Development of Novel Pectinase and Xylanase Juice Clarification Enzymes via a Combined Biorefinery and Immobilization Approach

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Development of Novel Pectinase and Xylanase Juice Clarification Enzymes via a Combined Biorefinery and Immobilization Approach

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

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Abstract

Hydrolytic enzymes, such as pectinase and xylanase, may be harnessed for numerous industrial applications in food industry. Therefore, economic factors such as achievement of optimum yields and overall production costs, in addition to biocatalyst instability, are the main obstacles to the industrial production and exploitation of enzymes. For example, microbially-derived enzymes are typically produced in fermenters using expensive growth media, which may account for 30 to 40% of the production cost, and such expense may be compounded further by downstream processing operations.

To counter such disadvantages, the major trend in industrial utilization of enzymes in cost-sensitive processes has been to immobilize such biocatalysts on a solid support, thereby mediating the key advantage of reusability. A complementary approach in recent years has been to target reduction in upstream processing costs in enzyme production by incorporation of negative cost raw materials into the medium composition. Food waste such as Brewers’ spent grain (BSG) constitutes an environmental problem in Ireland but has the potential to provide a continuous and renewable feedstock (carbon source) for production of industrial enzymes, thereby reducing production costs. However, a significant barrier to the exploitation of lignocellulose is the recalcitrant nature of the biomass, which usually requires expensive pre-treatment to release sugars. The key goal of this study was to pursue an integrated upstream and downstream approach to develop novel immobilized enzyme preparations for the juice industry. The aim of the project was achieved through the following measures:

• Screening and isolation of microorganisms which produced xylanases and pectinases possessing a physico-chemical profile of relevance to the fruit juice sector.

• Development of an optimal pretreatment strategy for BSG that would enhance their suitability as a carbon source for the upstream processing of the microbe which produced the enzymes.

• Optimisation of the upstream processing for the production of as the pectinase and xylanase catalysts.

• Investigation of a novel method for enzyme immobilization, and establishment of optimal process performance characteristics for use in juice clarification.
The following were major outputs of this work:

- Microwave pre-treatment was the best measure for maximizing the yield of reducing sugars from BSG. and demonstrated superior energy efficiency when compared to ultrasound pretreatment.
- The highest sugar yield (64.4±7 mg) was obtained when 1.0 g of biomass was pretreated with microwave energy of 600 w for 90 s, representing a relatively low energy input of 0.001 Kwh of electricity.
- Among twenty-nine (29) microbial isolates recovered from spoiled fruits, *Mucor hiemalis*, isolated from Bramley apple (*Malus domestica*), produced xylanpectinolytic enzymes with the highest specific activity. The highest enzyme activity (137 U/g, and 67 U/g BSG, for pectinase and xylanase, respectively) was achieved in a medium that contained 15 g of BSG, at pH 6.0, temperature of 30°C, and supplemented with 1.0 % xylan or pectin for inducing the production of xylanase or pectinase, respectively. The partially purified and concentrated enzymes were optimally active at 60°C and pH 5.0 (1602 U/ml of pectinase, and 839 U/ml of xylanase, respectively).
- Flake-shaped magnetic nanoparticles with average crystallite size of ~16 nm) were biosynthesized using *A. flavus*. Pectinase and xylanase were covalently immobilized on MNPs with efficiencies of ~84% and 77%, respectively.
- Deploying a coupling time up to 120 min, and an agitation rate of 213 rpm (pectinase) - 250 rpm (xylanase), a maximum enzyme activity recovery onto alginate beads was observed to be about 81-83% for both enzymes. Optimum enzyme loading and genipin (crosslinker) concentration were found to be 50 U/ml and 12 % (w/v), respectively. The immobilized enzyme preparations were suitable for up to 5 repeated process cycles, losing about 45% (pectinase) - 49% (xylanase) of their initial activity during this time. The maximum clarity of apple juice (%T_{660}, 84%) was achieved at 100 min when immobilised pectinase (50 U/ml of juice) and xylanase (20 U/ml of juice) were used in combination at 57°C.
Declaration

I certify that this thesis which I now 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 is prepared according to the regulations for graduate study by research of the “Technological University Dublin” and has not been submitted in whole or in part for another award in any other third level institution.

The work reported on in this thesis conforms to the principles and requirements of the TU Dublin’s guidelines for ethics in research.

TU Dublin 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 be duly acknowledged.

Signature

Date 22/12/2020

Shady Hassan
This dissertation is dedicated to both my parents ...

my mother, and my late father
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And to my wife, and daughter, without your constant support, and patience I would not have completed this work.
List of Abbreviations

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

EDX       Energy Dispersive X-ray

DNS       Dinitrosalicylic Acid

SSF       Solid State Fermentation

MNPs      Magnetic Nanoparticles

OFAT      One Factor at A Time

CCD       Central Composite Design

CCRD      Central Composite Rotatable Design

RSM       Response Surface Methodology

ANN       Artificial Neural Network
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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

Enzymes have long been known as biocatalysts, selectively catalysing thousands of metabolic processes, and usually acting under milder reaction conditions than traditional chemicals. They are used in numerous industries such as detergents, textile, cosmetics, leather processing, pharmaceuticals, biodiesel production, and waste-water treatment, and are a major tool in the production of modern food. In the food industry, enzymes are used to extract, produce and enhance the quality and quantity of a range of food products, spanning starch breakdown (α-amylase), reduction of proteins (especially glutenins) in bread flour (protease), improvement of dough stability for bread making (xylanase), cheese manufacturing (lipase, and lysozymes), viscosity reduction and imparting food texture (cellulose, hemicellulose), wine production (pectinase, urease), and meat tenderisation (bromelain, papain). The global enzyme market was valued at USD 4.62 billion in 2015 (Figure 1.1) (Grand View Research, 2016). Furthermore, the application scope is expected to grow at a CAGR of over 7.0% from 2016 to 2024 due to increasing use in the leather and bioethanol sectors.

![Figure 1.1. Industrial enzyme market (USD Million), 2013-2024 (Source: www.grandviewresearch.com)](image-url)
On its own, pectinase represents 70% of the global juices processing enzymes market (Dataintelo, 2019). The latter market is estimated to reach USD 671 million by the end of 2025, from USD 460.2 million in 2018, delivering a CAGR of 5.5% through the forecast period (2019-2025). By application, the enzymes used in apples is account for the largest share (30%), followed by oranges (20%), and three other main fruits combined, namely: Peach, Pineapple and Pear (>10%).

Enzymes for industrial use are largely produced through fungal or bacterial fermentation using expensive raw materials as a growth medium. Roughly, 30-40% of the overall production cost of industrial enzymes is estimated to come from the cost of the growth medium, which presents a major drawback for industrial enzyme producers. In addition to the high upstream cost of enzyme production, aspects such as purification, instability and low yields restricted the more widespread use of enzymes in industry. Enzyme immobilisation is the process of attaching an enzyme molecule to a solid support with the purposes of its reuse and easy product separation, thereby providing a means of cost reduction. Immobilisation techniques may also help conserve the beneficial properties of enzymes, thereby offering greater stability over a wide range of pH and temperature, and often improving activity and selectivity in reactions (Barbosa et al., 2014).

The use of lower-cost growth substrates for the production of enzymes may provide favourable enzyme production economics. Waste materials from a wide range of agro-industrial processes may be used as substrates for microbial growth, and subsequently production of a range of high value products, including enzymes of interest. The Food and Agriculture Organization of the United Nations (FAO) estimates that over 1.3 billion tonnes of lignocellulosic waste are generated from various food industries operating throughout the world (FAO, 2012). Besides contributing to pollution, such
wastes also represent a loss of valuable biomass and nutrients, thereby making the valorisation of food waste a priority topic for research. Each year the problem of food waste costs over €2 billion to the Irish economy (EPA, 2019). With composting as the only currently available solution, much of the food that still possesses certain essential components goes to waste. Utilisation of these agro-residues in bioprocesses has the dual advantage of providing alternative substrates for growth processes, as well as solving the disposal problems.

**Aim and Objectives:**

This project aims to address the above-mentioned issues by utilisation of brewer’s spent grain (BSG) as a growth medium component for production a novel and cost-effective hydrolytic enzyme cocktails (pectinase and xylanase). This will be achieved through the following objectives:

1. Investigation of the effects of novel technologies for pretreatment of lignocellulose, such as microwave and ultrasound, on the chemical composition of BSG.
2. Development of an optimal pretreatment strategy to provide maximum fermentable sugars for the upstream processing.
3. Screening fungal isolates from environmental samples for pectinase and xylanase production.
4. Investigation of the suitability of employing pretreated BSG as a fermentation media for pectinase and xylanase production.
5. Optimisation of fermentation processes for the production of xylanopeptinolytic cocktails.
6. Investigation of the suitability of inorganic nanoscale particles (e.g. magnetic nanoparticles) and a natural polymer (e.g. alginate beads) as immobilisation matrices for enhancing reusability, and operational-storage stability of the enzymes.
7. Reducing the total amount of the enzyme preparations used in the cost-sensitive market of apple juice.
Organisation of Thesis

This thesis begins with general introduction (Chapter 1), followed by chapter 2 of literature review that includes the current state and prospects of lignocellulosic biorefineries in Europe, new technologies for pretreatment of lignocellulose, and the valorisation of lignocellulose for enzyme production. Chapter 3 includes protocols of various studied pretreatments using microwave and ultrasound, in addition to the analytical and statistical methods employed in the experiments. Section A is focused on pretreatment strategy of BSG and includes two chapters, chapters 4 and 5 which discuss the effects of microwave and ultrasound pretreatments on BSG. Section B is dedicated to the upstream processing and the use of pretreated BSG as a fermentation medium for production of pectinases and xylanases by environmental isolates (chapter 6). Section C includes two chapters and investigates the efficacy of magnetic nanoparticles (chapter 7) and alginate beads (chapter 8) as immobilisation carriers for enhancing reusability, and operational-storage stability of enzymes. Chapter 8 also deals with the subsequent utilisation of the immobilised enzymes for apple juice clarification. The last chapter (9) summarises the findings of this study and details the future recommendations based on the conclusions from the work carried out.

In conclusion, this thesis makes a focused and comprehensive effort for biorefining of solid barley waste (BSG) generated as a by-product in brewery industry for the production of industrially relevant enzymes. The biorefining process start with BSG pretreatment, followed by the subsequent fermentation of BSG to produce pectinase and xylanase. The enzymes produced were then successfully immobilised onto novel magnetic nanoparticles and a natural hydrogel. Enzymes immobilised on the hydrogel were investigated for potential application in apple juice clarification.
Chapter 2

Literature Review

This chapter is a literature review of the current state and prospects of lignocellulosic biorefineries in Europe with insights into drivers, challenges, and opportunities of this nascent industry. Furthermore, this chapter discusses the traditional and new technologies for pretreatment of lignocellulose, in particular, microwave and ultrasound. Additionally, this chapter provides a comprehensive review about the valorisation of lignocellulose for enzyme production, with a focus on pectinase and xylanase.
The information included in this chapter has been published as peer reviewed review articles:


2.1 Introduction

The future development of Europe now faces Unprecedented challenges, spanning food security, climate change, and an over-dependence on non-renewable resources. Simultaneously, it must balance strategies that harness renewable resources to maintain environmental sustainability, while maintaining economic growth. To achieve this, in 2012, the European Commission (EC) launched the European bioeconomy strategy entitled “Innovating for sustainable growth: a bioeconomy for Europe”. Within this strategy, the modern bioeconomy is defined centrally by the production of biomass or the utilization of lignocellulosic wastes, with subsequent conversion into value-added products, such as bio-energy, as well as novel bio-based innovation. At the EU level, the current bioeconomy has an annual turnover of 2.3 trillion EURO and generates a total employment of 18.5 million people.

Biorefining is defined as the sustainable processing of biomass into a spectrum of marketable products (food, feed, chemicals, and materials) and energy (fuels, power and/or heat) (Muranaka et al., 2017). Representing a cornerstone of the bioeconomy, the goal of fully unlocking the value potential of lignocellulosic plant biomass in a cost-effective way remains elusive. Upstream aspects such as biomass type, transport logistics and the downstream value proposition offered by conversion products must be reconciled with the recalcitrance of the lignocellulosic structure: there is, as yet, no fully scalable yet cost-effective extraction method to unlock valuable sugars and lignin from this matrix, and this remains a key short-term research goal.

Lignocellulosic feedstock options for biorefinery use range from food/non-food crops to primary residues/secondary wastes from agroforestry. The S2Biom project has estimated that a total of 476 million tons of lignocellulosic biomass need to be secured
to fulfil demand for bio-based products by 2030 (S2Biom, 2016). The market for bio-based products is expected to be worth 40 million EURO by 2020, increasing to about 50 billion EURO by 2030 (average annual growth rate of 4%). Research in industry and academia has been galvanized to address the twin challenge of lignocellulosic breakdown and conversion into viable products, and consequently a myriad of publications featuring laboratory and pilot scale studies for pretreatment and conversion of lignocellulosic biomass into bioenergy and bioproducts are published each year (Meneses et al., 2020; Cheah et al., 2020; Mirmohamadsadeghi et al., 2021; Rahmati et al., 2020).

2.2 Lignocellulosic feedstocks

Integral to the biorefinery concept is accessing suitable feedstocks which are amenable to cost-effective processing. Biorefining is a capital-intensive industry with large capital expenditure (CAPEX) and requires knowledge of the feedstock resource base that is sustainably available at low cost to support a facility (Ulonska et al., 2018).

2.2.1 First generation

The first generation of feedstocks depended on easily accessible and edible fractions of food crops, with the main product being biofuel. Bioethanol may be produced from sugar (e.g. sugarcane, sugarbeet, and sweet sorghum) and starch (e.g. corn, and cassava) crops, while biodiesel is produced from oil seed crops (e.g. soybean, oil palm, rapeseed, and sunflower) (Khan et al., 2018). However, in recent years, serious criticisms have been raised about competition in land use that has arisen as a direct consequence of incentivizing energy and oil crops at the expense of food crops (Wesseler and Drabik, 2016).
2.2.2 Second generation

The growing controversy of ‘food versus fuel’, along with associated production economics, biofuel policies and sustainability trends, promoted the rise of a second generation of feedstocks based on lignocellulosic biomass. The latter include non-food, short rotation grasses that have high yield and suitability to marginal lands or poor soils (e.g. poplar, willow, eucalyptus, alfalfa, and grasses such as switch, reed canary, Napier and Bermuda), agricultural residues (e.g. forest thinning, sawdust, sugarcane bagasse, rice husk, rice bran, corn stover, wheat straw, and wheat bran), and agro-industrial wastes (e.g. potato and orange peel, spent coffee grounds, apple pomace, ground nut oil and soybean oil cake) (Muranaka et al., 2017). Critically, the latter are so-called negative cost waste materials from other industries, and so theoretically the value proposition has heightened appeal. However, such materials are also the most refractory to extraction of sugars (Figure 2.1).

Food industry by-products encompass wastes from various industries such as sugarcane bagasse (from sugar milling), pomace (pressing of tomato), apple and grapes (juice), olives (for oil), brewer’s spent grain (BSG - from beer-brewing), spent coffee grounds (coffee preparation), as well as citrus and potato peels. The global production of some of these humble wastes is significant. For example, BSG generated from beer-brewing has been estimated at 3.4 million tonnes annually in the EU alone, and over 4.5 million tons in USA as the largest craft beer producer (Chen et al., 2017).
Figure 2.1. Schematic diagram shows differences between lignocellulosic feedstocks from the first and second generation: sources, valorisation processes, and end products.
2.3 Biobased products market

Examples of potential bio-based products include biofuels (e.g. bioethanol, biodiesel, and biogas), biochemicals (e.g. industrial enzymes, and nutraceuticals), and biomaterials (e.g. biodegradable plastics) (Ravindran and Jaiswal, 2016). However, supported by specific EU policies, bioenergy and biofuels have received greater attention. By the year 2030, the EU aims to provide 25% of its transportation energy via biofuels derived from advanced biorefineries (2nd generation biorefineries). By this time, it also intends to replace 30% of oil-based chemicals by bio-based chemicals and supplant non-degradable materials with degradable materials. Interestingly, 80% of the EU bio-based infrastructure will be in rural areas, which are expected to support community development programs. However, developing sustainable markets for bio-based products, and raising the public awareness of this area will still be a challenge. Even so, it is expected that evolving market demand, combined with further EU policies acting to spur public awareness, will accelerate product development and encourage private sector investment.

The Bio-based consortium in the EU aims to replace 30% of overall chemical production with biomass-derived biochemicals by 2030 (BIC, 2012). According to the National Renewable Energy Laboratory in USA, the latter can be finished products or intermediates that then become a feedstock for further processing (Biddy et al., 2016). Biochemicals produced from the biorefining of lignocellulose include organic acids (e.g. citric, acetic, benzoic, lactic and succinic), microbial enzymes (e.g. amylase, cellulase, pectinase, xylanase, mannanase), and building blocks for bio-based polymers (e.g. phenylpropanoids, polyhydroxyalkanoates) (Kawaguchi et al., 2016; Kumar et al., 2016; Ravindran et al., 2018a). The projected production of some
lignocellulosic-based chemicals and materials in Europe (in 2020 and 2030) is summarized in Figure 2.2 (S2biom, 2016).

![Figure 2.2. Projected production of biobased chemicals and materials in Europe 2020/2030](image)

The global market for industrial enzymes is expected to increase at a compounded annual growth rate (CAGR) of 4.7% between 2016-2021 (growing from approximately $5.0 billion in 2016 to $6.3 billion in 2021; (Dewan, 2014)). Despite the tangible demand, enzymes are relatively expensive reagents, and this adds to the operational cost of processes that utilize them. Critical analysis of process plant economics for enzyme production reveals that almost 50% of the cost of production is associated with capital investment, while the cost of raw materials accounts for almost one third of such costs. Substitution or complementation of feedstocks with lignocellulosic sources can result in an increased return on investment.
2.4 Enzyme Production Using Lignocellulosic Food Waste

2.4.1 Lignocellulose as a Raw Material

Lignocellulosic biomass is a complex matrix that is relatively refractory to degradation. Sugars are locked in a recalcitrant structure that requires a pretreatment step to release them. Many conventional methods (e.g. chemical, physical, and biological methods) are used for pretreating lignocellulosic biomass. However, achieving a workable balance between pretreatment efficacy, cost and environmental sustainability is difficult (Hassan et al., 2019).

2.4.2 Structure of Lignocellulose

Plant biomass is composed mainly of polysaccharides (cellulose, hemicellulose) and lignin. Polysaccharides are polymers of sugars and therefore a potential source of fermentable sugars, while lignin can be used to produce chemicals. Generally, cereal residues (e.g. rice straw, wheat straw, corn stover, and sugarcane bagasse) contain a large fraction of lignocellulose substances and represent the favourite feedstock for biorefineries, while grasses, fruit and vegetable wastes have less lignocellulosic content.

The ECN Phyllis2 database (www.phyllis.nl) is an open literature facility which is readily available to users and documents the composition of biomass and waste. Furthermore, table 2.1 shows the chemical composition of different lignocellulosic feedstocks based on recent literatures published in 2016, 2017 and 2018. Biomass on a dry weight basis generally contains cellulose (50%), hemicellulose (10–30% in woods, or 20–40% in herbaceous biomass) and lignin (20–40% in woods or 10–40% in herbaceous biomass) (Sharma et al., 2015). However, these ratios between cellulose,
hemicellulose and lignin within a single plant will vary with different factors like age, harvesting season and culture conditions.

### Table 2.1 Chemical composition of different lignocellulosic feedstocks (% dry basis)

<table>
<thead>
<tr>
<th>Source</th>
<th>Cellulose</th>
<th>Hemicellulose</th>
<th>Lignin</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hardwood</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eucalyptus</td>
<td>44.9</td>
<td>28.9</td>
<td>26.2</td>
<td>(Muranaka et al., 2017)</td>
</tr>
<tr>
<td>Oak</td>
<td>43.2</td>
<td>21.9</td>
<td>35.4</td>
<td>(Yu et al., 2017)</td>
</tr>
<tr>
<td>Rubber wood</td>
<td>39.56</td>
<td>28.42</td>
<td>27.58</td>
<td>(Khan et al., 2018)</td>
</tr>
<tr>
<td><strong>Softwood</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spruce</td>
<td>47.1</td>
<td>22.3</td>
<td>29.2</td>
<td>(Yu et al., 2017)</td>
</tr>
<tr>
<td>Pine</td>
<td>45.6</td>
<td>24.0</td>
<td>26.8</td>
<td>(Yu et al., 2017)</td>
</tr>
<tr>
<td>Japanese cedar</td>
<td>52.7</td>
<td>13.8</td>
<td>33.5</td>
<td>(Muranaka et al., 2017)</td>
</tr>
<tr>
<td><strong>Grasses</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bamboo</td>
<td>46.5</td>
<td>18.8</td>
<td>25.7</td>
<td>(Chen et al., 2017)</td>
</tr>
<tr>
<td>Amur silver-grass</td>
<td>42.00</td>
<td>30.15</td>
<td>7.00</td>
<td>(Raud et al., 2016)</td>
</tr>
<tr>
<td>Natural hay</td>
<td>44.9</td>
<td>31.4</td>
<td>12.0</td>
<td>(De Caprariis et al., 2017)</td>
</tr>
<tr>
<td>Hemp</td>
<td>53.86</td>
<td>10.60</td>
<td>8.76</td>
<td>(Raud et al., 2016)</td>
</tr>
<tr>
<td>Rye</td>
<td>42.83</td>
<td>27.86</td>
<td>6.51</td>
<td>(Raud et al., 2016)</td>
</tr>
<tr>
<td>Reed</td>
<td>49.40</td>
<td>31.50</td>
<td>8.74</td>
<td>(Raud et al., 2016)</td>
</tr>
<tr>
<td>Sunflower</td>
<td>34.06</td>
<td>5.18</td>
<td>7.72</td>
<td>(Raud et al., 2016)</td>
</tr>
<tr>
<td>Silage</td>
<td>39.27</td>
<td>25.96</td>
<td>9.02</td>
<td>(Raud et al., 2016)</td>
</tr>
<tr>
<td>Szarvasi-1</td>
<td>37.85</td>
<td>27.33</td>
<td>9.65</td>
<td>(Raud et al., 2016)</td>
</tr>
<tr>
<td><strong>Agro-industrial waste</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Walnut shell</td>
<td>23.3</td>
<td>20.4</td>
<td>53.5</td>
<td>(De Caprariis et al., 2017)</td>
</tr>
<tr>
<td>Groundnut shell</td>
<td>37</td>
<td>18.7</td>
<td>28</td>
<td>(Álvarez et al., 2018)</td>
</tr>
<tr>
<td>Pistachio shell</td>
<td>15.2</td>
<td>38.2</td>
<td>29.4</td>
<td>(Subhedar et al., 2017)</td>
</tr>
<tr>
<td>Almond shell</td>
<td>27</td>
<td>30</td>
<td>36</td>
<td>(Álvarez et al., 2018)</td>
</tr>
<tr>
<td>Pine nutshell</td>
<td>31</td>
<td>25</td>
<td>38.0</td>
<td>(Álvarez et al., 2018)</td>
</tr>
<tr>
<td>Hazelnut shell</td>
<td>30</td>
<td>23</td>
<td>38.0</td>
<td>(Álvarez et al., 2018)</td>
</tr>
<tr>
<td>Coconut coir</td>
<td>44.2</td>
<td>22.1</td>
<td>32.8</td>
<td>(Subhedar et al., 2017)</td>
</tr>
<tr>
<td>Cotton stalk</td>
<td>67</td>
<td>16</td>
<td>13</td>
<td>(Kim et al., 2016)</td>
</tr>
<tr>
<td>Hemp stalk</td>
<td>52</td>
<td>25</td>
<td>17</td>
<td>(Kim et al., 2016)</td>
</tr>
<tr>
<td>Acacia pruning</td>
<td>49</td>
<td>13</td>
<td>32</td>
<td>(Kim et al., 2016)</td>
</tr>
<tr>
<td>Sugarcane peel</td>
<td>41.11</td>
<td>26.40</td>
<td>24.31</td>
<td>(Huang et al., 2016)</td>
</tr>
<tr>
<td>Rice husk</td>
<td>40</td>
<td>16</td>
<td>26</td>
<td>(Daza Serna et al., 2016)</td>
</tr>
<tr>
<td>Rice straw</td>
<td>38.14</td>
<td>31.12</td>
<td>26.35</td>
<td>(Huang et al., 2016)</td>
</tr>
<tr>
<td>Barley straw</td>
<td>35.4</td>
<td>28.7</td>
<td>13.1</td>
<td>(Liu et al., 2017)</td>
</tr>
<tr>
<td>Coffee grounds</td>
<td>33.10</td>
<td>30.03</td>
<td>24.52</td>
<td>(Huang et al., 2016)</td>
</tr>
<tr>
<td>Extracted olive pomace</td>
<td>19</td>
<td>22</td>
<td>40.0</td>
<td>(Álvarez et al., 2018)</td>
</tr>
<tr>
<td>Palm oil frond</td>
<td>37.32</td>
<td>31.89</td>
<td>26.05</td>
<td>(Khan et al., 2018)</td>
</tr>
<tr>
<td>Corn stover</td>
<td>43.97</td>
<td>28.94</td>
<td>21.82</td>
<td>(Huang et al., 2016)</td>
</tr>
<tr>
<td>Bamboo leaves</td>
<td>34.14</td>
<td>25.55</td>
<td>35.03</td>
<td>(Huang et al., 2016)</td>
</tr>
<tr>
<td>Hazel branches</td>
<td>30.8</td>
<td>15.9</td>
<td>19.9</td>
<td>(Liu et al., 2017)</td>
</tr>
</tbody>
</table>
2.4.3 Pre-treatment of lignocellulose

Pretreatment of lignocellulosic biomass is a necessary step to convert biomass into fermentable sugars and to enable enzymatic hydrolysis to break the lignin and hemicellulose structures and to free the buried cellulose (Sun et al., 2016). Pretreatment steps should be simple, eco-friendly, cost-effective and economically feasible (Ravindran et al., 2018b). In addition, the pretreatment process should not give rise to inhibitory compounds (affecting enzymatic hydrolysis and microbial fermentation) or loss in the fraction of interest (polysaccharide or lignin). Moreover, to date, there is no harmonised pretreatment strategy to suit all types of lignocellulosic biomass, and the pretreatment process depends mostly on the type of lignocellulosic biomass and the desired products. However, the use of a combination of two or more pretreatment strategies can significantly increase the efficiency of the process and represents an emerging approach in this field of study (Rahmati et al., 2020).

