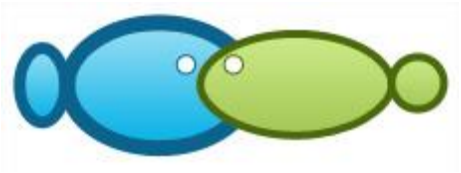

Review on bioprospecting of thermophilic enzymes from hot springs via Omics approaches

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Abstract. Thermophiles can live in high temperatures; their value stems primarily from their ability to produce thermoenzymes, which have a wide variety of applications in the market and can survive difficult industrial manufacturing processes. These thermophiles can be isolated from different sources. However, in this review, we focus on their presence in the Hot springs, which are unique natural environments presented worldwide; some of these hot springs have considerable amounts of industrial enzymes that can be extracted and produced. Nevertheless, the original culture-based approaches still challenge the isolation of these bacteria and their enzymes' identification. New techniques are arising to simplify this process, mainly the culture-independent procedures; one of these new processes is the metaomic approaches that introduce the genes (by metagenomics), transcripts (by metatranscriptomics), proteins (by metaproteomics), and metabolites (by metabolomics) from complex microbial communities to analyze the biochemical function and microbial interactions in situ, they improve our understanding and predicting the contaminant biotransformation capacities of microbial communities. For hot springs, culture-independent techniques allow a comprehensive analysis of microbial populations.
Key Words: metagenomics, metaproteomics, metatranscriptomics, thermoenzymes, thermophiles.

Introduction. Extreme thermophiles and thermophile bacteria are the organisms that can grow at elevated temperatures optimally; hydrothermal ventilation structures of geothermal activity, such as hot springs and ocean vents, were the most widespread environments for these organisms. These thermophiles have been isolated from terrestrial and aquatic ecosystems of elevated temperatures. These thermophiles also, can produce several enzymes used in different aspects of our lives due to their thermostability and thermoactivity (Yavuz 2003). Hence, isolating and identifying thermophilic bacteria from natural sources is essential in discovering new industrial enzymes.

However, enzymes commonly produce multiple environmentally friendly and sustainable products in industrial environments. There is an enhanced demand for enzymes that function well under the varying circumstances of numerous industrial procedures. Over the past centuries, with the technical advances in DNA and protein technology, distinct approaches have developed to satisfy these increasing requirements. One of these approaches is bioprospecting, which means looking for new enzymes in different settings that contain elevated natural diversity micro-organisms (Speda 2017).

Furthermore, although the ubiquity and complexity of microbial communities have been pretty well known for a long time, advances in high-throughput sequencing have created technological innovations that supplement culture-based strategies in both their molecular precision and usability to a large scientific world. The first culture-independent methods were based on low-performance bacterial 16S ribosomal rRNA gene sequencing, and the reliability and efficacy of its surveys dramatically improved with enhanced sequencing methods throughput (Tringe & Hugenholtz 2008).

Moreover, recently, approaches to genome-wide sequencing, including metagenomics and metatranscriptomics, have extended the scientific methods used to investigate microbiota. These meta-omic methods reveal the genomes, proteins, and

consequently, transcripts and metabolites of hundreds and hundreds of micro-organisms to examine molecular activity and system-level microbial interactions. All these responsive public assays offer unique ways of learning complex ecosystems (Segata et al 2013).

Therefore, hot springs now have increasing interest due to their microbial contents; in Malaysia, for instance, about 60 hot springs, 75% of which are in easily accessible areas; their temperature varies from 23 to 98°C (Sum et al 2010). In this review, we aim to look into the diversity and structure of micro-organisms in hot springs environments, highlight the modern research on industrial enzymes derived from hot springs micro-organisms and their commercial use, and understand the micro-organisms in hot springs using omics approaches.

Thermophiles types and environments. Thermophiles are micro-organisms that live in high temperatures, more than 45°C. These are present in numerous geothermal warmed regions of the world, such as deep-sea hydrothermal flows, hot springs, and rotting plant materials such as peat bogs and manure (Brock 2012); also, they have been isolated from hot desert soils and salty wetlands (Aanniz et al 2015), and from the deep sea with Antarctic biota, volcano zones, and other geothermal fields in whole as mentioned by Herbert (1992) who also emphasized that the industry sparked the quest for new species as it was recognized that the capacity of such micro-organisms to live in these harsh circumstances was directly linked to unique characteristics consisting primarily of specific enzymes and biochemical pathways. The main types of extremophiles are psychrophiles (low temperature), thermophiles (high temperature), barophiles (high pressure), acidophiles (low pH), alkaliphiles (high pH), and halophiles (high salinity). Since their appearance, thermophiles have provided researchers with a fascinating and demanding forum. Besides developing under harsh conditions, extremophiles may generate industrial-value compounds, such as antibiotics, enzymes, and hormones (Rampelotto 2013; Shivlata & Satyanarayana 2015).

Such intense microbial growing conditions are present in more common tropical habitats on the essential planet. Complex conditions may include the existence of strong chemical solvents or toxic metals. Extremophiles' development and taxonomy, especially thermophiles, are of growing interest. Thermophiles that exhibit ideal growing temperatures between 60 and 80°C are moderate thermophiles, usually mostly bacteria (Antranikian et al 2017).

Thermophiles that grow better at or above 80°C and cannot survive below 60°C are the hyperthermophiles, mainly Archaea (Antranikian et al 2017). Interestingly, certain species were extracted from regions with temperatures far hotter than their optimum temperature for development, e.g., *Hyperthermus butylicus* (Zillig et al 1990). This could apply to organisms isolated from temperatures well below their optimum growth temperature, such as *Archaeoglobus profundus* (Burggraf et al 1990).

