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Novel Enzymes for the Agro-food Industries

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Mestrado em Bioquímica Aplicada

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Doutor Tony Collins

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Resumo

As enzimas são catalisadores biológicos que aceleram reações químicas, sendo usadas diariamente numa ampla gama de indústrias, promovendo a melhoria da eficiência de processos, da relação custo-eficiência, assim como o impacto ambiental, para além de melhorar as características e a qualidade do produto. Há uma constante demanda por novas enzimas, de sequências, funções e características inovadoras. Neste projeto, 116 leveduras, maioritariamente provenientes de bio-resíduos acídicos, 11 fungos filamentosos de ambientes frios em Portugal e Islândia, 3 isolados bacteriais e 3 clones metagenómicos de amostras de solo Irlandês foram usados num rastreio por enzimas glicosil hidrolases de interesse industrial, nomeadamente, xilanases, celulases, pectinases e quitinases.

Métodos para um rastreio simplificado dos vários isolados a 16 °C, a pH 4, 7 e 9, foram investigados e desenvolvidos, tendo-se em particular, desenvolvido um novo substrato de xilanase. Este substrato demonstrou, através do ensaio DNS, ser apropriado para a medição de atividade xilanolítica, mas testes com outras xilanases são necessários para obter confirmação. Adicionalmente, este substrato foi ligado com sucesso ao corante remazol brilliant blue (RBB), obtendo-se um substrato cromogénico, possibilitando um rastreio de xilanases, em placas, altamente sensível e eficiente.

O rastreio dos isolados da coleção de culturas do CBMA permitiu a identificação de um grande número de isolados positivos para a presença de glicosil hidrolases, em que 31 demonstraram atividade exclusivamente a pH acídico. Estas enzimas podem ser de interesse para uso em várias aplicações, particularmente na indústria alimentar. Adicionalmente, 5 isolados mostraram ter atividade para todas as enzimas rastreadas a pH 4, podendo ter um potencial uso no tratamento de biomassa. Um clone metagenómico, com ADN metagenómico de solo Irlandês, positivo para atividade celulolítica, foi selecionado para análise adicional. O gene desta celulase foi amplificado com sucesso por PCR e digerido com enzimas de restrição apropriadas, mas tentativas de ligação num vetor de expressão e transformação para hospedeiro de expressão *E.coli* não foram bem sucedidas dentro do tempo limite do projeto. Estudos futuros deverão concentrar-se em expressar e caracterizar esta nova enzima.

Abstract

Enzymes are biological catalysts that accelerate the rate of chemical reactions in cells and are commonly used in a range of industries. They can improve the efficiency, cost-effectiveness and environmental impact of many processes and enhance the characteristics and quality of products. New enzymes with novel backbone sequences and novel functions and characteristics are constantly called for. In the present project, 116 yeasts from mainly acidic pH biowastes, 11 aquatic filamentous fungi from cold environments in Portugal and Iceland, 3 bacterial isolates and 3 metagenomic clones from Irish soil samples were screened for the production of glycoside hydrolase enzymes of industrial interest, namely xylanases, cellulases, pectinases and chitinases.

Methods were investigated and developed for a simplified screening of the various isolates at 16 °C, pHs 4, 7 and 9, with, in particular, the development of a novel xylanase substrate. The substrate was shown to be suitable for measurement of xylanase activity with the commonly used DNS assay but further tests with other xylanases are recommended to confirm this. Furthermore, the substrate was successfully coupled with remazol brilliant blue (RBB) dye, obtaining a chromogenic substrate and enabling a highly sensitive and efficient plate based screening of xylanases.

Screening of the CBMA culture collection isolates allowed for identification of a large number of glycoside hydrolase positive isolates, with 31 isolates showing desired activities exclusively at acidic pH. These enzymes may be of interest for use in various applications and, in particular, in food and beverages applications. In addition, 5 isolates were found to display all 4 activities screened for at pH 4, and these isolates may have potential for use in biomass treatment applications. A cellulase positive metagenomic clone with metagenomic DNA from Irish soil was selected for further analysis. The gene for this cellulase was successfully amplified and digested with restriction enzymes but attempts at ligation in an expression vector and transformation to an *E. coli* expression host proved unsuccessful within the time scope of the project. Further studies should be focused on overexpressing and characterising this novel enzyme.

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Abbreviations List

Abs – Absorbance

CBMA – Centro de Biologia Molecular e Ambiental

DNA – Deoxyribonucleic acid

GC % – Guanine-Cytosine content percentage

DNS – 3-amino-5-dinitrosalicylic acid

HPLC – High Performance Liquid Chromatography

LB – Lysogeny Broth

MOPS – 3-(N-morpholino)propanesulfonic acid

Mw – Molecular Weight

NSA – 3-amino-5-nitrosalicylic acid

OD – Optical Density

PCR – Polymerase Chain Reaction

PES – Polyethersulfone

rpm – revolutions per minute

SDS–PAGE – Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

Tm – Melting Temperature

upH2O – Ultrapure water

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Introduction

Enzymes in Industry

Enzymes are biological macromolecules that accelerate the rate of chemical reactions. They are highly specific, specialised by design for the catalysis of a specific reaction and outputting a specific result. This selectivity, in relation to both substrate and product stereochemistry (Jemli et al. 2014), warrants the development of enzymes as industrial alternatives surpassing traditional chemical catalysts. Enzymes are fast, efficient, and biodegradable and can operate under a variety of physicochemical conditions, according to their source. Moreover, they display a reduced environmental impact when compared to traditional chemical catalysts owing to a reduced use of process energy, raw materials and toxic components. Adding to this, their immobilization allows re-use, translating into cost reduction and performance improvement (Johannes et al 2006). Consequently, enzymes are already being used in a whole variety of industries.

The market for enzymes can be divided into high volume enzymes, for use in the food, feed, beverages, cleaning, textiles and biofuels industries; and high value, low volume specialty enzymes for use in diagnostics, research and development, and in the preparation of pharmaceuticals, fine chemicals, fragrances and flavours. Ultimately, enzymes enable the development of novel, improved and/or more economic and eco-friendlier end-products and bioprocesses (Barroca et al 2017). Global demand stands at ~7 billion USD and is expected to rise at a compound annual growth rate of ~5.7% from 2018 to 2024 (Srivastava et al., 2018). This rise is a consequence of the contemporary trend towards more sustainable and environmentally friendly processes and products.

Hydrolases

The International Union of Biochemistry and Molecular Biology (IUBMB) and the Enzyme Commission (EC) have divided enzymes into six main groups according to the type of reaction catalysed. Each enzyme has been assigned a code number, the EC number, consisting of four digits, separated by dots. The first digit (from 1 to 6) shows the main group to which the enzymes belong, and the remaining numbers represent a progressively finer classification of the enzyme (Webb, 1992).

Table I - Table detailing the classification of enzymes according to the Enzyme Commission (EC) number as recommended by the IUBMB. Information on the EC number, classification name and reactions catalysed are given.

EC Number	Classification	Reaction Catalysed
1	Oxidoreductases	Oxidation-reduction reactions between molecules.
2	Transferases	Transfer of functional group from one molecule to another.
3	Hydrolases	Bond cleavage via hydrolysis (a substitution reaction) with the addition of a water molecule.
4	Isomerases	Molecule conversion into isomers through intramolecular rearrangements.
5	Lyases	Non-hydrolytic catalysis of cleavage/removal of a molecule, leaving either a double bond or a ring structure.
6	Ligases	Promote formation of new bonds between two or more molecules.

Of the 6 classes of enzymes, hydrolases (group 3, EC 3.x.x.x) are the most predominantly used, (Srivastava et al, 2018). They are hydrolytic enzymes that break down a large molecule substrate into smaller molecule products. They are sub-divided into 13 groups in the EC classification system based on the type of bond hydrolysed. Some examples of common hydrolases include: glycosylases and nucleosidases (EC 3.2.x.x), esterases and lipases (EC 3.1.x.x) and proteases (EC 3.4.x.x). Hydrolases are commonly used in various industries, such as in the detergent, leather, textiles, pulp and paper, foods and feeds, dairy, and biofuels industries, where they allow for a reduced consumption of raw materials, energy, chemicals and water, and reduced production of waste and emission of greenhouse gases. (Dalmaso et al, 2015).

Glycosylases and Glycoside Hydrolases

Glycosylases (EC 3.2.x.x) are a hydrolase sub-group that act on glycosyl bond and are further subdivided into three sub-groups depending on the bond hydrolysed: EC 3.2.1.x: glycosidases, which hydrolyse O- and S-glycosyl compounds; EC 3.2.2.x: hydrolyse N-glycosyl compounds and EC 3.2.3.x: hydrolyse S-glycosyl compounds.

The glycosidases, also known as glycoside hydrolases (GH), or glycosyl hydrolases, (EC 3.2.1.x) are a group of enzymes that catalyse the hydrolysis of glycosidic linkages in complex sugars, degrading oligosaccharides and glycoconjugates into smaller oligo or monosaccharides (Henrissat & Coutinho, 2001).

Examples include cellulases (EC 3.2.1.4), pectinases (EC 3.2.1.15), amylases (EC 3.2.1.1) and xylanases (EC 3.2.1.8) which are routinely used in industry. These account for approximately 20% of total enzyme sales, and largely find use as commodity enzymes in the manufacture of food, feed, beverages, cosmetics in starch processing, textiles, biofuel production, (Linares-Pasten et al, 2014). and cleaning agents (Kurrataa'yun et al, 2015).

While these proteins are already generally used in industry and have been relatively well studied as compared to the speciality enzymes, the necessity for new glycoside hydrolases with novel specificities and properties, and for enzymes with new backbones, has arisen, to enable intellectual property protection. Indeed, studies of and industrial applications for glycoside hydrolases are expected to blossom and widen to new areas in the future. As essential enzymes in the conversion and modification of biomass, they are anticipated to play a predominant role in the current drive towards using this renewable resource as a substitute for petrochemical raw materials in the production of chemicals and other value-added products in the future.

The EC number corresponding to glycoside hydrolases is EC 3.2.1.x., which reveals information on the substrate and type of reaction catalysed but does not give information on structural or mechanistic relationships, (Webb, 1992), which motivated the classification into families in the Carbohydrate-Active enZYmes (CAZY) database (http://www.cazy.org/) according to primary structure homology of the catalytic domains, sequence similarity and devised to incorporate both structural and mechanistic characteristics of these enzymes (Henrissat & Davies 2000). Nowadays, this system lists 156 families for glycoside hydrolases. Information on these families and their members, as well as other families - glycosyltransferases, polysaccharide lyases and carbohydrate esterases - are available on the continuously updated CAZY database. With this classification system, enzymes with similar primary structures, and thus also similar three-dimensional structures, are assembled into the same family. Notwithstanding, due to evolutionary divergence to obtain new specificities, families can encompass enzymes with distinct substrate specificities and, interestingly enzymes that catalyse the same substrate are sometimes placed in different families (Henrissat & Davies 2000).

In the present study, four types of glycoside hydrolases, namely xylanases, cellulases, pectinases and chitinases, will be screened for and in the following sections a brief overview of the reaction catalysed, substrate and applications of each of these enzymes will be given.

Glycoside Hydrolases: Xylanases

Xylan is one of the most abundant polysaccharides in nature and is found in large quantities in wastes from agricultural and food industries, making up 15–30% of the cell wall content of hardwoods, 7–10% of softwoods, and up to 30% of annual plants. Xylanases, which hydrolyse xylan, are generally classified into CAZY glycoside hydrolase families 10 and 11 and are found to often have an inverse relationship between their pI and molecular mass values (Collins et al, 2002). They participate in the transformation of xylan into xylose and value-added xylo-oligomer products and offer new prospects for efficient treatment of hemicellulosic biowastes (Beg et al, 2001).

Xylan itself is a complex heteropolymer with variable structure dependent on the source, and typically contains a backbone of β-1,4 linked xylose units to which various side chains may be bonded as shown in Fig.1 Such a structure requires the action of several different enzymes in tandem, as also shown in Fig.1. Of these, the endo xylanases (EC 3.2.1.8) are the essential element, playing a key role in degrading xylan by cleaving the β-1,4 backbone of this complex plant cell wall polysaccharide (Butt et al. 2008). Indeed, the occurrence of multiple xylanases has been reported in many microorganisms, such as: bacteria (Chakdar et al, 2016) yeasts, fungi (Rytioja et al, 2014).

Xylanases are already applied in the making of beverages, mainly juices and beers (improving maceration, extraction, clarification and flavour, as well as in the extraction of coffee, plant oils and starch (Wong & Saddler 1992). In baking they have been found to improve the bread volume, crumb structure and reduce stickiness as well as increase the shelf life (Collins et al 2002a; Dutron et al 2004). There's also interest in their use in the production of liquid and gaseous fuels (Dodd et al, 2009), prebiotics and low-calorie products (Linares et al, 2018).

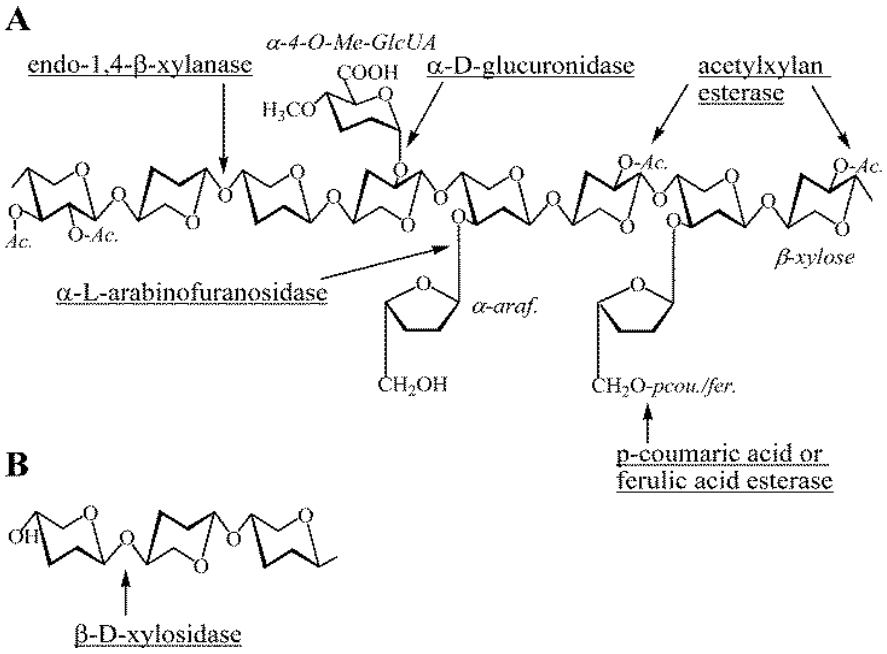


Figure 1 – Structure of Xylan and modes of action of xylanases. Xylan is a complex heterogeneous polysaccharide with a basic skeleton composed of a linear backbone of β-D-1,4 linked xyloses (pentose sugar) unit. The hydrolytic enzymes represented are endoxylanase (endo-1,4-xylanase (E.C.3.2.1.8)), xylosidase (xylan-1,4-xylosidase (E.C.3.2.1.37)), glucuronidase (α-D-glucuronidases (E.C.3.2.1.139)), arabinofuranosidase (α-L-arabinofuranosidase (E.C.3.2.1.55) and acetylxylan esterase (E.C.3.1.1.72). Taken from Collins et al (2005).

Glycoside Hydrolases: Cellulases

Cellulases are glycoside hydrolases which cleave cellulose, a linear polysaccharide of β (1→4) linked D-glucose units. While the most common renewable biological resource on Earth, cellulose is also a low-cost energy source based on energy content alone with the enzymes themselves being very abundant. Indeed, fungi, bacteria, plants and animals have been observed to have these enzymes, which typically hydrolyse the β-1,4 linkages of cellulose (Zhang, 2009). They include endo-acting endocellulases (endoglucanases) (EC 3.2.1.4), exo-acting cellobiohydrolases (EC 3.2.1.91) and cellobiases (β-glucosidases) (EC 3.2.1.21) which catalyse the segmentation of cellulose to smaller oligosaccharides and, finally, glucose as shown in Fig.2.

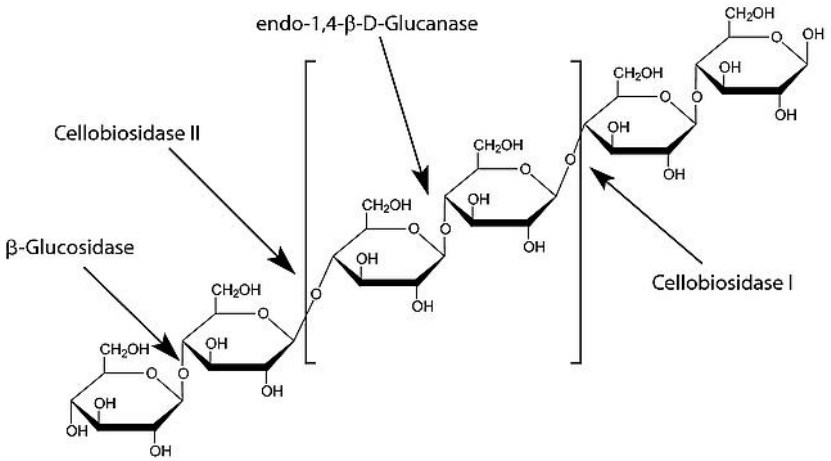


Figure 2 - Modes of action of cellulolytic enzymes on cellulose. Endoglucanases cleave cellulose chains internally; cellobiohydrolases or exocellulases cleave two to four units from the ends of exposed chains and release β-cellobiose; β-glycosidases hydrolyse the disaccharide β-cellobiose to glucose. Taken from www.sigmaaldrich.com/catalog/product/sigma/d9515?lang=pt®ion=PT

Cellulases have been commonly for improvement of fabric quality, softness and colour brightness (Kuhad et al. 2011). They have also found application in the detergents (removing cellulose derived stains), food and beverages (improving yields, maceration, clarification, aroma, taste, texture, among others) and pulp and paper industries (Behera et al, 2017). Their central part in the breakdown of biomass has culminated in their development for use in the bioconversion to biofuels and recently, to various chemicals (Payne et al. 2015).

Glycoside Hydrolases: Pectinases

Pectins are carbohydrates found in the cell wall and middle lamella of land plants, being very abundant in nature. Similar to xylan, they are heteropolysaccharides with a variable structure dependent on their source but with a basic structure consisting of a backbone of α(1–4)-linked D-galacturonic acids (pectic acid) or its methyl esters (pectin), and/or α(1,2)-linked-L rhamnose, and with this backbone being substituted to varying degrees with multiple types of neutral sugars (Mohnen, 2008). Enzymes involved in pectin catalysis are: pectin esterases (EC 3.1.1.11), hydrolases (EC 3.2.1.15, EC 3.2.1.67) and lyases (EC 4.2.2.10) (see Fig.3). Of the hydrolases, both endo- and exo-acting polygalacturonases act on pectic acid (EC 3.2.1.15) and polymethylgalacturonases act on pectin (EC 3.2.1.67), catalysing the cleavage of the backbone α -1,4-glycosidic linkages (see Fig. 3).

Pectinases were first commercialised in the 1930s for the preparation of wines and fruit juices, but became more versatile over time. While being much less well studied and used than other glycoside

hydrolases, biotechnological advances spurred the isolation and characterisation of novel pectinases, classifying them as important 'upcoming' enzymes of the commercial sector (Alimardani-Theuil et al, 2011). In addition to plants, pectinolytic enzymes are naturally produced by: bacteria, fungi, yeasts, insects, nematodes and protozoa and these pectinases, have also been applied in fruit processing and the production of juices, as well as in vegetable processing and wine making, as pectin present in the fruit and vegetables has the undesired effect of increasing viscosity and turbidity. As such, they improve fruit maceration and clarifying beverages as well as impacting on the product colour and flavour (Bhardwaj et al, 2017). In addition, they have also been suggested for use in the production of animal feed as well as in protoplast isolation, retting and degumming of fiber crops, the treatment of pectic wastewater, oil extraction and, finally, coffee and tea fermentation (Kashyap D. R. et al, 2001). Other reports indicate that pectinases can be used in the purification of viruses from plant tissues (Salazar et al, 1999).

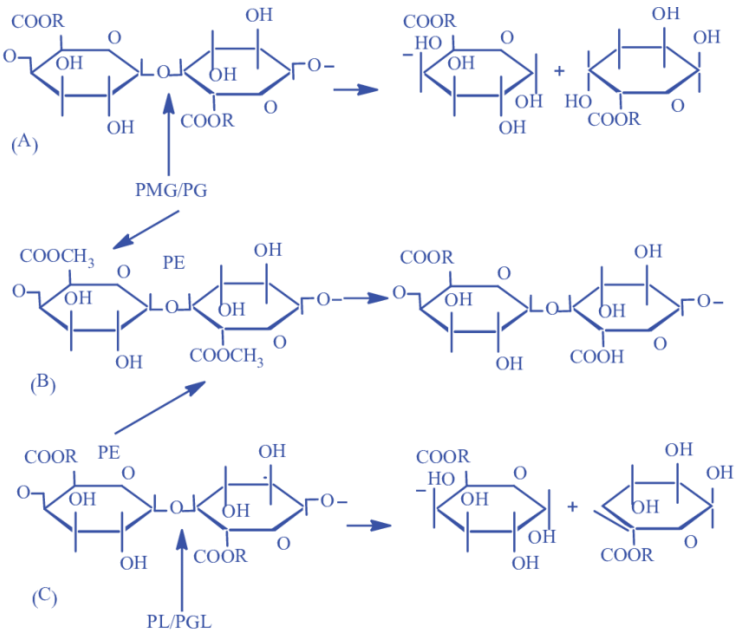


Figure 3 - Modes of action of pectinases: (a) Polygalacturonase (PG) hydrolyzes pectic acid to give saturated galacturonic acid. Polymethylgalacturonase (PMG) hydrolyzes pectin to give saturated methoxylated galacturonide; (b) Pectin esterase (PE) cleaves the methyl ester bond in pectin to give pectic acid. (c) Polygalacturonate lyase (PGL) action on pectin gives unsaturated galacturonic acid while Pectin lyase (PL) action leads to unsaturated methoxylated galacturonide, both enzymes degrade pectin through elimination reactions. Taken from Kantaraj et al., 2017.

