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Development of microbial consortium for biological pretreatment of lignocellulosic raw materials

PhD. Thesis

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TABLE OF CONTENT

Abbreviations

ANOVA	Analysis of Variance
CFU	Colony Forming Units
DP	Degree of depolymerization of carbohydrates
FPU	Filter paper unit (Cellulase enzyme activity)
GDS	Gram per dried substrate
HPLC	High-Performance Liquid Chromatography
Ils	Ionic liquids pretreatment
OD	Optical Density
Orgv	Organosolv pretreatment
PC	Principal component
PCA	Principal Component Analysis
RI	Refractive index
SHF	Separate hydrolysis and fermentation
SPSS	Statistical Analysis Software
SSF	Simultaneous saccharification and fermentation
YEPD	Yeast extract peptone dextrose

List of figures

List of tables

1. INTRODUCTION AND OUTLINE

1.1. Introduction

It has been predicted that the world's population may reach 9.7 billion in 2050 and 10.9 billion in the next 50 years (Roser, 2013). As a result of the increasing population and economic growth, by 2050, global energy consumption and energy-related carbon dioxide emissions will increase nearly 50% compared with 2020 (Energy Information Administration, 2021). Fossil fuel, natural gas, coal and nuclear energy cannot satisfy human beings' demands. The reliance on oil and gas makes the world economy dependent on the limited number of exporting countries and escalates gasoline prices. Moreover, CO_2 emissions as the result of burning fossil energy from the industrial zone or means of transport vehicles have been claimed as the main reasons for serious environmental issues such as global warming, and climate change which could damage the natural ecosystem. Thus, many governments are stimulating the utilization of renewable energies and resources to aim toward the three dimensions (three Ps) of sustainability, namely Profitability (affordable energy), Planet (climate change) and People (social stability). The renewable source will be a promising and sustainable source of energy alternatives to address the future environmental problem and energy scarcity. Different types of renewable energy are currently being extensively researched, namely solar, wind, geothermal, hydrothermal and biofuels. Among renewable energy sources, bioenergy (energy from bio-based sources) is the largest renewable energy present in the form of liquid fuels such as biofuel, diesel, or gasoline. In 2017, bioenergy accounted for 70% of renewable energy consumption (World Bioenergy Association, 2019).

Biomass can be obtained from many sources such as forestry or agriculture waste streams. There are three classifications of biofuel listed first-, second-, and third-generation biofuels. Edible biomasses such as starch and saccharose-based ones were employed as feedstock in first-generation biofuel, while non-edible feedstocks such as cellulose, hemicellulose, lignocellulose as well as algal biomass and gases were used as the substrates for second-generation and the third-generation biofuels, respectively. Agricultural residues such as wheat straw, rice straw, rice husk, switchgrass, etc. have been intensively studied for production of second-generation biofuel (Farkas et al., 2019; Jain et al., 2016a; Nikzad et al., 2013; Tohamy et al., 2019).

Lignocellulose is the complicated structure of lignin, cellulose and hemicellulose and other components of pectin, proteins, small molecules, and minerals. These components associated with non-covalent bonds and covalent cross-linkages in an intricate structure, contribute to the recalcitrance of lignocellulosic feedstock for bioconversion (Kumari and Singh, 2018). Thus, converting lignocellulose to fermentable sugars and ethanol requires three main steps, starting with the pretreatment, then hydrolysis and fermentation process. The pretreatment is considered the most important step in determining the whole process's effectiveness. In this step, lignocellulose structure is disrupted resulting in the breakage of lignin sheath, degradation of hemicellulose and reduction of cellulose crystallinity and polymerization, thus enhancing the enzyme’s accessibility to the cellulose during hydrolysis (Baruah et al., 2018; Mosier et al., 2005). Different pretreatment approaches are available for lignocellulosic sources include physical, chemical, physicochemical, biological, and combined pretreatment. Among these routes, biological pretreatment is known as safe and environmentally friendly method, because it has many advantages over others with cost efficiency, lower energy requirement, mild working condition and free of toxic chemicals (Sharma

et al., 2019). Additionally, there is a wide taxonomic array of microorganisms that can be used in biological pretreatment. Fungi are well-known microbes for the effects on lignocellulose substrate by their effective extracellular enzymes, especially species from Ascomycetes (*Aspergillus sp*., *Trichoderma sp*., *Penicillium sp*.), Basidiomycetes (*Schizophyllum sp*., *P. chrysosporium*) including white-rot fungi and brow-rot fungi (*Fomitopsis palustris*) (Dashtban et al., 2009). Basidiomycota have an outstanding lignin-degrading capacity, but most of their species are difficult to handle in the laboratory. On the contrary, species belonging to the Ascomycetes phylum are easier to manage and they can be found inhabiting soil and wood, using lignin as a food source (Dicko et al., 2020; Ferrari et al., 2021). Many bacteria including aerobic and anaerobic species used in pretreatment possess an array of enzymes such as xylanase, endo-glucanase (e.g. carboxymethyl cellulases), exo-glucanase (e.g. cellobiohydrolases), β-glucosidase (e.g. cellobiases) which were confirmed to enhance the digestibility of biomass by the removal xylan, reduction of cellulosic crystallinity (Chang et al., 2014; Guo et al., 2018; Li et al., 2009). Different cellulases and substrates have specific interactions and work together synergistically (Beguin and Aubert, 1994; Nidetzky et al., 1994). The gram-positive *Bacillus* strains, *Rhodococcus* strain and gram-negative *Pseudomonas* have the highest degrading efficiency of cellulosic materials (Paudel and Qin, 2015a). In addition, *Yarrowia lipolytica* is an excellent sample of microorganisms which can produce several types of metabolites for growth promotion (Gonçalves et al., 2014). *Pichia stipitis*, native xylose-fermenting yeast, was found as high xylanase producer in wheat bran as a substrate under suspended fermentation (Ding et al., 2018).

However, biological pretreatment still faces some drawbacks such as low degradation efficiency, taking long time, and the risks of carbohydrate loss (Sindhu et al., 2016). The promising approach to enhance the effectiveness of biological processes is utilization of microbial consortium, by taking advantage of synergistic action in the mix-culture technology. The appropriate pretreatment is presented through high adaptability, increasing degrading enzyme activities, control of pH and increasing in substrate utilization (Kalyani et al., 2013). Particularly, Haruta and co-workers (2002) proved the high degradation ability and stability of bacterial community from composting materials under harsh conditions. It was observed that bacterial consortium applied for cellulose degradation can expose effective interspecies interactions to maintain structural stability (Kato et al., 2008). Farkas and co-workers (2019) found that pretreatment of wheat bran using multi-cultural fungal consortium including *Aspergillus*, *Trichoderma* and *Penicillium* genus could result outstanding soluble carbohydrates in comparison to those of individual species. Yeast strains display some advantages to produce natural products from xylose such as aromatics and flavonoids, which can be used as supplements for microorganisms' metabolites (Zha et al., 2021). Numerous yeast genes were found to be associated with the tolerance of aromatic inhibitors which are generated through the conversion of lignocellulosic biomass (Bazoti et al., 2017; Jin and Cate, 2017).

The utilization of microbial consortium may have advantages over a single microorganism because of the potential of synergistic relationship between involved strains in the same habitats. The great challenge should be the organization of members of consortium for specific tasks to maximize the degradation rate and efficacy of process.

1.2. Outline

In the last decade, the application of microorganisms in every aspect of life has become promising due to their great advantages over the conventional process. The utilization of microbial communities comprised of various types of microbial species has become a new promising avenue to enhance the efficiency of bio-based processes. Connecting to this field, my PhD research focuses on the construction and tailoring of efficient microbial consortia for biological pretreatment of lignocellulosic biomass. The tasks aimed are followings:

- Effects of different individual strains and their consortia in degradation of lignocellulosic biomass
    - Bacteria strains and their consortia
    - Yeast strains and their consortia
    - Fungi strains and their consortia
    - Optimisation of operating conditions for microbial pretreatment with different microbial consortia
- Design and construction of different microbial consortia
    - Construction effective microbial comprised various microorganisms for biological pretreatment of lignocellulosic biomass
    - Evaluation of efficacy of the complex microbial consortia on the pretreatment of various types of lignocellulosic biomasses
- Application potential of newly developed microbial consortia – cases study
    - Investigation of new saccharification method in combination of microbial pretreatment with exogenous enzyme preparations
    - Ethanol fermentation of microbially pretreated biomass

2. LITERATURE REVIEW

2.1. Lignocellulose

Among the available bioenergy sources, lignocellulose is considered a potential feedstock for production of bioenergy and many other products including various chemicals, biofuels, enzymes (Isroi et al., 2011). The biofuel produced from lignocellulose is a promising alternative to fossil source, and it reduces reliance on fossil fuels and mitigate greenhouse gas emissions. Using crop residues instead of energy crops could contribute to avoiding competition of the use of land for biofuel or food farming that raised some ethical issues in the last some decades. Farming for energy purposes makes it possible to enable higher production per unit of land area, thus increasing land-use efficiency (Larson, 2008). Lignocellulosic biomass has increased great attention because of its abundance and cost-efficiency than conventional feedstock like sugar cane, corn, etc. Recent report has predicted that 1.3 billion tons of biomass can be produced annually in the US in the near term, and they mostly originate from agricultural and forestry sources (Perlack et al., 2005). Lignocellulosic biomass is rich in carbohydrates which can be converted into bioenergy during the aerobic and anaerobic digestion of carbohydrate polymers such as celluloses and hemicelluloses. Cellulose (40–60%) and hemicellulose (20-40%) are predominance, but aromatic polymer lignin also share about 10-24% in these feedstocks (Putro et al., 2016).

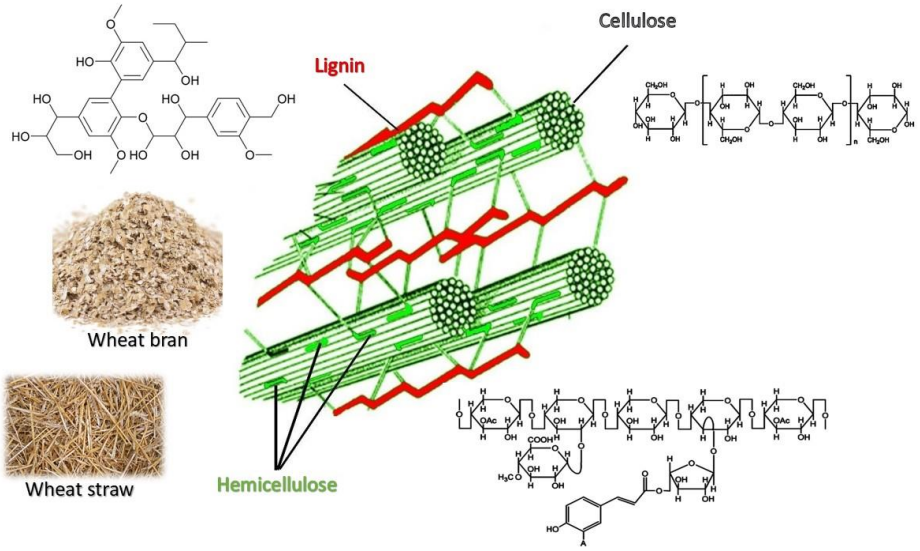

Figure 2.1 Hierarchy of structures of lignocellulosic biomass

The hierarchy of structure of lignocellulosic biomass is demonstrated in **Figure 2.1**. The diversity ratios of lignocellulose compositions vary from one plant species to another regarding the sources, and they are listed as hardwoods, softwoods and grasses. Additionally, factors such as age, growth stage and conditions are also accounted for the changes of ratios of composition within even single plant (Chen, 2014; Jeffries, 1994).

2.1.1. Cellulose

Cellulose is the most abundant organic compound on the earth. This linear biopolymer is comprised of up to 7 000-15 000 recurring D-glucose monomers ($C_6H_{10}O_5$) linked togethers via β(1→4) glycosidic bounds (Sampath et al., 2017). Moreover, it does not undergo coiling or branching, and its molecules are elongated and somewhat rigid rod-like structures (**Figure 2.2**).

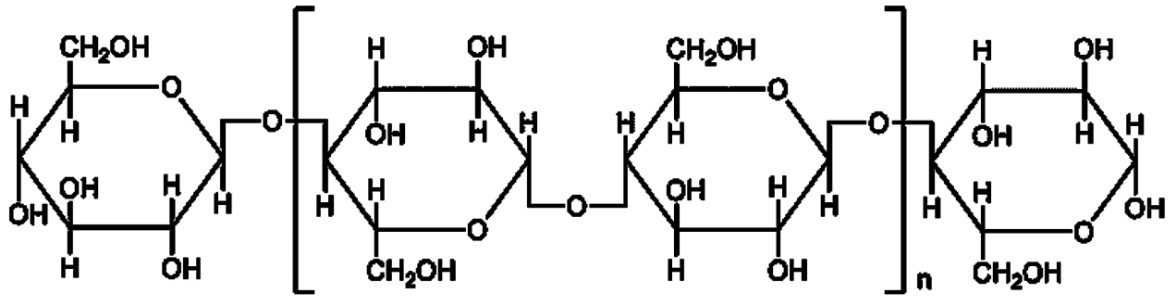

Figure 2.2 Cellulose structure (Terzopoulou et al., 2015)

Cellulose chains are held together by van der Waals forces and microfibril hydrogen bonds, which join together to form cellulose fibers, the structural components of the primary cell wall (Ching et al., 2015). Cellulose consists of crystalline parts together with some amorphous regions. On one hand, the crystalline cellulose has a well-organized structure of microfibrils, which are tightly bundled and bound together by a strong inter-chain hydrogen bond. In addition, cellulose that has a degree of polymerization between 1510 and 5500, may strengthen its crystallinity. On the other hand, amorphous cellulose is non-organized and takes up a small proportion, and it is easily degraded by enzymatic attack than crystalline cellulose (Pérez et al., 2002; Sampath et al., 2017).

2.1.2. Hemicellulose

Hemicelluloses are heterogenous polymers consisting of pentoses (D-xylose, D-arabinose), hexoses (D-glucose, D-mannose, D-galactose), and sugar acids. They consist of 500-3 000 sugar monomers linked via β-1,4- and occasionally β-1,3-glycosidic bonds (Ching et al., 2015). The structure of hemicellulose is shown in **Figure 2.3**. The composition of hemicellulose depends on the type of biomass. Like cellulose, most hemicelluloses serve as supporting material for cell walls. Hemicellulose that has a degree of polymerization between 50 and 200, is amorphous and easily degraded. Hemicellulose is commonly bound to other cell wall components such as cellulose, cell wall proteins, lignin, and phenolic compounds via covalent and hydrogen bonds as well as ionic and hydrophobic interactions.

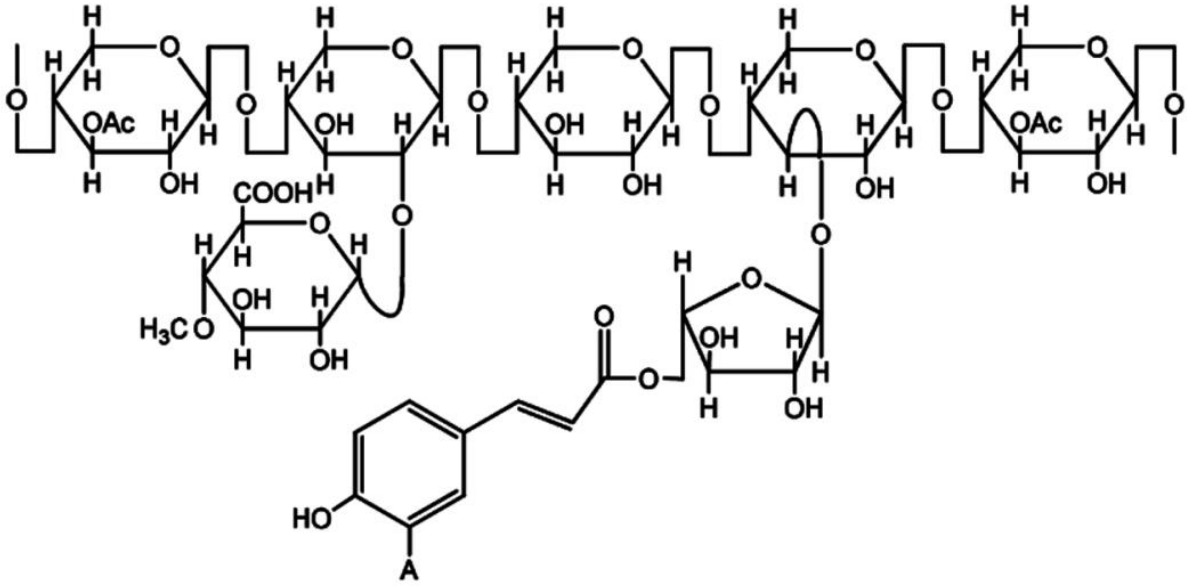

Figure 2.3 Hemicellulose structure (Terzopoulou et al., 2015)

2.1.3. Lignin

Lignin contains aromatic rings located in a long molecular chain. The structure of lignin is shown in **Figure 2.4**. It has a three-dimensional structure in which the phenylpropanoid units are linked together by carbon-carbon and aryl-ether bounds in an irregular arrangement. It results the recalcitrant and insoluble properties of lignocellulose. They also provide structural support, and impermeability and act as a physical barrier that protects against microbial enzyme action (Mussatto and Dragone, 2016). While this makes lignin to be potential biopolymer used in the production of impermeable bioplastic (Vu et al., 2020), whereas in our case, it may cause the big barrier.

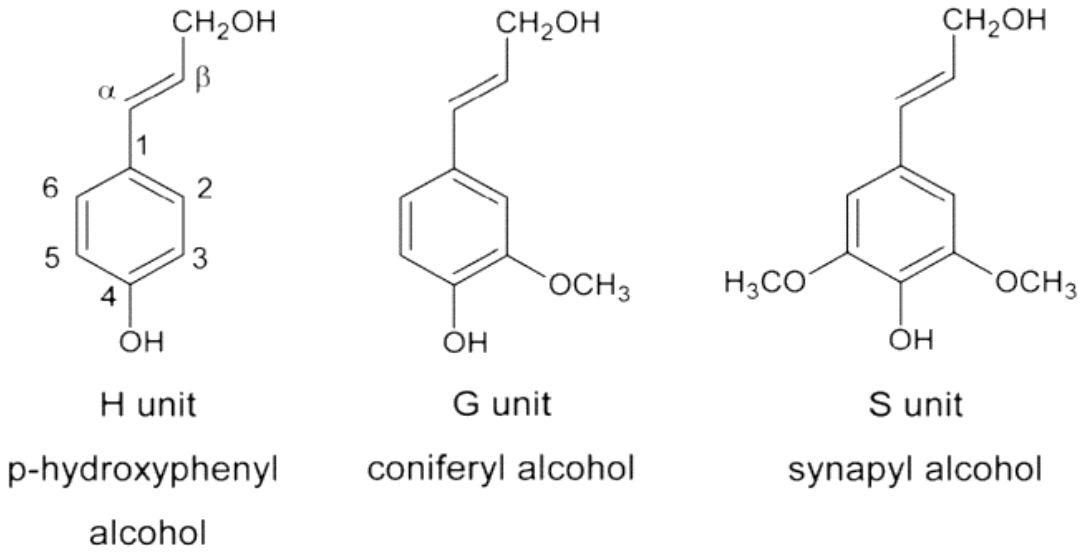

Figure 2.4 Three monomer types in lignin (Duval and Lawoko, 2014)

Three monolignols are named (i) coniferyl alcohol, (ii) coumaryl alcohol, and (iii) sinapyl alcohol, and they can form the phenylpropane units so called p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S), respectively (Cesarino et al., 2012; Lewis and Yamamoto, 1990). Lignin compositions are different not only between species but also between other tissues of individual plant. The coniferyl alcohol structure dominates in softwoods, while in hardwoods the ratio of sinapyl alcohol and coniferyl alcohol shows considerable variation, or the typical structure of p-hydroxyphenyl alcohol is found predominantly in lignin from grasses (Glasser, 1999; Holmgren et al., 2006). Different percentages of chemical groups such as methoxyl, hydroxyl, carbonyl, carboxyl etc. impart polarity to the lignin macromolecule. The dominant chemical groups are hydroxyl groups which are aliphatic or phenolic (Koda et al., 2005; Yang et al., 2016). As the most recalcitrant component in lignocellulosic fibers, lignin is extremely resistant to enzymes and chemical impacts (Tolbert et al., 2012). It does not dissolve in hot water, acids, either other solvents except alkalis (Ching et al., 2015; Feofilova and Mysyakina, 2016; Rahimi et al., 2014).

2.2. Pretreatment of lignocellulosic biomass

Biomass pretreatment aims to break down the recalcitrant structure of lignocellulosic biomass and to provide better enzymatic accessibility to the hemicellulose and cellulose chains which are converted into useful fermentable sugars.

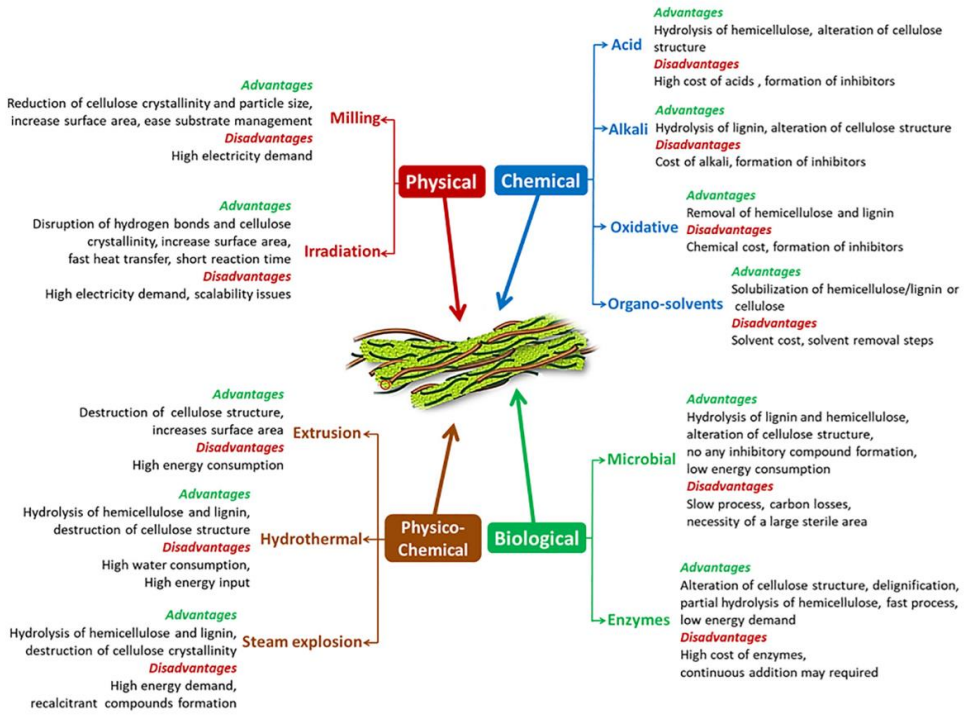

Figure 2.5 Different lignocellulose pretreatment approaches (Abraham et al., 2020)

To obtain the transformation of biomass into sugars, the removal of hemicellulose and lignin, reduction in the crystallinity of cellulose, as well as the increase of porosity and specific

surface area of biomass structure have to be fulfilled (Baruah et al., 2018). The effective pretreatment of lignocellulosic biomass should be focused on the maximization of sugar yield especially the production of pentoses and hexoses, maximization of enzymatic digestibility of the pretreated material preventing loss of sugary compounds; less production of inhibitory compounds, minimize the expenditure of energy cost and impact of environment (Alvira et al., 2010). The efficiency of pretreatment varies in different biomasses and their characteristics. Generally, available lignocellulosic pretreatment technologies are to be classified into various groups including physical, chemical, biological and their combination (**Figure 2.6**).

2.2.1. Physical pretreatment

Physical pretreatment involves using physical action to alter the structure of lignocellulose structure. One of the most popular methods is mechanical comminution such as grinding, milling, and chipping (Harmsen et al., 2010)which increase surface area of biomass by reducing its particle size and crystallinity degree. The size reduction can improve the accessibility of the biomass and increase its susceptibility to microbial and enzyme attacks, thus promoting biomass digestion during pretreatment. The common size of the materials is around 10-30 mm after chipping and 0.2-2 mm after milling or grinding (Sun and Cheng, 2002). The choice of physical methods strongly depends upon the biomass's moisture content (Neshat et al., 2017). In the mechanical approach, the power requirement varies based on the desired particle size and biomass characteristics. Cadoche and López (1989) estimated that the energy consumption of 30 kWh per ton of biomass needed for obtaining the biomass particles size ranged from 3-6 mm. The energy input is much higher than the theoretical energy content available in the biomass in most cases, which may add more expenses to the whole process (Kratky and Jirout, 2011).

The microwave irradiation route is also classified as physical pretreatment. During the process, microwave energy is transferred to the biomass, enabling its rapid heating with a minimal thermal gradient. As the consequence, the deviations in the dipole orientation of polar compounds increase the solubility of lignocellulosic biomass by altering the ultra-structure of cellulose or partially removing hemicellulose and lignin (Gabhane et al., 2011; Kumari and Singh, 2018). The main advantages of this method include high uniformity, short process time and less energy requirement compared to traditional heating. Jackowiak and co-workers (2011) indicated an increase in methane yield of 28% for an irradiated wheat straw with microwave at 115^{o}C compared to the untreated substrate. However, a side effect of this approach is the formation of inhibitory products like phenolics and furfural compounds. Thus, microwave irradiation is not fruitful individually but has been used for providing heat assistance for acid and alkaline pretreatment or for pretreating micro-organisms (inoculum) to suppress their methanogenic activity (Singhal and Singh, 2016).

Extrusion is a favourable method for the physical pretreatment of lignocellulosic biomass with moisture content over 15-20%. It includes mixing, heating and shearing of material, resulting in physical and chemical alteration of biomass. A high mechanical shear rate leads to the disruption of biomass structure during defibrillation and fibre shortening. The advantages of this approach are the lower energy needs and undischarged effluent which reduces the effluent disposal cost and

eliminates solid loss. In the study of Simona and co-workers (2013), they claimed the introduction of extrusion attributed to the enhancement of organic matter digestibility, resulting in the biogas/energy production increase and acceleration of the anaerobic digestion process. Similarly, in the thermal pretreatments, the formation of inhibitors also occurs in extrusion under certain high-pressure conditions.

Freezing is the recent novel approach for physical pretreatment developed to enhance the enzymatic conversion of lignocellulosic biomass. In this approach, biomass is frozen to -20^{o}C to break down its cell structures and create more porosity. Chang and co-workers (2011) found an increase in enzymatic digestibility of rice straw from 48% to 84% and a high yield of glucose of 377.91 g/kg of dry rice straw, following the freeze pretreatment. The freeze method has unique features including a significantly lower environmental impact and less hazardous proceeded chemicals. However, its main drawback is the intensive energy consumption, thus it has very less attention in the industrial scale.

2.2.2. Chemical pretreatment

Chemical pretreatments are classified into acid, alkaline, oxidative, and organo-solvent treatments. Meanwhile the sulfuric, hydrochloric, formic, and nitric acid are mostly used in acid pretreatment, whereas sodium hydroxide and ammonia are commonly used in alkaline pretreatment. The chemical pretreatments are purely initiated by chemical reactions for disruption of the biomass structure. The mode of action depends upon the chemical being used and the operating conditions in the pretreatment process.

Acid pretreatment

In this chemical pretreatment method, acids are used as catalysts which cause the disruption of Van der Walls, hydrogen and covalent bonds in the biomass. This treatment causes biomass disintegration and cell lysis, eliminating the hemicellulose portion from the lignocellulosic biomass. Concentrated acid allows high yields of sugars such as glucose from cellulose at low temperatures. The hydrolysis rate depends on the intrinsic properties of lignocellulose, which is slower for crystalline cellulose than amorphous hemicelluloses. However, using concentrated acids can produce inhibitor compounds which negatively affect the subsequent fermentation of the sugars and the risks of corrosion of the equipment, high consumption of the acid, toxicity to the environment and high energy needed for acid recovery (Jones and Semrau, 1984). The hydrolysis with diluted acid presents the advantages of lower acid consumption, but a higher temperature should be applied to achieve the proper yield of glucose from bio-polymer chains. Pretreated wheat straw with sulfuric acid at high temperatures, before mesophilic digestion, results in a significant increase (16%) in methane production (Taherdanak et al., 2016). The effect of diluted sulfuric acid pretreatment on water hyacinth was studied by Santos and co-workers (2018). An increase in biogas yield of 131% was observed in comparison with the control. Recently, organic acids (including maleic, succinic, oxalic, fumaric and acetic acids) were suggested as alternatives to inorganic ones to avoid machine corrosions and to lower energy demand for acid recovery. In the case of maleic acid, decomposition of glucose from hemicellulose is much lower than in the case

of concentrated acids. Organic acids would, thus, be better for biomass with high cellulose and lower hemicellulose contents such as aquatic plants (Rabemanolontsoa and Saka, 2015).

Alkaline pretreatment

Alkaline pretreatment enables delignification, causes a decrease in porosity, surface area and degree of polymerization of lignocellulosic biomass, and increases accessibility and digestibility of polysaccharides before enzymatic treatment. It is reported that the alkaline hydrolysis mechanism is based on saponification of the uronic ester linkages of 4-O-methyl-D-glucuronic acids attached to the xylan backbone, producing a charged carboxyl group and cleaving the linkages to lignin and other hemicelluloses (Sun and Cheng, 2002). The generally used alkaline reagents are sodium hydroxide (NaOH), potassium hydroxide (KOH), aqueous ammonia (NH_4OH), calcium hydroxide ($Ca(OH)_2$) and oxidative alkali. Chandra and co-workers (2012) found the enhancement of biogas and methane yield of 88 and 112%, respectively from wheat straw using NaOH pretreatment. Li and co-workers (2015) investigated the ammonia pretreatment on wheat straw, with a 40% increase in biogas yield attained. In comparison with other chemical pretreatment technologies, alkaline hydrolysis process conditions are relatively mild, working at lower temperature and pressure causing less sugar degradation than acid pretreatment but the reaction times take several hours or days, or even weeks for softwood (Bali et al., 2015).

Oxidative pretreatment

In the oxidative treatment, agents like hydrogen peroxide (H_2O_2) is used and it destructs the lignin and hemicellulose structures by breakage of aromatic nuclei, electrophilic substitutions, dislocation of side chains, and cleavage of alkyl aryl ether bonds (Paudel and Qin, 2015b). The degradation effect of oxidative pretreatment on agricultural wastes was studied by Almomani and co-workers (2019). They applied oxidation processes including ozone combined with H_2O_2 and Fe(II) on disintegration of three mixed agricultural solid wastes, and a maximum increase of 30% in methane yield and a 25% enhance in digestion process were achieved. In another study, the pretreatment of rice straw by H_2O_2 resulted an 88% increase in methane production (Song et al., 2013). The oxidative pretreatment method can help to improve cellulose retention and enzymatic digestibility and decrease the production of enzyme-inhibitors. The main drawbacks of this method are the high operational cost, and the acid formed in the process can act as inhibitor in the fermentation (Rabemanolontsoa and Saka, 2015).

Organosolv pretreatment

Organosolv pretreatment *(Orgv)* is the method which uses organic solvents such as ethyl alcohol, methyl alcohol, acetone, ethylene glycol and tetrahydrofurfuryl alcohol with and without the addition of a catalyst agent for the delignification of lignocellulosic materials. This method specifies lignocellulosic biomass with high lignin content, because it can break the internal bonds between lignin and hemicelluloses. Tang and co-workers (2017) used organic amine catalytic Orgv of corn stover with n-propylamine and 60% v/v ethanol as a solvent, resulting in about 81.7% delignification and 83.2% total sugar yield.

Pyrolysis pretreatment

Pyrolysis has also been used for the pretreatment of lignocellulosic materials, in which celluloses decompose rapidly to gaseous products and residual char at a temperature greater than 300^{o}C (Kilzer and Broido, 1965; Shafizadeh and Bradbury, 1979). Fan and co-workers (1987) pointed out that pyrolysis-treated biomass resulted in 80-85% conversion of cellulose to reducing sugars with 50% glucose higher in mild acid hydrolysis than untreated biomass. The decomposition effect is much slower and the product formed is less volatile at a lower temperature using this method (Kumar et al., 2009).

Ionic liquid pretreatment

Ionic liquid pretreatment (Ils) is recognized as a promising technology toward environmental sustainability. This route is evolved with the unique ability to dissolve whole biomass rather than individual components of plant cell walls under simultaneous action on reducing biomass cellulose amorphization, deacetylation of hemicellulose and delignification (Fukaya et al., 2008; Kumari and Singh, 2018). Pu and co-workers (2007) found that ILs ionic liquids form hydrogen bonds with cellulose at very high temperatures due to the presence of different anions such as formate, acetate, alkyl phosphonate and chloride, thus enhancing the solubility of ionic liquids in lignin. Among various available Ils, N-methyl morpholine-N-oxide monohydrate (NMMO) was claimed by Kabir and co-workers (2014) as the popular agent for the pretreatment of lignocellulosic biomass during anaerobic digestion. NMMO used by Teghammar and co-workers (2012) released higher methane yield when increasing the time of pretreatment and achieving maximal 400–1200% of methane yield from the IL-pretreated biomass compared to the control.

2.2.3. Physiochemical pretreatment

Physicochemical pretreatment is a combined approach of physical and chemical action to break down the hemicellulose or lignin polymers within lignocellulosic biomass before the aerobic and anaerobic fermentation. The following pretreatments belong to this group: steam explosion, ammonia fiber explosion, CO_2 explosion, ultrasonication, liquid hot water pretreatment and wet oxidation pretreatment. The general mechanism breaks down the hydrogen bonds between the complex polymers by heat, increasing the surface area accessible for the enzyme or microbial attack toward biomass (Rodriguez et al., 2017). The physicochemical pretreatment is performed over a wide temperature range from 50-250^{o}C. The pretreatment time is a critical factor in the process, in which prolonged heat exposure can lead to unexpected reactions of the formation of harmful inhibitors to the anaerobic digestion process (Fernández-Cegrí et al., 2012)

Steam explosion

Steam explosion (uncatalysed or catalysed) is one of the most applied pretreatment processes owing to the low use of chemicals and limited energy consumption. This method makes biomass more operative to enzyme (cellulase) attack due to the disintegration of structural

components of lignocellulose by steam-heating (thermo), shearing (mechano), and autohydrolysis of glycosidic bonds (chemical). The injection of a pressurized stream (160-270^{o}C, 20-50 bar) for short time and quick release to the atmosphere results in evaporation of succinct moisture and assimilation of the lignocellulosic matrix (Mabee et al., 2006). Steam explosion is advantageous because there is no need for chemicals, hence no polluting effect, low energy demand and low recycling cost of the waste steam, but the major downside is low saccharification yields and solubilisation along with inhibitory products (furfural and hydroxyl methyl furfural), losses carbohydrates (Taherzadeh and Karimi, 2008).

Ammonia fiber explosion

This pretreatment is related to the application of liquid ammonia and the stream explosion process. The parameters including water loading, ammonia loading, reaction temperature and residence time are the important factors of the process. This approach reduces the lignin content and removes some hemicellulose while decrystallising cellulose. A typical ammonia fibre explosion process is carried out with 1-2 kg ammonia/kg dry biomass at 90°C during 30 min (Teymouri et al., 2005). It is considered that ammonia fibre explosion may be a cost effect technique for the pretreatment which the possibility of ammonia recovery and no requirement of particle size reduction.

CO_2 explosion

This method is similar to steam and ammonia fibre explosion methods. Instead of using pressurized steam, compressed CO_2 is injected into the batch reactor and then liberated by explosive decompression. On one hand, the high processing cost is the major drawback of CO_2 explosions like ammonia fibre explosions. On the other hand, inhibitor formation is negligible in the process with a high conversion yield makes the CO_2 explosion method more advantageous, hence is preferred over other explosion-type methods (Zheng et al., 1995).

Ultrasonication

Ultrasonication pretreatment associates the addition of acid or base in its physical action, thus being classified in physicochemical pretreatment. The ultrasonic power can disrupt the cell wall by creating and collapsing monolithic cavitation bubble, elevating the alteration in chemical nature by the formation of free radicals (Mason and Peters, 2003). Therefore, the specific surface area of lignocellulose structure increases with the reduction of polymerization degree, increasing the biodegradability of lignocellulosic biomass.

Liquid hot water pretreatment

In liquid hot water pretreatment, high temperature and pressure are employed to keep water in a liquid state and biomass was soaked for 15 min without any chemical or catalyst. This results in an increase in cellulose digestibility and hemicellulose removal (Laser et al., 2002). This method has been found to be efficient for treating various types of lignocellulosic materials such as rice straw, sugar cane bagasse, corncobs, wheat straw, and corn stover (Banerjee et al., 2009; Rogalinski et al., 2008). Controlled pH between 4-7 was required in the pretreatment of corn stover

by liquid hot water method to prevent the sugar degradation and formation of inhibitors and optimized conditions of 190^{o}C for 15 min resulting in maximum hemicellulose solubilization, reported by Laser and co-workers (2002).

2.2.4. Biological pretreatment

Biological pretreatment is an attractive method to break down the recalcitrant structure of lignocellulosic biomass. This approach is safe and environmentally friendly, with low energy requirements and low formation rate of toxic materials. The naturally found wide taxonomic range of microorganisms can alter or degrade lignocellulose extracellularly by secreting hydrolytic enzymes and ligninolytic enzymes which depolymerizes lignin (Pérez et al., 2002). As the consequence, cellulose and hemicellulose are hydrolysed into monomeric sugars using the degrading microorganisms. Biological pretreatment methods are fungal pretreatment, microbial consortium, and enzymatic pretreatment. In this approach, microorganisms and enzymes are used as catalysts to modify lignin and degrade hemicellulosic content in the biomass, thus the cell wall structures open up, allowing subsequent hydrolysis of biopolymers. In many circumstances, the use of microorganisms is far more cost-effective than the use of hydrolytic enzymes. Microorganisms can secrete extracellular enzymes capable of breaking down harsh polymeric structures and convert high molecular weight compounds into lower mass compounds that can enter the fermentation process. There are several physical factors (moisture, incubation time, accessible surface area, etc.), chemical factors (pH, composition of culture media, source of carbon, cellulose crystallinity, enzymes and hydrolysates, etc.), biological factors (microorganisms and their consortia) which affect the rate of biomass degradation. However, unlike enzymatic hydrolysis, the uses of microbial strains required longer periods, stricter operating conditions and the possibility of growth of unwanted species (Sánchez and Cardona, 2008; Sun and Cheng, 2002; Tengerdy and Szakacs, 2003). Biological pretreatment has been demonstrated successfully for many lignocellulosic materials such as wheat and rice straw, corn stovers, and switch grass (Xu et al., 2010). An overview of biological pretreatment and its applications is shown in **Figure 2.6**.

