III. MICROORGANISMS AND ENVIRONMENT

CELLULOLYTIC MICROORGANISMS: AEROBIC, MICROAEROPHILIC, ANAEROBIC BACTERIA AND MICROBIAL CONSORTIA (Part II)

Yana Gocheva[1], Lyudmila Dimitrova[1], Venelin Hubenov[1], Lyudmila Kabaivanova[1], Plamen Angelov[2], Ivan Simeonov, Hristo Najdenski[1]

[1] *The Stephan Angeloff Institute of Microbiology at the Bulgarian Academy of Sciences*

[2] *Space Research and Technology Institute at the Bulgarian Academy of Sciences*

Abstract. In nature, cellulose, lignocellulose and lignin are major sources of plant biomass therefore their recycling is indispensable for the carbon cycle. The synergistic action of a variety of microorganisms is needed for recycling lignocellulosic materials. The capacities of microorganisms to assimilate complex carbohydrates, such as cellulose, hemicellulose and lignin, depend on the ability to produce the enzymes that work synergically. Populations growing in compost piles consist mainly of bacteria (including actinobacteria) and fungi. Polymers such as hemicellulose, cellulose, and lignin are only degraded once the more easily degradable compounds have been consumed. Afterwards, the lignocellulosic materials are partly transformed into humus. In the present review, numerous studies on the isolation of cellulose-degrading bacteria and fungi, their identification, enzymatic activities, and their ability to grow in the presence of lignocellulose and components of these industrial waste streams (phenolic compounds, sulfides, and dyes are analyzed and discussed. This is of particular interest to design future studies to isolate those bacteria that can specifically degrade cellulose matrix and more recalcitrant components such as lignin and aromatic lignin degradation products. Cultivation and characterization of microorganisms alone is not adequate without preservation techniques that do not alter the morphology, physiology or genetics of pure strains. Careful preservation is imperative for future research, teaching and industrial applications.

Keywords: cellulolytic microorganisms, bacteria, fungi, cellulose waste, biodegradation, culture preservation.

INTRODUCTION

In the near future, biotechnological processes that use microorganisms and/or their lignocellulolytic enzymes could lead to new, environmentally friendly technologies. According to Tuomela et al. [1], at the beginning of the biodegradation process mesophilic bacteria are predominant. Once the temperature has reached more than 40^{o}C, thermophilic bacteria and fungi appear in the biodegradable piles. When the temperatures reach more than 60^{o}C, the microbial activity decrease, and as the compost pile cools, mesophilic bacteria appear again. Except for 1% of anaerobic bacteria in the biodegradation process, which carry out much of the cellulolytic activity and so play a major role in the biodegradation of lignocellulosic materials, mostly biodegradation is carried out under aerobic conditions. Among the aerobic bacteria that have been isolated, actinobacteria probably play a major role in the degradation of lignocelluloses. These bacteria can degrade cellulose and solubilize lignin. However, it seems that thermophilic and thermotolerant fungi, known to have cellulolytic and ligninolitic activity, are critical to the biodegradation of lignocellulosic materials.

The involvement of diverse microorganisms (both bacteria and fungi) growing under mesophilic or thermophilic conditions is prerequisite to biodegradation of lignocellulose containing waste. A wide variety of microbial species are involved in the cellulose biodegradation process of lignocellulose containing waste, many of which have not even been identified. The composition of the microorganism communities depends, first and foremost, on the composition of components undergoing biodegradation and their relative contents in the mixtures (Table 1). Fungi and bacteria, had been found to participate also in wood degradation in the Antarctic coastal waters, albeit at a vastly reduced rate compared to warmer environments [3].

In the case of wastewater dung or wastewater sludge and residue, the material was firstly treated with calcium hydroxide and then fermented by filamentous bacteria, such as *Bacillus badius* or *Cellulomonas* spp., at controlled pH levels. Hiura and Maeda reported that the compost obtained may be used as a soil fertilizer [4].

For biodegradation of lignocellulose and dung mixture, the cultures have been isolated from thermophilic substrates obtained by lignocellulose and dung mixtures at 60-80°C. Asporous obligate autotrophic strains belonged to Gram-negative species growing at 60-80°C with a temperature optimum in the range of 70-75°C under microaerophilic conditions (5 kPaO_2). Judging by the DNA structures, these

doi.org/10.32006/eeep.2023.1.3653

strains were taxonomically close to hydrogen bacteria isolated from geothermal areas. All the strains were taxonomically close to *Bacillus schlegeli*, judging by homologies at the DNA level [5].

Table 1. Main microorganisms involved in aerobic biodegradation of lignocellulose waste (by Neklyudov et al.) [2]

Waste type	Microorganism
Dung or sludgy wastewater residue	*Bacillus badius, Cellulomonas*
Mixture of lignocellulose and dung	*Bacillus schlegeli*
Domestic organic waste	*Bacillus licheniformis* strain NH 1
Rice bran or potato waste	*Hunsenula, Aspergillus, Rhizopus, Mucor, Chlorella*
Domestic organic waste	*Bacillus* sp. (strain 0YK-01-600) *Aspergillus, Penicillium, Trichoderma, Myriodontium, Pleurotus*
Municipal waste	*Faenia rectivirgula, Saccharomonospora viridis, Streptomyces thermoviolaceus, Termoactinomyces thalophilus, T. vulgaris, Thermomonospora curvata*
Organic waste	*Protobacteria*
Shredded paper waste	*Cellulomonas* sp.
Citrus waste	*Bacillus licheniformus, B. macerans, B. stearothermophillus, Absidia corymbifera, Aspergillus fumigatus, Emericella nidulans, Penicillium diversum, Rhizomucor pusillis, Talaromyces thermophilus, Termomyces lanuginosus, B. licheniformis, Proteus vulgaris, Pseudomonas aeruginosa, P. fluorescens, P. luteola, Serratia marcescens, Aspergillus puniceus, A. ustus, Paecilomyces lilacinus, Coprinus lagopus*
Wheat straw	*Pleurotus sajror-caju, Trichoderma harzianum, Aspergillus niger, Azotobacter chroococcum*
Waste from waste disposal plants or sewage, supplemented with sawdust	*Salmonella*
Lignin-containing larch and asp waste	*Trametes villosus*
Beverage production and food waste	*Bacillus* sp. strain KHR-10 and *Cellulomonas* sp. strain KHR-15-MX
Refuse	*Penicillium, Bacillus, Termoactinomyces, Streptomyces*
Sterilized domestic waste	*Bacillus stearothermophilus*
Immature grass compost	*Trichoderma viridae, Bacillus* sp.

Citrus waste was degraded in compost humps put under a shed with addition of calcium hydroxide at a C: N ratio of 24, pH 6.3 and moisture content equal to 60%. Thermophilic microorganisms present during the thermophilic stage were largely represented by bacteria (*Bacillus licheniformis, B. macerans*, and *B. stearothermophilus*) and, to a lesser extent by fungi (such as *Absidia corymbifera, Aspergillus fumigatus, Emericella nidulans, Penicillium diversum, Rhizomucor pusillis, Talaromyces thermophilus,* and *Thermomyces lanuginosus*). Bacteria present at the terminal stage of composting included *B. licheniformis, B. macerans, Proteus vulgaris* from wheat straw, *Pseudomonas aeruginosa, P. fluorescens, P. luteola*, and *Serratia marcescens*, whereas fungal cultures remaining throughout this period of composting were most frequently represented by *Aspergillus puniceus, A. ustus, Paecilomyces lilacinus, and T. lanuginosus*, as well as by yeasts and basidiomycetes (possibly *Coprinus lagopus* and the like) [6].

Wheat straw processed for 40 days in the presence of *Pleurotus sajcaji, Trichoderma harzianum, Aspergillus niger* and *Azotobacter chroococcum* produces a compost suitable for soil fertilization or vermicomposting [7].

MICROBIAL COMMUNITIES ISOLATED FROM COMPOSTED MIXTURES

Thermophilic actinomycetes belonging to the species *Faenia rectivirgula, Saccharomonospora viridis, Streptomyces thermoviolaceus, Thermoactinomyces thalpophilus, T. vulgaris*, and *Thermomonospora cuvata* (130 strains) have been isolated from 76 specimens obtained by fermentation of municipal waste. The cultures were checked for the ability to form colonies. Biochemical studies demonstrated that the cultures degrade casein, cellulose, hypoxanthine, starch, and other substrates [8].

Solid municipal waste was found to contain mesophilic bacteria, yeasts, and filamentous fungi, bacterial spores, and representatives of the genera *Salmonella* and *Shigella*. The content of total fecal coliform species and fecal streptococci was taken as a fecal indicator of microbial communities. The study was performed using a pilot plant comprising a reactor and a system of aeration, which was capable of supplying oxygen to the mixture in the course of its composting. The results demonstrated that biodegradation is associated with autosterilization (due to the temperature increase of up to 60–65°C, taking place during the first stage of the process) and dramatic changes in the composition of the bacterial community. Thus, during the process, the content of *Escherichia coli* and fecal streptococci decreased from 2×10^7 and 1×10^7 to 3.1×10^3 and 1.5×10^3 respectively cells/g dry residue. The amount of yeasts/ filamentous fungi and mesophilic bacteria decreased from 4.5×10^6 and 5.8×10^9 to 2.6×10^3 and 1.8×10^7 respectively cells/g dry residue. On the other hand, the amount of bacterial spores, which increased in the beginning of the process, dropped by the end of week 3 times. *Salmonella* spp. were eliminated completely by day 25, when the temperature reached a level of 60°C. During the stage of cooling of the degraded mixture, however, the population of bacteria increased. Staphylococci were predominant during the mesophilic stage and in the beginning of the termophilic stage, whereas bacilli constitute the dominant type of bacteria at subsequent stages of biodegradation. The reappearance of rodlike microorganisms (capable of being pathogenic) at the stage of compost cooling is a serious obstacle to using the finished product in agriculture. However, sonication of the samples for 3 min destroyed these microorganisms. Conversely, Gram-positive bacteria (micrococci in particular) and spores of bacteria and fungi survive this treatment and their concentrations remain high enough [9].

