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Metagenome Analysis: a Powerful Tool for Enzyme Bioprospecting

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Abstract Microorganisms are found throughout every corner of nature, and vast number of microorganisms is difficult to cultivate by classical microbiological techniques. The advent of metagenomics has revolutionized the field of microbial biotechnology. Metagenomics allow the recovery of genetic material directly from environmental niches without any cultivation techniques. Currently, metagenomic tools are widely employed as powerful tools to isolate and identify enzymes with novel biocatalytic activities from the uncultivable component of microbial communities. The employment of next-generation sequencing techniques for metagenomics resulted in the generation of large sequence data sets derived from various environments, such as soil, the human body and ocean water. This review article describes the state-of-the-art techniques and tools in metagenomics and discusses the potential of metagenomic approaches for the bioprospecting of industrial enzymes from various environmental samples. We also describe the unusual novel enzymes discovered via metagenomic approaches and discuss the future prospects for metagenome technologies.

Keywords Bioprospecting · Metagenomics · Metatranscriptomics · Enzyme

Introduction

Enzymes are potent biocatalysts and enhance the rates of a large number of biological and chemical reactions. Currently, there is a high demand for the potent biocatalysts, which are

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considered as greener alternatives for high-value chemical synthesis. Majority of chemical synthesis now uses environmentally hazardous organic solvents and high energy [1, 2] as microbial enzymes do not possess any environmentally hazardous nature and thus provide "cleaner" solutions for the synthesis of bulk chemicals and compounds [3].

The exploitation of unexplored microbial communities for the identification and isolation of potent enzymes is an active area of research worldwide. Being the oldest type of life in earth, prokaryotic microorganisms possess remarkable physiological, metabolic and functional diversity and richest source of genetic diversity. Classic method to prospect this diverse gene information is by cultivating the microorganism and subsequent screening for the desired phenotype. However, about 99.9% of microorganisms in environmental niches cannot be cultivated by standard laboratory techniques [4].

Metagenomics is a technology to bypasses the requirement for the isolation or cultivation of microorganisms. Metagenomic technologies are based on the direct isolation of genomic DNA from environmental samples and found to be powerful tools for tapping the genetic and metabolic diversity of complex ecosystems [5]. The advent of this technology led to metabolic profiling of complex environmental samples [6–8] and identification of novel bioactive molecules and enzymes by the use of libraries constructed from isolated nucleic acids [9, 10].

Metagenomic technique involves two basic approaches for screening biomolecules from environmental samples: function-based and sequence-based screening of metagenomic libraries [11, 12]. In both cases, the metagenomic libraries are constructed by the cloning of fragmented genomic DNA in appropriate expression vectors such as plasmid, cosmid, lambda phage or fosmids that allow the expression of the desired genes. Depending on the desired target gene size, metagenomic libraries have been created using plasmids (15 kb), fosmids, cosmids (40 kb) or bacterial artificial chromosomes (40 kb) as expression vectors (Fig. 1). Metagenomic gene libraries can be used for the identification of novel enzymes encoded by a single gene or a small-sized operon, whereas large-sized insert libraries are required for the isolation of large biosynthetic gene clusters, which encode for complex pathways containing several genes [11]. After library construction, the resulting metagenomic clones are used to transform a heterologous host, which is in most cases *Escherichia coli*.

Excellent reviews are available in the literature on every aspect of metagenomics, including functional metagenomics [12], sequencing strategies [13] and applications in biotechnology
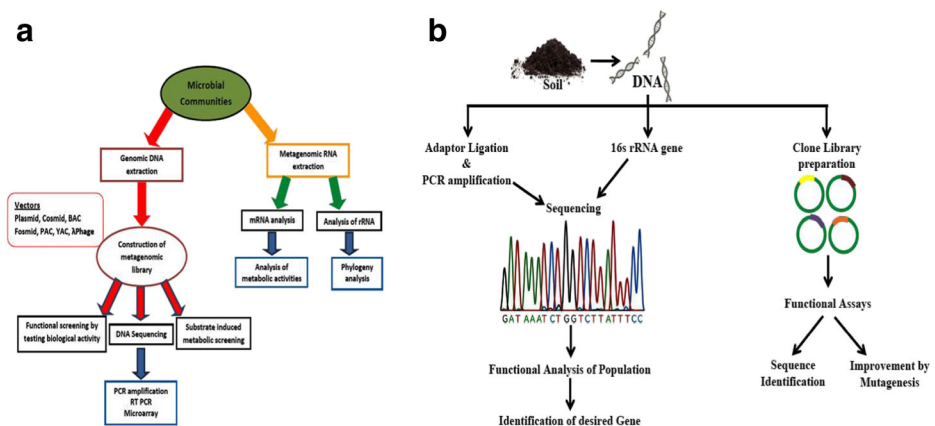
Fig. 1 **a** General strategies in metagenome mining of enzymes. **b** Function-based metagenomic screening

industry [14]. In this review article, an overview of metagenomic approaches is discussed as an emerging molecular technique with special reference to industrial enzyme prospecting and applications in industry.

Function-Based Metagenomic Screening

The common function-based strategies used in metagenomics include enzyme activity-based screens performed in culture plates, for example, starch-iodine test for amylase and cellulose screening assay for cellulase. The different function-based screening strategies are phenotypic detection, heterologous complementation of host strains and induced gene expression. In most of the cases, the phenotypic chromogenic screening strategies employ dyes and substrates of target enzymes incorporated in to the culture plate, where they detect the presence of specific biocatalyst. As gene sequence information is not required, functional screening is best for the identification of novel genes encoding novel enzymes [15, 16].

The heterologous complementation of host strains involves the use of complemented host strains which require the presence of target gene for growth under selective condition. For example, recently, screening of 446,000 clones of soil metagenomic library for genes that confer resistance to β-lactam and aminoglycoside antibiotic resulted in the identification of 10–13 restriction clones with novel resistance genes [17].

The main advantage of direct screening of metagenomic library is that prior knowledge of sequence is not required, and we may get novel gene sequence without any similarity to previously existing sequences. The main disadvantage of this screening is the chances for failure in gene expression mainly due to difficulties in promoter recognition, translational inefficiency, misfolding of proteins, defective post-translational modification of desired proteins, etc. This can be resolved by using vectors capable of accommodating large insert size, using vectors with broad host range which allow expression in multiple hosts and using rossetta *E. coli* strains which contain tRNA for rare amino acid codons [18, 19].

The third type of functional screening is substrate-induced gene expression screening which is a high-throughput screening which contains GFP as a reporter gene. The system is based on the fact that the GFP expression is induced by the presence of a specific metabolite produced by the clones. The GFP is under the control of tightly regulated promoter. Researchers were successful in isolating hydrocarbon-induced gene sequences from metagenomic library derived from ground water [19].

Function-based metagenomic screening has been used in the screening of several active biocatalysts. Here, we describe several published results of function-based screening of metagenomic clones. For the identification of novel glycosyl hydrolases, metagenomic fosmid libraries were screened from the cellulose-depleting microbial communities. The screening was based on the biocatalytic capability to hydrolyse p-nitrophenyl-β-D glucopyranoside and p-nitrophenyl-α-L-arabinopyranoside. This resulted in the recovery of two novel glycosyl hydrolases with high activity and had no similarity to any other reported glycosyl hydrolases [20]. Amylases are very important biocatalysts with several industrial applications. Function-based screening strategies have been used for the screening of several reported amylases. Another example for activity-driven metagenomic screening is the identification of a novel β-glucopyranoside from human microbiome, which is essential for human health [21, 22]. Functional screening of metagenome from extreme environment discovered salinity and alkali active novel cellulases and esterases [23, 24].