Glycoside Hydrolases: Chitinases

Chitin, a linear polymer of β-1,4-N-acetylglucosamine (GlcNAc), is the second most abundant biopolymer on the planet. (Shahidi et al. 2005). Chitin is found in the outer skeleton of insects, fungi,

yeasts, algae, crabs, shrimps, and lobsters, and in the internal structures of other invertebrates (Bhattacharya et al, 2007). It is is a white, hard, inelastic polysaccharide, and is a major contributer to pollution in coastal areas (Pangburn et al, 1984). For example, approximately 75% by weight of shellfish is disposed as waste and approximately 20-60% of this is chitin (Wang et al, 1997). Chitin and its associated materials have a broad number of uses in for example drug delivery, wound healing, potential role as dietary fiber and in waste water treatment (Muzzarelli et al, 1999). It exists in 2 allomorphic forms i.e. α-chitin and β-chitin, and these 2 forms vary in the packing and polarities of adjacent chains in the succeeding sheets (Bussink et al, 2007; Chen et al, 2010). The catabolism of chitin takes place in 2 steps, involving the initial cleavage of the chitin polymer by chitinases into chitin oligosaccharides and further cleavage to N-acetylglucosamine, and monosaccharides by chitobiases (Suginta et al, 2000).

Chitinases are glycosyl hydrolases with sizes ranging from 20 kDa to about 90 kDa (Bhattarchrya et al, 2007). They are produced by a wide range of organisms such as bacteria, fungi, yeasts, plants and arthropods. They have been divided into 2 main groups: endochitinases (E.C 3.2.1.14) and exochitinases (E.C. 3.2.1.29/30). The endochitinases randomly split chitin at internal sites, thereby forming, predominantely, the dimer di-acetylchitobiose and soluble low molecular mass multimers of GlcNAc such as chitotriose and chitotetraose (Sahai et al, 1993). The exo-chitinases have been further divided into 2 subcategories: chitobiosidases (E.C. 3.2.1.29), (Harman et al, 1993) which are involved in catalyzing the progressive release of di-acetylchitobiose from the non-reducing end of the chitin microfibril, and N-acetyl-glucosaminidase (E.C. 3.2.1.30), cleaving the oligomeric products of endochitinases and chitobiosidases, thereby generating monomers of GlcNAc (Sahai et al, 1993).

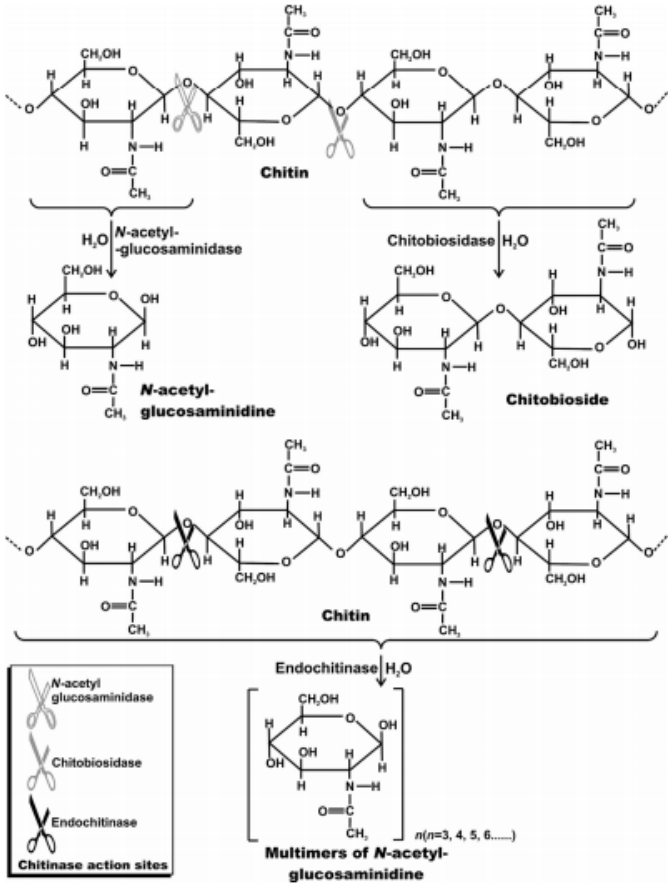


Figure 4 – Modes of action of the different types of chitinases. Taken from Das et al, 2016.

The products of chitinases, low molecular weight chitooligomers, can serve a broad range of industrial, agricultural and medical functions. They can have elicitor action and/or anti-tumor activity (Yuli et al, 2004) and N-acetylglucosamine has been investigated for the treatment of osteoarthritis (Shiro et al, 1996). Chitinases themselves have shown potential in the biocontrol of fungal phytopathogens (Mathivanan et al, 1998) and harmful insects, such as mosquitoes, and in plant defence systems against chitin-containing pathogens (Mendonsa et al, 1996). Chitin and chitinases are used by pathogens (mainly protozoans and metazoans) causing animal and human diseases. Several pathogens contain chitin coats, giving them protection, while others attack their host using chitinase, and to establish a successful infection or transmission from one vertebrate to another, they exploit the chitin-containing structures of the host (Shahabudin et al, 1999).

The Project BioProspect

The present study is encompassed in an ongoing project, titled Bioprospect, being developed at the laboratory where this work was carried out. BioProspect (Full title: Valorising CBMA biobanks: characterisation and bioprospection for novel value-added products) has as its goal the characterisation

and valorisation of the culture collection housed at the Centre of Molecular and Environmental Biology (CBMA). This culture collection is constituted by several hundred bacteria, yeasts and fungi from a variety of national and international environmental sources. Organisms have been isolated from such diverse environments as the biowastes of national and international fruit and vegetable processing industries, leaf litter debris of various Portuguese and Icelandic freshwater streams, leaf litter debris of Portuguese mine streams as well as from vineyards and the baking and wine manufacturing industries. Many of the CBMA culture collection organisms have been isolated from extreme environments, namely from acidic pH and low temperature environments. Actively growing biowaste decomposers were isolated from various fermenting vegetable (broccoli, cauliflower, artichoke, asparagus, salad mix, beans etc.) and fruit (strawberries, peaches, pears etc.) wastes and analysis of these biowastes indicated pHs between pH 2 (strawberry waste) and pH 9 (artichoke waste). Indeed, such a low pH points to the presence of acidophilic organisms adapted to the acidic environment. Similarly, microbial isolates were also obtained from litter debris in low temperature streams in both Portugal and Iceland (~5 °C) and acidic streams (pH ~4) near abandoned mines. These various isolates, and especially those from extreme environments, offer potentially rich sources of novel molecules adapted to their environments and of both fundamental and industrial interest. The overall objective of Bioprospect is to characterise the CBMA culture collection isolates in relation to their production of industrially relevant biomolecules, namely enzymes (cold adapted and acidophilic glycoside hydrolases), organic acid transporters and antimicrobial peptides. In the present study we focused on psychrophilic and acidophilic enzymes.

Psychrophilic enzymes

Psychrophilic enzymes are often defined as enzymes that originate from organisms with a maximal growth temperature below 20 °C and which grow well at temperatures around 0 °C. Furthermore, based on the growth temperature, psychrophilic organisms can again be sub-divided into two categories: psychrophiles (optimal growth temperature below 15 °C) and psychrotrophs or psychrotolerants (optimal growth temperature of around 20-25 °C)(Morita, R.Y. 1975). In general, psychrophilic enzymes display a high specific activity at low to moderate temperatures and a low thermal and chemical stability as compared to enzymes from organisms inhabiting warmer environments (Dalmaso et al, 2015). In addition, at a structural level, as compared to mesophilic and thermophilic homologs, they tend to possess various combinations of the following features: a decreased core hydrophobicity, increased surface hydrophobicity, lower arginine/lysine ratio, weaker

inter-domain and inter-subunit interactions, more and longer loops, decreased secondary structure content, more glycine residues, less proline residues in loops, more proline residues in α-helices, less and weaker metal-binding sites, a reduced number of disulphide bridges, fewer electrostatic interactions (H-bonds, salt-bridges, cation–pi interactions, aromatic–aromatic interactions), reduced oligomerisation and an increase in conformational entropy of the unfolded state. In addition, genomic comparisons of psychrophiles vs. thermophiles have also revealed that distinct biases in amino acid composition is a trademark of thermal adaptation (Saunders et al*.,* 2003; Siddiqui and Cavicchioli, 2006). All of these structural alterations would be expected to lead to the decreased stability reported for psychrophilic enzymes. Furthermore, they could also lead to an increased structural flexibility, thereby leading to the observed higher activity at low temperatures.

Cold-adapted enzymes are of industrial interest as they can provide economic benefits by being more productive than mesophilic or thermophilic homologues at low to moderate temperatures (e.g. 5-30 °C), thereby providing energy savings to the processes. They can minimize undesirable chemical reactions that can occur at higher temperatures, can be rapidly inactivated by heating, and can be used with heat sensitive substrates and/or products (Jeon et al*.,* 2009a). These properties are of relevance to the food and feed industry where it is important to avoid spoilage and change in nutritional value and flavour of the original heat-sensitive substrates and products (Russell, 1998; Gerday et al*.,* 2000; Cavicchioli et al*.,* 2002; De Pascale et al*.,* 2009). They are also useful for the molecular biosciences because of the need to use enzymes in sequential reactions, and the need to inactivate each enzyme after it has performed its function (Cavicchioli et al, 2011). Cold-adapted enzymes also have potential application in mixed aqueous-organic or non-aqueous solvents for organic synthesis. Their utility derives from their proposed inherent flexibility, which counteracts the stabilising effects of low water activity in organic solvents (Owusu-Apenten, 1999; Sellek and Chaudhuri, 1999; Gerday et al*.,* 2000).

Acidophilic Enzymes

Many extremophilic organisms inhabit environments with more than one extreme parameter, for example, extremophiles that thrive in the depth of the oceans, mines or close to hot springs. Acidophilic enzymes are found in organisms that thrive in low pH environments, with the most extreme surviving below pH 4 (Dumorné et al 2017). Studies thus far have indicated that these enzymes have adapted through a high proportion of acidic residues on their surface to cope with the high positive charges inherent to their environment. Furthermore, structural shielding of binding domains from

solvent exposure and increased binding to metallic co-factors have also been reported (Bönisch et al, 2002; Cooper et al, 1990). Acidophilic enzymes have several applications in the food and beverages and feed industries, such as in the preparation of 'acidic' breads including sour dough and rye bread, in fruit and vegetable juice preparation and wine making, in starch saccharification, baby foods and yoghurts (Sharma et al, 2016), as well as in biofuel and ethanol production.

Enzyme Screening

As indicated previously, one of the main objectives of the present study, and indeed of the project Bioprospect, is to screen the CBMA isolates for production of enzymes of industrial interest, with a focus on acidophilic and/or psychrophilic glycoside hydrolases. To achieve this, a simple, cheap and quick method is required to enable an efficient screening of a large number of isolates. Currently two approaches exist for screening cultures and environments for enzymes of interest, these being metagenomics approaches and culture based approaches.

Metagenomics for Enzyme Screening

Metagenomics is the study of genetic material isolated directly from an environment, pooling the genomes of the microbial community present, therefore constructing the metagenome. (López-López et al 2014). With metagenomics, DNA can be sequenced and analysed without the need for culturing individual wild-type organisms and hence allows for a greater access to the total functional and microbial compositions of these environments (Harkins & Jarvie 2007). Its development has been facilitated by rapid advances in next generation sequencing technology and associated bioinformatics. It is based on the extraction of total DNA from an environmental sample and subsequent purification (Bertrand et al 2005), sequencing and analysis, followed by database searches to identify homologs and putative function (sequence based metagenomics). In addition, a function-based metagenomics approach can be used. This is based on the cloning of the isolated DNA in appropriate vectors and laboratory hosts, to create metagenomic DNA libraries, and cultivation and screening of these for identification of specific enzyme functions (Sharma et al 2016).

Metagenomics however has its own limitations. Sequence based metagenomics targets specifically DNA sequences with homology to an enzyme of interest and hence denies the possibility of finding a truly novel enzyme as sequences found will always have a similarity with previously discovered proteins. Function-based metagenomics on the other hand may be hindered by an inability to produce a properly folded, stable and active enzyme in the screening host used. Most commonly, *E. coli* is used as screening host with some success, but lower success has been reported with gram positive and

yeast or fungal enzymes (Le Y. et al, 2011). In addition, due to its inability to grow efficiently at extremes of pH and low temperatures, it may not be suited to screening for acidophilic, psychrophilic and/or psychro-acidophilic enzymes. Furthermore, the power of metagenomics is in its ability to efficiently screen complex environmental samples whereas the CBMA collection is constituted by single isolates for which cultivation conditions have been identified, thus culture based approaches may be more suited for use in the present study.

Culture-based Enzyme Screening

Any culture-based approach for a determinate end hinges on the selection of an appropriate medium and incubation conditions to cultivate the organism(s) of interest. Although apparently simple to design, in practice success is difficult to attain. It has been long known that the number of cells in a sample surpass by a large margin the number of cells that can form colonies on a petri plate (Amaan, 1911). Estimations indicate that less than 1% of the organisms in soil can be cultured (Pham et al, 2012) while 0.01 to 0.1 % of all the microbial cells in marine environments form colonies on standard agar plates (Cho et al, 2004). It takes a really thorough approach, taking into account every aspect of the original environment to be able to properly and efficiently culture an organism (Stewart, 2012). Notwithstanding, researchers have devised over the years multiple rich and minimal media formulations to aid in achieving this end for use either as liquid cultures or, by adding agar to these very same media, for plate cultures.

Plate-based screening assays, when possible, are the method of choice as they enable a rapid, simplified, cost reduced method to identify enzymes of interest. They have been commonly used for a number of years with, in many cases, the original natural substrates being replaced by chromogenic substrates for more sensitive detection of specific activities. Most currently used plate assays are based on the breakdown of insoluble or partially soluble macrosubstrates and chromogenic substrates to smaller and more soluble products. Radial diffusion of these soluble products then leads to clearing zones or reduced/altered colour intensity for simplified identification of enzyme activity.

In the present study, plate-based screening will be used as, as indicated above, single isolates with known growth conditions will screened. A number of substrates and plate based assays have already been described for the enzymes to be screened in the present study and these different assays will be investigated and optimised here. In particular, a specific focus will be made in the present project on the development of in-house substrates and methods for screening and analysis of xylanase enzymes, as the high costs and poor availability of currently available xylanase substrates are somewhat

prohibitive. Another ongoing parallel project in the laboratory where the present project was carried out is focused on developing sustainable, environmentally friendly approaches based on enzyme technology for the conversion of waste biomass to value added products and namely various functional food/feed ingredients. A byproduct of this process has been found to be rich in a soluble xylan and another aim of the present project was thus to characterise this byproduct and develop it for use in xylanase screening and xylanase activity assays.

Objectives

The goals of this study are as follows:

- Develop and characterise an in-house substrate for xylanase screening and activity assays
    - Characterise substrate: purity, composition, size, degree of polymerisation
    - Develop for use in plate-based screening assay
    - Develop for use in xylanase activity assay
- Develop and optimise plate-based assays for screening for various glycoside hydrolases
    - Compare and develop plate assays for screening at low temperatures and/or extreme pHs
- Characterise and valorise the CBMA culture collection
    - Screen CBMA culture collection isolates for glycoside hydrolases (xylanases, cellulases, pectinases and chitinases) of industrial interest
    - Characterise enzymes of interest
- Overexpress in *E. coli* and characterise one enzyme of interest identified in the plate screening
    - PCR for gene isolation, restriction digestion and cloning in expression vector, protein production and characterisation.

Materials and Methods

Carbohydrate Rich Byproduct: Characterisation and Compositional Analysis

An ongoing parallel project focused on validating waste biomass has resulted in production of a carbohydrate rich byproduct. In the present project this byproduct will be analysed and characterised.

Compositional Analysis: Total Sugars (Dubois et al, 1956)

The total sugar content of the extracted biowaste byproduct was determined. The method employed is based on use of high temperatures and low pHs for dehydration of all monosaccharides present to hydroxymethyl furfural. Subsequent reaction of the hydroxymethyl furfural with phenol in acidic conditions leads to generation of an orange-yellow compound product which absorbs at 490 nm. For full details of protocol see Annexe I.

Compositional Analysis: Total Lipids (Bligh and Dyer, 1959)

A method first described in 1959 for the methanol-chloroform extraction and quantification by weight of purified lipids was used for determination of the lipid content of the carbohydrate rich byproduct as described in Annexe II.

Compositional Analysis: Ash Content (Laurens et al, 2012)

To determine the ash content of the carbohydrate rich byproduct, high temperatures were used to burn away the organic content and leave inorganic minerals for analysis by weight. See Annexe III for protocol.

Compositional Analysis: Protein Content

The Bradford assay was used for determination of the protein content as described in Annexe IV.

Compositional Analysis: Nitrogen Content

The Kjeldhal method was used for nitrogen content measurements which can be used for determination of the presence and quantification of nitrogen containing compounds such as amino acids, proteins, phenolic compounds etc. Byproduct samples are digested in sulphuric acid in the presence of a chemical catalyst, which results in the conversion of nitrogen to ammonia. This is followed by distillation of the ammonia into a trapping solution and ammonia quantification by titration with a standard solution of 2% - 4% boric acid. Digestion was performed in a FOSS Kjeldahl Digestor Unit, Tecator™/ Labtec™ 8 tubes. Analysis of the resulting ammonia was performed with a FOSS Kjeldahl Digestion Unit, KJELTEC™ 8400 Analyser Unit. The detailed protocol is given in Annexe V and

was performed at the Centro de Engenharia Biológica (CEB), UMINHO with assistance by Dr. Vitória Maciel

Compositional Analysis: Composition of Carbohydrate Component

Concentrated sulphuric acid treatment followed by high pressure liquid chromatography (HPLC) on a Rezex™ 8 µm ROA-organic acid H+(8%) column (Phenomenex) for separation of carbohydrate monomer was used to identify the carbohydrate content of the sugar component of the carbohydrate rich byproduct. HPLC was carried out on an Elite LaChrom (VWR Hitachi) chromatography system at 60 °C with detection by refractive index measurements with an Elite LaChrom L-2490 RI detector (VWR Hitachi). EZChrom Elite 3.3.2 SP2 software was used for data collection and analysis. For protocol, see Annexe VI.

Compositional Analysis: Molecular Weight of Carbohydrate Component

The molecular weight of the carbohydrate component of the byproduct was investigated by gel filtration chromatography on a PolySep™-SEC GFC-P Linear column (Phenomenex). HPLC was carried out on an Elite LaChrom (VWR Hitachi) chromatography system at 30 °C with detection by refractive index measurements with an Elite LaChrom L-2490 RI detector (VWR Hitachi). EZChrom Elite 3.3.2 SP2 software was used for data collection and analysis. The protocol used is described in Annexe VII.

Compositional Analysis: Degree of Polymerisation of Carbohydrate Component

For determination of the degree of polymerisation (DP) of the carbohydrate component of the byproduct a Rezex™ RSO-Oligosaccharide Ag+ (4) % HPLC column was used. HPLC was carried out on an Elite LaChrom (VWR Hitachi) chromatography system at 85 °C with detection by refractive index measurements with an Elite LaChrom L-2490 RI detector (VWR Hitachi). EZChrom Elite 3.3.2 SP2 software was used for data collection and analysis. For full protocol, see Annexe VIII.

Carbohydrate Rich Byproduct: Use as Xylanase Substrate in Screening and Assay

The high content of xylan in the byproduct indicated its potential as a substrate for xylanase screening and analysis and this was investigated here.

Carbohydrate Rich Byproduct for Xylanase Screening: Remazol Brilliant Blue – Xylan

Remazol Brillant Blue (RBB) was coupled to the xylan component of the byproduct to give RBB-xylan for use as a novel chromogenic screening substrate and the number of RBB molecules per molecule of

xylose determined as detailed in Annexe IX. The use of the novel prepared RBB-xylan as a chromogenic substrate for xylanase screening was then investigated as described below (RBB-Xylan for Xylanase Screening).

Carbohydrate Rich Byproduct: Use in Xylanase Activity Assay

Xylanase activity is frequently analysed using the Dinitrosalicylic Acid (DNS) assay (Miller, 1959) wherein the reducing ends released upon enzyme hydrolysis of a xylan substrate are measured. These reduce 3-amino-5-dinitrosalicylic acid (DNS) to 3-amino-5-nitrosalicylic acid (NSA) which under alkaline conditions is converted to a reddish brown coloured complex which has an absorbance maximum of 540 nm. In the present study the xylan rich byproduct was investigated for use as a substrate for xylanase analysis with the DNS assay using the protocol described in Annexe X.

Microbial Samples

A number of yeasts, fungi, bacteria and metagenome screening clones were made available for screening and analysis in this study. These isolates form part of the CBMA culture collection housed at the CBMA, University of Minho and were chosen due to their potential as sources of psychrophilic, acidophilic and/or psychro-acidophilic glycoside hydrolases of interest for this study.

Fungi

All 13 fungal isolates were kindly provided by Dr. Isabel Fernandes of the CBMA, University of Minho. Isolates had been identified by morphological analysis. Table II indicates the identification, source and habitat conditions of these isolates.

Table II –Fungal Isolates Screened in Study: Identification, Source Environment and Habitat Conditions

Species	Locality	GPS	Environment	T (°C)	pH
Heliscus lugdunensis	Jales Mines (Peliteira Stream), Portugal	41°26′N 7°33′W	Water Foams		
Tricladium splendens	Jales Mines (Peliteira Stream), Portugal	41°26′N 7°33′W	Leaves in Stream		
Triscelophorus sp2.	Brook (Hengill, Iceland)	64°03'N 021°18'W	Cotton Cloth Baiting in Brook	3.5	7.7
Triscelophorus sp2.	Brook (Hengill, Iceland)	64°03'N 021°18'W	Cotton Cloth Baiting in Brook	3.5	7.7
Triscelophorus sp2.	Brook (Hengill, Iceland)	64°03'N 021°18'W	Cotton Cloth Baiting in Brook	7.7	8.0
Triscelophorus sp2.	Brook (Hengill, Iceland)	64°03'N 021°18'W	Cotton Cloth Baiting in Brook	7.7	8.0
Triscelophorus sp2.	Brook (Hengill, Iceland)	64°03'N 021°18'W	Cotton Cloth Baiting in Brook	5.5	7.7
Triscelophorus sp2.	Brook (Hengill, Iceland)	64°03'N 021°18'W	Cotton Cloth Baiting in Brook	5.5	7.7
Triscelophorus sp2.	Brook (Hengill, Iceland)	64°03'N 021°18'W	Cotton Cloth Baiting in Brook	5.5	7.7
Triscelophorus sp2.	Brook (Hengill, Iceland)	64°03'N 021°18'W	Cotton Cloth Baiting in Brook	5.5	7.7
Varicosporium elodeae	Jales Mines (Peliteira Stream), Portugal	41°26′N 7°33′W	Water Foams		
Varicosporium elodeae	Jales Mines (Peliteira Stream), Portugal	41°26′N 7°33′W	Water Foams		
Articulospora tetracladia	Uranium Mines (Patanha Stream), Portugal	40°59'N 8°00'W	Leaves in Stream		

The aquatic hyphomycetes *Heliscus lugdunensis* and *Varicosporium elodeae* were isolated from natural foams in the waters of a stream, Peliteira stream, associated with the abandoned gold mines of Jales, Northern Portugal (41°26′N 7°33′W). *Tricladium splendens* was isolated from leaves collected in the same stream. All *Triscelophorus sp2* samples were isolated from 3 different streams in the Hengill volcanic region of Southern Iceland (64°03'N 021°18'W), with average temperatures of 3.5 °C, 5.5 °C and 7.7 °C through a baiting technique using cotton cloths incubated in the waters. *Articulospora tetracladia* was isolated from leaves in a stream, Patanha stream, near abandoned uranium mines near Alhões, Northern Portugal (40°59'N 8°00'W).

