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Review Article
Ecogenomics Based Microbial Enzyme for Biofuel Industry

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Abstract

Due to fastest depletion of fossil fuel assets and augmented greenhouse gases buildup in atmosphere owing to their consumption, therefore, biofuels have been considered as a substitute for energy and environment sustainability. Crop byproducts for instance, lignocellulosic waste material could act as viable approach for biofuel production. Lignocelluloses are the plentiful resource on the earth and application of microbial enzymes on it could lead to release of sugar molecules, which can be converted into ethanol. Microbial enzymes which hydrolyze cellulosic feed material considered as cost effective, which is a very important aspect in developing green energy sector. There are numerous effective enzymatic pathways reported for biomass conversion, potential enzymes for actual biomass digestion are yet to be investigated. Ecogenomics discipline act as microbial culture independent genome analysis in a precise environmental area. This discipline has been evolved as a powerful tool for unique microbial enzyme discovery at rare biomass exploitation level. With the development in sequencer technology and ecogenomic tools, microbial enzymes for biofuel production are becoming more lucrative. Novel enzymes in recent times developed for pretreatment and transformation of lignocellulosic waste materials are great possibilities with added explorations into enzymes from environment. This study emphasizes on omics tools as a promising attitude in the biofuel research field and its application towards green technology. Moreover, the newest research development and advancement in metagenomic sequencing, which is now considered as an effective tool for innovative enzyme unearthing and genome functional analysis in the biofuel production is also discussed in biofuel area under existing circumstances. Here, we insight few of the major constraints related to the finding of noble enzymes in metagenomic libraries and furthering how these might be reclamed with *in silico* methods.

Key words: Biofuel, bioprospection, enzymes, extremophiles, ecogenomics, omic tools

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INTRODUCTION

Microorganisms have been considered as the quickest evolutionary appliance amongst all living forms, moreover, are also considered as revolutionary organization for genome reintegration and restoration proficiency under the diverse conditions on this globe[1,2]. Scientists have provided evidences clearly demostrating that microbial cells, which are approximately (10^{30}) on this planet surpass the number of stars in cosmos by nine orders of scale[3]. Microbes are the only organisms, which are renowned to have adjusted themselves to thrive and flourish under stringent environmental conditions viz., extreme temperature variations, salinity, alkalinity, variable atmospheric pressure and above all flucatuations in pH. To overcome such severe and unfavorable conditions, the microbes survives proficiently by secreting enzymes or by changing internal cellular environment that could interfere the external environmental components[4]. Under the existing conditions, learning and analysis of microbial population thriving under severe conditions needs to be tackled by existing accessible tools and techniques. Although, the inadequate and incomplete genomic references for assessment and evaluation becomes an obstacle for advance assessment and exploiatation. The developments in Next Generation Sequencing (NGS)/pyrosequencing techniques leads the researchers to explore more massive sequences of an discrete and specific genomic material that might revolutionize the existing sequencing technology[5]. There are may niches, which are unexplored such as human, animal gut and highly extreme conditions could be a possible resource material where, ecogenomics tools (metagenomic DNA/nonredundant metagenomic DNA) might be exploited for concealed and unseen prospective of unusual and valuable microbiomes. Therefore, omic tools and techniques are considered to be helpful to revitalize and restore the biosynthetic equipments of uncultured or fastidious microorganisms in comparision to usual established cultivation approaches[6,7].

The ecogenomic technique involves the isolation of DNA (naked) directly from the environment, known as environmental DNA. Further, metagenomic libraries construction and screening of new and unique functional genes are main technical levels which leads to route of exploration and study at genomic level. In the existing and ongoing research scenario, ecogenomics technnique is being employed to produce and harvest the numerous marketable enzymes such as xylanase, laccase, amylase, chitinase, etc[8,9]. This review endorses the omics tool as a revolution and discovery in isolation of new and unique enzymes and bioactive metabolites from unexplored natural niches. Moreover, this technology also provides inputs at microbial enzyme level, which will play an essential responsibility in revolutionzing the green energy which fulfills the needs of biocatalist at feed stock level.

