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Review

Cold-environment metagenomics for discovering novel microorganisms and enzymes

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The environmental conditions on planet earth are extremely diverse, with enormous variations in pressure, pH, temperature and salt concentration. All these environments are inhabited by living organisms, particularly microorganisms which have adapted to the different extremes of environments. Among various extreme environments, low temperature is very common both in natural and man-made environments. Low temperature environment is a source for the isolation of cold adapted enzymes of commercial and industrial importance. In the recent past, metagenomics have been employed as a powerful approach for the isolation of novel genes, enzymes and bioactive molecules from the yet to be cultured component of microbial communities prevailing in various environments.

Key words: Metagenomics, cold environment, microorganism, enzyme.

INTRODUCTION

Microbial communities play a significant role for maintaining the ecological balance in any ecosystem. During the long journey of evolution, they have undergone changes at different levels for adaptation and thus show huge genetic diversity for exploration. A major part of the earth's surface has conditions which are extreme owing to one or the other environmental factor. Low temperature is very common among the extreme environments, both natural and man-made. About 80% of the biosphere and more than 90% of marine environments have temperature lower than 5°C (Margesin and Schinner, 1994; Brenchley, 1996). Extremely low temperature environments are generally inhabited by the cold adapted microorganisms which have the ability to grow and survive under harsh conditions. These cold adapted microorganisms, known as psychrophiles and psychrotrophs are excellent source of cold-active enzymes and biocatalysts which have great potential for industrial and biotechnological applications. But one of the limiting factors for isolation and identification of novel microorganisms, genes, enzymes, biocatalysts, bioactive molecules present in an environmental sample is that only a small fraction 0.1 to 1% of all kind of microorganisms present can be cultured by employing traditional or conventional laboratory methods (Amann et al., 1995). To conquer the problems and limitations associated with cultivation techniques the community genomes of all the microorganisms present in a given niche can be extracted and exploited by an approach called metagenomics (Handelsman et al., 1998). For quantitative description of microbial communities present in any environmental samples the application of metagenomics has been proposed to be the most accurate method (von Mering et al., 2007). This review highlights the recent studies on the diversity of unculturable microbial communities from cold environments and recent leads for discoveries of enzymes from cold areas using metagenomics approach.

BASICS OF METAGENOMICS

The basic steps involved in constructing and exploiting a metagenomics library are outlined in Figure 1. The environmental sample from cold environment (soil or ice core) is collected and then processed for isolation of total community DNA. The isolated DNA is called metagenome. This metagenome can be either used directly for sequencing or can be cloned into suitable vector to generate the metagenomic library or can be amplified by using universal primers to generate 16S rRNA gene library. For cloning the most frequently used

