Report to Stapledon Memorial Trust

Project title: Production of fermented products from grass juice and grass fibre

Period of Studentship: 1 June- 31 July, 2017

Student : Neelakshi Dutta Email: n.dutta@hotmail.co.uk

Host Institute: IBERS, Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Gogerddan, Aberystwyth, SY23 3EE

Supervisor: Dr. Sreenivas Rao Ravella Email: rsr@aber.ac.uk

Objectives and overview of the project

The aim of the work was to develop a novel process for producing value added products, from ryegrass juice and fibre. This work helps to develop an economically viable and sustainable biorefinery from grass. In addition, knowledge of design of experimental (DOE) concepts, fermentations related to grass biorefinery will be acquired.

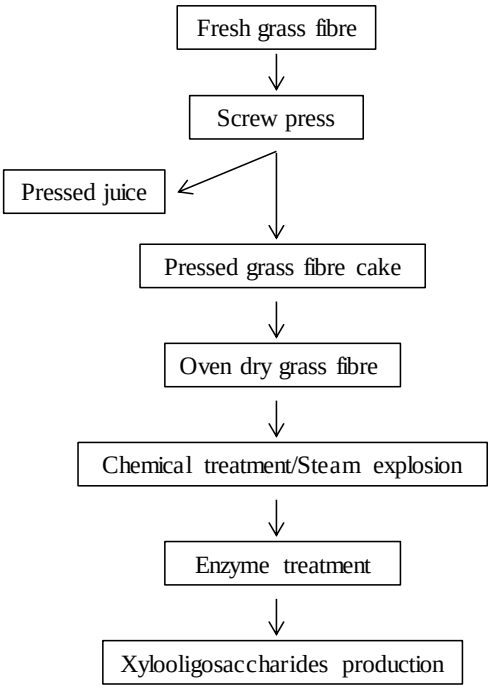
1. Introduction

The research work was first of its kind that has utilised low value ryegrass fibre and juice to produce value products- xylo-oligosaccharides (XOS) and ethanol, which are in great demand in the food, chemical and pharmaceutical industry. The work was novel and involved a number of innovative steps as detailed below.

The utilisation of grass fibre and juice as a raw material was itself novel in the sense that it was the waste product of grass biorefinery where the juice was used to produce a range of other commercial products including lactic acid, ethanol, protein, protein stabilizers etc. The fibre contains hemicellulose that can be converted to XOS. The grass juice can be used as fermentation broth to produce ethanol. Moreover, it is readily available in large amounts as the UK is covered with more than 60 % grasslands. Therefore, utilisation of this fibre for value added products will contribute to the bioeconomy in addition to the benefits derived from XOS production.

2. Experiments and materials:

The flow chart of the work is shown in the Figure:



2.1 Materials

Perennial ryegrass (Lolium perenne), age of 6 weeks, harvested from the plots of IBERS, Gogerddan, Wales in mid-June 2017, and were approximately 12 cm in height. They were directly transported to the laboratory as quickly as possible. The grass screw pressed and juice (H140617, ABERMAGIC with a total sugar of 56.3 g/L) and fibre were extracted separately. The grass fibres dried for 48 h at 65 °C before use.

2.2. Analytical Methods

2.2.1. Sugars and fermented product analysis

Liquid phase was filtered through 0.2 µm membranes (Syringe filter, 13 mm Nylon, Waters, Ann Arbor, MI) and analysed for arabinose, glucose, xylose and ethanol using high performance liquid chromatography (HPLC) coupled with a refractive index detector (Model 2031 Plus, Jasco, Japan). The HPLC samples were eluted with 0.005 M H2SO4 at 35 °C with a flow rate of 0.6 ml/min through the ROA Phenomenex column (150*7.80 mm) with guard.

2.2.2. Determination of total sugars

The amount of all sugars in solution was determined by autoclaving liquid samples in 4 % sulphuric acid for 1 hr at 121 °C to breakdown oligomers into monomeric sugars as described in NREL laboratory methods (Sluiter et al.,2008). Sugar recovery standard containing known sugar

concentrations were also autoclaved in parallel to estimate sugar losses during this post hydrolysis operation. Monomers were then quantified using a Jasco HPLC equipped with a refractive index (RI) detector and Bio-rad HPX column (Biorad). Then the total mass of oligomers was calculated according to Yang and Wyman (2008).

2.3. Pre-treatment

The pre-treatment of the grass fibres were carried thermo-chemically using acid (Phosphoric acid) as well base (Sodium hydroxide) at pilot level in 30 L steam explosion rig.

2.3.2. Pre-treatment at Pilot scale

Based on the preliminary work at bench scale, the steam explosion experiments were designed in order to optimise the process for maximum XOS production using sodium hydroxide as catalyst.

