient systems of metagenomic DNA cloning.

A problem is soon likely to appear of a huge amount of data whose analysis will be a problem. It will also be difficult to explore the functions of new genes and proteins and their role in a given ecological niche and in global cycles [Streit & Schmitz, 2004]. Consequently, new research and new methods of bacteria culturing should not be neglected as these have made it possible to multiply the ubiquitous *Pelagibacter* [Connon & Giovannoni, 2002; Giovannoni *et al.*, 2005] in culturing on diluted media and new bacteria from the genera *Acidobacteria* and *Planctomycetes* which have so far been resistant to laboratory growth.

Other applications of metagenomics, unrelated to the scope of this study, should also be borne in mind, *e.g.* sequencing the 40,000-year-old genome DNA from extinct cave bears.

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EKSPLORACJA METAGENOMÓW W CELU POZYSKANIA ENZYMÓW PRZYDATNYCH W BIOTECHNOLOGII ŻYWNOŚCI – ARTYKUŁ PRZEGLĄDOWY

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Metagenomika obejmuje analizę wszystkich genomów (metagenom) organizmów w danym środowisku. Zastosowanie analizy metagenomicznej w badaniach mikrobiologicznych pozwoliło zrozumieć fizjologię drobnoustrojów nienamnażanych z użyciem standardowych metod, tym samym umożliwiając pozyskiwanie nowych enzymów i metabolitów wtórnych.

Metody izolacji DNA muszą być ściśle dostosowane do rodzaju izolowanego materiału biologicznego i determinują jakość i wielkość otrzymanego DNA. Małe fragmenty DNA są zwykle wystarczające do analizy pojedynczych lub małych grup genów, podczas gdy duże inserty wymagane są do analizy szlaków metabolicznych, organizacji genomu czy sekwencjonowania dużych fragmentów DNA.

Obecnie wyróżnia się dwa rodzaje metod ekstrakcji genomowgo DNA. Pierwsza grupa metod polega na bezpośredniej ekstrakcji kwasów nukleinowych z próbki środowiskowej po uprzedniej lizie (*in situ*), a następnie oczyszczeniu otrzymanego DNA. Metody pośrednie polegają na wydzieleniu komórek bakteryjnych z próbki środowiskowej, a następnie lizie zawiesiny komórek i dalej ekstrakcji DNA.

W publikacji przedstawiono różne metody izolacji genomowego DNA ze środowiska naturalnego, metody jego klonowania oraz zaprezentowano przykłady pozyskiwania nowych enzymów z metagenomu.