ECOGENOMICS: "IS IT AN UNIQUE OMICS"

The notion that entire environmental microbiome can be explored and studied has revolutionized our concept and understanding of the ecology related to us. Ecogenomics attempts to define the copiousness and identity of microbiomes in a environmental sample. There are two technical ways to initiate ecogenomic exercise: Polymerase Chain Reaction (PCR, amplicon) sequencing and shotgun sequencing. An informative marker such as the 16S rRNA gene is used in amplicon sequencing. Shotgun sequencing is comprised of DNA that is extracted and randomly sheared into smaller fragments before sequencing. These technologies extend various forms of information and each offers exclusive advantages and trade-offs[10,11]. Gigantic number of microbes are unculturable, owing to lack of good techniques[12], ecogenomics approach has led to the discoveries that has been concealed from the conventional microbial culturing techniques[13]. Even though it is a multidimensional approach, the core of applied aspects of metagenomics is to express recovered genes in culturable heterologous host. A thriving and prosperous area of biotechnology is industrial exploiatation of potential microbes to manufacture effective antibiotics, enzymes for biofuels and other bioactive secondary compounds. Demand for commercial enzymes production using microbial sources has been employed industrially and is growing rapidly[14]. Application of metagenomics in industry also include identification of innovative and unusual biocatalysts, formulation of new antibiotics, tailored medicine and also bioremediation. Furthermore, microbes producing biosurfactant have also been efficaciously employed in the remediation of industrial, domestic and agricultural wastes, resulting in reduction of environmental pollution[15]. A huge chunk of information has been deciphered by metagenomics approach, such as microbial diversity, many types of uncharacterized secondary metabolites and complexity of biogeochemical cycles and it also promises to deliver innovative biocatalysts and biomolecules with miscellaneous applications[16,17,18]. Moreover, using metagenomics derived approach, RNase H1 crystal structure has also been determined, which showed a structure based mutational shift at active site of the motif[19]. Similarly, discovery of metagenomic extremophilic esterases having dissimilar active site compared to the esterases produced by a culturable microbes[20]. Hess *et al.*[21] also sequenced and

analysed 286 gigabytes of metagenomic DNA using Illumina GAIIx and HiSeq 2000 platforms technique from cow rumen microorganisms, described 27,775 putative carbohydrate active genes and expressing 90 candidate proteins, among which about 57% were enzymatically active against cellulosic biomass.

ECOGENOMIC LIBRARY CONSTRUCTION

The essential thing for a ecogenomic library construction is choice of selection of a suitable vector and a host but it may vary depending on types of environmental samples and rationale and aim of the database to be constructed.

Selection of suitable vector: Selection of a suitable and fitting vector is very cruicial in ecogenomic technology. One has to keep in mind about the gene clusters or genome has be transuded or transferred into the host cells and it should be expressed favorably. Choice of selection of a vector relies or determined on the characteristic or quality of the extracted DNA type and the purpose of study. It also require the concern of genomic size of insert fragments, vector copy number required, prospect host to be used and method of screening. The common examples of vectors used in laboratories are plasmid, cosmid, Bacterial Artificial Chromosomes (BAC) and fosmid.

A plasmid can be employed as a suitable vector to insert a small segment (around 15 kb) for separation of small sized operons or an independent genes[22]. While, extracting and purifying the DNA smaples, purity and retrieval of DNA segments should be considered. To construct a library containing sizable amount of DNA, sizeable genome segments or for encoding a complex biosynthetic pathway, a cosmid (upto a size of 35-45 kb)[22,23] and BAC (for about 200 kb)[24,25,26] are employed. For construction of huge library insert similar to a cosmid and fosmid vector are also employed[24,27,28], though the cosmid has higher cloning stability and efficiency when expressed in *Escherichia coli*.

Selection of suitable host: For well organized and proficient cloning or expression of a recombinant genes, choice of host strain is very important and crucial. While, selecting a host, competence, efficacy of gene expression, conversion process, plasmid stability and screening of target traits in the host cell should be taken into consideration. Presently, *Escherichia coli* is very common and widely employed host cell. Though, using *E. coli* as a popular host, there are many eukaryotic genes, which cannot express their functional proteins of biological importance in this bacteria owing to be prokaryote. Therefore, there is a huge necessity for development and creation of an innovative substitute effective host system[29,30]. There are many more microbes, such as *Pseudomonas* sp., *Streptomyces* sp. and Gram negative bacteria which are also employed as host for a suitable library construction[31,32,33]. The effectiveness and efficacy of the genetic screening could be significantly enhanced as the knowledge and expertise continues to grow and the newer host microbes are discovered or developed. This will help in discovery and finding of more functional novel genes of interest and ultimately lead to discovery of innovative and unusual bioactive molecules.