Substrate-induced gene expression (SIGEX) is an efficient tool for function-based screening of metagenomic clones. Uchiyama and Miyazaki [19] modified the SIGEX protocol and developed a reporter-based assay system called product-induced gene expression (PIGEX). The modified system includes a transcriptional activator, which is highly sensitive to the product of the desired enzyme. The downstream of the promoter region contains the reporter gene GFP. Another type of screening system has been developed and designated as metabolite-regulated expression (METREX) discovered by Williamson et al. [25]. This is a technology to screen metagenomic clones producing small active molecules, and the system contains a biosensor that detects the small diffusible signal molecules that activates the quorum sensing, which is also placed in the same cell as the vector bearing a metagenomic DNA fragment.

The potential of functional metagenomics is the identification of biological activity in a manner independent of sequence data; the main drawback of this technique is its dependence on the ability of the heterologous host to express the metagenomic gene efficiently, translation of messenger RNA (mRNA) in to functional protein and correct folding of the protein. *E. coli* is proved to express 40% of environmental DNA, but not suitable for actinomycete genes with high GC content [26]. In this context, the use of alternative hosts proved to be successful. For example, *E. coli*, *Streptomyces lividans* and *Pseudomonas putida* containing various antibiotic biosynthetic gene clusters produced variable levels of antibiotics [27], and six species of *Proteobacteria* differed in expression of metagenomic genes of interest [28].

Sequence-Based Metagenomic Screening

Another widely used strategy for screening metagenomic clones is the sequence-based approach. In this technique, metagenomic clones are screened using an oligonucleotide primer or probes for the target gene using the colony hybridization technique to shortlist the clones. The desired gene may also be amplified by PCR with specific or degenerate primers and cloned in appropriate expression vectors. This technique leads to discovery of novel sequences. However, these sequences may share similarity to pre-existing sequence. This technique opens the possibility of finding enzymes with high activity and efficiency [29].

This strategy has led to the identification of several novel enzymes like chitinase [30], hydrogenase [31], phosphatase [32], glycerol dehydratase [33], hydrazine oxidoreductase [34], etc. The sequencing strategy depends on the complexity of metagenomic community. If the complexity of the microbial community is high, new sequencing strategy has to be used. The advent of new sequencing technologies like 454-pyrosequencing has changed the scenario, and more representation of species in the metagenomic community is possible now. New computational tools are also required for analysing the bulk metagenomic sequence information [35].

Several publications reported the use of sequence-based approach for the screening of novel enzymes. Warnecke et al. [36] created metagenomic sequencing data from wood feeding termites. They generated about 71 million base pairs of sequence information. They could identify 700 domains of different glycosyl hydrolases, 45 different carbohydrate active enzymes, etc. Another example for large-scale analysis of metagenome data is the microbial population analysis of Sargasso Sea which generated 1.2 million previously unidentified genes and led to identification of rhodopsin-like photoreceptor in bacteria [37]. Hess et al. [38] deep sequenced the metagenomic library from cow rumen and analysed 268 gigabase of metagenomic DNA, and they could find several carbohydrate active enzymes.

High-Throughput Metagenomic Sequencing and Screening Strategies

The recent revolution in the field of DNA sequencing technologies has resulted in the significant decrease in the cost of sequencing and dramatic increase in the metagenome database. Improvements in sequencing technology have led to the introduction of next generation or cyclic array technologies which resolved the issues related to massive parallel sequencing. The array-based sequencing strategy can generate hundreds of millions of sequencing reads. The main next-generation sequencing platforms in the market are 454 genome sequencer from Roche Applied Science, SOLiD platform from Applied Biosystems, the Illumina Genome Analyser from Illumina and Ion Torrent/Ion Proton platform [39–41]. The shot metagenomic sequencing approach gave community-level sequence information in a highly complex environment with millions of different bacterial or eukaryotic species in soil, ocean, cow rumen, groundwater, etc. [42, 43].

Currently, microbial metagenomic platform uses targeted gene sequencing or shotgun metagenome sequencing [6, 39]. In the case of targeting the genomics, DNA is isolated from environmental samples using highly efficient DNA extraction and purification methods. The target genes are amplified using designed primers with oligonucleotide tags and also with sequencing adaptors which sequence the pooled multiple samples [41]. In shotgun sequencing approach, the genomic DNA is fragmented, end repaired and ligated with adaptors which allow amplification of template and subsequent sequencing generates large number of short reads, which can be further assembled and annotated with various computational tools and techniques [44].

The number of computational tools also has been evolved in the past decades to analyse the sequences originated from different sequencing platforms [45] (Table 1). The obtained protein-coding genes can be analysed with the help of protein data bank (NCBI, NR, Uniport) or domain database or Pfam [57]. Several enzyme databases are also available for searching Cazyme [58], esterase, laccase [59], metallo-β-lactamase [60], etc.

The experimental link from genome sequence to protein function is important and usually gives an overall idea about functional properties. Sometimes, metagenome annotations prove to be incorrect when validated experimentally. So, further high-throughput functional screening is required instead of time-consuming conventional screening methods. Conventional high-throughput screens use microtitre plates to screen large number of metagenomic clones. The advent of microarray-based technologies coupled with other advanced technologies like

Table 1 List of computational tools in metagenome analysis

Tool	Application	Reference
Mothur	Analyse the quality of sequencing reads and taxonomic classification	[46]
Printseq	Sequence trimming tool for quality control in the case where dinucleotide occurs	[47]
MEGAN	For taxonomic analysis and functional analysis. This tool is suitable for comparative metagenome	[48]
Metagenomeseq	Analyse the occurrence of 16S rRNA in metaprofiling	[49]
Metapath	Identification of biosynthetic pathway from metagenome data	[50]
BlastX	Analysis of translated sequence	[51]
PhyloSeq	Diversity and phylogenetic analysis	[52]
MG-RAST	Comparative metagenomics	[53]
Parallel-META	Taxonomic annotation of ribosome gene markers	[54]
CARMA	Phylogenetic analysis	[55]
antiSMASH	Identification of biosynthetic pathways	[56]

flow cytometry, cell compartmentalization and microfluidic devices resulted in the reduction of time in screening millions of clones [61–64]. Colin et al. [64] recently demonstrated the functional screening of a million-membered metagenomic library in microfluidic picolitre droplet compartments.

Omic Technologies Integrated with Metagenome Research

Metatranscriptomics

Metatranscriptomics is the RNA-based sequence information of microbial communities in a complex ecosystem [65]. The challenges associated with metatranscriptomics are the recovery of high-quality mRNA from environment samples and separation of mRNA from other RNA samples. The metatranscriptomic approaches in seawater community proved that this is an efficient method to elucidate the gene expression pattern and led to the discovery of novel genes [66]. Direct quantitation of mRNA transcript is feasible through metatranscriptomics [67]. The importance of ammonia-oxidizing archeae in soil ecosystem has been unravelled by Leininger et al. [68] through metatranscriptomics. Other successful metatranscriptomic approaches are the elucidation of novel pathway in archeae for methane oxidation and analysis of ocean surface water from the North Pacific Subtropical phytoplankton bloom in English Channel. Recently, Shi et al. [69] described the importance of small RNAs in environmental samples.