Depending on their fundamental growth temperatures, the thermophiles are grouped into three types (Baker et al 2001); the first type has adapted growth temperatures varying from 35 to 70ºC, and it is called moderate thermophiles; secondly, there are the extreme thermophiles that have optimum growth temperature of about 55 to 85ºC; finally, the ones with higher optimum growth temperatures between 75 to 113ºC are called hyperthermophiles. Their growth profile also classifies them as obligate thermophiles that grow between 65 to 75ºC but cannot grow below 40ºC. Facultative thermophiles can grow at about 37ºC, but their optimal growth temperatures are between 50 to 60ºC. Thermotolerant thermophiles can tolerate temperatures up to 45-50ºC and grow under 30ºC (Pask-Hughes & Williams 1977). Interestingly, thermophiles produce enzymes that can work at extreme temperatures to survive these harsh conditions (Brock 2001). Furthermore, because these enzymes work under high temperatures, there is an increased interest in their industrial application. Working under high temperatures reduces the contamination risk, increases substrate solubility, and increases reaction rates (Antranikian et al 2005). Besides molecular biology, some enzymes have broad applications, such as heat-stable DNA polymerases for a polymerase chain reaction (Baker et al 2001).

Thermophilic enzymes. The production of thermoenzymes from thermophiles has been a rich research topic for the last two decades. Still, the interest in thermophiles and how their proteins can function at high temperatures started as early as the 1960s by Brock & Freeze (1969). Studies found that there is an improvement in the atomic packing in thermophilic enzymes when compared to the mesophilic enzymes; also, there is an improvement in the packing quality and electrostatic interactions, and there is increased hydrophobicity in the protein core, which all improve the stability of protein folding, on the other hand, these enzymes possess a reduction in destabilizing forces such as decreased conformational flexibility or entropy of unfolding (Sammond et al 2016).

Thermophilic enzymes at high temperatures are also intrinsically firm and productive, offering significant biotechnological advantages over mesophilic or psychrophilic enzymes. In general, thermostability is associated with higher resistance to chemical denaturants like solvents or guanidinium hydrochloride (Rather et al 2018).

Thermophilic industrial enzymes. Isolated, purified thermostable enzymes from newer sources remain an exciting challenge (Godfrey & West 1996). The production of thermophiles at high temperatures is technologically and economically significant. It decreases the possibility of contamination by specific mesophiles, reduces viscosity, encourages mixing, and results in a high substrate solubility level. Nevertheless, the biomass produced by such species is typically shockingly small relative to their mesophilic equivalents. Reduced cell counts present problems for extensive- and small-scale development, challenging detailed enzyme studies (Krahe et al 1996). Special equipment and specific processes have been developed to improve thermophiles' and hyperthermophiles' fermentation (Schiraldi & De Rosa 2002). The enzymes obtained from some extremophiles are of considerable value in the biotechnology sector, capable of operating under circumstances that denature enzymes extracted from most typical species (Mattila et al 1991). Researchers are investigating micro-organisms that live in hot springs as potential sources of valuable biochemicals; screening marine micro-organisms known first for their environmental potential showed many remarkable biological molecules that contain rare proteins, anti-cancer agents, antialgal chemicals, and secreted sugars. Extracellular thermostable enzymes of significant value in the industry are proteases, xylanases, amylases, cellulases, pectinases, lipases, and DNA polymerase (Ladenstein & Antranikian 1998).

DNA polymerase. DNA polymerases play an essential role in DNA replication and repair of the cell by utilizing deoxyribonucleoside triphosphates (dNTPs) as substrates for genome replication (Burke & Lupták 2018). Increasing the understanding of these enzymes' function, especially in DNA sequencing and the polymerase chain reaction (PCR), significantly improves DNA amplification and sequencing methods. Also, there has been an increase in thermostable DNA polymerases since 1980, when the first type was isolated from *Thermus aquaticus* (Taq polymerase) and characterized. Since then, it has been widely used in DNA sequencing due to its thermostability; it facilitates the automated cycling of sequencing reactions and reduces the DNA template required for sequencing (Pavlov et al 2004). The absence of proofreading by Taq DNA polymerase is an issue for a few PCR procedures. DNA polymerase from *Thermococcus litoralis* *Thermococcus litoralis* has been reported to have an exonuclease-reading function (Godfrey & West 1996). Another heat-stable polymerase comes from the bacterium *Pyrococcus furiosus*; this bacterium grows optimally at 100°C, making it hyperthermophilic. Taq DNA polymerase is adequate for most PCR (Hamilton et al 2001).

Amylases. They are enzymes that catalyze starch and glycogen hydrolysis (Palmer & Bonner 2007). Bacterial α-amylases, along with the fungal glucoamylases, are the main enzymes in the production of glucose from starch; one of the first archaeal α-amylases discovered from the hyperthermophilic archaeon *Pyrococcus furiosus* exhibits optimal activity at 100°C (Elleuche et al 2014). Also, amylases are commonly employed in the cloth, pulp, food, and fermentation sectors, such as flour manufacture, fruit juices, glucose-fructose syrups, sweeteners, and alcoholic drinks (Haq et al 2010). During the

sweeteners' production from the starch, the temperature should be 50°C or more to avoid the browning effect and minimize the starch pastes' viscosity; thus, thermostable α-amylase is needed to preserve the process temperature (Castro et al 1999). Bacteria of the genus *Bacillus* are commonly used to commercialize thermostable α-amylases (Kubrak et al 2010). New studies are now done to produce amylases from thermophilic bacteria for these enzymes' commercial production; the PCR α-amylase gene was identified from *Anoxybacillus thermarum* FRM-RBK02 that was isolated from a hot spring in Remboken, Indonesia, this amylase having optimum activity at 80°C (Mantiri et al 2019).