Microbial activity in the course of paper pulp and shredded waste

Treatement of paper pulp and shredded waste in a partitioned reactor for 43 days resulted in a fourfold increase in the amount of microorganisms in the reaction mixture. However, the expected increase should have been 19 times, judging by the results of analysis of the kinetics of effluent CO_2. The difference indicated that biodegradation was associated with cell lysis. On day 22, microorganisms of genus *Cellulomonas* appeared. Chemical analysis demonstrated that 33% of cellulose-containing material was degraded during the process; no other pronounced chemical changes could be observed [10].

Cellulose, a polysaccharide consisting of linear β-1,4-linked D-glucopyranose chains, requires cellulases for its degradation. Cellulases, historically, have been divided into three major groups: endoglucanase (EC 3. 2.1.4), exoglucanase or cellobiohydrolase (EC 3.2.1. 91) and β-glucosidase (EC 3.2.1.21), and the synergistic actions of these enzymes is a widely accepted mechanism for cellulose hydrolysis [6, 11, 12]. These enzymes can either be free in aerobic microorganisms or grouped in a multicomponent enzyme complex – cellulosome in anaerobic cellulolytic bacteria [6].

Enormous amounts of agricultural, industrial and municipal cellulose wastes have been accumulating or used inefficiently due to the high cost of their utilisation processes [8, 13, 14]. Therefore, it has become of considerable economic interest to develop processes for effective treatment and utilisation of cellulosic wastes as a cheap carbon source. These resources are composed of leaves, stems and stalks from sources, such as corn fibre, corn stover, sugarcane bagasse, rice, rice hulls, woody crops and forest residues. Besides, there are multiple sources of lignocellulosic waste from industrial and agricultural processes, for example, citrus peel waste, coconut biomass, sawdust, paper pulp, industrial waste, municipal cellulosic solid waste and paper mill sludge [15].

In the last two decades, research has been aimed at developing new technologies and microbial strains to reduce the cost of cellulase production and improving the bioconversion of cellulose. Lignocellulose is a term for plant materials that are

composed of matrices of cellulose, hemicellulose, and lignin. Lignocellulose is a renewable feedstock for many industries. Lignocellulosic materials are used for the production of paper, fuels, and chemicals. Typically, industry focuses on transforming the polysaccharides present in lignocellulose into products resulting in the incomplete use of this resource. The materials that are not completely used make up the underutilized streams of materials that contain cellulose, hemicellulose, and lignin. These underutilized streams have potential for conversion into valuable products. Treatment of these lignocellulosic streams with bacteria, which specifically degrade lignocellulose through the action of enzymes, offers a lowenergy and low-cost method for biodegradation and bioconversion.

LIGNOCELLULOSE DEGRADING BACTERIA

Kraft pulping is the most widely used pulping process worldwide, and because of this, there is interest among researchers to isolate bacteria directly from pulp and paper mill industrial waste for use in biological treatment. Bacteria have been isolated from soil, wastewater, lignocellulose, and black liquor. These bacteria are capable of utilizing pulp and paper mill lignocellulosic waste to provide a variety of benefits including decolorizing pulp and paper mill effluent, producing low-molecular-weight compounds, and producing lignocellulose-degrading enzymes [16, 17, 18, 19, 20]. The majority of the bacteria isolated from pulp paper mill effluent are γ-proteobacteria (*Acinetobacter, Azotobacter, Citrobacter, Enterobacter, Escherichia, Halomonas, Klebsiella, Pantoea, Providencia, Pseudomonas,* and *Serratia*). Some of these strains belong to the *Enterobacteriaceae* family composed of Gram-negative facultative anaerobic bacteria which can ferment sugars to produce a variety of end products including lactic acid. Other Gram-negative bacteria, which were isolated from pulp paper mill effluent, belong to the α-*Proteobacteriaceae* family (*Novosphingobium* and *Pseudochrobactrum*) being characterized by some relevant metabolic capabilities – degradation of aromatic compounds. Two β-proteobacteria, which are also Gram-negative, were isolated from bamboo (*Cupriavidus* and *Pandoraea*). Additional bacteria isolated from pulp paper mill effluent belong to the *Bacillaceae* family (*Aneurinibacillus, Bacillus*, and *Paenibacillus*) which is composed of Gram-positive spore-forming bacteria that are also facultative anaerobes. Isolation of bacteria from other industrial streams may also provide organisms of value for the biological treatment of lignocellulose. Bacteria belonging to the *Flavobacterium, Bacteroides, Eubacterium, Clostridium, Bacillus,* and *Sphingomonas* genera as well as strains of *Proteus vulgaris* and *Streptococcus faecalis* have been shown to degrade azo dyes present in textile industry wastewaters [21]. Compost piles may also be a source of bacteria which can degrade lignocellulose. Members of the genera *Pseudomonas, Klebsiella, Nocardia, Streptomyces, Thermoactinomyces, Micromonospora*, and *Bacillus* have been isolated from compost piles and shown to be capable of degrading cellulose in the presence of lignin [1]. While the reported isolations have identified a variety of bacteria which can grow in the presence of lignocellulose and components of these industrial waste streams (phenolic compounds, sulfide, and dyes), it is of particular interest to design future studies to isolate those bacteria which can specifically degrade the more recalcitrant components such as lignin and aromatic lignin degradation products.

Growth conditions

The growth conditions for bacteria which can degrade and convert industrial lignocellulosic streams are also important. Future work should also focus on isolation of bacteria which can grow in industrially relevant conditions (low oxygen, high lignocellulose concentration, and basic pH and requires few additional nutrients). The amount of oxygen present is an important factor for growth of bacteria. Facultative organisms can grow in both aerobic and anaerobic environments. All of the fungi currently used for biological treatment of lignocellulose require oxygen. Anaerobic treatment of lignocellulose may be preferable because costs for supplementation of oxygen are eliminated and microorganisms are able to successfully remove high-strength organic effluents under anaerobic conditions [22-Pokhrel D., T. Viraraghavan 2004]. Chandra et al. isolated *Citrobacter freundii* and *Citrobacter* sp. from pulp paper mill effluent and demonstrated that these bacteria could also decolorize effluent (10% black liquor) under aerobic and microaerophilic conditions [23]. Paenibacillus sp. (AY952466), *Aneurinibacillus aneurinilyticus* (AY856831), and *Bacillus* sp. (AY952465) were isolated from pulp paper mill effluent and shown to degrade kraft lignin under microaerophilic conditions [17]. *Paenibacillus glucanolyticus* was isolated from black liquor and shown to be capable of

growth on black liquor under both aerobic and anaerobic conditions [18]. The pH at which these bacteria grow may provide an additional benefit over fungal biological treatment. While most fungi grow in neutral or slightly acidic pH, bacteria can grow in a wide range of pH. Black liquor and other industrial wastes vary in pH. Black liquor is extremely basic, ranging from pH 10 to 14. Lignin, the predominant component of black liquor, also increases in solubility at higher pH allowing for more efficient substrate access by enzymes [20]. *Paenibacillus glucanolyticus*, *Pandoraea* sp. B-6, *Providencia rettgeri*, and *Pseudochrobactrum glaciale* were shown to be capable of growth at pH 9 and 10, which is potentially important because development of bacterial treatment methods for using bacteria which can grow in unneutralized lignocellulosic streams could be very cost effective [17]. Bacteria which do not need additional nutrients for growth beyond what is provided by the lignocellulosic waste stream would also be advantageous in terms of minimizing costs for biological treatment. White-rot fungi, which have been used for biological treatment, require glucose or polysaccharides for growth. However, some bacteria have been shown to use lignocellulose components as the sole carbon source requiring no additional sugars or polysaccharides. *Novosphingobium* sp. B-7 was isolated by Chen et al. from eroded bamboo slips and can use kraft lignin as the sole carbon source for growth [24]. This strain was also capable of growing on lignin degradation products including guaiacol, vanillin, p-coumaric acid, cinnamic acid, ferulic acid, sinapic acid, and veratraldehyde. *Paenibacillus glucanolyticus* isolated from black liquor by Mathews et al. was shown to be capable of growth on black liquor, cellulose, hemicellulose, and lignin as the sole carbon source [18]. In addition to isolating bacteria that can degrade lignin or phenolics, bacteria can also be isolated from paper pulp mill effluent which metabolize polysaccharides. Those bacteria that can use both cellulose and hemicellulose (hexose and pentose sugars) are especially applicable for biological pretreatment. Utilization of mixed sugars is a unique metabolic property characteristic of some bacteria including *Escherichia coli, Klebsiella, Erwinia, Lactobacillus, Bacillus,* and *Clostridia* [25].

Lactobacillus plantarum had been reported recently to be a potential lignocellulosic biomass degrader since it had had the capability of producing versatile extracellular cellulolytic and hemicellulolytic enzymes – extracellular endoglucanase, exoglucanase, β-glucosidase, and mannanase [26]. Microorganisms such as *Saccharomyces* and *Xymomonas* have been modified to introduce pentose fermentation characteristics [25]. Ko et al. isolated *Paenibacillus campinansensis* from black liquor in the brownstock washers and found that it could degrade saccharides and polysaccharides [9].