Yeasts

The CBMA culture collection houses a large number of yeast isolates collected in the scope of a number of projects, namely the EU project TRANSBIO, from a number of different national and international agro-food industry biowastes. Actively growing biowaste decomposers have been isolated from various fermenting vegetables and fruit wastes with pHs between pH 2 and pH 9. In the present study 116 isolates were selected due to the low pHs of their source environment, namely biowastes of apples, green beans, fresh chard, processed chard, mixes of salads, peaches, pears, peppers and strawberries, with measured pHs from as low as 2 (strawberry biowaste) to 7.4. All isolates were kindly provided by Dr. Ricardo Franco-Duarte of the CBMA, University of Minho. Table III indicates the identification, source and habitat conditions of these isolates. Yeast identification was determined Internal Transcribed Spacer (ITS) sequencing.

Table III – Yeast Isolates Screened in Study: Identification, Biowaste Source and Biowaste pH. Gray cells in the pH columns: No Data.

Species	Environment	pH	Species	Environment	pH	Species	Environment	pH	Species	Environment	pH
Saccharomyces cerevisiae	Peach	~3.5	*Candida humilis*	Peach	~3.5 to 5.5	*Pichia fermentans*	Green Bean	~6.2 to 7.4	*Barnettozyma californica*	Chard F.	~5.5 to 6.8
Meyerozyma guilliermondii	Peach	~3.5	*Candida humilis*	Peach	~3.5 to 5.5	*Pichia fermentans*	Green Bean	~6.2 to 7.4	*Torulaspora delbrueckii*	Pepper	~3.6 to 6.8
Pichia fermentans	Peach	~3.5	*Candida humilis*	Peach	~3.5 to 5.5	*Wickerhamomyces anomalus*	Green Bean	~5.5 to 7.4	*Metschnikowia chrysoperlae*	Pepper	~3.6 to 6.5
Candida humilis	Peach	~3.5 to 5.5	*Aureobasidium pullulans*	Peach	~3.5	*Pichia kudriavzevii*	Green Bean	~5.5 to 6.5	*Candida tropicalis*	Pepper	~3.6 to 6.5
Kluyveromyces marxianus	Peach	~3.5	*Pichia occidentalis*	Peach		*Pichia fermentans*	Green Bean	~6.2 to 7.4	*Candida glabrata*	Chard P.	~3.6 to 6.8
Kluyveromyces marxianus	Peach	~3.5	*Candida tropicalis*	Peach	~3.5	*Pichia norvegensis*	Green Bean	~6.2 to 7.4	*Candida intermedia*	Chard P.	~3.6 to 6.8
Saccharomyces cerevisiae	Peach	~3.5 to 5.5	*Kazachstania gamospora*	Peach	~3.5 to 5.5	*Pichia fermentans*	Green Bean	~6.2 to 7.4	*Torulaspora delbrueckii*	Strawberry	~2
Kazachstania gamospora	Peach	~3.5 to 5.5	*Pichia exigua*	Peach		*Pichia orientalis*	Green Bean		*Pichia fermentans*	Mix of salad	~6.5 to 7
Saccharomyces cerevisiae	Peach	~3.5 to 5.5	*Candida humilis*	Peach	~3.5 to 5.5	*Pichia fermentans*	Green Bean	~6.2 to 7.4	*Candida tropicalis*	Mix of salad	~6.5
Pichia fermentans	Apple		*Candida humilis*	Peach	~3.5 to 5.5	*Wickerhamomyces anomalus*	Green Bean	~5.5 to 7.4	*Wickerhamomyces anomalus*	Mix of salad	~6.5
Hanseniaspora uvarum	Apple		*Candida humilis*	Peach	~3.5 to 5.5	*Saccharomyces cerevisiae*	Pear	~2 to 6.5	*Pichia orientalis*	Mix of salad	
Wickerhamomyces anomalus	Apple		*Kazachstania gamospora*	Peach	~3.5 to 5.5	*Candida tropicalis*	Pear	~2 to 6.5	*Candida solani*	Mix of salad	~6.5 to 7
Wickerhamomyces anomalus	Apple		*Candida stellimalicola*	Peach	~5.5	*Geotrichum candidum*	Pear	~2 to 6.5	*Candida solani*	Mix of salad	~6.5 to 7
Pichia fermentans	Mix of salad	~6.5	*Pichia kudriavzevii*	Apple		*Metschnikowia pulcherrima*	Pear	~2 to 6.5	*Pichia kudriavzevii*	Green Bean	~5.5 to 6.5
Candida solani	Mix of salad	~6.5 to 7	*Rhodotorula mucilaginosa*	Apple		*Geotrichum candidum*	Pear	~2 to 6.5			
Candida tetrigidarum	Mix of salad	~6.5	*Rhodotorula mucilaginosa*	Apple		*Wickerhamomyces anomalus*	Pear	~2.5 to 6.5			
Candida tropicalis	Mix of salad	~6.5	*Pichia kudriavzevii*	Apple		*Candida stellimalicola*	Pear	~2.5 to 6.5			
Candida tetrigidarum	Mix of salad		*Hanseniaspora uvarum*	Apple		*Hanseniaspora uvarum*	Pear	~2 to 6.5			
Candida solani	Mix of salad	~6.5 to 7	*Pichia orientalis*	Apple		*Geotrichum candidum*	Chard F.	~5.5 to 6.8			
Wickerhamomyces anomalus	Pepper	~3.6 to 7	*Candida tropicalis*	Pepper	~3.6 to 7	*Geotrichum candidum*	Chard F.	~5.5 to 6.8			
Pichia kudriavzevii	Pepper	~3.6 to 7	*Pichia fermentans*	Pepper	~3.6 to 7	*Geotrichum candidum*	Chard F.	~5.5 to 6.8			
Clavispora lusitaniae	Pepper	~3.6 to 7	*Clavispora lusitaniae*	Chard F.	~5.5 to 6.8	*Geotrichum candidum*	Chard F.	~5.5 to 6.8			
Geotrichum candidum	Pepper	~3.6 to 7	*Metschnikowia sinensis*	Pepper		*Geotrichum candidum*	Chard F.	~5.5 to 6.8			
Candida solani	Mix of salad	~6.5 to 7	*Pichia kudriavzevii*	Apple		*Pichia fermentans*	Pepper	~3.6 to 7			
Pichia norvegensis	Mix of salad	~6.5	*Hanseniaspora uvarum*	Apple		*Hanseniaspora uvarum*	Pepper	~3.6 to 6.8			
Candida solani	Mix of salad	~6.5 to 7	*Hanseniaspora uvarum*	Apple		*Pichia fermentans*	Pepper	~3.6 to 7			
Pichia kudriavzevii	Green Bean	~5.5 to 6.5	*Pichia fermentans*	Apple		*Hanseniaspora uvarum*	Pepper	~3.6 to 6.8			
Torulaspora delbrueckii	Green Bean	~6.2	*Pichia fermentans*	Apple		*Pichia kudriavzevii*	Pepper	~3.6 to 7			
Papiliotrema flavescens	Green Bean	~6.2 to 7.4	*Wickerhamomyces anomalus*	Apple		*Candida tropicalis*	Pepper	~3.6 to 7			
Metschnikowia pulcherrima	Pear		*Pichia fermentans*	Apple		*Clavispora lusitaniae*	Pepper	~3.6 to 7			
Candida stellimalicola	Pear	~2.5 to 6.5	*Saprochaete gigas*	Apple		*Candida humilis*	Pepper	~3.6 to 6.8			
Wickerhamomyces pijperi	Pear		*Pichia kudriavzevii*	Apple		*Wickerhamomyces anomalus*	Pepper	~3.6 to 7			
Wickerhamomyces pijperi	Pear	~5.5 to 6.8	*Rhodotorula mucilaginosa*	Mix of salad	~5.5 to 6.5	*Pichia kudriavzevii*	Pepper	~3.6 to 7			
Barnettozyma californica	Chard F.	~5.5 to 6.8	*Cyberlindnera jadinii*	Mix of salad	~6.5	*Candida tropicalis*	Pepper	~3.6 to 7			

Bacteria

In this study, four bacterial isolates (Table IV) from Irish Suppressor Soil samples collected from a harvested winter oats field (TO-WO) at an organic field trial site at the Teagasc Oak Park (TO) research facility (Co. Carlow, Ireland; http://www.agresearch.teagasc.ie/oakpark/), were also investigated. The soil contained organic and inorganic carbon, phosphate, nitrate, water and clay, and were at pH 6 - 6.5 and a temperature of 13-15 °C (Granja, 2017). Previous studies had indicated the potential for cellulase and xylanase activity with these isolates.

Table IV – Bacterial Isolates Screened in Study: Identification Code, Source and Habitat Conditions

Wild-type Isolate	Source Locality	GPS	Source Environment	Inorganic Nutrients	Temperature	pH
TO_7	Teagasc Oak Park, Carlow, Ireland	52°51'21.2"N 6°54'43.4"W	Winter Oats Field	Carbon, Phosphate, Nitrate, Water, Clay	13-15 °C	6-6.5
TO_14	Teagasc Oak Park, Carlow, Ireland	52°51'21.2"N 6°54'43.4"W	Winter Oats Field	Carbon, Phosphate, Nitrate, Water, Clay	13-15 °C	6-6.5
TO_B6	Teagasc Oak Park, Carlow, Ireland	52°51'21.2"N 6°54'43.4"W	Winter Oats Field	Carbon, Phosphate, Nitrate, Water, Clay	13-15 °C	6-6.5
TO_E11	Teagasc Oak Park, Carlow, Ireland	52°51'21.2"N 6°54'43.4"W	Winter Oats Field	Carbon, Phosphate, Nitrate, Water, Clay	13-15 °C	6-6.5

Metagenome Clones.

In addition to the wild-type isolates described above, three clones (Table V) from a metagenomic library of environmental samples collected at the harvested winter oats field at the Teagasc Oak Park (TO) research facility (Co. Carlow, Ireland; http://www.agresearch.teagasc.ie/oakpark/) were also investigated. These consisted of ~40 kbp fragments of metagenomic DNA inserted in the fosmid vector pCCERI-1Fos (Borchert et al. 2017) in the screening host *E. coli* EPI300-T1R (Granja, 2017). A previous sequence based metagenomics study had indicated a potential for novel cellulase genes in these clones (Granja, 2017) and here the objective was to confirm these initial observations by

screening for active cellulases. Table V indicates the source locality and characteristics of the original environmental samples.

Table V – Metagenome Clones Screened in Study: Identification Code, Source Environment and Habitat Conditions

Metagenome Clones	Source Locality	GPS	Environment	Inorganic Nutrients	Temperature	pH	Activity
12.D3	Teagasc Oak Park, Carlow, Ireland	52°51'21.2"N 6°54'43.4"W	Winter Oats Field	Carbon, Phosphate, Nitrate, Water, Clay	13-15 °C	6-6.5	Cellulase
90.K1	Teagasc Oak Park, Carlow, Ireland	52°51'21.2"N 6°54'43.4"W	Winter Oats Field	Carbon, Phosphate, Nitrate, Water, Clay	13-15 °C	6-6.5	Cellulase
88_J12	Teagasc Oak Park, Carlow, Ireland	52°51'21.2"N 6°54'43.4"W	Winter Oats Field	Carbon, Phosphate, Nitrate, Water, Clay	13-15 °C	6-6.5	Cellulase

Screening for Glycoside Hydrolase Activity: Plate Assays

2% (w/v) Yeast Peptone Dextrose (YPD) agar and 2% (w/v) Malt Extract Agar (MEA) media were used for growth and screening of yeasts and filamentous fungi, respectively. Bacterial isolates and metagenome clones were grown in LB medium (1% Tryptone, 0.5% NaCl, 0.5% Yeast Extract), with addition of 12.5 µg/mL chloramphenicol and 0.001% arabinose for the metagenomic clones. To these media appropriate natural substrates, dyed substrates and/or additional dyes, as described below, were added to assess for hydrolytic activity. Activity was assessed at pHs 4, 7 and 9 (see Table VI for details of buffers) with incubation at 16 °C.

Table VI – Buffers used at pH 4, 7 and 9.

pH 4 (McIlvaine Buffer)	pH 7 (MOPS Buffer)	pH 9 (Glycine-NaOH Buffer)
40% (v/v) 0.2 M Na2HPO4	20 mM MOPS	0.2 M Glycine
60% (v/v) 0.1 M Citric Acid	3M NaOH added dropwise until pH 7	3M NaOH added dropwise until pH 9

Cellulase Screening

A number of assays exist for plate screening of cellulases. Cellulytic activity is frequently detected using carboxymethyl cellulose (CMC), or cellulose gum, which is a water-soluble cellulose derivative often used to assess endoglucanase activity. When these are present, the CMC is cleaved forming a clearing zone (Brigitte et al, 2014). CMC can also be used with Congo Red, an azo dye which binds to cellulose through Van der Waals interactions between aromatic rings. When the cellulose is cleaved, the dye is unable to bind efficiently, forming a clear halo against a red background around the colonies (Meddeb-Mouelhi et al., 2014; Mazeau et al, 2012; Sazci et al, 1986). Furthermore, subsequent treatment with acetic acid has also been reported to enhance screening sensitivity. Finally, another screening method involve plate flooding with Lugol's of Gram's Iodine. Lugol's Iodine is a mixture of potassium iodide and iodine, and Gram's Iodine is a diluted version of this, both are commonly used for Gram Staining. These interact with polysaccharides by forming polyiodide chains that interact differently depending on the structure of the polysaccharides. As such, these can be used to dye CMC, which, when cleaved diffuse and bind less efficiently the dyes, forming a halo around the colonies against a brown background (Moulay, 2013; Meddeb-Mouelhi et al., 2014). In the present study, all 4 assays were examined and compared at various pHs as described in Annexe XI.

Pectinase Screening

Pectinolytic activity is most commonly assessed using pectin incorporated in agar plates. This substrate is soluble and when degraded by pectinases, the more soluble, smaller products diffuse and lead to formation of clearing zones. This substrate has also been used with Ruthenium Red, an inorganic dye that binds to the negative charges on intact pectin, binding less to cleaved pectin and therefore forming a clear halo against a pink/red background (Torres et al. 2011). Finally, similar to cellulase screening Lugol's and Gram's iodine can also be used for pectinase screening. All 4 protocols were used for pectinase screening and are detailed in Annexe XII.

Chitinase Screening

Chitinases are most commonly detected using colloidal chitin prepared by acid treatment of chitin. Plate media containing this substrate are opaque and upon substrate hydrolyses by chitinases, smaller more soluble products are formed which diffuse in the medium leading to transparent clearing zone (Hsu and Lockwood, 1975). It can also be used with the pH indicator dye Bromocresol Purple as chitin digestion leads to an increase in pH and this dye changes from yellow below pH 6.8 to purple above it

(Agrawal et al, 2012). These 2 methods were compared here and the protocols are detailed in Annexe XIII.

Xylanase Screening

Two types of plate assays are currently available for xylanase screening, these being based on use of the chromogenic substrates AZCL-xylan and RBB-xylan. AZCL-xylan is a chromogenic substrate prepared by cross-linking xylan with azurine dye. Isolates with xylanolytic activity cleave the surrounding dark coloured substrate, forming soluble fragments that disperse radially and give off a blue colour, forming a blue halo around the positive isolates (Agrawal & Kotasthane, 2012). RBB-xylan, is xylan dyed with Remazol Brilliant Blue (RBB), an anthraquinone dye (Bhat M.K., 2000, Biely et al, 1985). Agar plates containg this acquire a blue background and cleavage by xylanases generates smaller, more soluble fragments that spread radially, accumulating a blue colour at the edge while a clearing zone is formed directly around the colonies. These 2 methods were examined here for xylanase screening and protocol details are given in Annexe XIV.

Molecular Biology

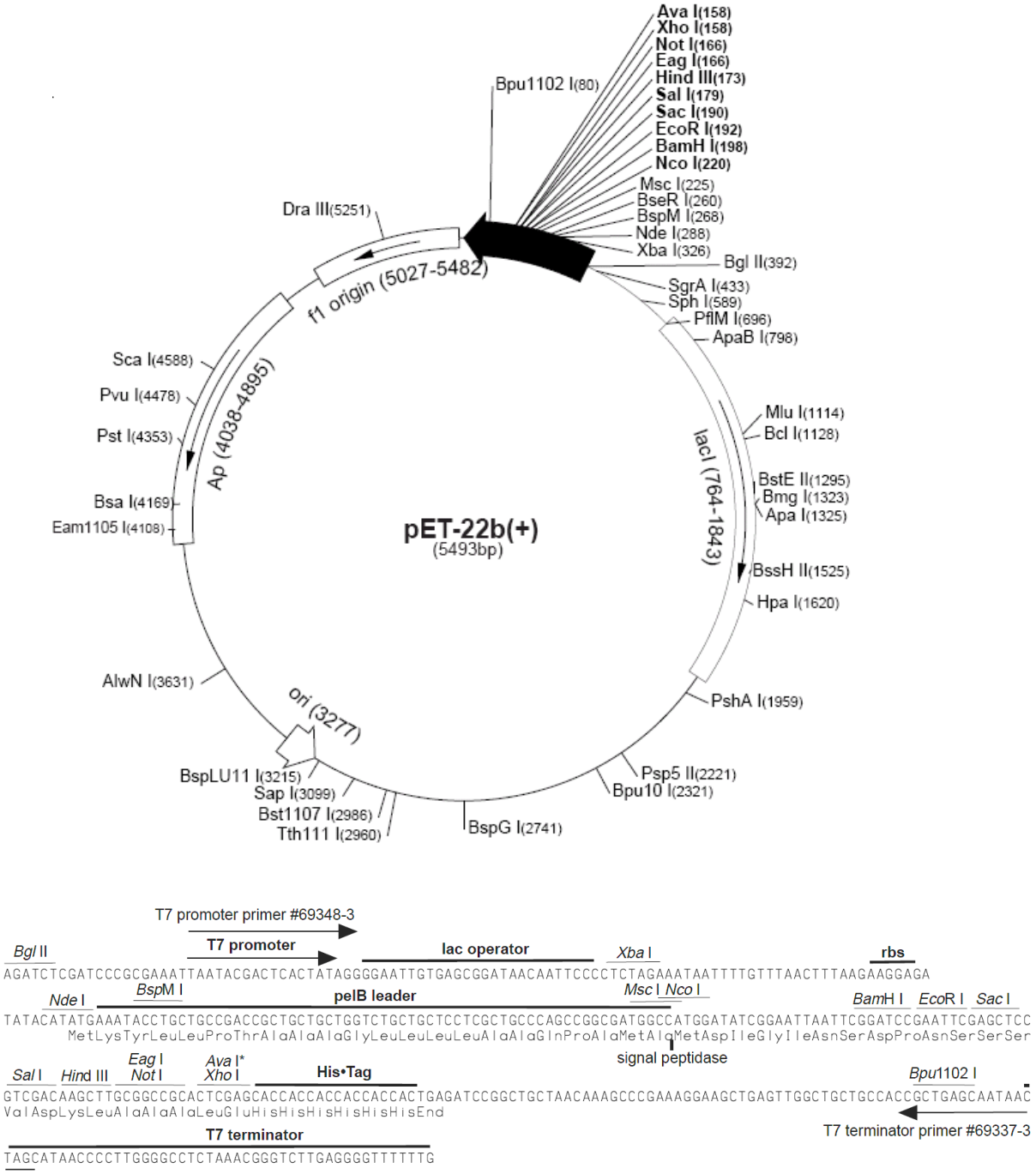
Prediction of Signal Peptides

Two different bioinformatics tools were used for DNA sequence analysis to determine the presence of potential protein secretion signal sequences: SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) and Phobius (http://phobius.sbc.su.se/).

The SignalP 4.1 server predicts the presence and location of signal peptide cleavage sites in amino acid sequences from different organisms: Gram-positive prokaryotes, Gram-negative prokaryotes, and eukaryotes. The method incorporates a prediction of cleavage sites and a signal peptide/non-signal peptide prediction based on a combination of several artificial neural networks. (Petersen et al, 2011). The DNA sequence was uploaded in FASTA format and the software run using standard settings. Phobius, acronym for prediction of transmembrane topology and signal peptides from the amino acid sequence of a protein. It predicts both the presence of signal peptides and transmembrane helices. (Käll et al, 2004). The DNA sequence was uploaded in FASTA format and the software run using standard settings.

PCR

Primers for PCR amplification of desired sequences were designed with the Snap Gene software program and optimised with the Primer 3 Plus webserver (http://primer3plus.com). Restriction digestion site for *Nde*I and *Bam*HI were incorporated in the 5´and 3´primers, respectively, for insertion in the corresponding restriction digestion sites of the expression vector pET22b(+) (Novagen) (Fig.5).



pET-22b(+) cloning/expression region

Figure 5 - A map of the expression plasmid pET-22b(+). Adapted from Novagen© (https://www.addgene.org).

PCR was carried out with Phusion High-Fidelity (HF) DNA Polymerase (from Thermo Scientific™) in a 96 well T100^{Tm} thermal cycler (Bio-Rad®). The PCR mix (Table VII) and PCR cycle program (Table VIII) used were optimised from the protocol recommended by Thermo Scientific. A 2 times concentrated master mix (Table IX) was prepared and used for all PCR cycles.