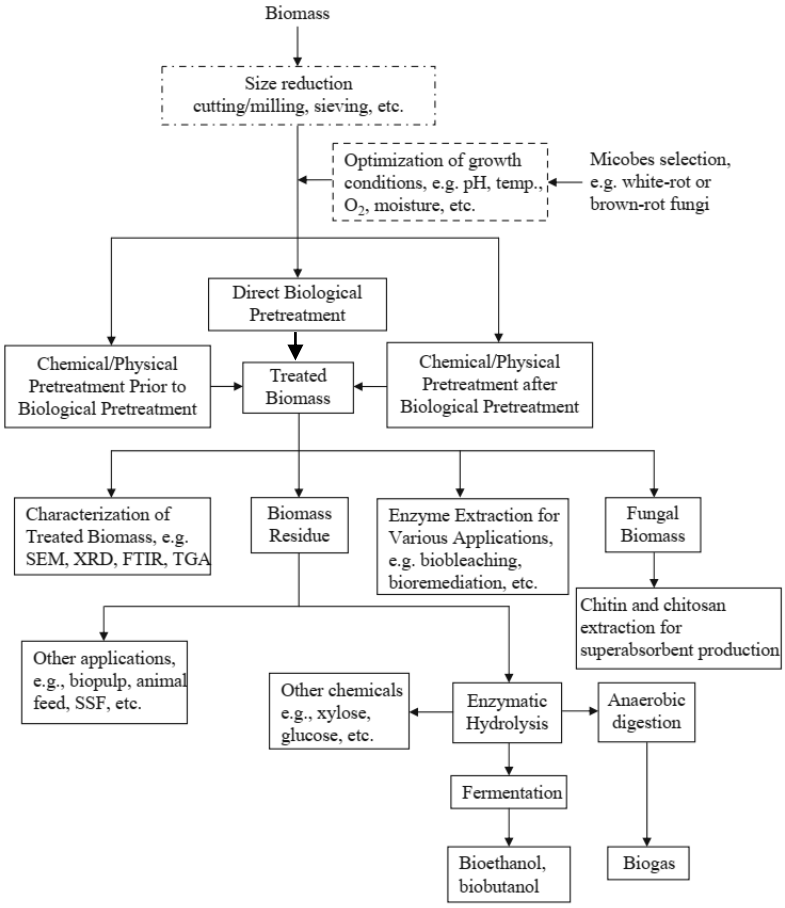

Figure 2.6 Overview of biological pretreatment and its applications
(Narayanaswamy et al., 2013)

Fungal pretreatment

Lignin roles as the main barrier for the plant against microorganism attacks with its high impermeability, resistance and recalcitrance nature. Unlike the degradation process of other lignocellulosic moieties such as proteins, polysaccharides, etc., the degradation of lignin structure involves scission of different inter-monomer structural linkages as well as phenyl-propane units. Therefore, it can be considered that the biological removal of encrusted lignin from the cell wall complex in wood and non-woody lignocelluloses by enzymatic attacks is difficult to accomplish. It was found some specific features of filamentous fungi in the phyla of Basidiomycota (basidiomycetes including white-rot fungi (*Schizophyllum sp.*, *Penicillium chrysosporium*), brown-rot fungi (*Fomitopsis palustris*) and few anaerobic species (*Orpinomyces* sp.) and Ascomycota (*Ascomycetes* (*Aspergillus sp.*, *Penicillium sp.*, *Trichoderma* species) (Liers et al., 2011). Besides, fungi also can degrade insoluble and crystalline cellulose due to their rich cellulase activities (Sánchez, 2009). White rot fungi are the most studied microorganisms for the pretreatment of lignocellulosic biomass. García-Torreiro and co-workers (2016) studied the pretreatment of four agricultural residues (wheat straw, corn stover, barley straw, and corncob) using the white-rot fungus *Irpex lacteus*. They found that all the pretreated substrates showed a relatively high lignin removal rate as well as an increase in glucan and xylan digestibility, in which

corn stover was considered a promising substrate with the highest degradation parameters recorded. Tangnu and co-workers (1981) claimed that *Trichoderma reesei* can produce considerable amounts of xylanases and β-glucosidase with high cellulase activities. Similarly, fungus *Trichoderma longibrachiatum* isolated from soil performed as a promising species in the solubilization of crystalline cellulose because it secrets three types of cellulases: endoglucanases (e.g. carboxymethyl cellulases), exo-glucanases (e.g. cellobiohydrolases), and β-glucosidases (e.g. cellobiases). These different cellulases and substrates have complex interactions that function in a synergistic manner (Beguin and Aubert, 1994; Nidetzky et al., 1996; Pérez et al., 2002; Zhou and Ingram, 2000) during hydrolysis. Not every species of filamentous fungi contains all the enzyme types, and the strategy of degradation can be different even when they have similar enzymatic systems, which implies various approaches to achieving high degradation of lignin (Reid, 1995). Additionally, fungal pretreatment can be performed in a solid or liquid state, but the former is preferred because it not only simulates the natural environment but also allows a higher substrate loading and does not generate liquid waste streams. Many factors may influence the fungal growth and lignin depolymerization such as moisture content, temperature, aeration rate and biomass particle size. An adequate particle size allows the optimal area of exposure without blocking the air flux, which is indispensable because of the highly aerobic nature of the oxidative process.

Bacterial pretreatment

Many bacteria are producing various biomass-degrading enzymes used in biological pretreatment. Bacteria grow faster than most fungi and degrade the lignin into small water-soluble fragments that can be converted into value-added products. Therefore, the selection of efficient bacterial species in the pretreatment is the crucial step for biological pretreatment.

Unlike lignin, cellulose and hemicellulose are comparatively easier to degrade. Several bacteria are well-known to be able to cellulase secretion such as *Cellulomonas fimi*, *Thermomonospora fusca*, *Paenibacillus campinasensis* (Ezeilo et al., 2017; Maki et al., 2010; Sharma et al., 2019). Some works reported gram-positive *Bacillus* as the high degraders of substrates incorporate from agricultural residue, including *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus coagulans* and *Bacillus cereus,* which were found to produce the array of cellulolytic enzymes such as cellobiase rich cellulase, endo-glucanase using agriculture residues as sole substrate (Aulitto et al., 2017; Bano et al., 2013; Dyk et al., 2009; Li Shu-bin, 2012).

Bacterial ligninolytic degradation has received comparatively little attention than fungal lignin degradation. Most fungal ligninolytic enzymes like LiP, MnP and VP enzymes play no roles in bacterial ligninolysis, reflecting the complexity of the fungal proteins and the lack of post-translational modifications in bacteria (de Gonzalo et al., 2016). A limited number of genera, including the *Pseudomonas*, *Rhodococcus,* and *Sphingobium* are capable of aromatic metabolism (Lubbers et al., 2019). Bacterial peroxidases and laccases are found in three groups namely actinomycetes, α-proteobacteria and γ-proteobacteria which were known to have a ligninolytic systems (Bugg et al., 2011). Ahmad and co-workers (2010) claimed that ligninolytic system composed of laccases or other non-peroxidases extracellularly secreted by *Pseudomonas putida* mt-2 and *Rhodococcus jostii* RHA was responsible for the depolymerization of lignin in lignocellulosic biomass.

Lignocellulose degrading enzymes

Natural cellulosic substrates (primarily plant cell wall polysaccharides) are composed of heterogeneous materials including cellulose and hemicellulose embedded in lignin. Thus, different enzymatic activities are found to be involved in the hydrolysis of lignocellulosic biomass. It is observed that microorganisms can produce a multiplicity of enzymes referred to as the enzymatic system which degrade cellulose and hemicellulose (Tomme et al., 1995). They alter or degrade lignocellulose extracellularly by hydrolytic enzymes to hydrolyse cellulose and hemicellulose; and ligninolytic enzyme to depolymerizes lignin. Lignin, as protective barrier of cellulose and hemicellulose from enzymatic hydrolysis, needs to be decomposed or modified in order to gain access to the polysaccharides. Xylan removal and lignin removal enhance cellulase accessibility to cellulose, reduce enzyme inhibition and reduce requirements of accessory enzymes (Sindhu et al., 2016).

- **Cellulolytic enzymes**: The cellulolytic enzyme systems consist of endoglucanase (EG, EC 3.2.1.4), exo-glucanase or cellobiohydrolase (CBH, EC 3.2.1.91), and β-glucosidase which belong to glycosyl hydrolase (GH) family (Henrissat and Davies, 1997). The synergistic action of these hydrolytic enzymes catalyses the cellulose into monomeric sugar units. Exo-glucanases hydrolyse the glycosidic linkages from chain ends of cellulose to release cellobiose to glucose, and β-glucosidase finally cleaves cellobiose to glucose (Himmel et al., 2018). Many bacteria and fungi are capable for production of extracellular cellulose-degrading enzymes that act on cellulose, resulting in the release of glucose and cellobiose. The carbohydrate-binding module of cellulases connects with the catalytic domain through a flexible linker. These modules play an important role in binding the enzyme to the crystalline cellulose and enhancing cellulase activity (Bayer et al., 1998).
- **Hemicellulolytic enzymes**: Hemicellulases can be categorized into glycoside hydrolase (GH) groups which act on the glycosidic bonds and carbohydrate esterase (CE) groups, hydrolyzing the ester bonds of acetate or ferulic acid. Like cellulases, the wide array of interdependent hemicellulases act synergistically during hemicellulose hydrolysis to form several monomeric sugars and also liberate cellulase (Sweeney and Xu, 2012). Enzymes like endo- and exo-xylanases and β-xylosidases are needed to hydrolyse the cross-linked hemicelluloses and to convert xylooligosaccharides to xyloses, respectively.
- **Ligninolytic enzymes**: Some fungi, bacteria and insects are known to secrete enzymes which can degrade lignin. Lignin degrading enzymes include laccase and various peroxidases such as manganese peroxidase (E.C. 1.11.1.7), lignin peroxidase (E.C. 1.11.1.7) and versatile peroxidase. Specifically, peroxidases are heme-containing glycoproteins which require hydrogen peroxide as an oxidant in peroxidation reactions, and degrade different aromatic structures. Laccase (E.C. 1.10.3.2), a copper-containing enzyme, catalyses the oxidation of phenolic units in lignin, phenolic compounds, and aromatic amines to radicals. The potential of laccase to degrade lignocelluloses can be enhanced by using some phenolic compounds such as 3-hydroxyanthranilic acid, 2,2 P-azino-bis (3-ethylthiazoline-6-sulfonate) as redox mediators (Saloheimo et al., 2002).

Factors affecting biological pretreatment

Biological pretreatment is an eco-friendly process which does not generate any inhibitors, but the fermentable sugar loss and time-consuming process are major challenges in biological approach. Optimization the process by selecting the most effective strain and proper cultivate conditions can make the process more efficient (van Kuijk et al., 2015). These are various process parameters affecting biological pretreatment, including physical factors (temperature, moisture, incubation time, substrate size, aeration rate, etc.), chemical factors (such as biomass type, pH, composition of culture media, cellulose crystallinity, inorganic and organic compounds, involved enzymes, etc.) and biological factors (such as species of microorganism, consortia of microorganisms, their interaction, inoculum concentration, etc.).

Incubation temperature: The temperature greatly impacts microbial growth and enzyme activities, and it varies with the different species. White rot ascomycetes fungi grow optimally around 39°C while the white-rot basidiomycetes grow optimally around 25 and 30°C. Bacteria can grow in a wider range from 4 to 60ºC. Generally, microorganisms are classified into four major groups psychrophiles (–15 to 10°C), mesophiles (20–45°C), thermophiles (41–80°C) and hyperthermophiles (65–112°C). Among them, the mesophilic fungi and bacteria are the most common and most studied microbes of which their optimum temperature ranges from 25 to 40°C. The microbial strains can generate heat and develop temperature gradients in the bioprocess which can destroy or inhibit microbial growth and metabolism. On a pilot scale, minimizing heat generation is the most challenge in designing and developing a bioreactor for suspended cultivation. The optimal temperature for biomass pretreatment is strongly dependent on the type of microorganisms and substrates (Isroi et al., 2011).

Incubation time: The recalcitrant structure of lignocellulose is the major limiting factor in biological pretreatment which requires a relatively long incubation time for efficient delignification compared to physical/chemical approaches. The incubation time greatly depends upon biomass composition and the microorganisms involved in the pretreatment process. Corn stalks treated with white rot fungi *Irpex lacteus* obtained the maximum hydrolysis yield of holocellulose after 60-day pretreatment (Zhong et al., 2011). Liong and co-workers (2012) pointed out that the 3 weeks of incubation with *Phanerochaete chrysosporium* was sufficient to degrade the recalcitrant structure of grass and release a sufficient amount of holocellulose. These previous studies also implied that polysaccharide loss increased with the prolongation of the treatment time. Therefore, optimization of the incubation time of biological pretreatment is necessary to achieve a balance between an increase in enzymatic saccharification efficiency and the consumption of polysaccharides during biological pretreatment (Du et al., 2011).

Moisture content: The moisture content plays a significant role in the establishment of microbial growth in the biomass and the enzyme production required for the degradation of lignocellulose which varies with biomass type and microorganism involved in the process (Sharma et al., 2019; Sindhu et al., 2016). It has been reported that optimum moisture content for many bacteria and fungi ranges from 40 to 70% on solid substrates (Raghavarao et al., 2003; Raimbault, 1998). Earlier studies conducted by Reid (1989) revealed that initial moisture content of 70–80% was optimal for producing lignin-degrading enzymes by most white rot fungi. Raimbault (1998) found that the optimum moisture of 40 and 80% were suitable for *Aspergillus niger* on rice and coffee

pulp respectively. Shi and co-workers (2008) reported biological pretreatment of cotton stalks using *Penicillium chrysogenum* where higher moisture content (75–80%) resulted in more lignin degradation than lower moisture content (65%).

Substrate size and aeration: Particle size plays a vital role in the biological pretreatment of lignocellulose (van Kuijk et al., 2015). Mechanical particle shape and size reduction increases the surface area, thus increasing the hydrolytic activity of various enzymes. Additionally, uniform air diffusion could improve the delignification rate by providing oxygenation, CO_2 removal, heat dissipation, humidity maintenance, and distribution of volatile compounds produced during metabolism (Isroi et al., 2011). Large particle size may limit microbial penetration and low air-diffusion, low interaction of water and metabolite intermediates into the particles whereas smaller particles adversely affect the interparticle gas circulation. Hence an optimum size particle has to be used for effective biological pretreatment.

pH: The pH has an important role in the growth and metabolic activities of microorganisms. It will change during microbial incubation (Marra et al., 2015) which may influence the lignocellulosic enzyme production (Sharma et al., 2019; Sindhu et al., 2016). Most white rot fungi grow well at pH range 4.0–5.0 and reduce the substrate's acidity during their growth (Agosin et al., 1985). Both decrease or increase in optimum pH during pretreatment results in reduction of enzyme activities.

Type of microorganisms: Fungal pretreatment using wood rot fungus is one of the effective methods for enzymatic saccharification. Brown rot fungi, *Gloeophyllum trabeum* produces enzymes which can depolymerize cellulose and hemicelluloses in wood and modify lignin in the brown residue (Gao et al., 2012). Pretreatment with fungi could increase enzymatic hydrolysis through lignin degradation. The results indicate that this pretreatment causes a partial defibrating effect on corn stover as well as partial removal of xylan and modification of the structure of lignin, resulting in disrupting the cell wall structure thereby increasing the accessibility of cellulase to lignocellulose structure. Some studies revealed the use of fungal consortium seems to perform better and faster degradability of biomass than a single culture. Asiegbu and co-workers (1996) performed delignification of spruce saw dust using *P. chrysogenum*, *Tinea versicolor* and *Pleurotus sajor-kaju*. When pure cultures were used, the delignification rate obtains a maximum of 5% while the consortium can reach 16% of lignin removal.

Enhancement of biological pretreatment using microbial consortium

Many microorganisms have a great capacity for lignocellulose degradation in biological pretreatment. In this approach, microbial species can hydrolysis enzymes to degrade the biomass in mild conditions. The maximum enzyme activity during the pretreatment is desirable to achieve a high degradation rate. However, it is not always possible to produce all enzymatic components from a single strain. Therefore, the use of microbial consortium is considered an effective and sustainable way of promoting lignocellulose degradation than monoculture approach. Microbial consortium is usually referred to as a group of diverse microorganisms that can act together in a community. Many microbial consortia are naturally found in the human body, in mild to extremely environments (e.g. hot springs, seabeds and subglacial melt), with more effective and efficient growth than mono population (Jorgensen et al., 2012; Paerl and Pinckney, 1996; Warren and

Kauffman, 2003). On the aspect of biodegradation of lignocellulose, the cooperation between different organisms could be suggested. Some bacteria and fungi can represent the key microorganisms in these consortia. They can break the recalcitrant bonding of biopolymers with better functions by balancing two or more tasks in mixed populations (Brenner et al., 2008).

Bacteria co-culture: Several attempts have been carried out in mixed culture of two or more bacteria for efficient enzymatic hydrolysis in pretreatment of lignocellulosic biomass. These strains belonging to *Clostridium*, *Cellulomonas*, *Bacillus*, *Thermomonospora*, *Ruminococcus* and *Streptomyces* which are capable to produce various cellulase enzymes (Sun and Cheng, 2002). Similarly, the microbial community of *Paenibacillus sp.*, *Aneurinibacillus aneurinilyticus*, and *Bacillus sp*. could achieve the higher enzyme levels than by the pure strains Chandra and co-workers (2007). Kato and co-workers (2004) also observed the improved cellulose degradation in mixed culture of *Clostridium straminisolvens* and three strains of aerobic isolates compared to that of the original microflora.

Fungal co-culture: Filamentous fungi are considered the best lignocellulose degraders. The application of two or more species of fungi in the biological pretreatment of lignocellulose has been practice for few decades. None of fungi can produce great amount of hydrolytic enzymes at a same time (Dashtban et al., 2009), thus cultivation of fungal co-culture is expected to obtain better output of bioprocesses. There are multiple evidences of improved cellulolytic and hemicellulolytic activities in fungal co-culture. Kausar and co-workers (2010) evaluated the potential decomposition of individual fungi isolated from rice straw and their co-culture. They found that the fungal co-culture showed more effective degradation of lignocellulose than the monoculture. Wen and co-workers (2005) found that mixed culture of *Trichoderma reesei* and *Aspergillus phoenicis* can secrete high levels of both total cellulase and β-glucosidase production at their optimum temperature 27^{o}C and pH 5.5, whereas single strains show the opposite level of cellulolytic enzymes secretion. Furthermore, a large amount of lignin degradation has also been reported so far by Chi and co-workers (2007) in co-culture of *Ceriporiopsis subvermispora* and *Pleurotus ostreatus*.

Fungal-bacterial co-culture: The microbial co-culture of bacteria and fungi is a potential approach which is inspired by microbial consortium from nature where different microorganisms live together, communicate to each other, and participate in the interconnected network of nutrition web within a microbial community. Co-culture of filamentous fungi and bacteria has been tested for biodegradation of organic pollutants, synthetic dyes, phenol, etc. It was found the superior biodegradation efficiency of selective microbial consortium in comparison to single microbial strains. A study on a dynamic consortium including white rot fungi and indigenous soil microbiota reveals the laccase and manganese peroxidase (MnP) of *Pleurotus* strains is not affected by soil microbiota and also shows high enzymatic activity in nonsterile soil (Lang et al., 1997). Machín-Ramírez and co-workers (2010) suggested that the combination of fungal and bacterial species could synergistically affect benzo[a] pyrene removal.

Utilization of yeast as supplement: Yeasts are far less studied for aromatic metabolism; however, they offer many advantages over bacteria, including resistance to phage infection and tolerance to extreme pH, high osmolarity (Yaguchi et al., 2020) and hydrolytic enzyme production (Ding et al., 2018). Some reports introduced *Candida sp*. as promising candidates for aromatic metabolism,

in which it can use phenol and catechol as sole sources of carbon and energy (Fialová et al., 2004; Gérecová et al., 2015; Krug et al., 1985). A literature review of Middelhoven (1993) gave the growth of fifteen Ascomycetous and thirteen Basidiomycetous yeast species on 84 benzene compounds, and 63 ones supported the growth of one or more yeast species. Besides, Ding and co-workers (2018) found high xylanase activities (5536 U/g substrates) produced by *Pichia stipitis* using corncob and wheat bran mixture under solid state fermentation. The extracellular xylanase was stable at pH 5-8 for 60 min by retaining 57% activity and at 50°C for 80 min by retaining 65% activity. Notwithstanding, the promising characteristic of yeast was addressed via its tolerance to the deficiency of nutrients and harsh conditions such as alkanes. *Yarrowia lipolytica* has been considered a suitable model for studies on yeast dimorphism since it produces pseudohyphae filaments in nitrogen-limited conditions (Coelho et al., 2010). *Y. lipolytica* does not produces ethanol, but it can grow in alkanes and hydrolyse triglycerides and fatty acids used as carbon sources (Desfougères et al., 2010). In addition, *Y. lipolytica* can degrade a variety of organic compounds, including aliphatic and aromatic hydrocarbons (Gonçalves et al., 2014)). The combination of yeast and other strains has been widely applied in the industry of wine production (Fleet, 2003; Renouf et al., 2006; Schallmey et al., 2004; Ward et al., 1995), food industry (Martin et al., 2001), biopolymer (Cheirsilp et al., 2003) and industrial enzyme as tannase (Aguilar et al., 2007). Therefore, it would be great potential to combine yeast with bacteria and/or fungi in microbial pretreatment to enhance the efficacy of the bioprocess.

Microbial co-culture adaptation: In industrial biotechnology, pure cultures are utilized to form the desired products. However, there are few applications of co-cultures in some specific fields such as waste-water treatment, biogas production, biological soil remediation (Rasul Chaudhry and Chapalamadugu, 1991) and the production of traditional foods such as cheese, yoghurt, sauerkraut, sourdough, kefir, salami, whisky, beer. The existence of co-culture based on their symbiosis relationship in Lichens, was observed 600 million years ago (Yuan et al., 2005). Lichens include more than 1500 species consisting of cyanobacteria and yeasts, which are a great example for the symbiotic relationship between different microorganisms (Rikkinen et al., 2002). Therefore, it can be evidence of the great benefit for partners in the symbiosis relationship. However, a major biological challenge in the postgenomic era has been untangling the composition and functions of microbes that inhabit complex communities, thus limiting applications of consortium. Selection of suitable microbes and optimization of biological processes are daunting tasks that do not only remain the survival of the active microbial consortium, but also achieve the success of bioprocesses. Specifically, different microorganisms may compete for the substrates in the same habitat. They tend to protect their substrates and defend their habitat against competitors. According to the study of Taniguchi and co-authors (1998), Acidogenic bacteria produce organic acids that suppress acid-intolerant organisms by reducing medium pH as well as by causing growth inhibition in microorganisms. Some strains of the genus *Lactobacillus* defend their habitat against other Gram-positive bacteria by the secretion of growth-inhibiting substances such as nisin or lactain F (Dalmau et al., 2002).

The selective microbial co-cultures can avoid the competition for substrates between species (Maki et al., 2010). In some cases, the symbiosis of different microorganisms may be caused by synergies of their different enzymatic systems and metabolic pathways (Yara et al., 2006). The benefits of co-culture in wine production include improved texture, taste, flavour and

microbial stabilization were reported by Benkerroum and co-workers (2005); Janssen and co-workers (2006) as well as Schwenninger and Meile (2004). This protection can be explained by the formation of growth-inhibiting substances like lactic acid, acetic acid or ethanol, which are responsible for decrease in pH value. The intensive protection may be achieved by the production of bacteriostatic or bactericidal substances such as nisin (Achemchem et al., 2006; Dalmau et al., 2002; Liu et al., 2006) which poses the capacity of modifying internal conditions such as oxygen availability, pH, substrates and product concentrations during fermentation processes.

The controlled cultivation of co-cultures enables the synergistic utilization of the metabolic pathways of the participating microorganisms. The optimal values various physiochemical parameters like pH, temperature, oxygen demand, substrate of individual microbes as well as product concentrations must be considered during the setting up microbe co-culture to achieve the satisfactory process efficiency (Bader et al., 2010).

2.3. Process of bioethanol production

The conversion of lignocellulosic biomass to bioethanol is a complex process that requires several steps including pretreatment, hydrolysis, fermentation of sugars, distillation and purification. First, lignocellulose is pretreated to alter the biomass's macroscopic and microscopic size and structure, and then the hydrolysis of the carbohydrate fraction to monosaccharides can be achieved faster with higher yield. And finally, these fermentable sugars are converted to ethanol by yeast culture and the distillation process will follow to separate water and alcohol from fermented mash. The entire biomass conversion process is summarized in **Figure 2.5**.

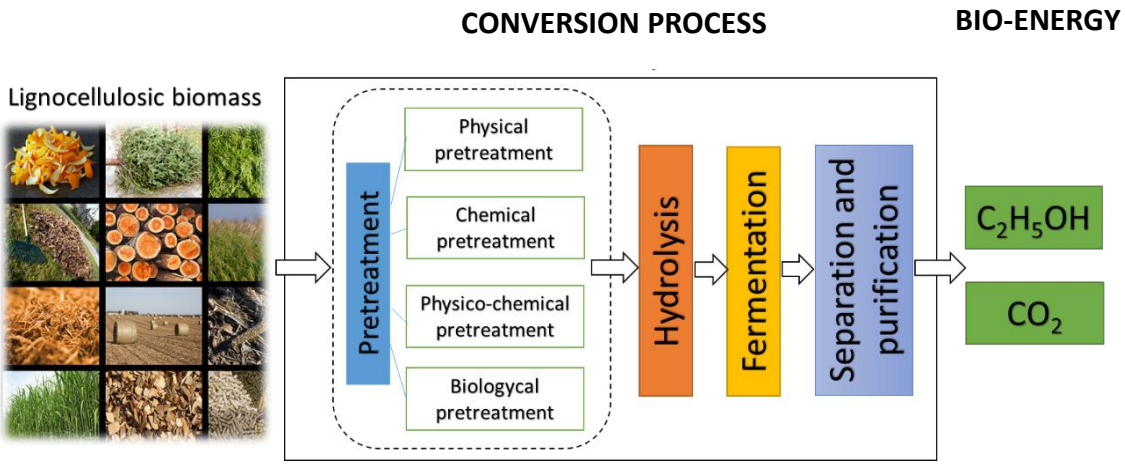

Figure 2.7 Ethanol conversion process from lignocellulosic biomass

Saccharification

After pretreatment of lignocellulose, hydrolysis process can take place to break down the polysaccharide-enriched materials to fermentable sugars. The two popular methods to hydrolyse polysaccharides are acidic (e.g., diluted sulphuric acid) and enzymatic (e.g., lignocellulose degrading enzymes). Enzymatic hydrolysis possesses major advantages over chemical route such

as higher yields, minimal by-product formation, low-energy requirements, mild operating conditions and low-chemical disposal cost (O'Dwyer et al., 2007). On the respect of enzymatic hydrolysis, the commercialized preparation is a mixture of different kinds of enzymes, commonly called cellulases produced my microorganisms. Generally, these enzymes can cleave the glycosidic linkages in carbohydrates. Cellulose is more stable than hemicellulose due to it crystalline structure, and thus it limits the efficiency of depolymerization. In order to enhance the enzymatic hydrolysis process, three following cellulase enzymes including endo-1,4-β-glucanases, exo-1,4-β-D-glucanases and β-D-glucosidase are popularly employed (Chosdu et al., 1993). The activity of cellulase enzyme is influenced by the concentration and source of the enzyme. The efficiency of enzymatic hydrolysis is also affected by conditions such as pH, time, temperature, concentration of substrate and enzyme dose (Hamelinck et al., 2005; Karimi et al., 2006; Zhu et al., 2008). One of advantages of the enzymic process is that doesn't cause corrosion problem in the reactors, resulting in high sugar yields. However, the main disadvantage of enzymatic hydrolysis is the expensive cost of enzymes, limiting its use in mass production of ethanol from biomass. It has been reported that without pretreatment, the sugar yields of enzymatic hydrolysis was 20% lower than theoretical quantity, whereas over 90% of sugar yields obtained with enzymatic saccharification after pretreatment (Ghosh and Ghose, 2003; Kumar et al., 2009).

Alcoholic fermentation

The general chemical pathway for conversion of natural glucose-based carbohydrates to bioethanol can be demonstrated in the **Equation 1**.

$$(C_6H_{10}O_5)_n + nH_2O \rightarrow nC_6H_{12}O_6 \rightarrow 2nC_2H_5OH + 2nCO_2 \qquad \text{(Eq.1)}$$

Saccharomyces cerevisiae is the most popular yeast to ferment sugar solution to ethanol due to its tolerance to high ethanol concentration and materials' inhibitors. In fermentation process, an additional nutrient is essential to provide organic nitrogen source and other compounds for the growth of microorganisms (Vu et al., 2015).

Many studies have been conducted to enhance the conversion of sugars into ethanol. There are two main approaches to fermentation:

- Separate hydrolysis and fermentation (SHF): microorganisms are added to the mixture to ferment the sugars after the hydrolysis finishes.

- Simultaneous saccharification and fermentation (SSF): the method in which carbohydrate biopolymer is broken down into sugary units and these sugars are fermented into ethanol by the microorganisms simultaneously. This method has better performance than SHF with short time, less equipment needed and risk of contamination minimized (Febrianti, 2017). However, there are still some backwards of this process, in which the optimal temperature of enzymatic hydrolysis and fermentation is not matched, 45-50^{o}C vice versus 28-35^{o}C, respectively. Additionally, some intermediate products can resist the growth of microorganisms (Kim and Holtzapple, 2006; Kong et al., 1992).

3. MATERIALS AND METHODS

3.1. Lignocellulose substrate

Wheat (*Triticum aestivum*) bran was purchased from Denes-Natural Kft. (Pecs, Hungary). The label chemical components of wheat bran were about 3.4% (w/v) fat (0.8% (w/v) saturated fatty acids), 56.2% (w/v) carbohydrates (5.0% (w/v) sugar), 16.3% (w/w) protein, 0.03% (w/w) salt. Wheat (*Triticum aestivum*) straw was collected from Jászberény village in Hungary. Wheat straw was dried and then cut into small pieces, then grounded and passed through an 80-mesh sieve. Wheat bran and wheat straw were kept separately in plastic bags in the desiccator for at least 24 hrs until used.

3.2. Microorganisms

Microorganisms (**Table 3.1**) were kindly provided by National Collection of Agricultural and Industrial Microorganisms (NCAIM, Institute of Food Science and Technology, MATE, Hungary).

Table 3.1 List of microorganisms

No.	Classification	Genus	Species	NCAIM number
1	Cellulolytic strains	*Bacillus*	*Bacillus subtilis*	B.01162
2			*Bacillus subtilis*	B.01212
3			*Bacillus licheniformis*	B.01223
4			*Bacillus licheniformis*	B.01231
5			*Bacillus cereus*	B.00076
6			*Bacillus cereus*	B.01718
7			*Bacillus coagulans*	B.01123
8			*Bacillus coagulans*	B.01139
9	Ligninolytic strains	*Rhodococcus*	*Rhodococcus erythropolis*	B.01914
10			*Rhodococcus erythropolis*	B.01952
11			*Rhodococcus opacus*	B.01915
12			*Rhodococcus fascians*	B.01608
13			*Rhodococcus fascians*	B.01614
14			*Rhodococcus sp.*	B.01916
15		*Pseudomonas*	*Pseudomonas putida*	B.01157
16			*Pseudomonas putida*	B.01494
17			*Pseudomonas putida*	B.01522
18	Filamentous fungi	*Aspergillus*	*Aspergillus niger*	F.00632
19		*Penicillium*	*Penicillium chrysogenum*	F.00814
20		*Trichoderma*	*Trichoderma viride*	F.00795
21	Yeast	*Yarrowia*	*Yarrowia lipolytica*	Y.85414
22			*Yarrowia lipolytica*	Y.00613
23			*Yarrowia lipolytica*	Y.00114
24			*Yarrowia divulgata*	Y.02062
25			*Yarrowia divulgata*	Y.05257
26		*Pichia*	*Pichia stipitis*	Y.00810
27			*Pichia stipitis*	Y.00888
28			*Pichia stipitis*	Y.00910
29			*Pichia stipitis*	Y.01047

These species included cellulolytic, ligninolytic, filamentous fungi, and yeast mostly classified into Hazard group 1 which no harmful to human's health, except *B. cereus* and *P. putida* in Hazard group 2 which need to be handled carefully in laboratory.

Cellulolytic and ligninolytic bacteria were refreshed for 24 hrs in nutrient medium (NCAIM 0025) containing 1 g/L yeast extract, 2 g/L meat extract, 5 g/L peptone, 5 g/L sodium chloride; fungi strains *A. niger* F.00632 and *T. viride* F.00795 were grown for 5 days on yeast extract peptone dextrose (YEPD) agar slants containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L agar, while *P. chrysogenum* F.00814 was grown on malt agar slants containing 30g/L malt, 5 g/L peptone and 20g/L agar before being used. Yeast species were refreshed for 24 hrs in yeast extract peptone dextrose (YEPD) agar slants until used.

3.3. Effect of bacteria, yeast and their consortia on the pretreatment of lignocellulose

The monoculture and microbial consortium constructed from the effective strains were cultivated in a basal medium containing wheat bran 2% (w/v). Basal medium was prepared with nutrient components (g/L) such as lactose, 5.0; NH_4NO_3, 5.0; KH_2PO_4, 1.0; NaCl, 1.0; $MgSO_4.7H_20$, 0.6; $CaCl_2$, 0.1; $FeCl_3$, 0.01. The pH was adjusted to pH 6.5 using 1M NaOH solution before autoclaving. After cooling down the flasks, the equivalent inoculum was added to 250 ml flask containing 150 mL of medium to obtain 10^5 CFU/mL. The biological pretreatment was conducted at 30 ± 2^oC for 7 days, 140 rpm agitation speed. Samples were taken at 24 hrs intervals, then centrifuged at 17.968 x g centrifugal force for 10 min at room temperature to remove the cells and supernatant. All samples were kept at -20^oC for further analysis.

3.4. Fungal biological pretreatment

Three fungal strains including *A. niger* F.00632, *P. chrysogenum* F.00814, *T. viride* F.00795 were selected as effective degraders according previous research of our colleagues (Farkas et al., 2019). Mono-strains and fungal consortia were incubated under a suspended pretreatment with at least 3 replicates **(Table 3.2)**.

Table 3.2 Description of the fungal consortia

No.	Synthetic community	Fungal species		
1	FA	*A. niger* F.00632		
2	FB		*P. chrysogenum* F.00814	
3	FC			*T. viride* F.00795
4	FAB	*A. niger* F.00632	*P. chrysogenum* F.00814	
5	FAC	*A. niger* F.00632		*T. viride* F.00795
6	FBC		*P. chrysogenum* F.00814	*T. viride* F.00795
7	FABC	*A. niger* F.00632	*P. chrysogenum* F.00814	*T. viride* F.00795

Five-day old fungal strains were transferred into sterilized glass tubes containing 5 ml Triton-X solution and dispersed with glass beads to separate fungal cells from the agar slants completely. Bucker chamber with Olympus Plan 40x/0.65 Ph2 objective was used to determine the number of fungal conidia. 10g of wheat bran was added to 250 ml Erlenmeyer flasks with liquid to solid ratio of 9:1. The pH value of the medium solution was adjusted to pH 6.5 by 1M NaOH solution. The flasks were sterilized at 121^{o}C for 30 min and cooled down at room temperature before cultivating microbes. Monoculture or mixed-cultures were added to flasks, then incubated at 28-30^{o}C and 140 rpm agitation speed for 7 days. The solid samples were periodically taken at every 24 hours, and they were mixed with 0.1M acetate buffer solution pH 4.5, centrifuged and filter before analysis.

3.5. Optimization of microbial pretreatment

3.5.1. Effect of culture medium and pH

The effect of culture medium and pH was evaluated using complex microbial consortium including filamentous fungi and ligninolytic bacteria. Culture media including 0,15M citrate buffer solution supplemented with mineral compounds (g/L) such as $NaNO_3$, 2.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 1.025; KCl, 0.5 and basal medium were studied. The pHs were adjusted to pH 4.5 and 6.5 with 1M NaOH or 1M HCl solution. The suspended pretreatment with liquid:solid ratio 9:1 and the initial inoculum ratio of fungi and bacteria 1:1 was applied.

3.5.2. Effect of liquid:solid ratio

Various liquid:solid ratios were tested to study the effects of moisture content on the pretreatment of lignocellulose using complex microbial consortium.

3.5.3. Effect of cultivation method

Co-culture of filamentous fungi *A. niger* F.00632 (FA) and lignocellulolytic bacterial co-culture *B. subtilis* B.01162 (A) and *P. putida* B.01522 (K*) were cultivated under suspended pretreatment (liquid:solid ratio of 9:1) or submerged pretreatment. Different cultivation routes including cultivation of fungi or bacteria co-culture 24 hrs were also investigated.

Table 3.3 Experimental design for evaluation the effect of cultivation method

			Inoculation time	
		Denoted	**0 hr**	**24 hrs**
Suspended	**I**	I-A	FA	K*-A
	II	II-A	K*-A	FA
	III	III-A	FA-K*-A	
Submerged	**I**	I-B	FA	K*-A
	II	II-B	K*-A	FA
	III	III-B	FA-K*-A	

The experimental design was summarized in **Table 3.3.** 90 ml of basal medium was added in 250 ml Erlenmeyer flask containing 10 g of dry wheat bran in suspended pretreatment, while in submerged condition, microbes were added in basal medium containing 2% (w/v) wheat bran.

3.6. Construction of complex microbial consortia

In order to select the consortium members, the bottom-up strategies are usually used. The microbial species were firstly screened and evaluated individually, then incorporated into community. Various microbial communities were constructed by combination of different strains of bacteria, fungi and yeast. The bacterial and yeast strains were freshly incubated for 24 hrs in a suitable culture medium, while the 5-day-old fungal spores were separated from agar slants by mixing with Triton X solution. Two-member, three-member and complex microbial consortia were constructed by adding 10^5 cells/gds of each strain into testing flasks at the ratios of 1:1, 1:1:1 and equal ratios of each member in communities, respectively. The microbial pretreatment was carried out similarly as procedure used in the case of individual species.

3.7. Effect of quality of lignocellulosic biomasses

Lignocellulosic substrates composed of wheat bran and wheat straw at different ratios were evaluated to study the effect of quality of substrates on the pretreatment efficiency. The distribution percentage of wheat bran and wheat straw was described in **Table 3.4**.

Table 3.4 Preparation various mixtures of lignocellulosic biomasses

Substrate	Wheat bran	Wheat straw
Percentage (%)	100	0
	75	25
	50	50
	25	75
	0	100

The substrates were pretreated by microbial consortia constructed artificially.

3.8. Saccharification and fermentation of pretreated biomass: cases study

The effects of substrate loading and enzyme dosage during the saccharification of pretreated substrates were evaluated in preliminary trial tests. Biologically pretreated wheat bran and soluble carbohydrates in the extract were enzymatically hydrolysed in 250 mL Erlenmeyer flasks containing 120 mL slurry with suitable substrate loadings at 50^{o}C and pH 5.0 for 4 hrs in a shaker with an agitation speed of 140 rpm. A commercial cellulase enzyme (Celluclast 1.5L) extracted from *Trichoderma reesei* was used in efficient enzyme dosages. The hydrolysis was

terminated by autoclaving the hydrolysates at 121^{o}C for 15 min. Then, pH of the mash was adjusted to pH 4.0 by sterilized 1M NaOH solution. The mash was supplemented with 5 mL nutrient solution containing 30 g/L glucose, 23 g/L $(NH_4)_2SO_4$, 11 g/L KH_2PO_4, 2.6 g/L $MgSO_4$, 34 mL/L trace metal solution and 5 g/L yeast extract. Commercial yeast preparation *Saccharomyces cerevisiae* Danstill A was firstly activated in warm distilled water containing 2% glucose at 32^{o}C for 20 min and then cultivated in YEPD medium. One day grown-yeast inoculum with cell concentration of 10^8 cell/gds was added to the substrate. The fermentation was carried out in static incubation and anaerobic conditions throughout the cultivation period of 7 days at 30^{o}C.