In recent years, several cellulolytic consortia have been enriched from different ecosystems, such as soils [27], composts [28], the phytophagous insects. Throughout the course of evolution, these insects have established symbiotic interactions with different microorganisms that perform cellulolytic activities and thus are highly efficient natural bioreactors. Some insects, such as termites, wood-feeding roaches, beetles, and leaf-cutting ants, can use lignocellulosic substrates as their main food source and are highly efficient at degrading cellulose to glucose as an energy source [29]. The experimental insect-phytophagous scarab (*Holotrichia parallela*) larvae – live in the soil, where they feed on plant roots and organic matter of low nutritive value. The hindgut of the larvae contains a wide diversity of cellulolytic and hemicellulolytic bacteria. The bacterial consortium showed high efficiency for rice straw degradation with short incubation time and could be considered a potential candidate for use in commercial biomass conversion. The authors [30] found that the degradability of rice straw was about 83.1% after three days of cultivation. The bacterial diversity and richness decreased during the consortium enrichment process, suggesting that convergent adaptation is driven by the selective pressure applied during the enrichment process. Furthermore, authors found that both aerobic and anaerobic bacteria coexisted steadily in the enriched consortium. This feature would be caused by the culture conditions; the upper phase of the culture system would supply oxygen for the aerobic bacteria, and the lower phase would be in anaerobic conditions. Microbial structure analysis showed that the lignocellulose, such as rice straw and filter paper, led to significant enrichment of the phyla *Proteobacteria* and *Spirochaetes*, classes *Clostridia*, *Epsilonproteobacteria*, and *Betaproteobacteria*, and genera *Arcobacter*, *Treponema*, *Comamonas,* and *Clostridium*. Inoculation of rice straw with a mixture of five bacterial strains (Aeromonas caviae, Shinella sp.p Rhizobium sp., Corynebacterium pseudotuberculosis and Streptomyces clavuligerus) at the proportion of 1:1:1:1 showed that inoculation functional bacterial agents accelerated the degradation of organic matter and coarse fiber content by 7.58%, 8.82%, which were due to the fact that key enzymes and core

microbes were stimulated. In addition, inoculation has reconstructed core microbes of producing lignocellulase, xylanase and manganese peroxidise and increased most core microbial abundance [31].

Spirochetes are the dominant phylum in the higher termite species, and metagenomic analysis has revealed that it is responsible for cellulose and hemicellulose utilization in the termite. Furthermore, another metagenomic study on cow rumen also revealed that the bacteria of the phylum *Spirochaetes* were always adherent to and degraded the plant fiber materials [32]. These results indicated that these two phyla might have strong cellulolytic activities in the enrichment consortium. In a comprehensive and deep analysis of an enriched rumen anaerobic consortium was established its lignocellulolytic abilities and confirmed by analyzing the depolymerization of bagasse by scanning electron microscopy, enzymatic assays, and mass spectrometry. Taxonomic analysis based on 16S rRNA sequencing elucidated the community enrichment process, which was marked by a higher abundance of *Firmicutes* and *Synergistetes* species [33].

Original studies of Jiao et al. demonstrated that enrichment culture combined with the microbial electrochemical system enhanced the low-temperature (20°C) anaerobic digestion of cow dung by 39,64 %. Enrichment culture combined with microbial electrochemical system increased the relative abundance of methanogenic archaea (Methanomassiliicoccus, Methanocorpusculum, unclassified Methanomicrobiaceae, Methanobacterium, Methanoculleus, Methanocalculus) and the relative abundance of cold-tolerant hydrolytic acidifying bacteria (unclassified Bacteroidetes, Treponema). The expressions of specific enzyme genes in the methanogenesis pathway were also enhanced, including acetyl-CoA synthetase, formylmethanofuran dehydrogenase, methanol cobalamin methyltransferase, etc. [34]. For the genus *Comamonas*, previous studies have shown that different *Comamonas* species of the class *Betaproteobacteria* have previously been reported in termites and eroded bamboo slips, and are known to be involved in lignin, cellulose, and hemicellulose degradation [35]. Applying aerobic cultivation conditions, Wenzel et al. isolated 119 cellulolytic strains from the gut of termite *Zootermopsis angusticollis*, which were assigned to 23 groups of aerobic, facultatively anaerobic or microaerophilic cellulolytic bacteria [36]. 16S rDNA restriction fragment pattern and partial 16S rDNA sequence analysis, as well as numerical taxonomy, were used for the assignment of the isolates. The Gram-positive bacteria of the actinomycetes branch could be assigned to the order *Actinomycetales* including the genera *Cellulomonas/ Oerskovia*, *Microbacterium* and *Kocuria*. The Gram-positive bacteria from the order *Bacillales* belonged to the genera *Bacillus, Brevibacillus* and *Paenibacillus*. Isolates related to the genera *Afipia, Agrobacterium/ Rhizobium, Brucella/Ochrobactrum, Pseudomonas* and *Sphingomonas/Zymomonas* from the α-proteobacteria and *Spirosoma*-like from the "*Flexibacteriaceae"* represented the Gram-negative bacteria. In a recent study active thermophilic cellulose-degrading microorganisms were identified from a full-scale anaerobic digester fed with maize by using metagenome-resolved protein stable isotope probing (protein-SIP). Metagenomic analysis revealed 238 different genes coding for carbohydrate-active enzymes, six of which were directly associated with cellulose degradation. The protein-SIP analysis identified twenty heavily labelled peptides deriving from microorganisms members of the order Clostridiales, e.g. Corynebacterium sp. [37**]**.

Isolation and characterization of pure cultures and microbial consortia degrading cellulose containing substrates

Lignocellulosic biomass (LCB) is an attractive source of carbon for the production of sugars and other chemicals. Due to its inherent complexity and heterogeneity, efficient biodegradation requires the actions of different types of hydrolytic enzymes. In nature, complex microbial communities that work efficiently and often synergistically accomplish degradation. The generation of a stable complex microbial consortium as a promising approach for efficient biomass decomposition needs relevant conservation and preservation [38]. In nature, many species of microorganisms coexist by interacting with each other.

A number of structurally stable multispecies consortia with high cellulose-degrading activity have been obtained by successive culture enrichments using agricultural biomass as the sole carbon source under meso- and thermophilic conditions. These symbiotic consortia can efficiently degrade various cellulosic materials, such as agro-industrial residues and pulp wastes. *Bacteroidetes* was the most abundant phylum in all samples. Within the *Bacteroidetes* phylum, *Bacteroidaceae* was the most abundant family in the rumen-derived enrichment cultures, whereas *Porphyromonadaceae* was the predominant one in the reactor-derived culture. Additionally, the enrichment procedure increased the relative abundance of

Ruminococcaceae (phylum: *Firmicutes)* in all cultures. T-RFLP profiles of the *mcrA* gene amplicons highlighted that the ruminal methanogenic communities were composed of hydrogenotrophic methanogens dominated by the order *Methanobacteriales* regardless of the host species. The methanogenic communities changed significantly during the enrichment procedure, but still the strict hydrogenotrophic *Methanobacteriales* and *Methanomicrobiales* were the predominant orders in the enrichment cultures. The bioaugmentation potential of the enriched methanogenic cultures will be evaluated in further studies [39].

A recent study by Wongwilaiwalin et al. concluded that a microbial consortium showed efficient degradation activity on potential biorefinery cellulosic substrates, including bagasse, rice straw, corn stover and industrial eucalyptus pulp sludge [40]. The consortium was structurally stable with the co-existence of eight major microbes, comprising anaerobic bacterial genera *Clostridium* and *Thermoanaerobacterium* along with an aerobic/facultative anaerobic *Rhodocyclaceae* bacterium, bacilli, and uncultured bacteria. Majority of the lignocellullolytic activities including endo-glucanase, xylanase and β-glucanase was present in the crude culture supernatant compared to the cell-bound fraction. Proteomic analysis of cellulose bound fraction of the crude extracellular enzyme revealed a multi-species lignocellulolytic enzyme system composed mainly of cellulosomal components and extracellular cellulases of clostridia along with hemicellulases and a β-glucanase from *Clostridium, Bacillus*, and *Thermobacillus* related origins. A psychrotrophic lignocelluloses degrading microbial consortium (LTF-27) was successfully obtained from cold perennial forest soil under facultative anaerobic static conditions. The consortium showed efficient degradation of rice straw, which cellulose, hemicelluloses and lignin lost 71.7%, 65.6% and 12.5% of its weigh, respectively, in 20 days at 15°C [41]. Lazuka et al. [42] demonstrated that it is possible to enrich and maintain a microbial consortium derived from termite gut microbiome in controlled anaerobic bioreactors, producing useful carboxylates from raw biomass. Their results suggest that the microbial community is shaped both by the substrate and the conditions that prevail during enrichment. However, when aseptic conditions are applied, it is also affected by the biotic pressure exerted by microorganisms naturally present in the substrate and in the surrounding environment.

Series of recent studies had indicated that enriched microbial communities, obtained from environmental samples through selective processes and efficient storage procedures can effectively contribute to lignocellulose degradation. Puentes-Téllez et al. [43] combined ecological theory and enrichment principles to develop an effective lignocellulose-degrading minimal active microbial consortia (MAMC). Then 65 compositional replicates of MAMC containing five species each were generated, which vary in the number of functional groups, metabolic potential, and degradation capacity. The characterization of the MAMC according to their degradation capacities and functional diversity measurements revealed that functional diversity positively correlated with the degradation of the most complex lignocellulosic fraction (lignin), indicating the importance of metabolic complementarity, whereas cellulose and hemicellulose degradation were either negatively or not affected by functional diversity. The screening method described here successfully led to the selection of effective MAMC, whose degradation potential reached up 96.5% of the degradation rates when all 18 species were present. A total of seven assembled synthetic communities were identified as the most effective MAMC. A consortium containing *Stenotrophomonas maltophilia*, *Paenibacillus sp*., *Microbacterium* sp., *Chryseobacterium taiwanense*, and *Brevundimonas sp*. was found to be the most effective degrading synthetic community [43].

