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**Agro-industrial Lignocellulosic Biomass:
A Sustainable Platform for the Production of
 α -amylase and xylanase**



**A THESIS SUBMITTED TO THE TECHNICAL UNIVERSITY OF DUBLIN
FOR THE AWARD OF
DOCTOR OF PHILOSOPHY**

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Abstract

Enzymes such as α -amylase and xylanase offer potential for numerous industrial applications such as food, beverage, and animal feed production, detergents, textile, cosmetics and biodiesel production. Economic factors such as achievement of optimum yields and production cost are the main deterrents in the industrial use of these enzymes. Enzymes are generally produced via microbial fermentation using expensive mediums which account for 30 to 40% of the production cost. Food industry wastes such as spent coffee waste (SCW) and Brewers' spent grain (BSG) are two of the most common food waste products expelled by the food and beverage industry here in Ireland and are environmental burden. However, these inexpensive food wastes can be utilized as a substrate for the production of enzymes, which can significantly reduce the production cost of the enzymes and also enhance the value of underutilized food waste. Based on the above facts, the aim of this study is to exploit the lignocellulosic food industry waste such as BSG and SCW for the production of industrially viable enzymes such as α -amylase and xylanase. Although high in polysaccharide content, the effective utilisation of SCW and BSG requires specific pretreatment measures that result in a decrease in recalcitrance by removal of the lignin fraction. The aim of the project was achieved through the following objectives:

- Identification and development of the best pretreatment strategy for SCW and BSG that would enhance their enzymatic digestibility and liberation of higher amount of fermentable sugars.
- Optimisation of SCW and BSG for the production of enzymes such as α -amylase and xylanase.

The effect of the pretreatments was primarily analysed as changes that occurred in the composition of SCW and BSG. A number of analytical techniques were used to characterise the pretreated waste samples, including FTIR, FESEM, XRD and DSC.

Overall, the following observations were made:

- Conc. phosphoric acid acetone pretreatment and ammonia fibre explosion (AFEX) was the best pretreatment measures in reducing recalcitrance in SCW. In lieu of devising further efficient pretreatments for SCW two strategies *viz.* two-step sequential pretreatment and ultrasound assisted pretreatment were developed and successfully tested for lignin and hemicellulose removal from the lignocellulosic substrate.
- Subjecting BSG to six different pretreatments revealed microwave assisted alkali pretreatment was the best in terms of reduction in lignin content.
- Screening test for four *Bacillus* sp. *viz.* *B. subtilis*, *B. megaterium*, *B. cereus* and *B. stearothermophilus* revealed that all the strains were amylase producing organisms. *B. stearothermophilus* amylase was found to be stable in a temperature range of 60°C to 100°C and in an alkaline pH range (5-10).
- Pretreated BSG was hydrolysed using cellulolytic enzymes and used as a growth medium supplement for cultivation of the thermophilic bacterium, *Bacillus stearothermophilus* in the production of α -amylase. Optimisation trials revealed that maximal amylase production (198.09 U/ml) occurred with a medium composition of starch (0.2% w/v), peptone (0.2% w/v), KCl.4 H₂O (0.02% w/v), MgSO₄.7 H₂O (0.01% w/v) and hydrolysate (0.22% v/v). A 1.3-fold increase in amylase activity was obtained following novel media composition.
- *B. stearothermophilus* α -amylase was successfully tested as an antibiofilm activity against three pathogens *viz.* *S. aureus* NCTC 1803, *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27835 inhibiting their biofilm producing capacity by 62%, 55% and 50% respectively.
- Pretreated SCW was used as the sole carbon source for xylanase production in solid state fermentation mode. The mycelial fungus, *Aspergillus niger*, was used as the fermentative microbe. Under optimised SSF conditions an enzyme activity of 6495.6 U/g of dry SCW was recorded, which was approximately 1.39-fold higher than a control culture (4649 U/g of dry SCW).
- The efficacy of the purified xylanase as a juice enrichment agent for strawberry, blueberry and raspberry pulp was also investigated. The xylanase produced by *A. niger* ATCC® was found to be an excellent fruit juice clarification agent.

Declaration

I certify that this report which I now submit to confirm that the thesis which I submit for examination for the award of Doctor of Philosophy is entirely my own work and has not been taken from the work of others, save and to the extent that such work has been cited and acknowledged within the text of my work.

This thesis has been prepared according to the regulations for postgraduate study by research of the “Dublin Institute of Technology” and has not been submitted in whole or in part for another award in any other third level institution.

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The institute has permission to keep, lend or copy this thesis in whole or in part on condition that such use of the material of the thesis is duly acknowledged.

Signature

Date 15/07/2019

Rajeev Ravindran

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List of Abbreviations

| | |
|----------|---|
| SCW | Spent Coffee Waste |
| BSG | Brewer's Spent Grain |
| NREL | National Renewable Energy Laboratory |
| FTIR | Fourier Transform Infra-red |
| FESEM | Field Emission Scanning Electron Microscopy |
| XRD | X-Ray Diffraction |
| DSC | Differential Scanning Calorimetry |
| AFEX | Ammonia Fibre Explosion |
| NCTC | National Collection of Type Cultures |
| ATCC | American Type Culture Collection |
| CCD | Central Composite Design |
| SSF | Solid State Fermentation |
| SDS-PAGE | Sodium Dodecyl Sulphate |
| EU | European Union |
| CRI | Crystallinity Index |
| CMC | Carboxymethyl Cellulose |
| EDTA | Ethylene Triamine Tetra Acetic Acid |

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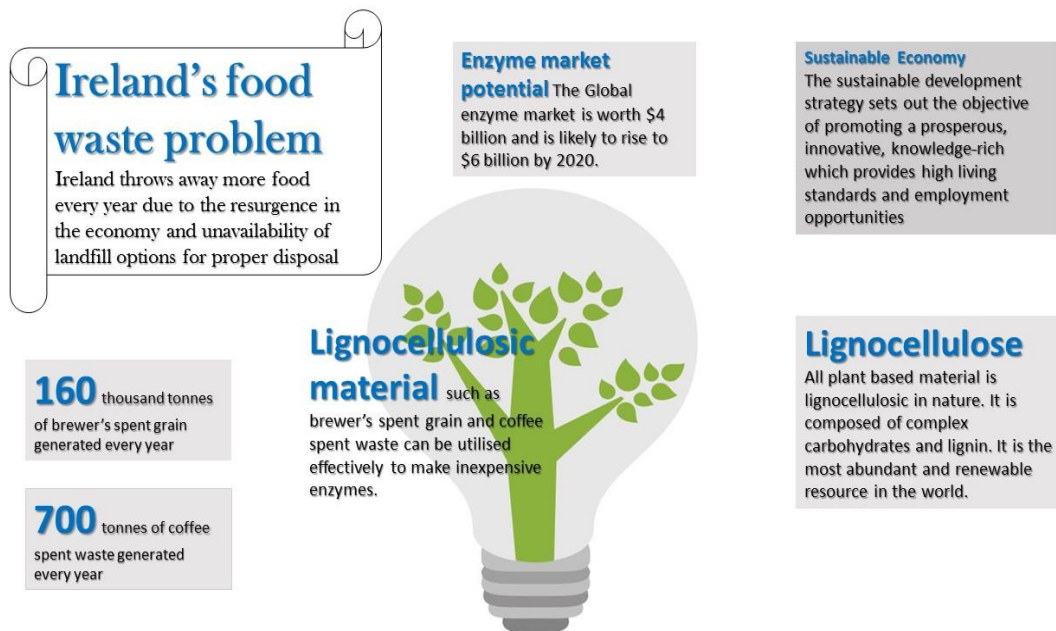
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Chapter 1

General Introduction



This chapter presents a brief explanation of the motivation behind the work, and a summary of the principal objectives.

1.1 Motivation

The global population is expanding at an exponential rate every year. There is a huge demand for food and energy to meet the needs of the society. Rapid urbanisation as compared to slow progress in the development of in effective waste management strategies leads to the accumulation of food waste. A study published by the EU in 2010 revealed that almost 90 million tonnes of food waste is expelled from the food manufacturing industry every year. Consequently, the revised EU waste legislation was adopted in the May of 2018 that calls on EU countries to act to reduce food waste at each stage of food supply chain. Furthermore, the member states of the EU have pledged to halve per capita food waste at the retail and consumer level by 2030. Food waste being high in nutritional content putrefies on accumulation turning out to be breeding grounds for disease causing organisms. This poses serious environmental issues and very few options exist today to deal with them. While preventive measures can be taken to reduce to the generation of food waste it is necessary to deal with the existing lot of accumulated food waste. The idea of converting food waste in to energy and other bio-based chemicals used for our day to day activities is an area of research with huge potential and opportunities. Lignocellulose is one of the most abundant yet underutilized bio-resource in the world. It is the best source of cheap carbohydrates. Apart from its abundance and easy availability, the fact that 75% of its composition is contributed by polysaccharide makes it a fiercely sought out raw material for the production of value-added products (bioethanol, biodiesel, biobutanol and enzyme production, bioactives compounds etc).

Enzymes are very efficient biocatalysts for various reactions and typically applied in many industries, and since last decade, the use of enzymes in industrial processes has

significantly increased. However, according to a recent report, 30-40% cost of these enzymes depends on the growth medium which is the major stumbling block in the current usage of enzymes in industrial processes. Effective utilization of inexpensive, renewable, abundant food-industry waste as cheaper media for production of industrially important enzymes could not only be helpful in lowering the cost of enzyme but also in enhancing the value of underutilized substrates thus improving the economy by solving dual purpose in a symbiotic manner.

However, the heterogeneous nature of biomass hinders them to be efficient nutrients for microbial growth for the production of bio-based chemicals including enzymes. Furthermore, regardless of lignocellulose being a cheap polysaccharide reservoir not many feasible techniques are available for mining its vast availability. Techniques pertaining to the usage of lignocellulosic food waste are so varied that there is no single solution to producing enzymes from lignocellulose. The most sustainable solutions are likely to be those that are designed according to the local conditions and demand. Other benefits such as high purity and reusability of enzymes are required alongside inexpensiveness are required to provide an advantage over conventional produced enzymes.

1.2 Aim and Objectives

Considering the above facts that a substantial part of industrial enzymes production cost is contributed by the cost of the fermentation medium and food-industry waste as a major economic and environmental burden, the aim of this thesis is to present the best procedures for converting lignocellulosic food waste such as spent coffee waste (SCW) and brewer's spent grain (BSG) into enzymes such as α -amylase and protease. In order to achieve the aim of the thesis following objectives have been identified:

1. Review of current state of technology.
2. Investigate the effects of pretreatments on the chemical composition of SCW and BSG and evaluating the formation of inhibitors during respective pretreatment.
3. Designing new and state of art pretreatment techniques intended for better enzymatic hydrolysis of SCW and BSG for the maximum liberation of fermentable sugars.
4. Screening of microorganisms for α -amylase and xylanase production.
5. Investigating the sustainability of employing pretreated SCW and BSG hydrolysate as a media component for amylase and xylanase production.
6. Application of α -amylase in antibiofilm removal and xylanase in fruit juice enrichment.

1.3 Organisation of Thesis

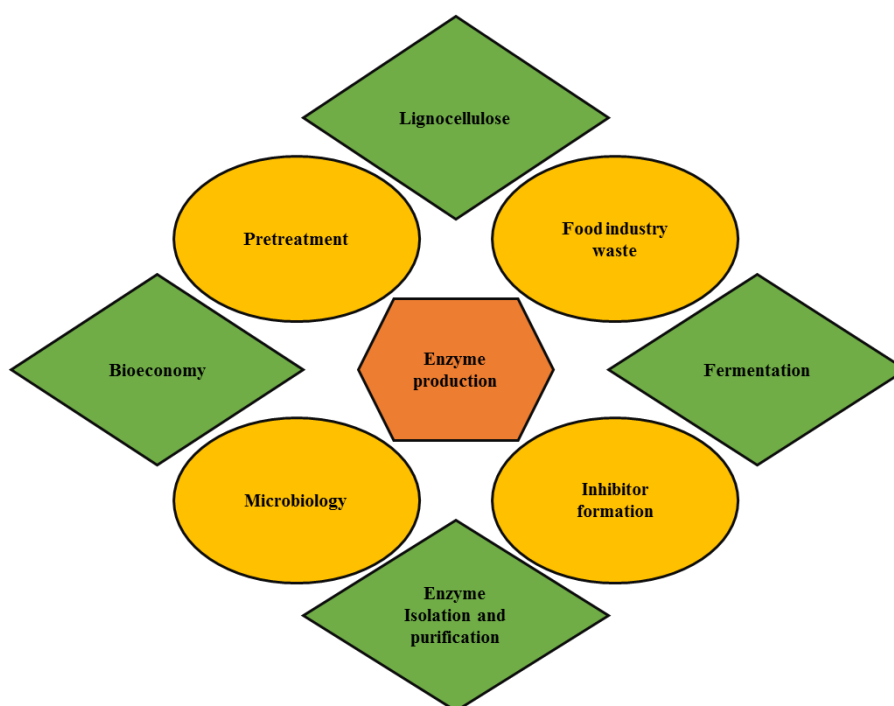
This thesis is designed in a manner to begin with a thorough investigation of the present state of current research on the valorisation of lignocellulosic food waste for enzyme production, the laws and the problems pertaining to the impediment of commercialization of the concept (Chapter 1 and 2). Chapter 3 presents protocols of various existing and newly designed pretreatments on the composition, structure, thermal properties and formation of inhibitors. Furthermore, it discusses the optimisation of enzymatic hydrolysis conditions with respect to SCW and BSG. Section A, focused on pretreatment strategy of selected lignocellulosic wastes and includes three chapters, where chapter 4 and 5 discusses the effects of conventional and customised pretreatments for SCW, while chapter 6 deals with BSG pretreatment and how they affect the release of sugar following enzymatic hydrolysis respectively. Section B is dedicated to enzyme production using lignocellulose waste. There are two chapters in this section. Chapter 7 discusses the observations pertaining the screening

of microorganisms for amylase production, utilisation of BSG hydrolysate for enzyme production, purification of the enzyme and its application as an antibiofilm agent. Chapter 8 investigates the efficacy of SCW as a fermentation medium for fungal xylanase production and subsequent utilisation of the purified enzyme for fruit juice enrichment. The last chapter summarises the findings of this study and provides a brief insight to the potential avenues for practical applications of enzymes produced by the utilisation of lignocellulosic waste.

In conclusion, this thesis makes a focused and comprehensive effort to identify the ‘usability’ of plant-based food waste for the production of industrially viable enzymes. Hurdles such as recalcitrance is addressed to develop innovative and novel technology for its removal. This is followed by processing of SCW and BSG through enzymatic hydrolysis to obtain sugar-rich hydrolysate. The viability of the hydrolysate as a potential media component for the production of amylase and xylanase was investigated. The enzymes produced were then successfully tested for potential industrial applications.

Chapter 2

Literature Review



This chapter is a literature review of the thesis and includes different aspects of lignocellulosic biomass targeting, in particular, plant-based wastes derived from the food processing industry. Furthermore, it provides a comprehensive idea about pretreatments, their classification, mode of action etc. Furthermore, the market potential of enzymes and the challenges pertaining to the utilisation of food processing waste for enzyme production and a brief summary of relevant recent research is included.

The information included in this chapter has been published in four different journals as critical review articles:

- Exploitation of food industry waste for high value products (2016). *Trends in Biotechnology*, 34(1), 58-69. (Invited Article)
- A comprehensive review on pre-treatment strategy for lignocellulosic food industry waste: challenges and opportunities (2016). *Bioresource Technology*, 92(0), 92-102. (Invited Article)
- Microbial enzyme production using lignocellulosic food industry wastes as feedstock: a review (2016). *Bioengineering*, 2016, 3(4), 30
- A review on bioconversion of agro-industrial wastes to industrially important enzymes (2018). *Bioengineering*, 5(4), 93.

2.1 Introduction

Lignocellulose is one of the most abundant yet underutilised bio-resource in the world. It is considered among the best source of cheap carbohydrates and applied as a potential substrate for the production of a range of high value products including bioethanol, enzymes and biogas. Apart from its abundance and easy availability, the fact that 75% of its composition is contributed by polysaccharide makes it a fiercely sought out raw material for the biofuel production (Sun & Cheng, 2002). Besides biofuels lignocellulose can be used as primal matter for the production of other value-added products such as enzymes.

In 2010, an article was published in the Ethanol Producer magazine entitled ‘The economics of enzyme production’ according to which the cost of producing cellulosic bioethanol is majorly dependent upon to the cost of enzymes used in the process. Although, this scenario has improved, there are still a few worries regarding harnessing the cost of production of bioethanol to becoming economically feasible. One such problem that needs to be addressed immediately is the carbohydrate source used for enzyme production. Theoretically, it is possible to recycle cheap carbohydrate sources from industries and use it as a sugar source for enzyme production. However, the heterogeneous nature of biomass carbohydrate sources hinders them to be efficient nutrients, leading to incompetent growth of the enzyme producing microorganisms. This is due to the fact that 5-C and 6-C sugars are absorbed by the microbe at different rates during fermentation (Abdel-Rahman et al., 2015). Furthermore, these carbohydrate sources comprise of other substances that may act as inhibitors of microbial growth, and leads to poor fermentation yields and subsequently raising the production costs for the desired products.

An efficient technique to remove components that inhibit microbial growth and enzymatic degradation of lignocellulose is a potential solution to increase its utility. With this aim, several studies have been dedicated to devising various pre-treatment methods with differing nature. The lignocellulosic substance undergoes a single or a sequential pre-treatment before its efficacy as a carbon source is evaluated.

2.2 Lignocellulosic Food Industry Waste

The food processing industry in the EU is progressing at a very fast pace. According to the report published by *FoodDrinkEurope* the European food and drink industry is the largest manufacturing sector in the continent and had a turnover of €1,048 billion in 2012 which was a 3.1% increase from 2011. Such a growing trend in the industry can give rise to more waste that is eventually left untreated due to lack of feasible options. Land filling remains the cheapest option for waste management by industries. Other options include incineration, which requires a lot of energy and, composting, a process that is quite slow. Improper treatment of these wastes leads to their putrefaction giving rise to toxic gases such as methane and leaching of other toxic liquids proving hazardous to the environment. Most of the waste generated from the food industry is lignocellulosic in nature, and thus can be potential substrates for the production of high value products. Products that can be produced from food and industry waste include fossil fuel alternatives such as ethanol, butanol, biogas, and fuel oil, food supplements such as prebiotics and bioactive compounds, volatile fatty acids and enzymes that are of commercial importance (Uçkun Kiran et al., 2014; Yin et al., 2014).

The European Commission has coined a new term, 'Bioeconomy' which focuses on addressing the environmental challenges that the world faces today. An article

published by the European Commission in 2009 states that EU produces an estimated 138 million tons of bio-waste from industries every year of which 40% is being used as landfill. The motivation behind bioeconomy is to reduce relying on natural resources by transforming the manufacturing sector. This can be achieved by promoting sustainable production of renewable resources from land, fisheries and aquaculture and their conversion into food, feed, fibre, bio-based products and bio-energy, while generating more jobs and giving rise to new industries (Commission, 2009).

2.3 Food Industry waste as a renewable resource

Food industry waste is particularly interesting for renewable energy researchers as it is mostly lignocellulosic in nature with high cellulose and lignin content (except animal-derived food waste). Many studies have reported on various technologies on the conversion of food waste such as apple pomace and brewer's spent grain into biofuel (Parmar and Rupasinghe, 2013; Liguori, Soccol et al., 2015). Cellulose and hemicelluloses upon enzymatic breakdown releases glucose and xylose, which can be converted into ethanol by fermentative microorganism (Das, Ravindran et al., 2012). Furthermore, lignin upon pyrolysis and anaerobic digestion yields H_2 and CH_4 (Azadi, Inderwildi et al., 2013). In the quest of renewable energy resources at the backdrop of rising oil prices one overlooks the fact that food waste is a reservoir of other value added chemicals. Recent studies suggest that the production of bulk chemicals from biomass waste is 3.5 times more profitable than converting it into biofuel (Tock et al.; 2012). Figure 2.1 provides a comprehensive overview of the different functionalised molecules that can be derived from food supply chain waste.

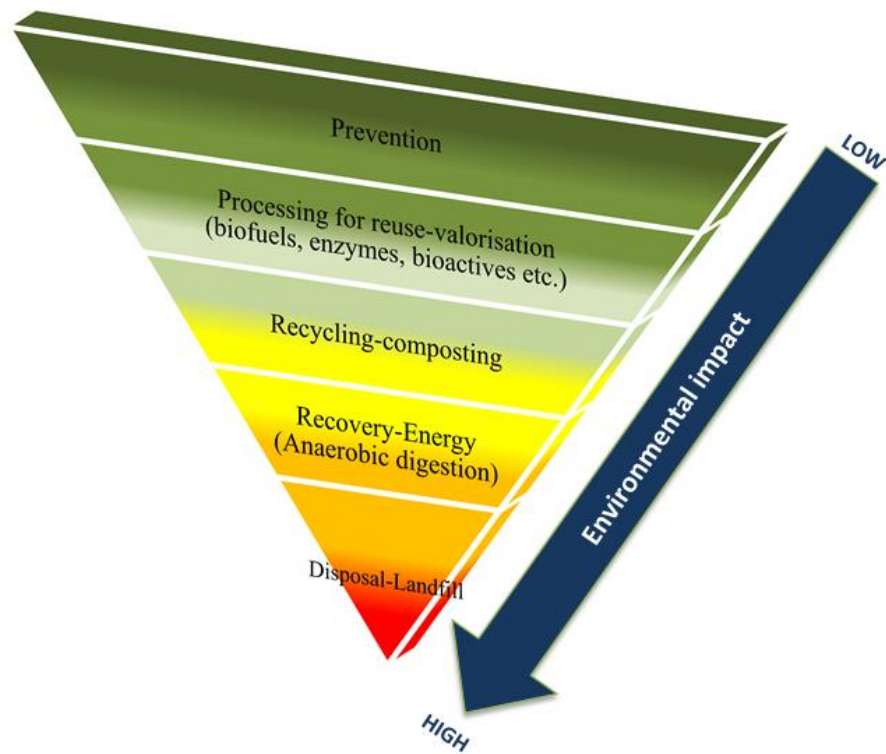


Figure 2.1 Hierarchy of waste processing (adopted and modified from Lin et al. (2013))

2.4 Market potential

The global enzyme industry is growing at a fast pace. It was worth almost \$4.8 billion in 2013 and is estimated that by 2018 will be worth a staggering \$7.1 billion (BCC research, 2014). Enzymes are predominantly used for the production of several products that we use in our day-to-day lives (Jegannathan & Nielsen, 2013). Besides, new-found interest in bioenergy has led to an increased demand for enzymes applicable in the biofuel sector (Phitsuwan et. al., 2013). Enzymes can be prepared for customized applications for different industrial processes with the help of recombinant DNA technology and protein engineering. According to a report published by the “National Renewable Energy Laboratory”, cellulosic ethanol prices are highly

dependent on the cost of enzymes that can break down complex carbohydrates into fermentable sugars. Therefore, decreasing the cost of enzymes can increase the market potential of biofuels and also other value-added products (NREL, 2010). Table 2.1 Pretreatment of different agricultural biomass for the production of industrially important enzymes. Table 2.1 provides a comprehensive idea about on the use of lignocellulosic materials as raw materials for enzyme production.

2.5 Enzymes Production using lignocellulosic food industry waste

2.5.1 Lignocellulose as a raw material

Lignocellulose is essentially a complex polymer which is made up of polysaccharides and phenolic polymers (lignin). All plant matter is composed of lignocellulose, and lignin is the most recalcitrant substance found in them. It imparts physical strength to the plant cell wall. It is a complex polymer made up of coniferyl alcohol, synapyl alcohol and *p*-hydroxyphenyl alcohol (Ragauskas et al., 2014). The presence of lignin in lignocellulose prevents its effective enzymatic degradation and subsequent utilization of fermentable sugars by microbes (Zeng et al., 2014). This calls for effective measures for lignin removal through various pretreatment techniques (Chang et al., 2000; Ghaffar et al., 2013). The crystalline nature of lignocellulose is another major hurdle in its efficient utilization. Crystallinity of the biomass material is imparted by the presence of crystalline cellulose, which, if not converted into its amorphous form, is not susceptible to enzymatic hydrolysis (Bansal et al., 2010; Gurgel et al., 2012).

Table 2.1 Pretreatment of different agricultural biomass for the production of industrially important enzymes (Ravindran et al., 2017)

| Enzyme | Feedstock | Pretreatment | Microbial strain | Application |
|--------------------|---|--|---|---|
| Amylase | Banana peel | Acid pretreatment | Catabolite repressed <i>Bacillus subtilis</i> KCC103, <i>Trichoderma reesei</i> | Baking, brewing, animal nutrition, aquaculture, biofuel, dishwashing and laundry detergents |
| β -glucanase | Oat meal, orange peel | Autoclaving, milling, fractionation, Acid pretreatment | <i>Rhizomucor miehei</i> CAU432, <i>Trichoderma viride</i> MBL, <i>Penicillium echinulatum</i> 9A02S1 | Brewing, bioethanol |
| Cellulase | Apple pomace, banana peel, mango peel, brewer's spent grain | Autoclaving, Milling | <i>Aspergillus niger</i> NRRL-567, <i>Trichoderma viride</i> GIM 3.0010 | Detergents, Bleaching, Deinking, refining, Starch modification, drainage |

| | | | | |
|-----------|--|-------------------------|--|--|
| | | | | improvement, decolourisation of dyes in effluent, cellulosic and starch- based ethanol, Biodiesel |
| Inulinase | Banana peel, wheat bran, rice bran, orange peel, bagasse, coconut oil cake, yacon | Hydraulic Pressure | <i>Saccharomyces</i> sp., <i>Pencillium</i> <i>rugulosum</i> (MTCC-3487), <i>Aspergillus kawachii</i> | Production of high fructose corn syrup |
| Invertase | Red carrot jam processing residue, Orange peel, pineapple peel waste, pomegranate peel, bagasse | Autoclaving, Milling | <i>S. cerevisiae</i> NRRL Y-12632, <i>Aspergillus niger</i> GH1, <i>Cladosporium cladosporioides</i> | Sucrose hydrolysis |
| Lactase | Fermented ragi | | <i>Lactobacillus acidophilus</i> | Dairy, preparation of lactose-free food products |

| | | | |
|------------------|---|--|---|
| Mannanase | Apple pomace and cotton seed powder mixture, fermented palm kernel cake, passion fruit peel | <i>Aspergillus niger</i> SN-09, <i>Aspergillus terreus</i> SUK-1, | Paper and pulp, textile, pharmaceuticals |
| Pectinase | Orange peel, deseeded sunflower head | <i>Penicillium oxalicum</i> PJ02, <i>Aspergillus niger</i> | Processing of starch and wine, juice processing Bleaching and deinking of paper, |
| Xylanase | Coffee by-products | <i>Penicillium</i> sp. CFR 303 | baking, animal nutrition Food, |
| Protease | Brewer's spent grain, tomato pomace, Jatropha seed cake, Corn steep liqueur | <i>Streptomyces malaysiensis</i> AMT-3, <i>Aspergillus oryzae</i> | pharmaceutical, animal feed, leather, diagnostics, waste management |
| Transglutaminase | Untreated corn grits, milled brewers rice, industrial fibrous soy | <i>Streptoverticillium mobaraense</i> , <i>Pythium</i> sp., | Meat processing, dairy products, baking, edible film, |

| | | | |
|--------------------|---|--|---|
| | residue, soy hull, malt bagasse | <i>Phytophthora</i> sp, <i>Bacillus</i> <i>circulans</i> BL32 | leather finishing, cosmetics |
| Laccase | Wheat bran | <i>Cerrena unicolor</i> C-139 | Bleaching, deinking of paper, polishing and preparation of textile |
| Lipase | Banana peel, potato peel, cassava peel | <i>Aspergillus niger</i> | Meat processing, detergents, degreasing, de- hairing of leather |
| Phytase | Orange and citrus peel | <i>Klebsiella</i> sp. DB- 3FJ711774.1 | Animal nutrition |
| Polygalactouronase | Orange peel, wheat bran | <i>Aspergillus sojae</i> M3 | 2% orange peel and 50mM HCl was found optimum for spore formation |

2.5.2 Structure of lignocellulose

Lignocellulose is made of lignin and carbohydrates like cellulose, hemicellulose, pectin, ash, salts and minerals.

Cellulose is a polysaccharide that is made up of D-glucose bonded to each by β (1 \rightarrow 4) linkage forming linear chains. Cellulose is mainly found in the cell wall of plant biomass. The plant cell wall is composed of microfibrils that are formed by cellulose chains bound together by hydrogen bonds. Microfibrils are insoluble long-chained compounds made up of 36 glucan chains held together by hydrogen bonds and each chain contains about 5,000 to 14,000 glucose molecules. Cellulose can exist in different polymorphs that are crystalline in nature. Natural cellulose is found in a polymorph structure called polymorph I. Three other polymorphs of cellulose have been attained via different pre-treatments *viz.* polymorphs II, III and IV. Cellulose also exists in amorphous form which is soluble and can be easily digested by enzymes (Kulasinski et al., 2014).

Hemicellulose is a heterogeneous polymer made up of short chains of polysaccharide molecules. They constitute 15-35% of the plant biomass and are composed of five different sugar monomers *viz.* D-xylose, L-arabinose (pentoses), D-galactose, D-mannose and D-glucose (hexoses). They are usually found in the form of O-acetyl-4-O-methylglucuronoxylans and O-acetyl-galactoglucomannans depending upon the source of biomass. Uronic acids, like α -D-glucuronic, α -D-4-O-methylgalacturonic and α -D-galacturonic acids have also been found in hemicellulose. Other sugars such as α -l-rhamnose and α -l-fucose may also be existent in small quantities. Hemicelluloses are linked to cellulose by hydrogen bonds and to lignin by covalent bonds (Sun et al., 2014).

Lignin is the most abundant constituent in plant biomass. It has an aromatic and amorphous nature and its structure varies according to the plant species it is derived from. Lignin monomers are basically phenyl-propane units that differ only in the substitution of methoxyl groups on the aromatic rings. The three main monolignols (lignin monomers) that form the lignin polymer are p-hydroxyphenyl alcohol (H), coniferyl alcohol (G), and synapyl alcohol (S). Softwoods usually contain more of the H subunit as compared to G and S, where as in grass varieties all the monolignols are found in equal proportions (Duval & Lawoko, 2014).

One of the most important limiting factors for making lignocellulose susceptible to microbial attack is the lignin content. Research has shown that the removal of lignin facilitates cellulose hydrolysis. The mechanism by which lignin obstructs hydrolysis of cellulose is still not completely understood. However, two reasons have been proposed that may be the lead to this phenomenon. Firstly, lignin is a structural polysaccharide which imparts strength to the plant cell wall by covalently linking to hemicellulose (Yuan et al., 2013). The covalent bonding between lignin and cellulose prevents the carbohydrate to be exposed for enzymatic hydrolysis. Secondly, woody biomass is harder to treat as compared to grass biomass. A comparison of lignin structures in both substrates reveals that the extent of cross-linking and the phenyl content lignin found in wood is far more complex than that of grassy substrates (Ververis et al., 2004).

Another property of lignin, which is detrimental for cellulose activity is its capacity to irreversibly adsorb enzymes. A study on the adsorption kinetics of enzyme to lignin and the effect of temperature revealed that higher temperatures accelerated the adsorption process attaining equilibrium. This may be a problem when conducting enzymatic hydrolysis at higher temperatures. The addition of surfactants was seen to

reduce the adsorption of cellulase to lignin (Tu et al., 2009). Certain pre-treatments have been observed to be more effective against lignin-enzyme adsorption as compared to others. For example, Kumar et al. (2013) reported that ammonia fibre expansion pre-treatment was superior over SO₂ and acid-pre-treatment in terms of cellulase adsorption capacity.

2.5.3 Pretreatment of Lignocellulose

The utilisation of cellulose in biomass as a carbon source via enzymatic hydrolysis is a complicated process. It involves the adsorption of enzymes on the substrate surface, the synergistic effects of other protein components on hydrolysis, and the release of hydrolysed product into the bulk liquid. A lot of factors influence this process and they can be broadly categorised into the structural features of lignocellulose and the mechanisms and interactions related to enzyme kinetics. Nonetheless, addressing the factors related to the structure of lignocellulose holds the key to efficient hydrolysis of substrate. The aspects that influence the difficulty in the degradation of biomass residues include lignin content, hemicellulose, available amorphous cellulose as compared to its crystalline counterpart, the degree of polymerisation, acetyl groups, plant protein-enzyme interaction, the association of cellulose with hemicellulose and lignin, the accessible surface area to enzymatic degradation and porosity and the residual surface area of biomass (Singh et al., 2015).

Pre-treatments are necessary practises to address these issues by bringing in structural and compositional changes in lignocellulose. This is achieved by exerting high pressure or temperature or a combination of both, treatment with corrosive chemicals such as acids and alkali or the usage of molecular disruption techniques such as

ultrasound and plasma etc. Pre-treatments can remove lignin and hemicellulose to a certain extent and increase the porosity and the concentration of amorphous cellulose.

2.5.4 Factors influencing the choice of pretreatment

There are a number of factors that need to be considered while employing a pre-treatment strategy for a particular biomass residue before it is used as a substrate for the fermentation. These factors include low capital and energy investments, overall effectiveness and applicability over a wide variety of substrates. Subjecting the lignocellulosic biomass to pre-treatment should not render the sample unusable for further use. Added advantages, such as the ability to retrieve the hemicellulose content in the aqueous solution, are desired. Most importantly, pre-treatments should lower the capital cost, the operational cost and the biomass cost. Besides all these factors, there are a few parameters that should be taken in consideration when pre-treatments are chosen for lignocellulose. These parameters depend upon the physical and chemical features of the biomass that help or resist enzymatic hydrolysis. The best pre-treatment result in end products which support the growth of desired microbes, not result in unemployable substrate molecules and also not give rise to inhibitory substances that hinder the growth or prove to be harmful to producers.

Crystalline cellulose exists in different polymorphs and is seen in microfibrils in plant cell wall. They are formed by long chains of (1, 4) β -D glucan bonded together by hydrogen bonds. The presence of hydrogen bonds between chains resists enzymatic and microbial attack while breaking them enhance depolymerisation (Chundawat et al., 2010). Studies have shown that during hydrolysis digestion of amorphous cellulose takes place before crystalline cellulose. The crystallinity of cellulose can be measured

by the different X-ray diffraction methods. The crystallinity index is used to measure the degree of crystallinity in biomass and pulps and is determined by the equation 2.1:

$$\text{Crystallinity index (CRI)}(\%) = \frac{(I_{002} - I_{am}) \times 100}{I_{002}} \quad \text{Equation 2.1}$$

I_{002} is the intensity of diffraction of 002 peak at $2\theta \approx 22.5^\circ$ and I_{am} is the scattering intensity of amorphous region ($2\theta \approx 18.7^\circ$) (Bansal et al. (2010)).

Degree of polymerisation of cellulose is an important parameter in determining biomass recalcitrance. Pre-treatment of lignocellulose as well as enzymatic hydrolysis is a depolymerisation process of cellulose (Meng & Ragauskas, 2014). Size exclusion chromatography (SEC) studies can reveal the degree of polymerization of a particular substrate sample. Melander and Vuorinen (2001) conducted a study to determine the depolymerisation of carboxy-methyl cellulose after enzymatic digestion using SEC. They detected the separated CMC hydrolysates as total carbon content and reducing sugars. The degree of depolymerisation was determined by dividing the number of anhydroglucose units by the reducing sugar concentration. Several studies have been focused on the efficacy of pre-treatments on the depolymerisation of cellulose. Steam explosion treatment of cotton stalks enhanced the saccharification of the substrate by reducing the degree of polymerization of cellulose (Huang et al., 2015).

The process of hydrolysis of cellulose greatly depends upon the adsorption of enzyme onto the substrate. This is determined by the accessible surface area, making it a limiting factor for efficient enzymatic digestion. The accessible surface area is in turn dependent upon particle size, porosity and pore volume. The measurement of surface area of the substrate can be carried out by Brunauer–Emmett–Teller method (Chen et al., 2011). According to this method increase in surface area in sugar cane bagasse can

be achieved by using dilute H₂SO₄ assisted by microwave heating. Variations in the pore size distribution can be determined by using differential scanning calorimetry. In general, the measure of porosity of the substrate is considered as a direct indication of the accessible surface area. Ye and Berson (2014) reported an enhanced rate of cellulose hydrolysis on increase in substrate binding surface area.

Acetyl groups form the backbone of hemicellulose structures in plant cell wall. Studies have shown that the presence of acetyl group can hinder the effective digestion of cellulose. The degree of acylation has been reported to be a crucial factor in cellulose hydrolysis (Jiang et al., 2014). Liquid hot water pre-treatment has found to be effective in removing the acetyl groups found in hemicellulose and converting them into acetic acid (Jiang et al., 2015). The acetyl groups may inhibit enzyme activity by interfering with the hydrogen bond formation between cellulose and cellulose binding domains. The negative effects of acetyl group inhibition can be eliminated by saponification (Balat et al., 2008).

2.5.5 Pretreatment techniques

The aim of pre-treatments is to facilitate or increase the efficacy of lignocellulose hydrolysis by improving accessibility towards cellulose-rich fractions. This is achieved by the removal of lignin and hemicellulose, factors which affect the availability of cellulose for microbial degradation. It also focuses on the purging other factors that have been detailed in an earlier section. The past three decades have witnessed a tremendous amount of research being done in the area of pre-treatments. Different techniques have been employed to investigate the efficiency as a pre-treatment measure. The synergistic effects on effective lignin removal have also been explored on a wide scale. Pre-treatment techniques can be classified in various ways. Depending on the pH that is maintained during the process pre-treatments can be

grouped in to three: acidic, neutral and alkaline (Singh et al., 2015). A comprehensive classification of pre-treatments involves the assembly of different techniques based on their mode of action. Accordingly, pre-treatment methods can be broadly categorised in to physical, chemical, physico-chemical and biological. As the name implies physico-chemical pre-treatments involves a combination of physical or biological methods with chemical strategies. Table 2.1 depicts a classification of various pre-treatments methods for lignocellulose suggested, based on extensive literature review.

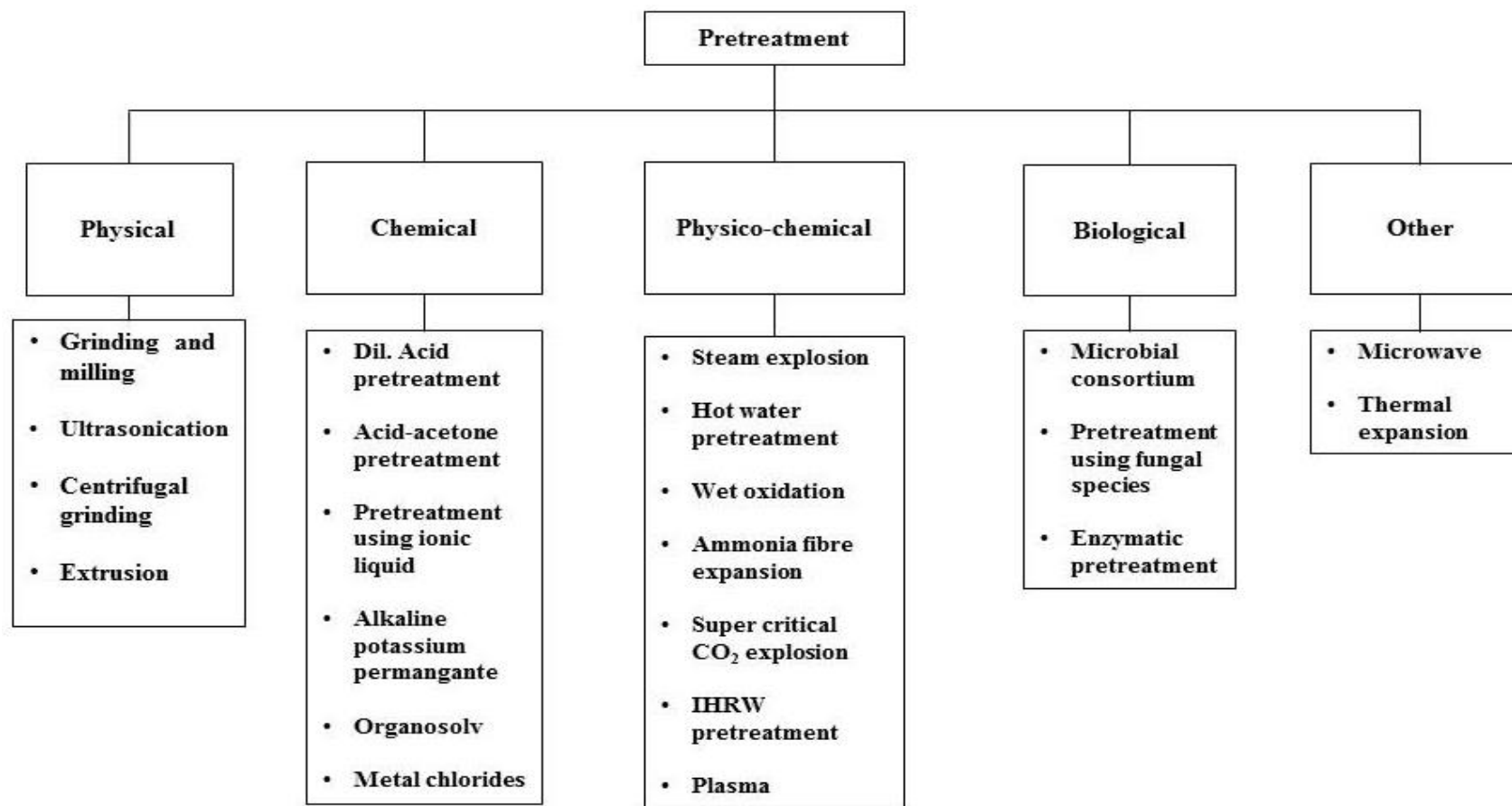


Figure 2.2 Classification of pre-treatments strategy for lignocellulosic biomass

Grinding is a size reduction technique used for biomass pre-treatment that increases the surface area and does not release any effluents. The size of the sample is preliminarily reduced by cutting and milling. Ultra-fine powder is collected by the use of a sieve. The choice of sieve depends upon the final particle size reduction that is aimed to be achieved. The effects of grinding include alterations in the degree of polymerisation, porosity, surface area and crystallinity. The final particle size mainly depends upon the conditions and intensity of grinding. Silva et al. (2012) achieved progressive particle size reduction by employing sieve based grindings, ball milling and jet milling. The enzymatic degradability of the substrate was improved by this process. Zakaria et al. (2015) used wet disk milling as a secondary pre-treatment step to extract sugars from recalcitrant oil palm mesocarp fibre. They found that wet disk milling increased the surface area accessible for the conversion of cellulose to glucose.

Ultrasonic pre-treatment employs ultrasound to breakdown the complex network of polymerisation in biomass. Cavitation produced due to the pulsating high frequency ultrasonic waves penetrates in to polysaccharides and disrupts the mesh of cross linking polymers facilitating better enzymatic degradation. The biomass suspension is irradiated for 10 minutes with ultrasound at 20 kHz and 200 KW before enzymatic treatment. Nakayama and Imai (2013) reported the increase in adsorption of cellulase when the authors used ultrasonic pre-treated Kenaf leaves for enzymatic hydrolysis. Employing pre-treatment designed by augmenting ionic liquids and ultra-sonication of rice straw. Ultrasonic pre-treatment assisted with FeCl_3 was found to improve the acid hydrolysis of cellulose thereby enhancing the crystallinity (Li et al., 2015).

Centrifugal grinding a modified grinding mechanism which utilises an advanced grinder. This technique results in size reduction of biomass to increase contact surface

area and reduce cellulose crystallinity leading to better exposure of polymers for hydrolysis. Centrifugal grinding is superior over ordinary grinding in such a way that it exerts multiple effects *viz.* impact and shear. Centrifugal grinding, done in successive steps, result in the reduction of particle to a great degree. In a particularly interesting study, Silva and Xavier (2011) examined the chemical composition of fractions obtained after passing grinded wheat straw through sieves of different sizes. They found out that larger fractions were mostly made up of cellulose while the smaller fractions had protein and ash in them.

Extrusion is a process where uniformly moistened biomass material is passed through a screw assembly and applied pressure upon under high temperature at a particular frequency. It requires a device called the extruder; the biomass can be mixed with different chemicals like sodium hydroxide, urea or thio-urea to increase the severity of the pre-treatment process. Lamsal et al. (2010) reported in higher reducing sugar yields using extrusion pre-treatment as opposed to grinding using wheat bran as substrate. Parameters, such as screw speed, barrel temperature and their collaboration has a substantial impact on sugar recovery from biomass. A comparative study between three pre-treatment strategies *viz.* dilute acid, alkali and extrusion was conducted using soybean hulls as the lignocellulosic substrate. It was found that post pre-treatment the glucose yield after enzymatic hydrolysis compared with untreated substrate increased by 69.6%, 128.7% and 132.2%, respectively (Yoo et al., 2011).

Dilute acid pre-treatment hydrolyses hemicellulose and makes cellulose more susceptible to enzymatic degradation. Strong acids, like sulphuric acid, in diluted form are used to treat the lignocellulosic biomass. The biomass samples are immersed in dilute acids and the temperature is raised to 160°C and maintained for 10 minutes. With dilute-acid pre-treatment a considerable reduction in galactosyl and xylosyl

groups (hemicellulose) can be achieved however, with a higher lignin content (Zhou et al., 2014). *Jatropha curcas* shells were used to study the effect of dilute sulphuric acid pre-treatment on hydrolysis. Almost 70% of enzymatic conversion of cellulose was obtained after following this pre-treatment protocol (Martín et al., 2015).

Acid-acetone pre-treatment takes advantage of acidolysis at high temperatures followed by cold shock. A quantified amount of biomass is mixed with concentrated phosphoric acid (not less than 85%) and kept in a rotary air-bath for 1h at 120 rpm and 50°C. The slurry is then mixed with pre-cold acetone and mixed thoroughly. After centrifuging the slurry for 10 minutes at 8000 rpm the supernatant is collected for reducing sugar analysis while solids are washed again thrice in distilled water and then used for enzymatic hydrolysis. Studies have shown that over 99% of the total cellulose content in acid-acetone pre-treated lignocellulose was available for enzymatic hydrolysis to obtain glucose (Qin et al., 2014).

Ionic liquids are thermally stable organic solvents that are capable of polar and non-polar organic, inorganic and polymeric compounds. They have low volatility, are non-flammable and can be recycled. This can be considered an advantage considering how cost intensive pre-treatments can be. Pre-treatments using ionic liquids do not require much energy as well. Some of the ionic liquids that are used for treating lignocellulose are an anionic chloride, formate, acetate or alkylophosphonate; moieties that can form strong hydrogen bonds with cellulose and other carbohydrates. Pre-treatment using ILs involves mixing it with the biomass at a ratio of 20:1 and then heating it for 120°C for 30 minutes. Deionised water is then added in to the mixture to form a ratio of 5:1. The IL/water mixture is then separated from the biomass by vacuum filtration. In a study involving energy cane bagasse, 7.9% or lignin removal was achieved using IL pre-

treatment. The insufficient removal of lignin was attributed to its location in the lignin-carbohydrate complex (Qiu et al., 2012).

Alkaline Potassium permanganate pre-treatment takes advantage of the strong oxidation property of potassium permanganate. Potassium permanganate is generally considered safe as compared to ozone and ionic liquids. Alkaline potassium permanganate solution (APP) has the ability to de-lignify biomass by oxidation with high carbohydrate retention. This is done by breaking the ester and ether bonds between lignin and carbohydrates. Corn cobs were used in a study employing APP pre-treatment as the lignin removal strategy. Researchers were able to find that the optimum time of exposure for maximum lignin removal was 6 hours. Also, it was observed that higher temperatures favoured the pre-treatment process (Ma et al., 2015).

Organosolv pre-treatment is normally performed by using strong inorganic acid catalyst like sulphuric acid which hydrolyses the bonds in lignin. The advantage of this process is the option to recover lignin as a value-added product. Additionally, solvent recovery requires minimal energy. Amiri et al. (2014) reported the use of aqueous ethanol containing 1% w/w of sulphuric acid for pre-treating rice straw with a solid-liquid ratio of 1:8. The temperature of the mixture was maintained at 150-180°C for 30-60 minutes. After cooling it in an ice bath, the pre-treated material was finally washed with 60% ethanol and kept drying overnight. The treatment of the biomass for 1 hour at 180°C resulted in 60% removal besides the partial dissolution of hemicellulose in the organic liquor. Developments in this mode of pre-treatments involve the use of other chemicals such as formaldehyde, acetylene, sulphuric acid catalysed ethanol and auto-catalysed ethanol (Chen et al., 2015).

Metal chloride pretreatment requires a high pressure reactor to perform the pre-treatment. Biomass is loaded in the reactor along with metal chlorides (FeCl₃, FeCl₂, CrCl₃, ZnCl₂, and AlCl₃). The reactants are heated to 170°C for 30 minutes. The pre-treated solids are removed via filtration. A study conducted by Chen et al. (2014) revealed that using metal chlorides for pre-treatment of lignocellulose results in the decrease in the total mass of the substrate. Most of the carbohydrate gets degraded in the acid environment and a major share of it appeared in the effluent. The hydrolysis of bamboo biomass by dilute hydrochloric acid in ionic liquid involving metal ions such as Na⁺, K⁺, Mg²⁺, Ca²⁺, Cu²⁺ and Fe³⁺ was conducted to study the effects. A maximum sugar yield of 67.1% was obtained at 100°C and Cu²⁺ was found to be the best metal ion for this process (Wang et al., 2014).

Plasma-assisted pre-treatment technique employs plasma to make changes on the surface of cellulose. Plasma treatment results in extirpation resulting in the increase in coarseness of cellulose. Plasma generated high reactive species such as HO[•] and H₂O₂ which degraded cellulose to obtain glucose. A study was conducted to find out the changes on the surface of cellulose when exposed to dielectric barrier discharge plasma. AFM and XPS were the analytical tools for investigating the surface bound changes. According to the data obtained from AFM, the corresponding changes can influence the adhesion of molecules (preferably protein and enzyme) on the substrate surface (Flynn et al., 2013). Experiments involving plasma pre-treated biomass for bioethanol production resulted in a maximum ethanol yield of 52% (Schultz-Jensen et al., 2011).

Steam-assisted fractionation of biomass materials (also called autohydrolysis) is one of the oldest and yet the most effective pre-treatment methods that facilitate enzymatic hydrolysis. The process involves subjecting the biomass to high pressure and

temperature for a certain amount of time after which the sample is rapidly decompressed resulting in the breakdown of the lignin-carbohydrate complex. However, a drawback that has been reported with this method is that with woody substrates the extent of delignification is very less leading to lesser enzymatic hydrolysis of cellulose (Kumar et al., 2012). A study using corn fibre as substrate employed steam explosion as the pre-treatment strategy with 6% SO₂ at 190°C. Accordingly, sequential SO₂ combined steam explosion followed by enzymatic hydrolysis resulted in a high polysaccharide conversion of 81% (Vivekanand, Olsen, Eijsink, & Horn, 2014). Sequential pre-treatment using steam explosion and 1% H₂SO₄ was found to assist enzymatic digestibility of cotton stalks and also resulted in high sugar-ethanol yield (Huang et al., 2015).

Hot water-assisted pre-treatment is ideal for lignocellulose substrates where the addition of chemicals for delignification can result in loss of useful biomass. This method does not require corrosion resistant reactors or chemicals and the formation of toxic compounds is almost absent (Jiang et al., 2015). The process is performed in a customized high-pressure reactor. 100 g of dried substrate is loaded in to the reactor along with a 900 ml of deionised water and 2g of NaOH. The stirring speed is maintained at 600 rpm with a temperature of 230°C. After pre-treatment the reactor is cooled in cold, running water and as the temperature lowers to 80°C the slurry is separated in solid and liquid fractions using vacuum filtration (Li et al., 2014).