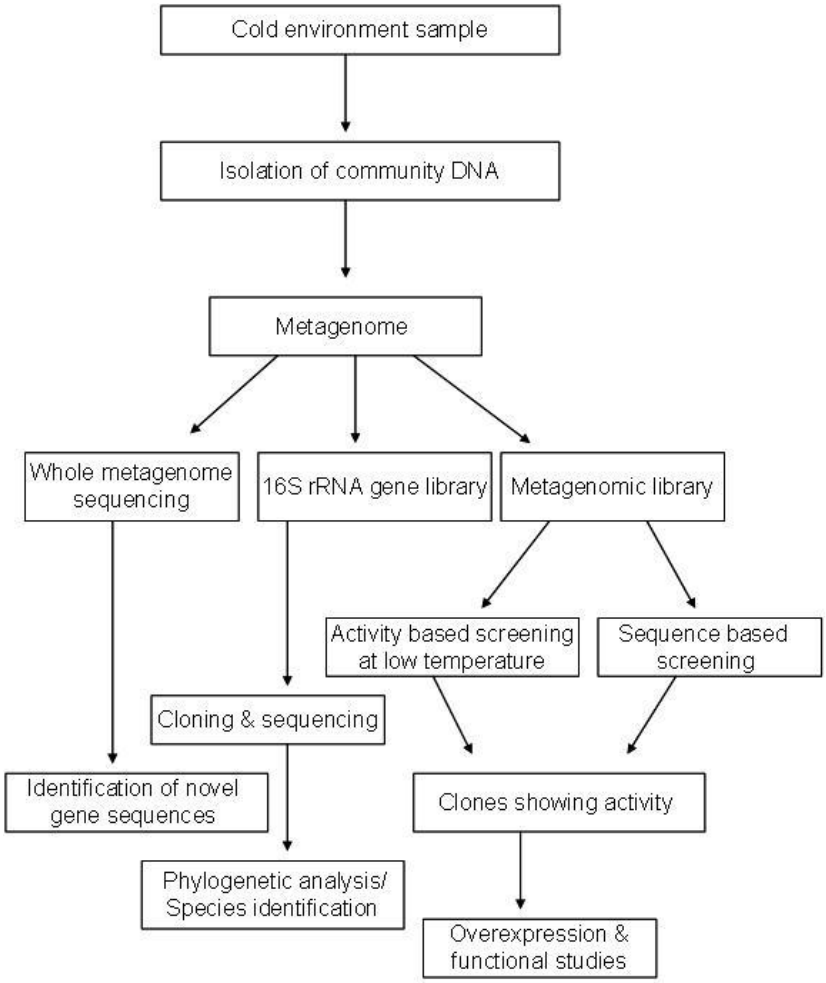

Fig 1. Metagenomics approach for identification of novel genes and enzymes from cold environment

Figure 1. Metagenomics approach for identification of novel genes and enzymes from cold environment.

vectors are the plasmids, but for large gene clusters other vectors, such as bacterial artificial chromosome (BAC), cosmid, and fosmid are being used. The library is then transferred to *Escherichia coli* as the most common host strain. Other hosts including *Pseudomonas putida, Streptomyces lividans, Rhizobium leguminosarum* and *Pichia pastoris* have also been used for cloning and heterologous expression. These clones can then be used for sequencing or screening for enzymes and secondary metabolite or bioactive molecules synthesized by the DNA fragment from cold environment samples. If high-throughput screens are available for the enzyme or biocatalyst one is looking for, then this method is enormously useful for isolating novel enzymes or biocatalyst from nonculturable microorganisms. For some of enzymes high-throughput screens are available but for many they need to be developed. Time has come where emphasis should be on developing fast, reliable and sensitive screens for more enzymes and biocatalysts for exploiting the metagenomics libraries for human benefit.

COLD ENVIRONMENT METAGENOMICS FOR EXPLORING PHYLOGENETIC DIVERSITY

The 16SrRNA gene is highly conserved between different species of bacteria and hence used for phylogenetic studies. Bacterial diversity and the abundance in cold environments for cultureable and nonculturable (metagenomic approach) have been described using 16S rRNA gene based analyses (Cheng and Foght, 2007; Simon et al., 2009a; Salwan et al., 2010; Kim et al., 2010; Shivaji et al., 2010).

The studies on screening of 16S rDNA clone library from permanently cold marine sediments by dot blot hybridization with group-specific oligonucleotide probes suggested the predominance of sequences related to δ-*Proteobacteria* (sulfate reducers), followed by the clones distantly related to sulfur-oxidizing symbiotic or free-living bacteria of the γ subclass of *Proteobacteria* (Ravenschlag et al., 1999). The bacterial diversity in two moraine lakes and two glacial meltwaters in the remote

