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Metagenomics: A Promising Approach to Assess Enzymes Biocatalyst for Biofuel Production

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ABSTRACT

Because of the diminishing fossil fuel reserves and increased CO_2 accumulation in the atmosphere due to their burning, biofuels have been looked as an alternative for sustainability and protecting the environment. Use of plant byproducts such as lignocellulosic material as feedstock would be a viable strategy avoiding the use of food (sugar) for bioethanol production. Lignocelluloses are the most abundant resource in soil, its susceptibility to enzymatic digestion favor the release of simple sugars which could be fermented to produce ethanol. The cellulolytic enzymes that hydrolyze cellulosic feedstocks economically are critical to develop the biofuel energy sector. Numerous highly effective pathways for degrading biomass have evolved but enzymes for effective digestion of biomass have been characterized only from few culturable organisms. These biomass-degrading organisms are ill suited for genetic engineering or industrial applications while conventional methods for identifying and cloning their individual enzymes are inefficient. The discipline of metagenomics as the culture independent genomic analysis of entire microorganisms in a particular environmental niche was evolved as an effort to discover novel microbial enzymes to improve biomass utilization. This review, focus on metagenomics as a promising approach for biofuel research and its application in the NGS era.

Key words: Biofuel, biomass, cellulases, enzymes, metagenomics, renewable energy

INTRODUCTION

Natural resources and environmental quality are in constant decline in parallel with the growth of the world population. There is an urgent in renewable fuels as means to sustainability and to reduce carbon dioxide emissions along with associated risks of climate change and global oceanic acidification. Use of ethanol as fuel has been considered as one of the most viable strategies. As a consequence, ethanol production capacity in the USA has more than tripled in the past 5 years and expanding from 3.4 in 2004 to 10 billion gallons in 2009 (Renewable Fuel Association; www.ethanolrfa.org). Moreover, the total renewable fuels requires for U.S. consumption is expected to increase from 9 billion gallons in 2008 to 36 billion ethanol-equivalent gallons in 2022 (EIA, 2012). Plant biomass derived glucose or sucrose have been considered as a superior source for bio-ethanol production in the United States and Brazil (Rosillo-Calle and Cortez, 1998). Eventually, food crops derived starch and sugar that are used for the production of ethanol competes with food supplies (Kim *et al*., 2008, 2006). The amount of municipal waste increases

every year with the growth of world population. The organic content of municipal waste is equates to 62% in Indonesia, 63.4% in China and 41.8% in India (Pandyaswargo *et al.*, 2012). Given the abundance and cost, these organic waste and the lingocellulosic plant biomass has been investigated as potential feedstocks for biofuel production.

In the last two decades, extensive efforts have been focused on the conversion of plant biomass into ethanol (Kumar *et al.*, 2009). There are two main processes involved in this conversion (1) Hydrolysis of cellulose in the plant biomass to produce reducing sugars and (2) Fermentation of the simple sugars into ethanol. Based on current technologies, the cost of ethanol production from cellulosic materials is relatively high mainly because of the low efficiency of the hydrolysis process (Sendich *et al.*, 2008; Wyman, 2007). Moreover, the enzymes currently employed for biomass conversion cannot meet the growing demand for economically viable biofuel due to their high cost, low activity and poor stability under the required operating conditions. Thus, continued development of novel enzymes for use in the production of biofuel is required (Barnard *et al.*, 2010).

The ecosystems have been completely mineralized with plant biomass. The native microbes in these ecosystems may provide novel genes relevant to the development of biofuel. The genes required for biofuel production includes, cellulases, hemicellulases and enzymes that facilitate sugar release from biomass (Lee *et al.*, 2008). Metagenomics is an advanced methodology which emerged in the late 1990s, by means of extracting all microbial genomic DNAs in a certain environmental habitat, constructing metagenomic libraries and screening for novel functional genes (Ferrer *et al.*, 2005b; Wang *et al.*, 2009). Metagenomics overcomes the disadvantages of isolation and cultivation procedures of the traditional microbial method and thus greatly broadens the space of microbial resource utilization. It has become one of the promising research tools for discovering novel enzymes to produce biofuel in an efficient way. This review discussed the recent research achievements and application of metagenomics for prospecting novel enzymes for biofuel production.