Metaproteomics

Metaproteomics is the study of total proteome expressed by the microorganisms within an ecosystem at a particular period of time. This technique can be used to explore the various microbial activities and complex metabolic pathways involved in soil ecosystem [70]. Ram et al. [71] analysed the proteome of natural acid mine drainage using the mass spectrometric technique. They could identify around 2000 proteins from the microbial community. Several metaproteomics studies were reported in the microbial community of activated sludge, phyllosphere, hindguts of termites, marine samples, etc. [72]. Tanca et al. [73] employed a metaproteogenomic approach combined with 16S rRNA gene sequencing, shotgun metagenomics and metaproteomics, to analyse the microbial communities of paired mouse caecal contents and faeces, to identify the changes in taxon-specific functions. Another study attempted to study the effect of a long-term metal exposure on sediment microbial communities using metaproteogenomics [74]. Various bioinformatics tools in this area have been reviewed by Seifert et al. [75].

Applications of Metagenomics in Enzyme Bioprospecting

Prospecting for Lignocellulolytic Enzymes

In order to produce biofuels from lignocellulosic biomass, efficient biomass-degrading enzymes need to be developed. Metagenomics has been widely used for the identification of hydrolytic enzymes like cellulase, xylanase, etc. Lignin-degrading enzymes are very important for biomass hydrolysis since the cross-linking by lignin in plant biomass limits the entry of

enzymes and affects the hydrolysis. Several lignin-degrading enzymes like manganese peroxidase and laccases have been developed through metagenomic discovery [76–78]. The hemicellulosic region of lignocellulosic biomass consists of xylan and mannan which, can be hydrolysed into monosaccharides by the action of xylanase (endo-β-1,4-xylanase (EC3.1.2.8) and mannase. With the help of functional metagenomic screening, several novel xylanases have been obtained from metagenomic DNA libraries [79, 80]. Low-temperature active xylanase was obtained from soil-derived metagenomic library [81]. Another highly active alkaline xylanase with broad pH range was obtained from the metagenomic library of microbiome extracted from termite's gut [82].

Cellulose is the most abundant component of plant biomass and is the most abundant polymer in the nature. Cellulose enzymes include endoglucanase, which randomly cleaves cellulose chain, and cellobiohydrolase, which acts on reducing or non-reducing ends of cellulose molecules and release glucose or cellobiose. β-Glucosidase cleaves cellobiose or cellodextrins to glucose. Recently, several novel cellulases with improved characteristics were identified through metagenomics [83]. By functional metagenomic screening, highly glucose-tolerant β-glucosidase was identified from marine metagenome and this offers industrial application due to less product inhibition [84]. Another novel β-glucosidase with novel properties has been isolated from soil metagenome library and exhibited low temperature tolerance and can withstand broad pH range (5.5–10.5) [85]. Several other potent cellulases obtained from metagenomic library are listed in Table 2. An endoglucanase was also identified with high tolerance to ionic liquids with high stability. Many other cellulases were identified through metagenomic screening which includes endoglucanases and β-glucosidases from different environmental DNA libraries, Cazy enzymes from gut of earthworms, etc. [76]. Novel β-glucosidases with glucose or ethanol tolerances are greatly required to make industrial bioprocesses more efficient. Gomes-Pepe et al. [94] reported the in silico/in vitro characterization of Bg10, a metagenomically derived homodimeric β-glucosidase that exhibited a V_{max} of 10.81 ± 0.43 μM min^{-1}, K_{cat} of 175.1 ± 6.91 min^{-1} and K_m of 0.49 ± 0.12 mM at a neutral pH and 37 °C. Another recent study by Pottkamper et al. [95] described the metagenomic analysis of microbial consortia enriched from rice straw adapted (RSA) compost. The study analysed the 16S pyrotag library and 5 Gbp of metagenomic sequence and showed that the phylum *Actinobacteria* was the predominant group among the bacteria in the RSA consortia, followed by *Proteobacteria*, *Firmicutes*, *Chloroflexi* and *Bacteroidetes*. The CAZyme profiling of the consortia revealed that CAZyme genes were also widely distributed within these bacterial phyla.

Table 2 List of some novel enzymes discovered through metagenomic approach

Enzyme	Vector/host	Environment	Reference
Esterase	Plasmid library/*E. coli*	Soil from river valley	[86]
Thermo-stable esterase	Fosmid library/*E. coli*	Mud sediment	[87]
Glycosyl hydrolase	Λ phage library/*E. coli*	Cow rumen	[88]
Halotolerant tannase	Plasmid library/*E. coli*	Soil (cotton field)	[89]
Serine protease	Plasmid and fosmid library	Dessert valley soil	[90]
Amylase	Cosmid library/*E. coli*	Soil from unplanted field	[21]
Endo-1,4-endoglucanase	YEP356/*E. coli*	Seaweed	[91]
Cellulase	*E. coli*	Anaerobic beer less	[8]
Glucoside hydrolase	*E. coli*	Forest soil	[92]
Laccase	pIndigo BAC5/*E. coli*	Seawater	[93]

Prospecting of Esterases

Lipases and esterase are hydrolytic enzymes which play an important role in food, biofuel and pharma industries. They are involved in ester hydrolysis, synthesis, transesterification, synthesis of fine chemicals, flavour compounds, etc. [25]. Many esterases with novel characteristics have been identified through function-based or sequence-based screening strategies from environmental metagenomic libraries (Table 2). A novel lipase belonging to a new family has been reported from intertidal flat metagenome, which is a cold-adapted lipase [96]. Peng et al. [97] constructed metagenomic library of Chinese marine sediments and screened for novel alkaline stable lipase with high specificity for buttermilk fat esters. Most of the metagenomically derived lipases possess resistance to solvents, salt tolerance, etc. Novel esterase Est16 from metagenome of diesel oil degradation region was recovered and possesses thermostability and active against wide range of substrates [25]. A novel esterase gene was cloned from the metagenome of the sediment of Soda Lake Dabusu. The 636-bp gene encodes a polypeptide of 211 amino acids and was recombinantly overexpressed, and the enzyme was highly cold-adapted and retains 70% of the activity at 0 °C, withstands broad pH range and is highly salt tolerant to 5 M NaCl [98].

Other Enzymes

Proteases which hydrolyse peptide bonds have numerous uses in food industry like tenderization of meat, in baking industry, etc. Metagenomic tools were successfully employed for the prospecting of proteases. Biver et al. [99] isolated alkaline stable serine proteases from forest soil metagenome library. Pushpam et al. [100] discovered a metagenome-derived protease from goat skin. β-Galactosidases are also used widely in food industry for the hydrolysis of lactose to glucose and galactose. A cold-active β-galactosidase was discovered from the metagenomic library of ikaite columns of SW Greenland, and Wang et al. [101] also isolated cold-adapted β-galactosidase from metagenome library (Table 2).

Marine Metagenome

A wide range of enzymatic activities have been identified from cultured marine microbes which show the potential for the discovery of novel enzymes from marine microbes, which spread over different parts of the globe. Recently identified non-specific nuclease, isolated from a bacteriophage which predates on the marine thermophile *Geobacillus* sp. 6K51, having an optimum temperature of 60 °C has been shown to have no known homology to any previously isolated nucleases [102]. On the other hand, the cold-adapted enzymes such as the lipases have been isolated from the γ-proteobacterium, *Pseudoalteromonas haloplanktis* [103]. Other recently reported enzymes include phospholipases [104], amylolytic enzymes [105] and agarases [106]. These enzymes are extensively being used in many biotechnological applications providing economic and energy benefits. The costs of enzyme preparation have been reduced as we have different options of enzymes according to our needs with optimum temperature at low and high temperatures and minimize undesirable chemical reactions especially at high temperatures [107]. These properties are significant for the food and feed industry to reduce spoilage and change in nutritional value and flavour of the original heat-sensitive substrates and products [108]. Comparative genome analyses suggested that marine psychrophilic enzymes have a flexible configuration, most probably due to a combination of changes in the overall amino acid composition,

which lose their rigidity and gain increased structural flexibility enhancing catalytic function at low temperatures [109]. Lipase and esterase are widely used in the food, laundry, textile, pulp and paper industries; production of biodiesel; and synthesis of fine chemicals. Furthermore, they are very easy to detect from a functional agar screening by using synthetic substrates [110].

