Environmental Protection Agency
An Ghníomhaireacht um Chaomhnú Comhshaoil

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The Use of Novel Enzymes for the Cleaner, Greener Production of Cellulosic Bioethanol

STRIVE

Environmental Protection Agency Programme

2007-2013

SSTI Strategy for Science, Technology and Innovation

Comhshaol, Pobal agus Rialtas Áitiúil
Environment, Community and Local Government

Environmental Protection Agency

The Environmental Protection Agency (EPA) is a statutory body responsible for protecting the environment in Ireland. We regulate and police activities that might otherwise cause pollution. We ensure there is solid information on environmental trends so that necessary actions are taken. Our priorities are protecting the Irish environment and ensuring that development is sustainable.

The EPA is an independent public body established in July 1993 under the Environmental Protection Agency Act, 1992. Its sponsor in Government is the Department of the Environment, Community and Local Government.

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- Office of Environmental Enforcement
- Office of Environmental Assessment
- Office of Communications and Corporate Services

The EPA is assisted by an Advisory Committee of twelve members who meet several times a year to discuss issues of concern and offer advice to the Board.

EPA STRIVE Programme 2007–2013

The Use of Novel Enzymes for the Cleaner, Greener Production of Cellulosic Bioethanol

Application of Novel Enzymes Derived from Thermoacidophiles to Second-Generation Biofuel Production

(2009-ET-MS-9-S2)

STRIVE Report

Prepared for the Environmental Protection Agency

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DISCLAIMER

The EPA STRIVE Programme addresses the need for research in Ireland to inform policymakers and other stakeholders on a range of questions in relation to environmental protection. These reports are intended as contributions to the necessary debate on the protection of the environment.

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Executive Summary

The development of renewable sources of liquid fuels as an alternative to fossil fuels is receiving increasing global attention and investment due to long-term environmental and economic concerns. Climate change is considered to be one of the most significant and challenging issues currently facing humanity. Increased atmospheric levels of greenhouse gases (GHGs), such as carbon dioxide, increase the amount of energy trapped in the atmosphere and associated global impacts include increased temperatures, melting of snow and ice, increasing global average sea level and extreme weather. The transportation sector currently accounts for approximately 50% of global oil consumption and produces roughly 25% of global-energy-related carbon dioxide emissions. In Ireland, transport is the third largest contributor to overall emissions at 18.9% of the total. Developing renewable energy is an integral part of climate change strategy. The United Nations Framework Convention on Climate Change and its Kyoto Protocol provide the basis for international action to address climate change. The Kyoto Protocol establishes binding targets, which in the case of Ireland is to limit emissions to no more than an annual average of 62.8 million tonnes carbon dioxide equivalents per annum during the period 2008–2012. The European Commission Roadmap for achieving a competitive, low-carbon economy by 2050 points to European Union (EU)-wide GHG emission reduction requirements of up to 80% by 2050. Initiatives taken to combat climate change include mandating increased use of renewable transport fuels. Directive 2009/28/EC on the promotion of the use of energy from renewable sources (Renewable Energy Directive), issued by the European Parliament in 2009, sets mandatory targets for EU Member States to ensure a share of 10% renewable energy in the transport sector in 2020. In the United States, the Renewable Fuels Standard, first established in 2005, mandates that the total volume of biofuels increases from 15 billion litres in 2006 to 136 billion litres in 2022. Other countries have also adopted blending targets, mandates and biofuel quotas.

In addition to reducing GHG emissions and contributing towards the protection of the environment, renewable energy also reduces dependency on fossil fuels, improves security of energy supply and provides green jobs to the economy. At present, crude oil supplies nearly all of the world's transportation fuel needs as well as a major portion of the material and chemical needs. The rising world population, depletion of fossil fuels, diminishing oil reserves and increased oil demand are therefore causing concerns about the security of energy supplies in the future. This, combined with increasing oil prices and vulnerability to any market disturbance has led to calls for reduced dependency on fossil fuels and diversification of fuel sources. Biofuels support economic growth by creating green jobs and new sources of income, thus contributing to national competitiveness and economic activity. A study on next-generation ethanol in Europe concludes that by producing ethanol for transport fuel from plant waste left after harvesting of crops, the EU could, with the right government policies, have a next-generation ethanol industry with sales of €31 billion by 2020. The *Strategy for Renewable Energy: 2012–2020*, published by the Department of Communications, Energy and Natural Resources in May 2012[1], identifies the major opportunity for economic growth and employment creation in Ireland afforded by the renewable energy sector. The employment growth potential of the renewable energy sector is also outlined in the *Action Plan for Jobs* strategy published by the Government in 2012[2]. Furthermore, *Food Harvest 2020*[3] refers to the alternative biofuel-related uses that exist for cereals, the additional income that a non-food crops industry would provide to Irish farmers and the contribution that

1. *Strategy for Renewable Energy: 2012–2020*. http://www.dcenr.gov.ie/NR/rdonlyres/9472D68A-40F4-41B8-B8FD-F5F788D4207A/0/RenewableEnergyStrategy2012_2020.pdf
2. *Action Plan for Jobs*, *2012*. http://www.djei.ie/ publications/2012APJ.pdf
3. *Food Harvest 2020*, http://www.agriculture.gov.ie/agri-foodindustry/foodharvest2020/

Irish farmers can make towards meeting government targets and policies in the bioenergy sector.

Currently, biofuels account for around 2% of total transport fuel and are almost all first-generation biofuels, produced primarily from food crops such as grains, sugar beet and oil seeds. In recent years, the viability of such first-generation biofuels has come under increasing criticism due to limited GHG reduction benefits, their contribution to rising food prices and impacts of direct and indirect land-use changes. Attention has therefore shifted to second-generation biofuels produced from lignocellulosic biomass feedstocks, such as crop residues (for example cereal straws, corn stover), agricultural processing by-products (rice hulls, sugar cane bagasse) and forest and sawmill residues. The use of non-food biomass avoids competition with food production and second-generation biofuels are also superior to first-generation biofuels in terms of energy balances, GHG emission reductions and land-use requirements. As a result, criteria set out in many support policies explicitly favour the use of second-generation biofuels and therefore emphasis is on achieving widespread deployment of such fuels.

The production of second-generation bioethanol from lignocellulose (cellulosic bioethanol) is, however, technically challenging as the main components of lignocellulose (cellulose, hemicellulose and lignin) are intimately associated with each other, resulting in a structure that is highly resistant to degradation. Current biochemical production processes involve an initial pretreatment step to disrupt the lignocellulose structure, followed by enzymatic hydrolysis of the cellulose and hemicellulose components to their constituent monomeric sugars which can then be fermented to produce bioethanol. Improving this process is essential to achieve widespread deployment of cellulosic bioethanol and maximising the efficiency of the pretreatment step is considered a key area for improvement. Several currently used pretreatment methods, for example dilute-acid pretreatment, involve using high temperatures (130–260°C) and acidic pH conditions. The severity of these conditions results in the formation of sugar degradation products that represent a loss of fermentable sugars and have a negative effect on subsequent process steps. Such pretreatment methods require high energy inputs and expensive construction materials (due to the corrosive conditions employed) and, following the pretreatment step, significant pH adjustment and washing of the pretreated biomass is necessary prior to the enzymatic hydrolysis step. The focus of this project was to develop a novel, cleaner, greener and more effective pretreatment method based on using thermoacidophilic lignocellulose-degrading enzymes in combination with a lower temperature (<100°C) and lower acid concentration. This would reduce the severity of pretreatment and have the following potential advantages:

- Reduced temperature and, hence, energy requirements;
- Reduced formation of degradation products;
- Reduced requirements for washing of the pretreated biomass;
- Reduced acid requirements;
- Reduced requirement for pH adjustment prior to hydrolysis;
- Lower corrosion potential and therefore lower capital costs;
- Reduced energy and acid costs;
- Improved safety; and
- Reduced environmental impact.

Screening of 65 micro-organisms, including bacteria, fungi and archaea, was undertaken to identify strains producing lignocellulose-degrading enzymes active under the high temperature and low pH conditions of the proposed pretreatment method. Based on application-relevant assessment of the enzymes produced, the five micro-organisms producing enzymes most suitable for the proposed pretreatment method were selected. These microbial strains are referred to as Strains A–E in order to protect potential intellectual property rights, which are currently being explored and the Environmental Protection Agency will be kept informed of any such intellectual property developments underpinned by this project. The enzymes produced by two of these strains were found

to be of particular interest on the basis of their high temperature optima (80–100°C) and substantial activity at low pH (<4). The enzymes produced by one of these micro-organisms were selected for further lab-scale pretreatment studies to determine the feasibility of the proposed enzymatic pretreatment method. When pretreatment was undertaken at 80°C for 3 h, using a low level of enzyme in 0.15% sulphuric acid, the amount of reducing sugars produced during the pretreatment step was similar to that produced by 0.25% sulphuric acid, indicating that, under the conditions used, a 40% reduction in acid usage was achieved by inclusion of low levels of the enzyme. Following pretreatment (80°C; 0.15% acid), straw pretreated with enzyme was more digestible than straw pretreated under similar conditions without enzyme, particularly in terms of xylose production. Enzymatic/Dilute-acid pretreatment was compared at lab scale to conventional dilute-acid pretreatment. At the enzyme dosage levels used, the enzymatic/dilute-acid pretreatment method produced less reducing sugars during the pretreatment step. However, the digestibility of the straw pretreated by both methods was comparable as judged by the amount of reducing sugars produced during subsequent hydrolysis. Further optimisation of the enzymatic pretreatment method using higher enzyme dosage levels is necessary to determine industrial applicability.

During this project significant advances were made towards developing a novel, cleaner and greener enzymatic pretreatment method, particularly with regard to identifying suitable enzymes. While further optimisation of this method using a more industrially relevant, increased enzyme dosage level is necessary to facilitate more extensive investigation of the efficiency of the method, the initial results observed with low enzyme dosage levels are promising. If satisfactory pretreatment is observed upon such optimisation, the developed pretreatment method, undertaken at a lower temperature of 80°C and using five times less sulphuric acid, potentially offers several environmental, technical and health and safety-related benefits as outlined above. In addition to the enzymes used to develop the enzymatic pretreatment method, the project also resulted in the identification of several other enzymes of potential application to improve both the pretreatment and subsequent hydrolysis steps of the process. Overall, the work undertaken demonstrates the potential of thermo(acido)philic enzymes to overcome many of the technical issues associated with the cellulosic bioethanol production process. The project has expanded University of Limerick expertise in this area and has built capacity for further applied research aimed at improving the cellulosic bioethanol production process using enzymes produced by thermoacidophiles. Improvement of this process is essential to facilitate wide-scale deployment of second-generation bioethanol, with subsequent benefits in terms of GHG emissions, energy security, job creation and economic growth. Such widespread deployment of second-generation biofuels is in line with global and European renewable energy strategy and policies and, on a national level, contributes to the development of a resource-efficient, sustainable green economy and to the overall objectives of the *National Renewable Energy Action Plan*[4], Strategy for Renewable Energy 2012–2020 and Food Harvest 2020.

4. *National Renewable Energy Action Plan.* http://www.dcenr.gov.ie/NR/rdonlyres/C71495BB-DB3C-4FE9-A725-0C094FE19BCA/0/2010NREAP.pdf

1 Introduction

1.1 Research Drivers

1.1.1 Protection of the environment, energy security and economic growth

The development of renewable sources of liquid fuels as an alternative to fossil fuels is receiving increasing global attention and investment due to long-term environmental and economic concerns. Renewable energy contributes towards the protection of the environment by reducing greenhouse gas (GHG) emissions and also reduces dependency on fossil fuels, improves security of energy supply and provides green jobs to the economy (EurActiv, 2009; Dennehy et al., 2010; NREAP, 2010).