Table VII - PCR Reaction Mix

Reagent	**Initial Concentration**	**Volume**	**Concentration per reaction**
Ultra-Pure H_2O	-	µL	-
Master Mix	2X	12.5 µL	1X
Forward Primer	20 µM	0.625 µL	0.5 µM
Reverse Primer	20 µM	0.625 µL	0.5 µM
DNA Template	ng/µL	µL	1-10 ng
Total Volume	-	25 µL	-

Table VIII - PCR Cycle

PCR Stage	**Temperature**	**Time**	**Cycles**
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	
Annealing	Phusion online *Tm* Calculator	30 s	30
Extension	72°C	40 s	
Final Extension	72°C	7 min	1
Storage	4°C	∞	1

Table IX – PCR Master Mix

Reagent	**Initial Concentration**	**Volume**	**Final Concentration**
Ultra Pure H_2O	-	108 µL	-
Phusion HF Buffer	5X	80 µL	2X
dNTPs	10 mM	8 µL	400 µM
Phusion DNA Polymerase	2 U/µL	4 µL	0.04 U/µL
Final Volume		200 µL	

Restriction Digestion and Purification

Restriction digestion of PCR products and expression vector (pET22b (+) Novagen©) were carried out to allow for later ligation. 1 µg DNA was digested for 4 h at 37°C with 1 unit of each restriction digestion enzyme, *NdeI* Fast Digest and *BamHI* (Thermo Scientific™), in 1x buffer Fast Digest Green Buffer (Thermo Scientific ™), final volume 5 µL.

Following restriction digestion, digested plasmid samples were subjected to 1% agarose gel electrophoresis and purified from the gels using a GeneJET Gel Extraction Kit® as recommended by the manufacturer (Thermo Scientific ™). Purification of the digested PCR products was carried out by PCR Clean-up using the NZYGelpure kit (Nzytech™).

Ligation and Transformation

Ligase (T4 Ligase) was used for insertion of the purified digested PCR products into similarly purified and digested expression vector at the restriction digestion sites *NdeI* and *BamHI* by catalysing linkage of the 3'- and 5'-ends of these. Ligates were then transferred to competent *E. coli* XL1-Blue cells for analysis. Protocol details are given in Annexe XV.

Colony PCR

Colony PCR was used to identify transformants containing the desired plasmid plus insert construct, carried out with Taq DNA Polymerase (from Thermo Scientific™) in a 96 well T100^{Tm} thermal cycler (Bio-Rad®). The PCR mix (Table X) and PCR cycle program (Table XI) used were optimised according to Thermo Scientific™ and the primers used. The primers employed were those used in the original PCR amplification of the gene of interest. A 1% agarose gel electrophoresis was run at 80 V for 40 to 50 minutes and stained with Midori Green (Grisp) (5µL per 100 mL dH_2O) and/or Xpert Green (Grisp) (5 µL per 100 mL Agarose-TAE buffer mix, with additional 5 µL in the running buffer) to confirm PCR product size and purity.

Table X - Colony PCR Mix

Reagent	Initial Concentration	Volume	Final Concentration per reaction
Ultra-Pure H_2O	-	19.125 µL	-
Taq Polymerase Buffer	10X	2.5 µL	1X
dNTPs	10 mM	0.5 µL	200 µM
Forward primer	20 µM	0.625 µL	0.5 µM
Reverse primer	20 µM	0.625 µL	0.5 µM
$MgCl_2$	50 mM	1 µL	2 mM
Taq DNA Polymerase	1 U/µL	0.625 µL	0.025 U/µL
Final Volume	-	25 µL	-

Table XI – Colony PCR Cycle

PCR Stage	Temperature	Time	Cycles
Initial Denaturation	95°C	10 min	1
Denaturation	95°C	30 s	30
Annealing	52°C	30 s	
Extension	68°C	1:30 min	
Final Extension	68°C	5 min	1
Storage	4°C	∞	1

Results and Discussion

In the present study, plate based screening substrates and methods were investigated and developed for screening of the CBMA culture collection and identification of psychrophilic, acidophilic and/or psychro-acidophilic glycoside hydrolases, namely xylanases, cellulases, chitinases and pectinases. The study can be divided into four main sections:

- Characterisation and development of 'in-house' xylan substrate for xylanase screening and xylanase activity assays
- Comparison of plate-based screening methods currently available
- Screening of CBMA library yeast, fungi and bacterial isolates for production of glycoside hydrolases of interest
- Overexpression and characterisation of one enzyme of potential industrial interest

In-house Substrate For Xylanase Screening and Activity Assays

An ongoing parallel project focused on valorising biomass has resulted in production of a carbohydrate rich byproduct which is believed to have potential as a substrate for xylanase screening and measuring xylanase activity. In the present study, the composition of this byproduct was determined and its use as xylanase substrate for screening and activity assay investigated.

Table XII shows the results of the compositional analysis of the carbohydrate-rich byproduct.

Table XII - Composition of the Carbohydrate-rich Byproduct.

Biowaste Xylan Composition	Xylose	Glucose	Proteins (Bradford Assay)	Nitrogen (Kjeldahl Method)	Lipids	Ash
	87% ±13% std	2% ± 0.3% std	0%	2% ± 0.04% std	0%	9%

The dried material was found to be easily dissolved and highly soluble in water and buffer solutions following overnight agitation and thus is advantageous over many xylan substrates from birchwood, beechwood and oat spelt which are partially insoluble. Total sugar was quantified with the phenol-sulphuric acid method in combination with acid treatment and HPLC analysis on a ROA-organic acid H+(8%) column which also allowed for identification of the carbohydrate monomers present (see Fig. 6). Both methods, HPLC and the Total Sugars assay, gave similar carbohydrate content values while HPLC indicated that this byproduct is composed majoritarily of xylose, with low amounts of glucose and

ash contaminants. The lack of other carbohydrate monomers and hence suggestion of a carbohydrate composed exclusively of xylose residues is in contrast to xylan substrates from birchwood, beechwood and oat spelt which are highly substituted to varying degrees with various side chain groups such as arabinose, glucuronic acid, methylglucuronic acid, acetylcoumaric acid etc. The composition of the ash was not determined here and it is suggested that future studies be focused on determining this by means of techniques such as e.g. atomic absorption/emission spectroscopy. In relation to protein content, interestingly, while the Kjeldahl method indicated the presence of nitrogen containing compounds, this being typically indicative of the presence of proteins, another method gave negative results. Analysis with the commonly used Bradford Assay (Bradford, 1976) indicated the absence of proteins (<1%), bearing in mind that the Bradford assay is specific for the presence of certain amino acids only, namely positive amino acids and possibly also aromatics. Therefore, it is suggested that further studies be carried out here to confirm the absence of proteins and to identify the source of the nitrogen atoms. For the former, further protein/amino acid assays such as the Lowry Assay (Lowry et al, 1951), albeit with the caveat that high sugar concentrations are reported to interfere with measurements, SDS-PAGE, HCL and dinitrofluorobenzene treatment, and the bicinchoninic acid assay should be investigated. In the case of confirmation of the absence of proteins, alternative nitrogen sources could be phenolic compounds points to the presence of other nitrogen-containing compounds, such as phenols, azides and nitrogen-based acids or oxides, among others.

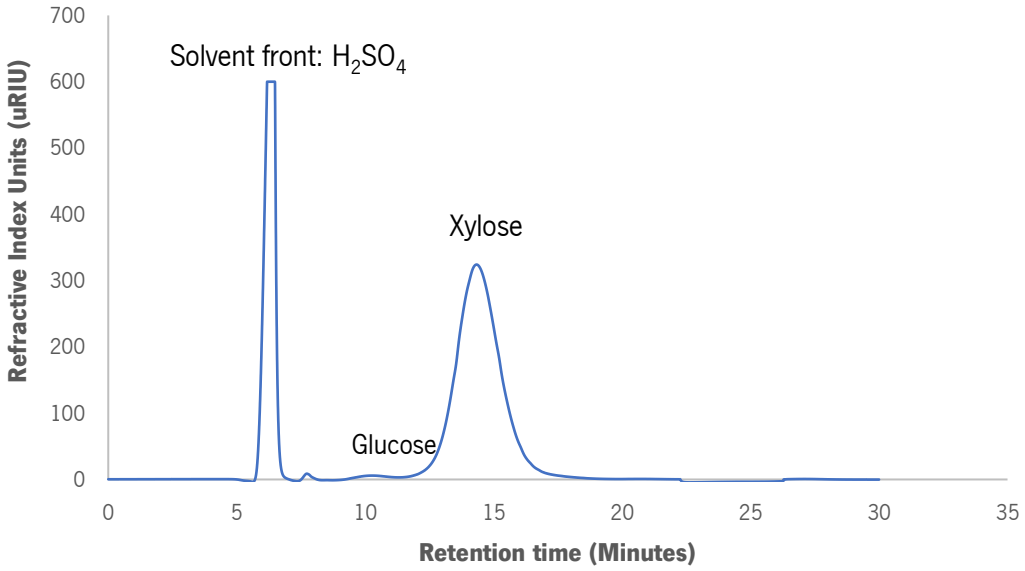


Figure 6 – Rezex™ 8 µm ROA-organic acid H+(8%) HPLC analysis of carbohydrate rich byproduct. Peak identity was determined by analysis of retention times of carbohydrate (glucose, xylose, lactose, fructose, glycerol), organic acid (acetic, succinic, lactic, citric, formic, malic, tartaric) and ethanol standards.

HPLC was further used to gain a better understanding of the characteristics of the xylan component of the byproduct. Gel filtration was carried out on a PolySep™-SEC GFC-P linear column which separates molecules based on molecular weight. Due to the unavailability of xylan standards, dextran and polyethylene glycol (PEG) standards were used to estimate xylan structure size in the byproduct. Figure 7 shows the results of the analysis where it can be seen that the carbohydrate fraction (xylan) eluted over a range of retention times indicative of a heterogenous compound composed of xylo-oligomers of variable lengths. Comparison with the standard curves indicated MWs of 1259 Da to 39 M Da PEG equivalents and 3981 Da to 125892 Da dextran equivalents. Calculation of the degree of polymerisation (DP) from these values indicates 9 to 265175 xylose monomers for calculation with the former and 27 to 839 xylose monomers for the latter. The retention time of maximum peak height corresponded to 25120 Da for PEG and 10000 Da for dextran, equivalent to DP of 167 and 67, respectively. However, care should be taken with these values as the standards used have a different composition and structure to xylan and are only used here for estimation and demonstration of the heterogeneity of the xylan product analysed. Indeed, while PEG is a linear polymer of ethylene oxide, dextran is indeed a carbohydrate, it is a polymer of glucose but does not have a totally linear form, being more serrated (Atamanov et al, 2016), which skews the correlation between chain length and retention time. Nevertheless, the study indicates that the xylan component of the byproduct is a

heterogenous compound composed of a range of high molecular weight xylo-oligomers. The other peak, of high intensity, corresponds to the inorganic components present in the sample, confirmed by a separate run, in the same conditions, of isolated ash content.

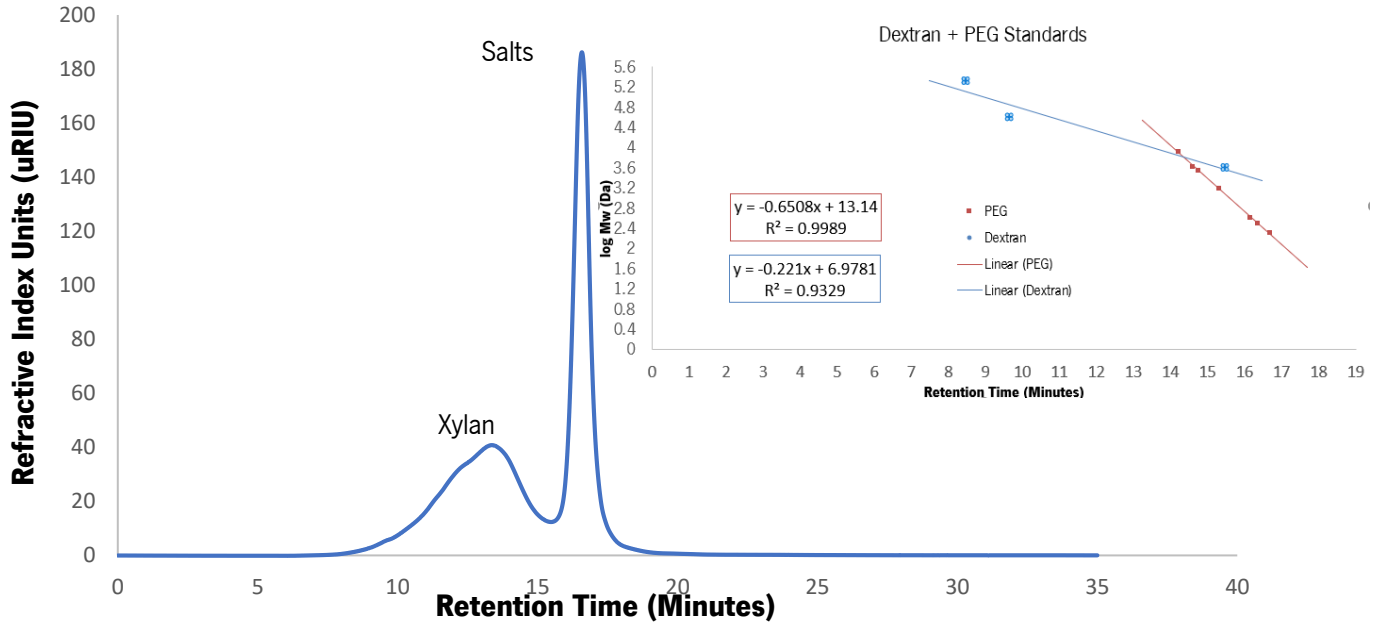


Figure 7 – Gel filtration HPLC on PolySep™-SEC GFC-P column of xylan rich byproduct. Peak identity was determined by total sugar and DNS analysis. Insert shows standard curves of dextran and polyethylene glycol (PEG) molecular weight standards versus retention time.

HPLC anaysis on a Rezex™ RSO-Oligosaccharide Ag+ (4)% column confirmed the high degree of polymerisation of the xylan compound as all carbohydrate present was eluted as one peak in the void volume (Figure 8). No low DP product is present as determined by comparison to xylo-oligomer standards of DP 1 to 6 combined with total sugar analysis and DNS analysis for reducing sugars. In addition, the column manufacturers indicate efficient separation of xylo-oligomers from DP1 to DP 18 with this HPLC column, thereby indicating the presence of higher DP xylan in the sample analysed. Two other peaks were identified and analysis confirmed the absence of carbohydrate and the potential for one of the peaks being due to salts present in the sample (ash). The second peak was not identified and further analysis is required to determine the identity of this.

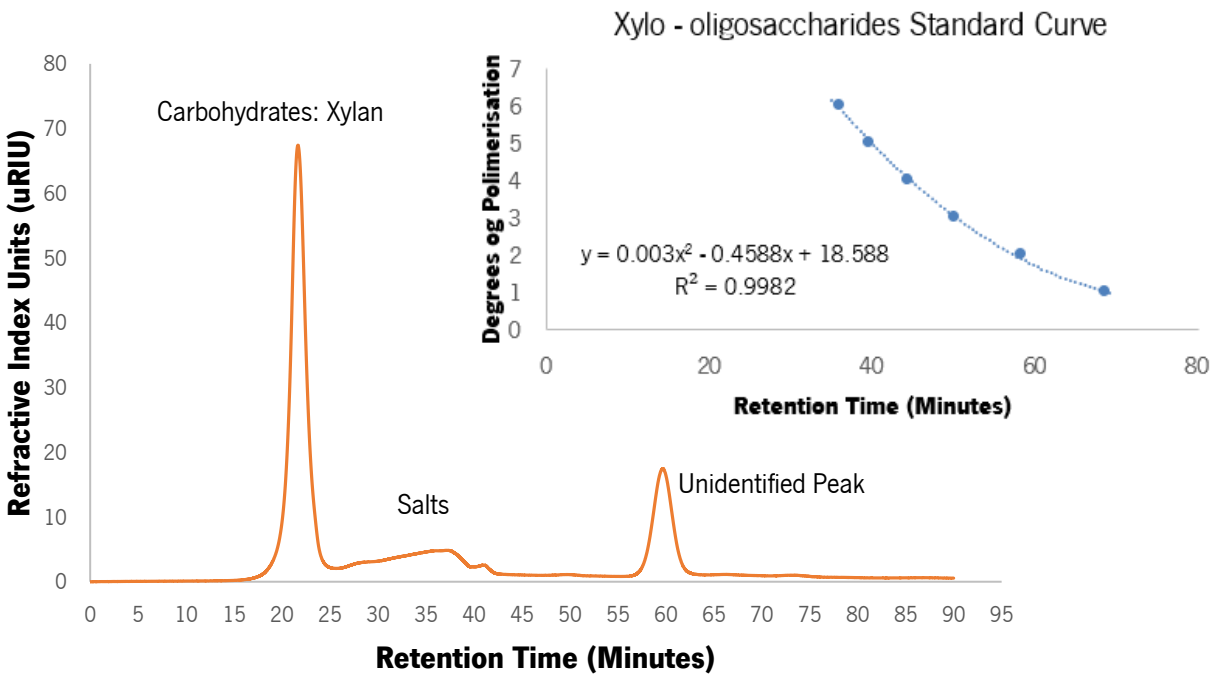


Figure 8 – Rezex™ RSO-Oligosaccharide Ag+ (4) % HPLC analysis of xylan rich byproduct, Peaks were identified by comparison to xylo-oligomer standard retention times and by total sugar and DNS analysis of individual peaks. Salt peak was identified by analysis of a 25 g/L NaCl solution.

The high xylan content, homogeneity in relation to monomer carbohydrate content, high solublility, relative purity and high molecular weight nature of the byproduct indicates its potential for use as a substrate in the DNS assay for xylanase activity. The effects of the substrate on the DNS assay and on measuring various concentrations of xylose reducing sugars were investigated. From Figure 9 it can be seen that increasing xylose concentrations do indeed lead to a linear increase in absorbance values, thereby indicating the suitability of the substrate for the assay. In addition, high reproducibility was observed with repeat assays. At high byproduct concentrations, above 15 mg/mL, deviation from non-linearity was observed, indicative of substrate saturation and was mainly due to high background absorbance for this substrate. Therefore, use of this substrate at concentrations below 15 mg/mL is required. The substrate was investigated in determining the specific activity of a xylanase enzyme using standard DNS assay conditions. Analysis indicated a specific activity of 1600 IU/mg with this substrate, as compared to 1000 IU/mg when using soluble birchwood xylan. The test was found to be highly reproducible with a standard deviation of ±2%. The higher specific activity observed here may be due to the specificity of the enzyme used, the xylanase activity being limited by substrate sidechains and hence could be expected to display a higher activity on this non-substituted substrate.

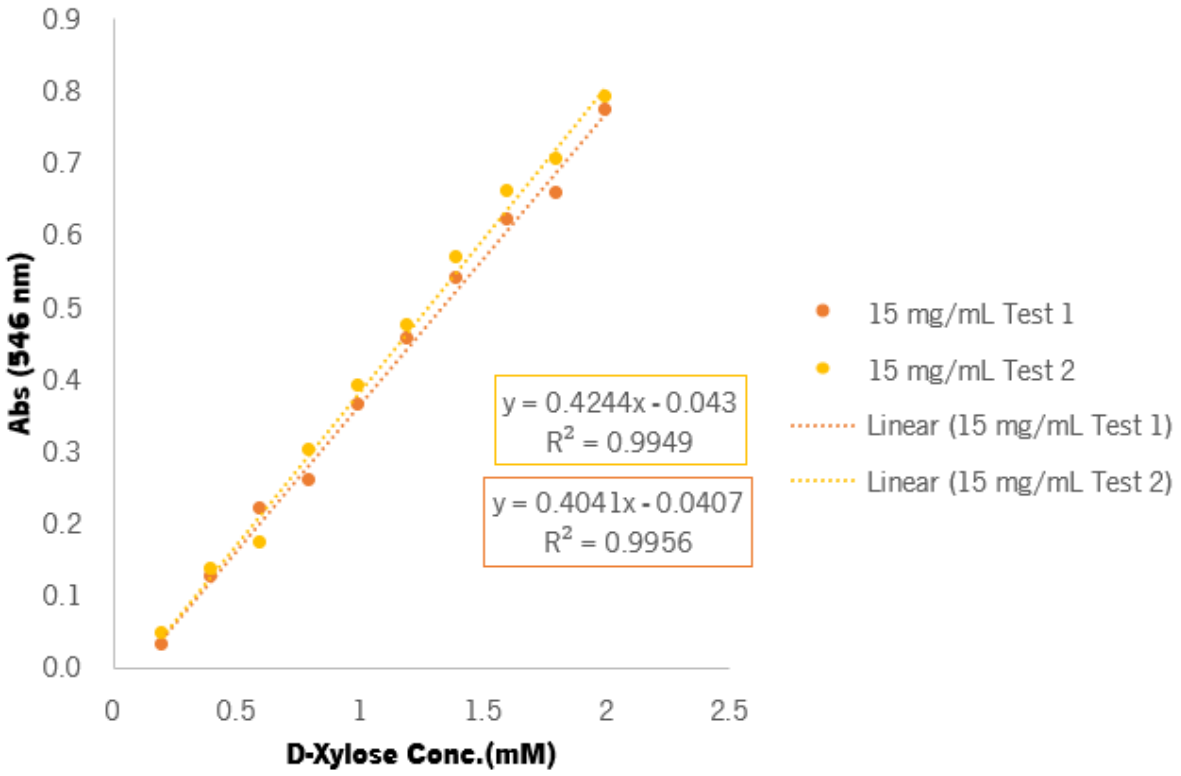


Figure 9 – Linear relationship between xylose reducing sugar concentration and DNS assay absorbance readings with 15 mg/mL of the xylan rich byproduct. The results for two repeat assays are shown.

The solubility of the xylan rich byproduct prevented its direct utilisation as a substrate in plate screening for xylanases. Therefore, it was chemically coupled with remazol brilliant blue (RBB) and characterised. The coupling process allowed a yield of 59% in terms of substrate produced, with only 17% RBB being incorporated and indicates the need for process optimisation. Product analysis indicated an average of one RBB molecule bound to every 29 molecules of xylose which is higher than the ratio of 1:20 reported with RBB-Xylan made from beechwood (Biely et al 1985). This latter xylan had a number-average molecular weight of 18600 Da and a ratio of xylose to 4-O-methylglucuronic acid of 7:1. It is suggested that the methylglucuronic acid side chains could enhance RBB binding with this substrate. Finally, the use of the RBB bound xylan rich byproduct substrate was investigated in plate assays and was found to give similar results to commercially available RBB-Xylan (Sigma). Figure 10 shows the results of an initial analysis with a positive and negative control. This product was used as screening substrate in the subsequent screening for xylanases.