Even environments such as the deep-sea floor, where only restricted numbers of cultivable bacteria have been identified, now emerge to be reservoirs for microbes with enzymatic activities with prospective biotechnological applications. For instance, a number of cultivable aerobic microbes producing a variety of enzymes including protease, amylase, lipase, chitinase, deoxy-ribonuclease and phosphatase have recently been isolated from the deep-subsea floor sediments from offshore the Shimokita Peninsula in Japan at a water depth of 1180 m [111]. The entire potential of these environments is still to be explored more as we know that this cultivable fraction represents only a little proportion of the total bacteria present in these environments. Hence, it is obvious that use of metagenomic or other culture-independent approaches together with robust heterologous expression systems is required to smoothen the progress of such an approach. A novel cold-active esterase called Est97 has been isolated by the metagenomic library screening of an Arctic intertidal zone [112], which retains 60% of relative activity at 20 °C, suggesting its utilization in cold biotransformation. MPlaG, a phospholipase A with lipase activity, was obtained from a metagenomic library from tidal flat sediments on the Korean west coast with maximum activity at 25 °C. It also has specific catalytic properties against olive oil and phosphatidylcholine, suggesting that MPlaG is a lipid-preferred phospholipase [113]. A fosmid library containing 7200 metagenomic clones was constructed from a deep-sea sediment sample from the South China Sea. A gene (H8) coding for an active esterase was identified from this library and expressed in E. coli. This esterase was found to be very suitable for industrial applications with optimal temperature and pH of 35 °C and 10.0, respectively, and tolerant to salinity [114]. A list of marine-derived metagenomic enzymes is presented in Table 3.

Metagenomics and Industrial Future

Recent reports suggested that the prospecting of industrial enzymes through metagenomics has surprisingly been increased. Metagenomic discovery also offers direct access to the diversity of microbial world and led to significant developments in industry-oriented research. The functional screening of metagenome library followed by pyrosequencing of the insert genes allows several-fold increase in the identification of novel genes with novel catalytic properties. Currently, several

Table 3 Novel enzymes discovered through marine metagenomic approach

Enzyme	Source	Reference
Esterase	Marine mud	[115]
α-Amylase	Marine sediment	[116]
β-Glucosidase	Hydrothermal spring	[117]
Laccase	Marine water	[118]
Proteases	Sub-Antarctic sediment	[119]
Xylanase	Antarctic seawater	[120]
Chitinase	Marine hot springs	[121]
Glycoside hydrolase	Deep sea	[122]
Feruloyl esterase	Antartic seawater	[123]
Aminopeptidase	Marine sediment	[124]

metagenomic-based biocatalysts have been patented and available in the market, for example, laccases, esterases and nitrile hydratase. The commercialization of metagenomically derived enzymes takes several years because of the complex steps involved in the screening and development [125]. In addition to that, the enzymes discovered through metagenomics should possess several characteristics for industrial production. They should withstand harsh reactions in industrial scale, broad pH range, temperature, salinity, varying solvent concentration, stereo-selectivity, high turnover rate, etc. [126]. Metagenome-derived industrially important enzymes can be improved through various tools and techniques. One can correlate the gene expression of desired gene and turnover rate of substrate conversion. This will lead to standardization of enrichment technique for the further improvement of the enzyme. In addition, the use of metatranscriptomic and metaproteomics approach efficiently screens highly active enzymes. Further, high-throughput enzyme screening strategies may further enhance the enzyme discovery through metagenomics. This will reduce the time for identification of suitable enzyme and subsequent establishment of an industrial process.

Conclusion

Metagenomics offers access to the genetic and metabolic diversity of the microbial communities and has led to significant developments in the area of enzyme and bioactive molecule prospecting and has revolutionized industrial production system with respect to the identification and isolation of novel biocatalysts. With the advent of next-generation sequencing technologies, millions of complex metagenomic sequence data were generated, which in turn led to the formulation of various computational tools for the efficient analysis and comparison of these data sets with respect to phylogenetic and metabolic diversity. This may aid in further improvements in the identification of novel biocatalysts and bioactive molecules. Currently, large-scale gene expression studies and proteome studies of microbial communities emerged to link the genetic diversity and metabolic activities of uncultivable microbes. Metatranscriptomics and metaproteomics in combination with metagenomics offer significant promise to elucidate the functional dynamics, activities and production capabilities of microbial consortia. Thus, metagenomic-assisted enzyme bioprospecting presents an opportunity to identify, isolate and develop novel biocatalysts that are highly significant to industrial bioprocess.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

References

1. Adrio, J. L., & Demain, A. L. (2003). Fungal biotechnology. *International Microbiology, 6*(3), 191–199.
2. Mackenzie, A. K., Naas, A. E., Kracun, S. K., Schückel, J., Fangel, J. U., Agger, J. W., et al. (2015). A polysaccharide utilization locus from an uncultured bacteroidetes phylotype suggests ecological adaptation and substrate versatility. *Applied and Environmental Microbiology, 8*(1), 187–195.

3. Alcalde, M., Ferrer, M., Plou, F. J., & Ballesteros, A. (2006). Environmental biocatalysis: from remediation with enzymes to novel green processes. *Trends in Biotechnology, 24*(6), 281–287.

4. Amann, R. J., Binder, B. L., Chisholm, S. W., Devereux, R., & Stahl, D. A. (1990). Combination of 16S rRNA targeted oligonucleotide probes with flow cytometry for analysing mixed microbial populations. *Applied and Environmental Microbiology, 56*(6), 1910–1925.

5. Biddle, J. F., Fitz-Gibbon, S., Schuster, S. C., Brenchley, J. E., & House, C. H. (2008). Metagenomic signatures of the Peru Margin subseafloor biosphereshow a genetically distinct environment. *Proceeding of the National Academy of Sciences of the United States of America, 105*(30), 10583–10588.

6. Tringe, S. G., von Mering, C., Kobayashi, A., Salamov, A. A., Chen, K., Chang, H. W., et al. (2005). Comparative metagenomics of microbial communities. *Science, 308*(5721), 554–557.

7. Mai, Z., Su, H., Yang, J., Huang, S., & Zhang, S. (2014). Cloning and characterization of a novel GH44 family endoglucanase from mangrove soil metagenomic library. *Biotechnology Letters, 36*(8), 1701–1709.

8. Yang, C., Xia, Y., Qu, H., Li, A., Liu, R., Wang, Y., & Zhang, T. (2016). Discovery of new cellulases from the metagenome by a metagenomics guided strategy. *Biotechnology for Biofuels, 9*, 138.

9. Simon, C., & Daniel, R. (2010). Construction of small-insert and large insert metagenomic libraries. *Methods in Molecular Biology, 668*, 39–50.

10. Steele, H. L., Jaeger, K. E., Daniel, R., & Streit, W. R. (2009). Advances in recovery of novel biocatalysts from metagenomes. *Journal of Molecular Microbiology and Biotechnology, 16*, 25–37.

11. Daniel, R. (2005). The metagenomics of soil. *Nature Reviews in Microbiology, 3*(6), 470–478.

12. Lam, K. N., Cheng, J., Engel, K., Neufeld, J. D., & Charles, T. C. (2015). Current and future resources for functional metagenomics. *Frontiers in Microbiology, 6*, 1196.