3.9. Analytical methods

3.9.1. Determination of degradation rate

After 7 day pretreatment, the remaining residue was quantitatively determined by gravimetric analysis (Qiu and Chen, 2012). Briefly, after biological treatments, the solid compound was suspended in water to remove adherent microorganism cells, then filtrates using Whatman filter paper (MN 612, Macherey-Nagel, Germany) and dried at 105°C for 24 hrs. The sample was then stored in a desiccator for 48 hrs to reach saturated moisture and weighed. Total weight loss was calculated based on the difference between initial weight of initial stage and of end of pretreatment.

3.9.2. Determination of reducing sugar

Reducing sugar concentration was determined using Somogyi-Nelson method (Dénes et al., 2013) by using copper reagents and arsenol molybdate. The working principle is the amount of deposro oxide deposits that react with arsenomolibdate which is reduced to molybdine blue. Finally, the absorbance of blue colour is measured. The amount of reducing sugar was determined by measuring the intensity of light detected using a spectrophotometer (Helios Gamma, Unicam, UK) at 540 nm. Glucose solutions with a series of known concentrations were prepared and used to create a calibration curve. Prior to measurement, the sample solution was diluted appropriately.

The reducing sugar accumulation ratio was defined as the difference between reducing sugar concentration produced from test sample over the reducing sugar concentration from the control sample. Reducing sugar accumulation ratio was calculated according to the **Equation 2**.

$$Reducing\ sugar\ accumulation = \frac{X\ mg\ sugar\ gds^{-1} of\ test\ sample}{Y\ mg\ sugar\ gds^{-1} of\ control\ sample} \quad \text{(Eq.2)}$$

3.9.3. Enzymatic activity assays

The crude enzyme solution was harvested in periods from 1 to 7 days, by centrifugation at 14000 rpm for 10 min at room temperature. The supernatants were used to assay enzyme activities including total cellulase, endo-glucanase, xylanase and β-glucosidase activities. Filter paper, carboxymethylcellulose (CMC) and birch xylan substrate were used to determine total cellulase,

endoglucanase and xylanase activities, respectively. Total cellulase activity was assayed by reacting 0.5 mL of crude enzyme solution with a 50 mg filter paper strip (1×6 cm equivalent size), using 1 mL of 0.05 M sodium citrate buffer, pH 7.0 and incubated at 50ºC for 1 hr (Yu et al., 2016). Endo-glucanase activity was measured at pH 7.0 at 50°C for 15 min of reaction, containing 1% (w/w) CMC in a 0.05 M citrate buffer solution. Total cellulase activity and endo-glucanase activity (IU/mL) were calculated according to the **Equation 3** (Shareef et al., 2015).

$$\text{FPase (or CMCase)activity } \left(\frac{\text{U}}{\text{ml}}\right) = \frac{\text{Abs}\times\text{d}\times V_r}{V_s\times\text{t}\times M_G\times\text{s}} \quad \text{(Eq.3)}$$

where Abs is the absorbance, d is the dilution factor, V_r is the volume of the reaction medium, V_s is the volume of the sample, t is the incubation time; s is the slope of the glucose calibration curve, M_G is the molecular mass of glucose.

In the case of xylanase, the reaction mixture contains the enzyme preparation with 1 ml of xylan substrate 1% (by weight) and 1 ml of buffer (50 mM citrate at pH 7.0), the reaction was done at 50ºC in a water bath for 10 minutes. After the incubation, the tubes were placed in boiling water for 15 min to stop the reaction. The mixture was then allowed to cool to room temperature before determination of reducing sugar using Somogyi-Nelson method (Farkas et al., 2019). The activity of xylanase was measured using the **Equation 4**.

$$\text{Xylanase activity (IU/mL)} = \frac{\text{Abs}\times\text{d}\times V_r}{V_s\times\text{t}\times M_X\times\text{s}} \quad \text{(Eq.4)}$$

where Abs is the absorbance, d is the dilution factor, V_r is the volume of the reaction medium, V_s is the volume of the sample, t is the incubation time; s: slope of the glucose calibration curve, M_X is the molecular mass of xylose.

Enzyme activity has been expressed in International Units (IU), as the amount of enzyme which released 1 µmol of corresponding sugar per minute under room temperature.

β-Glucosidase activity was assayed by determination of p-nitrophenol released from p-nitrophenol-β-D-glucopyranoside (PNPG) substrate (Shareef et al., 2015). 1 mL of PNPG (10 mM solution in 50 mM citrate buffer, pH 4.8) was added to 1 mL of the supernatant, then the mixture was incubated at 50 °C in a water bath for 10 min. The reaction was stopped by adding 1 mL of 1 M Na_2CO_3, followed by centrifugation to remove insoluble components. The absorbance of the released p-nitrophenol was measured spectrophotometrically at 410 nm. One unit of β-D-glucosidase activity was defined as the amount of 1 µmol of p-nitrophenol released per minute under the test conditions.

3.9.4. Determination of total phenolic content

The total phenolic content was measured according Folin-Ciocalteau method (Alvira et al., 2010). Briefly, 20 µL of sample and the serial standard solution of gallic acid were diluted with 158 mL of water, then 100 µL Folin-Ciocalteau reagent was added. The tubes were vortexed well and kept at room temperature for 8 min in dark conditions. Then 300 µL of 7.5% (w/w) sodium carbonate solution was pipetted into each tube to stop the reaction. After mixing well, samples

were incubated in the dark at room temperature for 2 hrs. Spectrophotometer was used to read the optical absorbance at 765 nm wavelength.

3.9.5. Determination of amino acid content

Amino acids containing phenolic or indolic groups like phenylalanine, tyrosine and tryptophan was detected by xanthoproteic test (Nigam and Omkar, 2003). Distilled water, 1% (v/v) tyrosine, 1% tryptophan, 1% phenylalanine and 5% egg white (albumin) solution were used as the control samples. 1 mL of sample solution was mixed well with 1mL concentrated nitric acid in test tubes before putting in boiling water for 30-60 seconds. Then the mixture was cooled down with tap water. Two mL of 40% (w/w) NaOH solution was added to initiate the reaction. The appearance of a yellow precipitate of xanthoproteic acid in the forms of salt of the tautomeric form of the nitro compound indicates the presence of aromatic groups in the proteins and amino acids.

3.9.6. HPLC analysis

Monosugars and ethanol content were analysed by high performance liquid chromatography (HPLC, Thermo Fisher Scientific Corporation, USA) equipped with RI detector. The analytical column was Aminex-87H from Bio-Rad (USA) and incubated at 45^{o}C. The mobile phase was 0.005M H_2SO_4 solution. The flow rate was 0.6 mL/min. Standard solutions of glucose, xylose for monomers; maltose, cellobiose for DP2 as well as ethanol were prepared in twice distilled water (ddH_2O) at a concentration of 500 mg/mL and 10 (v/v) %, respectively. All samples were centrifuged at 14000 rpm and were injected by automatic injector system. Both internal and external standards were injected to calculate the content of sugars and ethanol.

3.9.7. Determination of bioconversion rate

Saccharomyces can convert maltose and glucose into ethanol and release CO_2 in anaerobical fermentation. The fermented sugar concentration was worked out from the initial sugar concentration in the hydrolysate and the residual sugar concentration in the fermented broth. The other parameters related to ethanol fermentation were calculated based on the **Equation 4**.

$$Bioconversion\ rate\ (\%) = \frac{etOH}{Theoritical\ etOH} \times 100 \qquad \text{(Eq. 4)}$$

where etOH (ml/100ml) is amount of ethanol in the fermented mash and theoretical etOH is equivalent to 51% of fermented sugar concentration (maltose and glucose concentration in hydrolysate).

3.10. Statistical analysis

All experiments were run in triplicates. The data were processed in Microsoft Excel spreadsheet an expressed as the mean ± SE of different independent replicates. One-way analysis of variables (One-way ANOVA) followed by TUKEY post hoc multiple comparison tests was conducted using SPSS software (version 20.0) to test the differences between the variances. Data

were considered significant at $p < 0.05$ and reported as the mean ± SD (standard deviation). Mean values with different letters above the bars differ according to Tukey's test at $p < 0.05$.

The strength of a linear association between reducing sugar and weight loss was interpreted based on the covariance method, called Pearson's Correlation analysis.

Multivariate methods as cluster analysis with Euclidean shortest distance were applied to describe diversity patterns of hydrolysis capacity between strains and consortia.

The Principal Component Analysis (PCA) method was used for multi-variables. The correlation matrix between variables is calculated to transform orthogonal, creating new axes (eigenvectors) installed as the original variables' linear combination. The percentage variations of two principal components in investigated variable were obtained in the PCA diagram using SPSS 20.0. The contribution rates of each variable to PC1, PC2 and their interrelations were also performed.

4. RESULT AND DISCUSSION

4.1. Bacterial pretreatment of wheat bran

4.1.1. Cellulolytic Bacilli

Degradation efficiency by Bacillus monoculture

Dry weight loss and reducing sugar concentration could be used to estimate the bioconversion efficiency of microorganisms. According to **Figure 4.1**, the control has a weight loss of approximately 30% due to the autohydrolysis process during the sterilization using autoclaving. For *Bacillus* pretreatment samples, the weight losses of substrates are relatively high ranging from 54% to 60% except for samples from *B. licheniformis* B.01223 and B.01231 strains. The mass loss data indicated the advantages of biological pretreatment using *Bacillus* species for wheat bran as substrate, which gained higher efficiency than physical pretreatment or utilization of commercial enzymes according to Hell and co-workers (2015). Additionally, Zhang and co-workers (2016) claimed around 22% of biomass was lost after 6 days under submerged cultivation of *Bacillus* strain in fresh medium with 1% (w/v) rice straw as substrate.

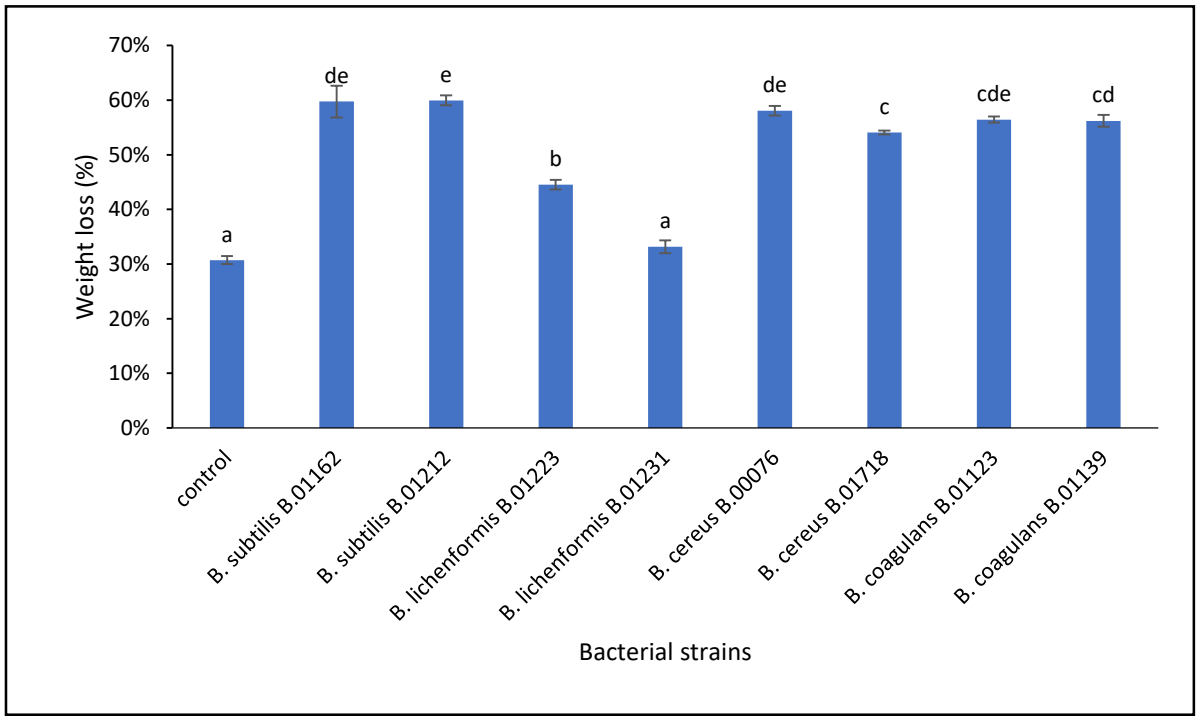

Figure 4.1 Dried weight loss of wheat bran after 7-day of cultivation of *Bacillus* strains

The weight losses seemed to be correlated with the released reducing sugars produced after the biological pretreatment (except with *B. subtilis* B.01212 strain) (**Figure 4.2**). The released reducing sugars were well-associated with the enzymatic activities of the microorganisms, which catalysed the glycosidic linkages of celluloses or hemicellulose to produce glucose, xylose or oligosaccharides with reducing ends and thus, increasing in concentration of soluble sugars.

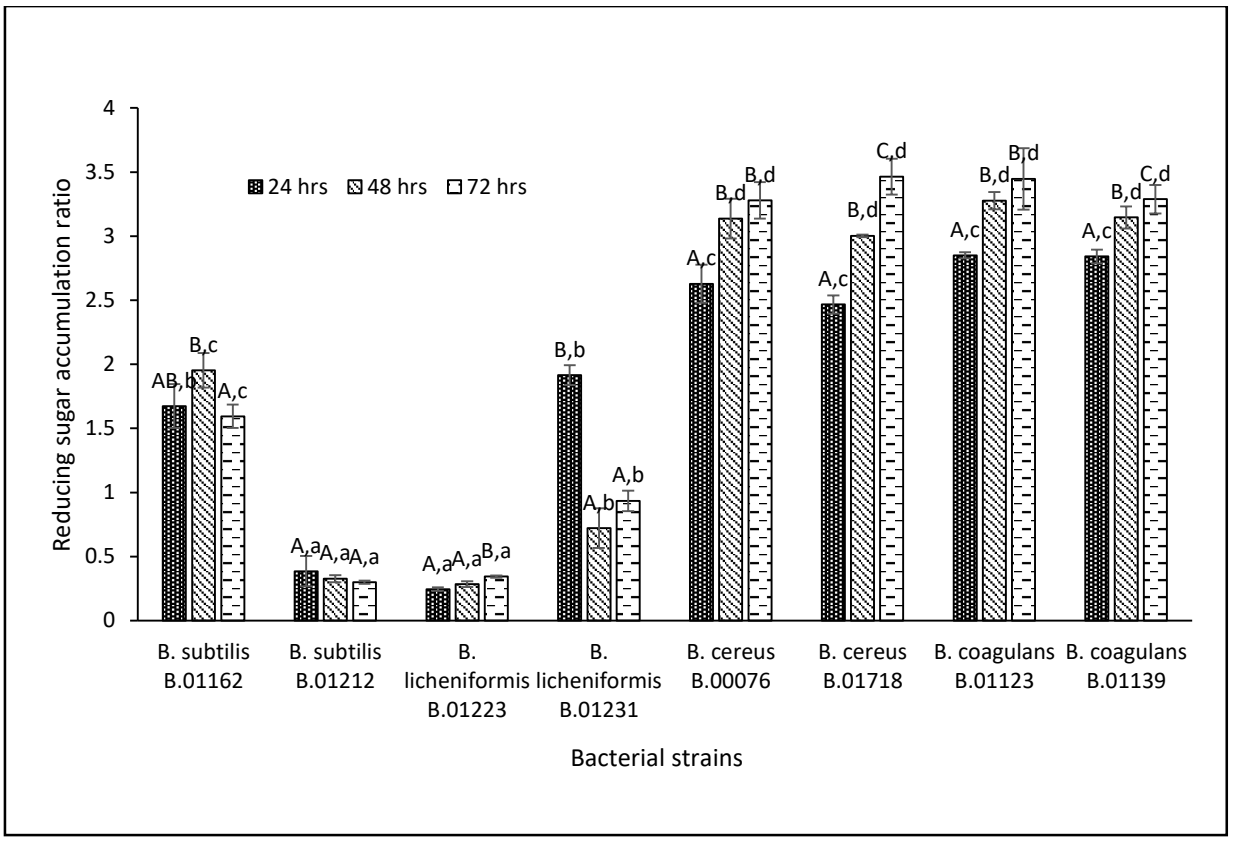

Figure 4.2 Reducing sugar accumulation ratio of *Bacillus* strains after 24, 48 and 72 hours of cultivation. Capital letters (A, B, C) indicate the difference by treatment time and lower-case letters (a, b, c, d, e) demonstrate difference by strains

Among investigated species, *B. cereus* and *B. coagulans* produced significantly higher amounts of reducing sugars than other strains ($p<0.05$). On the contrary, *B. subtilis* B.01212 and *B. licheniformis* B.01223 showed the lowest accumulation ratio of reducing sugar (0.385 and 0.246 at 24 hrs, respectively), indicating the lower efficiency of hydrolysis. In all samples, the reducing sugar accumulation ratio increased with time of incubation within the first 72 hrs, then significantly dropped on the day after. It might be explained by the negative effect on microbial metabolism caused by concentrated sugar in the culture medium (Reischke et al., 2014). Thus, the longer treatment leads to the loss in biomass from bacterial metabolism, which was also reported in previous works (Guo et al., 2018; Wan and Li, 2010).

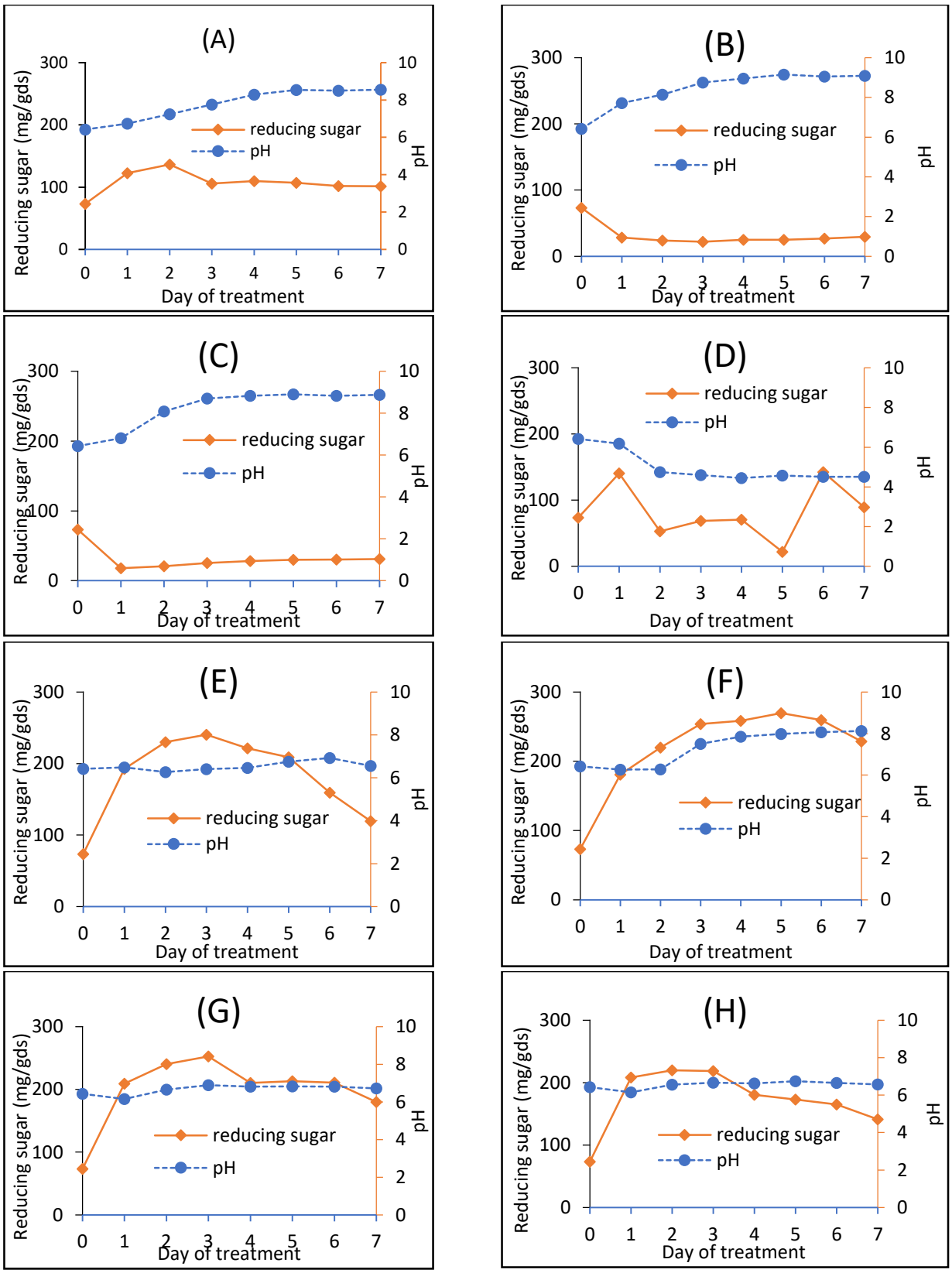

Figure 4.3 Correlation of pH and reducing sugar yield produced in pretreatment by individual *Bacillus* strains *B. subtilis* B.01162 (A), *B. subtilis* B.01212 (B), *B. licheniformis* B.01223 (C), *B. licheniformis* B.01231 (D), *B. cereus* B.00076 (E), *B. cereus* B.01718 (F), *B. coagulans* B.01123 (G), *B. coagulans* B.01139 (H)

In fermentation using microorganisms, the pH value is also a common indicator for their growth. **Figure 4.3** illustrates the correlation of pH value with the reducing sugars during single strain cultivation. It was found that pH of samples *B. licheniformis* B.01231, *B. cereus* B.00076 and *B. coagulans* B.01123 and B.01139 strains tended to constantly stay at a value of pH 7.0 or decreased due to the presence of organic acid during microbial metabolism. By contrast, pH value of the liquid extract from *B. subtilis* B.01162, B.01212, *B. licheniformis* B.01223, and *B. cereus* B.01718 strains increased by the cultivation time. In a previous study, Haruta and co-workers (2002) utilized microbial consortium to hydrolyse rice straw and observed the pH values of pH 6.0 within 4 days, before increasing to pH 8.0 – 9.0. Additionally, the pH profile during cultivations of *Bacillus* sp. SMIA-2 strain on sugarcane bagasse, carried by Ladeira and co-workers (2015), who revealed the increase of pH value at the end of the fermentation process to 8.5 from the initial value of pH 7.1.

Production of cellulolytic enzymes

To extensively elaborate the mechanism of cellulose degradation, the enzyme activities were assayed at 72 hrs of harvesting and the results were illustrated in **Figure 4.4**. Firstly, the total cellulase activities ranged from 0.038 IU/mL to 0.336 IU/mL. Among these strains, strains of *B. cereus* and *B. coagulans* achieved the highest cellulase with no significant difference, reaching above 0.300 IU/mL. *B. subtilis* B.01212 and *B. licheniformis* B.01223 showed low total cellulase activities of 0.038 U/ mL and 0.045 IU/mL, respectively. Other species (0.115 IU/mL by *B. licheniformis* B.01231 and 0.242 IU/mL by *B. subtilis* B.01162) posed moderate total cellulase activity in quantification. Similarly, high endoglucanase enzyme activities were also observed in strains that distinguished as the best total cellulase producers, including *B. cereus* B.00076, *B. cereus* B.01718, *B. coagulans* B.01123, *B. coagulans* B.01139 and *B. subtilis* B.01162 strains, with activities of 1.327 IU/mL, 1.303 IU/mL, 1.374 IU/mL, 1.325 IU/mL and 1.241 IU/mL, respectively. Studying the cellulose degradation activities of 11 strains isolated from water and soil samples of hot springs in India showed that strains of *Bacillus* genus presented similar enzymatic profiles of total cellulase activities and endo-glucanase activities (Gientka et al., 2017; Singh et al., 2019). They reported that the maximum cellulase activities and endo-glucanase were found after 72 hrs by the cultivations of *B. subtilis* BHUJPV-H12, *B. subtilis* BHUJPVH19, *B. subtilis* BHUJPV-H23 and *B. stratosphericus* BHUJPV-H5 strains. Ladeira and co-workers (2015) reported that *Bacillus sp*. was able to produce 0.290 IU/mL endo-glucanase activity at 50 ^{o}C incubation. In the present study, other strains produced enzymes at a much lower activity rate with 0.169 IU/mL by *B. licheniformis* B.01223 and 0.174 IU/mL by *B. licheniformis* B.01212 at 30 ^{o}C of incubation. The maximum activity of β-glucosidase was obtained by *B. cereus* B.01718, valued 0.129 IU/mL. *B. coagulans* B.01123, *B. coagulans* B.01139 strains showed the β-glucosidase activities of 0.118 IU/mL, while a comparable result of 0.108 IU/mL was obtained by *B. cereus* B.00076 and *B. subtilis* B.01162 strains. Although possessing the low total cellulase and endoglucanase but the high content of β-glucosidase produced by *B. subtilis* B.01212 strain in shake flask fermentation may attribute to the degradation of wheat bran.

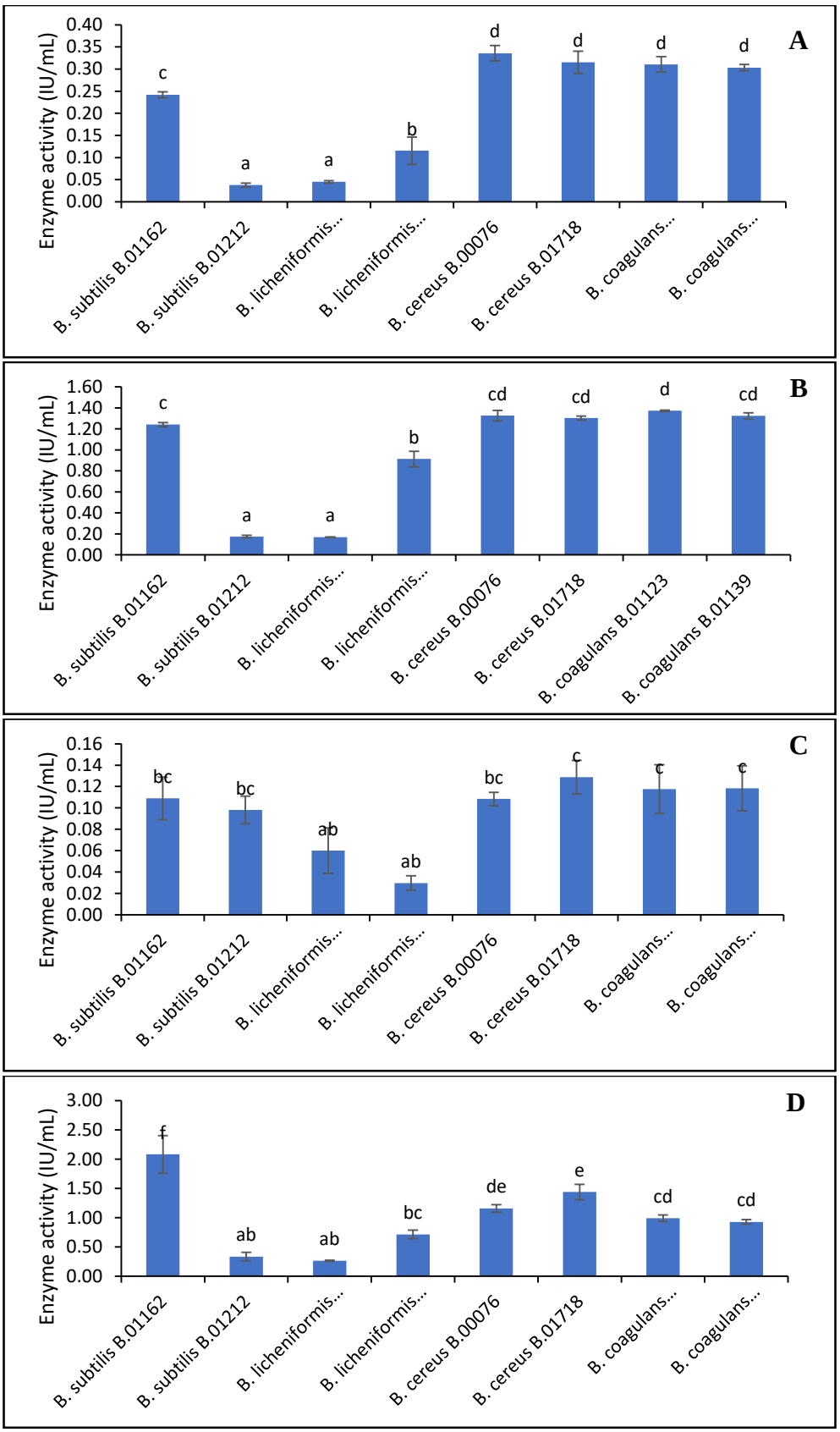

Figure 4.4 Total cellulase activity (A), endo glucanase activity (B), β-glucosidase activity (C) and xylanase activity (D) of *Bacillus* strains at 72 hrs of enzyme harvest

Agrawal and co-workers (2014) also claimed that the overall rate of bioreaction could be enhanced by high β-glucosidase activities which contributed to reducing of end-product inhibition. Hemicellulose removal by bacteria is one of the most promising approaches to enhance the digestibility of lignocellulolytic biomass. As one of the main heteropolymers of the hemicellulose, xylan with its hydrogen and covalent bonds forms a sheathing layer to cellulose, accounts for the structural integrity of lignocellulose. Therefore, the cleavage of xylan by xylanase enzymes can significantly increase cellulose accessibility (Hu et al., 2013; Moreira and Filho, 2016; Sindhu et al., 2016). Previous studies found the high xylanase activity using wheat bran as sole substrate by shake flask or suspended cultivation of *B. coagulans* (Choudhury et al., 2006; Heck et al., 2005). Roy and Habib (2009) discovered neutral xylanase secretion of *Bacillus cereus* using oat spelt xylan, birchwood xylan and beechwood xylan as sole substrate, and the maximum activities were reported at 40 ^{o}C and pH 6.0. In our study, *B. subtilis* B.01162, *B. cereus* B.00076, *B. cereus* B.01718, *B. coagulans* B.01123, *B. coagulans* B.01139 strains showed considerable xylanase activities in the cell-free culture supernatant. The maximum value was reached by the cultivation of *B. subtilis* B.01162 strain (2.081 IU/mL) for 72 hrs of shaking at 140 ppm. The other strains, *B. subtilis* B.01212, *B. licheniformis* B.01223 and *B. licheniformis* B.01231, produced lower xylanase activities and cellulase.

Classification of cellulolytic Bacilli

Cluster analysis technique using Cluster with Ward's method was used to group the strains of *Bacillus* aerobic bacteria based on the enzyme activity (total cellulase enzymes, endo-glucanase, β-glucosidase, xylanase enzymes) and the reducing sugar content. The total of 24 variances was classified into 3 groups A, B and C. Cluster A (includes *Bacillus cereus* and *Bacillus coagulans* species) showed the highest enzyme production. These strains also performed the promising lignocellulolytic biomass degradation capacity based on the high produced reducing sugar. *Bacillus subtilis* B.01162 strain was grouped in the cluster B, presenting the moderate capacity for hydrolytic enzyme production. The strains of *B. licheniformis* and *B. subtilis* B.0112 were clustered together to the cluster C, which gave insufficient effect on the hydrolysis process of wheat bran. These results were in line with previous works, which reported that *B. coagulans* and *B. cereus* could secrete a high level of cellulolytic activities towards lignocellulosic materials (Aulitto et al., 2017; Kovács et al., 2010). For the remaining cluster with *Bacillus licheniformis*, the decreasing cellulase activity could be explained by the presence of proteases. It was previously found that protease in mixed commercial feed enzyme additives may attack other enzymes such as cellulase and amylase, and decrease their efficiency (Wang and Hsu, 2006). Haab and co-workers (1990) explained that protease tightly binds to cellulase proteins, thus modifying homogeneously cellulase proteolytically. The low concentration of protease accounted for the extremely rapid loss of cellulase and xylanase activity (Whelan and Pembroke, 1989). Characteristics of each group were analysed and presented in **Figure 4.5.** In the first group (cluster A), bacterial strains showed superior cellulase enzymes and reducing sugar yield as compared to xylanase activity.

On the other hand, xylanase activity in cluster B varied less and remained at a high level but there was no significant effect on the lignocellulolytic degradation, which proved the moderate reducing sugar yield. The last one (cluster C) included two strains of *B. licheniformis* and *B. subtilis* B.01212 strain, which produced the lowest enzymatic activities and reduced sugar yields.

Overall, cluster analysis results appear to be useful for classifying numerous strains and selecting effective strains based on their bio-information. Regarding these analyses, different combinations of strains of *B. subtilis* B.01162, *B. coagulans* B.01123, *B. cereus* B.00076, *B. licheniformis* B.01223 and *B. licheniformis* B.01231, were constructed to evaluate their interaction as well as degradation capacity in co– and multi-cultures.

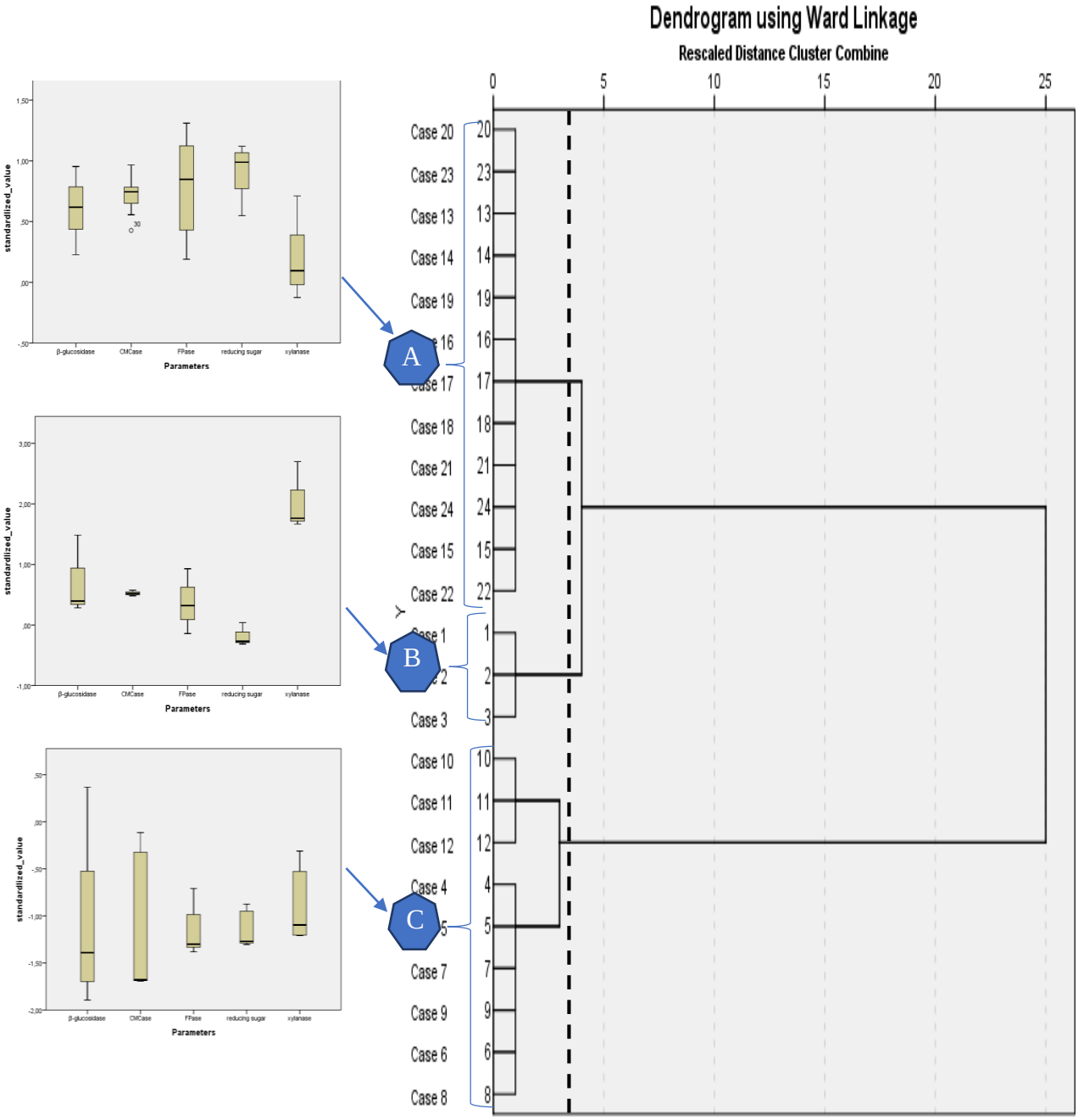

Figure 4.5 Cluster analysis and its characteristic using Ward's minimum variance, based on hydrolytic enzyme and reducing sugar in pretreatment by *Bacillus* strains

Enhancement of degrading enzyme using Bacillus consortia

According to the screening experiment, five strains of different *Bacillus* species were selected for the construction of eleven different consortia (**Table 4.1**).

Table 4.1 Description of the cellulolytic consortia

Type of Mix	Synthetic community	***Bacillus* strains**			
		I	**II**	**III**	**IV/V**
2-member consortia	AB	*B. subtilis* B.01162	*B. coagulans* B.01123		
	AC	*B. subtilis* B.01162		*B. cereus* B.00076	
	BC		*B. coagulans* B.01123	*B. cereus* B.00076	
3-member consortia	ABC	*B. subtilis* B.01162	*B. coagulans* B.01123	*B. cereus* B.00076	
	AB-D	*B. subtilis* B.01162	*B. coagulans* B.01123		*B. licheniformis* B.01223
	AB-E	*B. subtilis* B.01162	*B. coagulans* B.01123		*B. licheniformis* B.01231
	BC-D		*B. coagulans* B.01123	*B. cereus* B.00076	*B. licheniformis* B.01223
	BC-E		*B. coagulans* B.01123	*B. cereus* B.00076	*B. licheniformis* B.01231
	E-AB	*B. subtilis* B.01162	*B. coagulans* B.01123		*B. licheniformis* B.01231
	E-AC	*B. subtilis* B.01162		*B. cereus* B.00076	*B. licheniformis* B.01231
	E-BC		*B. coagulans* B.01123	*B. cereus* B.00076	*B. licheniformis* B.01231

The consortium's lignocellulolytic activities were investigated by determining the biomass degradation during 3 days of incubation period. The variety number of members of the microbial communities and the methods of incubation were claimed to have a significant effect in pretreatment of lignocellulose substrate ($p<0.05$). The consortium of *B. subtilis* B.01162 and *B. coagulans* B.01123 strain regarding 2-member mixture, and 3-member mixture including 2 above strains and *B. licheniformis* B.01223 strain added 24 hrs later after the first inoculation, gave the highest weight losses ranged from 51.74 – 53.1% (**Figure 4.6**). Zhang and co-workers (2016) reported that XDC-2 consortium gave the best weight loss of corn straw of 40% with high xylanase production capacity. Meanwhile, the individual strain of *Bacillus*, *Clostridium* and *Bacteroides* cultivation accounted for the lower percentage of solid cleavage, less than 85% of those of XDC-2 consortium.