Screening of ecogenomic library: The huge genetic resources treasures and unique bioactive molecules could be obtained from the ecogenomic library. However, methods of effectively screening functional genes responsible for potential enzymes from huge number of various microbes from environmental samples are still under progress. This is needed for regular update and development of high tech, huge sized library. At the moment, four screening programs are in use, viz., biological activity screening, compound configuration screening, DNA sequence screening and substrate-induced gene expression screening (SIGEX)[34].

Function based screening: Function based screening or biological activity screening implicates the identification and documentation of positive microbial clones exhibiting the required features. Using sequence or biochemical analysis the active clones are verified obtained through high throughput screening technologies. Since, biological activity screening does not rely upon the sequence data or sequence resemblance to known genes, this approach has led to the advancement and development of unique native products including protein genes products such as lyase[35] and amylase[36,37].

For screening of ecogenomic libraries, basically two types of function-based tactics have largely been employed. One is the direct detection of explicit or particular phenotypes of distinct and specific clones using chemical dyes. The results are observed based on insoluble product or colored derivatives of enzyme substrates into growth medium. For example, β-glucosidase activity on Luria Bertani agar plate containing esuculin hydrate and ferric ammonium citrate for detection of positive bacterial clones[38,39] or addition of tributyrin in the indicator agar for detection of lipolytic activity of selected microbes[40,41]. Another method is employing the host strains which need heterologous complementation by foreign genes for optimum growth under careful conditions. The recombinant clones embracing the targeted gene product produce the required gene product vigrously are able to grow[42].

BIOCONVERSION OF COMPLEX RESIDUES

The elementary and main component of plant biomass is lignocellulose and signify as important and copious amount of renewable source of carbon in this biosphere. The intricate arrangement and configuration consists chiefly of polymers of carbohydrate, such as cellulose, hemicellulose and lignin. In natural conditions, biodegradation of lignocellulose needs numerous enzymes excreted by miscellaneous microbiomes. These enzymes acts in a coordinated way and raid the intricate arrangement of lignocellulosic biomass[43,44,45]. A good number of research conducted on studies on intricate pathways involved in lignocellulose biodegradation advances, our information about elementary mechanisms and interaction among microorganisms in sustaining and conserving carbon balance in biogeochemical cycles (Table 1). Besides these vital informations, the researchers might also discover potential uncharacterized microorganisms and certain unique enzymes, which can increase the transformation of underused plant biomass products to biofuels, organic chemicals and other important ingredients for biorefinery productions[46].

The relative ecogenomic studies have been employed to explore the novel microbial communities in the diverse environments in terms of microbial taxonomy, their gene contents, besides their biochemical and metabolic capabilities[47,48]. Earlier culture independent and high-throughput sequencing techniques has been exercised to discover the complexity of metagenomes resulted from numerous lignocellulose degrading ecosystems, such as peat swamp forest[49], cow rumen[50,51], wallaby gut[52] and termite gut[53]. Comparison of soil ecogenomes from dissimilar and extreme biogeographical sites such as hot and cold deserts, unexplored forests and grasslands including tundra region has validated the exceptionality of microbial populations in terms of taxonomic multiplicity, moreover, high comparative and plenty of functional genes that might be connected to metabolic proficiency needed to handle with explicit environmental situations[54].

Presently, one foremost difficulties to a large scale manufacture of economical cellulosic biofuels is capability to competently and proficiently decompose cellulosic biomass into simple and fermentable carbon sources, such as glucose and xylose. The enzymatic saccharification of plant cell polymers such as cellulose and hemicellulose as an effective process to gain these simple sugars from complex biomass. This process is expensive and using the availabe fungal commercial enzyme combinations may lead to discovery of many more competent and tough lignocellulosic biomass degrading enzymes will reduce costs and upsurge the economic viability of this techniques[55].