Figure 10 - Use of novel RBB-xylan substrate produced in the present study for screening of xylanase production by fungal isolates. On the left is shown a xylanase producing fungi, on the right a negative control.

Screening for Glycoside Hydrolases

Plate based screening methods for screening of microbial isolates for glycoside hydrolases of industrial interest were investigated and compared in this study. Most currently used screening assays are based on the following for simplified detection of activities of interest:

- Use of opaque screening media with incorporation of insoluble or partially soluble substrates, which become transparent/less opaque upon hydrolysis and form 'clearing zones' around colonies.
- Use of agar media supplemented with a dye linked substrate (chromogenic dye) which change colour or colour intensity upon hydrolysis;
- Use of substrate supplemented agar media and dyeing of plates following incubation to detect reduction of substrate concentration or product formation

In the study, malt extract agar was used for screening of fungi and yeast peptone dextrose agar for yeast screening as these had already been show to allow for efficient growth of all isolates tested. To enable screening for enzymes active/stable at various pHs, media were supplemented with McIlvaine buffer for pH4, MOPS-NaOH buffer for pH 7 and Glycine-NaOH buffer for pH 9 screening. Different buffers were used for each pH as no single buffer was found that could be used at all pHs. Furthermore, in line with the secondary goal of finding cold-adapted enzymes, both fungi and yeasts were incubated at 16 °C. Bacterial isolates and metagenomics library clones were, however, grown at room temperature and 37 °C respectively, on LB plates. Pre-tests determined that bacteria should be grown for at least 7 days, yeasts required 14 days and filamentous fungi needed 22 days so as to observe activity. All isolates were screened at least 2 times for confirmation.

Xylanase Screening

To test for xylanolytic activity, two chromogenic substrates were investigated: AZCL-Xylan and the in-house prepared RBB-Xylan. Both substrates were equally effective and easy to use and gave similar screening results.

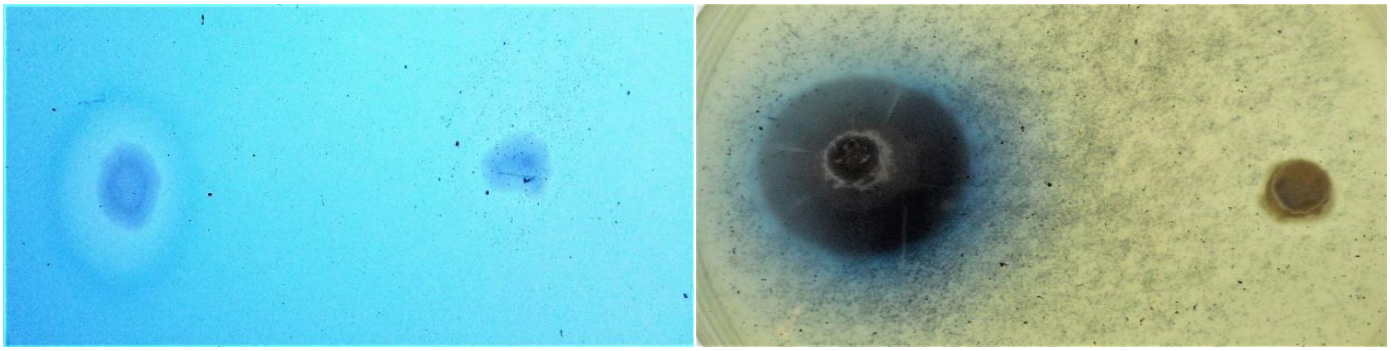


Figure 11 – Screening with 0.1% RBB-xylan (left) and 0.1% AZCL-xylan (right) of both a xylanase producing and xylanase negative fungal isolate on 2% malt extract agar at pH7 (left) and pH 4 (right).

Nevertheless, AZCL-Xylan is an insoluble substrate and hence practical difficulties were often encountered in equally dispersing this in plates (see Fig. 11). Furthermore, particle solubility was affected at alkaline pH and resulted in a reduced number of particles at pH 9 and hence reduced assay sensitivity.

Figure 12 – Malt Extract 2% (v/v) Agar plates with 0.1% (w/v) insoluble AZCL-Xylan (Megazyme) at different pHs: (from the left) pH 4, 7 and 9.

RBB-xylan colour intensity was also affected by pH and required higher (0.2%) substrate concentrations at acidic pHs, but did not interfere with screening and hence this substrate was used in subsequent screening.

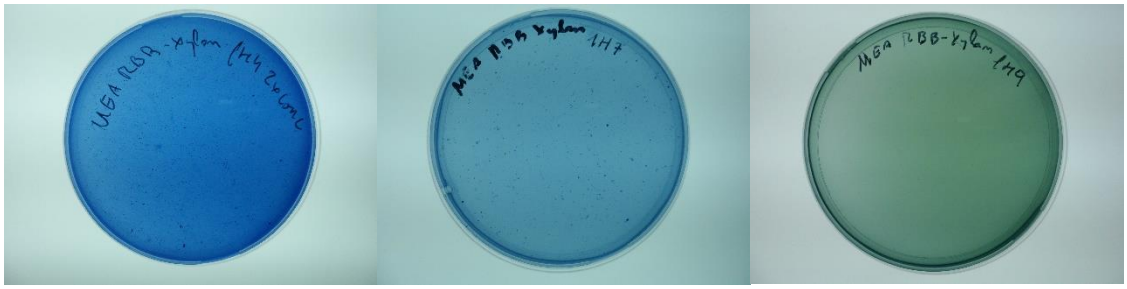


Figure 13 – Malt Extract 2% (v/v) Agar plates with soluble RBB-Xylan at different pHs: (from the left) RBB-Xylan at pH 4 (0.2% w/v), pH 7 (0.1% w/v) and pH 9 (0.1% w/v).

Cellulase Screening

Four different type of cellulase screening assays were investigated: CMC and observation for clearing zones; CMC and post-incubation staining with Congo Red, CMC and post-incubation staining with Lugol's Iodine, CMC and post-incubation staining with Gram's Iodine.

Use of the post-incubation staining methods led to practical difficulties with screening yeast strains due to inadvertent removal of colonies from the plates during the flooding and washing steps. Furthermore, Lugol's and Gram's Iodine stains led to appearance of many false positives as had been previously reported (Meddeb-Mouelhi et al, 2014). Indeed, use of these dyes with the malt extract agar or yeast peptone dextrose agar in the absence of CMC even led to false positive results as can be seen in Fig. 14. Use of weakly opaque CMC and observing for clearing zones was much simpler in practice than the other methods investigated, and while clearing zones were often difficult to clearly distinguish, requiring use of higher CMC concentrations (0.2%) and a background light for clearer observation, this test was selected as the method of choice for screening of yeasts and bacteria. In the case of the filamentous fungi, the use of CMC followed by post-incubation Congo Red staining is recommended.

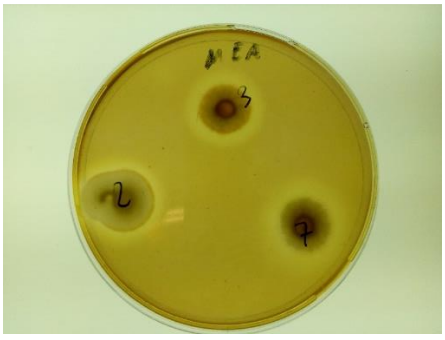


Figure 14 – Filamentous fungi showing apparent positive results (halos around colonies) on malt extract 2% (w/v) agar plate without any added CMC.

Pectinase Screening

For pectinase screening, 3 methods were investigated: use of the partially insoluble natural substrate pectin and observing for clearing zones; pectin and post-incubation staining with Ruthenium Red; pectin and post-incubation staining with Lugol's Iodine, pectin and post-incubation staining with Gram's Iodine.

Interestingly, use of Ruthenium Red was ineffective, with no positive results being observed, and led to binding to fungal hyphae. Lugol's and Gram's Iodine staining again led to false positives. In contrast, use of pectin, even though only weakly opaque, was successful, especially at higher concentrations (0.2%) and was simple to employ and therefore selected for further screening assays.

Chitinase Screening

For chitin screening, use of colloidal chitin and observing for clearing zones, and use of colloidal chitin with bromocresol purple and observing for colour change, were both investigated. As expected the latter, based on a colour change of the pH indicator bromocresol purple due to a pH increase, was unsuccessful due to the use of buffers in the screening media. While reduced opacity was observed for chitin plates prepared at pH 9, use of chitin in the medium and observing for clearing zones proved to be successful and was used in screening for chitinases.

Screening of CBMA Library Isolates

The plate screening assays developed above were used to screen for production of glycoside hydrolases in 116 yeast isolates, 13 aquatic hyphomycetes, 4 bacterial isolates and 3 metagenomic library clones of the CBMA culture collection. Xylanases (RBB-xylan), cellulases (CMC-cellulose, clearing zones), pectinases (citrus peel pectin, clearing zones in opaque media) and chitinases (colloidal chitin, clearing zones in opaque media) were screened for.

The detailed results for the screening of the yeast and fungal isolates are given in Annexes XVI; XVII, XVIII and XIX. These results are summarised in Table XIII. Most isolates displayed at least one hydrolytic activity in at least one condition investigated and only 4 isolates did not display any of the hydrolytic activities screened for at any pH.

Table XIII - Number of isolates showing the respective enzyme activities at pHs 4, 7 and 9.

Xylanases			Cellulases			Pectinases			Chitinases		
pH 4	pH 7	pH 9	pH 4	pH 7	pH 9	pH 4	pH 7	pH 9	pH 4	pH 7	pH 9
25	61	25	20	82	53	28	89	45	44	34	18

Xylanases

Annexe XVI gives the detailed results for the xylanase screening of the yeast and fungal isolates and Table XIV shows a summary of these results. Of the 116 yeasts isolated from vegetable or fruit biowaste habitats at a pH range from 2 to 7.4, only 18 displayed activity at pH 4, with 5 displaying it exclusively at this pH. 50 displayed xylanase activity at pH 7, with 32 exclusively at this pH; and 16 were positive at pH 9, with 4 exclusively. As xylan is typically present in the cell walls of fruits in vegetables, the presence of several yeasts with xylanolytic activity was expected, and the low pH of the habitats was expected to lead to identification of a higher content of low pH active enzymes. The positives observed at pH 9 were less expected. The aquatic hyphomycetes revealed themselves to be tolerant to a range of pHs and to produce xylanase(s) mostly active over the pH range investigated. Finally, two of the four bacterial isolates were confirmed to have xylanolytic activity at pH7, room temperature.

The 18 xylanase positive yeasts at pH 4 and especially the 5 exclusively active at this pH should be selected for further study. These may have potential in low pH food and beverages applications.

Table XIV – Number of positives identified for xylanase activity screening of yeasts and fungi.

	Total Positives	Xylanase Activity Positives			Exclusively Active			pH 4 and 7	pH 7 and 9	pH 4, 7 and 9	pH 4 and 9
		pH 4	pH 7	pH 9	pH 4	pH 7	pH 9				
Yeasts	61	18	50	16	5	32	4	8	7	3	2
Hyphomycetes	11	7	11	9	0	1	0	1	3	6	0

Analysis of the bacterial isolates and metagenomic clones from the Irish oats field samples allowed for identification of two positives (bacterial isolates T0_7 and TO_E14) at the neutral pH investigated.

Cellulases

Annexe XVII shows the detailed results for the fungal and yeast screening for cellulases and Table XV gives a summary of these results. It can be seen that there was a higher number of isolates with activity at pH 7, mirroring the results obtained when testing for xylanases. While the number of positives at pH 4 was similar as well, unexpectably there was a higher number of isolates displaying activity at pH 9. Many of these were active over a range of pHs; of the 43 positives at pH 9, 25 were

active at both pH 7 and 9, 11 were active at the 3 studied pHs and only 7 were active solely at pH 9. The filamentous fungi, as expected, all showed cellulytic activity but the majority displayed activity only at pHs 7 and 9.

Table XV – Number of positives identified for cellulase activity screening of yeasts and fungi.

	Total Positives	Cellulase Activity Positives			Exclusively Active			pH 4 and 7	pH 7 and 9	pH 4, 7 and 9	pH 4 and 9
		pH 4	pH 7	pH 9	pH 4	pH 7	pH 9				
Yeasts	79	19	72	43	0	28	7	8	25	11	0
Hyphomycetes	11	1	10	10	0	0	0	0	10	1	0

In the case of the wild-type bacterial isolates and metagenomic clones, two of the former (TO_B6 and TO_E11) and all three of the latter (12.D3, 90.K1, 88.J12) were found to be cellulase positive at pH 7, with no growth being observed at the other pHs tested. Indeed, the presence of cellulases and xylanases in the oats field samples was expected as this cereal is rich in the respective substrates xylan and cellulose. In relation, to the metagenomic clones, the positive cellulase activity observed for all isolates confirmed the sequence based metagenomics analysis which had indicated the presence of sequences with low homologies to known cellulases: 26% homology for 12.D3, 33% for 90.K1 and 63 % for 88.J12 (Granja, 2017).

Pectinases

Annexe XVIII shows the complete results for the fungi and yeast analysis for pectinases and Table XVI gives a summary of these results. Higher numbers of pectinase positives yeasts were observed as compared to xylanases and cellulases, which may be expected due to the known high pectin content of fruit and vegetable biowastes. In contrast, lower numbers of positives were seen for the fungi. Furthermore, even though the yeast isolates screened were from low pH habitats, the highest number of hits were at the neutral and basic pHs tested. In fact, in this study, only 1 isolate with activity exclusively at pH 4 was identified and this may have potential in fruit juice and wine manufacturing.

No positive results for pectinase screening were observed for any of the bacterial isolates or metagenomic clones analysed.

Table XVI – Number of positives identified for pectinase activity screening of yeasts and fungi.

	Total Positives	Pectinase Activity Positives			Exclusively Active			pH 4 and 7	pH 7 and 9	pH 4, 7 and 9	pH 4 and 9
		pH 4	pH 7	pH 9	pH 4	pH 7	pH 9				
Yeasts	90	27	84	41	0	42	5	7	16	19	1
Hyphomycetes	6	1	5	4	1	1	0	0	4	0	0

Chitinases

Annexe XIX gives detailed information on the chitinase screening results for the yeasts and fungi and Table XVII gives a summary of this. Interestingly, biowastes of fruits and vegetables are expected to have no or only low chitin content yet a high number of positives were observed for the yeast isolates and lower numbers for the fungi. Nevertheless, it is known that, in yeasts, chitin is found in septa, constriction rings and budding scars (Chaffin et al., 1998) while the cell walls of filamentous fungi consist of up to 20% or more chitin, and can be found throughout the whole cell wall of hyphae (Ruiz-Herrera, 1991). As such, chitinases have important physiological and biological roles, which include morphogenetic, autolytic and even nutritional and parasitic roles in these organisms, as well as remodelling of cell walls. Thus the activity detected may be related to the production of such chitinases for such functions.

No positive results for chitinase screening was observed for any of the bacterial isolates or metagenomic clones analysed.

Table XVII – Number of positives identified for chitinase activity screening of yeasts and fungi.

	Total Positives	Chitinase Activity Positives			Exclusively Active			pH 4 and 7	pH 7 and 9	pH 4, 7 and 9	pH 4 and 9
		pH 4	pH 7	pH 9	pH 4	pH 7	pH 9				
Yeasts	66	42	34	17	23	15	6	11	3	5	3
Hyphomycetes	3	2	0	1	2	0	1	0	0	0	0

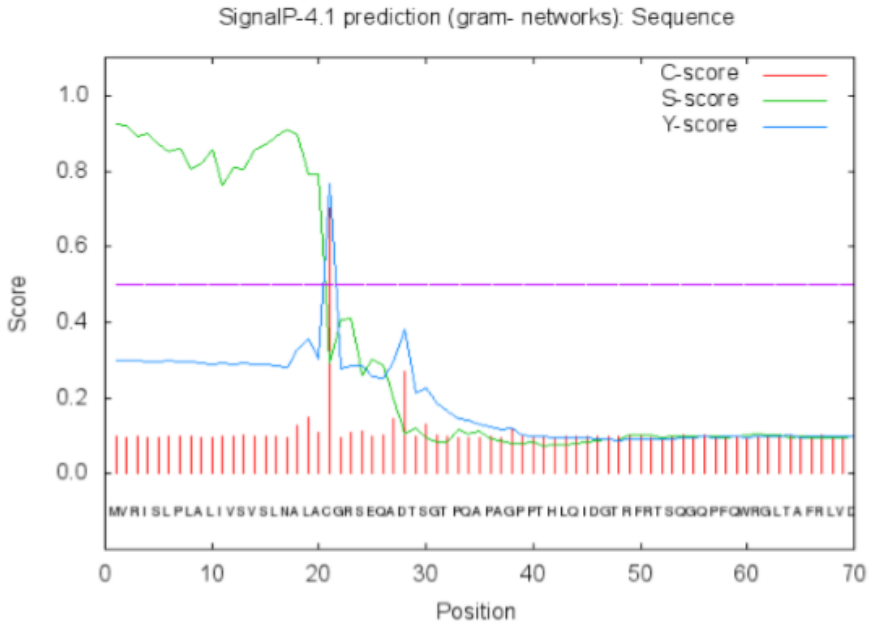
Molecular Biology

Having identified a number of enzymes of potential interest, the next step would be to produce and purify these and determine their physico-chemical characteristics. The confirmation of cellulase activity in this study for the three metagenomic clones for which the DNA sequence is already known would be expected to facilitate this process. One of these clones, clone 12.D3, was selected for further work due

to the low homology (26%) to known cellulases for this, and hence the potential for an enzyme with novel functions and characteristics. Here the initial work plan was to isolate the gene sequence by PCR, clone the gene in an expression vector, overexpress in *E. coli* and determine temperature and pH related activity and stability. Unfortunately due to experimental difficulties and time restraints this work was not completed during this project.

The sequence analysis software programs SignalP-4.1 and Phobius were used for determination of the potential presence of a signal sequence in the 12.D3 cellulase sequence. These indicated a gram negative host cleavage site between either amino acids positions 19-20 (Phobius, Fig. 16), or 20-21 (Signal P 4.1, Fig. 15), with a higher probablitiy for the latter due to the presence of 2 alanines at the -1 and -3 sites, this being heavily associated with signal peptide cleavage sites (Jain et al, 1994). A positive hit for a gram positive cleavage site between residues 27 and 28 was also observed with SignalP-4.1 but with a lower score than the Gram negative cleavage site.

```
# SignalP-4.1 gram- predictions
>Sequence
```




```
# Measure  Position  Value    Cutoff   signal peptide?
  max. C    21       0.701
  max. Y    21       0.767
  max. S     1       0.924
  mean S     1-20    0.854
       D     1-20    0.808   0.570   YES
Name=Sequence   SP='YES' Cleavage site between pos. 20 and 21: ALA-CG D=0.808 D-cutoff=0.570 Networks=SignalP-noTM
```

Figure 15 - SignalP prediction scores according to gram negative prediction parameters for 12.D3. C-value in red; S–value in green; Y-value in blue. Results indicate presence of a cleavage site between positions 20-21.

Prediction of 12.D3

```
ID   12.D3
FT   SIGNAL        1     19
FT   REGION        1      3       N-REGION.
FT   REGION        4     15       H-REGION.
FT   REGION       16     19       C-REGION.
FT   TOPO_DOM     20    435       NON CYTOPLASMIC.
//
```

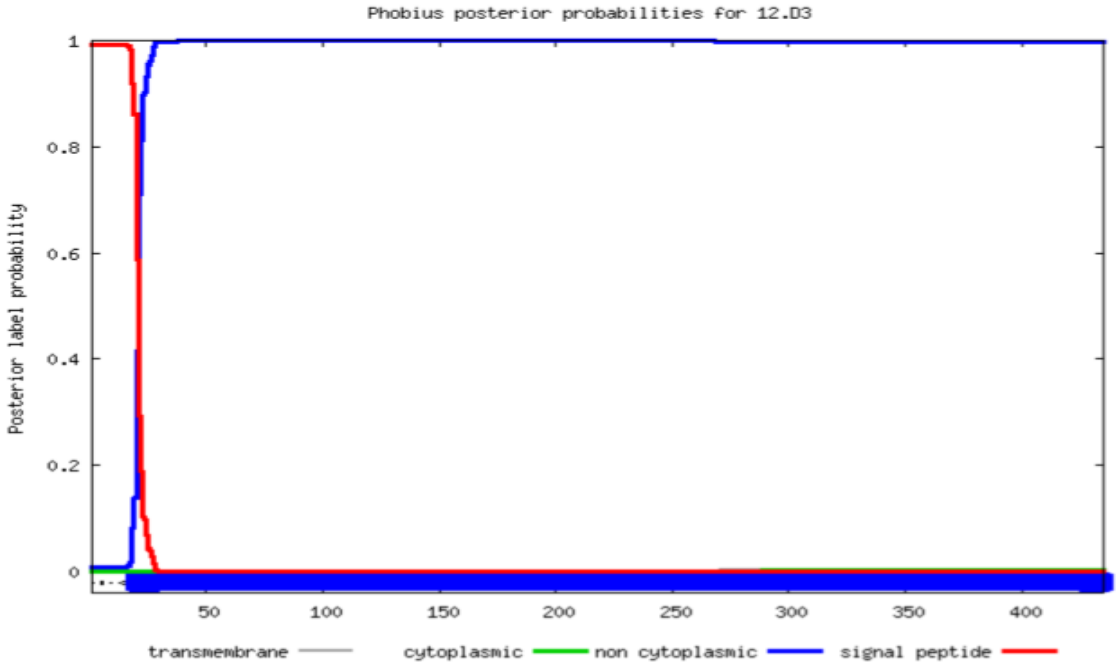


Figure 16 - Phobius prediction graph of signal peptides and protein topology for 12.D3. Presence of a cleavage site between positions 19-20.

Primers were designed for amplification of the 12.D3 cellulase sequence (see Table XVII), both the whole sequence, including the potential signal sequence, and the mature sequence, initiating with amino residue 22. Primers were designed to incorporate a *Nde*I and *Bam*HI restriction digestion sites at the 5' and 3'ends, respectively.

Table XVIII - Primers designed for amplification of the whole gene and the mature fragment only of the 12.D3 cellulase. The introduced restriction sites are underlined. FWD and REV are the forward (5´) and reverse (3´) primers.