Climate change is considered to be one of the most significant and challenging issues currently facing humanity. Increased atmospheric levels of GHGs, such as carbon dioxide (CO_2), increase the amount of energy trapped in the atmosphere and associated global impacts include increased temperatures, melting of snow and ice, increasing global average sea level and extreme weather (EPA, 2012, 2013). The transportation sector currently accounts for approximately 50% of global oil consumption and produces roughly 25% of global energy-related carbon dioxide emissions (IEA, 2010). In Ireland, transport is the third largest contributor to overall emissions, at 18.9% of the total (EPA, 2012). Developing renewable energy is an integral part of climate change strategy.

Biofuels reduce dependence on fossil fuels and improve security of supply (DCENR, 2012). At present, crude oil supplies nearly all of the world's transportation fuel needs as well as a major portion of the material and chemical needs (Foust et al., 2008). It is projected that the total world demand for oil will rise by 1% per year, while the oil import dependency of OECD–Europe[1] is expected to increase from 65% in 2007 to 83% in 2030 (Gnansounou, 2010). The rising world population, depletion of fossil fuels, diminishing oil reserves and increased oil demand are therefore causing concerns about the security of energy supplies in the future (Foust et al., 2008; Travers, 2010). This combined with increasing oil prices and vulnerability to any market disturbance has led to calls for reduced dependency on fossil fuels and diversification of fuel sources (Travers, 2010).

Biofuels also support economic growth by creating jobs and new sources of income (IEA, 2011). Global biofuel production increased from 16 billion litres in 2000 to more than 100 billion litres in 2010 (IEA, 2011). Bloomberg New Energy Finance (2011) forecasts second-generation biofuel production to increase more than 10% on average every year between 2010 and 2030. In 2010, about 40% ($351 million) of industrial biotech venture capital invested in the United States of America went into research on biofuels, with several large investments in second-generation processes (Gerecke and Pohl-Apel, 2012). Global new investment in biofuels in 2011 was $6.8 billion, with positive signs towards second-generation biofuel and it is forecasted that 95% of the total investment ($510 billion) in biofuel infrastructure will target second-generation facilities over 2011–2030 (Bloomberg New Energy Finance, 2011, 2012). A study on next-generation ethanol in Europe concludes that by producing ethanol for transport fuel from plant waste left after harvesting of crops, the European Union (EU) could, with the right government policies, have a next-generation ethanol industry with sales of €31 billion by 2020. The *Strategy for Renewable Energy: 2012–2020* published by the Department of Communications, Energy and Natural Resources in May 2012 identifies the major opportunity for economic growth and employment creation in Ireland afforded by the renewable energy sector (DCENR, 2012). The employment growth potential of the renewable energy sector is also outlined in the *Action Plan for Jobs* strategy published by the Government in 2012 (DJEI,

1. OECD-Europe comprises all European members of the Organisation for Economic Co-operation and Development (OECD) (not necessarily EU members). In 2012 these were Austria, Belgium, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Luxembourg, the Netherlands, Norway, Poland, Portugal, Slovak Republic, Slovenia, Spain, Sweden, Switzerland, Turkey and United Kingdom.

2012). Furthermore, *Food Harvest 2020* refers to the alternative biofuel-related uses that exist for cereals, the additional income that a non-food crops industry would provide to Irish farmers and the contribution that Irish farmers can make towards meeting government targets and policies in the bioenergy sector (DAFF, 2010).

1.1.2 The policy context – relevant European and national policy

The United Nations Framework Convention on Climate Change (UNFCCC) and its Kyoto Protocol provide the basis for international action to address climate change. The Kyoto Protocol establishes binding targets, which in the case of Ireland is to limit emissions to no more than an annual average of 62.8 million tonnes carbon dioxide equivalents per annum during the period 2008–2012, which equates to a 13% increase on the 1990 baseline year (EPA, 2012, 2013). The European Commission Roadmap for achieving a competitive, low-carbon economy by 2050 points to EU-wide GHG emission reduction requirements of up to 80% by 2050 (EPA, 2012). Initiatives taken to combat climate change include mandating increased use of renewable transport fuels. Directive 2009/28/EC on the promotion of the use of energy from renewable sources (Renewable Energy Directive, RED), issued by the European Parliament in 2009, sets mandatory targets for EU Member States to ensure a share of 10% renewable energy in the transport sector in 2020 (EU, 2009). In the United States, the Renewable Fuels Standard (RFS) mandates that the total volume of biofuels increases from 15 billion litres in 2006 to 136 billion litres in 2022 (IEA, 2010). Other countries have also adopted blending targets, mandates and biofuel quotas (IEA, 2011).

Renewable energy strategy and objectives in Ireland are set firmly in the global and European context and are in line with the ambitions set by the EU and the International Energy Agency (IEA) (DCENR, 2012). As outlined above, Ireland is legally obliged to achieve a target of 10% renewable energy in transport by 2020. This will require a steady, progressive and measurable increase in renewable energy use in the transport sector (DCENR, 2012). The National Biofuels Obligation scheme, introduced in 2010, requires companies that sell road transport fuel to have an average of 4% biofuels in their annual fuel sales. In 2011, the biofuel market in Ireland required over 200 million litres of biofuel. The 2009 RED requires each Member State to submit a National Renewable Energy Action Plan (NREAP), detailing the ongoing and planned measures to meet the mandatory 2020 NREAP describes a two-pronged strategy involving significant increases in the use of biofuels and the accelerated development and use of electric vehicles (NREAP, 2010). Biofuels in transport have increased from 0.03% in 2005 to 2.4% in 2010 in energy terms (EPA, 2012). The EU has also published an Energy 2050 Roadmap, which indicates that a substantial increase in renewable energy deployment in Europe will be required well over and above the 2020 targets (DCENR, 2012).

1.1.3 Development of second-generation biofuels

Currently, biofuels account for around 2% of total transport fuel (IEA, 2011) and are almost all first-generation biofuels, produced primarily from food crops such as grains, sugar beet and oil seeds (IEA, 2010). In recent years, the viability of such first-generation biofuels has come under increasing criticism due to limited GHG reduction benefits, their contribution to rising food prices and impacts of direct and indirect land-use changes (IEA, 2008; Agbor et al., 2011). Attention has therefore shifted to second-generation biofuels produced from lignocellulosic biomass feedstocks. Potential lignocellulosic biomass feedstocks include crop residues (for example cereal straws, corn stover), agricultural processing by-products (rice hulls, sugar cane bagasse), forest and sawmill residues, energy crops (*Miscanthus*, reed canary grass, switchgrass), cellulose wastes (newspaper, waste office paper) and municipal solid wastes (MSWs) (Sánchez and Cardona, 2008; Hayes and Hayes, 2009; Saha and Cotta, 2010). The use of non-food biomass avoids competition with food production and second-generation biofuels are also superior to first-generation biofuels in terms of energy balances, GHG emission reductions and land-use requirements (IEA, 2008). United States Environmental Protection Agency (US EPA) analysis showed that second-generation bioethanol generates 91% less GHG than fossil-based petrol or diesel in transport applications in comparison with 22% for corn-

based ethanol (Menon and Rao, 2012). As a result, criteria set out in many support policies explicitly favour the use of second-generation biofuels, for example definition of minimum GHG savings for biofuels, higher weighting of second-generation biofuels and social and environmental sustainability criteria in the RED. Similarly, the RFS specifies a blending mandate for second-generation biofuel from 2010 on, which requires an increase in the consumption of lignocellulosic ethanol to 60.6 billion litres per year in 2022 (IEA, 2010). Emphasis is therefore on the widespread deployment of second-generation biofuels. Biofuels are projected to provide 9% and 27% of total transportation fuel by 2030 and 2050, respectively (IEA, 2010, 2011). The IEA therefore projects a rapid increase in biofuel demand, particularly for second-generation biofuels, which are expected to account for roughly 90% of all biofuel in 2050 (IEA, 2010).

1.2 Lignocellulose

Lignocellulose, the major structural component of plant cell walls, is composed primarily of cellulose, hemicellulose and lignin. Cellulose and hemicellulose are polysaccharides composed of various sugars, while lignin is an aromatic polymer. The relative proportions of cellulose, hemicellulose and lignin present in lignocellulosic material vary significantly between different sources but are generally in the range 40–50% cellulose, 25–30% hemicellulose and 15–20% lignin (Sánchez, 2009; Menon and Rao, 2012). Cellulose is a linear, unbranched homo-polysaccharide made up of D-glucose subunits linked by β-1,4-glycosidic bonds (Walsh, 2002). Hemicelluloses are complex heterogeneous polysaccharides composed of various residues, including pentoses (D-xylose and D-arabinose), hexoses (D-mannose, D-glucose and D-galactose), uronic acids (4-*O*-methylglucuronic and glucuronic acids) and acetylated derivatives (Jorgensen et al., 2007). Hemicelluloses have a branched structure composed of a linear backbone, which may be a homo- or heteropolymer, to which single sugars or short chains of sugars are attached. In some cases, acetyl, feruloyl and cinnamoyl groups are esterified to these backbone or side-chain sugars (Jovanovic et al., 2009; Kumar et al., 2009). Lignin is a polyphenolic, amorphous, cross-linked polymer composed of *p*-hydroxyphenyl, guaiacyl and syringyl moieties (Weng et al., 2008; Simmons et al., 2010). Cellulose and non-cellulosic polysaccharides are intimately associated with each other and with lignin and other polymers, such as proteins, to form the structural framework of the plant cell wall. The resulting structure is naturally recalcitrant to biological degradation and represents a tough physical barrier which evolved to provide protection against pathogen attack (Williamson et al., 1998; da Costa Sousa et al., 2009).

The enzymes involved in the depolymerisation of cellulose are broadly referred to as cellulases. Three enzymes act synergistically to degrade cellulose – endo-1,4-β-glucanase, cellobiohydrolase and β-glucosidase. A larger range of enzymes is required for the hydrolysis of hemicellulose due to its diverse structure and the variety of chemical linkages involved. These include the depolymerising activities of endo-1,4-β-xylanase and β-xylosidase, which cleave the main xylan chain, and various debranching or accessory enzymes, which remove the side groups, for example α-glucuronidase, acetyl xylan esterase, α-L-arabinofuranosidase and ferulic acid esterase activities (Yeoman et al., 2010). The sites of enzymatic attack of the main enzyme activities involved in the hydrolysis of cellulose and xylan and details of these enzymes are shown in Fig. 1.1 and Table 1.1, respectively.

Straw (barley or wheat) was selected as feedstock for initial development of the proposed pretreatment method mainly on the basis of its low lignin content (14–21%) and abundant availability in Ireland and Europe. In addition, its suitability as a feedstock for the production of second-generation bioethanol has been confirmed and it represents one of the main feedstocks used in current near-commercial-scale operations.