Wet oxidation technique relies on the high pressure and temperature in aqueous conditions for the breakdown on lignocellulose. It employs oxidative agents and results in the formation of hydroxyl radicals which corrode in to lignin and carbohydrates. Experiments are conducted in a loop reactor with the over-head chamber filled with 12 bars of oxygen gas. After the reaction the solids are separated from the liquid by

vacuum filtration. Wet oxidation has been reported as a safe pre-treatment method as no harmful compounds result from it. Using clover and rye grass as substrate, a team of researchers were able to attain an enzymatic hydrolysis conversion efficiency of 93.6%. Furthermore, the overall glucose yield post-pre-treatment and hydrolysis was found to be 75.5% (Martín et al., 2008).

Ammonia fibre expansion (AFEX) is one of the most successfully tested lignocellulose pre-treatment methods for the production of fermentable sugars from biomass. Anhydrous or highly concentrated ammonia is added (ammonia loading) to wet substrate (water loading) at moderate temperatures and high pressure conditions. This condition is maintained for a while (residence time) before the pressure is released. This leads to the vaporisation of ammonia which can be collected, recycled and used again. AFEX pre-treatment is most suitable for preventing cellulase adsorption to lignin. A comparative study of AFEX pre-treatment on corn stover pre-soaked in hydrogen peroxide and normal substrate showed that using the former, the enzymatic digestibility was enhanced with values of 87.78% and 90.64% for glucan and xylose respectively (Zhao et al., 2014).

Super critical carbon dioxide (SCCO₂) is a modified steam explosion involving CO₂ instead of atmospheric air. A specified amount of biomass is wetted and loaded in a reactor after which the pressure within the reactor is built up by means of super critical CO₂. This condition is maintained for some time following which the pressure in the reactor is suddenly realised resulting in fractionation of the lignocellulosic substrate (Srinivasan & Ju, 2012). Super critical CO₂ diffuses in to the crystalline structure of cellulose and the explosion created subsequently disrupts the biomass cell wall and facilitates easy access to cellulytic enzymes towards the substrate. A maximum

glucose yield of 30% was obtained from corn stover samples after SCCO₂ pre-treatment and consequent hydrolysis (Narayanaswamy et al., 2011).

Integrated hydroxyl radicals and hot water (IHRWT) pre-treatment is a combination of hot water treatment and pre-treatment using hydroxyl radicals. Hydroxyl radicals have the capability to break hydrogen bonds in carbohydrates and lignin. In a study conducted by (Gao et al., 2015) hydroxyl radicals were generated in vivo by the Fenton or Haber-Wiess reaction by mixing H₂O₂ and FeSO₄ at concentrations of 0.018% and 11.9 mM respectively. The biomass was initially exposed to hot water pre-treatment (100°C, 30 minutes) which deteriorated the complex polymeric structures in lignocellulose to expose lignin and polysaccharides. As a result a glucose yield of 59.9 mg/g DM was obtained with a cellulose conversion rate of 88.1 % on enzymatic hydrolysis.

Biological pre-treatment systems rely on biological agents to delignify lignocellulose and make the process of enzymatic hydrolysis more convenient. Unlike physical and chemical pre-treatment methods, biological pre-treatments do not involve high temperature and/or pressure and does not require acids, alkali or any reactive species. Additionally, the process does not generate any undesirable products. However, the disadvantage of using biological pre-treatment strategies is that there is limited control over the whole process. Moreover, it is a slow process and thus time consuming. Nonetheless, one may employ these pre-treatment techniques for lesser capital costs and environmental benefits. Biological pre-treatment procedures may broadly be classified in to use of microbial consortium, fungal treatments and enzymatic treatments.

The idea of using mixed cultures for lignocellulose processing spawns from the fact that using a single species for lignin removal does not concur with the same process occurring in nature; lignocellulosic material is degraded as a cooperative effort of many microbial species regardless of the aerobic/anaerobic conditions. Employing a microbial consortium for delignification eliminates several drawbacks encountered in a single species treatment such as metabolite repression and feedback regulation (Zhang et al., 2011). Meanwhile, ensilage is a conventional technology used for the storage of animal feed. Naturally occurring bacteria produces enzymes that digest the lignocellulosic material providing substrates that is more easily digested by ruminant animals. *Lactobacillaceae* appears as the dominant species in the consortium of microbes that ferment biomass during ensilation. Although, the capability to degrade lignin is virtually absent, the effects on the biomass made by the microbial consortium improve the yield of fermentable sugars derived from the substrate (Chen et al., 2007). For this reason, ensilage storage cannot be counted upon as a stand-alone pre-treatment process.

Delignification of biomass using fungal species is an area that has witnessed a lot of research in recent years. ‘White rot fungi’ is a small group of basidiomycetic fungus that thrive on wood. They are capable of breaking down and demineralizing lignin. They do this with the help of one of their two enzyme systems *viz.* the oxidative lignolytic system which attacks the phenyl rings in lignin and the hydrolytic enzyme system degrades cellulose and hemicellulose to release fermentable sugars. The oxidative lignolytic system comprises of three major enzymes: lignin peroxidase, manganese peroxidase and laccase. The most common species of white rot fungi that has been employed for treatment of lignocellulose include *Phanerochaete chrysosporium*, *Phanerochaete carnosae*, *Ganoderma lucidum*, *Pleurotus ostreatus*,

Pleurotus pulmonarius, *Phanerochaete chrysosporium* and *Trametes* sp (Vicuña, 2000).

Brown rot fungi are another class of lignocellulose degrading fungi that uses enzymes to degrade cellulose and hemicellulose with minimal removal of lignin. It has been theorized that the removal of lignin by brown rot fungus follows a non-enzymatic pathway using hydroxyl radicals (Jensen et al., 2001). According to research done by Schilling et al. (2009) delignification is step undertaken by brown rot fungi as a pre-treatment of sorts: to facilitate saccharification. In another study conducted by Schilling et al. (2012) using different types of wood, the saccharification rates after pre-treatment increased three folds by using brown rot fungi (specifically *Gloeophyllum trabeum* and *Postia placenta*).

While fungal pre-treatment methods directly use white rot fungi to remove lignin from biomass, enzymatic pre-treatment procedures employ pure enzymes derived from the same organisms to achieve a common goal. Laccase, manganese peroxide and versatile peroxidase are enzymes that are used extensively to treat the lignocellulosic substrate. Laccases are phenol oxidases containing multiple copper ions and they catalyse the oxidation of phenols, anilines and aromatic thiols. Phenol removal by laccase enhances microbial growth, improves fermentation capability and reduces the time the organism spends in lag phase (Oliva-Taravilla et al., 2015). Manganese peroxidase is another enzyme produced by white rot fungi which takes advantage of the manganese in woody substrates. It is generally thought that the inability of brown rot fungi to produce this enzyme have compelled it depend on hydroxyl radicals and Fenton reaction to degrade lignin. Manganese peroxidase contains a heme component with a catalytic cycle and it is uniquely selective for Mn^{2+} ions for oxidation. The oxidation of Mn^{2+} to Mn^{3+} is coupled by the reduction of hydrogen peroxide to water. Mn^{3+} is a

highly reactive species capable of interactions with phenols, non-phenolic aromatic compounds and long chain unsaturated fatty acids leading to bond cleavage in lignin (Brown & Chang, 2014).

Thermal expansionary pre-treatment is a two-step process involving boiling of biomass at high pressure followed by rapid decompression. A process temperature ranges from 170-200°C is applied for a residence of 0-60 minutes. Torrefaction is kind of thermal pre-treatment which involved temperatures in the range of 200-300°C. Torrefaction can be categorised as wet and dry depending upon the presence and absence of water. Wet torrefaction involves hot compressed water at temperatures of 200-260°C in a pressure reactor. Dry torrefaction, also known as low temperature pyrolysis were the biomass is exposed to an environment with an inert gas such as nitrogen at temperatures ranging from 200 to 300°C. A study conducted to compare the effect of both torrefaction effects on the equilibrium moisture content of biomass revealed that pre-treated biomass is more hydrophobic than raw biomass (Acharjee et al., 2011).

Microwave pre-treatment is a modified version of thermal pre-treatment. As opposed to thermal pre-treatment, heat is provided to the biomass material directly in the form of microwave radiation which converts to thermal energy. The microwaves penetrate the material and heat the entire volume from the inside. The process is rapid and uniform in nature. The advantages of this process are not confined to uniform heating and lower time requirement but increased energy efficiency, controlled heating and excellent control over the whole process. Different microwave-assisted pre-treatments have been tried and tested and found to be very effective. About 46% of lignin was removed retaining 90% of glucose and 76% of hemicellulose when sweet

sorghum bagasse was subjected to microwave-assisted dilute ammonia pre-treatment (Chen et al., 2012).

2.5.6 Formation of inhibitors

Compounds formed during the pre-treatment process of lignocellulose may prove inhibitory to enzyme activity, microbial growth and metabolism. Potential inhibitory chemicals that can be released from cellulose, hemicellulose and lignin when employing thermochemical treatments are aliphatic acids such as acetic, formic and levulinic acid, furaldehydes such as furfural and 5-hydroxymethylfurfural (HMF) and, uronic acid, 4-hydroxybenzoic acid, vanillic acid, vanillin, phenol, cinnamaldehyde, and formaldehyde. Furan derivatives are formed by the thermal breakdown of pentose and hexose sugars. The two furan derived representatives, 2-furaldehyde (furfural) and hydroxyl methyl furfural arise from the decomposition of pentose and hexose sugars on treating lignocellulose with diluted sulphuric acid. Furan derivatives are detrimental to the activity of several enzymes that are involved in microbial metabolism such as hexokinase, aldolase, phosphofructokinase, triosephosphate dehydrogenase, and alcohol dehydrogenase (Behera et al., 2014).

Aliphatic acids are by-products of thermal augmented acid treatment of sugars. Lignocellulose hydrolysate derived aliphatic acids include acetic acid, formic acid and levulinic acid. Acetic acid is formed by two ways: the acetyl groups in hemicellulose are dissociated during hydrolysis to form acetic acid. Secondly, acetic acid is a by-product of the fermentation process (Taherzadeh et al., 1997). Formic acid is the dissociation product of furfural while HMF is the precursor for levulinic acid formation (Ulbricht et al., 1984). Phenolic compounds are a derivative of lignin degradation when lignocellulose undergoes thermal pre-treatment augmented by acid catalysis (Trajano et al., 2013). Phenolic compounds are also generated by the

breakdown of sugars. Several recent researches showed that phenolic compounds inhibits the growth of microorganisms; though, the precise process by which phenolic compounds inhibit microbial growth and fermentation process is yet not elucidated convincingly. The measurement of phenolic compounds in the pre-treated lignocellulose can be assessed by performing gas chromatography-mass spectroscopy (GC-MS), high performance liquid chromatography (HPLC) and liquid chromatography coupled with mass spectrometry (Jonsson et al., 2013).

2.5.7 Choice of Microorganism

Considerable research has been conducted to reveal several microorganisms belonging to the fungi, yeast, bacteria and actinomycetes categories. Screening of microorganisms is one of the most efficient means of finding new enzymes viable to the industry. This is particularly true in the case of thermophilic microorganisms that are isolated from exotic locations and subsequent extraction of enzymes. One of the greatest advantages of employing thermophilic microorganisms for enzyme production is reducing the risk of contamination due to bioprocessing operations being conducted at higher temperatures. Furthermore, elevated temperatures also result in lesser viscosity and greater solubility of substrates, subsequently resulting in increased product yields due to favourable displacement of the equilibrium in endothermic reactions (Vandenberghe et al., 2016).

Enzymes of microbial origin employed in the industry are commercially available as enzyme preparations. These preparations not only contain the desired enzyme, but also other metabolites of the production strain, along with additional preservatives and stabilizers that are food grade and comply with applicable regulatory standards. While evaluating the safety of an enzyme, the safety of the production strain remains the primary consideration. Toxigenic potential is defined as the ability of a microorganism

to produce chemicals (toxins) that can cause food poisoning (Arslan et al., 2014). Strains that are meticulously characterized to be non-pathogenic and non-toxigenic, particularly those with a history of being safe, are reasonable choices for the production of industrial enzymes (Pariza et al., 2014).

A majority of the industrially important enzymes has been derived from a rather small group of bacterial and fungal strains, primarily *Bacillus subtilis*, *Bacillus licheniformis*, *Aspergillus niger* and *Aspergillus oryzae*. These microbes have historically been used for the commercial production of various metabolites, leading to a thorough understanding of their characteristics and metabolic reactions, and have been documented to be efficient for industrial scale production. They can also be genetically manipulated easily and are known for their ability to overexpress proteins of interest in fermentation media. These features make these microorganisms extremely desirable as hosts for a variety of heterologous enzymes. Furthermore, genetic engineering has enabled several microorganisms with no history of use in the industrial production of native enzymes, such as *Escherichia coli* K-12, *Fusarium venenatum* and *Pseudomonas fluorescens* to be successfully utilized as hosts for expression of industrially important enzymes (Olempska-Beer et al., 2006).

Wild-type strains produce a variety of extracellular enzymes, which may naturally produce enzymes that have industrial importance. A common method exploited to find these microbes is bioprospecting. Microbes are collected from specific environmental niches and their ability to hydrolyze specific substrates is investigated. Subsequently, the best candidate is selected based on screening for the production of an enzyme of interest. Another method is analyzing the genetic composition using metagenomic tools. Probes and group-specific primers are employed to find new enzymes. The major drawback with this method is qualitative: the metabolic potential cannot be

measured since the isolation and culturing of the microorganism is not performed. Comparative genome analysis of microbial strains assists in the screening of prospective microbes in a short time. This facilitates the evaluation of the proteome of the microorganism (Youssef et al., 2013).

The concept of metabolic engineering was introduced by Bailey in 1991 and relates to ‘the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology (Nevoigt et al., 2008). A metabolic pathway (or pathways) associated with the production a desired chemical compound by fermentation is/are over-expressed in a cell by genetic engineering employing classical mutagenesis and selection and/or recombinant techniques. Cells are “evolved” in the laboratory to make them tolerant to high product concentrations by removing the normal genetic and biochemical regulation of the genes and enzymes associated with the pathway by genetic manipulation. Finally, a robust fermentation process is developed that allows mass production of the desired compound (Keasling et al., 2012).

While traditional metabolic pathway engineering approaches have been successful in producing engineered microbes, novel techniques need to be called upon to develop microorganism and processes that are cost-competitive with existing large-scale, low-cost chemical manufacturing processes, to produce the same compounds in high-volume and at low-cost. Techniques, like genome sequencing, and new fields of study, like bioinformatics, systems biology and metabolomics, have greatly helped researchers to embellish the competences of metabolic engineering over the past decades to deliver new, highly engineered organisms that are capable of high throughput performance using renewable resources as substrates, lowering the cost of production even further (Agrimi et al., 2012; Buschke et al., 2013).

2.6 Fermentation Strategies

2.6.1 Solid State Fermentation

Solid state fermentation has been used for the production of several industrial enzymes. This mode of fermentation encourages the growth of fungal species, such as ascomycetes, basidiomycetes and deuteromycetes, on solid substrates, and the development of conidiospores in particular (Batche et al., 2014). Most of the industrial enzymes are of fungal origin. The genetic expression of fungal organisms differs in solid state fermentation and submerged fermentation (Barrios-González et al., 2012). However, solid state fermentation has not been adopted for large-scale production because of its inability to standardize processes and limited reproducibility of results. Temperatures can rise to unprecedented levels with fewer measures to control them, which can denature the enzymes that are produced in the reactor. Aeration has been proposed as a measure to address this issue. This can, however, lead to water loss due to evaporation (Hölker et al., 2005).

Advances in fermentation technology have seen some success in formulating methods to make solid state fermentation an amenable process. Ito et al. (2013) constructed a non-air flow box (NAB) with a moisture permeable membrane. Water vapour escapes through the membrane keeping the substrate dry, resulting in uniform culture growth and rapid enzyme production with high reproducibility (Ito et al., 2013). Solid state fermentation promises high volumetric productivity with a high concentration of products, along with lesser effluent production, leading to minimal downstream processing (Singhania et al., 2010). However, a study involving the use of organic waste for the production of enzymes using the solid-state fermentation regime reported the emission of volatile organic compounds (VOC) such as CH₄, N₂O and NH₃ (Maulini-Duran et al., 2015).

2.6.2 Submerged Fermentation

The submerged fermentation strategy is the most sought-out method for large-scale production of enzymes. It involves water-based medium within the reactor, which helps in maintaining the pH and temperature and provides provisions for aeration and agitation within the vessel. A sterile environment can be maintained within the reactor with lesser chance of contamination. Submerged fermentation maintains a homogenous environment, with the reactor facilitating better control over process parameters. While this helps optimization studies and the even distribution of nutrients and oxygen to the growing microbe, the presence of negative factors such as butylated hydroxytoluene, hydrogen peroxide and metal ions, which induce oxidative stress, can undermine the whole operation (Roukas et al., 2016). Several filamentous fungal species are employed for the production of various enzymes and bioactive compounds. Although submerged fermentation is employed for the cultivation of these microbes in the industry, several studies prove that it is not the best method available (Gibbs et al., 2000). Submerged fermentation is not as economical as solid-state fermentation due to the requirement of large and sophisticated equipment. A comparative study involving the production of biodiesel revealed that the capital investment required for submerged fermentation was 78% more than that of the solid-state mode (Zhang et al., 2012).

2.6.3 Isolation, purification and recovery of enzymes

Once the production stage ends, enzymes are formed in the media in the form of crude extract. Although the crude extract exhibits the desired enzyme activity, low rates of reaction and the presence of impurities, such as media components used for microbial cultivation, metabolites and toxins released by the microorganisms, prevent its use as a commercial entity. Therefore, isolation and purification are the last steps of any

enzyme production process. Purifying the enzyme increases its specific activity and removes any unwanted factors from the finished product. Protein purification techniques have been an area of research for many decades owing to the product's commercial importance. Obtaining the finalized product requires strenuous measures, which start with cell disruption (depending on the source), extraction, fractionation and the final product.

2.6.4 Source of enzyme

All biological systems are a source of enzymes and there is bound to be considerable variation in the concentration of the concerned enzyme, its activity, stability, availability and presence of inhibitory factors. Traditional animal, plant and microbial sources have given way to genetically-engineered organisms with the introduction of recombinant DNA technology. Eukaryotic proteins cloned and expressed in bacterial hosts, such as *Escherichia coli* and *Bacillus subtilis*, may be located in different locations within the cell (cytoplasm, periplasmic space) or may be truly extracellular. Enzymes accumulating in the periplasm may be released into the fermentation media by changing the culture conditions (Fakruddin et al., 2013). However, recombination techniques allow the gene of interest to be equipped with an “affinity tag”, such as His-tag, which will help in the purification of the enzyme. This tag can later be removed by using highly-specific proteolytic enzymes (Young et al., 2012).

2.6.5 Isolation of enzymes

Isolating the protein from a solid source is a compromise between quality and quantity. The best isolation measure should facilitate the release of the enzyme of interest while leaving behind tough contaminants (nucleic acids, bacteria and viruses). Care should also be taken whereby the protein extracted is not degraded/denatured during the process. Homogenization is the most popular method for protein extraction from the

cellular environment. Another method used for cell disruption is ultrasonication. Ultrasonication facilitates the disruption of cells and exposes internal proteins to the growth medium. Ultrasonication techniques use high-frequency waves to cause cavitation on the microbial cell wall, thereby destroying it. However, prolonged exposure to ultrasound can denature the protein released upon cell lysis. Therefore, sonication cycles should be optimized in a manner where only cell disruption is achieved while the protein of interest is left intact (Liu et al., 2014). The cell disruption technique is usually followed by centrifugation or filtration aimed at the clarification of the extract prior to column chromatography.

Characteristics of the isolation medium are determined by the conditions that are necessary for the stability of the protein released. The main factors that govern the preparation of the isolation medium are pH, detergents, reducing agents, chelators or metal ions, proteolytic inhibitors and bacterial contamination. The pH is usually chosen to be the value in which the enzyme exhibits maximum activity. However, this may not always be the case. In the case of trypsin, maximum activity is attained at pH 8–9, while the enzyme is most stable at pH 3 (Pečová et al., 2013). Detergents are used to relieve the enzyme of bonds to membranes by hydrophobic interactions. Several of the detergents used for isolation (such as Triton X-100 and Sodium dodecyl sulfate, SDS) do not denature the globular proteins or affect their catalytic activity. The use of detergents is usually limited to the isolation medium. Detergents, being amphiphatic molecules, aggregate to form ‘micelles’ at the critical micelle concentration (CMC). This can interfere with the purification process during column chromatography. Therefore, the concentration of detergent used during isolation must be lesser than CMC (Malinsky et al., 2013).

Many enzymes have exposed thiol groups which can oxidize when the protein is released from the cytosol to the growth medium during isolation. This is prevented by the addition of reducing agents such as mercaptoethanol, dithiothreitol (DTT), or ascorbic acid. The concentration of these reducing agents can normally be kept as low as 10–25 mM, while keeping the internal disulphide bonds intact (Mathieson et al., 2013). Metal ions, proteases and bacterial contamination are three problems faced during enzyme isolation. The presence of metal ions leads to the enhanced oxidation of thiol groups and may form complexes with specific groups, which can cause problems. Heavy metals can be removed by treatment with chelating agents such as ethylene diamine tetra-acetic acid (EDTA) and ethylene glycol tetra-acetic acid (EGTA). EDTA is a buffer whose addition can change the pH of the buffer. Therefore, care should be taken to adjust the pH post addition of EDTA (Campden et al., 2015).

Proteases that are naturally present in the cell lysate pose a serious threat to the protein of interest. The simplest way to prevent the proteolytic action is by adding protease inhibitors. This can be a bit of a problem in large-scale processes, since proteolytic inhibitors are expensive. Nonetheless, other pragmatic measures to tackle this problem include the adsorption of proteases onto hydrophobic adsorbents and adjusting the pH to a value where proteases are rendered ineffective. Researchers have recently established that the addition of doxycycline indirectly inhibits proteolytic activity of tryptic peptidases (Kanada et al., 2012). The key to avoiding bacterial growth in enzyme preparation is following measures to ensure sterility. Some buffers, such as phosphate and acetate, among others, are more prone to supporting the growth of bacteria at neutral pH. The addition of antimicrobial agents to buffers whenever feasible is also a tactic to prevent contamination (Yang et al., 2013).

2.7 A review of enzymes produced using agro-industry waste

2.7.1 Enzymes that act on polysaccharides

Alpha amylases (endo-1, 4- α -d-glucanglucanohydrolase EC 3.2.1.1) are a family of enzymes that randomly cleave α -1, 4 linkages between adjacent glucose subunits in polysaccharides resulting in the release of short chain oligomers and α -limit dextrin. Alpha amylases find wide range of applications in bread and brewing industry, textile, paper and pulp industry and pharmaceuticals. (Sahnoun, Kriaa, Elgharbi, Ayadi, Bejar, & Kammoun, 2015). Alpha amylases are industrially produced via submerged fermentation using genetically improved *Bacillus* and *Aspergillus* species. A large spectrum of bacterial and fungal species has found to produce alpha amylase enzyme with different characteristics such as thermos-stability, halo-tolerance, psychotolerance and alkali-stability (Prakash, Vidyasagar, Madhukumar, Muralikrishna, & Sreeramulu, 2009; Roohi & Kuddus, 2014; Sen, Dora, Bandyopadhyay, Mohapatra, & Raut, 2014). Alpha amylase can also be synthesised following solid-state fermentation method (Sundarram & Murthy, 2014).

In lieu of decreasing the production cost, a lot of research has been dedicated to using lignocellulose as a viable carbon source for amylase production. Rajagopalan and Krishnan (2008) were able to achieve a maximum α -amylase enzyme activity of 67.4 U ml⁻¹ by adding sugarcane bagasse hydrolysate to the nutrient medium using catabolite de-repressed *Bacillus subtilis* KCC103 as the enzyme producer. Banana waste was used as substrate for the production of high titres of α -amylase using *B. subtilis* CBTK 106 (Krishna & Chandrasekaran, 1996). Meanwhile, Francis, Sabu, Nampoothiri, Ramachandran, Ghosh, Szakacs, et al. (2003) conducted optimization studies for the production of α -amylase using spent brewer's grain as substrate. *Aspergillus oryzae* was used as the fermentative microbe in solid state condition.

Amyloglucosidases (E.C. 3.1.2.3) are also known as glucoamylases and can cleave the α -1, 4 linkages found in starch to release glucose molecules. It is an exoamylase; it cleaves β -D glucose from the non-reducing ends of amylose, amylopectin and glycogen (James & Lee, 1997). Amyloglucosidase (AMG) also breaks α -1, 6 glycosidic bonds but at a slower rate (Espinosa-Ramírez, Pérez-Carrillo, & Serna-Saldívar, 2014). Amyloglucosidases exhibits optimum activity at a pH range of 4.5-5 and a temperature range of 40-60 °C (Kumar & Satyanarayana, 2009). AMG finds applications in the food, brewery and pasty industry (Diler, Chevallier, Pöhlmann, Guyon, Guilloux, & Le-Bail, 2015). *A. niger* and *A. oryzae* are common strains that are used by the industry for the production of commercial AMG (Espinosa-Ramírez, Pérez-Carrillo, & Serna-Saldívar, 2014; H. Singh & Soni, 2001). However, *Bacillus* sp., *Rhizopus* sp. and *Saccharomyces* sp. have been reported to synthesise AMG (Ali, Mahmood, Alam, & Hossain, 1989; Shin, Kong, Lee, & Lee, 2000).

Several lignocellulose residues have been used as an alternate carbon source for the production of AMG. A. Pandey, Selvakumar, and Ashakumary (1994) reported improved yields in the production of glucoamylase using *A. niger* on rice bran using additional nitrogen sources. Using *A. niger* in solid state fermentation conditions and wheat bran as substrate 5-6-fold increase in enzyme yield was obtained with productivity measures of 520–560 IU enzyme per gram dry substrate (Ashok Pandey, 1990). This was achieved by improved nutrient addition and better process parameter control. Agro-residues such as cassava, yam, banana, plaintain and banana peels have also been employed as alternate carbon sources for the production of fungal AMG (Adeniran, Abiose, & Ogunsua, 2010).

Cellulose: The depolymerisation of cellulose in to component glucose molecules require a combined hydrolysis of three key enzymes: endoglucanase (E.C. 3.2.1.4),

exoglucanase or cellobiohydrolase (E.C. 3.2.1.176) (E.C. 3.2.1.91) and β -glucosidase (E.C. 3.2.1.21). They are categorized in the glycoside hydrolase family and they catalyse the cleavage of glycosidic bonds (Juturu & Wu, 2014). Cellulases are enzymes of great commercial importance especially because of the inevitable role they play in bioethanol production (Singhania, Saini, Saini, Adsul, Mathur, Gupta, et al., 2014). Besides biofuels, cellulases find application in bread and brewing industry, textile and detergent industry and, paper and pulp industry (Ferreira, Margeot, Blanquet, & Berrin, 2014). A wide range of bacterial and fungal species produce cellulase enzyme. In bacteria, cellulases are found in the form of large, extracellular aggregates which are called cellulosomes (Doi & Kosugi, 2004). Some of the bacterial species which produce cellulosomes include *Clostridium thermocellum*, *Bacillus circulans*, *Proteus vulgaris*, *Klebsiella pneumonia*, *Escherichia coli* and *Cellulomonas* sp. Fungal species have also been found to synthesise cellulases (Juturu & Wu, 2014). Commercial cellulases are produced by improved strains of *Trichoderma reesei*. Examples of other fungal organisms that produce cellulase enzyme are *Schizophyllum commune*, *Melanocarpus* sp., *Aspergillus* sp., *Penicillium* sp., and *Fusarium* sp (Várnai, Mäkelä, Djajadi, Rahikainen, Hatakka, & Viikari, 2014).

Owing to its influence in the economics of bioethanol production cheaper methods to produce cellulase has been the focus of researchers over the past two decades (Klein-Marcuschamer, Oleskowicz-Popiel, Simmons, & Blanch, 2012). Several studies have been conducted to test the efficacy of lignocellulosic food waste as a suitable carbon source for cellulase production. Sun, Li, Zhao, and Peng (2013) evaluated the feasibility of using banana peel for cellulase production by *Trichoderma viride* GIM 3.0010 in solid-state fermentation. An optimized media was formulated using mango peel as carbon source for cellulase production by *Trichoderma reesei* (Saravanan,

Muthuvelayudham, & Viruthagiri, 2012). *Trichoderma reesei* QM9414 was used as the enzyme producer in a study involving brewer's spent grain as the substrate (Sim & Oh, 1990). In separate studies the effectiveness of apple pomace and corn steep liquor as carbon source for the production of cellulase was evaluated (Dhillon, Kaur, Brar, & Verma, 2012; Farid & El-Shahed, 1993).

Xylanases (E. C. 3.2.1.8, 1, 4- β -xylanxylanohydrolase) are enzymes that break down xylan which is an integral part of plant polysaccharide. Xylan is a complex polysaccharide made of xylose-residue backbone each subunit linked to each other by a β -1, 4-glycosidic bond (Ramalingam & Harris, 2010). Xylanases are produced by several bacterial and fungal species. Filamentous fungi that synthesise this enzyme are of particular interest because they secrete the enzyme in to the media in large quantities in comparison to bacteria (Knob, Beitel, Fortkamp, Terrasan, Fanchini, et al., 2013). Xylan, being a complex polysaccharide, requires a consortium of enzymes for total hydrolysis. Xylanases are an enzyme complex comprising of different enzymes that are specific to different substrates and cleave chemical bonds of different nature. This enzyme system consists of endoxylanases, β -xylosidases, ferulic acid esterase, p-coumaric acid esterase, acetylxylan esterase and α -glucuronidase. Endoxylanases and β -xylosidases are most extensively studied components of this system (Polizeli, Rizzatti, Monti, Terenzi, Jorge, & Amorim, 2005). Xylanases have wide applications in food industry, biomedical industry, animal feed industry, and bioethanol production (Das, Ravindran, Ahmed, Das, Goyal, Fontes, et al., 2012; Goswami & Pathak, 2013; Ramalingam & Harris, 2010).

The efficiency of lignocellulosic food waste as carbon source for the production of commercial xylanase has been studied by many researchers. Lowe, Theodorou, and Trinci (1987) experimented with wheat straw as a viable carbon source for the

production of anaerobic rumen fungus xylanase achieving high levels of xylanase activity. *A. niger* and *A. terreus* strains were employed on moistened wheat bran by Gawande and Kamat (1999) to attain 74.5 IU ml⁻¹ of xylanase. In another study, grape pomace was used as the substrate for the production of xylanase using *A. awamori* as the enzyme producer (Botella, Diaz, de Ory, Webb, & Blandino, 2007). Apple pomace, melon peel and hazelnut shell was used by Seyis and Aksoz (2005) for xylanase production from *Trichoderma harzianum* 1073 D3 achieving a maximum activity of 26.5 U/mg.

The importance of **inulinase** arises from the emergence of fructose and fructooligosaccharides as a safe sweetener compared to sucrose in the food and pharmaceutical industry. Inulinase acts upon inulin which is a polyfructose chain terminated by a glucose molecule. The fructose units in inulin is bonded together by β -2, 1-linkage (Vandamme & Derycke, 1983). Commercially, fructose syrup is produced by the combined activity of α -amylase and amyloglucosidase followed by glucose isomerase which converts glucose in to fructose. However, the best yield that can be procured from such a process is 45% of fructose, 50% of glucose and the rest being oligosaccharides. The activity of inulinase results in the complete conversion of the substrate to fructose (Zittan, 1981). Inulinase also finds applications in the production of bioethanol, citric acid, butanedoil and lactic acid as well.

Inulinases can be classified in to exo-inulinases (β -D-fructanfructohydrolase, EC 3.2.1.80) and endo-inulinases (2, 1- β -D-fructanfructohydrolase, EC 3.2.1.7) depending upon their modes of activity (Vijayaraghavan, Yamini, Ambika, & Sravya Sowdamini, 2009). Several bacterial and fungal species such as *Streptococcus salivarius*, *Actinomyces viscosus*, *Kluyveromyces fragilis*, *Chrysosporium pannorum*, *Penicillium sp.* and *Aspergillus niger* have been known to synthesise differ forms of

inulinase (Chi, Chi, Zhang, Liu, & Yue, 2009). The production of inulinase using lignocellulosic substrate has been investigated by many researchers. A. K. Gupta, Kaur, and Singh (1989) reported that when *Fusarium oxysporum* is grown on the aqueous extracts of roots of *Cichorium intybus* it produces extracellular inulinase which can be useful for the production of fructose. A newly isolated *Saccharomyces* sp. from spontaneously fermented sugar cane synthesised inulinase when grown on substrates such as banana peel, wheat bran, rice bran, orange peel and bagasse (A. Onilude, Fadaunsi, & Garuba, 2012). Coconut oil cake was used in study which involved the media optimization for inulinase production by employing *Penicillium rugulosum* (MTCC-3487) (Dilipkumar, Rajasimman, & Rajamohan, 2014). Sugar cane bagasse and yacon have also been used as substrates in different studies for inulinase production (Chesini, Neila, Parra, Rojas, Esquivel, Cavalitto, et al., 2013; Mazutti, Bender, Treichel, & Luccio, 2006).

Mannanases are a cluster of enzymes that degrade mannan which is an integral part of the plant cell wall. Mannan is a representative of hemicellulose which is the second most abundant polysaccharide found in plants. Three major enzymes are involved in the degradation of linear mannans viz. 1, 4- β -D mannohydrolases or β -mannanases (EC 3.2.1.78), 1,4- β -D mannopyranoside hydrolases or β -mannosidases (EC 3.2.1.25) and 1,4- β -D glucoside glucohydrolases or β -glucosidases (EC 3.2.1.21) (Chauhan, Puri, Sharma, & Gupta, 2012). β -mannanases show endo-hydrolysis activity by cleaving the internal glycosidic bonds resulting in the release of short chain β -1,4-manno-oligosaccharides (McCleary & Matheson, 1983). On the other hand, β -mannosidases possess exo-hydrolase activity attacking the polymer at the non-reducing terminal and degrading mannobiose into individual mannose units (Gomes, Terler, Kratzer, Kainz, & Steiner, 2007). β -glucosidase activity results in the excision of 1,4-glucofuranose

units from the non-reducing terminal ends of oligomers released from glucomannan and galactoglucomannan hydrolysis (Mamma, Hatzinikolaou, & Christakopoulos, 2004). Several *Bacillus* sp. including different *B. subtilis* strains have been reported to produce several mannan degrading enzymes. Among fungal organisms many *Aspergillus* sp. have been found to produce mannanase (Dhawan & Kaur, 2007). Other bacterial and fungal species such as *Clostridium* sp., *Penicillium* sp. and *Sterptomyces* sp. have also been known to synthesise mannanase (Chauhan, Puri, Sharma, & Gupta, 2012).

Due to its ability to effectively remove hemicellulose mannase has sparked an increasing interest in the paper and pulp industry (Clarke, Davidson, Rixon, Halstead, Fransen, Gilbert, et al., 2000). Mannases also find applications in the food, oil, feed and textile industries (Christgau, Andersen, Kauppinen, Heldt-Hansen, & Dalboege, 1994; Cuperus, Herweijer, Van Ooijen, & Van Schouwen, 2003; Naganagouda, Salimath, & Mulimani, 2009). Many researchers have studied the production of mannanase using lignocellulose and agro-food industry waste as a substrate. Yin, Liang, Li, and Sun (2013) used a mixture of apple pomace and cottonseed powder as raw material to produce β -mannanase by *Aspergillus niger* SN-09 in solid state fermentation. On optimization of process conditions an activity of 561.3 U/g was attained which was 45.7 % higher than that observed in basal medium. The efficacy of mixed substrate fermentation for mannanase production was investigated by Olaniyi, Osunla, and Olaleye using enzyme producers *Penicillium italicum* and *Trichosporonoides oedocephalis*. They experimented with lime, grape, tangerine and sweet orange peels in different concentrations and reported that mixed substrate fermentation resulted in appreciable enzyme activity. Fermented palm kernel cake was used in another study as the substrate for mannanase production by growing

Aspergillus terreus SUK-1 in a column reactor (Rashid, Samat, & Yusoff, 2013). Plantain peels, mango peels, sugar cane pulp, potato peels, soy bean meal, passion fruit peel, locust bean gum etc. have also been reported to be excellent choices of raw material for the production of β -mannosidase and β -glucosidases (J. M. Almeida, Lima, Giloni-Lima, & Knob, 2015; Gomes, Terler, Kratzer, Kainz, & Steiner, 2007; A. A. Onilude, Fadahunsi, Antia, Garuba, & Ja'afaru).

Lactase, otherwise known as β -d-galactohydrolase (EC 3.2.1.23), hydrolyses lactose into glucose and galactose (Duan, Sun, & Wu, 2014; Nakkharat & Haltrich, 2006). Lactose is the sugar which is found in milk. Humans produce this enzyme only as infants and this ability lessens as they grow older which leads to lactose intolerance (Kies, 2014). Hence, lactase is a very important enzyme in the dairy and food industry. Industrial lactase is produced by employing selected strains of *Kluyveromyces lactis* (Bonekamp & Oosterom, 1994). However, several articles can be found that report other organisms that produce lactase. *Candida pseudotropicalis* has been reported to produce lactase when grown on de-proteinized whey (de Bales & Castillo, 1979). *Trichoderma viride* ATCC 32098 produces a high thermostable lactase enzyme which shows 90% activity in a pH range of 3.0-7.5 (Seyis & Aksoz, 2004). Macris (1981) reported the production of extracellular lactase from *Fusarium moniliforme* using wheat bran solid medium. The addition of agriculture by-products such as molasses and whey increased enzyme yield.

A few studies have been done to increase the economic feasibility of lactase production by the addition of media components that are cheaply available and lignocellulosic in nature. A *Lactobacillus acidophilus* strain isolated from was reported to produce lactase enzyme even when the lactose content in the media was reduced to 0.75% supplemented with 1% ragi (Akolkar, Sajgure, & Lele, 2005). However, lactose was

found to be the best carbon source for lactase enzyme production. A study conducted by Mustranta, Karvonen, Ojamo, and Linko (1981) employed a mutant strain of *A. niger* for lactase production and attained threefold increase in enzyme yield. They observed that the mutated strain of *A. niger* produces high levels of lactase enzyme in a medium supplemented by wheat bran.

Beta Glucanases: 1, 3-1, 4- β -glucans are polysaccharides made up of glucose units that are found in endosperm cell walls of cereals such as barley, rye, sorghum, oats etc. (Celestino, Cunha, & Felix, 2006). Endo-1, 3-1, 4- β -glucanases are enzymes that can hydrolyse the β -1, 4 glycosidic linkages at the non-reducing ends of glucan to release cellobiosyl-D-glucose and 3-O- β -D-celotriosyl-D-glucose. This enzyme is particularly important in the brewing industry. β -glucanases along with a consortium of xylanase and cellulase facilitate the reduction of viscosity in fluids with higher solid content. This leads to less water consumption during production process, saves energy and eliminates the need for several raw materials (Tang, He, Chen, Zhang, & Ali, 2004). β -glucanases also finds application in the wine industry along with pectinases by facilitating a smooth and fast filtration process along with increasing the quality of the product (Villettaz, Steiner, & Trogus, 1984).

Several microorganisms have been tried and tested for the production of β -glucanase using lignocellulosic industry waste as substrate. *Rhizomucor miehei* CAU432 was grown under solid state fermentation conditions using oat meal as the sole carbon source to obtain a β -1,3-1,4-glucanase activity of 20 025 U g⁻¹ dry substrate which was the highest ever reported (Yang, Xiong, Yang, Yan, & Jiang, 2015). Irshad, Anwar, and Afroz (2012) reported the characterization of exo 1, 4- β glucanase from *Trichoderma viride* MBL by culturing the organism in solid state fermentation using orange peel waste as substrate. *Fusarium oxysporum*, *Bacillus subtilis*, *Penicillium*

echinulatum, *Sclerotium rolfsii* have also been reported to produce high levels of endo and exoglucanase when grown on biomass sources (Christakopoulos, Kekos, Kolisis, & Macris, 1995; Sachslehner, Nidetzky, Kulbe, & Haltrich, 1998; Tang, He, Chen, Zhang, & Ali, 2004; Zampieri, Guerra, Camassola, & Dillon, 2013).

Invertase, technically known as β -fructofuranosidase (EC.3.2.1.26) is a glycoprotein which catalyses the hydrolysis of sucrose in to glucose (dextrose) and fructose. Invertase exhibits optimum activity at a pH of 4.5 and temperature of 55 °C. *Saccharomyces cerevisiae* is the chief strain used in the production of invertase enzyme in the industry (Neumann & Lampen, 1967). Invertase is used for the production of invert sugar which was earlier done by acid hydrolysis. Acid hydrolysis of sucrose results in the 50% conversion of sucrose in to invert sugar. Moreover, acid hydrolysis product also contains impurities whose formation cannot be controlled during inversion. The use of invertase results in 100% inversion of sucrose without the formation of impurities (Kulshrestha, Tyagi, Sindhi, & Yadavilli, 2013). Since invertase is a commercially expensive enzyme, many researchers have attempted to use cheap carbon sources for its production. Rashad and Nooman (2009) used red carrot jam processing residue as a medium for solid state fermentation to characterise invertase synthesised by *S. cerevisiae* NRRL Y-12632. Hang and Woodams (1995) tested the efficacy of apple pomace as potential carbon source for the production of invertase from *Aspergillus foetidus* NRRL 337. Orange peel, pineapple peel waste, pomegranate peel, wheat bran, baggase waste etc. have all been tested and found to be excellent carbon source substitutes for invertase production employing different microbial species (Alegre, Polizeli, Terenzi, Jorge, & Guimarães, 2009; C Uma, Gomathi, Muthulakshmi, & Gopalakrishnan, 2010; C. Uma, Gomathi, Ravikumar,

Kalaiselvi, & Palaniswamy, 2012; Veana, Martínez-Hernández, Aguilar, Rodríguez-Herrera, & Michelena, 2014).

Pectinases are a class of enzymes that catalyse the disintegration of pectin containing compounds. Pectin compounds are an integral part of the plant cell wall. Pectinases can be classified in to two groups according to their mode of action. Pectin esterases catalyses the de-esterification of methyl groups found in pectin to produce pectic acid. Depolymerase enzymes cleaves the glycosidic bonds found in pectic acid to release various simpler compounds based on their mode of enzyme action. Protopectinases solubilizes protopectin in to highly polymerised form of soluble pectin (Sakai, Sakamoto, Hallaert, & Vandamme, 1993). Pectinases are used in the fruit juice industry and wine making for clarification and removal of turbidity in the finished product. It also intensifies the colour in the fruit extract while aiding in stabilization, clarification, and filtration (Servili, Begliomini, Montedoro, Petruccioli, & Federici, 1992).

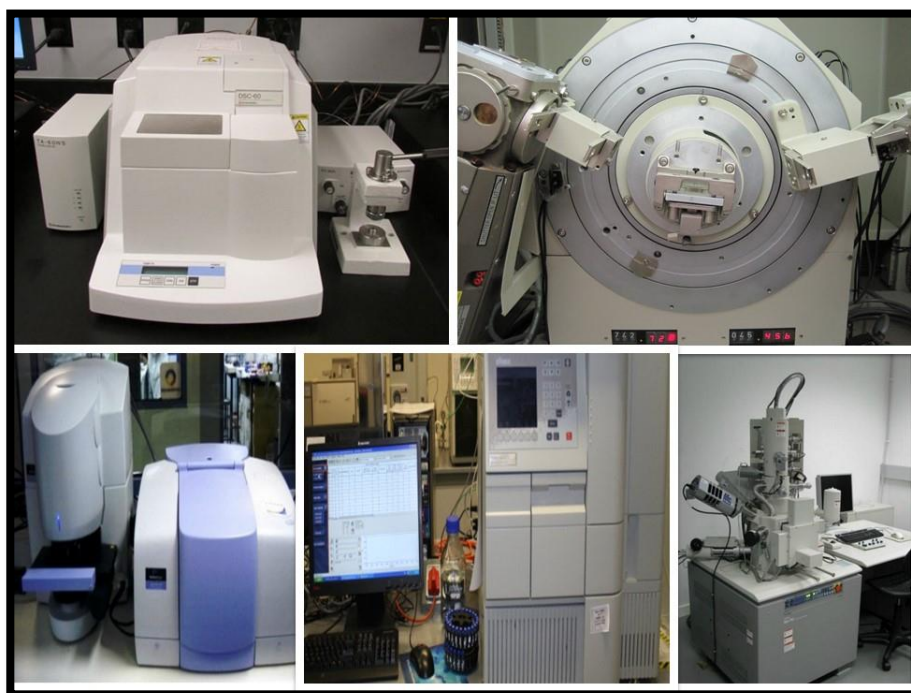
A lot of agro residue varieties have been tried and tested for the production of pectinases using different microbial species. Apple pomace and grape pomace have extensively used in studies to determine their efficacy as suitable substrates for pectinase production (Botella, Diaz, de Ory, Webb, & Blandino, 2007; Hours, Voget, & Ertola, 1988). S. A. Singh, Ramakrishna, and Appu Rao (1999) successfully optimised parameters for the downstream processing parameters for pectinase produced using fermented bran. A novel approach was devised by Raj Kashyap, Kumar Soni, and Tewari (2003) where they supplemented the fermentation media with neurobion (a multi-vitamin) and polygalacturonic acid while producing pectinase using wheat bran as the carbon source. Maximum enzyme activity was reported to be 8050 U/g dry substrate. A mixture of orange bagasse and wheat bran in the ratio (1:1)

employed as media for pectinase production using filamentous fungus *Penicillium viridicatum* RFC3 yielded highest enzyme activities of 0.7 and 8.33 U ml⁻¹ for endo- and exo-polygalacturonase and 100 U ml⁻¹ for pectin liase on performing the fermentation process in polypropylene packs (Silva, Tokuioshi, da Silva Martins, Da Silva, & Gomes, 2005). Seedless sunflower heads and barley spent grain are lignocellulosic food processing wastes among others that have been studied as potential media additives for pectinase production (C. Almeida, Brányik, Moradas-Ferreira, & Teixeira, 2003; Patil & Dayanand, 2006).

Pectinases have also been produced using a mixture of wastes. Biz, Finkler, Pitol, Medina, Krieger, and Mitchell (2016) developed an ingenious method to produce pectinases employing citrus peel and sugar cane bagasse in solid state fermentation mode while avoiding overheating problems. A pilot-scale packed bed reactor was used for this purpose. A uniform pectinase activity of 34-41 U/ml was obtained though out the bed. Combination of different biomass ensured temperature control while avoiding problems such a bed shrinkage, agglomerate formation etc. since sugar cane bagasse was highly porous in nature. *Aspergillus oryzea* was used as the fermentative microbe.

Chapter 3

Materials & Methods



This section of the thesis gives a comprehensive account of the scientific procedures and materials utilized for accomplishing the experimental work.

Table 3.1 Common chemicals and their sources

| | |
|--|---|
| Acetone: Sigma-Aldrich (now Merck) | Magnesium Sulphate |
| Agar: Fluka | Mannose: Sigma-Aldrich (now Merck) |
| Ammonia | Meat Extract: Sigma-Aldrich (now Merck) |
| Ammonium Hydroxide | |
| Arabinose: Sigma-Aldrich (now Merck) | Methanol: Merck |
| Calcium Chloride: Sigma-Aldrich (now Merck) | M9 minimal salts media: Sigma-Aldrich (now Merck) |
| Cellulose: Sigma-Aldrich (now Merck) | Ortho-phosphoric acid: Merck |
| Cellulase from <i>Trichoderma reesei</i> : Sigma-Aldrich (now Merck) | Peptone: Sigma-Aldrich (now Merck) |
| Citric acid: Sigma-Aldrich (now Merck) | Potassium Permanganate-Sigma-Aldrich (now Merck) |
| D(+) Galactose: Sigma-Aldrich (now Merck) | Soluble Starch: Sigma-Aldrich (now Merck) |
| D(+)-Glucose: Sigma-Aldrich (now Merck) | Sodium Acetate: Sigma-Aldrich (now Merck) |
| D(+)-Xylose: Fluka | Sodium Azide |
| Dinitrosalicylic acid-Sigma-Aldrich (now Merck) | Sodium citrate: Scharlau Chemie |
| Dipotassium phosphate: Sigma-Aldrich (now Merck) | Sodium Hydroxide: Sigma-Aldrich (now Merck) |
| Ethanol: Merck | Sodium nitrate: BDH Chemicals |
| EDTA: Sigma-Aldrich (now Merck) | Sodium nitrite: Sigma-Aldrich (now Merck) |
| Ferric Chloride: Sigma-Aldrich (now Merck) | Sulphuric Acid |
| Fructose: Sigma-Aldrich (now Merck) | Starch: Sigma-Aldrich (now Merck) |
| Galactose: BDH Chemicals | Sucrose: BDH Chemicals |
| Hemicellulase from <i>Aspergillus niger</i> | Xylan from beech wood-Sigma-Aldrich (now Merck) |
| Maltose: Sigma-Aldrich (now Merck) | |

3.1 Instruments

- Alliance HPLC (Waters, e2695 Separations module): Waters, Milford, MA.
- Guard column (50×7.8mm): Phenomenex, UK.
- Microwave oven: Sharp, Model R 244; Sharp electronics, UK.
- Millipore filter: Millipore, Bedford, MA.
- Rezex ROA-Organic Acid H+(8%) (350×7.8mm): Phenomenex, UK (particle size 8 µm).
- Shaking incubator: Innova 42, Mason Technology, Ireland.
- Ultrasonicator (ULTRASONIK™ 57X, Ney Dental Inc., USA).
- Perkin Elmer Spectrum GX FTIR (UATR) Microscope (USA)
- Siemens's BN D-500 X-ray diffractometer
- Shimadzu DSC-60 installed with TA-60WS software
- Field Emission Scanning Electron Microscopy (Hitachi SU-70)

3.2 Software

- Empower: Empower Software Solutions, Inc., Orlando, FL.
- Minitab® 16
- STATGRAPHICS Centurion XV: StatPoint Technologies, Inc., Warrenton, VA.

3.3 Analytical Techniques

3.3.1 Compositional analysis

Compositional analysis of the pretreated and native lignocellulose samples were performed by two stage acid hydrolysis according to National Renewable Energy Laboratory (NREL) protocol (Sluiter et al., 2005). The samples were subjected to acid hydrolysis using 72% H₂SO₄ at 30°C for 60 min. The mixture was then diluted to 4% H₂SO₄ concentration by adding deionised water and autoclaved for 60 min. The solids were filtered using a filtration crucible and dried at 105°C for 48h to remove all the

moisture content or until constant weight was achieved. The dried solids were then burned in blast furnace for 24h at 595°C to obtain acid insoluble lignin. The acid soluble lignin content in the liquid was determined using UV-VIS spectrophotometry at 205 nm.

3.3.2 Reducing sugar analysis

The reducing sugar concentration in the hydrolysate was estimated by dinitrosalicylic acid (DNS) method (Miller, 1959). Briefly, each hydrolysate (1 ml) was diluted 1:10 in dH₂O before addition of dinitrosalicylic acid reagent (1 ml) and dH₂O (2 ml). The mixture was submerged in a water bath for 5 min at 100 °C. The volume was made up to 10 ml by the addition of dH₂O (6 ml). Absorbance was measured at 540 nm.

3.3.3 Individual sugar and inhibitor analysis

The presence and quantification of monosaccharides was done on an Alliance High Performance Liquid Chromatography (HPLC) (Waters, e2695 Separation module, Waters, Milford, MA, USA) equipped with an auto-sampler and controller with dual pump was used (Jaiswal et al., 2012). The detection system consisted of a Waters 486 UV detector and a Waters 410 Differential Refractometer (RI detector) connected in series. The detector was maintained at room temperature. Empower[®] software was used for data acquisition and analysis. An aliquot (20 µL) was injected into a thermostatically controlled compartment set to 65 °C, followed by elution through a Rezex ROA-Organic acid H⁺ (8%) (350 mm × 7.8 mm, Phenomenex, Macclesfield, UK, particle size: 8 µm) column fitted with a guard column (50 mm × 7.8 mm, Phenomenex, Macclesfield, UK), at a flow rate of 0.6 ml·min⁻¹. The mobile phase was H₂SO₄ (5 mM). The run time for each sample injection was set at 30 min. All solutions were filtered through a 0.22 µm Millipore[®] filter (Millipore, Merck-Millipore, Billerica, MA, USA) before being injected into the instrument.