Mount Everest region through 16S rRNA gene library showed the 16S rRNA gene sequences belonging to the groups: *Proteobacteria, Cytophaga–Flavobacteria–Bacteroides, Actinobacteria, Planctomycetes, Verrucomicrobia, Fibrobacteres* and Eukaryotic chroloplast (Liu et al., 2006). The bacterial communities present in three subglacial samples were examined by amplifying 16S rRNA genes extracted from community DNA and from pure cultures. One sample had *Betaproteobacteria* (particularly *Comamonadaceae*), *Bacteroidetes* (particularly *Flavobacterium*) and *Actinobacteria,* while other two sample had *Betaproteobacteria*, and *Bacteroidetes.* Whereas in case of pure culture the group comprising *Bacteroidetes* (predominantly *Flavobacterium*), *Betaproteobacteria* (particularly *Comamonadaceae*), *Actinobacteria* were detected (Cheng and Foght, 2007). The phylogenetic diversity of microbial community present in a glacial ice sample was assessed by three approaches: analysis of a constructed 16S rRNA gene library, identification and classification of 16S rRNA gene sequences in the pyrosequencing- derived data set, and evaluation of this data set based on similarities to conserved proteins families and domains using the CARMA algorithm. The dominant phylogenetic groups established by all the three approaches were the phylum *Proteobacteria,* the class *Betaproteobacteria*, the family *Comamonadaceae* which includes many genera. The second most abundant phylogenetic group was the phylum *Bacteroidetes* followed by phylum *Actinobacteria* (Simon et al., 2009b). The nearest neighbors were 16S rRNA gene sequences of uncultured microorganisms from cold habitats as well as cultured genera found in permanently cold environments. The bacterial diversity analysis in the snow of the four glaciers Guoqu, Zadang, East Rongbuk and Palong No. 4 located in different climatic zones of the Tibetan Plateau using culture-independent molecular analysis of 16S rRNA gene clone library showed differences in the phyla present which were determined by surrounding environment. At Guoqu, members of *Bacteroidetes* and *β-Proteobacteria* were dominant, whereas at Zadang, the clones were affiliated to α-*, β*-and *γ-Proteobacteria*, *Actinobacteria* and *Bacteroidetes*. At East Rongbuk, the *γ-Proteobacteria* group was the most abundant, whereas at Palong No. 4, *α - Proteobacteria* and *Actinobacteria* were dominant (Liu et al., 2009). Bacterial diversity of three soil samples in the vicinity of Pindari glacier yielded *Actinobacteria, Firmicutes* and *Proteobacteria* as common phyla in all the three libraries. In addition to the two samples shared by phyla *Acidobacteria, Bacteroidetes, Gemmatimonadetes* and *Planctomycetes*, phyla *Chlamydiae, Chlorobi, Chloroflexi, Dictyoglomi, Fibrobacteres, Nitrospirae, Verrucomicrobia*, candidate division SPAM and candidate TM7s TM7a phylum were all present only in the third sample (Shivaji et al., 2010).

The type of microbial diversity in any cold location is generally influenced by the type of nutrients and environmental conditions prevailing in the near by area. During the metagenomics studies on bacterial diversity of the Tibetan Plateau it was reported that the Guoqu Glacier at the north of the Tanggula mountains is nearer to the desert zone with the lowest temperature and have more bacteria related to genera *Hymenobacter, Arthrobacter,* and *Polaromonas,* those are soil bacteria having strains isolated from cold environments. Whereas, in the Palong No. 4 Glacier located at the south warm region around vegetation, more bacteria were related to plant-associated general *Mesorhizobium, Pannonibacter, Phyllobacterium,* and *Rhizobium* only occurred in this glacier and only fewer bacteria were similar to psychrophilic or psychrotrophic bacteria. In the East Rongbuk Glacier there is less soil dust input and the abundance of soil bacteria was the low and most of them were related to aquatic bacteria may be due to the influence of the Indian monsoon and the number of lakes at the end of the glacier (Liu et al., 2009). Hence the selection of site/niche is of great concern while searching for new and novel microorganisms or enzymes. For isolation of psychrophilic microorganisms or cold adapted gene or enzymes the permanently cold environments/glaciers surrounded by low temperature environments should be explored. Sampling methods, transportation under low temperature, storage time before processing the sample for isolation of microorganisms also play a significant role in microbial diversity studies.