1.3 Production of Bioethanol from Lignocellulose via the Biochemical Route

Second-generation bioethanol can be produced by a biochemical process involving the enzymatic hydrolysis of pretreated lignocellulose with subsequent fermentation of the resulting sugars to ethanol, as shown in Fig. 1.2. The conversion of lignocellulose to

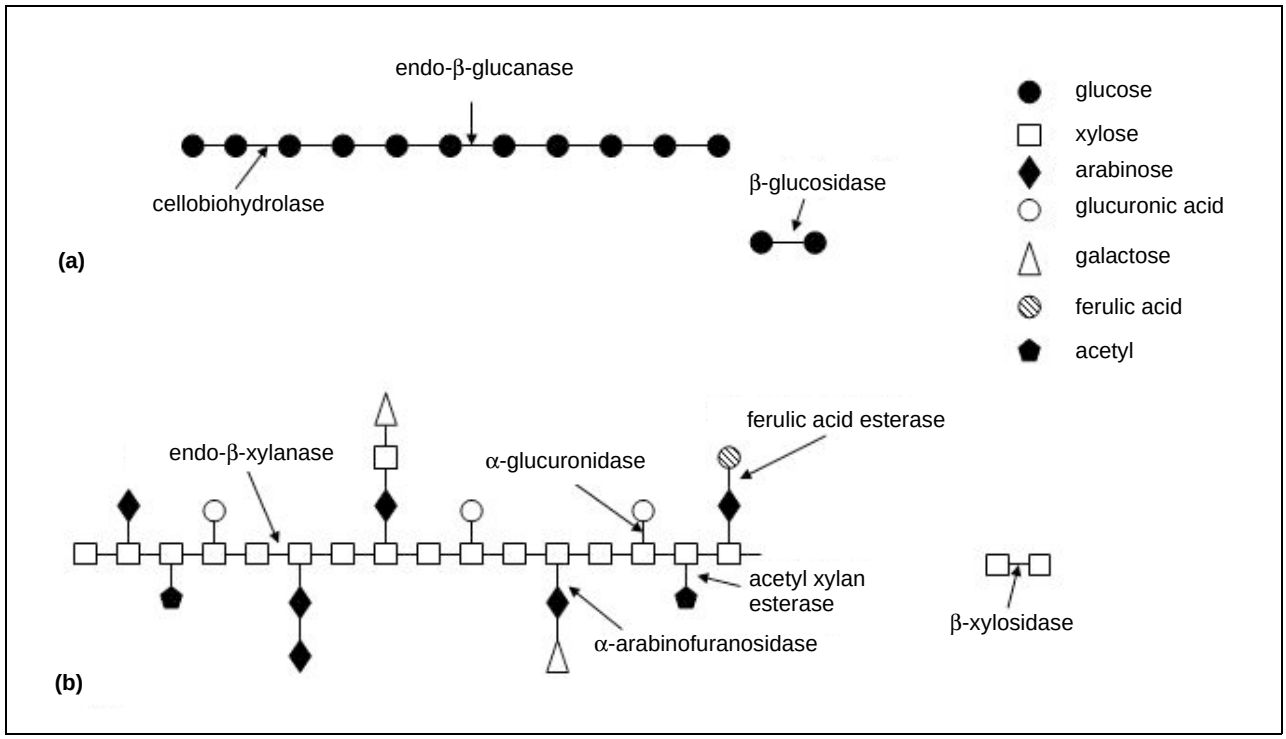

Figure 1.1. Simplified schematic representative structures of (a) cellulose and (b) xylan showing sites of enzymatic attack of the main enzyme activities involved in the hydrolysis of these structures.

Table 1.1. Enzymes involved in the hydrolysis of cellulose and xylan. Data from several online enzyme databases ((ExPASY, the Expert Protein Analysis System (http://www.expasy.org), BRENDA, the Comprehensive Enzyme Information System (http://www.brenda.uni-koeln.de), ExplorEnz – The Enzyme Database (http://www.enzyme-database.org)), (Saha, 2003; Decker et al., 2008).

Number	Name	Reaction catalysed
EC 3.2.1.4	Endo-1,4-β-glucanase	Endohydrolysis of β-1,4-glycosidic linkages in cellulose
EC 3.2.1.91	Cellobiohydrolase	Hydrolysis of β-1,4-glycosidic linkages releasing cellobiose from the chain ends
EC 3.2.1.21	β-Glucosidase	Hydrolysis of cellobiose producing glucose
EC 3.2.1.8	Endo-1,4-β-xylanase	Endohydrolysis of β-1,4-xylosidic linkages in xylans
EC 3.2.1.37	1,4-β-Xylosidase	Hydrolysis of xylobiose and other short-chain xylo-oligosaccharides releasing xylose units from the non-reducing end
EC 3.2.1.55	α-L-Arabinofuranosidase	Hydrolysis of terminal non-reducing α-L-arabinofuranoside residues in α-L-arabinosides
EC 3.2.1.139	α-Glucuronidase	Hydrolysis of α-1,2 bonds between glucuronic acid residues and xylose backbone units, resulting in the release of glucuronic acid from xylan
EC 3.2.1.72	Acetyl xylan esterase	Deacetylation of xylans and xylooligosaccharides; hydrolysis of acetyl ester groups from D-xylopyranosyl residues
EC 3.2.1.73	Feruloyl esterase (ferulic acid esterase)	Hydrolysis of the ester bond between arabinose side-chain residues and ferulic acid

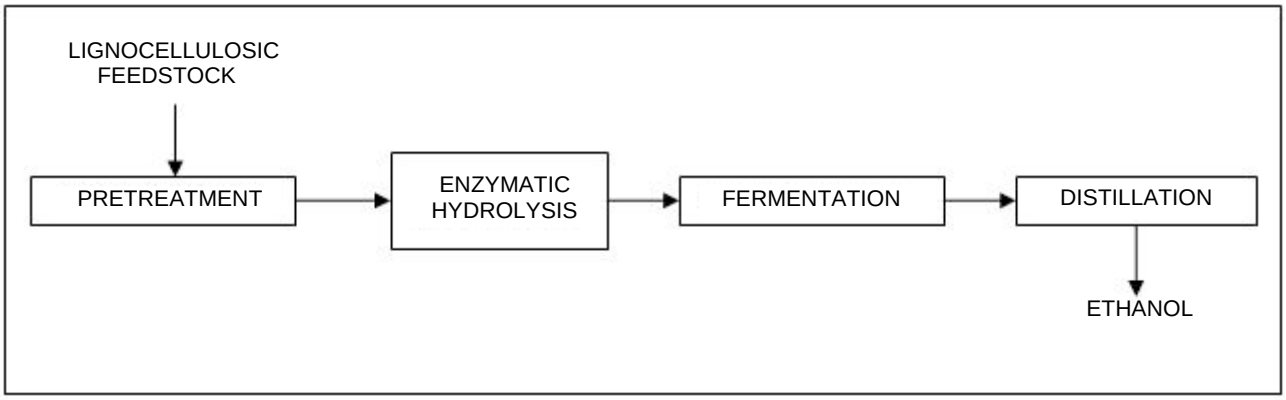

Figure 1.2. Overview of the process used for the production of second-generation bioethanol via the biochemical route.

ethanol involves the following major steps (Yang and Wyman, 2008; Kumar et al., 2009; Gamage et al., 2010):

1. Size reduction and pretreatment to modify the structure of lignocellulose and facilitate enzyme access;
2. Hydrolysis of cellulose (and depending on the pretreatment method, hemicellulose) to generate fermentable monomeric sugars;
3. Fermentation of the monomeric sugars by bacteria, yeast or filamentous fungi to form ethanol; and
4. Separation/Purification of the ethanol produced by distillation or other separation techniques.

The aim of pretreatment is to decrease the recalcitrance of biomass by modifying the lignocellulose structure, thereby producing a substrate which is more accessible to enzymes and can be more efficiently hydrolysed in the subsequent hydrolysis step (Margeot et al., 2009; Alvira et al., 2010). Pretreatment affects all other operations in the process and is considered to be one of the major costs (Mosier et al., 2005; da Costa Sousa et al., 2009; Alvira et al., 2010). A variety of different pretreatment technologies is available and these are based on different chemistries, operate under different conditions with respect to temperature, reaction time, etc., and have different effects on the composition and physico-chemical structure of lignocellulose (Johnson and Elander, 2008; Alvira et al., 2010). Each pretreatment method has its advantages and disadvantages, with none considered ideal. Several studies have identified dilute acid pretreatment, steam explosion with catalyst addition and liquid hot water pretreatment as the most promising pretreatments with dilute acid pretreatment and steam explosion considered closest to commercialisation and the pretreatments of choice in several near-commercialisation facilities (IEA, 2008; Sánchez and Cardona, 2008).

During hydrolysis, cellulose and hemicellulose are hydrolysed into their monomers to produce a hydrolysate suitable for fermentation (Mosier et al., 2005). Hydrolysis of cellulose and hemicellulose can be achieved by chemical (mainly acid) or enzymatic hydrolysis, with the enzymatic process considered to be the more effective and promising technology and, hence, is the most commonly used (Hahn-Hagerdal et al., 2006; Kumar et al., 2009; Gamage et al., 2010). The enzymatic hydrolysis of cellulose and hemicellulose to fermentable monosaccharides requires multiple enzyme activities. Endoglucanases, cellobiohydrolases and β-glucosidases are required to convert cellulose to glucose. The requirement for hemicellulase activity is dependent on the feedstock and pretreatment approach employed. Enzymatic hydrolysis is carried out at around 50°C and pH 5 using commercial enzyme complexes with multiple enzyme activities. In recent years, considerable progress has been made by enzyme manufacturers in reducing enzyme cost and in developing more efficient enzyme products (Foust et al., 2008).

From an economic perspective, sugars from both cellulose and hemicellulose should be used for bioethanol production and therefore it is favourable to use micro-organisms capable of fermenting both five- and six-carbon sugars in the fermentation step (Gamage et al., 2010). Distillation or distillation combined with adsorption is undertaken to recover the ethanol from the fermentation broth (Mosier et al., 2005). The solid part of the biomass remaining contains lignin, which may be burnt to provide heat and electricity for the process and/or may potentially be converted to higher-value products (Hahn-Hagerdal et al., 2006; Wyman, 2007). The use of lignin to provide heat and power for the ethanol production facility reduces the requirement for fossil or other external energy and results in a favourable energy balance and superior GHG emission features (Yang and Wyman, 2008).

Significant efforts in research, development and demonstration are currently being undertaken to facilitate commercialisation and wide-scale deployment of second-generation bioethanol. Several pilot and demonstration facilities have been developed with ambitious plans for commercialisation and the first large-scale plants demonstrating this technology are now coming into production (IEA, 2011).

1.4 Thermoacidophilic Enzymes in Second-Generation Bioethanol Production and Rationale for this Project

Thermoacidophiles are micro-organisms that grow at high temperatures (55–95°C) and low pH (0.0–4.0) (Bertoldo et al., 2004). Such micro-organisms are found mainly among the archaea, although some bacteria growing under these conditions have also been isolated. Examples include the archaea *Sulfolobus solfataricus* and *Picrophilus torridus*, with optimal growth conditions of 80°C, pH 3 and 60°C, pH 0.7, respectively, and the bacterium *Alicyclobacillus acidocaldarius*, which grows optimally at 60°C, pH 4 (Bertoldo et al., 2004; Miller and Blum, 2010). Enzymes produced by thermophiles (micro-organisms growing at >55°C) are generally active at high temperatures and are highly thermostable (Turner et al., 2007). Furthermore, proteins secreted into the acidic growth medium by thermoacidophiles and membrane proteins displayed on the outer surface of the cell are usually adapted to low pH and exhibit optimal activity under acidic pH conditions as well as stability upon prolonged exposure to low pH (Miller and Blum, 2010).