Target Gene	Primer	Sequence 5'-3'	Length (bp)	GC%	Melting Temperature
Cellulase 26%(Whole Sequence)	FWD 1	CCCCCATATGAGGATCTCG	19	58	56.1 °C
Cellulase 26% (Mature)	FWD 2	AACCCATATGTGCGGACGGT	20	55	61.9 °C
Cellulase 26%	REV	CAGGATCCACTTCACCCGAC	20	60	60.1 °C

Figure 17 presents the results for the PCR amplifications of the whole and 'mature' sequences of the 12.D3 cellulase gene at various annealing temperatures. It can be seen that successful amplification of both bands was observed, with optimal results at annealing temperatures of 57 °C.

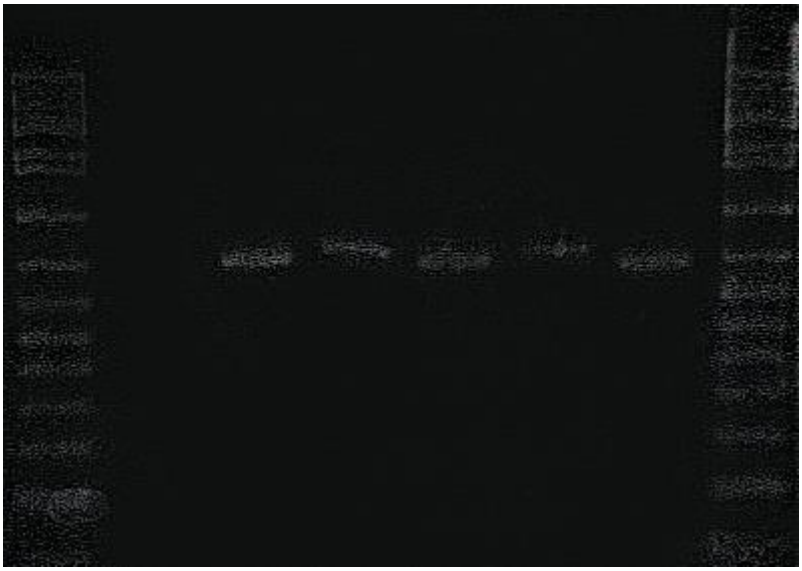


Figure 17 – 1% agarose gel of the PCR amplification of the cellulase gene of 12.D3. From left: Lanes 1 and 8 – Gene Ruler DNA Ladder Mix (Thermo Scientific™); Lane 2 – Whole gene, 55 °C; Lane 3 – 'Mature' sequence, 55 °C; Lane 4 – Whole gene, 57 °C; Lane 5 - 'Mature' sequence, 57 °C; Lane 6 – Whole gene, 60 °C; Lane 7 - 'Mature' sequence, 60 °C. The expected size of the whole gene sequence is 1308 bp (lanes 4 and 6) and of the mature sequence is 1248 bp (lanes 3, 5 and 7).

The PCR products and expression vector pET22b(+) were double digested with the restriction digestion enzymes *NdeI* and *BamHI* and following purification were analysed on a 1% agarose gel. Figure 18 shows the results for this analysis in which linear pET22b plasmid of 5493 bp and PCR products of the expected sizes are visible.

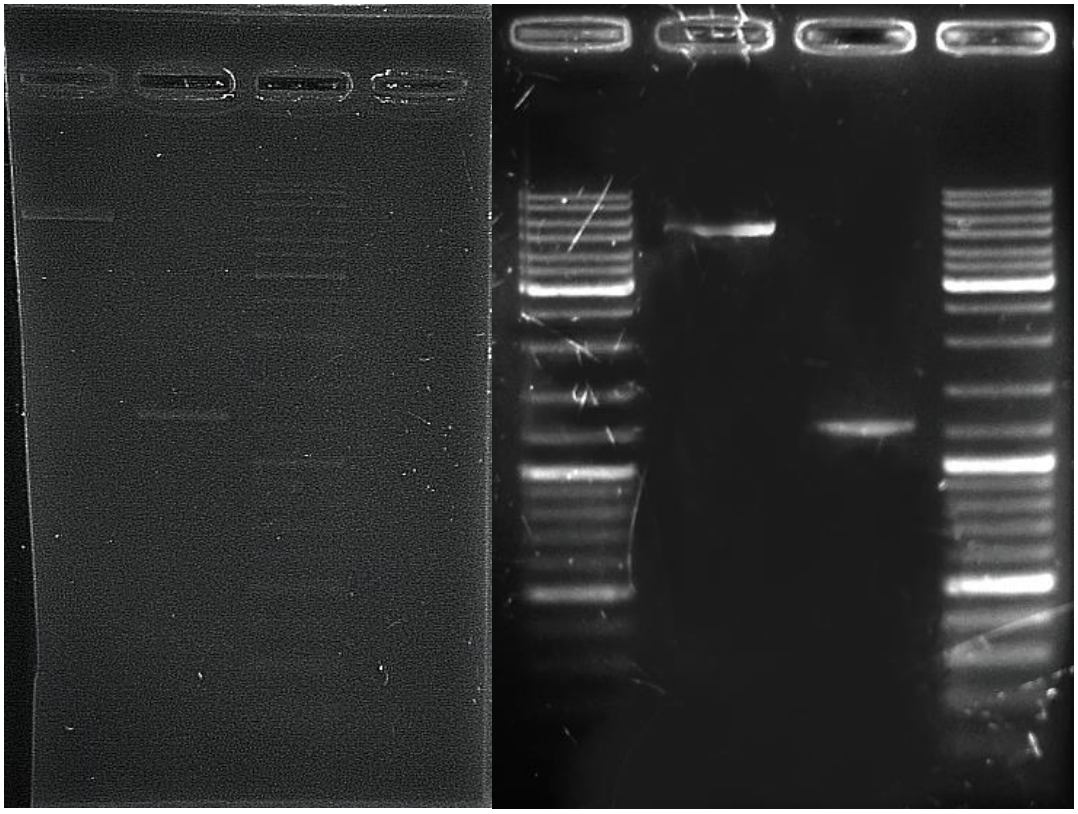


Figure 18 – 1% agarose gels of the digestion of pET22b expression plasmid (5493 bp) and either the full (1308 bp) or "mature" (1248 bp) sequences of the cellulase gene of 12.D3. Gel on the left: Lane 1 – linearised pET22b; Lane 2 – double digested full gene; Lane 3- Gene Ruler DNA Ladder Mix (Thermo Scientifc™); Lane 4 – Empty; Gel on the right: Lanes 1 and 4: Gene Ruler DNA Ladder Mix (Thermo Scientifc™); Lane 2 – linearised pET22b; Lane 3 – double digested "mature" cellulase gene.

Ligation

Ligation was carried out with various concentrations of plasmid insert (24 to 120 ng) and various insert to plasmid ratios (1:1, 1:3, 1:5), but unfortunately no transformants with the correct construct were obtained within the time limits of the project. On some occasions transformants were indeed observed but analysis by colony PCR and subsequent double digestion with *Nde*I and *Bam*HI indicated the absence of the desired construct. Future studies should be focused on preparing new competent cells and in repeating these experiments.

Conclusions and Future Work

Enzymes are highly specific biological catalysts that accelerate the rate of chemical reactions. They are Nature's own catalysts and their power and specificity of action, as well as a reduced environmental impact, makes them seemingly ideal tools for numerous applications. They bring cleaner and sustainable process alternatives to the chemical, food, pharmaceutical and agricultural industries and are continuously developing into new areas e.g. in the bioproduction of high value fine chemicals, nutraceuticals etc. (Blamey et al. 2017). Glycoside hydrolases are important commercial enzymes with widespread use as commodity enzymes in all three sectors of the industrial enzymes markets: food, feed and technical sectors. Novel glycoside hydrolases with novel characteristics and, importantly from a intellectual property protection point of view, with novel sequences, are called for. Importantly also, their role in biomass conversion is leading to the development of new, higher value application areas. In the present study we focused on screening microbial isolates for four types of glycoside hydrolases, namely xylanases, cellulases, chitinases and pectinases, due to the industrial potential of these particular enzymes.

The CBMA houses a culture collection constituted by a diverse range of bacteria, fungi and yeasts isolated from such diverse environments as fruit and vegetable industry biowastes, Portuguese and Icelandic freshwaters, mine waters, the wine and baking industry, Irish oats fields and Portuguese vineyards. In this study a number of these isolates were selected and screened for the production of psychrophilic, acidophilic and/or psychro-acidophilic glycoside hydrolases of industrial interest. The isolates selected for the study included: a total of 116 yeasts isolated from various fruit and vegetable biowastes with habitat pHs from as low as pH 2 (strawberry biowaste) to a maximum of pH 7.4; 11 filamentous fungi (aquatic hyphomycetes) isolated from various reduced temperature streams in Portugal and Iceland; and both 3 bacterial isolates and 3 metagenomic library clones from oats fields in Ireland which are expected to be rich in xylanase and cellulase producing organisms.

Methods were investigated and developed for a simplified screening of the various isolates at pHs 4, 7 and 9, with, in particular, the development of a novel chromogenic xylanase substrate. This substrate is a byproduct of an ongoing parallel project focused on using sustainable, environmentally friendly enzyme technology for conversion of waste biomass to value added products. Our analysis indicated this to be highly soluble, rich in a variable sized, high molecular weight and high DP xylan composed almost exclusively of xylose residues. Such a substrate offers advantages over many of the commercial xylans which are poorly soluble and characterised by extensive side chain branching with various

carbohydrate monomers. Insolubility difficults test reproducibility and side chain branching can interfere with xylanase activity. The substrate was shown to be suitable for measurement of xylanase activity with the commonly used DNS assay but further tests with other xylanases are recommended to confirm this. Furthermore, we successfully coupled the substrate with RBB dye, at a ratio of 1 RBB:29 xylose, to give a chromogenic substrate allowing for a highly sensitive and efficient plate based screening of xylanases. Indeed, this substrate gave similar results to commercially available high cost RBB-xylan and was used with success in our study to screen various yeasts, filamentous fungi and bacteria.

Our screening of the CBMA culture collection enables a better understanding of the characteristics of this collection, and combined with the other ongoing studies of the project 'Bioprospect' focused on screening for organic acid transporters and antimicrobial peptides should reveal its value. Most of the selected isolates displayed at least one hydrolytic activity in at least one condition investigated and only 4 isolates did not display any of the hydrolytic activities screened for at any pH. Furthermore, 31 isolates were found to display all 4 activities screened for; 5, 16 and 3 isolates were identified with all 4 activities at pHs 4, 7 and 9, respectively. Such isolates may be useful in biomass transformation and degradation. In addition, 5, 1, 25 isolates were found with, respectively, xylanase, pectinase and chitinase activity exclusively at pH 4, and could have potential for use in the food and beverages industries.

In the study, 3 *E. coli* metagenomic clones which had previously been identified by sequence based metagenomics to contain a cellulase like sequence were confirmed to be cellulase active in the present study. One of these clones, containing a gene with only 26% homology to a known cellulase, was selected for further studies. The gene sequences, both the complete and 'mature' forms, were successfully amplified by PCR and double digested with restriction enzymes but repeated ligation-transformaiton attempts were unsuccessful in obtaining transformants with the desired construct. Future studies should be focused on completing this work.

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Annexes

Annexe I – Total Sugars Quantification

Glucose Standard Preparation

1. Add 100 mg of glucose in 100 mL of water.
2. To 10 mL of the stock, add 90 mL of distilled water
3. Pipette out 0.2, 0.4, 0.6, 0.8 and 1 mL of the working standard into a series of test tubes.
4. Add 1 mL of phenol solution to each tube.
5. Add 5 mL of 96% sulphuric acid to each tube and shake well.
6. After 10 min shake the contents in the tubes and place in a water bath at 25–30°C for 20 min.
7. Read absorbance at 490 nm.

Protocol:

1. Weigh 100 mg of byproduct sample into a boiling tube.
2. Add 5 mL of 2.5 N HCl and incubate in a boiling water bath for three hours.
3. Cool to room temperature.
4. Neutralise solution by solid sodium carbonate addition until effervescence ceases.
5. Make up the volume to 100 mL with distilled water and centrifuge.
6. Pipette out 0.1 and 0.2 mL of the sample solution in two separate test tubes. Make up the volume in each tube to 1 mL with water.
7. Set a blank with 1 mL of water.
8. Add 1 mL of phenol solution to each tube.
9. Add 5 mL of 96% sulphuric acid to each tube, shake well and incubate at room temperature for 10 minutes.
10. Mix contents well and incubate in a water bath at 25–30 °C for 20 min.
11. Measure abosorbance at 490 nm.
12. Calculate the amount of total carbohydrate present in the sample solution by use of a glucose standard graph:

Absorbance corresponding to 0.1 mL of the test = X mg of glucose equivalent

100 mL of the sample solution contains = $\frac{x}{0.1}$ x 100 mg of glucose = % of total content in sugars present.

Annexe II – Total Lipids Assay

1. Homogenise 100 g of byproduct with 100 ml chloroform and 200 ml methanol in a Waring blender for 2 minutes with liquid nitrogen.
2. Add 100 ml chloroform to the mix and stir.
3. Add 100 ml distilled water.
4. Filter through a Whatman No.1 filter paper.
5. Transfer all filtrates to a graduated cylinder and leave for a few minutes to enable complete phase separation and clarification.
6. Record the volume of the lower chloroform layer containing the purified lipid (at least 150 ml) and the upper alcohol layer and remove the upper layer by aspiration.
7. Evaporate chlorophorm layer at room temperature overnight, inside a fume hood
8. After evaporation, weigh the lipids that remain, using as blank control a similarily treated water sample

Total Lipid Content = (Weight of lipid in aliquot X Volume of chloroform layer) / Volume of Aliquot

Annexe III – Ash Content

To determine the ash content of the carbohydrate rich byproduct, high temperatures were used to burn away the organic content and leave inorganic minerals for analysis by weight. Warning: Use of high temperatures, follow appropriate safety procedures. The method used was taken from Laurens et al, 2012.

1. Weigh 1 g of Freeze-dried xylan sample
2. Incubate at 550 °C for 16 h in a muffle furnace

% Ash = (Weight after heating/Initial weight) x 100

Annexe IV – Bradford Assay

The Bradford assay was used for determination of the protein content.

Bradford Assay (Bradford, 1976)

1. Add 100 µL of sample to a cuvette
2. Add 1 mL of Bradford Reagent
3. Incubate in the dark for 10 min at room temperature
4. Measure A595nm in the spectrophotometer against a blank with water.
5. Use a standard curve of bovine serum albumin (0-100 µg/mL) for determination of protein concentration.

Bradford Reagent: 100 mg Coomassie Brilliant Blue G-250 - 50 mL Ethanol 95% - 100 mL Phosphoric Acid 85% - Make up to 1 L dH_2O. Agitate overnight. Filter the solution twice. Store in the dark, at 4 °C.

Annexe V – Kjeldhal Assay

The Kjeldhal method was used for nitrogen content measurements which can be used for determination of the presence and quantification of nitrogen containing compounds such as amino acids, proteins, phenolic compounds etc.

Sample Preparation:

1. Weigh an equivalent to 5 g of a liquid xylan solution byproduct sample in Kjeldhal digestion tubes.
2. Add 10 mL of H_2SO_4 96% .
3. Add a Kjeldahl catalyst tablet, 3.5 g (KJELDAHL SE (K_2SO_4+SE)), Panreac).
4. Initiate Kjedahl Protocol.

Annexe VI – Carbohydrate Composition Analysis by HPLC

Concentrated sulphuric acid treatment followed by high pressure liquid chromatography (HPLC) on a Rezex™ 8 µm ROA-organic acid H+(8%) column (Phenomenex) for separation of carbohydrate monomer was used to identify the carbohydrate content of the sugar component of the carbohydrate rich byproduct. HPLC was carried out on an Elite LaChrom (VWR Hitachi) chromatography system at 60 °C with detection by refractive index measurements with an Elite LaChrom L-2490 RI detector (VWR Hitachi). EZChrom Elite 3.3.2 SP2 software was used for data collection and analysis.

1. Add H_2SO_4 to a final concentration of 4% to the byproduct samples
2. Autoclave at 121°C, 2 atm. pressure for 20 minutes
3. Centrifuge at 14000 x g for 15 min.
4. Discard pellet and filter supernatant through a PES 0.22 µm filter to HPLC vials.
5. Preheat HPLC column to 60 °C and equilibrate in 2.5 mM H_2SO_4
6. Load 20 µL sample to preequilibrated column.
7. Isocratic elution with 2.5mM H_2SO_4 as the mobile phase as follows:
    - 0 - 14 mins.: 0.15 mL/min
    - 14 - 35 mins.: 0.15 - 0.2 mL/min
    - 35 - 90 mins.: 0.2 mL/min

Annexe VII – HPLC Gel Filtration Chromatography

The molecular weight of the carbohydrate component of the byproduct was investigated by gel filtration chromatography on a PolySep™-SEC GFC-P Linear column (Phenomenex). HPLC was carried out on an Elite LaChrom (VWR Hitachi) chromatography system at 30 °C with detection by refractive index measurements with an Elite LaChrom L-2490 RI detector (VWR Hitachi). EZChrom Elite 3.3.2 SP2 software was used for data collection and analysis.

1. Filter byproduct sample through a PES 0.22 µm filter to HPLC vials.
2. Preheat HPLC column to 30 °C and equilibrate in ultrapure H_2O.
3. Load 20 µL sample to preequilibrated column.
4. Isocratic elution with ultrapure water as the mobile phase at 0.6 mL/min (PolySep) for a total run time of 35 minutes.

Standard curves using a 1 g/L concentration for each Dextran (4000, 40000 and 200000 Da) and also 1 g/L Polyethylene Glycol (200, 300, 400, 1500, 3350, 4000 and 8000 Da) standards were also prepared using the same experimental conditions.

Annexe VIII – Determination of Degree of Polymerisation of Carbohydrates

For determination of the degree of polymerisation (DP) of the carbohydrate component of the byproduct a Rezex™ RSO-Oligosaccharide Ag+ (4) % HPLC column was used. HPLC was carried out on an Elite LaChrom (VWR Hitachi) chromatography system at 85 °C with detection by refractive index measurements with an Elite LaChrom L-2490 RI detector (VWR Hitachi). EZChrom Elite 3.3.2 SP2 software was used for data collection and analysis.

1. Filter byproduct sample through a PES 0.22 µm filter to HPLC vials.
2. Preheat HPLC column to 85 °C and equilibrate in ultrapure H_2O.
3. Load 5 µL sample to preequilibrated column.
4. Isocratic elution with ultrapure water as the mobile phase at 0.15 mL/min (PolySep) for a total run time of 90 minutes.

The xylo-oligomer standards xylose (X_1), xylobiose (X_2), xylotriose (X3), xylotetraose (X_4), xylopentaose (X_5) and xylohexaose (X_6) each at a concentration of 1 g/L, were also analysed using the same experimental conditions.

Annexe IX – Remazol Brilliant Blue – Xylan Coupling and Analysis

Remazol Brillant Blue (RBB) was coupled to the xylan component of the byproduct to give RBB-xylan for use as a novel chromogenic screening substrate. The ratio of RBB to xylose molecules was then determined.

1. Add 2.5 g RBB dye (Sigma) and 2.5 g Xylan to 60 mL dH_2O and stir.
2. Add 20 mL of a 0.4 M Sodium Acetate solution (Merck), pH = 11, dropwise over 5 minutes with continuous stirring at room temperature.
3. Add 20 mL of a 1.875 M Sodium Hydroxide solution, pH = 13 to initiate coupling reaction.
4. Incubate for 90 minutes at toom temperature with continuous mixing.
5. Add 200 mL of 96% Ethanol to precipitate the RBB-Xylan.
6. Leave at -20 °C for 15 minutes.
7. Filter through a Macherey Nagel filter 4-12 µM pore size paper under vacuum.
8. Wash the precipitate sequentially with 1 L of wash solution (16 mM Sodium Acetate solubilized in Ethanol 70%). Filtrate should now be colourless.
9. Wash with 100 mL 70% Ethanol.
10. Wash with 50 mL Acetone.
11. Dry at room temperature overnight.

RBB to Xylose Ratio Determination

The prepared RBB-xylan was characterised in relation to the number of RBB molecules per molecule of xylose.

1. Dissolve 2 mg of RBB-Xylan in 10 mL of dH_2O (200 µg/mL)
2. Centrifuge to remove any particles in suspension
3. Dilute supernatant in dH_2O to RBB-xylan concentrations of 100 µg/mL and 50 µg/mL.
4. Measure absorbance at 595 nm with a Genesys 20 spectrophotometer (ThermoSpectronic). All measurements were made with triplicate samples.

Absorbance values were converted into Remazol Brilliant Blue molar concentration using the Beer-Lambert Law with a RBB Molar Extinction Coeficient at 595 nm (ε595 nm) of 9.25 X 10^3 M^{-1} cm^{-1}:

A (Absorbance) = c (Molar Concentration) x l (Pathlength) x ε (Molar Extinction Coeficient)

The ratio of the number of moles or RBB per moles of xylose were then calculated using the known concentration of xylan used and a xylose residue molecular mass of 133.12 g/mol.

Annexe X - Dinitrosalicylic Acid Assay for Xylanase Activity Measurement

DNS Solution Preparation

1. Add 10 g of 3,5 Dinitrosalicylic acid (Acros Organics) to 300 mL dH_2O, heat to dissolve.
2. Add 4 g of NaOH to 400 mL dH_2O for a 1 M solution.
3. Mix these two solutions with 300 g Potassium Sodium Tartrate (Merck)
4. Add dH_2O up to 1 L
5. Store in the dark at room temperature

Assay Limits: Maximum Concentration of Xylan Rich Byproduct

The maximum concentration of substrate that can be used with the standard DNS assay was evaluated as described:

1. Prepare a 0.1 M solution of Dissodium Hydrogen Phosphate (Na_2HPO_4) and a 0.1 M solution of citric acid.
2. 0.1 M McIlvaines buffer is then prepared by adding citric acid to Na_2HPO_4 until pH 6.5.
3. Prepare 5 mg/mL to 50 mg/mL solutions of byproduct in 0.1 M McIlvaine buffer.
4. Agitate at 200 rpm overnight at room temperature.
5. Centrifuge at 4 °C, 10 min, 3200 x g and retain supernatant at 4 °C.
6. To 200 µl of supernatant solutions add 50 µl of standard xylose concentrations of 1 to 10 mM xylose to reach a final xylose concentration in solution of 0.2 to 2 mM and vortex for 5 seconds
7. Add 1 mL DNS solution and vortex 5 seconds.
8. Develop reaction by incubating in a waterbath at 100 °C for 10 mins.
9. Cool on ice.
10. Measure absorbance at 546 nm. Against a blank wherein the xylose solution is substituted with buffer.