BIOFUELS

Biofuels have been considered as an alternative to fossil fuels which can be a liquid or gaseous in nature produced from carbon based feedstock such as plant biomass, natural oils, algae, animal fat, etc. Biofuels such as alcohols including ethanol, methanol, propanol butanol can be used as pure (100%) or blended with fossil fuels like gasoline as much as 15-20% alcohol (E15-20) by volume to operate the internal combustion engines (IEA, 2002). However, only methanol and ethanol are have been successfully blended with fossil fuels to operate Internal Combustion Engines (ICEs). Blended ethanol percentage up to 20% has been considered as acceptable fuel for use in farm machineries (Tangka *et al.*, 2011). Moreover, the pollutant emission from four stroke SI engine was decreased in case of 20% ethanol blend with gasoline was reported (Kiani *et al.*, 2010).

BIOMASS AND ENERGY SECURITY

Lignocellulosic biomass can be utilized to produce ethanol, a promising alternative eco-friendly energy source (Lynd *et al.*, 1999). Maize stover (leaves and stalks) constitutes a large part of agricultural biomass. Ethanol production from non-grain portions of plants is referred to as cellulosic or lignocellulosic ethanol. Lignocellulose is composed of 30% hemicellulose, 44% cellulose and 26% lignin (DOE, 2006). Bioethanol is one of the promising energy alternatives to minimize the negative environmental impacts generated by the use of fossil fuels (McMillan, 1997). It can

be produced from a wide range of raw materials that contains fermentable sugars. However, the utilization of energy-rich crops such as corn and sugar cane, as feedstock for ethanol generation, may jeopardize the food security in many countries and is a debated issue at the moment. Besides, the utilization of virgin resources enhances the total cost of large-scale production of ethanol. Therefore, biomass wastes such as corn fiber, waste wood, waste cardboard and paper sludge, molasses, bread residues, bagasse (Cazetta *et al*., 2007; Kadar *et al*., 2004; Teixeira *et al*., 1999) and the organic waste fractions are far attractive to be used as the cheap feedstocks in ethanol production.

BIOFUEL ENZYMES

Extensive research has been performed on biofuel enzymes production and their utilization in the bio-refining industries with the development of new technologies. Modern bio-refining industries have now begun to explore the advantages of utilizing these biofuel enzymes in their productions. With the current technological advances biofuel enzymes have been utilized to improve the refinery process leading to the growing market of biofuel enzymes. Thus, the total enzyme cost for enzymatic conversion of such lignocelluloses into biodiesel is estimated to be 0.73-1.49 euro kg^{-1} biodiesel with the current enzyme price and predicted to be 0.05-0.75 euro kg^{-1} with the supposed price of enzyme in future (Sotoft *et al*., 2010). Browsing for the novel enzymes is primarily dependent on two factors (1) Functional microbial screening (traditional screening) and (2) Discovering candidate genes from environmental niche (metagenomics). The flowchart describes the different approaches employed in the field of metagenomics for the finding of novel genes (Fig. 1). The fact that traditional screening of microbes for finding novel enzymes is one of the main limitations for the widespread application of enzymes in biotransformation process with brewing industries (Leresche and Meyer, 2006). More than 99% of microorganisms from natural