Proteases. They are enzymes that hydrolyze proteins; they have many classifications; depending on their optimum pH, they are classified into acidic, neutral, or alkaline groups; they may also be classified into aspartic, cysteine, glutamic, metallo, serine, and threonine depending on the amino acids present in their binding sites (Singh et al 2015). They are one of the most important and widely used industrial enzymes; they are present in all living organisms and hydrolyzes protein peptide bonds into peptides and amino acids (Muthu et al 2017).

Microbial proteases demonstrate one of the world's most significant and heterogeneous enzyme families, comprising about 65 percent of total enzyme production. They are considered the world's leading biotechnology, bioengineering, and industrial applications (Baweja et al 2016). These have various applications, such as ingredients in detergent formulations, industrial food and leather manufacturing, peptide synthesis, and pharmaceutical products (Mechri et al 2019). With the need for enzymes, reliable biocatalysts would be needed to endure harsh operating procedures (Haki & Rakshit 2003). Some haloalkaliphilic bacteria and actinomycetes have documented thermostable proteases (Thumar & Singh 2007; Dodia et al 2008). Compared to their mesophilic counterpart, thermophilic proteases have more alanine and leucine in their amino acid content; they are membrane-bound and have peptidase M48, peptidase M50, PDZ, and CBS domain (Vaidya et al 2018).

Xylanases. They are a large group of enzymes that produce xylose, a carbon source for cell metabolism, and plant cell infection by plant pathogens. Bacteria, algae, fungi, protozoa, gastropods, and arthropods produce it. They are used in the paper, pulp, and baking industries (De Vos 2006).

They are also used in starch hydrolysis, clarification of fruit, vegetable juices and wine, cheese ripening, dough fermentation, and bakery products (Dumorné et al 2017). Lately, thermostable xylanases have been discovered in many *Thermotoga* species (Elleuche et al 2014). Also, they were produced from thermophilic *Bacillus* strains from the Tunisian hot springs, the thermophilic anaerobic bacterium *Caldicoprobacter algeriensis* TH7C1(T), isolated from hydrothermal hot springs, the Gram-positive strain Rxl from the genus *Thermoanaerobacterium* from hot springs in Baoshan of the Yunnan Province, China, *Acidothermus cellulolyticus* 11B, isolated from hot springs in Yellowstone National Park, and the extremely thermophilic bacterium *Dictyoglomus thermophilum* Rt46B.1 from a New Zealand hot spring (Basit et al 2018).

Many requirements are essential for candidate xylanases; their molecular mass should be small to promote their migration in pulp fibers, they should be stable and dynamic at high temperatures and alkaline pH, they should not have a cellulolytic effect to prevent hydrolysis of cellulose fibers, and they should be produced at high yields and inexpensive (Niehaus et al 1999). New technologies are now used to improve the xylanase properties; structure-based site-directed mutagenesis on the N terminus of the xylanase structure resulted in nine mutations and disulfide bonds that will enhance the thermostability of this enzyme (Watanabe et al 2016).

Pectinases. They are a large enzyme group that divides pectic plant tissue polysaccharides into simpler molecules, such as galacturonic acid, through depolymerization and de-esterification reactions (Pedrolli et al 2009). They are is used in the fruit and textile industries, also in producing good quality paper, fermentation of

coffee and tea, oil extractions, and pectic wastewater treatment (Kashyap et al 2001). Numerous micro-organisms have produced thermostable pectinases like *Clostridium thermosulfurogenes*, *Sporotrichum thermophile*, *Aspergillus fumigatus*, and *Thermomucor indicae-seudaticae*, and from many species of *Bacillus*; also, *Aspergillus* is the primary source of thermophilic pectinolytic enzymes for industrial production (Dhiman et al 2013).

Lipases. They catalyze the hydrolysis of triacylglycerols to glycerol, diacylglycerols, mono glycerol, and free fatty acids. Bacterial lipases are categorized into eight families based on differences in their sequences of amino acids and biological properties (Liu & Kokare 2017). Lipases are used in detergent formulations, organic chemical paper manufacture, nutrition processing, production of biosurfactants, dairy, the oleochemical, agrochemical, beauty products, and medicinal manufacturing sectors (Liese et al 2006). Also, lipases were found in many species, such as *Bacillus prodigiosus*, *B. pyocyaneus*, *Pseudomonas fluorescens* (Hasan et al 2006), and *Bacillus* strain A30-1 (ATCC 53841) collected from Yellow Stone Park (Wang et al 1995), *Geobacillus* sp. TW1 in China (Li & Zhang 2005), and *Bacillus thermoglucosidasius* and *Bacillus coagulans* from the Setapak hot spring in Malaysia (Hamid et al 2003).

In addition, extremophile lipases have become an interesting topic in industrial biotechnology due to their strong resistance to organic solvents, proteases, detergents, thermal denaturation, and chaotropic agents (Hoesl et al 2011). Also, they are stable under high temperatures, in the presence of organic solvents, and over a broad pH spectrum (Sharma et al 2018).

Cellulases. They are the enzymes that hydrolyze β-1,4 links in cellulose chains and release oligosaccharides, cellobiosis, and glucose; various micro-organisms produce fungi and bacteria (Mojsov 2016). Thermophilic bacteria known to have thermostable cellulases are *Bacillus*, *Geobacillus*, *Caldibacillus*, *Acidothermus*, *Caldocellum*, and C*lostridium* (Patel et al 2019). The critical industrial uses of cellulases are in the clothing industry to 'biopolize' fabrics to create the stonewashed appearance of denim and household laundry detergents to enhance the softness of the cloth's clarity (Cavaco-Paulo 1998). Other uses include biofuels, which means the conversion of plant biomass into bioethanol, food, and brewing, pulp and paper (bio pulping), and animal feeds (Wang et al 2015); besides, they are used in waste management improvement of soils for agriculture (Biver et al 2014), and extraction of compounds from plants such as olive oil, pigments, and bioactive molecules (Sharma et al 2016).