3.3.4 Fourier Transform Infra-Red Spectroscopy analysis

FTIR spectroscopy is a mid-infrared spectroscopic analysis is a rapid and non-destructive technique for the qualitative and quantitative determination of biomass components in the mid-infrared region. The high infrared background absorbance of water is an obstacle when FTIR is employed in the analysis of wet, solid biomass. But ATR-FTIR allows attenuation of incident radiation and provides infrared spectra without water background absorbance. Sample preparation is critical because FTIR works well with individual components extracted from plant cell wall. FTIR provides information about certain components in the plant cell wall through absorbance bands. Table 3.1 summarises absorbance bands corresponding to the functional groups in biomass materials.

Table 3.2 Fourier Transform Infrared Absorbance Bands in Biomass Study

| Wave Number (cm ⁻¹) | Assignment/Functional Group | Polymer |
|---------------------------------|--------------------------------|----------------------------------|
| 875 | Glycosidic linkage | Hemicellulose |
| 990 | C-O valence vibration | Cellulose |
| 1035 | C-O, C=C, and C-C-O stretching | Cellulose, hemicellulose, lignin |
| 1200 | O-H bending | Cellulose, hemicellulose |
| 1215 | C-C+C-O stretching | Lignin |
| 1500 | Aromatic ring vibration | Lignin |
| 2840, 2937 | C-H stretching | Lignin (wood) |

FTIR spectroscopy studies were performed to observe any changes in the composition as a reflection of the variations in the functional groups in pretreated BSG at the backdrop of its raw counterpart. A Perkin Elmer Spectrum GX FTIR (UATR) Microscope (USA) (located in School of Food Science and Environmental Health, DIT) was employed for this study. The FTIR spectra was recorded from 3000 to 800 cm^{-1} with 32 scans at a resolution of 0.3 cm^{-1} in transmission mode (Raghavi et al., 2016).

3.3.5 X-ray diffraction

X-ray diffraction analysis of lignocellulosic biomass is conducted to analyse the crystallinity of the material. Atoms in a crystal are arranged in such as fashion that when X-ray beam hits the atom, the rays that are diffracted in all directions will be in phase. X-ray diffraction studies were conducted to analyse the changes in crystallinity of spent coffee waste and brewer's spent grain imparted by each pretreatment. This was done using a Siemens's BN D-500 X-ray diffractometer (located in FOCAS, DIT) with diffraction angles spanned from $2\theta=5^{\circ}$ - 50° . The radiation was generated at a voltage of 40 kV and current of 30 mA using Cu $K\alpha$ as the radiation source ($\lambda=0.154$ nm) (Binod et al., 2012).

3.3.6 Thermal Behavior analysis

Differential scanning calorimetry is a technique used to study the behaviour of polymers when they are heated. It provides an idea about the thermal transitions of the polymer such as glass transition, crystallisation and melting of crystalline materials (Figure 3.1). T_g (glass transient temperature) is the temperature at which amorphous component of the lignocellulose is converted from a brittle form into a rubbery, flexible form. Parameters such as specific heat, coefficient of thermal expansion, the

free volume and the dielectric constant changes rapidly. No enthalpy is associated with this process.

The glass transition event is followed by the crystallisation of the sample. Crystallisation is an exothermic process resulting in the release of heat to the surroundings. The crystallisation event is marked by a dip in temperature (T_c) and determines the latent heat of crystallisation (area under the curve). Meanwhile, increase in temperature results in a process called melting where the molecular chains can move freely. Melting is an endothermic process marked by T_m (melting temperature) and is defined as the temperature at the peak apex. The heat capacity of a polymer in the melt is higher than that of a solid crystalline polymer (lignocellulose).

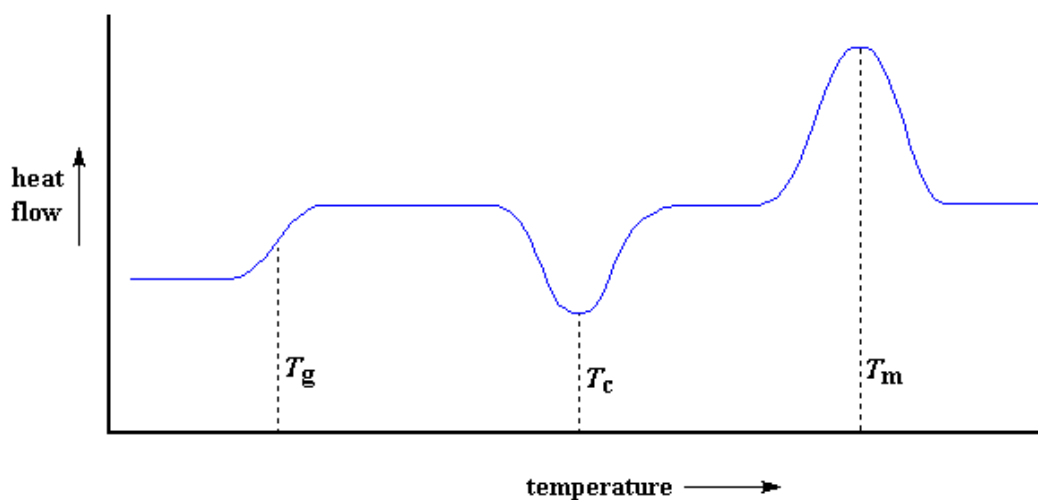


Figure 3.1 A typical DSC curve of a polymer

Differential scanning calorimetry (DSC) was used to study the changes in the thermal behaviour of spent coffee waste and brewers' spent grain before and after pretreatment. Shimadzu DSC-60 installed with TA-60WS software was the equipment used for this purpose (FOCAS, DIT). Each BSG sample (55 mg) was taken in an aluminium pan

with an empty pan used as a reference. All the measurements were carried out between 25°C and 500°C with a linear increase of 10°C/minute (Ferraz et al., 2013).

3.3.7 Scanning Electron Microscopy

FESEM is used to visualise very small topographical details on the surface of entire or fractioned objects. It may be used to study synthetic and naturally occurring polymeric structures. FESEM makes use of an electron beam instead of a light source and the reflected electrons is used re-construct the image of an object. Electrons are emitted from a field emission source in a high electron field gradient. These (primary) electrons are focussed on the object using electronic lenses in a high vacuum column. After bombarding the object, the (secondary) electrons are scattered in different directions and angles based on the surface morphology.

A detector catches these electrons and generates a corresponding electronic signal which is amplified and transformed into an image or video. The morphological structure of spent coffee waste and the effect of pretreatment on the same were analysed by performing field emission scanning electron microscopy (FESEM, Hitachi SU-70) in FOCAS, DIT. (Raghavi et al., 2016). Dried samples of the untreated and pretreated SCW were subject to FESEM, operating at the electron beam energy of 0.5 keV.

3.4 Optimisation of enzymatic hydrolysis of SCW

The enzymatic hydrolysis of SCW was performed by employing commercially available cellulase and hemicellulase purchased from Sigma-Aldrich (now Merck) Aldrich, Ireland. Response Surface Methodology (RSM) was applied to statistically optimise the reaction conditions for enzymatic hydrolysis of SCW to evaluate the main

effects, interaction effects and quadratic effects of five factors (biomass loading, enzyme loading (cellulase and hemicellulase) pH and time). A Central Composite Design (CCD) with five factors and five levels including five replicating centre point was created using STATGRAPHICS Centurion XV software (StatPoint Technologies Inc. Warrenton, VA, USA).

Table 3.3 Process variables and level in CCD for enzymatic hydrolysis of SCW

| Independent variables | Coded symbols | Levels | | | | |
|---------------------------------|---------------|--------|-----|-----|-----|-----|
| | | -2 | -1 | 0 | +1 | +2 |
| Biomass loading (g/50ml) | X_1 | 1 | 2 | 3 | 4 | 5 |
| Cellulase loading (ml/50ml) | X_2 | 0.3 | 0.6 | 0.9 | 1.2 | 1.5 |
| Hemicellulase loading (ml/50ml) | X_3 | 0.3 | 0.6 | 0.9 | 1.2 | 1.5 |
| pH | X_4 | 4.8 | 5.4 | 6.0 | 6.6 | 7.2 |
| Incubation time (h) | X_5 | 24 | 48 | 72 | 96 | 120 |

In order to find the optimum enzymatic conditions, following parameters were considered: biomass loading, cellulase (77.08 FPU/ml) and hemicellulase (72.23 U/ml) loading, incubation time and pH were selected. To optimise the enzymatic reaction, appropriate amount of SCW, cellulase, hemicellulase and 0.5% sodium azide was mixed in sodium citrate buffer of designated pH to achieve the required reaction mixture. Hemicellulase exhibited β -galactosidase activity with the ability to cleave β -1, 4 linkages between D-xylopyranosol units. The reaction volume was maintained at 50 ml and the temperature were set at 50°C. The reaction mixture was incubated for respective time period given by software (24, 48, 72, 96 and 120h). Replenishment of buffer was not required as the minimal changes were found at the end of the reaction. On completion of the experiments the suspensions were transferred

to 30 ml polypropylene tubes, centrifuged at 7000 rpm for 10 min. The supernatant was collected and stored at -20°C for further analysis.

3.5 Optimisation of enzymatic hydrolysis of BSG

The enzymatic hydrolysis of BSG was performed by employing commercially available cellulase and hemicellulase purchased from Sigma-Aldrich (now Merck), Ireland. The hydrolysis parameters were determined employing response surface methodology using STATGRAPHICS Centurion XV software. A central composite design was created which included five parameters and five levels with five replicating centre points. The parameters considered for this study included biomass loading, cellulase, hemicellulase, pH and time. Designated amounts of BSG, cellulase (77.08 FPU/ml), and hemicellulase (72.23 U/ml) was mixed in sodium citrate buffer (0.05M) and distilled water to maintain a reaction volume of 10 ml as presented in Table 3.4.

Table 3.4. Process variables and level in CCD for enzymatic hydrolysis of BSG

| Independent variables | Coded symbols | Levels | | | | |
|---------------------------------|---------------|--------|-----|-----|-----|-----|
| | | -2 | -1 | 0 | +1 | +2 |
| Biomass loading (g/50ml) | X_1 | 1 | 2 | 3 | 4 | 5 |
| Cellulase loading (ml/50ml) | X_2 | 0.3 | 0.6 | 0.9 | 1.2 | 1.5 |
| Hemicellulase loading (ml/50ml) | X_3 | 0.3 | 0.6 | 0.9 | 1.2 | 1.5 |
| pH | X_4 | 4.8 | 5.4 | 6.0 | 6.6 | 7.2 |
| Incubation time (h) | X_5 | 24 | 48 | 72 | 96 | 120 |

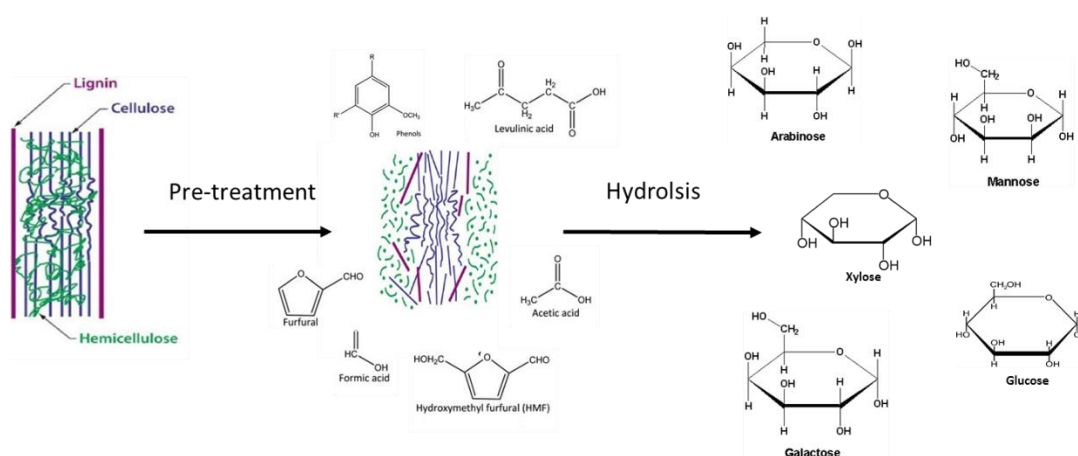
The temperature for all the experiments were set at 50°C. The reactions were carried out for different time periods (24, 48, 72, 96 and 120). On completion of each experiment the hydrolysate was collected by filtration and the reducing sugar content was measured using DNS method.

3.6 Statistical Analysis

All the experiments were carried out in triplicate and replicated twice unless stated. Significant differences were computed by employing analysis of variance (ANOVA) and multiple comparisons (Fischer's least significant difference test) by employing STATGRAPHICS Centurion XV software (StatPoint Technologies Inc. Warrenton, VA, USA). Value of $p < 0.05$ was considered as significant value.

Section I

Pretreatment of Lignocellulose



Lignocellulose is a common term used to describe the chemical composition of plant biomass. It is the most abundant renewable carbon resource in the world. It is composed of three polymers viz. lignin, cellulose and hemicellulose (hence the name lignocellulose). Lignocellulosic derivatives can be broken down into individual sugars. This fact is taken advantage of when using them as raw materials for production processes involving fermentation. However, lignocellulose also contains pectin and a higher fraction of lignin content compared to polysaccharides. This, along with high degree of crystalline cellulose prevents enzymes from accessing the polysaccharides. Pretreatment is techniques that eliminate these factors thereby facilitating enzymes to better access their respective substrates. The application of high temperature and pressure or harsh chemical treatments to break the covalent bonds that bind lignin to cellulose and hemicelluloses are common methods of lignocellulose pretreatments.

Chapter 4

Development of Novel Pretreatment for the Enhanced Enzymatic Hydrolysis of Coffee Spent Waste

In this chapter, a range of pretreatments namely, physical, chemical and physico-chemical followed by a sequential, combinatorial pretreatment strategy was applied to spent coffee waste (SCW) to attain maximum sugar yield. All the pretreated samples were analysed for total reducing sugar, individual sugars and generation of inhibitory compounds such as furfural and hydroxymethyl furfural (HMF) which can hinder microbial growth and enzyme activity. Native SCW was high in hemicellulose content. Galactose was found to be the predominant sugar in SCW. Results showed that sequential pretreatment yielded 350.12mg of reducing sugar/g of substrate, which was 1.7-fold higher than native SCW (203.4 mg/g of substrate). Organosolv pretreatment of SCW gave rise to the furfural formation in the liquor (12.42 mg/g). Furthermore, extensive delignification was achieved using sequential pretreatment strategy. XRD, FTIR, and DSC profiles of the pretreated substrates were studied to analyse the various changes incurred as opposed to native SCW.

Work described in this chapter has been published as peer review article:

Two-step sequential pretreatment for the enhanced enzymatic hydrolysis of coffee spent waste (2017). *Bioresource Technology*, 239, 276-284.

4.1 Introduction

Coffee is one of the most popular hot beverages and is the second most traded commodity in the world after petroleum. Global coffee production has increased by 17% from 2002 to 2014 due to its increased consumption. This gives rise to huge amounts of coffee processing residues leading to serious waste management issues (Campos-Vega et al., 2015). Spent coffee waste (SCW) is generated as a result of production of instant coffee from roasted coffee beans. In 2008/09 the EU introduced 'The New Waste Framework Directive' as a new approach to curb food waste such as SCW and as well as new processes for reuse and recycling (Ravindran & Jaiswal, 2016b). Additionally, the EU adopted a four directives in 2018 to solidify Europe's leading position in solid waste management. According to the Directive (EU) 2018/850, member states are to reduce waste disposal by landfilling. Additionally, the member states are to adopt waste management systems that are sustainable and value waste as a resource. This is on the backdrop of the Circular Economy Package adopted by the EU. SCW is rich in a variety of organic compounds such as carbohydrates, lignin, fatty acids and antioxidant compounds which makes it an excellent candidate for the production of value-added substances. It is also rich in phenolic compounds, caffeine, trigonelline, and Maillard reaction products. Furthermore, chlorogenic acid and gallic acid constitute bulk of polyphenols in SCW (Ballesteros et al., 2014). Commercially viable goods such as bioethanol, biodiesel, sugars, activated carbon, compost and sorbent material for metal ion removal fall into the spectrum of value-added products that can be derived from SCW (Go et al., 2016; Kwon et al., 2013; Utomo & Hunter, 2006).

SCW, being lignocellulosic in nature, is mainly rich in polysaccharides, lignin, lipids and a small fraction of proteins. The polysaccharides in SCW are composed of

cellulose and hemicellulose and comprise almost all of its dry weight. Cellulose is a polymer made of repeating units of glucose, while hemicellulose is heterogeneous in nature and composed of galactose, mannose and arabinose structurally arranged as arabinogalactan and galactomannan (Ballesteros et al., 2015). Although high in carbohydrate content, the extraction of monomeric sugars from SCW is a tedious task. Its ability to withstand high temperatures during roasting and extraction is indicative of its recalcitrant nature. Recalcitrance in SCW is imparted by the orderly structure of the component material along with the presence of lignin, a phenolic polymer. Pretreatments are essential strategies that can disrupt the structure of lignocellulose for the efficient utilisation of the inherent polysaccharides (Ravindran & Jaiswal, 2016a). Pretreatment methods are intended to increase the efficiency of lignocellulose hydrolysis by improving accessibility towards polysaccharide fractions. These strategies can be classified into physical, chemical, biological and combinatorial based on their modes of action. An efficient pretreatment strategy is generally simple to perform and releases highly fermentable products with the minimal formation of indigestible compounds. The polysaccharide fraction is least affected so that no inhibitory compounds are formed during the process. Furthermore, the energy and cost requirements should not be so high to render the whole process economically futile.

Several studies pertaining to the pretreatment of SCW for the production of value-added products have been described in literature. Rocha et al. (2014) employed ultrasonication as a pretreatment measure to produce bioethanol and biodiesel from SCW. Dilute acid hydrolysis was used as the pretreatment strategy in a study involving the extraction of sugars and lipids from SCW (Go et al., 2016). Pretreated SCW has also been used for enzyme production. Murthy and Naidu (2012) used acid and alkali pretreated SCW as a solid substrate medium for the production of xylanase

employing *Pencillium sp.* However, few studies are available that investigate the effects of pretreatment methods on the various aspects of SCW.

This study proposes a two-step pretreatment strategy to increase, reducing sugar recovery after subjecting SCW to enzymatic hydrolysis. Initially, eight different pretreatments were performed for SCW and the changes in its chemical composition, structural conformation, crystallinity and thermal behaviour was analysed and compared. All the pretreated SCW samples were then subjected to enzymatic hydrolysis (using celluclast and hemicellulase from *A. niger*) using optimised parameters. Furthermore, the incidence of inhibitory compounds after each pretreatment were also investigated. Finally, two of the most effective pretreatments determined in this investigation were combined and performed sequentially to increase the sugar recovery from the enzymatic hydrolysis of SCW.

4.2 Methodology

Spent coffee waste was generously supplied by a local Starbucks outlet in Dublin city. The SCW was dried at 80°C for 48h and stored at room temperature in a cool and dry place for further experiments (Kwon et al., 2013). All the chemicals such as cellulase from *Trichoderma reesei*, hemicellulase from *Aspergillus niger*, conc. H₂SO₄, alkaline potassium permanganate, ethanol, ferric chloride, conc. H₃PO₄, acetone and other chemicals required for experimentation were purchased from Sigma-Aldrich (now Merck), Ireland. Cellulase activity was assayed by following laboratory analytical procedures for the measurement of cellulase activity devised by National Renewable Energy Laboratory (Adney & Baker, 1996). Meanwhile hemicellulase activity was assayed followed protocols described by Rickard and Laughlin (1980). Cellulase

enzyme registered an enzyme activity of 77 FPU/ml while hemicellulase showed 72 U/ml enzyme activity.

4.2.1 Screening of various pretreatments for spent coffee waste

4.2.1.1 Dilute acid hydrolysis

Hydrolysis experiments were performed in an autoclave. SCW (1% w/v) was mixed with 10 ml of different concentrations of sulphuric acid (1%, 1.3% and 1.6%) in a 100 ml flask. The reaction mixture was subjected to different temperatures (10, 20 and 30 min) (Zheng et al., 2013). After pretreatment the solids were separated from liquids by centrifugation at 8000 rpm for 12 min and the supernatant was analysed for total and individual sugars and by-products such as acetic acid, furfural and hydroxymethyl furfural. The solids were dried and stored in cool and dry place until further analysis.

4.2.1.2 Steam explosion

The steam explosion pretreatment of SCW was performed as described by Huang et al. (2015) with certain modifications. Five grams of SCW were sprayed with de-ionized water to attain 50% moisture content (w/v) and then stored at room temperature. The samples were then loaded into a stainless-steel autoclave (TOMY, SS-325, USA). The temperature was raised and maintained at 121°C for 30 min after which the pressure was released by opening the pressure valve subjecting the biomass to an ‘explosion’. The steam exploded SCW was collected, dried and stored in sealed plastic bags for further analysis.

4.2.1.3 Ammonia Fiber explosion

The SCW (2.5 g) was soaked in 25 ml of NH₄OH and subjected to high pressure and temperature in an autoclave. The experiment was carried out at varying temperatures

(70–120 °C), water loadings (0.2–2.0 ml water/g dry biomass), and residence time (1–30 min). The treated biomass was removed from the autoclave and air-dried overnight (\approx 12 h) in a fume hood to remove residual ammonia. The treated samples were stored in sealed plastic bags in a cool and dry place (Shao et al., 2013).

4.2.1.4 Concentrated phosphoric acid pretreatment

Pretreatment of SCW was performed as described by Li et al. (2009). Five grams of SCW were mixed well with 50 ml of concentrated phosphoric acid (85%) in a 100 ml glass beaker. The mixture was incubated at 50 °C and 120 rpm for 1 h. After the reaction, the slurry was poured into 120 ml of pre-cold acetone and centrifuged at 8000 rpm. The supernatant was collected for analysis of inhibitory compounds. The solids were mixed with distilled water and centrifuged iteratively as a means of washing off the residual acid. After four repeated washing steps during the last washing step, the pH was adjusted to 6.0 with 10 M NaOH. The solids were dried and stored for further use.

4.2.1.5 Pretreatment using atmospheric air plasma

Five grams of spent coffee waste were placed in polyethylene terephthalate (PET) trays (0.15m \times 0.15m \times 0.035m) which were sealed in polymeric films of thickness 50 μ m thickness with very low gas transmission rates (oxygen transmission rate of 9.49×10^{-9} mol m⁻² s⁻¹ at 23 °C and 101325 Pa, Cryovac BB3050, Sealed Air, Ireland). These packages were placed in the inter electrode space of the dielectric barrier discharge set up. The SCW samples were subjected to non-thermal plasma in triplicates for 2 min, 4 min and 6 min at three discreet voltages of 60 kV, 70 kV and 80 kV and subsequently stored for 24 h at 10°C. The plasma reactor was installed in ventilated room and the

controlled was placed a minimum distance of 10m as safety measures. The pretreated SCW was then stored in a cool and dry place until further analysis.

4.2.1.6 Pretreatment using Ferric Chloride

Pretreatment of SCW using ferric chloride was performed according to the procedure described by Chen et al. (2015). SCW (1% w/v) was mixed in 50 ml of 0.1M FeCl₃ in an Erlenmeyer flask and the subjected to high pressure (15 psi) and temperature (120 °C) for 30 min in an autoclave. The mixture was immediately allowed to cool down and the solids were separated from the liquid. The solids were then washed several times with deionized water to remove any residual FeCl₃. The Fe (III) in the liquor was precipitated by gradually the neutralising the solution using 0.1M NaOH. Once the precipitate was removed the salt free liquor was subjected to HPLC analysis to identify and quantify individual sugars and hydroxyl-5-methyl furfural and furfural content.

4.2.1.7 Organosolv Pretreatment

Organosolv pretreatment was performed as described by Ostovareh et al. (2015) with minor modifications. The SCW (1% dry) was mixed in 25 ml of ethanol-water mixture (50-70% ethanol (v/v) in an Erlenmeyer flask. In all experiments, 1% of sulphuric acid (w/w) per gram substrate was added as a catalyst. The reaction temperature was set at 120 °C for 30 min. After the reaction the flasks were then placed in an ice chamber to cool the contents quickly to room temperature. The contents were filtered and washed with 250 ml of 50% ethanol mixture to extract the soluble products into the liquid phase. The pretreated substrate was separated from the liquid phase by filtration and was washed several times with distilled water until pH 7 was obtained. Ethanol was evaporated from the liquid fraction and recovered by condensation. To obtain a precipitated solid, the solid fraction, mainly contained lignin, was separated by

centrifugation. The pretreated SCW was dried and stored for enzymatic hydrolysis and further analysis.

4.2.1.8 Microwave assisted alkali pretreatment

Microwave-alkali pretreatment was carried out following the procedure described by Binod et al. (2012) with minor modifications. The biomass (10% (w/v)) were loaded to 1% NaOH (w/v) solution in a stoppered flask and subjected to microwave radiation at varying power settings of 850 W, 600 W, 300 W, and 180 W for different residence time varying from 30 to 120 s. After pretreatment, the biomass was thoroughly washed with distilled water till pH 6.0 and air dried. The dried solid residue was used for enzymatic hydrolysis and compositional analysis.

4.2.1.9 Sequential pretreatment of spent coffee waste

Based on the observations obtained from the extensive pretreatment study conducted using SCW, a two-step sequential pretreatment technique was devised to achieve enhanced lignin removal, coupled by increasing yield of reducing sugars and recovery of polysaccharide content. As a consequence, concentrated phosphoric acid acetone pretreatment was improvised to include the effects of AFEX pretreatment (Fig. 4.1). SCW was mixed with concentrated phosphoric acid in the ratio of 1:10 and incubated at 50 °C for 1 h in a shaker incubator at 120 rpm. The mixture was then mixed with ice cold acetone, which resulted in a slurry. The slurry was then centrifuged at 8000 rpm for 10 min to remove the solids from the liquid. The solids were washed several times to remove any reminiscent acid and acetone and then dried overnight at 80 °C. The dried SCW was weighed and 2.5g of the sample was mixed with 25 ml of ammonia. The mixture was then exposed to high temperature (120 °C) and pressure (15 lbs/sq. inch) in an autoclave for 30 min. The sequentially pretreated SCW was then kept in a

fume hood overnight to remove any residual ammonia following which it was dried and stored for further analysis.

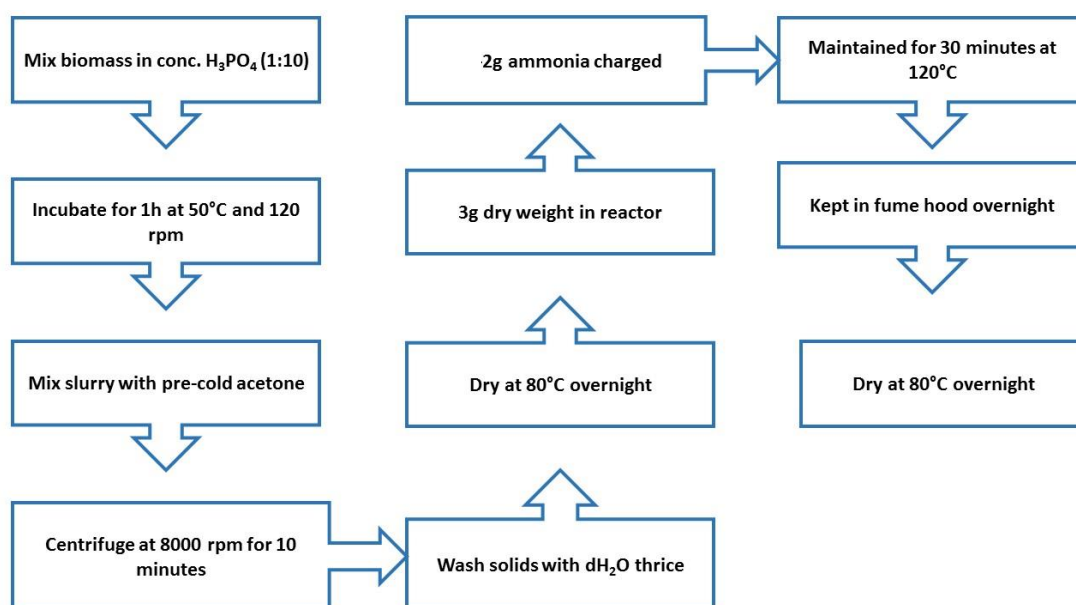


Figure 4.1 Sequential pretreatment flow diagram

4.2.2 Composition analysis

All the pretreated SCW samples along with its native counterpart was subjected to compositional analysis by following two stage acid hydrolysis according to the National Renewable Energy Laboratory (NREL) protocol (Sluiter et al., 2005). In brief, the SCW samples were hydrolysed in 72% H₂SO₄ at 30 °C for 60 min. The mixture was then diluted to 4% by adding deionised water and autoclaved for 60 min. The solids were filtered using a filtration crucible and dried at 105 °C for 48 h to remove all the moisture content. The dried solids were then burned in blast furnace for 24 h at 595 °C to obtain acid insoluble lignin. The acid soluble lignin content in the liquid was determined using spectrophotometry at 205 nm. The reducing sugar concentration in the hydrolysate was estimated by dinitrosalicylic acid (DNS) method (Miller, 1959). The identification and quantification of monosaccharides were done on an Alliance HPLC (Waters, e2695 Separation module) using a Rezex ROA-Organic

Acid H⁺ (8%) column, (350 x 7.8 mm; Phenomenex, UK) with 5 mM H₂SO₄ as the mobile phase at 65 °C maintaining a flow rate of 0.6 ml/min (Jaiswal et al., 2012).

4.2.3 Enzymatic hydrolysis

The enzymatic hydrolysis (celluclast and hemicellulose from *A. niger*) of SCW was performed according to the procedure mentioned in section 3.3.2.

4.2.4 Individual sugar, inhibition and organic acid analysis

The identification and quantification of monosaccharides and organic acids was done on following the protocol described in section 3.3.3.

4.3 Characterisation of native and pretreated substrates

4.3.1 Scanning electron microscopy

Scanning electron microscopy was performed in accordance to protocol described in section 3.3.7

4.3.2 X-ray diffraction

The changes in crystallinity of the pretreated and untreated substrates were studied following methodology detailed in section 3.3.5.

4.3.3 Fourier Transform Infrared Spectroscopy analysis

The changes in functional groups inflicted by pretreating the biomass was assessed using FTIR spectroscopy according to section 3.3.4.

4.3.4 Thermal Behavior

The thermal behaviour of the treated and raw samples was studied and compared using differential scanning calorimetry (DSC) by following methodology described in section 3.3.6

4.4 Results and discussion

4.4.1 Composition analysis of native spent coffee waste

The chemical composition of original SCW was determined and have been summarised in Table 4.2. SCW was abundant in four types of sugars *viz.* glucose, mannose, arabinose and galactose. Xylose was minimal in SCW. The presence of these sugars in high amounts indicated that the SCW is rich in galactomannan, arabinogalactan and cellulose structures. These findings are in consensus with other studies on the chemical composition of the SCW (Mussatto et al., 2011, Pujol et al., 2013). The chemical compositional analysis of the native substrate revealed that SCW is abundant in hemicellulose content (33.5g/100g) as compared to cellulose (8.6g/100g). Lignin amounted to 19.6g/100g of SCW. Of this, only 3.7g was contributed by acid soluble lignin. Variations in SCW compositions can be a result of the coffee bean raw material, the conditions that prevailed during roasting among other factors (Jooste et al., 2013).

4.4.2 Optimisation of enzymatic hydrolysis parameters using response surface methodology

The aim of the optimization study was to find the optimum parameters that would maximise reducing sugar yields. Total reducing sugar after enzymatic saccharification of pretreated biomass obtained from 30 experiments are listed in Table 4.1. The models

were compared based on the coefficient of determination (R^2) and adjusted coefficient of determination (R^2 -adj). The R^2 is defined as the regression of sum of squares proportion to the total sum of squares, which illustrates the adequacy of a model. R^2 ranges from 0 to 1 and R^2 values closer to 1, means the model is more accurate.

Table 4.1 CCD experimental designs for five independent variables, experimental and predicted values for total reducing sugar

| Biomass loading (g/50ml) X_1 | Cellulase loading (ml/50ml) X_2 | Hemicellulase loading (ml/50ml) X_3 | pH X_4 | Incubation Time (h) X_5 | Experimental total reducing sugar (mg/ml) | Predicted total reducing sugar (mg/ml) |
|-----------------------------------|--------------------------------------|--|-------------|------------------------------|---|--|
| 2.0 | 1.2 | 1.2 | 6.6 | 48 | 26.5 | 26.5 |
| 2.0 | 0.6 | 0.6 | 5.4 | 96 | 28.5 | 28.4 |
| 2.0 | 0.6 | 1.2 | 6.6 | 96 | 28.5 | 28.5 |
| 3.0 | 0.9 | 1.5 | 6.0 | 72 | 30.1 | 29.7 |
| 3.0 | 0.9 | 0.9 | 6.0 | 24 | 27.1 | 27.0 |
| 2.0 | 0.6 | 1.2 | 5.4 | 48 | 26.1 | 26.1 |
| 4.0 | 1.2 | 0.6 | 5.4 | 96 | 31.5 | 31.5 |
| 3.0 | 0.9 | 0.3 | 6.0 | 72 | 29.6 | 29.4 |
| 3.0 | 0.9 | 0.9 | 6.0 | 72 | 28.9 | 29.1 |
| 5.0 | 0.9 | 0.9 | 6.0 | 72 | 30.8 | 30.4 |
| 2.0 | 1.2 | 0.6 | 6.6 | 96 | 25.7 | 25.6 |
| 4.0 | 0.6 | 0.6 | 6.6 | 96 | 29.8 | 29.9 |
| 4.0 | 1.2 | 1.2 | 5.4 | 48 | 31.5 | 31.5 |
| 3.0 | 0.9 | 0.9 | 6.0 | 72 | 29.2 | 29.4 |
| 4.0 | 1.2 | 1.2 | 5.4 | 48 | 31.0 | 31.1 |
| 3.0 | 1.5 | 0.9 | 6.0 | 72 | 30.6 | 30.6 |
| 3.0 | 0.9 | 0.9 | 7.2 | 72 | 28.9 | 28.7 |
| 2.0 | 0.6 | 0.6 | 6.6 | 48 | 26.0 | 25.9 |
| 3.0 | 0.9 | 0.9 | 4.8 | 72 | 29.5 | 29.5 |
| 3.0 | 0.9 | 0.9 | 6.0 | 72 | 29.2 | 29.4 |
| 3.0 | 0.9 | 0.9 | 6.0 | 72 | 29.2 | 29.4 |
| 2.0 | 1.2 | 0.6 | 5.4 | 48 | 26.0 | 25.9 |
| 2.0 | 1.2 | 1.2 | 6.0 | 96 | 27.8 | 27.9 |
| 1.0 | 0.9 | 0.9 | 6.0 | 72 | 23.3 | 23.6 |
| 4.0 | 0.6 | 0.6 | 5.4 | 48 | 28.7 | 28.8 |
| 4.0 | 0.6 | 1.2 | 5.4 | 96 | 30.3 | 30.5 |
| 4.0 | 0.6 | 1.2 | 5.4 | 96 | 31.1 | 31.3 |
| 4.0 | 0.6 | 1.2 | 6.6 | 48 | 27.6 | 27.8 |
| 3.0 | 0.3 | 0.9 | 6.0 | 72 | 29.4 | 29.2 |
| 3.0 | 0.9 | 0.9 | 6.0 | 120 | 29.7 | 29.5 |

A value of 99.26% R^2 was observed in the present study while R^2 -adj was 97.62% illustrates the model adequately fits the data. The data obtained from the central composite design were fitted to second order polynomial equations. The polynomial equation for the model is given below:

$$\begin{aligned} \text{Reducing sugar (mg/ml)} = & 8.0815 + 4.0795X_1 - 6.346X_2 - 1.318X_3 + 0.218X_4 + 2.478X_5 \\ & - 0.592X_1^2 + 2.425X_1X_2 - 0.870X_1X_3 - 0.00515X_1X_4 + \\ & 0.0270X_1X_5 + 1.505X_2^2 + 1.722X_2X_3 - 0.0659X_2X_4 + 0.111X_2X_5 \\ & + 0.130X_3^2 + 0.0289X_3X_4 + 0.0902X_3X_5 - 0.00047X_4^2 - \\ & 0.0125X_4X_5 - 0.179X_5^2 \end{aligned}$$

where X_1 , X_2 , X_3 , X_4 and X_5 represents biomass loading, cellulase loading, hemicellulase loading, pH and incubation time, respectively. The significance of the coefficients of the models was determined by analysis of variance (ANOVA). ANOVA table showed that 13 effects have P-values less than 0.05, indicating that they are significantly different from zero at the 95.0% confidence level, indicating the considerable effect of these coefficients on reducing sugar yield. All the linear coefficients were found positive significant effect ($p < 0.05$) on the maximum reducing sugar yield from pretreated SCW. However, only biomass, cellulase loading, hemicellulase loading and time showed positive significant interaction effect ($p < 0.05$) on the maximum reducing sugar yield. Among quadratic coefficients only cellulase loading had positive effect on the maximising reducing sugar yield.

Three-dimensional response surfaces generated further gave insights on the interaction between the five factors tested (Figure 4.2). The contour plots revealed that there was significant interaction between each parameter taken in this study albeit in varying degrees. The highest interactions were recorded between SCW and time (Figure 4.2b) and, SCW and hemicellulase (Figure 4.2d). The highly significant interactions

between SCW and hemicellulase can be attributed to the high hemicellulose content in the biomass. As is evident from the graphs cellulase and hemicellulase enzymes has least interaction with each other (Figure 4.2a). The highest point on the three-dimensional plot determines the optimal value for each parameter to attain maximum reducing sugar yield. A maximum reducing yield was observed with high biomass loading (5g/50 ml), cellulase (1.5 ml/50ml), hemicellulase (0.37 ml/50ml), pH (6.7) and a short incubation time (24 h). Furthermore, the model predicted the maximum sugar yield to be 35.64 mg/ml of reaction volume. For the validation of the model a confirmation experiment was conducted using the optimised parameters. Experimentally obtained values of total reducing sugar amounted to 35.15 ± 0.2 mg/ml, which suggested that there was little disparity (< 5%) between predicted and observed values, and the model was adequate to predict the optimum parameters for saccharification of SCW. Since the time period for completion of the reaction was 24 h scale-up of this model is a feasible option (Sánchez, 2009).

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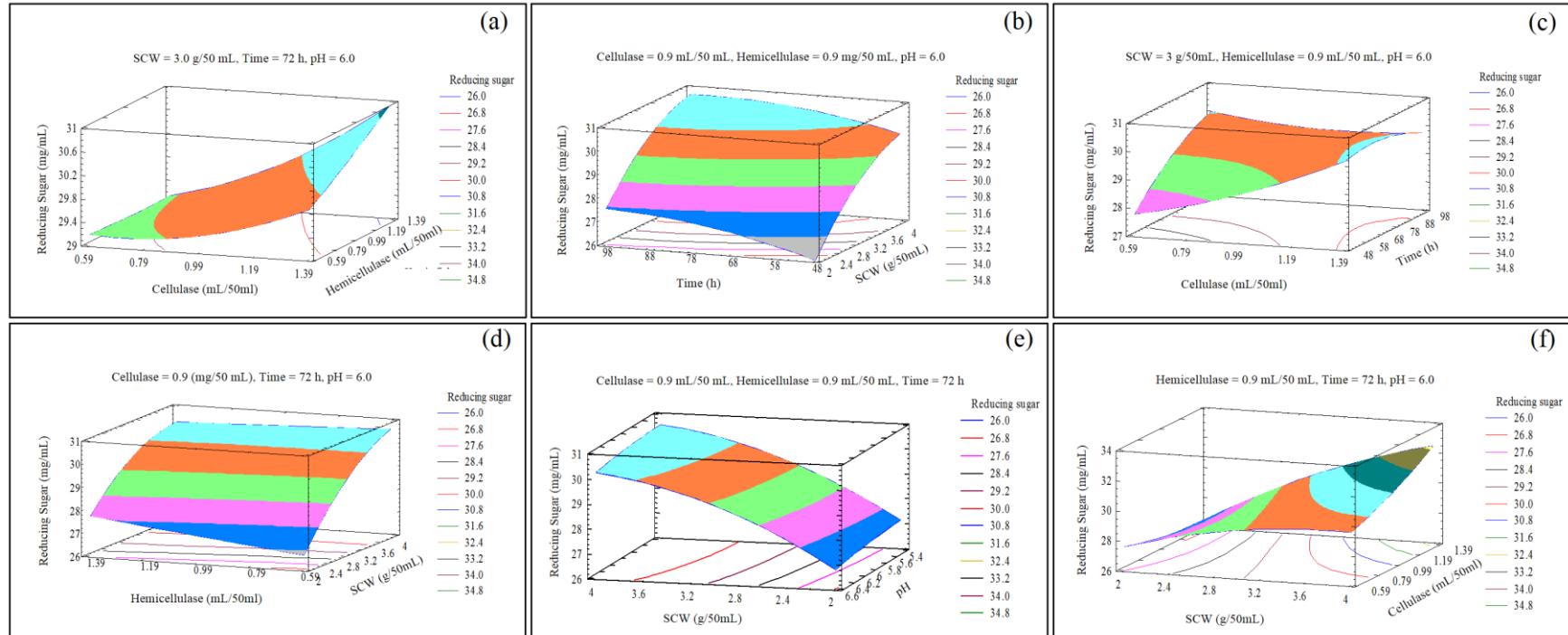


Figure 4.2. Response surface plots representing the effect of independent variables on reducing sugar yield (4.2a) the effect of cellulase and hemicellulase on reducing sugar yield when the response surface is fixed at CSG = 3.0 g/50 ml, time = 72 h and pH = 6.0; (4.2b) representing the effect of time and CSG on reducing sugar yield, when the response surface is fixed at cellulase = 0.9 ml/50 ml, hemicellulase = 0.9 mg/50 ml, pH = 6.0; (4.2c) representing the effect of cellulase, time on reducing sugar yield, when the response surface is fixed at CSG = 3 g/50ml, hemicellulase = 0.9 ml/50 ml, pH = 6.0; (4.2d) representing the effect of hemicellulase and CSG on reducing sugar yield, when the response surface is fixed at cellulase = 0.9 (mg/50 ml), time = 72 h, pH = 6.0; (4.2e) representing the effect of CSG and pH on reducing sugar yield, when the response surface is fixed at cellulase = 0.9 ml/50 ml, hemicellulase = 0.9 ml/50 ml, time = 72 h, and (3f) representing the effect of CSG and cellulase on reducing sugar yield, when the response surface is fixed at hemicellulase = 0.9 ml/50 ml, time = 72 h, pH = 6.0.

4.4.3 Influence of pretreatments on composition of SCW and reducing sugar yield

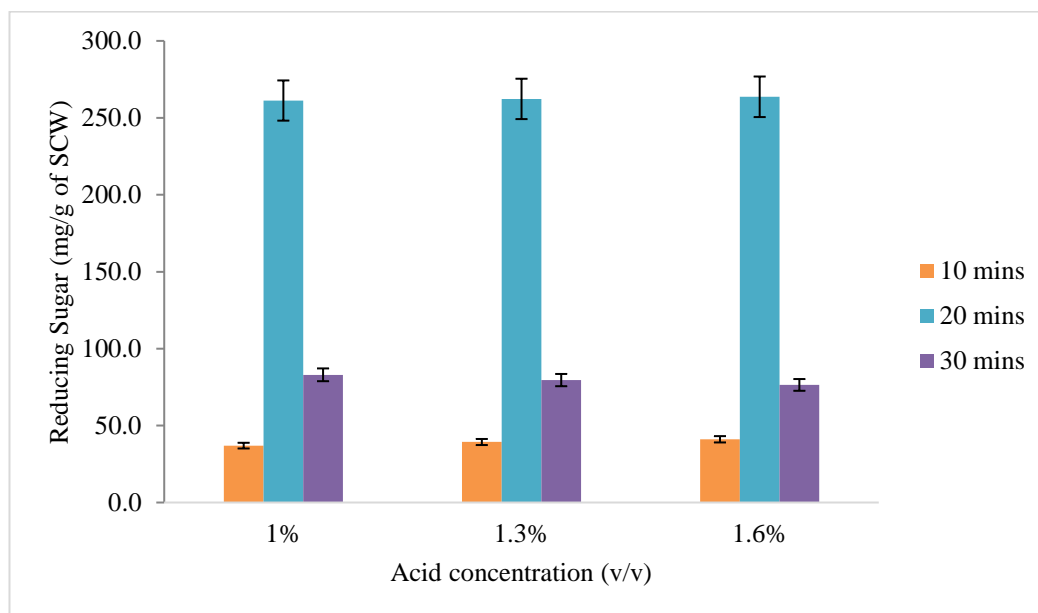
Eight pretreatments were tested at various conditions to study the effect of each strategy on the composition of SCW. The final chemical composition of the biomass post pretreatment is an important factor that governs the efficiency of enzymatic hydrolysis. For a lignocellulosic substrate to be effectively used in a bioconversion process it is necessary to relatively expose cellulose fibres along with possible removal of hemicellulose and lignin fractions. SCW was subjected to chemical composition analysis at each stage of pretreatment. The data obtained from the analysis have been summarised in Table 4.2.

Different experimental settings were tested for dilute acid hydrolysis (acid concentration 1%, 1.3% and 1.6%; time 10, 20 and 30 mins). High reducing sugar release was recorded for SCW pretreated with acid for 20 min at high temperature regardless of the concentration of the acid (Figure 4.3). This may be explained by the adverse impact of longer treatment times on the polysaccharide fraction of lignocellulosic biomass. Based on the reducing sugar yield employing an acid concentration of 1.6% for 20 min was found to be the best dilute acid hydrolysis strategy for SCW (263.68 mg/g of SCW). Compositional analysis of the biomass revealed that subjecting SCW to dilute acid hydrolysis resulted in the increase in cellulose content as well as removal of considerable fractions of hemicellulose. This was evident in the reduction in the galactan, mannan and arabinan content. However, it is worth noting that acid soluble lignin was also removed from the biomass as a result of dilute acid hydrolysis, which is characteristic to this pretreatment.

Air pressure plasma gives rise to highly reactant species such as ozone, hydroxyl ions and hydronium which react with lignocellulosic polymeric structures breaking it down into component molecules. Analysis of liquor obtained after pretreatment revealed the presence of reducing sugars, which supported this fact. Air pressure plasma pretreatments were conducted using the following settings: (voltages: 60, 70 and 80 kV; time: 2, 4 and 6 mins) Increasing the intensity of plasma resulted in the reduction of reducing sugar yield. Furthermore, pretreating SCW for longer time frames was found to be detrimental to the polysaccharide content of biomass as less reducing sugar was released following enzymatic hydrolysis. It was observed that the reducing sugar yield improved by exposing SCW to high intensity plasma for short time durations. Thus, subjecting the biomass to 80 kV for 4 min was found to be most effective plasma pretreatment strategy for SCW (268.68 mg of reducing sugar/g of SCW) (Figure 4.4). This pretreatment strategy conserved much of the hemicellulose fraction along with the removal of acid insoluble lignin.

Table 4.2 Component analysis of SCW after different pretreatments

| Pretreatment | Cellulose | Galactan | Arabinan | Mannan | AIL | ASL |
|-------------------------------|-----------|-----------|-----------|-----------|------------|----------|
| Plasma | 12.65±0.1 | 20.08±0.9 | 1.89±0.4 | 16.88±0.8 | 16.12±0.1 | 3.65±0.1 |
| Ferric Chloride | 10.74±0.9 | 18.76±0.2 | 2.24±1.2 | 24.51±1.3 | 12.32±0.45 | 3.11±0.5 |
| Organosolv | 7.04±3.1 | 30.12±0.4 | 3.02±0.3 | 31.54±0.5 | 14.45±0.12 | 2.89±0.6 |
| Microwave-alkali | 11.02±2.7 | 25.67±0.5 | 2.24±0.6 | 14.78±0.7 | 21.2±0.4 | 2.4±3.1 |
| Dil. Acid Hydrolysis | 15.37±0.2 | 3.84±0.5 | 0.476±1.1 | 5.88±2.4 | 25.67±0.3 | 1.17±2.1 |
| AFEX | 8.7±1.2 | 5.21±0.8 | 0.62±0.2 | 9.41±0.1 | 12.2±3.4 | 3.97±0.1 |
| Steam Explosion | 6.2±0.4 | 12.4±1.2 | 3.1±0.7 | 20.2±0.3 | 24.5±0.9 | 3.17±0.2 |
| Conc. Phosphoric Acid acetone | 18.14±0.5 | 2.4±0.3 | 0.42±0.6 | 4.75±0.1 | 21.43±0.4 | 1.06±0.4 |
| Sequential Pretreatment | 20.01±0.2 | 2.1±0.3 | 0.39±1.2 | 4.05±0.3 | 10.3±0.2 | 1.04±0.9 |
| Control | 8.6±0.1 | 13.7±0.3 | 1.7±0.2 | 21.1±0.5 | 27.12±0.4 | 3.89±0.3 |

**Figure 4.3 Effect of acid concentration and time on reducing sugar release**

Air pressure plasma gives rise to highly reactant species such as ozone, hydroxyl ions and hydronium which react with lignocellulosic polymeric structures breaking it down into component molecules. Analysis of liquor obtained after pretreatment revealed the presence of reducing sugars, which supported this fact. Air pressure plasma pretreatments were conducted using the following settings: (voltages: 60, 70 and 80 kV; time: 2, 4 and 6 mins) Increasing the intensity of plasma resulted in the reduction of reducing sugar yield. Furthermore, pretreating SCW for longer time frames was found to be detrimental to the polysaccharide content of biomass as less reducing sugar was released following enzymatic hydrolysis. It was observed that the reducing sugar yield improved by exposing SCW to high intensity plasma for short time durations. Thus, subjecting the biomass to 80 kV for 4 min was found to be most effective plasma pretreatment strategy for SCW (268.68 mg of reducing sugar/g of SCW) (Figure 4.4).

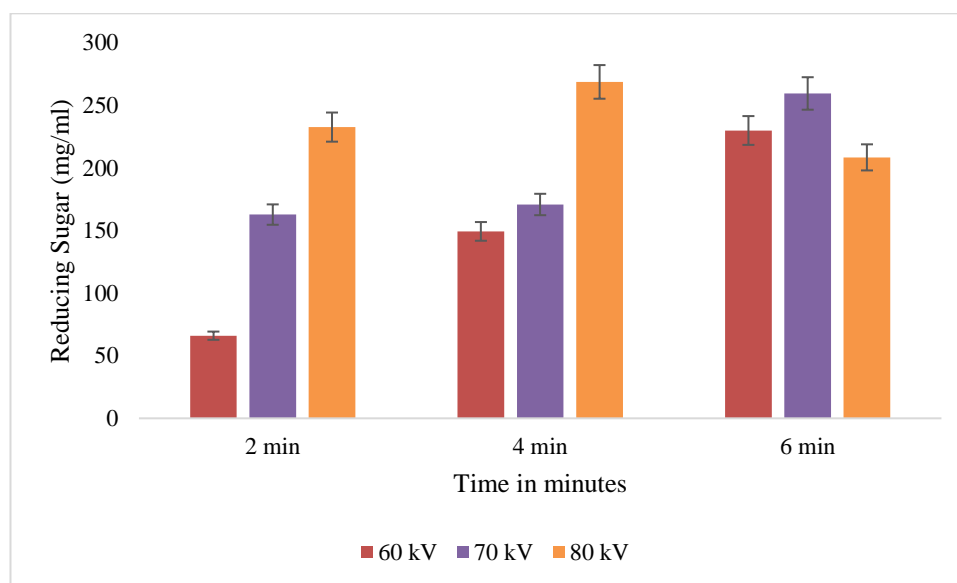


Figure 4.4 Effect of voltage and time on reducing sugar release

Microwave assisted alkali pretreatment was conducted in different settings of power and time (power setting: 850 W, 600 W, 300 W, and 180 W; time: 30s, 60s, 120s). Any treatment beyond 120s resulted in the burning and charring of SCW. This pretreatment was effective in the removal of acid soluble lignin from SCW. The cellulose and hemicellulose fractions were conserved microwave pretreated SCW. Subjecting SCW to microwave radiation at 850W for 30 s in the presence of alkali resulted in highest reducing sugar yield (258.3 mg/g of SCW). Figure 4.5 shows a comparative total reducing sugar content of SCW released after enzymatic hydrolysis of pretreated samples.

