Production of ethanol from apple pomace using *Saccharomyces cerevisiae, Trichoderma reesei* and *Myceliophthora thermophila* fungi

Anantveer Kailay

Masters of Science

Department of Bioresource Engineering

McGill University

Montreal, Quebec, Canada

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of Master of Science

2018

Contents

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BOD	Biological oxygen demand
CCD	Central composite design
°C	Degree Celsius
CFU	Colony forming units
MMN	Modified Melin-Norkans nutrient agar
SSF	Solid state fermentation
SmF	Submerged fermentation
M. thermophila	*Myceliophthora thermophila*
T. reesei	*Trichoderma reesei*
USDA	United States Department of Agriculture
PDA	Potato Dextrose Agar
RSM	Response surface methodology
S. cerevisiae	*Saccharomyces cerevisiae*
SAS	Statistical Analysis Software

ABSTRACT

In recent years, fuels from renewable sources have been gaining importance as an alternative to conventional fuels to reduce greenhouse gases and avail long term sustainability. Biofuels from fruit pomaces are gaining attention due to their richness in sugar content and their accessibility and low cost for the production of biofuels. The aim of this study is to provide a viable solution to the environmental problems caused by apple pomace waste through the optimization of bioethanol production from this available biomass. Two fungal strains, *Trichoderma reesei*, and *Myceliophthora thermophila* were used for saccharification of apple pomace and the sugars obtained from the saccharification process were fermented using the fungus *Saccharomyces cerevisiae* for the production of ethanol. The optimization steps for efficient saccharification were performed in order to study the significant factors such as temperature, moisture level, incubation time and the effect of supplementation of apple pomace with peptone as a nitrogen source. According to the results of the optimization process for saccharification, the highest values of sugar content were observed at a moisture content (wet basis) of 90% for *M. thermophila* and 85% for *T. reesei.* Based on the statistical analysis, the effect of temperature was not significant on sugar yield however the maximum yield was obtained at 28°C. 1% w/w peptone was found to be the most effective dose to increase the sugar yield for both the fungal strains tested for saccharification. The apple pomace treated with *T. reesei* and *M. thermophila* yielded 6.11 % (v/w) and 3.96% (v/w) amount of ethanol, respectively, after fermentation using *S. cerevisiae.* However, only 3.72% alcohol was obtained from the apple pomace treated with only *S. cerevisiae* as a control. Furthermore, the results revealed that using *T. reesei* with *S. cerevisiae* is an effective way for the production of bioethanol from apple pomace. Fermentation of apple sludge using *S. cerevisiae* was also carried out. Fermentation of apple filtered sludge resulted in 6.54% (v/w)

amount of ethanol using *S. cerevisiae* and fermentation of unfiltered sludge with similar conditions resulted in 8.05% (v/w) of ethanol at 72 hours.

RÉSUMÉ

Les combustibles provenant de ressources renouvelables deviennent de plus en plus importants comme alternatives aux combustibles conventionnels dans le cadre de la lutte contre les changements climatiques et dans la transition vers une économie durable. En outre, la teneur élevée en sucres, le faible prix et la disponibilité des marcs de fruits font de ceux-ci une source première intéressante pour la production de biocombustibles. Deux souches fongiques, *Trichoderma reesei* et *Myceliophthora thermophila*, ont été utilisées pour la saccharification du marc de pomme et les sucres obtenus lors du processus de saccharification ont été fermentés avec le champignon *Saccharomyces cerevisiae* pour la production d'éthanol. Les facteurs clefs déterminant l'efficacité du processus de saccharification, tels que la température, l'humidité, la durée de l'incubation et la supplémentation du marc avec de la peptone comme source d'azote furent identifiés. Selon les résultats, les teneurs en sucre les plus élevées furent obtenues avec une humidité de 90% pour *M. thermophila* et de 85% pour *T. reesei*. Tandis que le rendement maximal fut obtenu à une température d'incubation de 28°C, cette différence ne se releva pas significative lors de l'analyse statistique. Une concentration de peptone de 1% (p/p) fut la plus efficace pour augmenter le rendement de la saccharification, et cela, dans le cas des deux souches fongiques évaluées. Le marc de pomme traité avec *T. reesei* et *M. thermophila* a donné 6,11% (v / p) et 3,96% (v / p) de quantité d'éthanol, respectivement, après fermentation avec *S. cerevisiae*, tandis qu'une culture de *S. cerevisiae* seule (comme témoin) donna un rendement de 3,72% (v/p). Ces résultats démontrent que l'utilisation de cultures de *T. reesei* suivie de *S. cerevisiae* est une méthode efficace pour la production de bioéthanol. La fermentation de la boue filtrée avec une culture de *S. cerevisiae* a donné une concentration en éthanol de 6,54% (v / p) après 72 heures, tandis qu'une fermentation similaire avec une boue non filtrée a donné un rendement de 8,05% (v / p).

ACKNOWLEDGEMENT

Firstly, I owe the debt to my parents, Mr. Gurmeet Singh and Mrs. Kuldeep Kaur and my beloved sister Arashdeep Kaur. I am forever indebted to my parents for their endless support and encouragement.

I express my gratitude and moral obligation to my supervisor, Dr. Valérie Orsat,
Professor, Bioresource Engineering Department for her invaluable and inspiring guidance. I shall remain ever indebted for her care and affection during the course of the research work as well as in the preparation of this thesis.

I also express my thanks to the supportive professional associates, Dr. Darwin Lyew and Mr. Yvan Gariépy, for their continuous help during my research work. I am grateful to the computer facility provided by McGill IT Services.

I have been fortunate to come across my funny & good friends and I am happy to acknowledge the support and moral inspiration provided by Simer, Aman, Jaskaran, Nirbhay, Sunny, Mamta, Maryanne and Julien. Last but not the least, I duly acknowledge my sincere thanks to all those who love and care for me. Every name may not be mentioned but none is forgotten.

CHAPTER I: INTRODUCTION

Apple is the most important fruit crop in terms of production and it places second, after blueberries, in terms of market value in Canada. Though Canada has a very small share in the world's apple production, apple is still an important fruit crop in the Canadian fruit processing industry. Quebec alone produced 108,239 tons of apples in 2017 (Canada, 2018). About 30% of Canadian apples go through processing to produce juice, jams, pies, sauce and fresh cut. The apple processing industry produces 25-30% apple waste as pomace and 5-10% as sludge (G. Dhillon et al., 2011). Apple waste disposal is a major problem because of the large volume of waste being seasonally generated which is environmentally challenging to handle (Pfaltzgraff et al., 2013). Biomass is known to produce greenhouse gases when dumped into landfills and thus this method of handling biomass waste should be limited (Lim et al., 2016; Mirabella et al., 2014). Owing to its richness in sugar, fiber and phyto-nutrients, apple waste is seen as a source of various value-added products and for the potential production of biofuels. Major soluble simple sugars are present in apple waste and include fructose, sucrose and glucose. Like other lignocellulosic biomass, apple waste also contains cellulose, hemicellulose and pectin polysaccharides, which provide tremendous potential to be used as complex sugar sources for bioethanol production.

Various techniques have been tried over time to utilise this polysaccharide-rich biomass, but hydrolyzing and utilising it efficiently remains a problem as experienced with many other lignocellulosic substrates (Gama et al., 2015). Bioconversion of apple into bioethanol is a multistep process, which may require physical, chemical, enzymatic and biological treatments (Parmar & Rupasinghe, 2013). Pre-treatment of lignocellulosic biomass is conducted for better hydrolysis of the contained polysaccharides to yield higher amounts of fermentable simple sugars (Villas-Bôas, Esposito, & de Mendonca, 2003). Among the various existing pre-treatment techniques, the

biological conversion of apple pomace using micro-organisms, is preferably feasible due to its lower cost, its environmental stability and easy of process control. Many organisms are capable of breaking down the polymeric chain of cellulose and converting its simple sugars, via fermentation into bioethanol. Several bacteria and fungi have been studied on different lignocellulosic substrates. The current study includes the use of *T. reesei*, *M. thermophila* and *S. cerevisiae* for the biological treatment of apple pomace for bioethanol production.

The objective of this study is to explore the production of fermentable sugars from apple pomace/sludge for the production of bioethanol. This study provides insights regarding the interaction of different factors such as temperature, incubation time, moisture level, nitrogen supplementation for the release of fermentable sugars from apple pomace and sludge.

1.1 The specific objectives

1. Optimize the biological pre-treatment of apple pomace using fungal strains of *T. reesei* and *M. thermophila* for maximum sugar yield
2. Investigate the effect of peptone as a nitrogen supplement on the release of fermentable sugars from apple pomace
3. Standardize the fermentation of apple waste sludge for the production of ethanol
4. Investigate the effect of combination of different fungal strains (*T. reesei* + *S. cerevisiae)* and (*M. thermophila*+ *S. cerevisiae)* on the production of ethanol from saccharified apple residues.

CHAPTER II: REVIEW OF LITERATURE

2.1 History of fuels

During the course of evolution, humans have always been dependent on nature to fulfil their energy requirements. The very first type of energy production used by humans was in the form of fire from dry wood and leaves. This biomass-based fuel was humankind's primary fuel for thousands of years (Hubbert, 1949). With time, the development of civilisation allowed for advancements in technology and subsequent industrialisation which debuted in Europe (Huber, 2009). The search for cheap and convenient sources of energy, other than biofuels, led to the discovery and use of fossil fuels as an alternative to traditional fuels. Among these, coal and petroleum have been the most widely exploited (Panwar et al., 2011). Fossil fuels were considered inexhaustible and a sustainable resource at the time of major industrialisation during the 19th century (Rifkin, 2008). The rearrangement of economies and population lifestyles at the time resulted in an increased dependency on fossil fuels for energy (Novakov et al., 2003). Fossil fuels were cheaper and more efficient, as compared to previously used biofuels. They were also preferred as they left less particulate matter after combustion and were easy to handle. Fossil fuels were perceived as more reliable energy sources (Höök & Tang, 2013).

Equipment advancement further provided insights of the presence of large reserves of fossil fuels and advanced machinery eased their mining and extraction. Because fossil fuels were cost effective, technology involved in their extraction and refinement boomed and is in fact still developing (Rifkin, 2015). Fossil fuels still serve as a major source of energy. Use of fossil fuels is so crucial for today's generation that more than 80% of energy consumption worldwide is supplied by fossil fuels (Muradov, 2001). Modern lifestyles have become directly or indirectly dependent on them. Most production, food processing, transportation and employment are

dependent on energy and therefore everyday needs, such as clean water, food, and heating rely on electricity, often created through the consumption of fossil fuels (McGlade & Ekins, 2015).

2.2 Future energy requirements

Since their initial discovery, the finite nature of fossil fuels and their contribution to global warming have been realized (Golosov et al., 2014). Fossil fuel formation is a long process taking thousands of years to fix carbon from the environment while their consumption rapidly releases large amounts of carbon into the atmosphere. The atmospheric carbon dioxide has increased from 368.99 ppm in 1998 to 410.79 ppm in 2018 (Tans, 2018). The carbon released into the environment persists for thousands of years (Archer et al., 2009). The combustion of fossil fuels also releases a high amount of sulphates (Monticello & Finnerty, 1985). Sulphates have not shown any direct adverse effect on human health but sulphates lower the pH of water and soils (Moreno et al. 2009). Additional water and air pollution is caused throughout the mining process ultimately leading to global environmental warming (Lvovsky, 2000). Alternative fuels from lignocellulosic biomass are gaining increased public and research interests due to their renewable nature and having no net impact on the elevated levels of atmospheric carbon dioxide (Anwar et al., 2014; Schobert, 2013). Large portions of the population worldwide still use biomass as a primary fuel for their daily requirements such as cooking and heating (Johnson et al., 2013). Developed countries are also trying to mitigate the use of fossil fuels and increase the use of renewable resources such as solar, hydro and wind energies (Connolly, Mathiesen, & Ridjan, 2014). Many countries have made it mandatory to introduce bioethanol to their regular petroleum stocks as a transport fuel as for example in India and Australia the government announced the mandatory blending of at least 10% ethanol in their gasoline (Bangaraiah & Kumar, 2014; Johnson & Silveira, 2014; Niven, 2005).

2.3 Climate change and energy transformation

Climate change poses a serious threat to humanity and renewable resources are seen as an alternative to fossil fuels (Boyle, 1997). Wind, solar and hydro energies are already being used as a source of energy for electricity production. Worldwide, approximately 20 % of available electricity is being produced from such renewable resources. Biofuels such as bio-ethanol, bio-butanol and bio-diesel are considered as cleaner and renewable fuels, in comparison with traditionally derived fossil fuels (Ho et al., 2014). Unlike other renewable sources, these biofuels are available in liquid form and can be used for transportation purposes (Nigam & Singh, 2011). Biomass-derived biofuels are considered to have nearly zero net carbon emission as the carbon released through their use is compensated in a short interval by growing plants and the initial formation period is much shorter as compared to fossil fuels (Rahman et al., 2017).

2.4 Biofuels

Fuels from renewable sources are gaining in importance as an alternative to conventional fuel to reduce greenhouse gases and they could lead to long term sustainability. Biofuels are emerging as one of the most promising renewable fuels (Ghosh et al., 2016). The term biofuels emerged as a sustainable fuel source and produced from renewable or even waste organic sources (Zhang et al., 2016). Sugars synthesised by plants are available in simple and complex forms. Simpler forms include monomers and dimers of sugars such as glucose, fructose, and starch. Simple sugars can readily be converted to energy and energy producing biofuels (Rahman et al., 2017). Biofuels can be categorized as gaseous biofuels (hydrogen and methane, etc.) and liquid biofuels (ethanol and butanol, etc.). Furthermore liquid biofuels include the production of first generation and second generation biofuels (Pham & Balasubramanian, 2015; Zhang et al., 2016)

2.4.1 First generation biofuels

Biofuels produced directly from sugars are called first generation biofuel. As the plant parts with a higher content of simple sugars are widely used as food by humans and animals; competition, between their use as food or as fuel arose (Naik et al., 2010). Fuel production from simple sugar biomass such as sugarcane and corn kernel starch is controversial and questions the balance between food security and sustainability. Nonetheless, a high volume of such biofuels is currently produced in many countries, such as in the USA and Brazil, which represent the leaders in biofuel production (Naqvi & Yan, 2015). The USA aims for 30% of its liquid transportation fuel to be replaced by biofuels by 2030 (Somerville, 2007).

2.4.2 Second generation biofuels

Second-generation biofuels are made from biomass which includes any source of organic carbon that is renewed rapidly as part of the carbon cycle from plants but can also include biomass from animal products. Food and agricultural wastes are considered a primary source for second-generation biofuel as they no longer serve the purpose of feeding and can be diverted from filling landfills. Using biomass or agri-food waste, rather than food items like sugarcane and corn alleviates concerns generated by primary generation fuel sources leading to challenges with global food security (Ridley et al., 2001). Agri-food waste includes various sources at different stages of the food processing chain, from harvesting through processing to consumer food waste (Kiran et al., 2014). Agricultural waste includes a variety of plant biomass such as wheat straw, corn stover, and rice husk. Plant parts containing complex sugars are not usually used as food and can serve as a potential source for second generation biofuels. More complex polysaccharides such as cellulose, hemicellulose and pectin are used in this case (Carriquiry & Timilsina, 2011). In the monitoring of the life cycle of foods, about 1.3 billion tons of food is wasted per year worldwide. This includes,

waste from dairy products, fresh vegetables, fruits, bakery and meat, which contribute to about 35.5–69% of sugars and 3.9–21.9% proteins. Fruit and vegetable waste is mainly composed of easily digestible sugars and hemicellulose (75%), cellulose (9%) and lignin (5%), small amounts of proteins and fat, with 80–90% moisture content (Bouallagui, Touhami et al., 2005). Because of its abundance and low cost, food waste can be utilized as an important alternative substrate for the production of ethanol. With the increase in population and economic growth, the volume of food waste is expected to grow in the next 25 years (ElMekawy et al., 2015). Various studies have been performed to make efficient use of food waste for second generation biofuels (Kiran et al., 2014; Mirabella et al., 2014; Shalini & Gupta, 2010).

The most important step in the production of carbohydrate-based second-generation biofuels (bioethanol) is the hydrolysis of the available carbohydrates. This hydrolysis can be either an acid hydrolysis or an enzymatic hydrolysis (Matsakas & Christakopoulos, 2015). The literature found on the production of bioethanol from different types of agri-food wastes by microorganism, using different methods is presented in Table 2.1.

Table 2.1 Production of bioethanol from different types of agri-food wastes using different microorganisms.

	Type of agri-food waste	**Method of production**	**Microorganism used**	**Ethanol yield (as compared to maximum theoretical yield)**	**Reference**
1	Bread crust Rice grain (uncooked)	Continuous solid state fermentation	Dry yeast (*super camellia*)	100.9%±5.1% 108%±7.9%	(Moukamnerd et al., 2013)
2	Household food waste	Enzymatic hydrolysis and saccharification	*Myceliopthora thermophila*	9.27 g/l (actual ethanol yield)	(Matsakas & Christakopoulos, 2015)
3	Potato peel waste	Hydrolysis (enzymatic/acidic) followed by fermentation	*S. cerevisiae*	91.6%	(Arapoglou et al., 2010)
4	Kitchen waste	SSF	*S. cerevisiae* KF-7	88.9-91.2%	(Wang et al., 2017)
5	Pineapple waste	Acid hydrolysis followed by fermentation	*Clostridium acetobutylicum* B 527	butanol	(Khedkar et al., 2017)
6	Food waste (noodle waste)	Saccharification and fermentation	*S.cerevisiae* KF-35	96.8%	(Yang et al., 2014)

2.5 Lignocellulose composition

Biomass comprises of any organic renewable material including agricultural produce and waste, animal waste, wood and wood residues, marine plants and food waste. Lignocellulose is the primary component of plant biomass (Chen, 2005). It is comprised of lignin, cellulose, hemicellulose, pectin, ash, salts and minerals.

2.5.1 Cellulose

Cellulose is the most abundant polymer on earth with approximately 10^{11} tonnes of cellulose produced every year in the world mainly from higher plants and to a small extent from algae (Carroll et al., 2012). A high proportion (40-90%) of cellulose is present in all the plant based fibers (Wei & McDonald, 2016). It is a hydrophilic polysaccharide made of a homogeneous linear chain of glucose monomer units, linked by β-1,4-glycosidic bonds (Saini & Tewari, 2015). Most cellulosic materials possess crystalline (highly ordered) and amorphous (less ordered) domains that determine their chemical behavior, reactivity and water absorption. Crystalline cellulose micro fibrils are formed by the intra- and inter-molecular hydrogen bonds between hydroxyl groups on the glucose ring. The hydrogen bonding within and between cellulose chains, provide extra strength, stiffness, crystallinity and durability (Fig 2.1) (Wei & McDonald, 2016).

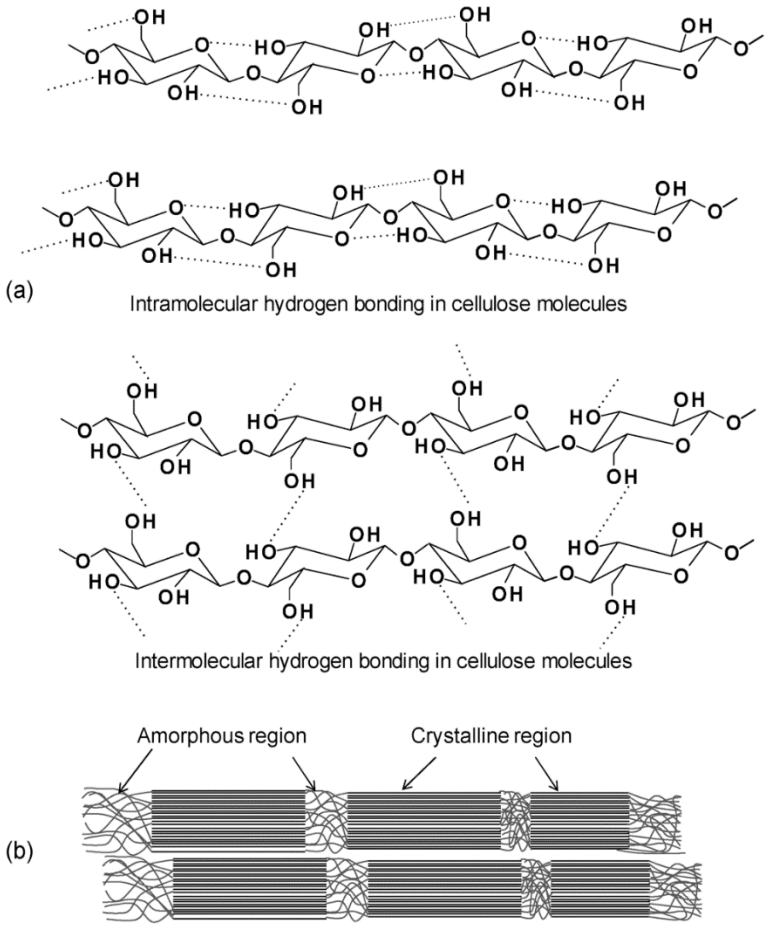


Fig 2.1 a) Hydroxyl groups of cellulose monomers showing intra- and inter-molecular hydrogen bonding; b) Cellulose molecules with amorphous and crystalline regions (Adapted from Wei and McDonald 2016).

2.5.2 Hemicellulose

Hemicellulose is present in lignocellulosic biomass and is also a polymer of saccharide units. It is heterogeneous in nature and is composed of chains which are shorter than with cellulose. The different monomer units which constitute hemicellulose are D-xylose, L-arabinose, D-galactose, D-mannose and D-glucose. Lignocellulose from hardwoods, softwoods and grasses contains (24-40%), (25-35%), (25-50%) of hemicellulose respectively. Cellulose binds to hemicellulose by

hydrogen bonding. Some other molecules such as alpha-L-rhamnose, alpha-L-fucose and alpha-glucuronic acids may also be present in small amounts, depending upon the biomass source (Jarvis, 1984).