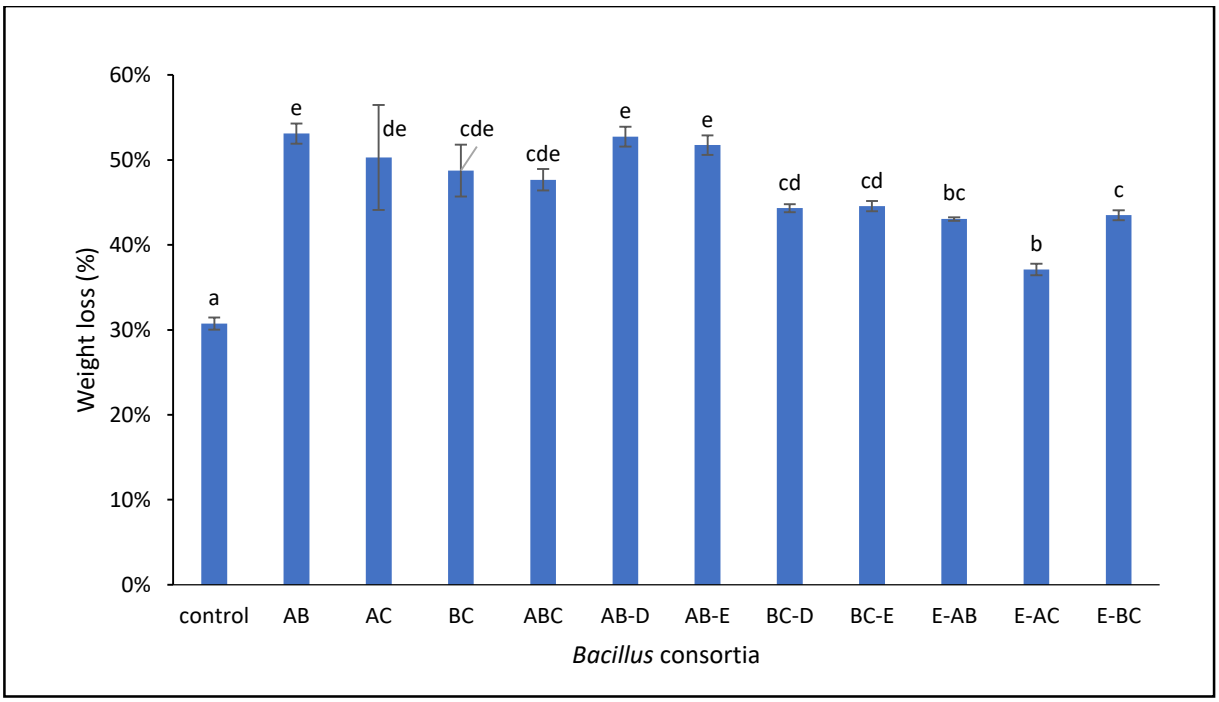

Figure 4.6 Dried weight loss of wheat bran after 7-day of cultivation of *Bacillus* consortia

The increasing pH from neutral to alkaline was observed in the first 3 days in hydrolysate of some consortia with high degradation rate. This fact was in agreement with prior work by Shruti and co-workers (2015) using microbial consortium isolated from wood rot and rice field soil sample for the degradation of rice straws, in which lignocellulose degradation increased with increasing pH value. However, it was also found that pH probably did not interfere the degradation rate by individual species. For instance, 60% of solid residue loss under 7 days of incubation by some pure *Bacillus* strains was found in different pH culture medium which range from pH 6.4-8.7. The competitive interactions among bacteria in nature are ubiquitous, even in the synergistic consortium. The relative decrease in reducing sugar yield was found in microbial consortia compared to individual strains (**Figure 4.7**). When wheat bran was used as substrate, species form consortium synergistically acted to modify on lignocellulose structure. Deng and Wang (2016) proved that bacteria in synergistic mixed cultures had higher metabolic activity than those in pure culture, showing their competition in nutrient consumption, thus led to the drop of fermented sugar yield during co-culture incubation.

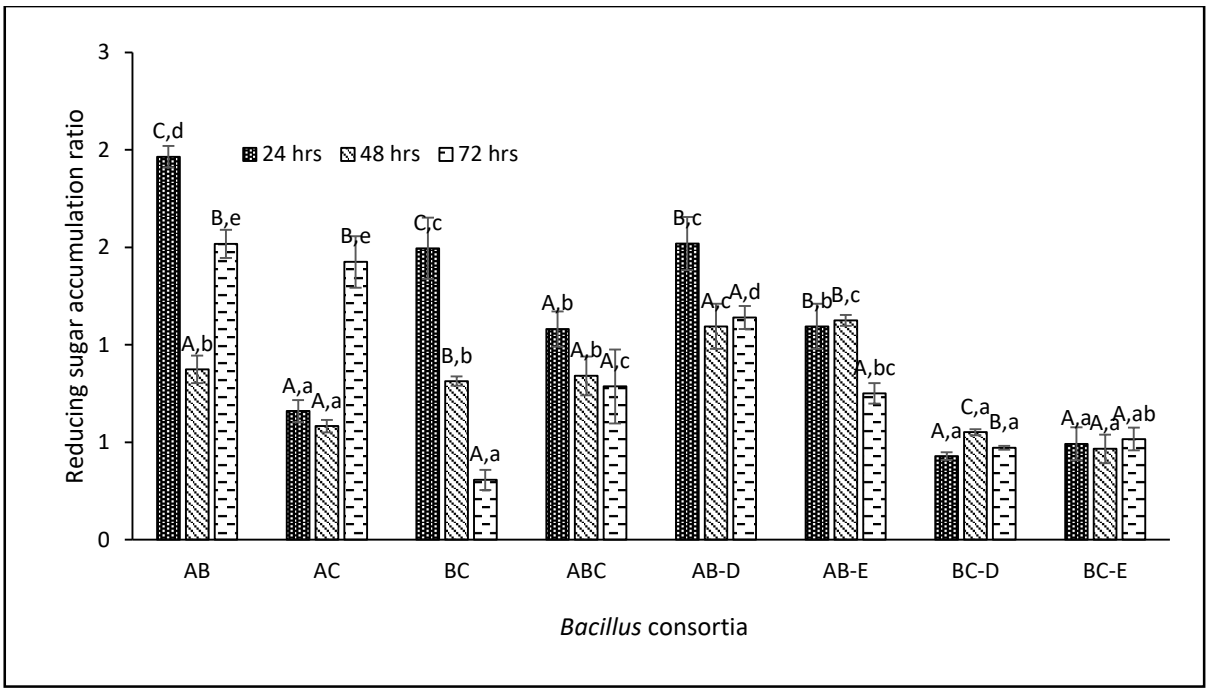

Figure 4.7 Reducing sugar accumulation ratio of *Bacillus* co-cultures after 24, 48 and 72 hours of cultivation. Capital letters (A, B, C) indicate the difference by treatment time and lower-case letters (a, b, c, d, e) demonstrate difference by strains

Additionally, two-step cultivation approach was also applied to determine the interaction between strains. The *B. licheniformis B.01223* strain (D) cooperated with AB consortium than other microbial communities when added 24 hrs later. 1-day-grown AB consortium took the dominant role in degrading consortium performance with strain D, resulting in a high degradation rate. In contrast, initially inoculated *B. licheniformis B.01223* strain before the cultivation of *B. subtilis* B.01162 and *B. coagulans* B.01123 strains did not reach the expectation for high degradation efficiency. Under those cultivation methods, it was observed that dominant strains which initially cultivated determined the degradation performance of applied microbial consortium. A minority population could become the most metabolically active strain, upon which the survival of the entire consortium depends (Brenner et al., 2008). Thus, a suitable cultivation strategy plays an important role in achieving the high degradation efficiency in pretreatment of lignocellulosic biomass using *Bacillus* consortium.

In another notice, the positive effect of complex microbial communities to enhance the enzymatic production in the bioconversion of lignocellulose to valuable products which also claim in several works (Haruta and Yamamoto, 2018; Jiménez et al., 2014; Wongwilaiwalin et al., 2010). Positive bacterial interaction promoted the production of total cellulase, endo-glucanase and xylanase compared to those by individual strain incubation. The enzymatic activities of selected species and their high impact consortia at 72 hrs of cultivation can be noted in the **Table 4.2**.

Table 4.2 Enzymatic production of various enzymes by monoculture and co-culture in the pretreatment hydrolysate at 72 hours of cultivation

STRAIN	Denoted by	Enzyme activities (IU/mL)			
		FPase	CMCase	β-glucosidase	Xylanase
B. subtilis B.01162	A	0.242^{b}	1.241^{b}	0.109^{a}	2.081^{c}
B. coagulans B.01123	B	0.311^{d}	1.374^{b}	0.118^{a}	0.989^{ab}
B. cereus B.00076	C	0.336^{de}	1.327^{b}	0.108^{a}	1.157^{b}
B. licheniformis B.01223	D	0.045^{a}	0.169^{a}	0.060^{b}	0.264^{a}
B. subtilis B.01162 - *B. coagulans* B.01123	AB	0.370^{ef}	2.153^{c}	0.030^{b}	2.692^{c}
B. subtilis B.01162 - *B. cereus* B.00076	AC	0.262^{bc}	1.513^{b}	0.026^{b}	2.140^{c}
B. coagulans B.01123 - *B. cereus* B.00076	BC	0.385^{f}	1.483^{b}	0.030^{b}	2.440^{c}
B. subtilis B.01162 - *B. coagulans* B.01123 - *B. cereus* B.00076	ABC	0.518^{g}	1.564^{b}	0.033^{b}	2.428^{c}
B. subtilis B.01162 – *B. coagulans* B.01123 - *B. licheniformis* B.01223	AB-D	0.300^{cd}	1.437^{b}	_	2.514^{c}

Mean values with different letters above the bars differ according to Tukey's test at $p<0.05$

The maximal filter paper enzyme activity and xylanase activity secreted in consortia of AB, BC and ABC were almost 2-fold higher and over 3-fold higher than that of pure cultures, respectively. The endo-glucanase activities of the listed consortia were found to be slightly higher than single cell cultivation, from 20-60%. The other mixtures of microbial communities did not show any improvement in cellulase production or even observing the negligible role of enzymatic activities in degradation process. The depression of β-glucosidase activity was also observed in pretreatment by microbial consortia. Dabhi and co-workers (2014) used a bacterial consortium comprising of *Pseudomonas* and *Bacillus* strains to degrade banana waste by solid state fermentation. The highest of 0.178 IU/mL total cellulase activity, 1.716 IU/mL of endo-glucanase activity were observed after 20 days and 0.602 IU/mL of β-glucosidase activity after 25 days. Singh and co-workers (2019b) developed thermophilic cellulose degrading consortia which produced mostly higher total cellulase activity than the individual strains, maximum value as 0.300 IU/mL at 72 hrs of incubation. However, these consortia failed to promote endo-glucanase and β-glucosidase activity, which were found very low compared to pure cell treatment. The ranges of enzyme activities were found of 0.012 IU/mL – 0.196 IU/mL and 0.1622–0.400 IU/mL for FPase and endo-glucanase (Gupta et al., 2012), respectively.

To elaborate more the degradation mechanism of these microbial consortia, principal component analysis (PCA) was employed to analyse the contributions of different cellulase

enzymes in the first 3 days (including total cellulase, endo-glucanase, β-glucosidase and xylanase) to the hydrolysis process and the results were depicted in **Figure 4.8**. FPase, CMCase, Xylanase were positively explained by the PC1 (66.47%) while PC2 (25.05%) loaded with β-glucosidase activity. The 2-member consortia including AB, AC, CD and 3-member consortia denoted as AB-C, AB-D located at the right quadrant with high scores of cellulolytic enzyme activities, while the remaining 6 consortia took place in other quadrants with the lowest intensities of hydrolytic enzymes. It appeared that the combination of high potential strains as *B. subtilis* B.01162, *B. coagulans* B.01123 and *B. cereus* B.00076 strains promoted the production of hydrolytic enzyme activities of FPase and xylanase than their individual cultivation. The role of β-glucosidase in degradation by the consortium was negligible, causing less efficiency in the removal of cellobiose, which may inhibit the hydrolytic actions of other cellulase enzymes. Enzymatic production capacities of different *Bacillus* consortia were presented in **Figure 4.8.** The crowd cluster with orange circle illustrated microbial flora that produced most of cellulase enzymes classified in PC1 (66.47%) as FPase, CMCase and xylanase.

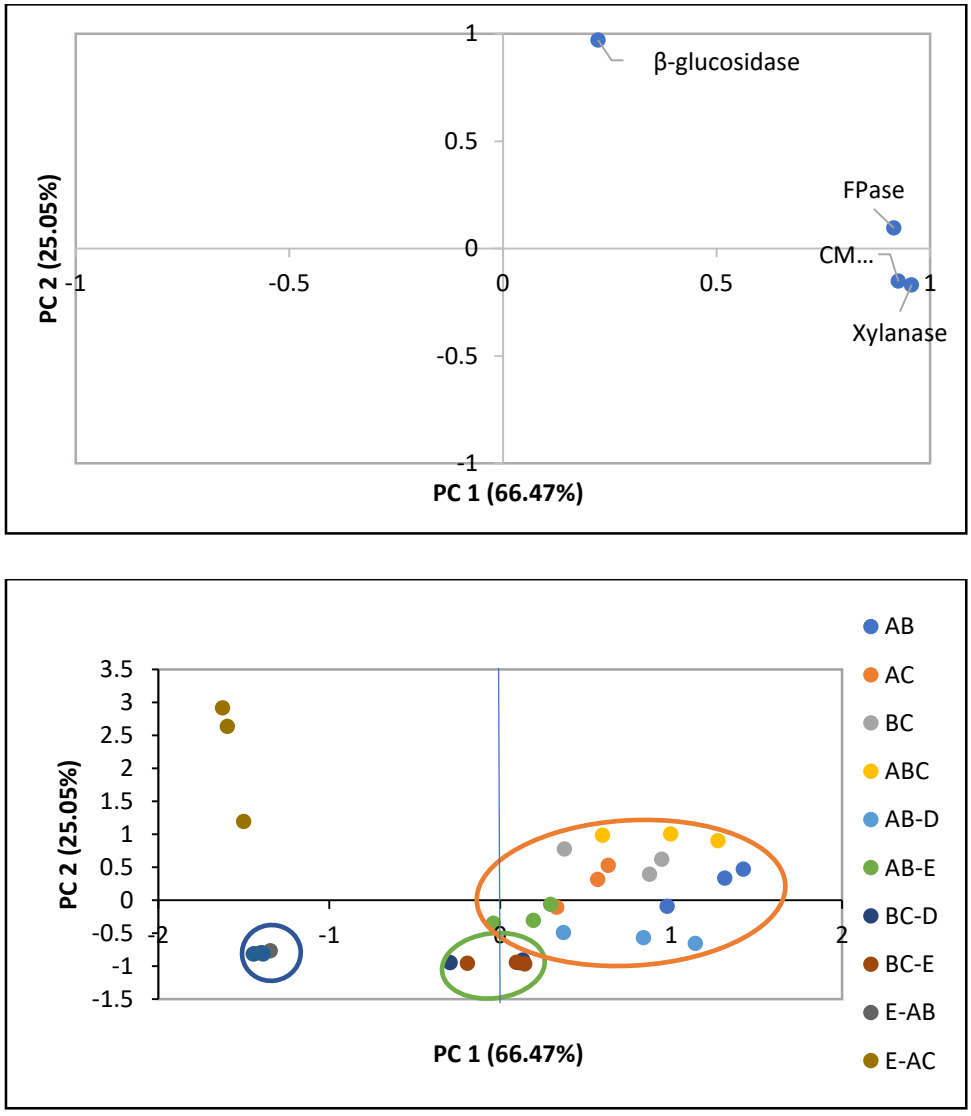

Figure 4.8 Principal component analysis (PCA) plot (A: component plot in rotated space; B: plot of regression factor on the first and second axes from PCA of 11 *Bacillus* consortia)

The high substrate weight loss was observed in pretreated sample containing fermented sugar yield under *Bacillus* co-cultures cultivation. The effective communities were graded with high scores for PC1 component, constituted by FPase, CMCase and xylanase activities. The low activities of β-glucosidase enzyme could lead to inefficient cleavage of cellobiose, thus limiting hydrolysis by cellulase enzymes secreted by co-cultures, which contributes to the decrease in reducing sugar yields. Despite the drawbacks caused by low endo-glucanase and β-glucosidase activities, the enhancement of important degradative enzymes such as xylanase and total cellulase activities secreted by the consortium demonstrated the promising role of cellulolytic strains in the biological pretreatment of lignocellulosic biomass. Among the genus *Bacillus*, some strains such as *B. subtilis, B. cereus, B. coagulans* possess high cellulolytic activity (Chantarasiri, 2015; Choudhury et al., 2006; Roy and Habib, 2009). The most important role of *Bacillus* species was their hydrolytic capacity through the synergistic action of enzyme array to form important products such as DP3, DP2 carbohydrate fraction, glucose and xylose (data are not shown). The total yield of reducing sugars produced by the consortium was lower than in the monoculture, and it decreased with the increase in the number of microorganisms in the communities. Generally, the simple sugars such as glucose, maltose are easily and prefer sugar to be utilized by microbes, thus this phenomenon can be explained by the quality of sugars released in the medium and the competitive consumption of nutrients by members in the same ecological system. More studies are needed to understand the interaction between members of the consortium, fermentation dynamics such as the growth of cells, consumption rate of sugars etc., and to control the quality of pretreated biomass. These experiments are in progress in our lab.

4.1.2. Ligninolytic bacteria

Degradation efficiency of ligninolytic strains

Rhodococcus and *Pseudomonas* were promising candidates for lignocellulose degradation. Ligninolytic strains including *Rhodococcus erythropolis* B.01914, B.11952; *Rhodococcus opacus* B.01915; *Rhodococcus fascians* B.01608, B.01614; *Rhodococcus sp*. B.01916, *Pseudomonas putida* B.01157, B.01494 and B.01522 were individually evaluated for degradation efficiency. After 7-daycultivation, the highest lignocellulosic weight loss was observed in pretreated samples by *R. erythropolis* B.01914, *R. opacus* B.01915, *R. fascians* B.01608, *P. putida* B.01157, B.01522.

Weight loss and total phenolic accumulation

Solid residue weight losses after pretreatment were demonstrated in **Figure 4.9.** It was observed that *R. erythropolis* B.01952, *R. fascians* B.01614 and *Rhodococcus sp.* B.01916 caused the lowest impact on lignocellulosic degradation than others after 7 days of the pretreatment. On the contrary, *R. erythropolis* B.01914, *R. opacus* B.01915 and *P. putida* B.01522 have the greatest effect on lignocellulose degradation, performed by the significant higher weight loss of solid residues ($p < 0.05$). The positive results of total phenolic content from extracted hydrolysate also confirmed the degradation capacity by mentioned strains. Phenol and its derivatives are aromatic compounds which structure on lignin, one of the most significant components of lignocellulose by mass and act as the protective layer. Therefore, to make biomass-to-fuel processes more

economically feasible, removing lignin and its derivatives, primarily phenolic compounds is necessary. Taylor and co-workers (2012) proved that isolated *R. erythropolis* from termite gut exhibited lignin metabolizing activity via the UV-vis assay using nitrated-lignin from wheat. Moreover, the increasing of *R. opacus* cell concentration up to 300-times when ethanol organosolv lignin used as sole carbon and energy source in 7 days proved its decomposing capability of lignin, claimed by Kosa and Ragauskas (2013).

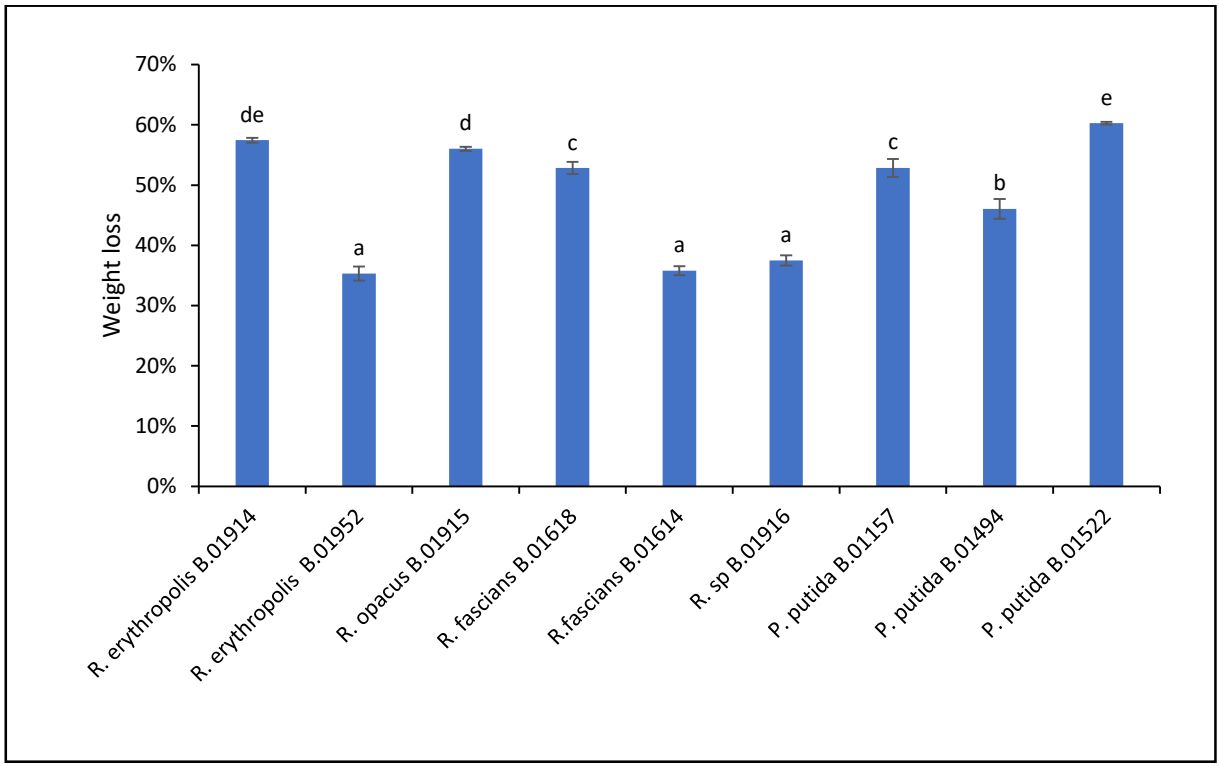

Figure 4.9 Dried weight loss of wheat bran after 7-day of cultivation of ligninolytic strains

The total phenolic accumulation by time of biological pretreatment was demonstrated in **Table 4.3**. The increasing of TPC with extended treatment period was observed in most case studies with different amplitudes. Specifically, TPC released by *P. putida* B.01522, *R. erythropolis* B.01914, *R. fascians* B.01608, *R. opacus* B.01915, *P. putida* B.01157 after 48 hrs of pretreatment increase dramatically, especially *P. putida* B.01522 which obtained the highest TPC within 72 hrs. Likewise, aromatic contents produced by *R. erythropolis* B.01914, *R. fascians* B.01608 were found to obtain the higher yields at the 5^{th} day of the pretreatment. The remaining strains have no significant effect on modify the lignin structure of lignocellulosic biomass due to the negligible changes in phenolic content in the extracted hydrolysates. According to previous studies, *Rhodococcus* species directly utilized lignin as sole carbon and generate bio-products such as aromatic dicarboxylic acids and vanillin. They also mentioned no metabolite observed in the first 72 hrs but after 5-7 days, treatment using *Rhodococcus* genus obtained the maximum metabolite production (Mycroft et al., 2015; Sainsbury et al., 2013)

Table 4.3 Total phenolic content (mg/ml) accumulation ratio of ligninolytic strains after 7-day incubation

Species	Incubation days						
	1	2	3	4	5	6	7
Rhodococcus erythropolis B.01914	1.38 ± 0.27	2.00 ± 0.08	2.10 ± 0.14	2.39 ± 0.20	2.87 ± 0.11	2.91 ± 0.14	2.83 ± 0.20
Rhodococcus erythropolis B.01952	0.85 ± 0.06	0.89 ± 0.05	0.92 ± 0.07	0.96 ± 0.06	1.13 ± 0.17	1.05 ± 0.10	1.16 ± 0.15
Rhodococcus opacus B.01915	1.01 ± 0.07	1.61 ± 0.13	1.67 ± 0.10	2.03 ± 0.15	1.91 ± 0.23	2.04 ± 0.09	2.06 ± 0.26
Rhodococcus fascians B.01608	1.09 ± 0.27	1.60 ± 0.09	1.65 ± 0.33	1.87 ± 0.11	2.48 ± 0.18	2.23 ± 0.12	2.64 ± 0.46
Rhodococcus fascians B.01614	1.15 ± 0.33	1.12 ± 0.14	1.19 ± 0.14	1.16 ± 0.14	1.34 ± 0.06	1.22 ± 0.13	1.38 ± 0.21
Rhodococcus sp. B.01916	0.79 ± 0.06	0.85 ± 0.06	0.85 ± 0.12	0.78 ± 0.09	0.94 ± 0.16	0.89 ± 0.04	0.85 ± 0.08
Pseudomonas putida B.01157	0.89 ± 0.18	1.59 ± 0.11	1.66 ± 0.03	1.68 ± 0.09	1.73 ± 0.21	1.47 ± 0.07	1.61 ± 0.30
Pseudomonas putida B.01494	1.00 ± 0.09	0.83 ± 0.06	0.84 ± 0.05	0.92 ± 0.09	0.90 ± 0.04	1.02 ± 0.08	1.05 ± 0.07
Pseudomonas putida B.01522	1.22 ± 0.13	1.93 ± 0.17	2.71 ± 0.06	2.72 ± 0.53	2.80 ± 0.41	2.86 ± 0.36	3.06 ± 0.10

Pseudomonas, *Rhodococcus* are well-known for production of extracellular enzymes which can break down lignin to synthesize bio-products (Jones et al., 2018; Xu et al., 2018). In this work, enzymatic activities were evaluated to elaborate the mechanism of lignocellulose deterioration by ligninolytic species (**Figure 4.10).** The total cellulase activities of *Rhodococcus* and *Pseudomonas* species were ranged from 0.099 to 0.269 IU/mL. Among these strains, *Pseudomonas* strains and *R. fascians* B.01614 achieved the highest cellulase activity (over 0,230 IU/mL) with no significant difference from each other. Lowest cellulosic enzyme activity was found in the extracted sample treated by *R. erythropolis* B.01914. The remaining species such as *R. erythropolis* B. 01952, *R. opacus* B.01915, *R. sp.* B.01916 performed moderate enzymatic activities. Xylanase is an important enzyme to remove xylan, cleave its cross-linkages with cellulose fibrils and lignin, make it more accessible for hydrolytic action, thus enhancing cellulose digestibility. The most effective strains in breaking down the hemicellulose structure can be mentioned including *R. erythropolis* B.01914, *R. opacus* B.01915 and *P. putida* B.01522 with enzyme activities of 1.716, 1.644 and 2.011 IU/mL, respectively. Moreover, *Pseudomonas putida*, with low glucose uptake, presents as a promising host for cellulases. Tozakidis and co-workers (2016) referred that *Pseudomonas putida* cells could express surface displayed cellulases which could directly be applied to the substrates. This makes it possible for cellulose hydrolysis to convert lignocellulosic biomass to biofuels and other bio-commodities.

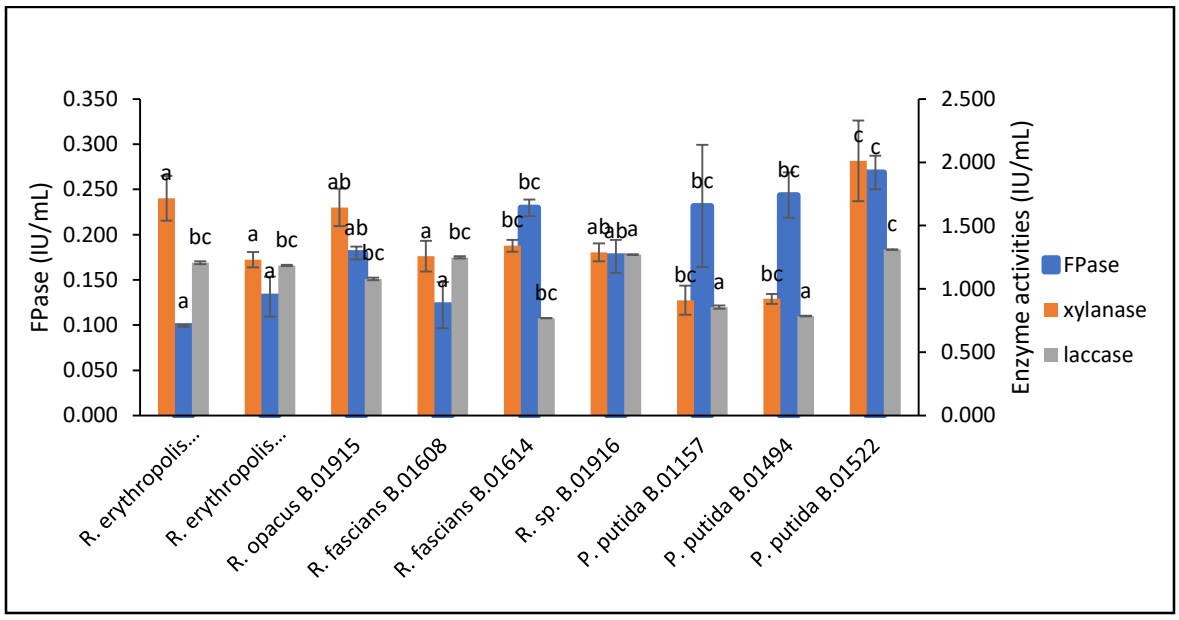

Figure 4.10 Comparison of enzyme production capacity of 8 lignin-degrading strains at 48 hrs of incubation

Rhodococcus and *Pseudomonas* are comparable in biotechnological applications due to their diverse range of metabolic capabilities. The bio-degradative pathways from these strains could initiate oxidative attack on aromatic ring, releasing central intermediates (Kim et al., 2018). Typically, laccase

enzyme catalyses one-electron oxidation of phenolics, aromatic amines, di-amines and other electron-rich substrates via the four-electron reduction of oxygen to water (Bento et al., 2010; Kunamneni et al., 2008). The most effective lignin degrading species such as *P. putida* B.01522, *R. erythropolis* B.01914, *R. fascians* B.01608 and *Rhodococcus sp.* B.01916 were addressed. Other *P. putida* species and *R. fascians* B.01614, however, showed poor performance in modifying lignin structure.

Generally, cellulolytic *Bacilli* species can produce higher cellulase activities in comparison to ligninolytic species. The best total cellulase observed in ligninolytic bacterial group was 0.269 IU/mL when *B. cereus* and *B. coagulans* act as the effective cellulolytic could produce cellulase activity over 0.3 IU/mL. *P. putida* B.01522, *R. erythropolis* B.01914 and *R. opacus* B.01915 could release high xylanase activity which performed comparatively with the best hemicellulose degrader in cellulolytic *Bacilli* genus. These facts indicate a promising cellulose degradation effect of co-cultures of cellulolytic and ligninolytic strains.

Correlation between reducing sugars and enzyme activities

The correlation between reducing sugar released by enzymatic hydrolysis of wheat bran was evaluated (**Figure 4.11**). A linear relationship between reducing sugar yield and degrading enzyme activities with strong magnitude was found between sugar yields with total cellulase (FPase) and laccase enzymes. Regarding former case, greater FPase was associated with greater reducing sugar in a significant linear relationship ($r = 0.571$, $p < 0.05$). Obviously, cellulases catalyse the decomposition of cellulose polysaccharide by breaking down β-1,4-glycosidic bond, thus boosting the conversion process of lignocellulosic biomass to fermented sugar products (Jayasekara and Ratnayake, 2019). On the contrary, negative association between laccase activity and reducing sugar was observed, increasing laccase activity led to reduction of sugar yield ($r = -0.898$, $p < 0.05$). It is known that in an oxidative polymerization process, laccases produce unstable radicals, which may significantly affect the hydrolytic activities. These consequences were in agreement with the study conducted by Rocha-Martín and co-workers (2018), which found only 0.1 mg laccase/glucan could negatively affect lignocellulosic substrates.

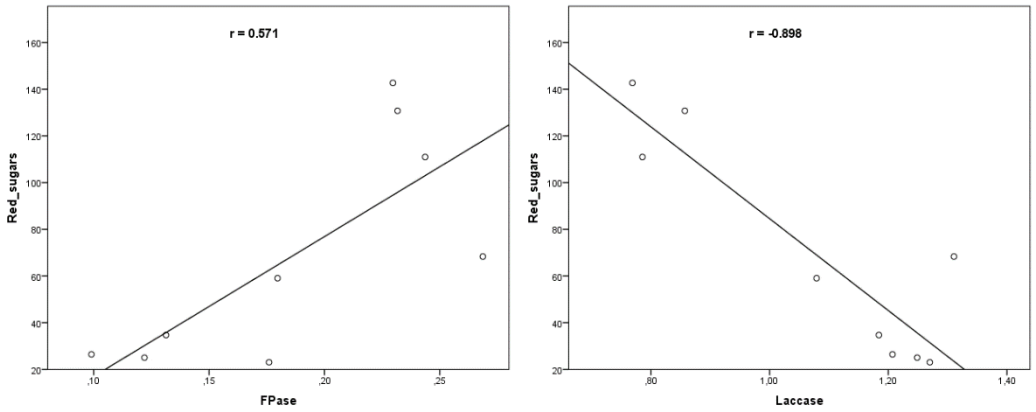

Figure 4.11 The correlation between degrading enzyme activities and sugar yield

Construction of ligninolytic strains

Six effective microorganisms including *R. erythropolis* B.01914 (A*); *R. opacus* B.01915 (C*); *R. fascians* B.01608 (D*); *R. fascians* B.01614 (E*); *P. putida* B.01157 (G*); *P. putida* B.01522 (K*) were selected to construct different lignin degrading consortia. The two-member and three-member consortia were constructed to evaluate the effect of mixed-culture of lignocellulolytic strains.

The biomass degradation capacity by lignocellulolytic consortia was investigated and evaluated during 7 days of the pretreatment process. The weight loss of solid residues after 7-day treatment by mixed cultures did not show any significant difference. These values range from 45% to 60%. The total phenolic accumulation change by time of pretreatment was presented in **Figure 4.12**.

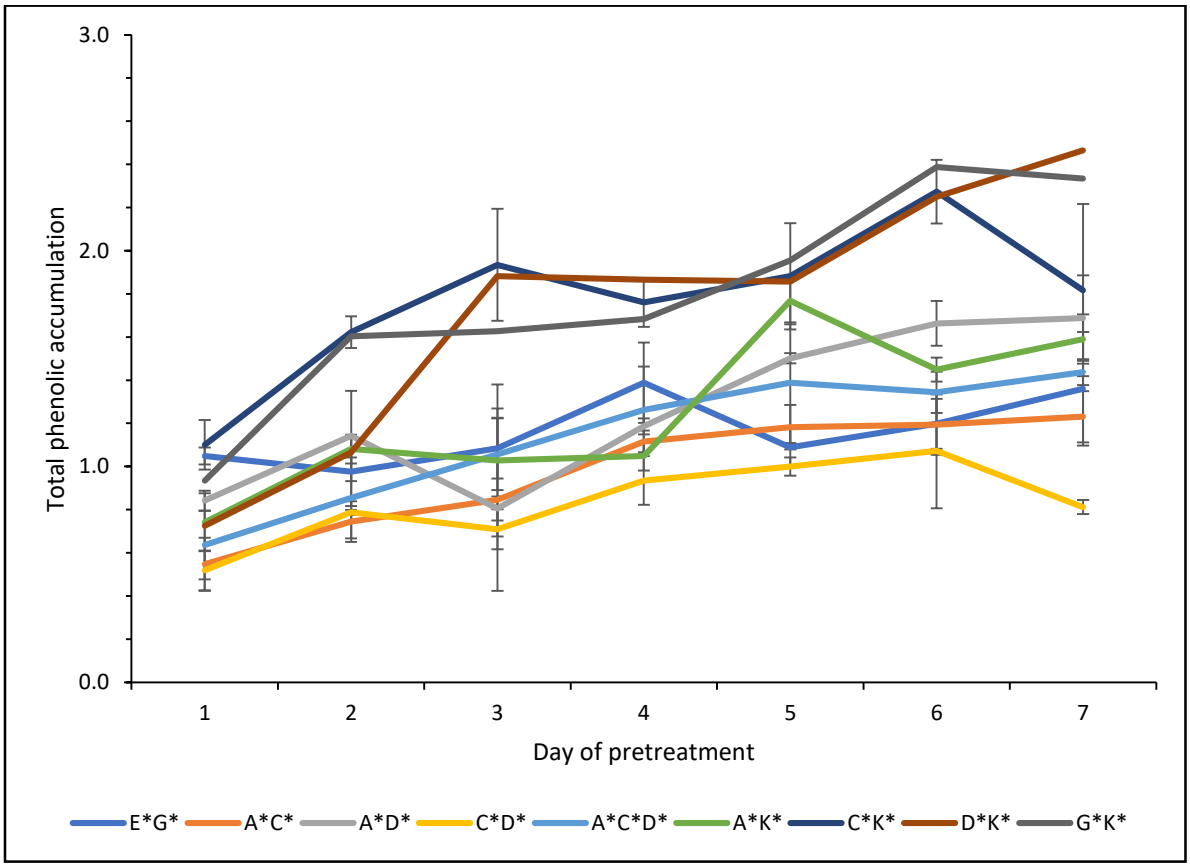

Figure 4.12 Total phenolic content generated by ligninolytic consortia

Various degradation characteristics were observed under cultivation of different microbial co-cultures. The co-culture of *P. putida* B.01522 with other species performed the outstanding aromatic contents. Ravi and co-authors (2017) claimed gram-negative bacteria *Pseudomonas* as effective degraders of lignocellulose components members of nature microbial consortia. *Pseudomonas and Rhodococcus* can produce enzymes capable of degrading and/or modifying lignin from lignocellulosic materials, which tested by Ahmad and co-workers (2010) using fluorescence and UV-vis assay. Co-

culture of *R. opacus* B.01915 and *P. putida* B.01522 showed a relatively high total phenolic content, with accumulation ratio up to 1.62. Regarding remaining consortia, ligninolytic co-cultures produce less TPC than individual cultivation. It can be improved by extend pretreatment period, stimulating microbial communities release more aromatic compounds.

pH values increase from neutral to alkaline after 48 hrs for most of consortia with high degradation rate, whereas negligible pH change under cultivation of individual species. Thus, pH probably did not interfere the degradation rate when using single strains or their combination in the biological pretreatment. However, the reduction of reducing sugar yield caused by co-culture was more serious than by single strain, which could be explained by the higher metabolic activity in synergistic mixed culture, showing their competition in nutrient consumption (Deng and Wang, 2016). This fact agreed with our previous results under cultivation of *Bacillus* co-culture. Among ligninolytic consortia, mixed culture of *R. opacus* B.01915 and *P. putida* B.01522 (C*K*) could release relatively high degrading enzyme activities than other bacterial mixtures (**Table 4.4**).