2.4.3.1 Conventional approaches for pretreatment of lignocellulosic biomass

Generally, each of the common pretreatment approaches that fall under the four categories of physical, chemical, physio-chemical and biological methods work differently to break the complex structure of the lignocellulosic material. As a result, different products and yields can be obtained from each pretreatment approach, and each method has its advantages and disadvantages that are summarized in Table 2.2. While some of the methods listed have successfully made the transition from research platform to the industrial stage, significant challenges remain (Rahmati et al., 2020), including in some cases the generation of environmentally hazardous wastes and/or high energy inputs; there is a pressing need for green technology solutions to this challenge (Capolupo and Faraco, 2016).
Table 2.2. Major advantages and disadvantages of each of the common pretreatment methods

<table>
<thead>
<tr>
<th>Pretreatment Method</th>
<th>Effects</th>
<th>Advantage</th>
<th>Disadvantage</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mechanical Milling</td>
<td>Reduce the particle size and crystallinity of lignocellulosic materials</td>
<td>Control of final particle size Make handling of material easy.</td>
<td>High energy consumption</td>
<td>(Devendra et al., 2015)</td>
</tr>
<tr>
<td>Extrusion</td>
<td>Shortening of fiber and defibrillation</td>
<td>operate at high solids loadings, low production of inhibitory compounds, short time</td>
<td>High energy consumption, effect is limited when no chemical agents are used, mostly effective on herbaceous type biomass</td>
<td>(Duque et al., 2017)</td>
</tr>
<tr>
<td>Acid</td>
<td>Hemicellulose and lignin fractionation</td>
<td>Enzymatic hydrolysis is sometimes not required as the acid itself may hydrolys the biomass to yield fermentable sugars</td>
<td>High cost of the reactors, chemicals are corrosive and toxic, and formation of inhibitory by-products</td>
<td>(Jönsson and Martín, 2016)</td>
</tr>
<tr>
<td>Alkaline</td>
<td>Lignin and hemicelluloses removal</td>
<td>Cause less sugar degradation than acid pretreatment</td>
<td>Generation of inhibitors</td>
<td>(Zhang et al., 2016)</td>
</tr>
<tr>
<td>Organosolv</td>
<td>Lignin removal and hemicellulose fractionation</td>
<td>Produce low residual lignin substrates that reduce unwanted adsorption of enzymes and allows their recycling and reuse</td>
<td>High capital investment, Handling of harsh organic solvents, formation of inhibitors</td>
<td>(Nitsos and Rova, 2017)</td>
</tr>
<tr>
<td>Oxidation</td>
<td>Removal of lignin and hemicelluloses</td>
<td>Lower production of by products</td>
<td>Cellulose is partly degraded, High cost</td>
<td>(Chandel and Da Silva, 2013)</td>
</tr>
<tr>
<td>Ionic liquid</td>
<td>Cellulose crystallinity reduction and partial hemicellulose and lignin removal</td>
<td>low vapor pressure designer solvent, working under mild reaction conditions</td>
<td>Costly, complexity of synthesis and purification, toxicity, poor biodegradability, and inhibitory effects on enzyme activity</td>
<td>(Yoo et al., 2017)</td>
</tr>
<tr>
<td>Liquid Hot Water</td>
<td>Removal of soluble lignin and Hemicellulose</td>
<td>The residual lignin put a negative effect on the subsequent enzymatic hydrolysis</td>
<td>High water consumption and energy input</td>
<td>(Zhuang et al., 2016)</td>
</tr>
<tr>
<td>AFEX</td>
<td>Lignin removal</td>
<td>High efficiency and selectivity for reaction with lignin</td>
<td>It is much less effective for softwood, Cost of ammonia and its environmental concerns</td>
<td>(Bajpai, 2016)</td>
</tr>
<tr>
<td>SPORL</td>
<td>Lignin removal</td>
<td>Effective against hardwood and softwood, and energy efficient</td>
<td>Pretreatment is preceded by biomass size-reduction</td>
<td>(Noparat et al., 2017)</td>
</tr>
</tbody>
</table>
The harsh chemicals and high conventional heating methods used for biomass pretreatment require extensive amounts of energy and are not environmentally friendly. Furthermore, these pretreatment strategies lead to the formation of numerous undesirable compounds, such as aliphatic acids, vanillic acid, uronic acid, 4-hydroxybenzoic acid, phenol, furaldehydes, cinnamaldehyde, and formaldehyde, which may all interfere with the growth of the fermentative microorganisms during fermentation (Ravindran and Jaiswal, 2016). This encouraged the movement from non-sustainable conventional pretreatments (e.g. chemical and physiochemical pretreatments) to sustainable green pretreatments (e.g. biological pretreatments). However, long treatment times, low yields and loss of carbohydrate during pretreatment are considered to be the major challenges in biological pretreatment by microorganisms (Saha et al., 2016). Furthermore, pretreatment processes can cost more than 40% of the total processing cost and represent the most energy intensive aspects in biomass conversion to value added products (Sindhu et al., 2016). Thus, the challenge of low efficiency production associated with green pretreatments encouraged the investigation of using large scale technologies that are now available on the market as scalable green solutions to achieve sustainable and efficient pretreatment process of lignocellulosic biomass.

2.4.3.2 Green approaches for pretreatment of lignocellulosic biomass

In recent years, the concept of “Green Chemistry” has gained increasing interest as a possible approach to the challenge of developing a viable biorefinery concept. Central to achieving this goal is the development of technology that uses raw materials more efficiently, eliminates waste and avoids the use of toxic and hazardous materials. Selected green methods currently being pursued for pretreatment of lignocellulosic biomass are summarised in Table 2.3.
## Table 2.3. Major advantages and disadvantages of selected green chemistry pretreatment methods.

<table>
<thead>
<tr>
<th>Pretreatment Methods</th>
<th>Effects</th>
<th>Advantage</th>
<th>Disadvantage</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deep eutectic solvents</td>
<td>lignin removal and hemicellulose fractionation</td>
<td>Green solvent, biodegradable and biocompatible</td>
<td>Poor Stability under higher pretreatment temperatures,</td>
<td>(Zhang et al., 2016)</td>
</tr>
<tr>
<td>Steam Explosion</td>
<td>lignin softening, particle size reduction</td>
<td>low capital investment, moderate energy</td>
<td>It is much less effective for softwood</td>
<td>(Pielhop et al., 2016)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>requirements and low environmental impacts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supercritical fluids</td>
<td>Cellulose crystallinity reduction and</td>
<td>Green solvent is used, it does not cause</td>
<td>Total utilities costs are high</td>
<td>(Daza Serna et al., 2016)</td>
</tr>
<tr>
<td></td>
<td>lignin removal</td>
<td>degradation of sugars, method is suitable for</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mobile biomass processor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microbes</td>
<td>Lignin and hemicellulose degradation</td>
<td>Environment friendly, selective degradation of</td>
<td>Very long pretreatment time (several weeks) due to slow</td>
<td>(Sun et al., 2016)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>lignin and hemicelluloses</td>
<td>yield</td>
<td></td>
</tr>
</tbody>
</table>

Although these green methods are environmentally friendly, problems exist regarding high production costs and poor efficiency, as well as lack of availability of commercial equipment suited to industrial scale processing. However, the more widespread adoption of such technology by the food industry, with anticipated decreases in initial capital cost and increased scale of operation, may encourage uptake for pretreatment of lignocellulosic biomass.
2.4.3.3 Emerging technologies for pretreatment of Lignocellulosic biomass

Chemical approaches conducted under extreme or non-classical conditions are currently a dynamically developing area in minimal food processing. Microwaves, and ultrasound are non-thermal food processing technologies that also being investigated for pretreatment of lignocellulosic biomass (Aguilar-Reynosa et al., 2017; Subhedar and Gogate, 2016).

2.4.3.3.1 Microwave Irradiation

Microwaves are an electromagnetic radiation with wavelengths ranging from 1 mm to 1 m. They are located between 300 and 300,000 MHz on the electromagnetic spectrum and are a nonionizing radiation that transfers energy selectively to different substances (Huang et al., 2016a). Microwaves have attracted renewed interest since the 1980s, when Gedye et al. (1986) reported the increase of hydrolysis, oxidation, alkylation, and esterification processes by energy efficient microwave heating. Researchers have reported good lignocellulosic pretreatment performance using microwave radiation over the past 30 years and have gradually moved from laboratory to pilot scale (Li et al., 2016). Currently, microwave-assisted pretreatment technologies of lignocellulosic biomass can be classified into two main groups: (a) Microwave-assisted solvolysis under mild temperatures (<200 °C) that depolymerises the biomass to produce value-added chemicals, and (b) Microwave-assisted pyrolysis of lignin without oxygen, under high temperatures (>400 °C) to convert biomass to bio-oil or biogases. Each of the two groups of technologies might be accomplished with catalysts. However, microwave-assisted pyrolysis is favored, largely due to energy shortages and the sustainability plans of most countries.

Compared with conventional heating, microwave radiation has significant advantages such as: (a) fast processing rate, short reaction time, (b) selectivity and uniform
volumetric heating performance (c) easy operation and energy efficiency and (d) low degradation or formation of side products. In addition, microwave hydrothermal pretreatment removes more acetyl groups in hemicellulose, which may be raised from the hot spot effect of microwave irradiation (Dai et al., 2017).

In the case of conventional heating, energy is transferred from the outside surface of the material inwards to the core of the material by conduction. Thus, overheating can occur on the outside surface whilst still maintaining a cooler inner region. Contrasting with this, microwaves induce heat at the molecular level by direct conversion of the electromagnetic energy into heat. Energy is therefore uniformly dissipated throughout the material.

Materials can be grouped into three categories according to their response to microwaves: insulators, absorbers, and conductors. Insulators are materials which are transparent to microwaves (e.g., glass and ceramics), conductors are materials which show high conductivity and thus reflect microwaves from the surface (e.g., metals), while absorbers or dielectrics are materials that can absorb microwaves and convert microwave energy into heat (Huang et al., 2016b). Most biomass is generally considered as low lossy materials, and they need to be supported with materials that achieve rapid heating, such as graphite, charcoal, activated carbons and pyrites.

Interestingly, Salema et al. (2017) studied the dielectric properties of different biomass from agriculture and wood-based industries (including oil palm shell, empty fruit bunch, coconut shell, rice husk, and sawdust) and reported that all were low loss dielectric materials. Such materials do not absorb microwaves well during microwave-assisted pyrolysis until the char is formed, and the microwave absorption will then be significantly higher.
In conventional heating methods, the lignocellulosic biomass is ground into small particles to prevent large temperature gradients and then heated by indirect heat conduction or high-pressure steam injection up to 160–250 °C. Therefore, fermentable sugar recovery and conversion might be affected by degradation of the hemicellulose into furfural or humic acids (Li et al., 2016). Alternatively, microwave heating is reported to enhance enzymatic saccharification through fibre swelling and fragmentation (Diaz et al., 2015) because of the internal uniform and rapid heating of large biomass particles. Almost no effect is observed in plant fibre material when treated with microwaves under temperatures that are equal to or below 100 °C (Chen et al., 2017).

The performance of microwaves depends on the dielectric properties of biomass which represent the ability of the material to store electromagnetic energy and to convert this energy into heat. Although, biomass usually is a low microwave absorber, the presence of relatively high moisture and inorganic substances can improve the absorption capacity of biomass (Li et al., 2016). The increasing commercial availability of flow-through microwave systems may be of relevance to lignocellulosic pretreatment.

Choudhary et al. (2012) evaluated the pretreatment of sweet sorghum bagasse (SSB) biomass through microwave radiation and reported that about 65% of maximal total sugars were recovered when 1 g of SSB in 10 ml water was subjected to 1000 W for 4 minutes. Scanning electron microscope analysis of microwave-assisted pretreatment of corn straw and rice husk in alkaline glycerol showed clear disruption of the plant cell structure (Diaz et al., 2015). Recently, Ravindran et al. (2018b) reported that microwave-assisted alkali pretreatment was the best pretreatment method for brewers’ spent grain (1g of BSG in 10 ml 0.5% NaOH was pretreated using 400 W for 60 seconds), as compared with dilute acid hydrolysis, steam explosion, ammonia fiber
explosion, organosolv and ferric chloride pretreatment. The authors found that BSG after microwave-assisted alkali pretreatment yielded 228.25 mg of reducing sugar/g of BSG which was 2.86-fold higher compared to untreated BSG (79.67 mg/g of BSG). Others have also found that microwave radiation for lignocellulosic pretreatment possesses the advantage of low capital cost, easy operation and significant energy efficiency (Kostas et al., 2017).

2.4.3.3.2 Ultrasound

Over 90 years ago, Wood and Loomis (1927) reported the effects of the ultrasonic treatment on cellular biomass, such as floc fragmentation, cell rupture and destruction. Ultrasound in the range of 20 kHz to 1 MHz is used in chemical processing, while higher frequencies are used in medical and diagnostic applications. Ultrasound pretreatment of biomass results in alteration of the surface structure and production of oxidizing radicals that chemically attack the lignocellulosic matrix (Luo et al., 2013). Additionally, ultrasound can disrupt α-O-4 and β-O-4 linkages in lignin (Shirkavand et al., 2016) which results in the splitting of structural polysaccharides and lignin fractions by formation of small cavitation bubbles (Kumar and Sharma, 2017). The bubbles formed grow to a certain critical size and then become unstable, collapsing violently, and achieving pressures up to 1,800 atmospheres and temperatures of 2,000–5,000 K (Kunaver et al., 2012). Hence, ultrasonic disruption may represent an effective green technology for the pretreatment of lignocellulosic biomass.

Kunaver et al. (2012) studied the utilization of forest wood wastes to produce valuable chemicals using high energy ultrasound at a power of 400 W and adjustable power output ranging from 20% to 100% and reported shorter reaction times (by a factor of up to nine). Sun et al. (2004) reported that ultrasound irradiated sugarcane bagasse achieved 90% hemicellulose and lignin removal at an ultrasound power of 100 W and
sonication time for 2 hours in distilled water at 55°C. The ultrasound was found to attack the integrity of cell walls, cleaving the ether linkages between lignin and hemicelluloses, and increasing the accessibility and extractability of the hemicelluloses. This is in agreement with another study for ultrasound-assisted alkaline pretreatment of sugarcane bagasse (1 g/25ml) using 400 W microwave power for 47.42 minutes in 2.89% NaOH and 70.15°C, where the theoretical reducing sugar yield recovered was about 92% (Velmurugan and Muthukumar, 2012).

Ultrasound-assisted, alkali pretreatment can enhance lignin degradation and enzymatic saccharification rates by breaking hydrogen bonds between molecules of lignocellulosics and lowering its crystallinity. However, the ultrasonic vibration energy is too low to change the surface conformation of the raw material biomass particles (Zhang et al., 2008). Subhedar et al. (2017) recently investigated the ultrasound-assisted delignification and enzymatic hydrolysis of three biomass types (groundnut shells, pistachio shell, and coconut coir) and reported an approximate 80–100% increase in delignification over conventional alkali treatments, where biomass loading was 0.5%, ultrasound power was 100 W and duty cycle was 80% for 70 minutes. Additionally, reducing sugar yields in the case of ultrasound-assisted enzymatic hydrolysis under optimised conditions of enzyme loading at 0.08% w/v, substrate loading at 3.0% w/v, ultrasound power of 60 W and duty cycle of 70% for 6.5h, were 21.3, 18.4 and 23.9 g/L for groundnut shells, pistachio shells, and coconut coir respectively, significantly more than that found for alkali hydrolysis (10.2, 8.1 and 12.1 g/L).

It was also reported that reducing sugar yield was increased by a factor of approximately 2.4 by the application of ultrasound at a power of 60 W and duty cycle
of 70 % for pretreatment of lignocellulosic wastepaper at substrate loading of 3.0% (w/v) and cellulase loading of 0.8% (w/v) for 6.5 hours (Subhedar et al., 2015). Moreover, acoustic cavitation was found to successfully decrease the crystallinity of the microcrystalline cellulose, enabling enhanced enzymatic digestibility (Madison et al., 2017).

Combining ultrasound with ammonia pre-treatment of sugarcane bagasse (sonication time of 45 minutes in 400 w power, 100% amplitude and 24 kHz frequency, biomass loading of 1 g per 10 ml of 10% ammonia and temperature of 80° C) resulted in a cellulose recovery of 95.78%, with 58.14% delignification (Ramadoss and Muthukumar, 2014). Additionally, the synergistic effect of combining ammonia with ultrasound reduced by-product formation, enabled the treatment to be conducted at moderate temperature and reduced cellulose crystallinity. This is with an agreement with recent work carried out on ultrasound-assisted dilute aqueous ammonia (2.0% w/v aqueous ammonia) pretreatment of corn cob, corn stover and sorghum stalk using ultrasound at 90 W power and 50 kHz frequency (Xu et al., 2017); the highest enzymatic hydrolysis sugar yield was approximately 81% in corn cob (70° C, 4h), 66% in corn stover (60° C, 2 h) and 57% in sorghum stalk (50° C, 4 h). Similarly, pretreatment of spent coffee waste by ultrasound assisted potassium permanganate (biomass loading of 1.0 g at 10 ml of 4% KMnO4 for 20 minutes, ultrasonic frequency of 47 kHz and power of 310 W) resulted in 98% cellulose recovery and 46% lignin removal (Ravindran et al., 2017).

2.4.3.3.3 Techno-economic feasibility

Equipment based on emerging technologies are available in the market and are used mainly in food processing industry. Example of these equipment includes: continuous
flow microwaves (Advanced Microwave Technologies, United Kingdom), and ultrasonic processors (Industrial Sonomechanics, United States).

Microwave use in chemical processing has been shown to be a technically and economically feasible alternative to conventional heating. Hasna (2011) evaluated the cost-benefit of using microwave drying in corrugated paperboard manufacturing as an alternative to conventional steam platens. It was concluded that the microwave capital cost ($7000 per kW) could be off-set against utilities and power savings (from $128.00 to $38.00 per hour), compared with conventional steam platens. Such savings were achievable in less than one year with an assumption that operation hours are 6000 per year. The author also reported additional benefits from using microwave drying in corrugated paperboard manufacturing, such as improved quality, reduced wastage, and minimum starch consumption. In a recent feasibility study on ginger processing to oleoresin, an ultrasound pretreatment step was introduced as a novel method to enhance extraction of chemical constituents from plant materials (Romis Consultants Ltd, 2017); however, the study did not focus on economics related to ultrasound specifically. Generally, the economic feasibility of emerging technologies is limited by the high cost of capital investment for new equipment. For commercial application of the emerging technologies in pretreatment of lignocellulosic biomass further feasibility studies will be needed considering the complexities of biorefining process, inter-dependence of pretreatment processes and the economics related to the market of the finished product.
2.4.4 Agro-industry wastes as fermentation media

Recent studies involving enzyme production using agro-industry wastes have primarily investigated the effect of pretreatment measures in influencing the enzymes yield. An interesting study conducted by Salim et al. (2017) deployed a new strain of *Bacillus* to study the production of four enzymes (α-amylase, protease, cellulase and pectinase) using different agricultural residues as growth substrates (soybean meal, sunflower meal, maize bran, maize pericarp, olive oil cake and wheat bran). Each residue was subjected to different pretreatment measures (acid/alkali, ultrasound and microwave treatments) to determine their effect on enzyme production. Chemical pretreatments were found to be superior to other techniques, with highest yields of protease and amylase achieved using alkali -treated corn pericarp. Another study conducted by Leite et al. (2016) studied the application of ultrasonication as a potential pretreatment for olive pomace to improve enzyme yield using solid state fermentation. They reported a 3-fold increase in xylanase activity and a 1.2-fold increase in cellulase activity employing *Aspergillus niger* as the producer microbe.

While media formulations based on agricultural wastes are heterogeneous by nature, studies have been conducted on optimization to improve yield. Gustavo et al. (2017) conducted an extensive study to optimize a solid-state fermentation process involving different agro-industrial wastes and *Rhizopus microsporus* var. *oligosporus* as the producer microbe. The application of four lignocellulosic substrates viz. wheat bran, wheat flour type II, soybean meal and sugarcane bagasse were assessed in their study as potential carbon sources for amylase production. Their findings suggested that the best individual substrate for amylase production was wheat bran.
Along with advances in fermentation technologies, studies have been conducted at lab-pilot scales employing different bacterial and fungal strains to determine the efficacy of enzyme production using lignocellulosic biomass (Pandey, 2003). Several studies have shown a rejuvenated interest in solid state fermentation for the production of various enzymes. Many of the attributes of agro-industrial residues are especially suited to this form of culture and can yield significant benefits in terms of sustainability. The latter include environmental friendliness and significant reduction in production costs.

2.4.4.1 Production of Xylanase

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 a xylose-residue backbone, with each subunit linked by a β-1, 4-glycosidic bond. The xylan backbone can be branched with D-glucuronic acid or D-arabinofuranoside (Harris and Ramalingam, 2010). Xylanases are produced by several bacterial and fungal species. Filamentous fungi that synthesise this enzyme are of particular interest because they secrete it into the media in large quantities in comparison to bacteria (Knob et al., 2013). Xylan, being a complex polysaccharide, requires a consortium of enzymes for total hydrolysis. 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 the most extensively studied components of this system (Polizeli et al., 2005). Xylanases have wide applications in industries as diverse as food, biomedical, animal feed and bioethanol (Harris and Ramalingam, 2010; Das et al., 2012; Goswami and Pathak, 2013).
The suitability of lignocellulosic food waste as a carbon source for the production of commercial xylanases has been studied by many researchers. Lowe et al. (1987) investigated wheat straw as a viable carbon source for the production of a xylanase derived from an anaerobic rumen fungus and achieved high levels of activity (0.507 IU/ml). 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 activity. In another study, grape pomace was used as the substrate for the production of xylanase using A. awamori as the enzyme producer (Botella et al., 2007). Highest xylanase activity (40 U/g) was achieved after 24h of incubation time. Addition of 6% glucose as an additional carbon source increased xylanase production significantly. Apple pomace, melon peel and hazelnut shell were used by Seyis and Aksoz (2005) for xylanase production from Trichoderma harzianum 1073 D3, achieving a maximum activity of 26.5 U/mg.

It is worth noting that Hg\(^{2+}\) might have an inhibitory effect on xylanase activity regardless the source of the enzyme. Da Silva et al. (2015) reported that the ions concentration of Hg\(^{2+}\) (2 mM), and Cu\(^{2+}\) (10 mM) were inhibitors for activity of xylanase from Trichoderma inhamatum. Another research (Guan et al., 2016) found that Hg\(^{2+}\) (2 mM), and Cu\(^{2+}\) (10 mM) inhibited the activity of xylanase from Cladosporium oxysporum, while Mg\(^{2+}\) enhanced the xylanase activity by 2%. Similarly, Khasin et al. (1993) reported that Zn\(^{2+}\), Cd\(^{2+}\), and Hg\(^{2+}\) were strong inhibitors for xylanase from Bacillus stearothermophilus T-6, while authors reported that the enzyme has no apparent requirement for cofactors.
2.4.4.2 Production of Pectinase

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 into two groups according to their mode of action. Pectin esterases catalyse the de-esterification of methyl groups found in pectin to produce pectic acid. Pectin depolymerase enzymes cleave the glycosidic bonds found in pectic acid to release various simpler compounds based on their mode of enzyme action. Protopectinases solubilize protopectin into highly polymerized forms of soluble pectin (Sakai et al., 1993). Pectinases are used in the fruit juice industry and wine making for clarification and removal of turbidity in the finished product. They also intensify the color in the fruit extract, while aiding in stabilization and filtration (Servili et al., 1992).

Many agricultural residue varieties have been tried-and-tested for the production of pectinases using different microbial species. Apple and grape pomace have been extensively used in studies to determine their efficacy as suitable substrates for pectinase production (Botella et al., 2007; Hours et al., 1988). A novel approach was devised by Kashyap et al. (2003) in which they supplemented the fermentation media with Neurobion® tablets (a multi-vitamin) and polygalacturonic acid, while producing pectinase using wheat bran as the carbon source. Maximum enzyme activity was reported to be 8,050 U/g dry substrate. A mixture of orange bagasse and wheat bran in the ratio (1:1) was employed as media for pectinase production using the filamentous fungus Penicillium viridicatum RFC3 and 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 lyase on performing the fermentation process in polypropylene packs (Silva et al., 2005). Seedless sunflower heads and barley spent grain are lignocellulosic food processing
wastes have also been studied as potential media additives for pectinase production (Almeida et al., 2003; Patil and Dayanand, 2006).

Pectinases have also been produced using a mixture of wastes. Biz et al. (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 throughout the bed. A combination of citrus peel and bagasse ensured temperature control while avoiding problems such as bed shrinkage and agglomerate formation since sugarcane bagasse is highly porous in nature. *Aspergillus oryzae* was used as the fermentative microbe.

It is noteworthy that published studies show that metal ions have a different impact on pectinase from different microorganisms. Anggraini et al. (2013) reported that the ions concentration of Zn$^{2+}$ (4 mM), Mg$^{2+}$ (4 mM), and Ca$^{2+}$ (6 mM) were inhibitor for activity of pectinase from *Aspergillus niger*. Recently, Xu et al. (2020) found that Ca$^{2+}$, Cu$^{2+}$, and Mn$^{2+}$ inhibited the activity of pectinase from *Aspergillus nidulans* by 14.8%, 12.8%, and 10.2% separately. On the contrary, Hassan et al. (2013) reported that Ca$^{2+}$ and Mn$^{2+}$ were the preferential cofactor of two pectinase enzymes belong to different families of pectinase produced by *Dickeya dadantii*. However, Dey and Banerjee (2014) found that there was no significant effect of 10 mM CaCl$_2$ on the activity of pectinase produced by *Aspergillus awamori* Nakazawa MTCC 6652.

### 2.5 Immobilisation of enzymes

Enzymes once suspended in an aqueous reaction medium are almost impossible to retrieve or recycle. Additionally, due to their proteinaceous nature, enzymes are highly unstable and require an aqueous environment to catalyse reactions (Joseph et al.,
Enzyme immobilisation is the process of attaching an enzyme molecule to a solid support with intentions for its reuse, and with the potential of minimizing loss in their catalytic activity, thereby dramatically improving process economics. Although theoretically promising, the practical experiences of enzyme immobilisation have not always lived up to expectations.

2.5.1 Carrier materials

Carrier materials can be classified into organic and inorganic according to their chemical composition. Organic enzyme carrier materials can be categorized further as being natural and synthetic, according to their mode of origin. The choice of carrier material for a particular enzyme molecule is often a question of trial-and-error but can be improved by possessing a strategic knowledge of the surface properties and functional groups borne on the enzyme. The same can be said about the choice of immobilisation method.