2.5.3 Lignin

One of the most abundant constituent of biomass, lignin is aromatic and amorphous in nature and has different structures depending upon the plant species (Eudes et al., 2014). The monomer unit of lignin is phenyl propane which exhibits minor structural differences in substitutions of methoxyl groups on its aromatic ring. The main monomer units of lignin are hydroxyphenyl alcohol, coniferyl alcohol and sinapoyl alcohol. Higher amounts of phydroxyphenyl alcohol than coniferyl alcohol and synapyl alcohol have been reported in soft wood trees (Penning et al., 2014). Lignin is one of the major factors that limits the hydrolyzation of lignocellulosic biomass. Lignin provides strength to the cell wall by covalently binding to hemicellulose and hindering the enzymatic digestion of cellulose. As it prevents enzymes from reaching the cellulose binding sites, plant tissues high in lignin such as woody biomass, are harder than grassy biomass to hydrolyze. Lignin has the capacity to absorb enzymes irreversibly and decrease the effectiveness of hydrolysis (Preston, 1974).

2.5.4 Pectins

Pectins are non-cellulosic hydrocolloids that help structure the plant cell wall. The major component of pectin is homogalacturonan, a polymer of methylated galacturonic acid. Rhamnogalacturonan 1 and 2, xylogalacturonan, arabinan and arabinogalactan are minor constituents of pectin (Ridley et al., 2001). As a naturally occurring hydrocolloid polymer, it has great economic impact due to its extensive use in the food industry as a gelling and thickening agent (Ding et al., 2017).

2.6 Second generation biofuels from apple pomace

2.6.1 Apple production

Apple is one of the most important fruit crops grown worldwide; falling only behind banana in terms of worldwide production. Overall production is increasing globally and is currently dominated by the Chinese production; where more than 58% of the world apples are produced (USDA, 2018). Although Canada's share is much less (0.5% of global production), apple is the most important fruit crop in Canada in terms of production and is the second most economically valuable crop after blueberries (Magyar et al., 2016).

2.6.2 Apple industry and waste production

In the food industry, apples are used for the production of various food items and value-added edible products. Food items available in the market include fresh fruits (70%) and 25-30% of processed fruit in the form of juice, cider, sliced apple, dried apple, frozen foods, candies, jellies and jams. More than 64% of total processed apple products consists of apple juice concentrate. Although the Canadian apple production is decreasing, apple production in general is experiencing a surge in demand on the global scale, especially in apple processing volumes. This processing leaves an enormous amount of apple waste globally with millions of tonnes of agro-industrial waste generated worldwide (Lin et al., 2013). Thousands of tonnes of apple pomace and apple pomace sludge are generated every year in Canada alone.

A total of 336,834 metric tonnes of apples were produced in 2010 in Canada. Apple waste accounts for 25.33% of total apple production and includes apple pomace, apple pomace sludge and fruit spoilage at various levels. Large scale industrial waste from apples can be divided into two types. One is belt rejection which occurs before processing, and the other is pomace and sludge generation that occurs after processing and contributes to the larger part of the total waste

produced. Belt rejection is where any partially or fully damaged, bruised, or otherwise spoiled items are sorted out at the initial post-harvest stages (Dhillon et al., 2012). This is usually done manually and the rejected fruit is thrown away into a waste bin. On the other hand, most of the waste is generated after processing the apple fruit and is in the form of pomace and sludge. The apple juice industry generates 25-30% of its waste as pomace and 5-10% as liquid sludge with 70-75% recovery of juice.

2.6.3 The composition of apple pomace

Apple pomace is the solid residue left after the extraction of apple juice which includes apple skin (95%), seeds (2-4%) and stems (1%). Apple pomace mainly consists of non-starch polysaccharides (35-60% dietary fibre) with a greater proportion of insoluble fibres (36.5%) and fewer soluble fibres (14.6%) (Sudha et al., 2007; Ziegler & Filer, 1996). These dietary fibres are mainly composed of pectins (5.50%–11.70%), cellulose (7.20%–43.60%), hemicelluloses (4.26%–24.40%), lignins (15.30%–23.50%) and gums (Bhushan et al., 2008). The produced pomace is low in simple sugars, as most are extracted through the juicing process. Pomace is also low in protein content, so while it is used as a supplementary ingredient as animal feed, this is not a significant use of the product, both from nutritional and economical standpoints (Chen et al., 1988). However, there is an enormous potential for these sugars to be made available for use in biofuel production which would decrease the environmental impact of discarding apple pomace into landfills and may offer an economically advantageous alternative (Kennedy et al., 1999).

2.6.4 Apple sludge

After obtaining juice from pressing the apple, the juice is left to stand undisturbed for clarification with the addition of bentonite (Fig 2.2). The sediments settle and the supernatant is obtained as clear apple juice. The sediment gathered at the bottom is a highly viscous liquid containing apple

juice and bentonite particles, called sludge (Bhushan et al., 2008). This sludge is high in both sugars and moisture content and has a higher content of simple sugars than pomace (Gassara et al., 2012). The high availability of simple sugars in sludge makes it readily usable for fermentation, however, the higher pectin concentration is more challenging to degrade.

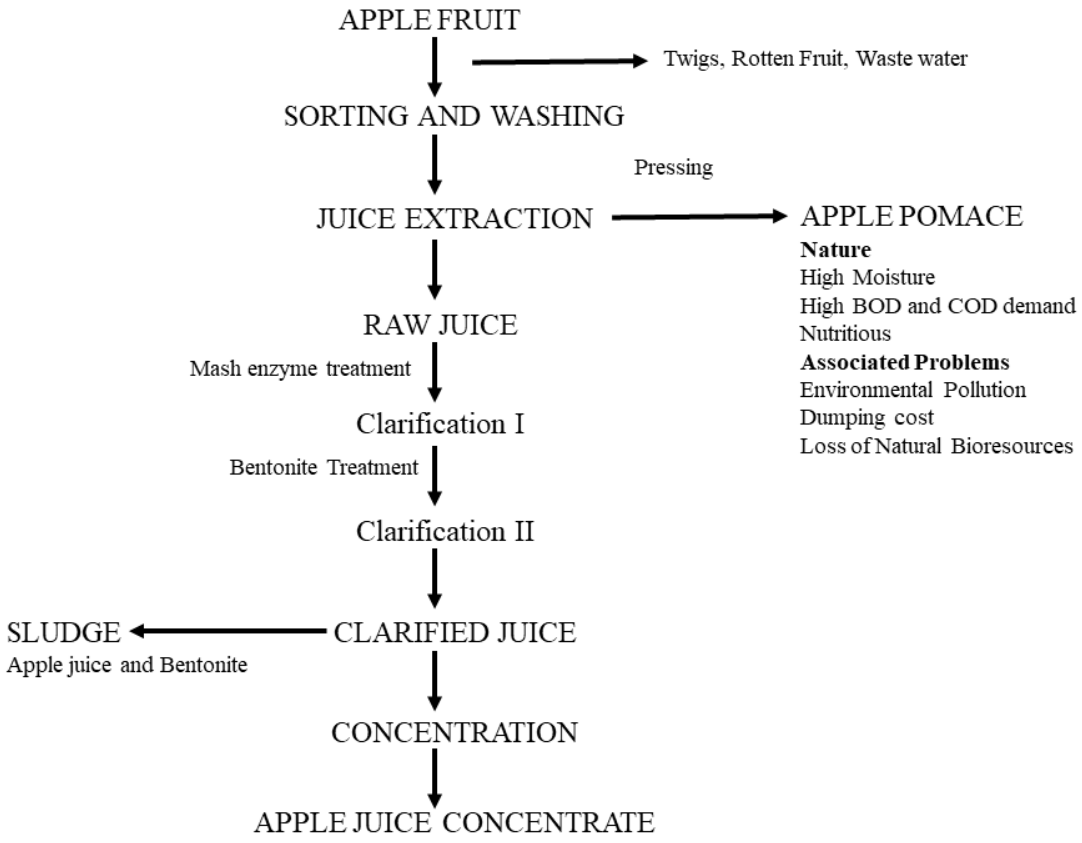


Fig 2.2 Flowchart showing the processing of apple fruits for juice concentrate (Adapted from Bhushan et al., 2008).

2.6.5 Apple pomace for bioenergy production

Apple pomace is a good source of pectin and other commercially important compounds such as antioxidants and its value-added use can reduce the residual waste of apple production. Pectins

extracted from apple pomace can be used as gelling agents in various food items such as jams, fillings and sweets, etc. (Dhillon et al., 2013). Apple pomace can also be processed into a dried powder which can be used in different edibles such as candies and flavouring bases. The most common commercial uses of apple pomace are for extracting pigments, producing vinegar, and processing dietary fibres and lactic acid (Baiano, 2014; Nawirska & Kwaśniewska, 2005).

The cellulose and pectin present in the pomace can be used as a substrate for enzymatic hydrolysis to yield fermentable sugars (Dos et al., 2012). One of the main methods to obtain energy from pomace is a two-step process that liberates, then ferments the sugars present into alcohol. Polysaccharides are first digested into simple sugars and then these sugars are fermented using selected microbes. Different methods of digestion of the polysaccharides have been reported and various organisms have been used for the secondary fermentation with varying results (Demers, 2012; Evcan & Tari, 2015; Gama et al., 2015; Parmar & Rupasinghe, 2013).

2.7 Conversion processes

Conversion of the cellulose present in lignocellulosic biomass into a more useful product is a complicated and time-consuming process. Even with a good carbon source, hydrolyzing cellulose is challenging due to its structural complexity in comparison to other saccharides while recovery of the desired product is not an easy process (Alvira et al., 2010). The efficiency and ease of hydrolysis of lignocellulosic biomass largely determines the ability of the substrate to be used as a source of biofuel. Initial breakdown of polysaccharides is the key factor that determines final yield (Pandey, 2011). Pretreatment is the primary step to hydrolyze/breakdown polysaccharides to simpler sugars before downstream processing. A lot of research has focused upon the testing and development of various methods of pretreatment for different biomasses (Fig 2.3). Some of these pretreatments have severe impacts on the environment, may require complex equipment and may

have complicated process parameters to monitor. The purpose of pretreatment is to breakdown the crystalline structure of cellulose, by breaking the inter-molecule bonds, to release glucose (Nathan et al., 2005). It does so by first breaking the hydrogen bonds between the surrounding lignin and hemicelluloses, thus increasing the surface area available for further cellulose breakdown. Pretreatment is chosen to increase the porosity and accessibility of the cellulose polysaccharide chain and accelerate the hydrolysis to yield glucose monomers (Singh et al., 2015). Pretreatments with high efficiency are not currently feasible as most techniques yield low sugar content at high cost (Alonso et al., 2010).

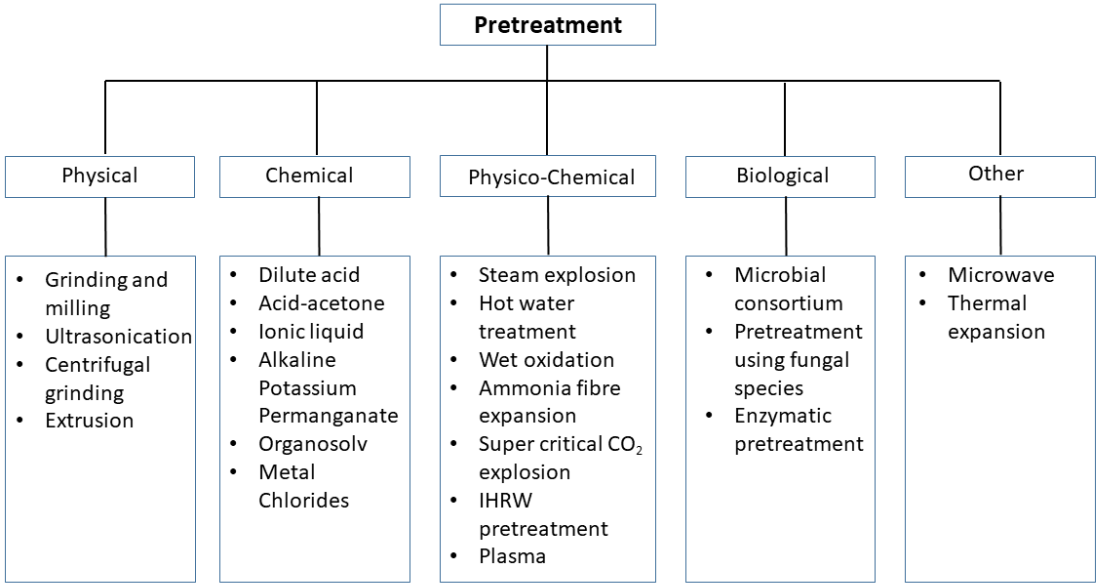


Fig 2.3 Classification of different methods for lignocellulosic biomass pretreatment (Adapted from (Ravindran & Jaiswal, 2016).

The different types of pretreatments can be classified as physical, chemical, physico-chemical, and biological.

2.7.1 Physical methods

Physical methods include grinding, microwaves, and ultrasound (Mosier et al., 2005). Grinding or milling increase the surface area of the biomass by reducing the particle size, however, particle size reduction does not break the substrate down to the molecular level and cannot liberate the bound glucose (Saini et al., 2015). The heating of biomass through microwave is an alternative that rapidly treats biomass with higher temperatures, which depends on the dielectric properties of the biomass substrate. Temperatures higher than 180 °C unfold cellulose's crystalline structure, however, cellulose can return to its crystalline form when the temperature is lowered. To avoid this, microwave treatment is often combined with some form of salt or acid.

2.7.2 Chemical methods

Chemical approaches include strong and weak acids, however a strong acid pre-treatment requires higher operational costs and specialized equipment (Silverstein et al., 2007). Chief among these problems is that of corrosion of the handling equipment caused by the acid. Weak acids do not tend to exhibit this equipment corrosion as readily but do not serve the purpose of hydrolysis appreciatively, as their degree of hydrolysis is very low (Yu et al., 2013). Use of ionic liquids and salts is another chemical means of pre-treatment, but due to their high cost compared to previous methods mentioned, they are not used as often. Low recovery of desired products that results from this treatment is another reason for its unpopularity. Organic solvents are a good method for the pre-treatment of biomass but again costs are high (Alonso et al., 2010).

Pre-treatment of biomass with ammonia has shown promise with sugar cane bagasse, corn stover, and cereal straws, as production of inhibitors is negligible and no particle size reduction is required. Pre-treatment with ammonia requires 1-2 kg of ammonia for 1 kg of dry biomass.

However, in this process, the recycling of ammonia is necessary due to its cost and environmental concerns (Zheng et al., 2009).

2.7.3 Biological methods

Biological pre-treatments involve the breakdown of biomass using microbial consortium, fungal treatment and enzymatic treatment. Naturally occurring bacteria such as *Lactobacillaceae* produces enzymes that are capable of digesting biomass to improve the yield of fermentable sugar but they are unable to degrade the lignin. Plant biomass is composed of complex structure interlaced by cellulose, lignin and hemicellulose. Lignin hydrolysis or delignification exposes woven cellulose providing opportunity for higher amount of cellulose for saccharification. However, some bacterial laccases characterized from *Azospirillum lipoferum* and *Bacillus subtilis* can also perform delignification (Kunamneni et al., 2007). Certain fungal species such as white, brown and soft-rot fungi are capable of digesting complex lignin polymers and hemicellulose components (Sahu & Pramanik, 2015a; Taherzadeh & Karimi, 2008). Some white-rot fungi (*P. chrysosporium, Pleurotus ostreatus, Pycnoporus cinnarbarinus, Trametes pubescens, Cyathus stercolerus* and *Ceriporiopsis subvermispora*) are reported to possess high delignification efficiency (R. Kumar et al., 2009; Marcolongo et al., 2014; Sahu & Pramanik, 2015b).

Due to the complex structure of lignocellulosic biomass, different enzymes are required for their efficient degradation process. Enzymatic hydrolysis is one of the most effective way for the digestion of complex polysaccharides into monosaccharides without the production of toxic by-products. The enzymes that are employed for the hydrolysis of cellulose and hemicellulose are classified as endo-*glucanases*, *exoglucanases*, and beta-*glucosidases* (Sahu, 2016); *hemicellulase* and *xylanase* enzymes are used for the hydrolysis of hemicellulose (Zawawy et al., 2011; Arantes & Saddler, 2011). Many microorganisms can produce enzymes (*cellulases*) that can hydrolyse the

complex structure of cellulose. Synthesis of these *cellulases* by various microorganisms has been thoroughly studied for a long time (Wilson, 2009). There are various pathways by which microorganisms can produce *cellulase* depending upon whether the organism grows via aerobic or anaerobic means (Devi & Kumar, 2017). The *cellulase* family of enzymes that can act differently on variable active sites of cellulose and based on their mode of action, have been divided into various groups (Beldman et al., 1988). Endoglucanases act at multiple sites of the polysaccharide chain of cellulose and release glucose and smaller polysaccharide chains of cellulose. *Exoglucanase* (cellobiohydrolases) hydrolyze the cellulose chain on both the reducing and non-reducing ends to release glucose or cellobiose. *Exoglucanases* act primarily against the crystalline form of cellulose and are inactive against cellobiose. *Cellodextrinases* are *exoglucanases* that specifically target cellobiose and cannot break down amorphous cellulose. Glucosidases breakdown cellobioses and cellodextrins from non-reducing sugars to liberate glucose. Cellobiose phosphorylases phosphorolytically breakdown cellobiose in a reversible process that liberates glucose. Cellodextrin phosphorylases catalyze the phosphorylic breakdown of cellodextrins, including sugars from cellotriose to cellohexose. Lastly, cellobiose epimerases release glucosylmannose by breaking down of cellobiose.

Various microbial strains such as *Trichoderma, Aspergillus, Cellulomonas, Streptomyces, Bacteriodes, Penicillum Altrernaria,* and *Bacillus* have been used for breakdown of lignocellulose through the production of efficient enzymes such as *xylanase, hemicellulase,* lignocellulolytic activity (synergistic effects of cellulolytic and lignolytic) (Devi & Kumar, 2017; Sun & Cheng, 2002). Microbial digestion requires less capital investment, lower energy input and is a more environmentally favourable treatment choice. However, it does require a longer treatment time period and commercial enzymes have been used often to speed up the breakdown to reduce the

time period required. Biological pre-treatment can be employed as a basic step for the pre-treatment of biomass with low lignin content or in combination with other pre-treatment methods based on the complexity of the biomass (Agbor et al., 2011).

2.8 Fermentation

Fermentation is used in various industries such as the food, pharmaceuticals and biofuels and is a crucial technique in the food industry for a variety of commodity production such as sauerkraut, beer and wine and for biomass conversion in general (Couto & Sanromán, 2006; Demain & Adrio, 2008; Mussatto & Teixeira, 2010). Depending upon the mode of operation, fermentation can be classified into two types: submerged fermentation (SmF) and solid state fermentation / cultivation (SSF) (Lin & Tanaka, 2006). Both SmF and SSF have advantages as well as disadvantages depending upon the type of biomass substrate being treated and the microorganisms used (Singhania et al., 2010). Solid and low moisture content substrates like straw, bran, paper pulp, bagasse and fruit pomace mainly use SSF as these substrates lack moisture during the fermentation process (Pandey, 2003a). SSF is the process of microbial growth and product formation from the substrate in the absence or near-absence of free water (solid state fermentation) (Young et al., 1980; Mudgett, 1986). SSF is most suited for microbes such as fungi and dry biomass substrates used in SSF can be easily recycled for further utilisation (Hölker et al., 004). SmF is used when the substrate contains free water and most of the target compound is submerged in the liquid. Some substrate examples include molasses, broth and sludge (Vidyalakshmi et al., 2009). SmF is the process where microorganisms grow in liquid media and it can only be performed with microorganisms requiring high moisture content such as specific bacteria (Sunnotel & Nigam, 2002). The product formed by SmF is mixed with the liquid already present in the reaction and can be easily removed, however, products at lower concentrations are hard to separate from SmF.

SSF is therefore preferred over SmF as it requires less handling, is easier to perform and cheaper than SmF (Ratna & Mustafa, 2011). SSF has been favoured over the years with the development of new strains of microorganisms by genetic engineering and has several advantages economically and operationally such as low energy and water requirements, less waste and easy separation (Sun & Cheng, 2002). The recovery of desired substrates through SSF has been reported to be 20-30% as compared to 5% in SmF (Brahmachari, 2016).

Fermentation processes are used for production of various products such as citric acid, ethanol, butanol, etc. (Hang & Woodams, 1982; Hang & Woodams, 1986; Khedkar et al., 2017). A wide variety of microorganism can be used for this purpose. A number of bacteria and fungi have been identified such as *Actinobacillus, Anaerobiospirillum, Clostridium, Saccharomyces, Aspergillus etc.*, and are recommended depending upon the final product desired (Song & Lee, 2006). The end products usually depend upon the type of substrate used. Some of the most commonly used substrates are crop waste, industrial fruit and food waste. Well studied and high yielding fungal strain *Saccharomyces cerevisiae* is the organism of interest for ethanol production in this study.

2.9 Factors affecting the biomass conversion processes

Growth of fungus can be limited by the nutritional factors, temperature, moisture content and the incubation time. Optimization of these factors is important to obtain higher levels of sugar yield through solid state fermentation (Kumar et al., 2014). Ratio of carbon and nitrogen is an important indicator of fungal growth in SSF. It is important to add a nitrogen source while the carbon source comes from starchy and cellulosic biomass materials. Different nitrogen sources such as peptone, yeast extract, tryptone, glycine, urea, ammonium chloride, ammonium sulphate and ammonium citrate have been utilized as nitrogen supplements for SSF (Uyar & Baysal, 2004). Success of overall process of SSF is also subject to the availability of moisture. The availability of water in

the substrate required for the fungal growth is expressed as the water activity (a_w). A higher a_w is required for the growth of most bacterial species; however filamentous fungi and some yeasts can grow at lower water activity (a_w). Microorganisms capable of growing under low a_w condition are considered suitable for the SSF (Oriol et al., 1988; Pandey & Vijayalakshmi, 1994). Under SSF, required levels of water activity can be determined by the type of microorganism, type of substrate and nature of the end product. An optimum level of a_w needs to be determined to avoid over-saturation or dryness of the substrate. High levels of a_w result in lower oxygen diffusion, decreased gas exchange, decreased porosity/intercellular spaces, decreased substrate degradation and increased risk of bacterial contamination. In contrast, lower a_w leads to lower substrate swelling and decreased microbial growth (Udo & Lenz, 2005; Lonsane et al., 1985; Raimbault, 1998; Ramesh & Lonsane, 1990). Every microorganism has its own optimal temperature for its growth and metabolism which makes temperature another critical factor for the success of SSF. During the process of solid state fermentation, a large quantity of heat is produced due to the metabolic activities of the microorganisms. Maintaining the suitable temperatures is not difficult under the laboratory conditions; however, for SSF at a larger scale, substrate concentration and microbial heat generation per unit volume are much higher than for liquid fermentation. Therefore means for constant heat removal is required to maintain the optimum temperature of the process (Lonsane et al., 1985; Pandey, 1992). Incubation time is another factor that is important for the complete hydrolysis process to take place. Optimization of incubation time is required for higher sugar yield and to lower the production of inhibitors (Pandey, 2003a, 2003b; Sun et al., 2010). This study has been conducted to maximize sugar yield from apple pomace through saccharification using two different fungi (*S. cerevisiae* and *M. thermophila*) and using obtained sugar substrate for subsequent ethanol production with the help of *Saccharomyces cerevisiae.*

CONNECTING STATEMENT FOR CHAPTER III

Based on the literature review in chapter II, the apple juice industry generates 25-30% of its waste as pomace. Apple pomace mainly consists of non-starch polysaccharides (35-60% dietary fibre) with a greater proportion of insoluble fibres (36.5%) and fewer soluble fibres (14.6%). The cellulose and pectin present in the pomace can be used as a substrate for enzymatic hydrolysis to yield fermentable sugars. Various fungal strains can be employed for the degradation of lignocellulosic biomass. Growth of fungus can be limited by the nutritional factors, temperature, moisture content and the incubation time. Optimization of these factors is important to obtain higher levels of sugar yield through solid state fermentation. A detailed information about the optimization of the growth conditions for fungi *Trichoderma reesei,* and *Myceliophthora thermophila* is discussed in chapter III.