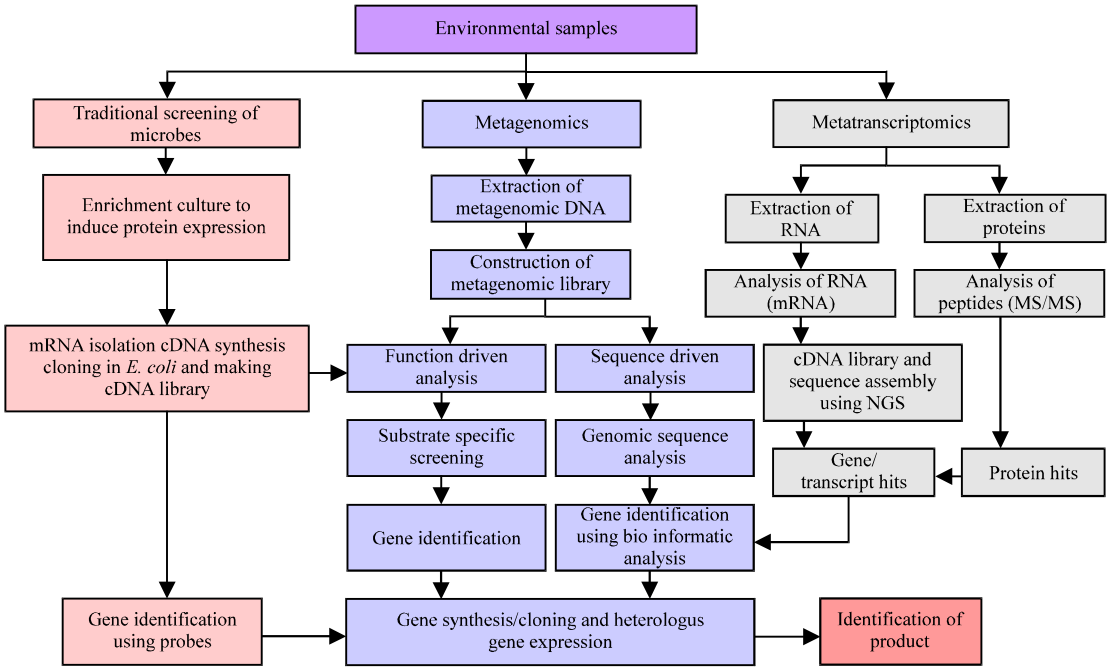

Fig. 1: General approaches for novel enzymes discovery from environmental sources

environments cannot be cultivated with current isolation system and using culture dependent methods may reduce the microbial resources which will affect the ecosystem (Torsvik and Ovreas, 2002). Identifying candidate genes from these ecosystems which have a high degree of microbial diversity, is challenging. Fortunately, Next-Generation Sequencing (NGS) technologies facilitate the discovery of relevant genes from these microbial ecosystem (Ansorge, 2009).

DIFFERENT STRATEGIES FOR PROSPECTING NOVEL ENZYMES

Enzymes are playing a major role in wide range of applications in industries (Kirk *et al*., 2002). There is a broad range of demands for novel enzymes to convert natural polymers including starch, cellulose and proteins, or the ones which are applicable in chemical and pharmaceutical industries. Finding suitable enzyme candidates depends on efficiency and sensitivity of screening strategies with the input of diverse candidate genes and organisms. The traditional method for finding novel enzymes by cultivation and subsequent screening of microbial strains is a standard and powerful approach (Ogawa and Shimizu, 1999). To date, many researchers have established collections of organisms from different environmental niche. Culturable organisms encompass only a tiny fraction of the genomic diversities existing in each environment. Hence, screening culturable microbes alone would limit the potential spectrum of new enzymes found for the bioprocess industry. Nevertheless, the fact that a large number of protein sequences deduced from the genomes of these cultured organisms still have no assigned function, gives a glimpse of a promising prospect for searching already sequenced genomes. By screening genomic libraries of culturable organisms, new genes and gene products can be identified for biotechnological applications. To overcome the deficiency of cultivation method, genomic and metagenomic-based strategies have been developed successfully as powerful approaches for identifying enzymes with fibrolytic activities from various terrestrial microbial ecosystems.

FUNCTION-BASED SCREENING OF MICROORGANISMS

Table 1 describes the chronological list of different fibrolytic enzymes identified through metagenomic approaches from different environmental sources for biofuel production. Over the years, culturable cellulase-producing bacteria have been isolated from a wide variety of sources such as composting heaps, decaying plant material from forestry, agricultural waste, the feces of ruminants, soil and organic matter and extreme environments. Function-based screening for cellulase production can be done by growing on microcrystalline cellulose as a sole source of carbon, followed by the extraction and analysis of 16S rRNA sequence to determine the cellulase producing species. In order to reach the pure isolate, an efficient plate-screening method is required. Moreover, function-based screening for cellulase activity is typically performed on plates containing carboxymethylcellulose (CMC) (Hankin and Anagnostakis, 1977).

Recently, Kasana *et al*. (2008) improved the plate-screening method by substituting the gram's iodine with hexadecyltrimethyl ammonium bromide or Congo red. However, the plate-screening method using diazo dye is not sensitive enough to screening due to poor enzyme activity. Thus, different screening methods were established with chromogenic/fluorogenic groups to achieve the quantitative methods with higher sensitivity (Maki *et al*., 2009). A major limitation in incorporation of fluorescent substrates into agar plates is the tendency of products failed to diffuse widely in the medium and therefore these kinds of compounds are not as readily used.