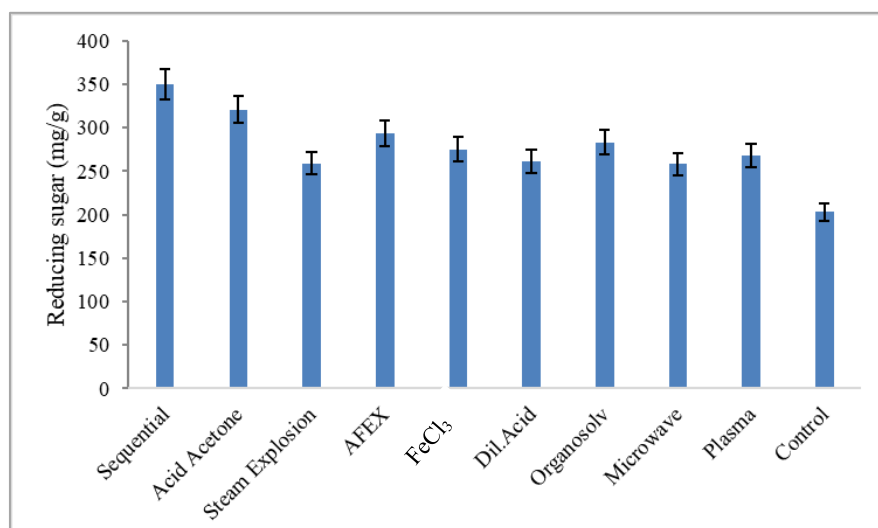


Figure 4.5 Total reducing sugar released after enzymatic hydrolysis of pretreated SCW

Ferric chloride pretreatment increases the ease of enzymatic hydrolysis of lignocellulosic biomass by the removal of lignin and hemicellulose content. Furthermore, it changes the chemical structure and functional groups in the substrate composition to enhance the adsorption of enzymes on the cellulose. Ferric chloride pretreatment removed considerable amounts of lignin from the SCW, while cellulose and hemicellulose fractions were left intact. A reducing sugar yield of 275.4 mg/g of pretreated SCW was obtained after enzymatic hydrolysis of ferric chloride pretreated

SCW. Steam explosion results in disruption of the structure and the solubilisation of the solids in SCW. The extent of solubilisation depends on the temperature applied during the process. The steam explosion of coffee waste resulted in marginal removal of non-carbohydrate content in SCW. The reducing sugar pertaining to steam exploded SCW post enzymatic hydrolysis amounted to 258.9 mg/g of pretreated SCW. Steam explosion was not a particularly effective pretreatment measure for SCW. This may be because the pretreatment was conducted in an autoclave in a comparatively mild setting (121°C and 15 psi) as compared to the common practice where biomass is treated with hot steam (180 to 240 °C) under pressure of approximately 150 to 500 psi followed by an explosive decompression of the biomass. Chiyanzy et al. (2015) reported that steam explosion is an effective treatment for SCW.

Organosolv pretreatment was found to be detrimental to the carbohydrate content in SCW. Cellulose fibres and varying amounts of hemicellulose along lignin leach into the organic solvent used in this pretreatment. The lignin can be precipitated by simple distillation, which also results in the revival of solvent used in the process (Zhao et al., 2009). The acid catalyst (1% w/w H₂SO₄) used in organosolv process is corrosive in nature and can give rise to inhibitory compounds when reacting with carbohydrate fractions in lignocellulose. There was a reduction in cellulose content (7.04g/100g of SCW) although the pretreatment removed a substantial amount of acid soluble lignin. A maximum reducing sugar of 283.12 mg/g of substrate was obtained when SCW pretreated using organosolv strategy was subjected to enzymatic hydrolysis.

Amongst all the pretreatments tested, concentrated acid acetone pretreatment was found to be the most effective in removing mannan and acid soluble lignin from SCW. This might be due to the presence of concentrated phosphoric acid, which has been found to disrupt hemicellulose structure leading to its removal (Siripong et al., 2016).

Meanwhile, AFEX pretreatment caused biomass swelling disrupting the fibres and thus breaking down linkages between lignin and carbohydrates thereby increasing the available surface area. However, this pretreatment did not affect the composition of SCW to a great extent, except for the removal of acid insoluble lignin. Of all the pretreatments tested, AFEX pretreated SCW had the least amount of acid-insoluble lignin (AIL) (12.2 g/100g of SCW). Uppugundla et al. (2014) in a study using pretreated corn stover for bioethanol production reported the decrease of AIL from 17.2g/100g of biomass to 12.2g/100g of biomass following AFEX pretreatment

Combining the varying effects of these two pretreatments was the motivation behind the sequential pretreatment strategy. Employing concentrated acid, acetone pretreatment resulted in the removal of hemicellulose and lignin content in SCW. This was followed up with the AFEX pretreatment at low temperature which resulted in the removal of acid insoluble lignin, while preserving the cellulose content (Table 1). Furthermore, by using sequential pretreatment a 1.7-fold (350.12 mg/g of SCW) increase was observed in the reducing sugar yield as compared to native SCW (203.4 mg/g of substrate).

4.4.4 Individual sugar, inhibitor and organic acid analysis

HPLC analysis of the hydrolysate obtained after enzymatic hydrolysis of each pretreated SCW identified four monosaccharides (glucose, mannose, galactose and arabinose) (Figure 4.6). Kwon et al. (2013) had reported the presence of these four sugars in a study involving the use of SCW for the co-production of bioethanol and biodiesel which validated the findings of the present study. Galactose was found to be the major sugar in the hydrolysate while xylose was not observed in the SCW, which could be understood by the fact that SCW is high in hemicellulose content. Ballesteros

et al. (2015) had reported similar findings in their study on the component sugars found in SCW. The liquor obtained after pretreatment was analysed for the presence of any individual sugars, organic acids and inhibitory compounds. All pretreatments released small but insignificant amounts of galactose and arabinose in the liquor. Interestingly, a considerable amount of furfural (12.42g/100g of SCW) was detected in the liquor obtained after organosolv pretreatment. The formation of furfural may be assisted by the presence of acid as catalysts coupled with high temperature in the organosolv process, which leads to the formation of furfural as a result of degradation of monosaccharides.

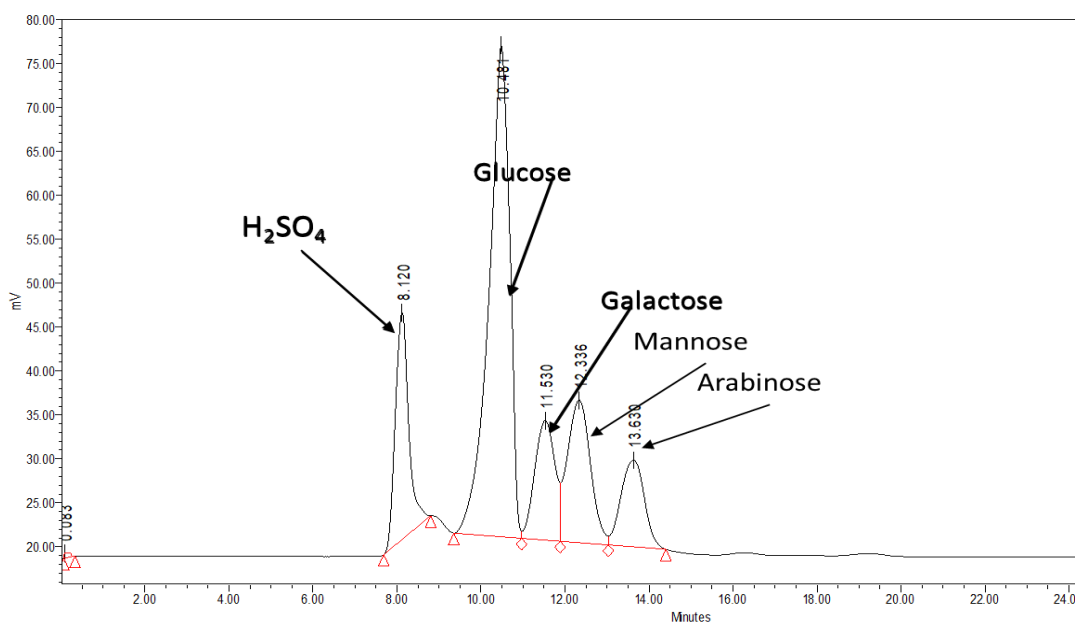


Figure 4.6 Individual Sugars in SCW hydrolysate

4.4.5 SEM, XRD and FTIR profiles of untreated and pretreated spent coffee wastes

SEM was employed to study the structural changes in SCW brought about the sequential pretreatment process. Fig. 4.6 reveals the changes pertaining to SCW before and after sequential pretreatment. SCW is different from other lignocellulosic

substrates in terms of particle shape, size and nature. It is highly porous, non-fibrous and exhibits a sheet like structure (Ballesteros et al., 2014). The main effects that can be observed in the SEM images of SCW is the reduction in size. Smaller particles can be easily identified in the pretreated SCW SEM image (Figure 4.7 A). This can be attributed to the efficiency of the pretreatment in the removal of hemicellulose structures which otherwise would have physically hindered the accessibility of the enzyme to cellulose. Furthermore, the hemicellulase fraction would have adsorbed the hydrolytic enzymes, resulting in low enzyme concentration. Extensive disintegration of cell structure of the biomass was achieved by the combination of two strategies resulted in a substantial increase in external surface area. All these factors contributed to a better saccharification of pretreated SCW.

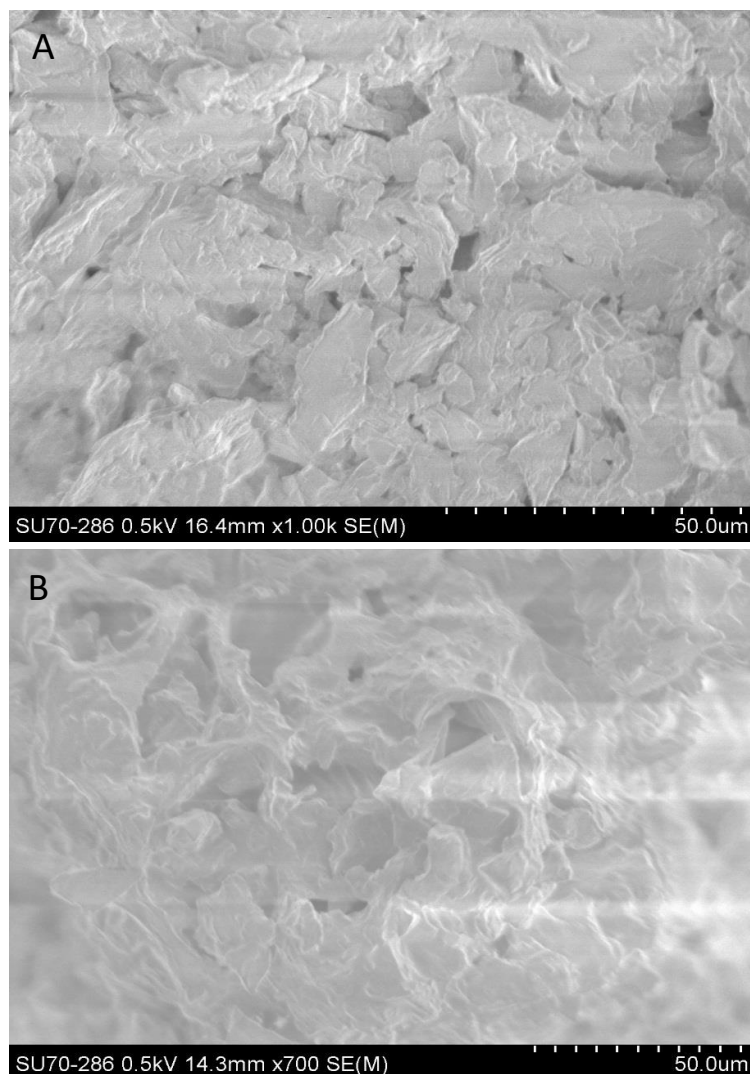


Figure 4.7 SEM images of sequentially pretreated (A) and native (B) SCW

The effects of the various pretreatments applied on SCW can be analysed by changes in functional group pertaining to the lignocellulosic biomass. These changes were analysed by FTIR. Figure 4.8 represents the changes in the composition and the functional groups of pretreated SCW as opposed to raw SCW. Additionally, Table 3.2 represents the important wavenumbers associated with functional groups commonly found in lignocellulosic biomass. The band at 897 cm^{-1} represents changes in the β -glycosidic linkages between cellulose and hemicellulose and any changes in this band represents the intermolecular degradation in the hemicellulose structure which occurs due to its removal. Decrease in absorbance at 1035 cm^{-1} is indicative of the stretching

of bonds C-O, C=C and C-C-O that exist between the polysaccharide and lignin fraction. The bands in the range 1000 cm^{-1} to 1200 cm^{-1} indicate cellulose regions. The decrease of intensity of bands at 1200 cm^{-1} suggests breakage of hydrogen bonds between cellulose and hemicellulose. The bands at 1335 cm^{-1} and 1400 cm^{-1} indicates vibrations of the C-H bond and bending of the lignin-polysaccharide plane. The adsorption peaks at 1509 , 1464 and 1422 cm^{-1} corresponds to lignin and any changes in this region specifies lignin degradation or removal. The bands 1730 cm^{-1} and 1750 cm^{-1} represents the ester moieties in hemicellulose. Diminishing of this peak is indicative of hemicellulose removal. The adsorption peak at 2920 cm^{-1} represents C-H stretching in cellulose and the broad peaks indicates breakage of hydrogen bonds. The broadening of the bands at $3000\text{-}3500\text{ cm}^{-1}$ indicates the stretching of -OH groups.

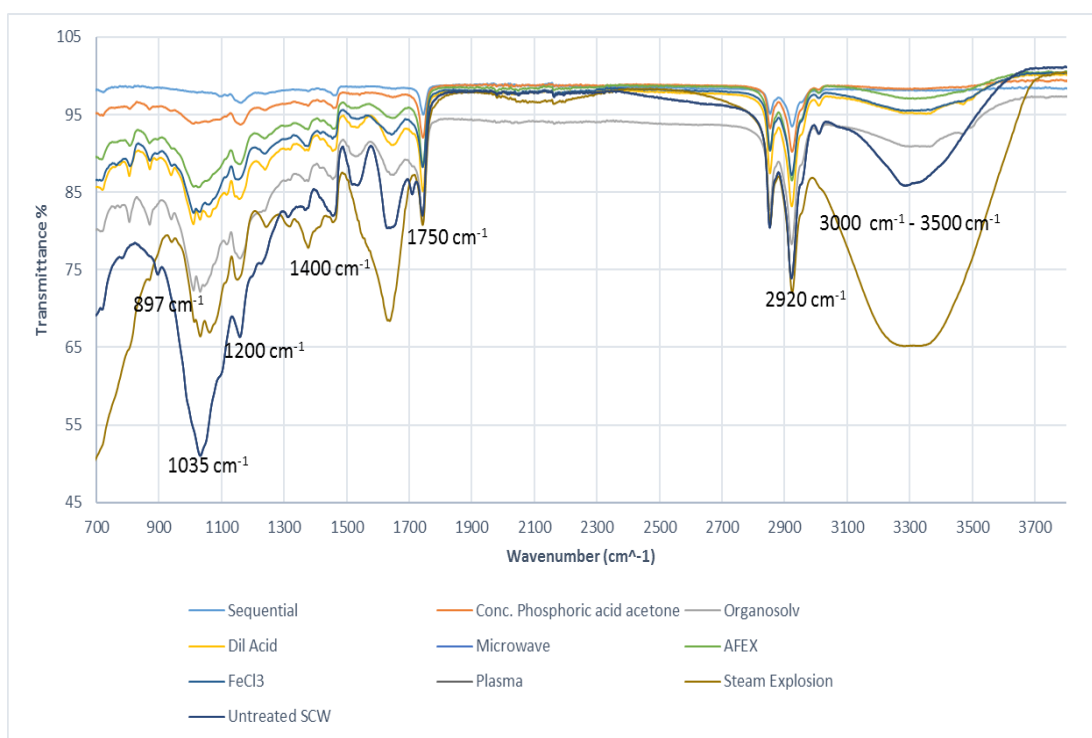


Figure 4.8 FTIR spectra of pretreated and untreated SCW

The FTIR spectrum corresponding to the sequentially pretreated SCW was devoid of any peaks mentioned in the paragraph above which indicated extensive breakage of bonds between cellulose, hemicellulose and lignin. Furthermore, peaks characteristic

to lignin, such as 1509, 1464 and 1422 cm^{-1} was absent in the spectrum. Conc. acid acetone pretreatment also showed similar characteristic in the FTIR spectrum as the sequential pretreatment. There was a stark reduction in peaks 897, 1035, 1200 and 1400 cm^{-1} in organosolv FTIR suggesting disruption in the cellulose and hemicellulose regions. However, the peak at 1750 cm^{-1} remained strong, indicating the presence of hemicellulose. Dilute acid pretreatment appeared to be a very effective strategy for SCW from the differences in peak heights as opposed to the FTIR spectrum of native SCW. AFEX and pretreatment with ferric chloride was also very effective in breaking glycosidic linkages and stretching of bonds between the polysaccharide and lignin fraction. Steam explosion also indicated strain in linkages between lignin and carbohydrates while retaining hemicellulose. Microwave pretreatment and plasma pretreatment conserved the hemicellulose regions as suggested by the peak at 1750 cm^{-1} .

The crystallinity of pretreated and native SCW was assessed by conducting X-ray diffraction. Figure 4.9 represents the changes in crystallinity in different pretreated samples as opposed to untreated SCW. The crystallinity of SCW is contributed by the presence of cellulose. A large fraction of cellulose present in SCW is crystalline in nature. The hemicellulose component of SCW contributes to the amorphous fraction of SCW as are easily susceptible to enzymatic degradation (Ballesteros et al., 2014). Furthermore, the lignin present in SCW also contributes to the amorphous nature of SCW. The increase in crystallinity can be attributed to the effectiveness of the pretreatment. The increase in peaks at the 15° and 22° is caused by the disorderliness of the structure due to the effects of pre-treatments (Pereira et al., 2011). The spectra of different pretreated samples showed an increasing trend in the crystallinity of the biomass with respect to the effectiveness of the treatment. Absence of distinct peaks

for amorphous region was observed as a general trend in the XRD spectra of all the pretreated SCW samples except for acid acetone pretreatment and alkali assisted microwave pretreatment. Sequentially pretreated SCW exhibited highest crystallinity compared to all the other pretreated SCW. Comparatively, microwave pretreated SCW exhibited lowest crystallinity among all the pretreated substrates with distinct peaks from amorphous and crystalline regions respectively. The increase in the crystalline nature of the pretreated samples can be attributed to the attrition in the amorphous components of SCW such as lignin and hemicellulose. Similar observations were reported by Binod et al. (2012) when employing short duration microwave pretreatment to improve the enzymatic hydrolysis of sugar cane bagasse. Also, Raghavi et al. (2016) reported an increase in biomass crystallinity as a result of various pretreatments.

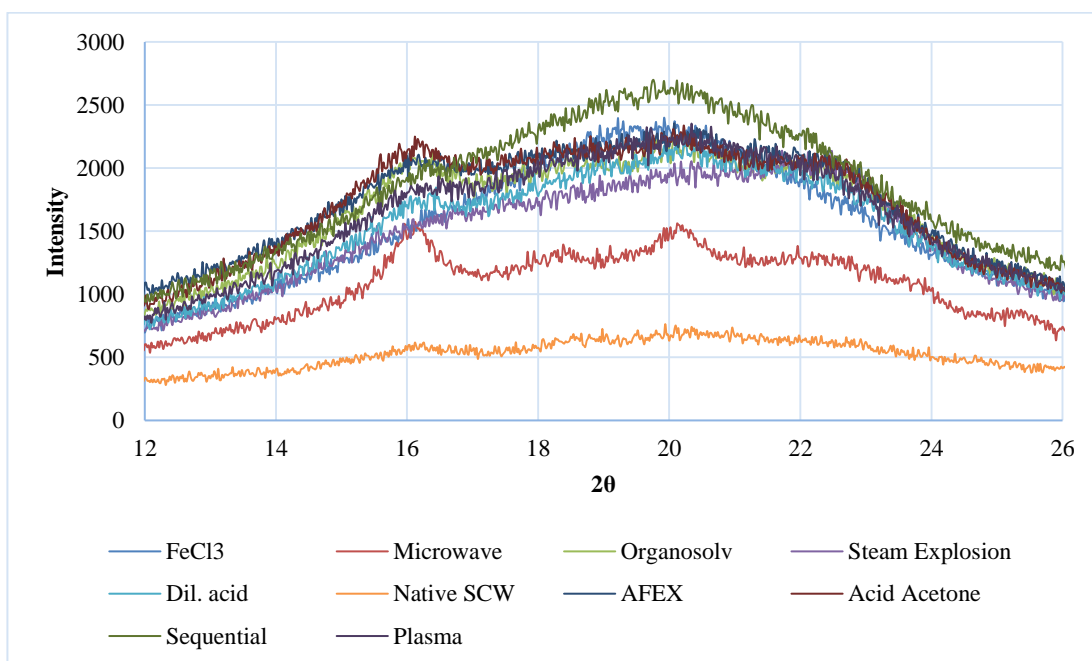


Figure 4.9 XRD spectra of untreated and pretreated SCW

4.4.6 Thermal behaviour study using differential scanning calorimetry

Differential scanning calorimetry is a technique used to determine the heat effects associated with phase transitions and chemical reactions. In this method, heat flow

differences in the sample and the reference (usually an empty aluminium pan) is recorded as a function of temperature. The temperature is increased at a constant rate both in the sample and reference. The heat flow is equivalent to enthalpy since the pressure is maintained constant. Figure 4.10 represents the changes in the thermal behaviour of the native and pretreated SCW between 20°C and 500°C which were obtained at a heating rate of 10°C/min under constant nitrogen atmosphere. The thermogram for native SCW exhibited an exothermic event between 20°C and 102.9°C which occurs due to the vaporisation of water and crystalline nature of the sample. The enthalpy associated with this event was found to be 114.99 J/g. This was succeeded by a phase transformation with a change in the heat capacity, which is a characteristic of every polymer. The glass transition temperature was recorded at 259.2°C. Different pretreated SCW has unique characteristics as far as their thermal behaviours were concerned. For example, microwave pretreatment exhibited an endothermic event and an exothermic event followed by a crystalline peak, which was specific to this type of sample. The thermograms of pretreated samples such as steam explosion, ferric chloride assisted treatment, plasma, organosolv, and AFEX were all similar to that of the native SCW except for the variations in enthalpy. However, thermograms of conc. phosphoric acid acetone treated biomass and SCW that underwent sequential pretreatment were similar to each other while being unique from the rest of the pretreatments. The thermograms of these two pretreatments reported glass transition followed by a crystallisation peak, melting peak and finally an ending transient.

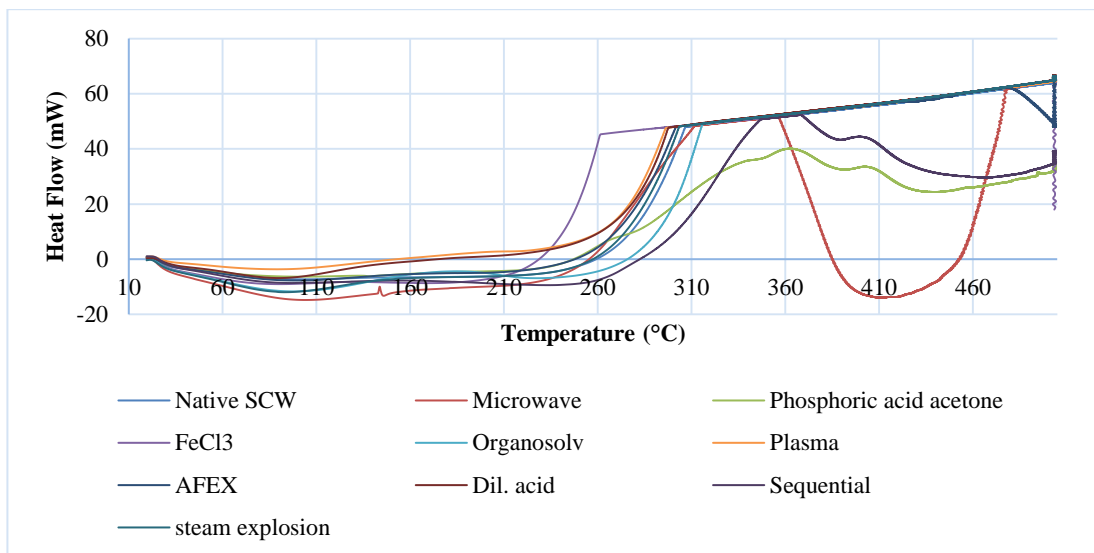


Figure 4.10 DSC thermogram of native and pretreated SCW

4.5 Conclusion

A sequential strategy to combine the effects of two pretreatments was found to be highly efficient in increasing the reducing sugar yield. A 1.7-fold increase in reducing sugar yield was obtained after enzymatic hydrolysis of sequential pretreated SCW as compared to native SCW. FTIR analysis revealed breakage of β -glycosidic bond between cellulose and hemicellulose. Lignin removal was also evident from the FTIR spectra of all the pretreated samples. XRD spectra revealed an increasing trend in crystallinity, which can be attributed to the removal of amorphous structures in SCW. SEM images of the biomass that was subjected to sequential pretreatment exhibited a crumbled structure as opposed to native SCW, which would have contributed in increasing the overall surface area. This can be a reason why enzymatic hydrolysis was enhanced due to sequential pretreatment.

Chapter 5

Evaluation of ultrasound assisted potassium permanganate pretreatment of spent coffee waste

In this chapter, novel pre-treatment for spent coffee waste (SCW) has been proposed which utilises the superior oxidising capacity of alkaline $KMnO_4$ assisted by ultrasonication. The pre-treatment was conducted for different exposure times (10, 20, 30 and 40 min) using different concentrations of $KMnO_4$ (1, 2, 3, 4, 5% w/v) at room temperature with solid/liquid ratio of 1:10. Pretreating SCW with 4% $KMnO_4$ and exposing it to ultrasound for 20 min resulted in 98% cellulose recovery and a maximum lignin removal of 46%. 1.7-fold increase in reducing sugar yield was obtained after enzymatic hydrolysis of $KMnO_4$ pretreated SCW as compared to raw. SEM, XRD and FTIR analysis of the pretreated SCW revealed the various effects of pretreatment. Thermal behaviour of the pretreated substrate against the native biomass was also studied using DSC. Ultrasound-assisted potassium permanganate oxidation was found to be an effective pretreatment for SCW and can be used as a potential feedstock pretreatment strategy.

Work described in this chapter has been published as peer review article:

Evaluation of ultrasound assisted potassium permanganate pre-treatment of spent coffee waste (2017). *Bioresource Technology*, 224, 680-687.

5.1 Introduction

Spent coffee waste (SCW) is the solid material obtained after brewing of instant coffee. It is rich source of lignocellulose. Over the past few years' global coffee production and consumption has been on the rise. It is estimated that the global coffee production in 2015/16 amounted up to 8,604 million tonnes (ICO, 2016). Several pretreatment methods have been devised for lignocellulose over the past decade (Ravindran & Jaiswal, 2016). Dilute acid hydrolysis, steam explosion, dilute alkali assisted microwave treatment, ammonia fibre explosion, wet oxidation, and conc. phosphoric acid-acetone pretreatment are some of the most popular and effect pretreatment strategies for lignocellulose. However, very few studies have been reported on pretreatment of spent coffee waste. Kwon et al. (2013) conducted a study based on SCW by subjecting it to dilute acid pretreatment and subsequent conversion to ethanol. Characterisation of polysaccharides extracted from alkali pretreated SCW was performed by Ballesteros et al. (2015). Hydrothermal pretreatment was selected as the pretreatment strategy by Conde and Mussatto (2015) for the isolation of polyphenols from SCW. Similarly, Scully et al. (2016) investigated spent coffee waste as a renewable source of bioactive compounds and industrially important sugars and considered hydrothermal pretreatment before subjecting to enzymatic saccharification process.

Ultrasonication pretreatment is a novel and effective pretreatment method for lignocellulose (Velmurugan & Muthukumar, 2012a). In an aqueous environment ultrasound waves induce cavitation which in bubbles form tend to collapse and expand with ultrasound waves until they collapse. This leads to the generation of heat with localised temperatures reaching 4730°C and pressures of 14700 psi. The collapse also

leads to the formation of reactive radical species (H, OH, HO₂, O₃ etc.). Ultrasonic reactors can be of probe (horn) type or water bath type. Water bath sonicators are mild in nature compared to probe sonicators (Bussemaker & Zhang, 2013). In a recently published study by Liyakathali et al. (2016) evaluated the effect of frequency and reaction time required for pretreating cane bagasse using ultrasound for bioethanol production. Correspondingly, permanganate oxidation is an effective method in lignin degradation (Bose et al., 1998). Potassium permanganate is inexpensive and potentially less toxic compared to acids and alkalis. It has been widely used for water purification. Although a pretreatment strategy employing potassium permanganate will lead to effluents consisting of the oxidizing agent it will gradually precipitate by generating manganese oxide rendering no harm to the environment (Ma et al., 2015).

In the present chapter, an attempt was made to combine the effects of ultrasound and potassium permanganate oxidation by using a water bath ultrasonic reactor. The optimum potassium permanganate concentration and reaction time were analysed to obtain high cellulose and hemicellulose recovery while achieving maximum lignin removal. Characterisation of the hydrolysate, physical characteristics, crystallinity and structure of the pretreated SCW was analysed by high performance liquid chromatography (HPLC), Field emission scanning electron microscopy (FESEM), X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR) respectively. Furthermore, the thermal behaviour of native as well as pretreated SCW was studied by performing differential scanning calorimetry (DSC).

5.2 Methodology

5.2.1 Pretreatment of SCW

5.2.1.1 Ultrasound-assisted potassium permanganate pretreatment

Dry SCW was dispersed in potassium permanganate solution in conical flasks with a biomass loading of 10% w/v. The reaction times were maintained for 10, 20, 30 and 40 min respectively. The effect of different potassium permanganate solutions (pH 11.5-12) were also studied ranging from 1%, 2%, 3%, 4% and 5%. The samples were subjected to ultrasound radiation in an ultrasonic bath at frequency of 47 kHz and an emission power of 310W (ULTRASONIK™ 57X, Ney Dental Inc., USA). All the experiments were performed at room temperature. The pre-treated residues were washed with distilled water, filtered and then dried at 80°C until constant weight was attained. The dried residues were subjected to compositional analysis to determine the optimal conditions for pretreatment using two step sequential acid hydrolysis (Sluiter et al., 2008). All the tests were done in triplicates.

5.2.1.2 Alkali assisted ultrasonication

For comparative studies, 1g of SCW was taken in a 100 ml conical flask and was suspended in 10 ml of NaOH in 1.0, 2.0, 3.0, 4.0 and 5.0% concentration (w/v). The contents were mixed well and sonicated for 30 min at 25 kHz frequency with an effective ultrasonic power of 150 W using an ultrasonicator (ULTRASONIK™ 57X, Ney Dental Inc., USA). The resultant liquid after ultrasonication was analysed for reducing sugar, hydroxymethyl furfural (HMF) and furfural. The residue after repeated washings with deionized water was dried in an oven at 50°C for 48 h and analysed for different components (Gabhane et al., 2014).

5.2.2 Enzymatic hydrolysis

Enzymatic hydrolysis was performed accordingly to protocol described in section 3.4

5.2.3 Compositional analysis

Raw and pretreated SCW were subjected to compositional analysis by following two-stage acid hydrolysis according to The National Renewable Energy Laboratory (NREL) protocol (Sluiter et al., 2005). The reducing sugar concentration in the hydrolysate was estimated by dinitro salicylic acid (DNS) method (Miller, 1959). The presence and quantification of monosaccharides, organic acids and any inhibitors in the form of furfural and hydroxymethylfurfural was done on an Alliance HPLC (Waters, e2695 Separation module) using a Rezex ROA-Organic acid H+ (8%) column, (350 x 7.8 mm; Phenomenex, UK) with 5 mM H₂SO₄ as the mobile phase at 65°C maintaining a flow rate of 0.6 ml/min (Jaiswal et al., 2012).

The percentage recovery and percentage removal were calculated using the following formula:

$$\% \text{ recovery} = W_{\text{pre}} / W_{\text{raw}} \times 100$$

$$\% \text{ removal} = 1 - \% \text{ recovery}$$

where, W_{pre} and W_{raw} are the weight of the corresponding component (cellulose, hemicellulose and lignin) in pre-treated and raw SCW (g) respectively (Ma et al., 2015).

5.2.4 Characterisation of raw and pretreated substrate

5.2.4.1 FTIR analysis

The changes in functional groups inflicted by pretreating the biomass was assessed using FTIR spectroscopy according to section 3.3.4.

5.2.4.2 X-ray diffraction

The changes in functional groups inflicted by pretreating the biomass was assessed using FTIR spectroscopy according to section 3.3.5.

5.2.4.3 Thermal behavior

The thermal behaviour of the treated and raw samples was studied and compared using differential scanning calorimetry (DSC) by following methodology described in section 3.3.6.

5.2.4.4 Scanning Electron Microscopy

The physical changes incurred in pretreated biomass in comparison with untreated biomass was observed by performing FESEM following procedure described in section 3.3.7

5.2.5 Statistical analysis

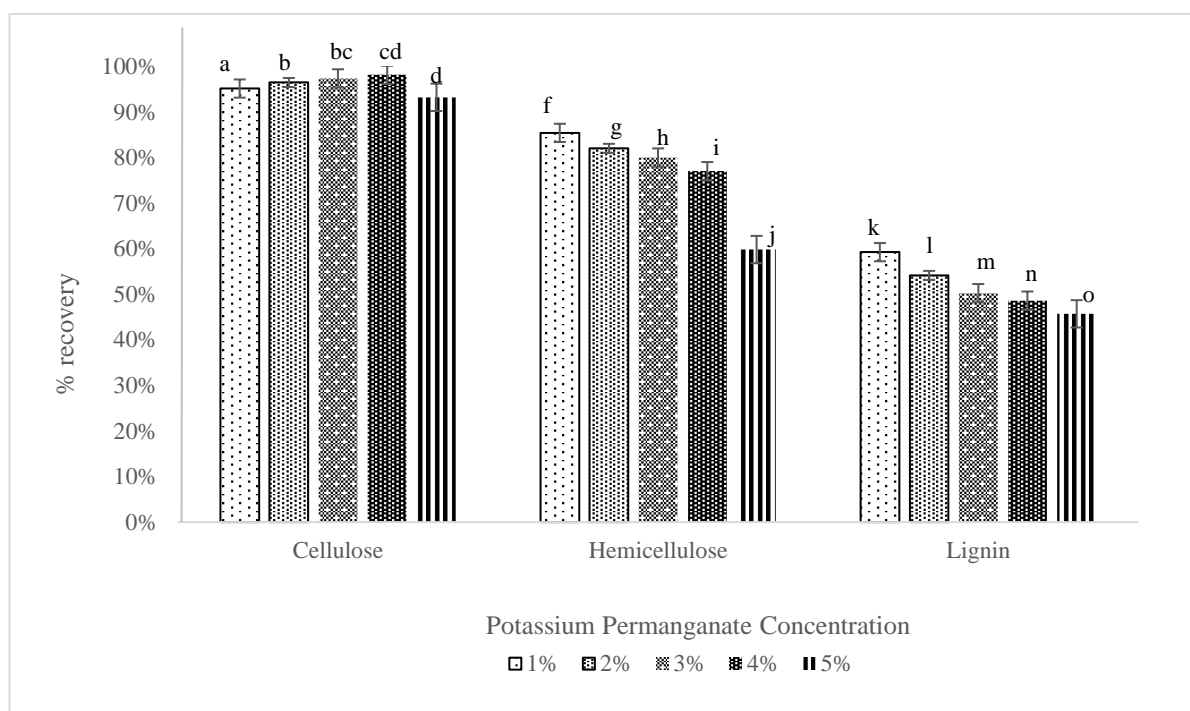
All the experiments were carried out in triplicate and replicated twice unless stated. Significant differences were computed by employing analysis of variance (ANOVA) and multiple comparisons (Fischer's least significant difference test) by employing STATGRAPHICS Centurion XV software (StatPoint Technologies Inc. Warrenton, VA, USA). Value of $p < 0.05$ was considered as significant value.

5.3 Result and discussion

5.3.1 Effect of pre-treatment on composition of spent coffee waste

The compositional analysis of native SCW revealed the presence of four major reducing sugars *viz.* glucose, mannose, arabinose and galactose. This indicated that SCW is rich in glucomannan, arabinogalactan and cellulose structures. SCW was abundant in hemicellulose content (33.5g/100g of dry SCW) as compared to cellulose (8.6g/100g of dry SCW). The total lignin content amounted to 31.1g/100g of dry SCW. The primary objective behind this study was to achieve maximum lignin removal along with maximum cellulose and hemicellulose recovery. This is because lignin removal will improve the degree of enzymatic hydrolysis of lignocellulosic biomass. The lignin removal strategies in this pretreatment process *viz.* potassium permanganate concentration and ultrasonication time were optimised based on cellulose and hemicellulose recovery coupled with lignin removal. The effect of potassium permanganate concentration on the recovery of lignin and polysaccharides have been illustrated in Figure 5.1. Potassium permanganate reacts with lignin to form carboxylic methyl esters. Lignin is more susceptible to oxidation by potassium permanganate compared to the polysaccharide fraction of lignocellulose. Potassium permanganate concentration is directly linked to oxidation capacity and plays an important role in delignification (Gellerstedt, 1992). The changes in the recovery of cellulose incurred was found to be significant with respect to increasing potassium permanganate concentration ($p < 0.05$). Maximum recovery of cellulose was achieved by using 4% (w/v) potassium permanganate solution. However, lignin and hemicellulose recoveries were significantly impacted by the presence of potassium permanganate ($p < 0.05$). Lignin recovery progressively decreased from 59.2% to 46.7% with increasing concentration of potassium permanganate. Likewise, the hemicellulose recovery (85%

to 60%) was also detrimentally affected especially after the concentration of potassium permanganate was increased beyond 4%. Higher concentrations of potassium permanganate resulted in the oxidation of more ester and ether linkages in the hemicellulose fraction. This may be due to the amorphous nature of hemicellulose making it susceptible to extensive solubilisation when the concentration of the oxidising agent was increased.



Means not sharing the same letter are significantly different (LSD) at $P < 0.05$ probability level for respective lignocellulosic components (cellulose, hemicellulose and lignin).

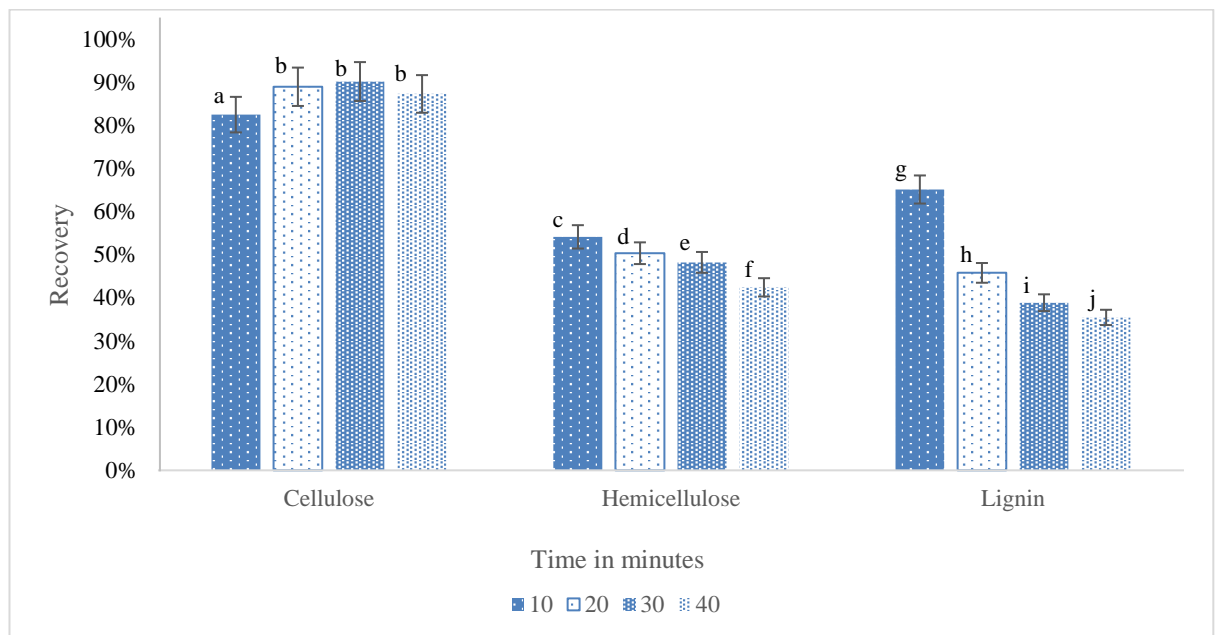
Figure 5.1 Effect of potassium permanganate concentration on the recoveries of SCW residues after pretreatment

Several studies have reported ultrasonication as an effective pretreatment strategy for lignocellulosic biomass. Sonication probes are commonly used as the ultrasonication instrument at high frequencies (Subhedar & Gogate, 2014). However, this study employed a sonication water bath, which is mild as compared to the probe sonicator. Nonetheless the treatment strategy did have an effect on SCW. The effect of time on

pre-treated SCW is shown in Figure 5.1 Effect of potassium permanganate concentration on the recoveries of SCW residues after pretreatment. Cellulose recovery increased with increase in time. The same was not observed in the case of hemicellulose and lignin. The resistance of cellulose to pretreatment conditions can be attributed to its crystalline nature. Velmurugan and Muthukumar (2012b) reported high losses in cellulose and hemicellulose content in sugarcane bagasse as a result of over exposure to strong ultrasound frequencies. However, in this experiment the loss of biomass component due to prolonged exposure to ultrasound radiation was overcome since the longest treatment time only lasted 40 min while utilising a mild sonication instrument. The variations in exposure time had a significant impact on recovery of cellulose ($p < 0.05$). Cellulose recovery amounted up to 87.25% while that of hemicellulose and lignin was 42.42% and 35.45% respectively after 40 min of ultrasonication. Moreover, statistical analysis of the effect of exposure time on the cellulose, hemicellulose and lignin recovery revealed that there is a significant difference between the recoveries of the three major components at 95% confidence interval.

The removal of lignin content can be attributed to the effect of cavitation and the oxidation of the ester and ether bonds which had a deleterious effect on the amount of soluble as well as insoluble lignin content. This phenomenon was also reported by García et al. (2012) when they studied the effect of ultrasound on the properties of alkaline lignin. In an aqueous solution ultrasound waves give rise to highly reactant species such as hydroxyl ions, hydronium ions, ozone which interact lignocellulosic components (cellulose, hemicellulose and lignin) and results in its decay. Exposing lignocellulose to longer periods of ultrasound waves resulted in extensive removal of lignin and hemicellulose. Although lignin removal was at maximum after 40 min the

optimum time for pretreatment was set at 20 min as there was no significant increment in cellulose content after 20 min of pretreatment. All experiments were performed at room temperature thus eliminating the requirement of any heating element and contributing to cost reduction of the overall process.



Means not sharing the same letter are significantly different (LSD) at $P < 0.05$ probability level for respective lignocellulosic components (cellulose, hemicellulose and lignin).

Figure 5.2. Effect of ultrasound exposure on the recoveries of SCW residues after pretreatment

Furfural and HMF are formed as a result of thermochemical treatment of lignocellulose. The absence of inhibitors can be justified by the nature of this pretreatment strategy since it was devoid of any heat or strong acids. This study was important since detoxification processes to remove inhibitors can add up to the cost of ethanol fermentation by a factor of 22% (Lau et al., 2008).

5.4 Characterisation of pretreated spent coffee waste

5.4.1 Fourier transform infrared spectroscopy

The structural and compositional changes in the pretreated SCW was observed and measured by SEM, XRD and FTIR. FTIR spectra (Figure 5.3) was different for

pretreated and raw SCW which indicated differences in the structure of cellulose in both samples. The bands 875 to 930 cm^{-1} indicate glycosidic bonds in cellulose and hemicellulose (Sills & Gossett, 2012). The reduction in intensity of peaks representing glycosidic bonds was observed in pretreated SCW compared to untreated SCW. This can be due to the disruption of these bonds during the pretreatment process, resulting in the release of monosaccharides. The absorbance of band at 1035 cm^{-1} was observed in the pretreated SCW sample which indicates C-O, C=C and C-C-O stretching between polysaccharides and lignin decreased after pretreatment (Tamaki & Mazza, 2011). Also, decrease in peak height at 1200 cm^{-1} (O-H bending in cellulose and hemicellulose) suggested the breakage of hydrogen bonds between cellulose and hemicellulose. The band 1280 cm^{-1} represents C-H bending and is crucial in determining the degree of crystallinity of cellulose (Binod et al., 2012). The new pretreatment strategy was able to remove cellulose crystallinity from the SCW which is evident in the decrease in peak absorbance at 1280 cm^{-1} , representing C-H bending in crystalline cellulose. The bands at 1335 cm^{-1} and 1380 cm^{-1} represent C-H vibrations and bending between lignin and the polysaccharide fractions. Bands 1425 cm^{-1} and 1465 cm^{-1} indicate C-H plane deformation and C-H deformation in lignin respectively (Xu et al., 2013). The bands 1730 cm^{-1} and 1750 cm^{-1} suggest ketone/aldehyde stretch and free ester in hemicellulose (Ruiz et al., 2013). These signals were weaker in the pretreated SCW which suggested that these bonds between lignin and polysaccharides were probably cleaved by the pretreatment step. C-H stretching in cellulose is represented by the band 2920 cm^{-1} and the broad peaks indicate the breakage of hydrogen bonds leading to the rupture of methyl and methylene portions.

The crystallinity of cellulose has been used for several decades to study the changes incurred in the structure post pretreatment. Cellulose crystallinity plays an important role in enzymatic digestibility. Lignocellulose structure can be divided into crystalline and amorphous regions. The crystallinity of lignocellulose is contributed largely by the cellulose fraction while hemicellulose and lignin regions contribute to the amorphous nature. The amorphous nature of hemicellulose makes it highly susceptible to enzymatic hydrolysis. Furthermore, high degrees of harshness of pretreatment result in partial or complete removal of hemicellulose.

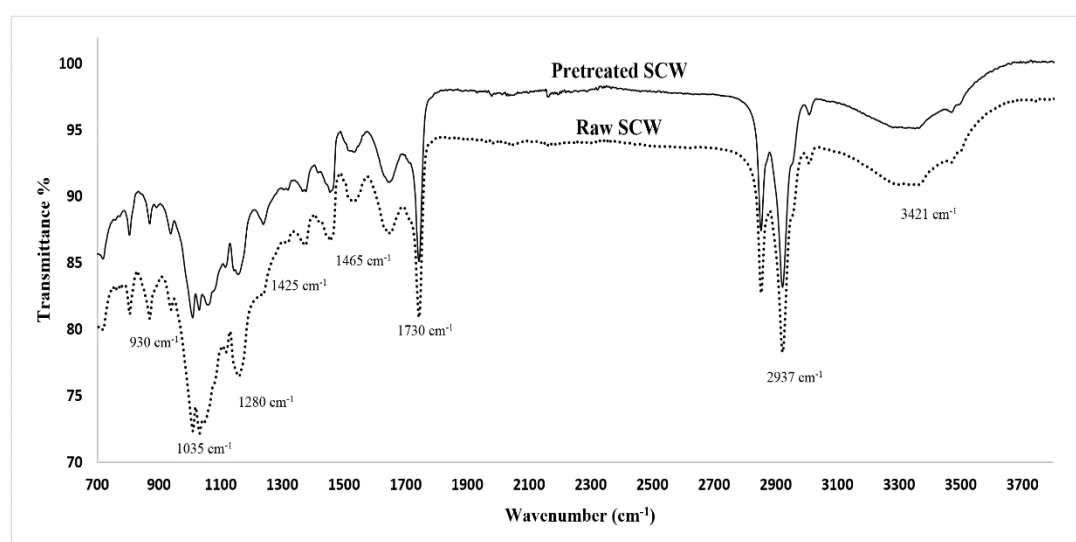


Figure 5.3. FTIR Spectrum of ultrasound assisted KMnO_4 pretreated SCW and raw SCW

5.4.2 X ray diffraction

The XRD spectra of the native and pretreated SCW was compared to evaluate the differences in the crystallinity of the respective biomass. Untreated SCW was found to be less crystalline as compared to treated samples. A unique crystalline peak was observed in both XRD patterns which was contrasting compared to XRD spectra of other lignocellulosic materials Fig. 5.4. The XRD spectra of SCW suggested the presence of higher amount of amorphous material as compared to crystalline counterparts. This can be justified by the studies from the compositional analysis

which has been discussed in earlier sections of this paper. Earlier studies on spent coffee waste reported that crystallinity in SCW is attributed to the presence of cellulose (Ballesteros et al., 2014). The peak at the 16° is an indication of the amorphous structures present in SCW (in this case, hemicellulose). There was a noticeable dip in the peak intensity in the pretreated SCW spectrum at 16° which is an indication of the hemicellulose removal. A difference in peaks between the native biomass and its pretreated counterpart at 22° is an indication of the effectiveness of the pretreatment, caused due to the increase in disorderliness of the structure of the biomass. Raghavi et al. (2016) in their study involving sugar cane bagasse reported a similar increase in biomass crystallinity which was imparted by the increase in cellulose content. Furthermore, an increase in crystallinity signals the removal of amorphous components such lignin and hemicellulose leaving behind the crystalline cellulose. Ballesteros et al. (2015) had reported similar results using alkali pretreatment on SCW.