CHAPTER III: Study of growth conditions for fungi *Trichoderma reesei*, and *Myceliophthora thermophila* using apple pomace as a substrate to maximize sugar yield

Abstract

Apple pomace is a processing waste produced after the extraction of the juice from apples and it represents approximately 25-35 % of the weight of the fresh apples. Efficient utilization of the waste product can result in value added products as well as solve the problem related to its disposal. This study has been planned to understand and optimize the effect of factors such as temperature, incubation time, moisture and nitrogen supplementation on biological pre-treatment for maximizing sugar yield from apple pomace. We have selected two fungal strains *Trichoderma reesei* and *Myceliophthora thermophila* for biological pre-treatment of the substrate. Optimized moisture was found to be 90% for *M. thermophila* and 85% for *T. reesei*. The range of temperatures tested have come out to be insignificant, however, maximum yield was obtained at 28°C. The saccharification of apple pomace by both the fungal strains of *T. reesei* and *M. thermophila* increased progressively with incubation time increasing from 3 to 11 days, with a significant high on the 7^{th} day for *T. reesei* and on the 9^{th} day for *M. thermophila* while the maximum was reached on the 11^{th} day bearing no significant increment between 9^{th} and 11^{th} day ($p<0.05$). 1% w/w peptone was found to be the most effective nitrogen supplementation dose to increase the sugar yield for both the fungal strains tested.

3.1 Introduction

Apple pomace is the waste produced after the extraction of juice from apples and it contains peel, seeds and remaining solid parts formed after juice extraction. It represents approximately 25-35 % of the weight of the processed fresh apples (Joshi & Attri, 2005). Every year 17-24 million metric tonnes of waste are produced worldwide (Van & Pletschke, 2013). Most of the waste is disposed

of to landfills and causes major environmental and health issues due to its richness in carbohydrates, dietary fibres and minerals, high fermentable sugar, high moisture content (70–75 %), high chemical oxygen demand (COD, 10,000 mg/L) and biological oxygen demand (BOD) (Bhushan et al., 2008; Parmar & Rupasinghe, 2013). Treatment of this waste, combined with the production of value added products can solve the problem of disposal and will add revenue to the bio-economy. Apple pomace is rich in cellulose and hemicellulose which can be enzymatically hydrolysed to sugar monomers and then fermented to ethanol (Parmar & Rupasinghe, 2013; Pranita, 2015; Vendruscolo & Ninow, 2014). Commercial enzymes can be used to hydrolyze lignocellulosic biomass into fermentable saccharides (Wen & Chen, 2005). However, the high cost of commercial cellulose enzymes makes the process non-economical (Wen & Gallaher, 1988). However, the cost of *cellulase* can be significantly reduced by producing *cellulase* directly from the apple pomace with the production of cellulolytic enzymes by a number of bacteria and fungi. Biological pre-treatments with various fungi and bacteria require less capital investment, lower energy input and are more environmentally favourable treatment choices. Choice of the proper microorganism and optimization of growth conditions and supplementation of medium with adequate amount of a nitrogen source are the most important factors for the successful fermentation of apple pomace (Kumar et al., 2014; Mahawar & Sahgal, 2013; Uyar & Baysal, 2004). *Trichoderma* has been considered the most productive organism for cellulose degradation and *Myceliophthora* has evolved as a promising alternative to *Trichoderma* (Alexander, 2011). In the present study, we have selected both the fungal strains *Trichoderma reesei*, and *Myceliophthora thermophila* to understand and study the effect of biological pre-treatment factors such as temperature, incubation time, moisture and nitrogen supplementation for maximizing sugar yield using a full factorial experimental design.

3.2 Materials and Methods

3.2.1 Apple pomace

Apple pomace was provided by A. Lassonde, a juice and beverage manufacturing company (Rougemont, QC), with 1% (w/w) rice husk which was added during the processing of apples as a general practice to favour juice extraction during pressing. Once received, the apple pomace was frozen until used for the experiments. The moisture of the apple pomace was analyzed by oven drying at 105°C for 48 hours (Samuelsson et al., 2006). 10 g of frozen sample was thawed and distilled water was added as per the moisture content requirements for this study. Pomace was transferred to 125 ml glass jars (Fig 3.1). Five levels were tested for each of the variables studied, namely temperature, moisture level and incubation period. The experiment was performed in replicates of five for each combination.

3.2.2 Fungus culture

The cultures of fungi, *T. reesei*, and *M. thermophila* were obtained from the Agricultural Research Service of the USDA (United States Department of Agriculture). Both the fungi were grown on PDA (Potato Dextrose Agar) medium for 4 days at 37°C for spore formation as per the instructions provided with the strains. After spore formation, the cultures were maintained at 4°C until further use. The colony forming units (CFU) were calculated using serial dilutions. All fungal cultures were sub-cultured every 2 weeks and incubated at 37°C and subsequently stored at 4°C for inoculum preparation.

3.2.3 Sample preparation and inoculations

All equipment and lab wares were sterilized at 121°C for 30 min using an autoclave. A population of $10^8 – 10^9$ spores calculated with haemocytometer was used to inoculate 10 g of the apple pomace substrate and then incubated at variable temperatures (24, 26, 28, 30 and 32°C), at different moisture contents (70, 75, 80, 85 and 90%), and various incubation times (3, 5, 7, 9 and 11 days). Based on the optimum temperature, moisture and incubation time, the influence of nitrogen source (peptone) was evaluated at 0.5%, 1%, and 2% w/w.

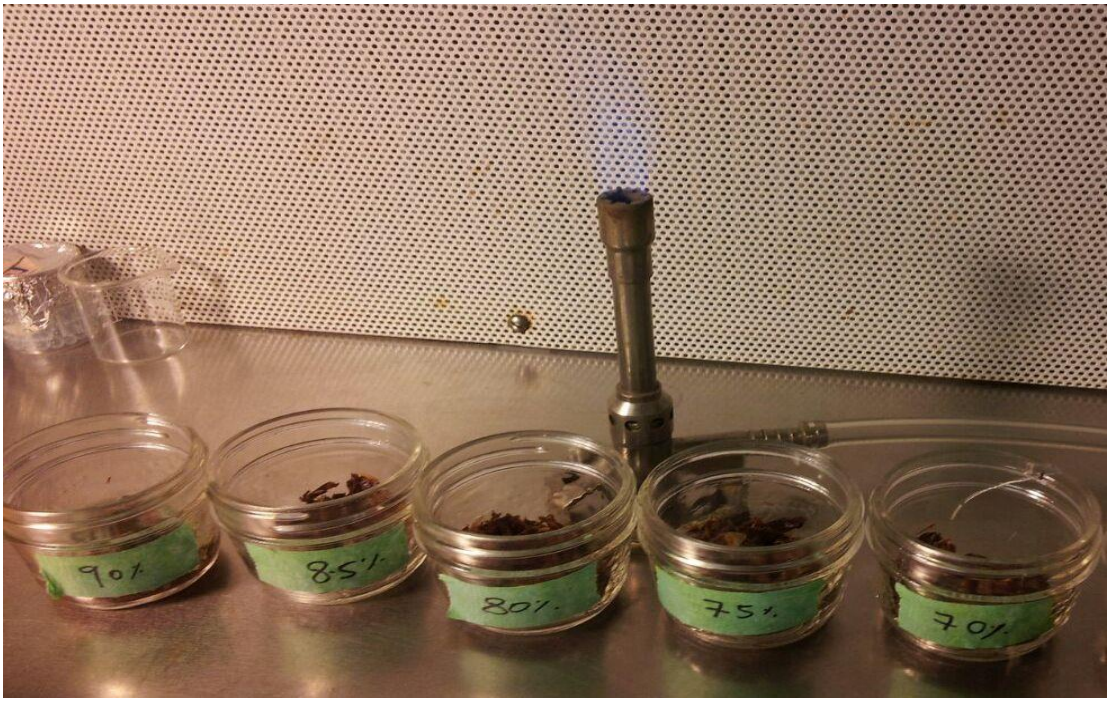


Fig 3.1 Apple pomace samples marked for incubating at different moisture levels (90%, 85%, 80%, 75%, and 70%).

3.2.4 Sugar measurement

Total sugar content (Brix values) was measured using a hand-held mini refractometer r^2 model manufactured by Reichert (Kim et al., 2014). Brix has been used as an approximate measure of

sugar content in various products in the fruit industry and is most widely used in general chemistry (Bhosale, 2017; Jacobson, 2006). Brix value represents the approximate sugar content of 1 gram in 100 grams of solution as a percentage by mass. The amount of sugar in the pomace was first measured before the beginning of the experiment. Measurements were taken every 2 days until 11 days to study the saccharification of the apple pomace by the selected fungus.

3.2.5 Experimental design and statistical analysis

To determine the combination of temperature, moisture and time period for higher sugar yield from apple pomace, a full factorial experimental design was used with three independent variables (temperature, moisture, time). This design was preferred to evaluate the interaction between different factors. The response function was sugar yield (Brix value) as a function of temperatures (24, 26, 28, 30 and 32°C), different moisture levels (70, 75, 80, 85 and 90%), and different incubation periods (3, 5, 7, 9 and 11 days). The experiment was performed with five replicates for all the variables combination. Design and data analysis were carried out using JMP software (SAS Institute, 2014). An analysis of variance (ANOVA) was performed and significance was verified at $p \leq 0.05$. Five levels for each factor (temperature, incubation time and moisture content) were taken in replicates of five.

3.3 Results

In the present experiment, we studied the fungal growth conditions that may favour the increase in sugar yield from the apple pomace treatments. Based on the statistical analysis, it was observed that the moisture level and the incubation time significantly impacted the sugar yield, however the impact of temperature remained insignificant in the case of *T. reesei* (Table 3.1).

Table 3.1) Statistical analysis of the tested factors on saccharification of apple pomace carried out by a) *T. reesei* b) *M. thermophile*.

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Temperature (degree C)	1	1	0.82022	2.1611	0.1421
Moisture (%)	1	1	535.84904	1411.846	<.0001*
Temperature (degree C)*Moisture (%)	1	1	0.05750	0.1515	0.6972
Time (days)	1	1	752.85969	1983.622	<.0001*
Temperature (degree C)*Time (days)	1	1	0.25281	0.6661	0.4147
Moisture (%)*Time (days)	1	1	121.22010	319.3886	<.0001*
Temperature (degree C)*Moisture (%)*Time (days)	1	1	0.09786	0.2578	0.6118

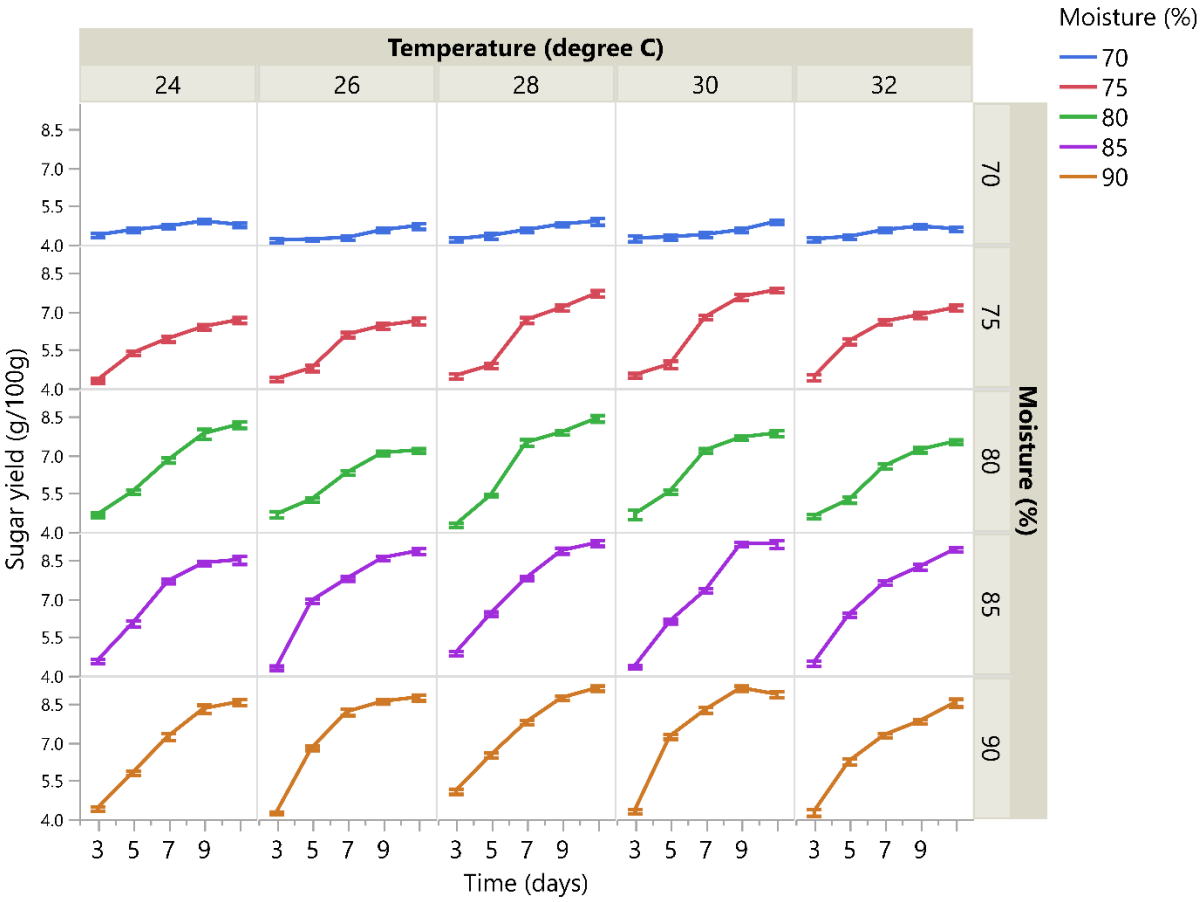
Table 3.1 a)

Effect Tests

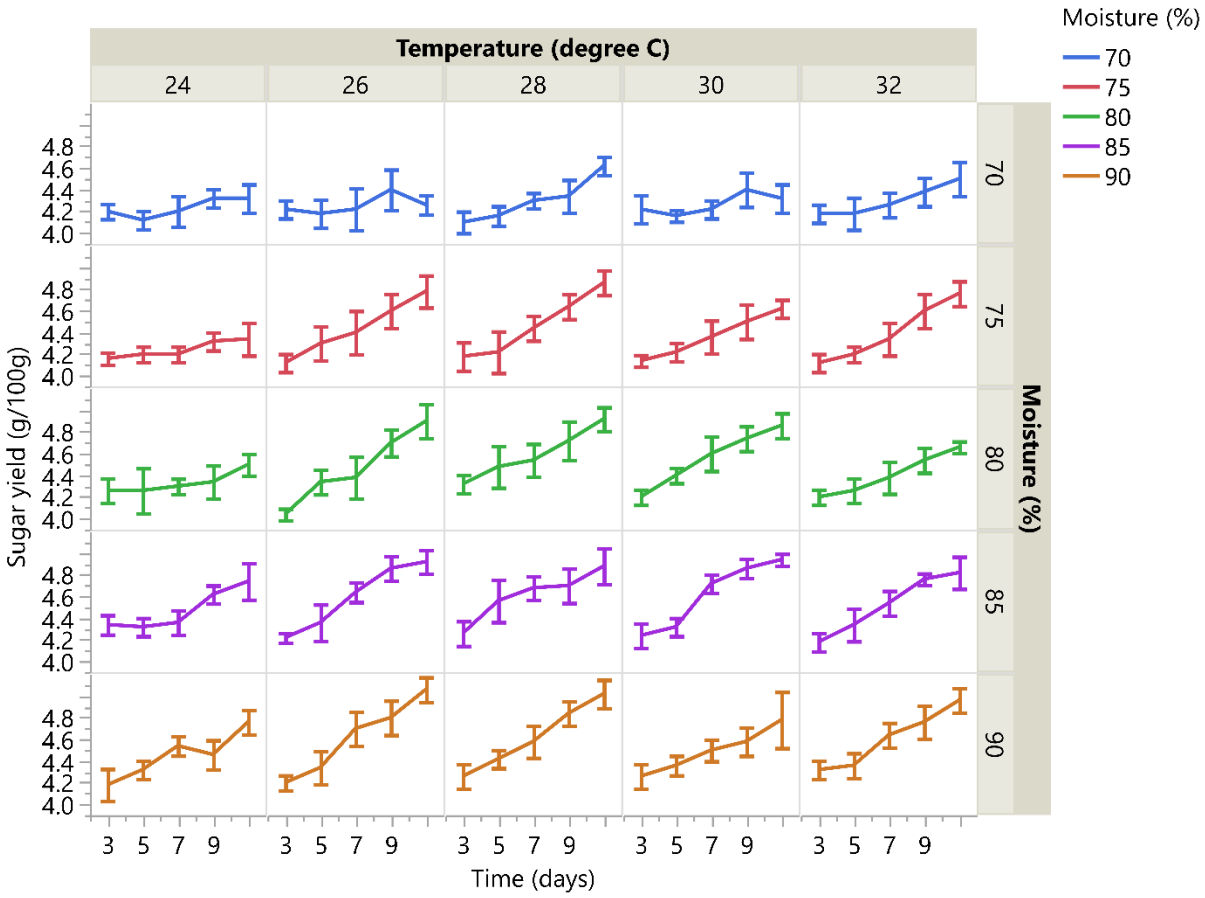
Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Temperature (degree C)	1	1	0.441800	18.8207	<.0001*
Moisture (%)	1	1	7.220000	307.5727	<.0001*
Temperature (degree C)*Moisture (%)	1	1	0.000400	0.0170	0.8962
Time (days)	1	1	22.257792	948.1842	<.0001*
Temperature (degree C)*Time (days)	1	1	0.240100	10.2283	0.0015*
Moisture (%)*Time (days)	1	1	1.313316	55.9474	<.0001*
Temperature (degree C)*Moisture (%)*Time (days)	1	1	0.044402	1.8915	0.1695

Table 3.1 b)

The combined effect of temperature, moisture and time has been reported statistically insignificant for the saccharification process in the case of both fungi used. Moisture and time interaction is significant in both the cases whereas temperature and time interaction has been observed insignificant in case of *T. reesei* while statistically significant for *M. thermophila*. Moisture and temperature interactions have resulted in an insignificant effect on sugar yield in both fungal cases (Fig 3.2).


Moisture (%)
70
75
80
85
90
Temperature (degree C)
24
26
28
30
32
Moisture (%)
Sugar yield (g/100g)
8.5
7.0
5.5
4.0
3 5 7 9
Time (days)

3.2a)



3.2b)

Fig 3.2 Graph showing the combined effect of moisture content at 5 levels (70, 75, 80, 85 and 90%), time at 5 levels (3, 5, 7, 9 and 11 days), and temperature at 5 levels (24°C, 26°C, 28°C, 30°C, 32°C), on sugar yield for (a) Fungal strain *T. reesei* and (b) Fungal strain *M. thermophila.*

3.3.1 Optimum incubation time for sugar yield

The evolution of the sugar content was affected by the time of the process as the measured brix value increased with time while on the 9th day it was 8.4% and on the 11th day it was 8.8% for the fungal strain *T. reesei.* For *M. thermophile*, the Brix values obtained were 4.8% and 5.0% on the 9th and 11th day respectively. However, the increment in sugar content after 9th day was

insignificant as verified by student's t-test at $p \leq 0.05$, while the maximum was nonetheless reached on the 11th day. As shown in Figure 3.3a and 3.3b, the saccharification of apple pomace by both fungal strains *T. reesei* and *M. thermophila* increased progressively with incubation time from 3 to 11 days and reached a maximum on the 11th day. Following the 9th day, however the increase in sugar yield was not significant in both cases.