Meta-omic approaches. Because thermophiles grow in harsh conditions, there is a difficulty in culturing them on standard biological media. There is a need to study communities of microbial organisms directly in their natural environments, bypassing the need for isolation and lab cultivation of individual species (Mirete et al 2016); meta-omics techniques appear to have broad microbial ecology applications because they provide unparalleled observations into the organismal and functional structure in natural society in situ (Figure 1). The main types of novel high-throughput molecular methods are metagenomics, metatranscriptomics, metaproteomics, and metabolomics (Rodríguez et al 2015). While metagenomics and metatranscriptomics make it possible for an in-depth biodiversity assessment, metaproteome analyses directly measure the proteins found in an environmental sample, providing functional information at the intracellular level (Bastida et al 2012).

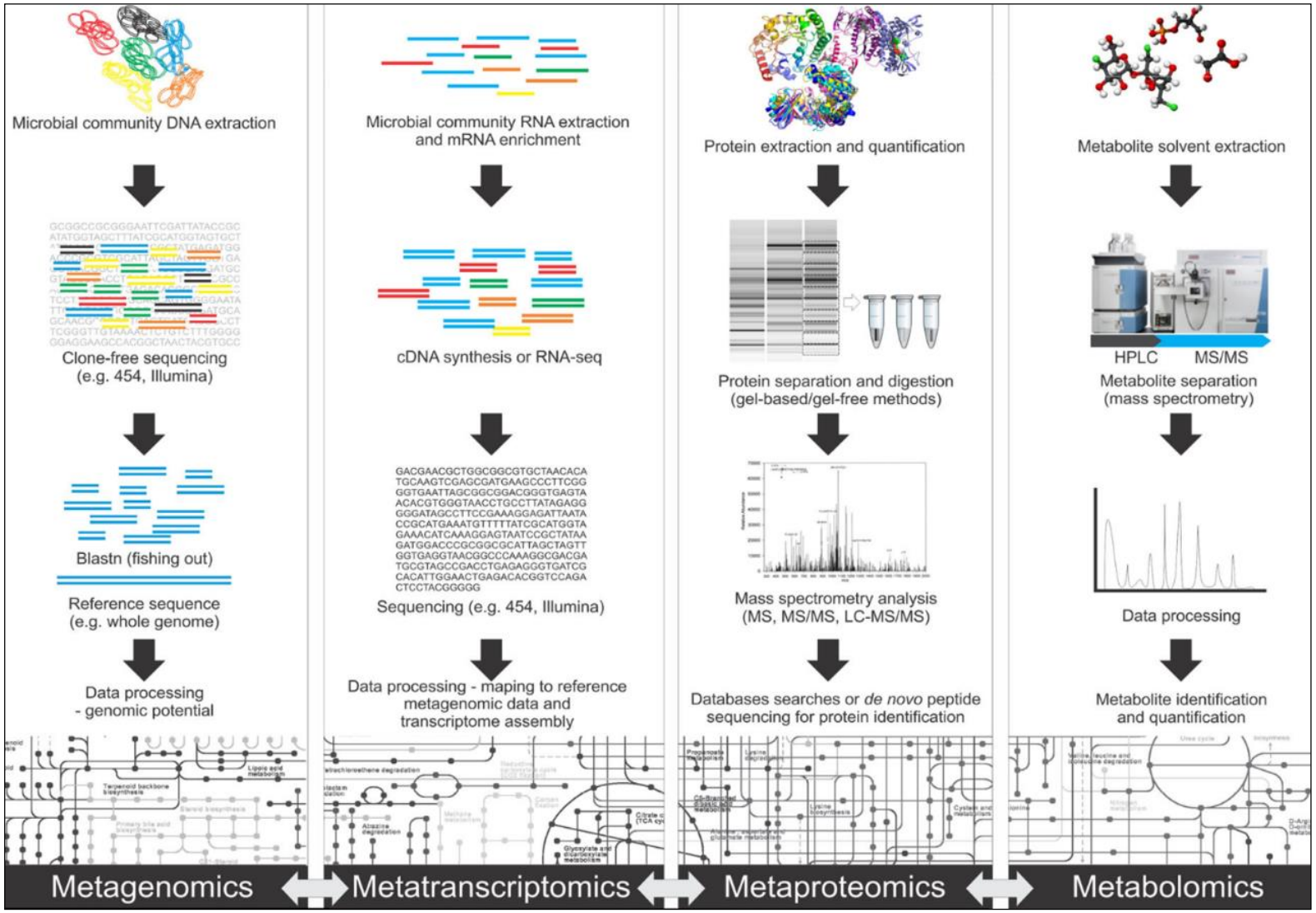

Figure 1. The diagram shows the key steps in metagenomic, metatranscriptomic, metaproteomic, and metabolomic approaches (Rodríguez et al 2015).

Metagenomics. Also known as environmental genomics, defined as "the use of whole-genome shotgun approaches to sequence genomes from entire communities of microbes in environmental samples of water, air, and soil", it allows us to know more about millions of species of bacteria, and the discovery of new viruses mainly bacteriophages that were identified from water samples (Klug et al 2006). Metagenomics, this mining strategy for the biotechnology and pharmaceutical industries, has been disclosed as promising (DeCastro et al 2016). It has two phases: meta(analysis), a form of the mathematical study of the effects of two different analysis methods, and genomics, which investigates genetic structure (Rondon et al 2000). It isolates genomic DNA directly from an environmental sample. It is extracted through sequencing high-throughput viz shotgun sequencing, 454 pyrosequencing, minimizing the loss of critical elements while cultivation, and utilizing its methods to evaluate the structure and functional ability of the microbial community. Sequence-based screening is often used for genes or areas of developing systemic analysis, distribution, etc., while functional-based screening assesses microbial environmental communities' functional capacity (Baweja et al 2016).