13. Escobar-Zepeda, A., Vera-Ponce de León, A., & Sanchez-Flores, A. (2015). The road to metagenomics: from microbiology to DNA sequencing technologies and bioinformatics. *Frontiers in Genetics, 6*, 348.

14. Coughlan, L. M., Cotter, P. D., Hill, C., & Alvarez-Ordóñez, A. (2015). Biotechnological applications of functional metagenomics in the food and pharmaceutical industries. *Frontiers in Microbiology, 6*, 672.

15. Ferrer, M., Beloqui, A., Timmis, K. N., & Golyshin, P. N. (2009). Metagenomics for mining new genetic resources of microbial communities. *Journal of Molecular Microbiology and Biotechnology, 16*(1–2), 109–123.

16. Riesenfeld, C. S., Schloss, P. D., & Handelsman, J. (2004). Metagenomics: genomic analysis of microbial communities. *Annual Reviews of Genetics, 38*, 525–552.

17. Donato, J. J., Moe, L. A., Converse, B. J., Smart, K. D., Berklein, F. C., McManus, P. S., & Handelsman, J. (2010). Metagenomic analysis of apple orchard soil reveals antibiotic resistance genes encoding predicted bifunctional proteins. *Applied and Environmental Microbiology, 76*, 4396–4401.

18. Perner, M., Ilmberger, N., Köhler, H. U., Chow, J., & Streit, W. R. (2011). Emerging fields in functional metagenomics and its industrial relevance: overcoming limitations and redirecting the search for novel biocatalysts. In F. J. de Bruijn (Ed.), *Handbook of molecular microbial ecology II: metagenomics in different habitats* (pp. 483–498). Hoboken: Wiley.

19. Uchiyama, T., Abe, T., Ikemura, T., & Watanabe, K. (2005). Substrate induced gene-expression screening of environmental metagenome libraries for isolation of catabolic genes. *Nature Biotechnology, 23*, 88–93.

20. Beloqui, A., Nechitaylo, T. Y., López-Cortés, N., Ghazi, A., Guazzaroni, M. E., Polaina, J., et al. (2010). Diversity of glycosyl hydrolases from cellulose depleting communities enriched from casts of two earthworm species. *Applied and Environmental Microbiology, 76*, 5934–5946.

21. Voget, S., Leggewie, C., Uesbeck, A., Raasch, C., Jaeger, K. E., & Streit, W. R. (2003). Prospecting for novel biocatalysts in a soil metagenome. *Applied and Environmental Microbiology, 69*, 6235–6242.

22. Yun, J., Kang, S., Park, S., Yoon, H., Kim, M. J., Heu, S., & Ryu, S. (2004). Characterization of a novel amylolytic enzyme encoded by a gene from a soil-derived metagenomic library. *Applied and Environmental Microbiology, 70*, 7229–7235.

23. Elend, C., Schmeisser, C., Leggewie, C., Babiak, P., Carballeira, J. D., Steele, H. L., et al. (2006). Isolation and biochemical characterization of two novel metagenome-derived esterases. *Applied and Environmental Microbiology, 72*, 3637–3645.

24. Pereira, M. R., Mercaldi, G. F., Maester, T. C., Balan, A., & de Macedo Lemos, E. G. (2015). Est16, a new esterase isolated from a metagenomic library of a microbial consortium specializing in diesel oil degradation. *PloS One, 10*(7), e0133723.

25. Williamson, L. L., Borlee, B. R., Schloss, P. D., Guan, C., Allen, H. K., & Handelsman, J. (2005). Intracellular screen to identify metagenomic clones that induce or inhibit a quorum-sensing biosensor. *Applied and Environmental Microbiology, 71*, 6335–6344.

26. Gabor, E. M., Alkema, W. B., & Janssen, D. B. (2004). Quantifying the accessibility of the metagenome by random expression cloning techniques. *Environmental Microbiology, 6*, 879–886.

27. Martinez, A., Kolvek, S. J., Yip, C. L., Hopke, J., Brown, K. A., MacNeil, I. A., & Osburne, M. S. (2004). Genetically modified bacterial strains and novelbacterial artificial chromosome shuttle vectors for

constructing environmentallibraries and detecting heterologous natural products in multiple expression hosts. *Applied and Environmental Microbiology, 70*, 2452–2463.

28. Craig, J. W., Chang, F. Y., Kim, J. H., Obiajulu, S. C., & Brady, S. F. (2010). Expanding small-molecule functional metagenomics through parallel screening of broad-host-range cosmid environmental DNA libraries in diverse proteobacteria. *Applied and Environmental Microbiology, 76*, 1633–1641.

29. Lee, H. S., Kwon, K. K., Kang, S. G., Cha, S. S., Kim, S. J., & Lee, J. H. (2010). Approaches for novel enzyme discovery from marine environments. *Current Opinion in Biotechnology, 21*, 353–357.

30. Hjort, K., Bergström, M., Adesina, M. F., Jansson, J. K., Smalla, K., & Sjöling, S. (2010). Chitinase genes revealed and compared in bacterial isolates, DNA extracts and a metagenomic library from a phytopathogen suppressive soil. *FEMS Microbiology Ecology, 71*, 197–207.

31. Schmidt, O., Drake, H. L., & Horn, M. A. (2010). Hitherto unknown [Fe-Fe]-hydrogenase gene diversity in anaerobes and anoxic enrichments from a moderately acidic fen. *Applied and Environmental Microbiology, 76*, 2027–2031.

32. Villamizar, G. A., Nacke, H., & Daniel, R. (2017). Function-based metagenomic library screening and heterologous expression strategy for genes encoding phosphatase activity. *Methods in Molecular Biology, 1539*, 249–260.

33. Knietsch, A., Bowien, S., Whited, G., Gottschalk, G., & Daniel, R. (2003). Identification and characterization of coenzyme B12-dependent glycerol dehydratase- and dioldehydratase-encoding genes from metagenomic DNA libraries derived from enrichment cultures. *Applied and Environmental Microbiology, 69*, 3048–3060.

34. Li, M., Hong, Y., Klotz, M. G., & Gu, J. D. (2010). A comparison of primer sets for detecting 16S rRNA and hydrazine oxidoreductase genes of anaerobic ammonium-oxidizing bacteria in marine sediments. *Applied Microbiology and Biotechnology, 86*, 781–790.

35. Tyson, G. W., Chapman, J., Hugenholtz, P., Allen, E. E., Ram, R. J., Richardson, P. M., Solovyev, V. V., Rubin, E. M., Rokhsar, D. S., & Banfield, J. F. (2004). Community structure and metabolism through reconstruction of microbialgenomes from the environment. *Nature, 428*, 37–43.

36. Warnecke, F., Luginbühl, P., Ivanova, N., Ghassemian, M., Richardson, T. H., & Stege, J. T. (2007). Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. *Nature, 450*, 560–565.

37. Venter, J. C., Remington, K., Heidelberg, J. F., Halpern, A. L., Rusch, D., Eisen, J. A., et al. (2004). Environmental genome shotgun sequencing of the Sargasso Sea. *Science, 304*, 66–74.

38. Hess, M., Sczyrba, A., Egan, R., Kim, T. W., Chokhawala, H., Schroth, G., et al. (2011). Metagenomic discovery of biomass-degrading genes and genomes from cow rumen. *Science, 331*, 463–467.

39. Weinstock, G. M. (2012). Genomic approaches to studying the human microbiota. *Nature, 489*, 250–256.

40. Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S. M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J. A., Smith, G., & Knight, R. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME Journal, 6*, 1621–1624.