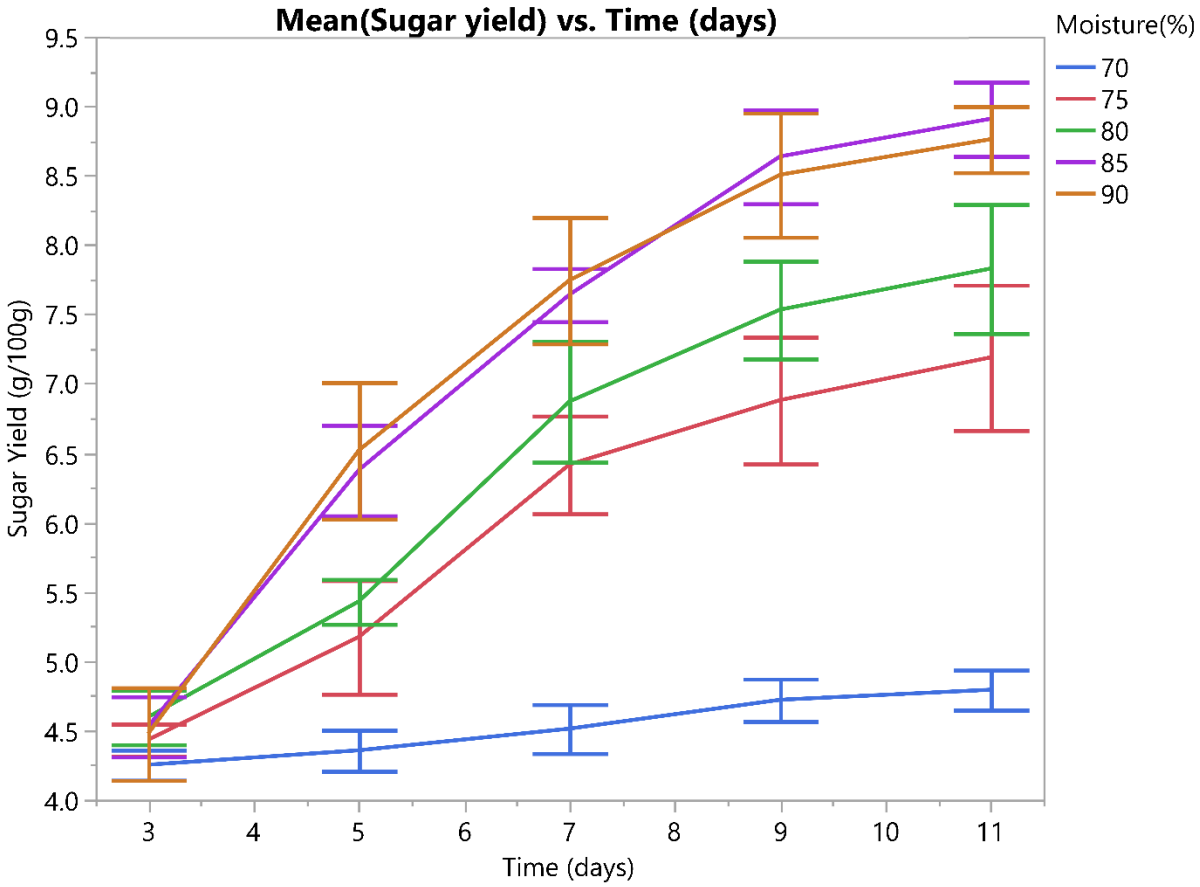


Fig 3.3a) Effect of incubation time on sugar yield at different moisture levels for fungus *T. reesei* irrespective of the temperature used.

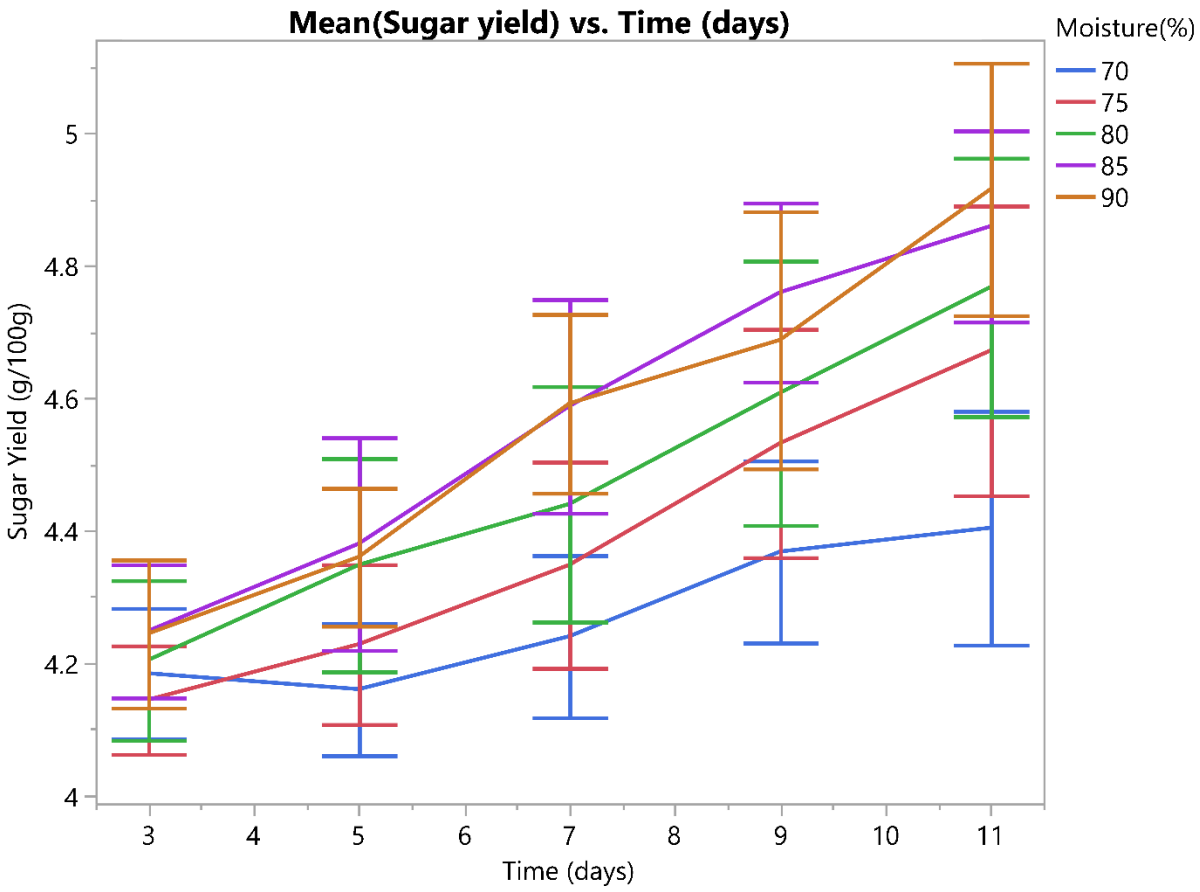


Fig 3.3b) Effect of incubation time on sugar yield at different moisture levels for fungus *M. thermophila* irrespective of the temperature used.

3.3.2 Effect of the medium's moisture level on the saccharification process

Moisture content being a critical factor for fungal growth was seen as a controlling factor for the level of saccharification of the apple pomace during fungal growth on the pomace. The highest values of sugar content were observed at a moisture content (wet basis) of 90% for *M. thermophila* and 85% for *T. reesei.* Lower and higher moisture contents than 90% for *M. thermophila* and 85% for *T. reesei* were leading to a decreased sugar yield for both the fungal strains (Fig 3.3a and 3.3b).

3.3.3 Effect of incubation temperature on sugar yield

Lower temperatures can hinder the growth of microbes leading to lower outputs. Higher temperatures dry out the samples leading to lower moisture content which results in hindered growth of the fungus (Sun et al., 2010). Thus, a range of incubation temperatures (24, 26, 28, 30 and 32°C) was tested for promoting fungal growth and its related sugar yield. The range of temperatures tested led to an insignificant effect, while maximum yield was obtained at 28°C for both *M. thermophila* and *T. reesei*. Based on the statistical analysis, the effect of temperature was not significant on sugar yield (Fig 3.4a and 3.4b). In the case of *M. thermophila,* only effect reported was at 24°C as a significant low. No other temperatures tested observed a significant difference. Temperatures from 26°C to 30°C showed no significant effect on the sugar yield for *M. thermophila.*

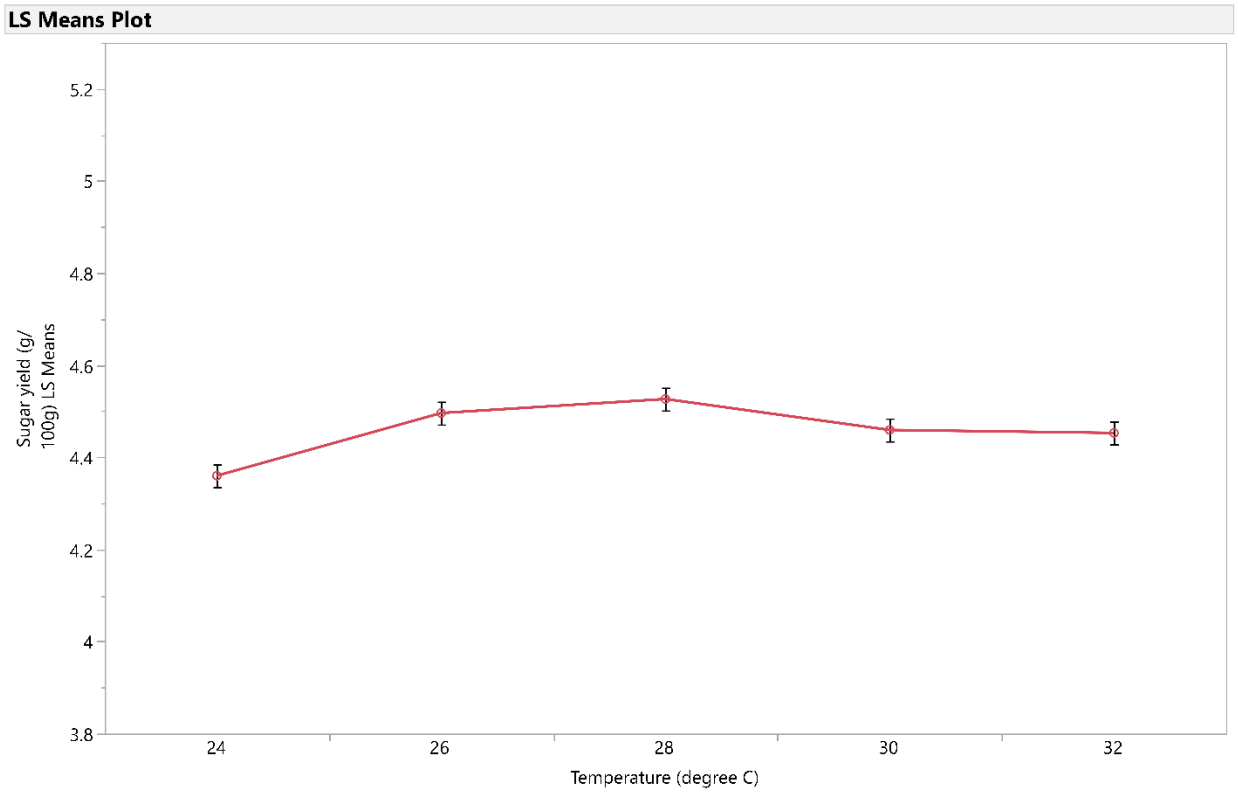


Fig 3.4a) Effect of temperature on sugar yield using fungus *M. thermophila.*

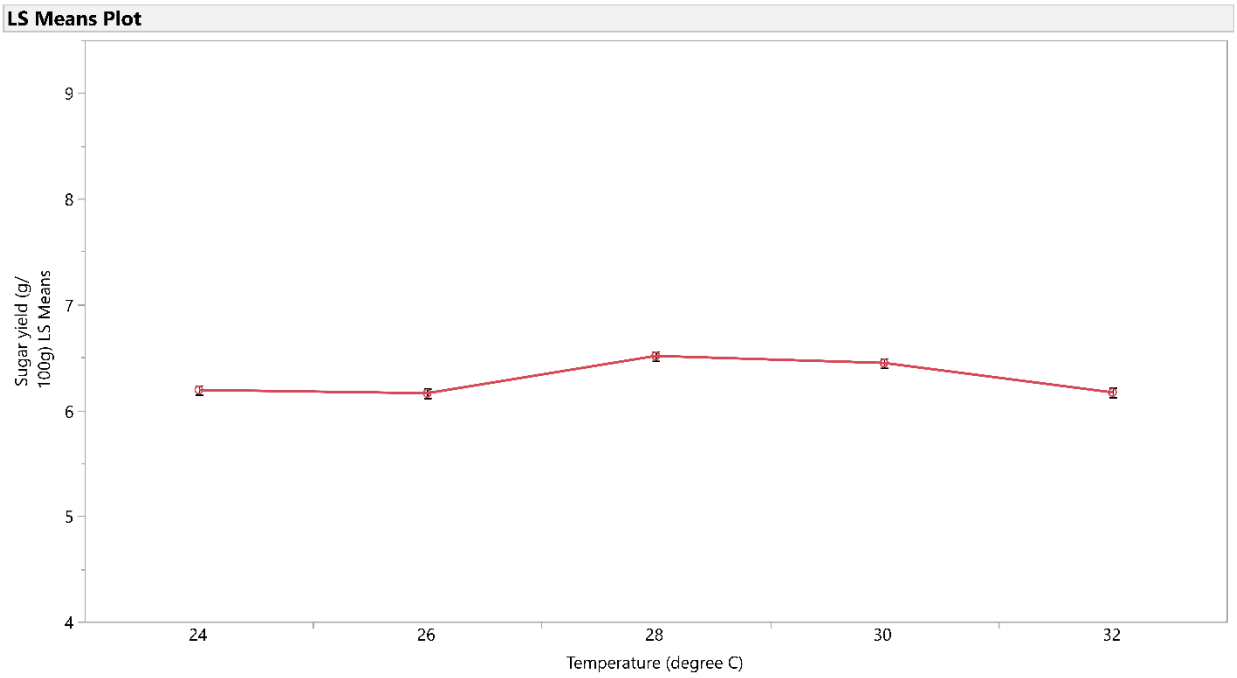


Fig 3.4b) Effect of temperature on sugar yield using fungus *T. reesei.*

3.3.4 Effect of supplementation of apple pomace with peptone as a nitrogen source

Three different levels (0.5%, 1%, and 2%) of nitrogen source (Peptone) were tested for their effect on sugar yield from apple pomace at 85% moisture content and 30°C temperature. Supplementation of apple pomace with a nitrogen source resulted in a significant increase in the sugar yield ($p<0.05$) as compared to control (Fig 3.5). 1% w/w peptone treatment had a significantly higher yield among the levels tested using t-test at $p<0.05$ (Table 3.2). For fungal strain *M. thermophila*, using 1% w/w peptone, a higher sugar yield measured was at a Brix value of 6.32%, obtained on the 9th day of incubation. However, the Brix value was 4.85% for the substrate without the supplementation of peptone (control) on the 9th day. On the other hand for the fungal strain *T. reesei*, the maximum sugar yield (Brix = 9.65%) was obtained on the 7th day of incubation as compared to the similar yield (Brix = 8.77%) obtained for the control on the 9th day of incubation.

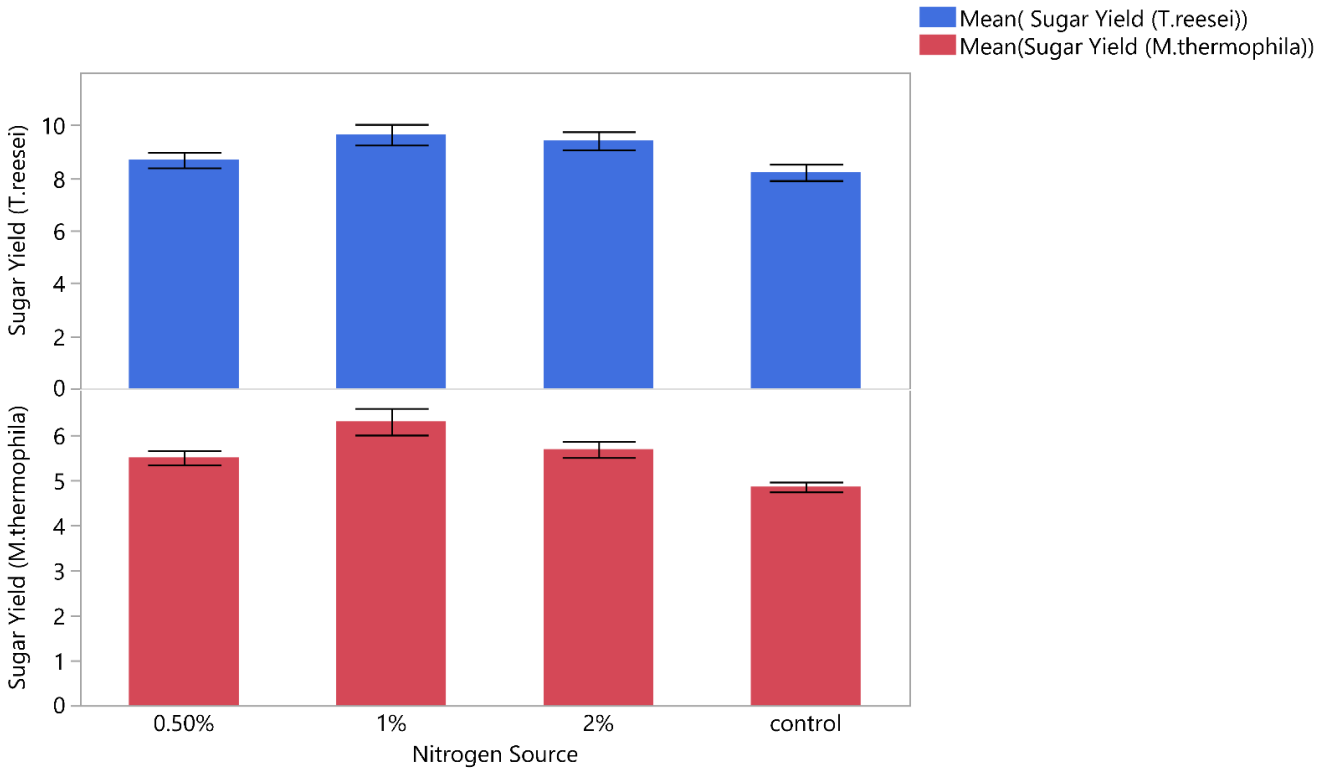


Fig 3.5 Bar chart showing the effect of nitrogen source (peptone w/w) on the sugar yield using fungus *T. reesei* and *M. thermophila*.

Table 3.2 Statistical analysis showing 1% nitrogen supplementation as the most favourable dose for sugar yield a) *T. reesei* b) *M. thermophila*.

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	8.9916667	0.065828	136.59	<.0001*
Nitrogen Source[0.50%]	-0.308333	0.114018	-2.70	0.0137*
Nitrogen Source[1%]	0.6583333	0.114018	5.77	<.0001*
Nitrogen Source[2%]	0.425	0.114018	3.73	0.0013*

Table 3.2 a)

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	5.5833333	0.038819	143.83	<.0001*
Nitrogen Source[0.50%]	-0.083333	0.067237	-1.24	0.2295
Nitrogen Source[1%]	0.7166667	0.067237	10.66	<.0001*
Nitrogen Source[2%]	0.1	0.067237	1.49	0.1525

Table 3.2 b)

3.4 Discussion

Apple pomace is seen as a potential fuel source for bioenergy production due to its sugar rich nature. The cellulose, when broken down into glucose, and the pectin contained in the pomace can be fermented to ethanol (Dos et al., 2012). One of the main methods to yield energy from pomace is a two-step process that first releases then ferments the sugars present into alcohol. Polysaccharides are first digested into simple sugars and then these sugars are fermented. Hydrolyzing the polysaccharides to simpler sugars before downstream processing can be done by a variety of pre-treatments. Numerous techniques of pre-treatment are being developed for effective hydrolysis of biomass (Alonso et al., 2010). These techniques can be categorised as physical, chemical, physico-chemical and biological treatment of biomass. Biological pathway for treatment of biomass was considered for this study. Different microbes such as bacterial and fungi can be used as biological agents for the biomass treatment. Filamentous fungi particularly *T. reesei* and *M. thermophila* appear to be suitable organisms as the pomace substrate provides an adequate natural habitat for their growth (Florencio et al., 2016). Filamentous fungi have the tendency to enter pores of the substrate and bind firmly to the solid substrate. High moisture conditions favour fungal growth however filamentous fungi have high efficiency at lower water activity making them naturally optimum for solid substrates (Raimbault, 1998). This study has investigated the activity of the selected filamentous fungi in solid state fermentation and has yielded a significant amount of sugars. *T. reesei* is an ascomycete fungus used as a source of *cellulases* and *hemicellulases* in the industry to breakdown complex polysaccharides. This fungal strain has emerged as a model system for the study of lignocellulosic degradation (Martinez et al., 2008; Nevalainen et al., 1994). The cellulolytic fungus *T. reesei* has been extensively studied for its *cellulase* production for substrates such as rice straw, apple distillery waste, and paper waste

(Domingues et al., 2000; Ellilä et al., 2017; Friedrich et al., 1987; Ju & Afolabi, 1999; Kogo et al., 2017; Sternberg, 1976). Another fungal strain *M. thermophila* is also a filamentous fungus which was, based on its morphological characters, previously known as *Crysosporium lucknowense* which has been reported as an important alternative for the production of various enzymes for industrial applications (Visser et al., 2011). This fungus has recently raised attention for its industrial application for *cellulase* enzyme production, but has not yet been exploited for apple waste processing.

This study has investigated the hydrolysis of cellulose from apple pomace using *T. reesei* and *M. thermophila*. The results have shown the degradation of cellulosic biomass to release fermentable sugars by both the fungal strains. However, *T. reesei* has yielded more sugars than *M thermophila. T. reesei* is considered the highest *cellulase* producing microbe. *M. thermophila* has shown competitive production of enzymes in terms of activity as compared to *T. reesei* on commercial scale. However current study suggests that *T. reesei* still leads *M. thermophila* for cellulose hydrolysis. Although *T. reesei* has been previously used for *cellulase* production from various substrates, its use for apple pomace and sludge hydrolysis had not yet been explored (Ortiz et al., 2015; Xin & Geng, 2010).

An optimum moisture level and incubation time is required for the solubility and distribution of nutrients in the substrate while maintaining porosity and stickiness of the medium for the appropriate growth of selected fungal species. Low moisture content leads to less swelling of the substrate and high water tension resulting in reduced solubility of nutrients, however higher moisture content decreases porosity, oxygen transfer and diffusion, which increases stickiness leading to higher number of aerial hyphae. In the present study the optimum moisture level was recorded as 90% for *M. thermophila* and 85% for *T. reesei.* A decrease in sugar yield was recorded

for both the fungal strains at lower and higher moisture contents than the optimum. Less incubation time produces lower yield due to incomplete degradation of the substrate whereas longer incubation periods can lead to the production of inhibitors (Gao et al., 2008; Pandey, 2003a; Pasanen et al., 2000; Sun et al., 2010). We have noticed similar findings where the sugar yield was less for first few days and then started rising significantly. After the 9^{th} day of incubation, the increment was recorded as statistically insignificant at $p<0.05$.