Also, metagenomics is divided into two essential methods; a structural metagenomic approach allows the study of the composition and dynamics of the population in a particular ecosystem, thus providing a broader explanation of the interactions between the different components that create a society and are necessary to decode its members' ecological or biological functions. The other type is the functional metagenomic approach; it aims to identify the active genes and generate expression libraries with metagenomic clones followed by activity-based screenings (Figure 2) (Alves et al 2018). Next Generation Sequencing (NGS) techniques have dramatically enhanced this approach; Illumina and Roche 454 are the leading systems in high-temperature environments. Illumina performs well per run and produces read lengths of up to 300bp. On the other hand, since Roche 454 provides more extended readings and is deemed simpler to map to a reference genome, it is more costly and has lower throughput (DeCastro et al 2016).

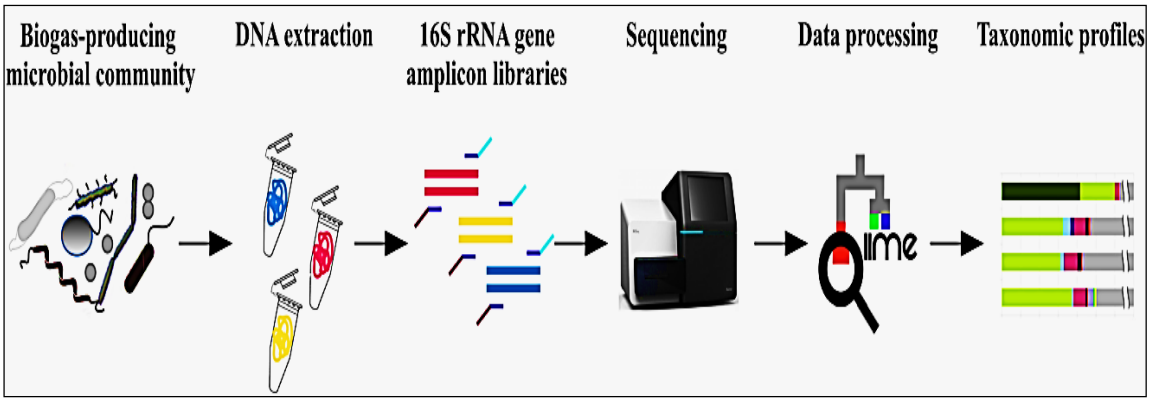

Figure 2. It shows the process for functional characterization of microbial biogas populations using metagenome sequence data. Complete DNA was extracted from biogas reactor samples to construct whole metagenome shotgun libraries sequenced on high-throughput sequencing platforms. The sequencing data were quality-checked and functionally defined based on single-read sequences to predict the underlying biogas population's functional profiles. Furthermore, MAGs were compiled using a metagenome assembly followed by a binning method, and then their metabolic capacity was assessed (Hassa et al 2018).

Metagenomic studies of the hot springs aim to understand their microbial ecology and identify the novel genes that are responsible for high-temperature tolerance; one of these studies was done in hot spring Tattapani, Himachal Pradesh in India, and the main results revealed that this spring is rich in the bacterial phylum Proteobacteria and other bacterial phyla were also presented which are *Thermodesulfobacteria*, *Firmicutes*, *Deinococci-Thermus*, *Bacteriodates* and *Aquificea,* gene isolation responsible for thermal tolerance in these bacteria can help develop high-temperature tolerance in crop plants (Mohanrao et al 2016).

Metaproteomics. It aims to screen and identify proteins presented within the ecosystem at a given time (Wilmes & Bond 2004). This will provide more information about microbial communities' structure, function, and dynamics, which is essential to a better understanding of the metabolic activity, microbial recruitment, competition in nutrient resources, and ecosystemic distribution of defense systems (Hettich et al 2013).

Successful assessment of metaproteomics depends on three factors: valuable protein extraction from an environmental sample, protein or peptide separation before detection, and eventually, high-throughput clearcut protein and peptide recognition mainly by mass spectrometry analysis and database searching (Siggins et al 2012; Wilmes et al 2015). Some protein extraction methods mainly used in the analyses of environmental samples are guanidine hydrochloride (GuHCl), bacterial protein extraction reagent (B-PER), and sequential citrate-phenol (SCP) (Leary et al 2014).

Protein separation is a very critical step to reduce the complexity of the samples; this can be done mainly by two techniques: gel-based methods that include one-dimensional (1D) or two-dimensional (2D) electrophoresis, which separates proteins according to their size or isoelectric point (Brunelle & Green 2014), on the other hand, gel-free methods depend on the expansion of chromatography techniques to separate proteins; high-pressure liquid chromatography (HPLC) is more accessible and practical for separating peptide mixtures than electrophoresis, but the most frequent choice for the proteomic study is reversed-phase liquid chromatography (RPLC) (Xiao et al 2017; Hinzke et al 2019).

Mass spectrometry (MS) is a critical step for the identification of the proteins, followed by database searches or *de novo* peptide sequencing to identify the proteins; after that, data interpretation - which is the incorporation of established proteins and metabolic pathways into functional processes - will be the final step (Figure 3) (Srivastava et al 2019).