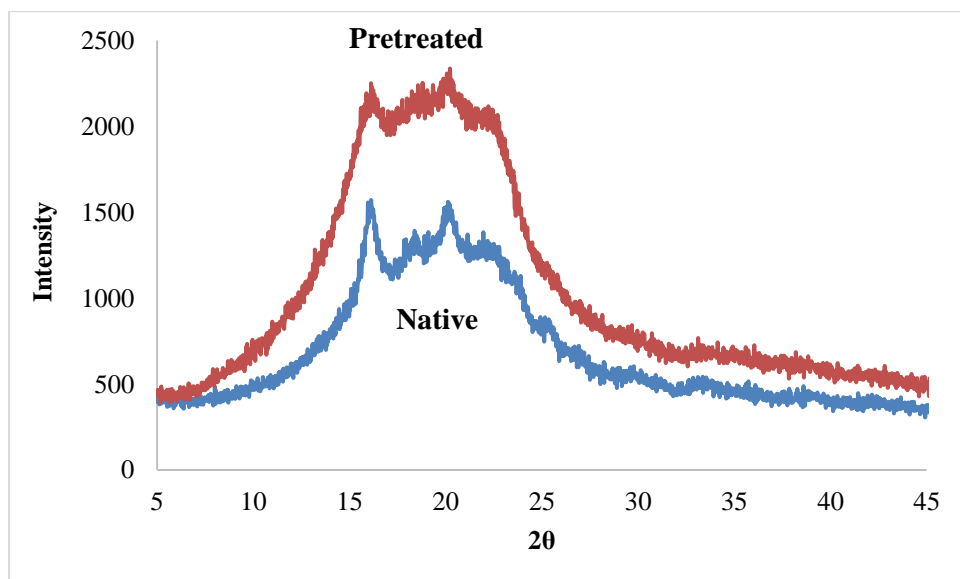


Figure 5.4 XRD spectra for native and pretreated SCW

5.4.3 Differential Scanning Calorimetry

Differential scanning calorimetry determines the heat effects associated with phase transitions and chemical reactions as a function of temperature. The difference in heat flow in the sample and the reference (usually an empty aluminium pan) is recorded as a function of temperature. The temperature is increased at a constant rate both in the sample and reference. Since the pressure is maintained constant, the heat flow is equivalent to enthalpy. The DSC thermogram (Fig. 5.5) represents the thermal characteristics of the native and the pretreated SCW between 20°C and 500°C which were obtained at a heating rate of 10°C/min. The thermogram for raw SCW exhibited an exothermic event which initiated at 20°C and peaked at 102.9°C. This event can be associated with the vaporisation of water. The enthalpy associated with this process was found to be 114.99 J/g. This was succeeded by a phase transformation with a change in the heat capacity which is a characteristic of every polymer. The glass transition temperature was recorded at 259.2°C. Interestingly, the pretreated SCW exhibited two events: an exothermic phase followed by an endothermic phase. The exothermic phase was observed with a peak at 94.8°C. The enthalpy associated with this event amounted to be 79.49 J/g. In contrast with the raw SCW, a crystallisation change of 485.34 J/g. Melting events were not observed for pretreated SCW which suggests the absence of any impurities.

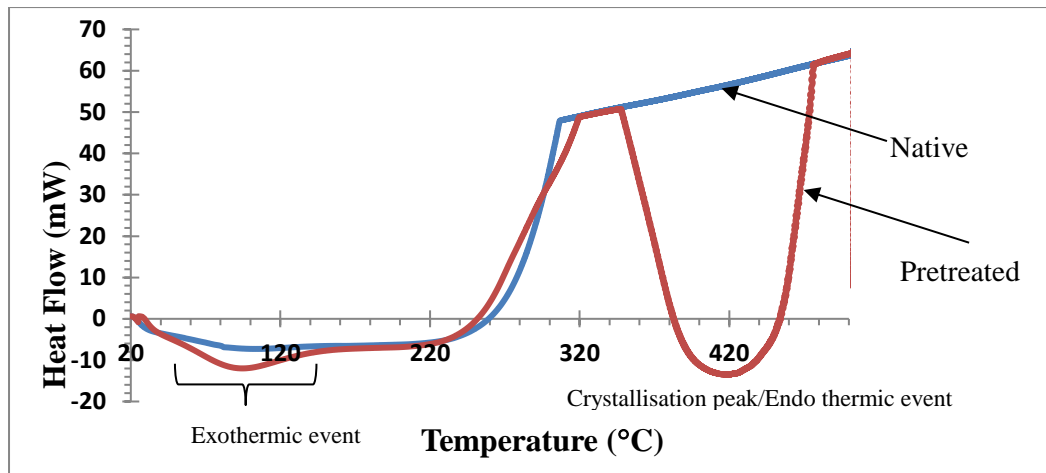


Figure 5.5 DSC thermogram of native and pretreated SCW

5.4.4 Scanning Electron Microscopy

SEM analysis of the raw and pretreated SCW revealed the changes in the physical nature imparted as a result of pretreatment. Earlier studies have shown that SCW is widely porous in nature and resembles thin sheets of saw dust (Ballesteros et al., 2014). Any size reduction in the biomass structure can be attributed to the effect of the cavitation caused by the ultrasound waves. The particles in the pretreated biomass is crumbled in nature compared to the native SCW (Fig. 5.6). This exposed the inner parts of the biomass leading to biomass degradability. Furthermore, this also led to the increase in enzyme access total surface area.

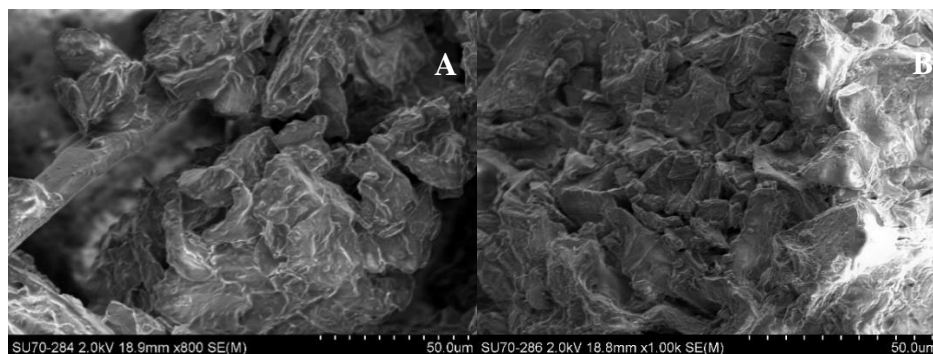


Figure 5.6 SEM image of the spent coffee waste (SCW) (A) native and (B) pretreated (4% KMnO_4 , 47 kHz frequency)

5.5 Conclusion

The evaluation of ultrasound assisted potassium permanganate pretreatment revealed that this method is very effective for lignin and hemicellulose removal from spent coffee waste. Significant lignin removal (45.70%) was achieved with a KMnO_4 concentration of 4% and ultrasound exposure time of 20 min. Additionally this process was mild enough to safeguard the polysaccharide fraction (hemicellulose in particular). A 1.7fold increase in reducing sugar yield was obtained when pretreated SCW was subjected to enzymatic hydrolysis compared to untreated SCW. The most important feature of ultrasound assisted potassium permanganate treatment is that no heat was used as part of this process thus rendering this technique a simple, fast and effective pretreatment strategy.

Chapter 6

Evaluation of the effects of pretreatment on properties of brewers spent grain

In this chapter, a range of physical, chemical and physico-chemical pretreatments were examined to study the effect of each pretreatment on increasing reducing sugar yield from the brewer's spent grain (BSG). Changes in chemical composition of native as well as pretreated biomass was studied. Enzymatic hydrolysis was conducted based on a response surface methodology study performed to obtain maximum reducing sugar. Accordingly, a maximum reducing yield was observed with high biomass

loading (1g/10 ml), cellulase (158.76 µl/10ml), hemicellulase (153.3 µl /10ml), pH (5.4) and an incubation time (120h). Brewers spent grain was high in cellulose (19.21g/100g of BSG) and lignin content (30.84g/100g of BSG). Glucose and xylose was found to be the predominant sugars in BSG. Microwave assisted alkali pretreatment was found to be the most effective pretreatment for BSG. The pretreatment was conducted at 10W was for a time period of 30s. Upon enzymatic hydrolysis, microwave assisted alkali pretreated BSG yielded 228.25 mg of reducing sugar /g of BSG which was 2.86-fold higher compared to native BSG (79.67 mg/g of BSG). This pretreatment strategy was also successful in de-lignifying BSG. The changes in functional groups, crystallinity and thermal behaviour was studied by means of FTIR, XRD and DSC respectively.

Work described in this chapter has been published as a peer review article:

A comparative analysis of pretreatment strategies on the properties and hydrolysis of brewers' spent grain (2018). *Bioresource Technology*, 248, 272-279.

6.1 Introduction

Brewer's spent grain is a by-product of the brewing industry. It is obtained from barley and is essentially the outer pericarp seed coat layer of the malted barley grain that remains after the mashing process (Mussatto et al., 2006). It is structurally heterogeneous in nature and consists of husk, pericarp and fractions of endosperm (Forssell et al., 2008). Almost 30% of the starting malted grain end up as BSG by the end of the brewing process. Every hectolitre of beer generated 15-20 kg of BSG which corresponds to 34 million tonnes of wet BSG generated annually in Europe (Xiros & Christakopoulos, 2012). Although rich in polysaccharides, proteins and lignin, which can be used for industrial exploitation BSG is generally used as animal feed. 'The New Waste Framework Directive' was introduced by the EU in 2008/09 as a new approach to curb food waste such as BSG and as well as new processes for reuse and recycling

(Ravindran & Jaiswal, 2016b). BSG is mainly composed of cellulose hemicellulose and lignin along with considerable amounts of proteins and lipids. The chemical composition of BSG along with its high fibre content enables it to act as a potential feedstock for several commercial processes with applications in biotechnology, thermochemical and biochemical engineering such as renewable energy, substrate cultivation, enzyme production, bread making, ethanol, butanol and xylitol, activated carbon, charcoal, lignin, oligo saccharides etc. (Ferraz et al., 2013).

As mentioned in the earlier section BSG is lignocellulosic in nature and is mainly composed of polysaccharides, lignin, lipids and a small fraction of proteins. The polysaccharides in BSG are represented by cellulose (formed by repeating units of β -D-glucopyranose) and hemicellulose (heteropolymer formed by non-cellulose sugars such as mannose, xylose, galactose and arabinose) and comprise almost 45% of total dry weight. Lignin, a highly branched phenyl propane polymer, forms the rest of the BSG composition (Mussatto et al., 2006). The effective utilisation of BSG for valorisation requires techniques to disrupt the naturally ordered structure and remove lignin. Pretreatments are essential strategies that can achieve this aim and facilitate the exposure of the polysaccharides for efficient utilisation (Ravindran & Jaiswal, 2016a).

Pretreatment methods are essential in increasing the efficiency of processes that involve the valorisation of lignocellulosic materials. They can broadly be classified into physical, chemical, biological and combinatorial with respect to the mechanism behind the process. An efficient pretreatment strategy essentially should be simple, cost effective, devoid of corrosive materials and should not give rise to indigestible or inhibitory compounds. Furthermore, the fraction of interest (polysaccharide or lignin)

should be safeguarded and should not result in its considerable loss. Lastly, the whole process must be economically feasible.

The effect of several pretreatment studies have been studied in different studies as a part of valorisation of brewers spent grain. Macheiner et al. (2003) used microwave radiation as a pretreatment measure for BSG for increasing the efficiency of enzymatic hydrolysis. Accordingly, 25% release of saccharides was achieved after 4h of incubation at 50°C. An interesting study was published by Wolters et al. (2016) where BSG was used for the production of erinacine C production, a secondary metabolite produced by a medicinal fungus *Hericium erinaceus*. Acid hydrolysis using 0.2M H₂SO₄ was employed as the pretreatment measure in this study. A novel pretreatment for BSG was devised by Zhang and Zang (2016) where they used calcined red mud to pre-treat BSG for subsequent hydrogen production. Liguori et al. (2015) utilised acid-alkali pretreated BSG for ethanol production with 55% efficiency. However, a comprehensive analysis on the effects of different physical and chemical pretreatments on the composition of BSG and efficiency of enzymatic hydrolysis are yet to be performed.

This chapter presents the various attributes of the changes undergone by BSG when it was subjected to six different pretreatments. The changes in its chemical composition, structural conformation, crystallinity and thermal behaviour was analysed and compared upon subjecting BSG to different pretreatment strategies. The pretreated BSG as well as native BSG samples were then subjected to enzymatic hydrolysis using optimised parameters. The pretreatment liquor was analysed for the incidence of monosaccharides and inhibitory compounds.

6.2 Methodology

Brewer's spent grain was generously donated by Metalman Brewing Co., a brewery in Waterford. The BSG was dried at 60°C for 48h and thereafter ground and sieved using a 350 µm sieve. It was then stored at room temperature in a cool and dry place for further experiments. All the chemicals such as cellulase from *Trichoderma reesei*, hemicellulase from *Aspergillus niger*, conc. H₂SO₄, alkaline potassium permanganate, ethanol, ferric chloride, and other chemicals required for experimentation were purchased from Sigma-Aldrich (now Merck), Ireland. Cellulase activity was assayed by following laboratory analytical procedures for the measurement of cellulase activity devised by National Renewable Energy Laboratory (Adney & Baker, 1996). Meanwhile hemicellulase activity was assayed followed protocols described by Rickard and Laughlin (1980). Cellulase enzyme registered an enzyme activity of 77 FPU/ml while hemicellulase showed 72 U/ml enzyme activity.

6.2.1 Screening of various pretreatments of BSG

6.2.1.1 Dilute acid hydrolysis

BSG (1% w/v) was mixed with 10 ml of sulphuric acid (1%, 2% and 3% v/v) in a 100 ml flask. The reaction mixture was subjected to 121°C for 10, 20 and 30 min in an autoclave (Zheng et al., 2013). The solids were separated from liquids by centrifugation after pretreatment and the filtrate was analysed for the release of any individual sugars and by-products such as acetic acid, furfural and hydroxymethyl furfural. The solids were dried and stored in cool and dry place until further analysis.

6.2.1.2 Steam Explosion

Steam explosion of BSG was performed as described by Huang et al. (2015) with certain modifications. Five grams of BSG was moistened with de-ionized water to attain 50% moisture content (w/v). The samples were then loaded in to a stainless-steel autoclave. The temperature was raised and maintained at 121°C for 30 minutes after which the pressure was released by opening the pressure valve subjecting the biomass to an ‘explosion’. The steam exploded BSG was collected, dried and stored in sealed plastic bags for further analysis.

6.2.1.3 Ammonia Fiber Explosion (AFEX)

Ammonia Fibre Explosion was performed by soaking 2.5 g of BSG with 25 ml of NH_4OH and then subjecting it to high pressure and temperature in an autoclave. The experiment was carried out at 120°C, water loading of 2.0 g water/g dry biomass, for a residence time of 30 min. The treated biomass was removed from the autoclave and air-dried overnight (about 12 h) in a fume hood to remove residual ammonia. The treated samples were stored in a cool and dry place (Shao et al., 2013).

6.2.1.4 Pretreatment using Ferric Chloride

Ferric chloride pretreatment was implemented on BSG according to the procedure described by Chen et al. (2015) with minor modifications. BSG (10% w/v) was mixed in 50ml of 0.1M FeCl_3 in an Erlenmeyer flask and the subjected to high pressure (15 psi) and temperature (120°C) for 30 minutes in an autoclave. The solids were separated from the liquid after allowing the mixture to cool. The solids were then washed five times with deionized water to remove any residual FeCl_3 . The Fe (III) in the liquor was precipitated by gradually the neutralising the solution using 0.1M NaOH. Once

the precipitate was removed the salt free liquor was subjected to HPLC analysis to identify and quantify any individual sugars present as well as hydroxyl-5-methyl furfural and furfural content. The solids were dried and stored for enzymatic hydrolysis and further analysis.

6.2.1.5 Organosolv Pretreatment

Organosolv pretreatment was performed as described by Ostovareh et al. (2015) with minor modifications. 1% (w/v) dry BSG was mixed in 25 ml of ethanol-water mixture (60% ethanol (v/v)) in an Erlenmeyer flask. In all experiments, 1% of sulphuric acid (w/w) per gram substrate was added as a catalyst. The reaction temperature was set at 100 °C for 30 min. After the reaction the flasks were then placed in an ice chamber to cool the contents quickly to room temperature. The contents were centrifuged at 8000 rpm for 12 minutes to separate the solids from the liquids. The solids were washed with 250 ml of 50% ethanol mixture to extract the soluble products into the liquid phase. The pre-treated substrate was then washed several times with distilled water until pH 7 was obtained. Ethanol was evaporated from the liquid fraction and recovered by condensation leaving behind a precipitate which mainly contained lignin. The pretreated BSG was dried and stored for enzymatic hydrolysis and further analysis.

6.2.1.6 Microwave assisted alkali pretreatment

Microwave-alkali pre-treatment was carried out following the procedure described by Binod et al. (2012) with minor modifications. A domestic microwave (Sharp/R-269 KM) was employed for this purpose. 1% (w/v) biomass was loaded to 0.5% NaOH (w/v) solution in a stoppered flask and subjected to microwave radiation at varying power settings of 10 W, 50 W, and 100 W for different residence time varying from

30s, 60s and 120s. After pre-treatment, the biomass was thoroughly washed with distilled water till pH 6.0 and dried in air. The dried solid residue was used for enzymatic hydrolysis and compositional analysis.

6.2.2 Compositional analysis

Compositional analysis of pretreated and untreated BSG was performed according to the protocol described in section 3.3.1

6.2.3 Enzymatic hydrolysis of BSG

Enzymatic hydrolysis of BSG was performed by applying optimized parameters according to section 3.5

6.2.4 Individual sugar inhibitor and organic acid analysis

The liquor obtained after each pretreatment was analysed for the presence of monosaccharides organic acids, acetyl or any inhibitory compounds such as furfural and hydroxymethyl furfural following protocol described in section 3.3.3.

6.2.5 Characterization of native and pretreated substrate

6.2.5.1 X-ray diffraction

The changes in crystallinity of the pretreated and untreated substrates were studied following methodology detailed in section 3.3.5.

6.2.5.2 FTIR analysis

The changes in functional groups inflicted by pretreating the biomass was assessed using FTIR spectroscopy according to section 3.3.4.

6.2.5.3 Thermal behavior

The thermal behaviour of the treated and raw samples was studied and compared using differential scanning calorimetry by following methodology described in section 3.3.6.

6.3 Results and discussion

6.3.1 Optimisation of enzymatic hydrolysis parameters using RSM

The parameters for enzymatic hydrolysis of BSG was optimised by using response surface methodology. This ensured that maximum reducing sugar was released hydrolysis experiments. The total reducing sugars obtained after the 30 experiments conducted have been listed in table 6.1. The model was compared based on coefficient of determination (R^2) and adjusted coefficient of determination (R^2 -adj). R^2 is regression of sum of squares proportional to the sum of squares. The value of R^2 ranges from 0 to 1 and a value closer to one indicates that the model is accurate.

Table 6.1 CCD experimental designs for five independent variables, experimental and predicted values for total reducing sugar

| Biomass loading (g/10ml) X_1 | Cellulase loading (μ l/10ml) X_2 | Hemicellulase loading (μ l/10ml) X_3 | Incubation Time (h) X_4 | pH X_5 | Experimental total reducing sugar (mg/ml) | Predicted total reducing sugar (mg/ml) |
|-----------------------------------|--|--|------------------------------|-------------|---|--|
| 0.8 | 0.30 | 0.24 | 96 | 5.4 | 16.74 | 16.81 |
| 0.8 | 0.30 | 0.24 | 48 | 6.6 | 13.27 | 13.34 |
| 0.8 | 0.30 | 0.12 | 96 | 6.6 | 16.08 | 15.93 |
| 0.6 | 0.15 | 0.18 | 72 | 6.0 | 15.21 | 15.30 |
| 0.6 | 0.45 | 0.18 | 72 | 6.0 | 14.14 | 14.10 |
| 0.6 | 0.45 | 0.18 | 72 | 6.0 | 14.14 | 14.10 |
| 0.8 | 0.30 | 0.12 | 48 | 5.4 | 13.84 | 13.69 |
| 0.6 | 0.75 | 0.18 | 72 | 6.0 | 13.09 | 13.18 |
| 0.8 | 0.60 | 0.24 | 96 | 6.6 | 14.44 | 14.51 |
| 0.8 | 0.60 | 0.12 | 48 | 6.6 | 12.73 | 12.58 |
| 0.6 | 0.45 | 0.60 | 72 | 6.0 | 13.19 | 13.72 |

| | | | | | | |
|-----|------|------|-----|-----|-------|-------|
| 0.8 | 0.60 | 0.12 | 96 | 5.4 | 15.64 | 15.49 |
| 0.4 | 0.60 | 0.24 | 96 | 5.4 | 14.14 | 14.20 |
| 0.4 | 0.60 | 0.12 | 48 | 5.4 | 11.73 | 11.58 |
| 0.6 | 0.45 | 0.18 | 24 | 6.0 | 10.40 | 10.49 |
| 0.6 | 0.45 | 0.18 | 72 | 4.8 | 14.48 | 14.57 |
| 0.6 | 0.45 | 0.18 | 120 | 6.0 | 15.69 | 15.78 |
| 0.6 | 0.45 | 0.18 | 72 | 6.0 | 14.14 | 14.10 |
| 0.4 | 0.60 | 0.12 | 96 | 6.6 | 13.56 | 13.41 |
| 0.4 | 0.30 | 0.12 | 48 | 6.6 | 12.25 | 12.10 |
| 0.6 | 0.45 | 0.18 | 72 | 6.0 | 14.14 | 14.10 |
| 0.2 | 0.45 | 0.18 | 72 | 6.0 | 12.76 | 12.85 |
| 0.6 | 0.45 | 0.30 | 72 | 6.0 | 13.81 | 13.46 |
| 0.4 | 0.30 | 0.12 | 96 | 5.4 | 15.85 | 15.70 |
| 0.6 | 0.45 | 0.18 | 72 | 7.2 | 13.28 | 13.37 |
| 1.0 | 0.45 | 0.18 | 72 | 6.0 | 15.33 | 15.42 |
| 0.4 | 0.30 | 0.24 | 96 | 6.6 | 14.42 | 14.49 |
| 0.4 | 0.30 | 0.24 | 48 | 5.4 | 12.08 | 12.14 |
| 0.4 | 0.60 | 0.24 | 48 | 6.6 | 11.13 | 11.20 |
| 0.8 | 0.60 | 0.24 | 48 | 5.4 | 12.68 | 12.75 |

An R^2 value of 98.92 and adj- R^2 value of 96.5 illustrated that the model adequately fitted the data. The data obtained from the experiment were fit into a second order polynomial equation. The polynomial equation depicted the relationship between various parameters used in the model and is mentioned below:

$$\begin{aligned} \text{Reducing sugar (mg/ml)} = & 0.711 + 3.05458X_1 - 0.002755X_2 + 0.00879306X_3 + \\ & 0.211115 X_4 + 1.38194X_5 + 0.2375X_1^2 - 0.0833X_1 X_2 + 0.0239X_1X_3 - 0.0052X_1X_4 + \\ & 0.0312X_1X_5 + 0.0158X_2^2 + 0.0166X_2X_3 - 0.0371X_2X_4 + 0.0011X_2X_5 - 0.0352X_3^2 + \\ & 0.0289X_3X_4 + 0.0104X_3X_5 - 0.0417X_4^2 - 0.0126X_4 X_5 - 0.0881X_5^2 \end{aligned}$$

X_1 , X_2 , X_3 , X_4 and X_5 represent biomass loading, cellulase, hemicellulase, pH and incubation time respectively. Analysis of variance (ANOVA) was used to determine the significance of the coefficients of the models. The ANOVA table indicated that 7 effects had P value less than 0.05 rendering them significantly different from the confidence interval spanning from zero to 95.0%. This also illustrated that these

factors had considerable influence on the reducing sugar yield. All the linear coefficients were found to have a positive effect on the reducing sugar yield. However, positive significant interaction effects on reducing sugar release were exhibited only by biomass loading and time.

Three-dimensional response plots were generated to understand the interactions between different variables as well as to determine the optimal level of each variable for maximum response (Fig. 6.1). This gave further insights on the interaction between the five factors tested. The contour plots were indicative of significant interaction between each parameter considered in this study. The highest point on the three-dimensional plots represents the optimum conditions for maximum reducing sugar release. Accordingly, a maximum reducing yield was observed with high biomass loading (1g/10 ml), cellulase (158.76 μ l/10ml), hemicellulase (153.3 μ l /10ml), pH (5.4) and an incubation time (120h). The model predicted the maximum sugar yield to be 19.42 mg/ml when using the optimised parameters for enzymatic hydrolysis.

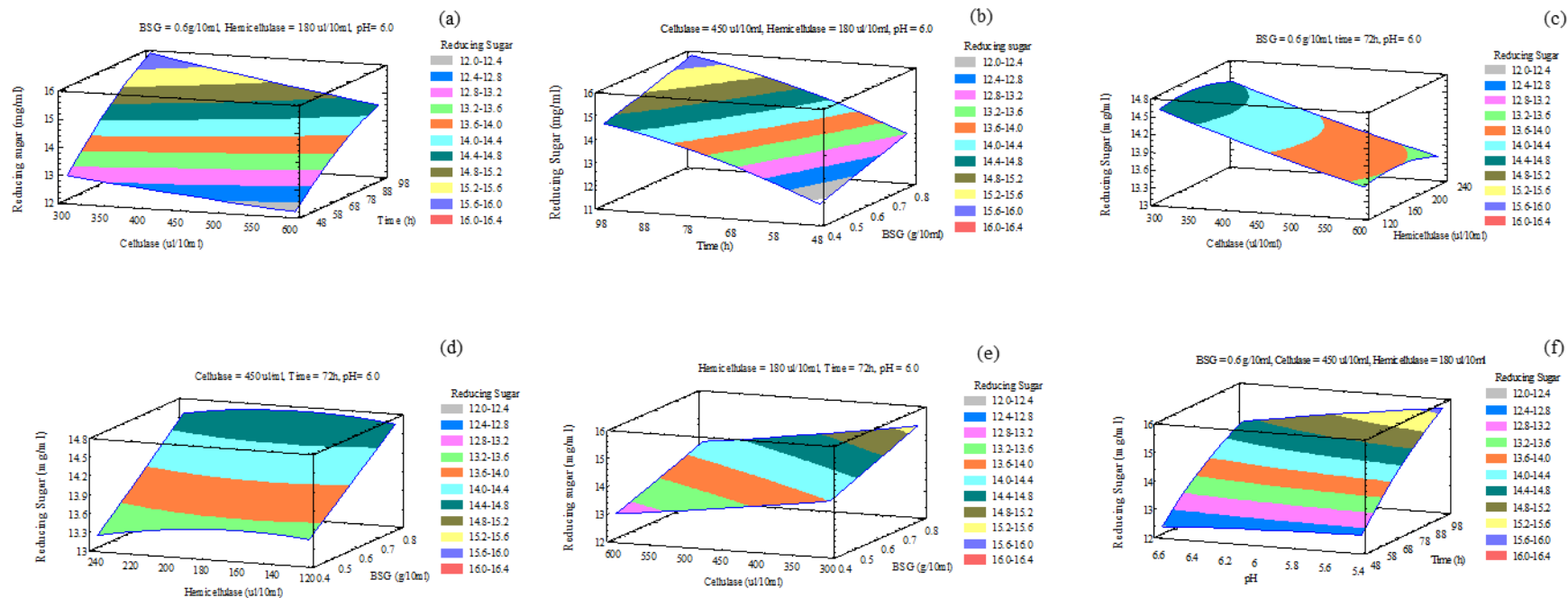


Figure 6.1 Response surface plots representing the effect of independent variables on reducing sugar yield (6.1a) the effect of cellulase and time on reducing sugar yield when the response surface is fixed at BSG = 0.6 g/10 ml, hemicellulase = 180 μ l/10 ml and pH = 6.0; (6.2b) representing the effect of time and BSG on reducing sugar yield, when the response surface is fixed at cellulase = 450 μ l /10 ml, hemicellulase = 180 μ l/10 ml, pH = 6.0; (6.3c) representing the effect of cellulase and hemicellulase on reducing sugar yield, when the response surface is fixed at BSG = 0.6 g/10 ml, time = 72h, pH = 6.0; (6.4d) representing the effect of hemicellulase and BSG on reducing sugar yield, when the response surface is fixed at cellulase = 450 μ l /10 ml, time = 72 h, pH = 6.0; (6.5e) representing the effect of BSG and cellulase on reducing sugar yield, when the response surface is fixed at hemicellulase = 180 μ l /10 ml, time = 72 h, and pH = 6.0 (6.6f) representing the effect of time and pH on reducing sugar yield, when the response surface is fixed at BSG = 0.6 g/10 ml, hemicellulase = 180 μ l /10 ml, cellulase = 450 μ l/10 ml.

A reducing sugar concentration of 18.61 ± 0.5 mg/ml confirmed that the model was valid for the enzymatic hydrolysis of BSG due to little disparity ($< 5\%$) between predicted and observed values.

6.3.2 Influence of pretreatments on composition of BSG and reducing sugar yield

Pretreatments breakdown lignocellulose creating disorder in an otherwise orderly structure with or without the removal of inherent components. The effect of each pretreatment varies according to its mode of action (for example: physical, chemical, physico-chemical and biological) (Ravindran & Jaiswal, 2016a). The final chemical composition of the biomass post pretreatment is an important factor that governs the efficiency of enzymatic hydrolysis. For a lignocellulosic substrate to be effectively used in a bioconversion process it is necessary to relatively expose cellulose fibres along with possible removal of hemicellulose and lignin fractions. BSG was comprised of 19.21g of cellulose (glucose), 26.94g of hemicellulose and 30.48g per 100g of dry biomass (Table 6.2). The values obtained were more or less similar to composition of BSG described elsewhere. Any variation can be attributed to the source of collection of BSG. The composition of BSG can vary with respect to the barley variety, harvest time and mashing conditions (Forssell et al., 2008; Robertson et al., 2010). The aim of this study was to increase the cellulose content there by facilitating better recovery of reducing sugars on enzymatic hydrolysis. In this study BSG was subjected to six different pretreatments which were of physical and chemical in nature. After each pretreatment the biomass was subjected to chemical composition analysis. Furthermore, all the pretreated samples were subjected to hydrolysis employing polysaccharide hydrolysing enzymes and optimised parameters.

Table 6.2 Compositional analysis of untreated and pretreated samples of BSG

| Pretreatment | Cellulose (glucose) | Galactan | Arabinan | Xylan | Mannan | AIL | ASL |
|---------------------------------|--------------------------------|-----------------|-----------------|--------------|---------------|------------|------------|
| AFEX | 21.32±0.9 | 0.40±0.3 | 2.91±0.4 | 11.23±0.3 | 0.30±0.1 | 21.10±0.1 | 7.26±0.4 |
| Steam Explosion | 19.31±0.45 | 0.83±0.12 | 6.57±0.3 | 15.41±0.7 | 0.43±0.5 | 21.87±0.7 | 7.01±0.6 |
| Dil. Acid hydrolysis | 35.43±1.1 | 0.35±0.9 | 2.54±0.75 | 13.52±0.4 | 0.21±0.3 | 21.76±0.4 | 4.42±0.9 |
| Ferric Chloride | 17.99±0.7 | 0.45±0.4 | 5.28±0.8 | 3.61±0.9 | 0.21±0.2 | 20.61±0.5 | 5.69±0.21 |
| Organosolv | 26.42±0.4 | 0.39±0.1 | 2.96±0.15 | 6.45±0.4 | 0.25±0.7 | 11.68±1.4 | 5.89±0.43 |
| Microwave-alkali | 43.67±1.2 | 0.70±0.6 | 5.35±0.2 | 11.53±0.2 | 0.46±0.54 | 9.09±0.65 | 2.77±0.9 |
| Control | 19.21±1.3 | 1.06±0.4 | 7.99±0.9 | 17.21±0.7 | 0.68±0.1 | 23.36±1.2 | 7.12±0.4 |

Cellulose, Galactan, Arabinan, Xylan, Mannan, acid soluble and acid insoluble lignin are presented as g/100g of dry BSG

In general, all the pretreatments tested were effective in increasing the efficiency of enzymatic hydrolysis. This was evident from the increase in reducing sugar release of pretreated BSG samples (Fig. 6.2). Dilute acid hydrolysis was conducted for BSG following different experimental settings. There was almost 50% loss in biomass post pretreatment. The effect of acid concentration and reaction time on the effectiveness of acid hydrolysis for BSG was studied by employing three acid concentrations (1%, 2% and 3%) and three different time settings (10, 20, and 30 min). The solids obtained after pretreatment were subjected to enzymatic hydrolysis. Longer treatment times resulted in reduction in reducing sugar yield (Fig. 6.3). Best results were achieved when BSG was treated with 3% H₂SO₄ for 20 min (208.78 mg/g of BSG). Compositional analysis of the biomass revealed that subjecting BSG to dilute acid hydrolysis resulted in the increase in cellulose content (35.43g/100g of BSG) as well as removal of considerable fractions of hemicellulose. This was evident in the reduction in the xylan, galactan, mannan and arabinan content. Also, there was obvious reduction in the acid soluble lignin fraction (4.42g/100g of BSG).

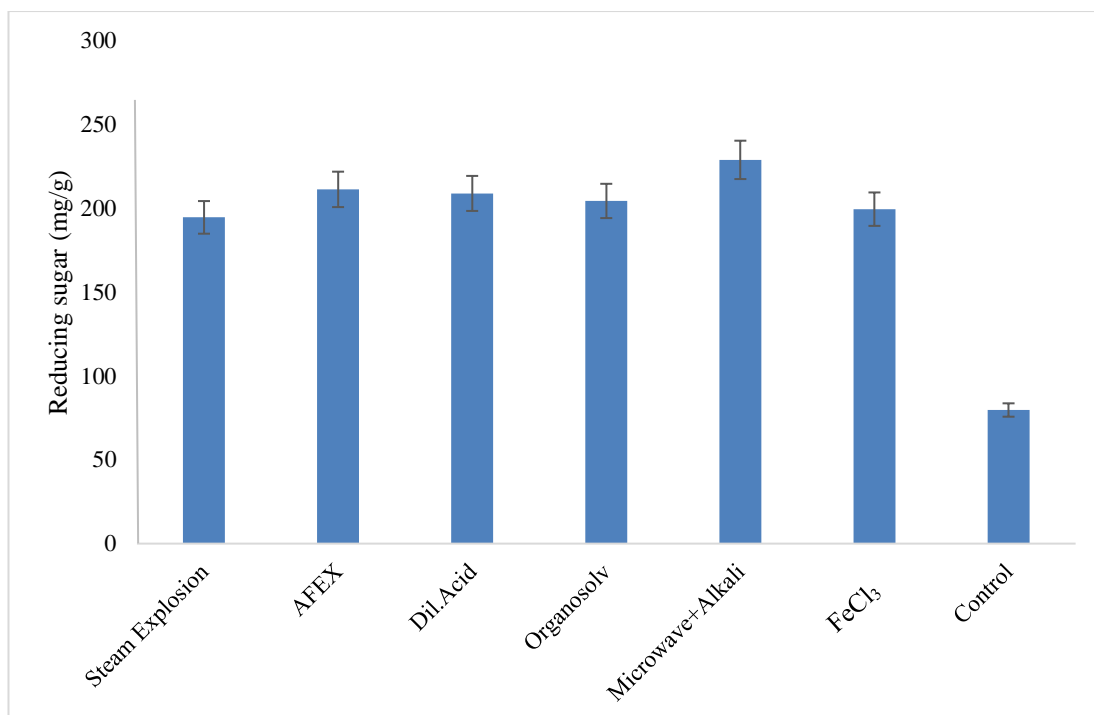


Figure 6.2 Total reducing sugar released after enzymatic hydrolysis of pretreated BSG

Microwave assisted alkali pretreatment was found to be the most effective pretreatment for BSG. Three different power settings (10W, 50W, 100W) and three-time durations (30s, 60s, 120s) were employed to study the individual effects on each parameter on reducing sugar yield conducted at different settings of power and time. Irradiating BSG at 10W for 60s was found to be the setting for this pretreatment strategy (Fig. 6.4). After the pretreatment the cellulose content in the BSG increased (43.67g/100g dry wt.) while the hemicellulose and lignin fractions diminished (Table 3) which was by far the best cellulose recovery obtained among all the treatments studied. Furthermore, subjecting BSG to microwave radiation presence of alkali resulted in highest reducing sugar yield (228.78 mg/g of BSG) (Figure 6.2).

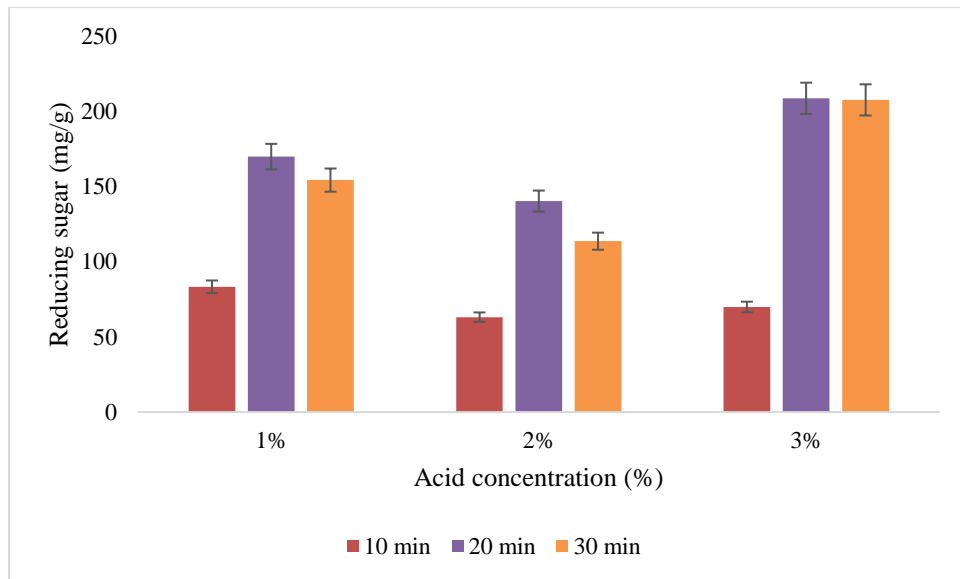


Figure 6.3 : Dilute acid hydrolysis (effect of acid concentration and time on reducing sugar release)

Steam explosion of BSG did not result in the increase of cellulose content or substantial lignin removal from the biomass. However, there was increase in reducing sugar content post enzymatic hydrolysis compared to native BSG. The mode of action of this pretreatment results in structural disruption of lignocellulose by sudden decompression there by revealing internal structures for enzymatic digestion (Jönsson & Martín, 2016). Steam explosion was conducted in an autoclave which limited the temperature and pressure used in this study to 121°C and 15 psi respectively. A reducing sugar concentration of 194 mg/g of BSG was obtained when steam exploded BSG was subjected to enzymatic hydrolysis. AFEX pretreatment results in the melting of lignin and re-deposition of the same on the biomass. After treatment BSG appeared to be dark in colour due to this phenomenon. The residual lignin prevents the evaporation of water and residual ammonia. Hence the pretreated BSG was left in the fume hood overnight for maximum ammonia removal. Similar observations were reported by Lee et al. (2010) when they subjected coastal Bermuda grass to AFEX

pretreatment. AFEX pretreatment resulted in a reducing sugar release of 211.2 mg/g of BSG post enzymatic hydrolysis (Figure 6.2).

Organosolv pretreatment employs primary alcohols along with high temperatures are a pretreatment measure for delignification of plant biomass. Methanol and ethanol are employed for this purpose due to their low cost and availability. The advantage of this pretreatment is that the solvent and the lignin dissolved by it can be recovered using simple distillation methods. (Zhao et al., 2009). The acid catalyst used in organosolv process is corrosive in nature and can give rise to inhibitory compounds when reacting with carbohydrate fractions in lignocellulose. However, in this case no inhibitory compounds were formed. Furthermore, there was considerable delignification (16.89g/100g of BSG) and increment in cellulose content (26.42g/100g of BSG) (Table 6.2). Reducing sugar yields amounted to 204.3 mg/g of substrate when BSG pretreated using organosolv strategy was subjected to enzymatic hydrolysis (Figure 6.2).

The effect of ferric chloride pretreatment is generally insignificant with respect to delignification. This pretreatment results in the degradation of the polysaccharide fraction into the pretreatment liquor (Chen et al., 2015). There was obvious loss in weight of biomass after pretreatment. The cellulose content in the pretreated biomass amounted to 17.99g/100g of BSG while the hemicellulose content was found to be 9.55g/100g of BSG. The total lignin content (26.3 mg/100g of BSG) remained the same more or less compared to raw BSG (Table 6.2). 199.4 mg/g of reducing sugar was obtained after enzymatic hydrolysis of BSG pretreated with ferric chloride (Figure 6.2).

6.3.3 Individual sugar, inhibitor and organic acid analysis

The hydrolysate obtained after the enzymatic hydrolysis experiment was analysed to identify reducing sugars released from BSG. These sugars were identified to be arabinose, xylose, mannose, galactose and glucose. Glucose and xylose were found to be the most abundant monosaccharides in BSG making this type of food waste a good feedstock for bioethanol production (Robertson et al., 2010). The liquor obtained after each pretreatment were subjected to HPLC analysis to identify the presence of individual sugars as well as inhibitory compounds such as furfural and hydroxymethyl furfural. All the pretreatment tested resulted in the release of small amounts of different component sugars into the pretreatment liquor. A notable observation was the formation of furfural when BSG was subjected to dilute acid pretreatment (1.7g/100g of BSG). Furfural is formed as degradation product of xylose component in lignocellulose. A study conducted by Djioleu and Carrier (2016) revealed that the furfural formation from xylose is directly linked to the temperature applied during dilute acid hydrolysis.

6.3.4 XRD and FTIR profiles of BSG

Fourier Transform Infrared spectroscopy (FTIR) is used to characterise the chemical structure of lignocellulosic biomass by identifying the functional groups present in the sample. Figure 6.4 represents the FTIR spectra for untreated and pretreated BSG samples. The peak at 1739 cm^{-1} represents ester bonds or carboxylic linkages in lignin and hemicellulose. This peak was evident in untreated BSG but diminished in pretreated BSG samples according to the effectiveness of the pretreatment. The peak 1526 cm^{-1} represents C=C bonds in the aromatic ring of lignin. On the other hand, the peak observed at 1247 cm^{-1} corresponds to aryl-alkyl ether bonds (C-O-C). While these bands were present (albeit less prominent) in steam exploded, organosolv, ferric

chloride and dilute acid pretreated BSG no trace of it was observed in AFEX and microwave assisted alkali pretreated samples. The peaks 895 cm^{-1} and 1053 cm^{-1} are directly related to the C-O stretching and C-H vibrations that are a characteristic of cellulose content in BSG. All the BSG samples (pretreated and native alike) exhibited these peaks in their respective spectra (Santos et al., 2015).

The inherent components in lignocellulosic biomass can crystalline or amorphous in nature. Crystallinity in any plant biomass is attributed by cellulose. Hemicellulose and lignin are amorphous in nature. The crystallinity of all the BSG samples was assessed by X-ray diffraction. The changes in crystallinity pertaining to all the BSG samples with respect to pretreatment can be observed in Figure 6.5. Increase in crystallinity is an indication of increase of cellulose content and in turn effectiveness of the pretreatment (Binod et al., 2012). The increase in peaks at the 15° and 22° is caused by the disorderliness of the structure due to the effects of pre-treatments (Pereira et al., 2011).

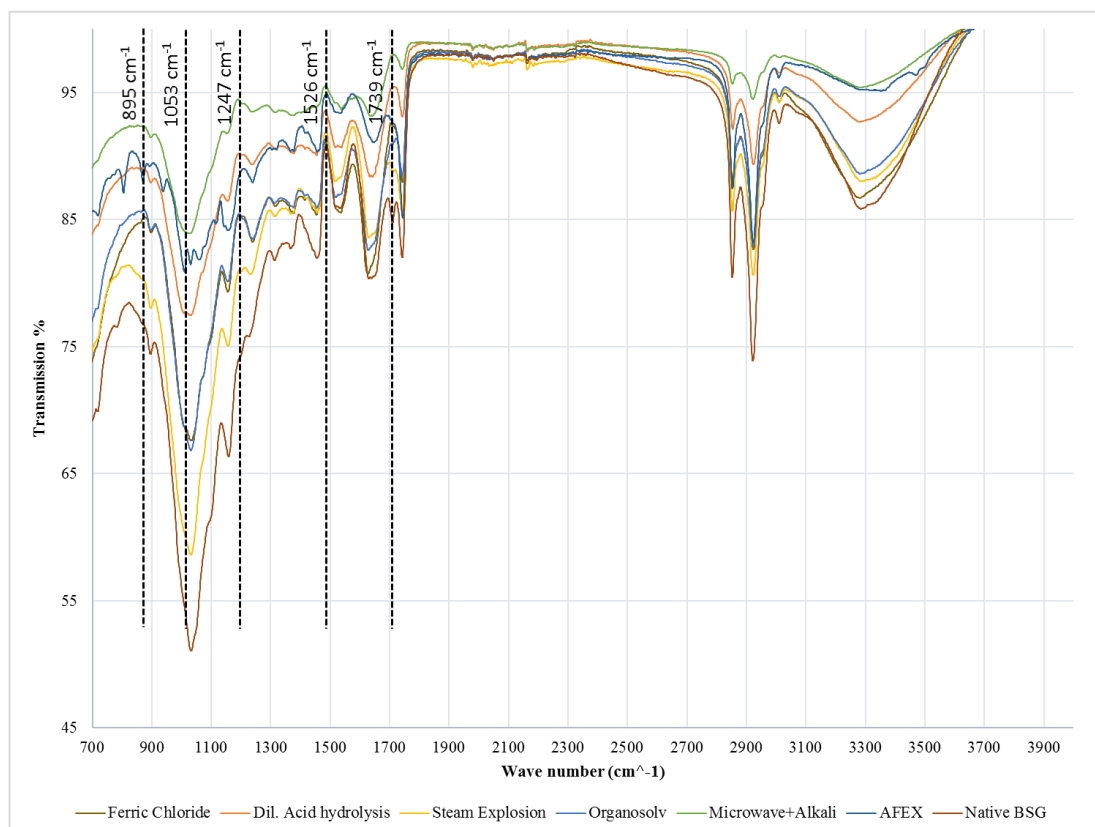


Figure 6.4 FTIR spectra and native and pretreated BSG

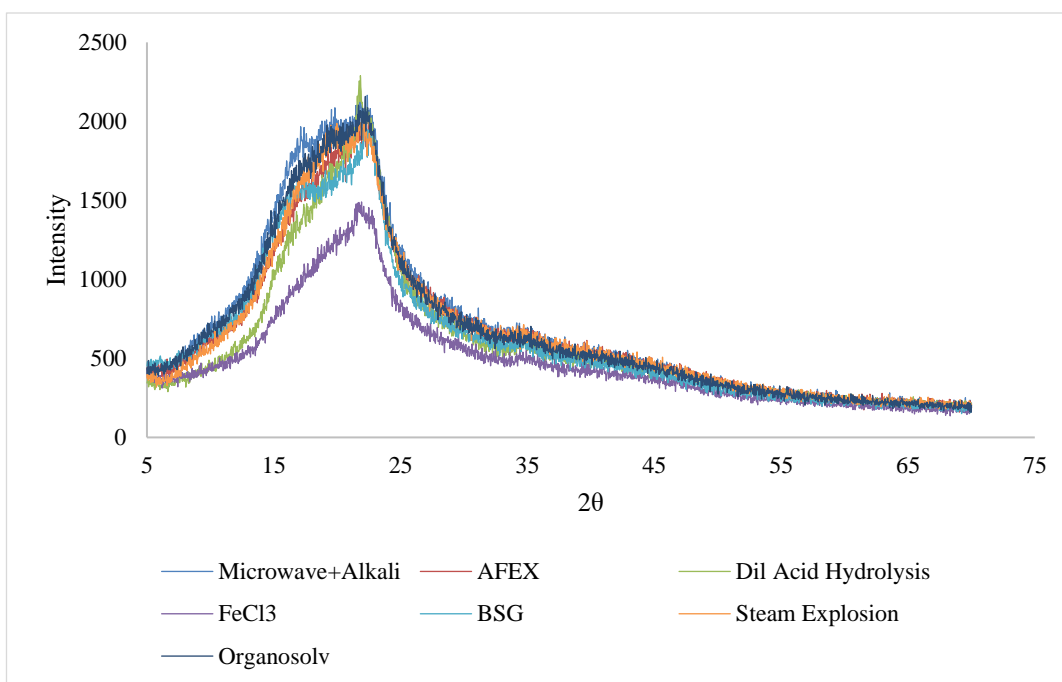


Figure 6.5 XRD spectra of native and pretreated BSG

XRD spectra of all the pretreated BSG revealed an absence in distinct amorphous regions. This can be attributed to the attrition of hemicellulose and lignin fractions in pretreated BSG samples. Microwave assisted alkali pretreated BSG exhibited highest crystallinity compared to all the other pretreated BSG. Comparatively, BSG samples that underwent ferric chloride pretreatment exhibited lowest crystallinity which may be explained by the low cellulose content observed after pretreatment.

6.3.5 Thermal behaviour study using differential scanning calorimetry

Differential scanning calorimetry (DSC) is a technique used to study the behaviour of materials as a function of temperature or time. Melting points, crystallisations and chemical reactions are some of the properties that can be studied using DSC. This technique measures the heat flow in a sample when it is heated, cooled or held at a constant temperature. A sample may undergo various phase changes during heating or

cooling. The phase changes undertaken by pretreated and impetrated samples have been represented in Figure 6.6. The temperature of the sample was raised from 20°C to 500°C at a heating rate of 10°C/min at constant nitrogen atmosphere. An empty aluminium pan was used as reference. Pretreated samples such as steam explosion, dilute acid hydrolysis, ferric chloride pretreatment and organosolv, along with native BSG exhibited a similar trend in their thermogram suggesting that they were similar in their composition. An exothermic event can be observed for all the samples with extensive mass loss between a temperature ranges of 20°C to 320°C. This temperature range marked several processes which gave rise to compounds such as carbon monoxide, carbon dioxide and other pyrolysis products. Some of the processes that occur within this temperature range is the degradation of lignin by the fragmentation of linkages between the phenyl propane units, protein degradation and decomposition of the polysaccharide fraction (Alriols et al., 2009; Sun et al., 2001). This was followed by an endothermic event that spanned between the temperature range of 300°C to 430°C and beyond. Microwave pretreated BSG sample exhibited a thermal behaviour which included a crystallisation peak between the temperature range of 360°C to 480°C. Interestingly, the thermogram of AFEX pretreated BSG was unique due to the sharp fall in heat flow which represented a transient end at a very early stage of the experiment (355°C).