Microbes require a nitrogen source for their growth and multiplication. Apple pomace is rich in sugars but low in nitrogen. To provide an appropriate environment for microbes and increase the efficiency of the sugar yield, we have also tested different concentrations of peptone as an external nitrogen source. Providing an external nitrogen source can facilitate the growth of fungi and a 1% w/w peptone addition was found to be the most effective dose to increase the sugar yield for both the fungal strains tested. In the case of the fungus *T. reesei*, the maximum sugar yield was also achieved in less time as compared to the samples without added peptone (control). Similar results were obtained for different fungal strains grown on bacteriological agar and modified Melin-Norkans nutrient agar (MMN) with added nitrogen sources (Albers et al., 1996; France & Reid, 1984). Different levels of nitrogen supplements were found significant over the control. 1% peptone supplementation was significant among all the other levels tested in this study.

3.5 Conclusion

Overall this study has been performed to optimize different variables that can affect the growth of fungal stains and their efficiency for yielding fermentable sugars. The parameters (temperature, time and moisture) were optimized for yielding the maximum fermentable sugars in the substrate by the use of fungi *T. reesei* and *M. thermophila*. The results showed that the fungal stain *T. reesei* is more efficient for optimal sugar yield as compared to *M. thermophila.*

CONNECTING STATEMENT FOR CHAPTER IV

In chapter III, the parameters such as temperature, time, moisture and nitrogen supplementation were optimized for yielding the maximum fermentable sugars in the substrate by the use of fungi *T. reesei* and *M. thermophila*. These fungal strains can be used to develop a low-cost alternative solution to bioethanol production from apple waste. 20% of the total energy potential of the apple pomace can be recovered through the ethanol production. The parameters optimized in chapter III, were used for the production of fermentable sugars through the enzymic hydrolysis and processed by using *Saccharomyces cerevisiae* (one of the most widely used yeast for ethanol fermentation) to yield bio-ethanol. The details of ethanol production process have been discussed in chapter IV.

CHAPTER IV. Production of ethanol from apple pomace treated with *T. reesei* and *M. thermophila* and apple sludge using *S. cerevisiae*

Abstract

By-products of the apple processing industry are rich in both soluble and insoluble carbohydrates and can be utilized as substrates for the production of bioethanol. This study has been performed to investigate the effects of different fungal strains and co-culturing on bioethanol production and to attempt to develop a low-cost alternative solution to bioethanol production from apple waste. The addition of *S. cerevisiae* in the apple pomace samples pre-treated with *T. reesei* resulted in 6.11 % (v/w) amount of ethanol after 72 hours. The inoculation of *M. thermophila* treated samples with *S. cerevisiae* yielded 3.96% (v/w) amount of ethanol from the apple pomace after 72 hours. In comparison, 3.72% alcohol was obtained from the apple pomace treated with only *S. cerevisiae* as a control. *S. cerevisiae* treated apple sludge (liquid settlement from the juice tanks) resulted in 6.54% (v/w) and 8.05% (v/w) amount of ethanol at 72 hours for the filtered and the unfiltered fermented sludges respectively.

4.1 Introduction

Apple pomace and sludge are by-products of the apple industry that are rich in both soluble and insoluble carbohydrates and can be excellent substrates for the production of bioethanol (Vendruscolo, Albuquerque, Streit, Esposito, & Ninow, 2008). Apple pomace can have a potential value as a renewable energy fuel as dry biomass or for bio-ethanol production. Ethanol production from apple pomace represents 20% of the total energy potential of the pomace that can be recovered (Hang et al., 1982; Jewell & Cummings, 1984). The by-products of the apple industry possess major sugars such as fructose, glucose and sucrose and major polysaccharides such as

cellulose, hemicellulose and pectin. Similar to all other lignocellulosic biomasses, close physical and chemical associations between lignin, cellulose and hemicelluloses limit the efficient use of apple processing by-products (Villas-Bôas et al., 2003). A shield of cell wall pectin matrix around cellulose and hemicelluloses also decrease its enzymatic digestibility (Carpita & Gibeaut, 1993; Oechslin et al., 2003; Ryden & Selvendran, 1990). Therefore an alternate pre-treatment is required to enhance biomass digestibility for hydrolysis of cellulose to sugar monomers such as glucose and fermentation of the available sugars to bio-ethanol (Zheng et al., 2009). The fermentable sugars produced by enzyme hydrolysis can be processed by using *Saccharomyces cerevisiae* (one of the most widely used yeast for ethanol fermentation) to yield ethanol. Pre-treatment is an important step to hydrolyse lignocellulose into glucose, however the production of cellobiose and glucose reduces the efficiency of saccharification by inhibiting the activity of *cellulase* enzymes. In this study, apple pomace and apple sludge were used as a fermentation media for bioethanol production. Parameters used to maximize the sugar yield in the study presented in Chapter III, from the growth of two fungal strains *Trichoderma reesei* and *Myceliophthora thermophila,* were further used to subsequently mix the apple pomace substrate with *Saccharomyces cerevisiae* for ethanol production. Simultaneous saccharification and fermentation was performed in the same vessel for both the fungal-enzymatic hydrolysis of apple pomace into sugars and conversion of the released fermentable sugars to ethanol. In parallel, fermentation of apple sludge was performed directly by using *Saccharomyces cerevisiae.* Overall, the goal of this study was to investigate the effects of different fungal strains and co-culturing on bioethanol production and an attempt to develop a low-cost alternative solution to bioethanol production from apple waste.

4.2 Material and Methods

4.2.1 Substrate

Apple pomace as described in Chapter III was used in this experiment. Apple pomace served as the substrate for both selected fungal strains for the saccharification process reaching maximum sugar levels at 8.6g/100g and 4.9 g /100g for *T. reesei* and *M. thermophila* respectively as illustrated in Chapter III for maximum sugar yield. When the optimized time for both the fungi was reached (7^{th} day for *T. reesei* and 9^{th} day for *M. thermophila*), *S. cerevisiae* was added to the saccharified pomace to commence the fermentation process (Fig 4.1).

Apple sludge provided by A. Lassonde (Rougemont, Qc), a juices and beverages manufacturing company, was also used in this study. Sludge of apple juice is the fraction of juice with suspended particulate matter obtained after clear juice recovery. The obtained sludge was further filtered using a muslin cloth to separate the particulate matter. Both the filtered and unfiltered sludge were used as substrates to compare the availability of sugars to *S. cerevisiae* for fermentation. Initial sugar contents of the sludge were measured using the Brix refractometer as described in section 3.2.4. Initial alcohol content was also measured for the sludge as explained further in section 4.2.5. 125 ml glass jars were used for the processing of the apple pomace. In the case of apple sludge, petri plates were used to increase the surface area for higher substrate and organism interaction.

4.2.2 Microorganism

The cultures of fungi, *Trichoderma reesei* and *Myceliophthora thermophila* and the commercial bakery yeast *Saccharomyces cerevisiae*, were obtained from the Agricultural Research Service of the USDA (United States Department of Agriculture). The methodology of culture media preparation and fungal growth as described in Chapter III for *Trichoderma reesei*, and

Myceliophthora thermophila was followed. The yeast *Saccharomyces cerevisiae* was incubated in PDA-broth (100 ml) and incubated at 37ºC for 24h as suggested by the yeast provider. PDA media was prepared, autoclaved, poured into petri dishes and allowed to solidify under a laminar flow hood. The plates were streaked with the organism using the inoculum loop and were incubated at 37ºC for 3-4 days. Sub-culturing of the microorganism was done every two days to maintain the culture.

4.2.3 Sample preparation and inoculations for ethanol production

Saccharomyces cerevisiae inoculum used was prepared by suspending and scraping 4 days old culture to 100 mL beakers, containing 10 mL of distilled water. The beakers were put on a shaker at 32°C for 1h at 100 rpm before being used. All the equipment and lab-ware used was sterilized before the experiment. Apple pomace samples saccharified as per the parameters optimised in the previous study were handled carefully in the laminar air flow chamber. Apple pomace used for the fermentation experiment was treated with fungal strains *T. reesei* at 28°C and 85% moisture levels for 7 days of incubation, and with *M. thermophila* at 28°C and 90% moisture levels for 9 days of incubation, after which *S. cerevisiae* was added at 1%v/w. 1% v/w *S. cerevisiae* was also added in untreated apple pomace as a control. In the case of apple sludge, each petri dish containing 10ml sludge (filtered and unfiltered) was inoculated with 1% v/w *S. cerevisiae* inoculum. All the samples after inoculation were maintained at 30ºC for fermentation up to 96 hours as suggested by previous studies (Chatanta et al., 2008; Ergun & Mutlu, 2000; T Roukas, 1996). Samples for measuring alcohol concentration were taken every 24 hours. All the experiments were done in six replicates (Trinh et al 2008).

4.2.4 Estimation of ethanol yield

The estimation of ethanol production was performed by the potassium dichromate method. An accurately measured amount of potassium dichromate and silver nitrate in H_2SO_4 was added to the solution of alcohol. With the reaction of the sample with the potassium dichromate, ethanol is oxidized to acetic acid: $C_2H_5OH + [O] \rightarrow CH_3COOH$. The contact of alcohol vapor with the orange potassium dichromate solution, changes its color from orange to green. The level of alcohol in the sample is directly related to the degree of the color change. Four beakers (250 ml) were prepared by mixing 5 ml of 0.25 M potassium dichromate, 1 drop of 0.1 M silver nitrate and 5 ml of 6M sulfuric acid. Reaction was continued by adding 0%, 2.5%, 5.0% and 10.0% of alcohol to each of the four beakers followed by adding 39.0 ml of water to each beaker. The reaction produced a blue-green colour. The spectrophotometer was set to 560 nm to absorb blue-green visible light as this colour is produced in the reaction, thus the absorbance values were recorded at a wavelength of 560 nm. After plotting the calibration curve (Fig 4.2), the absorbance values of the samples taken from the experiment were used for determining the percentage of ethanol yield (Sumbhate & Jadon, 2012). Student t-test and Tukey's test were used to analyse the data obtained and compared the output against the control. All significance levels were considered at $p \leq 0.05$.

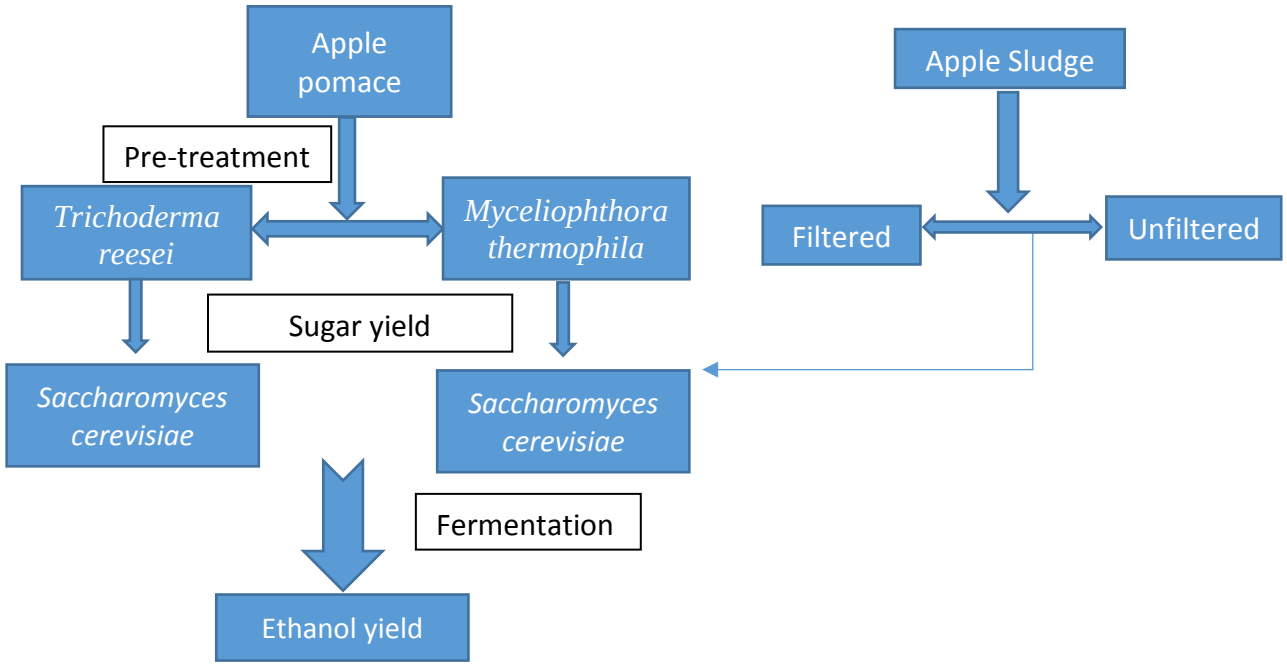


Fig 4.1 Process of producing ethanol from apple pomace and apple sludge.

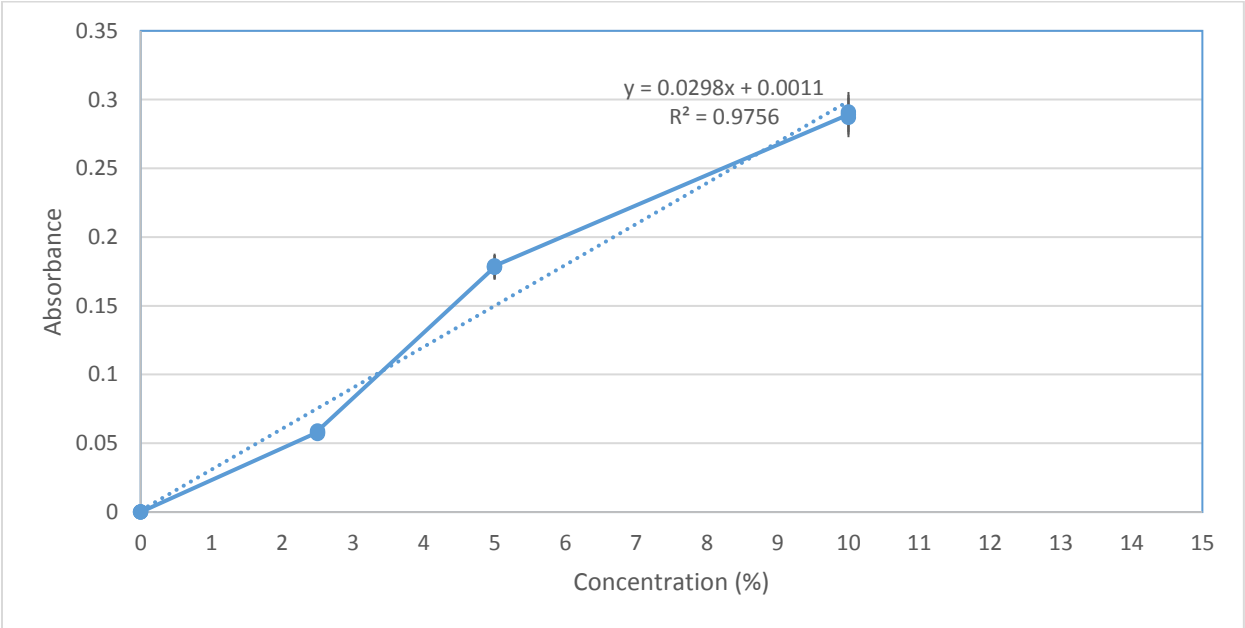


Fig 4.2 Calibration curve for determining the percentage of ethanol yield.

4.3 Results

4.3.1 Production of ethanol from apple pomace and apple sludge using different micro-organisms

4.3.1.1 Sugar content

S. cerevisiae was added to the pre-treated apple pomace, optimized in the experiment presented in Chapter III. After addition of *S. cerevisiae*, the amount of sugars in the pomace samples was measured regularly every 24 hours for 96 hours (Fig 4.3). The loss of the sugar content was rapid for the first 48 hours in all the pomace samples. After 48 hours the rate of decrease in the amount of sugars lowered. Sugar content in apple pomace previously treated with *T. reesei* after inoculation with *S. cerevisiae* had dropped to 4.5g/100g from an initial value of 8.6g/100g which later came to 2.3g/100g after 96 hours as compared to 7.6g/100g after 96 h for the control pomace sample treated with *T. reesei* without the *S. cerevisiae* inoculation. In the case of pomace treated with *M. themophila*, the amount of sugar was 3.4 and 1.9 g/100g after 48 hours and 96 hours respectively after an initial amount of 4.9 g /100g (Fig 4.3) when treated with *S. cerevisiae*. The control sample treated with *M. thermophila* without *S. cerevisiae* inoculation did not show much depreciation in sugar and remained at 4.5g/100g.

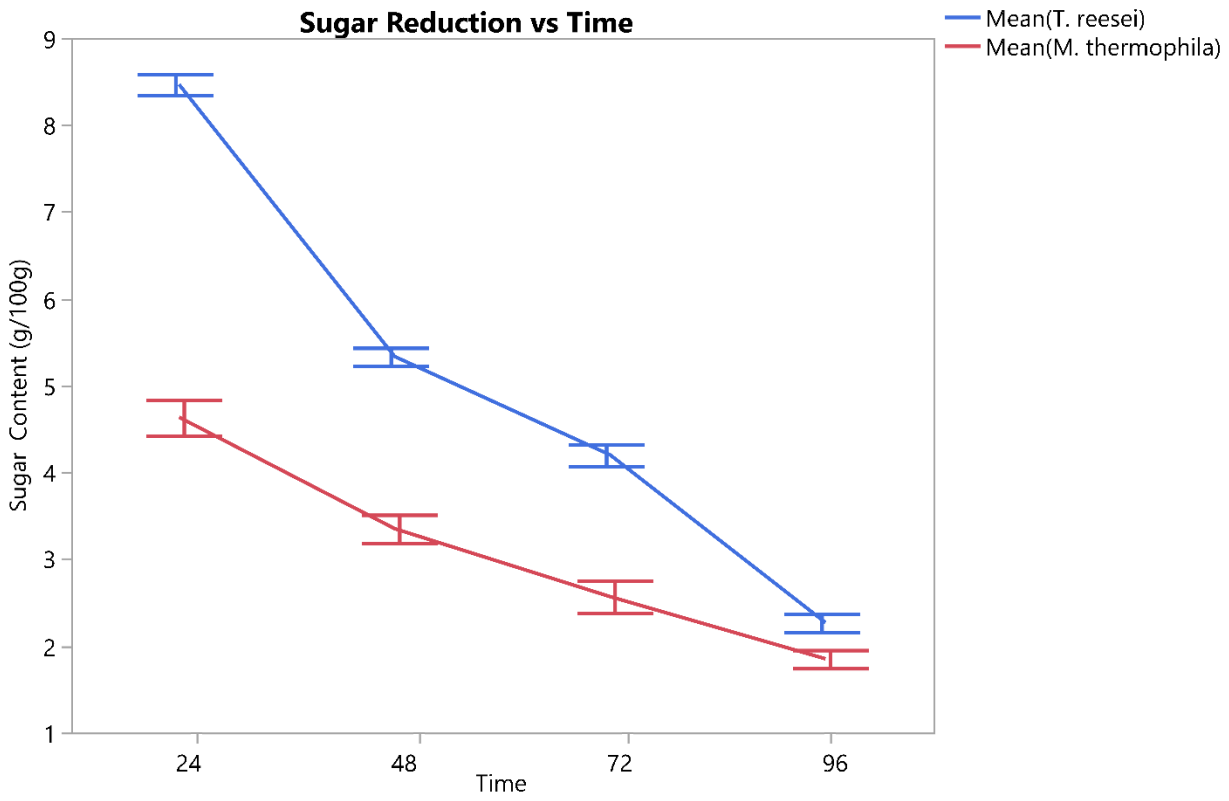


Fig 4.3 Effect of addition of *S. cerevisiae* on the sugar content obtained from apple pomace pre-treated with fungi *T. reesei* and *M. thermophila.*

S. cerevisiae was inoculated to the filtered and unfiltered apple sludge. Amount of sugars in the samples was monitored every 24 hours till 96 h. The rate of loss of sugar content was quick for the first 48 hours in both the filtered and unfiltered sludges. The rate of loss of sugars in the unfiltered sludge continued to remain almost the same for 72 hours after inoculation. After 72 hours, the amount of sugar in the unfiltered sludge started depleting faster, though still at a low rate. The initial sugars for the sludge were high at a value of 13g/100g and 13.5g/100g for filtered and unfiltered sludge respectively. The sugar content in the sludge decreased throughout the test period. The sugars in the filtered sludge fell to 6.3g/100g losing 51.54% of the initial sugar content after 72h. Whereas unfiltered sludge had 6.1g/100g after 72 hours losing up to 54.8% of the initial

sugars (Fig 4.4). They had dropped to an amount of 4.7g/100g and 4.8 g/100g after 96 hours as compared to control which remained unchanged throughout the experiment.