Several publications have identified the potential benefits of using thermostable enzymes in the production of bioethanol from lignocellulose, with emphasis mainly on the hydrolysis step (Turner et al., 2007; Viikari et al., 2007; Hess, 2008; Miller and Blum, 2010). Hydrolysis at higher temperatures using thermostable enzymes is associated with lower viscosity, improved mixing properties, better substrate solubility, high mass transfer rate and lowered risk of contamination, while high temperatures also facilitate enhanced enzyme penetration and cell-wall disorganisation (Turner et al., 2007; Viikari et al., 2007). The high stability of thermostable enzymes facilitates enhanced hydrolysis performance, longer hydrolysis times and greater flexibility with respect to process configurations. An additional benefit of thermostable enzymes in lignocellulose hydrolysis is their reported higher specific activity, which could potentially decrease the amount of enzyme product required. These benefits could result in decreased hydrolysis costs (Viikari et al., 2007).

Miller and Blum (2010) suggest the potential to improve current pretreatment processes based on extreme heat and very low pH by adjusting to less extreme conditions with supplementation of a thermophilic and acidophilic enzyme such as the endoglucanase produced by *Sulfolobus solfataricus* with optimal activity at pH 1.8 and 80°C. United States Patent 7,727,755 (Enzyme and Methodology for the Treatment of a Biomass) describes the production of extracellular hemicellulase and cellulase activities by *Alicyclobacillus acidocaldarius* ATCC 27009. The hemicellulase activity displays optimum activity at 80°C and a pH of less than 2 and the patent identifies its usefulness in the pretreatment of a biomass to reduce the severity of pretreatment. However, experimental results from such a pretreatment approach are not reported (http://www.uspto.gov/).

1.5 Aims of this Study

The current project focused on the potential application of enzymes from thermoacidophiles in the pretreatment step. Several pretreatment methods, for example dilute-acid pretreatment, involve high temperatures (130–260°C) and acidic conditions and disadvantages associated with such methods include (Sánchez and Cardona, 2008; Kumar et al., 2009; Alvira et al., 2010; Agbor et al., 2011):

- The formation of sugar degradation products which represent a loss of fermentable monosaccharides and are inhibitory to glycolytic enzymes and fermenting micro-organisms;
- The requirement for detoxification/washing of pretreated biomass to remove such inhibitors resulting in additional cost and potential sugar loss;
- The condensation and precipitation of solubilised lignin components, which decreases digestibility;
- Energy intensive, often requiring steam-generation and pressurised systems;
- Acid at high temperature creates a corrosive environment necessitating expensive corrosion-resistant construction materials; and
- pH neutralisation is often necessary prior to the downstream enzymatic hydrolysis which results in the generation of gypsum, creating a disposal issue.

The project proposed developing an alternative novel pretreatment method based on an enzymatic pretreatment approach at less extreme conditions of temperature (<100°C) and lower acid concentration, with supplementation of appropriate lignocellulosic enzymes derived from a thermoacidophilic micro-organism (Fig. 1.3). This would reduce the severity of pretreatment and have the following potential advantages:

- Reduced acid requirements;
- Reduced temperature and hence energy requirements;
- Reduced formation of degradation products/inhibitors;
- Reduced requirements for washing/detoxification of the pretreated biomass;
- Reduced requirement for neutralisation prior to hydrolysis and hence reduced issues in relation to the disposal of neutralisation salts;
- Lower corrosion potential and therefore lower capital costs;
- Reduced energy and acid costs; and
- Improved safety and reduced environmental impact.

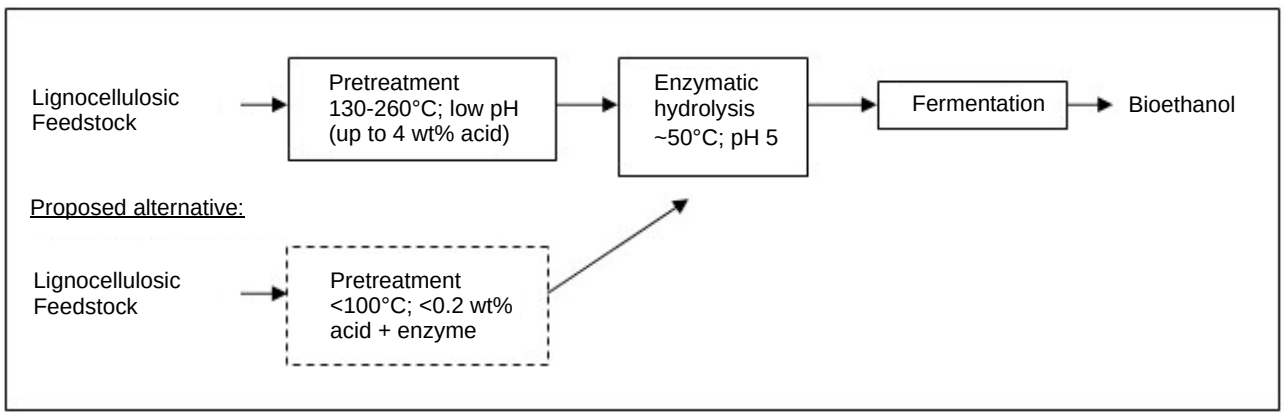

Figure 1.3. Overview of the process used for the production of second-generation bioethanol via the biochemical route with the alternative proposed pretreatment method shown in the dashed box.

2 Research Approach, Actions and Results

The commercial enzymes that are currently available for use in the enzymatic hydrolysis step generally display optimum activity in the range 45–60°C and from pH 4 to 6 and are therefore unsuitable for use in the proposed pretreatment method. Screening of a range of micro-organisms to identify lignocellulose-degrading enzymes maximally active at lower pH and higher temperatures for use in the proposed pretreatment approach was therefore necessary and is described in Section 2.1. Based on application-relevant assessment of the enzymes produced during screening, the micro-organism producing the most suitable enzymes was identified and the efficiency of the developed enzymatic pretreatment method was determined, as described in Section 2.2.

2.1 Screening of Micro-Organisms for Target Enzymes

The main enzymes of interest for the proposed application are endo-β-glucanase and endo-β-xylanase. Additional enzymes of interest are β-glucosidase, β-xylosidase, α-L-arabinofuranosidase, acetyl xylan esterase and ferulic acid esterase. Micro-organisms potentially capable of producing these lignocellulosic-degrading enzymes with high activity at high temperature and low pH were identified by searches of relevant scientific literature, patents and the enzyme information system BRENDA (http://www.brenda.uni-koeln.de), as well as analysis of published genome sequences and direct screening of environmental samples. Selected microbial strains were grown in various screening media, with the aim of inducing production of lignocellulosic-degrading enzyme activities. Appropriate enzyme assays for detection of the activities of interest were undertaken at relatively high temperature and low pH to target enzymes active under the conditions of the proposed pretreatment method. Screening of 65 micro-organisms, including environmental samples, fungi, bacteria and archaea, resulted in the identification of 19 strains producing endo-β-glucanase activities and 21 strains producing endo-β-xylanase activities of potential interest for the proposed application. In addition, several of these strains were found to produce β-glucosidase, β-xylosidase, α-D-galactosidase, α-L-arabinofuranosidase, acetyl xylan esterase and ferulic acid esterase activities. Application-relevant assessment of the crude enzymes produced was undertaken and the five strains producing the most suitable enzymes for the proposed application were identified and designated Strains A, B, C, D and E.

Upon determination of the effect of temperature and pH on enzyme activity, the cellulase and xylanase activities produced by Strains A–E were found to display a high proportion of their maximum activity at high temperature and relatively low pH (Figs 2.1 and 2.2).

Lab-scale lignocellulosic pretreatment studies were carried out to determine the ability of the crude enzyme mixtures produced by these fungi to hydrolyse straw lignocellulose at high temperature in the presence of acid.

For Strains A, B and C, pretreatment studies were undertaken by measuring the amount of reducing sugars released over time from 10% (w/v) chopped straw in 0.1%, 0.15% or 0.2% (w/v) sulphuric acid at 70°C. The enzyme inclusion level was 22–27 units cellulase activity/g straw, where enzyme activity was quantified by a standard reducing sugar assay (Miller, 1959) at 70°C and pH 4 and one unit is defined as the amount of enzyme that catalyses the production of 1 µmol reducing sugar per minute under the assay conditions. The pH of the resulting pretreatment samples ranged from 4 to 4.5 (0.1% acid), 2.9 to 3.5 (0.15% acid) and 2 to 3.2 (0.2% acid). The amounts of reducing sugars released after 30 min and 3 h are shown in Fig. 2.3 in comparison with the amount of reducing sugars released by higher concentrations of sulphuric acid only. The results observed indicate that the crude enzymes produced by Strains A, B and C are capable of hydrolysing straw lignocellulose at high temperature in the presence of acid, with the enzymes produced by Strain B resulting in the highest release of

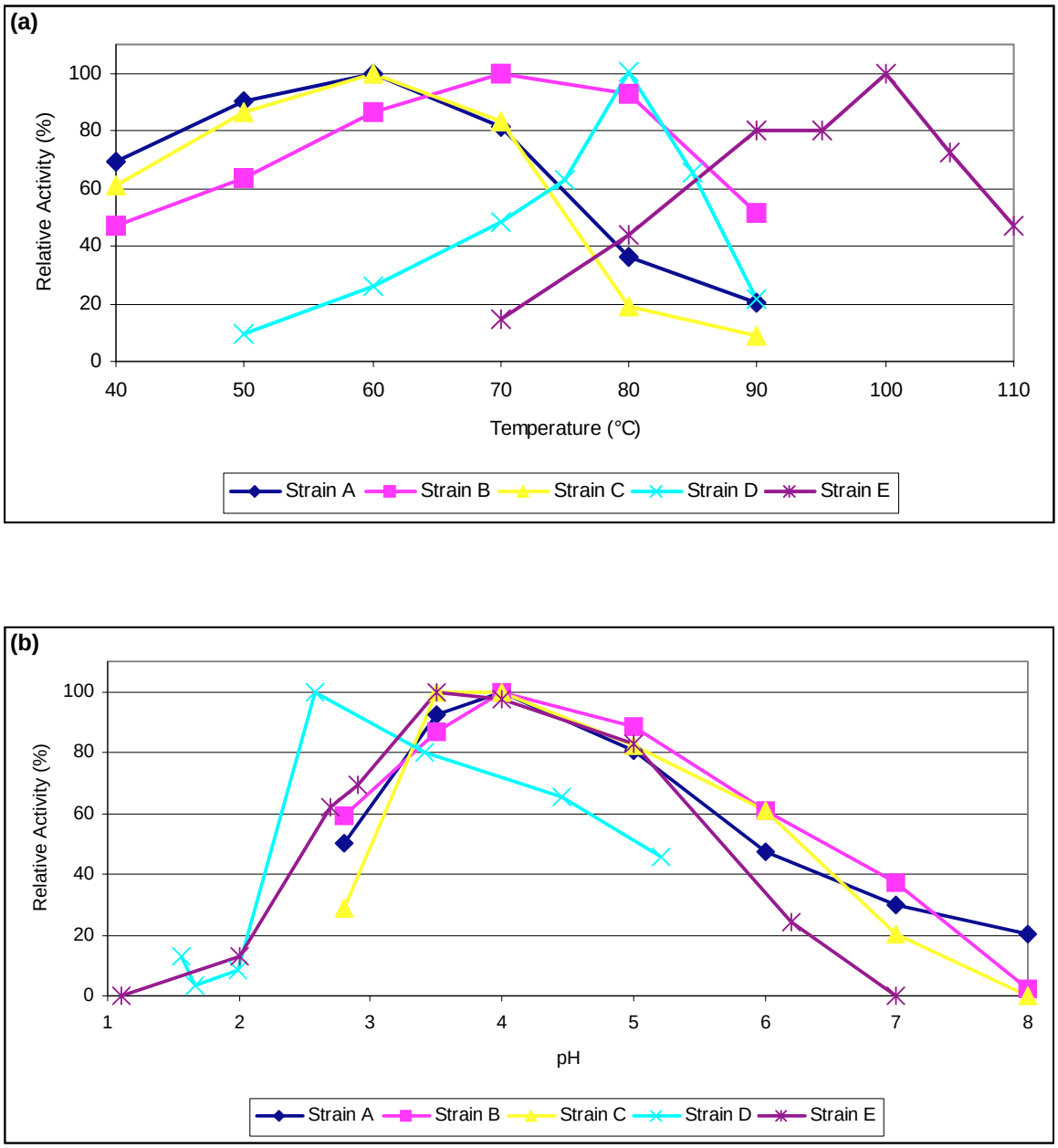

Figure 2.1. Effect of (a) temperature and (b) pH on the cellulase activities produced by Strains A–E. Data are expressed as a percentage of the maximum value. Each value represents mean ± SD (n = 3). The effect of temperature on enzyme activity was determined at pH 4 and the effect of pH on enzyme activity was determined at 60°C.