COLD ENVIRONMENT METAGENOMICS AS SOURCE OF NOVEL ENZYME

In the last 3 to 4 decades lot of emphasis have been given to the study of extremophilic microorganisms which thrive under different kinds of extreme and represent the vast diversity of unexplored genetic pool. The cold environments are inhabited by cold adapted microorganisms known as psychrophiles and psychrotrophs which are taxonomically, biochemically, physiologically and metabolically diverse. These cold adapted bacteria are vital to the biogeochemistry and functioning of all ecosystems prevailing under low temperature environments. The cold environments are extremely diverse and the microorganisms are exposed to extremes in temperature, pressure, light and nutrient availability. These cold environments are likely to possess highly different microbial communities with potentially unique biochemical and molecular profile depending on the location. The microbial enzymes from such environments are supposed to have quite diverse biochemical, physiological and molecular properties, which have allowed the adaptation and survival of microbial communities to such harsh conditions. There is great potential for the discovery of new and novel enzymes

Table 1. Enzymes discovered through metagenomic from cold environments.

Enzyme activity	Habitat	Screening	Temperature optima (°C)	pH optima	Mol weight (kDa)	References
β-Galactosidase	Daqing oil field	Activity based	38	7.0	78.6	(Wang et al., 2010)
Esterase	Deep-sea sediment	Activity based	50–55	10.0–11.0	28.9	(Park et al., 2007)
Lipase	Oil contaminated soil	Activity based	30	7.0	53.2	(Elend et al., 2007)
Lipase	Activated sludge	Activity based	10	7.5	35.6	(Roh and Villatte, 2008)
Lipase	Mangrove sediment	Activity based	35	8.0	32.0	(Couto et al., 2010)
Lipase	Baltic sea sediment	Activity based	35	---	35.4	(Hardeman and Sjoling, 2007)
Amylase	Soil	Activity based	35-40	6.5	38.0	(Sharma et al., 2010)
Xylanase	Waste lagoon	Activity based	20	7.0	45.9	(Lee et al., 2006)
Xylanase	Goat rumen contents	Sequence based	30	6.5	52.0	(Wang et al., 2011)
Lipase	Deep-sea sediment	Activity based	25	8.0	33.6	(Jeon et al., 2009a)
Lipase	Intertidal flat sediments	Activity based	30	8.0	---	(Kim et al., 2009)
Lipase rEstAT1 rEstAT11	Arctic sediment	Activity based	30	8.0	33.4 and 34.7	(Jeon et al., 2009b)
DNA polymerase I	Glacial ice	Activity based	--	--	--	(Simon et al., 2009a)

enzymes from cold environments as cold adapted enzymes like lipase, proteases, cellulases, amylases, xylanase has been isolated and characterized from various microorganisms inhabiting cold environments (Lee et al., 2006; Joseph et al., 2008; Zhang and Zeng, 2008; Kasana, 2010; Kasana and Gulati, 2011).

Using metagenomics approach the enzymes β-Galactosidase, esterase, lipase, amylase, xylanase and DNA polymerase I have been isolated from cold environments (Table 1). The characteristics of various enzymes isolated from different location are listed (Table 1). Most of these enzymes from cold environments have optimum temperature for activity around 30°C (so they are cold active) with some others showing optima at lower or higher temperature (Table 1). In all the cases activity based screening was employed except in one recent study where sequence based screening was used for isolation of a novel cold-active xylanase gene from the environmental DNA of goat rumen contents (Wang et al., 2011). The low-temperature-active enzyme genes from various metagenomics libraries have been successfully heterologously expressed by using *E. coli* as host and subsequently biochemically characterized. The gene cloning and heterologous expression of different enzymes from various cold environment metagenome is listed in the Table 2. The cold adapted *lipo1* (Lipase) gene isolated from activated sludge metagenomic library showed 32% amino acid identity to *Pseudomonas mendocina* lipase (Roh and Villatte, 2008), while another cold adapted *lipCE* (Lipase) gene displayed 88% amino acid identity to *Pseudomonas fluorescens* lipase (Elend et al., 2007).