Steam explosion (30L) rig at BEACON biorefining facility

Results: sugars from grass fibre

DT data\2017 07 11	Glucose	Xylose	Arabinose	TOTAL GXA mg/ml	TOTAL GXA %
Steam exploded grass 1	39.24	14.85	0.92	55.01	5.50
Steam exploded grass 2	47.34	2.59	0.00	49.93	4.99
Steam exploded grass 3	49.77	4.00	0.00	53.77	5.38
Steam exploded grass 4	38.96	16.52	0.00	55.47	5.55
Grass 1 hydrolysate	1.49	9.69	1.89	13.06	1.31
Grass 2 hyrolysate	2.36	12.31	2.31	16.99	1.70
soak acid	0.00	0.00	0.00	0.00	0.00
soak alkali	0.61	0.00	0.00	0.00	0.00

2.4. Enzymatic hydrolysis of pre-treated grass fibres

In order to produce XOS with suitable DP, 5 g pre-treated grass fibres in 250 ml laboratory bottles was further analysed by commercialized xylanase (Novozymes, NS22083) with activity of 2500 U/g substrate and mixture of two xylanase (Novozymes, NS22083 and NS22002). After adjusting pH to 5.5 with citrate phosphate buffer to final volume of 100 ml, the xylanase was added to pre-treated grass fibres and the bottles were incubated at 150 rpm and 50 °C. 1 ml sample was taken at regular intervals of 24 h and stored at freezer for future use. The formation of XOS was monitored by HPLC and high performance anion exchange chromatography with pulsed amperometric detection system (HPAEC-PAD).

2.5 Isolation of yeasts for fermentation experiments

Yeasts were isolated from grapes (*Vitis* sp.) and strawberries (*Fragaria* sp. L.) collected from the local market in Aberystwyth, UK, using Rose Bengal agar medium with chloramphenicol. After isolation yeasts were further sub cultured on YEPD (Yeast Extract Peptone Dextrose) medium.

2.6 Identification of yeasts:

Based on genomic DNA (D1/D2 domain of LSU rRNA gene) analysis yeasts were identified as follows

Strain ID	Putative Genus Species	Query coverage	Identity
N1	Starmerella bacillaris isolate (Candida zemplinina)	65	77
N2	Metschnikowia aff. chrysoperlae P34A005	74	99
ST1	Unidentified Metschnikowia	69	98
	Metschnikowia ziziphicola strain XY201	74	96
	Metschnikowia sinensis strain XY103	68	98
ST2	Metschnikowia reukaufii strain 3FAR2	67	97
BTG1	Candida tropicalis strain SSm-39	97	98
BTG2	Candida tropicalis strain YZ1	83	97
S1	Metschnikowia aff. chrysoperlae	87	99
M2	Metschnikowia aff. chrysoperlae	88	99

2.7 Fermentation of glucose and grass juice using novel yeast isolates.

Fermentations using yeast strains M1, M2, M3, M4, N1, N2, N3, S1 were carried out to produce ethanol from glucose. Only strains M2, N2, N3, S1, were successful in producing ethanol. Hence these strains in addition to strain Y and R were selected for fermenting grass juice into ethanol. Moreover, it was found that not only were these novel strains successful in fermenting grass juice into ethanol but also that the grass juice was found to be a suitable substitute medium for xylitol production from xylose.

3. Economic and market potential:

IBERS scientists are in the top seven in research areas of agricultural, veterinary and food sciences in UK. Scientists at IBERS have successfully commercialised agricultural rye grasses for many years and recently, at the breeding centre, they have developed a high sugar rich (30-40 % sugar) grass that can be potentially converted into bio-based products. The aim of IBERS research is to help farmers in developing pasture management systems, which can allow different grasses to be produced for their

desired levels of livestock whilst having the option of producing a surplus for biobased products. Therefore, grassland could be multi-functional (for both biobased products and livestock production).
IBERS is home to the National Plant Phenomics Centre and the **BEACON** centre of excellence for biorefining; a £ 20 million partnership between Aberystwyth, Bangor and Swansea universities. BEACON is a winner of European Union RegioStar award in 2014. The BEACON centre developed at IBERS has been using this developed high sugar grass for biorefining. BEACON team also works with up to 140 businesses located in Wales

In Europe, total grassland area is 16,358,000 ha. Generally, 85 % is used as livestock and animal feeding, while remaining 15 % (2454,000 ha) called as surplus grassland which generates a 20 million tonne dry matter grass fibre per annum at the rate of 8 tonne dry matter per ha. This grass biomass contains about 820 million tonne sugar in form of glucan, xylan and arabinan. Bio-processing of the grass fibres has been used successfully to produce fibre for the construction industry, high-protein animal fodder, biogas and ethanol. Grasses are the source of high value materials such as proteins and fibres as well as valuable chemicals such as lactic acids, amino acids and xylooligosaccharides.

4. Experience gained during this project and impact

The EU provides recommendations, in the document entitled "The Bioeconomy Enabled – a Roadmap to a Thriving Industrial Biotechnology Sector in Europe", to enable a 50 billion Euro industrial biotechnology market in Europe by 2030, and the main EU recommendation is training and improving skills of individuals in the industrial biotechnology sector.

Whilst working at BEACON part of the IBERS biorefining research team, not only were the objectives outlined before fulfilled but also the following were acquired during the process of the project completion namely, getting a good feel for and broadening competences in industrial biotechnology up to pilot level and understanding whole concept of biorefining and its economics

The BEACON+ is an award-winning project, which has assisted more than 150 businesses and undertook collaborative R&D projects with 50 businesses. The research that BEACON+ has undertaken with businesses is helping to foster the transition to a low carbon society, presenting significant environmental and business impacts.

Acknowlegements
We would like to thank the Stapledon Memorial Trust for sponsoring this project to work at IBERS, Gogerddan, Aberystwyth University. We are very grateful to Dr Sreenivas Rao for supervision and also would like to acknowledge the contribution of BEACON Biorefining team, especially Dave Thomas, Joe, Damon and Paul for providing technical support.