Kinet et al. [44] designed an anaerobic thermophilic cellulolytic microbial consortium able to degrade cellulose under conditions related to anaerobic digestion process. A cellulolytic consortium was isolated from a composting plant in order to boost the initial hydrolysis step encountered in anaerobic digestion. Improvement of the cellulose degradation, as well as biogas production, was observed for the cultures inoculated with the exogenous consortium. Main microbial strains determined were strictly anaerobic and belong to the *Clostridia* class. During cellulose anaerobic degradation, pH drop induced a strong modification of the microbial population. Despite the fact that richness and evenness were very weak, the exogenous consortium was able to adapt and to maintain the cellulolytic degradation potential. Among various waste management practices, anaerobic digestion is proved as a useful method to transform food waste, producing renewable energy/biofuel and bio-fertilizers [45]. In a recently publishe review Basak et al. assessed the metabolic roles of microbial communities of lignocellulolytic

microbiomes (LMs) and their complex interactions with the indigenous anaerobic digester microbiome as a pivotal in implementing bioaugmentation. Multiple meta-omics are the frontline approaches to investigating gene functions, metabolic roles, and the ecological niches of LMs [46].

It is well known that pretreatment of lignocellulosic biomass is crucial to promote its fragmentation, increase its surface area and solubility, and lower the cellulose crystallinity and lignin content for sustainable biorefinery. Conventional pretreatment processes have several drawbacks, including high operational costs, corrosion of equipment, and generation of toxic effluents and by-products. To offset the negative impacts of these limitations on biofuel production, Kumar et al [47] discussed and critically compared various eco-friendly approaches for the efficient conversion of biomass to ensure high yields of biofuels as a commercial solution. Moreover, a range of microbes and enzymes have been highlighted that effectively utilize lignocellulosic biomass to obtain energy and convert its complex polymeric structure into a biodegradable one, facilitating its subsequent valorization. Furthermore, the importance of multi-omics approaches was discussed to gain functional insights into the lignocellulolytic microbial communities and their interspecies symbiosis during the hydrolysis of organic biomass.

Cortes-Tolalpa et al. [48] examine the wheat straw degradation potential of synthetic microbial consortia composed of bacteria and fungi. Growth of and enzyme secretion by monocultures of degrader strains were studied in aerobic cultures using wheat straw as the sole carbon and energy source. To investigate synergism, co-cultures were constructed from selected strains and their performance was tested in comparison with the respective monocultures. In monoculture, each organism – with a typical enzymatic profile – was found to mainly consume the cellulose part of the substrate. The authors demonstrated that the strain, *Flavobacterium ginsengisoli* so9, displayed an extremely high degradation capacity, as measured by its secreted enzymes. Among 13 different co-cultures, five presented synergisms. These included four bacterial bicultures and one bacterial–fungal triculture. The highest level of synergism was found in a *Citrobacter freundii/Sphingobacterium multivorum* biculture. As compared to both monocultures, this bacterial pair showed significantly increased enzymatic activities, in particular of cellobiohydrolases, mannosidases, and xylosidases. In another series of experiments, inocula enrichment was performed using an innovative habitat-based selection approach to improve wheat straw anaerobic digestion efficiency. The procedure was carried out by sequentially re-inoculating the primary microbial community seven times in subsequent anaerobic reactors containing untreated wheat straw. Re-inocula were performed at different re-inoculum times (24, 48, and 96 h) by moving a porous support mimicking a rumen structure. The authors found that microbial communities were dominated by fermenting, hydrogen-producing bacteria and Archaea component (7%) [49].

Recently archaeal and bacterial consortia in a two-stage system with wheat straw as a substarte were identified for the first time by Kabaivanova et al. 2022. The authors' study revealed that *Proteiniphilum saccharofermentans* likely contributes to the majority of biohydrogen production, comprising 28.2% and 45.4% of the microbial community in the first and second bioreactors, respectively. The methane-producing reactor contained *Archaeal* representatives such as *Methanobacterium formicicum* (0.71%), *Methanosarcina spelaei* (0.03%), *Methanothrix soehngenii* (0.012%), and *Methanobacterium beijingense* (0.01%) [50].

Two stable, thermophilic mixed cellulolytic consortia were enriched from an industrial scale biogas fermenter. The two consortia, marked as AD1 and AD2, were used for bioaugmentation in laboratory scale batch reactors. They enhanced the methane yield by 22–24%. Next generation sequencing method revealed the main orders being *Thermoanaerobacterales* and *Clostridiales* and the predominant strains were *Thermoanaerobacterium thermosaccharolyticum*, *Caldanaerobacter subterraneus*, *Thermoanaerobacter pseudethanolicus* and *Clostridium cellulolyticum*. The effect of these strains, cultivated in pure cultures, was investigated with the aim of reconstructing the defined cellulolytic consortium. The addition of the four bacterial strains and their mixture to the biogas fermenters enhanced the methane yield by 10–11% but it was not as efficient as the original communities indicating the significant contribution by members of the enriched communities present in low abundance.

Poszytek et al. [10] reported that the cooperation and synergism of the isolated microorganisms in the Microbial Consortium with High Cellulolytic Activity consortium (MCHC) enhance their degradation abilities, and the use of a combination of microorganisms is more efficient than using monocultures (pure cultures), especially when the conditions change during the hydrolysis process. Microbial consortia are

usually better adapted to pH and temperature changes and tend to show higher resistance to the presence of heavy metals, toxic organic compounds or contamination by other strains [30]. These authors studied degradation of rice straw by cooperative microbial activities. They reported that microbial consortium enriched from the hindgut of *Holotrichia parallela* larvae via continuous subcultivation (20 subcultures in total) possess strong cellulolytic activity and depredate rice straw about 83.1% after three days of cultivation, indicating it. The diversity analysis results showed that the bacterial diversity and richness decreased during the consortium enrichment process and this process could lead to a significant enrichment of phyla *Proteobacteria* and *Spirochaetes*, classes *Clostridia, Epsilonproteobacteria*, and *Betaproteobacteria*, and genera *Arcobacter*, *Treponema, Comamonas*, and *Clostridium*. Some of these are well known as typical cellulolytic and hemicellulolytic microorganisms.

In another study it was found that the degree of thermophilic anaerobic biodegradation of organic waste in a bioreactor can reaches 74% depending of substrate loading. The metagenomic analysis shows that bacteria predominated and archaeal share was 1.37% of the micobial content. Most of bactreia belonged to class *Clostridia* (32.9%), followed by *Bacteroidia* (21.5%), *Betaproteobacteria* (11.2%), *Gammaproteobacteria* (6.1%), and *Alphaproteobacteria* (5%). The most prominent genera among them were *Proteiniphilum*, *Proteiniborus*, and *Pseudomonas*. The genera *Methanocorpusculum, Methanobacterium, Methanomassiliicoccus, Methanoculleus,* and *Methanosarcina* were the most abundant among archaea [51].

The use of microbial consortia in the biodegradation of lignocelluloses could reduce problems such as incomplete synergistic enzymes, end-product inhibition, and so on [50]. In a comparative study, Arias et al. 2020 [53] assessed the effect of different swine manure (SM)/corn stover (CS) mixtures based on total solids (TS) content with respect to hygienization, microbial community dynamics and methane yields on batch anaerobic co-digestion performance. Different ratios of SM and CS with TS content between 0.69 and 6% digested at 75 day revealed SM had the greatest methane yield.

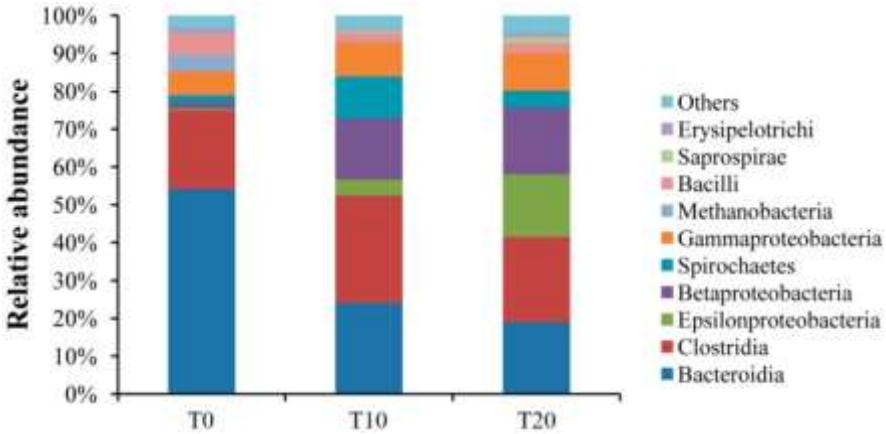

Fig. 1. Bacterial composition of the communities in those three groups (Class level). Note: T0, T10, and T20 means that the number of transfers in each was 0, 10, and 20, respectively [30].

The use of bacterial consortia able to degrade hemicellulose, with a focus on anaerobic ones, appears to represent a viable strategy to enhance biodegradation rates. However, and rather surprisingly, studies on the structure and composition of lignocellulolytic communities are rarely conducted under anoxic conditions. A study of switchgrass-degrading anaerobic bacteria, enriched from tropical forest soils, revealed dominant organisms to consist of members of the *Firmicutes, Bacteroidetes* and *Alphaproteobacteria* [54]. Another study, which enriched bacteria from sugarcane bagasse compost under aerobic (static) conditions, revealed the co-occurrence of two dominant anaerobic genera, *Clostridium* and *Thermoanaerobacterium*, together with aerobic bacilli next to as yet uncultured bacteria [40]. Previous studies have also enriched microorganisms on different plant biomass along successive transfers [55, 56]. For instance, some of these enrichments were designed to favour anaerobic fermentation (methanogenesis) with the concomitant production of biomethane from pretreated wheat straw [57].