41. Loman, N. J., Misra, R. V., Dallman, T. J., Constantinidou, C., Gharbia, S. E., Wain, J., & Pallen, M. J. (2012). Performance comparison of bench top high throughput sequencing platforms. *Nature Biotechnology, 30*, 434–439.

42. Gans, J., Wolinsky, M., & Dunbar, J. (2005). Computational improvements reveal great bacterial diversity and high metal toxicity in soil. *Science, 309*, 1387–1390.

43. Kuczynski, J., Lauber, C. L., Walters, W. A., Parfrey, L. W., Clemente, J. C., Gevers, D., & Knight, R. (2012). Experimental and analytical tools for studying the human microbiome. *Nature Review in Genetics, 13*, 47–58.

44. Margulies, M., Egholm, M., Altman, W. E., Attiya, S., Bader, J. S., Bemben, L. A., et al. (2005). Genome sequencing in microfabricated high-density picolitre reactors. *Nature, 2005*(437), 376–380.

45. Wheeler, D. A., Srinivasan, M., Egholm, M., Shen, Y., Chen, L., McGuire, A., et al. (2008). The complete genome of an individual by massively parallel DNA sequencing. *Nature, 452*, 872–876.

46. Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology, 75*, 7537–7541.

47. Schmieder, R., & Edwards, R. (2011). Quality control and preprocessing of metagenomic datasets. *Bioinformatics, 27*, 863–864.

48. Huson, D. H., & Weber, N. (2013). Microbial community analysis using MEGAN. *Methods in Enzymology, 531*, 465–485.

49. Paulson, J. N., Stine, O. C., Bravo, H. C., & Pop, M. (2013). Differential abundance analysis for microbial marker-gene surveys. *Nature Methods, 10*, 1200–1202.

50. Liu, B., & Pop, M. (2011). MetaPath: identifying differentially abundant metabolic pathways in metagenomic datasets. *BMC Proceedings, 5*, S9.

51. Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research, 25*, 3389–3402.

52. McMurdie, P. J., & Holmes, S. (2014). Shiny-phyloseq: web application for interactive microbiome analysis with provenance tracking. *Bioinformatics, 31*, 282–283.

53. Meyer, F., Paarmann, D., D'Souza, M., Olson, R., Glass, E. M., Kubal, M., et al. (2008). The metagenomics RAST server—a public resource for the automatic phylogenetic and functional analysis of metagenomes. *BMC Bioinformatics, 9*, 386.

54. Su, X., Pan, W., Song, B., Xu, J., & Ning, K. (2014). Parallel-META 2.0: enhanced metagenomic data analysis with functional annotation, high performance computing and advanced visualization. *PloS One, 9*, e89323.

55. Krause, L., Diaz, N. N., Goesmann, A., Kelley, S., Nattkemper, T. W., Rohwer, F., et al. (2008). Phylogenetic classification of short environmental DNA fragments. *Nucleic Acids Research, 36*, 2230–2239.

56. Medema, M. H., Blin, K., Cimermancic, P., de Jager, V., Zakrzewski, P., Fischbach, M. A., et al. (2011). antiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. *Nucleic Acids Research, 39*, W339–W346.

57. Fajardo, J. E., & Fiser, A. (2013). Protein structure based prediction of catalytic residues. *BMC Bioinformatics, 14*, 63.

58. Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., & Henrissat, B. (2009). The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Research, 37*, D233–D238.

59. Sirim, D., Wagner, F., Wang, L., Schmid, R.D., & Pleiss, J. (2011). The Laccase Engineering Database: a classification and analysis system for laccases and related multicopper oxidases. *Database (Oxford) 2011*, bar006. https://doi.org/10.1093/database/bar006.

60. Widmann, M., & Pleiss, J. (2014). Protein variants form a system of networks: microdiversity of IMP metallo-beta-lactamases. *PloS One, 9*, e101813.

61. Najah, M., Mayot, E., Mahendra-Wijaya, I. P., Griffiths, A. D., Ladame, S., & Drevelle, A. (2013). New glycosidase substrates for droplet-based microfluidic screening. *Annals of Chemistry, 85*, 9807–9814.

62. Meier, M. J., Paterson, E. S., & Lambert, I. B. (2015). Use of substrate-induced gene expression in metagenomic analysis of an aromatic hydrocarbon-contaminated soil. *Applied and Environmental Microbiology, 82*, 897–909.

63. Vidal-Melgosa, S., Pedersen, H. L., Schückel, J., Arnal, G., Dumon, C., Amby, D. B., et al. (2015). A new versatile microarray-based method for high throughput screening of carbohydrate-active enzymes. *Journal of Biological Chemistry, 290*, 9020–9036.

64. Colin, P. Y., Kintses, B., Gielen, F., Miton, C. M., Fischer, G., Mohamed, M. F., Hyvönen, M., Morgavi, D. P., Janssen, D. B., & Hollfelder, F. (2015). Ultrahigh-throughput discovery of promiscuous enzymes by picodroplet functional metagenomics. *Nature Communications, 7*(6), 10008.

65. He, S., Kunin, V., Haynes, M., Martin, H. G., Ivanova, N., Rohwer, F., et al. (2010). Metatranscriptomic array analysis of 'Candidatus Accumulibacter phosphatis'-enriched enhanced biological phosphorus removal sludge. *Environmental Microbiology, 12*, 1205–1217.

66. Frias-Lopez, J., Shi, Y., Tyson, G. W., Frias-Lopez, J., Shi, Y., Tyson, G. W., Coleman, M. L., Schuster, S. C., Chisholm, S. W., & Delong, E. F. (2008). Microbial community gene expression in ocean surface waters. *Proceedings of the National Academy of Sciences of the United States of America, 105*, 3805–3810.

67. Zhou, J., He, Z., Yang, Y., Deng, Y., Tringe, S. G., & Alvarez-Cohen, L. (2015). Highthroughput metagenomic technologies for complex microbial community analysis: open and closed formats. *MBio, 6*(1), e02288–e02214.

68. Leininger, S., Urich, T., Schloter, M., Schwark, L., Qi, J., Nicol, G. W., et al. (2006). Archaea predominate among ammonia-oxidizing prokaryotes in soils. *Nature, 442*, 806–809.

69. Shi, Y., Tyson, G. W., & DeLong, E. F. (2009). Metatranscriptomics reveals unique microbial small RNAs in the ocean's water column. *Nature, 459*, 266–269.

70. Zampieri, E., Chiapello, M., Daghino, S., Bonfante, P., & Mello, A. (2016). Soil metaproteomics reveals an inter-kingdom stress response to the presence of black truffles. *Scientific Reports, 6*, 25773.

71. Ram, R. J., Verberkmoes, N. C., Thelen, M. P., Tyson, G. W., Baker, B. J., Blake, R. C., et al. (2005). Community proteomics of a natural microbial biofilm. *Science, 308*, 1915–1920.

72. Kleiner, M., Wentrup, C., Lott, C., Teeling, H., Wetzel, S., Young, J., Chang, Y. J., Shah, M., VerBerkmoes, N. C., et al. (2012). Metaproteomics of a gutless marine worm and its symbiotic microbial community reveal unusual pathways for carbon and energy use. *Proceedings of the National Academy of Sciences of the United States of America, 109*, E1173–E1182.

73. Tanca, A., Manghina, V., Fraumene, C., Palomba, A., Abbondio, M., Deligios, M., Silverman, M., & Uzzau, S. (2017). Metaproteogenomics reveals taxonomic and functional changes between cecal and fecal microbiota in mouse. *Frontiers in Microbiology, 14*(8), 391.