reducing sugars. At the optimum acid concentration for enzymatic hydrolysis (0.1%), the amount of reducing sugars released by the crude enzymes from Strain B after 30 min and 3 h is comparable with or greater than that released by 2.5% acid only (Fig. 2.3). This indicates that at 70°C, a 25-fold reduction of acid usage could potentially be achieved by incorporation of these crude enzymes in the acid/straw mixture. Increased enzymatic hydrolysis could potentially be achieved by inclusion of higher levels of enzyme activity.

For Strains D and E, similar pretreatment studies were undertaken at 80°C and 95°C, respectively. For Strain D, incubation of the crude enzyme (3.6 units cellulase activity/g straw, where enzyme activity was quantified by standard reducing sugar assay at 80°C and pH 4) with an acid/straw mixture containing 0.2% (w/v)

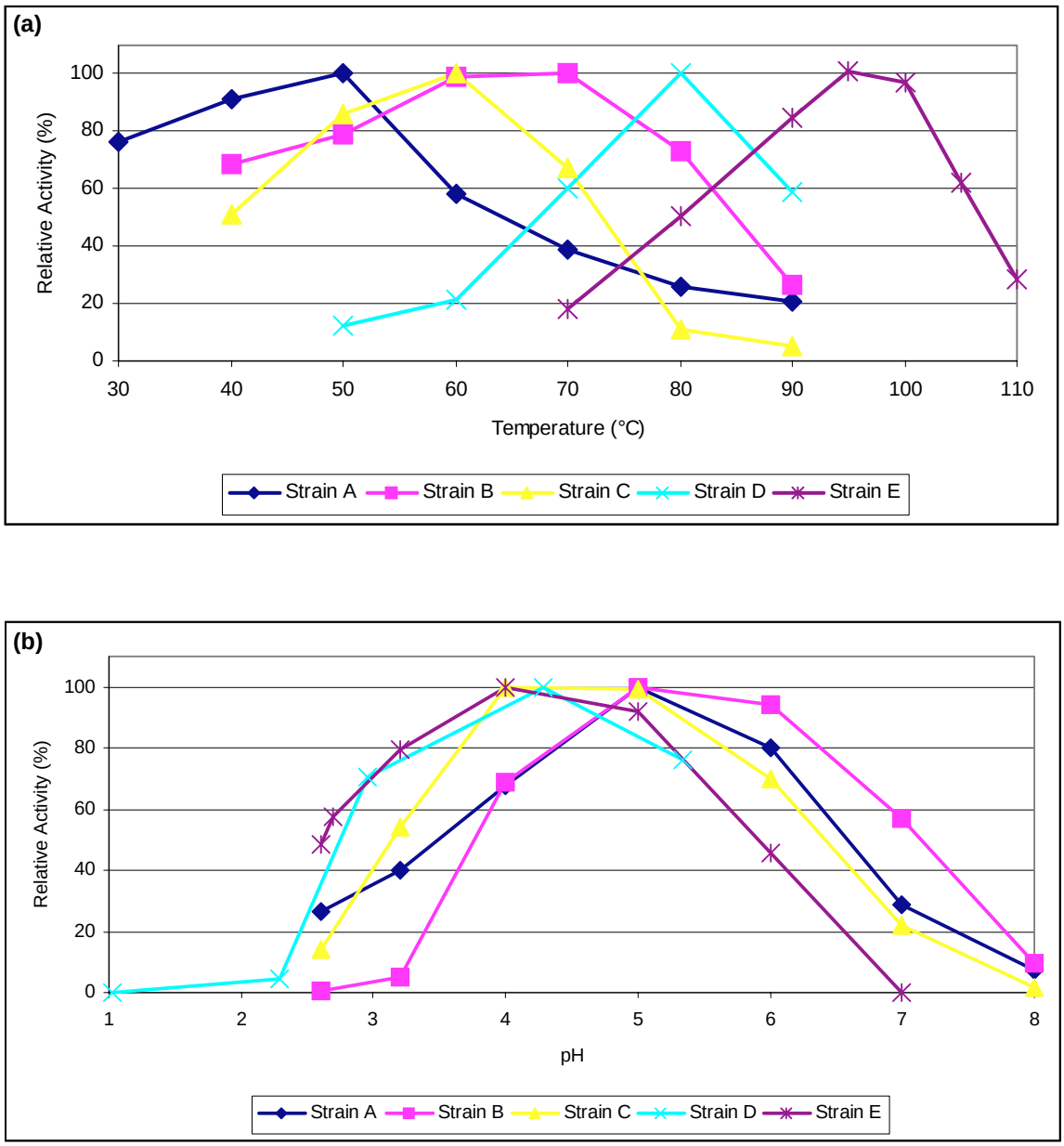

Figure 2.2. Effect of (a) temperature and (b) pH on the xylanase activities produced by Strains A–E. Data are expressed as a percentage of the maximum value. Each value represents mean ± SD (n = 3). The effect of temperature on enzyme activity was determined at pH 4 and the effect of pH on enzyme activity was determined at 60°C.

sulphuric acid at 80°C resulted in the release of approximately three times the amount of reducing sugars relative to that released by acid only. For Strain E, addition of dilute crude enzyme at a low activity level to 10% (w/v) straw in 0.1% (w/v) sulphuric acid at 95°C resulted in the release of 1.5 times more reducing sugars relative to that released by acid only after 3 h. It is noteworthy that the enzyme dosage levels used in these experiments are significantly less than would be desirable for industrial application, as outlined later in Section 2.2, and that increased enzymatic hydrolysis is likely to be achieved using more industrially relevant enzyme dosage levels. The results observed indicate that the crude enzymes produced by Strains D and E are capable of hydrolysing straw lignocellulose under conditions of low pH and high temperature even at the low enzyme dosage levels used. Strain D was selected for the further work packages undertaken to assess the

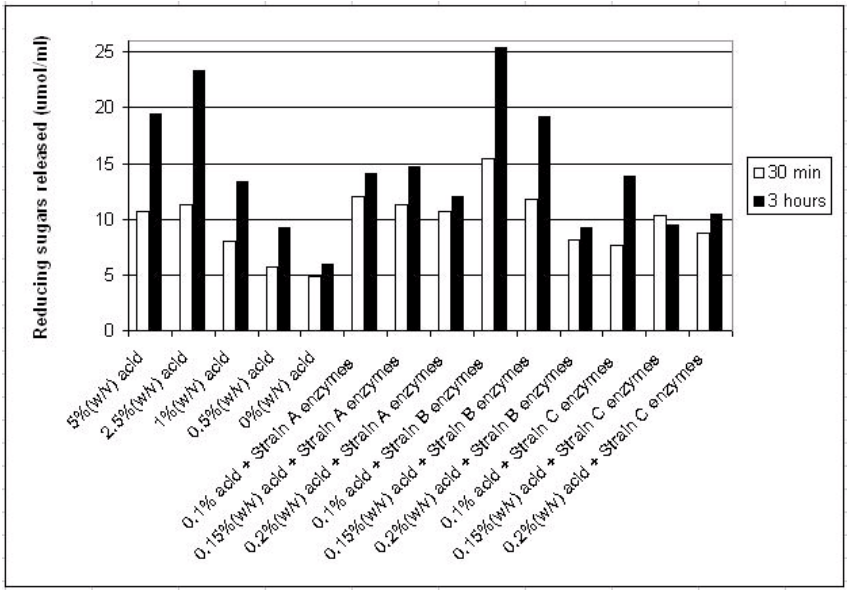

Figure 2.3. The amount of reducing sugars released from 10% (w/v) chopped straw at 70°C by 5%, 2.5%, 1%, 0.5% and 0% (w/v) sulphuric acid in comparison with that released by lower concentrations of acid (0.1%, 0.15% or 0.2% (w/v) sulphuric acid) in combination with the crude enzymes produced by Strains A, B and C.

feasibility of the proposed enzymatic/dilute-acid pretreatment method.

2.2 Assessment of the Potential Suitability of the Enzymes Produced by Strain D for the Proposed Enzymatic/Dilute-Acid Pretreatment Method

In the bioethanol production process, pretreatment must make the lignocellulosic substrate more accessible and susceptible to digestion by the enzymes used in the subsequent hydrolysis step, so as to provide as much monomeric sugars as possible for fermentation to bioethanol. Evaluation of the efficiency of the proposed enzymatic/dilute-acid pretreatment method was therefore undertaken by assessing the digestibility of the pretreated material, as outlined in Fig. 2.4. Initial characterisation studies undertaken showed that Stain D produces more than one cellulase activity including a cellulase with activity at low pH (~2.3), which is the cellulase activity of most interest for the proposed pretreatment application. To facilitate extensive lab-scale enzymatic/dilute-acid pretreatment studies using this enzyme, work was undertaken to produce the enzyme in large quantities by recombinant means. While a cellulase enzyme displaying maximum activity at 80°C and pH 4 was successfully cloned and expressed in *Escherichia coli*, exhaustive attempts to achieve recombinant production of the target cellulase with high activity at low pH were unsuccessful. Therefore, the application-relevant assessment studies were undertaken using natively produced crude enzyme, which, due to low levels of native enzyme production, was concentrated 143 to 215-fold by ultrafiltration prior to use.