Korenblum et al. [52] described the dynamics of the phylogenetic composition and abundance of the

bacterial communities developing on the recalcitrant biomass. In addition, it was confirmed the (hemi) cellulolytic activities of isolated members of the communities, which were able to grow anaerobically on carboxymethyl cellulose (CMC) and xylan. Until now, several cellulolytic consortia have been enriched from different ecosystems, such as soils composts and so on. Microbial structure analysis showed that the lignocellulose, such as rice straw and filter paper, led to significant enrichment of the phyla *Proteobacteria* and *Spirochaetes*, classes *Clostridia*, *Epsilonproteobacteria,* and *Betaproteobacteria*, and genera *Arcobacter*, *Treponema*, *Comamonas*, and *Clostridium* [58]. The authors constructed a stable microbial consortium able to degrade biomass from Napier grass and rice straw. A slight increase of bacterial diversity from day 5 to the end of the culture (day 19) was observed. This could be due to an alteration of oxygen level in the culture system from fully aerobic to anoxic condition where low oxygen level occurred at the bottom of the culture caused by static incubation of culturing tubes. Thus, aerobic bacteria were promoted at the beginning and then at the end of the culture, the diversity was higher because of the presence of both aerobic and anaerobic bacteria in the system. Co-existence of cellulolytic and non-cellulolytic microbes was observed in this consortium.

Taxonomic analysis of the sequencing dataset revealed the variation in overall microbial community profiles among the Np-LMC samples. At the phylum level, diverse bacterial phyla with different oxygen requirements, namely *Proteobacteria, Firmicutes*, and *Bacteroidetes*, comprising mainly the orders *Burkholderiales*, *Clostridiales*, and *Bacteroidales*, were found with similar representations (25–30%) throughout the cultivation period as the predominant bacteria in the consortium. However, the ratio of phylum *Proteobacteria* was slightly increased after 5 days of incubation, and then reduced continuously until day 19. The reduction of *Proteobacteria* during cultivation was accompanied by increased representation of *Bacteroidetes* and *Firmicutes*.

Biomass degradation efficiency was examined by determining the extent of Napier grass and rice straw degradation within 10 days of treatment. The consortium was able to enhance the decomposition rate of both non-pretreated and alkaline pretreated biomass, but to different extents. Alkaline pretreated Napier grass and rice straw were effectively degraded by the consortium by 28 ± 3% and 43 ± 4%, respectively, after 3 days of incubation and then to 39 ± 6% and 54 ± 6%, respectively, at the end of incubation (10 days) under the static anoxic conditions. The native biomass was also decomposed by the consortium, but with a slower rate than the pretreated ones. The degradation of native rice straw reached 37 ± 4% at the end of the experiment, whereas the non-pretreated Napier grass was only slightly decomposed by 30 ± 2% on day 10. Game et al. [59] isolated cellulolytic microorganisms from naturally decomposing organic matter collected from different locations. These isolates were studied for cellulolytic activity and compatibility with each other and a consortium of efficient cellulolytic microorganisms was developed.

Finally, newly developed cellulolytic microbial consortium was evaluated for its composting efficiency on rural and urban waste in open pit method. The bacterial and fungal population in composting pits increased gradually and highest population was recorded in initial phase of composting i.e. between 60 to 90 days of composting in test consortium. Test consortium reduced the composting period of rural waste by 22.68% while that of urban waste by 18.39% over uninoculated control [60]. Some previous studies also showed that the coexistence of anaerobic cellulolytic and aerobic non-cellulolytic bacteria (which scavenge metabolites from cellulose) is crucial for cellulose degradation, and this phenomenon is often detected at various sites when cellulose degradation occurs. The aerobic bacteria would consume oxygen by utilizing substrates contained in peptone and yeast extract, and would supply the anaerobic environment, reduce the concentration of celloligosaccharides, and neutralize the pH value for the anaerobic bacteria and archae, which would accelerate the cellulose degradation process [55]. Zhang et al. [61] showed that efficient degradation of cellulose could be achieved in batch cultures inoculated with rumen microorganisms. The rumen enriched cultures showed the best cellulose hydrolysis efficiency of 81% and the maximum hydrogen yield of 178.16 mL L^{-1} at pH 6.5. Acetate, propionate, and butyrate were the major liquid products at all pH values. Smaller amounts of ethanol and lactate were produced during the conversion of cellulose into hydrogen. The relative abundances of bacterial populations changed during the pH value transition. Xing et al. [62] identified by enzyme analysis and Illumina MiSeq sequencing that the rich core lignocellulolytic enzymes secreted by the abundant and diverse rumen bacteria and fungi contributed to the persistent degradation of lignocellulosic wastes. Members of the Clostridiales order and Basidiomycota phylum were found to be the dominant lignocellulolytic bacteria and fungi, respectively. It could thus be inferred that the main lignocellulose degradation processes were a series of catalytic reactions under the actions of lignocellulolytic enzymes secreted from bacteria and fungi.

Table 2**.** Microbial consortia involved in biodegradation of cellulose

Substrate	**Microbial consortia**	**Temperature, pH**	**Cultivation time**	**Biodegradation of cellulose, %**	**References**
Filter paper strip	*Arconacter* , genera *Comamonas* and *Escherichia*	40 °C	3 days	85%	[30]
Rice straw	*Prevotella, Escherichia Methanobrevibacter, Treponema, Comamonas, Escherichia, Arcobacter,* and *Bacteroides***,** *Arconacter*	40 °C	3 days	82.9%	[30]
Sugarcane	*Stenotrophomonas maltophilia, Paenibacillus sp., Microbacterium sp., Chryseobacterium taiwanense, and Brevundimonas sp*.	28 °C	96 hours	40-50%	[43]
Wheat straw	Synthetic microbial consortia composed of bacteria and fungi	55°C	72 hours	Increased biodegradation potential, enzymes activities	[43]
Filter paper	Anaerobic thermophilic cellulolytic microbial consortium, isolated form Composting plants	55°C	7 days	98,7%	[44]
Crystalline cellulose	Anaerobic thermophilic cellulolytic microbial consortium, isolated form Composting plants	55°C	7 days	98,7%	[44]
Cellulose and corn stover	Two stable, thermophilic mixed cellulolytic consortia were enriched *Thermoanaerobacterales* and *Clostridiales*	55°C	35 days	No data	[27]
Napier grass	*Proteobacteria, Firmicutes, and Bacteroidetes, Burkholderiales, Clostridiales, Bacteroidales,*	40°C	10 days		[58]
Wheat straw 1	~40 % (bacteria) and ~60 % (fungi)	25°C pH 7.2	45 days	51.92±0.41 %	[55]
Wheat straw 2	~40 % (bacteria) and ~60 % (fungi)	25°C pH 9.0	45 days	38.60 %-	[55]
Switchgrass	~40 % (bacteria) and ~60 % (fungi)	25°C pH 7.2	45 days	47.67%-	[55]
Corn stover	~40 % (bacteria) and ~60 % (fungi)	25°C pH 7.2	45 days	62.79±4.69 %	[55]
Eucaliptus Pulp sludge	aerobic, microaerophilic and anaerobic strains, bacteria (*Rhodocyclaceae, Thermobacterium thermosacharolyticum,Clostridium*)	50°C	7 days	77%	[40]
Rice strew	aerobic, microaerophilic and anaerobic strains, bacteria (*Rhodocyclaceae, Thermobacterium thermosacharolyticum,Clostridium*	50°C	7 days	75.3%	[40]
Sugar cane Bagasse	aerobic , microaerophilic and anaerobic strains, bacteria (*Rhodocyclaceae, Thermobacterium thermosacharolyticum,Clostridium*	50°C	7 days	59.4%	[40]
Straw	rumen enriched cultures *Bacteroidetes,Firmicutes, Spirochaetae*	37°C	5 days	81%	[61]