Xylan Rich Byproduct for Xylanase Activity Assay

The substrate was then investigated for use in measuring xylanase activity with the standard DNS assay. The xylanase used here was the cold adapted GH8 xylanase pXyl from *Pseudoalteromonas haloplanktis* (Collins et al, 2002a).

1. Dissolve 15 mg/mL of lyophilized byproduct in 0.1 M McIlvaine buffer (pH 6.5), agitate overnight at room temperature. Centrifuge at 4 °C, 10 min 3200 x g. Discard pellet and store the supernatant at 4 °C.

2. Add 200 µL supernatant solution to 1.5 mL Eppendorf tubes (4 tubes, the blank and triplicates) and preincubate at 25 °C.

3. Add 50 µL of a 0.80 µg/mL xylanase solution to each tube except the blank and vortex 5 seconds
4. Incubate at 25 °C for 5 min.
5. Stop reaction by addition of 500 µL DNS Reagent and vortex 5 seconds
6. Prepare the blanks by adding 500 µL DNS Reagent, vortex 5 seconds then add 50 µL of the working stock enzyme solution and vortex 5 seconds;
7. Heat the reaction in a boiling water bath at 100 °C for 10 minutes

Note: here it is important that the lids of the tubes are perforated as the solution will boil;

8. Cool all tubes in ice
9. Read the Abs_{546}

A standard curve of xylose concentrations from 0.2 mM to 2 mM was also prepared using the same conditions and used in combination with the following equation to determine the number of xylose equivalent reducing ends liberated and the xylanase activity in International Units per mL (IU/mL). One IU/mL of xylanase activity is defined as the amount of enzyme which liberates 1 µmole of xylose equivalents per minute per mL.

$$\frac{IU}{mL} = \frac{Abs_{546}+0.0407}{0.4041} \times 1000 \times \frac{200\ (\mu L)_{Reaction}}{1000} \times \frac{1}{5\ (min)_{Reaction}} \times \frac{1000}{50\ (\mu L)_{Enzyme}}$$

Enzyme activity is displayed as the specific activity i.e. number of IU per mg of protein.

Annexe XI – Cellulase Plate Screening Assays

CMC for Cellulase Screening

1. Prepare either 2% (w/v) YPD or MEA media with 2% (w/v) CMC (Fischer Scientific) and appropriate buffers (see Table VI).
2. Autoclave medium for 20 minutes at 121°C, 2 atm. pressure.
3. Pour plates under aseptic conditions.
4. Inoculate with culture isolates. For fungal isolates, agar sections of fungal growth were placed directly in plates. Yeast and bacterial isolates were inoculated by single cfu streaks.
5. Incubate at 16 °C for 22 days for fungi, 14 days for yeasts and 7 days for bacterial isolates.
6. Observe for zones of clearing around colonies.

Congo Red for Cellulase Screening

Following incubation on CMC plates, plates can be stained with Congo Red for cellulase detection:

1. Prepare a 0.1% (w/v) Congo Red (Sigma) solution.
2. After incubation, flood plates with 10 mL of Congo Red solution.
3. Incubate at room temperature for 30 minutes.
4. Carefully remove solution and flood plates with a 1M NaCl solution.
5. Incubate at room temperature for 30 minutes.
6. Repeat steps 4 and 5.
7. Remove NaCl solution and observe for clear halos against a red background.
8. Add 10 mL of 5% (v/v) acetic acid solution to plates to improve halo visibility if necessary.

Lugol's Iodine/Gram's Iodine for Cellulase Screening

1. Prepare a Gram's Iodine solution by making a 1:3 dilution of Lugol's Iodine solution (Prolab Diagnostics) in water.
2. Following completion of culture incubation, flood plates with 10 mL of Lugol's or Gram's Iodine.
3. Incubate at room temperature for 2 minutes.
4. Remove the solution and observe for halos.

Annexe XII – Pectinase Plate Screening Assays

Pectin for Pectinase Screening

1. Prepare either 2% (w/v) YPD or MEA media with 2% (w/v) citrus peel pectin (Acros Organics) and appropriate buffers (see Table VI).
2. Autoclave medium for 20 minutes at 121°C, 2 atm. pressure.
3. Pour plates under aseptic conditions.
4. Inoculate with culture isolates. For fungal isolates, agar sections of fungal growth were placed directly in plates. Yeast and bacterial isolates were inoculated by single cfu streaks.
5. Incubate at 16 °C for 22 days for fungi, 16 days for yeasts and 7 days for bacterial isolates.
6. Observe for zones of clearing around colonies.

Ruthenium Red for Pectinase Screening

Following incubation on Pectin plates, they can be stained with Ruthenium Red for cellulase detection

1. Prepare a 0.05% (w/v) Ruthenium Red (Sigma) solution.
2. Following incubation of pectin plates, flood plates with 10 mL of Congo Red solution.
3. Incubate at room temperature for 30 minutes.
4. Carefully remove solution and check for zones of clearing around single colonies.

Lugol's Iodine/Gram's Iodine for Cellulase and Pectinase Screening

Following completion of incubation on pectin plates, plates can be stained with Lugol's or Gram's iodine for pectinase detection as described above for cellulases.

Annexe XIII – Chitinase Plate Screening Assays

Colloidal Chitin Preparation (Hsu and Lockwood, 1975).

1. Add 50 g of chitin from shrimp shells (TCI Chemicals) to 150-300 mL of cold 12M HCl, with constant stirring for 1 hour, until total dissolution of the chitin.
2. Filter the solution using a strainer into 2L of ice-cold dH_2O and leave the solution at 4 °C overnight for chitin precipitation.
3. Centrifuge the resulting chitin solution at 11,000 rpm for 8 minutes at 4 °C. Wash the pellets repeatedly with water to remove residual HCl until the pH of the supernatant rises to 3.5.
4. Store the chitin at 4 °C or lyophilize it and store at room temperature.

Colloidal Chitin for Chitinase Screening

1. Prepare either YPD or MEA 2% (w/v) media with 1% (w/v) Colloidal Chitin, and appropriate buffers (see Table VI).
2. Autoclave medium for 20 minutes at 121°C, 2 atm. pressure.
3. Pour plates under aseptic conditions.
4. Inoculate with culture isolates. For fungal isolates, agar sections of fungal growth were placed directly in plates. Yeast and bacterial isolates were inoculated by single cfu streaks.
5. Incubate at 16 °C for 22 days for fungi, 14 days for yeasts and 7 days for bacterial isolates.
6. Observe for zones of clearing around colonies.

Bromocresol Purple for Chitinase Screening (Agrawal et al, 2012)

1. Prepare either YPD or MEA 2% (w/v) media with 1% (w/v) Colloidal Chitin with 0.15 g/L Bromocresol Purple (Merck) solution and appropriate buffers (see Table VI).
2. Autoclave medium for 20 minutes at 121°C, 2 atm. pressure.
3. Pour plates under aseptic conditions.
4. Inoculate with culture isolates. For fungal isolates, agar sections of fungal growth were placed directly in plates. Yeast and bacterial isolates were incoulated by single cfu streaks.
5. Incubate at 16 °C for 22 days for fungi, 14 days for yeasts and 7 days for bacterial isolates.
6. Observe for a purple zone around colonies.

Annexe XIV – Xylanase Plate Screening Assays

AZCL-Xylan for Xylanase Screening

1. Prepare screening media supplemented with 0.1% (w/v) AZCL-Xylan (Megazyme) and appropriate buffer (see Table VI).
2. Autoclave medium for 20 minutes at 121°C, 2 atm. pressure.
3. Pour plates under aseptic conditions.
4. Inoculate with culture isolates. For fungal isolates, agar sections of fungal growth were placed directly in plates. Yeast and bacterial isolates were inoculated by single cfu streaks.
5. Incubate at 16 °C for 22 days for fungi, 14 days for yeasts and 7 days for bacterial isolates.
6. Observe for blue zones around colonies.

RBB-Xylan for Xylanase Screening

1. Prepare screening media supplemented with 0.1% (w/v) RBB-Xylan and appropriate buffer (see table VI).
2. Autoclave medium for 20 minutes at 121°C, 2 atm. pressure.
3. Pour plates under aseptic conditions.
4. Inoculate with culture isolates. For fungal isolates, agar sections of fungal growth were placed directly in plates. Yeast and bacterial isolates were inoculated by single cfu streaks.
5. Incubate at 16 °C for 22 days for fungi, 14 days for yeasts and 7 days for bacterial isolates.
6. Observe for zones of clearing around colonies.

Annexe XV – Ligation and Transformation

Ligation

Combine the following in an Eppendorf tube for 1:1, 1:3 and 1:5 Vector Insert ratios

1. 100 ng digested purified vector.
2. 1:1 ratio – 24 ng digested purified insert.
3. 1:3 ratio – 72 ng digested purified insert.
4. 1:5 ratio –120 ng digested purified insert.
5. 2.5 µL of 10x T4 DNA Buffer (Thermo Scientific™).
6. 1 µL T4 DNA Ligase (5 U/µL) (Thermo Scientific™).
7. upH20 to a final volume of 25 µL
8. Incubate overnight at 4 °C and subsequently at room temperature for 1 h.

Transformation (Inoue et al. 1990)

1. Defrost 200µL of pre-prepared *E. coli* XL1-Blue competent cells on ice (10 to 20 min.)
2. Add 1-10 µL of the DNA ligate (10 to 100 ng).
3. Swirl the tubes gently and incubate on ice for 30min.
4. Heat-shock cells in a water bath at 42 °C for 45 seconds and immediately transfer to ice for 10-15 minutes.
5. Add 800 µL of fresh LB broth and incubate for 1 h at 37 °C, 200 rpm.
6. Centrifuge for 3 min. at 3000 ×g, room temperature.
7. Remove 850 µL of the supernatant and gently resuspend the pellet.
8. Spread-plate the remaining 150 µL solution on LB agar plates containing 200 µg ampicillin/mL for pET25b positive constructs.
9. Incubate overnight at 37°C.
10. A positive transformation control (pUC18 plasmid) a negative control of either upH_2O or solely digested pET22b(+) plasmid should also be carried out.

Annexe XVI – Results for Xylanase Screening of Yeasts and Filamentous Fungi of the CBMA Culture Collection

Species	Biowaste	pH	Xylanase Activity		
			pH 4	pH 7	pH 9
Heliscus lugdunensis		Acidic	-	+	-
Tricladium splendens		Acidic	+	+	+
Triscelophorus sp2.		7.7	+	+	+
Triscelophorus sp2.		7.7			
Triscelophorus sp2.		8.0	+	+	+
Triscelophorus sp2.		8.0			
Triscelophorus sp2.		7.7	-	+	+
Triscelophorus sp2.		7.7	+	+	+
Triscelophorus sp2.		7.7	-	+	+
Triscelophorus sp2.		7.7	-	+	+
Varicosporium elodeae		Acidic	+	+	-
Varicosporium elodeae		Acidic	+	+	+
Articulospora tetracladia		Acidic	+	+	+
Saccharomyces cerevisiae	Peach	~3.5	-	+	-
Meyerozyma guilliermondii	Peach	~3.5	-	+	-
Pichia fermentans	Peach	~3.5	-	+	+
Candida humilis	Peach	~3.5 to 5.5	-	-	-
Kluyveromyces marxianus	Peach	~3.5	-	+	-
Kluyveromyces marxianus	Peach	~3.5	-	+	+
Saccharomyces cerevisiae	Peach	~3.5 to 5.5	-	+	+
Kazachstania gamospora	Peach	~3.5 to 5.5	-	+	+
Saccharomyces cerevisiae	Peach	~3.5 to 5.5	+	+	+
Pichia fermentans	Apple		-	-	+
Hanseniaspora uvarum	Apple		-	+	+
Wickerhamomyces anomalus	Apple		-	-	-
Wickerhamomyces anomalus	Apple		-	+	+
Cyberlindnera jadinii	Mix of salad	~6.5	-	+	-
Barnettozyma californica	Mix of salad	~6.5 to 7	-	-	-
Cyberlindnera jadinii	Mix of salad	~6.5	-	-	+
Candida tropicalis	Mix of salad	~6.5	-	-	-
Suhomyces xylopsoci	Mix of salad		-	-	-
Candida sclani	Mix of salad	~6.5 to 7	-	-	-
Candida sclani	Mix of salad	~6.5 to 7	-	-	-
Pichia norvegensis	Mix of salad	~6.5	-	-	-
Candida sclani	Mix of salad	~6.5 to 7	-	-	-
Pichia kudriavzevii	Green Bean	~5.5 to 6.5	-	-	-

Species	Biowaste	pH	Xylanase Activity		
			pH 4	pH 7	pH 9
Papillotrema flavescens	Green Bean	~6.2 to 7.4	-	-	-
Metschnikowia sp.	Pear		+	-	-
Candida stellimaliicola	Pear	~2.5 to 6.5	-	-	-
Wickerhamomyces pijperi	Pear		-	-	-
Wickerhamomyces sp.	Pear	~5.5 to 6.8	+	-	-
Barnettozyma californica	Chard F.	~5.5 to 6.8	-	+	-
Barnettozyma californica	Chard F.	~5.5 to 6.8	+	+	+
Torulaspora delbrueckii	Pepper	~3.6 to 6.8	-	+	-
Metschnikowia	Pepper	~3.6 to 6.5	+	+	-
Candida glabrata	Pepper	~3.6 to 6.5	-	-	-
Candida glabrata	Chard P.	~3.6 to 6.8	+	+	-
Candida intermedia	Chard P.	~3.6 to 6.8	-	-	-
Torulaspora delbrueckii	Strawberry	~2	-	+	-
Candida humilis	Peach	~3.5 to 5.5	-	+	-
Candida humilis	Peach	~3.5 to 5.5	-	-	-
Candida humilis	Peach	~3.5 to 5.5	-	-	-
Aureobasidium pullulans	Peach	~3.5	-	-	-
Pichia occidentalis	Peach		-	+	-
Candida tropicalis	Peach	~3.5	-	+	-
Kazachstania gamospora	Peach	~3.5 to 5.5	+	+	-
Pichia manshurica	Peach		+	+	-
Candida humilis	Peach	~3.5 to 5.5	-	-	-
Candida humilis	Peach	~3.5 to 5.5	-	-	-
Candida humilis	Peach	~3.5 to 5.5	-	-	-
Kazachstania gamospora	Peach	~3.5 to 5.5	-	+	-
Pichia manshurica	Peach	~5.5	-	-	-
Pichia kudriavzevii	Apple		-	-	-
Rhodotorula mucilaginosa	Apple		-	-	-
Rhodotorula mucilaginosa	Apple		-	+	-
Pichia kudriavzevii	Apple		-	+	-
Hanseniaspora uvarum	Apple		-	-	-
Candida orientalis	Apple		-	+	-
Pichia kudriavzevii	Apple		-	+	-
Hanseniaspora uvarum	Apple		+	-	+
Hanseniaspora uvarum	Apple		+	-	+
Pichia fermentans	Apple		+	-	-

Species	Biowaste	pH	Xylanase Activity		
			pH 4	pH 7	pH 9
Torulaspora delbrueckii	Green Bean	~6.2	-	+	-
Pichia fermentans	Apple		-	-	-
Wickerhamomyces anomalus	Apple		-	+	-
Pichia fermentans	Apple		-	-	-
Saprochaete gigas	Apple		-	-	-
Pichia kudriavzevii	Apple		-	-	-
Rhodotorula mucilaginosa	Mix of salad	~5.5 to 6.5	+	+	-
Cyberlindnera jadinii	Mix of salad	~6.5	+	+	-
Pichia fermentans	Mix of salad	~6.5 to 7	-	-	-
Candida tropicalis	Mix of salad	~6.5	+	+	-
Wickerhamomyces anomalus	Mix of salad	~6.5	-	-	-
Pichia orientalis	Mix of salad		-	-	-
Candida sclani	Mix of salad	~6.5 to 7	-	+	-
Candida sclani	Mix of salad	~6.5 to 7	+	+	-
Pichia fermentans	Green Bean	~5.5 to 6.5	-	-	-
Pichia fermentans	Green Bean	~6.2 to 7.4	+	-	-
Pichia fermentans	Green Bean	~6.2 to 7.4	-	-	-
Wickerhamomyces anomalus	Green Bean	~5.5 to 7.4	-	+	-
Pichia kudriavzevii	Green Bean	~5.5 to 6.5	-	-	-
Pichia fermentans	Green Bean	~6.2 to 7.4	-	-	-
Pichia norvegensis	Green Bean	~6.2 to 7.4	-	+	-
Pichia fermentans	Green Bean	~6.2 to 7.4	-	-	-
Pichia orientalis	Green Bean		-	-	-
Pichia fermentans	Green Bean	~6.2 to 7.4	+	+	+
Wickerhamomyces anomalus	Green Bean	~5.5 to 7.4	-	+	-
Saccharomyces cerevisiae	Pear	~2 to 6.5	-	+	-
Candida tropicalis	Pear	~2 to 6.5	-	+	-
Geotrichum candidum	Pear	~2 to 6.5	-	-	-
Metschnikowia pulcherrima	Pear	~2 to 6.5	-	-	-
Geotrichum candidum	Pear	~2 to 6.5	-	+	-
Wickerhamomyces anomalus	Pear	~2.5 to 6.5	-	-	-
Candida stellimaliicola	Pear	~2.5 to 6.5	-	+	-
Hanseniaspora uvarum	Pear	~2 to 6.5	-	-	-
Geotrichum candidum	Chard F.	~5.5 to 6.8	-	+	-
Clavispora lusitaniae	Chard F.	~5.5 to 6.8	-	+	-
Geotrichum candidum	Chard F.	~5.5 to 6.8	-	+	-

Species	Biowaste	pH	Xylanase Activity		
			pH 4	pH 7	pH 9
Geotrichum candidum	Chard F.	~5.5 to 6.8	-	-	-
Geotrichum candidum	Chard F.	~5.5 to 6.8	-	-	-
Pichia fermentans	Pepper	~3.6 to 7	-	-	-
Clavispora lusitaniae	Chard F.	~5.5 to 6.8	-	+	-
Metschnikowia sinensis	Pepper		+	-	-
Pichia fermentans	Pepper	~3.6 to 7	-	-	-
Hanseniaspora uvarum	Pepper	~3.6 to 6.8	-	-	-
Pichia fermentans	Pepper	~3.6 to 7	-	-	-
Hanseniaspora uvarum	Pepper	~3.6 to 6.8	-	+	-
Pichia kudriavzevii	Pepper	~3.6 to 7	-	-	-
Candida tropicalis	Pepper	~3.6 to 7	-	-	-
Clavispora lusitaniae	Pepper	~3.6 to 7	-	-	-
Candida humilis	Pepper	~3.6 to 6.8	-	-	-
Wickerhamomyces anomalus	Pepper	~3.6 to 7	-	+	+
Pichia kudriavzevii	Pepper	~3.6 to 7	-	-	+
Candida tropicalis	Pepper	~3.6 to 7	-	-	-
Wickerhamomyces anomalus	Pepper	~3.6 to 7	-	-	+
Pichia kudriavzevii	Pepper	~3.6 to 7	-	-	+
Clavispora lusitaniae	Pepper	~3.6 to 7	-	+	-
Geotrichum candidum	Pepper	~3.6 to 7	-	+	-
Candida tropicalis	Pepper	~3.6 to 7	-	-	-

Labels: Yellow cells: Filamentous Fungi; Orange Cells with a "+": Positives; Cells with a "–": Negatives. Gray cells: No Data

Annexe XVII – Results for Cellulase Screening of Yeasts and Filamentous Fungi of the CBMA Culture Collection

Species	Biowaste	pH	Cellulase Activity		
			pH 4	pH 7	pH 9
Heliscus lugdunensis		Acidic	-	-	-
Tricladium splendens		Acidic	-	+	+
Triscelophorus sp2.		7.7	-	+	+
Triscelophorus sp2.		7.7			
Triscelophorus sp2.		8.0	-	+	+
Triscelophorus sp2.		8.0			
Triscelophorus sp2.		7.7	-	+	+
Triscelophorus sp2.		7.7	-	+	+
Triscelophorus sp2.		7.7	-	+	+
Triscelophorus sp2.		7.7	-	+	+
Varicosporium elodeae		Acidic	-	+	+
Varicosporium elodeae		Acidic	-	+	+
Articulospora tetracladia		Acidic	+	+	+
Saccharomyces cerevisiae	Peach	~3.5	-	+	+
Meyerozyma guilliermondii	Peach	~3.5	-	+	-
Pichia fermentans	Peach	~3.5	-	-	-
Candida humilis	Peach	~3.5 to 5.5	-	+	-
Kluyveromyces marxianus	Peach	~3.5	-	+	-
Kluyveromyces marxianus	Peach	~3.5	+	+	+
Saccharomyces cerevisiae	Peach	~3.5 to 5.5	-	+	+
Kazachstania gamospora	Peach	~3.5 to 5.5	-	-	-
Saccharomyces cerevisiae	Peach	~3.5 to 5.5	+	+	+
Pichia fermentans	Apple		+	+	+
Hanseniaspora uvarum	Apple		+	+	-
Wickerhamomyces anomalus	Apple		-	+	-
Wickerhamomyces anomalus	Apple		-	+	-
Cyberlindnera jadinii	Mix of salad	~6.5	+	+	-
Barnettozyma californica	Mix of salad	~6.5 to 7	-	+	+
Cyberlindnera jadinii	Mix of salad	~6.5	+	+	+
Candida tropicalis	Mix of salad	~6.5	+	+	+
Suhomyces xylopsoci	Mix of salad		+	+	+
Candida solani	Mix of salad	~6.5 to 7	-	+	+
Candida solani	Mix of salad	~6.5 to 7	-	+	+
Pichia norvegensis	Mix of salad	~6.5	-	+	+
Candida solani	Mix of salad	~6.5 to 7	-	+	+
Pichia kudriavzevii	Green Bean	~5.5 to 6.5	-	+	-