Initial assessment of the enzymatic/dilute-acid pretreatment method using the crude enzymes from Strain D was undertaken, as outlined in Fig. 2.4, using 0.15% (w/v) acid at 80°C for a pretreatment time of 3 h. The enzyme dosage level of crude enzyme employed was 8.3 units/g straw, 15.2 units/g straw and 2.8 units/g straw when enzyme activity was quantified at 80°C on carboxymethylcellulose (CMC) at pH 2.3, CMC at pH 4 and xylan at pH 4, respectively. The pH of the pretreatment samples was 2.3–2.9. For comparison purposes, pretreatment was also undertaken under similar conditions without the enzyme. Upon quantification of the amount of reducing sugars released during the pretreatment step, inclusion of the enzyme was found to result in the release of approximately twice the amount of reducing

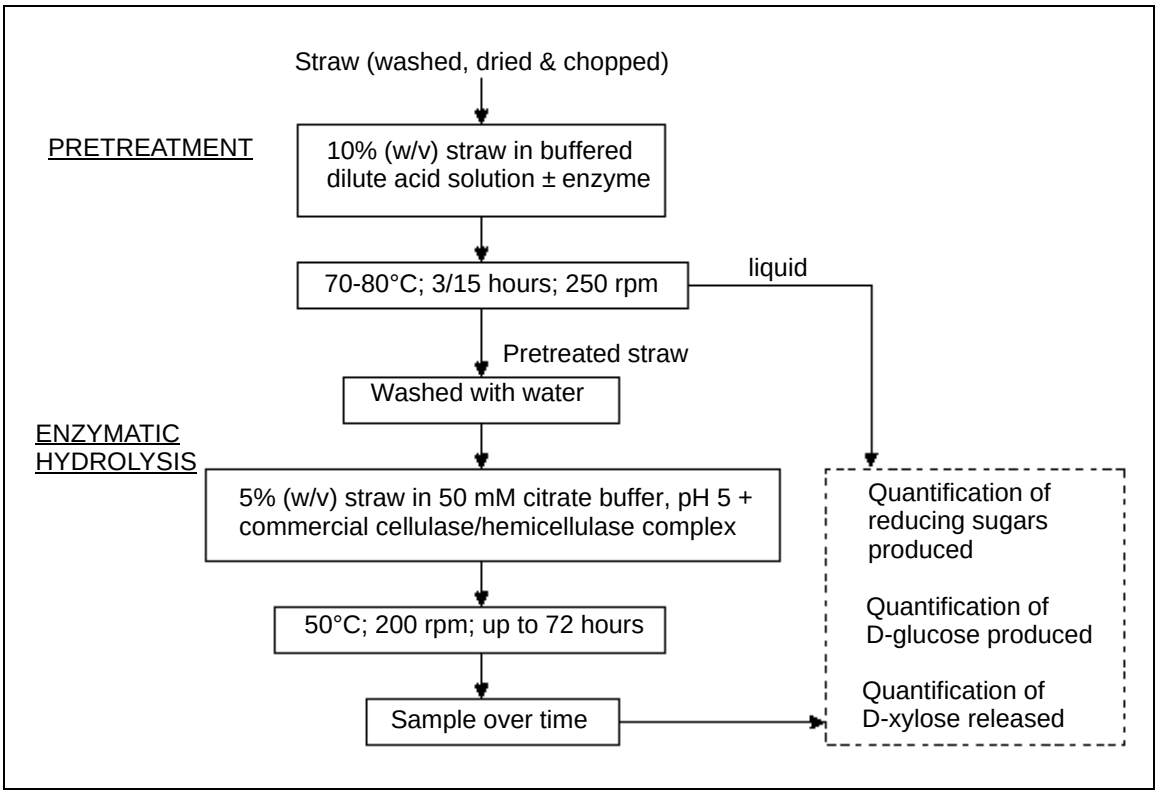

Figure 2.4. Overview of lab-scale method used to determine the feasibility of enzymatic/dilute-acid pretreatment.

sugars, indicating that the crude enzyme is capable of hydrolysing the straw under the conditions used. The digestibility of the pretreated straw was subsequently determined by enzymatic hydrolysis with excess commercial enzyme cocktail, as outlined in Fig. 2.4. The amount of reducing sugars, D-glucose and D-xylose produced over time during the hydrolysis period from the straw pretreated with and without enzyme are shown in Fig. 2.5. The straw pretreated with enzyme was more digestible than the straw pretreated without enzyme, as indicated by the higher amount of reducing sugars produced from the former throughout the hydrolysis period (Fig. 2.5a). Specific quantification of the amount of the monomeric sugars D-glucose and D-xylose produced indicates that production of D-glucose from straw pretreated with enzyme and without enzyme is similar (Fig. 2.5b), while a significant improvement in the production of D-xylose was achieved upon inclusion of the enzyme in the pretreatment step (Fig. 2.5c). After 5.5 h hydrolysis, approximately three times more D-xylose was detected in the hydrolysate from the enzyme-pretreated straw indicating that the xylan component of the enzyme-pretreated straw was more readily digested than that of the straw pretreated with acid only.

Further pretreatment studies were undertaken to determine the effect of pretreatment time. Increased production of reducing sugars and D-xylose was observed after 15 h pretreatment compared with 3 h pretreatment for samples pretreated either with or without enzyme. Studies were also undertaken to determine the effect of including (i) 10% ethanol, or (ii) PEG 8000[2] (5 g/l) in the enzymatic/dilute-acid pretreatment method. For all pretreatment studies undertaken, the results observed followed a similar trend to those outlined above with improved production of reducing sugars and D-xylose observed from the straw pretreated with enzyme and similar levels of D-glucose produced from straw pretreated with or without enzyme. A significant improvement in subsequent digestibility was not observed upon inclusion of 10%

2. PEG 8000, poly(ethylene) glycol, molecular weight 8,000.

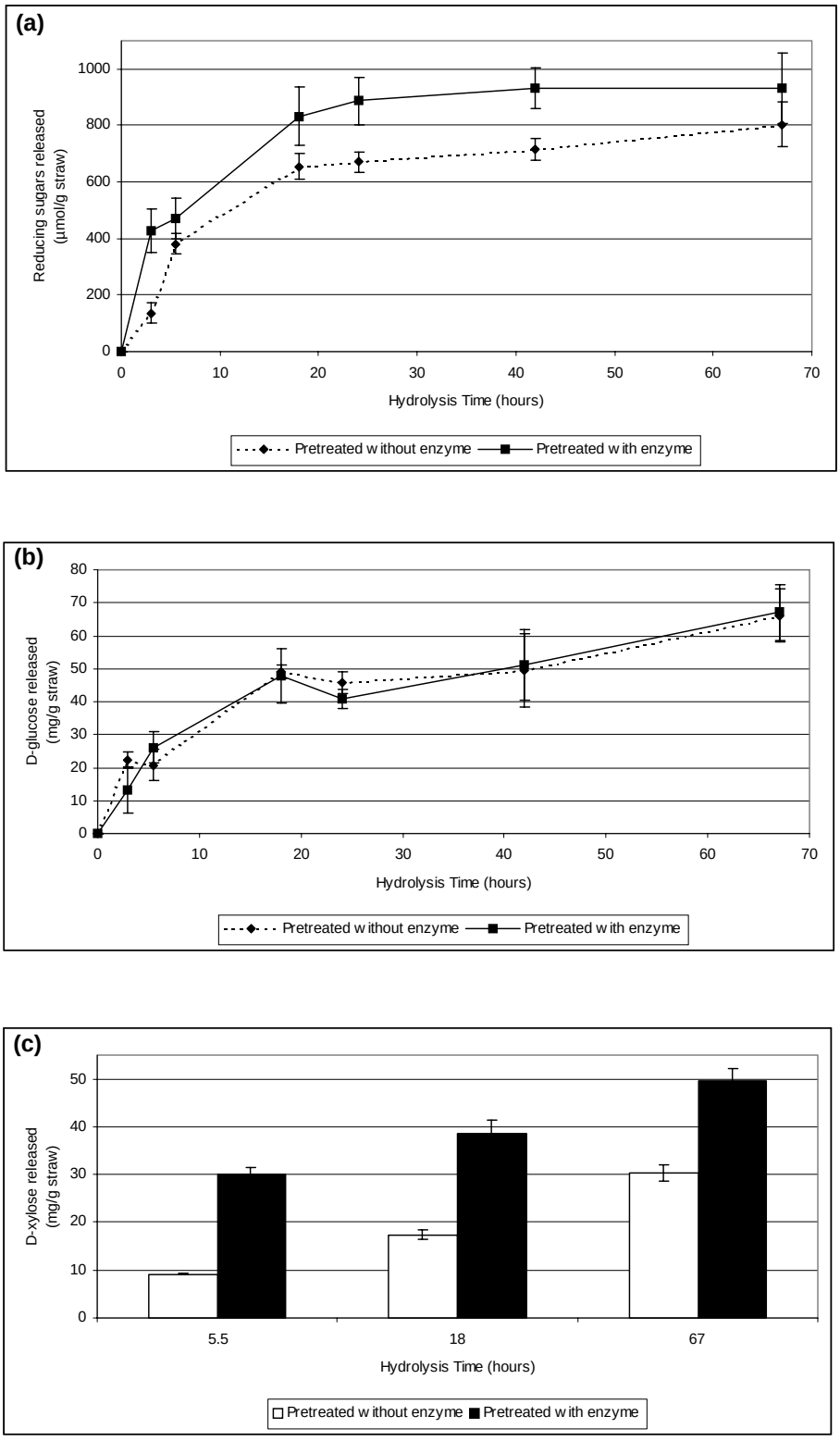

Figure 2.5. Assessment of the enzymatic digestibility of straw pretreated by enzymatic/dilute-acid pretreatment method for 3 h in comparison with that of straw pretreated under similar conditions with omission of Strain D crude enzyme activity. (a) Amount of reducing sugars released during enzymatic hydrolysis; (b) amount of D-glucose released during enzymatic hydrolysis; and (c) amount of D-xylose released during enzymatic hydrolysis. Each value represents mean ± SD of values obtained for three separate hydrolysis samples.

ethanol or PEG 8000 in the enzymatic pretreatment method.

The enzymatic/dilute-acid pretreatment method using crude enzyme activity from Strain D was compared on a lab scale to conventional dilute-acid pretreatment (121°C; 0.75% sulphuric acid), using straw as the feedstock. At the low enzyme dosage levels used, the enzymatic/dilute-acid pretreatment method resulted in the production of significantly less reducing sugars during the pretreatment step than conventional dilute-acid pretreatment. However, the digestibility of the straw pretreated by both methods was comparable as judged by the amount of reducing sugars produced during hydrolysis. More specific analysis by quantification of D-glucose and D-xylose revealed that a significantly greater amount of D-glucose was produced from the straw pretreated using the conventional dilute-acid approach while a significantly greater amount of D-xylose was produced from the straw pretreated using the enzymatic/dilute-acid pretreatment method.

Due to the low levels of enzyme produced natively by Strain D and the difficulties encountered in achieving a high-fold concentration of the crude enzyme, the enzyme dosage levels used in the enzymatic/dilute-acid pretreatment experiments undertaken are significantly less than would be desirable for this application. This is evident upon comparison of the enzyme dosage levels used in the present study with those recommended for optimally formulated commercial enzyme products currently marketed for the hydrolysis of pretreated lignocellulose. The enzyme dosage levels of Strain D used for enzymatic/dilute-acid pretreatment were approximately 6–8 units/g straw, 15–19 units/g straw and 3 units/g straw when enzyme activity was quantified at 80°C on CMC at pH 2.3, CMC at pH 4 and xylan at pH 4, respectively. The recommended enzyme dosage levels for hydrolysis of a pretreated lignocellulosic substrate using a currently available commercial enzyme complex is equivalent to 20–86 units/g straw and 5–20 units/g straw when enzyme activity is quantified on CMC and xylan, respectively, under the optimum temperature and pH conditions of the enzyme complex. The employed dosage of cellulase with activity in the pH range at which the enzymatic pretreatment was undertaken is therefore roughly equivalent to only 8–35% of the recommended commercial enzyme dosage level. A substantial improvement in the efficiency of the enzymatic/dilute-acid pretreatment method could therefore potentially be achieved by using a higher, more industrially relevant enzyme dosage level.