Table 4.4 Enzymatic production by monoculture and co-culture of lignin-degrading strain in the pretreatment hydrolysate at 72 hours of cultivation

Strains and consortium	Code	Enzyme activity (IU/mL)		
		FPase	Laccase	TPC
R. erythropolis B.01914	A*	0.10 ± 0.00	1.21 ± 0.18	2.00 ± 0.01
R. opacus B.01915	C*	0.18 ± 0.01	1.08 ± 0.15	1.61 ± 0.01
R. fascians B.01608	D*	0.12 ± 0.03	1.25 ± 0.12	1.60 ± 0.01
P. putida B.01157	G*	0.23 ± 0.07	0.86 ± 0.11	1.59 ± 0.01
P. putida B.01522	K*	0.27 ± 0.02	1.31 ± 0.32	1.93 ± 0.00
R. erythropolis B.01914-*R. opacus* B.01915	A*C*	0.04 ± 0.01	1.00 ± 0.04	0.75 ± 0.16
R. erythropolis B.01914-*R. fascians* B.01608	A*D*	0.06 ± 0.03	0.89 ± 0.03	1.14 ± 0.09
R. opacus B.01915-*R. fascians* B.01608	C*D*	0.17 ± 0.04	0.79 ± 0.10	0.79 ± 0.21
R. erythropolis B.01914-*R. opacus* B.01915-*R. fascians* B.01608	A*C*D*	0.04 ± 0.01	1.02 ± 0.05	0.85 ± 0.01
R. erythropolis B.01914-*P. putida* B.01522	A*K*	0.08 ± 0.03	0.90 ± 0.04	1.08 ± 0.19
R. opacus B.01915-*P. putida* B.01522	C*K*	0.22 ± 0.04	0.92 ± 0.06	1.62 ± 0.07
R. fascians B.01608-*P. putida* B.01522	D*K*	0.17 ± 0.06	0.94 ± 0.06	1.06 ± 0.07
P. putida B.01157-*P. putida* B.01522	G*K*	0.14 ± 0.00	0.90 ± 0.03	1.60 ± 0.20
R. fascians B.01614- *P. putida* B.01157	E*G*	0.16 ± 0.08	1.45 ± 0.17	0.85 ± 0.01

Among investigated ligninolytic consortia, consortium C*K* possessed a comparative total cellulase with those secreted by *P. putida* B.01157 (G*) and *P. putida* B.01522 (K*), activity of 0.22 IU/mL, 0.23 IU/mL and 0.27 IU/mL, respectively. The 2 and 3-member consortia of *Rhodococcus* such as *R. erythropolis* B.01914-*R. opacus* B.01915 (A*C*), *R. erythropolis* B.01914-*R. fascians* B.01608 (A*D*) and *R. erythropolis* B.01914-*R. opacus* B.01915-*R. fascians* B.01608 (A*C*D*) show the poor

hydrolytic effect on the lignocellulosic biomass with 4-7 times lower hydrolytic activity than under cultivation of *Pseudomonas putida* B.01522 (K*). In term of laccase enzyme, lignin degrading enzyme produced from monocultures have higher activity than those from co-cultures. For instance, co-culture of *R. opacus* B.01915 and *P. putida* B.01522 produced laccase enzyme with activity of 1.62 IU/mL whereas *R. erythropolis* B.01914 and *P. putida* B.01522 produced laccase efficiently, activity of 2.00 and 1.93 IU/mL, respectively. The differences in individual stability of the different laccase isoforms or substrate specificity could probably account for the reduction of laccase activity in co-culture (Flores et al., 2010). Among these strains and consortia, *Pseudomonas putida* B.01522 performs as the best laccase producer, in which laccase activity was up to 1.6 times higher than the remaining microorganisms. This result was relevant to other studies that claimed the rich lignin-degrading activity of *Pseudomonas putida* for degrading a wide range of aromatic compounds (Bugg et al., 2011; de Gonzalo et al., 2016).

Contrary to cellulolytic strains in previous experiments, the co-culture of lignin-degrading strains of *Rhodococcus* and *Pseudomonas* did not perform sufficiently in enhancing degrading enzyme activities. Nevertheless, in term of soluble fraction, higher concentration of fermented sugars were observed from their co-cultures. **Figure 4.13** demonstrated the monosaccharides and oligosaccharides released by ligninolytic strains and their consortia after 48 hrs of the lignocellulose pretreatment process. The maximum glucose yield produced from ligninolytic co-culture was 4-fold higher than the greatest yield employed from cultivation of pure-strain. Thus, the potential role of lignin-degrading consortia should be put into consideration to discover their capacity to alter lignocellulose substrates in the combination with other species in the same habitat.

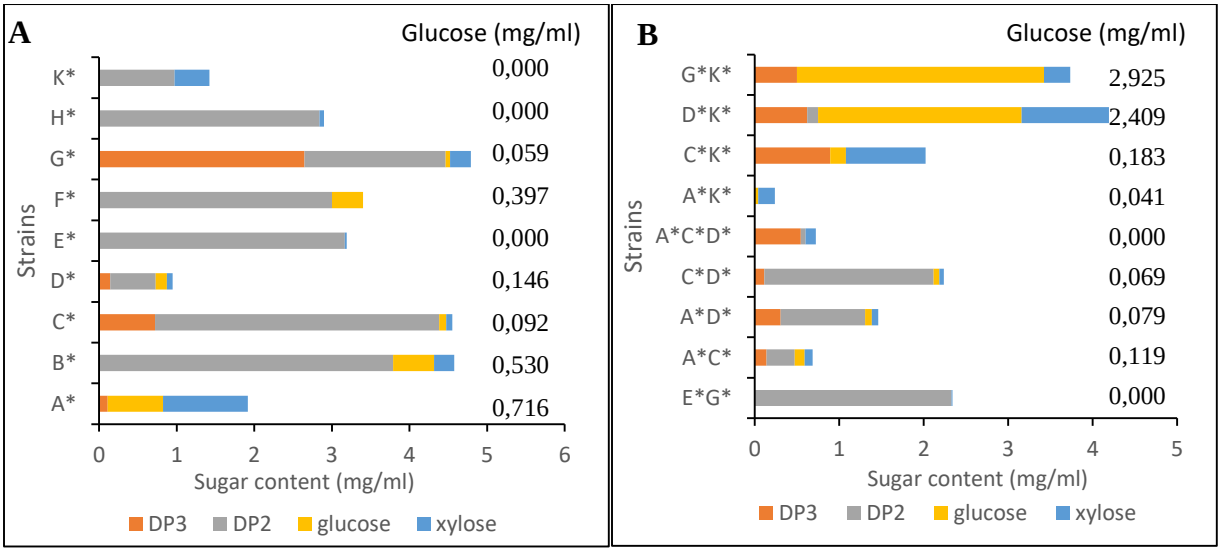

Figure 4.13 The sugar conversion of pretreated lignocellulosic biomass using ligninolytic strains (A) and their co-cultures (B)

4.1.3. Construction of the mixed cultures of cellulolytic and ligninolytic strains

Effective cellulolytic and ligninolytic strains and co-cultures were selected to construct the complex microbial consortia. Cellulolytic species include *B. subtilis* B.01162, *B. coagulans* B.01123, *B. cereus* B.00076 and ligninolytic species include *R. opacus* B.01915, *R. fascians* B.01608, *R. fascians* B.01614, *P. putida* B.01157 and *P. putida* B.01522. The degradation efficiency was investigated during 72 hrs of incubation period. Cluster analysis was applied to classify bacterial communities based on their similar degradation profiles (**Figure 4.14**).

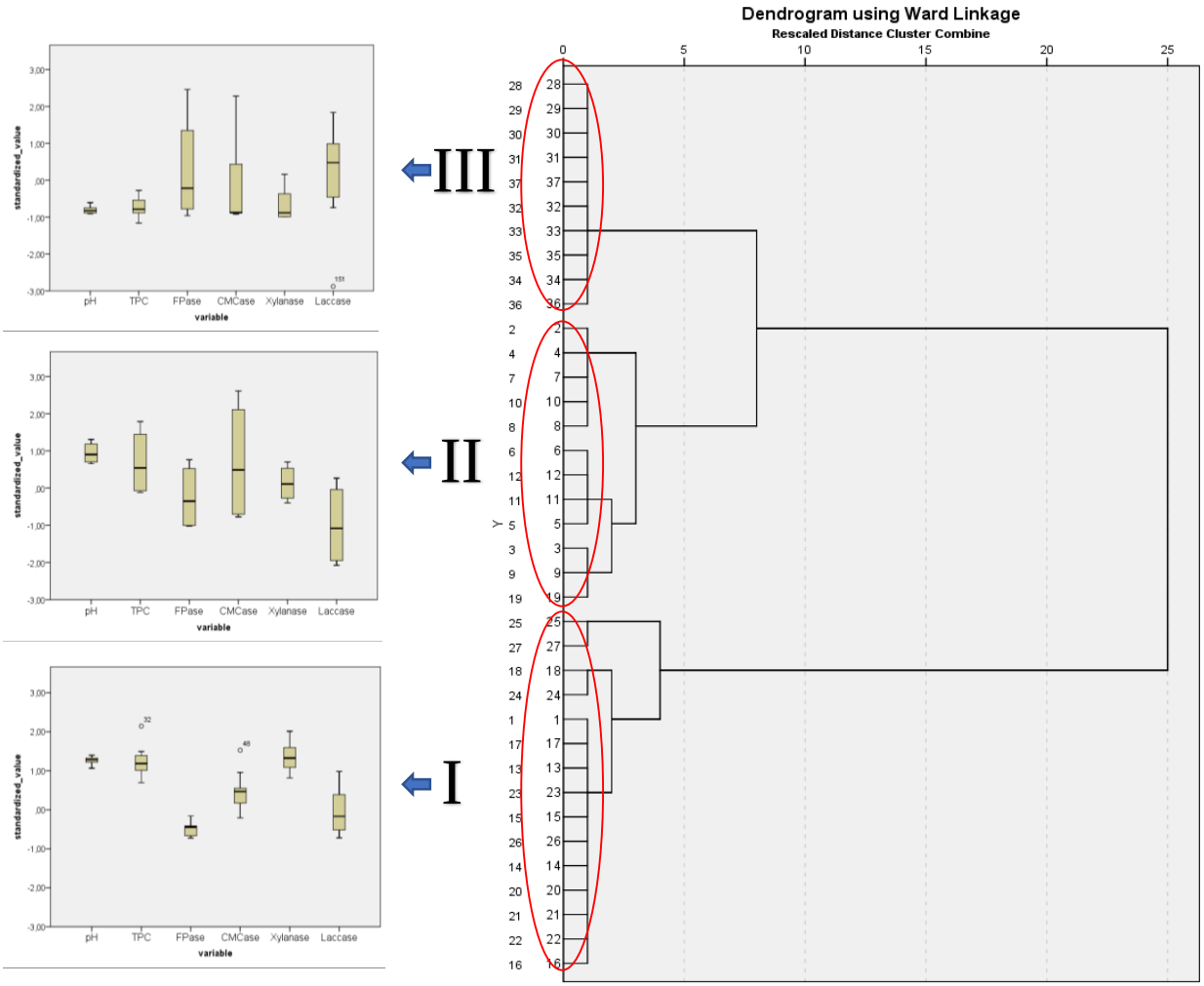

Figure 4.14 Cluster analysis and its characteristic using Ward's minimum variance, based on various parameters in pretreatment by bacterial consortia

37 variances presenting in three clusters were found and these cluster were denoted as cluster I, II and III. The degradation effect of selected microorganisms and their combination was presented in **Table 4.5.** The mixtures of C*K and D*K with various cellulolytic *Bacillus* consortia were grouped in cluster I. Cluster II, including cellulolytic *Bacillus* and their mixture with *Pseudomonas putida* B.01522 and cluster III was composed by remaining species.

Table 4.5 Degradation efficiency of co-cultures of cellulolytic and ligninolytic strains

Cluster	Species	Denoted	Cluster	pH	TPC accumulation
I	*P. putida* B.01522	K*	54.98 ± 1.81	8.79 ± 0.10	1.15 ± 0.06
	R. opacus B.01915-*P. putida* B.01522	C*K*	55.71 ± 2.68	8.81 ± 0.02	1.45 ± 0.22
	R. opacus B.01915-*P. putida* B.01522-*B. subtilis* B.01162	C*K*-A	53.89 ± 1.10	8.89 ± 0.04	1.43 ± 0.15
	R. opacus B.01915-*P. putida* B.01522-*B. subtilis* B.01162-*B. coagulans* B.01123	C*K*-AB	51.42 ± 1.92	8.83 ± 0.04	1.36 ± 0.34
	R. opacus B.01915-*P. putida* B.01522-*B. subtilis* B.01162-*B. cereus* B.00076	C*K*-AC	52.70 ± 0.80	8.89 ± 0.13	1.19 ± 0.13
	R. opacus B.01915-*P. putida* B.01522-*B. coagulans* B.01123-*B. cereus* B.00076	C*K*-BC	53.30 ± 3.01	8.68 ± 0.07	1.30 ± 0.28
	R. fascians B.01608-*P. putida* B.01522	D*K*	53.68 ± 1.57	5.65 ± 0.01	0.59 ± 0.07
	R. fascians B.01608-*P. putida* B.01522-*B. subtilis* B.01162	D*K*-A	53.49 ± 0.40	9.00 ± 0.02	1.24 ± 0.02
	R. fascians B.01608-*P. putida* B.01522-*B. subtilis* B.01162-*B. coagulans* B.01123	D*K*-AB	48.82 ± 1.31	8.94 ± 0.03	1.33 ± 0.32
	R. fascians B.01608-*P. putida* B.01522-*B. subtilis* B.01162-*B. cereus* B.00076-	D*K*-AC	52.24 ± 2.53	8.86 ± 0.00	1.33 ± 0.15
	R. fascians B.01608-*P. putida* B.01522-*B. coagulans* B.01123-*B. cereus* B.00076	D*K*-BC	51.56 ± 1.74	8.65 ± 0.23	1.69 ± 0.62
	R. fascians B.01608-*P. putida* B.01522-*B. subtilis* B.01162-*B. coagulans* B.01123-*B. cereus* B.00076	D*K*-ABC	47.00 ± 5.59	8.86 ± 0.13	1.30 ± 0.02
	R. fascians B.01614-*P. putida* B.01522	E*K*	45.69 ± 2.57	8.04 ± 0.10	0.88 ± 0.03
	P. putida B.01157-*P. putida* B.01522	G*K*	48.89 ± 1.48	8.51 ± 0.05	1.39 ± 0.27
	R. fascians B.01614-*P. putida* B.01157	E*G*	45.70 ± 3.94	7.91 ± 0.19	0.85 ± 0.01
II	*B. subtilis* B.01162	A	33.83 ± 1.62	5.65 ± 0.01	0.47 ± 0.09
	B. subtilis B.01162-*B. coagulans* B.01123	AB	33.12 ± 4.63	5.67 ± 0.03	0.53 ± 0.03
	B. subtilis B.01162-*B. cereus* B.00076	AC	33.45 ± 1.83	5.67 ± 0.09	0.59 ± 0.01

	B. coagulans B.01123-*B. cereus* B.00076	BC	30.72 ± 0.93	5.70 ± 0.03	0.56 ± 0.05
	B. subtilis B.01162-*B. coagulans* B.01123-*B. cereus* B.00076	ABC	28.70 ± 1.50	5.77 ± 0.01	0.46 ± 0.03
	P. putida B.01522-*B. subtilis* B.01162-	K*-A	37.88 ± 1.74	5.81 ± 0.12	0.63 ± 0.16
	P. putida B.01522-*B. cereus* B.00076-	K*-C	36.73 ± 1.37	5.67 ± 0.19	0.79 ± 0.21
	P. putida B.01522-*B. subtilis* B.01162-*B. coagulans* B.01123	K*-AB	34.83 ± 0.08	5.87 ± 0.01	0.58 ± 0.06
	P. putida B.01522-*B. subtilis* B.01162-*B. cereus* B.00076	K*-AC	35.58 ± 1.61	5.67 ± 0.09	0.69 ± 0.03
	P. putida B.01522-*B. coagulans* B.01123-*B. cereus* B.00076	K*-BC	34.10 ± 1.74	5.79 ± 0.05	0.53 ± 0.05
	P. putida B.01522-*B. subtilis* B.01162-*B. coagulans* B.01123-*B. cereus* B.00076	K*-ABC	33.19 ± 4.76	5.77 ± 0.09	0.53 ± 0.01
	R. opacus B.01915-*P. putida* B.01522-*B. subtilis* B.01162-*B. coagulans* B.01123-*B. cereus* B.00076	C*K*-ABC	42.15 ± 1.28	8.5 ± 0.14	1.56 ± 0.24
III	*P. putida* B.01157-*P. putida* B.01522-*B. subtilis* B.01162	G*K*-A	34.46 ± 2.53	6.08 ± 0.13	0.60 ± 0.01
	B. coagulans B.01123-*P. putida* B.01157-*P. putida* B.01522-*B. subtilis* B.01162	G*K*-AB	33.79 ± 1.51	6.02 ± 0.13	0.60 ± 0.07
	P. putida B.01157-*P. putida* B.01522-*B. subtilis* B.01162-*B. cereus* B.00076	G*K*-AC	32.84 ± 0.29	6.06 ± 0.14	0.71 ± 0.09
	P. putida B.01157-*P. putida* B.01522-*B. coagulans* B.01123-*B. cereus* B.00076	G*K*-BC	32.12 ± 0.34	5.76 ± 0.05	0.58 ± 0.01
	P. putida B.01157-*P. putida* B.01522-*B. subtilis* B.01162-*B. coagulans* B.01123-*B. cereus* B.00076	G*K*-ABC	34.63 ± 0.26	5.75 ± 0.00	0.71 ± 0.09
	R. fascians B.01614-*P. putida* B.01157-*B. subtilis* B.01162-	E*G*-A	33.54 ± 1.50	5.88 ± 0.00	0.68 ± 0.15
	R. fascians B.01614-*P. putida* B.01157-*B. subtilis* B.01162-*B. coagulans* B.01123	E*G*-AB	33.26 ± 1.52	5.75 ± 0.02	0.63 ± 0.08
	R. fascians B.01614-*P. putida* B.01157-*B. subtilis* B.01162-*B. cereus* B.00076	E*G*-AC	33.74 ± 0.68	5.88 ± 0.00	0.71 ± 0.08
	R. fascians B.01614-*P. putida* B.01157-*B. coagulans* B.01123-*B. cereus* B.00076	E*G*-BC	33.08 ± 0.10	5.77 ± 0.02	0.73 ± 0.03
	R. fascians B.01614-*P. putida* B.01157-*B. subtilis* B.01162-*B. coagulans* B.01123-*B. cereus* B.00076	E*G*-ABC	33.28 ± 0.95	5.80 ± 0.02	0.64 ± 0.04

Degradation rate of bacterial mixed cultures

Various consortia appeared to have a significant effect in pretreatment of lignocellulose substrate ($p < 0.05$). Specifically, hydrolytic bacteria can alter polysaccharide structure under different degradation pathways. As consequence, ligninolytic consortia caused higher solid losses than cellulolytic consortia (**Table 4.5**). *Bacillus subtilis* B.01162 and its co-cultures with *B. coagulans* B.01123, *B. cereus* B.00076 accounted for 28.7-33.8% of solid weight loss, whereas ligninolytic mixtures caused the weight loss percentage ranged from 45.7-55%. Growing cellulolytic and ligninolytic species in the same habitats, we found a relatively higher solid loss under the cultivation of some specific microbial mixtures in the presence of C*K*, D*K* and cellulolytic consortia (Cluster I). In contrast, either K*, G*K*, or E*G* seemed not to have synergistic action with *Bacillus* cellulolytic species to deteriorate the harsh structure of lignocellulosic biomass, through the low percentage of weight loss residues.

Concerning total phenolic accumulation, we also discovered the strong correlation between a ratio of the aromatic compound and weight loss in the case of ligninolytic pretreatment. Typically, the total phenolic accumulation ratios produced by mixtures of C*K* and D*K* with cellulolytic strains (Cluster I) were observed at an outstanding level compared to the remaining communities, approximately 2-3 folds higher than those in cluster II, III. The enhancement in the production of aromatic compounds revealed the co-existence of multiple strains and their synergistic relationship in the same habitats. Synergistic relationship of microbes in community gets rid of catabolic limitations, improve streams towards desired chemicals, or enhance microbial resistance to toxicity (Singh et al., 2019) and show synergistic functions by collaborated partners. Li and co-workers (2019) found the highest lignin degradation rate was 23.2% for the single strain fermentation and 33.6% for the three co-cultured strains.

Moreover, while efficient consortia in cluster I worked on the alkaline pH values ranging from 8.5-9.0, whereas slight acidic pH values of 5.0-6.0 were found in consortia in other clusters. When evaluating degrading capacity of *Streptomyces viridosporus* on lignocellulosic biomass, Pometto and Crawford (1986) found lower substrate weight loss at neutral to slightly acidic pH, also correlated to lignin mineralization instead of lignin solubilization which strongly occurred at alkaline pH values. An increase in pH from neutral to alkaline is consistent with the previous work of Shruti and co-workers (2015), in which they reported the degradation of rice straw increased with pH values using microbial complex isolated from rotting and field soil. Competitive interaction between members in the same habitat was also mentioned.

Enzymatic performance of combined cultures of cellulolytic and ligninolytic strains

The enzymatic activities of bacterial co-cultures of cellulolytic and ligninolytic strains was demonstrated in **Figure 4.15**.

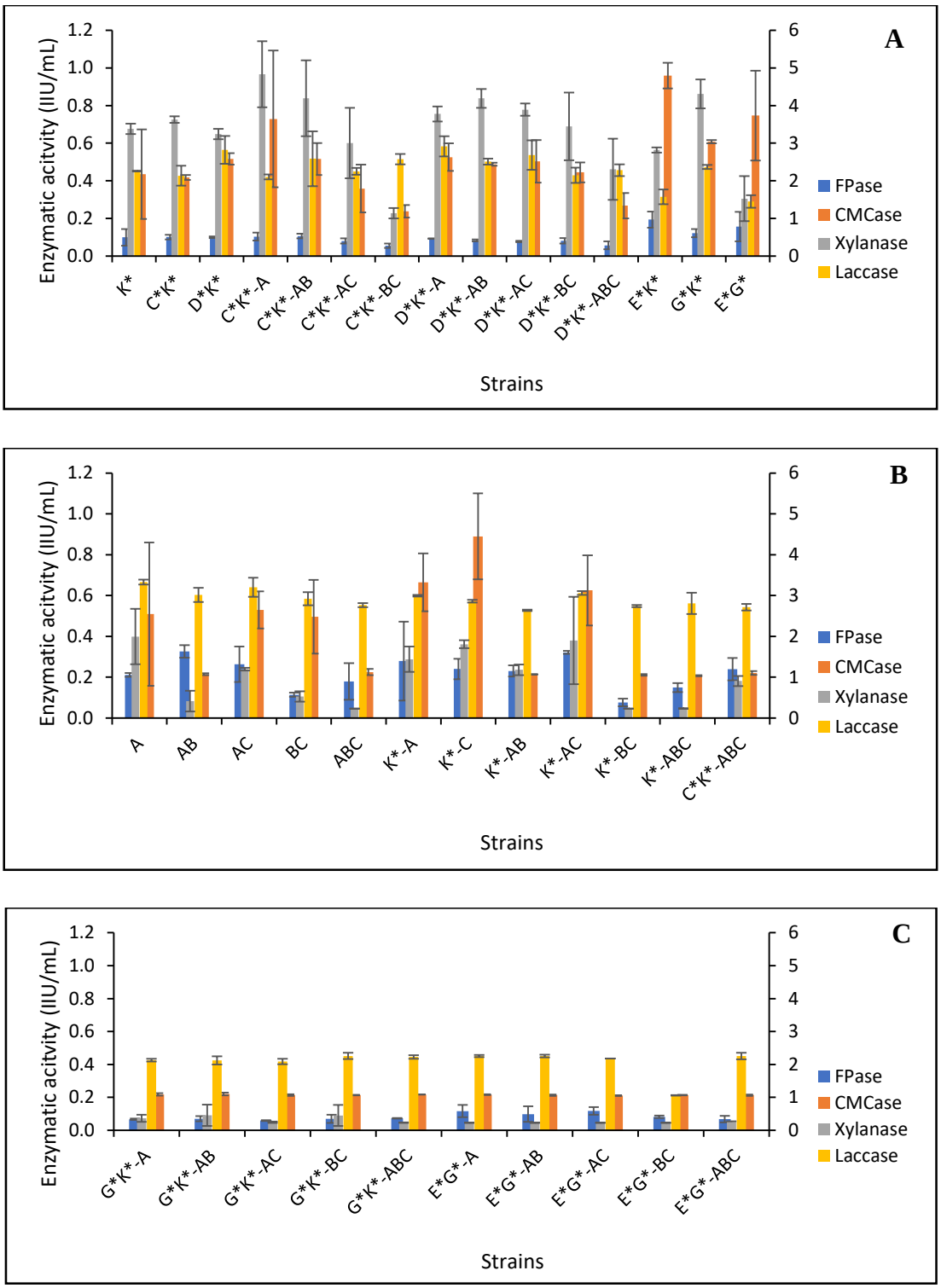

Figure 4.15 Enzymatic properties of three clusters I (A), cluster II (B) and cluster III (C)

The ligninolytic consortia were classified into cluster I due to their great performances on high enzyme activities of endo-glucanase, xylanase and laccase. Ligninolytic species C*K*; D*K* coordinate with *Bacillus* strains and showed a more effective effect on breaking down the lignin structure, facilitating the enzymatic attack on cellulose and hemicellulose structure. The highest total cellulase enzyme activity was found in cellulolytic consortia AB and mixed culture

of *P. putida* B. 01522 (K*) and 2 *Bacillus* species (K*-AC) with values of 0.213 and 0.206 IU/mL. On the other hand, the degradation effect of cellulolytic consortia and other collaborations of K*, G*K*, E*G* with cellulolytic species were dominated by laccase. Endo-glucanase activities of these mixtures were less active than other communities. Thus, K* and cellulolytic co-culture was highly evaluated for its degradation capacity due to degrading enzyme system of each member.

Sugar conversion

The sugar fractions after 72 hrs of biological pretreatment using a mixture of microbes were analysed and illustrated in three clusters with typical degradation profiles (**Figure 4.16**).

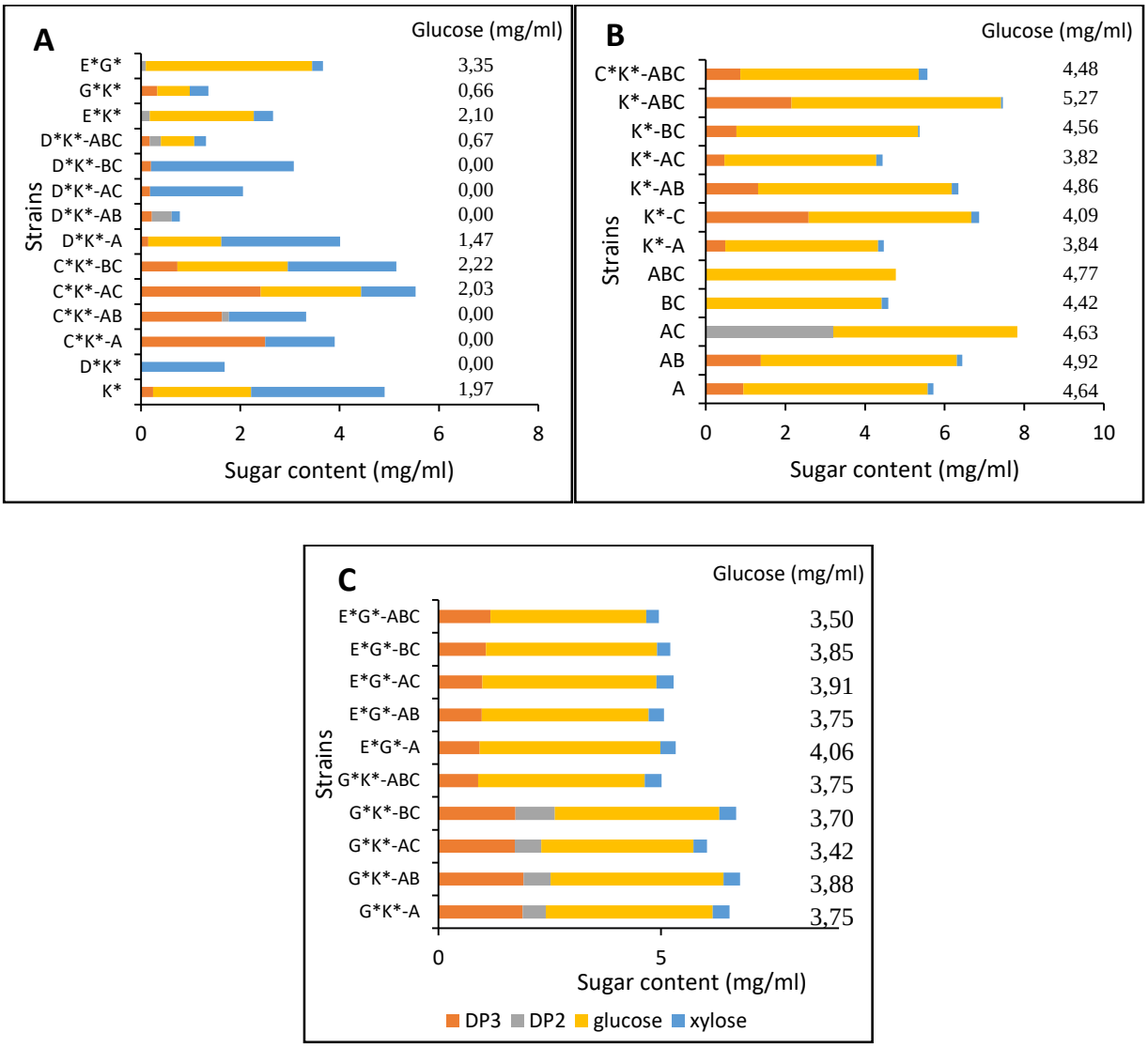

Figure 4.16 The sugar conversion of pretreated lignocellulosic biomass by bacterial consortia in three clusters (cluster I (A), cluster II (B), cluster III (C))

Mono sugars as glucose and xylose were the desired products after the pretreatment. Glucose was found as a dominant oligosaccharide in extracted hydrolysates after cultivation of species classified in cluster II and cluster III, opposite to those in cluster I which possessed higher degrading enzyme activities. Consortia in cluster I were characterized with higher solid loss after pretreatment and great amount of TPC, which indicated the strong deteriorate activity of ligninolytic species. It was assumed that these consortia in cluster I can produce the high xylanase activity. However, they also consume a great amount of glucose which was released during the pretreatment for their metabolism. In comparison with monocultures, communities might be able to weather periods of nutrient limitation better due to the diversity of metabolic modes available in a mix of species and their communication among individuals enables the division of labour that results in exhibit complex function (Fay, 1992). The highest activities of total cellulase enzymes and moderate endo-glucanase enzymes were found in cluster II. The enhancement of enzymatic activities as well as degradation efficiency were observed in case of synthetic microbial consortia of ligninolytic and cellulolytic strains in cluster II, III.

4.2. Fungal pretreatment of lignocellulosic biomass

Degrading efficiency by filamentous fungi

The results of lignocellulose residue weight loss after pretreatment by fungi was summarized in **Figure 4.17**. As can be seen in the figure, there was a significant difference in solid weight loss of wheat bran treated by *Aspergillus niger* B.01162 (FA) with those treated by co-culture of *A. niger* F.00632 and *Trichoderma viride* F.00795 (FAC), while no significant difference between remaining filamentous fungi and their consortia ($p < 0.05$). Filamentous fungi have been considered the most effective degraders for decaying lignocellulosic residues because they can secrete various degrading enzymes, including cellulolytic, hemicellulolytic and ligninolytic enzymes and they interact synergistically during hydrolysis (Beguin and Aubert, 1994; Nidetzky et al., 1994; Pérez et al., 2002; Zhou and Ingram, 2000)

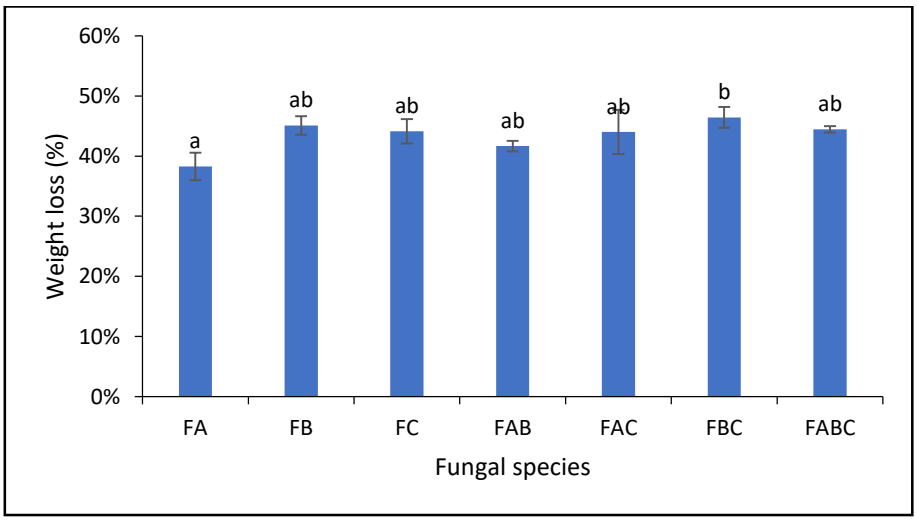

Figure 4.17 Weight loss of wheat bran by the pretreatment of fungi strains and their consortia

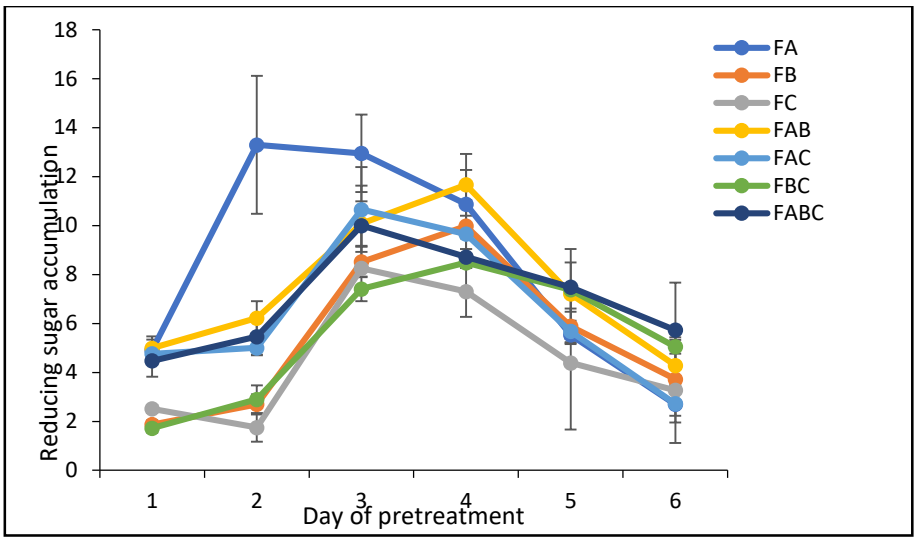

Figure 4.18 Reducing sugar vs. time of pretreatment by fungi

The reducing sugar accumulation ratio produced by filamentous fungi was presented in **Figure 4.18**. The result indicated that *A. niger* F.00632 could release the maximum reducing sugar yield after 48 hrs under suspended pretreatment, whereas other fungus and their co-culture needed more time to reach their peak of sugar yield. For instance, 72 hrs is needed for *T. viride* F.00795, co-cultures of *A. niger* F.00632-*T. viride* F.00795 and *A. niger* F.00632-*P. chrysogenum* F.00814-*T. viride* F.00795 achieved the maximum reducing sugar yields, while the remaining species including *P. chrysogenum* F.00814, co-cultures *A. niger* F.00632-*P. chrysogenum* F.00814 and *P. chrysogenum* F.00814-*T. viride* F.00795 needed 96 hrs. The reducing sugar yield generally decreased after reaching the peak in most cases. In comparison with bacteria, filamentous fungi can produce a significant high amount of reducing sugar yield than other species. It can be explained by the effective degrading enzyme system secreted by filamentous fungi fed with lignocellulosic substrates.

Production of degrading enzyme

Degrading enzymes produced by fungal species including total cellulase, endo-glucanase, xylanase and laccase were evaluated and summarized in **Table 4.6**. It was noted that the total cellulase activities ranged from 0.26 IU/gds to 0.41 IU/gds. Among the investigated species, co-culture of *A. niger* F.00632 and *T. viride* F.00795 achieved the highest hydrolysis capacity, total cellulase, endo-glucanase and xylanase activities of 0.411, 1.827 and 12.990 IU/gds, respectively. Filamentous fungi such as *A. niger*, *P. chrysogenum* and *T. viride* were well-known for the hydrolytic capacity due to their efficient degrading enzymes. However, we found the less degradation efficiency using fungal co-culture due to a low total cellulase activity from the hydrolysate extracted by co-culture of *P. chrysogenum* F.00814 and *T. viride* F.00795, whereas consortium of *A. niger* F.00632 and *P. chrysogenum* F.00814 has low endo-glucanase and xylanase activities. On contrary, mixture of *A. niger* F.00632 and *P. chrysogenum* F.00814 (FAB) produced maximal laccase activity of 5.877 IU/gds, followed by two-member consortia of *A. niger* F.00632 and *T. viride* F.00795 (FAC) of 4.215 IU/gds and monoculture of *A. niger* of 3.95 IU/gds.

Table 4.6 Enzyme production by fungi species and their consortium

Codes	Strains	Enzyme activities (IU/gds)			
		FPase	**CMCase**	**Xylanase**	**Laccase**
FA	*A. niger* F.00632	0.30 ± 0.06	1.67 ± 0.24	10.57 ± 1.95	3.95 ± 0.46
FB	*P. chrysogenum* F.00814	0.27 ± 0.03	1.53 ± 0.26	10.49 ± 1.30	2.67 ± 0.54
FC	*T. viride* F.00795	0.31 ± 0.13	1.43 ± 0.49	11.81 ± 3.68	3.03 ± 0.74
FAB	*A. niger* F.00632-*P. chrysogenum* F.00814	0.32 ± 0.02	1.34 ± 0.17	9.03 ± 0.72	5.88 ± 0.08
FAC	*A. niger* F.00632-*T. viride* F.00795	0.41 ± 0.04	1.83 ± 0.84	12.99 ± 4.96	4.21 ± 1.02
FBC	*P. chrysogenum* F.00814-*T. viride* F.00795	0.26 ± 0.03	1.70 ± 0.58	11.05 ± 2.21	3.64 ± 0.61
FABC	*A. niger* F.00632-*P. chrysogenum* F.00814-*T. viride* F.00795	0.32 ± 0.08	1.62 ± 0.50	10.63 ± 3.22	3.19 ± 0.77

Many authors has pointed out many benefits when using fungal co-cultures instead of mono-cultures such as improving adaptability and substrate utilization. Maheshwari and co-workers (1994), Kavitha and Nagarajan (2011), Ejechi and Obuekwe (1994) reported the increasing cellulolytic activity by *Aspergillus niger* and other fungal strains. Our results are in agreement with these studies with higher cellulase and hemicellulase activities under cultivation of co-cultures of *A. niger* F.00632 and *T. viride* F.00795 than those secreted by mono strains. Additionally, laccase produced by mixtures of filamentous fungi showed higher activity than by individual species. Previous studies found that co-culture of white-rot fungus *Funalia floccosa* LPSC 232 and *Penicillium commune* GHAIE86 (Rodríguez et al., 2019) or co-culture of *Trametes maxima* and *Paecilomyces carneus* (Cupul et al., 2014) accounted for the enhancement of laccase activity. The complementary action of degradation enzymes to facilitate the biodegradation of rice straw was reported by Kausar and co-workers (2010), resulting in the deterioration of lignin barrier, cellulose and hemicellulose to simple sugars.