74. Gillan, D. C., Roosa, S., Kunath, B., Billon, G., & Wattiez, R. (2015). The long-term adaptation of bacterial communities in metal-contaminated sediments: a metaproteogenomic study. *Environmental Microbiology, 17*(6), 1991–2005.

75. Seifert, J., Herbst, F. A., Halkjaer Nielsen, P., Planes, F. J., Jehmlich, N., Ferrer, M., & von Bergen, M. (2013). Bioinformatic progress and applications in metaproteogenomics for bridging the gap between genomic sequences and metabolic functions in microbial communities. *Proteomics, 13*(18–19), 2786–2804.

76. Wang, C., Dong, D., Wang, H., Müller, K., Qin, Y., Wang, H., & Wu, W. (2016). Metagenomic analysis of microbial consortia enriched from compost: new insights into the role of Actinobacteria in lignocellulose decomposition. *Biotechnology for Biofuels, 29*(9), 22.

77. Fang, Z., Li, T., Wang, Q., Zhang, X., Peng, H., Fang, W., et al. (2010). A bacterial laccase from marine microbial metagenome exhibiting chloride tolerance and dye decolorization ability. *Applied Microbiology and Biotechnology, 89*, 1103–1110.

78. Ferrer, M., Beloqui, A., & Golyshin, P. N. (2010). Screening metagenomic libraries for laccase activities. *Methods in Molecular Biology, 668*, 189–202.

79. Brennan, Y., Callen, W. N., Christoffersen, L., Dupree, P., Goubet, F., Healey, S., et al. (2004). Unusual microbial xylanases from insect guts. *Applied and Environmental Microbiology, 70*, 3609–3617.

80. Lee, C., Kibblewhite-Accinelli, R., Wagschal, K., Robertson, G., & Wong, D. (2006). Cloning and characterization of a cold-active xylanase enzyme from an environmental DNA library. *Extremophiles, 10*, 295–300.

81. Hu, Y., Zhang, G., Li, A., Chen, J., & Ma, L. (2008). Cloning and enzymatic characterization of a xylanase gene from a soil-derived metagenomic library with an efficient approach. *Applied Microbiology and Biotechnology, 80*, 823–830.

82. Liu, N., Yan, X., Zhang, M., Xie, L., Wang, Q., Huang, Y., et al. (2011). Microbiome of fungus-growing termites: a new reservoir for lignocellulase genes. *Applied and Environmental Microbiology, 77*(1), 48–56.

83. Fang, Z., Fang, W., Liu, J., Hong, Y., Peng, H., Zhang, X., et al. (2010). Cloning and characterization of a beta-glucosidase from marine microbial metagenome with excellent glucose tolerance. *Journal of Microbiology and Biotechnology, 20*, 1351–1358.

84. Duan, C. J., Xian, L., Zhao, G. C., Feng, Y., Pang, H., Bai, X. L., et al. (2009). Isolation and partial characterization of novel genes encoding acidic cellulases from metagenomes of buffalo rumens. *Journal of Applied Microbiology, 107*, 245–256.

85. Henne, A., Schmitz, R. A., Bomeke, M., Gottschalk, G., & Daniel, R. (2000). Screening of environmental DNA libraries for the presence of genes conferring lipolytic activity on *Escherichia coli. Applied and Environmental Microbiology, 66*, 3113–3116.

86. Ferrer, M., Golyshina, O. V., Chernikova, T. N., Khachane, A. N., Martins Dos Santos, V. A., Yakimov, M. M., et al. (2005). Microbial enzymes mined from the Urania deep-sea hypersaline anoxic basin. *Chemical Biology, 12*, 895–904.

87. Rhee, J. K., Ahn, D. G., Kim, Y. G., & Oh, J. W. (2005). New thermophilic and thermostable esterase with sequence similarity to the hormone-sensitive lipase family, cloned from a metagenomic library. *Applied and Environmental Microbioogy, 71*, 817–825.

88. Palackal, N., Lyon, C. S., Zaidi, S., Luginbuhl, P., Dupree, P., Goubet, F., et al. (2007). A multi-functional hybrid glycosyl hydrolase discovered in an uncultured microbial consortium from ruminant gut. *Applied Microbiology. Biotechnology, 74*, 113–124.

89. Yao, J., Chen, Q., Zhong, G., Cao, W., Yu, A., & Liu, Y. (2014). Immobilization and characterization of tannase from a metagenomic library and its use for removal of tannins from green tea infusion. *Journal of Microbiology and Biotechnology, 24*, 80–86.

90. Neveu, J., Regeard, C., & Dubow, M. S. (2011). Isolation and characterization of two serine proteases from metagenomic libraries of the Gobi and Death Valley deserts. *Applied Microbiology and Biotechnology, 91*, 635–644.

91. Martina, M., Bivera, S., Steels, S., Barbeyronb, T., Jamb, M., Portetelle, D., Michelb, G., & Vandenbo, M. (2014). Functional screening of a metagenomic library of seaweed-associated microbiota: identification and characterization of a halotolerant, cold active marine endo-β-1,4-endoglucanase. *Applied and Environmental Microbiology, 80*(16), 4958–4967.

92. Hua, M., Zhao, S., Zhang, L., Liu, D., Xia, H., Li, F., & Chen, S. (2015). Direct detection, cloning and characterization of a glucoside hydrolase from forest soil. *Biotechnology Letters, 37*(6), 1227–1232.

93. Fang, Z., Li, T., Wang, Q., Zhang, X., Peng, H., Fang, W., et al. (2011). A bacterial laccase from marine microbial metagenome exhibiting chloride tolerance and dye decolorization ability. *Applied Microbiology and Biotechnology, 89*, 1103–1110.

94. Gomes-Pepe, E. S., Machado Sierra, E. G., Pereira, M. R., Castellane, T. C. L., & Lemos, E. G. M. (2016). Bg10: a novel metagenomics alcohol-tolerant and glucose-stimulated GH1 β-glucosidase suitable for lactose-free milk preparation. *PloS One, 11*(12), e0167932. https://doi.org/10.1371/journal.pone.0167932.

95. Pottkamper, J., Barthen, P., Ilmberger, N., Schwaneberg, U., Schenk, A., Schulte, M., et al. (2009). Applying metagenomics for the identification of bacterial cellulases that are stable in ionic liquids. *Green Chemistry, 11*, 957–965.

96. Kim, E. Y., Oh, K. H., Lee, M. H., Kang, C. H., Oh, T. K., & Yoon, J. H. (2009). Novel cold-adapted alkaline lipase from an intertidal flat metagenome and proposal for a new family of bacterial lipases. *Applied and Environmental Microbiology, 75*, 257–260.

97. Peng, Q., Wang, X., Shang, M., Huang, J., Guan, G., Li, Y., et al. (2014). Isolation of a novel alkaline-stable lipase from a metagenomic library and its specific application form ilk fat flavour production. *Microbial Cell Factories, 13*, 1.

98. Wang, G., Wang, Q., Lin, X., Ng, T. B., Yan, R., Lin, J., & Ye, X. (2016). A novel cold-adapted and highly salt-tolerant esterase from *Alkalibacterium* sp. SL3 from the sediment of a soda lake. *Scientific Reports, 6*, 19494.

99. Biver, S., Portetelle, D., & Vandenbol, M. (2013). Characterization of a new oxidant-stable serine protease isolated by functional metagenomics. *Springer Plus, 2*, 410.