3 Overall Project Conclusions and Recommendations

3.1 Project Conclusions

The focus of this project was to develop an alternative pretreatment method for use in the production of second-generation bioethanol. The proposed enzymatic/dilute-acid pretreatment method, based on the use of thermoacidophilic enzymes, aims to improve the efficiency of the pretreatment step and to overcome many of the technical, economical, environmental and health and safety-related disadvantages associated with current practice.

The first task of the project involved identification of micro-organisms producing lignocellulose-degrading enzymes suitable for use in the proposed pretreatment method, production of these enzymes and application-relevant assessment studies to identify the most suitable candidates. Screening of 65 micro-organisms, including fungi, bacteria, archaea and environmental isolates, resulted in the identification of 19 strains producing endo-β-glucanase activities and 21 strains producing endo-β-xylanase activities of potential interest for the proposed application. In addition, several of these strains were found to produce β-glucosidase, β-xylosidase, α-D-galactosidase, α-L-arabinofuranosidase, acetyl xylan esterase and ferulic acid esterase activities. Based on the results of application-relevant studies undertaken, five strains (designated Strains A–E) were identified as producing enzymes most suitable for the proposed application. Strain D was selected for further studies to determine the feasibility of the proposed enzymatic/dilute-acid pretreatment method. It is noteworthy that the enzymes produced by Strain E are also potentially very suitable for the proposed application. However, these enzymes were only identified during the last round of screening undertaken at a later stage in the project, at which time, significant work focusing on Strain D had already been undertaken.

Lab-scale enzymatic/dilute-acid pretreatment was undertaken using natively produced Strain D crude enzyme activity and the subsequent digestibility of the pretreated material was determined by quantification of the amount of fermentable sugars produced upon hydrolysis with excess commercially available cellulase/hemicellulase complex. The results indicate that the crude enzymes produced by Strain D are capable of hydrolysing straw lignocellulose under the conditions used (80°C; 0.15% acid) and that enzymatic/dilute-acid pretreatment using these enzymes has a beneficial effect on the subsequent digestibility of the straw relative to pretreatment under similar conditions without inclusion of enzyme. Improved production of xylose accounted for the observed increase in digestibility. The enzymatic/dilute-acid pretreatment method using Strain D crude enzyme activity was compared on a lab scale to conventional dilute-acid pretreatment (121°C; 0.75% sulphuric acid), using straw as feedstock. At the low enzyme dosage levels used, the enzymatic/dilute-acid pretreatment method resulted in the production of less reducing sugars during the pretreatment step than conventional dilute-acid pretreatment. However, the digestibility of the straw pretreated by both methods was comparable, as judged by the amount of reducing sugars produced during hydrolysis. More specific analysis, by quantification of D-glucose and D-xylose, revealed that a significantly greater amount of D-glucose was produced from the straw pretreated using the conventional dilute-acid approach, while a significantly greater amount of D-xylose was produced from the straw pretreated using the enzymatic/dilute-acid pretreatment method. These preliminary results indicate that the enzymatic pretreatment approach may potentially be particularly suitable for use in conjunction with xylose (C5) fermentation, an area that has recently been the focus of considerable research efforts.

The authors were unable to produce large quantities of enzyme from Strain D due to difficulties in recombinant expression and problems were encountered in achieving a high-fold concentration of the natively produced enzyme. Consequently, the enzyme dosage level used in the pretreatment experiments undertaken here was lower than would be desirable for this

application at industrial scale. To facilitate a meaningful determination of pretreatment efficiency and accurate comparison to other pretreatment methods, the enzyme dosage level for the enzymatic pretreatment method should be at least equivalent to the enzyme dosage levels recommended for optimally formulated commercial enzyme products currently marketed for the hydrolysis of pretreated lignocellulose. The enzyme dosage levels used in the enzymatic pretreatment studies undertaken equate to only approximately 8–35% of the recommended dosage for one such commercial enzyme product. A substantial improvement in the efficiency of the enzymatic/dilute-acid pretreatment method could therefore potentially be achieved by using a higher, more industrially relevant, enzyme dosage level. This would also facilitate more realistic comparison with conventional dilute-acid pretreatment.

3.1.1 Key outputs from the project

- The work undertaken demonstrates the potential of thermoacidophilic enzymes to overcome many of the technical issues associated with the cellulosic bioethanol production process and, hence, contribute to overall process improvement. This would facilitate more widespread deployment of cellulosic bioethanol, with subsequent benefits in terms of GHG emissions, energy security and economic growth.

- Significant advances were made towards developing an enzymatic/dilute-acid pretreatment method, particularly with regard to identifying suitable enzymes. While further optimisation of this method using a more industrially relevant, increased enzyme dosage level is necessary to facilitate more extensive investigation of the efficiency of the method, the initial results observed with low enzyme dosage levels are promising. If satisfactory pretreatment is observed upon such optimisation, the developed pretreatment method, undertaken at a lower temperature of 80°C and using five times less sulphuric acid potentially offers several environmental, technical and health and safety-related benefits over currently used pretreatment methods.

- In addition to the enzymes from Strain D used to develop the enzymatic pretreatment method, the project also resulted in the identification of several other enzymes of potential application, both in the pretreatment and subsequent hydrolysis steps of the process, as outlined in Section 3.2. The use of such enzymes in the cellulosic bioethanol production process may potentially contribute to overall process improvement.

- The project has expanded expertise in this area within the research group and the University of Limerick and has built capacity for further applied research aimed at improving the cellulosic bioethanol production process using enzymes produced by thermoacidophiles. To this end, the research undertaken in this project has laid the foundation for a follow-on research project that aims to exploit the initial proof of concept data generated. Further knowledge and experience in working with thermoacidophiles and their novel enzymes has been accrued which may potentially be exploited in other industrial applications to achieve more sustainable use of resources and contribute towards the protection and improvement of the environment.

3.2 Recommendations

3.2.1 General recommendations

Research that contributes to overcoming the technical, economic and environmental issues associated with the production of cellulosic bioethanol should be continued. Such improvement of this production process is desirable to facilitate wide-scale deployment of cellulosic bioethanol with subsequent benefits in terms of GHG emissions, energy security and economic growth. Climate change and energy security are considered to be two of the most significant and challenging issues currently facing humanity. The widespread deployment of cellulosic bioethanol will contribute to solving both these issues in a meaningful way by reducing dependence on fossil fuels and achieving GHG emission reductions. US EPA analysis showed that second-generation bioethanol generates 91% less GHG than fossil-based petrol or diesel in transport applications. Furthermore,

widespread deployment of second-generation bioethanol is in line with global and European renewable energy strategy, objectives and policies and will assist nations in the practical implementation of relevant national/international global policy documents/agreements. On a national level, widespread deployment of cellulosic bioethanol is in line with the overall objectives of the *National Renewable Energy Action Plan*, the *Strategy for Renewable Energy 2012–2020* and *Food Harvest 2020* and contributes to economic growth and the development of a resource-efficient, sustainable green economy. The development of a second-generation biofuel industry could fulfil the *Food Harvest 2020* vision of providing revenue streams (plus employment) to farmers (and processors) for non-food crops while contributing towards meeting government targets and policies. A potential €31 billion second-generation bioethanol industry is forecast for the EU alone by 2020. The development of thermoacidophilic enzyme products of beneficial use in this industry could underpin sustainable, export-oriented employment in enzyme manufacture, and more broadly in biofuel manufacture.

3.2.2 Recommendations for future work based on technical findings from project

- Further research of the enzymatic/dilute-acid pretreatment method with enzymes from Strain D should use higher, more industrially relevant enzyme dosage levels. Large amounts of enzyme, for use in such studies, could potentially be produced by recombinant means or natively by large-scale culture of the strain. More extensive optimisation studies should then be undertaken to determine the pretreatment conditions (temperature, acid concentration, enzyme dosage level, time) resulting in maximum digestibility of straw lignocellulose. In addition to digestibility, the optimised enzymatic/dilute-acid pretreatment method should be assessed in terms of the formation of degradation products/inhibitors and the results compared with conventional dilute-acid pretreatment. For industrial acceptance, assessment of the economic feasibility of enzymatic/dilute-acid pretreatment should also be undertaken

- Investigation of the potential application of the enzymes produced by Strain E in enzymatic/dilute-acid pretreatment should be carried out. These enzymes, described in Section 2.1, are potentially very suitable for this application, on the basis of their high temperature optima (95–100°C) and exceptional thermal stability. Due to the higher temperature optima of these enzymes relative to those produced by Strain D, the enzymatic/dilute-acid pretreatment could be undertaken at a higher temperature of 95–100°C. Increased thermal hydrolysis of lignocellulose is likely to be achieved at this temperature, improving the efficiency of pretreatment while still resulting in a significant reduction in energy usage relative to conventional dilute-acid pretreatment.

- Further optimisation of enzymatic/dilute-acid pretreatment should be undertaken. This could potentially be achieved by (i) incorporating various additives reported in the literature to improve lignocellulose disruption and/or enzymatic hydrolysis, and (ii) incorporating additional enzyme activities to promote enhanced enzymatic hydrolysis of lignocellulose. Several such lignocellulose-degrading enzymes, displaying activity at high temperature and low pH were identified during the screening studies undertaken during this project. Optimised enzyme cocktails containing such enzymes in addition to cellulase and xylanase activities should be developed. In particular, efforts should focus on incorporating ferulic acid esterase activity in the enzymatic pretreatment method as this enzyme disrupts the structure of lignocellulose by hydrolysing crosslinks between xylan and lignin and is considered a key enzyme in biomass degradation.

- While the research undertaken in the current project focused on the application of thermoacidophilic enzymes in an enzymatic/dilute-acid pretreatment method, thermoacidophilic enzymes are also of potential interest in the enzymatic hydrolysis step, as outlined in Section 1.4. Enzymatic hydrolysis is generally carried out using currently available

commercial enzymes at around 50°C and pH 5. Disadvantages of this approach include decreased enzyme performance due to heat inactivation during the hydrolysis period and the need to significantly reduce temperature and increase pH after pretreatment (Rosgaard et al., 2007; Alvira et al., 2010). Enzyme manufacturers, for example DSM, favour more thermostable enzymes for this application. Processing and economic advantages of using thermostable enzymes for the enzymatic hydrolysis step include:

- Thermostable enzymes exhibit higher specific activity and enhanced stability, likely reducing the amount of enzyme needed and improving hydrolysis performance (Turner et al., 2007; Viikari et al., 2007; Yeoman et al., 2010);
- Thermostable enzymes are more resistant to inhibitors and proteolysis (Kumar, 2012);
- Hydrolysis at higher temperatures is associated with lower viscosity, improved substrate solubility, improved flowability, easier mixing/pumping and reduced mass transport costs (Turner et al., 2007; Viikari et al., 2007; Yeoman et al., 2010);
- Reduced risk of microbial contamination (Turner et al., 2007; Viikari et al., 2007; Yeoman et al., 2010; Kumar, 2012);
- Improved substrate accessibility and biomass disorganisation due to high temperature (Turner et al., 2007; Kumar, 2012);
- Reduced requirement and energy cost for cooling after pretreatment (Kumar, 2012); and
- Reduced viscosity potentially facilitates higher biomass solids loadings resulting in higher sugar and ethanol concentrations (Kumar, 2012).

Several of the enzymes identified during the screening work undertaken in this project are potentially suitable for application in the high temperature hydrolysis/prehydrolysis of pretreated material and their use in this step of the process could potentially contribute to improving the overall efficiency of the bioethanol production process. Research should be undertaken to fully investigate their potential use for this application.