Phenomenal conversion of complex lignocellulosic biomass into bioalcohol is considered as the proven consequence of cell wall degrading enzymes[8,56]. Such enzymes have been tagged as commercial enzymes dominating the bio-energy market, giving a healthy turnover, which is anticipated to reach approximately $4.4 billion by 2015[57]. In this direction an advanced approach at bioprospection level is constantly desired where, novel cellulolytic microbes those are associated with high quality biomass can harvest the volume of biofuel at commercial level. In recent past biofuel research has been revolutionized with extremophilic bacterium (*Caldicelluloseruptor bescii*) which is experienced

Table 1: Plant biomass degrading enzymes using ecogenomics approaches

Enzymes	Ecogenomic DNA sources	Insert size (kb)	Vectors	No. of screened clones	References
Amylase	Groundwater table junction soil	2-7	Plasmid	30,000	94
Amylase	Environmental	-	Lambda	50,000	95
Agarase	Unplanted field soil	25-40	Cosmid	1523	6
Amylase	Soil and compost from garden	1.5-6.5	Plasmid	31,967	56
Cellulase	East African lake water	2-10	Lambda	114,000	78
Cellulase	Unplanted field soil	25-40	Cosmid	1523	6
Cellulase	Soda lake, Egypt	2-6	Lambda	37,000	96
Cellulase	Rabbit caecum	22-47	Cosmid	32,500	14
Chitinase	Coastal seawater	1.8-4.2	Lambda	75,000	97
Cyclodextrinase	Microbes from bovine rumen	~5.5	Lambda	14,000	15
Endo β-1-4-glucanase	Microbes from bovine rumen	~5.5	Lambda	14,000	15
Esterase	Lake water, East Africa	2-10	Lambda	130,000	78
Esterase	Microbes from bovine rumen	~5.5	Lambda	14,000	15
Esterase	Soil contaminated with crude oil	25-40	Cosmid	2,500	13
Esterase	Drinking water biofilm	25-40	Cosmid	1,600	13
Esterase	Environmental soils	30-40	Fosmid	60,000	98
Pectate lyase	Unplanted field soil	25-40	Cosmid	1,523	6
Xylanase	Insect gut	3-6	Lambda	1,000,000	62
Xylanase	Dairy farm manure	4-10	Lambda	5,000,000	99
1,4-α-glucan branching enzyme	Unplanted field soil	15-23	Cosmid	1,523	6