100. Pushpam, P. L., Rajesh, T., & Gunasekaran, P. (2011). Identification and characterization of alkaline serine protease from goat skin surface metagenome. *AMB Express, 1*, 3.

101. Wang, K., Lu, Y., Liang, W. Q., Wang, S. D., Jiang, Y., Huang, R., et al. (2012). Enzymatic synthesis of galacto-oligosaccharides in an organic- aqueous biphasic system by a novel beta-galactosidase from a metagenomic library. *Journal of Agricultural and Food Chemistry, 60*, 3940–3946.

102. Song, Q., & Zhang, X. (2008). Characterization of a novel non-specific nuclease from thermophilic bacteriophage GBSV1. *BMC Biotechnology, 8*, 43.

103. de Pascale, D., Cusano, A. M., Autore, F., Parrilli, E., di Prisco, G., Marino, G., & Tutino, M. L. (2008). The cold-active Lip1 lipase from the Antarctic bacterium *Pseu*member of a new bacterial lipolytic *doalteromonas haloplanktis* TAC125 is a member of a new bacterial lipolytic enzyme family. *Extremophiles, 12*(3), 311–323.

104. Nishihara, M., Kamata, M., Koyama, T., & Yazawa, K. (2008). New phospholipaseA1-producing bacteria from a marine fish. *Marine Biotechnology, 10*(4), 382–387.

105. Yoon, S. A., Ryu, S. I., Lee, S. B., & Moon, T. W. (2008). Purification and characterization of branching specificity of a novel extracellular amylolytic enzyme from marine hyperthermophilic *Rhodothermus marinus*. *Journal of Microbiology and Biotechnology, 18*(3), 457–464.

106. Fu, X. T., Lin, H., & Kim, S. (2008). Purification and characterization of a novel beta-agarase, AgaA34, from *Agarivoransalbus* YKW. *Applied Microbiology and Biotechnology, 78*(2), 265–273.

107. Cipolla, A., Delbrassine, F., DaLage, J. L., & Feller, G. (2012). Temperature adaptations in psychrophilic, mesophilic and thermophilic chloride-dependent alpha-amylases. *Biochimie, 94*, 1943–1950.

108. Florczak, T., Daroch, M., Wilkinson, M. C., Bialkowska, A., Bates, A. D., Turkiewicz, M., et al. (2013). Purification, characterisation and expression in *Saccharomyces cerevisiae* of Lip G7 an enantioselective, cold-adapted lipase from the Antarctic filamentous fungus *Geomyces* sp. P7 with unusual thermo-stability characteristics. *Enzyme and Microbial Technology, 53*, 18–24.

109. Liszka, M. J., Clark, M. E., Schneider, E., & Clark, D. S. (2012). Nature versus nurture: developing enzymes that function under extreme conditions. *Annual Reviews in Chemistry and Biomolecular Engineering, 3*, 77–102.

110. Jeon, J. H., Kim, J. T., Kim, Y. J., Kim, H. K., Lee, H. S., Kang, S. G., et al. (2009). Cloning and characterization of a cold-active lipase from a deep-sea sediment metagenome. *Applied Microbiology and Biotechnology, 81*, 865–874.

111. Kobayashi, T., Koide, O., Mori, K., Shimamura, S., Matsuura, T., et al. (2008). Phylogenetic and enzymatic diversity of deep subseafloor aerobic microorganisms in organics- and methane-rich sediments off Shimokita Peninsula. *Extremophiles, 12*(4), 519–527.

112. Fu, J., Leiros, H. K., DePascale, D., Johnson, K. A., Blencke, H. M., & Landfald, B. (2013). Functional and structural studies of a novel cold-adapted esterase from an Arctic intertidal metagenomic library. *Applied Microbiology and Biotechnology, 97*, 3965–3978.

113. Lee, M. H., Oh, K. H., Kang, C. H., Kim, J. H., Oh, T. K., Ryu, C. M., et al. (2012). Novel metagenome-derived, cold adapted alkaline phospholipase with superior lipase activity as an intermediate between phospholipase and lipase. *Applied Microbiology and Biotechnology, 78*, 4959–4966.

114. Zhang, Y., Hao, J., Zhang, Y., Chen, X., Xie, B., Shi, M., Zhou, B., Zhang, Y., & Li, P. (2017). Identification and characterization of a novel salt-tolerant esterase from the deep-sea sediment of the South China Sea. *Frontiers in Microbiology, 8*, 441.

115. Gao, W., Wu, K., Chen, L., Fan, H., Zhao, Z., Gao, B., Wang, H., & Wei, D. (2016). A novel esterase from a marine mud metagenomic library for biocatalytic synthesis of short-chain flavour esters. *Microbial Cell Factory, 15*, 41.

116. Nair, H. P., Vincent, H., Puthusseri, R. M., & Bhat, S. G. (2017). Molecular cloning and characterization of a halotoleranta-amylase from marine metagenomic library derived from Arabian Sea sediments. *Biotech, 3*(7), 65.

117. Schroder, C., Elleuche, S., Blank, S., & Antranikian, G. (2014). Characterization of a heat-active archaeal beta glucosidase from a hydrothermal spring metagenome. *Enzyme and Microbial Technology, 57*, 48–54.
118. Fang, Z. M., Li, T. L., Chang, F., Zhou, P., Fang, W., Hong, Y. Z., et al. (2012). A new marine bacterial laccase with chloride-enhancing, alkaline-dependent. Activity and dye decolorization ability. *Bioresource Technology, 111*, 36–41.
119. Olivera, N. L., Sequeiros, C., & Nievas, M. L. (2007). Diversity and enzyme properties of protease-producing bacteria isolated from sub-Antarctic sediments of Isla de Los Estados, Argentina. *Extremophiles, 11*, 517–526.
120. Collins, T., Meuwis, M. A., Stals, I., Claeyssens, M., Feller, G., & Gerday, C. (2002). A novel family 8 xylanase, functional and physic-chemical characterization. *Journal of Biological Chemistry, 277*, 35133–35139.
121. Hobel, C. F., Hreggvidsson, G. O., Marteinsson, V. T., Bahrani-Mougeot, F., Einarsson, J. M., & Kristjansson, J. K. (2005). Cloning, expression, and characterization of a highly thermo-stable family 18 chitinase from *Rhodothermus marinus*. *Extremophiles, 9*(1), 53–64.
122. Wierzbicka-Wos, A., Bartasun, P., Cieslinski, H., & Kur, J. (2013). Cloning and characterization of a novel cold-active glycoside hydrolase family enzyme, with beta-glucosidase, beta-fucosidase and beta-galactosidase activities. *BMC Biotechnology, 13*, 22.
123. Aurilia, V., Parracino, A., & D'Auria, S. (2008). Microbial carbohydrate esterases in cold adapted environments. *Gene, 410*, 234–240.
124. Huston, A. L., Methe, B., & Deming, J. W. (2004). Purification, characterization, and sequencing of an extracellular cold-active aminopeptidase produced by marine psychrophile Colwellia psychrerythraea strain 34H. *Applied and Environmental Microbiology, 70*, 3321–3328.
125. Fernández-Arrojo, L., Guazzaroni, M. E., López-Cortés, N., Beloqui, A., & Ferrer, M. (2010). Metagenomic era for biocatalyst identification. *Current Opinion in Biotechnology, 21*, 725–733.
126. Spickermann, D., Kara, S., Barackov, I., Hollmanns, F., Schwaneberg, U., Duenkelmanns, P., & Leggewie, C. (2014). Alcohol dehydrogenase stabilization by additives under industrially relevant reaction conditions. *Journal of Molecular Catalysis and Enzymes, 103*, 24–28.