Screening of culturable microbes may require knowledge on a specific enzyme with determined activity whether it is exoglucanases with activity on microcrystalline cellulose or endoglucanases

Table 1: List of fibrolytic enzymes identified for biofuel development through metagenomics approaches

Enzymes	Source	Screening method	Sequencing method	References
Glycoside hydrolases	Anaerobic digestion sludge	Sequence based	Sanger sequencing	Xia *et al*. (2013)
Cellulase; xylanase	Higher termite	Function based (fosmid library)	Sanger sequencing	Nimchua *et al*. (2012)
Xylanase	Sugarcane bagasse	Function based	PCR cloning	Weerachavangkul *et al*. (2012)
Cellulases	Soil	Function based (fosmid vector)	Sanger sequencing	Xiong *et al*. (2012)
Cellulase; xylanase	German grassland soil	Function based (fosmid vector)	-	Nacke *et al*. (2012)
Xylanase	Compost sample from pig manure and mushroom culture	Function based (fosmid vector)	Sanger sequencing	Jeong *et al*. (2012)
Xylanase	Bovine rumen	Function based (BAC vector)	Sanger sequencing	Gong *et al*. (2012)
Cellulases	Enrichment culture of shipworm	Function based (fosmid vector)	Pyrosequencing	Ilmberger *et al*. (2012)
Xylanase	Sea-bottom	Sequence based	Pyrosequencing -454 GS FLX	Hung *et al*. (2011)
Laccase	Surface water of the sea	Sequence based (PCR)	Sanger sequencing	Fang *et al*. (2011)
Glycoside hydrolases	Decaying poplar biomass under anaerobic conditions	Function based	Sanger sequencing	Li *et al*. (2011)
Endoglucanase	Soil	Function based (BAC vector)	Sanger sequencing	Liu *et al*. (2011a)
Cellulase	Yak rumen	Function based (cosmid vector)	-	Bao *et al*. (2011)
Esterase	Antarctic soil	Function based (fosmid vector)	Sanger sequencing	Fu *et al*. (2011)
Cellulase	Cow rumen	Sequential and functional screening	Shotgun sequencing	Hess *et al*. (2011)
β-glucosidase	Alkaline polluted soil	Function based	Sanger sequencing	Jiang *et al*. (2011)
Xyloglucanase	Bovine rumen	Activity-based	-	Findley *et al*. (2011)
Esterase	Arctic soil	Function based	Sanger sequencing	Yu *et al*. (2011)
β-Galactosidase	Oil field soil	Function based	Sanger sequencing	Wang *et al*. (2010)
Cellulase	Compost	Function based	Pyrosequencing -454 GS FLX	Allgaier *et al*. (2010)
Xylanase	Holstein cows rumen	Function based	Sanger sequencing	Zhao *et al*. (2010)
Endoglucanase	Human gut	Multi-step functional screening	Pyrosequencing -454 GS FLX	Tasse *et al*. (2010)
β-glucosidase	Sludge from biogas reactor	Function based	Sanger Sequencing	Jiang *et al*. (2010)
Exoglucanase	Earthworms	Function based	Sanger sequencing	Beloqui *et al*. (2010)
Lipases/esterases	Peat-swamp forest soil	Functional and sequence based	Pyrosequencing-454 GS FLX	Bunterngsook *et al*. (2010)
Esterase	Rhizosphere	Function based	Sanger sequencing	Lee *et al*. (2010)
Amylase	Human gut	Multi-step functional screening	Pyrosequencing on -454 GS FLX	Tasse *et al*. (2010)
Amylase	Soil (Astaka)	Function based	Sanger sequencing	Sharma *et al*. (2010)
Endoglucanase; Exoglucanase; β-glucosidase	Contents of buffalo rumen	Function based	Sanger sequencing	Duan *et al*. (2009)
Endoglucanase; β-glucosidase	Forest soil, elephant dung, cow rumen, rotted tree	Function based	Sanger sequencing	Wang *et al*. (2009)
Endoglucanase	Compost	Function based	Sanger sequencing	Pang *et al*. (2009)
Glycoside hydrolases	Cow rumen	Sequence based	Pyrosequencing-454 GS FLX	Brulc *et al*. (2009)
Endoglucanase	Buffalo rumen	Function based (cosmid vector)	-	Liu *et al*. (2009)