The matrix chosen for enzyme immobilisation should ideally be affordable and eco-friendly, as well as easily available. It should be inert to any chemical reactions and should not interfere with the enzymatic process. It should be stable over a wide temperature and pH range, while protecting the attached enzyme. A carrier material that can accommodate a large amount of enzyme is highly desirable. As is obvious, all these properties are usually not observed in any single type of enzyme immobilisation matrix. Table 2.4 represents some of the common matrices used for the immobilisation of different enzymes.
Table 2.4 Enzyme support materials and their properties

<table>
<thead>
<tr>
<th>Carrier type</th>
<th>Carrier material</th>
<th>Properties</th>
<th>Enzyme</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organic (Natural)</td>
<td>Poly(3-hydroxybutyrate-co-hydroxyvalerate)</td>
<td>Biocompatibility, biodegradability, strength, easy reabsorption, non-toxicity, eco-friendliness</td>
<td>Candida rugosa lipase</td>
<td>Cabrera-Padilla et al., 2012</td>
</tr>
<tr>
<td>Chitosan</td>
<td>Inexpensive, natural polyaminosaccharide, non-toxic, biocompatible and biodegradable</td>
<td>β-Galactosidase</td>
<td></td>
<td>Biró et al., 2008</td>
</tr>
<tr>
<td>Collagen</td>
<td>Form of the porous structure, permeable, hydrophilic and biocompatible</td>
<td>Alkaline phosphatase</td>
<td></td>
<td>Hanachi et al., 2015</td>
</tr>
<tr>
<td>Organic (Synthetic)</td>
<td>Paper-based carriers</td>
<td>Inexpensive, non-toxic, biocompatible and biodegradable, abundantly available</td>
<td>Glucose oxidase biosensor</td>
<td>Nery and Kubota, 2016</td>
</tr>
<tr>
<td>Inorganic</td>
<td>Zeolite</td>
<td>Molecular sieves, microporous, crystalline</td>
<td>Lysosyme, papain, trypsin</td>
<td>Díaz and Balkus, 1996; Xing et al., 2000</td>
</tr>
<tr>
<td>Hydrotalcite</td>
<td>Anion exchange properties</td>
<td>Lipozyme-TL IM</td>
<td></td>
<td>Yagiz et al., 2007</td>
</tr>
<tr>
<td>Mesoporous silica</td>
<td>Highly porous, adsorption, immobilization influenced by electrostatic interaction</td>
<td>Lysosyme</td>
<td></td>
<td>Carlsson et al., 2014</td>
</tr>
<tr>
<td>Gold nanoparticles</td>
<td>Controllable particle size, structure, non-toxic, hydrophobic/hydrophilic and biocompatible</td>
<td>Trypsin</td>
<td></td>
<td>Kalska-Szostko et al., 2012</td>
</tr>
<tr>
<td>Organic–inorganic hybrid</td>
<td>Alginate/silica biocomposites</td>
<td>Inorganic porous network, control over diffusion properties</td>
<td>β-galactosidase</td>
<td>Coradin and Livage, 2003</td>
</tr>
</tbody>
</table>

The boom of nanotechnology has encouraged an increasing interest in magnetic nanoparticles as supports for enzymes (Bilal et al., 2018; Bilal et al., 2019; Tavares et al., 2015), due to their small size and large surface area. Furthermore, conventional support materials can be coupled with magnetic nanoparticles to facilitate easier product separation (Woo et al., 2015). However, biocompatibility problems may be
an issue when dealing with magnetic nanoparticles, and food applications have largely favoured the use of natural polymers for enzyme immobilisation. In this regard, alginate hydrogel is a popular polymer of natural polysaccharides that is widely used as a simple and inexpensive carrier for enzymes. However, a problem faced with this material is the leakage of enzyme in an aqueous media which in turn reduce the activity of immobilized enzymes. This can be greatly reduced by addition of cross-linking agents (of which the most common is glutaraldehyde and genipin) prior to immobilisation (Tsai and Meyer, 2014).

2.5.1.1 Magnetic nanoparticles

Superparamagnetic nanoparticles have a size range of 10–20 nm and possess a high magnetization value, resulting in an excellent separation efficacy when used in enzyme immobilization processes. Besides facile separation of superparamagnetic nanoparticles using external magnetic fields, they have very large surface area-to-volume ratios that increases the reactivity at the molecular level via enhanced enzyme loading (Bilal et al., 2018). Therefore, magnetic nanoparticles are understandably attracting substantial attention as promising enzyme carriers.

Consequently, a myriad of publications featuring laboratory-scale studies for immobilisation of enzymes on magnetic nanoparticles have been published in recent decades (Bilal et al., 2018; Bilal et al., 2019; Furtado et al., 2018; Seenuvasan et al., 2018). Apart from the millilitre-scale bench studies, proof-of-concept pilot studies at liter scale were attempted to explore a realistic enzymatic reactor with a magnetic separation system that enables the recovery of enzymes immobilised on magnetic nanoparticles. Such reactors were investigated at volumes of 1 L (Lindner et al., 2013), 20 L (Alftrén, 2014; Zlateski et al., 2014), and 100 L (Moldes-Diz et al., 2018). These
studies suggest that a separation efficiency of 99% is possible for the separation of magnetic particles from reactors in a volume range of 1.0–100 L (Lindner et al., 2013; Moldes-Diz et al., 2018). However, large scale manufacturers would definitely require reactors at larger capacities that are not available nowadays.

Commercial availability of industrial reactor with magnetic separation system is not the only challenge facing implementation of magnetically retrievable enzymes in food manufacturing facilities. In fact, safety of consumers and consumer’s acceptance of nano-enabled products are key challenges that need to be overcome by stakeholders (enzyme producers, food manufacturers, and regulatory authorities).

A recent review of the literature on the toxic effects of magnetic nanoparticles both in vitro (cell lines) and in vivo (Invertebrates/Vertebrates) by Malhotra et al. (2020) found that magnetic nanoparticles tend to degrade in the body. The aforementioned finding suggests that there is a need to understand not only the potential toxicity of the nanoparticles but also the potential toxicity of the degraded products. Cellular uptake of magnetic nanoparticles and their subsequent degradation can disturb the intracellular levels of iron which in turn can influence cellular iron metabolism and leading to the overproduction of reactive oxygen species (Mulens-Arias et al., 2020, Fu et al., 2014). On the other hand, another review paper found no potential toxicity associated with the oral administration of iron oxide nanoparticles in experimental animals when surveyed published studies (McClements and Xiao, 2017). Hence, at this juncture, predictions for the future of the magnetic nanoparticles in food sector will carry a degree of uncertainty. It is worth mentioning that consumers may be exposed to iron oxide nanoparticles unintentionally through the consumption of the food pigment E172 as there are no specifications concerning the fraction of nanoparticles in these pigments (Voss et al., 2020).
2.5.1.2 Alginate beads

Alginites are commercially available as sodium alginate from brown seaweeds, including *Laminaria hyperborea*, *Laminaria digitata*, and *Macrocystis pyrifera* (Abhilash and Thomas, 2017). Alginate can also be extracellularly produced by bacteria, including *Azotobacter vinelandii* and *Pseudomonas spp.* (Urtuvia et al., 2017).

Alginate is an ingredient used by the food industry as stabilizers, thickeners, and emulsifiers (Brownlee et al., 2009); and it is sold in bulk for food applications at prices as low as $5 kg^{-1}$ (Hay et al., 2013). Hence, alginate beads are widely studied as an accessible, non-toxic, and inexpensive matrix for entrapment of industrially important enzymes. However, enzyme entrapment can have significant drawbacks, including enzyme leakage and substrate-product diffusion limitations (Liu, 2017). To overcome these drawbacks, the enzymes can be bound to alginate covalently using a cross-linker such as glutaraldehyde (Barbosa et al., 2014). In addition to glutaraldehyde, genipin from the fruits of *Gardenia jasminoides* Ellis has been investigated as an efficient and biocompatible crosslinker, and when combined with alginate provides a ‘food-friendly’ matrix for enzyme immobilization (Tacias-Pascacio et al., 2019).

2.5.2 Enzyme Immobilization methods

The choice of best immobilisation method for a particular enzyme should be determined based on certain characters that are specific to both enzyme and carrier material. Generally, two methods are used for enzyme immobilization, namely: entrapment and support binding. The latter can be physical (e.g. adsorption) or chemical (e.g. covalent binding, and cross linking).
Neither physical adsorption nor entrapment involve strong bonding the enzyme to the carrier material. Hence, adsorption and entrapment immobilisation methods share the same disadvantage of leaching of the enzyme from the carrier material in an aqueous reaction mixture (Freitas et al., 2012; Homaei et al., 2013). In the case of adsorption immobilisation, bonding strength is weak, while the method of entrapment immobilisation does not involve chemically bonding the enzyme to the carrier material. Entrapment can be described as the engulfing of the enzyme inside a matrix, such as within a gel or fibre. Hence, the diffusion of a macromolecular substrate to the enzyme may present a serious limitation in the case of small matrix pore size.

Covalent binding basically fixes the enzyme molecule onto the carrier on a permanent basis through strong covalent bonds. This minimizes the problem of leaching of the enzyme found in adsorption-based biocatalysts when suspended in reaction mixtures. However, owing to the strong covalent binding of the enzyme to the carrier material, the conformation (shape) of the enzyme might be altered, resulting in a major loss of activity (Costa et al., 2005).

Cross linking of enzymes is another innovative method to immobilise enzymes without the help of a carrier material. Here, every enzyme molecule acts as a carrier for each other. Cross linked enzyme aggregates (CLEAS) eliminate some of the disadvantages associated with carrier materials and have been reported to possess very high loading rate. However, CLEAs tend to have several disadvantages compared to other immobilisation strategies. They are not mechanically strong and depending on the process may not be deemed fit for industrial application (Cui et al., 2015).
2.5.3 Application of Immobilised Enzymes in Food and Beverage Industry

Apart from the pharmaceutical industry and some chemical industries, the food sector is targeting production of cost sensitive products in large quantities and tends to use packed bed column reactors rather than stirred batch reactors (Basso and Serban, 2019). Hence, continuous flow operations are typically preferred over batchwise processing mode, whenever applicable. A successful example of immobilized enzymes used at industrial scale in continuous process is the use of D-glucose/xylose isomerase for production of high fructose corn syrup. In fact, immobilized glucose isomerase was a game changer for the market of industrial enzymes, accounting for 20% of the total industrial enzyme sales in 1990 (Robinson, 2015). Alongside glucose isomerase, other immobilised enzymes such as β-galactosidase, and lipase have been successfully commercialized. Despite the steady growth that industrial enzymes witnessed since 2000, few new immobilized enzymes have been successfully introduced.

2.6 Enzymatic treatments in fruit juice production

Depectinization is an essential part of fruit juice production to increase the efficacy of fruit pulp extraction, and juice clarification (Dey and Banerjee, 2014). However, different enzymatic treatments are required for each fruit juice according to consumer preferences. For instance, intensive depectinization is required for clarifying juices from apples, berries, or grapes to achieve complete juice clarification. On the other hand, only mild enzymatic treatment is enough to improve fruit pulp extraction while maintaining cloudy juices from orange and pineapple, which are considered more desirable consumer products. Commercial enzymes are currently used in fruit juice production as enzymatic cocktails (Dal Magro et al., 2020). In combination with pectinase, cellulase is usually used in enzymatic liquefaction and fruit pulp extraction.
prior to juice clarification (Sharma et al., 2017; Will et al., 2000). Moreover, destarching is also essential when unripe starch-rich fruits (e.g. apples) are used for juice processing (Dey and Banerjee, 2014). Hence, $\alpha$-amylase is required to eliminate the additional haziness of juice due to the presence of starch.

Fruits such as apples, and pineapples contain xylan-rich hemicellulose, and hence the treatment of the corresponding juices with xylanase is also needed for juice clarity (Bhardwaj et al., 2019). In the case of citrus fruits (e.g. grapefruit), debittering by naringinase is required to remove the perceptible bitter taste to consumers (Bodakowska-Boczniewicz and Garnarek, 2019). Glucose oxidase may be used for preventing changes in color and taste during storage of fruit juice (Fernandes, 2018).

To the best of the author's knowledge, both powder and liquid commercial preparations of juice extraction and clarification enzymes (e.g. pectinase, xylanase) are based on free enzymes, and immobilized forms have only been explored in laboratory scale studies. The food industry segment dominates the market of industrial enzymes by 35% of total market share (major producers being Novozymes and Dupont) and is expected to expand at a compounded annual growth rate (CAGR) of more than 6% between 2020 and 2025 (Research and Markets, 2020).
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.
3.1 General

3.1.1 Commercial sources of common reagents

- Agar: Fluka
- Bovine serum albumin: Sigma-Aldrich
- Calcium Chloride: Sigma-Aldrich
- Cellulase from *Trichoderma reesei*: Sigma-Aldrich
- Citric acid: Sigma-Aldrich
- Congo red: Sigma-Aldrich
- Coomassie G-250: Sigma-Aldrich
- D(+) Glucose: Sigma-Aldrich
- D(+) Xylose: Fluka
- D-Galacturonic acid: Sigma-Aldrich
- Dinitrosalicylic acid: Sigma-Aldrich
- Genipin: Sigma Aldrich
- Glutaraldehyde: Sigma Aldrich
- Hemicellulase from *Aspergillus niger*: Sigma Aldrich
- Iron(II) chloride: Sigma-Aldrich
- Iron(III) chloride: Sigma-Aldrich
- Lactophenol blue solution: Sigma-Aldrich
- Pectin from citrus peel: Sigma-Aldrich
- Potato Dextrose Agar: Sigma Aldrich
- Rochelle salt: Sigma Aldrich
- Sodium Acetate: Sigma-Aldrich
- Sodium Alginate: BDH Chemicals
- Sodium citrate: Scharlauch Chemie
- Sodium Hydroxide: Sigma-Aldrich
- Xylan from beech wood: Sigma-Aldrich
- All reagents and chemicals used for the study were of analytical grade.

3.1.2 Instruments

- Field Emission Scanning Electron Microscopy (Hitachi SU-70, Japan).
- Perkin Elmer Spectrum GX FT-IR (UATR) Microscope, USA.
- Siemens’s BN D-500 X-ray diffractometer, Germany.
- Microwave oven: Sharp, Model R 244; Sharp electronics, UK.
- UV-1800 UV-VIS spectrophotometer (Shimadzu Scientific Instruments, USA).
- Quava Mini Ultrasonic System: Kaijo, USA.
- X-Max silicon drift detector (Oxford Instruments, UK).

3.1.3 Software
- Microsoft Office: Microsoft, Inc., USA.
- NeuralPower® (CPC-X Software, version 2.5, USA).
- Origin Pro 8 software (Microcal Software Inc., USA).
- STATGRAPHICS Centurion XV: StatPoint Technologies, Inc., Warrenton, VA.

3.1.4 Analytical Techniques

3.1.4.1 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., 2008). 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 a blast furnace for 24h at 595°C to obtain acid insoluble lignin. The acid soluble lignin content in the liquid was determined using spectrophotometry at 205 nm.
3.1.4.2 Reducing sugar analysis

The reducing sugar concentration in the hydrolysate was estimated by the 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.

3.1.4.3 Enzymatic hydrolysis

To perform the hydrolysis, 1 g of BSG, 158.76 µl of cellulose (77 FPU/ml), and 153.3 µl hemicellulase (72 U/ml) were mixed in sodium citrate buffer (0.05 M) and distilled water at pH 5.4 (total volume 10mL) and heated to 50°C for 120 h (Ravindran et al., 2018b). After treatment, the hydrolysate liquors were collected, and stored at 4°C for further compositional analysis.

3.1.4.4 Assay of Total Protein

Total protein concentration was determined by the Bradford assay using the absorbance values of the dye at 595 nm using bovine serum albumin (BSA) as the standard (Bradford, 1976).

3.1.4.5 Assay of enzyme activity

Xylanase, and pectinase activities were assayed by determining the liberated reducing sugars (xylose and D-galacturonic acid, respectively) resulting from the enzymatic hydrolysis of the corresponding substrates (xylan, and pectin, respectively) using 3,5-dinitrosalicylic acid reagent (Miller, 1959). The supernatant from the culture broth was served as the source of the enzyme), a standard curve was graphed for each sugar
(xylose, and D-galacturonic acid), and the absorbance of the solution was measured at 540 nm. One unit of enzyme activity (U) was defined as the amount of enzyme that produces 1.0 μmol of reducing sugar per ml per minute (μmol min⁻¹) under the assay conditions.

3.1.4.6 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. However, 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.

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 FT-IR (UATR) Microscope (USA) was employed for this study The FTIR spectra was recorded from 4000 to 400 cm⁻¹ with 32 scans at a resolution of 0.3 cm⁻¹ in transmission mode (Raghavi et al., 2016).

3.1.5 Molecular Identification of Fungi

Initially, the genomic DNA was extracted using PrepMan® Ultra Sample Preparation Reagent following the instructions of the manufacturer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The internal transcribed spacer (ITS) regions of
ribsomal DNA were then amplified using universal primers: ITS1 (forward, 5’-CTTGGTCATTAGAGGAA GTAA-3’), ITS4 (reverse, 5’-TCCTCCGCTTA-TTGATATGC-3’), and MyTaq™ DNA Polymerase Kit (Bioline, London, UK) (M’barek et al., 2019). The PCR products were then purified using ExoSAP-IT® (Thermo Fisher Scientific Inc., Waltham, MA, USA), followed by Sanger sequencing (LGC Genomics GmbH, Berlin, Germany). Finally, the resulting nucleotide sequences were processed with the software Chromas (V 2.6.6, Technelysium, Brisbane, Australia) and compared using a Basic Local Alignment Search Tool (BLAST) with sequences deposited in the National Center for Biotechnology Information (NCBI) Nucleotide database (blast.ncbi.nlm.nih.gov).

3.1.6 Statistical analysis

All the experiments were carried out in triplicate and repeated 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

Pretreatment of lignocellulosic biomass to overcome its intrinsic recalcitrant nature prior to the production of valuable chemicals has been studied for nearly 200 years. Research has targeted eco-friendly, economical, and time-effective solutions, together with a simplified large-scale operational approach. Commonly used pretreatment methods, such as chemical, physico-chemical, and biological techniques are still insufficient to meet optimal industrial production requirements in a sustainable way. Recently, advances in applied chemistry approaches conducted under extreme and non-classical conditions has led to possible commercial solutions in the marketplace (e.g. Microwave, and Ultrasound technologies). These new industrial technologies are promising candidates as sustainable green pretreatment solutions for lignocellulosic biomass utilization in a large scale biorefinery.
Chapter 4

The application of microwave and ultrasound pretreatments for enhancing saccharification of Brewers' Spent Grain (BSG)

In this chapter, the potential of microwave and ultrasound was evaluated for energy efficiency and environmental compatibility in the pretreatment of BSG prior to fermentation. The highest sugar yield was obtained (64.4±7 mg) when 1 g of biomass was pretreated with microwave energy of 600 W for 90 s, with an energy input amounting to 0.001 Kwh of electricity. FTIR spectra of the pretreated substrates was studied to analyse the various changes incurred as opposed to native BSG.

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

https://doi.org/10.1016/j.biteb.2019.100371
4.1 Introduction

Globally, beer is the primary consumed alcoholic beverage, and the fifth most consumed beverage overall. Brewer's spent grain (BSG) is the solid barley waste produced during mashing of malted barley and is separated from the wort in beer brewing. The average global by-production of BSG is 39 million tonnes annually, with approximately 3.4 million tonnes of BSG produced annually in Europe alone (Lynch, 2016). However, the BSG has a high moisture content (up to 80%) and polysaccharides (40–50 % on dry weight basis) which make it susceptible to microbial growth and spoilage after few days of storage. Thus, it is used immediately as an animal feed or is disposed in landfill sites.

This notwithstanding, since BSG is rich in sugars, it holds potential as a cheap feedstock option for biorefinery applications (Hassan et al., 2018a). However, achieving enzymatic hydrolysis of sugars in BSG requires energy-intensive pretreatment processes to fractionate the lignocellulosic complex matrix and improve digestibility (Hassan et al., 2018b). Interestingly, both ultrasound and microwave have potential as energy efficient green technologies for pretreatment of lignocellulosic biomass prior to further conversions.

Microwave-assisted pretreatment of lignocellulose has been reported to be a time and energy efficient process that does not lead to generation of inhibitors (affecting enzymatic hydrolysis and/or microbial fermentation) or superficial overheating of biomass (Aguilar-Reynosa et al., 2017). The efficiency of microwave-assisted pretreatment of lignocellulose may be attributed to the energy absorbing properties of such carbon materials, and the heating properties of the microwave electromagnetic fields. Ultrasound technology has also been investigated for pretreatment of
lignocellulose, with advantages of reduction in duration of treatment and chemicals required (Baruah et al., 2018). Cavitation bubbles are formed during the ultrasound-assisted pretreatment that leads to fractionation of lignocellulose, and thus, an increase in the accessibility to hydrolytic enzymes. Therefore, this study aimed to compare the effectiveness of microwave and ultrasound-assisted pretreatments of BSG for further use as a microbial growth substrate. Microwave-assisted pretreatment of BSG was carried out at varying power levels (100, 250, 440, 600, and 1000 W) for 30, 60, and 90 s. Additionally, BSG was pretreated using different ultrasound frequencies (25, 35, 45, 130, and 950 kHz) for 20, 40, and 60 min.

4.2 Methodology

Brewer’s spent grain (BSG) was generously donated by a local brewery in Dublin, Ireland. Once received, BSG was dried at 60 °C for 48 h, then milled, and sorted using a 350-µm sieve. It was then maintained at ambient temperature in a dry place for further experiments. All chemicals required for experimentation were purchased from Sigma Aldrich (Ireland), and were of analytical grade. Beechwood xylan and pectin from citrus peel were used to assay xylanase and pectionase activities, respectively. The cellulase from *Trichoderma reesei* (aqueous solution, ≥700 units/g) showed 77 FPU/ml when assessed by a protocol developed by NERL National Renewable Energy Laboratory (Adney and Baker, 1996). Hemicellulase was from *Aspergillus niger* (powder, 0.3-3.0 unit/mg solid). A hemicellulase stock solution at a concentration of 10 g/l was prepared by dissolving hemicellulose powder in sodium acetate buffer (pH 4.8, 500 mM), and then the protocol developed by Rickard and Laughlin (1980) was used to assay activity (72 U/ml).
4.2.1 Pretreatment of substrate (BSG)

4.2.1.1 Microwave pretreatment

A domestic microwave oven with a frequency setting at 50 Hz and adjustable power outputs was used. Fixed solid loading experiments were carried out by immersing five grams of biomass in 50 ml of deionized water in a 200-mL flask. The flask was placed at the center of a rotating circular plate in the microwave oven. Pretreatments were carried out at 100, 250, 400 and 600 W, for 30, 60, and 90 seconds. The mixture was then centrifuged, oven-dried, and the biomass stored for further analysis.

4.2.1.2 Ultrasound pretreatment

Three different ultrasound baths were used: (a) a multi-frequency ultrasonic bath with selectable frequencies of 25 and 45 kHz (Elma Schmidbauer, Transonic TI-H-10, output 550 W), (b) a multi-frequency ultrasonic bath with selectable frequencies of 35 and 130 kHz (Fisher Bio-block Scientific, Transonic TI-H-10, output 750 W), and (c) an ultrasonic tank with a frequency of 950 kHz (Kaijo, Quava Mini Ultrasonic System, output 100 W). Fixed solid loading experiments were carried out by immersing five grams of biomass in 50 ml of deionized water in a 200-mL flask. Pretreatments were carried out at 25, 35, 45, 130 and 950 kHz for 20, 40, and 60 minutes. The mixture then centrifuged, oven-dried and the biomass stored for further analysis.

4.2.2 Enzymatic hydrolysis

The enzymatic hydrolysis of BSG was performed according to the procedure outlined in section 3.1.4.3.
4.2.3 Total reducing sugars

The reducing sugar concentration in the hydrolysate was estimated following the protocol described in section 3.1.4.2.

4.2.4 Fourier Transform Infrared Spectroscopy analysis

The changes in functional groups caused by pretreating the biomass was assessed using FTIR spectroscopy according to section 3.1.4.6.

4.3 Results and Discussion

4.3.1 Pretreatment of BSG for recovery of fermentable sugars

4.3.1.1 Microwave pretreatment

Microwave heating can replace the conventional heating in disruption of the complex structures of lignocellulosic biomass and achieve higher production, minimal energy loss, and lower cost (Hassan et al., 2018a). Thus, microwave-assisted pretreatments were carried out at 100, 250, 400, 600 and 100 W, for 30, 60, and 90 seconds. Table 4.1 shows the effect of microwave power on sugar yield from pretreated BSG. The maximum sugar yield (64.4 ± 7.0 mg/g of BSG) was obtained at 600 W after 90 sec, as compared to 24.5± 0.3 mg of reducing sugar obtained from every 1g of native BSG before pretreatment.

Analysis of variance and Fischer’s Least Significant Difference (LSD) were performed to determine whether the changes in fermentable sugars derived from BSG brought about by microwave-assisted pretreatments at different power levels were significantly different to each other.
Table 4.1: Effect of microwave power on sugar yield from BSG

<table>
<thead>
<tr>
<th>Time (s)</th>
<th>100</th>
<th>250</th>
<th>440</th>
<th>600</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ±SE</td>
<td>Mean ±SE</td>
<td>Mean ±SE</td>
<td>Mean ±SE</td>
<td>Mean ±SE</td>
</tr>
<tr>
<td>30</td>
<td>24.4 ± 0.2</td>
<td>27.8 ± 8.5</td>
<td>37.2 ± 1.2</td>
<td>42.1 ± 3.7</td>
<td>50.9 ± 0.7</td>
</tr>
<tr>
<td>60</td>
<td>37.9 ± 1.2</td>
<td>41.4 ± 8.2</td>
<td>46.4 ± 1.0</td>
<td>64.4 ± 6.3</td>
<td>51.2 ± 1.6</td>
</tr>
<tr>
<td>90</td>
<td>38.2 ± 0.2</td>
<td>41.3 ± 0.7</td>
<td>46.3 ± 4.7</td>
<td>64.4 ± 7.0</td>
<td>51.1 ± 2.1</td>
</tr>
</tbody>
</table>

* Where reducing sugar yields for native BSG = 24.5± 0.3 mg/g of biomass.

* Only means underlined or/and bolded are significantly different at different power levels or/and different treatment time intervals, respectively.

ANOVA revealed that all the microwave-assisted pretreatments were significantly different (P<0.05) at different power levels. However, not all the microwave-assisted pretreatments were significantly different when comparing fermentable sugars derived from BSG pretreated by microwave at different time intervals but constant power level. For example, the microwave-assisted pretreatments for BSG at 100 W for 60, and 90s were statistically similar as far as fermentable sugars were concerned. The same was observed for the microwave-assisted pretreatments for BSG at 440 W for 60, and 90s.

Nevertheless, the results from microwave-assisted pretreatments for BSG at higher power (600 or 1000 W) for short time (30s) were significantly different when compared to their counterparts for longer time (60, and 90s). The same was observed for the microwave-assisted pretreatments for BSG at 250 W. Furthermore, the microwave-assisted pretreatment at 440 W was found to be statistically similar to pretreatment at 600 W if pretreatment time was 60 or 90s.