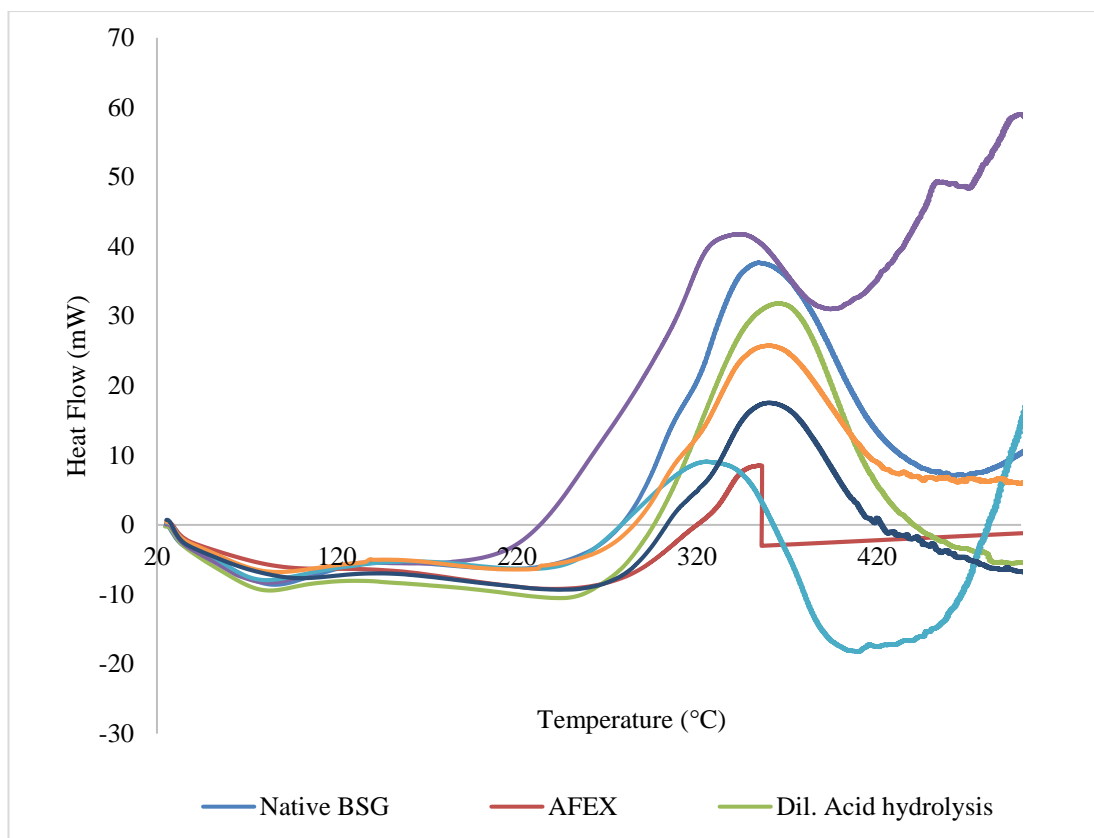


Figure 6.6 DSC thermogram of native and pretreated BSG

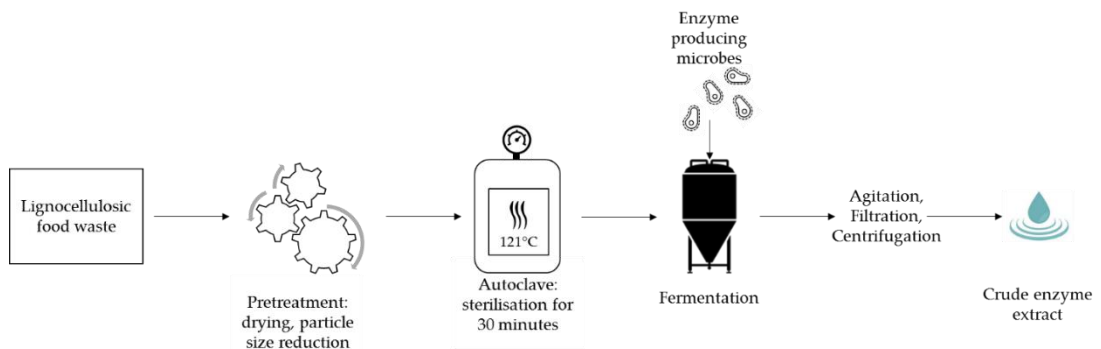
6.4 Conclusion

The present study analyses the effect of various pretreatments on brewers' spent grain. Microwave assisted alkali pretreatment was highly successful in the removal of recalcitrance from BSG. Employing this pretreatment 228.78 mg of reducing sugar /g of BSG which was 2.86-fold higher than its native counterpart. AFEX was found to be the second best pretreatment for BSG in terms of reducing sugar yield (211.2 mg/g of BSG). Dilute acid pretreatment was also successful in considerable reducing sugar release (208.8 mg/g of BSG) using 1% acid concentration and hydrolysis time of 30 min. However, this pretreatment gave rise to furfural, an inhibitory compound (1.7g/100g of BSG). Crystallinity study using XRD revealed that crystallinity of pretreated BSG samples (except ferric chloride pretreatment) were more than the native BSG samples suggesting increase in cellulose content. All these results

suggested that BSG which is an industrial by-product is with great potential as a feedstock for the production of value added products.

Section II

Enzyme Production using Lignocellulosic Food Waste



Amylases are enzymes that hydrolyse starch into release diverse products such as dextrins and oligomeric compounds made of repeating glucose structures. Amylases constitute a class of enzymes that account to approximately 25% of the enzyme market. The availability of thermostable enzymes gives rise to new possibilities for industrial processes. Thermostable enzymes are isolated from thermostable microorganisms and have found considerable commercial applications due to their overall inherent stability.

Chapter 7

Evaluation of brewer's spent grain hydrolysate as a substrate for production of thermostable α -amylase produced by *Bacillus stearothermophilus*

Brewer's spent grain (BSG) is a by-product of the beer industry. Being highly nutritious in nature and it can harbour and support microbial growth. The current scenario where BSG is produced in large quantities call for novel technologies for its efficient valorisation. In this study, BSG was hydrolysed using cellulolytic enzymes and used as a growth medium supplement for cultivation of the thermophilic bacterium, Bacillus stearothermophilus in the production of α -amylase. A central composite design involving five parameters and four levels viz. starch, peptone, KCl.4H₂O, and MgSO₄ along with BSG hydrolysate was used to derive the optimal media composition. The fermentation was conducted using shake flasks for 36h at a temperature of 50°C and pH 7.0 at 220 rpm. Optimisation trials revealed that maximal amylase production (198.09 U/ml) occurred with a medium composition of starch (0.2%w/v), peptone (0.2% w/v), KCl.4 H₂O (0.02% w/v), MgSO₄.7 H₂O (0.01% w/v) and hydrolysate (0.22% v/v). A 1.3-fold increase in amylase activity was obtained following novel media composition. All the factors considered in the study were found to be significant. The enzyme was purified by three step purification strategy, characterised and tested for anti-biofilm activity.

Work described in this chapter has been submitted as a research article:

Evaluation of brewer's spent grain hydrolysate as a substrate for production of thermostable α -amylase by *Bacillus stearothermophilus* (2018). *Bioresource Technology Reports* 5, 141-149.

7.1 Introduction

Brewer's spent grain (BSG) is a by-product of the brewery industry. It is highly nutritious and rich in carbohydrates, protein, lignin, fibre and lipids. Every hectolitre of beer can result in 20 kg of BSG being produced accounting to the ~85% of the total wastes generated (Łaba et al., 2017). In Ireland, 760 million litres of beer are produced annually which may lead to the accumulation of approx. 152,000 tonnes of BSG (Association, 2016). BSG constitutes of 20-30% dry matter and is susceptible to microbial spoilage due to its high nutritional content. Since BSG is high in protein, fibre and the presence of trace elements such as silicon, magnesium, phosphorus and sulphur it finds wide applications as animal feed (Gregori et al., 2008). However, this only accounts to a small fraction of the total BSG produced making it a huge environmental burden on the brewery industry. This calls for innovative, sustainable and commercially attractive processes for its efficient valorisation.

The race to develop sustainable technology over the past several decades has led to the utilisation of agro-based industry wastes such as BSG for its exploitation or bioconversion into commercially important compounds (Ravindran & Jaiswal, 2016). The nutritious content and food grade quality of BSG makes it a valuable resource for the development of relevant biotechnological processes (Ravindran et al., 2018). Since BSG is abundantly available, numerous technologies have been developed to extract value-added compounds BSG which belong to the nature of carbohydrates, proteins or lipids. Due to its diverse composition, BSG perfectly fits into the concept of biorefinery where multiple processes of chemical, enzymatic and biotechnological in nature can be incorporated into a single production facility (Ravindran & Jaiswal, 2016).

Due to the inherent chemical nature of BSG, it supports microbial growth and has thus been widely used for the production of enzymes. BSG has extensively been applied as a potential raw material for the production of enzymes such as amylases, amyloglucosidases, cellulases and hemicellulases. Most studies focus on the utilisation of mycelial fungi for such applications. Among bacteria, *Bacillus* sp. has gained considerable interest due to its ability to produce carbohydrate degrading enzymes that are of industrial importance. Some of these species such as *B. subtilis*, *B. amyloliquefaciens*, *B. cereus*, *B. licheniformis* and *B. stearothermophilus* are important due to their ability to produce α and β amylases (Chen et al., 2015; Prajapati et al., 2015; Vaikundamoorthy et al., 2018). Amylases derived from *Bacillus* sp. find wide applications in the industry ranging from the production of fructose and glucose syrup to bakery, detergents, textile and fuel ethanol production (Souza, 2010). Amylases hold 30% of the global enzyme market share, having largely replaced chemical hydrolysis of starch (Lal et al., 2017). Many BSG and BSG-based products have been utilised as a media component for the production of α -amylases (Hashemi et al., 2011).

An integral component of demonstrating the economic feasibility of adopting BSG for fermentation processes is the identification of novel markets for enzyme products that can provide a sustainable return on investment. Biofilms are formed by prokaryotic microorganisms capable of producing exopolysaccharides (EPS) around the cell. Many biofilm-producing organisms are pathogenic in nature, including *Vibrio cholerae*, multi-drug resistant *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (Costerton et al., 1999; Hall-Stoodley & Stoodley, 2005; Mihailescu et al., 2014). Biofilms produced by such microbes can cause disruption and reduction in efficiency of industrial processes. Furthermore, these pathogenic strains are responsible for 10-20% of all the hospital acquired infections (Jamal et al., 2018). Conventional

preventive measures against biofilm producers include chemical biosurfactants, surface modifications and natural substances such as honey and oils. (Sadekuzzaman et al., 2015). Exopolysaccharides produced by these microbes safeguard them from attacks by antibiotics and other antimicrobial agents. However, recent studies have shown enzymes as a promising technology in the prevention of EPS production and biofilm formation. Some of the enzymes studied for this purpose include lysozyme, proteinase K, trypsin and amylase (Shukla & Rao, 2013). These enzymes have been studied individually and as cocktails as a deterrent for biofilm production (Kalpana et al., 2012).

This study investigates the applications of BSG hydrolysate as a potential media component for the production of a thermostable α -amylase by *B. stearothermophilus*. The growth medium composition for amylase production was optimised by the application of response surface methodology. This was followed by purification of the enzyme via a three-stage strategy. The enzyme was then characterised to determine the optimum pH and temperature for activity. Finally, initial proof-of-principle was shown for the use of the α -amylase from *B. stearothermophilus* as an anti-biofilm agent using multiple bacterial strains of medical significance.

7.2 Methodology

7.2.1 Microorganism

Four *Bacillus* strains viz. *B. subtilis*, *B. cereus*, *B. megaterium* and *B. stearothermophilus* were revived from the microbiology repository maintained in the School of Food Science and environmental health, Dublin Institute of Technology, Ireland. The strains were revived in nutrient broth media at 37°C and 50°C (for *B. stearothermophilus*) for 18h at 150 rpm. The cultures were regularly subcultured and maintained at 4°C.

7.2.2 Screening for amylase producing *Bacillus* strains

Four strains viz. *Bacillus subtilis*, *Bacillus cereus*, *Bacillus megaterium* and *Bacillus stearothermophilus* were cultured on starch agar plates which contained 3 mg/ml meat extract, 5 mg/ml peptic digest, 2 mg/ml starch and 15 mg/ml of agar. The final pH of the media was adjusted to 7.2. The plates were incubated at optimal growth conditions for each strain (37 °C for *B. subtilis* and *B. megaterium*, 30 °C for *B. cereus* and 50 °C for *B. stearothermophilus*). After incubating the plates for 24h the plates were flooded with Gram's iodine and the plates with zones of clearance were identified and maintained for amylase production (Sethi et al. 2013).

7.2.3 Preparation of M9 media and amylase production

M9 media was prepared by adding 5.64 g of the powder in 100 ml distilled water. The solution was sterilised and stored. To prepare M9 minimum salts media the 20 ml of the above prepared solution was diluted with sterile distilled water to make up a volume of 100 ml. 2 ml of 20% starch solution was aseptically added into the media along with 0.2 ml of sterile 1.0 M MgSO₄ solution and if desired 0.1 ml sterile 1.0 M CaCl₂ solution. The flasks were autoclaved and cooled, after which they were inoculated with 1% inoculum of 18h old *Bacillus* sp. cultures. The flasks were incubated at 37 °C for *B. subtilis* and *B. megaterium*, at 30°C for *B. cereus* and at 50°C for *B. stearothermophilus*. The pH was maintained at 6 for all cultures. Samples were drawn at different intervals (0h, 12h, 24h, 30h, 36h and 48h). The growth profile of each strain was created at 600 nm using UV-visible spectrophotometry. The broth containing extracellular proteins was centrifuged at 10000 rpm for 10 min at 4°C to remove the residual solids and were analysed for amylase activity (Song and Wei 2010).

7.2.4 Amylase production using starch, peptone and meat extract

Media for enzyme production comprised of 0.2% starch, 0.5% peptone, and 0.3% meat extract (SPM). The medium (50 ml) was inoculated with 1 ml of inoculum of 18h old *Bacillus* sp. cultures and incubated at different temperatures with respect to each *Bacillus* strain as mentioned in the above section. Samples were harvested at different intervals (as mentioned earlier). After amylase production the culture broth was centrifuged at 10000 rpm for 10 min at 4 °C and stored for further analysis. All analyses were conducted within 48h.

7.2.5 Lipid extraction from BSG

The total lipid content in BSG was reduced using a modification of the protocol devised by Niemi et al. (2012). Briefly, 10g of BSG was weighed, dispensed into a cellulose thimble and stoppered with cellulose wool. A Soxhlet apparatus was set up with 100 ml of acetone in a round bottom flask and anti-bumping granules added. The extraction was conducted in reflux cycles for 5h. The lipid-free BSG was then dried and stored for further experiments.

7.2.6 Microorganism and amylase production

Bacillus stearothermophilus LZT020 strain was supplied by the microbiology repository at the School of Food Science and Environmental Health, Dublin Institute of Technology. For seed culture, frozen glycerol stock maintained at –80°C was used to inoculate liquid medium containing (in g/l) peptone (3.0), yeast extract (1.5), beef extract (1.5) and NaCl (2.5). Cultures were maintained in 100 ml Erlenmeyer flasks maintaining a working volume of 10%. The pH of the medium was adjusted to 7.0. The culture was grown in at 50°C for 18h maintaining an agitation rate of 200 rpm.

7.2.7 Analytical methods

7.2.7.1 Amylase assay

For performing the amylase assay, 50 μ l of soluble starch (1% (w/v) solution made in phosphate buffer (50mM, pH 6) was incubated with analyte samples at 50°C for 5 min. The reaction was stopped by adding 3, 5-dinitrosalicylic acid reagent. Sample absorbance was measured at 546 nm (Miller, 1959). Glucose was used as standard. One unit of enzyme was defined as the amount of enzyme required to release 1 μ mol of reducing sugar equivalent from soluble starch in one min under assay conditions. Specific activity was expressed in U/mg of protein. Protein estimation was carried out by the Bradford method using BSA as standard (Bradford, 1976).

7.2.7.2 Effect of pH and temperature on amylase activity

Activity of alpha amylase was determined at different temperature ranging from 0 °C to 100 °C with the interval of 10 °C. Substrate was incubated for 3 min at desired temperature ranging from 0 °C to 100 °C. Maximum activity obtained was taken as 100% and accordingly percentage relative activity was calculated. Percentage relative activity was plotted against respective temperature. Alpha amylase is relatively stable within a range of pH 4-9 under standard assay condition. Sodium acetate buffer was used for determination of optimum pH (4-9). Relative activity determined considering maximum enzyme activity as 100%.

7.2.8 Pretreatment and enzyme hydrolysis of BSG

BSG was first subjected to microwave assisted alkali pretreatment and subsequently hydrolysed following the procedure mentioned in section 6.2.1.6 and 3.5 respectively.

7.2.9 Optimisation of novel media composition for amylase production

A response surface methodology design was created to optimise the media composition for production of amylase. A central composite design (CCD) was implemented to estimate the regression parameters to fit a second-degree polynomial regression model for a given response. The polynomial calculated the relationship between estimated response Y and all the independent variables involved, X_i (starch, peptone, KCl, 4 H₂O, MgSO₄ · 7 H₂O and hydrolysate). The significance of each factor considered in the study was determined by analysis of variance, where P values were calculated. Any factor with $P < 0.05$ was considered statistically significant. All statistical analyses were carried out using Statgraphics Centurion XV software version 15.1.02 (StatPoint Technologies Inc. Warrenton, VA, USA). Table 7.1 represents the factors and ranges considered in this study. Fermentation experiments were conducted at 50°C for 36h at 220 rpm while maintaining a working volume of 20%. The hydrolysate was autoclaved separately from the rest of the reaction mixture and was then aseptically added to prevent the Milliard reaction (Mottram et al., 2002).

Table 7.1 Process variables and different levels in CCD

| Independent variables | Coded symbols | Level | | | | |
|---------------------------|---------------|-------|------|------|------|------|
| | | -2 | -1 | 0 | -1 | -2 |
| Starch (% w/v) | X_1 | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 |
| Peptone (% w/v) | X_2 | 0.2 | 0.4 | 0.6 | 0.8 | 1.0 |
| KCl (% w/v) | X_3 | 0.01 | 0.02 | 0.03 | 0.04 | 0.05 |
| MgSO ₄ (% w/v) | X_4 | 0.01 | 0.02 | 0.03 | 0.04 | 0.05 |
| Hydrolysate % v/v) | X_5 | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 |

7.2.10 Control Experiment

Control experiments for amylase production by *B. stearrowthermophilus* were set up according to protocols devised by Al-Qodah (2006). Fermentation was conducted in 250 ml Erlenmeyer flask maintaining a reaction volume of 20%. The flasks were incubated at 55°C in a shaking incubator rotating at 220 rpm for 36h. The culture supernatant was collected by centrifugation at 12,000 rpm for 30 mins at 4°C and used for further analysis.

7.2.11 Dry cell weight analysis

The effect of fermentation medium supplemented by BSG hydrolysate on the production of biomass was studied by conducting dry cell weight analysis. Briefly, temperature-resistant plastic universal bottles (20 ml capacity) was dried over in a hot air oven at 105°C with the lids separately retained. These were then carefully removed from the oven and placed in a desiccator jar. Once the universals were cooled they were weighed and the weights were recorded. Next, 15 ml aliquot of each test and control fermentation cultures were decanted into the pre-weighed universal. The universals were then centrifuged at 15,000 rpm for 30 minutes at 4°C to pellet the cells. The supernatant was carefully transferred into sterile bottles and stored for further experiments. Meanwhile, the universals with cell pellets were dried at 105°C in a hot air oven for 24 hours and then placed in a desiccator jar. Finally, each universal was re-weighed, and the dry cell weight was calculated in mg/ml.

7.2.12 Purification of α -amylase

Cell removal was performed by centrifugation at 15,000 rpm for 30 min at 4°C. The cell free supernatant was then subjected to precipitation using ammonium sulphate (60% (w/v)). The precipitate was re-suspended in 10 ml of 0.1 M sodium phosphate

buffer (pH 7.0). This was followed by salt removal-buffer exchange by diafiltration (10 kDa MWCO, Amicon Corporation, Lexington, Mass., U.S.A.). Anion exchange chromatography was performed following the protocol devised by Chakraborty et al. (2000) with slight modifications. A DEAE-Sepharose Fast Flow column (2.6 cm × 50 cm) was used for this purpose. The column was equilibrated with 20 mM Tris-HCl buffer (pH 8.0) before loading the sample. Elution was performed using 20 mM Tris-HCl containing 0.5 M NaCl (pH 8.0) and 2 ml fractions were collected at 15 ml/h and absorbance was measured at 280 nm. The active fractions were pooled and concentrated by ultrafiltration (10 kDa cut-off).

7.2.13 Screening of pathogens for biofilm production capacity

Pathogenic bacterial strains *Staphylococcus aureus* NCTC 1803, *S. aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 were obtained from the DIT microbiology repository. The strains were revived by growing them over night in LB broth at 37°C for 18h. Each strain was then plated on brain-heart infusion agar supplemented with 3.6% sucrose and 0.08% Congo red dye. The plates were incubated for 48h at 37°C following which they were inspected for biofilm formation.

7.2.14 Biofilm formation assay

The effect of thermostable α -amylase on the growth of pathogenic organisms and its capacity to inhibit biofilm formation were determined by UV-VIS spectrophotometric assays. The assay was conducted following the protocol reported by Vaikundamoorthy et al. (2018) with slight modifications. The crude α -amylase enzymes were taken in different volumes ranging from 50, 100, 150, 200 and 250 μ l/ml were suspended in LB broth (pH 7.0) containing bacterial suspension at 10^6 CFU/ml. Meanwhile, the efficacy of commercial amylase as an antibiofilm agent was also assessed. Different

volumes of the commercial amylase were added to the bacterial suspensions ranging from 20 to 100 µl/ml of LB broth and its effect of biofilm production was determined.

The biofilm assay was conducted in 96-well microtiter plates. The wells were filled with 100 µl of LB broth following which the microtiter plate was then incubated at 37°C for 24h for biofilm formation. Meanwhile, wells with LB broth and without enzymes were used as control. Following incubation, the culture was discarded, and the plates were washed repeatedly with distilled water. The wells were then stained with 125 µl of 0.1% crystal violet and incubated at room temperature for 15 min., after which the contents of the wells were discarded. For quantification purposes, 125 µl of acetic acid (30%) was added to each well and incubated at room temperature for 15 min. The contents were then transferred to a separate 96-well microtiter plate and absorbance was measured at 550 nm. Acetic acid was used as a blank. Biofilm inhibitory concentration (BIC) was defined as the lowest concentration that produced significant reduction in biofilm formation (spectrophotometric equivalent) when compared to control wells.

7.3 Results and discussion

7.3.1 Screening of amylase producing *Bacillus* sp.

Screening test conducted for amylase producing *Bacillus* strains revealed that all the strains considered in this study were capable of producing amylase. This was evident from the zone of clearance observed after flooding the starch agar plates of individual strains with Gram's iodine (Fig.7.1).

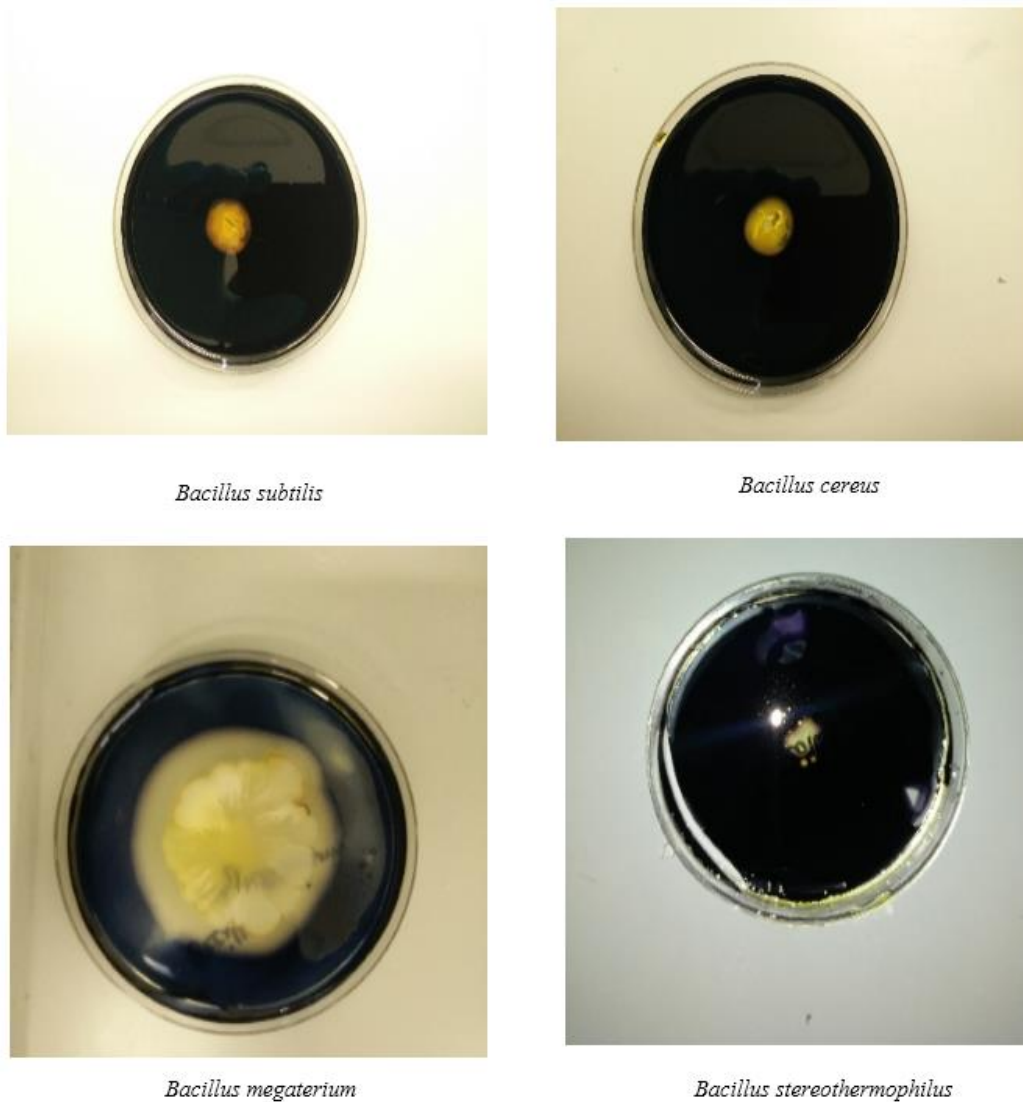


Figure 7.1 Zones of clearance observed after flooding starch agar plates with Gram's iodine

Fig. 7.2 represents the growth profiles of all the strains cultured in both media. All the *Bacillus* strains studied followed a sigmoid growth profile achieving maximum growth at the 24h and thereafter gradually shifting into senescence phase. General observations revealed that SPM medium was a better medium for growth of amylase producing *Bacillus* strains (Fig. 7.2 A) compared to M9 minimal salts media (Fig. 7.2 B). *B. subtilis* achieved highest growth in starch, peptone, and beef extract media while the same was achieved by *B. megaterium* in M9 minimal salts media. A similar trend was also observed with respect to amylase activity (Fig. 7.3 A). Higher activity was observed in starch, peptone and meat extract medium as compared to M9 medium. All

the strains tested exhibited maximum amylase activity at 36h (Fig 3a&b). Maximum amylase activity of 144.6 U/ml was registered by *B. subtilis* in the 36h employing SPM medium. In comparison, *B. megaterium* exhibited highest activity using M9 media (125.77 U/ml). *B. stearothermophilus* exhibited maximum enzyme activity of 80.44 U/ml in SPM medium and 81.60 U/ml in M9 medium (Fig 7.2A & 7.2B).

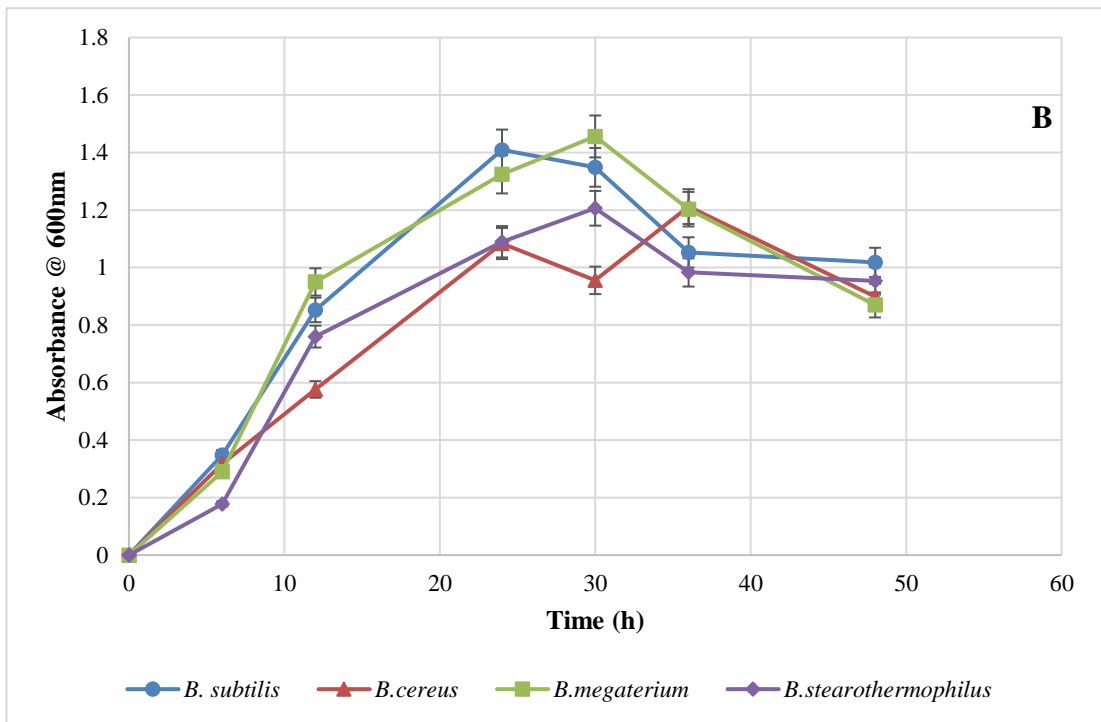
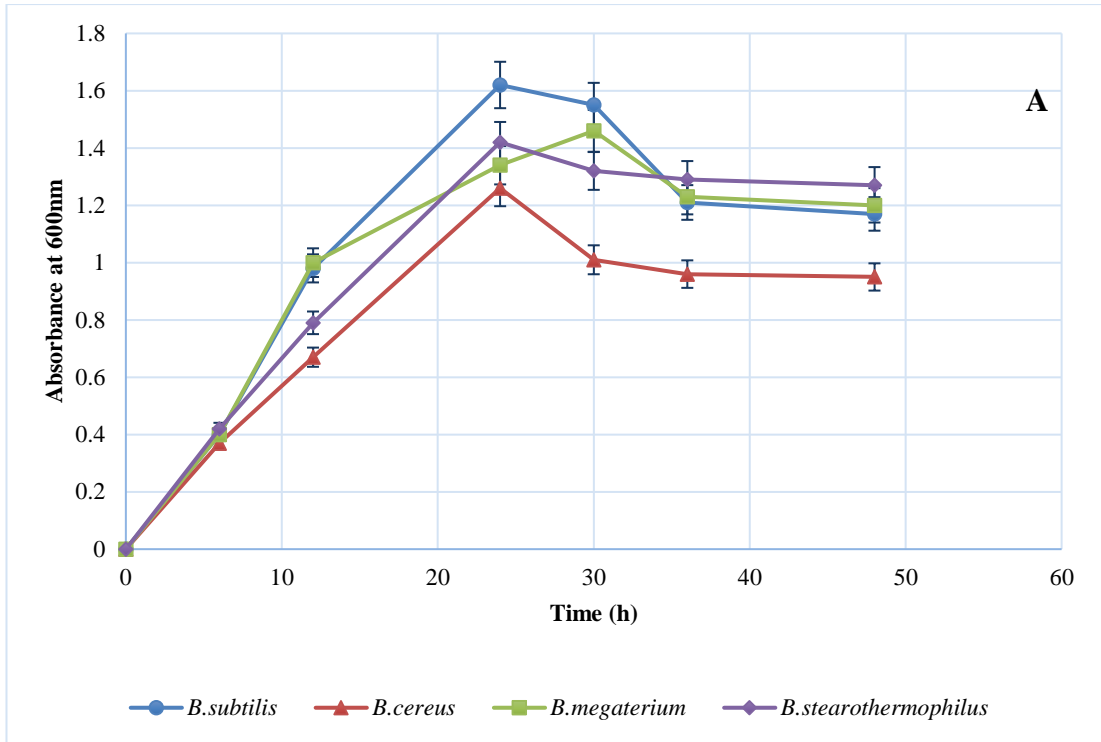


Figure 7.2 Growth profiles of amylase producing *Bacillus* strains in (A) starch, peptone, and beef extract media and (B) M9 minimal salts media

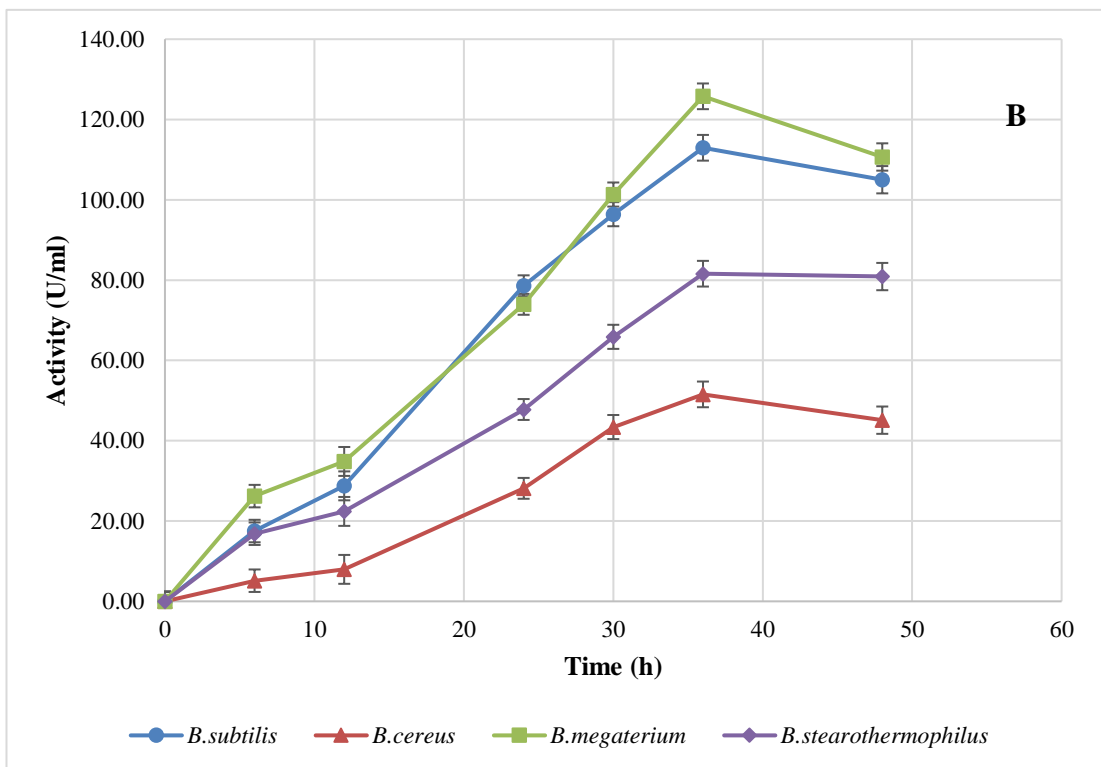
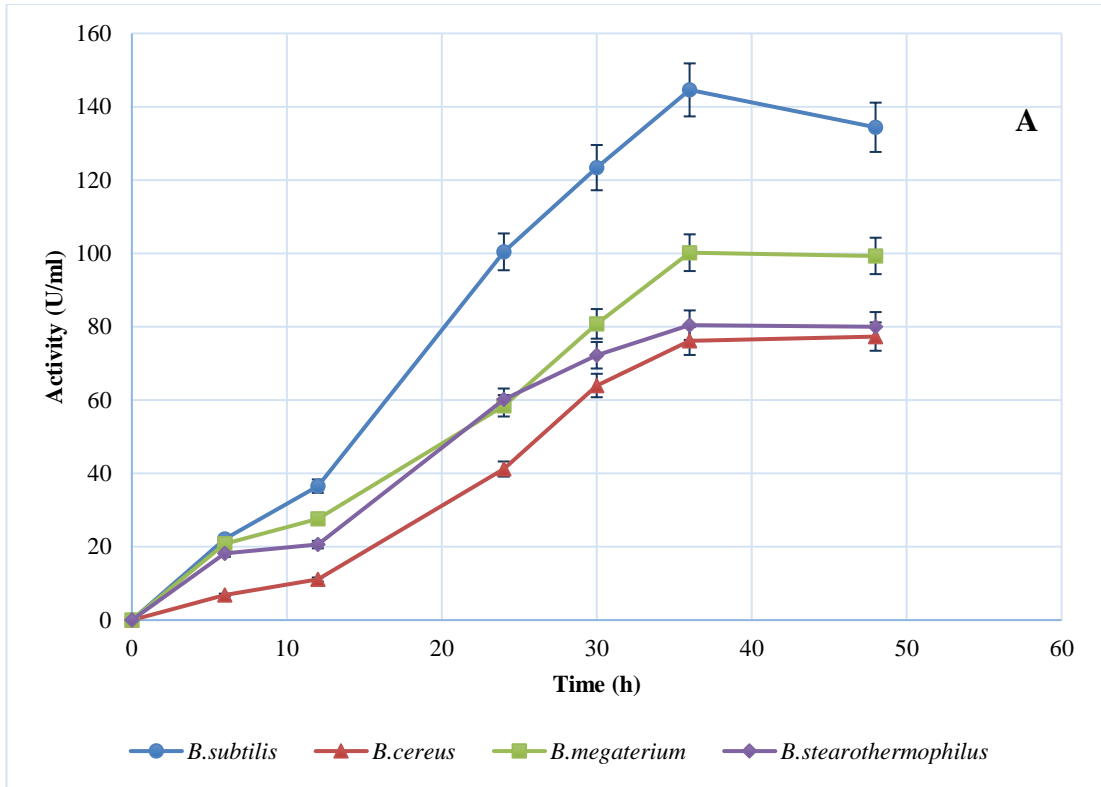


Figure 7.3 Amylase activity profile of *Bacillus* strains in (A) starch, peptone, and beef extract media and (B) minimal salts media

The pH and temperature stability (Fig 7.4 and Fig. 7.5 respectively) of the amylase derived from each strain cultured in SPM media was analysed. Amylase derived from *B. subtilis* and *B. megaterium* were stable over the same pH range. The optimum pH for *B. subtilis* and *B. megaterium* amylase activity was found to be 6. Similar observations were reported by Demirkan (2011) and Oyeleke, Auta et al. (2010) for *B. subtilis* and *B. megaterium* respectively. Interestingly, *B. megaterium* amylase was stable over a pH range of 6-8. *B. cereus* derived amylase exhibited maximum activity at pH 5 and was stable from pH 5-8 and thereafter declined as the pH increased to alkaline range. Trivedi et al. (2006) also reported similar characteristics for amylase derived from *B. cereus*. The most notable observation was the pH stability of *B. stearothersophilus* amylase over a wide range of pH falling in the alkaline range.

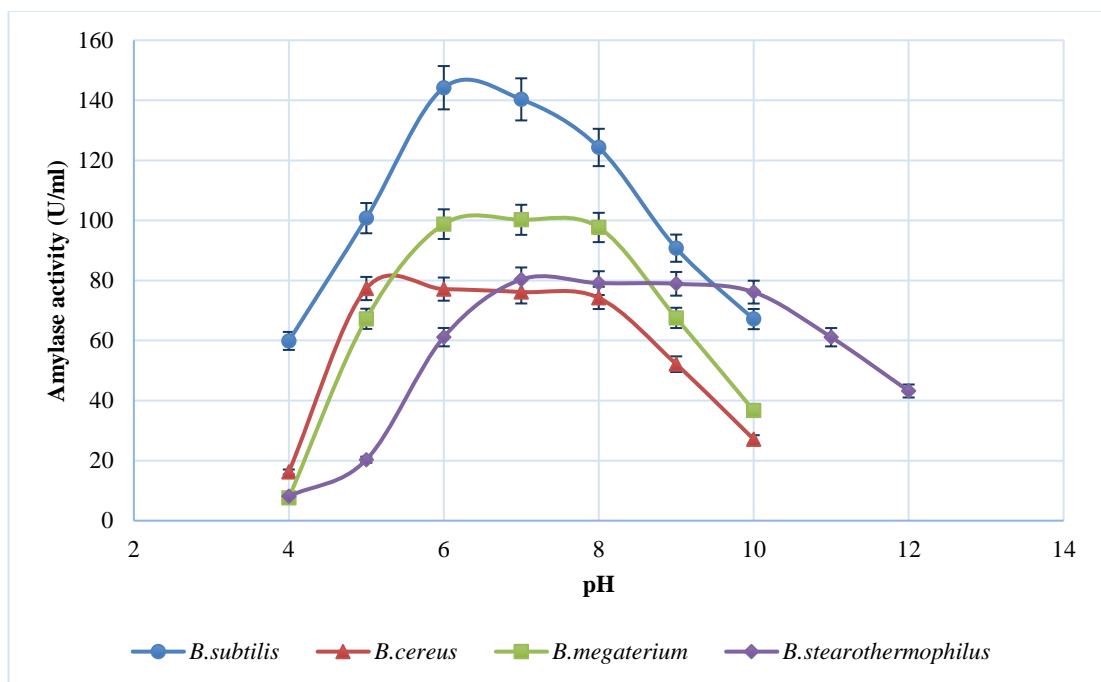


Figure 7.4 pH profile for amylase derived from *Bacillus* strains

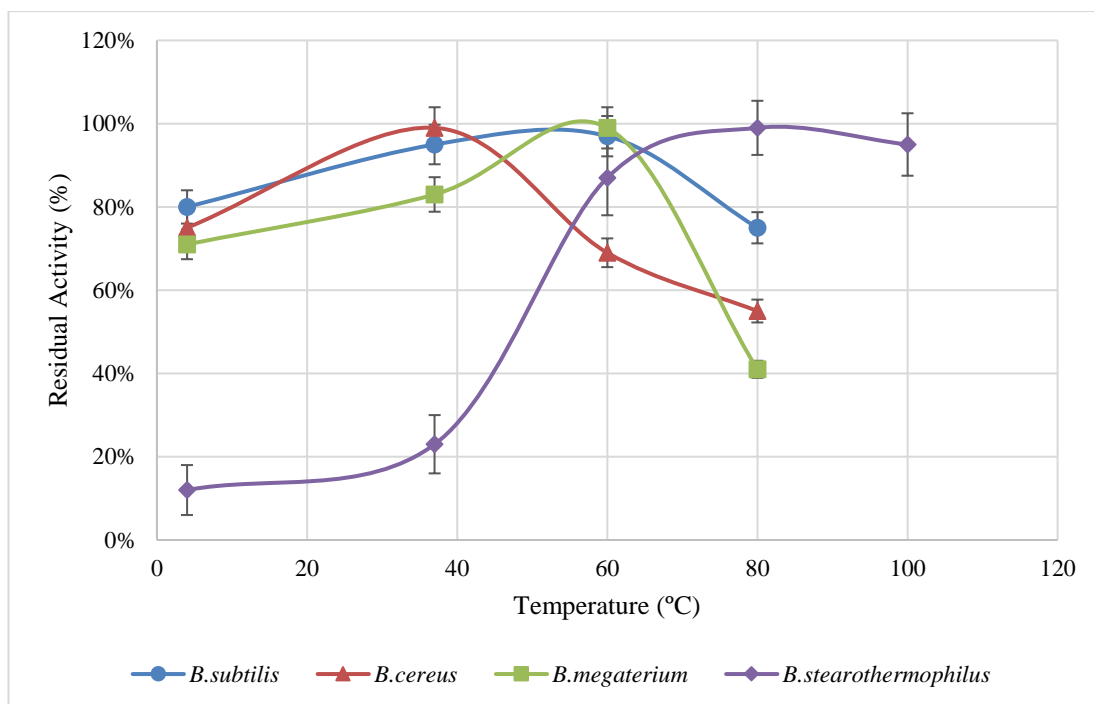


Figure 7.5 Temperature profile for amylase derived from *Bacillus* strains

The amylase enzymes derived from three species studied viz. *B. subtilis*, *B. cereus* and *B. megaterium* exhibited stability in the mesophilic range (10°C to 40°C). The residual activity of *B. cereus* enzyme declined past the point of 40°C. However, the enzymes from *B. subtilis* and *B. megaterium* were stable at a temperature as high as 60°C. Interestingly, the amylase derived from *B. stearothermophilus* was most stable at higher temperature ranges (50°C-90°C).

7.3.2 Utilisation of BSG hydrolysate as media component for amylase production

In this study, BSG hydrolysate which was rich in fermentable sugars was tested as a media component for the production of thermostable α -amylase using *B. stearothermophilus* LZT020. This organism was chosen as the enzyme producer due to its ability to produce thermostable amylase. Sugar composition analysis of the BSG substrate indicated glucose (2.5 mg/ml), xylose (2.1 mg/ml), mannose (1.3 mg/ml), arabinose (1.0 mg/ml) and galactose (4.1 mg/ml) and total reducing sugar content was

20 mg/ml. The optimal concentration of each media component was investigated by response surface methodology. Peptone, starch, KCl. 4H₂O and MgSO₄.7H₂O and BSG hydrolysate were the media components considered in this study. The concentrations of BSG hydrolysate, peptone, starch, KCl. 4H₂O and MgSO₄ were varied according to the experimental strategy. The amylase activity obtained after 30 experimental trial is shown in Table 7.2.

Table 7.2 CCD experimental design for five independent variables, experimental and predicted values for amylase activity

| Starch (%, w/v) X₁ | Peptone (%, w/v) X₂ | KCl (%, w/v) X₃ | MgSO₄ (%, w/v) X₄ | Hydrolysate (%, v/v) X₅ | Observed amylase activity (U/ml) | Theoretical amylase activity (U/ml) |
|--|---|---|--|---|---|--|
| 0.4 | 0.4 | 0.04 | 0.04 | 0.2 | 48.34 | 49.97 |
| 0.4 | 0.8 | 0.02 | 0.02 | 0.4 | 61.35 | 59.65 |
| 0.4 | 0.8 | 0.02 | 0.04 | 0.2 | 58.14 | 59.78 |
| 0.4 | 0.4 | 0.02 | 0.02 | 0.2 | 104.56 | 107.02 |
| 0.2 | 0.8 | 0.04 | 0.02 | 0.4 | 37.49 | 34.97 |
| 0.3 | 0.6 | 0.03 | 0.01 | 0.3 | 121.68 | 121.34 |
| 0.1 | 0.6 | 0.03 | 0.03 | 0.3 | 84.29 | 85.60 |
| 0.2 | 0.4 | 0.04 | 0.02 | 0.2 | 104.89 | 106.52 |
| 0.2 | 0.4 | 0.04 | 0.04 | 0.4 | 26.54 | 24.01 |
| 0.4 | 0.8 | 0.04 | 0.02 | 0.2 | 70.19 | 71.83 |
| 0.2 | 0.8 | 0.04 | 0.04 | 0.2 | 83.79 | 84.59 |
| 0.2 | 0.4 | 0.02 | 0.02 | 0.4 | 106.34 | 104.64 |
| 0.3 | 0.6 | 0.05 | 0.03 | 0.3 | 37.99 | 39.30 |
| 0.3 | 0.6 | 0.03 | 0.03 | 0.3 | 131.93 | 127.78 |
| 0.3 | 0.6 | 0.03 | 0.03 | 0.3 | 129.01 | 127.78 |
| 0.3 | 1.0 | 0.03 | 0.03 | 0.3 | 112.78 | 114.08 |
| 0.4 | 0.8 | 0.04 | 0.04 | 0.4 | 56.44 | 53.92 |
| 0.2 | 0.8 | 0.02 | 0.02 | 0.2 | 126.10 | 127.73 |
| 0.3 | 0.6 | 0.03 | 0.03 | 0.3 | 124.37 | 127.78 |
| 0.3 | 0.6 | 0.03 | 0.05 | 0.3 | 85.99 | 87.30 |
| 0.4 | 0.4 | 0.02 | 0.04 | 0.4 | 76.29 | 74.59 |
| 0.2 | 0.4 | 0.02 | 0.04 | 0.2 | 75.24 | 76.87 |
| 0.2 | 0.8 | 0.02 | 0.04 | 0.4 | 81.54 | 79.02 |
| 0.3 | 0.2 | 0.03 | 0.03 | 0.3 | 114.09 | 113.75 |
| 0.3 | 0.6 | 0.03 | 0.03 | 0.3 | 126.79 | 127.78 |
| 0.4 | 0.4 | 0.04 | 0.02 | 0.4 | 28.24 | 26.54 |
| 0.3 | 0.6 | 0.01 | 0.03 | 0.3 | 98.88 | 98.54 |
| 0.3 | 0.6 | 0.03 | 0.03 | 0.5 | 20.98 | 28.95 |
| 0.5 | 0.6 | 0.03 | 0.03 | 0.3 | 52.18 | 51.84 |
| 0.3 | 0.6 | 0.03 | 0.03 | 0.1 | 92.69 | 85.69 |

The model generated a regression equation which explained the relationship that existed between the amylase yield and all the parameters considered in the experimental design which is provided below:

$$\begin{aligned} \text{Amylase activity (U/ml)} = & 13.3337 + 475.416 \times X_1 - 31.1953 \times X_2 + 5923.18 \times X_3 - \\ & 5094.32 \times X_4 + 725.692 \times X_5 - 1476.61 \times X_1^2 - 85.0465 \times \\ & X_1 \times X_2 + 2461.56 \times X_1 \times X_3 + 51 \quad 61.56 \times X_1 \times X_4 + \\ & 494.906 \times X_1 \times X_5 - 86.6518 \times X_2^2 + 2350.47 \times X_2 \times X_3 + \\ & 3200.46 \times X_2 \times X_4 - 18.078 \times X_2 \times X_5 - 147161. \times X_3^2 + \\ & 50884.4 \times X_3 \times X_4 - 7499.06 \times X_3 \times X_5 - 58660.6 \times X_4^2 + \\ & 9225.94 \times X_4 \times X_5 - 1761.61 \times X_5^2 \end{aligned}$$

where X_1 , X_2 , X_3 , X_4 and X_5 represents starch, peptone, KCl. $4H_2O$, $MgSO_4$ and BSG hydrolysate, respectively. Analysis of variance was conducted to determine the significance of the all the parameters considered in the model. Table 7.3 represents the analysis of variance with respect to parameters, combined effects and the P-value assigned by the model to each of them considering a confidence interval spanning from 0 to 95%. Any parameter or combined effect which was assigned a -value less than 0.05% was considered to be significant in terms of influence on amylase activity.

Table 7.3 Analysis of variance obtained for amylase activity

| Source | Sum of Squares | Df | Mean Square | F-Ratio | P-Value |
|-------------------------------|----------------|----|-------------|---------|---------|
| X ₁ | 1710.15 | 1 | 1710.15 | 72.91 | 0.0001 |
| X ₂ | 0.163839 | 1 | 0.163839 | 0.01 | 0.0002 |
| X ₃ | 5263.54 | 1 | 5263.54 | 224.4 | 0.0001 |
| X ₄ | 1737.66 | 1 | 1737.66 | 74.08 | 0.0001 |
| X ₅ | 4828.66 | 1 | 4828.66 | 205.86 | 0.0001 |
| X ₁ ² | 5814.35 | 1 | 5814.35 | 247.88 | 0.0001 |
| X ₁ X ₂ | 46.2906 | 1 | 46.2906 | 1.97 | 0.1936 |
| X ₁ X ₃ | 96.9484 | 1 | 96.9484 | 4.13 | 0.0726 |
| X ₁ X ₄ | 426.267 | 1 | 426.267 | 18.17 | 0.0021 |
| X ₁ X ₅ | 391.891 | 1 | 391.891 | 16.71 | 0.0027 |
| X ₂ ² | 320.364 | 1 | 320.364 | 13.66 | 0.005 |
| X ₂ X ₃ | 353.58 | 1 | 353.58 | 15.07 | 0.0037 |
| X ₂ X ₄ | 655.55 | 1 | 655.55 | 27.95 | 0.0005 |
| X ₂ X ₅ | 2.09161 | 1 | 2.09161 | 0.09 | 0.772 |
| X ₃ ² | 5775.04 | 1 | 5775.04 | 246.21 | 0.0001 |
| X ₃ X ₄ | 414.276 | 1 | 414.276 | 17.66 | 0.0023 |
| X ₃ X ₅ | 899.774 | 1 | 899.774 | 38.36 | 0.0002 |
| X ₄ ² | 917.618 | 1 | 917.618 | 39.12 | 0.0001 |
| X ₄ X ₅ | 1361.89 | 1 | 1361.89 | 58.06 | 0.0001 |
| X ₅ ² | 8275.4 | 1 | 8275.4 | 352.8 | 0.0001 |
| Total error | 211.105 | 9 | 23.4561 | | |
| Total (corr.) | 33533.1 | 29 | | | |

The coefficient of determination (R^2) was calculated to determine if the model adequately fitted the data. The closeness of R^2 to 1.0 is an indication of the strength of the model and how well it can predict a response. Statistical analysis helps in the determination of parameters that generate signals that are larger than the noise. In a model where the sample size is not very large in comparison with the number of parameters, the adjusted R^2 corrects the R^2 value. A disproportionate number of terms with respect to the sample size manifests in a noticeably smaller adj- R^2 value compared to the R^2 value. The model predicted an R^2 value of 99.37% and an adj- R^2 value of 97.97% indicating a good agreement between experimental and predicted values of amylase activity.