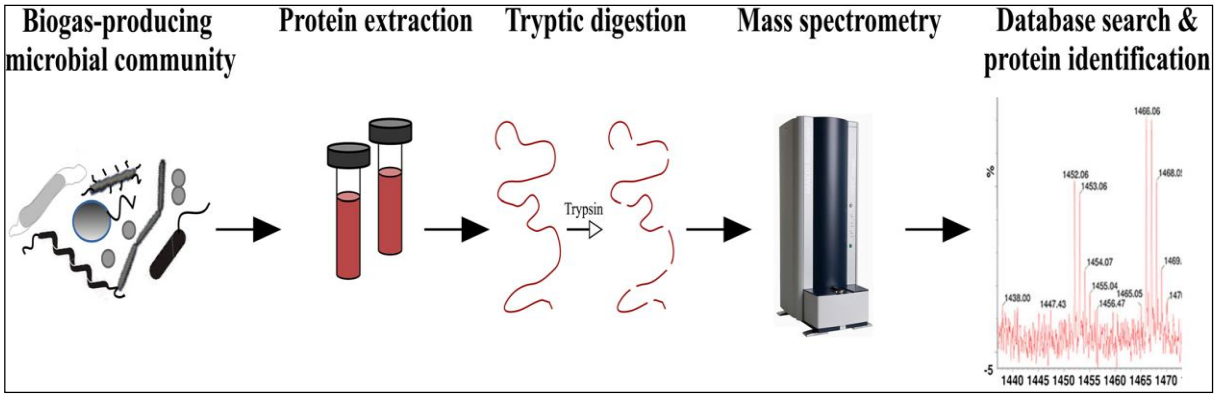

Figure 3. Samples of the microbial biogas culture, protein extraction, tryptic digestion of proteins, mass spectrometry of the resulting peptides, and database searching using mass spectrometry data to classify proteins within the metaproteome examined are all part of the metaproteomics workflow (Hassa et al 2018).

Metaproteomics ensures the rapid and precise identification of biocatalysts in environmental samples and allows mining microbiomes for novel proteins from previously uncultured organisms. It is considered an agile approach to enzyme discovery (Wilmes & Bond 2004). One example of this approach in hot springs was a study done on the Mammoth Hot Springs in Yellowstone National Park that aimed to understand the specific metabolic pathways utilized by *Sulfurihydrogenibium*-dominated filamentous microbial mats; the results showed that 533 out of 665 unique proteins were associated with the *Sulfurihydrogenibium* pangenome B001 (Dong et al 2019).

Metatranscriptomics. It refers to the total content of gene transcripts: the RNA copies of these genes in a community, considered a unique entity, at a specific moment of sampling. The metatranscriptomic analysis identifies the whole microbial population's

gene expression profiles, depending on the compilation of transcripts synthesized under different environmental conditions (Booijink et al 2010).

It enhances our knowledge of microbial environmental communities' structure, function, metabolic activity, adaptive mechanisms, and regulation (Embree et al 2014). Differences in gene expression are observed by quantifying and comparing the transcriptome composition between samples of a time series or various tissues or cell types (Wang et al 2009). The main steps of metatranscriptomics are: firstly, to extract and then identify total RNA from the sample. Fragment scanning, database creation, and related consistency checking for the qualified RNA is the second step, followed by the sequencing to the eligible library, mainly utilizing the Illumina sequencing platform. Finally, the raw data collected from the sequencing can be used for the bioinformatics analysis (Figure 4) (Peimbert & Alcaraz 2016).

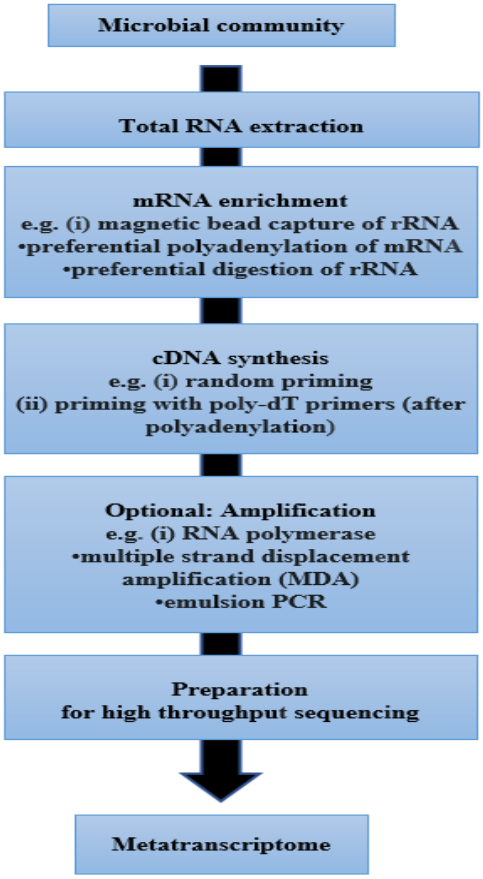

Figure 4. High-throughput sequencing workflow for metatranscriptomics from microbial communities (e.g., 454 pyrosequencing) (Warnecke & Hess 2009).

Bioinformatic analyses of the metatranscriptome will manage extensive data sets through a series of steps called a pipeline or a workflow like Galaxy, KNIME, Chipster, and Snakemake (Lott et al 2017). The main steps include filtering the reads; selecting the library between aligning reads to a reference sequence and doing *de novo* assembly; annotation with BLAST, KASS (Kegg), or M5NR; statistical analysis to give meaning to the data; and finally, sharing the data by uploading the original, assembled, and annotated data sets (Solbiati & Frias-Lopez 2018). Metatranscriptomics can be used to obtain unprecedented insights into community members in situ physiological properties as inferred from transcription profiles and investigate the actively transcribed messenger RNA. Metatranscriptomics research on hot springs enhances our knowledge of different gene-encoding energy metabolism enzymes and indicates their significance in the survival of microbial hot spring organisms (Tripathy et al 2016).

Metabolomics. Metabolomics is the large-scale study of metabolites within cells, biofluids, tissues, or organisms; it also includes the analysis of metabolism substrates and products affected by hereditary and environmental factors. Metabolites and their concentrations represent the underlying biochemical behavior and cell/tissue status (Guijas et al 2018; Nalbantoglu 2019). Mainly, there are two approaches for metabolomics: the untargeted or global approach, which tests as many metabolites as possible without any intentional bias from several biological samples (Figure 5B). The targeted approach measures metabolite sets when a particular biochemical query should be addressed; this method is commonly used in drug metabolism pharmacokinetics and examines the impact of medicinal or genetic alterations on a specific enzyme (Figure 5A) (Hyötyläinen & Wiedmer 2013).