Overall, the statistical analysis suggested that microwave power has significant impact on pretreatment process as compared to pretreatment time in most cases. Increasing microwave power had a positive effect on fermentable sugar yield; pretreatment time
can be reduced by increasing microwave power. However, for longer pretreatments (60, 90s) the highest fermentable sugar yield was produced at 600 W power level, and increasing microwave power level to 1000 W had a negative effect on fermentable sugar yield.

This agrees with results obtained by Binod et al. (2012) who evaluated the microwave-assisted pretreatments of sugarcane bagasse at different power levels (100, 180, 300, 450, 600 and 850 W). The authors reported that the highest sugar yield was obtained from sugarcane bagasse at 600 W microwave power using either microwave-alkali (MAL) pretreatment or microwave-alkali followed by acid (MAA) pretreatment. Furthermore, Mithra et al. (2017) recommended the same microwave power (600 W) after optimization of microwave-assisted dilute acid pretreatment for peels of root crops and vegetables. The authors found that microwave power had the greatest influence in fermentable sugar yield as compared to other factors studied, such as dilute sulphuric acid concentration, and irradiation time. Similar results that found 600 W microwave power to be optimal were reported by Kuittinen et al. (2016) who studied the effect of two microwave power levels (600 and 1200 W) on pretreatment of Norway spruce under high pressure-temperature. Recently, Tiwari et al. (2019) investigated the application of microwave-alkali pretreatment at different microwave power levels (100 to 600 W) and times (1 to 6 min) on banana peel waste to achieve optimal fermentable sugars. Interestingly, the study found that the maximum reducing sugar (0.561 g/g of biomass) was obtained after microwave-assisted alkali pretreatment at 600 W for 2 min. It is worth noting that most studies integrate chemical pretreatment with microwave pretreatment to improve process efficiency, in spite of disadvantages of chemical pretreatments. In this study, microwave was investigated as
the sole pretreatment technology for lignocellulose to achieve an energy-efficient and chemical-free process.

Microwave electromagnetic fields target the polar part of the biomass selectively, leading to a rise in temperature in the internal parts of lignocellulose. Increasing temperature also affects the degree of lignocellulose polymerization and crystallization so that biomass becomes more accessible to hydrolytic enzymes. However, excessive heating (resulting from high microwave power or long exposure time) can lead to formation of “hot spots”, degradation of useful components like glucose and the formation of unwanted components such as furfural, formaldehyde, acetaldehyde, and dihydroxyacetone. Thereby, it is essential to find a proper microwave power and exposure time to achieve efficient pretreatment.

4.3.1.2 Ultrasound pretreatment

Ultrasound pretreatments were carried out at varying frequencies (and ultrasound power): 25 (550 W), 35 (750 W), 45 (550 W), 130 (750 W) and 950 kHz (100 W) for 20, 40, and 60 minutes. Table 4.2 shows the effect of ultrasound frequency on sugar yield from pretreated BSG. The maximum sugar yield (39.9 ± 6.1 mg/g of BSG) was obtained at low ultrasound frequency (25 kHz, 550 W) after 60 min. (compared to 24.5 ± 0.3 mg of reduced sugar obtained from every 1g of native BSG before pretreatments).

Analysis of variance and Fischer’s Least Significant Difference (LSD) were performed to determine whether the changes in fermentable sugars derived from BSG brought about by ultrasound-assisted pretreatments at different power levels were significantly different to each other. ANOVA revealed that all the ultrasound-assisted pretreatments were significantly different (P<0.05) at different frequencies of each power levels.
Moreover, most of the ultrasound-assisted pretreatments were significantly different (P<0.05) at different frequencies and different power levels.

Table 4.2: Effect of ultrasound frequency on sugar yield from BSG

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Reducing Sugar Yield (mg/g of biomass)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>US Power 550</td>
</tr>
<tr>
<td>US Frequency</td>
<td>Mean ±SE</td>
</tr>
<tr>
<td>20</td>
<td>32.1 ± 7.0</td>
</tr>
<tr>
<td>40</td>
<td>33.6 ± 0.0</td>
</tr>
<tr>
<td>60</td>
<td>39.9 ± 6.1</td>
</tr>
</tbody>
</table>

* Where reducing sugar yields for native BSG = 24.5± 0.3 mg/g of biomass.

* * Only means underlined or/and bolded are significantly different at different ultrasound frequencies or/and different treatment time intervals, respectively.

However, some pretreatments at different ultrasound frequencies were insignificantly different at a constant pretreatment time. For example, the ultrasound pretreatment at a frequency of 25 KHz (550 W) did not feature any significant difference in fermentable sugars compared to ultrasound pretreatment at frequencies of 130 KHz (750 W) and 950 KHz (100 W) after 20 min of pretreatment. Likewise, the ultrasound pretreatments at frequencies of 45 KHz (550 W) and 130 (750 W) did not feature any significant difference in fermentable sugars compared to ultrasound pretreatments at frequencies of 35 KHz (750 W) and 950 KHz (100 W) after 40 min. The same was observed for the ultrasound-assisted pretreatments after 60 min.

Ultrasound-assisted pretreatment at 35 KHz (750 W) was statistically similar at different time point. The same was observed for the ultrasound-assisted pretreatment after 40 and 60 min at an ultrasound frequency of 25 KHz (550 W) and 45 KHz (550 W), and after 20 and 40 min at ultrasound frequencies of 130 KHz (750 W) and 950 KHz (100 W). Overall, the statistical analysis suggested that ultrasound pretreatment at a frequency as low as 25 KHz is always significantly different from pretreatments
at higher frequencies (regardless of the ultrasound power levels) at a given pretreatment time. Additionally, pretreatment time can be reduced by decreasing the ultrasound frequency. The literature is dominated by pretreatments at low-ultrasound frequencies (<50 kHz) because of the short acoustic cycle which allow the growth, radial motion and collapse of bubbles. However, considering the longer required pretreatment time and lower reducing sugar yield of ultrasound pretreatment, as compared to microwave pretreatment, the latter method was selected for all subsequent studies.

Ultrasound used for pretreatment of lignocellulosic biomass disrupted the complex structures of lignocellulosic biomass due to the cavitation effect. Cavitation bubbles are produced in a liquid medium as a result of ultrasound waves, and then collapse to produce a strong mechanical effect on lignocellulosic solid biomass, as well as producing free radicals such as H• and HO• from the liquid medium. Moreover, the “hot spots” that develop on the surface of the solid biomass (as a result of the heat generated during the collapse of the cavitation bubbles) can introduce chemical changes in the lignocellulosic complex structure. It has previously been reported that longer ultrasound pretreatment times may lead to greater sugar recovery (Rehman et al., 2014). However, exposure to long times of ultrasound pretreatment may cause adverse effects due to collision and aggregation between the particles, and this also increases energy consumption during the process. Therefore, it is essential to find a proper ultrasound power and frequency that may achieve an efficient process within the shortest exposure time.
4.3.2 FTIR characterization of pretreated BSG

The chemical changes in the functional groups of the BSG were studied based on FTIR analysis (Figure 4.1). The microwave pretreated BSG displayed significant decreases in band intensities at characteristic peaks for cellulose, hemicellulose and lignin (Table 4.3) as compared to ultrasound-pretreated and native BSG.

![FTIR spectra of native, microwave pretreated, and ultrasound pretreated BSG.](image)

Figure 4.1. FTIR spectra of native, microwave pretreated, and ultrasound pretreated BSG.

Although all BSG samples (pretreated and native alike) exhibited a band at 1033 cm$^{-1}$ which is characteristic of cellulose content in BSG (Silbir et al., 2019) in their respective spectra, microwave pretreated BSG showed the highest transmittance (i.e., the lowest absorption intensity). Similar to the peak at 1033 cm$^{-1}$, microwave pretreated BSG showed the highest transmittance at different bands that represent different functional groups. These results are related to the fact that microwave pretreatment was more efficient in changing the chemical composition of BSG.
### Table 4.3. FTIR peak assignments for lignocellulose

<table>
<thead>
<tr>
<th>Wave number (Cm⁻¹)</th>
<th>Assignment</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>C–O stretching, C–C stretching of cellulose</td>
<td>(Szymanska-Chargot and Zdunek, 2013)</td>
</tr>
<tr>
<td>1015</td>
<td>C–O stretching, C–C stretching of pectin</td>
<td>(Szymanska-Chargot and Zdunek, 2013)</td>
</tr>
<tr>
<td>1020</td>
<td>C–C, C–OH, C–H ring and side group vibrations</td>
<td>(Fan et al., 2012)</td>
</tr>
<tr>
<td>1033</td>
<td>C–O stretching, C–C stretching of cellulose</td>
<td>(Szymanska-Chargot and Zdunek, 2013)</td>
</tr>
<tr>
<td>1040</td>
<td>C–O stretching, C–C stretching of xyloglucan</td>
<td>(Szymanska-Chargot and Zdunek, 2013)</td>
</tr>
<tr>
<td>1049</td>
<td>C–OH bending</td>
<td>(Adapa et al., 2011)</td>
</tr>
<tr>
<td>1058</td>
<td>C=O stretching of hemicellulose and lignin</td>
<td>(Wang et al., 2018)</td>
</tr>
<tr>
<td>1063</td>
<td>C–O–C asymmetrical stretching of cellulose, hemicellulose</td>
<td>(Sills and Gossett, 2012)</td>
</tr>
<tr>
<td>1072</td>
<td>C–O stretching, C–C stretching of xyloglucan</td>
<td>(Szymanska-Chargot and Zdunek, 2013)</td>
</tr>
<tr>
<td>3010</td>
<td>O–H stretching vibration</td>
<td>(Abidi et al., 2011)</td>
</tr>
<tr>
<td>3020</td>
<td>O–H stretching vibration</td>
<td>(Abidi et al., 2011)</td>
</tr>
<tr>
<td>3070</td>
<td>O–H stretching vibration</td>
<td>(Abidi et al., 2011)</td>
</tr>
</tbody>
</table>

Similar results were also reported by other researchers (Silbir et al., 2019) who used FTIR to identify the functional groups present in BSG samples. Moreover, the results were consistent with reported data in the literature (Hou et al., 2019) for other lignocellulosic biomass pretreated with microwave.

### 4.4 Conclusions

Microwave power has a significant impact on pretreatment of BSG as compared to pretreatment time, and pretreatment time can be reduced by increasing microwave power. However, for longer pretreatments (60, 90s) the highest fermentable sugar yield was produced at 600 W power level and increasing microwave power level to 1000 W
had a negative effect on fermentable sugar yield. On the other hand, ultrasound pretreatment of BSG at a low frequency of 25 KHz achieved the maximum fermentable sugar yield compared with that obtained after ultrasound pretreatments at higher frequencies and at an optimum pretreatment time of 60 minutes.

However, microwave pretreatment of BSG at the optimum conditions (600 w for 90 s) achieved higher fermentable sugar yield (64.4±7 mg / 1 g of BSG) as compared to ultrasound pretreatment of BSG (39.9±6.1 mg / 1 g of BSG) at the optimum conditions (frequency of 25 kHz, power of 550 W, and time of 60 min.). Where reducing sugar yields for native BSG before pretreatment was 24.5± 0.3 mg/g of biomass.

Ultrasound achieved a significant increase in fermentable sugar yield as compared to the native BSG. However, the long pretreatment time (1 hour) is a critical limiting factor in industrial application. Thus, response surface methodology (RSM) is used in the next chapter for optimization of the multiple variables of ultrasound pretreatment of BSG and to investigate the ability to reduce pretreatment time. Moreover, chemical composition, morphological structure and thermal behavior of BSG before and after pretreatment was studied and compared.
Chapter 5

Optimisation of ultrasound pretreatment of Brewers' Spent Grain (BSG)

This chapter deals with the optimization of the ultrasound (US) pretreatment of brewer's spent grain (BSG) using response surface methodology (RSM). In this study, the influence of ultrasound (US) power (%), time, temperature and biomass loading on fermentable sugar yield from BSG was studied at the optimum conditions (frequency of 25 kHz, power of 550 W). The optimal conditions were found to be 20% US power, 60 min, 26.3°C, and 17.3% w/v of biomass in water. Under these conditions, an approximate 2.1-fold increase in reducing sugar yield (19.1±0.4 mg/g of biomass) was achieved, relative to native BSG (8.9±0.6 mg/g of biomass). Since only water was used, there was no need for neutralization after US pretreatment for the recovery of sugars. HPLC, FTIR, SEM and DSC were performed to analyze and characterize the native and pretreated BSG.

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

5.1 Introduction

According to a Brewers of Europe report published in 2017, Europe is the second largest beer producer in the world after China, with over 8,490 breweries producing over 400,168 million hectoliters of beer in 2016 (BOE, 2017). Brewers’ spent grain (BSG) is the most plentiful agro-industrial waste generated from the beer-brewing process, and about 3.4 million tonnes of BSG are produced annually in the EU (McCarthy et al., 2013). Typically, spent grain is the insoluble part of the barley grain, separated during the mashing process before fermentation of the soluble liquid wort (Lynch et al., 2016). BSG represents about 85% of the total by-products, and about 2 Kg of BSG are generated per 10 liters of beer. BSG composition offers considerable options for developing value-added products, but the complex structure of lignin and high crystallinity of cellulose reduces its accessibility to hydrolytic enzymes. Thus, a pretreatment step is essential prior to enzymatic hydrolysis. However, the common conventional methods for pretreatment have major drawbacks, including high energy consumption, the necessity to use harsh chemicals, production of inhibitors or low efficiency in large scale production (Hassan et al., 2018a).

The application of ultrasound technology for pretreatment of lignocellulosic biomass holds some potential for large scale processes. Ultrasonic pretreatment of plant material in liquid media results in cavitation phenomena, whereby microbubbles are formed which grow and then violently collapse at a critical size, converting sonic into mechanical energy. Such cavitation effects generate high temperature, pressure and violent shear forces, which lead to the formation of the ‘hot-spot’ effect in the liquid, and the generation of free radicals (Bundhoo and Mohee, 2017). Such effects may be postulated to aid in disruption of the lignocellulosic complex structure, decreasing the
degree of crystallinity of the cellulose, and increasing the accessible surface area to improve enzymatic hydrolysis (Suresh et al., 2014).

Recently, chemical pretreatment methods combined with ultrasonic technique have received some attention (Wang et al., 2018). However, very few reports discuss the ultrasound pretreatment of lignocellulosic material using water as solvent, and optimization of such processes has not been attempted. He et al. (2017) compared the ultrasound pretreatment (300 W, frequency of 28 kHz) of wood in soda solution, acetic solution, and distilled water. The authors reported an increase in sample crystallinity up to 35.3% in the case of soda solution, and up to 35.5% in the case of distilled water or acetic solution, which was due to physiochemical structure changes. Water is safe to handle and is a sustainable green solvent. Moreover, using only water as an alternative for the conventional acid and alkaline pretreatment conditions will mitigate the risk of recovery and effluent treatment of these harsh chemicals.

The critical parameters for optimization of an ultrasound-mediated process for biomass pretreatment include ultrasonic power (Song et al., 2015), biomass loading (Sasmal et al., 2012), treatment time, (Sasmal et al., 2012; Rehman et al., 2014; Song et al., 2015) and temperature (Rehman et al., 2014). Optimization of the pretreatment process by one-factor at a time in a multifactorial process is time consuming and does not consider the interactive effects between the variables. Thus, Response Surface Methodology (RSM) is widely used for the statistical modeling and optimization of the multiple variables of pretreatment processes (Flores-Gómez et al., 2018).
The aim of the present study is to investigate the effect of ultrasonic (US) pretreatment on Brewers' spent grain (BSG) in an aqueous medium under optimized conditions to achieve the highest cellulose (glucose) and hemicellulose (xylose) recovery for bioethanol production, in addition to maximizing lignin recovery for use in the sugar-lignin platform biorefinery (Hassan et al., 2019, 2018b).

5.2 Methodology

Brewer’s spent grain (BSG) was generously donated by a local brewery in Dublin, Ireland. Cellulase from *Trichoderma reesei* (aqueous solution, ≥700 units/g), and hemicellulase from *Aspergillus niger* (powder, 0.3-3.0 unit/mg solid) were purchased from Sigma Aldrich (Ireland), and were of analytical grade.

5.2.1 Pretreatment of BSG

5.2.1.1 Experimental design

For experimental design and optimization of ultrasound pretreatment of BSG, a Central Composite Design (CCD) of Response Surface Methodology (RSM) were employed (Manorach et al., 2015). The effects of four pretreatment variables (biomass loading, temperature, ultrasound power, time) each at five levels (Table 5.1) on total sugar yield from BSG after saccharification were studied; using a CCD with five replicates at the center points, requiring 30 experiments. Experimental runs were randomized in order and carried out in triplicate. These factors were further optimized using RSM. Experimental design was carried out using STATGRAPHICS Centurion XV software (StatPoint Technologies Inc. Warrenton, VA, USA). A total of 30 experiments as given by the experimental design were carried out to investigate the effect of varying conditions of ultrasound (Fisher Scientific TI-H-10 Ultrasonic bath) pretreatment on the enzymic hydrolysis of BSG. After pretreatment, the biomass was
collected, air-dried, and stored for further enzymatic hydrolysis, compositional analysis and characterization.

Table 5.1. Experimental range and levels of the independent variables

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<tr>
<th>Code</th>
<th>Variables</th>
<th>Range and levels</th>
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</thead>
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<td>A</td>
<td>Solids/Liquids ratio (%)</td>
<td>-2 10 15 20 25</td>
</tr>
<tr>
<td>B</td>
<td>Temperature (°C)</td>
<td>20 30 40 50 60</td>
</tr>
<tr>
<td>C</td>
<td>Ultrasound Power (%)</td>
<td>20 40 60 80 100</td>
</tr>
<tr>
<td>D</td>
<td>Time (min)</td>
<td>20 30 40 50 60</td>
</tr>
</tbody>
</table>

5.2.1.2 Model development and optimization

After conducting the experiments, a second-order polynomial regression model, as given by Eq. (1), was generated and analysed by the statistical software STATGRAPHICS Centurion XV software (StatPoint Technologies Inc. Warrenton, VA, USA) to define the response in terms of the independent variables.

\[ Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \]  
(1)

Where \( Y, \beta_0, \beta_i, \beta_j, \beta_{ij}, X \) represent process response (total sugar yield), linear coefficients, quadratic coefficients, interaction coefficients and coded independent variables (biomass loading, temperature, ultrasound power, and time), respectively. The regressors (\( \beta_0, \beta_i, \beta_j, \) and \( \beta_{ij} \)) provide a quantitative measure of the significance of linear effects, quadratic of factors and interactions between factors. The significant differences between each pretreatment with respect to the components of BSG were analyzed by performing analysis of variance (ANOVA) and multiple comparisons (Fischer’s least significant difference test); Values of \( p < 0.05 \) were considered as significant.
5.2.2 Enzymatic hydrolysis

The enzymatic hydrolysis of BSG was performed according to the procedure mentioned in section 3.1.4.3.

5.2.3 Characterization of raw and pre-treated substrate

5.2.3.1 Compositional analysis

Compositional analysis of pretreated and untreated BSG was performed according to the protocol described in section 3.1.4.1.

5.2.3.2 Total reducing sugars

The reducing sugar concentration in the hydrolysate was estimated following the protocol described in section 3.1.4.2.

5.2.3.3 Fourier Transform Infra-Red Spectroscopy analysis

The changes in functional groups caused by pretreating the biomass was assessed using FTIR spectroscopy according to section 3.1.4.6.

5.2.3.4 Scanning electron microscopy

The morphological structure of BSG before and after pretreatment was observed by performing field emission-scanning electron microscopy FE-SEM (Hitachi SU-70). Dried samples of the untreated and pretreated BSG were subjected to FE-SEM at an electron beam energy of 0.5 keV (Raghavi et al., 2016).

5.2.3.5 Thermal behavior

The thermal behavior of BSG before and after pretreatment was studied and compared using differential scanning calorimetry (DSC). The thermal analysis instrument (Shimadzu DSC-60) was controlled by TA-60WS software. For carrying out thermal
analysis, 55 mg of each BSG sample was placed in an aluminium pan, and an empty pan was used as a reference. The measurements were carried out between 25°C and 500°C, with a linear increase of 10°C per min (Ballesteros et al., 2014).

5.3 Results and discussion

5.3.1 Effect of pretreatment on composition of BSG

5.3.1.1 Composition of BSG

Polysaccharides represented as much as 53% of the dry weight of BSG. BSG contained xylan (38%), glucan (15%), and lignin (10%). This is largely in agreement with the values previously reported in the literature for this biomass type (Lynch et al., 2016). The hemicellulose and cellulose content of BSG as described in the literature varies between 19-40 % and 9-25% per dry matter, respectively (Xiros et al., 2008). The chemical composition of BSG is known to vary with barley cultivars, cultivation conditions, harvest time, malting practices, mashing regime, the presence of hops or adjuncts, and brewing conditions associated with lager and ale fermentations (Mussatto, 2014).

5.3.1.2 Modelling and optimization of ultrasound pretreatment

The total sugar yield from enzymatic hydrolysis was used in Response Surface Methodology to optimize the ultrasound-assisted pretreatment. The model adequacy was confirmed based on the coefficient of determination (R²) and adjusted coefficient of determination (R²-adj). Precision range for R² is from 0 to 1.0, considering that a value closer to 1.0 means that the model is more accurate. A value of 98.28 % R² was observed in the present work, while R²-adj was 96.68 %, illustrating that the model adequately fits the data.
The polynomial equation (2) describing the total sugar yield behaviour is:

\[
\text{Reducing sugar (mg/ml)} = 2.18129 - 0.1184 \times A - 0.009875 \times B + 0.135458 \times C - 0.0658167 \times D - 0.00243 \times A^2 + 0.0003025 \times A \times B + 0.0012275 \times A \times C + 0.002905 \times A \times D - 0.00053875 \times B^2 + 0.00132438 \times B \times C - 0.00117125 \times B \times D + 0.0008125 \times D^2
\]  

(2)

The significance of the coefficients of the model was determined by ANOVA. The ANOVA table (Table 5.2) showed that 12 effects have P-values less than 0.05, indicating that they are significantly different from zero at the 95.0% confidence level, and implying a considerable effect of these coefficients on reducing sugar yield. The predicted levels of sugar yield in pretreated BSG using the equation (2) are given in Table 5.3, along with experimental data.

Table 5.2. ANOVA table for the quadratic model of ultrasound pretreatment

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<th>Source</th>
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<th>Mean square</th>
<th>F-Ratio</th>
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### Table 5.3. Experimental conditions and results of central composite design

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Figure 5.1: Response surface plots representing the effect of independent variables on reducing sugar yield: (a) Effect of solid-to-liquid ratio and temperature on reducing sugar yield when the response surface is fixed at ultrasound power = 60% and time = 40 min; (b) Effect of solid-to-liquid ratio and ultrasound power on reducing sugar yield when the response surface is fixed at temperature = 40°C and time = 40 min; (c) Effect of solid-to-liquid ratio and time on reducing sugar yield when the response surface is fixed at temperature = 40°C and ultrasound power = 60%; (d) Effect of temperature and ultrasound power on reducing sugar yield when the response surface is fixed at Solid-to-liquid ratio = 15% (w/v) and time = 40 min; (e) Effect of temperature and time on reducing sugar yield when the response surface is fixed at solid-to-liquid ratio = 15% (w/v) and ultrasound power = 60%; and (f) Effect of ultrasound power and time on reducing sugar yield when the response surface is fixed at solid-to-liquid ratio = 15% (w/v) and temperature = 40°C.
Additionally, response surface plots were generated as a function of two factors at a time in order to understand the main and interaction effects of different variables, and to determine the optimal level of each variable for maximum response (Figure 5.1). A maximum reducing yield was observed with low ultrasound power (20%) and temperature (26°C); as well as high biomass loading (17g/100 mL), and long pretreatment time (60 min). Under optimum conditions, the model predicted the maximum sugar yield to be 388 mg/mL of reaction volume.

After the optimized pretreatment of the native BSG, and following saccharification, 74% of sugars in BSG were recovered, while no degradation of lignin was observed (remaining as 10.6 g per 100 g of raw BSG). Improvement in saccharification of BSG without lignin degradation may be attributed to the type of solvent used in this pretreatment step (water, with no chemicals), and the mechano-acoustic (physical) effects of ultrasound that increase surface erosion and pore size, and therefore the accessibility of biomass to hydrolytic enzymes (Bussemaker and Zhang, 2013). Obtaining high saccharification yields from lignocellulose, while maintaining a high amount of lignin after the pretreatment available for further valorization, can maximize the utilization of lignocellulose.

For the validation of the model, a confirmation experiment was conducted using the optimized parameters, and the experimentally obtained values of total reducing sugar amounted to 325 ± 1.0 mg/mL. Thus, since the experimentally obtained values of total reducing sugar from native BSG amounted to 151 ± 0.6 mg/mL, this model provided a 2.1-fold higher reducing sugar yield.

Ultrasonic frequency has a significant effect on ultrasound pretreatment, due to its influence on the critical size of the cavitation bubble. Lower frequency ultrasound (20–
100 kHz) can produce more violent cavitation, resulting in higher localized temperatures and pressures at the cavitation site, as well as more effective shock waves (Hilares et al., 2018); water molecules dissociate into oxidative radicals that oxidize and degrade organic molecules. Therefore, low frequency ultrasound is commonly used in biomass processing for intense physical effects, such as cell disruption (Tang and Sivakumar, 2015).

Using similar low frequency ultrasound for pretreatment of biomass was reported by other researchers (Rehman et al., 2014) who used ultrasonication at an operating frequency of 20 kHz (power 750 W) and employing 20 % of amplitude for enhancing sulfuric acid pretreatment of rice straw. Moreover, the results were consistent with reported data in the literature (Subhedar and Gogate, 2013) for newspaper pretreated with ultrasound (at frequency of 20 KHz) combined with alkali (NaOH) solution (concentration of 1 N). Eblaghi et al. (2016) also employed ultrasonic irradiation at a frequency of 35 kHz combined with alkaline solution (3% NaOH concentration) for pretreatment of sugarcane bagasse.