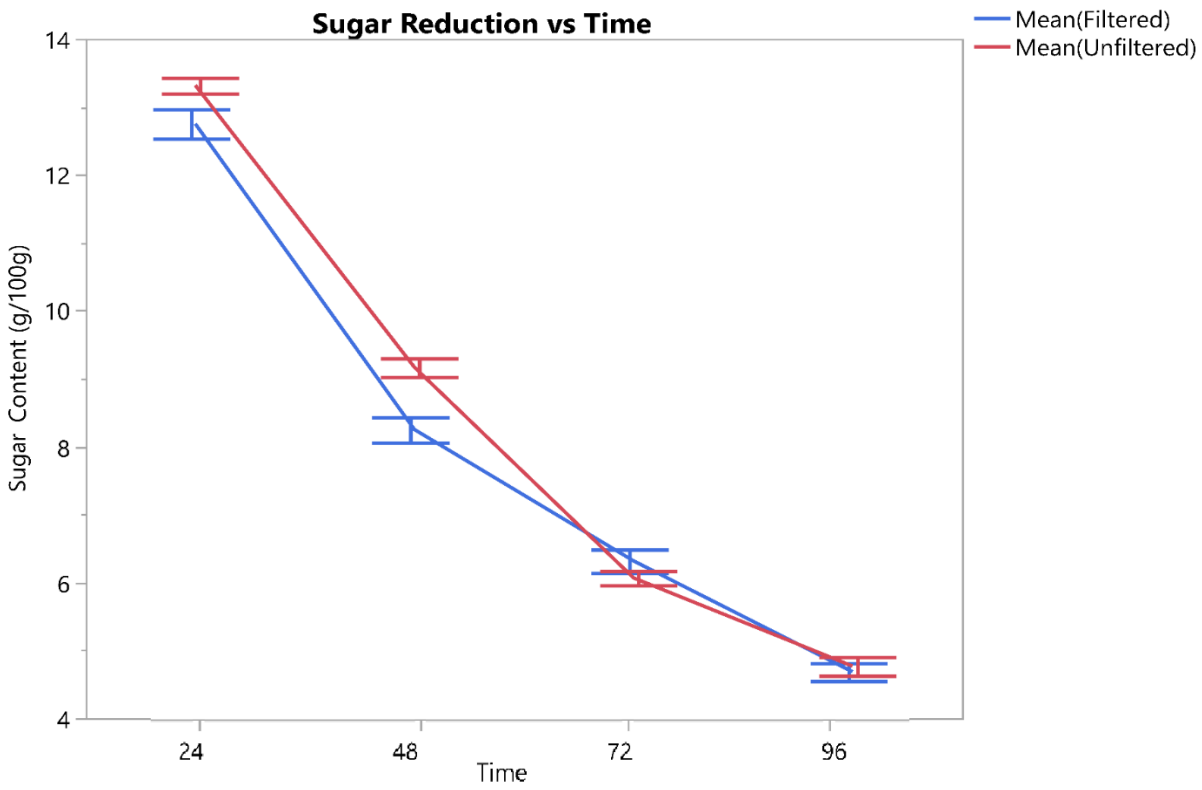


Fig 4.4 Effect of addition of *S. cerevisiae* on the sugar content obtained from unfiltered and filtered apple sludges.

4.3.1.2 Alcohol content

The alcohol content obtained after inoculation of apple pomace with different fungal cultures was observed by chemically induced colour change using a spectrophotometer at 560 nm. A standard curve was established prior to that using known amounts of alcohol (Fig 4.2). Amount of alcohol in the samples was measured every 24 hours after inoculation with *S. cerevisiae.*

Alcohol concentration was on a slow rise within the initial 48 hours in the case of apple pomace. From 48 to 72 hours the alcohol concentration rose more vigorously. In apple pomace pre-treated with *T. reesei* the alcohol content increased from 2.37% after 48 hours to 6.11% after 72 hours of fermentation. Whereas it increased from 2.16% to 3.96% in the pomace samples pre-treated with *M. thermophila* for the same period of time. Apple pomace samples without any inoculation with *S. cerevisiae* responded with no or insignificant amounts of alcohol. Apple pomace without any treatment when inoculated with only *S. cerevisiae* resulted in alcohol concentrations of 1.93% and 2.27% after 48 hours and 72 hours respectively after inoculation. The alcohol concentration after 72 hours in the case of pomace treated with *M. thermophila* was higher than the concentration at 96 hours. High alcohol concentration and low fermentable sugars available for the organisms may have caused the inhibition of the fermentation process. Similar results have been reported by Chatanta et al., (2008). Whereas in samples treated with *T. reesei* the concentration of alcohol remained on the rise although very low. The apple pomace treated with *T. reesei* and *M. thermophila* yielded 6.11 % (v/w) and 3.96% (v/w) amount of ethanol, respectively, followed by fermentation using *S. cerevisiae* after 72 hours of inoculation (Fig 4.5).

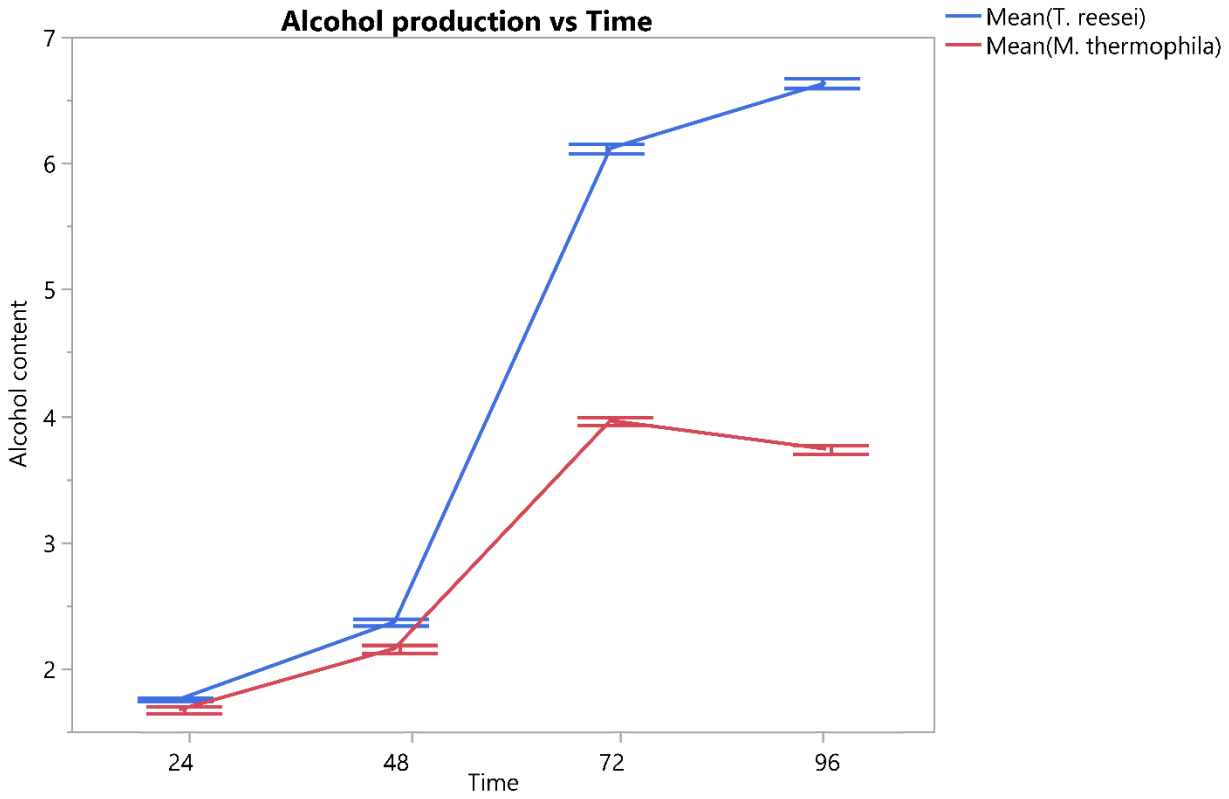


Fig 4.5 Effect of incubation time on the alcohol yield (%) obtained from apple pomace pre-treated with fungi *T. reesei* and *M. thermophila* and subsequently fermented with *S. cerevisiae.*

The alcohol concentration increased rapidly for 72 hours for fermentation of filtered apple sludge resulting in 6.54% (v/w) amount of ethanol using *S. cerevisiae* while fermentation of the unfiltered sludge with similar conditions resulted in 8.05% (v/w) of ethanol at 72 hours (Fig 4.6). After 72 hours of inoculation, the alcohol concentration started falling down in both the cases as explained by Chatanta et al., (2008). In the control samples of sludge containing no inoculum, no amount of fermented alcohol was observed.

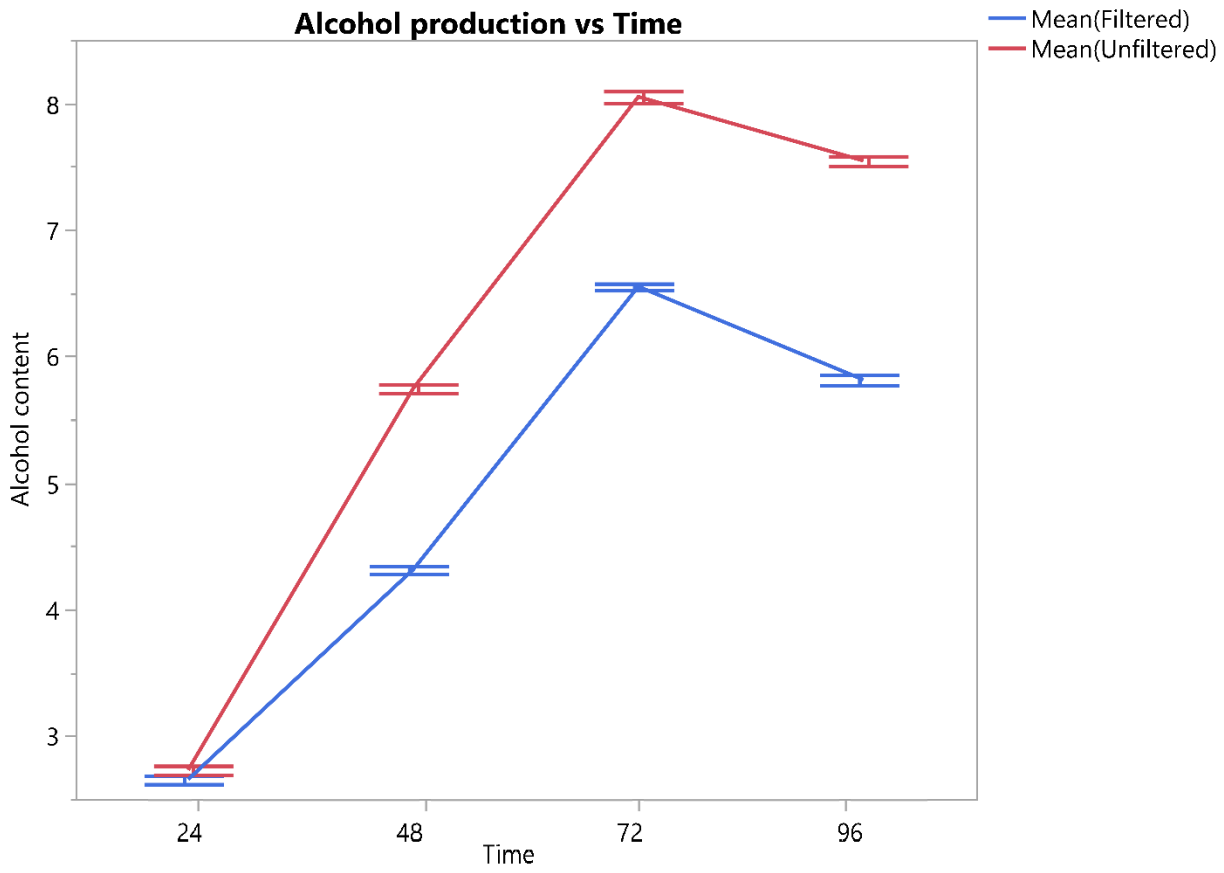


Fig 4.6 Effect of incubation time on the alcohol yield (%) obtained from unfiltered and filtered apple sludge fermented with *S. cerevisiae*.

4.4 Discussion

The addition of *S. cerevisiae* in the apple pomace samples pre-treated with *T. reesei* and *M. thermophila* resulted in 38.37% and 36.92% decrease of sugar content within 48 hrs of incubation and 51.16% and 46.94% respectively after 72 hrs. After 96 hrs of incubation, the sugar content decreased by 73.25% and 61.22% respectively. Initial decrease in sugar content shows the conversion of sugars to alcohols due to the fermentative action of the *S. cerevisiae* fungus. As compared to the pomace pre-treated with *M. thermophila*, *T. reesei* pre-treated pomace resulted in significantly higher sugar utilization by the fungus *S. cerevisiae*. In the case of the unfiltered sludge, it resulted in 54.81% decrease in sugar content after 72 hrs of incubation. The filtered and unfiltered sludges were not pre-treated with different fungal species as this substrate could not support solid state fermentation due to its higher water content. There is a potential for future studies on apple sludge by identifying suitable organisms for submerged treatment and more efficient ethanol production. The concentration of alcohol for apple pomace pre-treated with *M. thermophila* and *T. reesei* remained very low during the initial 48 hrs of incubation and then there was a steep boost in alcohol production until 72 hours. The change in alcohol production may be due to the growth of fungus and its transition phase (Meletiadis & Verweij, 2001). The combination of *T. reesei* + *S. cerevisiae* resulted in 6.11 % (v/w) amount of ethanol from apple pomace at 72 hours. The culturing of pomace with of *M. thermophila* and subsequently with *S. cerevisiae* resulted in 3.96% (v/w) amount of ethanol from apple pomace at 72 hours. On the other hand, 3.72% alcohol was obtained from the apple pomace treated with only *S. cerevisiae* as a control which shows the improved and effective sugar yield from apple pomace by *T. reesei* and *M. thermophila*. For the sludge fermentation, the alcohol concentration increased rapidly for 72 h. Fermentation of apple filtered sludge resulted in 6.54% (v/w) amount of ethanol using *S. cerevisiae*

while the fermentation of unfiltered sludge with similar conditions resulted in 8.05% (v/w) of ethanol at 72 hours.

After 72 hours the alcohol concentration started falling for all the samples except for the pomace pre-treated with *T. reesei*. Indeed, in the case of *T. reesei*, the simultaneous saccharification may have mitigated the inhibition effect of alcohol (Chatanta et al., 2008). *T. reesei* has more efficiency than *M. thermophila* for releasing fermentable sugars from the pomace as observed in Chapter III. It was observed that the apple pomace pretreated with *T. reesei* resulted in significant increase in the alcohol yield as compared to the control (direct fermentation of apple pomace with *S. cerevisiae*). However, pre-treatment using *M. thermophila* resulted in an insignificant ($p > 0.05$) increase in alcohol yield. The results are supported by reports on the higher efficiency of *T. reesei* to produce *endo*- and *exo-glucanases* (Bollok & Reczey, 2000; Grassin & Fauquembergue, 1996; Miettinen-Oinonen & Suominen, 2002) to effectively release sugars during the pre-treatment of apple pomace. In other studies, *T. reesei* showed 100% degradation of lignocellulose at pH 4.8, temperature 28°C with an incubation time of 7 days (Velkovska, Marten, & Ollis, 1997). *T. reesei* has been reported as an effective producer of *cellulase* which can efficiently hydrolyse cellulose in different substrates (Muthuvelayudham & Viruthagiri, 2006). The simultaneous saccharification and fermentation can lead to higher ethanol yields by supplying sugars for fermentation and reducing inhibition of the fermentation process. The results obtained from optimization studies demonstrated that mixed culture fermentation, which contained two or more microorganisms is important for effective bioethanol production (Ballesteros & Ballesteros, 2004). However, Doelle et al., (1991) reported that a high concentration of ethanol is dependent on the strain of organisms used, the chemical composition of the substrate and the conditions under which the fermentation takes place. Their results showed the gradual decrease of sugars during the fermentation,

coinciding with an increase in ethanol production. Similar studies have been performed by Hang & Cooley (1981) using apple pomace as a substrate for ethanol production. Solid state fermentation of apple pomace was performed for 96 h at 30°C without shaking or stirring. After 24 h the sugar content was reduced from an initial concentration of 10.2% to less than 0.4%, and the final concentration of alcohol was observed greater than 4.3%, which represents approximately 89% fermentation efficiency. The bioethanol production, using mixed cultures of *T. harzianum, A. sojae* and *S. cerevisiae,* resulted in higher bioethanol production (Sharada et al., 2013). Triantafyllos (1996) reported the rapid increase of ethanol concentration during the first 12 h of fermentation using *S. cerevisiae* and gradual increase at a slow rate to reach a maximum (43.5 g/litre) after 24 h of incubation. 26.84 g/L bioethanol was reported at temperature of 30°C, for fungi *S. cerevisiae* and *Pachysolen tannophilus* at an incubation time of 48 h and from the steam pretreated kinnow waste and banana peels by simultaneous saccharification and fermentation using co-cultures of *S. cerevisiae* G and *P. tannophilus* MTCC 1077 (Sharma & Bansal, 2007). A maximum ethanol yield of 48.19 g/l with fermentation efficiency of 61.42% by immobilized co-culture of *S. cerevisiae* I + *P. stipites* was also reported (Pathania, 2016) . The yield of 0.685 g/g of reducing sugar per gram of pretreated sugarcane-tops biomass as substrate and 11.365 g/L of bioethanol was reported after the fermentation of the hydrolysate using *S. cerevisiae* with efficiency of about 50% (Sindhu et al., 2011). The enzymatic hydrolysis of corn meal by commercial enzymes (*a-amylase and glucoamylase*) and the simultaneous ethanol fermentation of the hydrolysates by *S. cerevisiae* resulted in 0.50 g/g ethanol (Anita Singh et al., 2013). Another study conducted using mixed yeast culture (*S. cerevisiae* ITV-01 and *Scheffersomyces* (formerly Pichia) *stipitis* NRRL Y-7124) and sugarcane bagasse hydrolysate supplemented with sugarcane molasses resulted in 0.45 g/g ethanol yield (Gutiérrez-Rivera et al., 2015). A study by Khosravi and Shojaosadati (2003) showed the

production of 2.5% (wt./wt.) ethanol without saccharification and 8% (wt./wt.) with saccharification under a solid-state fermentation process from apple pomace by *S. cerevisiae* at a moisture content of 75% with initial sugar concentration of 26% (wt./wt.), and a nitrogen content of 1% (wt./wt.). Alcoholic fermentation of apple juice, pomace extract, and pomace extract added with sucrose provided after fermentation showed 6.90%, 4.30%, and 7.30% ethanol, respectively with the addition of sucrose (Nogueira & Wosiacki, 2005).

4.5 Conclusion

In the present study, we were able to obtain a yield of 6.11 % (v/w) ethanol from apple pomace using *T. reesei* + *S. cerevisiae* after 72 hrs. The culturing of pomace with *M. thermophila* and subsequently with *S. cerevisiae* resulted in 3.96% (v/w) amount of ethanol from apple pomace after 72 hrs. Whereas, 3.72% alcohol was obtained from the apple pomace treated with only *S. cerevisiae* as a control which shows the improved and effective sugar yield from apple pomace by the consortia of *T. reesei* and *M. thermophila*. Fermentation of apple unfiltered sludge resulted in higher amount of ethanol using *S. cerevisiae* than the filtered sludge under similar conditions after 72 hrs. This is the first study to report the production of ethanol from apple pomace and apple sludge using the fungal strains, *T. reesei* and *M. thermophila* for saccharification and subsequently using the obtained sugars for the production of ethanol using *S. cerevisiae*.

CHAPTER V: GENERAL DISCUSSION AND CONCLUSION

Environmental concerns about the disposal of agro industrial waste and its utilization for the production of different metabolites with a higher commercial value have become a major focus of research over the past few decades (Vendruscolo et al., 2008; Villas-Bôas et al., 2003). The industrial processing of apples mainly results in the production of juice, jelly, and pulp. Solid residue from juice production, the apple pomace, is generated during fruit pressing and represents around 30% of the original fruit (Villas-Bôas et al., 2003). Apple pomace is posing environmental concerns due to its degradability and also its use as an animal feed is limited due to its low nutritive value, high perishability and high cost of transportation. The efficient use of apple pomace for the production of value added products has been proposed by many researchers (Berovič & Ostroveršnik, 1997; Favela-Torres, Volke-Sepúlveda, & Viniegra-González, 2006; Shrikot, Sharma, & Sharma, 2004; Z. Zheng & Shetty, 2000), such as organic acids (Shojaosadati & Babaeipour, 2002), aroma compounds (Bramorski & Revah, 1998; Foo & Lu, 1999; Medeiros & Soccol, 2000; Tsurumi & Takeda, 2001), and natural antioxidants (Foo & Lu, 1999; Lu & Foo, 2000). Even following extraction of these valuable products, a considerable fraction of the biomass will remain as lignocellulosic residue warranting its use for ethanol production (Ngadi & Correia, 1992a, 1992b; Paganini & Wosiacki, 2005; Shojaosadati & Babaeipour, 2002).

Cellulose and hemicellulose can be hydrolyzed into their simpler sugar counterparts by a great diversity of microorganisms such as bacteria and fungi. The capacity of hydrolyzing cellulose is widely distributed among fungal species and some bacteria such as Clostridiales (anaerobic) and Actinomycetales (aerobic). The extra-cellular complex enzyme system includes a variety of enzymes known as *endoglucanases*, *exoglucanases*, *hemicellulases*, *chitinases*, pectin *lyases*, and *lichenases* (Bayer et al., 2004). Anaerobic cellulolytic fungi produce free *cellulases*, which can

hydrolyze the lignocellulosic substrates by penetrating the hyphal extensions into the lignocellulosic substrates and hydrolyze the substrate (Chang & Yao, 2011). Filamentous fungi are preferred as they can be directly inoculated onto cellulosic biomass and they do not require strictly anaerobic conditions. In this study, *T. reesei* and *M. thermophila* were selected as subject organisms for the pre-treatment of the biomass. The filamentous growth habit of these fungi facilitates separation of cell mass from the broth and the inoculation of non-sterile biomass is more practical since many fungal strains produce plentiful conidiospores which facilitate rapid multiplication of the fungus and could be useful for inoculation at a larger scale (Stevenson & Weimer, 2002).

Various fungi such as *Aspergillus, Rhizopus, Monilia, Neurospora,* and *Fusarium* have been reported to convert cellulose directly to ethanol (Deshpande et al., 1986; Gong et al., 1981; Ajay Singh & Kumar, 1991; Skory et al., 1997). For more effective outputs, combination of different microbes, which can convert cellulose to reducing sugars and then to ethanol, are often used. This way a higher amount of reducing sugar can be obtained and then can be fermented by a different microbe for improved ethanol yields. This study used both the filamentous fungi *T. reesei* and *M. thermophila* for yielding sugars from apple pomace for subsequent fermentation to ethanol. *S. cerevisiae* was used to ferment those sugars into alcohol. *S. cerevisiae* is the most commonly used fungus for fermentation. *T. reesei* has been reported as the most efficient producer of endo and exo-glucanase (Miettinen-Oinonen & Suominen, 2002). Cellulases are the primary microbial enzymes for hydrolysis of cellulose and are composed of three predominant activities: endo-1, 4-β-glucanase, exo-1, 4-β-glucanase, and β-glucosidase also known as cellobiase. Similar results were obtained in this study, where the pomace substrate treated with *T. reesei* produced higher

amount of sugars than *M. thermophila* which subsequently led to a higher alcohol yield during the fermentation process.