A metagenome-derived and cold-active lipase LipCE with admirable properties was isolated, purified and characterized. The optimum temperature of LipCE was 30°C, but it displayed 28% residual activity at 0°C and even 16% at −5°C. LipCE hydrolyzed stereo-selectively ibuprofen-*p*NP ester with a high preference for the (*R*) enantiomer of >91% ee and it demonstrated selectivity for esters of primary alcohols, whereas esters of secondary or tertiary alcohols were nearly not converted (Elend et al., 2007). The lipolytic enzyme isolated from activated sludge metagenomic library exhibited the highest activity at pH 7.5 and 10°C (Roh and Villatte, 2008). The novel cold adapted alkaline lipase LipEH166 showing no amino acid similarity to any known lipolytic enzyme except in the consensus region which was isolated from an intertidal flat metagenome. It was suggested that LipEH166 and its homologues belong to a new family of lipolytic enzymes (Kim et al., 2009). The optimum activity of the purified lipase EML1 from cold-sea

Table 2. Relationship of enzymes from cold environment metagenome to already known enzymes.

Gene (enzyme)	Source	Length (amino acid)	Highest identity	% Identity	Expressed in	References
xynGR40 (Xylanase)	Goat rumen contents	--	Hypothetical xylanase from *Bacteroides eggerthii* DSM 20697	67	*E. coli*	(Wang et al., 2011)
zd410 (β-Galactosidase gene)	Daqing oil field	--	*Planococcus* sp. "SOS Orange"	39	*P. pastoris*	(Wang et al., 2010)
xyn8 (Xylanase)	Waste lagoon		Insect gut xylanase	42	*E. coli*	(Lee et al., 2006)
EML1 (Lipase)	Deep-sea sediment	304	LipG from uncultured bacterium	34	*E. coli*	(Jeon et al., 2009a)
EM2L8 (Esterase)	Deep-sea sediment	267	Biotin biosynthetic genes (BioH) of *Kurthia*	38	*E. coli*	(Park et al., 2007)
lipCE (Lipase)	Oil contaminated soil	476	*P. fluorescens*	88	*E. coli*.	(Elend et al., 2007)
lipo1 (Lipase)	Activated sludge	324	*P. mendocina*	32	*E. coli*	(Roh and Villatte, 2008)
*lipA (*Lipase)	Mangrove sediment	283	Uncultured bacterium	52	*E. coli*	(Couto et al., 2010)
h1Lip1 (Lipase)	Baltic sea sediment	325	*P. putida* esterase	54	*E. coli*	(Hardeman and Sjoling, 2007)
pAMY (Amylase)	Soil sample	302	Uncultured bacterium	80	*E. coli*	(Sharma et al., 2010)
LipEH166 (Lipase)	Intertidal flat sediments		--	--	*E. coli*	(Kim et al., 2009)

sediment samples was at pH 8.0 and 25°C, respectively, and importantly enzyme displayed more than 50% activity at 5°C indicating its exploitation for cold use (Jeon, et al., 2009a). The purified β-galactosidase ZD410 from soil metagenomic library displayed optimal activity at 38°C and 54% of the maximal activity at 20°C and 11% close to 0°C. Compared to earlier known β-galactosidases, ZD410 had many novel properties like high activity at low temperatures, the optimal pH of 7.0, tolerance to low concentration (10 mM) of Na^+, K^+ or Ca^{2+}, high enzyme activity for lactose (Wang et al., 2010). The purified recombinant xylanase XynGR40 exhibited high activity at low temperatures remaining active even at 0°C (10% of the activity), had relatively better stability at mesophilic temperatures and a higher catalytic efficiency than other known GH 10 cold active xylanases indicating its potential for basic research and industrial applications (Wang, et al., 2011). The isolation and characterization of various novel cold-adapted enzymes highlight and support the potential of the cold environment metagenomics in discovering future enzymes and biocatalysts.

CONCLUSION

There is enduring need for a broad range of novel enzymes and biocatalysts which are required for various industrial and biotechnological applications. The cold environments are extremely diverse in nature with fluctuation in temperature, radiation and pressure. These environments present largely "untapped" bioresource and the microorganisms flourishing, there have great potential for isolation of new and novel enzymes. Overcoming the limitation of culturing the microorganisms in laboratory, the metagenomics has started making its impact in discovering the new and novel molecules. In future the emphasis should be given for preparing and exploiting the metagenomic libraries from unexplored, unique niches from extreme environments.

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