Table 1: Continue

Enzymes	Source	Screening method	Sequencing method	References
Esterase/lipase	Deep sea sediment	Function based	Sanger sequencing	Jeon *et al*. (2009)
Endoglucanase	Content of cow rumen	Function based	-	Shedova *et al*. (2009)
Endoglucanase	Aquatic community and soil	Cosmid	Sanger sequencing	Pottkamper *et al*. (2009)
Glucanase-xylanase	Soil (Korean)	Function based	Sanger sequencing	Kim *et al*. (2008) and Nam *et al*. (2009)
Xylanase	Soil	Function based	Sanger sequencing	Hu *et al*. (2008)
Mannanase-xylanase-glucanase	Cow rumen fluid	Function based	Sanger sequencing	Palackal *et al*. (2007)
Endoglucanase	Contents of hindgut of higher termite	Fosmid and plasmid	Sanger sequencing	Warnecke *et al*. (2007)
Esterase	Deep sea sediments	Function based (fosmid vector)	Sanger sequencing	Park *et al*. (2007)
Cellulase	Rabbit cecum	Function based	Sanger sequencing	Feng *et al*. (2007)
Cellulase	Soil	Function based (cosmid vector)	Sanger sequencing	Voget *et al*. (2006)
Esterase	Biofilms growing with a drinking water	Function based (cosmid vector)	Sanger sequencing	Elend *et al*. (2006)
Esterase	Pools of environmental soils	Function based	-	Kim *et al*. (2006)
Cellulase	Sargasso Sea	Fosmid library screening Sequencing	Sanger sequencing	Cottrell *et al*. (2005)
Cyclodextrinase; Esterase; Endo-β-1, 4-glucanase	Bovine rumen	Function based	-	Ferrer *et al*. (2005a)
Xylanase	Insect gut	Function based	Sanger sequencing	Brennan *et al*. (2004)
Cellulase	Soda lake sediments (Egypt)	Function based	-	Grant *et al*. (2004)
Agarase; Amylase; Cellulase; Pectate lyase	Soil	Function based (cosmid vector)	Sanger sequencing	Voget *et al*. (2003)
Cellulase; Esterase	Lake water (East Africa)	Function based	-	Rees *et al*. (2003)

with activity on carboxymethyl cellulose (CMC). In order to find the novel protein, different assay procedures have been applied to screen the recombinant proteins in *E. coli* (Duan *et al*., 2009; Li *et al*., 2006). Expression screening is an efficient method to specify the novel cellulose producing bacteria among the microbial population in the complex mibrobiome such as rumen, termite digestome, pulp and paper mill effluent sediments, etc. Using different screening methods, a variety of cellulases with novel features have been identified or are still being identified.

Wang *et al*. (2008) reported a novel cellulose degrading *Paenibacillus* sp., strain B39 by using the 16S rRNA gene sequencing analysis. The cellulolytic enzyme showed promising thermostability and acidic tolerance in CMC assay and hence was considered potent to be used in industrial applications like the cellulose hydrolysis. Furthermore, a novel cellulase-producing *P. campinasensis* BL11 strain was isolated from black liquor of the Kraft pulping process. *P. campinasensis* BL11 is a spore-forming bacterium which was found to grow over a wide range of pH and temperatures. The physiological properties and ability of producing the free glycosyl hydrolases make this strain a potential candidate for bio-refining industry (Ko *et al*., 2007). Li *et al*. (2008) reported a thermostable cellulase was found in newly isolated *Bacillus subtilis*, extracted from a hot spring. The high temperature environment allowed for the production of a thermostable endocellulase with an optimum temperature activity at 50°C. It was found to retain 70% of its maximum activity at 75°C after incubation for 30 min on CMC assay. The strain offers a thermostable enzyme for potential application in bio-refining industry.