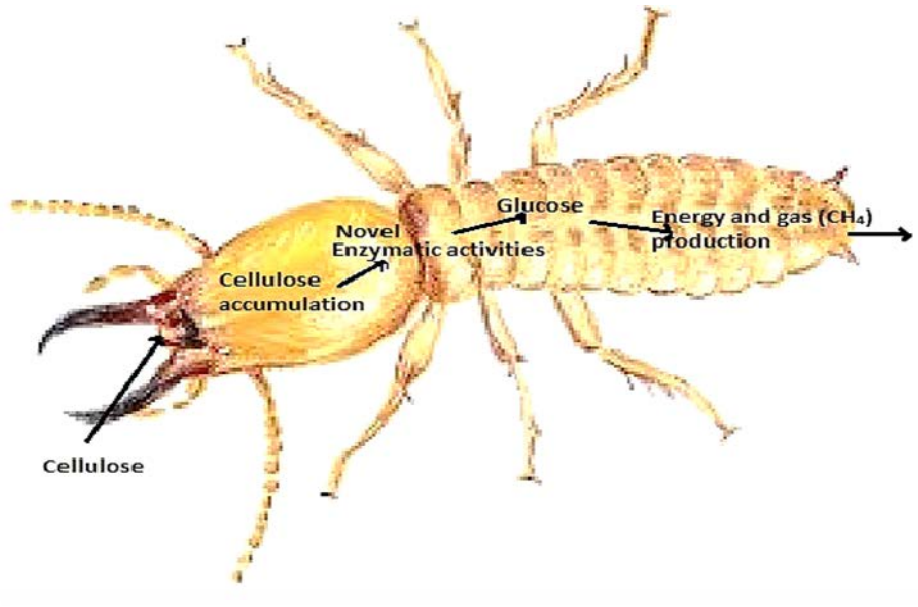

Fig. 1: Termite as a model bioreactor

to possess a predominant cellulolytic enzyme system, reported to have two fold activity over the *Trichoderma reesei* fungal strain of normal habitat/landscape[58,59,60]. With a mutual goal of bio prospecting, researchers have explored termites as the potential source of cellulolytic enzymes, identified several novel enzymes (glycosyl hydrolases) from one of the common termites (*Nasutitermes cornige)*. Thus, the set of genes has been characterized from *Nasutitermes corniger* responsible for complex biomass degradation[61,62,63,64] (Fig. 1). Some other important mileston reported by Research group of Bioinformatics at NIAS, Japan, where bifunctional cellulolytic gene sequences from goat rumen liquid has been appended to the list of important bio-prospects. Figure 1 shows the conversion of complex cellulose into simple biomass materials through the novel enzymes of termite digestive system, which could be used as possible future biofuel.

As of September, 2011[65], Genomes OnLine Database (GOLD) version 4.0, claims information for 11,472 sequencing projects, of which 2907 have been completed and their sequence data has been deposited in a public repository. Out of these complete projects, 1918 are finished and 989 are permanent drafts. Moreover, GOLD is comprised of more than 300 metagenome studies associated with thousands of rare metagenome libraries.

Functional screening of other metagenome libraries which confer the numerous cellulolytic enzymes have been purified and characterized, these strains are known as cellulolytic clones of rare microbiome procured from extreme hot lake water, rabbit's cecum and other extreme environmental samples. Among the other contemporaries, Duan *et al.*[66] cited novel endoglucanases with optimal activity at low pH (4.5) exhibits stability over a broad range of pH (3.5-10.5). The another landmark, marine metagenome comprises the enzymatic series of high value (β-glucosidase-Bgl1A), which confer the maximum glucose tolerance, reported as great success for its sensivity test against high product yield[67]. Jiang *et al.*[68] reported isoform of β-glucosidase (Bgl1D) from soil metagenome, which shows higher activity at low temperature and high ionic liquid titre, also it is reported for its action at a liberal range of pH (5.5-10.5). In recent findings, a newly reported β-glucosidase (unglu135B12) derived from the rumen of cattle is known as a potent enzyme for saccharification of lignocellulose[69].

MICROBIAL GENOME UNDER ECOLOGICAL UPSETS

Since the last few decades, improvements in cultivation independent approach have potentially contributed to our understanding of microbial diversity and community structure in the extreme environment. Though, cultivation dependent approaches have delivered the significant results and the rising number of organisms obtained thereby have allowed for detailed surveys of their physiology and genetics[68,70]. Moreover, the role of microorganisms in revolution of industrial biology is well known, let it be any field, food, feed, dairy, environmental, pharmaceutical, beverages and many more. Involvement of novel microbes and their newest enzyme has revolutionalized the todays industry[71].

Thermophilic fungal endogluconases (*Talaromyces emersonii*) has been reported to have antagonistic effects on microcrystalline cellulose (Avicel) or carboxymethyl cellulose (CMC)[72,73]. These enzymes can be bioengineered for its efficient implication in recalcitrant cell wall degradation as desired in industrial inclusive parapemters e.g., three fungal cellulases were shuffled by means of homologous protein shuffling (SCHEMA-a computational algorithm) resulting in 33-fold enhancement of half life at 63° C while, the activity was maintained within the range of the parent enzymes[74]. Likewise, shuffling of four cellulose gene families from termites led to the enhancement of optimum temperature from 45-55° C but, there was no alteration in the activity[75,76]. The conversion of the chitin binding domain to a hyper thermophilic endoglucanase (*Pyrococcus horikoshii*) doubled its activity at 85° C toward avicel[77].