Sugar profile of the hydrolysates

The soluble sugars present in the final hydrolysate were identified and quantified by high-pressure liquid chromatography. The results of this analysis were demonstrated in **Figure 4.19**. As can be seen, filamentous fungus efficiently produced a variety of sugars such as six-carbon sugar (glucose), a five-carbon sugar (xylose) and oligosaccharides such as maltose and cellobiose which were also present in the lignocellulose hydrolysates. Under cultivation of individual strains, *P. chrysogenum* F.00814 and *T. viride* F.00795 showed an outstanding total sugar yield than *A. niger* F.00632.

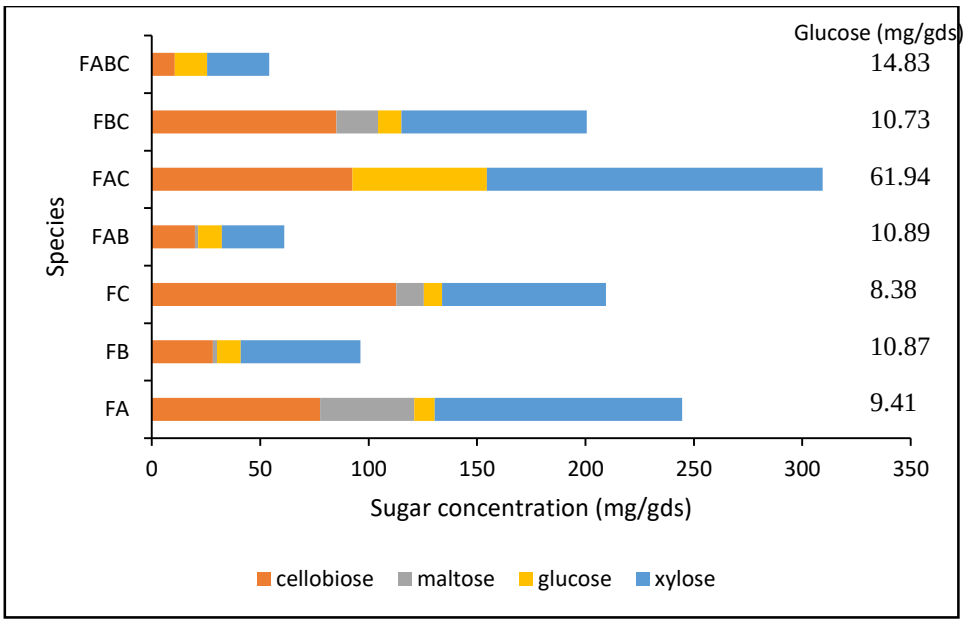

Figure 4.19 The sugar conversion of pretreated biomass by fungi and their consortia

In particular, these two strains exhibited a higher yield of cellobiose and xylose concentrations, and comparative glucose yield versus those produced by *A. niger* in suspended condition. The highest conversion rate was observed in samples treated by co-culture of *A. niger* F.00632 and *T. viride* F.00795, in which glucose concentration was 4-7 fold higher than in others. The maximum xylose yield of 61.944.21 mg/gds was also produced by this consortium. These positive results associated with synergistic action of three fungal cellulases on sugar conversion. On the other hand, a decrease of cellobiose and xylose in the fermented sugar yield was more evident in the co-culture of *A. niger* F.00632 - *P. chrysogenum* F.00814 and the tri-culture of three fungi species. Since these above consortia posed lower hydrolytic and laccase enzyme activities than others, it is postulated that insufficient collaborated action between members was responsible for the poor conversion of cellulose and hemicellulose.

4.3. Utilization of yeast as supplements

Eight yeast strains including *Yarrowia lipolytica* Y.85414; Y.00613; Y.00114, *Yarrowia divulgata* Y.02062; Y.05257, *Pichia stipitis* Y.00810; Y.00888; Y.00910; Y.01047 and the co-cultures of yeasts Y.02062-Y.00888 and Y.02062-Y.01047 were screened to evaluate the effect of yeast to the pretreatment efficiency.

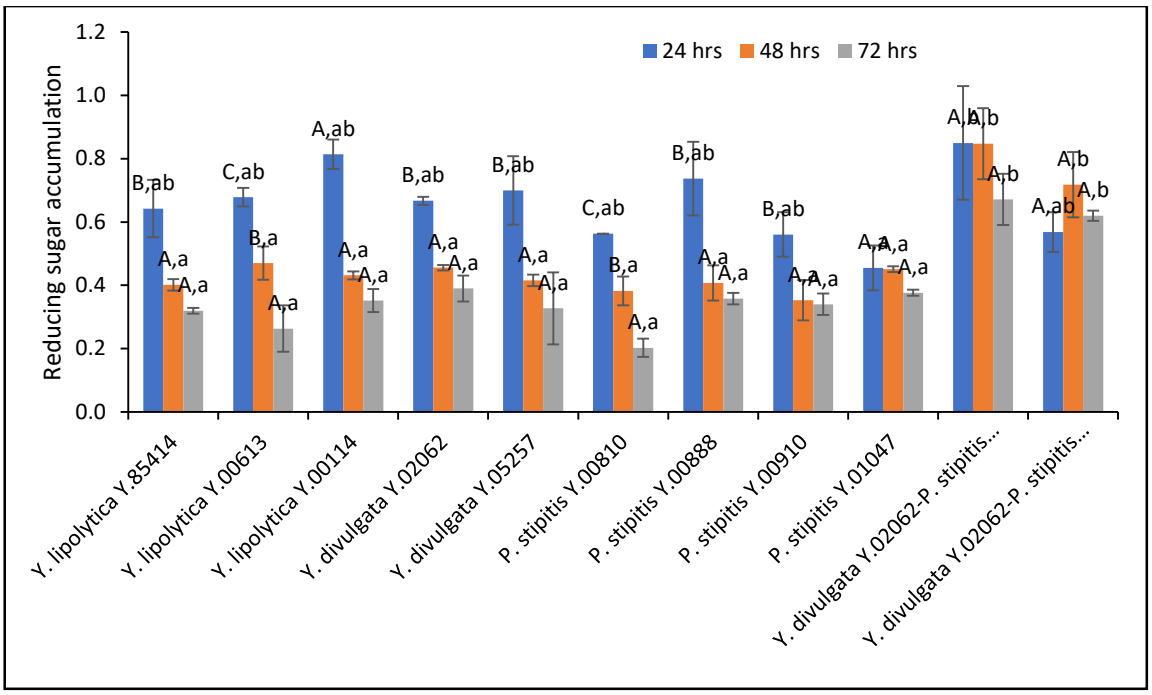

Figure 4.20 Reducing sugar accumulation ratio of yeast strains and their consortium after 24, 48 and 72 hrs of cultivation

The reducing sugar accumulation ratio was presented in **Figure 4.20**. As can be seen from the figure, individual yeast strains and their co-culture showed various degradation, and treatment time showed a significant effect on reducing sugar yields. Among yeast strains, *Y. lipolytica* Y.00114 showed the highest reducing sugar accumulation ratio after 24 hrs of the pretreatment with a value of 0.81. Day after witnessed the sharp decrease of reducing sugar on the 2nd and the 3rd day of the process, 1.4 and 1.9-times lower, respectively. Interestingly, great sugar yields were detected in samples pretreated by yeast consortia. In these cases, reducing sugar yields did not depend on pretreatment duration. In another work, reducing sugar yields tended to stay stable or with negligibly fluctuated amplitudes. Solid weight loss of lignocellulose biomass and pH values were summarized in **Table 4.7**. No significant difference of solid residue after 3-day process using yeast species. Acidic pH values were observed in most samples.

Yeast was highly evaluated for its hydrolytic capacity by its capacity to produce xylanase enzymes. Combining yeast with other microorganisms could enhance the bioprocess efficiency due to yeast's capacity to generate amino acids, which could be used as a supplement for other strains in the microbial communities. Therefore, one of my tasks is to determine xylanase activities and detect amino acids for selecting yeast strains as members of effective complex microbial consortia used for biological pretreatment. The hydrolysis capacity of yeast was determined by xylanase assay. Among investigated yeast species, the highest xylanase activities were observed in samples cultivated with *Y. divulgata* Y.02062, Y.05257 with values of 0.35 IU/gds and 0.38 IU/gds, respectively. The remaining species showed no significant differences on degradation effect. To investigate the potential capacity of yeast co-culture in pretreatment of lignocellulose, three yeast strains *Y. divulgata* Y.02062, *P. stipitis* Y.00888 and *P. stipitis* Y.01047, which have

equivalent growth capacity in the YPD culture medium, were chosen to collaborate with each other.

Higher xylanase activity in a treated sample using the co-culture of *Y. divulgata* and *P. stipitis* was found than those from pure cell incubation. For instance, co-cultures of *Y. divulgata* Y.02062-Y.00888 and *P. stipitis* Y.02062-Y.01047 secreted xylanase activity with enzyme titers 0.48 IU/gds and 0.33 IU/gds, respectively. Moreover, the optical quantitative analysis revealed that yeast co-culture could release a larger amount of amino acids than single cells due to the greater color intensity of aromatic benzene ring underwent nitration at water boiling temperature.

Table 4.7 Degradation profiles by yeast

Species	Weight loss (%)	Xylanase (IU/gds)	pH
Y. lipolytica Y.85414	24.85 ± 2.00	0.31 ± 0.02	5.91 ± 0.05
Y. lipolytica Y.00613	24.13 ± 0.17	0.25 ± 0.10	5.70 ± 0.05
Y. lipolytica Y.00114	23.30 ± 2.29	0.29 ± 0.03	5.44 ± 0.03
Y. divulgata Y.02062	23.50 ± 2.99	0.35 ± 0.20	5.88 ± 0.23
Y. divulgata Y.05257	20.15 ± 5.94	0.38 ± 0.03	5.95 ± 0.39
P. stipitis Y.00810	25.49 ± 4.20	0.19 ± 0.01	5.68 ± 0.12
P. stipitis Y.00888	24.54 ± 3.62	0.25 ± 0.05	6.15 ± 0.28
P. stipitis Y.00910	27.38 ± 2.73	0.25 ± 0.09	5.62 ± 0.10
P. stipitis Y.01047	25.77 ± 1.25	0.22 ± 0.09	6.06 ± 0.77
Y. divulgata Y.02062-*P. stipitis* Y.00888	29.64 ± 1.58	0.48 ± 0.05	6.28 ± 0.39
Y. divulgata Y.02062-*P. stipitis* Y.01047	24.06 ± 1.87	0.33 ± 0.09	5.29 ± 0.05

The sugar conversion was performed in **Figure 4.21**. The best conversion rate was found in the case of co-cultures of two yeast species including *Y. divulgata* and *P. stipitis*. Among individual yeasts, *P. stipitis* genus seemed to have a better performance on fermented sugar production than *Y. divulgata*, in general. The derivate products after pretreatment such as glucose and xylose are used to evaluate the efficiency of the process. Therefore, the outstanding yield of mono sugar was an indication of the potential degradation effect of yeast co-cultures in biological pretreatment. The maximum glucose concentration of 67.36 mg/gds was measured in pretreated lignocellulose substrates by co-culture of *Y. divulgata* Y.02062 and *P. stipitis* Y.00888, followed by *P. stipitis* Y.00910. A relatively high amount of cellobiose was observed in pretreated wheat bran with yeast mixed cultures compared with monocultures after 72 hrs of pretreatment. The increase of each soluble sugar could accumulate to the final yield of fermented sugars, stimulating ethanol production.

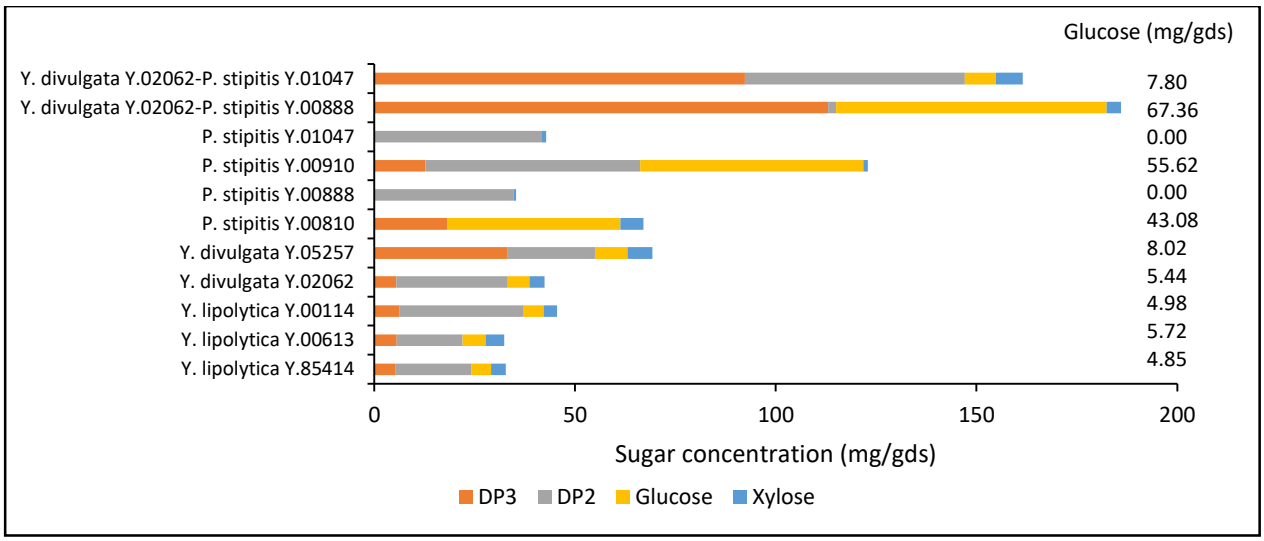

Figure 4.21 The sugar conversion of pretreated lignocellulosic biomass using yeast and their co-cultures

4.4. Optimization of operating parameters

Synthetic microbial consortia consisted of two or more defined microbial populations were assembled in a well-characterized and controlled environment. An effective consortium can not only carry out the desired functions but also sustain cell growth in a stable and robust way. We created a synthetic microbial ecology according to bottom-up approach (from simple to complex) and individual members from common environmental origins were selected. The bottleneck of effective co-culture of different species are different genetic makeup, enzymatic components and ecological niche. Despite having complexity to grow in the same culture medium, several attempts have been made to understand the microbial community of communication, their secretions, adaptation and possible application on pretreatment. Environmental factors have been shown to affect the rate of biomass degradation and play an important role in changing the physicochemical structure of lignocellulosic biomass. Therefore, several operating factors which affect the biological pretreatment such as cultivation methods, pH, medium culture, etc. were investigated. The trial tests were carried out using individual species, then optimized operating conditions were applied to pretreatment using microbial co-culture.

4.4.1. Effect of culture medium and pH

Culture medium and its pH have significant role in growth and metabolic activities of microorganisms. In this experiment, two pH values (4.5 and 6.5) and two culture medium (citrate phosphate buffer and basal medium) were applied for fungal pretreatment in submerged cultivation. Farkas and co-workers (2019) used citrate phosphate buffer as culture medium, she found the acidic pH ranged from 4.0 to 6.0 was preferable for filamentous fungi to obtain lignocellulose degrading enzymes with high activities at the low ratio of culture medium to solid

substrates (from the ratio of 5:1 to 7:1). Meanwhile, submerged bioprocess was best suited bacteria that required high moisture content (Ravichandran and Vimala R, 2012) and at neutral pH, maximum production of hydrolytic enzyme was achieved (Bano et al., 2013). Supplements in culture medium can affect to fungal growth and degradation of biomass (Wan and Li, 2010). Messner and co-workers (1998) found addition of minerals in basal medium contributed to the formation of fungal biomass and facilitation of fungal colonization in the deeper areas of feedstocks. Therefore, in this experiment the optimized process conditions for the growth of different microbial populations at the same habitat to achieve an effective lignocellulosic pretreatment were figured out.

The degradation rate of wheat bran using *A. niger* F.00632 in submerged process was demonstrated in **Figure 4.22**. There was no significant difference in the weight loss as well as sugar content of pretreated substrates at different pH values or culture medium. However, reducing sugar yield was highly achieved in basal medium instead of citrate buffer under fungal pretreatment.

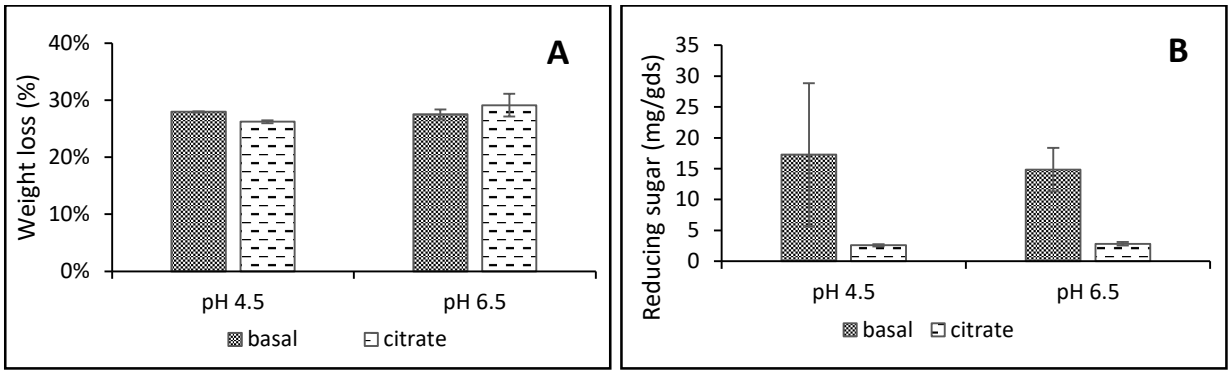

Figure 4.22 Degradation efficiency in biological pretreatment by fungi using different culture medium and pH values: weight loss (A), reducing sugar yield (B)

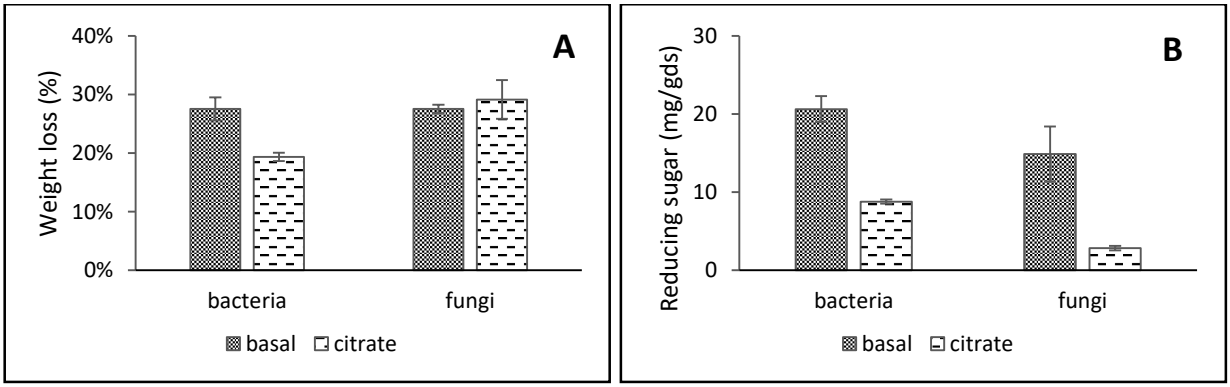

Figure 4.23 Degradation efficiency by bacterial co-cultures using different culture medium (A: Weight loss, B: reducing sugar yield)

The effect of different culture mediums on bacterial pretreatment was also evaluated. The co-culture of *Pseudomonas putida* B.01522 and *Bacillus subtilis* B.01162 selected from lignocellulosic and cellulolytic genus was cultivated in submerged medium using citrate phosphate buffer or basal medium at pH 6.5. **Figure 4.23** illustrated the different degradation characteristics using bacterial co-culture of *P. putida* B.01522-*B. subtilis* B.01162 and filamentous fungi. Results of weight loss and reducing sugar yield indicated basal medium promoted microbial metabolic, resulting in the improved degradation rate and hydrolysis efficacy.

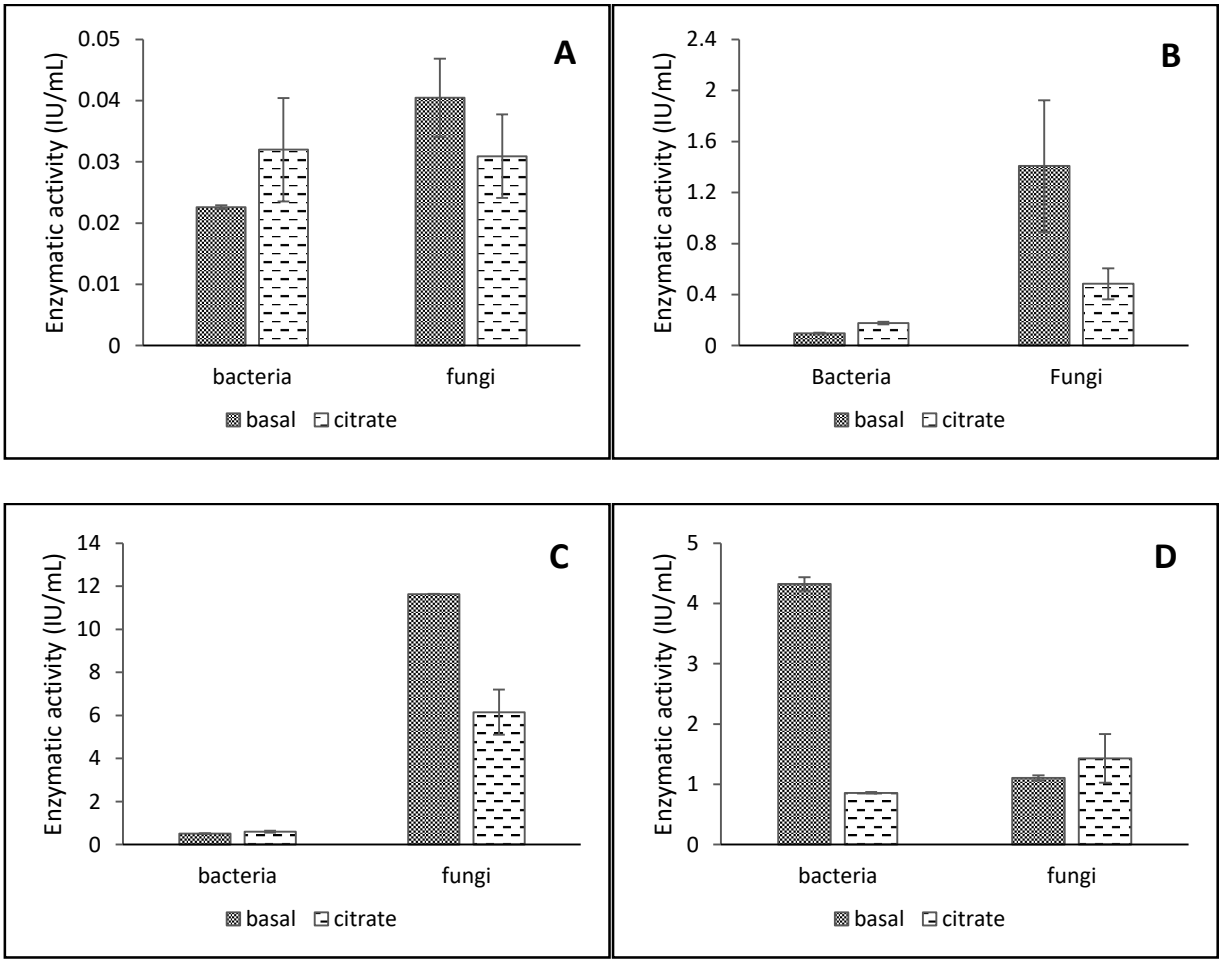

Figure 4.24 Comparison of enzymatic characteristics under cultivation of bacteria and fungi in submerged pretreatment (A: FPase, B: CMCase, C: xylanase and D: laccase)

Figure 4.24 demonstrated the effect of enzymatic activity on decomposition of lignocellulose under cultivation of bacterial co-culture or fungi. Three hydrolytic enzymes were tested including total cellulase, endoglucanase and xylanase. In general, fungi showed higher hydrolase activity in all case studies. Specifically, total cellulase enzymes extracted from fungi showed higher activity than those extracted from bacteria in basal media, 0.04 IU/mL versus 0.022 IU/mL. However, no significant difference of FPase activity was found between two culture media. In terms of endo-glucanases and xylanases, the highest enzymatic activities were found in hydrolysates extracted from samples pretreated by fungi. A significant improvement in hydrolytic enzymes from the basal medium as to citrate buffer medium was observed, with endoglucanase

activities of 1.407 to 0.484 IU/mL and xylanase activities of 11.637 to 6.151 IU/mL, respectively. On the other hand, bacteria reached the maximum laccase activity in the basal medium, but no significant difference was found between two approach using citrate-phosphate buffer. As consequence, the culture media of basal medium at initial pH of 6.5 were applied to further studies.

4.4.2. Effect of the liquid:solid ratio

The ratio of liquid:solid is one of the dominant keys for microbial growth, which involved the response of microbial cells to water potential stress. Microbes could adjust the different components of their total potential stress to achieve thermodynamic water potential equilibrium with their environment (Harris, 1981). We expected to construct efficient microbial consortia from a diversity of microbes which favour to different growing conditions. Therefore, optimizing liquid:solid ratio for microbial pretreatment is the critical point in this study. Regarding fungal bioprocess, Zadrazi and co-cultures (2000) claimed that excess moisture could inhibit fungal growth, especially in the deep layers with little air and mycelia. Higher moisture content also reduces solid loading for biological pretreatment. Another side effect of too low moisture is to hamper fungal delignification without providing sufficient water for fungal growth (Wan and Li, 2012). The other approach involved to bacteria, high liquid content, is required to produce high bioactive compounds. Various ratios of liquid:solid of 4:1, 5:1, 6:1, 7:1, 8:1 and 9:1 were investigated to evaluate the effect of microorganisms, especially bacterial co-culture and filamentous fungi on degradation efficiency. Our results were relevant to previous studies that claimed that at the moisture content of around 70%, fungi and bacteria were enriched, and possessed higher metabolite production and enzyme activities (Xia and Cen, 1999).

The effect of liquid:solid ratio on the pretreatment by bacterial co-culture composed of *P. putida* and *B. subtilis* and filamentous fungi *A. niger* was demonstrated in **Figure 4.25**.

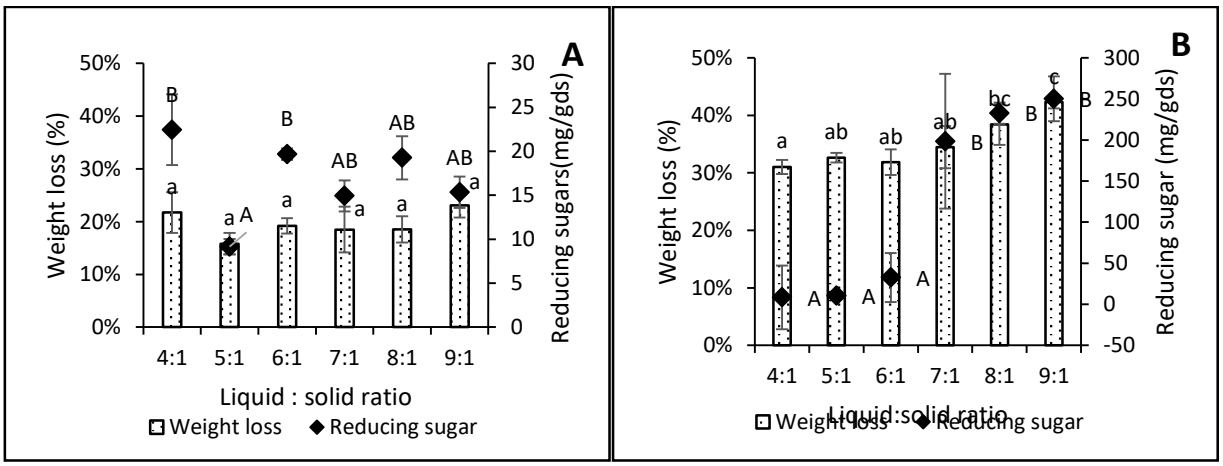

Figure 4.25 Effect of moisture in degradation efficiencies in biological pretreatment using bacteria (A) and fungi (B)

Regarding bacterial pretreatment, the weight loss of pretreated lignocellulosic biomass didn't show any significant difference at any liquid:solid ratio (**Figure 4.25 A**). On the contrary, it was found that the liquid content has a strong effect on fungal pretreatment. Specifically, relatively higher weight loss was observed at liquid:solid ratio of 9:1; in other words, an increased liquid content accounted for higher loss of pretreated lignocellulosic biomass. Previous studies suggested that most white rot fungi have an initial humidity of 70-80%, the optimal level for lignin degradation. Shi and co-cultures (2008) observed that after 14-day cultivation of cotton stalks by *P. chrysosporium*, lignin degradation of 27.6% was obtained at the moisture content of 75%, which was 7% efficiency higher than at moisture of 65% and similar to those at the moisture of 80%. Asgher and co-workers (2006) studied the solid-phase culture of *P. chrysosporium* on maize cobs with moisture content ranged from 40% to 90%. They found that the highest ligninase activity was obtained at 70% moisture content. During the fungal pretreatment of maize straw with *C. subvermispora*, no fungal growth and fungal degradation occurred when the moisture content was 45%. Regarding fungal pretreatment, the highest reducing sugar yield ranged from 198.42-250.05 mg/gds was observed at liquid:solid ratio of 7:1, 8:1 and 9:1 (**Figure 4.25B**). The low liquid content could restrict the degradation capacity of fungi, showing an insufficient reducing sugar content. Lonsane and co-workers (1992) referred that the slow and inefficient metabolic and enzymatic activity by microbes under low moisture which reduce the solubility of nutrients from a solid substrate, low substrate swelling and higher water tension. Thus, it is crucial to provide optimized water content and control the water activity of the bioprocess (Nigam and Pandey, 2009).

4.4.3. Effect of cultivation methods

The performance of bacterial-fungal consortium was assessed in terms of weight loss, reducing sugar and enzyme activities required for conversion of lignocellulosic biomass. Research studies on suspended cultivation of microorganisms have shown that co-culture of bacteria and fungi have higher enzyme activities and degradation rate than submerged cultivation.

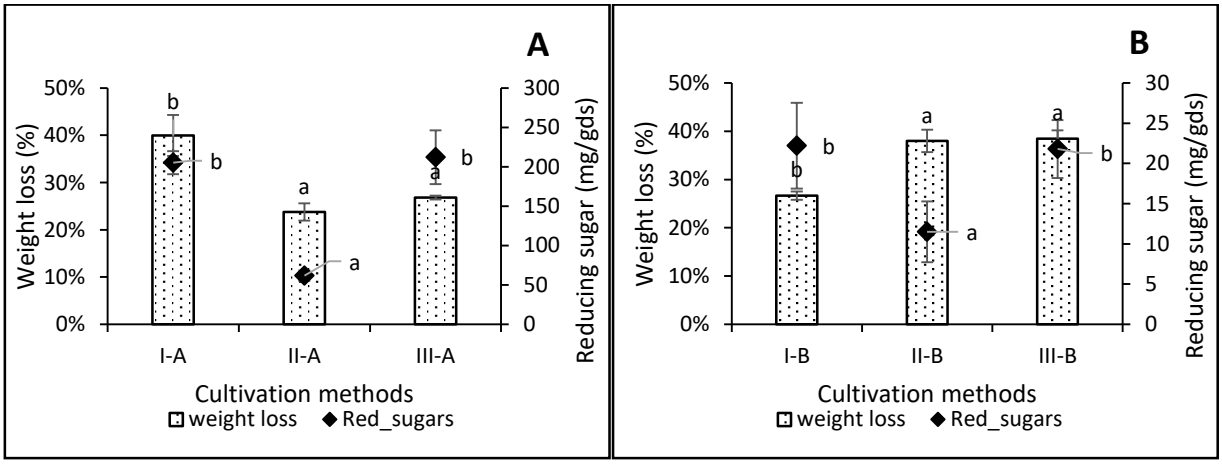

Figure 4.26 Effect of two cultivation methods on degradation efficiency in biological pretreatment of lignocellulosic biomass: Suspended (A) and submerged (B) pretreatment

A previous study by Jain and co-workers (2016b) found that bacterial-fungal consortia enriched from switch grass bales could produce enzyme mixtures required for the microbial deconstruction of ammonia-pretreated sweet grass. The degradation rate and reducing sugar production in two cultivation methods were illustrated in **Figure 4.26**.

In the suspended pretreatment, firstly-inoculated *A. niger* followed by bacterial co-culture 24 hrs later resulted in high weight loss of 40% after 72 hrs pretreatment (**Figure 4.26A**). Oppositely, the enrichment of bacteria co-culture before the fungal cultivation restricted the growth filamentous fungi, probably due to nutrient depletion from bacterial metabolite process. The suspended cultivation observed higher endo-glucanase activity when fungi added at the first stage instead of bacterial co-culture, and showed no significant difference with simultaneous microbial inoculation (**Figure 4.26A**). Addition, ten times higher of reducing sugar yield was found under suspended cultivation in comparison to submerged approach. Firstly-inoculated *A. niger* or simultaneous cultivation of microbes achieved reducing sugar yield of 3.3-times higher than firstly inoculated bacterial co-culture approach, resulting in an efficient breaking down of lignocellulose biomass after 72 hrs pretreatment.

Similar reducing sugar profiles under submerged cultivation was observed. However, firstly-inoculated *A. niger* approach did not gain proper degradation rate (weight loss of 27%) and showed disadvantages compared to other routes (**Figure 4.26B**). It could be consumed that submerged cultivation is a favor to bacteria, thus the approach of firstly-fungi inoculation likely restricts the degradation rate. The endo-glucanase as well as xylanase activity, were found higher in extracted liquid cultivated by bacteria in the first stage than in other methods in submerged pretreatment (**Figure 4.27B**).

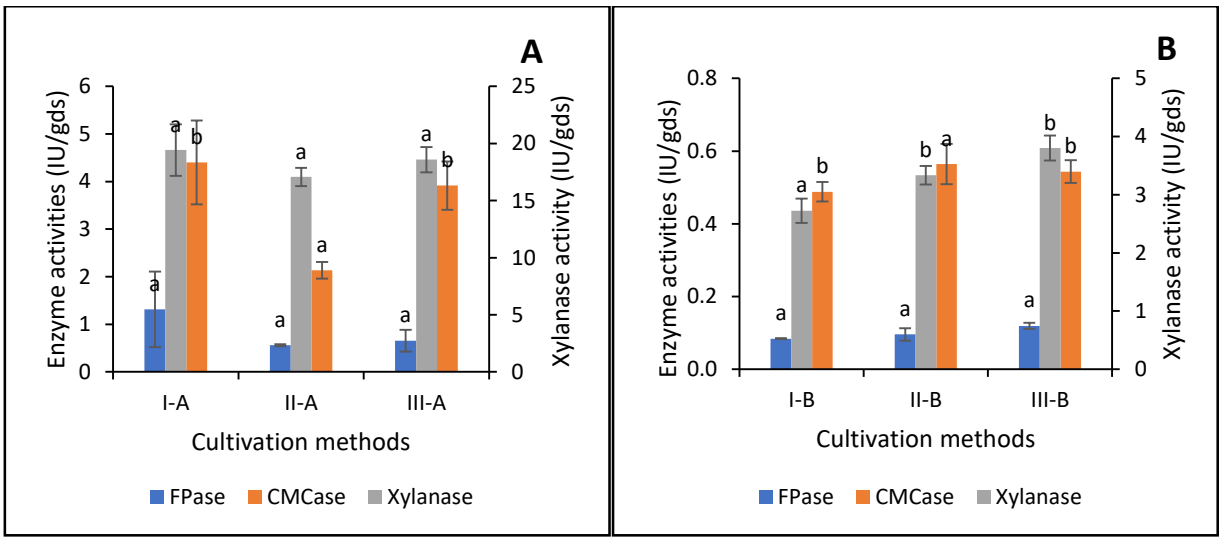

Figure 4.27 Effect of two cultivation methods on enzymatic production in biological pretreatment of lignocellulosic biomass (A suspended and B submerged pretreatment)

Values of pH in culture medium during 72 hrs pretreatment was reported, determined by the synergistic effect of species living in the same habitat. For instance, prior cultivation of *A. niger* 24 hrs before adding bacteria maintains pH neutral for the culture medium in suspended fermentation. On the contrary, a pH drop was observed in the submerged medium using a similar cultivation method. Simultaneous cultivation of both fungi and bacteria or prior cultivation of bacteria to fungi are responsible for the slightly acidic property which was proved by the dominant growth of bacteria in the consortium. The addition of bacteria co-culture after 24 hrs of fungal inoculation could produce more acidic compounds in a submerged medium.

4.5. Development of the effective microbial consortia

4.5.1. Promising approach

Microbial biodiversity is always crucial for any ecosystem as they play the vital role in dynamic equilibrium, which has been attributed to communication systems of agricultural ecosystem. Microbial consortia have more advantages than an individual inoculum, due to the synergistic relationship of microorganisms that co-exist in the same habitat. We found that mixed culture of cellulolytic Bacilli and ligninolytic bacteria promoted hydrolytic efficiency as enhancement of degrading enzyme activities and sugar yields. Besides, degradation capacity of fungi under suspended pretreatment was highly evaluated, either are they join in co-culture or work individually. Additionally, yeast has a promising capacity on sugar conversion. Therefore, three bacterial strains including *Bacillus subtilis* B.01162, *Rhodococcus opacus* B.01915 and *Pseudomonas putida* B.01522, three fungi as *A. niger*, *P. chrysogenum* and *T. viride* and two yeast strains *Yarrowia divulgata* Y.02062 and *Pichia stipitis* Y.00888 were employed for construction of complex microbial consortia. Each species possessed unique degradation characteristics, however, the survival and continue success of biologically active microbial consortia in highly competitive and hostile environment is still challenging. Sixteen microbial co-cultures were developed under optimized suspended pretreatment, then evaluated to select efficient consortium which can obtain multiple benefits in downstream process.

Cluster analysis with Ward's minimum variance was used to classify these strains and microbial consortia based on the degrading parameters including weight loss, reducing sugar and enzyme activities (total cellulase enzyme, endo-glucanase and xylanase enzymes).