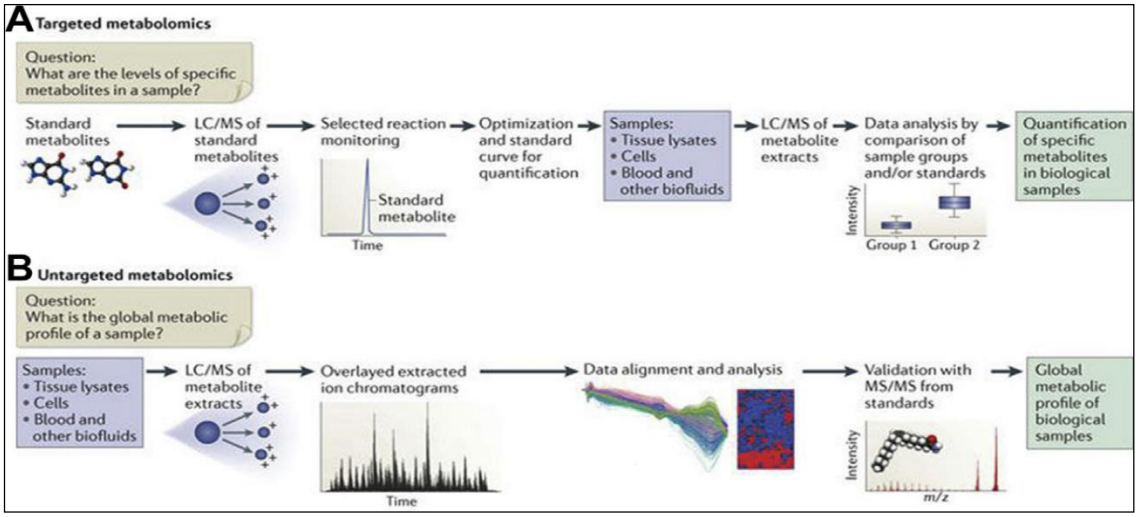

Figure 5. The targeted and untargeted workflow for LC–MS-based metabolomics. (A) Normal metabolites are used to develop targeted approaches. Afterward, the metabolites are obtained from tissue lysates, cells, semen, and other biofluids. The metabolites are examined, and the data output allows for quantifying these metabolites using previously established standard methods. (B) In an untargeted process, biological samples, like tissue lysates, cells, blood, and other biofluids, are isolated using liquid chromatography. Such samples are then analyzed using mass spectrometry to obtain data. Bioinformatic software is used to process the data and establish a global metabolic profile of biological samples (Patti et al 2012).

Experimental design directs the entire metabolomics process, crucial for formulating research objectives and ensuring that the collected samples accurately represent the biological systems of interest. Subsequent to this, sample preparation involves the collection and preservation of samples to minimize variability and degradation. In the last step, extraction, liquid or gas chromatography is used to separate metabolites based on their chemical properties, or solid-phase extraction (SPE) is used to selectively isolate metabolites using sorbent materials, which improves the quality and reliability of the data. After that, several techniques will undergo the data analysis, namely liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry(GC-MS), and nuclear magnetic resonance (NMR) spectroscopy; the final steps are data processing and statistical analysis (Tan et al 2016; Dayalan et al 2019). Metabolomics studies can provide a quantitative metabolite analysis and include qualitative information about the organisms' metabolic activities (Koek et al 2011). Metabolomic study results for the Mushroom Spring, Yellowstone National Park showed 58 metabolites; this offers insight into the metabolites' dynamics to consider group reactions to these changing environmental conditions (Kim et al 2015).

Hot springs worldwide. Although there is no widely accepted conventional definition of hot springs, they can usually be described as a geothermal manifestation where warm water in the form of spring flows out of the earth. Thompson & Turk (2005) described hot springs as warm groundwater flowing naturally to the ground. Sen et al (2010) described

hot springs as springs where the water temperature is substantially above that region's average annual air temperature (Sen et al 2010; Nazaruddin et al 2015).

Several local hot springs were studied as a source of thermophilic bacteria. The variety of the hot springs is enormous. Most microbiologists trying to remove thermophiles from hot environments seems worthwhile as this environment has proved to be the home for helpful enzyme-producing bacteria. It is possible to use biochemical and molecular techniques to define the bacteria and the enzymes that are expressed (Zuridah & Norazwin 2011).

As hot springs can be a source of bacteria with valuable enzymes, eight thermophilic isolates have been isolated from Dusun Tua hot springs and studied for their enzymatic activities of essential enzymes, which are protease, lipase, amylase, cellulase, pectinase, and xylanase. The results show that all the isolates had at least three vital enzyme activities; one has intense enzyme activity for all enzymes, so this isolate needs to be investigated more for potential biotechnological application (Msarah et al 2018).

Eighty-four bacterial isolates were collected from multiple hot springs in Saudi Arabia's southern region, Al-Majardah, Al-Khubah, and Al-Ardah, and 78 exhibited expressions for one or more enzymes. *Bacillus aerius*, *Bacillus licheniformis*, and *Bacillus sonorensis* were identified through molecular recognition and phylogenetic analysis of potential isolates, which can produce the target enzymes - amylase, protease, and lipase, respectively. The physicochemical properties of these hot springs appear to be a virtual environment for thermophilic bacteria that produce hydrolytic enzymes (Alrumman et al 2018).