The ultrasonic treatment is also known to be influenced by solvent properties (Bussemaker and Zhang, 2013): water can produce more favorable conditions for cavitation than solvents with higher viscosity, surface tension or density. According to the literature, increases in biomass loading, temperature, sonication time, or the US power applied to the reaction mixture results in an increased rate of the reaction only up to a certain point; further increases in these parameters do not result in greater disruption effects (Bussemaker and Zhang, 2013; Karimi et al., 2014).
5.3.1.3 FTIR analysis

The chemical changes in the functional groups of the BSG were studied based on FTIR analysis (Figure 5.2). The pretreated BSG displayed significant decreases in band intensities at characteristic peaks for cellulose (3290 Cm\(^{-1}\)), hemicellulose (1030 Cm\(^{-1}\)) and lignin (1240 Cm\(^{-1}\)) (Table 5.4). Similar reduction in intensity of peaks representing functional groups of BSG after ultrasound-assisted pretreatment of lignocellulose were reported by Ravindran et al. (2017). These observations indicate that ultrasound treatment disturbed the lignocellulosic structure of BSG, increasing the yield of the reducing sugar.

![FTIR spectra of native and pretreated BSG.](image)

**Figure 5.2:** FTIR spectra of native and pretreated BSG.
Table 5.4. FTIR peak assignments for lignocellulose

<table>
<thead>
<tr>
<th>Wavenumber (Cm⁻¹)</th>
<th>Assignment</th>
<th>References</th>
</tr>
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<td>1030</td>
<td>C-O stretching vibration of hemicelluloses</td>
<td>(Peng et al., 2015)</td>
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<td>1160</td>
<td>C-O stretching vibration of acetyl xylan</td>
<td>(Peng et al., 2015)</td>
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<td>1240</td>
<td>C−O stretching of syringyl lignin</td>
<td>(Kalia, 2018)</td>
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<td>1380</td>
<td>phenolic OH and aliphatic C−H in methyl groups</td>
<td>(Coletti et al., 2013)</td>
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<td>C−H bending vibration of chitosan</td>
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<td>vibration of C=O</td>
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</tr>
<tr>
<td>2920</td>
<td>stretching of C=O</td>
<td>(Kalia, 2018)</td>
</tr>
<tr>
<td>3290</td>
<td>vibration of OH group of cellulose</td>
<td>(Jawaid et al., 2017)</td>
</tr>
</tbody>
</table>

5.3.1.3 Scanning electron microscopy

SEM analysis of the raw (control) and pretreated BSG revealed surface modifications in response to pretreatment (Figure 5.3). The untreated BSG had an undulated and crumbled surface as a result of the brewing process. The ultrasound pretreated BSG showed a porous surface of uneven and non-uniform cavities. This may be due to the modifications in the external fibers arising from the effect of the cavitation due to ultrasound waves (Ravindran et al., 2017). Gabhane et al. (2014) compared alkali-microwave and alkali-sonication pretreatment of lignocellulosic biomass and reported that ultrasound pretreatment resulted only in fibrillation of the cell wall of the pretreated biomass; however, microwave pretreatment led to complete tissue collapse.
Ultrasound pretreatment increased the total surface area of BSG that was exposed to enzyme activity and enhanced the enzyme accessibility.

Figure 5.3: Micrographs by scanning electron microscopy (SEM) of native (on Left), and the modified structure of the pretreated BSG (on Right). Magnification, 1000-fold.

5.3.1.3 Differential scanning calorimetry

Differential scanning calorimetry determines the difference in the heat flow associated with heating, cooling, or isothermal conditions of the sample, compared with a reference, and as a function of temperature. The DSC thermogram (Figure 5.4) represented the thermal characteristics of the native and the pretreated BSG between 20°C and 500°C, which were obtained at a heating rate of 10°C/min. Both native and pretreated BSG exhibited a similar trend in their thermo-gram profile, suggesting that they were approximately similar in their composition. An exothermic event was evident between a temperature range of 20–300°C. This temperature range was associated with several processes which gave rise to compounds such as carbon monoxide, carbon dioxide and other pyrolysis products. In addition, an endothermic event was observed between a temperature range of 300–500°C. Both native and
pretreated BSG exhibited a thermal behaviour that included a crystallization peak at the temperature of 370°C.

![DSC thermogram of native and pretreated BSG.](image)

**Figure 5.4: DSC thermogram of native and pretreated BSG.**

### 5.4 Conclusion

The optimal conditions for ultrasound-assisted pretreatment of BSG were found to be 20% US power, 60 min, 26.3°C, and 17.3% w/v of biomass in water. BSG pretreatment under the optimal ultrasonication conditions resulted in a 2.1-fold higher reducing sugar yield, relative to native BSG. These results suggest that performing a design of experiments (DOE) to optimise ultrasound pretreatment of BSG achieved higher fermentable sugar yield (2.1-fold, relative to native BSG) as compared to the one-factor-at-a-time (OFAT) approach used in previous chapter for optimisation of ultrasound pretreatment that achieved 1.6-fold increase in fermentable sugar yield, relative to native BSG.
However, the same long pretreatment time (60 min.) is required in both of the design of experiments (DOE) or the one-factor-at-a-time (OFAT) approach to achieve maximum fermentable sugar yield from BSG using ultrasound pretreatment. Moreover, exposure to long times of ultrasound pretreatment may cause adverse effects due to collision and aggregation between the particles, and also increase energy consumption during the process. Therefore, considering the longer required pretreatment time of ultrasound pretreatment, as compared to microwave pretreatment, the latter method was selected as an energy efficient and effective pretreatment step for BSG for all subsequent fermentation studies.
Xylanases (E. C. 3.2.1.8, 1, 4-β-xylanylanohydrolase) are enzymes that break down xylan, while pectinases are a class of enzymes that break down pectin. Both xylan and pectin are integral parts of the plant cell wall. Thus, pectinases and xylanases are used in fruit juice and other numerous biotechnological processes. Several studies have shown a rejuvenated interest in solid state fermentation for the production of these microbial enzymes. Many of the attributes of agro-industrial residues are especially suited to this form of culture and can yield significant benefits in terms of sustainability. The latter include environmental friendliness and significant reduction in production costs.
Chapter 6

Pectinase and xylanase production by fungi isolated from spoiled fruit

In this chapter, the utility of brewer’s spent grain (BSG) as substrate for production of pectinase and xylanase using fungi was investigated. Fungi were isolated from nineteen (19) spoiled fruits belonging to different varieties and screened for production of Xylanopectinolytic enzymes. Out of twenty-nine (29) isolates recovered, Mucor hiemalis isolated from Bramley apple (Malus domestica) produced xylanopectinolytic enzymes with higher specific activity and were selected for production studies. The highest enzyme activity (137 U/g, and 67 U/g BSG, for pectinase and xylanase, respectively) was achieved in a medium that contained 15 g of BSG, at pH 6.0, temperature of 30°C, and supplemented with 1.0 % xylan or pectin for inducing the production of xylanase or pectinase, respectively. The partially purified and concentrated pectinase and xylanase were optimally active at 60°C and pH 5.0 (1602 U/ml of pectinase, and 839 U/ml of xylanase, respectively). Thus, the present study suggests that BSG is an effective substrate for production of microbial enzymes such as pectinase and xylanase.

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

https://doi.org/10.1016/j.biteb.2019.100371
6.1 Introduction

Although the large majority of industries still rely on chemical catalysts, there is an increasing demand for biocatalysts since their introduction in the 1960's. The global market for biocatalysts increased from approximately $2 billion dollars in 2004 (Joseph et al., 2008) to $3.3 billion dollars in 2010 (Sarrouh et al., 2012), and increasing to 7 billion dollars by 2017: it is expected to reach $10.5 billion dollars by 2024 (Editorial, 2018).

Xylanases (E. C. 3.2.1.8, 1, 4-β-xylanxylanohydrolase) are enzymes that break down xylan, which is the second most abundant polysaccharide that constitutes an integral part of plant cell wall. While xylanases are usually used in pulp and paper industries for bleaching of cellulose pulp as an alternative to chlorine treatment (Walia et al., 2017), xylanases have also found other wide ranging commercial applications in areas such as animal feed, bakery processing, food and drinks, and lignocellulose degradation (Malhotra and Chapadgaonkar, 2018). Many countries including Canada, Denmark, Finland, Germany, Japan, Ireland and USA are involved in the industrial production of commercial xylanases (Polizeli et al., 2005).

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 are used in the fruit juice industry and wine making for clarification and removal of turbidity in the finished product. While pectinases are usually used in fruit and vegetable processing for treatment of pulp to produce juices with higher yield, low viscosity and high clarity (Verma et al., 2018), xylanases have found other uses in animal feed, tea and coffee processing, extraction of vegetable oil, and lignocellulose degradation (Garg et al., 2016).
Commercial applications require cheaper enzymes. Interestingly, waste materials from a wide range of agro-industrial processes may be used as substrates for microbial growth, and subsequently the production of a range of high value products, including enzymes of commercial interest (such as pectinases and xylanases). Such an approach may significantly reduce the cost of enzyme production. In addition, utilization of these agro-residues in bioprocesses has the dual advantage of providing alternative substrates, as well as solving their disposal problems.

Brewers’ spent grain (BSG) is the main by-product of the brewing industry and represents an abundant agro-industrial waste. Many cereal brans with similar chemical composition and structure to BSG have been used for the production of commercial enzymes in solid-state-fermentation. This encouraged the investigation of BSG as an alternative substrate for production of enzymes (Anal, 2017).

Although researchers investigated the utilisation of BSG for production of xylanase, this work represents the first study on utilisation of BSG for pectinase production. BSG has been used for production of xylanases by fungi such as F. oxysporum (Xiros and Christakopoulos, 2009), Penicillium glabrum (Knob et al., 2013), Penicillium janczewskii (Terrasan and Carmona, 2015), yeast such as Moesziomyces spp. (Faria et al., 2019) and bacteria such as: Anoxybacillus sp. (Alves et al., 2016), B. cereus (Łaba et al., 2017), B. subtilis (Moteshafi et al., 2016), and Bacillus amyloliquefaciens XR44A (Amore et al., 2015).

Fungi represent the main producers for most of the industrial lignocellulose-degrading enzymes, and screening in the present study focused exclusively on this group. Therefore, this chapter aims to investigate the effectiveness of pretreated BSG as a growth substrate in the production of xylanpectinolytic enzymes by fungi.
6.2 Methodology

Brewer’s spent grain was generously donated by a brewery in Dublin. The BSG was dried at 60°C for 48h and thereafter ground and sorted 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, Ireland. Cellulase activity was assayed by following laboratory analytical procedures for the measurement of cellulase activity devised by National Renewable Energy Laboratory (Adney and 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 Pretreatment of substrate (BSG)

Microwave-assisted pretreatment of BSG was performed according to the optimum conditions described in section 3.2.1.1.

6.2.2 Isolation and screening of xylanpectinolytic enzyme-producing fungi

6.2.2.1 Isolation of fruit-rotting fungi

Samples of nineteen different spoiled fruits (Pome, Stone, and Soft fruits) were randomly collected in January and February 2019 from a commercial pack-house facility in Dublin, Ireland. The type of fruits collected was dependent upon availability; fruits (and varieties) namely: apple (Bramley, and Gala), blackberry (Tupi), blueberry (Emerald, and Duke), grape (Crimson, Melody, Tawny, Sugraone, and Sugarthirteen,
Starlight), raspberry (Kweli, Imara, and Framboises), strawberry (Calinda, Rociera, Sensation, and Marquis), and cherry (Regina). The fruits were collected in separate sterile polythene bags and transferred to the laboratory for the study. Suspected fungal growth visible on the fruits was aseptically transferred to potato dextrose agar (PDA) plates and incubated at 25°C until fungal growth developed on the medium surface. Isolates were serially sub-cultured until pure and transferred to PDA slants for storage at 5°C. Isolates were identified to genus level based on macroscopic appearance and microscopic characteristics under x40 objective lens of a light microscope (after staining with a lactophenol cotton blue dye), and compared with a standard mycological atlas (Watanabe, 2010).

6.2.2.2 Molecular Identification
The best xylan and pectin degrader among all the fungi was identified to the species level by molecular techniques according to the procedure mentioned in section 3.1.5.

6.2.2.2 Qualitative screening of xylanopectinolytic enzyme-producing fungi
The presence of xylanases and pectinases was detected as described earlier (Félix et al., 2016) using differential, substrate-containing media. Briefly, the various substrates [0.5% (w/v) xylan, and 0.5% (w/v) pectin, respectively] were independently added to a medium containing 0.5% (w/v) malt extract and 1.5% (w/v) agar. Each isolate was inoculated individually on enzyme assay plates and incubated at 25°C for 7 days. The activities were detected by the formation of a halo around the mycelium after flooding the plates with Congo red (1.0 mg/ml) for 15 min, discarding the solution, and washing cultures with 1.0 M NaCl. The appearance of hydrolysis zones (halo) around the fungal growth was observed against the red background and considered as positive result. All
fungal isolates that showed a positive result were further assessed for their enzymatic activities via a quantitative assay.

**6.2.2.3 Quantitative determination of xylanopectinolytic enzymes produced by fungi**

Following qualitative screening, the fungal isolates were further screened for yield potential by quantitative determination in submerged fermentation using basal liquid media (Mandels and Weber, 1969) supplemented with 1.0% (w/v) of either xylan or pectin for determination of xylanase and pectinase, respectively (Deep et al., 2014). For each experiment, 50ml of basal liquid media was poured into a 250ml Erlenmeyer conical flask and then was autoclaved at 15 psi and 121°C for 20 minutes. After cooling, each flask was inoculated with a PDA agar plug of growth (0.5 cm) which had previously been cultivated for 7-days. Flasks were incubated in a rotary incubator (150 rpm) at 30°C for seven days. After incubation, the supernatants were collected separately by filtration using Whatman filter paper (No.1) followed by centrifugation at 10,000 rpm at 4°C for 10 minutes. The supernatants were used for protein estimation and enzyme activity assay as crude enzyme.

**6.2.3 Production of xylanopectinolytic enzymes using BSG under different conditions**

The best xylan and pectin degrader among all the fungi tested was selected for solid-state fermentation (SSF) using BSG as a fermentation medium. Pretreated BSG was used as the main carbon source (5.0 g), moisturized (77.5%) with basal medium (Mandels and Weber, 1969) as described by Gautam et al. (2018) and supplemented with 1.0% xylan or pectin for inducing the production of xylanase or pectinase, respectively. The approach of “one-factor-at-one-time” was employed for
optimization of different cultural parameters for enzyme production, where during each run a single factor was varied and other factors were kept at a constant level. The factors studied were BSG concentration (5.0, 10, 15, 20 and 25 % w/v), pH (4.0, 5.0, 6.0, 7.0 and 8.0), and temperature (20, 25, 30 and 37 °C). The optimized culture conditions derived from these experiments were then used for production of enzyme.

Following media sterilization by autoclaving at 121 °C for 30 min, five 7-mm-diameter agar plugs of actively growing fungi were used as inoculum. Flasks were incubated at 30 °C (except for temperature studies) for 7 days, and then 50 ml of distilled water was added to each flask. Substrate in flasks was crushed with a glass rod, and then shaking was applied at 150 rpm for 60 min in an incubator shaker. Following shaking, the flasks were harvested by squeezing the BSG, and the supernatant was further centrifuged at 10,000 rpm at 5.0°C for 15 minutes. The cell-free supernatants were treated as crude enzyme and were used for protein estimation and enzyme activity assay.

6.2.3.1 Partial purification and concentration of pectinases and xylanases
Crude enzymes were centrifuged at 12,000 g for 15 min. The resulting cell-free supernatants were fractionated by ammonium sulfate between 50–80% saturation to find the optimum saturation. Extracts were further purified by centrifugal ultrafiltration using a 10-kDa MW cut-off membrane.

6.2.3.1 Determination of pH and temperature profiles
In order to determine the optimal pH for enzyme activity, the xylanase and pectinase were studied over a pH range of 2.0-11.0 at 50 °C with 1.0% xylan and 1.0% pectin,
respectively. To determine the optimal temperature for activity, the enzymes were tested at different temperatures between 20 and 80 °C (More et al., 2011), and at the optimum pH of each enzyme.

6.2.4 Protein and enzyme assays

6.2.4.1 Assay of Total Protein

Protein concentration was estimated following the protocol described in section 3.1.4.4.

6.2.4.2 Assay of enzyme activity

Enzyme activity was estimated following the protocol described in section 3.1.4.5.

6.3 Results and discussion

6.3.1 Isolation and screening of xylanopeptinolytic enzyme-producing fungi

6.3.1.1 Isolation xylanopeptinolytic enzyme-producing fungi

Twenty-nine fungal isolates were obtained from spoiled-fruit samples of 19 different varieties (Table 6.1). The isolates were distributed in three genera, namely: Mucor spp. (19 isolates), Pencillium spp. (6 isolates), and Aspergillus spp. (4 isolates). All isolates were selected for subsequent pectinase and xylanase production screening.

The low pH of fruit gives fungi a competitive advantage over bacteria. Moreover, fungi can spread on commodities in cold storage (Moss, 2008). Some fungi have the ability to produce pectinolytic enzymes to soften the plant tissue causing rot, and others infect through wounds caused during handling and packing of the fruit. Aspergillus spp., Pencillium spp., and Mucor spp. are among the commonly encountered species causing spoilage of fruit (Moss, 2008).
Table 6.1: List of fruit-spoilage fungi isolated from infected fruits

<table>
<thead>
<tr>
<th>Code</th>
<th>Fungal Isolate</th>
<th>Fruit</th>
<th>Fruit Variety</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB1</td>
<td>Mucor sp.</td>
<td>Apple</td>
<td>Bramely</td>
</tr>
<tr>
<td>AB2</td>
<td>Mucor sp.</td>
<td>Apple</td>
<td>Bramely</td>
</tr>
<tr>
<td>AG1</td>
<td>Penicillium sp.</td>
<td>Apple</td>
<td>Gala</td>
</tr>
<tr>
<td>GC1</td>
<td>Mucor sp.</td>
<td>Grape</td>
<td>Crimson</td>
</tr>
<tr>
<td>GM1</td>
<td>Aspergillus sp.</td>
<td>Grape</td>
<td>Melody</td>
</tr>
<tr>
<td>GM2</td>
<td>Mucor sp.</td>
<td>Grape</td>
<td>Melody</td>
</tr>
<tr>
<td>GM3</td>
<td>Penicillium sp.</td>
<td>Grape</td>
<td>Melody</td>
</tr>
<tr>
<td>GT1</td>
<td>Aspergillus sp.</td>
<td>Grape</td>
<td>Tawny</td>
</tr>
<tr>
<td>GS01</td>
<td>Mucor sp.</td>
<td>Grape</td>
<td>Sugraone</td>
</tr>
<tr>
<td>GS1</td>
<td>Penicillium sp.</td>
<td>Grape</td>
<td>Starlight</td>
</tr>
<tr>
<td>GS2</td>
<td>Penicillium sp.</td>
<td>Grape</td>
<td>Starlight</td>
</tr>
<tr>
<td>GS3</td>
<td>Mucor sp.</td>
<td>Grape</td>
<td>Starlight</td>
</tr>
<tr>
<td>GST1</td>
<td>Aspergillus sp.</td>
<td>Grape</td>
<td>Sugarthirteen</td>
</tr>
<tr>
<td>BT1</td>
<td>Mucor sp.</td>
<td>Blackberry</td>
<td>Tupi</td>
</tr>
<tr>
<td>BE1</td>
<td>Mucor sp.</td>
<td>Blueberry</td>
<td>Emerald</td>
</tr>
<tr>
<td>BD1</td>
<td>Mucor sp.</td>
<td>Blueberry</td>
<td>Duke</td>
</tr>
<tr>
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<td>Mucor sp.</td>
<td>Blueberry</td>
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</tr>
<tr>
<td>RI1</td>
<td>Penicillium sp.</td>
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<td>Imara</td>
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<td>Raspberry</td>
<td>Kweli</td>
</tr>
<tr>
<td>RF1</td>
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<tr>
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<td>Calinda</td>
</tr>
<tr>
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<td>Mucor sp.</td>
<td>Strawberry</td>
<td>Rociera</td>
</tr>
<tr>
<td>SR2</td>
<td>Aspergillus sp.</td>
<td>Strawberry</td>
<td>Rociera</td>
</tr>
<tr>
<td>SS1</td>
<td>Mucor sp.</td>
<td>Strawberry</td>
<td>Sensation</td>
</tr>
<tr>
<td>SM1</td>
<td>Mucor sp.</td>
<td>Strawberry</td>
<td>Marquis</td>
</tr>
<tr>
<td>CR1</td>
<td>Mucor sp.</td>
<td>Cherry</td>
<td>Regina</td>
</tr>
</tbody>
</table>
6.3.1.2 Screening of xylanopectinolytic enzyme-producing fungi

In the current study, three candidates were obtained from qualitative screening as pectinase producers (Mucor sp. AB1, Penicillium sp. GS1 and GS2) and three candidates as xylanase producers (Penicillium sp. GS1, Mucor sp. AB1 and RI2.). Among these, Mucor sp. coded AB1 displayed the highest extracellular pectinase and xylanase activity after the quantitative determinations (Table 6.2) and was, therefore, retained for all subsequent studies. In a previous study, Mucor sp. has been reported as a higher polygalacturonase producer than Aspergillus sp. (Thakur and Gupta, 2012) when authors compared the activity of polygalacturonase produced by Aspergillus sp., Byssochlamys sp., and Mucor sp. However, the literature is dominated by studies that employ Aspergillus sp. for production of xylanopectolytic enzymes (Ravindran et al., 2018a).

Table 6.2: Secondary evaluation of xylanases and pectinases production by fruit-spoilage fungi

<table>
<thead>
<tr>
<th>Code</th>
<th>Fungal Isolate</th>
<th>Xylan-supplemented medium</th>
<th>Pectin-supplemented medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total Cell-</td>
<td>Xylanase activity (u/ml)</td>
</tr>
<tr>
<td>AB1</td>
<td>Mucor sp.</td>
<td>28.1 ± 0.7</td>
<td>9.8 ± 0.2</td>
</tr>
<tr>
<td>GS1</td>
<td>Penicillium sp.</td>
<td>23.3 ± 0.4</td>
<td>7.4 ± 0.1</td>
</tr>
<tr>
<td>GS2</td>
<td>Penicillium sp.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RI2</td>
<td>Mucor sp.</td>
<td>21.4 ± 0.6</td>
<td>7.2 ± 0.1</td>
</tr>
</tbody>
</table>

There are relatively few publications on production of xylanase and pectinase by fungi that belong to the species of the genus Mucor. Daling et al. (2008) reported in their patent the production of xylanases by Mucor rouxii under aerobic fermentation in media containing xylan as inducer. Behnam et al. (2016) reported the production of xylanases by Mucor indicus (43.1 U/g of dry substrate) and Mucor hiemalis (43.8 U/g of dry substrate) on wheat bran as fermentation medium; these authors found that the
optimum fermentation conditions were a temperature of 40 and 43.4 °C, moisture percent of 49.8 and 54.2 %, and incubation time of 51.3 and 53.2 h for *Mucor indicus*, and *Mucor hiemalis*, respectively.

The pectinolytic enzyme (polygalacturonase) was found to be optimally produced by *Mucor circinelloides* ITCC 6025 after 48 h of fermentation at 30°C and pH 4.0 with pectin methyl ester (1% w/v) as carbon source (Thakur et al., 2010). Moreover, Sharma et al. (2013) reported the production of polygalacturonase by the same isolate of *Mucor circinelloides* ITCC 6025 on medium containing pectin (1% w/v) as sole carbon source after fermentation for 48 h at 30°C and pH 4.5.

### 6.3.1.3 Molecular Identification of *Mucor sp.* AB1

*Mucor sp.* AB1 isolate used in the current study was identified to the species level by analyzing the sequences of the ITS region of ribosomal DNA (ITS1-5.8S-ITS2) and pairwise sequence alignment using the BLAST algorithm. The resultant nucleotide sequence in this study (Table 6.3) matched 100% with the published sequence of *Mucor hiemalis* (Accession No. JQ912672.1) available in GenBank.

#### Table 6.3. Nucleotide sequences of *Mucor hiemalis*

<table>
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<tr>
<th></th>
<th>TAGAGGAAGT</th>
<th>AAAAGTCGTA</th>
<th>ACAAGGTTC</th>
<th>CGTAGGTGA</th>
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<td>70</td>
<td>GTTTAAATT</td>
<td>TCAAGGTTTG</td>
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<td>AGCATAT</td>
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</tr>
</tbody>
</table>
6.3.2 Production of Xylanopectinolytic Enzymes using BSG

6.3.2.1 Optimization of production

Microwave pretreated BSG at 600 w for 90 s was used for solid-state fermentation by
*Mucor hiemalis*. Various process parameters influencing pectinases and xylanases
production viz., substrate loading (5, 10, 15, 20, and 25 g), incubation temperature (20,
25, 30, and 37°), and pH (4, 5, 6, 7, and 8) were studied.

Figure 6.1 shows the effect of substrate loading, incubation temperature, and pH on
pectinase and xylanase production by *Mucor hiemalis*. Enzyme activities of 137 U/g,
and 67 U/g BSG, for pectinase and xylanase, respectively were achieved in medium
that contained 15 g of BSG, at pH 6.0, and at a temperature of 30°C.

Temperature and pH play an important role in the synthesis of microbial enzymes. The
optimum temperature for production of pectinase and xylanase by *Mucor hiemalis* was
30° C. Temperature beyond 30°C led to a decrease in enzyme specific activity. Most
fungi are mesophiles with optimum growth temperatures between 25 and 30°C (Dix
and Webster, 1995).

The same optimum temperature was reported by Thakur et al. (2010) for production
of polygalacturonase by *Mucor circinelloides* ITCC 6025 and was also reported by
Ahmad et al. (2009) for production of Xylanase by *Aspergillus niger*. Similar
temperatures were reported for optimal production of xylanase, ranging from 28° C
by *Aspergillus niger* (Bhushan et al., 2012) to 32.5° C by *Fusarium sp*. BVKT R2
(Ramanjaneyulu and Reddy, 2016).
Furthermore, increasing pH from 4.0 to 6.0 increased the production of pectinase and xylanase. However, enzyme specific activity then decreased until pH 8.0. This can be attributed to the fact that most fungi grow optimally at a low pH between 5.0 and 6.0. In earlier studies, Bhushan et al. (2012) and Anthony et al. (2003) reported similar
results while, according to Ramanjaneyulu and Reddy (2016) and Terrone et al. (2018) a pH value of 5.0 was an optimum.

The optimum biomass loading for production of pectinases and xylanases by *Mucor hiemalis* was 15 g per 250-ml conical flask, with any further increases suppressing enzyme activity. In addition to the availability of more nutrients, more inducers are available to the fungus at the optimum substrate concentration that in turn enhance the production of enzymes (Ahmed et al., 2016). In an earlier study, Kavya and Padmavathi (2009) found that maintaining the substrate concentration (wheat bran) at 10g/250 ml conical flask yielded the maximum production of xylanase by *Aspergillus niger*. However, the optimum substrate concentration for enzyme production varies widely with varying substrate type, and the water absorbed onto the substrate surface (Pandey, 1992).