Ecogenomic tool couples the conservative approaches and enhances the analytical approach for better understanding of biomass degrading organisms from diverse environmental niches. Further, it provides the strong base for establishing enzyme engineering of rare microbiomes[21,78,79,80]. Global energy scarcity could be circumvented with immediate mandate for enzymes with improved catalytic performance or tolerance to process specific parameters. Biotechnology takes a crucial lead in the development of biocatalysts for use in green energy generation[37]. The ecogenomics approach along with synthetic biology has helped in bioprospection of cellulolytic enzymes with efficient activity and stability[81,82]. Such unique omics takes benifit of genetic advancement and biochemical diversity present in the microorganisms of diverse environmental resources and gives a series of noble technologies destined of screening for new catalytic activities from environmental samples with potential omic tool. Moreover, certain biased approaches at the level of heterologous protein expression in *Escherichia coli* together with the misfit of cloning vectors such as non optimal cloning vectors for the metagenomic library constructon. Such technical gaps are under research scan and could be furthered with alternative approach to assure the higher production of desired enzymes from rare resource system[83].

Bioprospecting ecogenomes have been revolutionized non cultivated microbial world which has been identified for new enzymes with multigenic background. Modernization of tools, techniques and prime limitations under the impression of the biotechnological potential uncultivated bacteria are easily being realized by direct DNA cloning rescued from the rare microbial community[37].

The synergetic implementation of multiple approaches can overcome the bottleneck arising from inefficient screening of enzymatic activities. It has been addressed from several perspectives; however, the limitations related to biased expression in heterologous hosts has been investigated at

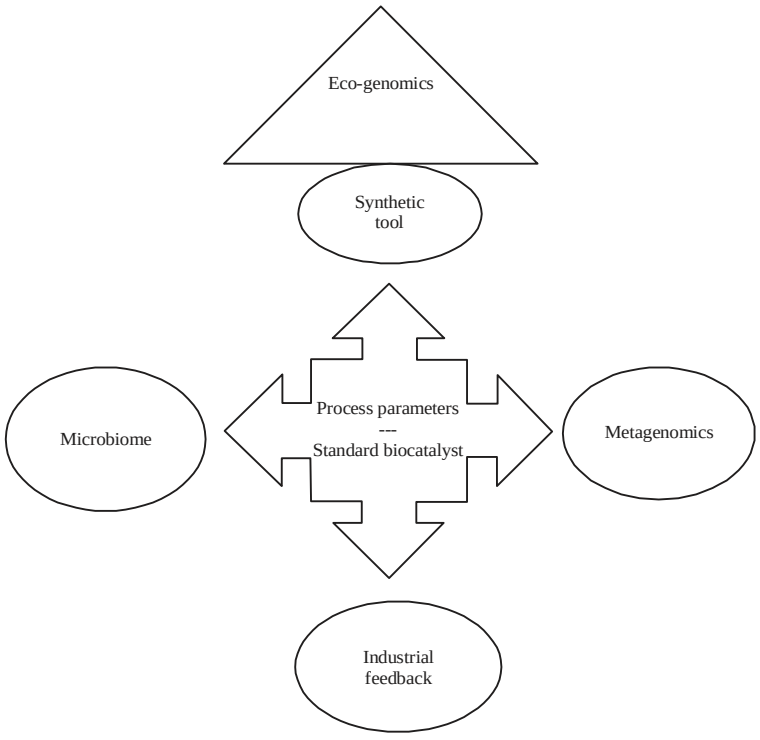

Fig. 2: Eco-genomics: Inputs to harvest the industrial biocatalyst

larger scale[84]. The ecogenomic library construction and subsequent screening approaches allow discovery of the candidate genes encoding the desired catalytic activities for biofuel production. Here, a model in Fig. 2 is depicted for the gene screening approaches for desired enzymes from cultivable or yet to be cultivated microorganisms. Figure 2 shows the ecogenomics and its multiple parameters for biocatalytic harvest system. Ecogenomics approaches are involved for recovering of such genes those are expected to have encoding the desired catalytic activity for industrial processes. The characterized sequence for enzymes from cultured or non-cultured microorganisms could be cloned and expressed. Set parameters such as activity, stability, specificity, efficiency, etc., are got optimized at industrial level using protein coherent design and *in vitro* evolution techniques. Ecogenomics are comprised of activity based exercises, which include the construction of expression libraries and its subsequent activity run or sequence based screening. Sequence based screening again involve either the DNA primers designing for conserved regions of characterized protein families (protein data base) or data mining of genes encoding significant biocatalysts recognized in sequences from next generation sequencing projects. A complementary approach which has been treated as synthetic biology can be potential solutions to the existing boundaries in activity based ecogenomic approaches. Advancement of approaches for the recombining of new bacterial hosts and molecular biology tools assure the exponential finding of relevant enzymes at commercial level.