The use of microorganisms for the hydrolysis of biomass is more environment friendly, energy efficient and can be performed at milder conditions as compared with chemical and physicochemical pretreatments (Plácido & Capareda, 2015). There are numerous parameters such as moisture, temperature, nitrogen supplementation pH, particle size, substrate accessibility which may play an important role for microbial saccharification (Dionisi & Paton, 2015). Although all of them have not been included in this study, we have analyzed for moisture, temperature, nitrogen supplementation and incubation time. Unfiltered sludge has yielded higher alcohol content as compared to pomace due to the presence of higher simple sugars than pomace (Gassara et al., 2012). The high availability of high simple sugars and moisture content in sludge makes it readily usable for direct fermentation to produce ethanol.

Conclusion:

This study was conducted using *M. thermophila* and *T. reesei* fungi as biological pre-treatments to breakdown the cellulose present in apple pomace. Both fungi served the purpose as expected by yielding increasing sugar content from the cellulose present in the pomace. The levels of different factors of moisture, nitrogen supplement and time, corresponding to maximum sugar yield were reported. On reaching the maximum yield, the pomace along with the fungi, served as the substrate to *S. cerevisiae* for fermentation. *T. reesei* resulted in higher release of fermentable sugars from the pomace as compared to *M. thermophila* which subsequently has resulted in higher amount of ethanol obtained with *S. cerevisiae* fermentation as compared with *M. thermophila.*

CHAPTER VI: REFERENCES

Agbor, V. B., Cicek, N., Sparling, R., Berlin, A., & Levin, D. B. (2011). Biomass pretreatment: fundamentals toward application. *Biotechnology advances*, *29*(6), 675-685.

Albers, E., Larsson, C., Lidén, G., Niklasson, C., & Gustafsson, L. (1996). Influence of the nitrogen source on Saccharomyces cerevisiae anaerobic growth and product formation. *Applied and environmental microbiology*, *62*(9), 3187-3195.

Alonso, D. M., Bond, J. Q., & Dumesic, J. A. (2010). Catalytic conversion of biomass to biofuels. *Green Chemistry*, *12*(9), 1493-1513.

Alvira, P., Tomás-Pejó, E., Ballesteros, M., & Negro, M. (2010). Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. *Bioresource Technology*, *101*(13), 4851-4861.

Anwar, Z., Gulfraz, M., & Irshad, M. (2014). Agro-industrial lignocellulosic biomass a key to unlock the future bio-energy: a brief review. *Journal of radiation research and applied sciences*, *7*(2), 163-173.

Arapoglou, D., Varzakas, T., Vlyssides, A., & Israilides, C. (2010). Ethanol production from potato peel waste (PPW). *Waste Management*, *30*(10), 1898-1902.

Archer, D., Eby, M., Brovkin, V., Ridgwell, A., Cao, L., Mikolajewicz, U., Montenegro, A. (2009). Atmospheric lifetime of fossil fuel carbon dioxide. *Annual Review of Earth and Planetary Sciences*, *37*, 117-134.

Baiano, A. (2014). Recovery of biomolecules from food wastes—a review. *Molecules*, *19*(9), 14821-14842.

Ballesteros, M., Oliva, J., Negro, M., Manzanares, P., & Ballesteros, I. (2004). Ethanol from lignocellulosic materials by a simultaneous saccharification and fermentation process

(SFS) with Kluyveromyces marxianus CECT 10875. *Process Biochemistry, 39*(12), 1843-1848.

Bangaraiah, P., & Kumar, P. A. (2014). Bioethanol as an alternative energy resource. *International Journal of Pharma and Biosciences, 5*(1).

Bayer, E. A., Belaich, J.-P., Shoham, Y., & Lamed, R. (2004). The cellulosomes: multienzyme machines for degradation of plant cell wall polysaccharides. *Annu. Rev. Microbiol., 58*, 521-554.

Beldman, G., Voragen, A., Rombouts, F., & Pilnik, W. (1988). Synergism in cellulose hydrolysis by endoglucanases and exoglucanases purified from Trichoderma viride. *Biotechnology and bioengineering, 31*(2), 173-178.

Berovič, M., & Ostroveršnik, H. (1997). Production of Aspergillus niger pectolytic enzymes by solid state bioprocessing of apple pomace. *Journal of Biotechnology, 53*(1), 47-53.

Bhosale, A. A. (2017). Detection of Sugar Content in Citrus Fruits by Capacitance Method. *Procedia Engineering, 181*, 466-471. doi:https://doi.org/10.1016/j.proeng.2017.02.417

Bhushan, S., Kalia, K., Sharma, M., Singh, B., & Ahuja, P. S. (2008). Processing of apple pomace for bioactive molecules. *Critical reviews in biotechnology, 28*(4), 285-296.

Bollok, M., & Reczey, K. (2000). Cellulase enzyme production by various fungal strains on different carbon sources. *Acta Alimentaria, 29*(2), 154-168.

Boyle, G. (1997). *Renewable energy: power for a sustainable future*: Taylor & Francis.

Brahmachari, G. (2016). *Biotechnology of Microbial Enzymes: Production, Biocatalysis and Industrial Applications*: Academic Press.

Bramorski, A., Christen, P., Ramirez, M., Soccol, C. R., & Revah, S. (1998). Production of volatile compounds by the edible fungus Rhizopus oryzae during solid state cultivation on tropical agro-industrial substrates. *Biotechnology letters, 20*(4), 359-362.

Canada, S. (2018). Estimates, production and farm gate value of fresh and processed fruits1 Retrieved from https://www150.statcan.gc.ca/t1/tbl1/en/tv.action?pid=3210036401&pickMembers%5B0%5D=1.8&pickMembers%5B1%5D=2.1&pickMembers%5B2%5D=4.4

Carpita, N. C., & Gibeaut, D. M. (1993). Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *The Plant Journal, 3*(1), 1-30.

Carriquiry, M. A., Du, X., & Timilsina, G. R. (2011). Second generation biofuels: Economics and policies. *Energy Policy, 39*(7), 4222-4234.

Carroll, A., Mansoori, N., Li, S., Lei, L., Vernhettes, S., Visser, R. G. F., Trindade, L. M. (2012). Complexes with Mixed Primary and Secondary Cellulose Synthases Are Functional in Arabidopsis Plants. *Plant Physiology, 160*(2), 726.

Chang, T., & Yao, S. (2011). Thermophilic, lignocellulolytic bacteria for ethanol production: current state and perspectives. *Applied Microbiology and Biotechnology, 92*(1), 13.

Chatanta, D., Attri, C., Gopal, K., Devi, M., Gupta, G., & Bhalla, T. (2008). Bioethanol production from apple pomace left after juice extraction. *Internet J. Microbiol, 5*(2).

Chen, H. (2005). Biotechnology of lignocellulose. *sl: sn*, 73-141.

Chen, H., Rubenthaler, G., & Schanus, E. (1988). Effect of apple fiber and cellulose on the physical properties of wheat flour. *Journal of Food Science, 53*(1), 304-305.

Connolly, D., Mathiesen, B. V., & Ridjan, I. (2014). A comparison between renewable transport fuels that can supplement or replace biofuels in a 100% renewable energy system. *Energy, 73*, 110-125.

Couto, S. R., & Sanromán, M. A. (2006). Application of solid-state fermentation to food industry—a review. *Journal of Food Engineering, 76*(3), 291-302.

Demain, A. L., & Adrio, J. L. (2008). Strain improvement for production of pharmaceuticals and other microbial metabolites by fermentation *Natural Compounds as Drugs Volume I* (pp. 251-289): Springer.

Demers, A. (2012). Enzymatic hydrolysis of cellulosic biomass for the production of second generation biofuels. *Project Number DDB.*

Deshpande, V., Keskar, S., Mishra, C., & Rao, M. (1986). Direct conversion of cellulose/hemicellulose to ethanol by Neurospora crassa. *Enzyme and microbial technology, 8*(3), 149-152.

Devi, M. C., & Kumar, M. S. (2017). Production, optimization and partial purification of cellulase by Aspergillus niger fermented with paper and timber sawmill industrial wastes. *Journal of microbiology and biotechnology research, 2*(1), 120-128.

Dhillon, G., Brar, S., Verma, M., & Tyagi, R. (2011). Enhanced solid-state citric acid bio-production using apple pomace waste through surface response methodology. *Journal of applied microbiology, 110*(4), 1045-1055.

Dhillon, G. S., Kaur, S., & Brar, S. K. (2013). Perspective of apple processing wastes as low-cost substrates for bioproduction of high value products: A review. *Renewable and Sustainable Energy Reviews, 27*, 789-805.

Dhillon, G. S., Kaur, S., Brar, S. K., & Verma, M. (2012). Potential of apple pomace as a solid substrate for fungal cellulase and hemicellulase bioproduction through solid-state fermentation. *Industrial Crops and Products, 38*, 6-13.

Ding, S., Wang, R., Shan, Y., Li, G., & Ou, S. (2017). Changes in pectin characteristics during the ripening of jujube fruit. *Journal of the Science of Food and Agriculture*.

Dionisi, D., Anderson, J. A., Aulenta, F., McCue, A., & Paton, G. (2015). The potential of microbial processes for lignocellulosic biomass conversion to ethanol: a review. *Journal of Chemical Technology & Biotechnology, 90*(3), 366-383.

Domingues, F., Queiroz, J., Cabral, J., & Fonseca, L. (2000). The influence of culture conditions on mycelial structure and cellulase production by Trichoderma reesei Rut C-30. *Enzyme and microbial technology, 26*(5-6), 394-401.

dos Santos Lima, F. C., da Silva, F. L. H., Gomes, J. P., & da Silva Neto, J. M. (2012). Chemical composition of the cashew apple bagasse and potential use for ethanol production. *Advances in Chemical Engineering and Science, 2*(04), 519.

El-Zawawy, W. K., Ibrahim, M. M., Abdel-Fattah, Y. R., Soliman, N. A., & Mahmoud, M. M. (2011). Acid and enzyme hydrolysis to convert pretreated lignocellulosic materials into glucose for ethanol production. *Carbohydrate Polymers, 84*(3), 865-871.

Ellilä, S., Fonseca, L., Uchima, C., Cota, J., Goldman, G. H., Saloheimo, M., Siika-Aho, M. (2017). Development of a low-cost cellulase production process using Trichoderma reesei for Brazilian biorefineries. *Biotechnology for biofuels, 10*(1), 30.

ElMekawy, A., Srikanth, S., Bajracharya, S., Hegab, H. M., Nigam, P. S., Singh, A., Pant, D. (2015). Food and agricultural wastes as substrates for bioelectrochemical system (BES):

the synchronized recovery of sustainable energy and waste treatment. *Food Research International, 73*, 213-225.

Ergun, M., & Mutlu, S. F. (2000). Application of a statistical technique to the production of ethanol from sugar beet molasses by Saccharomyces cerevisiae. *Bioresource Technology, 73*(3), 251-255.

Eudes, A., Liang, Y., Mitra, P., & Loque, D. (2014). Lignin bioengineering. *Curr Opin Biotechnol, 26*, 189-198. doi:10.1016/j.copbio.2014.01.002

Evcan, E., & Tari, C. (2015). Production of bioethanol from apple pomace by using cocultures: Conversion of agro-industrial waste to value added product. *Energy, 88*, 775-782.

Favela-Torres, E., Volke-Sepúlveda, T., & Viniegra-González, G. (2006). Production of Hydrolytic Depolymerising Pectinases. *Food Technology & Biotechnology, 44*(2).

Florencio, C., Cunha, F. M., Badino, A. C., Farinas, C. S., Ximenes, E., & Ladisch, M. R. (2016). Secretome analysis of Trichoderma reesei and Aspergillus niger cultivated by submerged and sequential fermentation processes: enzyme production for sugarcane bagasse hydrolysis. *Enzyme and microbial technology, 90*, 53-60.

Foo, L. Y., & Lu, Y. (1999). Isolation and identification of procyanidins in apple pomace. *Food chemistry, 64*(4), 511-518.

France, R., & Reid, C. (1984). Pure culture growth of ectomycorrhizal fungi on inorganic nitrogen sources. *Microbial Ecology, 10*(3), 187-195.

Friedrich, J., Cimerman, A., & Perdih, A. (1987). Mixed culture of Aspergillus awamori and Trichoderma reesei for bioconversion of apple distillery waste. *Applied Microbiology and Biotechnology, 26*(3), 299-303.

Gama, R., Van Dyk, J. S., & Pletschke, B. I. (2015). Optimisation of enzymatic hydrolysis of apple pomace for production of biofuel and biorefinery chemicals using commercial enzymes. *3 Biotech, 5*(6), 1075-1087.

Gao, J., Weng, H., Zhu, D., Yuan, M., Guan, F., & Xi, Y. (2008). Production and characterization of cellulolytic enzymes from the thermoacidophilic fungal Aspergillus terreus M11 under solid-state cultivation of corn stover. *Bioresource technology, 99*(16), 7623-7629.

Gassara, F., Ajila, C., Brar, S. K., Verma, M., Tyagi, R., & Valero, J. (2012). Liquid state fermentation of apple pomace sludge for the production of ligninolytic enzymes and liberation of polyphenolic compounds. *Process Biochemistry, 47*(6), 999-1004.

Ghosh, S., Chowdhury, R., & Bhattacharya, P. (2016). Sustainability of cereal straws for the fermentative production of second generation biofuels: A review of the efficiency and economics of biochemical pretreatment processes. *Applied Energy*.

Golosov, M., Hassler, J., Krusell, P., & Tsyvinski, A. (2014). Optimal taxes on fossil fuel in general equilibrium. *Econometrica, 82*(1), 41-88.

Gong, C.-S., Chen, L.-F., Flickinger, M. C., Chiang, L.-C., & Tsao, G. T. (1981). Production of ethanol from D-xylose by using D-xylose isomerase and yeasts. *Applied and environmental microbiology, 41*(2), 430-436.

Grassin, C., & Fauquembergue, P. (1996). Application of pectinases in beverages *Progress in Biotechnology* (Vol. 14, pp. 453-462): Elsevier.

Gusakov, A. V. (2011). Alternatives to Trichoderma reesei in biofuel production. *Trends in Biotechnology, 29*(9), 419-425. doi:https://doi.org/10.1016/j.tibtech.2011.04.004

Gusakov, A. V., Salanovich, T. N., Antonov, A. I., Ustinov, B. B., Okunev, O. N., Burlingame, R., Sinitsyn, A. P. (2007). Design of highly efficient cellulase mixtures for enzymatic hydrolysis of cellulose. *Biotechnology and bioengineering, 97*(5), 1028-1038.

Gutiérrez-Rivera, B., Ortiz-Muñiz, B., Gómez-Rodríguez, J., Cárdenas-Cágal, A., González, J. M. D., & Aguilar-Uscanga, M. G. (2015). Bioethanol production from hydrolyzed sugarcane bagasse supplemented with molasses "B" in a mixed yeast culture. *Renewable Energy, 74*, 399-405.

Hang, Y., Lee, C., & Woodams, E. (1982). A solid state fermentation system for production of ethanol from apple pomace. *Journal of Food Science, 47*(6), 1851-1852.

Hang, Y., Lee, C., Woodams, E., & Cooley, H. (1981). Production of alcohol from apple pomace. *Applied and environmental microbiology, 42*(6), 1128-1129.

Hang, Y., & Woodams, E. (1986). Solid state fermentation of apple pomace for citric acid production. *MIRCEN journal of applied microbiology and biotechnology, 2*(2), 283-287.

Ho, D. P., Ngo, H. H., & Guo, W. (2014). A mini review on renewable sources for biofuel. *Bioresource Technology, 169*, 742-749.

Hölker, U., Höfer, M., & Lenz, J. (2004). Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. *Applied Microbiology and Biotechnology, 64*(2), 175-186.

Hölker, U., & Lenz, J. (2005). Solid-state fermentation—are there any biotechnological advantages? *Current Opinion in Microbiology, 8*(3), 301-306.

Höök, M., & Tang, X. (2013). Depletion of fossil fuels and anthropogenic climate change—A review. *Energy Policy, 52*, 797-809.

Hu, J., Arantes, V., & Saddler, J. N. (2011). The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect? *Biotechnology for biofuels, 4*(1), 36.

Hubbert, M. K. (1949). *Energy from fossil fuels*: Moses King.

Huber, M. T. (2009). Energizing historical materialism: Fossil fuels, space and the capitalist mode of production. *Geoforum, 40*(1), 105-115.

Jacobson, J. L. (2006). *Introduction to wine laboratory practices and procedures*: Springer Science & Business Media.

Jarvis, M. C. (1984). Structure and properties of pectin gels in plant cell walls. *Plant, Cell & Environment, 7*(3), 153-164.

Jewell, W. J., & Cummings, R. J. (1984). Apple pomace energy and solids recovery. *Journal of Food Science, 49*(2), 407-410.

Johnson, F. X., & Silveira, S. (2014). Pioneer countries in the transition to alternative transport fuels: Comparison of ethanol programmes and policies in Brazil, Malawi and Sweden. *Environmental Innovation and Societal Transitions, 11*, 1-24.

Johnson, M. A., Pilco, V., Torres, R., Joshi, S., Shrestha, R. M., Yagnaraman, M., Canuz, E. (2013). Impacts on household fuel consumption from biomass stove programs in India, Nepal, and Peru. *Energy for Sustainable Development, 17*(5), 403-411.

Joshi, V., & Attri, D. (2005). Solid state fermentation of apple pomace for the production of value added products. *Pollution in Urban Industrial Environment*, 180.

Ju, L. K., & Afolabi, O. A. (1999). Wastepaper Hydrolysate as Soluble Inducing Substrate for Cellulase Production in Continuous Culture of Trichodermareesei. *Biotechnology progress, 15*(1), 91-97.

Kennedy, M., List, D., Lu, Y., Foo, L., Newman, R., Sims, I., . . . Fenton, G. (1999). Apple pomace and products derived from apple pomace: uses, composition and analysis *Analysis of plant waste materials* (pp. 75-119): Springer.

Khedkar, M. A., Nimbalkar, P. R., Gaikwad, S. G., Chavan, P. V., & Bankar, S. B. (2017). Sustainable biobutanol production from pineapple waste by using Clostridium acetobutylicum B 527: Drying kinetics study. *Bioresource technology, 225*, 359-366.

Kim, D.-M., Cho, E. J., Kim, J. W., Lee, Y.-W., & Chung, H.-J. (2014). Production of cellulases by Penicillium sp. in a solid-state fermentation of oil palm empty fruit bunch. *African Journal of Biotechnology*, *13*(1), 145-155. doi:http://dx.doi.org/10.5897/AJB12.2970

Kim, S., & Dale, B. E. (2004). Global potential bioethanol production from wasted crops and crop residues. *Biomass and Bioenergy, 26*(4), 361-375.

Kiran, E. U., Trzcinski, A. P., Ng, W. J., & Liu, Y. (2014). Bioconversion of food waste to energy: a review. *Fuel, 134*, 389-399.

Kogo, T., Yoshida, Y., Koganei, K., Matsumoto, H., Watanabe, T., Ogihara, J., & Kasumi, T. (2017). Production of rice straw hydrolysis enzymes by the fungi Trichoderma reesei and Humicola insolens using rice straw as a carbon source. *Bioresource Technology, 233*, 67-73.

Kumar, G., Sahgal, M., Bharti, M. K., Pandey, A. K., & Singh, A. (2014). Optimization of Various Parameters for Utilization of Apple Pomace Amended with Molasses by Indigenous Yeast Isolates. *National Academy Science Letters, 37*(6), 529-533.

Kumar, R., Mago, G., Balan, V., & Wyman, C. E. (2009). Physical and chemical characterizations of corn stover and poplar solids resulting from leading pretreatment technologies. *Bioresource Technology, 100*(17), 3948-3962.

Kunamneni, A., Ballesteros, A., Plou, F. J., & Alcalde, M. (2007). Fungal laccase—a versatile enzyme for biotechnological applications. *Communicating current research and educational topics and trends in applied microbiology, 1*, 233-245.

Lim, S. L., Lee, L. H., & Wu, T. Y. (2016). Sustainability of using composting and vermicomposting technologies for organic solid waste biotransformation: recent overview, greenhouse gases emissions and economic analysis. *Journal of Cleaner Production, 111*, 262-278.

Lin, C. S. K., Pfaltzgraff, L. A., Herrero-Davila, L., Mubofu, E. B., Abderrahim, S., Clark, J. H., . . . Dickson, F. (2013). Food waste as a valuable resource for the production of chemicals, materials and fuels. Current situation and global perspective. *Energy & Environmental Science, 6*(2), 426-464.

Lin, Y., & Tanaka, S. (2006). Ethanol fermentation from biomass resources: current state and prospects. *Applied Microbiology and Biotechnology, 69*(6), 627-642.

Lonsane, B., Ghildyal, N., Budiatman, S., & Ramakrishna, S. (1985). Engineering aspects of solid state fermentation. *Enzyme and microbial technology, 7*(6), 258-265.

Lu, Y., & Foo, L. Y. (2000). Antioxidant and radical scavenging activities of polyphenols from apple pomace. *Food chemistry, 68*(1), 81-85.

Lvovsky, K. (2000). Environmental costs of fossil fuels: a rapid assessment method with application to six cities.

Magyar, M., da Costa Sousa, L., Jin, M., Sarks, C., & Balan, V. (2016). Conversion of apple pomace waste to ethanol at industrial relevant conditions. *Applied Microbiology and Biotechnology, 100*(16), 7349-7358.

Mahawar, M. K., Singh, A., & Sahgal, M. (2013). Ethanol production from apple pomace using enzymatic treatment and yeast strains. *BIOINFOLET-A Quarterly Journal of Life Sciences, 10*(3a), 884-887.

Marcolongo, L., Ionata, E., La Cara, F., Amore, A., Giacobbe, S., Pepe, O., & Faraco, V. (2014). The effect of Pleurotus ostreatus arabinofuranosidase and its evolved variant in lignocellulosic biomasses conversion. *Fungal Genetics and Biology, 72*, 162-167.

Martinez, D., Berka, R. M., Henrissat, B., Saloheimo, M., Arvas, M., Baker, S. E., Cullen, D. (2008). Genome sequencing and analysis of the biomass-degrading fungus Trichoderma reesei (syn. Hypocrea jecorina). *Nature biotechnology, 26*(5), 553-560.

Matsakas, L., & Christakopoulos, P. (2015). Ethanol production from enzymatically treated dried food waste using enzymes produced on-site. *Sustainability, 7*(2), 1446-1458.

McGlade, C., & Ekins, P. (2015). The geographical distribution of fossil fuels unused when limiting global warming to 2 [deg] C. *Nature, 517*(7533), 187-190.