4.5.2. Construction of complex microbial consortia

The three clusters denoted as A, B, and C were classified among 16 variances. Fungi and their consortium were grouped in Cluster A, Cluster B including bacteria, yeast, and co-culture of fungi and yeast, Cluster C contains microbial consortia of bacteria and fungi, yeast as well as complex cultures of bacteria, fungi and yeast together. The degradation characteristics of various microbial communities were presented in **Table 4.8**.

Table 4.8 Degradation parameters of wheat bran substrate in the cultivation of mono and mixed culture in the pretreatment

Cluster	Microbes	Codes	Weight loss (%)	Reducing sugar accumulation	pH
Cluster A	*A. niger* F.00632	**F1**	42.32 ± 1.13	3.78 ± 0.41	4 ± 0.17
	A. niger F.00632 + *P. chrysogenum* F.00814 + *T. viride* F.00795	**F2**	44.45 ± 0.54	10 ± 1.63	4.23 ± 0.67
Cluster B	*B. subtilis* B.01162 + *P. putida* B.01522	**B1**	23.07 ± 2.3	0.23 ± 0.03	5.89 ± 0.02
	R. opacus B.01915 + *P. putida* B.01522	**B2**	33.93 ± 1.63	0.31 ± 0	8.81 ± 0.02
	B. subtilis B.01162 + *R. opacus* B.01915 + *P. putida* B.01522	**B3**	32.82 ± 0.67	0.32 ± 0.01	8.83 ± 0.04
	Y. divulgata Y.02062	**Y1**	23.5 ± 2.99	0.31 ± 0.14	5.88 ± 0.23
	Y. divulgata Y.02062 + *P. stipitis* Y.00888	**Y2**	29.64 ± 1.58	0.67 ± 0.08	6.28 ± 0.39
	A. niger F.00632 + *Y. divulgata* Y.02062	**FY**	35.14 ± 3.72	1.05 ± 0.17	4.81 ± 0.11
Cluster C	*B. subtilis* B.01162 + *P. putida* B.01522 + *A. niger* F.00632	**BF**	26.83 ± 0.39	3.21 ± 0.52	5.56 ± 0.03
	B. subtilis B.01162 + *P. putida* B.01522 + *Y. divulgata* Y.02062	**BY**	35.45 ± 2.65	3.5 ± 0.26	5.99 ± 0.01
	B. subtilis B.01162 + *P. putida* B.01522 + *A. niger* F.00632 + *Y. divulgata* Y.02062	**BFY1**	36.15 ± 3.65	1.72 ± 0.36	5.65 ± 0.03
	B. subtilis B.01162 + *P. putida* B.01522 + *A. niger* F.00632 + *P. chrysogenum* F.00814 + *T. viride* F.00795 + *Y. divulgata* Y.02062	**BFY2**	28.47 ± 1.3	1.62 ± 0.94	5.89 ± 0.01
	R. opacus B.01915 + *P. putida* B.01522 + *A. niger* F.00632 + *Y. divulgata* Y.02062 + *P. stipitis* Y.00888	**BFY3**	39.11 ± 2.93	2.19 ± 0.47	5.91 ± 0.34
	B. subtilis B.01162 + *R. opacus* B.01915 + *P. putida* B.01522 + *A. niger* F.00632 + *Y. divulgata* Y.02062 + *P. stipitis* Y.00888	**BFY4**	36.23 ± 2.74	1.48 ± 0.36	5.62 ± 0.04
	R. opacus B.01915+*P. putida* B.01522+*A. niger* F.00632 + *P. chrysogenum* F.00814+*T. viride* F.00795+*Y. divulgata* Y.02062+*P. stipitis* Y.00888	**BFY5**	33.85 ± 1.28	1.78 ± 0.24	5.95 ± 0.01
	B. subtilis B.01162 + *R. opacus* B.01915 + *P. putida* B.01522 + *A. niger* F.00632 + *P. chrysogenum* F.00814 + *T. viride* F.00795 + *Y. divulgata* Y.02062 + *P. stipitis* Y.00888	**BFY6**	36.39 ± 1.72	1.83 ± 0.16	5.71 ± 0.02

The characteristics of each group were analysed and presented in **Figure 4.28**. The high hydrolytic enzyme activities and superior reducing sugar yields were found in cluster A, where the total cellulases varied less and remained at a higher level than other enzymes. A strong collaboration of fungal hydrolytic enzyme system account for the coherence on biomass decomposition, along with high reducing sugar yields. Meanwhile, microbial consortia belonging to cluster B showed moderate reducing sugar concentration and less polysaccharide loss than fungal pretreatment. Positively, microbes in this group account for high hydrolytic enzymes. The remaining group, including co-culture of bacteria with other species and complex consortium of bacteria, fungi and yeast, produced the lowest degrading enzymes and reducing sugar yield. The result of cluster analysis can demonstrate the typical degrading characteristics of microbial consortia.

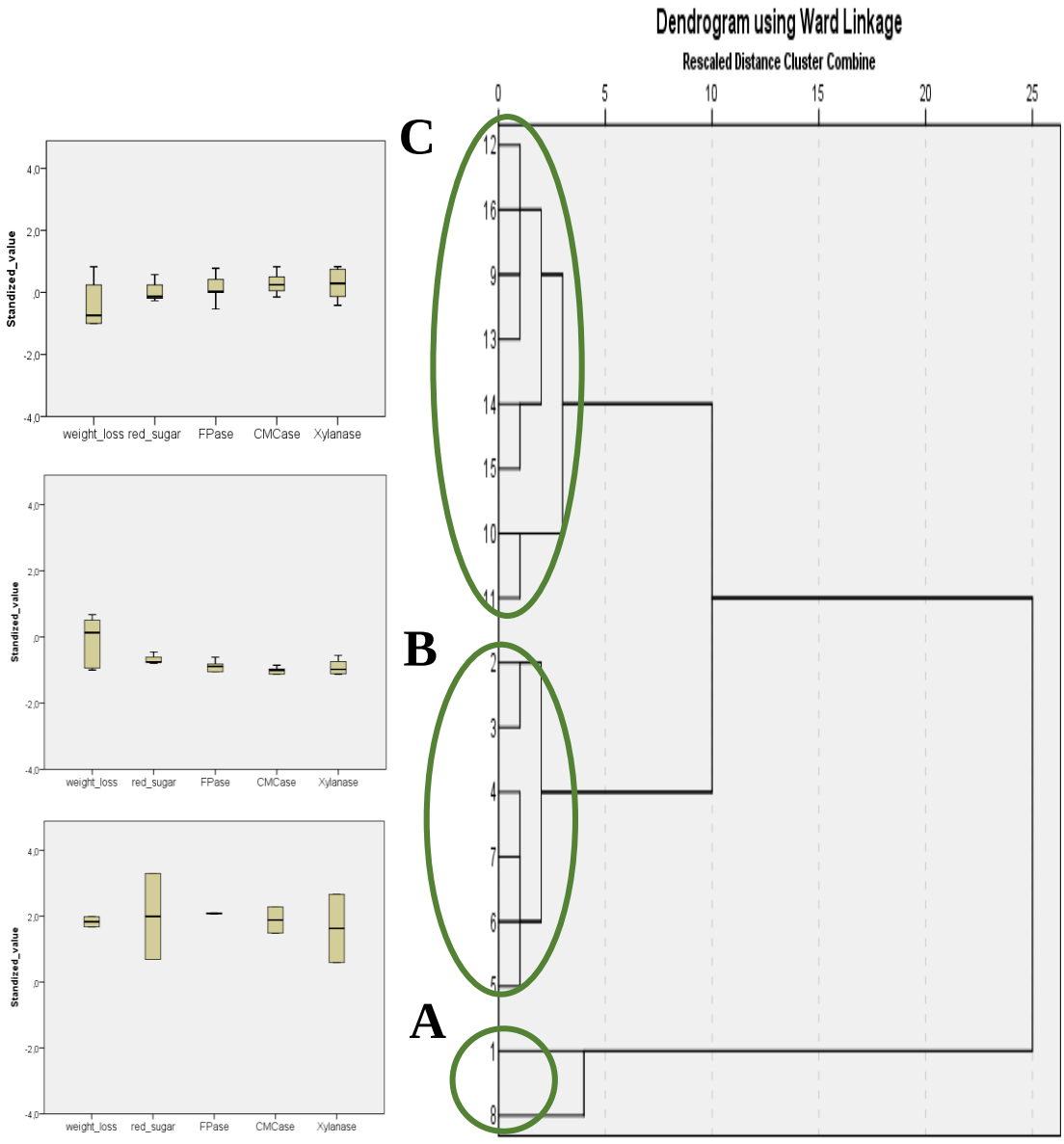

Figure 4.28 Cluster analysis of degrading criteria and their characteristics in the pretreatment by the complex consortia using Ward's method

Group 1: Fungi and their co-cultures

The suspended condition of liquid:solid ratio of 9:1 was sufficient for the growth of fungal population. *A. niger*, *T. viride*, *P. chrysogenum* showed the outstanding performance of lignocellulose degradation. These were in agreement with previous authors who reported an interactive effect on decay lignocellulosic residues by rich enzyme systems secreted from fungi (Haab et al., 1990; Kang et al., 2004; Pirota et al., 2013; Tsao et al., 2000). Our result was in line with study of Pirota and co-workers (2013), in which solid to liquid ratio played a significant impact on the secretion of the cellulolytic enzyme by *Aspergillus niger*. The highest endo-glucanase recovery (35.7 U/g) was observed at solid: liquid ratio of 1:9 under suspended fermentation (Pirota et al., 2013). Rodríguez and co-workers (1996) suggested that lignin degradation by laccase produced by soil-isolated *P. chrysogenum* to oxidize aromatic compound. On the other aspect, few bacterial strains could display lignin-degrading ability, some show unsatisfactory capacity or need morphology engineering to achieve desired degrading efficiencies (Tsegaye et al., 2018).

The maximum substrate weight losses were reported at around 42.32% and 44.45% under the cultivation of *A. niger* and fungal community of *A. niger* F.00632, *P. chrysogenum* F.00814 and *T. viride* F.00795, respectively. Nonetheless, a remarkable decline in pH value was observed during the pretreatment by filamentous fungi as a consequence of the biodegradation process (Ali et al., 2020). Filamentous fungi have been considered as the most effective degraders for decaying lignocellulosic residues because they can secrete various degrading enzymes, including cellulolytic, hemicellulolytic and ligninolytic enzymes and they interact synergistically during hydrolysis (Beguin and Aubert, 1994; Nidetzky et al., 1994; Pérez et al., 2002; Zhou and Ingram, 2000). In this study, enzymatic activities secreted by filamentous fungi showed better performance than other species was observed. Specifically, *A. niger* and the co-culture of *A. niger*, *P. chrysogenum* and *T. viride* achieved the maximum value of total cellulase activity of 2.88 and 2.91 IU/gds, respectively. Additionally, to obtain the highly efficient deterioration of lignocellulosic substrates, breaking down hemicellulose structure is considered an important step to make cellulose accessible for enzymatic hydrolysis. The fungal consortium also performed as the best xylanase producer. The improvement of straw saccharification efficiency by co-culturing of degrading microorganisms was reported by Taha and co-workers (2015), who found double-fold higher enzymatic activities released by fungal isolates instead of bacteria.

Group 2

In terms of bacterial pretreatment, the combination of a cellulolytic strain (*Bacillus subtilis* B.01162) and ligninolytic strains (*Rhodococcus opacus* B.01915 and *Pseudomonas putida* B.01522) accounted for an enhancement of reducing sugars compared to co-culture of *Bacillus* and *Pseudomonas* species. Similarly, mixed culture of *Yarrowia divulgata* Y.02062 and *Pichia stipitis* Y.00888 showed better performance of bioconversion capacity when effectively converted two-folds higher amount of sugar concentration than individual strain. The pH values of bacterial species were increasing during the pretreatment, which could be explained by the release of ammonia and form ammonium in an aqueous solution (Vu et al., 2022).

The highest total cellulase activities of 0.41 IU/gds was observed in case of *P. putida* B.01522-*B. subtilis* B.01162 co-culture among bacterial consortia. It was found that endo-glucanase activity of bacterial co-culture including *B. subtilis* B.01162, *P. putida* B.01522 and *R. opacus* B.01915 has no significant difference to 2-member consortium above. The improvement of xylanase activities was observed when adding more members to the bacterial habitat, 17.66 IU/gds by the 3-member consortium compared to 5.26 IU/gds by the 2-member consortium. However, microbes in this cluster released insufficient reducing sugar yield along with low enzymatic activities, resulting less impact on breaking down lignocellulose than those in other clusters.

Group 3

Soluble sugar accumulated in the complicated cultures was relatively higher in the respective monocultures or simple co-cultures (**Table 4.8**). In the microbial community including various types of microorganisms, bacteria play the important role on enhancement of lignocellulose bioconversion into small fragments such as soluble sugars. After 72 hrs of cultivation, co-cultures of bacteria-fungi and bacteria-yeast performed high sugar accumulation ratio of 3.21 to 3.5, while fungi-yeast co-culture accounted for ratio of 1.05.

The FPase activities of complex cultures ranged from 1 to 1.69 IU/gds, which surpassed the total cellulase secreted by bacteria-yeast co-culture. Similarly, endo-glucanase enzyme which hydrolysed the glycosidic bonds from cellulose chain ends was mostly observed in strains distinguished as the best cellulase producers, as fungal species and mixed cultures of fungi, bacteria, and yeast. These microbial communities also secreted promising xylanase activities, ranged from 21 IU/gds to 51 IU/gds.

Newly developed microbial consortia

The construction of complex microbial communities including fungi, bacteria and yeast is a new and promising avenue to reduce metabolic burden and execute multiple tasks simultaneously via division of labour which monoculture fails. The improved degradation capacity by synthesized consortium revealed that members in consortium could co-exist and coordinate with each other to achieve efficient decay process.

Performance of co-culture: weight loss, reducing sugar, their interactions

Newly developed microbial co-cultures were cultivated under suspended conditions. It can be observed that filamentous fungi as *Aspergillus niger* F.00632, *Penicillium chrysogenum* F.00814 and *Trichoderma viride* F.00795 have the grandest degrading capacity among investigated communities, performing the relatively high reducing sugar yield. Biological pretreatment is associated with the release of soluble sugars, resulting a positive linear relationship

between solid residue weight losses, and reducing sugar yield ($r = 0.596$, $p < 0.05$) (Pearson's correlation coefficient test) (**Figure 4.29**).

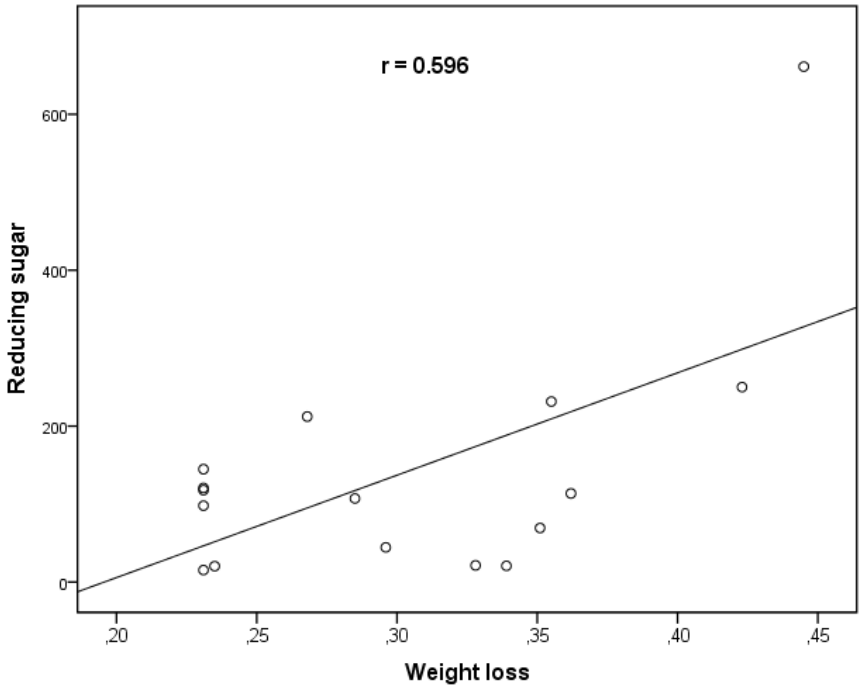

Figure 4.29 The correlation between weight loss and reducing sugars from pretreated wheat bran

Enzymatic degradation

As can be concluded from the enzymatic assay, the degradation abilities of pure cell on celluloses and hemicelluloses are generally limited, except *A. niger* with high hydrolytic activities which is responsible to degrade recalcitrant structures of lignocellulose substrates **(Figure 4.30)**. Previous studies implied that pure-culture isolates can degrade substrates with simple structure and composition such as artificial xylan and carboxymethyl starch, despite possessing the high lignocellulolytic activities and impossible to degrade natural lignocelluloses (Kato et al., 2004; Levin et al., 2006).

Microbial consortia composed of different types of species revealed unique characteristics, associated with the interactions between them. An effective microbial community is defined if its members can co-exist and collaborate to each other, produce sufficient degrading enzymatic activities. The addition of yeast into the co-culture of bacteria or bacteria and fungi attribute to the increase of xylanase from 31.96 to 48.85 IU/gds and was responsible for a higher weight loss of solid residues. Application of same fungi and yeasts, BFY2 consortium with *Bacilli* and *Pseudomonas* genus exhibited a slight increase in total cellulase, and endo-glucanase but a drop in xylanase activities occurred while BFY5 consortium containing ligninolytic co-culture could produce higher hydrolytic enzyme activities. BFY4 containing only one fungus with three bacterial strains and two yeast species showed a remarkable results of degrading enzyme activities, which were equivalent of enzyme activities produced by BFY5.

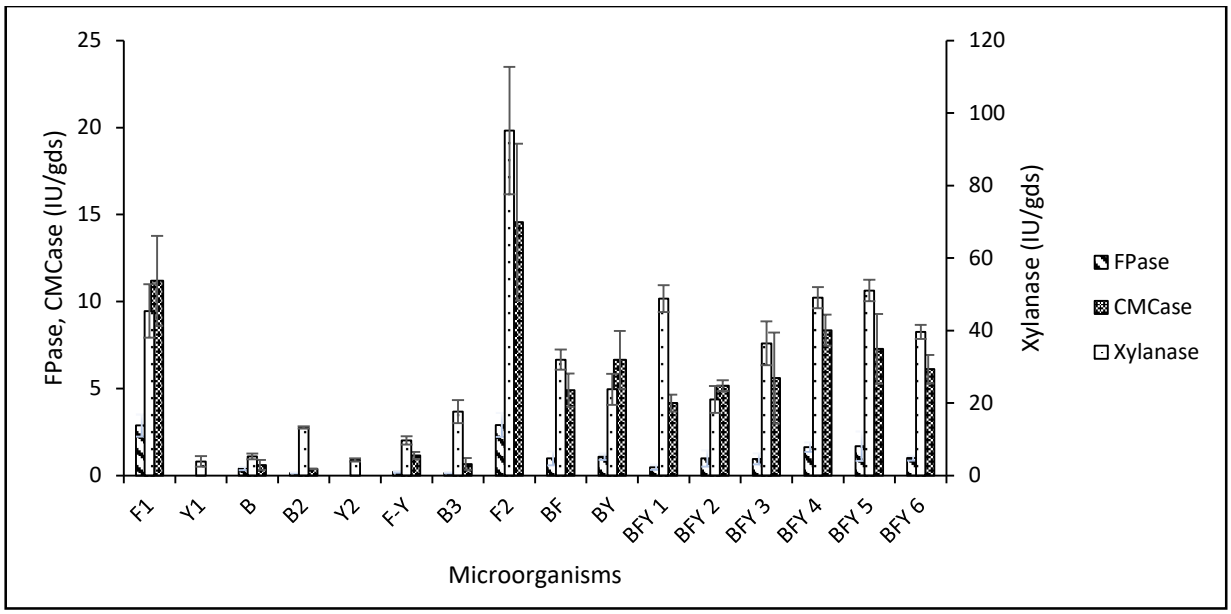

Figure 4.30 Enzyme production by microbes at 72 hrs of pretreatment

Reduction of reducing sugars

However, the reduction on reducing sugar yield was noticed in the complex consortium, which could be explained by the competitive metabolite exchange of microbes for their growth when multiple species compete for a single resource (McCarty and Ledesma-Amaro, 2019; Shahab et al., 2020; Wang et al., 2020; Xu and Yu, 2021). In agreement, Deng and Wang (2016) proved the higher metabolic pathway by synergistic co-cultures than pure culture, resulting in the competition of members for nutrients and as the consequence, fermented sugar yield was reduced by the time of treatment.

Sugar profile of the hydrolysates

The degradation effect includes the hydrolysis of cellulose and hemicellulose and the conversion of hydrolysed products into fermented sugars. The sugar conversions using mono-strains and the microbial consortium were different according to members' interaction in the same communities. After 72 hrs of pretreatment, the low conversion of oligosaccharides such as cellobiose, maltose or maltotriose and monosaccharides such as glucose, and xylose in the hydrolysates of wheat bran pretreated by fungi or bacteria consortia was observed. Doubtless, bacterial utilisation had less impact on deteriorating the structure of recalcitrant lignocellulose due to the deficient production of hydrolytic enzymes. Filamentous fungi, however, had an inadequate sugar conversion even though they conducted a large weight loss of lignocellulose substrate and possessed a high reducing sugar yield. Notwithstanding, a combination of bacterial co-culture of *B. subtilis* and *P. putida* with filamentous fungi simultaneously could improve the degree of degradation. Regardless of the enhancement of hydrolytic enzymes and reducing sugar yield compared with bacterial treatment, lower content of fermentable sugars was found. Recent works by Kim and co-workers (2018, 2012) reported the conversion possibility of cellobiose to another

oligosaccharide or degradation into an additional monosaccharide (possibly glucose) by *Bacillus subtilis*, probably leading to the reduction of fermented sugar yield.

It can be encountered that co-culture of yeast with fungi or bacteria release astonishing soluble sugar yield after 72 hrs pretreatment, resulting in glucose yield of 102-103 mg/gds and xylose yield of around 47-66 mg/gds. The highest glucose concentration of 235.91 mg/gds was determined in the hydrolysate by a consortium denoted as BFY2, which included the mixture of filamentous fungi and other species (**Figure 4.31**). Zhang and co-workers (2021) indicated *Yarrowia divulgata* could produce metabolites for growth promotion of other species. Another yeast strain, *Pichia stipitis*, was reported to secrete xylanase to promote the degradation process (Ding et al., 2018). Fermented sugar yields released by microbial communities depended on specific interactions and interrelationships amongst populations. Nevertheless, the existence of yeast in the communities of fungi and bacteria played an important role in enhancing the conversion efficiency of lignocellulose to fermentable sugars.

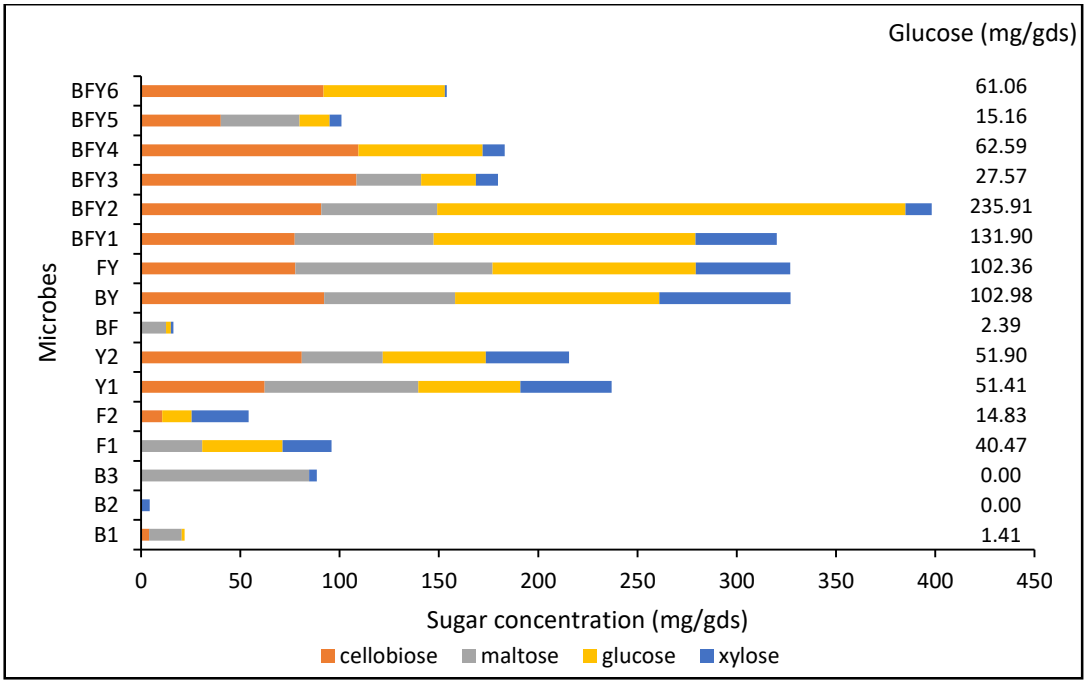

Figure 4.31 The sugar conversion of pretreated lignocellulosic biomass

4.5.3. Performance of newly developed microbial consortia

Our preliminary results indicated that mixed cultures of fungi, bacteria and yeast had a positive effect to enhance the degradation of lignocellulosic biomass. Two microbial consortia denoted as BFY4 (including three bacterial strains of *B. subtilis* B.01162, *R. opacus* B.01915, *P. putida* B.01522; one filamentous fungi as *A. niger* F.00632 and two yeast strains as *Y. divulgata* Y.02062, *P. stipitis* Y.00888) and BFY5 (including *R. opacus* B.01915 + *P. putida* B.01522 + *A. niger* F.00632 + *P. chrysogenum* F.00814 + *T. viride* F.00795 + *Y. divulgata* Y.02062 + *P. stipitis* Y.00888) were studied to evaluate the degradation effect on various types of lignocellulose

substrates. The mixture substrates contain variety ratios of wheat straw and wheat bran biomass were prepared.

Based on **Figure 4.32**, the degradation rates of two microbial consortia were similar. The reduction of wheat straw composition in the substrate mixture accounted for the increased degradation rate. In order to increase in the digestibility of cellulose and hemicellulose, removing or breaking down recalcitrant lignin is crucial. Since the concentration of lignin in wheat straw is often significantly higher than that of wheat bran, 26 % versus 5.5-6.0 % of the dry substrate (Guo et al., 2018; Kaprelyants et al., 2019), lower degradation efficiency was found in biomass substrate containing high wheat straw composition.

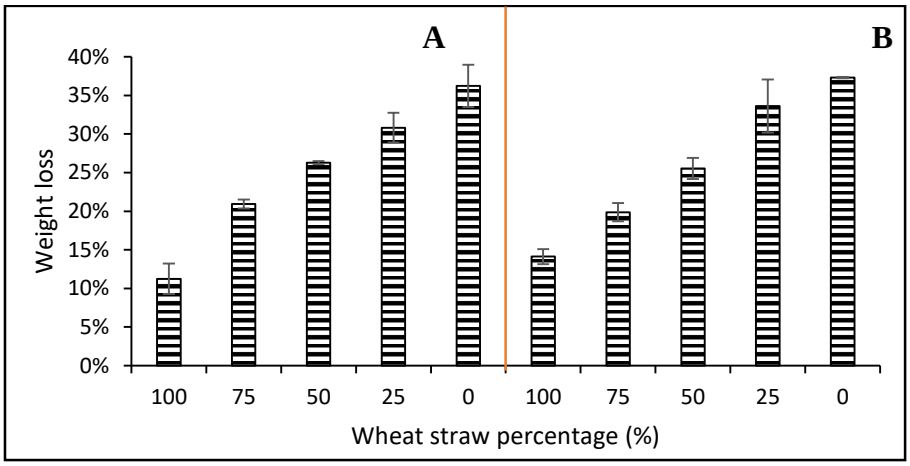

Figure 4.32 Weight loss of lignocelluloses using microbial consortia BFY4 (A), BFY5 (B)

The effect of pretreatment time on digestibility using microbial consortium was evaluated from 1-7 days. As shown in **Table 4.9**, the sugar accumulation generated by substrate containing 100% wheat straw reached the peak of 3.19 after 48 hrs and 2.09 after 72 hrs under cultivation of BFY4 and BF5, respectively. The mixture of wheat bran and wheat straw substrates at the ratio of 25:75 and 50:50 obtained the highest accumulation of sugars on the 4^{th} day and the 3^{rd} day of the pretreatment then reducing sugar yield decreased the day after. On the contrary, substrates containing large amounts of wheat bran over 75% could generate more reducing sugar when extending the treatment time. The effect of hydrolysis was also evaluated by measuring the pH of the culture medium. Acidic pH was noted after a long period of treatment. On the last day of the process, the substrate containing only wheat bran showed a pH value of 5.0 and a pH value of 3.0 was observed for the wheat straw substrate. The acidic pH of filtrate hydrolysate may be associated with the cleavage of acetyl groups from hemicellulose chains, indicating the severity of the treatment after a considerable time (Tang et al., 2019).

Table 4.9 Reducing sugar accumulation ratio under pretreatment of various lignocellulosic biomass using microbial consortia

	Ratio of substrates (%)		Time of pretreatment (days)						
	Wheat bran	**Wheat straw**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
BFY4	0	100	1.58 ± 0.27	3.19 ± 0.65	2.12 ± 0.63	2.07 ± 0.21	1.31 ± 0.44	1.12 ± 0.28	1.36 ± 0.17
	25	75	1.57 ± 0.15	0.99 ± 0.10	0.89 ± 0.24	1.66 ± 0.29	1.63 ± 0.31	0.74 ± 0.21	0.58 ± 0.13
	50	50	0.96 ± 0.20	1.48 ± 0.43	2.25 ± 0.42	1.95 ± 0.13	1.58 ± 0.43	0.73 ± 0.28	0.78 ± 0.28
	75	25	1.00 ± 0.05	1.11 ± 0.13	2.37 ± 0.41	2.46 ± 0.31	3.75 ± 0.43	4.72 ± 1.38	7.16 ± 1.65
	100	0	0.62 ± 0.07	0.80 ± 0.21	1.48 ± 0.36	5.32 ± 0.29	3.09 ± 0.41	6.11 ± 0.56	8.21 ± 2.45
BFY5	0	100	1.63 ± 0.23	2.00 ± 0.49	2.09 ± 0.65	0.79 ± 0.17	0.77 ± 0.29	1.29 ± 0.18	1.57 ± 0.15
	25	75	1.15 ± 0.14	0.87 ± 0.22	1.19 ± 0.37	2.09 ± 0.33	0.90 ± 0.11	1.23 ± 0.41	0.60 ± 0.16
	50	50	1.56 ± 0.11	1.77 ± 0.13	2.46 ± 0.48	1.94 ± 0.19	1.00 ± 0.05	0.88 ± 0.22	1.02 ± 0.13
	75	25	1.35 ± 0.07	1.40 ± 0.50	1.95 ± 0.50	2.71 ± 1.02	3.15 ± 1.21	5.58 ± 1.71	5.67 ± 1.67
	100	0	0.66 ± 0.15	0.94 ± 0.19	1.69 ± 0.22	3.94 ± 0.89	5.75 ± 1.06	5.20 ± 1.51	5.79 ± 1.15

The production of hydrolytic enzymes is inducible and was influenced by the nature of lignocellulosic substrates in the pretreatment. Thus, the selection of input sources for the bioprocess is very important. Hydrolytic enzymes including FPase, CMCase, xylanase and laccase were evaluated after 72 hrs of the pretreatment process (**Figure 4.33**). Using microbial consortia, substrates of 100% wheat straw exhibited the most potential degradation capacity. Particularly, FPase, xylanase and laccase activities were markedly higher in the wheat straw alone than in the mixture of wheat straw and wheat bran. The highest FPase (0.213 IU/gds) and xylanase (6.564 IU/gds) activities were achieved with BFY4 consortium in the case of wheat straw substrate (**Figure 4.33A**). These values were about 2.4-fold and 1.3-fold higher, respectively, in comparison with values from the mixture of wheat straw and wheat bran in the ratio of 75:25. The BFY5 consortium also showed a promising decay capacity when wheat straw was used alone, especially with outstanding xylanase activities (7.588 IU/gds) in comparison to other (**Figure 4.33B**). Our results are in agreement with the studies of Kang and co-workers (2004), who claimed the maximum FPase and xylanase activities were obtained when using only rice straw treated with *Aspergillus niger* KK2. Also, all enzymes including FPase, β-glucosidase, xylanase and β-xylosidase except CMCase were found to increase in the cultivation of *Trichoderma reesei* MCG77 using beet pulp (Considine et al., 1988). Besides, CMCase activity reached the maximum value of 0.928 IU/gds and 0.872 IU/gds when the medium contained wheat bran pretreated with BFY4 and BFY5 consortia, respectively. High activities of FPase and CMCase were found in case of substrates used in the same proportion (in the ratio of 50:50), which contributes to the high accumulation of reducing sugars. Enzymatic activities reduced from 27% to 57% (FPase), and from 13% to 53% (CMCase) when BFY4 consortium was inoculated to the medium containing 25% wheat straw and 75% wheat straw, respectively. Decrease in CMCase activity of 38% was also observed when BFY5 consortium was used in same conditions.

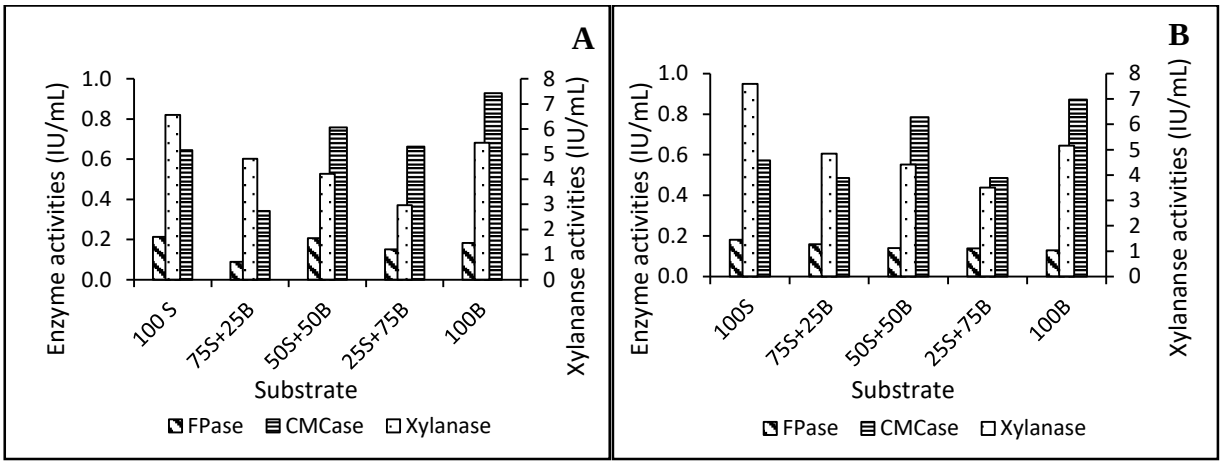

Figure 4.33 Enzyme activities after 72 hrs of lignocellulose pretreatment by microbial consortia BFY4 (A) and BFY5 (B)

The concentration of hexoses (glucose) and pentoses (xyloses) produced by microbial consortia BFY4 and BFY5 was depicted in **Figure 4.34**. Although xylose cannot be fermented by *Saccharomyces cerevisiae*, some other yeasts such as *Pichia stipitis*, *Candida shehatae* naturally worked on xylose, gaining acceptable efficiency (Ding et al., 2018; Htet et al., 2018).

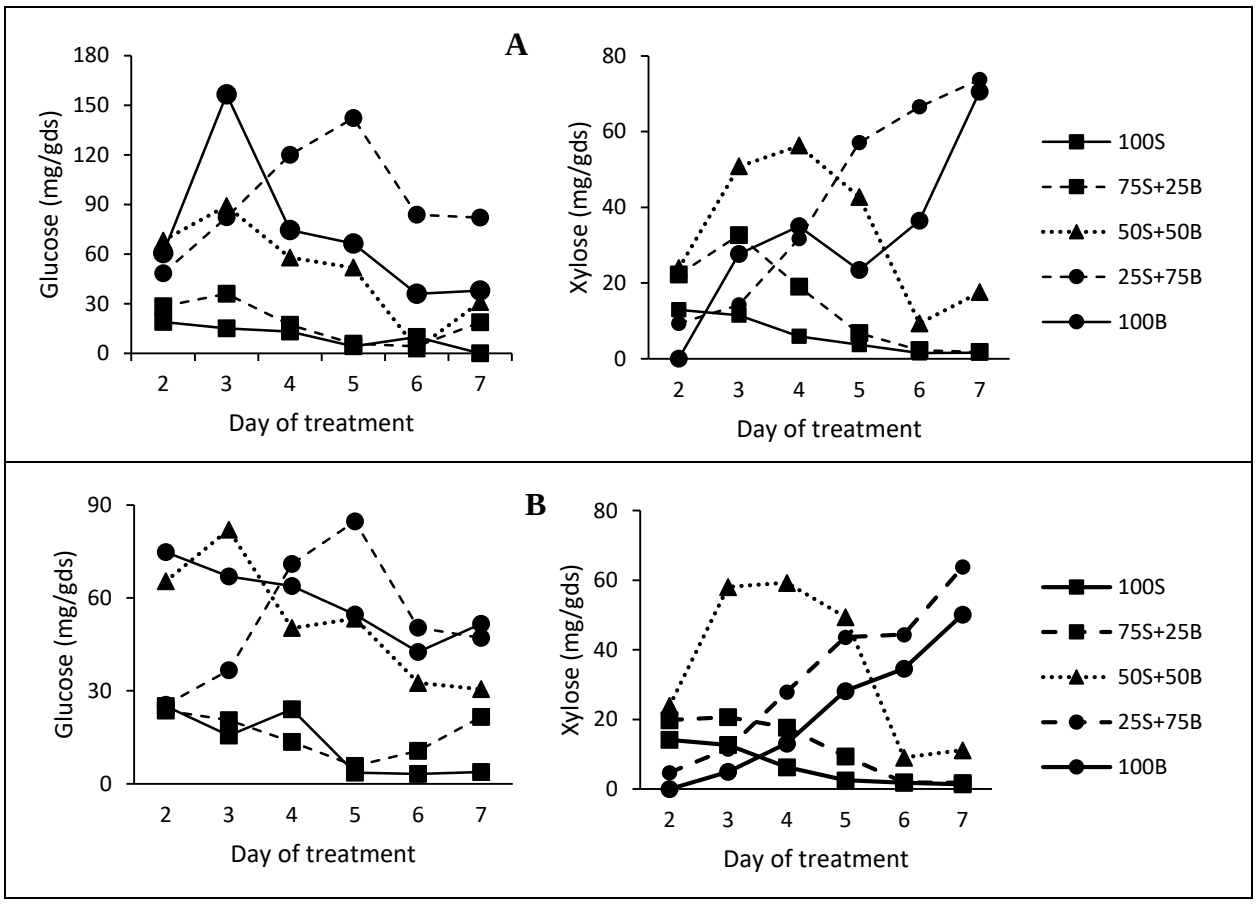

Figure 4.34 Sugar conversion of different mixtures of substrate under cultivation of BFY4 (A) and BFY5 (B)

Co-cultures of yeast and bacteria were developed to ferment different sugars from lignocellulosic hydrolysis, achieving a high yield of ethanol and improving ethanol tolerance (Fu et al., 2009; Rouhollah et al., 2007). A higher amount of glucose was released by the cultivation of BFY4 than BFY5, which was observed in the hydrolysate of pretreated substrates containing over 50% of wheat bran (**Figure 4.34B**). Complex consortia BFY4 released the maximum glucose yield of 156.47 mg/gds, and 89.02 mg/gds after 72 hrs from substrates containing only wheat bran and the mixture of wheat bran and wheat straw at an equal ratio, respectively (**Figure 4.34A**). Likewise, the highest glucose concentration was observed on the 5th day of the pretreatment of the substrate containing 25% wheat straw and 75% wheat bran, with the amount of 142.16 mg/gds (BFY4) and 84.73 mg/gds (BFY5). On the other aspects, BFY4 and BFY5 showed a similar effect on xylose production. The amount of xylose reached the highest value at 72 hours of the pretreatment, when the ratio of substrates was 1:1. Then, after 96 hours, the xylose content dramatically reduced till the end of the process. However, substrates containing over 75% wheat bran produced more xylose when extending the treatment time. It can be explained by the lower lignin content of wheat bran as well as by the increase in the secretion of cellulases with process going. Our results were in line with the study of Chang and co-workers (2018), in which they found an increase in mono sugar yield when extending the duration time of the post-hydrolysis process. The long pretreatment time is necessary to convert crystalline parts of lignocellulosic

biomass into amorphous cellulose which can easily be hydrolysed to hexoses during the pretreatment process. Due to the degradation effect of microbial consortium BFY4, it was utilized for the potential application of cases study of saccharification and fermentation in further research. Besides, different cases of lignocellulosic biomass pretreated by a diversity of strains and microbial consortia were also investigated.