Thermophilic bacteria were isolated from Tarabalo's hot spring in India. *Bacillus* sp. were known as bacteria that could withstand high temperatures based on their morphology, biochemistry, and 16S rRNA gene sequencing. A BLAST search of the series revealed the highest resemblance to *Bacillus amyloliquefaciens* (99 percent similarity). Protease activity was increased in this strain. The study found that the extracted *Bacillus* sp. was a valid thermophile and could produce thermostable protease for pharmaceutical and industrial applications (Panda et al 2013). Eight strains of moderately thermophilic bacteria with observable galactosidase activities were obtained from a saline hot spring in Odaito, Japan. The representative strains BEK6 and BEK11 had 97.1 percent and 96.6 percent nucleotide sequence similarity with *Bacillus aeolius* and *Bacillus alveayuensis*, respectively, and exhibited catalase and oxidase activity. The strains grow in a medium containing 10% NaCl. In 3 percent NaCl, the strain BEK11 displayed reasonably strong protease and amylase activities, implying that these enzymes may have industrial applications under salinity stress (Kawasaki et al 2011).

Table 1 summarizes the worldwide hot springs as a source of novel enzymes. New techniques and approaches are arising to study the whole genome content of a particular habitat without using the cultivation-based method. Metagenomic sequencing is now widely used; for example, it was used to determine the microbial diversity in the second hottest hot spring in Malaysia, namely the Sungai Klah hot spring; the temperature of the water sample was 80^{o}C, a result, 96.8% of the16SrRNA gene fragments were allocated to bacteria. In comparison, 0.91% belonged to Archaea, 35 phyla were presented, and the major phylum was Firmicutes, accounting for 37.15%. In general, the fragments were classified into 67 classes, 120 orders, 206 families, and 358 genera, which shows the diversity of the microbial population in this hot spring (Chan et al 2015). On the other side, whole-genome sequencing of the thermophilic *Thermus* sp. CCB US3 UF1, known as an essential source of thermophilic enzymes extracted from a hot spring in Malaysia, reveals valuable knowledge of this genus which can be used in industrial and biotechnological fields (Teh et al 2015).

A novel glutaredoxin gene segment was discovered in a metagenomic library from soil sediment obtained from a hot spring in Tapovan District, Chamoli, Uttarakhand, India. An open reading frame analysis found a 420-nucleotide coding sequence. This location encodes a 139-amino-acid protein with a homologous relationship with glutaredoxin from *Thermus* sp., with 84% query coverage. Multiple sequence alignment reveals a glutaredoxin domain with a redox-active CXXC motif in the shape of the catalytic motif CLYC, indicating that this is a novel glutaredoxin gene (Rawat et al 2018).

Environmental DNA was collected from a hot spring field in Niujie, Eryuan, Yunnan province, China, to establish a metagenomic bacterial artificial chromosome (BAC) library. This library yielded eight esterase/lipase genes in three novels. A lipase gene with 622 amino acid residues has been discovered. This lipase has a high tolerance for methanol, according to its enzymatic properties. This novel lipase gene, lip-1, has many potentials for biodiesel production because of its reduced preparation cost, excellent durability, and methanol tolerance (Yan et al 2017).

Table 1

Summary of hot springs and their thermoenzymes content

Hot springs name	*Enzymatic activity screened*	*Type of bacteria*	*References*
Labis, Dusun Tua, Ulu Legong and Ranau	Phytase	Gram –	Alias et al (2017)
Gadek, Labis, Pedas, Sg. Klah, Selayang	Lipase	*Bacillus* species *Geobacillus* species	Zuridah & Norazwin (2011)
Dusun Tua	Protease, lipase, amylase, cellulase, pectinase, and xylanase	Gram – (bacilli)	Msarah et al (2018)
KSA (Al-Majardah, Al-Khubah and Al-Ardah)	α-amylase Protease, Lipase	*Bacillus aerius, Bacillus licheniformis*, *Bacillus sonorensis*	Alrumman et al (2018)
India (Tarabalo)	Protease	*Bacillus amyloliquefaciens*	Panda et al (2013)
Japan (Odaito, Hokkaido)	Protease, amylase	*Bacillus aeolius*, *Bacillus alveayuensi*	Kawasaki et al (2011)
Thailand (Chiang Mai)	Superoxide dismutase (SOD)	*B. stearothermophilus*	Sookkheo et al (2002)
Armenian (Akhurik, Arzakan, Bjni, Hankavan, Jermaxbjur, Jermuk, Tatev, Uys, Karvachar, Zuar) and Nagorno-Karabakhian (Karvachar, Zuar)	Lipase, protease, amylases	*Anoxybacillus*, *Bacillus*, *Brevibacillus*, *Geobacillus*, *Paenibacillus*, *Sporosarcina*, *Ureibacillus*, *Thermoactinomyces*	Panosyan (2017)
Philippines (Badekbek mud spring in Bokod and Dalupirip hot spring in Itogon)	Xylanase	*Geobacillus*, *Thermonema*, *Meiothermus, Anoxybacillus*, *G. thermoparaffinivorans*, *G. thermoleovorans*	Daupan & Rivera (2015)
Turkey (Seferihisar Karakoc hotspring)	Carboxylesterase	*Anoxybacillus* sp. PDF1	Ay et al (2011)
Azad Kashmir, Pakistan (Tattapani hot spring)	Extracellular α-amylase, CMCase, FPase, xylanase, protease and lipase, and intracellular CMCase and FPase	Thermophilic bacterium (TP-2)	Zahoor et al (2016)

Conclusions. Thermophiles and extreme thermophiles are a significant source of new enzymes commonly used in industry, so there is a growing interest in isolating and classifying them; new techniques help not only to isolate these bacteria but also in determining how they can survive in harsh conditions by studying the genes that are responsible for extreme enzymes production. Studies on hot springs reveal that they harbor important thermophile species that can produce critical thermophilic enzymes. Further studies are needed to utilize these habitats as a source for thermophilic enzymes used in the industry.

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