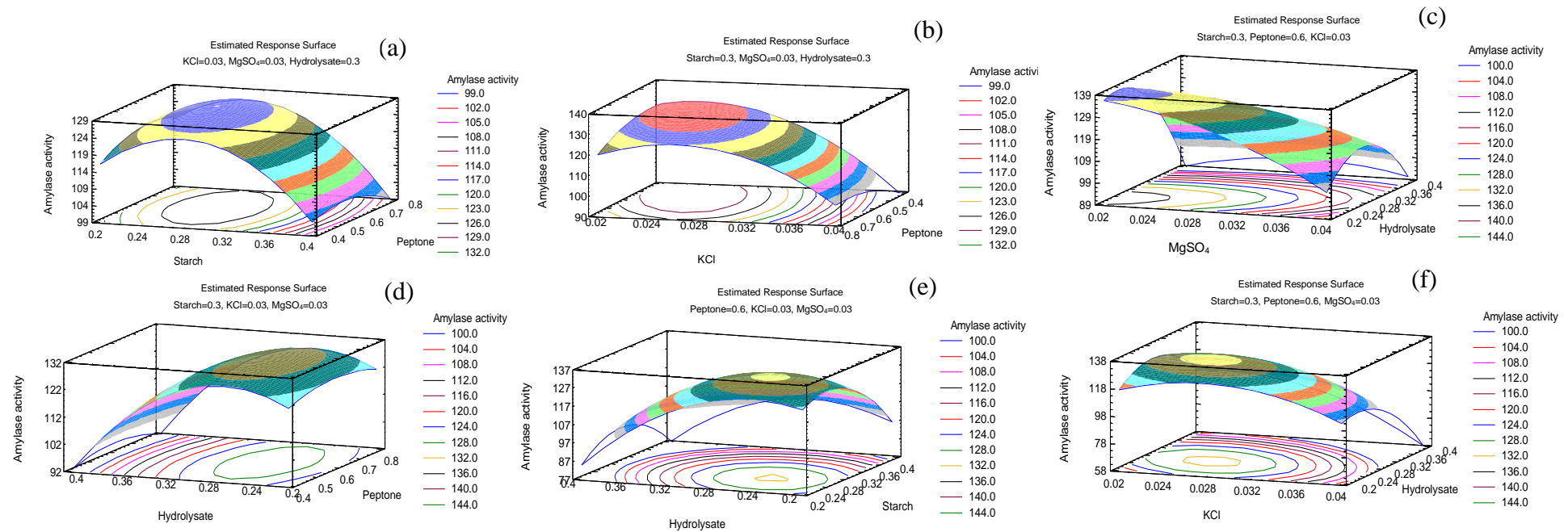


Figure 7.6 Response surface plots representing the effect of independent variables on amylase activity: (1a) the effect of starch and peptone when the response surface is fixed at KCl = 0.03%, MgSO₄ = 0.03 and hydrolysate = 0.3% (1b) the effect of KCl and peptone when the response surface is fixed at starch= 0.3%, MgSO₄ = 0.03 and hydrolysate = 0.3% (1c) the effect of MgSO₄ and hydrolysate when the response surface is fixed at starch= 0.3%, KCl= 0.03 and MgSO₄= 0.03% (1d) the effect of starch and hydrolysate when the response surface is fixed at peptone= 0.6%, MgSO₄ = 0.03% and KCl= 0.03% (1e) the effect of hydrolysate and starch on amylase activity when response surface is fixed at peptone=0.6%, KCl=0.03% and MgSO₄=0.03% (1f) the effect of hydrolysate and KCl on amylase activity when response surface is fixed at starch=0.3%, peptone=0.6%, MgSO₄=0.03%

All the factors considered in the model were found to have a significant effect and enhanced the production of amylase activity. Inorganic salts like MgSO₄ and KCl have been reported to have a positive influence on α -amylase production by *B. stearothermophilus* (Srivastava & Baruah, 1986). Furthermore, starch and other polysaccharides positively influences α -amylase production by *Bacillus* sp. (Gangadharan et al., 2008). The P-values also indicated that some of the interactive effects of the media components were significant with respect to α -amylase production.

The interactive effects of all the media components on the amylase activity was analyzed by generating 3-D response surface plots (Fig 7.6). In all the cases the lower concentration of BSG hydrolysate tended to enhance amylase activity. Maximum amylase activity was achieved at a BSG hydrolysate concentration range of 0.2 to 0.24 %. Any increase in hydrolysate concentration in the media beyond this range was found to be detrimental to enzyme activity. A higher hydrolysate concentration was found to be inhibitory for amylase production. This might be due to catabolite repression due to the presence of glucose in the hydrolysate (Ravindran et al., 2018). In a recent study, Ouattara et al. (2017) had reported incidences of catabolite repression due to the presence of glucose in the fermentation media when *Bacillus* sp. was used as the enzyme producer. Although a higher concentration of glucose can result in catabolite repression, the presence of this monosaccharide in the fermentation medium enhances the growth of the bacteria. In fact, lower concentrations of maltose and glucose have been reported to enhance amylase activity in *B. stearothermophilus* (Rahmati et al., 2017). A higher biomass production does not necessarily have to translate into similar enzyme production kinetics. Simair et al. (2017) conducted an

extensive study to determine the best carbon and nitrogen sources for *Bacillus sp.* BCC 01-50 amylase. Although there was no significant difference in biomass formation glucose augmented medium compared to that containing starch, the latter resulted in higher amylase activity.

To study the effects of BSG hydrolysate on biomass formation dry cell weight studies were conducted. Consequently, a 22% increase in biomass was observed in the experimental trial when compared to control. These all point to the fact that low concentrations of BSG hydrolysate is favourable for cell biomass formation and α -amylase production. An increase in peptone content also positively influences the amylase activity. Rajagopalan and Krishnan (2008) had reported similar findings in a study involving the effects of peptone concentration in media on the production of α -amylase by *Bacillus subtilis* KCC103.

The RSM experimental design generated optimized levels for every factor upon analyzing the observed amylase activity after each trial. Accordingly, the optimized values for each media component was as follows: starch 0.2% (w/v), peptone 0.2% (w/v), KCl.4 H₂O 0.02% (w/v), MgSO₄.7H₂O 0.01% (w/v) and hydrolysate 0.22% (v/v). The model predicted an enzyme activity of 203.3 U/ml on testing the optimized conditions. Validation experiments following optimized media composition resulted in 198.09 U/ml of amylase activity. Minimal disparity between predicted and observed values (<5%) confirmed that the model was valid and also indicated that it was adequate enough to predict the optimum values for variables considered for amylase production. Furthermore, the amylase yield obtained after optimizing the media formulation was 1.3-fold higher than that observed after control fermentation (152.3 U/ml).

7.3.3 Purification of thermostable *B. stearothermophilus* amylase

Enzymes are commonly purified by a multi-step process. These steps may be specific or non-specific depending upon the purification strategy adopted. Increasing the number of steps in protein purification strategy can result in a low final yield (Bajpai, 2014). Ion exchange chromatography is a common technique used for the purification of α -amylase. In this study, a three-step strategy was adopted to purify the thermostable α -amylase. The summary of the purification stages has been provided in table 7.4. The crude enzyme which exhibited a total activity of 9152 U and specific activity of 34.63 U/mg was subjected to ammonium sulphate precipitation (65% w/v) at 4°C. This step resulted in a purification of 1.24-fold and theoretical yield of 76%. This was followed by an ultrafiltration/diafiltration step which led to an increase in purification fold by 12.44-fold. The final purification strategy involved ion exchange chromatography where the concentrate was loaded on to DEAE-Sepharose Fast Flow column and eluted using 0.5 M NaCl via isocratic mode after removal of unwanted proteins. The active fractions were collected and concentrated by diafiltration. The last stage of purification resulted in the final enzyme preparation achieving 13.09-fold purification and a theoretical yield of 25.1%. As shown in Fig. 7.7, a single, salt-eluted peak (A_{280}) was obtained in ion-exchange chromatography. The theoretical yield and purification folds can be comparable with earlier published studies. Chakraborty et al. (2000) in studies involving *B. stearothermophilus* for amylase production had reported a yield of 56% and purification fold of 6.7.

Table 7.4 Purification of α -amylase from *B. stearothermophilus*

| Step | Total activity (U) | Total protein (mg) | Specific activity (U/mg) | Purity (fold) | Theoretical Yield (%) |
|-------------------------------|---------------------------|---------------------------|---------------------------------|----------------------|------------------------------|
| Cell Supernatant | 9152 | 264 | 34.63 | 1.00 | 100 |
| Ammonium sulphate precipitate | 6956 | 161 | 43.15 | 1.24 | 76.0 |
| Ultra/Diafiltration | 5508 | 13 | 431.13 | 12.44 | 58.0 |
| DEAE Sepharose Fast Flow | 2297 | 5 | 453.45 | 13.09 | 25.1 |

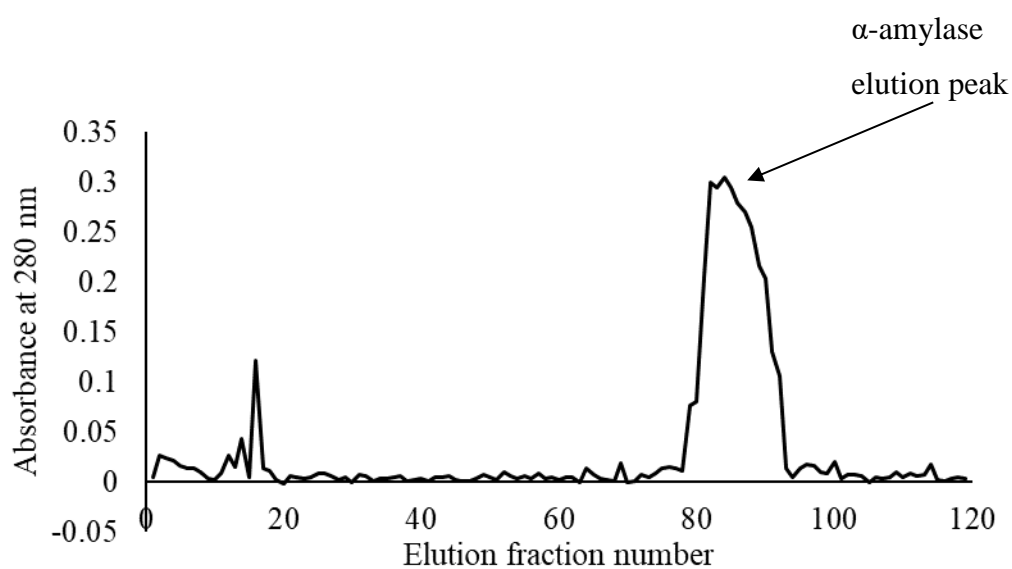


Figure 7.7 Elution profile of α -amylase from DEAE Sepharose Fast Flow

Similarly, Fincan and Enez (2014) reported a purification fold of 65 and yield of 46% employing a four-step purification strategy that involved size exclusion chromatography (Sephadex G-100) followed by ion exchange chromatography employing DEAE cellulose. The three-stage purification strategy adopted in this study for thermostable α -amylase enabled the authors to perform biochemical characterization to determine the optimum pH and temperature for enzyme activity. Furthermore, the purification fold and yield of the α -amylase was high with less number of purification stages. This purification procedure can be easily adopted for large-scale purification.

The molecular weight of denatured thermostable α -amylase was determined by relative mobility of standard proteins on SDS-PAGE. The relative molecular mass of the enzyme was estimated via 10% SDS-PAGE and appeared to be 64 kDa (Fig 3). The enzyme appeared as a single protein band suggesting that it was a homogenous monomer with a single polypeptide chain. This was in agreement to reports published elsewhere. For example, Fincan and Enez (2014) conducted an extensive study that purified and characterized α -amylase produced by *Geobacillus stearothermophilus* where molecular weight of thermostable amylase was found to be 63 kDa.

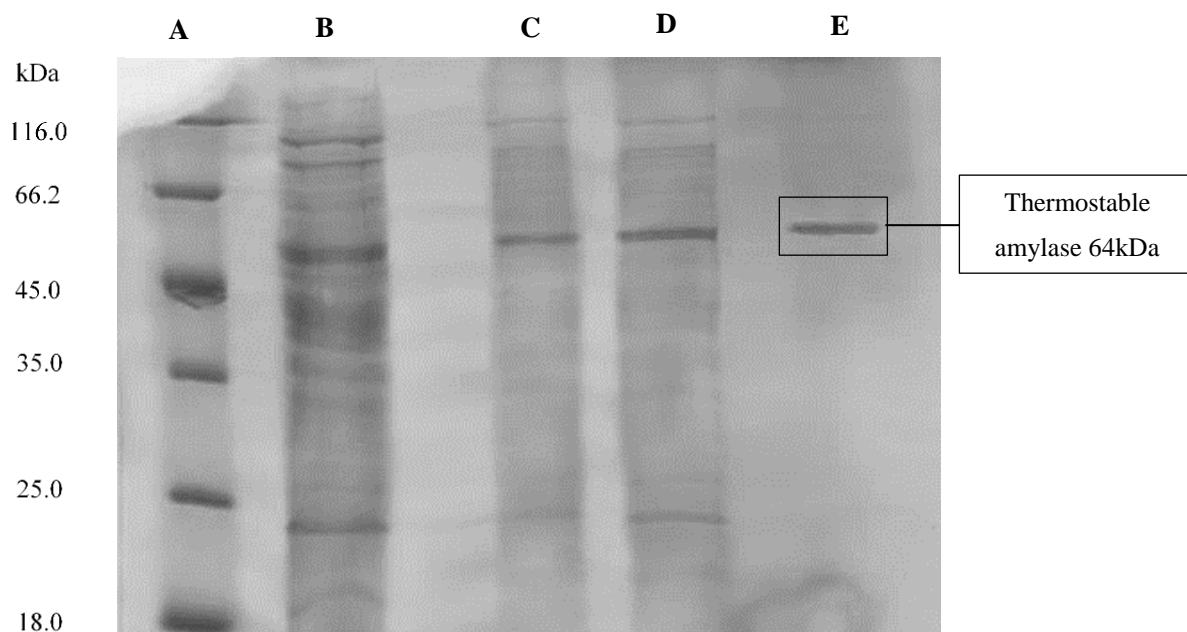


Figure 7.8 SDS-PAGE illustrates different stages of purification: (A) molecular size markers (B) Cell free supernatant (crude enzyme), (C) enzyme after ammonium sulphate precipitation (D) Enzyme after ultrafiltration/diafiltration (E) enzyme after DEAE-Sepharose Fast Flow Chromatography (64kDa)

7.3.4 Antibiofilm activity of α -amylase and determination of inhibitory concentration

Antimicrobial agents ineffective against biofilm producing bacteria due to their ability to produce exopolysaccharides (EPS). This forms a barrier around the bacterial cell protecting it within the biofilm. Enzymes such as proteases and lysozyme have been proven to be effective antibiofilm agents (Mirsada et al., 2018; Vaikundamoorthy et al., 2018). Carbohydrate degrading enzymes such as cellulases and amylases along with alginate lyase have been shown to reduce biofilm activity in *Pseudomonas* strains. This polysaccharide structure is necessary for the bacterial cell for pellicle formation (Alkawash et al., 2006). Additionally, polysaccharide intercellular adhesin (PIA), extracellular-DNA, proteins, and amyloid fibrils form the extracellular polymeric structures of staphylococcal biofilm. Therefore, in this study, three strains

viz. *S. aureus* NCTC 1803, *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27835 were screened for biofilm production capacity. Black colonies with crystalline consistency was an indication of biofilm formation (Fig. 7.9) (Lee et al., 2016).

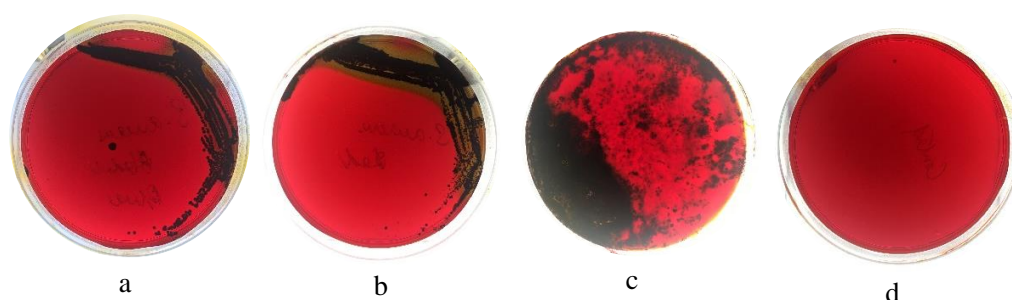


Figure 7.9 Biofilm formation in BHI agar supplemented with Congo red: the appearance of black crystalline colonies indicate exopolysaccharide production (a) *S. aureus* NCTC 1803 (b) *S. aureus* ATCC 25923 (c) *P. aeruginosa* ATCC 27835 (d) control

In the case of commercial amylase (specific activity 439.0 U/mg), 20 μ l of the amylase was able to inhibit biofilm production by all the three strains (Fig 7.10 a). The crude enzyme (specific activity 36.63 U/mg) was able to achieve considerable disruption in biofilm formation in all the pathogenic organisms tested. The BIC for crude enzyme varied among all the bacterial strains tested (Fig 7.10 b). The BIC for *S. aureus* NCTC 1803 and *S. aureus* ATCC 25923 was found to be 150 μ l (198.09 U/ml). Meanwhile the BIC for *P. aeruginosa* ATCC 27853 was 100 μ l. Thereafter, increasing the concentrations of the amylase led to the decrease in biofilm inhibition. This trend was observed in both crude amylase and commercial amylase trails. Employing crude amylase resulted in inhibiting biofilm formation of the all the strains tested by 62%, 55% and 50% respectively. A similar observation was reported by Kalpana et al. (2012) that involved crude, purified and commercial amylase derived from *Bacillus subtilis* S8-18 as an antibiofilm forming agent against pathogenic bacterial strains.

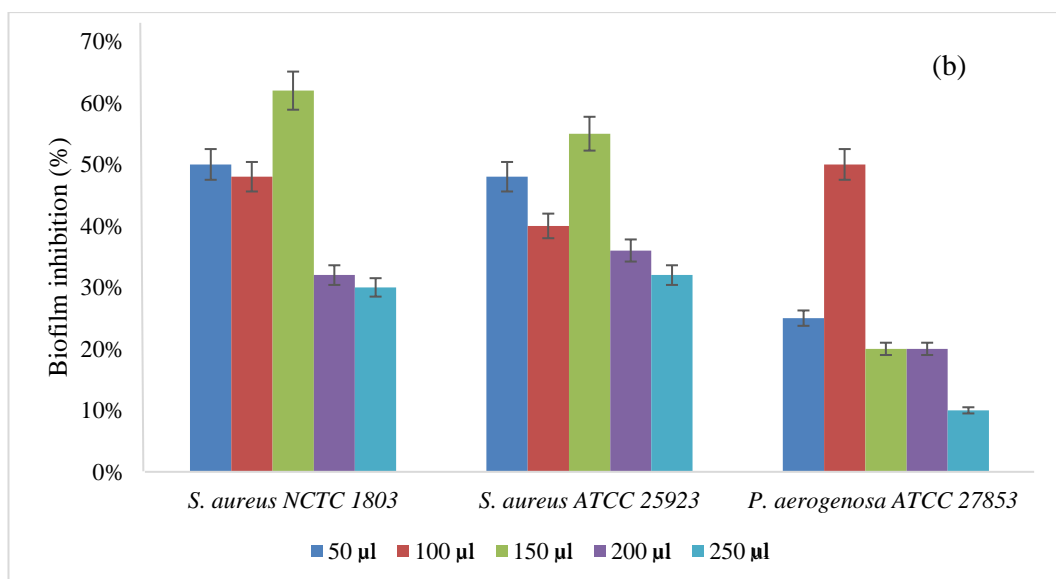
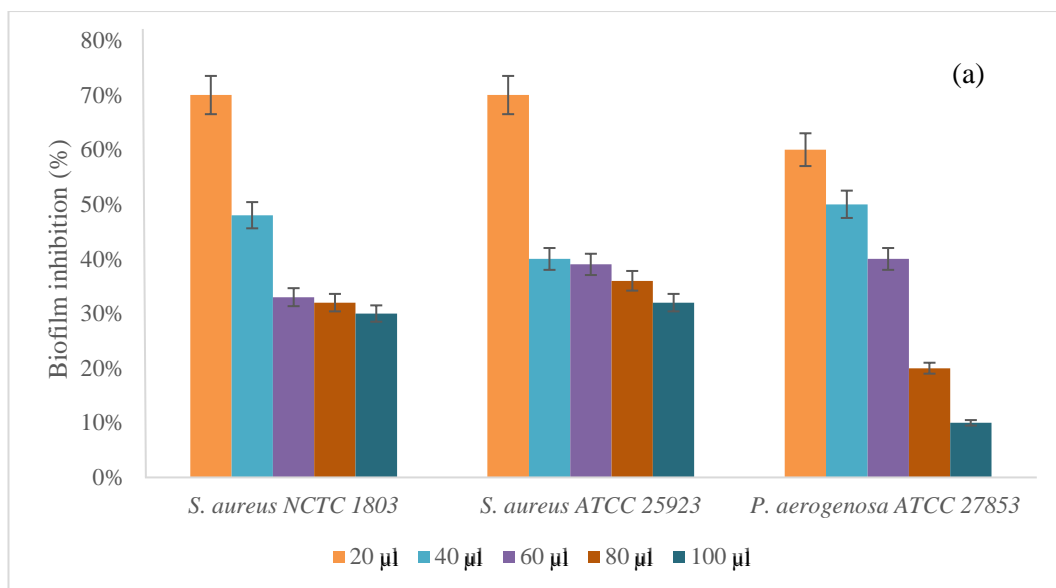


Figure 7.10 Quantitative determination of BIC for commercial α -amylase enzyme (a) and (b) crude α -amylase from *B. stearothermophilus*

Exopolysaccharides provide strength to the bacterial cell wall while preventing the entry of antimicrobial agents in to cells (Czaczyk & Myszka, 2007). Circumventing this problem calls for novel solutions. α -amylase have been widely studied as a potential anti-biofilm agent by several researchers. Craigen et al. (2011) found that α -amylases from four different sources viz. *Bacillus*, *Aspergillus*, sweet potato and saliva

was effective against biofilm producing capacity of *Staphylococcus aureus*. Other studies have reported that proteases are better at preventing biofilm formation and antimicrobial activity compared to α -amylases (Mitrofanova et al., 2017; Molobela et al., 2010). Some studies report the use of an antimicrobial enzyme cocktail including proteases, lysozyme, oxidative enzymes, amylases etc. to prevent or remove biofilm formation. However, the presence of protease can lead to the degradation of other enzymes leading to partial removal of biofilm (Kalpana et al., 2012). Hence this study was solely focused on the effect of thermostable *B. stearothermophilus* α -amylase on biofilm formation.

7.4 Conclusion

In this study, for the first time BSG hydrolysate was used to successfully formulate media for the production of thermostable α -amylase by *B. stearothermophilus*. The presence of hydrolysate in small quantities enhanced the growth and enzyme yield of *B. stearothermophilus*. This resulted in an increase of enzyme yield by 1.3-fold. The enzyme produced was purified by three-step purification strategy. This resulted in a 13.09-fold purification with a yield of 25%. On characterizing the enzyme, α -amylase exhibited maximum activity at a pH range of 7-8 and temperature range of 60°C to 70°C. Furthermore, *B. stearothermophilus* α -amylase was successfully tested as an antibiofilm activity against three pathogens viz. *S. aureus* NCTC 1803, *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27835 inhibiting their biofilm producing capacity by 62%, 55% and 50% respectively. Hence this study concludes that BSG hydrolysate can be a potential media component for α -amylase production.

Chapter 8

Spent coffee waste as a potential media component for xylanase production employing *Aspergillus niger* ATCC® 6275

*Xylanases are enzymes that hydrolyse xylan into xylose or oligomeric mixtures. It has been widely used in the bleaching of paper as well as in baking and juice clarification. Spent Coffee Waste (SCW) is an important by-product of the coffee industry. It is highly rich lipids, proteins and polysaccharides. In this study SCW was used as the sole carbon source for xylanase production in solid state fermentation mode. Mycelial fungi, *Aspergillus niger* was used as the fermentative microbe. A Box-Behnken design was constructed using three parameters viz. temperature, initial moisture content and log no. of spores to determine the optimal fermentation condition for xylanase production. The data obtained was fitted into the model and an optimum level was obtained on which nutrient supplementation for the fermentation was optimised. A Plackett-Burman design was used to screen ten nutrient components. Further observations revealed that yeast extract, K_2HPO_4 and $MgSO_4$ exerted maximum influence on the xylanase production. The media for fermentation was further optimised using a Box-Behnken design. The best fermentation conditions for xylanase production was found to be incubation at 30°C with an initial moisture content of 70% and using an inoculum of 6.5×10^6 spores/g of dry SCW. Furthermore, the design of experiments revealed that maintaining a medium composition of 0.2g of yeast extract, 0.04g of K_2HPO_4 and 0.03g of $MgSO_4$ increased xylanase production. Under optimised SSF conditions an enzyme activity of 6495.6 U/g of dry SCW was registered which was approximately 1.39-fold higher than control (4649 U/g of dry SCW). The efficacy of the purified xylanase as a juice enrichment agent for strawberry, blueberry and raspberry pulp.*

8.1 Introduction

Lignocellulosic residues are plant-based materials that are generated in large quantities by the agriculture industry. They are rich in carbohydrates and lignin, with small amounts of other potentially valuable compounds such as proteins, lipids and polyphenols. They have gained considerable interest as a cheap carbon source in fermentation processes (Anwar et al., 2014). The polysaccharide fraction of lignocellulose can be broadly classified into cellulose and hemicellulose, depending upon the composition. After cellulose, hemicellulose is the most abundant carbohydrate fraction in lignocellulosic materials and constitutes 30% of the plant cell walls (Das & Ray, 2016). Xylan is one of the main components that comprise hemicellulose. Xylan is degraded enzymatically by several bacterial and fungal species by synthesising a specific group of enzymes called xylanases. Xylanases are hemicellulolytic enzymes that specifically degrade β -1, 4-xylans, and find wide application in areas such as juice and wine clarification, food processing and paper pulping (Irfan et al., 2014). They are also used in combination with cellulase in the digestion of silage to improve nutritional qualities. Xylanase is commercially produced in large scale by the utilisation of xylan as the key carbon source in fermentation. This process adds to the cost of production. Xylan can arguably be replaced by cheap agricultural, lignocellulosic residues which are abundantly available thereby reducing enzyme production costs.

Most enzymes are commercially produced in large scale by following submerged fermentation strategy due to ease of process control. However, solid state fermentation is an alternative fermentation strategy that has numerous advantages, such as high

volumetric productivity, less effluent generation, simplistic instrumentation and equipment, and most importantly, low capital investment and operational cost (Ajijolakewu et al., 2017; Irfan et al., 2014). Filamentous fungi are excellent producers of lignocellulytic enzymes such as cellulases and xylanases and are perfect strains for enzyme production via the solid-state fermentation mode; combined with a lignocellulosic substrate holds advantages of low-cost operation coupled with high enzyme titre (Ang et al., 2013). Some of the common lignocellulosic residues employed in SSF-based enzyme production studies include rice bran, wheat bran, corn cob, sugar cane bagasse (Irfan et al., 2014). Coffee by-products are one such lignocellulose residue which is produced in abundance by the growing global coffee industry. Some of the by-products derived from coffee are coffee husk, silver skin, pulp and spent coffee. While coffee husk is high in cellulose and lipid content, spent coffee is high in hemicellulose content, in addition to representing a valuable reservoir of cellulose, lipids, proteins and polyphenols. This makes spent coffee an ideal substrate for enzyme production (Ballesteros et al., 2014). Despite the high nutritional value, not many studies are available that utilise spent coffee in SSF mode for the production of xylanases. Murthy and Naidu (2012) published an interesting study where coffee by-products were used in SSF mode to produce xylanases employing *Pencillium* sp. CFR 303 as the enzyme producer.

In the present study, we have attempted to utilise spent coffee waste (SCW) as the sole carbon source for xylanase production. *Aspergillus niger* ATCC® 6275 was employed as the enzyme producing fungus. The process parameters for SSF mode were optimised by the application of response surface methodology. The xylanase was subsequently purified by a three-step strategy following and preliminary

characterization performed. The purified xylanase was finally tested as a potential juice enrichment agent for three fruit pulp varieties.

8.2 Methodology

8.2.1 Lipid extraction from SCW

The total lipids were extracted from SCW by following the protocol described by (Ahangari & Sargolzaei, 2013). Briefly, 10g of SCW was weighed and taken in a cellulose thimble which was then stoppered with cellulose wool. A Soxhlet apparatus was set up with 100 ml of petroleum ether in a round bottom flask and anti-bumping granules. Extraction was conducted for 6h in reflux cycles or until constant weight of petroleum ether-oil mixture was attained. The petroleum ether was then evaporated off leaving behind the freshly extracted SCW lipids. The weight of the lipids recovered was recorded and the lipid-free SCW was stored for further experiments.

8.2.2 Screening of fungal species for xylanase production capacity

Four fungal strains *viz.* *Aspergillus fumigatus* (A1), *Paecilomyces variotii* (A2), *Penicillium roqueforti* (A3) and *Aspergillus niger* (A4) were selected for screening of xylanase producing capacity. Czapek Dox Agar supplemented with 1% xylan was inoculated with the strains and allowed to grow for 72h. The plates were then flooded with 1% Congo red and allowed to stand for 15 min. The plates was washed with 1% NaCl and then observed for zones of clearance. *A. niger* exhibited largest zone of clearance and was thus selected for further studies.

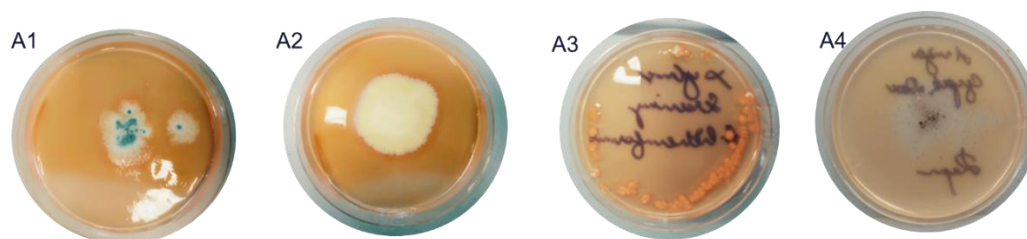


Figure 8.1 Screening of fungal strains for xylanase production capacity

8.2.3 Microorganism and xylanase production

Aspergillus niger (ATCC® 6275) was obtained from the microbiology repository at the School of Food Science and Environmental Health, DIT. The microorganism was grown on potato dextrose agar (PDA) slants at 30°C for five days and subsequently stored at 4°C. The microbe was screened for xylanase production capability by culturing it on Czapek Dox agar plates supplemented with 1% xylan and incubation for 72h at 30°C. The plates were stained with 1% Congo red for 20 min followed by de-staining using 1M NaCl. The plates were checked for zones of clearing (Kalim & Ali, 2016). For fermentation purposes, the inoculum was prepared by the protocol described by (Pal & Khanum, 2010). Briefly, a spore preparation was prepared by re-suspending spores from the slants in 0.1% sterile Tween-80. This was used a master suspension from which dilutions were prepared to achieve the required spore concentration.

8.2.4 Substrate preparation and solid-state fermentation

SCW was subjected to organosolv pre-treatment as mentioned in a previous study conducted at our facility (Ravindran et al., 2017). Five grams of pre-treated SCW was taken in a 250 ml Erlenmeyer flask supplemented with original medium for solid-state fermentation experiments consisting of (in g/g of dry SCW) $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, (0.01); $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, (0.05); KH_2PO_4 , (0.5); and industrial yeast extract, (0.05). The contents

of the flask were autoclaved at 121°C for 30 min followed by which it was inoculated with 1 ml of inoculum ($\sim 1 \times 10^6$ spores/ml). Fermentation was carried out for 120h followed by enzyme extraction (Park et al., 2002). SSF was conducted for 96h. After each trial the fermented samples were subjected to an enzyme extraction process and assayed for xylanase activity and soluble protein content.

8.2.5 Enzyme extraction and assay

On completion of each fermentation experiment, the contents of each flask were re-suspended by the addition of citrate buffer (0.05M, pH 5.0). A solid-to-liquid ratio of 1 g initial dry substrate/ 12 ml of buffer was maintained for xylanase extraction. This mixture was homogenised for 20 min and centrifuged for 1,200 rpm for 30 min at 4°C. The solids were separated from the liquids by centrifugation followed by filtration through Whatman No. 1 filter paper to remove any particulate matter. The filtrate was collected and then assayed for xylanase activity. Xylanase activity was estimated as follows: 50 µl of enzyme solution was mixed with 100 µl of 1% Birchwood xylan prepared in 0.05 M citrate buffer (pH 4.8). The mixture was incubated for 20 min at 50°C (Park et al., 2002). This was followed by determination of reducing sugar by the dinitrosalicylic acid method (Miller, 1959). Xylose was used as a standard. One unit was defined as the amount of enzyme that releases 1µmol of xylose in 1 min under assay conditions.

8.2.6 Optimisation of process parameters

8.2.6.1 Optimisation of physical parameters

In this study the three physical parameters pertaining to solid state fermentation *viz.* temperature, moisture content and inoculum size (log no. of spores) was optimised by the application of response surface methodology. A Box Behnken design was

incorporated into the study taking into account the three factors and three different levels (Table 8.5). A total of 15 experiments were conducted in triplicate- and the average xylanase activity was taken as the response. A second order polynomial equation was generated and analysed by statistical software.

Table 8.1 Variables and level for Box-Behnken Design for the optimisation of physical parameters

| Factors | Coded Symbols | Basic level | Variation level | Value of factor | Coded value |
|------------------------|----------------|-------------|-----------------|-----------------|-------------|
| Temperature | X ₁ | 30 | 5 | 25 | -1 |
| | | | | 30 | 0 |
| | | | | 35 | +1 |
| Moisture content (%) | X ₂ | 70 | 2 | 68 | -1 |
| | | | | 70 | 0 |
| | | | | 72 | +1 |
| Log (number of spores) | X ₃ | 6.5 | 1 | 5.5 | -1 |
| | | | | 6.5 | 0 |
| | | | | 7.5 | +1 |

8.2.6.2 Plackett-Burman design for identification of significant variables

A Plackett-Burman design was adopted to supplement SCW with media components that supported the production of xylanase enzyme. Plackett-Burman is essentially a two-factorial design which identifies the physico-chemical parameters required to increase the levels of xylanase production. This design of experiments strategy assumes that there are no interactions between variables. The variables are investigated at two levels, -1 (low level) and +1 (high level), spaced by a large interval. The experimental design conducts n+1 screening trials for n variables. In this study, 10 potential media components *viz.* KH₂PO₄, NaCl, MgSO₄, Yeast Extract, Peptone, (NH₄)₂SO₄, NH₄Cl, CaCl₂, FeCl₃ and KCl were included to determine which one enhanced the production of xylanase by *A. niger* ATCC® 6275 via solid state

fermentation (Table 8.2). Minitab®17.0.1. was used to generate the experimental design. The effects of individual variables on the production of xylanase was calculated by the following equation:

$$E = (\Sigma M_+ - \Sigma M_-) / N$$

where E is the effect of the parameter and M₊ and M₋ are responses (xylanase activity) of each experimental trail where parameters are at their highest (M₊) and lowest (M₋) levels while N is the number of trials.

Table 8.2 Nutrient supplements for the screening of nutrients using Plackett-Burman method

| Nutrient Code | Compound | (+) Level (%) (g/g of dry substrate) | (-) Level (%) (g/g of dry substrate) |
|---------------|---|--------------------------------------|--------------------------------------|
| A | KH ₂ PO ₄ | 0.2 | 0.1 |
| B | NaCl | 0.1 | 0.05 |
| C | MgSO ₄ | 0.1 | 0.05 |
| D | Yeast Extract | 0.2 | 0.1 |
| E | Peptone | 0.2 | 0.1 |
| F | (NH ₄) ₂ SO ₄ | 0.2 | 0.1 |
| G | NH ₄ Cl | 0.2 | 0.1 |
| H | CaCl ₂ | 0.1 | 0.05 |
| I | FeCl ₃ | 0.1 | 0.05 |
| J | KCl | 0.1 | 0.05 |

8.2.7 Optimisation of media composition

As with optimisation of physical parameters for SSF optimisation, a Box-Behnken design was adopted to optimise the concentration of media components identified in

the Plackett-Burman screening study. Three parameters and three levels were taken into account to generate the model (Table 8.3).

Table 8.3 Variables and level for Box-Behnken Design for the optimisation of nutrient supplements

| Independent variables | Coded Symbols | Levels | | |
|---------------------------------|----------------|--------|-----|-----|
| | | -1 | 0 | +1 |
| Yeast Extract | Y ₁ | 0 | 0.5 | 1.0 |
| K ₂ HPO ₄ | Y ₂ | 0 | 0.1 | 0.2 |
| KCl | Y ₃ | 0 | 0.1 | 0.2 |

8.2.8 Purification of xylanase

Xylanase was purified using a modification of the protocol devised by (Pal & Khanum, 2011b). Crude xylanase obtained after solid state fermentation was subjected to 65% w/v (NH₄)₂SO₄ precipitation and stored overnight at 4°C. The solution was then centrifuged at 15,000 rpm for 30 min. The precipitate obtained was dissolved in 0.05M citrate buffer (pH 6.0). Desalination was performed by dialysis using a cellulose tubing (molecular mass cut-off 10 kDa) against citrate buffer for 24h with frequent changes of buffer. The xylanase was further concentrated by diafiltration using a 10 kDa MWCO Amicon Ultra-15 centrifugal filter unit (Millipore, Germany) and centrifugation at 5,000 rpm for 10 min at 4°C. The final purification step involved anion exchange chromatography using a DEAE-cellulose column (1.5 cm × 6.0 cm) which was equilibrated with 0.05M citrate buffer (pH 6.0). After elution of unbound proteins, the bound proteins were collected with a step elution of varying

concentrations from 0.1~0.5M KCl in the same buffer by collecting 3 ml fractions while maintaining a flow rate of 30 ml/h. The active fractions were pooled and concentrated by ultrafiltration (10 kDa cut-off).

8.2.9 Application of xylanase for fruit juice clarification

8.2.10 Preparation of puree

Ripened varieties of three berries *viz.* strawberries (*Fragaria ananassa*), blueberries (*Cyanococcus*) and raspberries (*Rubus idaeus*) were purchased from a local market in Dublin city. They were thoroughly washed and inspected for any microbial growth and stored at 4°C for not more than 2 days. The berries were macerated using a blender until a smooth puree was obtained. The puree was then filtered using a cheese cloth to separate the pulp from the juice.

8.2.11 Juice enrichment by xylanase treatment

The effectiveness of the purified xylanase for juice clarification was inspected by determining the incubation time, the incubation temperature and enzyme dosage. The dosage used for juice clarification was optimised between the ranges of 5 to 25 U/g of fruit pulp. The incubation time and temperature required for optimal reducing sugar release, juice yield and clarification were determined by conducting experimental trials at different temperatures, ranging from 30°C to 60°C for different time periods (30-120 min). For enzyme dosage and incubation experimental trials, pulp devoid of any enzyme at a retention time of 0 was taken as control. However, pulp maintained at 27°C was used as control for temperature optimisation experiments. After each experiment, the enzymes were deactivated by subjecting the pulp to 80°C for two minutes, after which the pulp was strained through a cheese cloth to obtain juice after enzymatic treatment.

8.2.12 Determination of reducing sugar and clarity

The clarity in the pulp was measured by determining the percentage transmittance. A UV-VIS spectrophotometer (Perkin Elmer Lambda 900, US) was employed for this purpose. Transmittance was measured at a wavelength of 660 nm. Distilled water was used as reference. Juice clarity was estimated as a measure of percentage transmittance. The release of reducing sugar from puree following enzymatic treatment was measured by using dinitrosalicylic acid method (Miller, 1959). For yield measurement, the enzymatically treated fruit pulp was filtered using filter paper (Whatman No. 1). The filtrate was measured, and the yield was expressed as % w/v (Pal & Khanum, 2011a).

8.3 Results and Discussion

8.3.1 Optimisation of fermentation parameters

SCW was initially subjected to lipid extraction process followed by organosolv pretreatment as a part of the upstream processing strategy. The detailed observations of these experiments have been published in literature elsewhere as part of different studies performed in our facility (Ravindran et al., 2018; Ravindran et al., 2017). According to our study, 13.4% of the total weight of dry SCW was contributed by lipids (results not shown). Furthermore, SCW was found to be high in hemicellulose content. This fraction of the biomass was subjected to organosolv pretreatment to enhance xylanase production.

Solid state fermentation for xylanase production using pre-treated SSF was conducted by the application of a Box-Behnken Design. Three parameters *viz.* temperature, moisture content and inoculum size were taken into consideration for optimisation. Table 8.4 represents the variables, and each variation level. The theoretical and

observed xylanase activity with respect to the Box-Behnken design has been provided in Table 1c. The models were compared based on the coefficient of determination (R^2) and adjusted coefficient of determination (R^2 -adj). The R^2 is the regression of sum of squares proportion to the total sum of squares and illustrates the adequacy of a model. R^2 ranges from 0 to 1, and a value closer to 1 indicates that the model is considerably accurate. A value of 99.65% R^2 was observed in the present study, while R^2 -adj was 97.04%, illustrating that the model adequately fitted the data. The results obtained from the Box Behnken design were fitted to a second order polynomial equation. The polynomial equation for the model is given below:

$$\begin{aligned} \text{Xylanase activity (U/g)} = & - 1.04829 \times 10^6 + 2508.5 \times X_1 + 27204.3 \times X_2 + 17259.2 \times X_3 - \\ & 20.65 \times X_1^2 - 18.2 \times X_1 \times X_2 - 12.15 \times X_1 \times X_3 - 184.813 \times X_2^2 - \\ & 88.125 \times X_2 \times X_3 - 778.0 \times X_3^2 \end{aligned}$$

where X_1 , X_2 and X_3 represents temperature ($^{\circ}\text{C}$), moisture content [% (w/w)] and inoculum size (log no. of spores/g of dry substrate).

Table 8.4 Box-Behnken experimental design for SSF Optimisation employing three independent variables, experimental and predicted values for xylanase activity

| Trail no. | Temperature (°C) | Moisture Content (%) | Log (no. of spores) | Observed xylanase activity (U/g) | Predicted xylanase activity (U/g) |
|------------------|-------------------------|-----------------------------|----------------------------|---|--|
| 1 | 25 | 68 | 6.5 | 4215 | 4281 |
| 2 | 30 | 68 | 7.5 | 4653 | 4572 |
| 3 | 30 | 72 | 5.5 | 4113 | 4194 |
| 4 | 25 | 70 | 7.5 | 5504 | 5520 |
| 5 | 35 | 72 | 6.5 | 4358 | 4293 |
| 6 | 35 | 70 | 5.5 | 3477 | 3461 |
| 7 | 25 | 70 | 5.5 | 4190 | 4174 |
| 8 | 25 | 72 | 6.5 | 5557 | 5491 |
| 9 | 30 | 70 | 6.5 | 5724 | 5724 |
| 10 | 30 | 70 | 6.5 | 5724 | 5724 |
| 11 | 35 | 70 | 7.5 | 4548 | 4564 |
| 12 | 30 | 72 | 7.5 | 5016 | 5066 |
| 13 | 35 | 68 | 6.5 | 3744 | 3810 |
| 14 | 30 | 68 | 5.5 | 3045 | 2995 |
| 15 | 30 | 70 | 6.5 | 5724 | 5724 |

Significant parameters were determined by conducting analysis of variance (ANOVA) (Table 8.5). Meanwhile, Fischer’s statistical test of ANOVA determines the influence of each factor and their significance. The ANOVA table showed that eight effects have P-values less than 0.05, indicating that they are significantly different from zero at the 95.0% confidence level, confirming considerable influence of these coefficients on xylanase activity.

Table 8.5 Analysis of variance obtained for xylanase activity

| Source | Sum of Squares | Df | Mean Square | F-Ratio | P-Value |
|-------------------------------|----------------------|----|----------------------|---------|---------|
| X ₁ | 1.39×10 ⁶ | 1 | 1.39×10 ⁶ | 191.23 | 0.0001 |
| X ₂ | 1.43×10 ⁶ | 1 | 1.43×10 ⁶ | 196.77 | 0.0001 |
| X ₃ | 3.00×10 ⁶ | 1 | 3.00×10 ⁶ | 411.16 | 0.0002 |
| X ₁ X ₁ | 984052 | 1 | 984052 | 135.03 | 0.0001 |
| X ₁ X ₂ | 132496 | 1 | 132496 | 18.18 | 0.008 |
| X ₁ X ₃ | 14762.3 | 1 | 14762.3 | 2.03 | 0.2139 |
| X ₂ X ₂ | 2.02×10 ⁶ | 1 | 2.02×10 ⁶ | 276.88 | 0.0003 |
| X ₂ X ₃ | 124256 | 1 | 124256 | 17.05 | 0.0091 |
| X ₃ X ₃ | 2.23×10 ⁷ | 1 | 2.23×10 ⁶ | 306.67 | 0.0002 |
| Total error | 36437.8 | 5 | 7287.55 | | |
| Total (corr.) | 1.07×10 ⁷ | 14 | | | |

Three dimensional plots were constructed to investigate the interactions between different variables, and to determine the optimal level of each parameter for maximum response. Response surface plots were generated as a combination of two parameters, while keeping the third parameter constant. Three-dimensional response surfaces generated further gave insights into the interaction between the three factors tested. The 3-D plots related to this optimisation study have been provided in Fig 8.2. The highest point on each plot reveals the optimum value for each variable that facilitated a higher xylanase activity. The model predicted that the best conditions for xylanase production would be maintaining a temperature of 30°C, an initial moisture content of 70% (w/w) and an inoculum size of 6.5×10^6 (log no. of spores/ml). On performing the validation experiment, a high xylanase activity of $5,780 \pm 0.2$ U/g of dry SCW was

recorded. The model predicted a xylanase activity of 6068 U/g of SCW, which suggested minimum disparity (<5%) between predicted and observed values. This also indicated that the model was adequate to predict the optimum values for variables considered for xylanase production when adopting a SSF mode. Similar observations were reported in other studies investigating lignocellulosic residues for xylanase production via SSF. For example, Pal and Khanum (2010) were able to achieve a 4.5-fold increase in xylanase activity using various lignocellulosic substrates and by moistening the media to 70%.

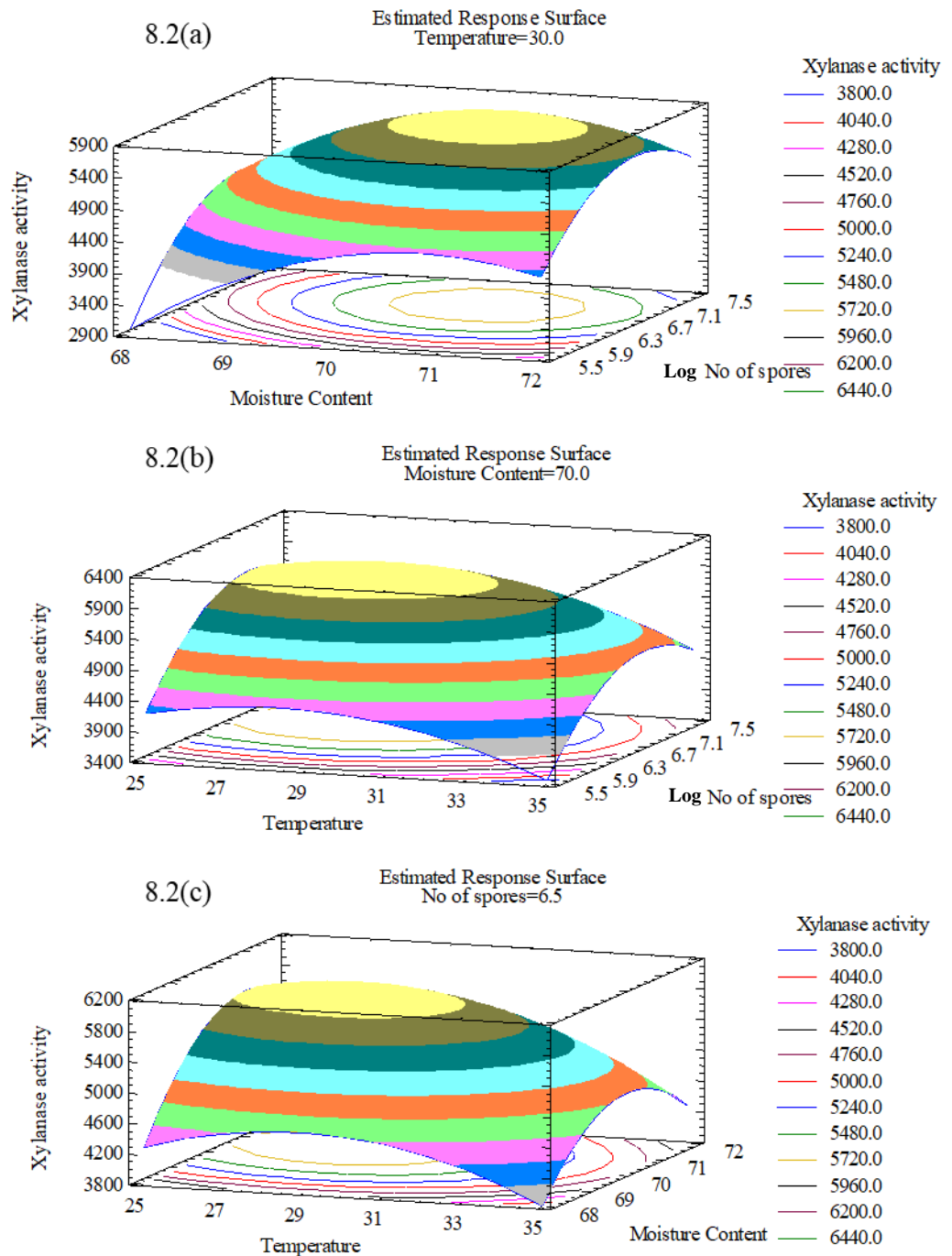


Figure 8.2 Response surface plots representing the effect of independent variables on xylanase activity: (8.2a) the effect of inoculum size (log no. of spores) and moisture content (%) when response surface is fixed at temperature = 30°C (8.2b) the effect of inoculum size (log no. of spores) and temperature (°C) when response surface is fixed at moisture content = 70% (8.2c) the effect of temperature (°C) and moisture content (%) when response surface is fixed at inoculum size (log no. of spores) = 0.5.

8.3.2 Optimisation of media components

8.3.2.1 Screening of media components

Screening of media components was conducted by employing a Plackett-Burman Design of experiments to determine their influence on the yield of xylanase produced by *A. niger* ATCC®6275 employing a solid-state fermentation mode. These included organic and inorganic nitrogen sources, together with metallic salts, and amounting to a total of 10 constituents. Table 8.6 show the different components and their levels used in the Plackett Burman design. Codes A to J were assigned to each component. From analysis performed, it was determined that SCW supplemented with yeast extract, MgSO₄ and KH₂PO₄ (trial no. 7) resulted in the highest xylanase activity. Analysis of variance of the design revealed that these three media components had a significant effect on the production of xylanase by *A. niger*. A regression equation was generated by design software which related all the media components to the xylanase activity by a first order polynomial which is given below:

$$\text{Xylanase activity (U/mg)} = 4507 - 239 A - 941 B + 275 C - 166 D - 201 E + 308 F \\ + 256 G + 260 I + 125 J$$

A model F-value of 15.07 suggested that the model was significant. A determination coefficient (R²) of 97.66% indicated that the fitted model could be used to predict the media supplements which influenced xylanase production. The analysis of variance showed that yeast extract, MgSO₄ and KH₂PO₄ had a pronounced influence on the production of xylanase enzyme (P<0.05). The findings of the Plackett-Burman study were in agreement with other studies based on xylanase production. For example, MgSO₄ has been reported by several researchers to have a positive influence on xylanase production by bacterial and fungal species alike (Geetha & Gunasekaran,

2010; Long et al., 2017). A recent study published by Silva et al. (2016) revealed that higher levels of xylanase production were achieved using KH_2PO_4 in combination with corn stover when *Penicillium crustosum* was used as the xylanase producer. Therefore, further studies were conducted employing the three media components to determine the optimum combinations by means of a Box-Behnken Design.