4.6. Application of newly developed microbial consortia

Bioconversion of lignocellulosic biomass to second generation biofuel is the most promising approach to overcome the disadvantages of fossil fuel, reduce greenhouse gas emission (Farrell et al., 2006). The production of ethanol involves the hydrolysis of celluloses and hemicellulose into monomeric sugars, which further fermented into ethanol by fermenting organisms. In this study, enzymatic hydrolysis and fermentation were performed sequentially. The hydrolysis process (saccharification) was run for 4 hrs at 50^{o}C using exogenous cellulase enzyme, but before that, enzyme dosage and substrate loading were optimized in advance to maximize the saccharification efficiency. After hydrolysed under optimal conditions, pretreated biomass was anaerobically fermented in 7 days using *Saccharomyces cerevisiae*.

4.6.1. Optimization saccharification process

Different levels of substrate and enzyme dosage were optimized. The co-culture of *A. niger* F. 00632, *B. subtilis* B.01162 and *P. putida* B.01522 was utilized in the pretreatment of wheat bran substrate. Different saccharification efficiency when applying various ranges of substrate loading (**Table 4.10)** were found. Generally, solid residue weight loss after saccharification was significantly higher than after pretreatment, proving the degradation effect of hydrolysis enzymes on the polysaccharide composition of the substrate. The increase of substrate loading from 3% to 7% accounted for a corresponding increase in reducing sugar yield, ranging from 228.43-291.34 mg/gds. There was a significant difference in degradation rate between substrate loading of 3% and 7%, and they showed no difference with substrate loading of 5%. Significant higher soluble sugar yields were found in the liquid extract from hydrolysed samples instead of pretreated samples, specifically, 2-5 times higher in maltose and glucose yield and more than 10 times higher in xylose yield. Among various substrate amounts, substrate loading of 5% produced the highest fermentable sugars.

Table 4.10 Effect of substrate loadings in saccharification efficacy of pretreated biomass

	Pretreated sample	Substrate loadings		
		3%	5%	7%
Weight loss (%)	26.83 ± 0.39	33.04 ± 0.00	31.68 ± 1.03	25.31 ± 4.01
Reducing sugar (mg/gds)	80.76 ± 13.43	228.43 ± 0.00	248.03 ± 5.77	291.34 ± 65.08
DP2	37.29 ± 0.00	_	47.19 ± 3.95	25.53 ± 1.03
Maltose	50.06 ± 15.03	128.25 ± 0.00	121.07 ± 5.69	119.57 ± 2.38
Glucose	42.59 ± 6.17	180.93 ± 0.00	225.31 ± 12.94	200.55 ± 7.22
Xylose	12.39 ± 4.76	142.84 ± 0.00	168.08 ± 7.75	140.22 ± 4.16

This finding was in accordance with the study by Rosgaard and co-workers (2007), in which they found a higher hydrolytic efficiency when reducing pretreated barley straw concentration to 5% w/w of dry matter. Therefore, based on the above results, a substrate loading of 5% was chosen as an optimal parameter for lignocellulose saccharification.

Table 4.11 Effect of enzyme dosages in saccharification efficacy of pretreated biomass

		Enzyme dosages (FPU/gds)			
	Pretreated sample	**5**	**10**	**20**	**40**
Weight loss (%)	26.83 ± 0.39	33.69 ± 1.92	29.58 ± 2.21	28.3 ± 4.37	17.01 ± 1.99
Reducing sugar (mg/gds)	80.76 ± 13.43	280.64 ± 5.61	288.37 ± 27.68	298.45 ± 14.96	362.82 ± 14.02
DP2	_	_	12.35 ± 5.03	_	_
Maltose (mg/gds)	50.69 ± 2.29	153.89 ± 3.58	126.07 ± 12.57	131.97 ± 4.67	127.03 ± 5.72
Glucose (mg/gds)	48.31 ± 8.71	340.03 ± 15.57	374.30 ± 18.39	401.04 ± 5.46	439.34 ± 37.52
Xylose (mg/gds)	20.16 ± 1.91	226.24 ± 6.09	230.33 ± 4.49	244.92 ± 7.94	258.32 ± 5.81

Various enzyme dosages ranging from 5 to 40 FPU/gds were analysed. The hydrolytic effect on pretreated biomass using different enzyme dosages was summarized in **Table 4.11**. The lowest weight loss was observed in samples hydrolysed with high cellulase concentration. It was found that lowering the enzyme dosage could improve the degradation rate, as judged by the increase of weight loss of pretreated substrates after 24 hrs saccharification. The use of cellulolytic enzyme of 40 FPU/gds produced the highest amount of reducing sugar yield of 362,82 mg/gds. During the saccharification, the cellulolytic enzyme hydrolysed biomass into fermentable sugars. The disaccharides (maltose) and monosaccharides (glucose/xylose) were detected in hydrolysed samples as 2-3 fold higher and more than 10 times higher than those from pretreatment, respectively. The highest maltose concentration was observed under enzyme activity of 5-10 FPU/gds. Likewise, the addition of enzyme, however, reduced the DP2 sugar yields. In this study, no significant difference in mono-sugar yields when using a range of enzyme dosages from 10 to 40 FPU/gds was found. Therefore, the cellulase activity of 10 FPU/gds was selected and used as an operating parameter for the next experiments. The use of moderate enzyme activity not only stimulates the hydrolysis process but also reduces process costs, which are mostly due to the high cost of enzymes.

4.6.2. Saccharification of pretreated wheat bran using mono- and co-cultures

Pretreated samples with BFY4 were hydrolysed under optimal conditions. The saccharification efficiency of lignocellulosic samples pretreated by different microbial co-cultures was evaluated. The microbial communities were described in **Table 4.12**.

Table 4.12 List of microbial consortia used in the pretreatment of lignocellulosic biomass

Consortium	Microorganisms		
	Fungi	**Bacteria**	**Yeast**
C1	*Aspergillus niger* F.00632	_	_
C2	*Aspergillus niger* F.00632	*Bacillus subtilis* B.01162 - *Pseudomonas putida* B.01522	–
C3	*Aspergillus niger* F.00632	_	*Yarrowia divulgata* Y.02062
C4	–	*Bacillus subtilis* B.01162 - *Pseudomonas putida* B.01522	–
C5	–	*Bacillus subtilis* B.01162 - *Pseudomonas putida* B.01522	*Yarrowia divulgata* Y.02062
C6	*Aspergillus niger* F.00632	*Bacillus subtilis* B.01162 - *Pseudomonas putida* B.01522	*Yarrowia divulgata* Y.02062
C7 (BFY4)	*Aspergillus niger* F.00632	*Bacillus subtilis* B.01162 - *Pseudomonas putida* B.01522 - *Rhodococcus opacus* B.01915	*Yarrowia divulgata* Y.02062 - *Pichia stipitis* Y.00888
C8	*Aspergillus niger* F.00632 - *Penicillium chrysogenum* F.00814 - *Trichoderma viride* F.00795	*Bacillus subtilis* B.01162 - *Pseudomonas putida* B.01522	*Yarrowia divulgata* Y.02062
C9	*Aspergillus niger* F.00632 - *Penicillium chrysogenum* F.00814 - *Trichoderma viride* F.00795	*Bacillus subtilis* B.01162 - *Pseudomonas putida* B.01522 - *Rhodococcus opacus* B.01915	*Yarrowia divulgata* Y.02062 - *Pichia stipitis* Y.00888
C10	*Aspergillus niger* F.00632 - *Penicillium chrysogenum* F.00814 - *Trichoderma viride* F.00795	*Bacillus subtilis* B.01162 - *Pseudomonas putida* B.01522	–

Five-percentage pretreated lignocellulose loading was hydrolysed for 4 hrs at pH 5.0 and incubation temperature 50^{o}C using exogenous cellulase concentration of 10 FPU/gds. The result of enzymatic saccharification was demonstrated in **Figure 4.35**. It can be inferred that the release of reducing sugar by enzymatic hydrolysis gained higher conversion, due to the prior deterioration of polysaccharide structures under pretreatment. Lignocellulosic structure is vulnerable after microbial attack during pretreatment, exposing cellulose surface areas which is beneficial for hydrolysis (Bak et al., 2009; Taniguchi et al., 2005; Zhao et al., 2012). High production of reducing sugar pointed out efficient hydrolysis by cellulase on microbial pretreated wheat bran, which differed greatly among samples. The maximum reducing sugar accumulation ratio was observed for samples treated by complex consortium C7 (BFY4) and it showed a significant difference with pretreated samples by bacterial co-culture C4. Among these samples, consortia composed of filamentous fungi, bacteria and yeast strains could produce relatively high sugar content, as similar to fungal-bacterial or fungal-yeast co-culture with a reducing sugar accumulation ratio over 1.6 in the pretreatment step.

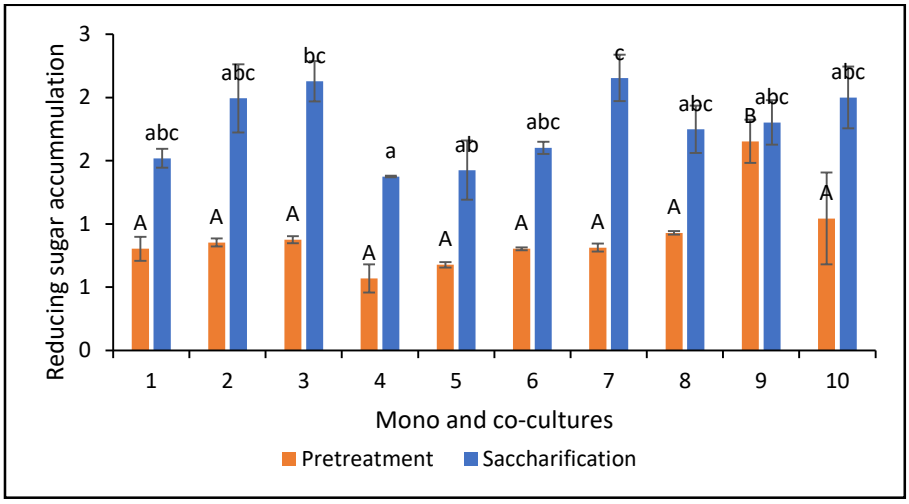

Figure 4.35 Comparison of the reducing sugar accumulation ratio in hydrolysates after 72 hrs pretreatment then after 4 hrs hydrolysis of lignocellulosic biomass

4.6.3. Ethanol fermentation of biologically pretreated wheat bran

Ethanol fermentation was carried out by commercial *Saccharomyces cerevisiae* after hydrolysis of pretreated lignocelluloses, the fermentation was run under anaerobic conditions for 7 days. The fermentation profiles of pretreated substrates by mixed cultures of less than 3 members were demonstrated in **Figure 4.36**. The bioconversion rate related to the relevant microbes was presented in **Figure 4.38 A**. It was found that the fermentable sugars such as maltose and glucose dropped drastically as the result of ethanol conversion by yeast. After 1 week of alcoholic fermentation, approximately 30-54% of the carbohydrates were still in the mash residues. We observed a similar fermentation rate for pretreated samples by co-culture of *A. niger* F.00632 with *B. subtilis* B.01162 – *P. putida* B.01522 (C2) and co-culture of *A. niger* F.00632 and *Y. divulgata* Y.02062 (C3), in which ethanol yield reached the peak at 3rd day, then decreasing the day after. The bioconversion rate on the 3rd day of C2, C3 were 82.82% and 94.59%, respectively.

Likewise, lignocellulose pretreated by single strains of fungi (C1) or bacteria (C4) and co-culture of bacteria and yeast (C5) showed a reduction in ethanol yield at the 2nd day of fermentation. Among these cases, the sample treated with C5 continued to release ethanol till the last day of the fermentation period, the maximum bioconversion rate of 92.38% was observed on the 5th fermentation day while consortium C1 possessed the highest bioconversion rate of ethanol of 99% on the 3rd day. The fermentation results of lignocellulose pretreated by complex microbial consortia (four members and above) were demonstrated in **Figure 4.37** and their bioconversion rate was presented in **Figure 4.38B**.

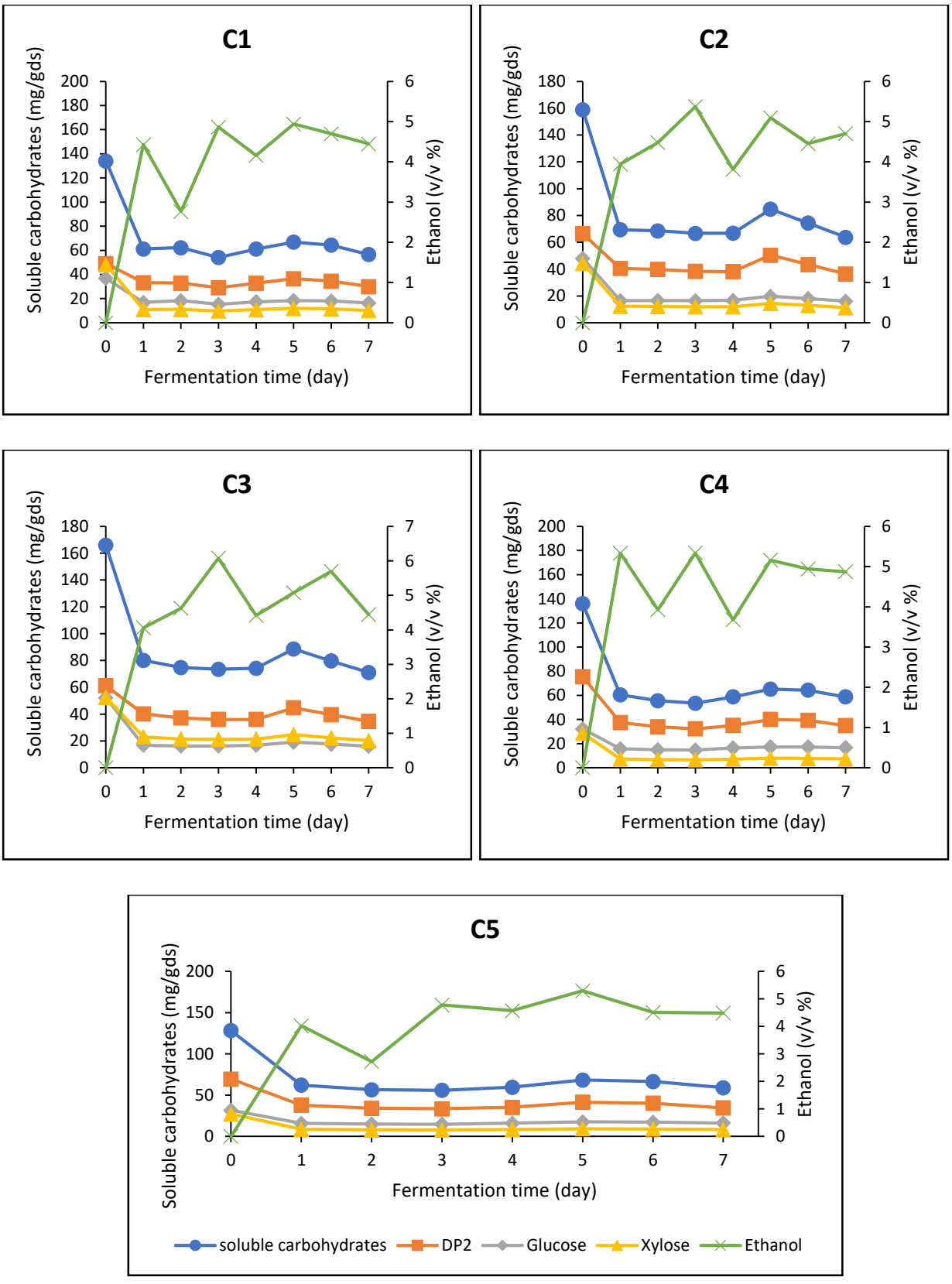

Figure 4.36 Saccharification and ethanol production using mono-cultures and co-cultures (maximum 3 members) pretreated wheat bran hydrolysates

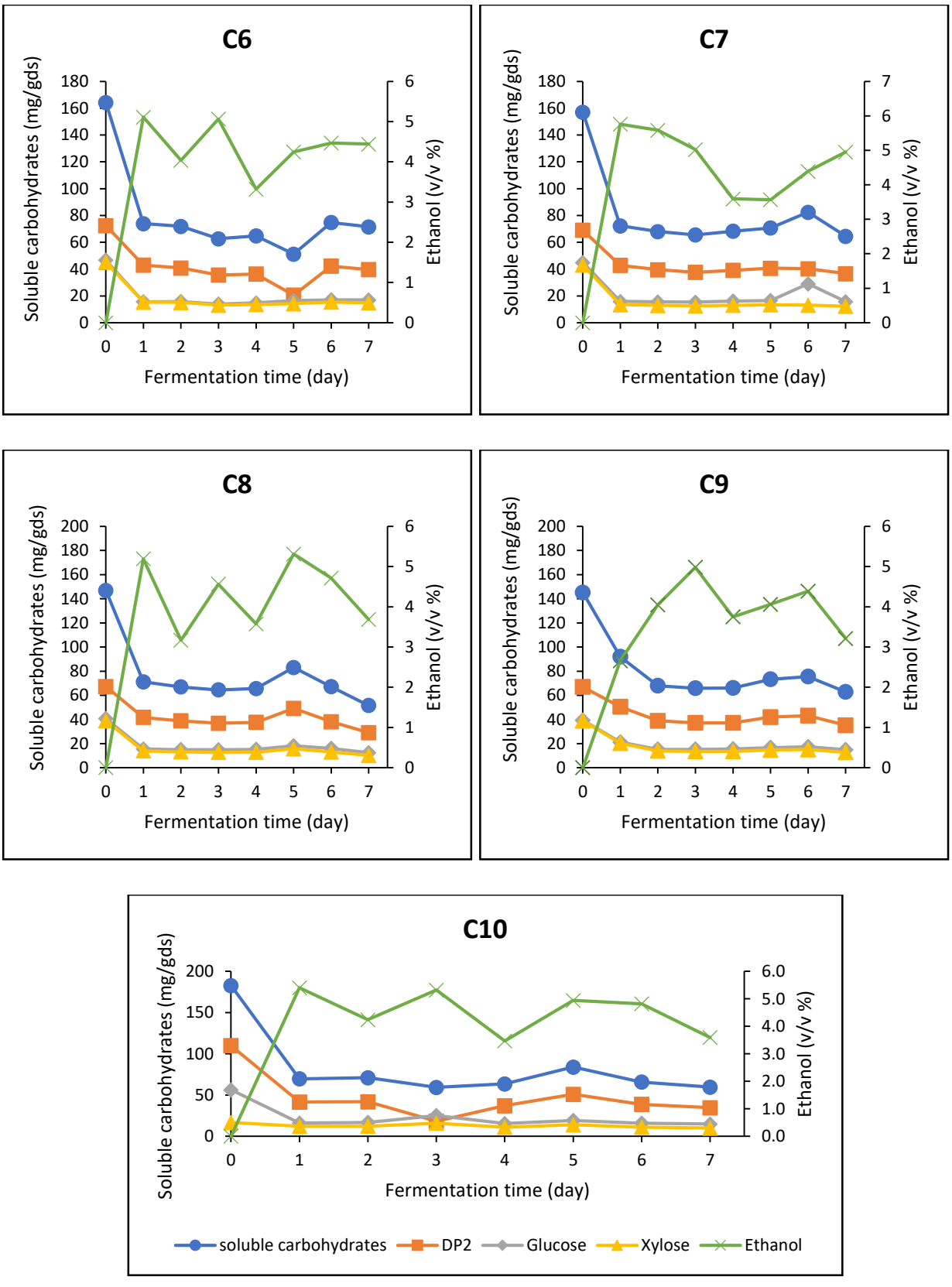

Figure 4.37 Saccharification and ethanol production using complex consortia (from 4 members and above) pretreated wheat bran hydrolysates

It was obvious that the highest ethanol concentration of 5.7 (v/v%) with an 89.21% conversion rate was observed on the first fermentation day in the case of consortium C7, the ethanol conversion rate reduced gradually when increasing time for fermentation till 5th day. C6, C8, and C10 comprised the same bacterial members and exhibited similar fermentation profiles in which ethanol yield reached the peak of 5.11, 5.19 and 5,40 (v/v%) after 24 hrs of fermentation, respectively. There was a fluctuation of alcoholic concentration after reaching the peak. It can be inferred that the effect of yeast on the pretreatment process played the important role in the bioconversion of biomass substrate into ethanol. For instance, C10 with the lack of yeast, resulted in the reduction of fermentation efficiency in comparison to C6, and C8. Microbial consortium C9 comprised of 3 filamentous fungi, 3 bacterial species and 2 yeast strains was applied to lignocellulosic biomass in the pretreatment process, then pretreated biomass was fermented by *Saccharomyces cerevisiae*, resulting in the enhancement of bioconversion rate till it reached the peak of 82.72% at the 3rd fermentation day before decreasing in the following days. We found the different effect alcoholic fermentation between samples treated with fungal monoculture and co-culture. For instance, samples treated by consortium C7 containing *A. niger* obtained the highest ethanol conversion rate of 89.21% on the 1st fermentation day while those treated by consortium C9 containing three filamentous fungi achieved the maximum ethanol conversion rate of 82.72% after 3 days of fermentation.

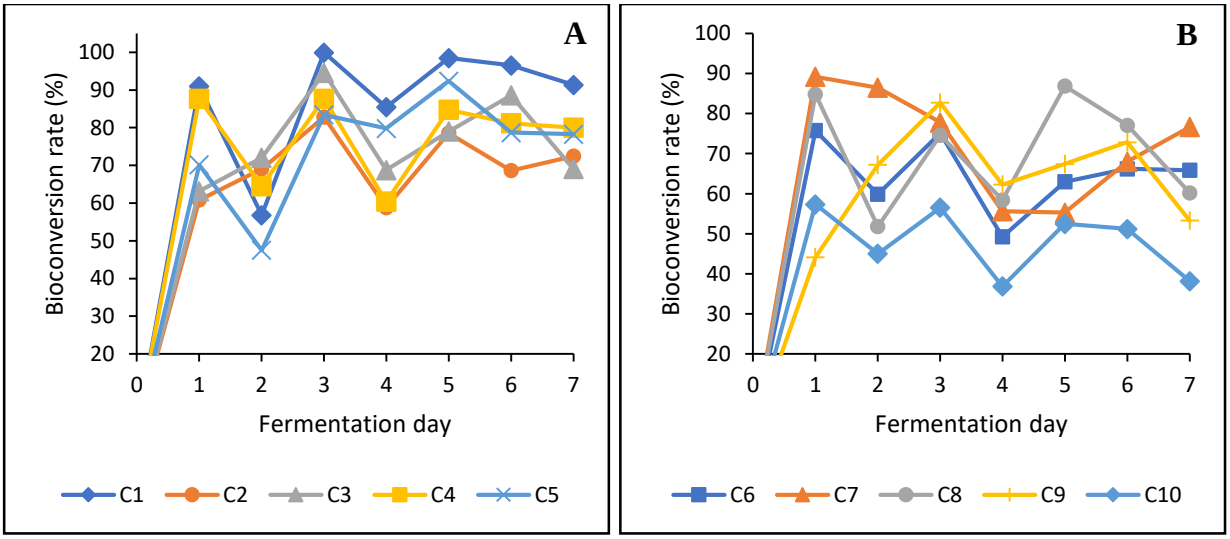

Figure 4.38 Bioconversion rate with microbial consortia using pretreated wheat bran hydrolysates

5. NOVEL CONTRIBUTION

- Seventeen strains of bacteria, three mould strains and nine yeast strains were obtained and screened to check the biological treatment of biomass, and three *Bacillus* strains (*Bacillus subtilis* B.01162, *Bacillus coagulans* B.01123 and *Bacillus cereus* B.00076), one *Pseudomonas* strain (*P. putida* B.01522) and one *Rhodococcus* strain (*R. opacus* B.01915) were shown to be the best degraders with high enzyme activities. These strains were selected for development of consortia for biological pretreatment of lignocellulosic raw materials.
- The 3-member consortium comprised the *Bacillus subtilis* B.01162, *Bacillus coagulans* B.01123 and *Bacillus cereus* B.00076 bacterial strains was found to be very promising degraders with high cellulase activities and efficient digestibility of solid substrates. Significant increase in the production of both cellulolytic and ligninolytic enzymes compared to monocultures was observed in the case of pretreatment of wheat bran substrate by co-culture of *P. putida* B.01522 and *R. opacus* B.01915 in submerged medium. Co-culture of yeast comprised by *Y. divulgata* and *P. stipilis* resulted the higher xylanase activity as well as amino acids than monocultures. Although, the combination of *A. niger* with *T. viride* F.00795 exhibited the outstanding hydrolysis capacity of total cellulase, endo-glucanase and xylanase (0.411, 1.827 and 12.990 IU/gds, respectively) in the case of suspended pretreatment.
- Different microbial communities with various quality and quantity microbes were constructed and degrading profiles were studies. Generally, higher solid loss was observed in the cases of ligninolytic consortia (mould) than in the cases of cellulolytic consortia (bacterial), along with the increase in total phenolic compounds. Consortia of *R. opacus* B.01915-*P. putida* B.01522, *R. fascians* B.01608-*P. putida* B.01522, *R. fascians* B.01608-*P. putida* B.01522-*B. subtilis* B.01162-*B. cereus* B.00076 and *R. opacus* B.01915-*P. putida* B.01522-*B. coagulans* B.01123- *B. cereus* B.00076 performed the high degradation capacity in the case of lignocellulose. The highest total cellulase enzyme activity was found in the cases of consortia of *B. subtilis* B.01162-*B. coagulans* B.01123 and *P. putida* B.01522-*B. subtilis* B.01162-*B. cereus* B.00076. These values were 0.213 IU/mL and 0.206 IU/mL, respectively.
- The parameters for the pretreatment of lignocellulosic biomass using complex consortia were optimized and they are basal medium, pH 6.5, *liquid:solid* ratio 9: 1. suspended pretreatment was proved more effective than the submerged approach. Numerically, 10 times higher reducing sugar yield after 72 hrs of pretreatment were obtained in the case of suspended biological pretreatment than in the case of submerged one.
- In the case of suspended pretreatment with the microbial consortium, meanwhile the fungi played the most important role in degrading lignocellulosic, whereas the bacteria itself showed less impact to the lignocellulose degradation, however, its combination with fungi or yeast was capable of boosting pretreatment efficiency. In addition, the presence of yeast in the microbial consortia could improve the enzyme production and sugar conversion. The highest glucose concentration of 235.91 mg/gds was determined in the hydrolysate after 72 hrs pretreatment by a consortium comprised of *B. subtilis* B.01162, *P. putida* B.01522, *A. niger* F.00632, *P. chrysogenum* F.00814, *T. viride* F.00795, *Y divulgata* Y.02062.

- In the case of saccharification, 5% (w/w) substrate load and 10 FPU/gds enzyme dosage were found to be optimal parameters to archive the maximum fermentable sugar yield. The highest ethanol concentration of 5.7% (v/v) with 89.21% conversion rate at the first day of fermentation was obtained in the case of pretreatment of wheat bran with 7-member microbial consortium consisted of *Aspergillus niger* F.00632, *Bacillus subtilis* B.01162, *Pseudomonas putida* B.01522, *Rhodococcus opacus* B.01915, *Yarrowia divulgata* Y.02062 and *Pichia stipitis* Y.00888. The fermentation efficiency was enhanced and the conversion of recalcitrant lignocellulose was accelerated by the synergistic actions of suitable fungi, bacteria and yeast in the same habitats.

6. SUMMARY

This study applied biological pretreatment of lignocellulosic biomass with the careful selection of suitable microbial consortiums before saccharification and fermentation processes. Different types of microorganisms, including bacteria, fungi and yeast were screened individually and then constructed in co-cultures. The parameters of degradation rate, hydrolytic and ligninolytic enzyme activities, sugar conversion, etc. were measured to determine the degradation efficiencies by microbial pretreatment.

All the microbial strains were received from NCAIM. Bacteria were classified into 2 groups, cellulolytic and ligninolytic species, which were refreshed aerobically in nutrient medium for 24 hrs before being cultivated into flasks containing 3% lignocellulose in basal medium. The fungus was cultivated on YPD agar slants at room temperature for 5 day-growth and used for suspended pretreatment. The cultivation of monoculture or co-cultures occurred at 28-30^{o}C for 7 days.

Bacillus strains including *B. subtilis*, *B. cereus*, *B. coagulans* were considered the best degraders with relatively high weight loss ranging from 54% to 60% after 7 days of submerged pretreatment. The correlation between weight loss and reducing sugars was introduced in pretreatment by single *Bacillus* strains but did not apply to their mixed culture. Among these species, *B. cereus* and *B. subtilis* release the highest yield of reducing sugars ranging from 240-252 mg/gds after 72 hrs pretreatment, and 72 hrs was also the optimal time for most efficient *Bacillus* strains to achieve the peak of reducing sugar yields. *B. subtilis* B.01162, *B. coagulans* B.01123 and *B. cereus* B.00076 showed the highest enzyme production and their collaboration in 2-member consortia showed the synergistic relationship with the great enhancement of cellulolytic enzyme activities, specifically FPase and xylanase, which were almost 2-fold higher and over 3-fold higher than that of pure cultures. However, the role of β-glucosidase in degradation by the consortium was negligible.

In order to select lignin-degrading strains, various species of *Rhodococcus* and *Pseudomonas* were screened. Among them, *R. erythropolis* B.01914, *R. opacus* B.01915 and *P. putida* B.01522 have the greatest effect on lignocellulose degradation, performed by the significantly higher weight loss of solid residues ($p<05$). They also secreted significant high xylanase activities of 1.716, 1.644 and 2.011 IU/mL, respectively. Reducing sugar and enzymatic activities, especially total cellulase and laccase, produced by lignin-degrading strain showed a linear relationship with a strong magnitude ($r^2 > 0.6$). The co-culture of ligninolytic strains, however, caused the reduction of reducing sugar yields. The high degrading enzymes such as total cellulose and laccase were observed in *P. putida* B.01522 and its co-culture with *R. opacus* B.01915 in comparison to pretreatment by other strains. On the other aspect, the co-culture of ligninolytic strains could break down the biopolymer structure, and release a higher concentration of fermented sugars which were able to be converted into valuable products.

Selected cellulolytic and ligninolytic strains were constructed in various bacterial communities, which significantly affected the degradation of lignocellulose substrates. In general, ligninolytic consortia accounted for a higher solid loss than cellulolytic consortia, a high percentage of weight loss, and an increase of total phenolic compounds in some microbial mixtures

in the presence of *R. opacus* B.01915-*P. putida* B.01522 (C*K*) and *R. fascians* B.01608-*P. putida* B.01522 (D*K*). In addition, these consortia showed a more effective effect on breaking down the lignin structure, facilitating the enzymatic attack on cellulose and hemicellulose structure while the others' degradation effects were dominated by laccase. The highest total cellulase enzyme activity was found in cellulolytic consortia AB and mixed culture of *P. putida* B.01522 (K*) and 2 *Bacillus* species (K*-AC), values of 0.213 and 0.206 IU/mL. On contrary, glucose yield was highly released in the extracted hydrolysate of species with lower degrading enzyme activities.

Role of yeast in the bioprocess could be highly evaluated when applying their co-culture in the pretreatment of lignocellulosic biomass. It was found that co-cultures of *Y. divulgata* and *P. stipitis* (Y.02062-Y.00888 and Y.02062-Y.01047) gave xylanase activity with enzyme titers 0.48 IU/gds and 0.33 IU/gds, respectively. They also released a larger amount of amino acids than single cells using quantitive analysis and produced a significantly high amount of fermented sugar. The maximum glucose concentration of 67.36 mg/gds was measured in pretreated lignocellulose substrates by co-culture of *Y. divulgata* Y.02062 and *P. stipitis* Y.00888.

Three species of filamentous fungi including *A. niger* F.00632, *P. chrysogenum* F.00814 and *T. viride* F.00795 were investigated in suspended pretreatment, using the basal medium with the ratio of liquid:solid as 9:1, acidic pH of 4.5. Among them, *A. niger* could need a short processing time of 48 hrs to achieve the best reducing sugar yield while others took a longer time. Also, it showed the comparative degrading enzyme activities compared to fungal co-cultures. The most effective fungal co-culture is *A. niger* F.00632-*T. viride* F.00795 with outstanding hydrolysis capacity, in which enzyme activities of total cellulase, endo-glucanase and xylanase were 0.411, 1.827 and 12.990 IU/gds, respectively. Additionally, these consortia could release a high amount of glucose which a 4-7 times higher than in other case studies.

Factors such as culture medium, pH value, moisture and cultivation methods were studied to figure out the best operating parameters for biological pretreatment. In submerged pretreatment, basal medium stimulated the production of more reducing sugar and higher hydrolytic enzyme activities under fungal or bacterial pretreatment than utilization of citrate buffer. It was found that pH didn't significantly affect the degradation efficiency. Suspended pretreatment was more favourable for filamentous cultivation than utilization of bacteria. It was observed that the increase of moisture content under fungal cultivation caused greater solid loss after 72 hrs of pretreatment and at liquid:solid ratio of 7:1, 8:1, 9:1, reducing sugar yield was recorded around 200 mg/gds while at moisture content less than 75%, the highest yield was only 32.53 mg/gds. The complex culture of fungi and bacteria was applied to investigate the effects of incubation order, in which strains were cultivated simultaneously or at different times. Two opposite degradation profiles under suspended and submerged pretreatment were found when each species have its favor habitat conditions. 10 times higher reducing sugar yield was released in suspensed pretreatment than in the submerged approach. The cultivation route in which fungi were cultivated 24 hrs before the addition of bacteria accounted for the higher weight loss but no significant differences in reducing sugar yields or enzymatic activities. Therefore, simultaneous incubation of microbes was selected to construct complex microbial consortia in suspended pretreatment.

The construction of complex microbial communities including fungi, bacteria and yeast is a new and promising avenue to reduce the metabolic burden and execute multiple tasks simultaneously. They have their unique characteristics, associated with the interactions between members in the same habitat. A greater weight loss of lignocellulose treated with mixed cultures than with simple culture was obtained, revealing its strong correlation with reducing sugar yield produced during the biological pretreatment. Bacteria itself showed less impact on the lignocellulose degradation, however, its combination with fungi or yeast was capable of boosting pretreatment efficiency. The role of yeast in microbial communities is to improve the hydrolytic enzyme activities and sugar conversion. It was found that the co-culture of yeast with fungi or bacteria generated a tremendous concentration of glucose and xylose, around 102-103 mg/gds and 47-66 mg/gds, respectively. The highest glucose concentration of 235.91 mg/gds was determined in the hydrolysate after 72 hrs pretreatment by a consortium comprised of *B. subtilis* B.01162, *P. putida* B.01522, *A. niger* F.00632, *P. chrysogenum* F.00814, *T. viride* F.00795, *Y divulgata* Y.02062.

The effect of substrates on biological pretreatment by complex consortia was investigated. As a consequence, BFY4 and BFY5 showed a similar degradation rate to each investigated substrate. Mixtures of wheat bran and wheat straw substrate at the ratio of 25:75 and 50:50 obtained the highest accumulation of sugars on the 4^{th} day and the 3^{rd} day of the pretreatment. High hydrolytic enzyme activities were also observed in these above substrates. Consortium BFY4 could stimulate the bioprocess by producing the maximum yield of glucose of 156.47 mg/gds, and 89.02 mg/gds after 72 hrs from substrates containing only wheat bran and the mixture of wheat bran and wheat straw at an equal ratio.

After pretreatment, 4 hrs of hydrolysis with commercial cellulase at 50^{o}C was conducted, followed by ethanol fermentation using *Saccharomyces cerevisiae*. The 3 levels of substrate loadings as 3, 5, and 7% (w/w) and 4 levels of enzyme dosage as 5, 10, 20 and 40 FPU/gds were tested to select optimal criteria for saccharification. It was found that the maximum fermented sugar yield was obtained at substrate loading of 5% and they showed no significant difference in sugar yields at various enzyme dosages which ranged from 10-40 FPU/gds. Therefore, substrate loading of 5% (w/w) and enzyme dosage of 10 FPU/gds were used in the saccharification process. Among samples pretreated by different strains and consortia, the maximum reducing sugar accumulation ratio achieved by consortium C7 comprised of *A. niger* F.00632, *B. subtilis* B.01162, *P. putida* B.01522, *R. opacus* B.01915, *Y. divulgata* Y.02062, *P. stipitis* Y.00888.

After hydrolysis, pretreated samples were anaerobically fermented in 7 days. Glucose and maltose yield dropped during fermentation due to ethanol conversion by yeast. A diversity of optimal periods to obtain the peak of bioconversion rate was found when utilizing different species in lignocellulose pretreatment. The highest ethanol concentration of 5.7% (v/v) with an 89.21% conversion rate was observed on the first fermentation day in the case of consortium C7. The fermentation efficiency was enhanced and the conversion of recalcitrant lignocellulose was accelerated by the synergism relationship of suitable fungi, bacteria and yeast in the same habitats.

Overall, in this study, we discover the synergistic relationship of microorganisms including fungi, bacteria and yeast, which play an important role to enhance the degradation efficiency in biological pretreatment. The suspended process was proved to stimulate the growth of species in

the same habitats and gave a markable bioconversion rate of lignocellulose to fermented sugars. However, we could not achieve a sufficient ethanol yield in the fermentation process. The knowledge of this study could be used as primary background for scaling up and improving the bioprocesses.

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