ECOGENOMICS AND NANOTECHNOLOGY

The size of nanoparticles ranges from 1-100 nm and display distinctive optical, magnetic, thermodynamic and catalytic properties[85,86]. Such properties of nanoparticles with their uniform size, facilitate them to improve enzyme constancy, strength, action, competence and work performances in bioprospecting application[87,88]. Immobilization of enzyme by nanostructured shippers may substantially upsurge biocatalyst shelf life and cutting the budget of enzymatic process. Inventive, newest and possibly extra competent biocatalyst achieved using ecogenomic approach is feasible by involving supplementary changes in enzyme structure to achieve amplified productivity and constancy in piolet scale bioreactors. Other fesibilities, such as merging nanotechnology and metagenomics is dependent on the base that numerous new secondary metabolites of vital commercial and pharmaceutical products using microbial sources. Novel nano-enabled aspects of ecogenomics, microbial genomic study is a commencement to manufacture more effectual vaccines, diagnostics and particularly for treating multi resistant microbial strains. Synthesis of novel and magic bullet antibiotics including early identification and examining of disease using molecular based recognition tools such as nanobiosensors and isothermal gene amplification[89].

Such protection to novel enzymes can be delivered by evolving nanobiotechnological approaches. Using interdisciplinary tactic, combining of techniques like genomics (primarily functional metagenomics), nanobiotechnology and protein bioengineering could leads to manipulation of metabolic efficacy of biomedical and environmental research aspects[90]. This in turn will generate resource for balanced and proficient resource management and also in biotechnological applications. Additional exploitation of environmental microorganisms is also projected to upsurge the manufacture of renewable and effectual biofuels and high value bioproducts employed in industry and natural product based drug innovation[91,92,93].

CONCLUSIONS AND FUTURE ASPECTS

Irrespective of the cultivability of microbes, conventional ecogenomics technique provided a constructive insight into wide range of minute community diversity in the ecosystem. Conversely, to study the whole genome of every microbial species is still a big challenege for ecogenome research owing to the complexity of microbial species and diversity. Conventional molecular techniques allow the study of microbial diversity in the ecosystem, this was the vicinity where, research work was formerly limited by classical microbiological and biotechnological methods with some disadvantages. For this reason, molecular tools become more and more acceptable and putative in microbiological and technological laboratories. To overcome some disadvantages of molecular biology techniques, omics tools solved the problem to some extent. Scientists and researchers all over the globe from existing ecogenomics face problems owing to inadequate efforts at natural variation level in diverse microbial populations. Evaluation and scrutiny of ecogenomics is making a straight influence on our current understanding of microbial assortment, biology and production of unusual natural secondary metabolites. Subsequently, certifying how finest is the sample and concluding whether, an isolated sample is true representative still remains a big challenge. Technical questions those are not indicated in this review could be concluded and eventually be discussed as future tasks: Are DNA extraction techniques destined to minimize contamination and to ensure that a

community's genome is adequately represented have yet to be considered in ecogenomics? Are expression systems for functional metagenomics completely robust and flexible to express candidate genes in ecogenomes? Future strategies involve the collaboration among novel technologies (bionanosensors, proteomic tools) in order to enhance the exploration of innovative genes expressed in environmental condition. Less efficiency and squat functioning of currently prevailing enzymes for greenfuels production has led to limited industrial role. In such situation, the ecogenomic statistics provides a novel unmapped genomic information booty that could augment enzyme account by finding valuable and new microbial enzymes. There have been various well designed screening techniques in ecogenomics that has been executed to highlight non culturable microorganisms and their possible and probable employement in biofuel advancement, concidering their precise roles in their respective ecosystem. To provide further promices in cultivation of uncultured microorganisms, techniques like metatranscriptomics and metaproteomics are the state of the art development in ecogenomics technology. With the advent of existing sophisticated tools and technological developments in next generation sequencing technologies, extraordinary and significant reduction in price and in large data sequencing within quick time has credited ecogenomics as novel technique to retrieve the inaccessible microbes from environment.

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