A highly stable thermophilic, cellulolytic bacterium was isolated from swine waste and identified as *Brevibacillus* sp., strain JXL. Liang *et al*. (2009) reported an isolated enzyme showed broad spectrum substrate specificities such as crystalline cellulose, CMC, xylan, cellobiose, glucose and xylose and the protein appeared highly thermostable in nature. Furthermore, a salt-activated endoglucanase was isolated from a *Bacillus* strain, alkaliphilic *B. agaradhaerens* which was shown to have increased thermostability with optimal pH range with the addition of 0.2M NaCl (Hirasawa *et al*., 2006). In addition, microbes are capable of producing complex proteins that supporting the hydrolysis of polysaccharides, such as the cellulosome, xylosome and multifunctional enzymes are currently gain a lot in enzymes discovery. Perez-Avalos *et al*. (2008) reported, a bifunctional endoglucanase/endoxylanase enzyme from *C. flavigena* has potential industrial application. Similarly, a multifunctional enzyme was isolated from a wood-boring marine bivalve (*L. pedicellatus*) symbiotic bacteria *T. turnerae* T7902. The enzyme has two catalytic and carbohydrate-binding domains to bind both cellulose and chitin. Besides, it has cellobiohydrolase and endoglucanase activities to degrade complex polysaccharides. Also these enzymes have acid-tolerant, mesophilic character with potential ability to reduce viscosity of CMC approximately 40%, displaying promising characteristics for the industrial application (Ekborg *et al*., 2007).

It is necessary to characterize the isolated enzyme with efficient hydrolysis system to use in industrial applications. Isolation and characterization of cellulase producing microbial organisms is an important aspect in biofuel research. Researchers have been looking for novel genes from unculturables using metagenomics approaches. The newly isolated enzymes must possess the ability to withstand enzymatic activity in biotransformation processes with higher resistance capacity to harsh environmental conditions such as wide range of pH and temperatures. Also the enzymes must decrease the cost of conversion of complex polysaccharides into ethanol by minimizing enzyme utilization in biotransformation process through retaining its enzymatic nature. In functional screening, for finding the novel enzyme from the complex microbiome, a metagenomics library has to be created and screened functionally with a specific character.

IN SILICO SEQUENCED BASED SCREENING

Karr *et al.* (2012) reported the world's first complete phenotype prediction from a genotype of world's smallest free-living bacterium *Mycoplasma genitalium*. Thus, *in silico* sequence-based screening for the candidate genes relies on known conserved sequences and hence it is failed to be able to detect different new genes. However, unlike the functional-based screening, the sequence-based screening can reveal target genes irrespective of the completeness of the gene's sequence. Here, with the development of new sequencing technology, such as the next-generation sequencing, has changed the limitation of gene cloning. The first metagenome project successfully reported by Tyson *et al.* (2004) explored the microbial communities of the drainage from acid mines. Nowadays, metagenome projects using new sequencing technologies not only generate greater total base pair reads but also have more even coverage of species within the community (http://www.genomesonline.org/). There has been many online bioinformatics databases available to analysis the metagenomic data (Huson *et al.*, 2007) but still need new bioinformatic tools for data mining basing on sequence homology, protein structures, catalytic sites and specific activities. With protein prediction and classification tools, protein structural models can be designed to study the folding mechanism of proteins. Based on their structural folding, the creation of putative active sites and their function can be predicted (Cantarel *et al.*, 2009; Claudel-Renard *et al.*, 2003; Henrissat, 1991; Nair and Rost, 2004; Selengut *et al.*, 2007).

Several publications are available on prospecting metagenome for finding cellulolytic genes and their active enzymes that will be useful in development of biofuel sector (Gilbert *et al.*, 2012). Schluter *et al.* (2008) sequenced a metagenome library of the microbial community from an agricultural biogas plant. They have generated 141 million base pairs sequences and found a group of bacteria play a dominant role in methanogenesis and were identified genes encoding cellulolytic functions from among the *Clostridia* spp. (Warnecke *et al.*, 2007) sequenced a metagenome library of hindgut microbiome data from the largest family of wood-feeding termites (Termitidae) and generated 71 million base pairs of sequence data. Using the global alignment method, they identified a rich diversity of putative cellulases and hemicellulases with more than 700 domains homologous to glycoside-hydrolase catalytic enzymes corresponding to 45 different carbohydrate active enzymes (CAZy) families. Currently more than 2500 glycoside hydrolases have been identified and classified into 115 families. The enzyme family contains members from bacteria, fungi and plants with different characteristics and they have substrate specifications in hydrolysis reactions. The lignocellulolytic enzymes such as cellulases and hemicellulases belong to a group of enzymes known as glycoside hydrolases (Dashtban *et al.*, 2009).