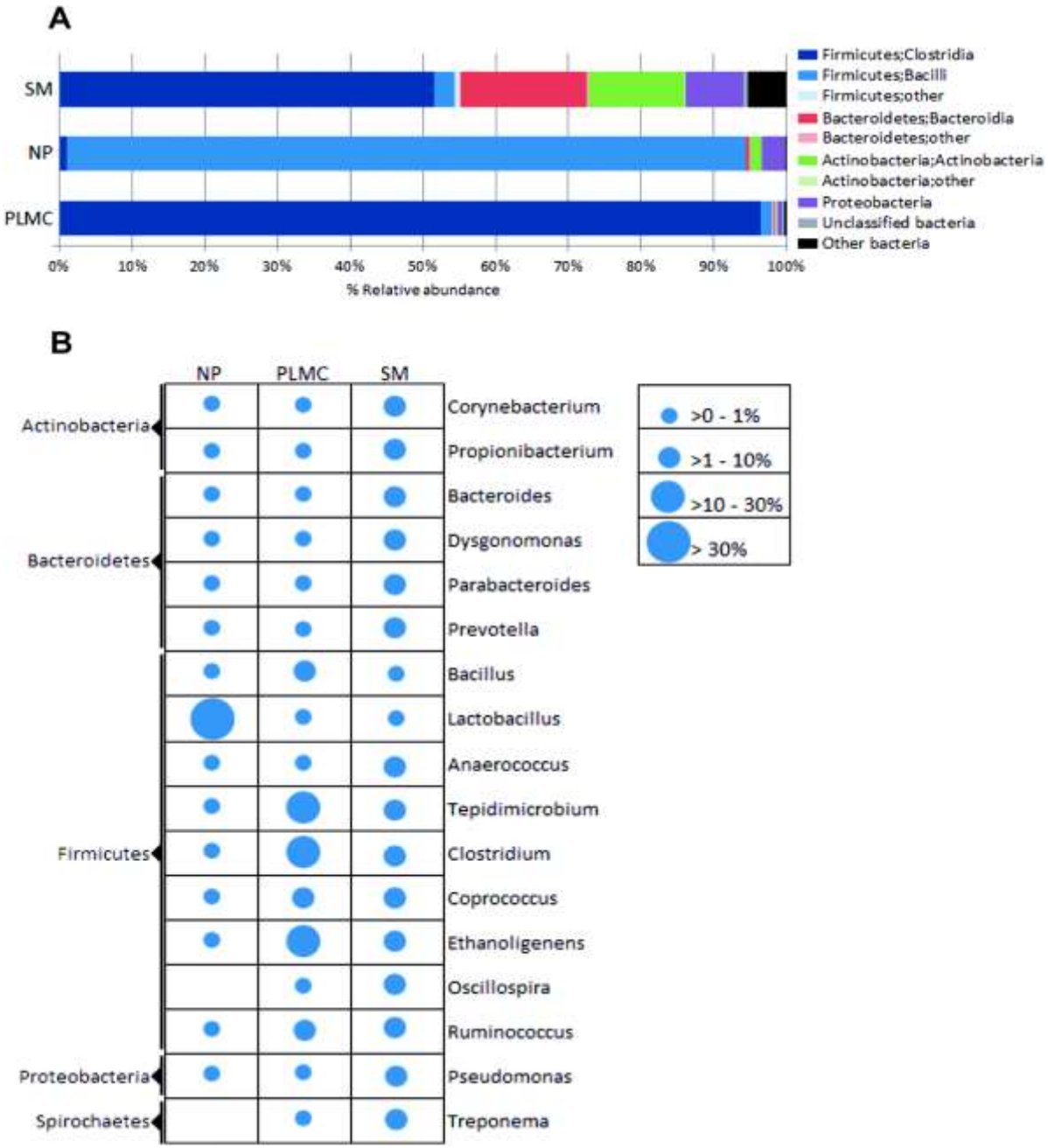

Fig. 2**.** Distribution of bacterial taxa in the cellulolytic consortia and the cellulosic substrates based on the % of relative abundance according to tagged 16S rRNA gene sequencing. (A) Phylum/class, (B) genus: the circle size indicates the relative abundance in genus level calculated as percentage of the total diversity [63].

Focusing on a more refined taxonomic level, *Actinobacteria* in the *genus Propionibacterium* were the most abundant in SM, followed by F*irmicutes* (order *Clostridiales* in genera *Corynebacterium* and *Prevotella)*; however, they accounted for less than 15% of the total diversity, reflecting the highly heterogeneous nature of the microbial community [63].

CRYOPRESERVATION AND CRYOPROTECTANTS IN LONG-TERM PRESERVATION

Cultivation and characterization of microorganisms alone is not adequate without preservation techniques that do not alter the morphology, physiology or genetics of pure strains. Careful preservation is imperative for future research,

teaching and industrial applications. Several methods have been successfully used for the preservation of microorganisms: repeated sub-culturing, preservation on agar beads, oil overlay of slant-grown cultures use of silica gel and other sterile supports, cryopreservation and lyophilization. Among these, cryopreservation and lyophilization are highly utilized for culture collections and industry, and a discussion of technical aspects and the pros and cons of both the methods is warranted [64]. In a comprehensive review Srivastava et al. focusses on the different methods available for preservation of microbial strains for short to long term. In addition, specific preservation techniques for certain microbes and problems and concerns in the routinely used preservation techniques and revival of preserved microbes have been dealt in detail [65].

Cryoprotectants protect the cells from cryo-injuries during cryopreservation. The cryoprotective additives (CPAs) used in the frozen storage of microorganisms (viruses, bacteria, fungi, algae, and protozoa) include a variety of simple and more complex chemical compounds, but only a few of them have been used widely and with satisfactory results: these include dimethylsulfoxide (Me_2SO), glycerol, blood serum or serum albumin, skimmed milk, peptone, yeast extract, saccharose, glucose, methanol, polyvinylpyrrolidone (PVP), sorbitol, and malt extract. Pairwise comparisons of the cryoprotective activity of the more common CPAs used in cryomicrobiology, based on published experimental reports, indicate that the most successful CPAs have been Me_2SO, methanol, ethylene glycol, propylene glycol, and serum or serum albumin, while glycerol, polyethylene glycol, PVP, and sucrose are less successful, and other sugars, dextran, hydroxyethyl starch, sorbitol, and milk are the least effective. DMSO and glycerol are the most common protectants added to bacterial cells to enhance cryopreservation. However, low concentrations of DMSO might result in cellular toxicity. Glycerol prevents hydrogen bonding between water molecules and thus prevents formation of intracellular ice crystals during freezing [64]. For long-term storage of individual microorganisms, cryopreservation and lyophilization are two major methods used; however, cryopreservation is generally preferred over lyophilization due to potential cell damage during the drying process [67].

The term cryopreservation refers to the preservation of biological materials at cryogenic temperatures, generally 80 °C, (dry ice) or 196 °C, (liquid nitrogen). Low temperature protects proteins and DNA from denaturation and damage and slows the movement of cellular water. Consequently, biochemical and physiological activities of the cells are essentially halted and cells are protected for long periods of time. Preservation of cells at -20 °C is not recommended for long-term preservation. Preservation at -80 °C is adequate, but -196 °C is considered ideal because the chances of DNA mutations are almost zero at that temperature. During cryopreservation, cryovials can be stored immersed in liquid nitrogen (at -196 °C) or in its vapour phase (-135 to -150 °C). Storage in vapor phase is considered better because it prevents the entry of liquid phase nitrogen into the cryovials, protecting against bursting and viral contamination [68].

Lyophilization

Freeze-drying or lyophilization is the preferred long-term preservation method due to the low cost of maintenance and ease of transportation of lyophilized cultures. Lyophilization gives satisfactory results for the preservation of many bacteria, yeast and sporulating fungi, but does not adequately preserve non-sporulating fungi (vegetative hyphae), some species of yeast (*Lipomyces, Leucosporidium, Brettanomyces, Dekkera, Bulleera, Sporobolomyces*) and certain bacteria (*Aquaspirillum serpens, Clostridium botulinum, Helicobacter pylori)* [68].

Besides lyophilization (unsuitable for the majority of basidiomycetes), cryopreservation at low temperatures seems to be a very efficient way to attain this goal. Especially the storage in liquid nitrogen has been considered as the best and most widely applicable preservation technique available for fungi, which seems to surpass all others in the ability to preserve genomic and phenotypic features. It is a safe and reliable method for a long-term maintenance of most fungal species, especially those not amenable to freeze-drying. But neither this cryopreservation method is applicable to preservation of all fungal cultures in the present form. In the development of alternative storage methods, it is important to consider their cost and accessibility [69]. Microbial communities enriched from diverse environments have shown considerable promise for the targeted discovery of microorganisms and enzymes for bioconversion of lignocellulose to liquid fuels. While preservation of microbial communities is important for commercialization and research, few studies have examined storage conditions ideal for preservation [70- Yu C et al. 2015]. Little work has been done to evaluate the effects of

cryoprotective agents on community structure. Ideally, the composition of microbial communities after storage should be the same as their initial states [69].

A group of preservation methods included cryopreservation with the cryoprotective agents DMSO and glycerol, and cryopreservation without cryoprotective agents. Revived communities were examined for their ability to decompose switchgrass under high-solid and thermophilic conditions. In one early study mixed dried rice straw with chicken, pig, and cattle feces under thermophilic conditions was used to create a compost community that was then used to inoculate Whatman filter paper. This enrichment yielded a stable microbial community that included *Thermobacillus*, which remained stable for at least 1 year when stored at −80 °C in a medium [0.1 % yeast extract, 0.5 % peptone, 0.5 % $CaCO_3$, 0.5 % NaCl, and H_2O (pH8.0)) with 20% (v/v) glycerol [28]. The relative abundances of *Acidobacteria*, *Bacteroidetes*, *Proteobacteria* and *Verrucomicrobio* were not affected by the storage methods suggesting that they can be preserved in either DMSO or glycerol at −80 °C. The relative abundances *of Chloroflexi* and *Planctomycetes* stored in DMSO and glycerol-treated samples were lower than the control and inoculum samples [70]. Proper preservation methods should not significantly alter the composition of microbial communities after preservation. Preservation of samples in the absence of cryoprotectant resulted in variable changes in community composition. Samples preserved with DMSO and glycerol did experience a consistent shift in community composition though dominant microorganisms were retained in the active community. Despite shifts in the community with storage, the samples were active upon revival under thermophilic and high-solid conditions. The results suggest that the presence of microorganisms may be more important than their relative abundance in retaining an active microbial community [70]. Even different strains of the same species may show different responses in terms of survival and durability with the same preservation strategy. Due to the vast diversity of microbial life and the time-consuming nature of preservation research, it is not possible to optimize the preservation of all the species of the same genus or all the strains of same species.

Anaerobic preservation techniques

The state of anaerobic preservation techniques requires even more attention because only a few culture collections in the world are dealing with anaerobic preservation. In the future, microbiologists should focus on the development and optimization of robust preservation methods for strict anaerobes and archaea in order to ensure the longterm viability of these microorganisms [64].

Both cryopreservation and lyophilization have advantages and disadvantages, and the response of preservation varies by species. Even different strains of the same species may respond differently to the same preservation method. The viability and longevity of microorganisms under preservation depends on some critical factors: (1) composition of the suspension and rehydration medium, (2) type of cryoprotectant used, (3) rate of cooling and thawing, (4) growth stage of the culture, (5) cell size and type, lipid content, water content, and initial density of cells [68, 71].

CONCLUSIONS

The recent review underlines the synergy between enzymes produced by different microorganisms to overcome the lack of efficient conversion by a single strain probably because many consortia of bacterial and archae strains may produce high levels of some important enzymes required for efficient conversion. Enourmous microbial consortia with different cellulose degrading activity are already isolated from various ecological niches including a wide variety and functionally active microorganisms. Moreover, the enrichment culture techniques are powerful tool for obtaining microbial consortia with desired cellulolytic properties. Many microbial consortia could possess stable cellulolytic activity during many passages but changes were observed in their microbial structure and cellulolytic activity depending on many factors such as cultivation temperature, pH, metal ions, etc. Experimental approaches for cryopreservation and cryoprotection are presented and discussed aiming to preserve the microbial cellulolytic activity.