### 6.3.2.2 Partial purification and concentration

Following removal of biomass by centrifugation, the pectinase and xylanase produced by *Mucor hiemalis* were purified by ammonium sulphate precipitation. An ammonium sulphate fraction at 80% saturation resulted in a 95% and 76% yield for pectinase and xylanase, respectively. The ammonium sulfate-enriched fraction of pectinase and xylanase were further concentrated to 14-fold and 12-fold respectively by ultrafiltration (Table 6.4).
Table 6.4. Summary of purification of pectinases and xylanases

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Protein (mg)</th>
<th>Total Activity (U)</th>
<th>Specific Activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PE</td>
<td>XY</td>
<td>PE</td>
<td>XY</td>
<td>PE</td>
</tr>
<tr>
<td>Crude</td>
<td>289.51</td>
<td>214.14</td>
<td>2057.72</td>
<td>996.25</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.65</td>
<td>4.65</td>
<td></td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>230.07</td>
<td>136.83</td>
<td>1943.62</td>
<td>760.21</td>
<td>94.46</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8.45</td>
<td>5.56</td>
<td>1.19</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>15.62</td>
<td>12.51</td>
<td>1496.48</td>
<td>687.15</td>
<td>72.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>95.82</td>
<td>54.93</td>
<td>13.48</td>
</tr>
</tbody>
</table>

* Where: PE = Pectinases, and XY = Xylanases.

6.3.2.3 Effect of temperature and pH on enzyme activity

Figure 6.2 depicts the effect of different temperatures and pH on the enzyme activity of the enriched pectinase and xylanase from *Mucor hiemalis*.

![Figure 6.2. Effect of temperature, and pH on pectinase and xylanase activity.](image)

The pectinase activity steadily increased with an increase in the temperature up to 50°C and pH up to 5.0, with no significant increase at 60°C and pH 6.0, before declining at 70°C and pH 7.0 or higher. Contrasting with this, the xylanase activity steadily increased with an increase in the temperature up to 60°C and pH up to 5.0 before
declining at 70°C and pH 6 or higher. This temperature-optima at 60°C is similar to that reported for xylanase obtained from *Penicillium glabrum* (Knob et al., 2013) and *Trichoderma harzianum* (Ahmed et al., 2012), as well as the pectinase obtained from *Aspergillus fumigatus* (Okonji et al., 2019). However, slightly higher optimal temperatures have been reported for other xylanases, such as 70°C for the purified xylanase from *Rhizomucor pusillus* (Robledo et al., 2016) and *Chaetomium thermophilum* (Ahmed et al., 2012). Moreover, slightly lower optimal temperatures have been reported for other pectinases, such as 55°C for the purified polygalacturonase from *Rhizomucor pusillus* (Siddiqui et al., 2012) and 50°C for the purified polygalacturonase from *Aspergillus niger* MCAS2 (Khatri et al., 2015).

These results are in accord with that reported by Gautam et al. (2018) that maximum activity of the xylanase from *Chizophyllum commune* ARC-11 was at pH 5. Similar results have been reported for polygalacturonase from *Rhizomucor pusillus* (Siddiqui et al., 2012), as well as xylanases by *Fusarium* sp. BVKT R2, and *Trichoderma harzianum* (Khatri et al., 2015). However, slightly higher optimal pH values have been reported for other pectinases and xylanases, such as pH 6.0 for pectinase from *Neurospora crassa* (Polizeli et al., 1991), or xylanase from *Chaetomium thermophilum* (Ahmed et al., 2012) and *Rhizomucor pusillus* SOC-4A (Robledo et al., 2016). Moreover, pH values of up to 6.5 have been reported as the optimum pH for xylanase (Terrone et al., 2018), and pectinase (Rasheedha Banu et al., 2010) from *Penicillium chrysogenum*. On the other hand, lower optimal pH has been reported for other xylanases, such as pH 3.0 for the xylanase from *Penicillium glabrum* (Knob et al., 2013).
6.4 Conclusion

When the pretreated BSG was used for xylano-pectolytic enzyme production by *Mucor hiemalis* under optimized conditions (15g biomass/250ml conical flask, pH 6.0, and temperature of 30°C), the enzyme activity was 137 U/g, and 67 U/g BSG, for pectinases and xylanases, respectively. These results suggest that microwave pretreatment is a viable technology for increased valorisation of BSG, and *Mucor hiemalis* was a potential producer of pectinases and xylanases using BSG fermentation. The partially purified and concentrated pectinase and xylanase were optimally active at 60°C and pH 5.0 (1602 U/ml of pectinase, and 839 U/ml of xylanase, respectively). Further work on immobilisation of the produced enzymes is discussed in the following chapter.
Enzymes once suspended in an aqueous reaction medium are almost impossible to retrieve or recycle. 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. The binding to a support material can be temporary or permanent depending upon the chemical bond formed between the enzyme and the support. Covalent binding method basically fixes the enzyme molecule on to the substrate on a permanent basis through strong covalent bonds. This solves the problem of leaching of the enzyme found in adsorption-based biocatalysts when suspended in aqueous media. With the problem leaching solved, covalently immobilised enzymes can be used to great effect in reaction mixtures that are aqueous in nature. Proper immobilisation of an enzyme on to a support is dependent on the properties of the enzyme and carrier material. This section investigates the use of different carriers for enzyme immobilisation.
Chapter 7

Magnetic nanoparticles as carrier for enzymes immobilization

In this chapter, superparamagnetic iron oxide nanoparticles (MNPs) were synthesized via exposure of fungal cell filtrate from Aspergillus flavus to aqueous iron ions. The morphology of MNPs was found to be flakes-like, as confirmed by Field Emission Scanning Electron Microscopy (FESEM), while the average crystallite size was ~16 nm, as determined by X-ray diffraction (XRD). Energy dispersive X-ray (EDX) analysis was performed to confirm the presence of elemental Fe in the sample. Pectinase and xylanase were covalently immobilized on MNPs with efficiencies of ~84% and 77%, respectively. Furthermore, the residual activity of the immobilized enzymes was about 56% for pectinase and 52% for xylanase, after four and three consecutive use cycles, respectively.
7.1 Introduction

Nanoparticles (1–100 nm in size) have gained prominence in the modern era, as they constitute the building blocks for tailored nanocomposites that can be exploited in a wide range of novel applications. In 2017, nanocomposites had a market value amounting to nearly $2 billion (USD) worldwide, and this is projected to increase to over $7 billion (USD) by 2022 at a compounded annual growth rate (CAGR) of 29.5% during the forecast period of 2017 to 2022 (BCC Research, 2019). Driven by global environmental challenges, the industrial revolution in the 21st century is likely to be based on renewable biological resources. In this regard, bio-nanotechnology has emerged as a promising field of research that builds on nanotechnology and biotechnology. Interestingly, the 2016 Nobel Prize in chemistry went to Stoddart, Sauvage and Feringa for design and synthesis of nanoscale machines (The Royal Swedish Academy of Sciences, 2016), which exemplifies the high relevance of this field.

Immobilized enzymes are of prime importance for some commercial processes, with the key advantage of biocatalyst recycling often being complemented by higher operational stability, and a lower risk of product contamination with enzyme (Reis et al., 2019). However, depending on the immobilization method employed, such advantages can be offset by additional process costs, up to 25 times that of the free enzyme (Rasor, 2008). Therefore, there is considerable interest in exploring the potential of nanoparticles as a low-cost platform for enzyme immobilization. Of all the nanoparticles studied so far, magnetic nanoparticles (MNPs) are of interest for enzyme immobilization owing to their ease of separation in a reaction and chemical inertness (Bilal et al., 2018).
Moreover, the advent of microbially-produced MNPs has created a paradigm shift in enzyme immobilization technology, providing favorable process economics and offering the additional advantage of mild reaction conditions for their production (Cueva and Horsfall, 2017; Moon et al., 2010). Therefore, microbial production of nanoparticles is rapidly becoming a favoured approach in this field (Verma et al., 2019). In this respect, filamentous fungi have been used chiefly in the synthesis of metal nanoparticles due to their wide array of extracellular enzymes and high metal tolerance (Silva et al., 2016).

A review of the literature in this field indicates a degree of heterogeneity in terms of fungal species being investigated in the biosynthesis of magnetic nanoparticles (e.g. *Fusarium oxysporum* (Bharde et al., 2006), *Fusarium incarnatum* (Mahanty et al., 2019a), *Trichoderma asperellum* (Mahanty et al., 2019a), *Phialemoniopsis ocularis* (Mahanty et al., 2019a), *Verticillium sp.* (Bharde et al., 2006), *Aspergillus niger* (Abdeen et al., 2016; Chatterjee et al., 2020), *Aspergillus oryzae* (Tarafdar and Raliya, 2013), and *Aspergillus japonicus* (Bhargava et al., 2013)). However, to the best knowledge of the authors, no work in this field has yet been reported using *Aspergillus flavus* for synthesis of MNPs. Therefore, the present study investigates the use of *A. flavus* to produce MNPs and explores their application in enzyme immobilization. To date, MNPs have been extensively studied for immobilization of pectinase (Dal Magro et al., 2019; Fang et al., 2016; Kharazmi et al., 2020; Mosafa et al., 2014; Nadar and Rathod, 2018; Sojitra et al., 2017) and xylanase (Amaro-Reyes et al., 2019; Hamzah et al., 2019; Kumar et al., 2018; S. Kumar et al., 2016; Kumari et al., 2018; Landarani-Isfahani et al., 2015; Liu et al., 2014; Mehnati-Najafabadi et al., 2019; Singh et al.,
2018) for easy separation and reuse. Therefore, pectinase and xylanase were used as model enzymes in this study for immobilization on MNPs.

7.2 Methodology

Pectinase (912 U/ml) and xylanase (455 U/ml) were produced from Mucor hiemalis AB1 (GenBank accession number: JQ912672.1) as described in chapter 6. Enzyme activity was determined by the dinitrosalicylic acid (DNS) method of Miller (1959) using pectin (citrus peel) or xylan (beechwood) as standard. Unless stated, all other chemicals including pectin, xylan, ferric chloride, ferrous chloride, glutaraldehyde solution, Sabouraud dextrose broth and agar were purchased from Sigma Aldrich (Ireland), and were of analytical grade.

7.2.1 Isolation of Aspergillus sp. SR2

The fungus, Aspergillus sp. SR2, was isolated from spoiled strawberry (variety: Rociera) as described in chapter 6. In the latter, the isolated strain was preliminarily identified to the genus level based on macroscopic morphology and microscopic features. The isolate was stored at 4°C on SDA slopes (Sabouraud dextrose agar; 40 g/l dextrose, 10 g/l mycological peptone, and 15 g/l agar) for further study.

7.2.2 Molecular Identification of Aspergillus sp. SR2

The molecular identification of the isolate was performed according to the procedure mentioned in section 3.1.5.
7.2.3 Biosynthesis of magnetic nanoparticles

The protocol reported by Mahanty et al. (2019) was followed with minor modifications. Briefly, the fungal isolate was grown in potato dextrose broth (PDB; 250 ml Erlenmeyer flask with a working volume of 20%) with orbital shaking (120 rpm, 25 °C). After 14 days of incubation, the culture fluid was separated from fungal biomass by filtration through Whatman filter paper no. 1, followed by centrifugation at 5,000 rpm for 5 min. The final filtrate (10 ml) was later utilized for the reduction of iron salts in aqueous solution (5 ml) consisting of 2 mM Iron (III) and 1 mM Iron (II) chloride mixture (2:1). The reaction mixture was agitated for 5 minutes at room temperature, and color changes were observed and considered as a positive result for the development of MNPs. Next, the MNPs were collected by centrifugation at 12,000 rpm for 15 min and washed three times with deionized water. Finally, MNPs were obtained after magnetic separation and immediately re-dispersed in deionized water and sonicated (Fisher Scientific TI-H-10 Ultrasonic bath, output 750 W) for 1 hour to separate the MNPs into individual particles. The culture filtrate (without iron salts) and salt solution (without culture filtrate) were considered as positive and negative controls, respectively.

7.2.4 Characterization of MNPs

The absorption spectra of MNPs were measured in the wavelength range from 200 to 800 nm using a UV-1800 UV-VIS spectrophotometer (Shimadzu Scientific Instruments, Columbia, USA). Powder X-Ray diffraction (XRD) analysis was carried out with a Siemens D500 diffractometer (Berlin, Germany) to study the crystal structure of the MNPs. XRD data was analyzed using the Origin Pro 8 software (Trial version, Microcal Software Inc., USA), and the average crystallite size of the MNPs
was estimated using the Scherrer equation (Scherrer, 1918). The morphology (particle shape) of the MNPs were observed using a Hitachi SU 70 (Hitachi High Technologies, Japan) field emission scanning electron microscope (FESEM). The FESEM system was equipped with an X-Max silicon drift detector (Oxford Instruments, UK) for energy dispersive X-ray (EDX) analysis to confirm the presence of iron in the particles, as well as to characterize the other elementary composition of the particles.

7.2.5 Immobilization of biocatalysts on MNPs

A two-step procedure was followed to immobilize pectinase and xylanase on MNPs adopted from previously reported methods, with minor modifications (Ghadi et al., 2015; Kaur et al., 2020). The first step was the activation of the MNPs (50 mg) by suspension in glutaraldehyde solution (1.0 M, 5 ml) for 2 h at 37°C under constant mechanical stirring. The next step involved the covalent binding of the glutaraldehyde-activated MNPs (50 mg, MNPs@GLU) to pectinase or xylanase (1 mL) by incubating the suspension for 24 h at 37°C under constant mechanical stirring. Finally, enzyme-functionalized MNPs (MNPs@GLU-ENZ) were obtained after magnetic separation and washed with deionized water to remove loosely or unbound proteins. The immobilization yield (Kumari et al., 2018), and activity recovery (Zhou et al., 2013) of immobilized pectinase or xylanase, were calculated as follows:

\[
\text{Immobilization yield (\%) } = \left( \frac{C_{\text{init}} - C_{\text{fin}}}{C_{\text{init}}} \right) \times 100
\]

where \( C_{\text{fin}} \) is the amount of protein in the supernatant (enzyme solution) after magnetic separation and \( C_{\text{init}} \) is the total protein available for immobilization, as determined by the Bradford assay (Bradford, 1976).
Activity recovery (%) = \( \frac{A_{\text{immob}}}{A_{\text{init}}} \times 100 \)

where \( A_{\text{immob}} \) is the immobilized enzyme activity and \( A_{\text{init}} \) is the initial (free) enzyme activity as determined by the Miller DNS assay (Miller, 1959).

7.2.6 Evaluation of the nano-immobilized enzymes (MNPs@GLU-ENZ)

The optimum reaction pH of MNPs@GLU-ENZ was determined over the range between 2.0 and 11.0 using glycine-HCL buffer (0.1 M, pH 2.0), citrate buffer (0.1 M, pH 3.0-6.0), phosphate buffer (0.1 M, pH 7-8) and glycine-NaOH buffer (0.1 M, pH 9-11); while the optimum temperature was investigated between 20 °C and 80 °C. To evaluate the storage stability, MNPs@GLU-ENZ were held for 30 days at 4 °C (Xiao et al., 2016). For a reusability assessment, MNPs@GLU-ENZ were recovered with magnetic separation after each cycle of use, washed with deionized water, and then a new cycle was run under the same conditions for a total of 6 cycles (Mosafa et al., 2014). The enzyme activity in the first cycle was assigned a relative activity of 100% (at temperature of 60 °C and pH of 5), and relative activity was calculated for the successive cycles. All experiments were performed in triplicate.

7.3 Results and discussion

7.3.1 Molecular Identification of *Aspergillus* sp. SR2

*Aspergillus* isolate used in the current study was previously isolated in our lab from spoiled strawberry (variety: Rociera). Molecular identification of fungal isolate was carried out by analyzing the sequences of the ITS region of ribosomal DNA (ITS1-5.8S-ITS2) and pairwise sequence alignment using the BLAST algorithm. The resultant nucleotide sequence in this study (Table 7.1) matched 100% with the
published sequence of *Aspergillus flavus* (Accession No. MN238861.1) available in GenBank. The aforementioned taxon is one of the most common fungi that are found on strawberry fruit, as previously reported (Saleem, 2017).

**Table 7.1. Nucleotide sequences of Aspergillus flavus SR2**

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<tr>
<th>1</th>
<th>GAGGAAGTAA</th>
<th>AAGTCGTAAC</th>
<th>AAGGTTTCGG</th>
<th>TAGGTGAACC</th>
<th>TGGCGGAAGGA</th>
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</tr>
</tbody>
</table>

### 7.3.2 Biosynthesis of magnetic nanoparticles

Biosynthesis of MNPs using culture filtrate of *A. flavus* was initiated rapidly after treatment with iron salts, which was visualized by observing the change in color of the reaction mixture from yellow to black (Fig. 7.1), while no visible color change was observed for either positive or negative controls.
Figure 7.1. Shows the culture filtrate of *A. flavus* (A), iron salts solution (B), black coloration due to mixing of solutions A+B for 5 min (C), separation of MNPs from reaction mixture using an external magnet (D), and the magnetic behavior of dried MNPs in the presence of a magnet (E).

Few studies to date have investigated the capability of fungi for the production of magnetic nanoparticles, and most of these studies have focused on *Aspergillus* species, such as *Aspergillus niger* (Abdeen et al., 2016; Chatterjee et al., 2020), *Aspergillus oryzae* (Tarafdar and Raliya, 2013), and *Aspergillus japonicus* (Bhargava et al., 2013). In the aforementioned studies on *Aspergillus* species, different iron salts were utilized, such as potassium ferricyanide and potassium ferrocyanide salts solution (Bhargava et al., 2013), ferrous sulfate and ferric chloride salts solution (Abdeen et al., 2016; Chatterjee et al., 2020), and ferric chloride solution (Tarafdar and Raliya, 2013). Thus, our study brings a novel approach for the biosynthesis of MNPs by *Aspergillus* species where filtrate of *A. flavus* reduced the ferrous chloride and ferric chloride salts solution. According to the available literature, the exact mechanism for the
biosynthesis of metal oxide NPs by fungi has not yet been elucidated. However, the biomineralization process could have occurred via reducing iron metal ions by extracellular redox enzymes from *A. flavus* (Dorcheh and Vahabi, 2016).

### 7.3.3 Characterization of MNPs

The detailed characterization of the mycosynthesized MNPs was performed using UV–vis spectrophotometry, Field Emission Scanning Electron Microscopy (FESEM), Energy dispersive X-ray (EDX) and X-ray diffraction (XRD). The UV-vis absorption spectra (Fig. 7.2) show a characteristic absorption band at 310 nm, confirming the formation of MNPs (Saranya et al., 2017).

![Figure 7.2. UV-vis spectrum of MNPs.](image)

Furthermore, the EDX spectrum and elemental mapping as shown in Fig. 7.3 detected the presence of Fe and O, which indicated the stoichiometric formation of Fe$_3$O$_4$
nanoparticles. The Fe and O element mapping further confirmed that the two constituent elements were well-distributed over the sample. However, the spectrum also revealed small traces of impurities which presumably originated from the iron salts and the *A. flavus* filtrate used for the synthesis of MNPs (Rahman et al., 2017).

Figure 7.3. MNPs observed in EDX spectrum (A), electron micrograph region at 200 µm (B), distribution of Fe in elemental mapping (C), and distribution of O in elemental mapping (D).

The SEM images (Fig. 7.4) of MNPs show clusters of flakes-like morphology. Similar morphology was also observed by Chatterjee et al. (2020), who studied MNPs produced by *Aspergillus niger* BSC-1. Bharde et al. (2006) also reported the biosynthesis of irregular MNPs using *Fusarium oxysporum*. The observed flakes-like
morphology could be due to the binding activity of biomolecules of *A. flavus* on the growing particles of Fe$_3$O$_4$ (Kavitha et al., 2017). Additionally, there is a possibility of improper drying of the MNPs using the vacuum drying technique (GeneVac EZ-2 Plus evaporator, Genevac Ltd, Ipswich, UK). Thus, one possible solution would be to use vacuum freeze-drying of liquid nanoparticle dispersions to produce lyophilized MNP powder which may minimize aggregate formation, that could lead to high or modified particle size distribution. Above all, MNPs tends to agglomerate into larger clusters in order to reduce their surface energy (Campos et al., 2015), in a phenomenon known as Ostwald ripening (Huang et al., 2014). Thus, XRD was used to estimate the average crystallite size of individual (primary) particles.

*Figure 7.4. SEM micrograph shows irregular flakes-like clusters.*

Fig. 7.5 shows the XRD patterns for MNPs with six characteristic peaks for Fe$_3$O$_4$ marked by their indices (220), (311), (400), (422), (511), and (440). The results of phase analysis agree with the reference magnetite Fe$_3$O$_4$ pattern from the Joint Committee on Powder Diffraction Standards (JCPDS card number 19-0629), and published literature (Fatima et al., 2018; Yew et al., 2017). Due to the size of the small crystallites, only one major peak at 35.3° corresponding to the (311) plane was
detected, while other peaks for magnetite were less distinctive (Loh et al., 2008). Therefore, the full width at half maximum (FWHM) of the (311) peak was used to calculate the crystallite size. The average crystallite size of the MNPs was ~16 nm, as estimated by the Scherrer equation. Comparisons with the literature revealed that MNPs derived from fungi were in the size range of 10 to 40 nm (Bhargava et al., 2014; Chatterjee et al., 2020). MNPs smaller than the critical size of 20 nm are known to be superparamagnetic, with a high magnetization value (Namanga et al., 2013). The aforementioned superparamagnetic iron oxide nanoparticles are of industrial interest due to their low toxicity, and good biocompatibility (Xu et al., 2014). Thus, superparamagnetic iron oxide nanoparticles are versatile carriers for realizing enzyme immobilization that facilitates catalyst separation.

![XRD spectrum of MNPs.](image)

**Figure 7.5. XRD spectrum of MNPs.**

### 7.3.4 Immobilization of biocatalysts on MNPs

Direct immobilization of pectinase and xylanase on MNPs is not effective because of surface oxidation and rejection (Masi et al., 2018). Thus, MNPs were subjected to
glutaraldehyde treatment for the activation of MNPs which facilitates the formation of the Schiff base linkage between the aldehyde group of glutaraldehyde and the terminal amino group of enzymes (Costa et al., 2016). Finally, the activated MNPs (MNPs@GLU) were treated with pectinase or xylanase individually to produce enzyme-functionalized MNPs (MNPs@GLU-ENZ). Immobilization yields of pectinase and xylanase functionalized MNPs were about 84% and 77%, respectively. For pectinase, the immobilization yield of this work (84%) was much higher than that (51%) previously reported by Mosafa et al. (2014) using silica-coated magnetite nanoparticles via a sol–gel approach. The use of the latter may result in composite particles with an ill-defined structure due to the formation of aggregates (Chung et al., 2009), and this may affect the enzyme immobilisation yield. Another reason could be that these enzymes are of different origin, and therefore are most likely to be structurally different. It follows that the immobilisation yield would most probably be different. This may also be the reason why a previous study reported a higher yield (92%) of xylanase from Bacillus sp. NG-27 when immobilized on glutaraldehyde activated magnetic nanoparticles (Kumari et al., 2018). It is known that a wide number of factors can affect the immobilization yield using this method, including surface area activation, stirring speed, enzyme load, MNP proportion, and enzyme-MNP coupling time (Costa et al., 2016).

The recovery of enzyme activity remaining in immobilized pectinase and xylanase were about 74% and 68%, respectively. For pectinase, the activity recovery of this work (74%) was much lower than that (92%) previously reported by Nadar and Rathod (2018) using amino-activated magnetite nanoparticles, and the 85% activity recovery reported by Muley et al. (2018) using glutaraldehyde-activated magnetic
nanoparticles. Also, the latter study reported higher activity recovery of xylanase (76%) compared to the activity recovery of xylanase (68%) in the present work. However, Perwez et al. (2017) reported similar xylanase activity (69%) when immobilized on 3-aminopropyl triethoxysilane-activated magnetic nanoparticles. The use of 3-Aminopropyl triethoxysilane (APTES) for particle functionalization prior to glutaraldehyde cross-linking (Muley et al., 2018; Nadar and Rathod, 2018) has previously proved to be a viable approach to achieve improved enzyme immobilisation. However, we tried to minimize the use of toxic compounds to achieve a ‘greener’ immobilisation process, and hence silanization by APTES was avoided. It is worth noting that covalent immobilization may lead to changes in the native structure of the enzymes, causing serious loss of enzymatic activity (Cen et al., 2019). Moreover, improving the covalent immobilization method would not only ultimately increase the activity recovery, but also suppress the enzyme leaching from the surface of MNPs (Rehm et al., 2016).

### 7.3.5 Evaluation of the nano-immobilized enzymes (MNPs@GLU-ENZ)

The effect of temperature on the activity of free and immobilized enzymes was evaluated by assaying the enzyme activity at different temperatures (20 - 80°C) and pH 5.0. The results shown in Fig. 7.6a indicated that immobilization did not change the optimal temperature of pectinase and xylanase (50°C and 60°C, respectively). This agrees with the typical temperatures for optimum activation of commercial pectinase (50 °C) and xylanolytic thermostable enzymes (60 °C). Additionally, the immobilized pectinase and xylanase retained 80% activity over a wider temperature range of 30-80°C and 40-80°C, respectively, compared to the free enzymes (retained more than 80% activity over range of 30-70°C and 50-70°C, respectively). The improved
retained enzyme activity of the immobilized enzymes above the optimum temperature, with no shift in the optimum temperature, might be ascribed to the covalent bonding to MNPs, which in turn prevented the conformational change of enzymes at high temperature (Muley et al., 2018).

Figure 7.6. Panels (A–C) shows the effect of temperature (A), pH (B), storage time (C), and recycle count (D) on relative activity of immobilized pectinase and xylanase in comparison with free enzyme.

The influence of pH on the activity of free and immobilized enzymes was studied by assaying at varying pH values ranging from 2.0 to 11.0 at 50ºC. The results shown in Fig. 7.6b indicated that immobilization did not change the optimal pH (5.0) of pectinase and xylanase. This agrees with the typical pH (5) for optimum activation of
commercial pectinase and xylanase. Moreover, the pH activity scope of the immobilized pectinase was expanded, retaining more than 80% activity over a wider pH range of 4.0–8.0, compared with that of the free form (pH range of 5.0-7.0). One possible reason for this could be the intact conformational structure of enzyme in the polymeric network of nanocarrier (Kharazmi et al., 2020). On the other hand, the optimal pH of immobilized xylanase was slightly shifted toward the neutral range compared to the free form. Immobilized xylanase retained more than 80% activity in a pH range of 4.0–7.0 compared with that of the free form (pH range of 3.0-6.0). The slight shift of pH activity for immobilized xylanase might be attributed to partitioning effects of polyionic matrices (Liu et al., 2014).