- Some of the enzymes identified during the screening undertaken may be of potential interest for other industrial applications. For example, in the pulp and paper industry, the use of esterases and xylanases in biopulping and bioleaching of pulps is associated with reduced chlorine consumption during bleaching. Many of the enzyme activities identified could also potentially be used in animal feed to improve the digestibility of feed components. High thermal stability, as observed for several of the enzymes identified in the current study, is a key feature for animal feed application as animal feed is commonly heat conditioned and pelleted at high temperatures after enzyme addition.
- Cellulosic bioethanol can potentially achieve significant GHG reductions in the transport sector, contribute to energy security and economic growth, and assist nations in the practical implementation of relevant national/international global policy documents/agreements. Further research and development aimed at overcoming the technical, economic and environmental issues associated with the production process are necessary to achieve the widespread deployment of cellulosic bioethanol required to meet this vision.

Table 3.1. Summary of recommendations for implementation and uptake of research findings by policy makers, regulators, process designers and researchers

General recommendations:	
Second-generation bioethanol	• Continuation of research that contributes to overcoming the technical, economic and environmental issues associated with the production of second-generation bioethanol. This will facilitate widespread deployment of second-generation bioethanol with associated benefits in terms of: – reducing greenhouse gas emissions – climate change mitigation – implementation and fulfilment of relevant national/international global policy documents/ agreements – reducing dependency on fossil fuels – improving energy security – creation of green jobs and economic growth – development of a resource-efficient, sustainable green economy
Technical recommendations:	
Enzymatic/Dilute-acid pretreatment	• Optimisation of method developed with enzymes from Strain D using industrially relevant enzyme dosage levels and extensive comparison of the optimised method with conventional dilute-acid pretreatment • Investigation of the potential application of the enzymes produced by Strain E in enzymatic/dilute-acid pretreatment • Further optimisation of the efficiency of enzymatic/dilute-acid pretreatment method by using optimally formulated cocktails of lignocellulose-degrading enzymes identified during screening with/without additional additives
Enzymatic hydrolysis/ prehydrolysis at high temperature	• Investigation of the potential suitability of the enzymes identified during screening for this application
Other industrial applications	• Investigation of the potential suitability of selected enzymes identified during screening for other industrial applications.

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Acronyms and Annotations

CMC	Carboxymethylcellulose
CO_2	Carbon dioxide
EPA	Environmental Protection Agency
EU	European Union
GHG	Greenhouse gas
IEA	International Energy Agency
MSW	Municipal solid waste
NREAP	National Renewable Energy Action Plan
OECD	Organisation for Economic Co-operation and Development
PEG	Poly(ethylene) glycol
RED	Renewable Energy Directive
RFS	Renewable Fuel Standard
UNFCCC	United Nations Framework Convention on Climate Change

Appendix 1 Research Dissemination

Conference Paper and Poster

Boyce, A. and Walsh, G., 2012. Potential application of *Phanerochaete chrysosporium* enzymes in the dilute-acid pretreatment stage of bioethanol production. *In:* Olabi, A.G. and Benyounis, K.Y. (Eds) *Proceedings of the 5th International Conference on Sustainable Energy and Environmental Protection (Part 1)*. Dublin City University, Dublin, Ireland. pp. 131–136.

Boyce, A. and Walsh, G., 2012. Investigation of the potential suitability of the enzymes produced by the fungus *Thermoascus aurantiacus* for the pretreatment of lignocellulose for bioethanol production. *Proceedings of the 20th European Biomass Conference and Exhibition*. Milan, Italy. pp. 1658–1661.

Conference Poster

Boyce, A. and Walsh, G., 2012, Assessment of the potential suitability of the enzymes produced by an environmental isolate for the pretreatment of lignocellulose for bioethanol production. *The World Congress on Industrial Biotechnology and Bioprocessing*. Florida, USA.

Conference Poster

Boyce, A. and Walsh, G., 2012. Screening of selected microorganisms for thermo(acido)philic enzymes of potential use in the production of second-generation bioethanol. Environ 2012. *The 22nd Irish Environmental Researchers' Colloquium*. University College Dublin, Dublin, Ireland. p. 150.

An Ghníomhaireacht um Chaomhnú Comhshaoil

Is í an Gníomhaireacht um Chaomhnú Comhshaoil (EPA) comhlachta reachtúil a chosnaíonn an comhshaol do mhuintir na tíre go léir. Rialaímid agus déanaimid maoirsiú ar ghníomhaíochtaí a d'fhéadfadh truailliú a chruthú murach sin. Cinntímid go bhfuil eolas cruinn ann ar threochtaí comhshaoil ionas go nglactar aon chéim is gá. Is iad na príomh-nithe a bhfuilimid gníomhach leo ná comhshaol na hÉireann a chosaint agus cinntiú go bhfuil forbairt inbhuanaithe.

Is comhlacht poiblí neamhspleách í an Ghníomhaireacht um Chaomhnú Comhshaoil (EPA) a bunaíodh i mí Iúil 1993 faoin Acht fán nGníomhaireacht um Chaomhnú Comhshaoil 1992. Ó thaobh an Rialtais, is í an Roinn Comhshaoil, Pobal agus Rialtais Áitiúil.

ÁR bhFREAGRACHTAÍ

CEADÚNÚ

Bíonn ceadúnais á n-eisiúint againn i gcomhair na nithe seo a leanas chun a chinntiú nach mbíonn astuithe uathu ag cur sláinte an phobail ná an comhshaol i mbaol:

- áiseanna dramhaíola (m.sh., líonadh talún, loisceoirí, stáisiúin aistrithe dramhaíola);
- gníomhaíochtaí tionsclaíocha ar scála mór (m.sh., déantúsaíocht cógaisíochta, déantúsaíocht stroighne, stáisiúin chumhachta);
- diantalmhaíocht;
- úsáid faoi shrian agus scaoileadh smachtaithe Orgánach Géinathraithe (GMO);
- mór-áiseanna stórais peitreail;
- scardadh dramhuisce;
- dumpáil mara.

FEIDHMIÚ COMHSHAOIL NÁISIÚNTA

- Stiúradh os cionn 2,000 iniúchadh agus cigireacht de áiseanna a fuair ceadúnas ón nGníomhaireacht gach bliain
- Maoirsiú freagrachtaí cosanta comhshaoil údarás áitiúla thar sé earnáil - aer, fuaim, dramhaíl, dramhuisce agus caighdeán uisce
- Obair le húdaráis áitiúla agus leis na Gardaí chun stop a chur le gníomhaíocht mhídhleathach dramhaíola trí comhordú a dhéanamh ar líonra forfheidhmithe náisiúnta, díriú isteach ar chiontóirí, stiúradh fiosrúcháin agus maoirsiú leigheas na bhfadhbanna.
- An dlí a chur orthu siúd a bhriseann dlí comhshaoil agus a dhéanann dochar don chomhshaol mar thoradh ar a ngníomhaíochtaí.

MONATÓIREACHT, ANAILÍS AGUS TUAIRISCIÚ AR AN GCOMHSHAOL

- Monatóireacht ar chaighdeán aeir agus caighdeáin aibhneacha, locha, uiscí taoide agus uiscí talaimh; leibhéil agus sruth aibhneacha a thomhas.
- Tuairisciú neamhspleách chun cabhrú le rialtais náisiúnta agus áitiúla cinntí a dhéanamh.

RIALÚ ASTUITHE GÁIS CEAPTHA TEASA NA HÉIREANN

- Cainníochtú astuithe gáis ceaptha teasa na hÉireann i gcomhthéacs ár dtiomantas Kyoto.
- Cur i bhfeidhm na Treorach um Thrádáil Astuithe, a bhfuil baint aige le hos cionn 100 cuideachta atá ina mór-ghineadóirí dé-ocsaíd charbóin in Éirinn.

TAIGHDE AGUS FORBAIRT COMHSHAOIL

- Taighde ar shaincheisteanna comhshaoil a chomhordú (cosúil le caighdéan aeir agus uisce, athrú aeráide, bithéagsúlacht, teicneolaíochtaí comhshaoil).

MEASÚNÚ STRAITÉISEACH COMHSHAOIL

- Ag déanamh measúnú ar thionchar phleananna agus chláracha ar chomhshaol na hÉireann (cosúil le pleananna bainistíochta dramhaíola agus forbartha).

PLEANÁIL, OIDEACHAS AGUS TREOIR CHOMHSHAOIL

- Treoir a thabhairt don phobal agus do thionscal ar cheisteanna comhshaoil éagsúla (m.sh., iarratais ar cheadúnais, seachaint dramhaíola agus rialacháin chomhshaoil).
- Eolas níos fearr ar an gcomhshaol a scaipeadh (trí cláracha teilifíse comhshaoil agus pacáistí acmhainne do bhunscoileanna agus do mheánscoileanna).

BAINISTÍOCHT DRAMHAÍOLA FHORGHNÍOMHACH

- Cur chun cinn seachaint agus laghdú dramhaíola trí chomhordú An Chláir Náisiúnta um Chosc Dramhaíola, lena n-áirítear cur i bhfeidhm na dTionscnamh Freagrachta Táirgeoirí.
- Cur i bhfeidhm Rialachán ar nós na treoracha maidir le Trealamh Leictreach agus Leictreonach Caite agus le Srianadh Substaintí Guaiseacha agus substaintí a dhéanann ídiú ar an gcrios ózóin.
- Plean Náisiúnta Bainistíochta um Dramhaíl Ghuaiseach a fhorbairt chun dramhaíl ghuaiseach a sheachaint agus a bhainistiú.

STRUCHTÚR NA GNÍOMHAIREACHTA

Bunaíodh an Ghníomhaireacht i 1993 chun comhshaol na hÉireann a chosaint. Tá an eagraíocht á bhainistiú ag Bord lánaimseartha, ar a bhfuil Príomhstiúrthóir agus ceithre Stiúrthóir.

Tá obair na Gníomhaireachta ar siúl trí ceithre Oifig:

- An Oifig Aeráide, Ceadúnaithe agus Úsáide Acmhainní
- An Oifig um Fhorfheidhmiúchán Comhshaoil
- An Oifig um Measúnacht Comhshaoil
- An Oifig Cumarsáide agus Seirbhísí Corparáide

Tá Coiste Comhairleach ag an nGníomhaireacht le cabhrú léi. Tá dáréag ball air agus tagann siad le chéile cúpla uair in aghaidh na bliana le plé a dhéanamh ar cheisteanna ar ábhar imní iad agus le comhairle a thabhairt don Bhord.

Science, Technology, Research and Innovation for the Environment (STRIVE) 2007-2013

The Science, Technology, Research and Innovation for the Environment (STRIVE) programme covers the period 2007 to 2013.

The programme comprises three key measures: Sustainable Development, Cleaner Production and Environmental Technologies, and A Healthy Environment; together with two supporting measures: EPA Environmental Research Centre (ERC) and Capacity & Capability Building. The seven principal thematic areas for the programme are Climate Change; Waste, Resource Management and Chemicals; Water Quality and the Aquatic Environment; Air Quality, Atmospheric Deposition and Noise; Impacts on Biodiversity; Soils and Land-use; and Socio-economic Considerations. In addition, other emerging issues will be addressed as the need arises.

The funding for the programme (approximately €100 million) comes from the Environmental Research Sub-Programme of the National Development Plan (NDP), the Inter-Departmental Committee for the Strategy for Science, Technology and Innovation (IDC-SSTI); and EPA core funding and co-funding by economic sectors.

The EPA has a statutory role to co-ordinate environmental research in Ireland and is organising and administering the STRIVE programme on behalf of the Department of the Environment, Heritage and Local Government.

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