REFERENCES

1. Tuomela M, M. Vikman, A. Hatakka, M. Itävaara. Biodegradation of lignin in a compost enviroment: a review. Bioresour. Technol., Vol. 72, 2000, 169-183.

2. Neklyudov, G., N. Fedotov, and A.N. Ivankin. Intensification of composting processes by aerobic microorganisms: A review, Appl. Biochem. Microbiol., Vol. 44, 2008, 1, 6-18

3. Björdal, C.G.; P.K. Dayton. First evidence of microbial wood degradation in the coastal waters of the Antarctic. Sci. Rep., Vol.10, 2020, 12774.

4. Hiura, K. and T. Maeda. JP Appl. No. 0702589, 1995.

5. Haruta S., Z. Cui, Z. Huang, M. Li, M. Ishii, Y. Igarashi. Construction of a stable microbial community with high cellulose-degradation ability. Appl. Microbiol. Biotechnol., Vol. 59, 2002, 529-534.

6. Lynd L., P. Weimer, W.I. van Zyl. Pretorius. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol. Molec. Biol. Rev., Vol.66, 2002, 3, 506-577.

7. Mathews S.L., J. Pawlak, A.M. Grunden. Bacterial biodegradation and bioconversion of industrial lignocellulosic streams. Appl. Microbiol. Biotechnol. Vol. 99, 2015, 6, 2939-2954.

8. Kim K., Y. Scung-soo, O. Young et al. Isolation and characteristics of *Trichoderma harzianum* FJ1 producing cellulases and xylanase. J. Microb. Biotechnol., Vol. 13, 2003, 1-8.

9. Ko C., W. Chen, C. Tsai, W. Jane, C. Liu, J. Tu. *Paenibacillus campinasensis* BA11: a wood material-utilizing bacterial strain isolated from black liquor. Bioresour. Technol., Vol. 9, 2007, 2727-2733.

10. Poszytek K., M. Ciężkowska, A. Skłodowska, L. Drewniak. Microbial consortium with high cellulolytic activity (MCHCA) for enhanced biogas production. Frontiers Microbiol., Vol. 7, 2016.

11. Bhat M. Cellulases and related enzymes in biotechnology. Biotechnol. Adv., Vol. 18, 2000, 355-383.

12. Bayer E., J. Belaich, Y. Shoham, et al. The cellulosomes: multienzyme machines for degradation of plant cell wall polysaccharides. Ann. Rev. Microbiol., Vol. 58, 2004, 521-554.

13. Lee Y., B. Kim., B. Lee et al. Purification and characterization of cellulose produced by *Bacillus amyoliquefaciens* DL-3 utilizing rice hull. Bioresource Technol., Vol. 99, 2008, 378-386.

14. Chukwuma, O.B., M. Rafatullah, H.A. Tajarudin, N. Ismail. Lignocellulolytic enzymes in biotechnological and industrial processes: A Review. Sustainability Vol. 12, 2020, 7282.

15. Sadhu S., P. Saha, S. Sen et al. Production, purification and characterization of a novel thermotolerant endoglucanase (CMCase) from *Bacillus* strain isolated from cow dung. Springer Plus, 2013, 2, 10.

16. Anwar F., S. Hussain, S. Ramzan, F. Hafeez, M. Arshad, M. Imran, Z. Maqbool, N. Abbas. Characterization of reactive red-120 decolorizing bacterial strain *Acinetobacter junii* FA10 capable of simultaneous removal of azo dyes and hexavalent chromium. Water Air Soil Pollut., 2014, 225-2017.

17. Chandra R., R. Singh. Decolourisation and detoxification of rayon grade pulp paper mill effluent by mixed bacterial culture isolated from pulp paper mill effluent polluted site. Biochem. Eng. J., Vol. 61, 2012, 49-58.

18. Mathews S., J. Pawlak, A. Grunden. Isolation of *Paenibacillus glucanolyticus* from pulp mill sources with potential to deconstruct pulping waste. Bioresour. Technol. Vol. 164, 2014, 100-105.

19. Singh S., B. Singh, R. Chandra. Biodegradation of phenol in batch culture by pure and mixed strains of *Paenibacillus* sp. and *Bacillus cereus*. Pol. J. Microbiol., Vol. 58, 2009, 4, 319-325.

20. Shi Y., L. Chai, C. Tang, Z. Yang, Y. Zheng, Y. Chen, Q. Jing. Biochemical investigation of kraft lignin degradation by *Pandoraea* sp. B-6 isolated from bamboo slips. Bioprocess Biosyst. Eng., Vol. 36, 2013, 1957-1965.

21. Stolz A. Basic and applied aspects in the microbial degradation of azo dyes. Appl. Microbiol. Biotechnol., Vol. 56, 2001, 69-80.

22. Pokhrel D., T. Viraraghavan. Treatment of pulp and paper mill wastewater - a review. Sci. Total Environ., 2004, 333, 37-58.

23. Chandra R., Abhishek A., Sankhwar M. Bacterial decolorization and detoxification of black liquor from rayon grade pulp manufacturing paper industry and detection of their metabolic products. Bioresour. Technol., 2011, 102, 6429-643.

24. Chen Y., L. Chai, C. Tang, A. Yang, Y. Zheng, Y. Shi, H. Zhang. Kraft lignin biodegradation by *Novophingobium* sp. B-7 and analysis of the degradation process. Bioresour Technol., 2012, 123, 682-685.

25. Saha B. Hemicellulose bioconversion. J. Ind. Microbiol. Biotechnol., Vol. 30, 2003, 279-291.

26. Zabidi, N.A.M.; H.L. Foo, T.C. Loh, R. Mohamad, R.A. Rahim. Enhancement of versatile extracellular cellulolytic and hemicellulolytic enzyme productions by *Lactobacillus plantarum* RI 11 isolated from Malaysian food using renewable natural polymers. Molecules, 2020, 25, 2607.

27. Feng Y., Y. Yu, X. Wang, Y. Qu, D. Li, W. He, B.H. Kim. Degradation of raw corn stover powder (RCSP) by an enriched microbial consortium and its community structure. Bioresour. Technol. 2011, 102, 742–747.

28. Haruta S., Z Cui, Z. Huang, M. Li, M. Ishii, Y. Igarashi. Construction of a stable microbial community with high cellulose-degradation ability. Appl. Microbiol. Biotechnol., 2002, 59, 529–534.

29. Sun J.Z., M.E Scharf. Exploring and integrating cellulolytic systems of insects to advance biofuel technology. Insect Sci. 2010, 17, 163–165.

30. Sheng P., J. Huang, Z. Zhang, D. Wang, X. Tian, J. Ding. Construction and characterization of a cellulolytic consortium enriched from the hindgut of *Holotrichia parallela* Larvae. Int. J. Mol. Sci., Vol.17, 2016, 10, 1646.

31. Wu, D., Z. Wei, X. Gao, J. Wu, X. Chen, Y. Zhao, L. Jia, D. Wen. Reconstruction of core microbes based on producing lignocellulolytic enzymes causing by bacterial inoculation during rice straw composting. Bioresour. Technol., Vol. 315, 2020, 123849.

32. Hess, M., A. Sczyrba, R. Egan, T.W. Kim, H. Chokhawala, G. Schroth, S. Luo, D.S. Clark, F. Chen, T. Zhang, et al. Metagenomic discovery of biomass-degrading genes and genomes from cow rumen. Science, Vol. 331, 2011, 463-467.

33. Tomazetto, G. A.C. Pimentel, D. Wibberg, N. Dixon, F.M. Squina. Multi-omic directed discovery of cellulosomes, polysaccharide utilization loci, and lignocellulases from an enriched rumen anaerobic consortium. Appl. Environ. Microbiol. Vol. 86, 2020, e00199-20**.**

34. Jiao, Y., Y. Yuan, C. He, L. Liu, X. Pan, & P. Li. Enrichment culture combined with microbial electrochemical enhanced low-temperature anaerobic digestion of cow dung. Bioresource Technol., 2022, 360, 127636.

35. Zheng, Y., L. Chai, Z. Yang, Y. Chen, Y. Shi, Y. Wang. Environmentally safe treatment of black liquor with *Comamonas* sp. B-9 under high-alkaline conditions. J. Basic Microbiol., Vol. 54, 2014, 152-161.

36. Wenzel M., I. Schönig, M. Berchtold, P. Kämpfer, H. König. Aerobic and facultatively anaerobic cellulolytic bacteria from the gut of the termite *Zootermopsis angusticollis*. J. Appl. Microbiol., Vol. 92, 2002, 32-40.

37. Poulsen, J.S., N. de Jonge, W.V. Macêdo, F.R. Dalby, A. Feilberg, & J.L. Nielsen. Characterisation of cellulose-degrading organisms in an anaerobic digester. Bioresource Technol., Vol. 351, 2022, 126933.

38. Shikata A., J. Sermsathanaswadi, P. Thianheng, S. Baramee, C. Tachaapaikoon, R. Waeonukul, P. Pason, K. Ratanakhanokchai, A. Kosugi. Characterization of an anaerobic, thermophilic, alkaliphilic, high lignocellulosic biomass-degrading bacterial community, ISHI-3, isolated from biocompost. Enz. Microbial Technol., Vol. 118, 2018, 66-75.

39. Ozbayram E., S. Kleinsteuber, M. Nikolausz, B. Ince, O. Ince. Enrichment of lignocellulose-degrading microbial communities from natural and engineered methanogenic environments. Appl. Microbiol. Biotechnol., Vol.102, 2018, 2, 1035-1043.

40. Wongwilaiwalin S., U. Rattanachomsri, T. Laothanachareon, L. Eurwilaichitr, Y. Igarashi, V. Champreda. Analysis of a thermophilic lignocellulose degrading microbial consortium and multi-species lignocellulolytic enzyme system. Enzyme Microb. Technol., Vol.47, 2010, 6, 283-290.