Storage stability of the free and immobilized enzymes was evaluated at 5-day intervals by storing them at 6 °C for 30 days. As shown in Fig. 7.6c, the immobilization with magnetic nanoparticles improved the stability of enzymes. The immobilized pectinase and xylanase retained about 56% and 53% of their initial activity after 30 days and 25 days, respectively. This compares with the free pectinase and xylanase, which retained about 43% and 49% of their initial activity after 25 days and 20 days, respectively. These results indicated that the immobilization with magnetic nanoparticles improved conservation and stabilization of the structural conformation of enzymes which preventing possible distortion effects on the active sites of enzyme (Sojitra et al., 2017).

From a process economics perspective, the reusability aspect of immobilized enzymes is a key consideration for industrial applicability. Thus, the useful lifetime of immobilized enzymes in the present work was assessed by subjecting the preparations
to 6 successive process cycles (Fig. 7.6d). After one cycle was over, the immobilized enzymes were removed from the reaction medium with an external magnetic field and reused again to check durability. The residual activity of immobilized enzymes was 56% for pectinase and 52% for xylanase, after four and three consecutive cycles, respectively. The activity loss of immobilized enzyme probably could be due to enzyme denaturation due to recurrent encountering of substrate to the active site of immobilized enzyme after each successive use (Sahu et al., 2016). Besides, the leakage of enzymes from the MNPs, or probably the weak magnetization of MNPs due to the high temperature or being coated by components from the reaction mixture (Mahanty et al., 2019b). In fact, previous studies have shown that the activity of hydrolytic enzymes immobilized on MNPs tends to drop down to 20% after the second reuse (Amaro-Reyes et al., 2019). One solution could be encapsulation of MNPs by chitosan not only for functionalization of MNPs but also to minimize leaching of MNPs into the reaction medium (Parandhaman et al., 2017). In such a case, enzymes can be immobilized on the surface of chitosan and achieve the magnetic separation and recycling of the immobilized enzymes from the reaction medium.

7.4 Conclusion

Superparamagnetic magnetite nanoparticles were biosynthesized using *A. flavus*. The particles were found to be flakes-like, with average crystallite size of ~16 nm. Pectinase and xylanase were individually immobilized on the surface of MNPs. The immobilized enzymes were more stable than the free ones at wider ranges of pH and temperatures. Furthermore, reusability and storage stability of the enzymes were improved by immobilization. Despite these promising results, application of MNPs nowadays in the food industry is challenged by the current controversy around
potential toxicity and possible health effects due to the risk of product contamination with MNPs. However, considering the promising results in this chapter, the next chapter investigates safer alternatives from natural resources to replace MNPs (herein used a carrier) and glutaraldehyde (herein used a cross linking agent). Such approach opens route for numerous potential applications in the food industry, in particular for fruit juice clarification.
Chapter 8

Alginate hydrogel beads as carrier for enzymes immobilization

In this chapter, immobilization of pectinase and xylanase from m. hiemalis on genipin-activated alginate beads was optimised, followed by the application of immobilised enzymes in apple juice clarification. Optimum activity recovery obtained were about 81% and 83% for pectinase and xylanase, respectively. Optimum enzyme load and genipin concentration were found to be 50 U/ml, and 12% (w/v), respectively. Using a coupling time up to 120 min, agitation rate of 213 rpm for pectinase and 250 rpm for xylanase, maximum activity recovery was observed. Beads prepared at optimum immobilisation conditions were suitable for up to 5 repeated uses, losing only about 45% (for immobilized pectinase) and 49% (for immobilized xylanase) of their initial activity. The maximum clarity of apple juice (%T₆₆₀, 84%) was found to be taking 100 min when pectinase (50 U/ml of juice) and xylanase (20 U/ml of juice) were used in combination at 57°C.

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

8.1 Introduction

Unlike animal counterparts, plant cells are surrounded by an extracellular matrix known as the cell wall which comprises polysaccharide and protein polymers. Protein accounts for only 5-10% of this structure, whereas polysaccharides constitute 90-95% of the cell wall (Jacq et al., 2017). Such polysaccharides are predominantly pectin, hemicellulose, and cellulose (Broxterman and Schols, 2018). In a typical cell of hardwood, such as apple, the wall possesses high pectin levels, and the predominant hemicellulose fraction is xylan (Donev et al., 2018). However, structural non-cellulosic polysaccharides, such as pectin and xylan, are indigestible by human digestive enzymes in the upper gastrointestinal tract (Tappia et al., 2020). Additionally, the presence of colloidal particles of pectin and xylan results in an undesirable cloudiness in apple juice (Kuddus, 2018). Hence, pectinase and xylanase enzymes have been commonly applied to clarification in the apple juice industry (Garg et al., 2016; Nagar et al., 2012; Sharma et al., 2017).

A key concern with the use of enzymes in cost-sensitive food processing operations is the relative expense of such biocatalysts. In this context, immobilization technology affords the key advantage of enzyme re-use, and can also enhance their operational and/or storage stability (Cao, 2011). Among various immobilization techniques available, while the use of covalent binding to solid insoluble carriers has extensively appeared in the literature (Novick and Rozzell, 2005), its use within the food sector is relatively under-developed.

The main advantage of the covalent approach is the strength of enzyme binding to a solid phase, theoretically minimizing in-process enzyme leachate from the carrier
Natural polymers such as alginate beads have received considerable attention due to their potential applications in the food and pharmaceutical industries (Martău et al., 2019). The continued search for adoption of natural materials in food processing has also pointed to the exploitation of genipin (from gardenia fruit *Gardenia jasminoides*) as a potentially safer alternative to the conventional crosslinker, glutaraldehyde, for activation of alginate beads prior to covalent binding to enzymes (Tacias-Pascacio et al., 2019). To the best of our knowledge, enzyme immobilization with genipin-activated alginate beads for juice clarification has received little attention. Thus, this work investigates the covalent coupling of pectinase and xylanase to genipin-activated alginate beads for application in apple juice clarification. The immobilized enzyme preparations were subsequently evaluated in terms of reusability and operational-storage stability.

An artificial neural network was employed to achieve the maximum activity recovery (%) of pectinase and xylanase, as well as maximum apple juice clarification using the immobilized enzymes. Multiple studies have demonstrated that computational modeling (e.g. artificial neural network) is more accurate than statistical modeling (e.g. response surface methodology) in enzyme immobilization and juice clarification processes (Talib et al., 2019; Youssefi et al., 2009). For instance, trained ANN models have been successfully employed to optimize the immobilization process of lipase from *Candida rugosa* on Amberjet® 4200-CI (Fatiha et al., 2013), and cellulase from *Trichoderma viride* on Eudragit® L-100 (Zhang et al., 2012). Moreover, trained ANN models have been successfully employed to optimize apple juice clarification by ultrafiltration (Gökmen et al., 2009), suggesting that incorporation into the present
study could be beneficial, the results of the algorithms were compared by minimized root mean squared error (RMSE) and maximized coefficient of determination ($R^2$).

8.2 Methodology

8.2.1 Enzymes

Pectinase (912 U/ml) and xylanase (455 U/ml) were produced from *Mucor hiemalis* AB1 (GenBank accession number: JQ912672.1) as described in chapter 6. Enzyme activity was determined by the dinitrosalicylic acid (DNS) method of Miller (1959) using pectin (citrus peel) or xylan (beechwood) as standards. Unless stated, all the chemicals used in this work were commercial products of analytical grade (Sigma-Aldrich, Ireland).

8.2.2 Enzyme immobilisation

8.2.2.1 Experimental design and data acquisition for ANN modelling

Optimizations were based on the protocol established by Khairudin et al. (2015). Experimental design was carried out using STATGRAPHICS Centurion XV software (StatPoint Technologies Inc. Warrenton, VA, USA). A four-factor-five-level central composite rotatable design (CCRD) was used to evaluate the enzyme activity recovery (%). The selected CCRD model consisted of four factors, viz. genipin concentration (%) for alginate bead activation, enzyme loading (U/ml), coupling time (min), and agitation rate (rpm). The factors and their levels were obtained through preliminary tests and based on previous results from the literature (Pal and Khanum, 2011a). Table 8.1 summarizes the range and levels of the four factors.
Table 8.1. Independence factors and corresponding levels for enzyme immobilization

<table>
<thead>
<tr>
<th>Code</th>
<th>Factors</th>
<th>Units</th>
<th>levels</th>
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<td></td>
<td></td>
<td></td>
<td>-α</td>
</tr>
<tr>
<td>A</td>
<td>Genipin</td>
<td>%, w/v</td>
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</tr>
<tr>
<td>B</td>
<td>Enzyme load</td>
<td>U/ml</td>
<td>50</td>
</tr>
<tr>
<td>C</td>
<td>Coupling time</td>
<td>min</td>
<td>30</td>
</tr>
<tr>
<td>D</td>
<td>Agitation rate</td>
<td>rpm</td>
<td>50</td>
</tr>
</tbody>
</table>

The CCD contained 16 factorial points, 8 axial points, and 6 central points with α value fixed at 2.0 for a total of 30 experiments, as shown in the model matrix (Section 8.3, Table 8.3). Once the experiments were performed, the experimental dataset (30 experiments) were randomly divided into two sets - training set and testing set - whereas experimental values at predicted optimum conditions were used as the validating set.

8.2.2.2 Covalent immobilization of pectinase and xylanase

Initially, alginate beads were prepared by manually dropping sodium alginate solution (3%, w/v) into the hardening solution (calcium chloride, 0.2 M) using a peristaltic pump (Bhushan et al., 2015). The beads were collected using a filter funnel through a Whatman® (No. 1) paper after 3 hours of gentle stirring on a magnetic stirrer, and maintained in the gelling solution (CaCl₂, 0.02 M) overnight at 4°C to harden. Afterwards, the beads were washed with deionized water and further activated by mixing with genipin solutions of varying concentrations, ranging from 3 to 12% (w/v) in citrate buffer (0.05 M, pH 5.0), and gently stirred to ensure a homogeneous coating of cross-linker. Finally, the beads were removed by filtration and washed with distilled water to remove the unbound genipin.
The resulting activated beads were used as carrier in enzyme immobilization experiments. The immobilization of pectinase and xylanase was performed by orbital mixing (50–250 rpm) of an equal volume (1:1 ratio) of enzyme solution (50–450 IU/ml) with activated beads for different durations (30–150 min). The beads were removed by filtration and washed with distilled water until no enzyme activity could be detected in the washings. The enzyme activity recovery (AR, %) was calculated using the following equation (Zhou et al., 2013):

\[
\text{Enzyme activity recovery (\%)} = \left( \frac{A}{A_{\text{init}}} \right) \times 100
\]

where A is the activity of immobilized enzyme on beads and \( A_{\text{init}} \) is the initial (free) enzyme activity.

### 8.2.2.3 Evaluation of the immobilized enzymes

The optimum reaction pH of the immobilized enzymes was measured in the range between 2.0 and 11.0 using glycine-HCl buffer (0.1 M, pH 2.0), citrate buffer (0.1 M, pH 3.0–6.0), phosphate buffer (0.1 M, pH 7.0–8.0) and glycine-NaOH buffer (0.1 M, pH 9.0–11.0); and the optimum temperature was measured in the range between 20 °C and 80 °C. To evaluate the storage stability, immobilized enzymes were held for 30 days at 4 °C. For a reusability assessment, immobilized enzymes were recovered by magnetic separation after each cycle of use and washed with deionized water, and then a new cycle was run under the same conditions for a total of 6 cycles. The enzyme activity in the first cycle was assigned a value of 100%, and relative activity was calculated for the successive cycles. All experiments were performed in triplicate.
8.2.3 Enzymatic treatment of apple juice

8.2.3.1 Experimental design and data acquisition for ANN modelling

A four-factor-five-level central composite rotatable design (CCRD) that required 30 experiments was used to evaluate the juice clarification (%). The selected CCRD model consisted of four factors, viz. pectinase loading (U/ml of apple juice), xylanase load (U/ml of apple juice), holding time (min), and temperature (°C). The factors and their levels were obtained through preliminary tests and based on previous results from the literature (Ravindran et al., 2019). Table 8.2 summarizes the range and levels of the four factors.

Table 8.2. Independence factors and corresponding levels for clarification of apple juice

<table>
<thead>
<tr>
<th>Code</th>
<th>Variables</th>
<th>Units</th>
<th>levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>-α</td>
</tr>
<tr>
<td>A</td>
<td>Pectinase load</td>
<td>U/ml of apple juice</td>
<td>10</td>
</tr>
<tr>
<td>B</td>
<td>Xylanase load</td>
<td>U/ml of apple juice</td>
<td>10</td>
</tr>
<tr>
<td>C</td>
<td>Holding time</td>
<td>min</td>
<td>40</td>
</tr>
<tr>
<td>D</td>
<td>Temperature</td>
<td>°C</td>
<td>40</td>
</tr>
</tbody>
</table>

The CCRD contained 16 factorial points, 8 axial points, and 6 central points, with α value fixed at 2.0 for a total of 30 experiments, as shown in the model matrix (in Section 8.3, Table 8.6). Once the experiments were performed, the experimental dataset (30 experiments) were randomly divided into two sets - training set and testing set - while experimental values at predicted optimum conditions were used as the validating set.
8.2.3.2 Clarification of apple juice using immobilized enzymes

Fresh apple fruits (*Malus domestica*) of Royal Gala variety (without any visual defects) at commercial maturity were purchased from a local market (Dublin, Ireland). The apples were washed with tap water, chopped into small pieces, and later macerated in a domestic blender, without addition of water, until a homogenous juice was obtained. The concentrated juice was then pasteurized for 1 hour at 60°C (Padma et al., 2017). The filtered juice (pH 5.0) was used for the clarification studies.

Figure 8.1 illustrates the laboratory scale set up of a packed-bed reactor using a glass column for enzymatic clarification of apple juice using the immobilized enzymes (pectinase and xylanase) on alginate beads.

![Figure 8.1. Schematic diagram of the experimental set-up for clarification of apple juice](image)
The enzymatic clarification experiments were performed by subjecting 25 ml of apple juice to different concentrations of pectinase and xylanase (10-50 U/ml of juice) for varying duration of holding times (40–120 min) within the range of temperatures between 40 °C and 60 °C. Finally, the enzyme beads were removed, and treated apple juice centrifuged (10,000 rpm, 15 min), followed by filtration using Whatman no 1 filter paper, and this juice filtrate was used for further analysis. The clarity of juice was expressed as percentage transmission (%T) that was determined using a UV-1800 UV-VIS spectrophotometer (Shimadzu Scientific Instruments, Columbia, USA) at a wavelength of 660 nm, and using distilled water as a reference (Dey and Banerjee, 2014).

### 8.2.4. Artificial Neural Network (ANN) modelling and analysis

The commercial artificial intelligence software, NeuralPower® (CPC-X Software, version 2.5, Carnegie, PA, USA) was employed for ANN modelling and analysis. The networks were trained in a supervised learning environment by different learning algorithms (incremental back propagation, IBP; batch back propagation, BBP; quickprob, QP; and Levenberg-Marquardt algorithm, LM). Multilayer normal feed-forward was used to predict the response and the hyperbolic tangent function (a.k.a. tanh) used as transfer function in the hidden and output layers. To determine the optimal network topology, only one hidden layer with varying number of neurons was used to develop different networks. The comparison between the models were assessed using root mean square error (RMSE) and correlation coefficient (R²). The lower the RMSE and the closer R² is to 1, the more precise the model. Models were further assessed using a testing dataset to predict the unseen data (data not used for ANN training).
For process optimization, three different optimization algorithms were employed, namely rotation inherit optimization (RIO), particle swarm optimization (PSO), and genetic algorithm (GA). After determination of optimum conditions, experimental validation was carried out to calculate the percentage error between the experimentally measured values and the ANN predicted value using the formula (Zhang et al., 2020) as follows:

\[
\text{Error} \, (\%) = \left(\frac{P' - P}{P}\right) \times 100
\]

where, \(P'\) is the enzyme activity recovery predicted using ANN, and \(P\) is the observed enzyme activity recovery measured in the experiment.

8.3 Results and discussion

8.3.1 Enzyme immobilisation

8.3.1.1 The ANN model training

A neural network with optimal number of neurons is required to avoid over- or undertraining of the training dataset. If neurons are lower than the optimum range, undertraining would result in a poor fit to the training dataset. On the other hand, increasing the number of hidden neurons above the optimum range may lead to overfitting, as the network may end up memorizing the training data. Although this would result in very good fit to the training dataset, the model would have poor generalization ability to handle testing and unseen datasets.

The larger subset (\(n=25\)) comprising more than 80% of the available experimental data was used for the ANN training and model building. To determine the optimal topology
for the networks, the number of neurons in the hidden layer was varied from 1 to 7. Subsequently, the decision on the optimum topology was based on the minimum RMSE (and the closer $R^2$ to 1) of testing set values. Figure 8.2 illustrates the performance of the network for training data versus of the number of neurons in the hidden layer using different learning algorithms.

![Figure 8.2](image)

**Figure 8.2.** The performance of different learning algorithms (Incremental backpropagation algorithm, IBP; Batch backpropagation algorithm, BBP; Quick propagation algorithm, QP; and Levenberg-Marquardt algorithm, LM) for training data versus of the number of neurons in the hidden layer for predicting the activity recovery of pectinase (A) and xylanase (B) onto alginate beads.

According to the RMSE, the network with 3 hidden neurons produced the optimum performance when any of the four algorithms (IBP, BBP, QP and LM) was employed. Therefore, the optimum topology of the networks (Figure 8.3) was 4-3-1 (four neurons in the input layer, three neurons in the hidden layer and one neuron in the output layer).
Figure 8.3. The illustration of multilayer normal feed-forward neural network. The neural network having three inputs of variables (genipin, enzyme load, coupling time, and agitation rate), one hidden layer with three neurons (nodes) and one output of response (enzyme activity recovery).

8.3.1.2 Selection neural network model

The model architecture of 4-3-1 was selected as the best topology for the four learning algorithms. Moreover, as shown in figure 8.4, LM and QP were at maximum $R^2$, while its RMSE were at the lowest value in comparison with the other algorithms for predicting the activity recovery (%) of pectinase and xylanase, respectively.

Figure 8.4. Comparison of different learning algorithms (Incremental backpropagation algorithm, IBP; Batch backpropagation algorithm, BBP; Quick
propagation algorithm, QP; and Levenberg-Marquardt algorithm, LM) with 6 neurons in the hidden layer for predicting the activity recovery of pectinase and xylanase onto alginate beads.

Backpropagation is an extensively used family of supervised training algorithms based on the error-correction learning rule and can be implemented in either incremental or batch mode (Kahraman, 2012). Backpropagation algorithm has been improved for a faster training process (‘quick propagation- QP- algorithm’; Awolusi et al., 2018), and enhanced performance (‘Levenberg Marquardt – LM - algorithm’; Reddy et al., 2018). It was reported that QP gave the best performance in modeling the enzymatic synthesis of betulinic acid ester when compared with IBP, BBP, and LM (Moghaddam et al., 2010). On the other hand, Adnani et al. (2011) employed the LM algorithm for lipase-catalyzed synthesis of sugar alcohol ester. It is worth noting that there is no ideal algorithm per se that will give the best results in the training of any dataset, and the result of training is highly dependent on the architecture of the network, the training algorithm, the size of training dataset and data noise levels (Jacobsson, 1998).

Table 8.3 displays the ANN predicted values for the training datasets using LM-4-3-1 for pectinase activity recovery and QP-4-3-1 for xylanase activity recovery. The results revealed the close correlation between the experimental and the predicted values. The $R^2$ and RMSE metrics were used to evaluate the developed models. The $R^2$ value was 0.99 for both models, where RMSE values were 1.37 and 1.48 for pectinase and xylanase immobilization models, respectively. The obtained $R^2$ of the two models is very close to 1, indicating a good adjustment between the observed and predicted
values. Moreover, the obtained low RMSE values of the two models did not show significant disparity, indicating relatively similar performance.

Table 8.3. Experimental design showing the observed and predicted values of enzyme (pectinase or xylanase) activity recovery (%) as output for training dataset.

<table>
<thead>
<tr>
<th>Run</th>
<th>Independent Variables</th>
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<th>Enzyme activity recovery (%)</th>
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<td></td>
<td></td>
<td></td>
<td>Pectinase</td>
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<tr>
<td></td>
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<td>Observed</td>
</tr>
<tr>
<td>1</td>
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<td>18.66</td>
</tr>
<tr>
<td>2</td>
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<td>3</td>
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<td>31.74</td>
</tr>
<tr>
<td>25</td>
<td>6 250 90 250</td>
<td></td>
<td>43.75</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>R²</th>
<th></th>
<th>RMSE</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Training Data</td>
<td>0.99</td>
<td></td>
<td>1.37</td>
<td>1.48</td>
</tr>
</tbody>
</table>

128
A subset (n=5) comprising just above 15% of the available experimental data was used for ANN testing to predict the unseen data (data not used for ANN training). Hence, the trained ANNs was tested against the corresponding testing datasets to assess the predictive power of the developed ANN models. Table 8.4 displays the ANN predicted values for the testing datasets using LM-4-3-1 for pectinase immobilization and QP-4-3-1 for xylanase immobilization.

Table 8.4. Experimental design showing the observed and predicted values of enzyme (pectinase or xylanase) activity recovery (%) as output for testing dataset.

<table>
<thead>
<tr>
<th>Run</th>
<th>Independent Variables</th>
<th>Response</th>
<th>Enzyme activity recovery (%)</th>
<th>Pectinase</th>
<th>Xylanase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
<td>Observed</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>150</td>
<td>120</td>
<td>100</td>
<td>46.07</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>250</td>
<td>250</td>
<td>150</td>
<td>64.49</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>250</td>
<td>90</td>
<td>150</td>
<td>32.41</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>150</td>
<td>120</td>
<td>100</td>
<td>75.91</td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>350</td>
<td>60</td>
<td>200</td>
<td>15.92</td>
</tr>
<tr>
<td></td>
<td>R^2</td>
<td>0.99</td>
<td></td>
<td></td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>RMSE</td>
<td>1.73</td>
<td></td>
<td></td>
<td>1.86</td>
</tr>
</tbody>
</table>

The R^2 value was 0.99 for both models, where RMSE values were 1.73 and 1.86 for pectinase and xylanase immobilization models, respectively. The obtained R^2 indicated that the regression predictions perfectly fit the data. In addition, the obtained RMSE values showed a small difference between training and testing datasets (0.36 for pectinase immobilization model, and 0.38 for xylanase immobilization model), indicating good generalization capability and accuracy of the trained ANN models.
8.3.1.3 Optimization of enzyme immobilization using trained ANNs

The optimum conditions for enzyme (pectinase and xylanase) immobilization were determined by comparing three different algorithms, which were rotation inherit optimization (RIO), particle swarm optimization (PSO), and genetic algorithm (GA). However, there was no significant difference in values of enzyme immobilization (%) predicted by the three different algorithms. The predicted conditions for optimum pectinase activity recovery (82.56 %) were 50 U/ml xylanase with 12% of genipin crosslinker, with a coupling time of 120 min and agitation rate of 213 rpm. Similarly, the predicted conditions for optimum xylanase activity recovery (83.89 %) were also 50 U/ml xylanase with 12% of genipin crosslinker and coupling time of 120 min, but with an agitation rate of 250 rpm. The grid color charts of pectinase and xylanase activity recovery (%) are shown in figures 8.5 and 8.6, respectively.

Pal and Khanum (2011) reported a slightly higher RSM-predicted activity recovery of xylanase (89.5%) on alginate beads compared to our ANN-predicted values (84%) using 8.31% glutaraldehyde crosslinker, 250 U/ml of xylanase from Aspergillus niger, coupling time of 120 min and an agitation rate of 200 rpm. On the other hand, Abdel Wahab et al. (2018) reported a slightly lower RSM-predicted activity recovery for pectinase (80.43%) comparing to our ANN-predicted values (83%) using 5% polyethyleneimine (PEI), 1.5% glutaraldehyde, 15 U/ml of pectinase from Aspergillus awamori, and a coupling time of 6 h.
Figure 8.5. Grid color charts representing the effect of independent variables on pectinase activity recovery (%): (a) Effect of genipin concentration and pectinase load on activity recovery when coupling time and agitation rate are fixed at 120 min and 213 rpm, respectively; (b) Effect of genipin concentration and coupling time on activity recovery when pectinase load and agitation rate are fixed at 50 U/ml and 213 rpm, respectively; (c) Effect of genipin concentration and agitation rate on activity recovery when pectinase load and coupling time are fixed at 50 U/ml and 120 min, respectively; (d) Effect of pectinase load and coupling time on activity recovery when genipin concentration and agitation rate are fixed at 12 % (w/v) and 213 rpm, respectively; (e) Effect of pectinase load and agitation rate on activity recovery when genipin concentration and coupling time are fixed at 12 % (w/v) and 120 min, respectively; and (f) Effect of coupling time and agitation
rate on activity recovery when pectinase load and genipin concentration are fixed at 50 U/ml and 12 % (w/v), respectively.

Figure 8.6. Grid color charts representing the effect of independent variables on xylanase activity recovery (%): (a) Effect of genipin concentration and xylanase load on activity recovery when coupling time and agitation rate are fixed at 120 min and 250 rpm, respectively; (b) Effect of genipin concentration and coupling time on activity recovery when xylanase load and agitation rate are fixed at 50 U/ml and 250 rpm, respectively; (c) Effect of genipin concentration and agitation rate on activity recovery when xylanase load and coupling time are fixed at 50 U/ml and 120 min, respectively; (d) Effect of xylanase load and coupling time on activity recovery when genipin concentration and agitation rate are fixed at 12 % (w/v) and 250 rpm, respectively; (e) Effect of xylanase load and agitation rate
activity recovery when genipin concentration and coupling time are fixed at 12 % (w/v) and 120 min, respectively; and (f) Effect of coupling time and agitation rate on activity recovery when xylanase load and genipin concentration are fixed at 50 U/ml and 12 % (w/v), respectively.

From Fig. 8.5a and Fig. 8.6a, it is observed that with increase in genipin concentration, immobilization efficiency will also theoretically increase, as more attachment points become available for enzyme immobilisation on the alginate beads. However, increasing the enzyme load (pectinase or xylanase) will not always lead to an increase in immobilisation efficiency, presumably due to insufficient attachment points of genipin for enzyme. Similar results were reported by Sukri and Munaim (2017) where the highest xylanase activity recovery was achieved when the alginate beads were activated by a higher concentration of glutaraldehyde and a lower enzyme loading. As one might expect, a longer coupling time and higher genipin concentration resulted in higher immobilization efficiency at constant enzyme loading, as shown in Fig. 8.5b and Fig. 8.6b. On the other hand, longer coupling time and higher enzyme loading did not result in higher immobilization efficiency at constant genipin concentration, as shown in Fig. 8.5d and Fig. 8.6d. The effect of agitation rate on the immobilization efficiency (Fig. 8.5c and Fig. 8.6c) had a smaller effect compared to the effect of other variables, most probably as mixing serves the single purpose of generating a homogeneous suspension of bead- and enzyme solution. Such a homogeneous suspension improves contact of the free enzyme with the beads, which results in higher immobilization efficiency. Hence, as the agitation rate increases above the optimum rate, immobilization efficiency shows no significant improvement. Sukri et al., (2020) reported an equivalent result, reporting that an agitation rate of 200 rpm resulted in an
optimum activity recovery (83.93%) of xylanase (200 U) on alginate beads activated by glutaraldehyde (12%, w/w), but no improvements could be achieved in using a higher rate.