Table 8.6 Experimental design for the screening of nutrients using Plackett-Burman method

| Blocks | A | B | C | D | E | F | G | H | J | Activity/g of dry substrate(U/g) |
|--------|----|----|----|----|----|----|----|----|----|----------------------------------|
| 1 | 1 | -1 | 1 | 1 | -1 | 1 | -1 | -1 | -1 | 5451 |
| 2 | -1 | -1 | -1 | 1 | 1 | 1 | -1 | 1 | 1 | 5692 |
| 3 | -1 | 1 | 1 | 1 | -1 | 1 | 1 | -1 | 1 | 4281 |
| 4 | 1 | 1 | -1 | 1 | -1 | -1 | -1 | 1 | 1 | 1697 |
| 5 | -1 | -1 | 1 | 1 | 1 | -1 | 1 | 1 | -1 | 4467 |
| 6 | 1 | 1 | 1 | -1 | 1 | 1 | -1 | 1 | -1 | 3488 |
| 7 | 1 | -1 | 1 | 1 | -1 | -1 | 1 | 1 | 1 | 6034 |
| 8 | -1 | 1 | 1 | -1 | 1 | -1 | -1 | -1 | 1 | 3611 |
| 9 | -1 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | -1 | 4860 |
| 10 | 1 | 1 | -1 | 1 | 1 | -1 | 1 | -1 | -1 | 3457 |
| 11 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | -1 | 1 | 4118 |
| 12 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 4567 |

8.3.2.2 Optimisation of media components

A three parameter-two level Box-Behnken design was adopted for the optimisation of media composition for xylanase production using solid state fermentation mode. From the initial screening study, yeast extract, MgSO₄ and KH₂PO₄ were chosen as the three media supplements for further experiments. The parameters and levels were inputted into statistical software Statgraphics Centurion XV which generated a set of 15 experimental trials. Fermentation was conducted for 96 h in shake flasks containing five grams of pretreated SCW supplemented with media additives. The trials were performed in duplicate and the enzymes extracted from each trial were assayed for xylanase activity and protein concentration (Table 8.7). The models were compared based on the coefficient of determination (R²) and the adjusted coefficient of determination (adj-R²). An R² value of 99.07 and an adj-R² of 96.26% were observed, which illustrates that the model adequately fitted the data. A second order polynomial equation was generated by the model into which the data was fitted, and is given below:

$$\begin{aligned} \text{Xylanase activity (U/g)} = & 6175.58 + 14326.7 \times Y_1 - 4426.84 \times Y_2 - 18034.1 \times Y_3 - \\ & 160357 \times Y_1^2 + 25463.2 \times Y_1 \times Y_2 - 3500.13 \times Y_1 \times Y_3 + 323.685 \\ & \times Y_2^2 + 29240 \times Y_2 \times Y_3 - 54692.1 \times Y_3^2 \end{aligned}$$

where Y₁, Y₂ and Y₃ represent the media components KH₂PO₄, yeast extract and MgSO₄ respectively.

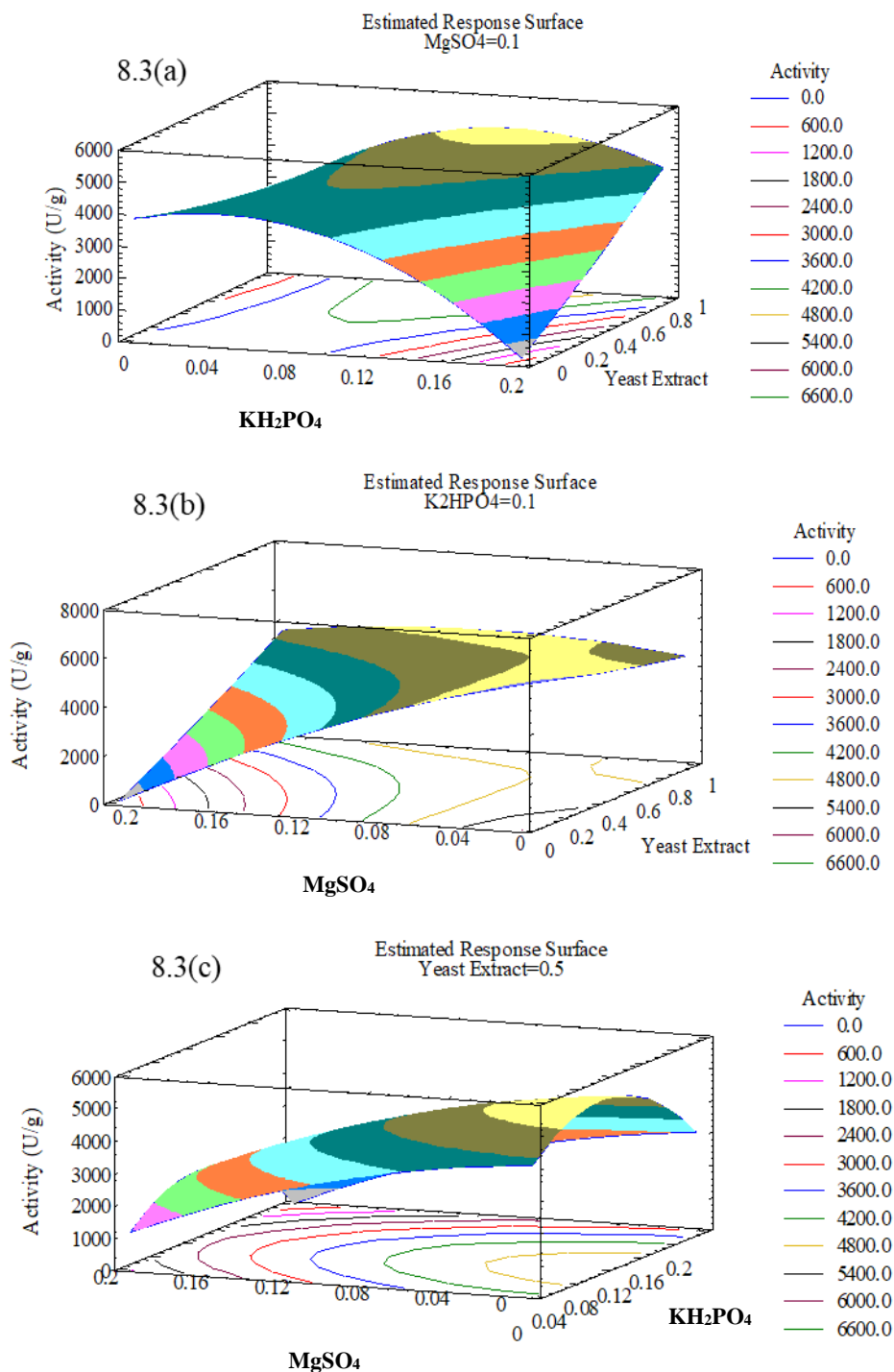


Figure 8.3 Response surface plots representing the effect of independent variables on xylanase activity: (8.3a) the effect of KH_2PO_4 and yeast extract on xylanase activity when response surface is fixed at $MgSO_4 = 0.1$ g/g of SCW (8.3b) the effect of $MgSO_4$ and yeast extract on xylanase activity when response surface is fixed at $KH_2PO_4 = 0.1$ g/g of SCW (8.3c) the effect of KH_2PO_4 and $MgSO_4$ on xylanase activity when response surface is fixed at yeast extract = 0.5 g/g of SCW

Table 8.7 Box-Behnken experimental design for SSF optimisation employing three independent variables, experimental and predicted values for xylanase activity

| Trial no. | K₂HPO₄ (g/g of SCW) | Yeast Extract (g/g of SCW) | MgSO₄ (g/g of SCW) | Xylanase observed (U/g) | Xylanase theoretical (U/g) |
|------------------|--|-----------------------------------|--------------------------------------|--------------------------------|-----------------------------------|
| 1 | 0.2 | 0.0 | 0.1 | 56 | 206 |
| 2 | 0.2 | 1.0 | 0.1 | 4149 | 4120 |
| 3 | 0.1 | 0.5 | 0.1 | 4222 | 4222 |
| 4 | 0.1 | 0.0 | 0.0 | 6065 | 6005 |
| 5 | 0.0 | 0.5 | 0.2 | 1083 | 1173 |
| 6 | 0.2 | 0.5 | 0.0 | 3130 | 3040 |
| 7 | 0.1 | 0.0 | 0.2 | 259 | 140 |
| 8 | 0.1 | 1.0 | 0.2 | 4371 | 4431 |
| 9 | 0.0 | 0.5 | 0.0 | 4012 | 4043 |
| 10 | 0.0 | 1.0 | 0.1 | 2796 | 2646 |
| 11 | 0.1 | 0.5 | 0.1 | 4222 | 4222 |
| 12 | 0.1 | 1.0 | 0.0 | 4329 | 4448 |
| 13 | 0.0 | 0.0 | 0.1 | 3796 | 3825 |
| 14 | 0.1 | 0.5 | 0.1 | 4222 | 4222 |
| 15 | 0.2 | 0.5 | 0.2 | 61 | 30 |

Analysis of variance was conducted to determine the significance of coefficients of the parameters considered in the experimental design. The ANOVA table (Table 8.8) showed that seven variables were found to be significant, considering the P-values being less than 0.05 and indicating that they are significantly different from zero at the 95.0% confidence level. This also meant that these variables have considerable influence on xylanase activity. Interestingly, the interaction effects ($p < 0.05$) of yeast extract with KH_2PO_4 and MgSO_4 had a positive influence on enzyme activity. Among quadratic coefficients only yeast extract had a positive effect on maximising the xylanase activity. Three dimensional plots were generated by the model to understand the interactions between different variables and to determine the optimal level of each parameter for maximum response (Fig 8.3). A maximum xylanase activity of 6,495.6 U/g of activity was achieved by performing solid state fermentation while maintaining

the media composition of yeast extract (0.2g/g of substrate), KH₂PO₄ (0.04g/g of substrate) and MgSO₄ (0.03g/g of substrate). The model predicted a maximum xylanase activity of 6,225 U/g of substrate. The experimentally obtained values and the value predicted by the model had little disparity between them (<5%), which suggested that the model was adequate to predict the optimum measures for maximum xylanase activity. As a consequence of the optimisation protocol a 1.39-fold increase in xylanase activity was observed compared to original media (4,649 U/g of dry SCW).

Table 8.8 Box-Behnken experimental design for SSF optimisation employing three independent variables, experimental and predicted values for xylanase activity

| Source | Sum of Squares | Df | Mean Square | F-Ratio | P-Value |
|-------------------------------|----------------------|----|----------------------|---------|---------|
| Y ₁ | 2.30×10 ⁶ | 1 | 2.30×10 ⁶ | 114.26 | 0.0001 |
| Y ₂ | 3.74×10 ⁶ | 1 | 3.74×10 ⁶ | 185.62 | 0.0002 |
| Y ₃ | 1.73×10 ⁷ | 1 | 1.73×10 ⁶ | 858.69 | 0.0003 |
| Y ₁ Y ₁ | 9.49×10 ⁶ | 1 | 9.49×10 ⁶ | 471.45 | 0.0324 |
| Y ₁ Y ₂ | 6.48×10 ⁶ | 1 | 6.48×10 ⁶ | 321.95 | 0.0021 |
| Y ₁ Y ₃ | 4900.35 | 1 | 4900.35 | 0.24 | 0.6427 |
| Y ₂ Y ₂ | 24178.1 | 1 | 24178.1 | 1.2 | 0.3232 |
| Y ₂ Y ₃ | 8.55×10 ⁶ | 1 | 8.55×10 ⁶ | 424.54 | 0.0041 |
| Y ₃ Y ₃ | 1.10×10 ⁶ | 1 | 1.10×10 ⁶ | 54.84 | 0.0007 |
| Total error | 100695 | 5 | 20139 | | |
| Total (corr.) | 4.88×10 ⁷ | 14 | | | |

8.3.3 Purification of xylanase from *A. niger*

Xylanases are usually purified by a multi-step process. A higher number of steps adopted in the purification process resulted in lower yields of the enzyme in most cases (Bajpai, 2014). Purification of xylanase needs to be achieved in order to perform complete biochemical analysis and molecular studies. Several studies have employed DEAE cellulose/Sepharose for the purification of xylanase from fungal species (Lappalainen et al., 2000; Lv et al., 2008). In this study, a three-step purification strategy was adopted to purify *A. niger* ATCC®6275 xylanase. A summary of the purification process is described in Table 8.9.

Table 8.9 Purification of xylanase from *A. niger*

| Step | Total activity (U) | Total Protein (mg) | Specific activity (U/mg) | Purification fold | Yield |
|-----------------------------|---------------------------|---------------------------|---------------------------------|--------------------------|--------------|
| Crude extract | 167250 | 5009 | 33 | 1.0 | 100 |
| Ammonium sulphate | 155309 | 1412 | 110 | 3.3 | 72 |
| Ultrafiltration | 93336 | 173 | 540 | 16.2 | 56 |
| Ion-exchange chromatography | 39036 | 68 | 572 | 17.1 | 23 |

The crude enzyme extract exhibited a total activity of 167,250 U/ml and specific activity of 5,009 U/mg. The crude extract was subjected to (NH₄)₂SO₄ precipitation (65% w/v) at 4°C which resulted in 72% recovery and 3.3-fold purification. The precipitates were then recovered by dissolving them in 0.05M citrate buffer (pH 6.0) and subjected to dialysis against the same buffer (1:10 dilution). Subsequently, the partially purified xylanase was concentrated via diafiltration using a 10 kDa

centrifugal filter unit. This was followed by ion exchange chromatography (DEAE-Sepharose fast flow column) involving step elution of 0.5M KCl after the removal of unbound proteins. The xylanase eluted at a concentration of 0.2M KCl. The final enzyme recovery and purification fold of the enzyme was 23% and 17.1, respectively. Pal and Khanum (2011b) conducted an extensive study to purify and characterise thermostable xylanase from *A. niger* DFR-5. A four-step strategy was employed which encompassed ion-exchange chromatography followed by gel permeation chromatography, which resulted in a final yield of 38.9% and purification fold of 36.97. Higher yields (67.5%) have been reported for *A. niger* xylanase cloned into *E. coli*, deploying a three-step method using metal ion affinity and ion exchange chromatography (Elgharbi et al., 2015). The molecular mass of purified xylanase was determined by relative mobility of standard proteins on SDS-PAGE (10%) to be 36 kDa (Fig. 8.4). In a study conducted by Takahashi et al. (2013) on the production and purification of xylanase from *A. niger*, the purified enzyme was found to be with a molecular weight of 35 kDa.

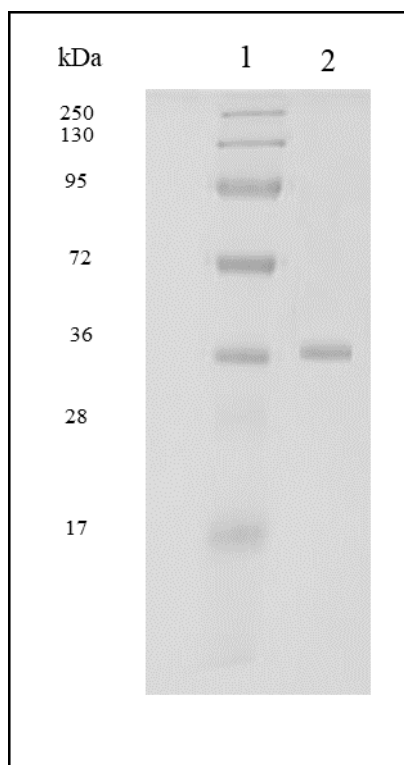


Figure 8.4 SDS-PAGE (1) molecular size markers (2) purified xylanase. Step elution was performed by various salt concentrations (0.1, 0.2, 0.3, 0.4 and 0.5 M KCl)

8.3.4 Effect of pH and temperature on enzyme activity

The optimum pH and pH stability of an enzyme are important parameters in determining its potential commercial applications. Xylanases derived from *A. niger* have been reported to be functional at an acidic to neutral pH range (Betini et al., 2009). In the present study, the optimum pH of xylanase was determined to be 5.0 (Fig. 8.5 a), which is used in juice clarification (Pal & Khanum, 2011a). Extensive pH stability studies were conducted to analyse the robustness of the xylanase. The enzyme was pre-incubated in a pH range of 4.0-6.5 and the activity was measured at pH 5. No decline in activity was observed in this process. However, incubating the enzyme at pH<4.0 or pH>6.5 resulted in decreased activity. The stability of the xylanase was assessed at various temperatures ranging from 20°C to 50°C (Fig 8.5 b). The optimum

temperature for maximum enzyme activity was found to be 35°C. The enzyme showed no signs of thermostability as increasing the temperature beyond 40°C resulted in the decrease in xylanase activity. Xylanases derived from *A. niger* ATCC 6275 have been reported to exhibit high activity only in the mesophilic range (Prasertsan et al., 1997).

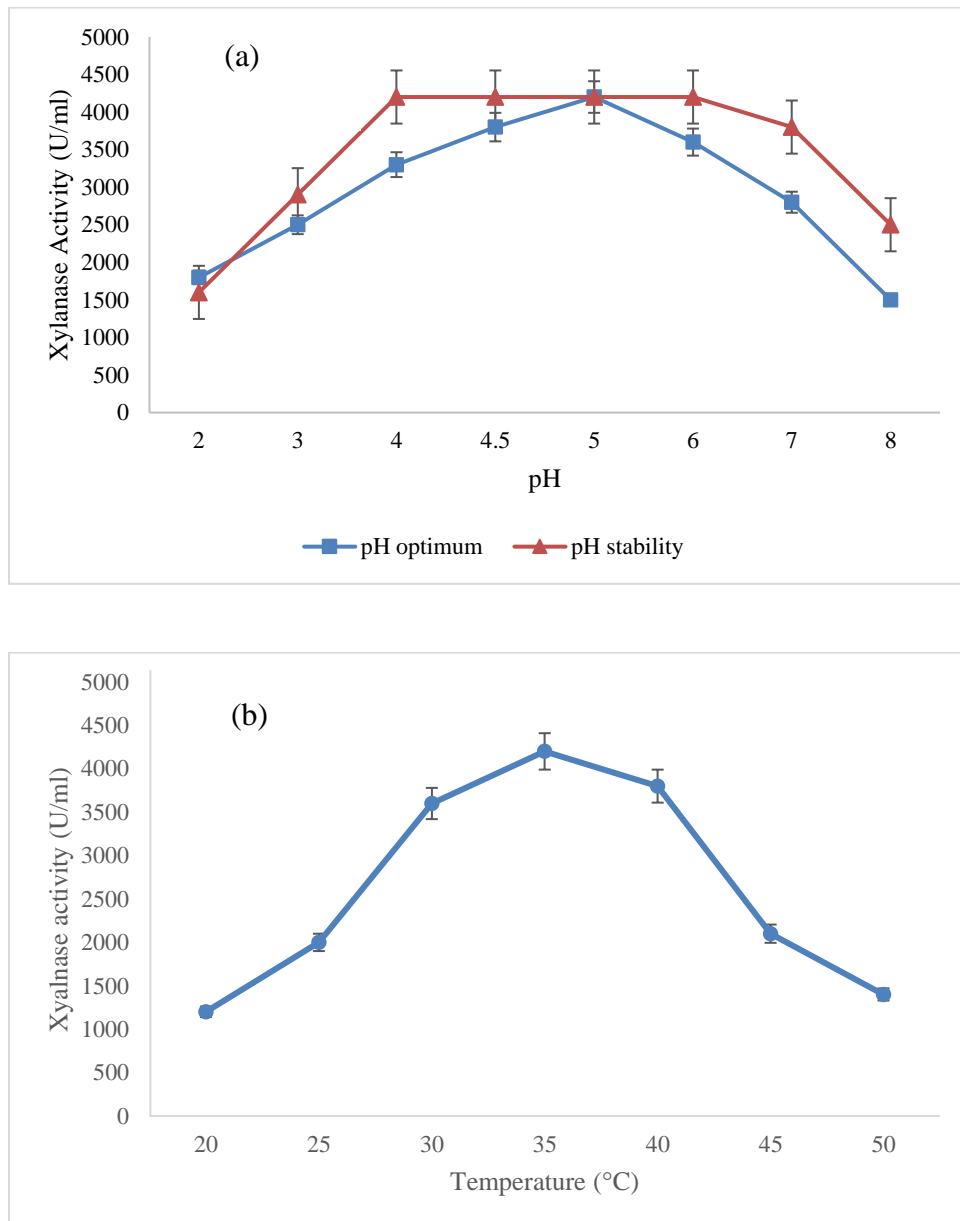


Figure 8.5 a) Effect of pH on activity and stability of xylanase (b) effect of temperature on activity of xylanase

8.3.5 Application of xylanase in fruit juice enrichment

The potential application of purified *A. niger* xylanase was investigated for the enrichment of strawberry, blueberry and raspberry pulps. Enzyme activity decreases its water holding capacity of the fruit pulp by digesting the cell wall materials which increases the yield and clarity of the fruit pulp. Therefore, three variables *viz.* enzyme dosage, incubation time and incubation temperature were included in this study. The efficacy of the enzyme as an enhancement measure was examined by estimating the variations in juice yield (%), clarity (%) and reducing sugars (%).

8.3.6 Effect of enzyme dosage

The optimum enzyme dosage for clarification of the different fruit pulp juices was determined. A range of concentrations varying from 5 to 25 U/gram fruit pulp were investigated at room temperature (25°C) for 60 min. Fruit pulp treated with heat inactivated xylanase was taken as 100% for each experiment. The optimum enzyme dosage with respect to reducing sugar content, clarity and juice yield differed with respect to each variety of fruit pulp (Fig 8.6). For example, the reducing sugar content, clarity and yield increased as the dosage was increased up to 10~15 U/gfp. While a dosage of 10 U/gfp was found to be ideal for strawberry, 15 U/gfp was found to be the optimum for blueberry and raspberry with respect to achieving higher reducing sugar content, yield and clarity. Fruits commonly consist of a primary cell wall which is composed of cellulose, hemicellulose and embedded lignin, and in certain cases, pectin. The middle lamella functions as the connecting agent that holds the cells together (Goulao & Oliveira, 2008). Enzymatic treatment of fruit pulp results in the hydrolysis of the cell wall making juice recovery easier. Increasing the enzyme dosage results in clarification of fruit juice due to the exposure of internal charged moieties in

pulp proteins which occurs as the hemicellulose is digested. However, this also promotes electrostatic interactions, subsequent flocculation and settling resulting in decrease in clarity (Kim & Bunz, 2006). This might be the reason why high enzyme dosage is detrimental to the clarification of fruit pulp (Nagar et al., 2012).

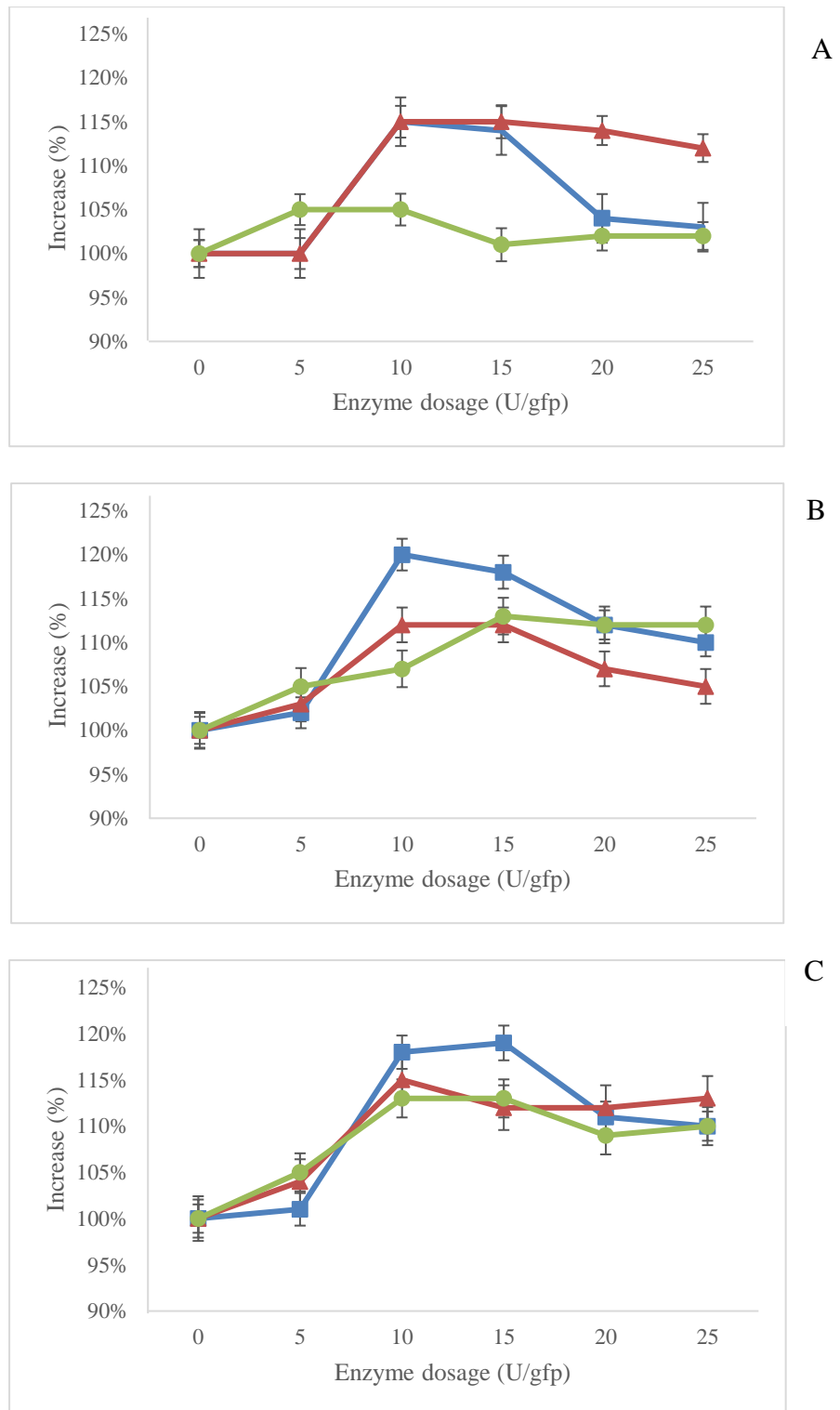


Figure 8.6 Effect of enzyme dosage on juice enrichment with respect to reducing sugar content (■) clarity (▲) and yield (●) (A) of strawberry, (B) blueberry and (C) raspberry

8.3.7 Effect of temperature

Temperature is an important variable when considering the application of enzymes for commercial purposes. The incubation temperature of enzymatic enrichment of fruit pulp was studied. A fixed dosage of 10 U/gfp was considered for this study while varying the temperature between 30~60°C. The enzymatic treatment was performed for 60 min. A control experiment maintaining the same dosage and incubation time was conducted at room temperature (25°C). A temperature of 35°C was found to be optimal for all three fruit pulp varieties (strawberry, blueberry and raspberry) tested in terms of reducing sugar content, clarity and yield (Fig. 8.7). This was in agreement with the optimum temperature of the xylanase determined earlier in this study. The yield and clarification of the fruit pulp increases as temperature increases due to the digestion of polysaccharides by xylanase. (Nagar et al., 2012).

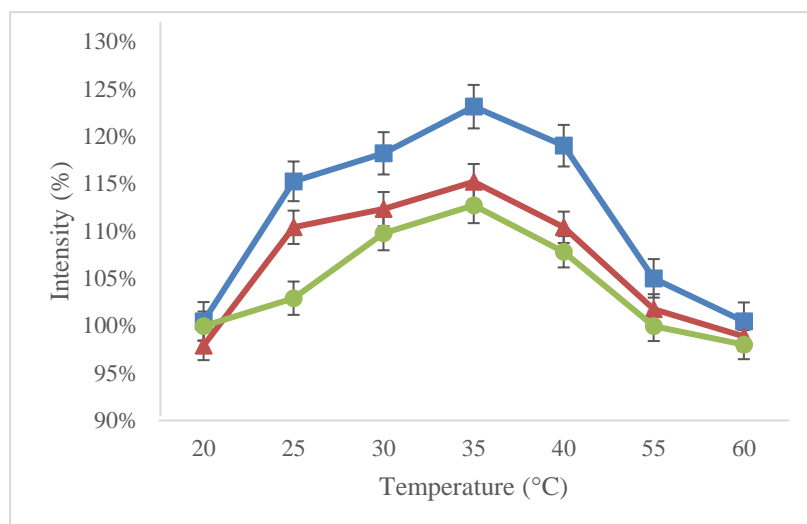
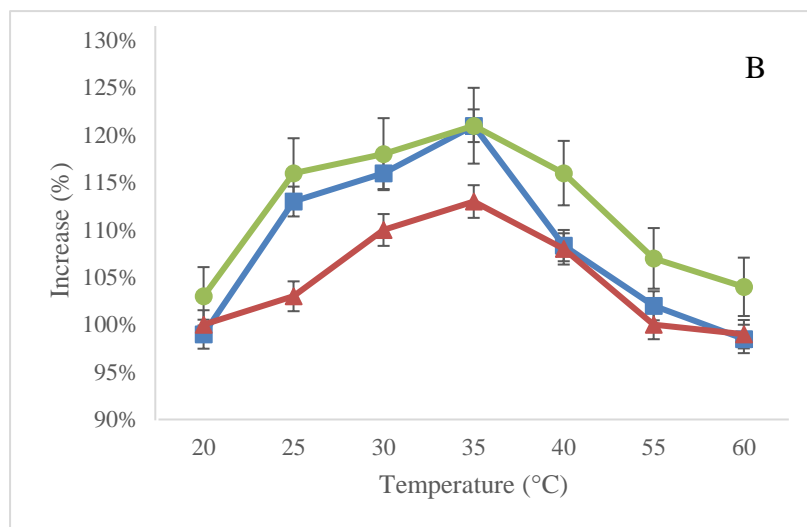
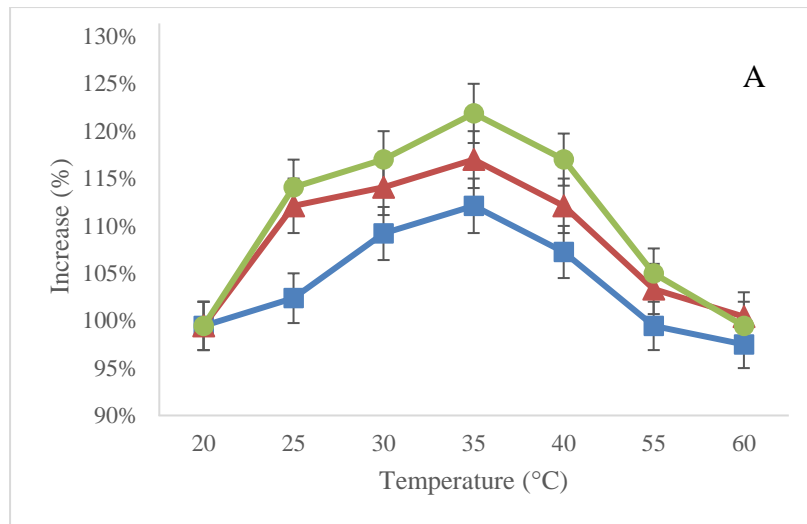


Figure 8.7 Effect of temperature on juice enrichment with respect to reducing sugar content (■) clarity (▲) and yield (●) of (a) strawberry (b) blueberry and (c) raspberry

8.3.8 Effect of incubation time

Optimisation of incubation time for enzymatic treatment of fruit pulp was investigated. Dosage was fixed at 10 U/gfp and experiments were done at room temperature (25°C). An experimental trial with fruit pulp treated by xylanase for 0 min was taken as control (100% benchmark). Fig 8.8 represents the results obtained from this study. An increase in incubation time resulted in an initial increase in clarity of the fruit pulp, but an extended incubation period resulted in a decrease in clarity. The optimum incubation time for strawberry fruit pulp was found to be 90 min., contrasting with that blueberry (60 -90 min.) and raspberry (60 min.). the decrease in clarity as the incubation time was increased may be due to the formation of haze particles by protein-carbohydrate or protein-tannin complexes (Tajchakavit et al., 2001).

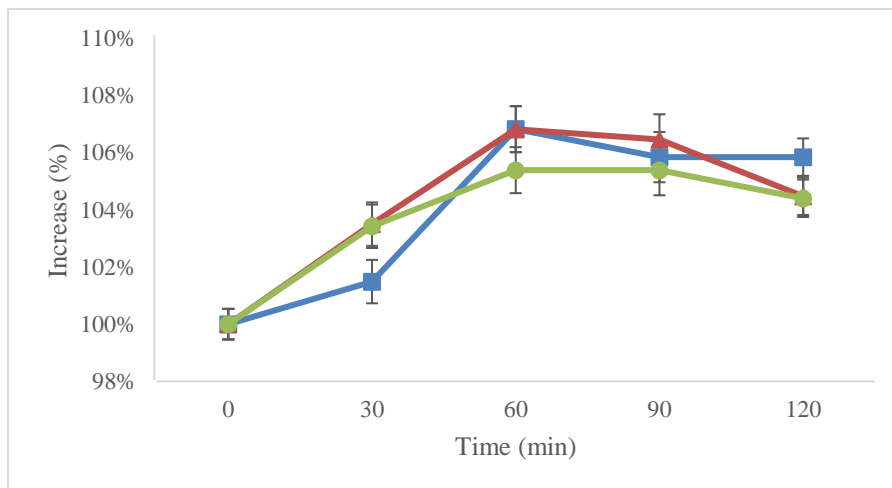
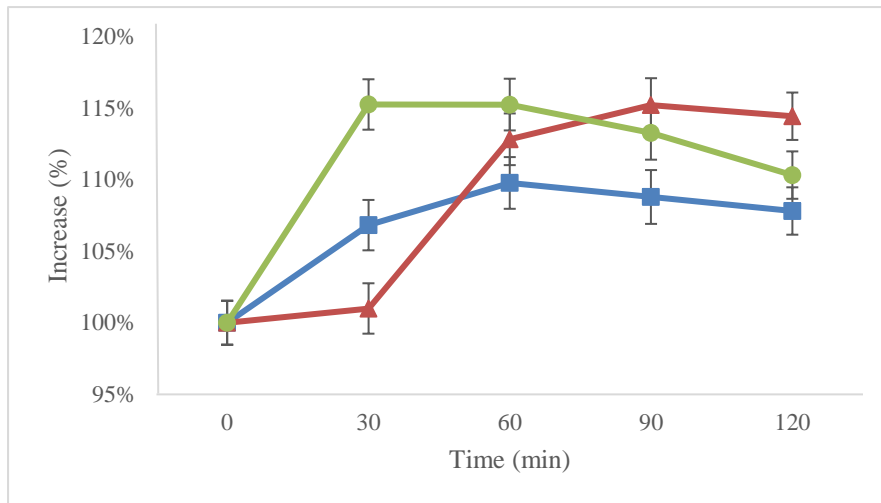
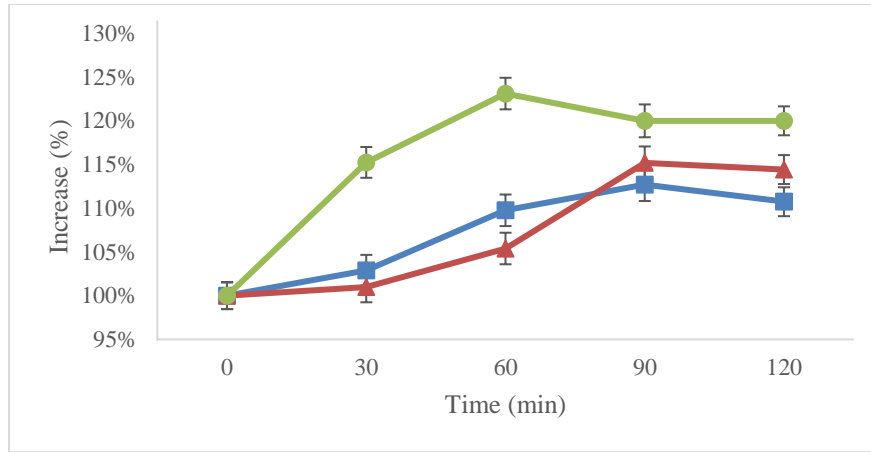


Figure 8.8 Effect of incubation time on juice enrichment with respect to reducing sugar content

(■) clarity (▲) and yield (●) of (a) strawberry, (b) blueberry and (c) raspberry

8.4 Conclusion

In this study, we studied the production of xylanase using SCW as the substrate in solid state fermentation mode. From our observations, conducting SSF at a temperature of 30°C, an initial moisture content of 70% (w/w) and an inoculum size of 6.5×10^6 (log no. of spores/ml) resulted in high xylanase activity. Additionally, supplementing the fermentation medium with yeast extract, $MgSO_4$ and KH_2PO_4 further increases xylanase yield. A maximum activity of 6495.6 U/g of dry SCW was achieved following optimised SSF protocol. Furthermore, the xylanase produced by *A. niger* ATCC® was found to be an excellent fruit juice clarification agent. Process scale-up and further recovery techniques need to be investigated to attain commercial feasibility.

General Conclusions

Future Recommendation

This chapter includes conclusions of present work and discusses recommendations of future work.

9.1 General Conclusion

Agricultural residues such as a brewer's spent grain and spent coffee waste are a rich source of carbon and are produced in abundance in Ireland. These cheap carbon sources can be put to good use by replacing traditional carbon sources in fermentation operations. This project focused on the efficient incorporation of SCW and BSG into the upstream processes of enzyme production. This was achieved by developing innovative methods for efficient removal of recalcitrance by studying the effect of existing technologies on the aforementioned food industry wastes.

The aims of the project were achieved in four stages. The first stage involved an extensive exploration into the existing and established pretreatment technologies employed commercially to determine the efficacy of each method with respect to each food industry waste. Furthermore, new methods were developed based on the newly emerging technologies such as atmospheric air pressure plasma and ultrasonication. The second step involved screening of bacterial and fungal species for their enzyme producing capacity. This was followed by the third step, which the optimisation and application of the two fermentation modes: submerged and solid-state fermentation strategies for the production of amylase and xylanase enzymes, respectively. The final stage investigated the potential of the enzymes in commercial applications.

As a part of the pretreatment study, SCW and BSG were characterised to determine the fraction of cellulose, hemicellulose and lignin that contribute to the total weight. Additionally, the changes imparted by each pretreatment on the composition of SCW was determined. The extensive pretreatment study revealed that organosolv pretreatment conserved the hemicellulose fraction of SCW. Hence, organosolv pretreated SCW was chosen as the appropriate carbon source for supplementing

xylanase production media owing to its high hemicellulose content. Furthermore, optimised hydrolysis protocols for SCW and BSG were developed to determine the efficiency of each pretreatment with respect to the release of reducing sugars. Meanwhile, microwave-assisted alkali pretreatment was highly successful in the removal of recalcitrance from BSG. Employing this pretreatment, 228.78 mg of reducing sugar /g of BSG was obtained, which was 2.86-fold higher than its native counterpart.

Four *Bacillus* sp., viz. *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus cereus* and *Bacillus stearothermophilus* were screened for their amylase producing capabilities. All of the four strains screened were found to produce α - amylase. *B. stearothermophilus* was selected as the primary enzyme producer, due to its ability to produce thermostable amylase. Submerged fermentation mode was adopted for the production of thermostable α -amylase. Fermentation media with BSG hydrolysate as the media component was optimised by employing response surface methodology. The novel media composition resulted in an increased enzyme yield of 198.09 U/ml, which was 1.3-fold higher than control fermentation (152.3 U/ml). The thermostable amylase was subjected to a three-stage purification strategy to obtain a purified enzyme, achieving a yield of 25.1% and 13.09 purification fold.

Meanwhile, four fungal strains viz. *Aspergillus fumigatus*, *Paecilomyces variotii*, *Penicillium roqueforti* and *Aspergillus niger* were subjected to screening studies to determine the xylanase producing capacity. The zones of clearance formed by each strain were used as measure xylanase production capacity. Consequently, *A. niger* was selected as the enzyme producing microbe for the later section of this study. Organosolv pretreated SCW was subjected to solid state fermentation mode for the production of xylanase. Before this could be achieved, fermentation conditions as well

as media composition needed to be optimised. The fermentation condition for SSF utilising SCW was optimised by the application of a Box-Behnken design. Meanwhile, a Plackett-Burman screening study was performed to identify the media components that enhanced xylanase production by *A. niger* in SSF mode. Yeast extract, MgSO₄ and KH₂PO₄ were found to significantly support xylanase production. The concentrations of these three components with respect to the SCW content were further optimised to achieve a final xylanase activity of 6495.6 U/g of dry SCW, which was 1.4-fold higher than control (4649 U/g of dry SCW). The xylanase was purified via a three-step strategy to attain a final specific activity of 572 U/mg. The purified enzyme was characterised and found to be most active at an acidic pH and mesophilic temperature range.

The enzymes obtained after fermentation of the lignocellulosic food industry wastes considered in this study were tested for potential applications. The increase in biofilm-related infections, as well as incidence of the same in process plant equipment, has prompted researchers to devise novel and effective measures to control surface attached bacteria. Therefore, the thermostable amylase obtained from *B. stearothermophilus* was tested as a potential anti-biofilm forming agent against three pathogenic bacterial strains. The crude amylase was found to effectively prevent the growth and biofilm formation of all the three strains tested. On the other hand, the xylanase enzyme obtained from *A. niger* was used in a food industry application as a fruit pulp enrichment agent. The purified xylanase was successful in increasing the reducing sugar content, clarity and yield of the fruit juices processed from strawberries, blueberries and raspberries. The findings of this study conclusively demonstrate the applicability of lignocellulosic food industry wastes.

Although the aims of this project were successfully achieved, more studies can be conducted in this area to further the possibilities of this work. A few of the recommendations have been mentioned below.

9.2 Future recommendations

Although the aims of this project were successfully achieved, more studies can be conducted in this area to further the possibilities of this work. A few of the recommendations have been mentioned below.

9.2.1 Screening and isolation of novel microbial strains for enzyme production

The enzyme producers employed in this study were obtained from the microbiology repository maintained in the School of Food Science and Environmental Health. Superior strains that naturally degrade lignocellulosic food waste can be isolated, characterised and screened for the production of lignolytic enzymes. Another aspect that was not considered in this study was the utilisation of recombinant strains, as well as catabolite-repressed mutants for the production of enzymes. In general, lignocellulose hydrolysates are high in reducing sugars, mainly glucose. The kinetics of enzyme production do not necessarily go hand-in-hand with biomass production. The ability of naturally occurring microbial strains to produce lignolytic enzymes are hindered by the presence of glucose in the media. This calls for the need for catabolite repressed or recombinant strains. Furthermore, the objectives of this project can be extended to include non-lignolytic enzymes, such as proteases.

9.2.2 In-depth analysis of substrate utilisation and scale up of fermentation process

This study has not investigated the nature of uptake of nutrients by the microorganism for enzyme production. A comprehensive mass balance analysis based on the assimilation of lignocellulosic component by the enzyme producer and its effect of biomass production and enzyme production kinetics can be analysed to obtain an all-inclusive picture on the final enzyme yield. Furthermore, all the fermentation experiments conducted in this study were performed at shake flask level. The media components and process parameters having been optimised, the next stage calls for scale-up of the for fermentation to bench top and subsequently pilot scale.

9.2.3 Innovation in enzyme application by immobilisation

Enzymes once suspended in an aqueous reaction medium are almost impossible to retrieve or recycle. Additionally, due to their proteinaceous nature enzymes are highly instable and require an aqueous environment to catalyse reactions. Enzymes can catalyse reactions in different physical states: as individual molecules, in collection with other moieties, and fastened to surfaces. This unique property of enzymes is made use of while ‘immobilising enzymes’. Enzyme immobilisation is the process of attaching an enzyme molecule to a solid support with intentions of its reuse, production and purification without loss in their catalytic activity thereby dramatically improving process economy. It also enables the use of insoluble enzymes suspended in hydrophobic organic media by optimising the enzyme dispersion and improving accessibility towards the substrate without the aggregation of hydrophilic protein particles. Although theoretically promising, practical experiences of enzyme immobilisation have not always been fruitful as per expectations. Substantial design

approaches and parameters need to be considered while selecting the mode of immobilisation. Innovation in enzyme immobilisation has resulted in interesting and efficient strategies to purify enzymes. Enzymes can be specifically immobilised on to a matrix by a range of covalent or non-covalent interactions. In certain cases, the purification step as a whole can be avoided by adopting to form cross linked enzyme aggregates (CLEA).

In conclusion, it has been comprehensively determined that lignocellulosic food industry waste such as spent coffee waste and brewer's spent grain can be used as potential raw materials for the production of commercially important enzymes. Commercialisation of this technology can be achieved by studying the economic feasibility and scale up options which in itself comprise as a topic that will require extensive research.

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Peer Review Publications

1. Ravindran, R., Williams, G. A. & Jaiswal, A. K. (2018). An investigation on spent coffee waste as a potential media component for xylanase production employing *Aspergillus niger* ATCC® 6275. *Biotechnology Reports* (Submitted to Journal).
2. Ravindran, R., Williams, G. A. & Jaiswal, A. K. (2018). Evaluation of brewer's spent grain hydrolysate as a substrate for production of thermostable α -amylase by *Bacillus stearothermophilus*. *Bioresource Technology Reports* (Reviewer comments submitted).
3. Ravindran, R., Hassan, S. S., Williams, G. A. & Jaiswal, A. K. (2018). A review on bioconversion of agro-industrial wastes to industrially important enzymes. *Bioengineering-MDPI*, 5(4), 93; doi:10.3390/bioengineering5040093.
4. Ravindran, R., Jaiswal, S., Abu-ghannam, N. & Jaiswal, A. K. (2018). A comparative analysis of pretreatment strategies on the properties and hydrolysis of Brewers' spent grain. *Bioresource Technology*, 248, 272-279.
5. Ravindran, R., Jaiswal, S., Abu-Ghannam, N. & Jaiswal, A. K. (2017). Two-step sequential pretreatment for the enhanced enzymatic hydrolysis of coffee spent waste. *Bioresource Technology*, 239, 276–284.
6. Ravindran, R., Jaiswal, S., Abu-Ghannam, N. & Jaiswal, A. K. (2017). Evaluation of ultrasound assisted potassium permanganate pre-treatment of spent coffee waste. *Bioresource Technology*, 224, 680–687.
7. Ravindran, R. & Jaiswal, A. K. (2016). Microbial enzyme production using lignocellulosic food industry wastes as feedstock: A Review, *Bioengineering*, 3(4), 30; doi:10.3390/bioengineering3040030.
8. Ravindran, R. & Jaiswal, A. K. (2016). Exploitation of food industry waste for high value products. *Trends in Biotechnology*, 34(1), 58-69.

9. Ravindran, R. & Jaiswal, A. K. (2016). A comprehensive review on pre-treatment strategy for lignocellulosic food industry waste: challenges and opportunities. *Bioresource Technology*, 92(0), 92-102.

Oral/ Poster Presentations

1. Ravindran, R., Williams, G. A. & Jaiswal, A. K. (2018). Optimisation of solid-state fermentation using spent coffee waste as substrate for xylanase production. 2nd International Conference on Bioresource Technology for Bioenergy, Bioproducts & Environmental Sustainability, 16 - 19 September, 2018, Melia Sitges, Spain.
2. Ravindran, R., Williams, G. A. & Jaiswal, A. K. (2018). Thermostable α -amylase production by *Bacillus stearothermophilus* utilising brewer's spent grain hydrolysate. 2nd International Conference on Bioresource Technology for Bioenergy, Bioproducts & Environmental Sustainability, 16 - 19 September, 2018, Melia Sitges, Spain.
3. Ravindran, R. & Jaiswal, A. K. (2018). Effect of various pretreatments on the composition and hydrolysis of spent coffee waste. 2nd International Conference on Bioresource Technology for Bioenergy, Bioproducts & Environmental Sustainability, 16 - 19 September, 2018, Melia Sitges, Spain.
4. Ravindran, R., Jaiswal, S., & Jaiswal, A. K. (2018). Effect of different pretreatments on the composition and sugar recovery from spent coffee waste. LignoBiotech V Symposium, the Fifth Symposium of Biotechnology Applied to Lignocelluloses. August 29- September 1, 2018, Helsinki, Finland.
5. Ravindran, R., & Jaiswal, A. K. (2018). Optimisation of thermostable α -amylase production from *B. stearothermophilus* using Brewer's spent grain as a carbon source. LignoBiotech V Symposium, the Fifth Symposium of Biotechnology Applied to Lignocelluloses. August 29- September 1, 2018, Helsinki, Finland.
6. Ravindran, R., Sarangapani C., Jaiswal, A. K. & Abu-Ghannam, N. (2018). Novel non-thermal plasma treatment for improving enzymatic hydrolysis of

brewer spent grain. ECO-BIO, Challenges in Building a Sustainable Biobased Economy, March 4-7, 2018, Dublin, Ireland.

7. Ravindran, R., Sarangapani C. & Jaiswal, A. K. (2016). Improved enzymatic hydrolysis of lignocellulose through oxidative removal of lignin by air pressure plasma. 1st International Conference on Bioresource Technology for Bioenergy, Bioproducts & Environmental Sustainability, October 23-26, 2016, Barcelona, Spain.
8. Ravindran, R., Sarangapani C. & Jaiswal, A. K. (2016). Mimicking the Fenton reaction using atmospheric air pressure plasma for lignocellulose pre-treatment. 1st International Conference on Bioresource Technology for Bioenergy, Bioproducts & Environmental Sustainability, October 23-26, 2016, Barcelona, Spain.
9. Ravindran, R. & Jaiswal, A. K. (2016). Metal chloride assisted plasma pre-treatment strategy of lignocellulose for the production of amylase. 4th Symposium on Biotechnology applied to Lignocelluloses, June 19-22, 2016, Madrid, Spain.
10. Ravindran, R. & Jaiswal, A. K. (2016). Ultrasound assisted alkaline potassium permanganate pre-treatment of spent coffee waste. Presented at “*International Conference on Renewable Resources and Biorefineries (ICRRB 2016)*”, Jan 18-19, 2016, London, UK.
11. Ravindran, R. & Jaiswal, A. K. (2016). Sequential and combinatorial pre-treatment strategy of lignocellulose for the enhanced enzymatic hydrolysis of spent coffee waste. Presented at “*International Conference on Renewable Resources and Biorefineries (ICRRB 2016)*”, Jan 18-19, 2016, London, UK.

Book Chapters

1. Ravindran, R., & Jaiswal, A. K. (2018). Current advances in immobilization techniques of enzymes. In: Inamuddin & A. M. Ansari (Eds.). *Enzymatic and Microbial Fuel Cells*. Millersville, PA, USA, Materials Research Forum LLC (in press).
2. Ravindran, R., & Jaiswal, A. K. (2018). Enzymes in bioconversion and food processing. In: M. Kuddus (Ed.). *Enzymes in Food Technology: Improvement and Innovation*. Springer Nature, Singapore (pp. 19-40).
3. Ravindran, R., & Jaiswal, A. K. (2017). Toxicological aspects of irradiated foods. In: Ferreira, I, C. F. R., Antonio, A. L. & Verde, S. C. (Eds.). *Food Irradiation Technologies: Concepts, Applications and Outcomes*. Cambridge, UK, The Royal Society of Chemistry (pp. 337 - 351).
4. Ravindran, R., Sharma, S. & Jaiswal, A. K. (2016). Enzymes in processing of functional foods ingredients and nutraceuticals. In: Martirosyan D. M. (Ed.). *Functional Foods for Chronic Diseases (Vol 1)*. Dallas, TX, USA, D&A Inc. (pp. 360-385).
5. Ravindran, R., Sharma, S. & Jaiswal, A. K. (2016). Enzymes in food processing: Impact on food products attributes. In: Jaiswal, A. K. (Ed.). *Food Processing Technologies: Impact on Product Attributes*. Boca Raton, Florida, USA, CRC Press. (pp. 695-722).