41. Zheng, G., T. Yin, Z. Lu, S. Boboua, J. Li, W. Zhou. Degradation of rice straw at low temperature using a novel microbial consortium LTF-27 with efficient ability. Bioresour. Technol., Vol. 304, 2020, 123064**.**

42. Lazuka A., L. Auer, M. O'Donohue, G. Hernandez-Raquet. Anaerobic lignocellulolytic microbial consortium derived from termite gut: enrichment, lignocellulose degradation and community dynamics. Biotechnol. Biofuels, Vol. 11, 2018, 284.

43. Puentes-Téllez J. and P. Falcao. Construction of effective minimal active microbial consortia for lignocellulose degradation. Microb. Ecol., Vol.76, 2018, 2, 419-429.

44. Kinet R., J. Destain, S. Hiligsmann, P. Thonart, L. Delhalle, B. Taminiau, G. Daube, F. Delvigne. Thermophilic and cellulolytic consortium isolated from composting plants improves anaerobic digestion of cellulosic biomass: Toward a microbial resource management approach. Bioresource Technol., Vol. 189, 2015, 138-144.

45. Negri, C., M. Ricci, M. Zilio, G. D'Imporzano, W. Qiao, W., R. Dong, F. Adani. Anaerobic digestion of food waste for bio-energy production in China and Southeast Asia: A review. Renew. Sustain. Energy Rev., Vol. 133, 2020, 110138**.**

46. Basak, B., Y. Ahn, R. Kumar, J.H. Hwang, K.H. Kim, & B.H. Jeon. Lignocellulolytic microbiomes for augmenting lignocellulose degradation in anaerobic digestion. Trends Microbiol., Vol.30, 2022, 1, 6-9.

47. Kumar, R., T.H. Kim, B. Basak, S.M. Patil, H.H. Kim, Y. Ahn et al. Emerging approaches in lignocellulosic biomass pretreatment and anaerobic bioprocesses for sustainable biofuels production. J. Cleaner Product., 2022, 333, 130180**.**

48. Cortes-Tolalpa L., J. Salles, J. van Elsas. Bacterial synergism in lignocellulose biomass degradation – complementary roles of degraders as

influenced by complexity of the carbon source. Front. Microbiol., Vol. 8, 2017, 1628.

49. Ferraro, A., G. Massini, V.M. Miritana, S. Rosa, A. Signorini, & M. Fabbricino. A novel enrichment approach for anaerobic digestion of lignocellulosic biomass: Process performance enhancement through an inoculum habitat selection. Bioresource Technol., Vol. 313, 2020, 123703.

50. Kabaivanova L., V. Hubenov, L. Dimitrova, I. Simeonov, H. Wang, P. Petrova. Archaeal and bacterial content in a two-stage anaerobic system for efficient energy production from agricultural wastes. Molecules, Vol. 27, 2022, 5, 1512.

51. Kabaivanova, L., P. Petrova, V. Hubenov, I. Simeonov. Biogas production potential of thermophilic anaerobic biodegradation of organic waste by a microbial consortium identified with metagenomics. Life, Vol.12, 2022, 5, 702.

52. Korenblum E., D. Jiménez, J. van Elsas. Succession of lignocellulolytic bacterial consortia bred anaerobically from lake sediment. Microbial Biotechnology, 2016, 9 (2), 224-234.

53. Arias, D.E., C. Veluchamy, K.E. Dunfield, M.B. Habash, B.H. Gilroyed. Hygienization and microbial metabolic adaptation during anaerobic co-digestion of swine manure and corn stover. Bioresource Technol. 2020, 306, 123168.

54. DeAngelis K., J. Fortney, S. Borglin, W. Silver, B. Simmons, T. Hazen. Anaerobic decomposition of switchgrass by tropical soil-derived. MBio., Vol. 3, 2012, 19.

55. De Lima Brossi M., D. Jiménez, L. Cortes-Tolalpa, J. van Elsas. Soil-derived microbial consortia enriched with different plant biomass reveal distinct players acting in lignocellulose degradation. Microbial Ecol., Vol.71, 2015, 3, 616-627.

56. Porsch K., B. Wirth, E. Tóth, F. Schattenberg, M. Nikolausz. Characterization of wheat straw-degrading anaerobic alkali-tolerant mixed cultures from soda lake sediments by molecular and cultivation techniques. Microb. Biotechnol., 2015, 8, 801-814.

57. Sträuber H., F. Bühligen, S. Kleinsteuber, M. Nikolausz, and K. Porsch. Improved anaerobic fermentation of wheat straw by alkaline pre-treatment and addition of alkali-tolerant microorganisms. Bioengineering, 2015, 2, 66-93.

58. Kanokratana P., S. Wongwilaiwalin, W. Mhuantong, S. Tangphatsornruang, L. Eurwilaichitr, and V. Champreda. Characterization of cellulolytic microbial consortium enriched on Napier grass using metagenomic approaches. J. BioSci. BioEng., Vol.125, 2018, 4, 439-447.

59. Game B., C. Deokar, P. More. Efficacy of newly developed microbial consortium for composting of rural and urban wastes. Int. J. Curr. Microbiol. Appl. Sci., Vol.6, 2017, 6, 626-633.

60. Kato S., S. Haruta, Z. Cui, M. Ishii, Y. Igarashi. Effective cellulose degradation by a mixed-culture system composed of a cellulolytic *Clostridium* and aerobic non-cellulolytic bacteria. FEMS Microbiol. Ecol., Vol. 51, 2004, 133-142.

61. Zhang D., P. Clauwaert, A. Luther, D. Barreiro, W. Prins, D. Brilman, F. Ronsse. Sub- and supercritical water oxidation of anaerobic fermentation sludge for carbon and nitrogen recovery in a regenerative life support system. Waste Manag., Vol. 77, 2018, 268-275.

62. Xing, B.S., Y. Han, X.C. Wang, J. Wen, S. Cao, K. Zhang, Q. Li, H. Yuan. Persistent action of cow rumen microorganisms in enhancing biodegradation of wheat straw by rumen fermentation. Sci. Total Environ. 2020, 715, 136529.

63. Wongwilaiwalin S., W. Mhuantong, V. Champreda, S. Tangphatsornruang, P. Panichnumsin, K. Ratanakhanokchai, C. Tachaapaikoon. Structural and metabolic adaptation of cellulolytic microcosm in co-digested Napier grass-swine manure and its application in enhancing thermophilic biogas production. RSC Advances, Vol. 8, 2018, 5, 29806-29815.

64. Prakash O., Y. Nimonkar, Y. Shouche. Practice and prospects of microbial preservation. FEMS Microbiol. Lett., 2012, 339 (1), 1-9.

65. Srivastava, A.K., H. Chakdar, P.K. Sahu, & M. Kumar. Conserving microbial diversity: practices, trends and beyond. Indian J., Vol. 35, 2022, 3, 338- 342.

66. Hubálek Z. Protectants used in the cryopreservation of microorganisms. Cryobiol., Vol.46, 2003, 3, 205-229.

67. Heylen K., S. Hoefman, B. Vekeman, J. Peiren, P. De Vos. Safeguarding bacterial resources promotes biotechnological innovation. Appl. Microbiol. Biotechnol., Vol.94, 2012, 3, 565-574.

68. Smith D., J. Ryan, E. Stackebrandt. The ex situ conservation of microorganisms: aiming at a certified quality management. Biotechnology (Doelle HW & DaSilva EJ, eds), Encyclopedia of Life Support Systems (EOLSS), Developed under the Auspices of UNESCO, EOLSS Publisher, Oxford, UK, 2008.

69. Eichlerová, I. and L. Homolka. Preservation of basidiomycete strains on perlite using different protocols. Mycoscience, Vol.55, 2014, 6, 439-448.

70. Yu C., A. Reddy, C. Simmons, B. Simmons, S. Singer, J. van der Gheynst. Preservation of microbial communities enriched on lignocellulose under thermophilic and high-solid conditions. Biotechnol. Biofuels, Vol.8, 2015, 1.

71. Chian R-C. Cryobiology: an overview. Fertility Cryopreservation (Chian R-C & Quinn R, Eds), 2010, 1-9.

Assist. Prof. Yana Gocheva, PhD
Department of Biotechnology
The Stephan Angeloff Institute of Microbiology
Acad. G. Bonchev Str., Bl. 26
1113 Sofia, Bulgaria
e-mail: yana2712@gmail.com

Assist. Prof. Lyudmila Dimitrova, PhD
Department of Infectious Microbiology
The Stephan Angeloff Institute of Microbiology
Acad. G. Bonchev Str., Bl. 26
1113 Sofia, Bulgaria
e-mail: lus22@abv.bg

Assist. Prof. Venelin Hubenov, PhD
Department of Biotechnology
The Stephan Angeloff Institute of Microbiology
Acad. G. Bonchev Str., Bl. 26
1113 Sofia, Bulgaria
e-mail: vhubenov@microbio.bas.bg

Prof. Hristo Najdenski, DVM, DSc, Corresponding Member of BAS
Department of Infectious Microbiology
The Stephan Angeloff Institute of Microbiology
Acad. G. Bonchev Str., Bl. 26
1113 Sofia, Bulgaria
e-mail: hnajdenski@abv.bg

Prof. Lyudmila Kabaivanova, PhD
Department of Biotechnology
The Stephan Angeloff Institute of Microbiology
Acad. G. Bonchev Str., Bl. 26
1113 Sofia, Bulgaria
e-mail: lkabaivanova@yahoo.com

Prof. Plamen Angelov, PhD
Department Aerospace Information
Space Research and Technology Institute
Acad. G. Bonchev Str., Bl. 1
1113 Sofia, Bulgaria
e-mail: pangelov@space.bas.bg

Assoc. Prof. Ivan Simeonov, PhD
Department of Biotechnology
The Stephan Angeloff Institute of Microbiology
Acad. G. Bonchev Str., Bl. 26
1113 Sofia, Bulgaria
e-mail:issim@abv.bg