Since, the termites eat cellulose in its various forms as plant fiber, a series of efforts on metagenomics and metatranscriptomics from termites were reported, discovering genes encoding GHs, GTs and CBMs (Liu *et al.*, 2011b; Scharf and Tartar, 2008; Tartar *et al.*, 2009; Todaka *et al.*, 2007; Warnecke *et al.*, 2007), as well as components involved in the lignin modification (Beis *et al.*, 2010; Coy *et al.*, 2010). Hence, these midgut based genes, enzymes, proteins and co-factors produced by the termite and its symbionts (bacteria, yeast and protozoa) were named as the digestome (Scharf and Tartar, 2008). Warnecke *et al.* (2007) identified 700 genes encoding GH family accessory proteins in a metagenomic library from the higher termite *Nasutitermes* sp. and identified 13 enzymes by liquid chromatography with tandem mass spectrometry from its digestome. Recently, Burnum *et al.* (2011) identified 886 proteins including 22 glycosyl hydrolases from the proteome of the higher termite *N. corniger*. *C. gestroi* is classified as a lower termite belonging to the Rhinotermitidae family (Kirton and Brown, 2003) which was introduced to Brazil in the early

years of the past century and regarded as the most important urban and building pest of the country now. Gene expression patterns related to metabolic pathways and cellulose degrading enzymes such as endo-β-1,4-glucosidase and β-1,4-glucosidase were identified by transcriptome analysis (Leonardo *et al*., 2011). Moreover, Bacterial isolates from the European Corn Borer *Ostrinia nubilalis* and the coleopteran Colorado Potato Beetle *Leptinotarsa decemlineata* midguts were identified and characterized. A complete enzymatic characterization revealed moderate cellulase properties in all but one isolate and high xylanase activity in the four Colorado Potato Beetle isolates. Different enzymatic patterns in terms of optimal pH, substrate use and degradation times were observed and several isolates were selected as promising cellulose producers at extreme pH conditions (Vilanova *et al*., 2012).

Rapid and efficient enzymatic degradation of biomass-derived polysaccharides is currently a major challenge for biofuel production. A prerequisite is the availability of enzymes that hydrolyze cellulose, hemicellulose and other polysaccharides into fermentable sugars at conditions suitable for industrial use. The most widely used cellulases and hemicellulases are produced by fungal species and they are most effective over a range of temperature. At these temperatures, complete saccharification of biomass polysaccharides requires long reaction times, during which hydrolysis reactors are susceptible to contamination. One way to overcome these obstacles is to raise the reaction temperature, thereby increasing hydrolytic rates and reducing contamination risks. However, implementing higher reaction temperatures requires the deployment of enzymes that are more thermostable than the available preparations from mesophilic fungi (Berka *et al*., 2011).

The fungi are playing a predominant role in lignocelluloses conversion in bio-refineries. Lignocellulolytic enzymes-producing fungi are widespread and include species from the ascomycetes (e.g., *T. reesei*), basidiomycetes including white-rot fungi (e.g., *P. chrysosporium),* brown-rot fungi (e.g., *Fomitopsis palustris*) and finally a few anaerobic species (e.g., *Orpinomyces* sp.) which degrade cellulose in gastrointestinal tracts of ruminant animals. Biomass degradation by these fungi is performed by complex mixtures of cellulases, hemicellulases (Ljungdahl, 2008) and ligninases reflecting the complexity of the materials (Dashtban *et al*., 2009). Enzymes from thermophilic fungi often tolerate higher temperatures than enzymes from mesophilic species and some show stability at 70-80°C (Margaritis *et al*., 1986). Notably, it has been reported the cellulolytic activity of some thermophilic species was several times higher than that of the most active cellulolytic mesophiles (Tansey, 1971). Furthermore, biomass-degrading enzymes from thermophilic fungi consistently demonstrate higher hydrolytic capacity despite the fact that extracellular enzyme titers are typically lower than from more conventionally used species (Berka *et al*., 2011). An endoglucanase gene was cloned from the fungus *Phialophora* sp., gene encodes a bimodular cellulase composed of an N-terminal carbohydrate-binding module and a C-terminal glycoside hydrolase catalytic module (Zhao *et al*., 2012a). Recombinant endoglucanase enzyme produced in *Pichia pastoris* exhibited maximal activity at pH 4.0-5.0 and 70°C, retained 40% of the maximal activity at pH 2.0 and was stable at pH 2.0-10.0. Sequence-structure analysis indicated that the distinct β-sheet in EgG5 in place of a linking loop in *Trichoderma* sp., C-4 endoglucanase might be the reason (Zhao *et al*., 2012b). This study shows an excellent endoglucanase activity with improved enzyme thermostability potential in bioconversion of lignocellulosic materials (Zhao *et al*., 2012a).