The model validation was carried out by running at the predicted conditions (Table 8.5). As a result of three successive runs, only slight variation (1.5-1.6%) in the value of enzyme immobilization was observed, suggesting that the optimal conditions for immobilization of both enzymes generated by the ANN algorithms were reliable and valid.

Table 8.5. Experimental validation of the optimization values predicted by ANN for enzyme (pectinase or xylanase) activity recovery (%).

<table>
<thead>
<tr>
<th>Replicates</th>
<th>Pectinase</th>
<th>Xylanase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Predicted</td>
<td>Observed</td>
</tr>
<tr>
<td>1</td>
<td>82.56</td>
<td>80.57</td>
</tr>
<tr>
<td>2</td>
<td>81.96</td>
<td>81.92</td>
</tr>
<tr>
<td>3</td>
<td>81.45</td>
<td>83.14</td>
</tr>
<tr>
<td>Mean</td>
<td>81.33</td>
<td>Mean</td>
</tr>
<tr>
<td>Error (%)</td>
<td>1.52</td>
<td>Error (%)</td>
</tr>
</tbody>
</table>

8.3.1.4 Evaluation of the immobilized enzymes

Immobilized enzymes were evaluated by studying their key required operational parameters (temperature, pH, storage and recycle stability) in comparison with free forms (Figure 8.7).
Figure 8.7. Panels (A–C) shows the effect of temperature (A), pH (B), storage time (C), and recycle count (D) on relative activity of immobilized pectinase and xylanase in comparison with free enzyme.

As shown in Figure 8.7a, the immobilization did not change the optimal temperature of xylanase (60°C) at pH 5. However, the optimum temperatures of the free and immobilized pectinase were 50°C and 60°C, respectively. Such a forward shift in the temperature optimum of immobilized pectinase by 10°C could be the result of improved enzyme rigidity after covalent binding on alginate beads (Ortega et al., 2009). To study the pH-dependent activities of the free and immobilized enzymes, the temperature of assay mixtures was maintained at 60°C while pH values were varied from 2.0 to 11.0 (Figure 8.7b). Although the immobilized enzymes retained the optimal pH (5.0) of their free pectinase and xylanase counterparts, the pH scope of the
immobilized pectinase was expanded, and it retained more than 80% activity over a wider pH range of 4.0–8.0 than that of the free form (pH range of 5.0-7.0). Similarly, the immobilized xylanase exhibited improved pH stability, and retained greater than 80% activity over a wider pH range of 3.0–7.0 than that of the free form (pH range of 4.0-6.0). These results may be attributed to the free protein undesired aggregation that is prevented by the covalent bonding of the enzyme onto alginate beads during the immobilization process (Mostafa et al., 2019).

The data in Figure 8.7c show the storage stability of enzymes immobilized onto alginate beads over 30 days at 4°C. Immobilized pectinase and xylanase retained 60% and 51%, respectively, of their initial activity after 30 days of storage. In contrast, the free pectinase and xylanase lost more than 46% and 51%, respectively, of their initial activity after 20 days of storage. The multiple re-use capability of immobilized enzymes can be achieved by recovering the beads from the reaction mixture, thereby reducing costs. As shown in Figure 8.7d, the residual activity of the immobilized enzymes was 55% (pectinase) and 51% (xylanase), after five consecutive cycles. The activity loss of the immobilized enzyme may be due to a combination of inactivation and enzyme leakage from the support (Mohamad et al., 2015).

8.3.2 Enzymatic treatment of apple juice

8.3.2.1 The ANN model training

The larger subset (n=25) comprising more than 80% of the available experimental data was used for the ANN training and model building. To determine the optimal topology for the networks, the number of neurons in the hidden layer was varied from 1 to 7. A decision on the optimum topology was subsequently based on the minimum RMSE.
(and the closer $R^2$ to 1) of the training set values. Figure 8.8a illustrates the performance of the network for testing data versus the number of neurons in the hidden layer using different learning algorithms. According to the RMSE, the network with 3 hidden neurons produced the optimum performance when any of the four algorithms (IBP, BBP, QP and LM) was employed. Therefore, the optimum topology of the networks (Figure 8.8b) was 4-3-1 (four neurons in the input layer, three neurons in the hidden layer and one neuron in the output layer).

Figure 8.8. The left panel (a) shows the performance of different learning algorithms (Incremental backpropagation algorithm, IBP; Batch backpropagation algorithm, BBP; Quick propagation algorithm, QP; and Levenberg-Marquardt algorithm, LM) for training data versus the number of neurons in the hidden layer for predicting the apple juice clarification (%) using pectinase and xylanase immobilized onto alginate beads. The right panel (b) shows schematic diagram of the optimal multi-layer, normal feed-forward neural network architecture for apple juice clarification.

### 8.3.2.2 Selection neural network model

The model architecture of 4-3-1 was selected as the best topology for the four learning algorithms. Figure 8.9 presents the predictions using different learning algorithms with
optimum architecture (4-3-1) versus the observed values of the juice clarification (%) which were obtained in the laboratory.

![Graphs showing scatter plots of predicted versus observed juice clarification for different learning algorithms.](image)

**Figure 8.9.** The scatter plots of the predicted juice clarification versus the observed juice clarification for training dataset that shows the performed R$^2$ and RMSE of different learning algorithms at optimal neural network architecture (4-3-1).

The comparison of the RMSE proved that the BB with 4 nodes in input, 3 nodes in hidden, and 1 node in the output layer (BB-4-3-1) presented the minimum RMSE, while its R$^2$ was at the maximum value. As illustrated, the RMSE was 1.21, and the R$^2$ was 0.998 which indicated the great predictive accuracy of the model. Therefore, BB-4-3-1 was selected as the final optimum provisional model of the juice clarification for an evaluation test. Table 8.6 displays the ANN predicted values for the testing datasets using BB-4-3-1 for juice clarification (%). It is worth noting that the batch algorithms...
are effective in training small datasets with small network topologies (Plagianakos et al., 2001).

Table 8.6. Experimental design showing the observed and predicted values of juice clarification (%) as output for training dataset.

<table>
<thead>
<tr>
<th>Run</th>
<th>Independent Variables</th>
<th>Response Juice clarification (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Training Data</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
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<td>5</td>
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<td>24</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>25</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>

R²  
RMSE  

0.99  
1.22
A subset (n=5) comprising just above 15% of the available experimental data was used for ANN testing to predict the unseen data (data not used for ANN training). Hence, the trained ANNs was tested against the corresponding testing datasets to assess the predictive power of the developed ANN models (Table 8.7). The \( R^2 \) value was 0.99, where RMSE value was 1.84 for the trained ANN. Such an \( R^2 \) value indicates that the regression predictions perfectly fit the data. In addition, the obtained RMSE values showed a small difference between training and testing datasets (0.62), indicating good generalization capability and accuracy of the trained ANN model (BB-4-3-1).

**Table 8.7. Experimental design showing the observed and predicted values of juice clarification (%) as output for testing dataset.**

<table>
<thead>
<tr>
<th>Run</th>
<th>Independent Variables</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>-----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>Testing Data</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>( R^2 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RMSE</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**8.3.2.3 Optimization of juice clarification process using trained ANNs**

The optimum points for juice clarification were determined by comparing three different algorithms, namely rotation inherit optimization (RIO), particle swarm optimization (PSO), and genetic algorithm (GA). However, there was no significant difference in values of enzyme immobilization (%) predicted by the algorithms. The
predicted conditions for juice clarification (85.62%) were 50 U of pectinase/ml of juice and 20 U of xylanase/ml of juice for 100 min at 57 °C. Thus, our findings are in line with previous literature (Rai et al., 2004; Sin et al., 2006) confirming that enzymatic treatment for juice clarification is greatly influenced by enzyme loading, holding time and temperature. After the experimental validation of the model using the optimization conditions, it was found that the observed value (84.33±0.32%) was close to that predicted (85.62%), suggesting the appropriateness of the developed ANN model.

Similarly, Singh and Gupta (2004) achieved apple juice clarification of 85% (%T$_{650}$) using polygalacturonase from *Aspergillus niger* (15 IU/ml) in the presence of 0.01% gelatin at 45 °C and with a 6 h holding time. The most effective clarification (%T$_{660}$, 97%) was reported by Dey and Banerjee (2014) with 1% polygalacturonase from *Aspergillus awamori* Nakazawa (9.87 U/mL) and 0.4% α-amylase from *A. oryzae* (899 U/mL), in the presence of 10 mM CaCl$_2$ at 50 °C and with a 2 h holding time. Also, Dey et al. (2014) reported the maximum transmittance of 93% (%T$_{660}$) in clarified apple juice upon enzymatic treatment using polygalacturonase from *Aspergillus awamori* Nakazawa (9.87 U/ml) at 50 °C and a 2 h holding time. Gaomei et al. (2011), reported in their patent a novel technology for clarifying apple juice. In the latter invention, the maximum clarity of apple juice (%T$_{660}$, 93%) was achieved when cider boiled for 5-10 min and then treated with pectinase (60 U/gram cider, dissolved in buffer solution of pH=3.5) for 2h at 50°C.

Additionally, researchers have previously reported that the treatment with xylanase positively contributes to the clarity of apple juice. For instance, the treatment of juice
with xylanase (15 IU/g of apple pulp) from *Bacillus pumilus* SV-85S lead to a clarity in terms of % transmittance of approximately 42 (%T\textsubscript{660}) at 40 °C, with a 30 min holding time (Nagar et al., 2012). Adigüzel and Tunçer (2016) reported the maximum transmittance of about 18% (%T\textsubscript{660}) in clarified apple juice upon enzymatic treatment using xylanase from *Streptomyces sp.* AOA40 (12.5 U/ml of apple juice) at 60 °C and a 90 min holding time. Also, Phadke and Momin (2015) reported a maximum transmittance of 20% (%T\textsubscript{650}) in clarified juice upon enzymatic treatment using xylanase from *Bacillus megaterium* (20 U/g of apple pulp) at 37 °C and a 4 h holding time.

Madhu et al. (2015) achieved apple juice clarification of approximately 42%, and 49% (%T\textsubscript{650}) using enzyme cocktails (cellulase, pectinase and xylanase) from *P. exigua* and *A. niger*, respectively, at 60°C and a 50h holding time. The study of Pal and Khanum (2011b) explored a synergistic effect of xylanase, pectinase and cellulase to improve clarity of pineapple juice, achieving about 81% (%T\textsubscript{650}) clarity.

Figure 8.10 demonstrates the importance of effective parameters on apple juice clarification as an output of the model. The importance values of the parameters were pectinase loading > xylanase loading > temperature > holding time in the selected range of the variables. Thus, the effects of the two most important parameters (pectinase and xylanase loadings) on apple juice clarification are presented in Figure 8.11, where temperature and time were kept constant at the optimal values (100 min, and 57 °C, respectively). As shown in Figure 11, apple juice clarity increased with an increase in pectinase rather than xylanase loading at optimum reaction conditions (100
min, and 57 °C). This can be attributed to the fact that apples are particularly rich sources of pectin rather than xylan.

**Figure 8.10. Importance of effective parameters on apple juice clarification.**

**Figure 8.11. Three-dimensional surface plot of pectinase load and xylanase load effects on apple juice clarification. The other variables (temperature and time) were kept constant at the optimal values.**
Finally, table 8.8 summarizes application data sheets of different commercial enzyme preparations available in the market for apple juice clarification and suggests a role for the use of immobilized enzyme beads developed in our study.

**Table 8.8. Application of different commercial enzyme preparations in apple juice clarification**

<table>
<thead>
<tr>
<th>Company</th>
<th>Product</th>
<th>Description</th>
<th>Dosage</th>
<th>Application</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RAPIDASE</td>
<td>Pectinases from <em>A. niger</em> and <em>A. aculeatus</em></td>
<td>20-40 ml/1,000 L of Juice</td>
<td>3.5-5.5</td>
<td>(DSM, 2015)</td>
</tr>
<tr>
<td>DSM</td>
<td>Pectinase</td>
<td>Pectinesterase, polygalacturonase, pectinlyase from <em>A. niger</em></td>
<td>2-20 g/ton</td>
<td>3.5-6.0</td>
<td>(Biovet)</td>
</tr>
<tr>
<td>Biovet</td>
<td>Panzym Pro Clear</td>
<td>Polygalacturonase from <em>A. niger</em> and <em>A. aculeatus</em></td>
<td>20-50 ml/1,000 L of Juice</td>
<td>-</td>
<td>(Eaton, 2015)</td>
</tr>
</tbody>
</table>

8.4 Conclusion

The artificial neural network (ANN) modelling was adopted to simulate and predict the activity recovery of enzymes (pectinase and xylanase) when immobilized onto genipin-activated alginate beads, as well as their application in apple juice clarification. The predicted conditions for optimum pectinase activity recovery (~83%) were 50 U/ml pectinase with 12% of genipin crosslinker and a coupling time of 120 min (agitation rate of 213 rpm). On the other hand, the predicted conditions for optimum xylanase activity recovery (~84%) were also 50 U/ml xylanase with 12% of genipin crosslinker and coupling time of 120 min, but at an agitation rate of 250 rpm. A maximum activity recovery was observed to be about 81-83% for both enzymes. Moreover, the predicted conditions for juice clarification (85.62%) were 50 U/ml of pectinase, 20 U/ml of xylanase for 100 min at 57 °C. It was found also that the observed value (84.33± 0.32%) was close to that predicted (85.62%). The developed model indicated pectinase loading as the most important factor, having a dramatic influence
on apple juice clarification. Enzyme beads prepared at optimum immobilization conditions were suitable for up to 5 repeated uses, losing only ~45% (pectinase) and ~49% (xylanase) of their initial activity.
General Conclusions

Future recommendations

This chapter includes conclusions of present work and discusses the area of future recommendations.
9.1 General conclusions

The overall aim of the project was to utilise brewer’s spent grain (BSG) for production of industrially important enzymes, which are in turn expected to contribute to BSG waste recycling and reduce the cost of enzymes. BSG generated from beer-brewing has been estimated at 3.4 million tonnes annually in the EU alone, and over 4.5 million tons in USA as the largest craft beer producer. Novel enzymes identified in this work were immobilised to enable enzyme re-use, extend storage time, and also potentially achieve higher stability under varying temperature and pH. Finally, the immobilised enzyme preparations were tested for proof-of-concept in apple juice clarification process.

In line with the aim of developing a pre-treatment strategy that is devoid of any chemicals, a novel extraction treatment for BSG was identified and optimised based on the energy-efficient technologies of microwave heating and ultrasound irradiation. For pretreatment of brewer's spent grain, BSG was subjected to microwave and ultrasound pretreatment in separate one-factor-at-a-time (OFAT) experiments to study the effect of different variables on fermentable sugar yields from BSG. Microwave pretreatment of BSG at the optimum condition (600 w for 90 s) achieved a higher fermentable sugar yield (64.4±7 mg / 1 g of BSG) as compared to ultrasound pretreatment of BSG (39.9±6.1 mg / 1 g of BSG) at the optimum condition (frequency of 25 kHz, power of 550 W, and time of 60 min.). The reducing sugar yield for native BSG used in this study before pretreatment was 24.5± 0.3 mg/g of biomass.

However, the derived ultrasound pretreatment time (1 hour) during the OFAT experiments was relatively long. Such a long duration may adversely affect the uptake of such technology by industry. Thus, response surface methodology (RSM) was used
for optimization of the multiple variables of ultrasound pretreatment of BSG and to investigate the possibility for reducing the pretreatment time. Additionally, chemical composition, morphological structure and thermal behavior of BSG before and after pretreatment were studied and compared.

The optimal conditions for ultrasound-assisted pretreatment of BSG were found to be 20% US power of 550 W, 60 min, 26.3°C, and 17.3% w/v of biomass in water. BSG pretreatment under the optimal ultrasonication conditions resulted in a 2.1-fold higher reducing sugar yield, relative to native BSG. These results suggest that performing a design of experiments (DOE) to optimise ultrasound pretreatment of BSG achieved a higher fermentable sugar yield (2.1-fold, relative to native BSG) as compared to the one-factor-at-a-time (OFAT) approach used in previous experiments for optimisation of ultrasound pretreatment (that achieved 1.6-fold increase in fermentable sugar yield, relative to native BSG).

However, the same long pretreatment time (60 min.) was required for optimisation of ultrasound pretreatment which can lead to adverse effects (due to collision and aggregation between the particles) and increased energy consumption during the process. Therefore, microwave pretreatment was selected as an energy efficient and effective pretreatment step for BSG for all subsequent fermentation studies.

In order to discover novel enzyme activities, twenty-nine fungal isolates were obtained from spoiled-fruit samples of 19 different varieties. The isolates were distributed across three genera, namely: *Mucor* spp. (19 isolates), *Penicillium* spp. (6 isolates), and *Aspergillus* spp. (4 isolates). All isolates were selected for subsequent pectinase and xylanase production screening. Out of the isolates recovered, *Mucor*
*hiemalis* isolated from Bramley apple (*Malus domestica*) was found to produce xylano-pectinolytic enzymes with higher specific activity.

The highest enzyme activity (137 U/g, and 67 U/g BSG, for pectinase and xylanase, respectively) was achieved in a medium that contained 15 g of BSG/250ml conical flask, at pH 6.0, temperature of 30°C, and supplemented with 1.0 % xylan or pectin for inducing the production of xylanase or pectinase, respectively. Thus, BSG represent an attractive feedstock for microbial fermentation–based biomanufacturing.

Following removal of biomass by centrifugation, pectinase and xylanase produced by *Mucor hiemalis* were purified by ammonium sulphate precipitation, resulting in a 95% and 76% yield for pectinase and xylanase, respectively. The ammonium sulfate-enriched fraction of pectinases and xylanases were further concentrated to 14-fold and 12-fold respectively by ultrafiltration. The partially purified and concentrated pectinase and xylanase were optimally active at 60°C and pH 5.0 (1602 U/ml of pectinase, and 839 U/ml of xylanase). Thus, the present study suggests that BSG is an effective substrate for production of microbial enzymes such as pectinase and xylanase.

The enzymes obtained after fermentation of BSG were immobilised on novel magnetic nanoparticles and a natural hydrogel. Superparamagnetic magnetite nanoparticles (MNPs) were biosynthesized using *Aspergillus flavus* isolated from spoiled strawberry (*Fragaria ananassa* Duchesne, Variety: Rociera). The morphology of MNPs was found to be flake-like, as confirmed by Field Emission Scanning Electron Microscopy (FESEM), while the average crystallite size was ~16 nm, as determined by X-ray diffraction (XRD). Energy dispersive X-ray (EDX) analysis was performed to confirm the presence of elemental Fe in the sample. Pectinase and xylanase were covalently
immobilized on MNPs with efficiencies of ~84% and 77%, respectively. Compared to the free enzymes, the immobilized enzymes were found to exhibit enhanced tolerance to variation of pH and temperature and demonstrated improved storage stability. Furthermore, the residual activity of the immobilized enzymes was about 56% for pectinase and 52% for xylanase, after four and three consecutive use cycles, respectively. However, rapid and efficient separation of magnetic nanoparticles from the reaction medium to prevent unintended leaching of potentially toxic nanoparticles into consumer products remains a challenge.

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As an alternative food-friendly approach, this project also investigated sodium alginate from the brown algae laminaria hyperborea as a carrier and genipin from gardenia fruit Gardenia jasminoides as a cross linking agent for immobilization of pectinase and xylanase from Mucor hiemalis. The artificial neural network (ANN) modelling was adopted to simulate and predict the efficiency of enzyme immobilization onto genipin-activated alginate beads, as well as their application in apple juice clarification. The predicted conditions for optimum pectinase activity recovery (~83%) were 50 U/ml pectinase with 12% of genipin crosslinker and a coupling time of 120 min (agitation rate of 213 rpm). A maximum activity recovery was observed to be about 81-83% for both enzymes. On the other hand, the predicted conditions for optimum xylanase activity recovery (~84%) were also 50 U/ml xylanase with 12% of genipin crosslinker and coupling time of 120 min, but at an agitation rate of 250 rpm.

The following table provides a comparison between MNP and alginate beads as carriers for pectinase and xylanase:
Table 9.1. Comparison between MNP and alginate beads as carriers for pectinase and xylanase

<table>
<thead>
<tr>
<th>Features</th>
<th>Magnetic nanoparticles</th>
<th>Alginate beads</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pectinase</td>
<td>Xylanase</td>
</tr>
<tr>
<td>Fractional enzyme activity (%)</td>
<td>74%</td>
<td>68%</td>
</tr>
<tr>
<td>Optimal temperature (20 - 80°C)</td>
<td>50°C</td>
<td>60°C</td>
</tr>
<tr>
<td>Optimal pH (2.0 to 11.0)</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Half-life of the enzyme at 4-6 °C</td>
<td>30 days</td>
<td>25 days</td>
</tr>
<tr>
<td>Reusability</td>
<td>4 cycles</td>
<td>3 cycles</td>
</tr>
</tbody>
</table>

Moreover, the predicted conditions for juice clarification (85.62%) were 50 U/ml of pectinase, 20 U/ml of xylanase for 100 min at 57 °C. It was found also that the observed value (84.33± 0.32%) was close to that predicted (85.62%). The developed model indicated pectinase loading as the most important factor, having a dramatic influence on apple juice clarification. Enzyme beads prepared at optimum immobilization conditions were suitable for up to 5 repeated uses, losing only ~45% (pectinase) and ~49% (xylanase) of their initial activity. The immobilized enzymes are of industrial relevance in terms of biocompatibility, recoverability and operational-storage stability.

From the above mentioned results, one can conclude that our in-house pectinase and xylanase recyclable preparations can improve juice appearance through a cost-effective enzymatic clarification process at bench-scale. The obtained experimental proof of concept should encourage investment into pilot-scale demonstration in a move towards full commercial scale. Moreover, the use of alginate beads as enzyme carriers represents a feasible option for juice producers, as such an approach will require only installing a stainless steel perforated mesh basket or a retaining stainless-steel filter at the outlet of the enzyme treatment tank for bead retention.
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

9.2.1 Other potential lignocellulosic wastes

This work investigated the application of selected emerging technologies such as ultrasound and microwave as promising technologies in the pretreatment of BSG prior to microbial fermentation. Similar to the work undertaken in this thesis, an interesting continuation of this work would be investigating ultrasound and microwave as potential technologies for sustainable pretreatment of other lignocellulosic wastes and subsequent microbial fermentation. Other potential lignocellulosic wastes from various industries includes sugarcane bagasse (from sugar milling), pomace (pressing of tomato), apple and grapes (juice), spent coffee grounds (coffee preparation), as well as citrus and potato peels. This calls for the need for investigating those lignocellulosic feedstock options for fermentation processes.

9.2.2 Isolation of pectin and xylan degrading bacteria

Out of twenty-nine (29) isolates recovered in this study, Mucor hiemalis isolated from Bramley apple (Malus domestica) produced acidic pectinolytic and xylanolytic enzymes with higher specific activity. Enzymes produced were successfully used after immobilisation for clarification of apple juice that is acidic in nature due to presence of acids, mainly malic acid. The objectives of this project can be extended to include the production of other hydrolytic enzymes, such as cellulase, amylase, and ligninase.
Furthermore, pectinase and xylanase have also potential applications in alkaline medium such as biobleaching of paper pulp, and the bioscouring of cotton fabrics. Production of alkaline enzymes require isolation of pectin and xylan degrading bacteria which in itself comprise as a topic of extensive research.

Moreover, the advent and proliferation of recombinant DNA technology has enabled scientists to clone and mass produce enzymes of any origin in microbes to fit the demand of various industries. Strain improvement via mutation is another technology widely employed in industry to increase the yield of enzyme production. Such advanced approach has proven to be important to industry and represent possible direction for future research.

9.2.3 The application of magnetic nanoparticles in food industry

This project provided a proof of concept study that iron oxide nanoparticles from Aspergillus flavus (isolated from Strawberry) have potential as enzyme carrier. However, this study has not investigated the safety of magnetic nanoparticles and their use in food industry applications. Therefore, cellular uptake, trafficking and potential toxicity of ingested MNPs in the human gastrointestinal route would warrant further in vitro-in vivo investigations prior to application of MNPs in food industry.

In fact, there is a lack of adequate standard methods for nanoparticles testing under reproducible and realistic conditions to predict the fate and toxicity of MNPs in the human gastrointestinal tract and the external environment. Thus, only after going through safety trials of MNPs, potential applications of MNPs in food applications is recommended.
Back to the big picture, it is worth noting that successful commercialisation of lignocellulose-based biorefining requires policy support, optimisation of feedstock supply chain, and assessing the economic feasibility in a large-scale production scenario, which itself comprises further directions for future research. Although politics and economics were not included in the project scope, below are some important considerations to support the biorefining nascent industry in the EU.

**9.2.4 Policies**

European Commission initiatives, such as Projects-for-Policy (P4P), aims to use results from research and innovation projects to shape policy making. In this context, P4P (2018) published reports have recommended policy measures to unlock the unexploited potential of industrial waste streams, and to enhance circular utilisation of resources. Moreover, independent alliances, such as the European Bioeconomy Alliance, have requested revision of the bioeconomy strategy to ensure that biorefineries and related technologies become an integral part of EU level policies. Recently, the commission expert group on bio-based products in the EU reported that progress in the development of a renewables-based economy is at risk of being slower than the rest of the world in achieving the targeted shift to a renewables-based economy. As a result, the expert group also recommended the revision of the EU bioeconomic strategy.

**9.2.5 Sustainable feedstock logistics**

The results suggest that brewing waste (BSG) can be utilised for production of industrially important enzymes such as pectinase and xylanase. However, from a commercial point of view, the logistical challenge surrounding the feedstock supply recommend several future research directions. Areas for future research should include
the development of integrated logistical models to remove supply chain bottlenecks, the availability of machinery which is capable of handling large amounts of feedstock, the mapping of biomass inventories, and the establishment of a centralized regional hub for biomass collection and storage.
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Poster / Oral Presentations


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2018  GRSO1005 Introduction to statistics
2019  GRSO1012 Research Integrity

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2018  FDSC40250 Hot topics in the agri-food sector
2018  FDSC40160 Innovation and Creativity for the Agri-Food Researcher
2018  Career Development and Management the Agri Food Sector

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