Medeiros, A. B., Pandey, A., Freitas, R. J., Christen, P., & Soccol, C. R. (2000). Optimization of the production of aroma compounds by Kluyveromyces marxianus in solid-state fermentation using factorial design and response surface methodology. *Biochemical Engineering Journal, 6*(1), 33-39.

Meletiadis, J., Meis, J. F. G. M., Mouton, J. W., & Verweij, P. E. (2001). Analysis of Growth Characteristics of Filamentous Fungi in Different Nutrient Media. *Journal of Clinical Microbiology, 39*(2), 478-484. doi:10.1128/JCM.39.2.478-484.2001

Miettinen-Oinonen, A., & Suominen, P. (2002). Enhanced production of Trichoderma reesei endoglucanases and use of the new cellulase preparations in producing the stonewashed effect on denim fabric. *Applied and environmental microbiology, 68*(8), 3956-3964.

Mirabella, N., Castellani, V., & Sala, S. (2014). Current options for the valorization of food manufacturing waste: a review. *Journal of Cleaner Production, 65*, 28-41.

Monticello, D., & Finnerty, W. (1985). Microbial desulfurization of fossil fuels. *Annual Reviews in Microbiology, 39*(1), 371-389.

Moo-Young, M., Lamptey, J., & Robinson, C. (1980). Immobilization of yeast cells on various supports for ethanol production. *Biotechnology letters, 2*(12), 541-548.

Moreno, P., Aral, H., & Vecchio-Sadus, A. environmental impact and toxicology of suphate, conference paper (2009).

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y., Holtzapple, M., & Ladisch, M. (2005). Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology, 96*(6), 673-686.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y., Holtzapple, M., & Ladisch, M. (2005). Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresour Technol, 96*(6), 673-686. doi:10.1016/j.biortech.2004.06.025

Moukamnerd, C., Kawahara, H., & Katakura, Y. (2013). Feasibility study of ethanol production from food wastes by consolidated continuous solid-state fermentation.

Mudgett, R. (1986). Solid-state fermentations.

Muradov, N. (2001). Hydrogen via methane decomposition: an application for decarbonization of fossil fuels. *International Journal of Hydrogen Energy, 26*(11), 1165-1175.

Mussatto, S. I., & Teixeira, J. (2010). Lignocellulose as raw material in fermentation processes. *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology (Méndez-Vilas, A., Ed.), 2*, 897-907.

Muthuvelayudham, R., & Viruthagiri, T. (2006). Fermentative production and kinetics of cellulase protein on Trichoderma reesei using sugarcane bagasse and rice straw. *African Journal of Biotechnology, 5*(20).

Naik, S. N., Goud, V. V., Rout, P. K., & Dalai, A. K. (2010). Production of first and second generation biofuels: a comprehensive review. *Renewable and Sustainable Energy Reviews, 14*(2), 578-597.

Naqvi, M., & Yan, J. (2015). First-Generation Biofuels. *Handbook of Clean Energy Systems*.

Nawirska, A., & Kwaśniewska, M. (2005). Dietary fibre fractions from fruit and vegetable processing waste. *Food chemistry, 91*(2), 221-225.

Nevalainen, H., Suominen, P., & Taimisto, K. (1994). On the safety of Trichoderma reesei. *Journal of Biotechnology, 37*(3), 193-200.

Ngadi, M., & Correia, L. (1992a). Kinetics of solid-state ethanol fermentation from apple pomace. *Journal of Food Engineering, 17*(2), 97-116.

Ngadi, M., & Correia, L. (1992b). Solid state ethanol fermentation of apple pomace as affected by moisture and bioreactor mixing speed. *Journal of Food Science, 57*(3), 667-670.

Nigam, P. S., & Singh, A. (2011). Production of liquid biofuels from renewable resources. *Progress in energy and combustion science, 37*(1), 52-68.

Niven, R. K. (2005). Ethanol in gasoline: environmental impacts and sustainability review article. *Renewable and Sustainable Energy Reviews, 9*(6), 535-555.

Nogueira, A., Santos, L. D., Paganini, C., & Wosiacki, G. (2005). Evaluation of the alcoholic fermentation of aqueous extract of the apple pomace. *Semina: Ciências Agrárias, 26*(2), 187-194.

Novakov, T., Ramanathan, V., Hansen, J., Kirchstetter, T., Sato, M., Sinton, J., & Sathaye, J. (2003). Large historical changes of fossil-fuel black carbon aerosols. *Geophysical Research Letters, 30*(6).

Oechslin, R., Lutz, M. V., & Amadò, R. (2003). Pectic substances isolated from apple cellulosic residue: structural characterisation of a new type of rhamnogalacturonan I. *Carbohydrate Polymers, 51*(3), 301-310.

Oriol, E., Raimbault, M., Roussos, S., & Viniegra-Gonzales, G. (1988). Water and water activity in the solid state fermentation of cassava starch by Aspergillus niger. *Applied Microbiology and Biotechnology, 27*(5-6), 498-503.

Ortiz, G. E., Guitart, M. E., Cavalitto, S. F., Albertó, E. O., Fernández-Lahore, M., & Blasco, M. (2015). Characterization, optimization, and scale-up of cellulases production by trichoderma reesei cbs 836.91 in solid-state fermentation using agro-industrial products. *Bioprocess and Biosystems Engineering, 38*(11), 2117-2128. doi:10.1007/s00449-015-1451-2

Paganini, C., Nogueira, A., Silva, N. C., & Wosiacki, G. (2005). Utilization of apple pomace for ethanol production and food fiber obtainment. *Ciência e Agrotecnologia, 29*(6), 1231-1238.

Pandey, A. (1992). Recent process developments in solid-state fermentation. *Process Biochemistry, 27*(2), 109-117.

Pandey, A. (2003a). Solid-state fermentation. *Biochemical Engineering Journal, 13*(2), 81-84.

Pandey, A. (2003b). Solid-state fermentation. *Biochemical Engineering Journal, 13*(2-3), 81-84.

Pandey, A. (2011). *Biofuels: alternative feedstocks and conversion processes*: Academic Press.

Pandey, A., Ashakumary, L., Selvakumar, P., & Vijayalakshmi, K. (1994). Influence of water activity on growth and activity of Aspergillus niger for glycoamylase production in solid-state fermentation. *World Journal of Microbiology and Biotechnology, 10*(4), 485-486.

Panwar, N., Kaushik, S., & Kothari, S. (2011). Role of renewable energy sources in environmental protection: a review. *Renewable and Sustainable Energy Reviews, 15*(3), 1513-1524.

Parmar, I., & Rupasinghe, H. V. (2013). Bio-conversion of apple pomace into ethanol and acetic acid: Enzymatic hydrolysis and fermentation. *Bioresource Technology, 130*, 613-620.

Pasanen, A.-L., Kasanen, J.-P., Rautiala, S., Ikäheimo, M., Rantamäki, J., Kääriäinen, H., & Kalliokoski, P. (2000). Fungal growth and survival in building materials under fluctuating moisture and temperature conditions. *International Biodeterioration & Biodegradation*, *46*(2), 117-127.

Pathania, S. (2016). *BIOFUEL PRODUCTION FROM APPLE POMACE-A MAJOR HORTICULTURAL WASTE OF HIMACHAL PRADESH*. UHF, NAUNI.

Penning, B. W., Sykes, R. W., Babcock, N. C., Dugard, C. K., Klimek, J. F., Gamblin, D., Carpita, N. C. (2014). Validation of PyMBMS as a High-throughput Screen for Lignin Abundance in Lignocellulosic Biomass of Grasses. *BioEnergy Research*. doi:10.1007/s12155-014-9410-3

Pfaltzgraff, L. A., Cooper, E. C., Budarin, V., & Clark, J. H. (2013). Food waste biomass: a resource for high-value chemicals. *Green Chemistry, 15*(2), 307-314.

Pham, T. P. T., Kaushik, R., Parshetti, G. K., Mahmood, R., & Balasubramanian, R. (2015). Food waste-to-energy conversion technologies: Current status and future directions. *Waste Management, 38*, 399-408.

Plácido, J., & Capareda, S. (2015). Ligninolytic enzymes: a biotechnological alternative for bioethanol production. *Bioresources and Bioprocessing*, *2*(1), 23.

Pranita A. Gulhane, A. V. G., Kalyani Kadu. (2015). Apple Pomace: A Potential Substrate for Ethanol Production. *International Journal of Research Studies in Biosciences*, *3*(6), 5.

Preston, R. D. (1974). *The physical biology of plant cell walls*: London.: Chapman & Hall.

Rahman, F. A., Aziz, M. M. A., Saidur, R., Bakar, W. A. W. A., Hainin, M., Putrajaya, R., & Hassan, N. A. (2017). Pollution to solution: Capture and sequestration of carbon dioxide (CO 2) and its utilization as a renewable energy source for a sustainable future. *Renewable and Sustainable Energy Reviews*, *71*, 112-126.

Raimbault, M. (1998). General and microbiological aspects of solid substrate fermentation. *Electronic Journal of Biotechnology*, *1*(3), 26-27.

Ramesh, M., & Lonsane, B. (1990). Critical importance of moisture content of the medium in alpha-amylase production by Bacillus licheniformis M27 in a solid-state fermentation system. *Applied Microbiology and Biotechnology*, *33*(5), 501-505.

Ratna, S., & Mustafa, B. (2011). Solid substrate fermentation (SSF) and submerged fermentation (SmF) of aspergillus versicolor A6 using pineapple waste as substrate for pectinase production.

Ravindran, R., & Jaiswal, A. K. (2016). A comprehensive review on pre-treatment strategy for lignocellulosic food industry waste: Challenges and opportunities. *Bioresource Technology*, *199*, 92-102. doi:http://dx.doi.org/10.1016/j.biortech.2015.07.106

Ridley, B. L., O'Neill, M. A., & Mohnen, D. (2001). Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry*, *57*(6), 929-967.

Rifkin, J. (2008). Industrial Revolution.

Rifkin, J. (2015). Industrial Revolution. *Retrieved in March 2015 from http://thethirdindustrialrevolution. com*.

Roukas, T. (1996). Continuous ethanol production from nonsterilized carob pod extract by immobilizedSaccharomyces cerevisiae on mineral kissiris using a two-reactor system. *Applied Biochemistry and Biotechnology*, *59*(3), 299-307.

Roukas, T. (1996). Ethanol production from non-sterilized beet molasses by free and immobilized Saccharomyces cerevisiae cells using fed-batch culture. *Journal of Food Engineering*, *27*(1), 87-96.

Ryden, P., & Selvendran, R. R. (1990). Structural features of cell-wall polysaccharides of potato (Solanum tuberosum). *Carbohydrate Research*, *195*(2), 257-272.

Sahu, S. (2016). *Conversion of Cotton Gin Waste to Bioethanol: Pretreatment, Hydrolysis and Fermentation.*

Sahu, S., & Pramanik, K. (2015a). Bioconversion of Cotton Gin Waste to Bioethanol *Environmental Microbial Biotechnology* (pp. 267-288): Springer.

Sahu, S., & Pramanik, K. (2015b). Delignification of cotton gin waste and its optimization by using white rot fungus Pycnoporus cinnabarinus. *Journal of Environmental Biology*, *36*(3), 661.

Saini, J. K., Saini, R., & Tewari, L. (2015). Lignocellulosic agriculture wastes as biomass feedstocks for second-generation bioethanol production: concepts and recent developments. *3 Biotech*, *5*(4), 337-353.

Samuelsson, R., Burvall, J. and Jirjis, R., 2006. Comparison of different methods for the determination of moisture content in biomass. Biomass and Bioenergy, 30(11), pp.929-934

Saravanan, P., Muthuvelayudham, R., & Viruthagiri, T. (2013). Enhanced production of cellulase from pineapple waste by response surface methodology. *Journal of engineering, 2013*.

Schobert, H. (2013). *Chemistry of fossil fuels and biofuels*: Cambridge University Press.

Shalini, R., & Gupta, D. (2010). Utilization of pomace from apple processing industries: a review. *Journal of food science and technology, 47*(4), 365-371.

Sharada, R., Venkateswarlu, G., Venkateshwar, S., & Rao, M. A. (2013). PRODUCTION OF CELLULASE-A REVIEW. *International Journal of Pharmaceutical, Chemical & Biological Sciences, 3*(4).

Sharma, N., Kalra, K., Oberoi, H. S., & Bansal, S. (2007). Optimization of fermentation parameters for production of ethanol from kinnow waste and banana peels by simultaneous saccharification and fermentation. *Indian Journal of Microbiology, 47*(4), 310-316.

Shojaosadati, S., & Babaeipour, V. (2002). Citric acid production from apple pomace in multi-layer packed bed solid-state bioreactor. *Process Biochemistry, 37*(8), 909-914.

Shrikot, C., Sharma, N., & Sharma, S. (2004). Apple pomace: An alternative substrate for xylanase production by na alkalophilic Bacillus macerans by using solid-state fermentation. *Journal of Microbial World, 6*, 20-26.

Silverstein, R. A., Chen, Y., Sharma-Shivappa, R. R., Boyette, M. D., & Osborne, J. (2007). A comparison of chemical pretreatment methods for improving saccharification of cotton stalks. *Bioresource Technology, 98*(16), 3000-3011.

Sindhu, R., Kuttiraja, M., Binod, P., Janu, K. U., Sukumaran, R. K., & Pandey, A. (2011). Dilute acid pretreatment and enzymatic saccharification of sugarcane tops for bioethanol production. *Bioresource Technology, 102*(23), 10915-10921.

Singh, A., & Kumar, P. (1991). Fusarium oxysporum: status in bioethanol production. *Critical reviews in biotechnology, 11*(2), 129-147.

Singh, A., Sharma, P., Saran, A. K., Singh, N., & Bishnoi, N. R. (2013). Comparative study on ethanol production from pretreated sugarcane bagasse using immobilized Saccharomyces cerevisiae on various matrices. *Renewable Energy, 50*, 488-493.

Singh, J., Suhag, M., & Dhaka, A. (2015). Augmented digestion of lignocellulose by steam explosion, acid and alkaline pretreatment methods: a review. *Carbohydrate Polymers, 117*, 624-631.

Singhania, R. R., Sukumaran, R. K., Patel, A. K., Larroche, C., & Pandey, A. (2010). Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases. *Enzyme and microbial technology, 46*(7), 541-549.

Skory, C. D., Freer, S. N., & Bothast, R. J. (1997). Screening for ethanol-producing filamentous fungi. *Biotechnology letters, 19*(3), 203-206.

Somerville, C. (2007). Biofuels. *Current Biology, 17*(4), R115-R119.

Song, H., & Lee, S. Y. (2006). Production of succinic acid by bacterial fermentation. *Enzyme and microbial technology, 39*(3), 352-361. doi:https://doi.org/10.1016/j.enzmictec.2005.11.043

Sorek, N., Yeats, T. H., Szemenyei, H., Youngs, H., & Somerville, C. R. (2014). The Implications of Lignocellulosic Biomass Chemical Composition for the Production of Advanced Biofuels. *BioScience, 64*(3), 192-201. doi:10.1093/biosci/bit037

Sternberg, D. (1976). *Production of cellulase by Trichoderma.* Paper presented at the Biotechnology and bioengineering symposium.

Stevenson, D., & Weimer, P. (2002). Isolation and characterization of a Trichoderma strain capable of fermenting cellulose to ethanol. *Applied Microbiology and Biotechnology, 59*(6), 721-726.

Sudha, M., Baskaran, V., & Leelavathi, K. (2007). Apple pomace as a source of dietary fiber and polyphenols and its effect on the rheological characteristics and cake making. *Food chemistry, 104*(2), 686-692.

Sumbhate, S. V., Nayak, S., Goupale, D., Tiwari, A., & Jadon, R. S. (2012). Colorimetric method for the estimation of ethanol in alcoholic-drinks. *Journal of Analytical Techniques, 1*(1), 1-6.

Sun, H., Ge, X., Hao, Z., & Peng, M. (2010). Cellulase production by Trichoderma sp. on apple pomace under solid state fermentation. *African Journal of Biotechnology, 9*(2).

Sun, Y., & Cheng, J. (2002). Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresource Technology, 83*(1), 1-11.

Sunnotel, O., & Nigam, P. (2002). Pectinolytic activity of bacteria isolated from soil and two fungal strains during submerged fermentation. *World Journal of Microbiology and Biotechnology, 18*(9), 835-839.

Taherzadeh, M. J., & Karimi, K. (2008). Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review. *International journal of molecular sciences, 9*(9), 1621-1651.

Tans, E. D. a. P. (2018). Trends in Atmospheric Carbon Dioxide. Retrieved from https://www.esrl.noaa.gov/gmd/ccgg/trends/gl_data.html

Tsurumi, R., Shiraishi, S., Ando, Y., Yanagida, M., & Takeda, K. (2001). Production on flavor compounds from apple pomace. *Journal of the Japanese Society for Food Science and Technology (Japan)*.

Trinh, C. T., Unrean, P., & Srienc, F. (2008). Minimal Escherichia coli cell for the most efficient production of ethanol from hexoses and pentoses. Applied and environmental microbiology, 74(12), 3634-3643

USDA, F. (2018). Global leading apple producing countries in 2017/2018 (in 1,000 metric tons). Retrieved from https://www.statista.com/statistics/279555/global-top-apple-producing-countries/

Uyar, F., & Baysal, Z. (2004). Production and optimization of process parameters for alkaline protease production by a newly isolated Bacillus sp. under solid state fermentation. *Process Biochemistry, 39*(12), 1893-1898.

Van Dyk, J., Gama, R., Morrison, D., Swart, S., & Pletschke, B. (2013). Food processing waste: Problems, current management and prospects for utilisation of the lignocellulose component through enzyme synergistic degradation. *Renewable and Sustainable Energy Reviews, 26*, 521-531.

Velkovska, S., Marten, M. R., & Ollis, D. F. (1997). Kinetic model for batch cellulase production by Trichoderma reesei RUT C30. *Journal of Biotechnology, 54*(2), 83-94.

Vendruscolo, F., Albuquerque, P. M., Streit, F., Esposito, E., & Ninow, J. L. (2008). Apple pomace: a versatile substrate for biotechnological applications. *Critical reviews in biotechnology, 28*(1), 1-12.

Vendruscolo, F., & Ninow, J. L. (2014). Apple pomace as a substrate for fungal chitosan production in an airlift bioreactor. *Biocatalysis and Agricultural Biotechnology, 3*(4), 338-342.

Vidyalakshmi, R., Paranthaman, R., & Indhumathi, J. (2009). Amylase production on submerged fermentation by Bacillus spp. *World Journal of Chemistry, 4*(1), 89-91.

Villas-Bôas, S. G., Esposito, E., & de Mendonca, M. M. (2003). Bioconversion of apple pomace into a nutritionally enriched substrate by Candida utilis and Pleurotus ostreatus. *World Journal of Microbiology and Biotechnology, 19*(5), 461-467.

Visser, H., Joosten, V., Punt, P. J., Gusakov, A. V., Olson, P. T., Joosten, R., . . . Emalfarb, M. A. (2011). RESEARCH: Development of a mature fungal technology and production platform for industrial enzymes based on a Myceliophthora thermophila isolate, previously known as Chrysosporium lucknowense C1. *Industrial Biotechnology, 7*(3), 214-223.

Wang, Y.-F., Tan, L., Wang, T., Sun, Z.-Y., Tang, Y.-Q., & Kida, K. (2017). Production of ethanol from kitchen waste by using flocculating Saccharomyces cerevisiae KF-7. *Environmental technology, 38*(3), 316-325.

Wei, L., & McDonald, A. G. (2016). A review on grafting of biofibers for biocomposites. *Materials, 9*(4), 303.

Wen, L.-F., Chang, K., Brown, G., & Gallaher, D. (1988). Isolation and characterization of hemicellulose and cellulose from sugar beet pulp. *Journal of Food Science, 53*(3), 826-829.

Wen, Z., Liao, W., & Chen, S. (2005). Production of cellulase by Trichoderma reesei from dairy manure. *Bioresource Technology, 96*(4), 491-499.

Wilson, D. B. (2009). Cellulases and biofuels. *Current Opinion in Biotechnology, 20*(3), 295-299.

Xin, F., & Geng, A. (2010). Horticultural waste as the substrate for cellulase and hemicellulase production by Trichoderma reesei under solid-state fermentation. *Applied Biochemistry and Biotechnology, 162*(1), 295-306.

Yang, X., Lee, S. J., Yoo, H. Y., Choi, H. S., Park, C., & Kim, S. W. (2014). Biorefinery of instant noodle waste to biofuels. *Bioresource Technology, 159*, 17-23.

Yu, Q., Zhuang, X., Lv, S., He, M., Zhang, Y., Yuan, Z., . . . Tan, X. (2013). Liquid hot water pretreatment of sugarcane bagasse and its comparison with chemical pretreatment methods for the sugar recovery and structural changes. *Bioresource Technology, 129*, 592-598.

Zhang, K., Pei, Z., & Wang, D. (2016). Organic solvent pretreatment of lignocellulosic biomass for biofuels and biochemicals: a review. *Bioresource Technology, 199*, 21-33.

Zhang, Z., O'Hara, I. M., Mundree, S., Gao, B., Ball, A. S., Zhu, N., . . . Jin, B. (2016). Biofuels from food processing wastes. *Current Opinion in Biotechnology, 38*, 97-105. doi:http://dx.doi.org/10.1016/j.copbio.2016.01.010

Zheng, Y., Pan, Z., & Zhang, R. (2009). Overview of biomass pretreatment for cellulosic ethanol production. *International journal of agricultural and biological engineering, 2*(3), 51-68.

Zheng, Z., & Shetty, K. (2000). Solid-state bioconversion of phenolics from cranberry pomace and role of Lentinus edodes β-glucosidase. *Journal of Agricultural and Food Chemistry, 48*(3), 895-900.

Zhong-Tao, S., Lin-Mao, T., Cheng, L., & Jin-Hua, D. (2009). Bioconversion of apple pomace into a multienzyme bio-feed by two mixed strains of Aspergillus niger in solid state fermentation. *Electronic Journal of Biotechnology, 12*(1), 2-3.

Ziegler, E. E., & Filer Jr, L. (1996). Present knowledge in nutrition. *International Life Sciences Institute-Nutrition Foundation*. Retrieved from http://agris.fao.org/agris-search/search.do?recordID=XF2016001304