The microbiome of the rumen, including fungi, protozoa, bacteria and archaea that are mostly obligate anaerobes, is entirely responsible for digestion of plant fiber in herbivores. In bioinformatics research group at NIAS, have identified novel bifunctional cellulolytic gene

sequences from goat rumen liquid and adherent fraction (data not published). To develop a cost effective process to produce second generation biofuels, goat rumen metagenomics is a promising approach for identifying novel enzymes with higher specificities towards cellulosic biomass. The novel cellulase and xylanase enzymes from goat rumen microbiome have been overexpressed in *E. coli* to characterize the protein. The expressed bifunctional enzymes showed equal or higher activity against celluloses and hemicelluloses on CMC assay. Further characterization has been under process to produce these enzymes in the bioreactors and evaluate their stability and activity on a pilot scale. Metagenomics is one of the fastest advancing fields in modern microbiology. It facilitates the access to the genomes of entire communities of bacteria, viruses and fungi. Metagenomics is a promising approach to broadening the understanding of the microbial diversity, ecology, evolution and functioning of the microbial world.

CURRENT SCENARIO AND FUTURE PROSPECTS

There is an online resource for monitoring metagenome projects worldwide named Genome Online Database (GOLD) (http://www.genomesonline.org/). As of September 2011, GOLD contains information for 340 metagenome projects associated with 1927 metagenome samples (Pagani *et al*., 2012). The year 2011 is an important landmark in the history of genome sequencing projects with the registration and tracking of 10K genome projects. The most important developments in the metagenome projects are coupled with the growth of the metagenome database (Pagani *et al*., 2012). Thus, supercomputing infrastructures and bioinformatics pipelines are necessary for large-scale metagenome projects (Teeling and Glockner, 2012).

Since, the commercial metagenomics endeavor in the late 1990s (Recombinant Biocatalysis Ltd. and TerraGen Discovery Inc.) these approaches have been taken up by several of the biotechnological start-up companies. Verenium corporation (http://www.verenium.com) is one of the acknowledged leader in the field of metagenomics based cloned enzymes in wide range of enzyme classes. By using next generation sequencing technologies, sequencing of complex microbiomes has been facilitates the discovery of bioactive molecules. The commonly available method for identifying genes in metagenomic reads are similarity searches using the reference databases. However, using this reference based approach is not a valid approach when the reference database failed to include the novel gene sequences. Therefore many computational methods have been developed to identify the novel gene which is also called *ab initio* gene prediction. Many algorithms are being used to make structural models to analysis the gene structure. Based on these models, a great number of *ab initio* gene prediction programs have been developed or are still being in set up with improvements to date (Picardi and Pesole, 2010). Moreover with metagenome expression libraries, fragmented metagenomic DNA gene expression can be examined in a suitable host system. Researchers can access the unknown genes and their encoded enzymes with this library screening.

CONCLUSION

The scarcity of energy with the fossil fuels continues to explore the alternative fuel resources. Biofuels have been considered as an alternative energy source throughout the world. Microbial enzymes are known to be used as biocatalysts in bio-refinery industries however, only a few enzymes are currently employed for commercial applications. The inefficiency and low activities of currently available enzymes for production of biofuels has limited their industrial application. In this scenario, the metagenomic data provides a new unexplored treasure of genomic wealth that can enhance the enzyme inventory by the discovery of novel useful enzymes. Number of functional

screening approaches in metagenomics has been implemented to emphasize uncultivated microbes and their potential application in biofuel development, concerning their specific functions in their environments. Metatranscriptomics and metaproteomics are the newest development in metagenomics that gives further promises for functional screening of uncultivated microbes. With the current technological advances in the next generation sequencing technologies, remarkable price drop in sequencing large data within short time has greatly makes metagenomics as a great tool to access the inaccessible organisms.

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