Species	Biowaste	pH	Cellulase Activity		
			pH 4	pH 7	pH 9
Torulaspora delbrueckii	Green Bean	~6.2	-	+	+
Papiliotrema flavescens	Green Bean	~6.2 to 7.4	-	-	+
Metschnikowia sp.	Pear		-	+	-
Candida stellimalicola	Pear	~2.5 to 6.5	-	-	-
Wickerhamomyces pijperi	Pear		-	-	-
Wickerhamomyces sp.	Pear	~5.5 to 6.8	-	-	-
Barnettozyma californica	Chard F.	~5.5 to 6.8	-	+	-
Barnettozyma californica	Chard F.	~5.5 to 6.8	-	+	-
Torulaspora delbrueckii	Pepper	~3.6 to 6.8	-	+	-
Metschnikowia	Pepper	~3.6 to 6.5	-	+	-
Candida glabrata	Pepper	~3.6 to 6.5	-	+	+
Candida glabrata	Chard P.	~3.6 to 6.8	-	+	+
Candida intermedia	Chard P.	~3.6 to 6.8	-	+	+
Torulaspora delbrueckii	Strawberry	~2	-	+	-
Candida humilis	Peach	~3.5 to 5.5	-	+	+
Candida humilis	Peach	~3.5 to 5.5	-	+	-
Candida humilis	Peach	~3.5 to 5.5	-	+	+
Aureobasidium pullulans	Peach	~3.5	-	+	+
Pichia occidentalis	Peach		-	-	-
Candida tropicalis	Peach	~3.5	+	+	-
Kazachstania gamospora	Peach	~3.5 to 5.5	-	-	+
Pichia manshurica	Peach		-	-	-
Candida humilis	Peach	~3.5 to 5.5	-	-	+
Candida humilis	Peach	~3.5 to 5.5	-	-	+
Candida humilis	Peach	~3.5 to 5.5	-	-	-
Kazachstania gamospora	Peach	~3.5 to 5.5	-	-	+
Candida stellimalicola	Peach	~5.5	-	-	-
Pichia kudriavzevii	Apple		-	+	-
Rhodotorula mucilaginosa	Apple		-	+	+
Rhodotorula mucilaginosa	Apple		-	-	-
Pichia kudriavzevii	Apple		-	-	-
Hanseniaspora uvarum	Apple		-	-	-
Pichia orientalis	Apple		-	-	-
Pichia kudriavzevii	Apple		-	+	-
Hanseniaspora uvarum	Apple		+	+	+
Hanseniaspora uvarum	Apple		+	+	+

Species	Biowaste	pH	Cellulase Activity		
			pH 4	pH 7	pH 9
Pichia fermentans	Apple		-	-	-
Pichia fermentans	Apple		-	+	-
Wickerhamomyces anomalus	Apple		-	+	+
Pichia fermentans	Apple		-	-	-
Saprochaete gigas	Apple		-	-	-
Pichia kudriavzevii	Apple		-	-	-
Rhodotorula mucilaginosa	Mix of salad	~5.5 to 6.5	+	+	-
Cyberlindnera jadinii	Mix of salad	~6.5	+	+	-
Pichia fermentans	Mix of salad	~6.5 to 7	-	-	-
Candida tropicalis	Mix of salad	~6.5	+	+	-
Wickerhamomyces anomalus	Mix of salad	~6.5	-	-	-
Pichia orientalis	Mix of salad		-	-	-
Candida solani	Mix of salad	~6.5 to 7	-	+	-
Candida solani	Mix of salad	~6.5 to 7	+	+	-
Pichia kudriavzevii	Green Bean	~5.5 to 6.5	-	-	-
Pichia fermentans	Green Bean	~6.2 to 7.4	+	-	-
Pichia fermentans	Green Bean	~6.2 to 7.4	-	-	-
Wickerhamomyces anomalus	Green Bean	~5.5 to 7.4	-	+	-
Pichia kudriavzevii	Green Bean	~5.5 to 6.5	-	-	-
Pichia fermentans	Green Bean	~6.2 to 7.4	-	-	-
Pichia fermentans	Green Bean	~6.2 to 7.4	-	-	-
Pichia orientalis	Green Bean		-	-	-
Pichia fermentans	Green Bean	~6.2 to 7.4	+	+	+
Wickerhamomyces anomalus	Green Bean	~5.5 to 7.4	-	+	-
Saccharomyces cerevisiae	Pear	~2 to 6.5	-	+	-
Candida tropicalis	Pear	~2 to 6.5	-	+	-
Geotrichum candidum	Pear	~2 to 6.5	-	-	-
Metschnikowia pulcherrima	Pear	~2 to 6.5	-	-	-
Geotrichum candidum	Pear	~2 to 6.5	-	+	-
Wickerhamomyces anomalus	Pear	~2.5 to 6.5	-	-	-
Candida stellimalicola	Pear	~2.5 to 6.5	-	+	-
Hanseniaspora uvarum	Pear	~2 to 6.5	-	-	-
Geotrichum candidum	Chard F.	~5.5 to 6.8	-	+	-
Clavispora lusitaniae	Chard F.	~5.5 to 6.8	-	+	-
Clavispora lusitaniae	Chard F.	~5.5 to 6.8	-	+	-

Species	Biowaste	pH	Cellulase Activity		
			pH 4	pH 7	pH 9
Geotrichum candidum	Chard F.	~5.5 to 6.8	-	-	-
Geotrichum candidum	Chard F.	~5.5 to 6.8	-	-	-
Pichia fermentans	Pepper	~3.6 to 7	-	-	-
Clavispora lusitaniae	Chard F.	~5.5 to 6.8	-	+	-
Metschnikowia sinensis	Pepper		+	-	-
Pichia fermentans	Pepper	~3.6 to 7	-	-	-
Hanseniaspora uvarum	Pepper	~3.6 to 6.8	-	-	-
Pichia fermentans	Pepper	~3.6 to 7	-	-	-
Hanseniaspora uvarum	Pepper	~3.6 to 6.8	-	+	-
Pichia kudriavzevii	Pepper	~3.6 to 7	-	-	-
Candida tropicalis	Pepper	~3.6 to 7	-	-	-
Clavispora lusitaniae	Pepper	~3.6 to 7	-	-	-
Candida humilis	Pepper	~3.6 to 6.8	-	-	-
Wickerhamomyces anomalus	Pepper	~3.6 to 7	-	+	+
Pichia kudriavzevii	Pepper	~3.6 to 7	-	-	+
Candida tropicalis	Pepper	~3.6 to 7	-	-	-
Wickerhamomyces anomalus	Pepper	~3.6 to 7	-	-	+
Pichia kudriavzevii	Pepper	~3.6 to 7	-	-	+
Clavispora lusitaniae	Pepper	~3.6 to 7	-	+	-
Geotrichum candidum	Pepper	~3.6 to 7	-	+	-
Candida tropicalis	Pepper	~3.6 to 7	-	-	-

Labels: Yellow cells: Filamentous Fungi; Orange Cells with a "+": Positives; Cells with a "–": Negatives. Gray cells: No Data

Annexe XVIII – Results for Pectinases Screening of Yeasts and Filamentous Fungi of the CBMA Culture Collection

Species	Biowaste	pH	Pectinase Activity		
			pH 4	pH 7	pH 9
Heliscus lugdunensis		Acidic	-	-	-
Trichoderma splendens		Acidic	+	-	-
Trisecloglohcrus sp2.		7.7	-	+	+
Trisecloglohcrus sp2.		7.7			
Trisecloglohcrus sp2.		8.0	-	+	+
Trisecloglohcrus sp2.		8.0			
Trisecloglohcrus sp2.		7.7	-	+	+
Trisecloglohcrus sp2.		7.7	-	+	-
Trisecloglohcrus sp2.		7.7	-	-	-
Trisecloglohcrus sp2.		7.7	-	-	-
Varicosporium elodeae		Acidic	-	-	-
Varicosporium elodeae		Acidic	-	-	-
Articulospora tetracladia		Acidic	-	+	+
Saccharomyces cerevisiae	Peach	~3.5	-	+	+
Meyerozyma guilliermondii	Peach	~3.5	-	+	+
Pichia fermentans	Peach	~3.5	-	-	-
Candida humilis	Peach	~3.5 to 5.5	-	-	+
Kluyveromyces marxianus	Peach	~3.5	-	+	+
Kluyveromyces marxianus	Peach	~3.5	-	+	+
Saccharomyces cerevisiae	Peach	~3.5 to 5.5	-	+	+
Kazachstania gamospora	Peach	~3.5 to 5.5	-	+	-
Saccharomyces cerevisiae	Peach	~3.5 to 5.5	+	+	+
Pichia fermentans	Apple		-	+	-
Hanseniaspora uvarum	Apple		+	+	+
Wickerhamomyces anomalus	Apple		+	+	+
Wickerhamomyces anomalus	Apple		+	+	+
Cyberlindnera jadinii	Mix of salad	~6.5	-	-	+
Barnettozyma californica	Mix of salad	~6.5 to 7	-	+	-
Cyberlindnera jadinii	Mix of salad	~6.5	-	+	+
Candida tropicalis	Mix of salad	~6.5	+	+	+
Suhomyces wickerhamii	Mix of salad		-	+	-
Candida sclani	Mix of salad	~6.5 to 7	-	+	-
Candida sclani	Mix of salad	~6.5 to 7	-	+	-
Pichia norvegensis	Mix of salad	~6.5	-	+	-
Candida sclani	Mix of salad	~6.5 to 7	-	+	-
Pichia kudriavzevii	Green Bean	~5.5 to 6.5	-	+	-

Species	Biowaste	pH	Pectinase Activity		
			pH 4	pH 7	pH 9
Torulaspora delbrueckii	Green Bean	~6.2	-	+	+
Papiliotrema flavescens	Green Bean	~6.2 to 7.4	-	+	-
Metschnikowia sp.	Pear		-	+	+
Candida stellimalicola	Pear	~2.5 to 6.5	-	-	-
Wickerhamomyces pijperi	Pear		-	+	-
Wickerhamomyces sp.	Pear	~5.5 to 6.8	-	-	-
Barnettozyma californica	Chard F.	~5.5 to 6.8	-	+	-
Barnettozyma californica	Chard F.	~5.5 to 6.8	-	+	-
Torulaspora delbrueckii	Pepper	~3.6 to 6.8	-	+	-
Metschnikowia	Pepper	~3.6 to 6.5	-	+	-
Candida glabrata	Pepper	~3.6 to 6.5	-	+	-
Candida glabrata	Chard P.	~3.6 to 6.8	-	-	-
Candida intermedia	Chard P.	~3.6 to 6.8	-	+	-
Torulaspora delbrueckii	Strawberry	~2	-	+	-
Candida humilis	Peach	~3.5 to 5.5	-	+	-
Candida humilis	Peach	~3.5 to 5.5	-	+	+
Candida humilis	Peach	~3.5 to 5.5	-	+	-
Aureobasidium pullulans	Peach	~3.5	-	-	-
Pichia occidentalis	Peach		-	+	-
Candida tropicalis	Peach	~3.5	+	+	-
Kazachstania gamospora	Peach	~3.5 to 5.5	-	+	-
Pichia manshurica	Peach		-	-	-
Candida humilis	Peach	~3.5 to 5.5	-	+	-
Candida humilis	Peach	~3.5 to 5.5	-	+	-
Candida humilis	Peach	~3.5 to 5.5	+	+	+
Kazachstania gamospora	Peach	~3.5 to 5.5	-	+	-
Candida stellimalicola	Peach	~5.5	-	+	+
Pichia kudriavzevii	Apple		-	+	-
Rhodotorula mucilaginosa	Apple		-	+	+
Rhodotorula mucilaginosa	Apple		-	+	-
Pichia kudriavzevii	Apple		+	+	-
Hanseniaspora uvarum	Apple		-	+	-
Pichia orientalis	Apple		-	+	-
Pichia kudriavzevii	Apple		+	+	-
Hanseniaspora uvarum	Apple		+	+	+
Hanseniaspora uvarum	Apple		+	+	+

Species	Biowaste	pH	Pectinase Activity		
			pH 4	pH 7	pH 9
Pichia fermentans	Apple		-	-	-
Pichia fermentans	Apple		-	-	-
Wickerhamomyces anomalus	Apple		-	+	-
Pichia fermentans	Apple		-	-	-
Saprochaete gigas	Apple		-	-	-
Pichia kudriavzevii	Apple		-	-	-
Rhodotorula mucilaginosa	Mix of salad	~5.5 to 6.5	-	+	-
Cyberlindnera jadinii	Mix of salad	~6.5	-	+	-
Pichia fermentans	Mix of salad	~6.5 to 7	+	+	+
Candida tropicalis	Mix of salad	~6.5	+	+	+
Wickerhamomyces anomalus	Mix of salad	~6.5	-	+	-
Pichia orientalis	Mix of salad		-	-	-
Candida sclani	Mix of salad	~6.5 to 7	-	+	-
Candida sclani	Mix of salad	~6.5 to 7	-	+	-
Pichia kudriavzevii	Green Bean	~5.5 to 6.5	-	+	-
Pichia fermentans	Green Bean	~6.2 to 7.4	-	+	-
Wickerhamomyces anomalus	Green Bean	~5.5 to 7.4	-	+	-
Pichia kudriavzevii	Green Bean	~5.5 to 6.5	-	+	-
Pichia fermentans	Green Bean	~6.2 to 7.4	-	+	-
Pichia fermentans	Green Bean	~6.2 to 7.4	-	+	-
Pichia norvegensis	Green Bean	~6.2 to 7.4	-	-	-
Pichia fermentans	Green Bean	~6.2 to 7.4	-	-	-
Pichia orientalis	Green Bean		-	+	-
Pichia fermentans	Green Bean	~6.2 to 7.4	+	+	+
Wickerhamomyces anomalus	Green Bean	~5.5 to 7.4	-	-	+
Saccharomyces cerevisiae	Pear	~2 to 6.5	-	-	-
Candida tropicalis	Pear	~2 to 6.5	+	+	-
Geotrichum candidum	Pear	~2 to 6.5	-	-	-
Metschnikowia pulcherrima	Pear	~2 to 6.5	-	-	-
Geotrichum candidum	Pear	~2 to 6.5	-	-	-
Wickerhamomyces anomalus	Pear	~2.5 to 6.5	+	+	+
Candida stellimalicola	Pear	~2.5 to 6.5	+	+	+
Hanseniaspora uvarum	Pear	~2 to 6.5	-	+	+
Geotrichum candidum	Chard F.	~5.5 to 6.8	-	-	-
Clavispora lusitaniae	Chard F.	~5.5 to 6.8	-	-	-
Candida sclani	Mix of salad	~6.5 to 7	-	+	-
Candida sclani	Mix of salad	~6.5 to 7	-	+	-
Candida tropicalis	Mix of salad	~6.5	+	+	+
Candida sclani	Mix of salad	~6.5 to 7	-	+	-
Pichia kudriavzevii	Mix of salad	~6.5 to 7	+	+	+
Candida tropicalis	Mix of salad	~6.5	+	+	+
Wickerhamomyces anomalus	Mix of salad	~6.5	+	+	+
Pichia kudriavzevii	Mix of salad	~6.5 to 7	+	+	+
Clavispora lusitaniae	Mix of salad	~6.5 to 7	-	+	-
Geotrichum candidum	Mix of salad	~6.5 to 7	-	-	-
Candida tropicalis	Mix of salad	~6.5 to 7	+	+	+

Species	Biowaste	pH	Pectinase Activity		
			pH 4	pH 7	pH 9
Geotrichum candidum	Chard F.	~5.5 to 6.8	-	-	-
Geotrichum candidum	Chard F.	~5.5 to 6.8	-	-	-
Pichia fermentans	Pepper	~3.6 to 7	+	-	+
Clavispora lusitaniae	Chard F.	~5.5 to 6.8	-	-	-
Metschnikowia sinensis	Pepper		+	+	+
Pichia fermentans	Pepper	~3.6 to 7	-	+	+
Hanseniaspora uvarum	Pepper	~3.6 to 6.8	-	+	+
Pichia fermentans	Pepper	~3.6 to 7	-	-	+
Hanseniaspora uvarum	Pepper	~3.6 to 6.8	-	-	+
Pichia kudriavzevii	Pepper	~3.6 to 7	+	+	-
Candida tropicalis	Pepper	~3.6 to 7	+	+	-
Clavispora lusitaniae	Pepper	~3.6 to 7	+	+	-
Candida humilis	Pepper	~3.6 to 6.8	+	+	+
Wickerhamomyces anomalus	Pepper	~3.6 to 7	+	+	+
Pichia kudriavzevii	Pepper	~3.6 to 7	-	+	+
Candida tropicalis	Pepper	~3.6 to 7	-	+	+
Wickerhamomyces anomalus	Pepper	~3.6 to 7	+	+	+
Pichia kudriavzevii	Pepper	~3.6 to 7	+	+	+
Clavispora lusitaniae	Pepper	~3.6 to 7	-	+	-
Geotrichum candidum	Pepper	~3.6 to 7	-	-	-
Candida tropicalis	Pepper	~3.6 to 7	+	+	+

Labels: Yellow cells: Filamentous Fungi; Orange Cells with a "+": Positives; Cells with a "–": Negatives. Gray cells: No Data

Annexe XIX – Results for Chitinase Screening of Yeasts and Filamentous Fungi of the CBMA Culture Collection

Species	Biowaste	pH	Chitinase Activity			Species	Biowaste	pH	Chitinase Activity			Species	Biowaste	pH	Chitinase Activity			Species	Biowaste	pH	Chitinase Activity		
			pH 4	pH 7	pH 9				pH 4	pH 7	pH 9				pH 4	pH 7	pH 9				pH 4	pH 7	pH 9
Helicosus lugdunensis		Acidic	-	-	-	*Torulaspora delbrueckii*	Green Bean	~6.2	+	+	-	*Pichia fermentans*	Apple		+	-	-	*Geotrichum candidum*	Chard F.	~5.5 to 6.8	-	-	+
Tricladium splendens		Acidic	-	-	-	*Papiliotrema flavescens*	Green Bean	~6.2 to 7.4	+	+	-	*Pichia fermentans*	Apple		+	-	-	*Geotrichum candidum*	Chard F.	~5.5 to 6.8	-	-	-
Triscelophorus sp2.		7.7	+	-	-	*Metschnikowia sp.*	Pear		+	-	-	*Wickerhamomyces anomalus*	Apple		-	-	-	*Pichia fermentans*	Pepper	~3.6 to 7	-	-	-
Triscelophorus sp2.		7.7				*Candida stellimalicola*	Pear	~2.5 to 6.5	+	-	-	*Pichia fermentans*	Apple		-	-	-	*Clavispora lusitaniae*	Chard F.	~5.5 to 6.8	-	-	-
Triscelophorus sp2.		8.0	-	-	-	*Wickerhamomyces pijperi*	Pear		-	-	-	*Saprochaete gigas*	Apple		-	-	-	*Metschnikowia sinensis*	Pepper		-	+	-
Triscelophorus sp2.		8.0				*Wickerhamomyces sp.*	Pear	~5.5 to 6.8	-	-	-	*Pichia kudriavzevii*	Apple		-	-	-	*Pichia fermentans*	Pepper	~3.6 to 7	-	+	-
Triscelophorus sp2.		7.7	+	-	-	*Barnettozyma californica*	Chard F.	~5.5 to 6.8	-	+	+	*Rhodotorula mucilaginosa*	Mix of salad	~5.5 to 6.5	-	+	-	*Hanseniaspora uvarum*	Pepper	~3.6 to 6.8	-	-	-
Triscelophorus sp2.		7.7	-	-	-	*Barnettozyma californica*	Chard F.	~5.5 to 6.8	-	+	+	*Cyberlindnera jadinii*	Mix of salad	~6.5	-	-	-	*Pichia fermentans*	Pepper	~3.6 to 7	-	-	-
Triscelophorus sp2.		7.7	-	-	+	*Torulaspora delbrueckii*	Pepper	~3.6 to 6.8	+	+	+	*Pichia fermentans*	Mix of salad	~6.5 to 7	-	-	-	*Hanseniaspora uvarum*	Pepper	~3.6 to 6.8	+	-	-
Triscelophorus sp2.		7.7	-	-	-	*Metschnikowia*	Pepper	~3.6 to 6.5	-	+	-	*Candida tropicalis*	Mix of salad	~6.5	+	+	-	*Pichia kudriavzevii*	Pepper	~3.6 to 7	-	-	-
Varicosporium elodeae		Acidic	-	-	-	*Candida glabrata*	Pepper	~3.6 to 6.5	-	+	-	*Wickerhamomyces anomalus*	Mix of salad	~6.5	-	+	-	*Candida tropicalis*	Pepper	~3.6 to 7	-	+	-
Varicosporium elodeae		Acidic	-	-	-	*Candida glabrata*	Chard P.	~3.6 to 6.8	+	+	+	*Pichia orientalis*	Mix of salad		-	-	-	*Clavispora lusitaniae*	Pepper	~3.6 to 7	+	-	-
Articulospora tetracladia		Acidic	-	-	-	*Candida intermedia*	Chard P.	~3.6 to 6.8	+	+	-	*Candida solani*	Mix of salad	~6.5 to 7	+	+	-	*Candida humilis*	Pepper	~3.6 to 6.8	-	-	-
Saccharomyces cerevisiae	Peach	~3.5	+	-	-	*Torulaspora delbrueckii*	Strawberry	~2	-	-	+	*Candida solani*	Mix of salad	~6.5 to 7	-	+	-	*Wickerhamomyces anomalus*	Pepper	~3.6 to 7	-	+	-
Meyerozyma guilliermondii	Peach	~3.5	-	-	-	*Candida humilis*	Peach	~3.5 to 5.5	-	+	-	*Pichia kudriavzevii*	Green Bean	~5.5 to 6.5	-	-	-	*Pichia kudriavzevii*	Pepper	~3.6 to 7	-	-	-
Pichia fermentans	Peach	~3.5	-	-	-	*Candida humilis*	Peach	~3.5 to 5.5	+	+	-	*Pichia fermentans*	Green Bean	~6.2 to 7.4	+	-	-	*Candida tropicalis*	Pepper	~3.6 to 7	-	-	-
Candida humilis	Peach	~3.5 to 5.5	+	-	-	*Candida humilis*	Peach	~3.5 to 5.5	-	-	+	*Pichia fermentans*	Green Bean	~6.2 to 7.4	+	-	-	*Wickerhamomyces anomalus*	Pepper	~3.6 to 7	+	-	-
Kluyveromyces marxianus	Peach	~3.5	-	-	-	*Aureobasidium pullulans*	Peach	~3.5	+	+	+	*Wickerhamomyces anomalus*	Green Bean	~5.5 to 7.4	-	+	-	*Pichia kudriavzevii*	Pepper	~3.6 to 7	-	-	-
Kluyveromyces marxianus	Peach	~3.5	+	+	-	*Pichia occidentalis*	Peach		-	-	-	*Pichia kudriavzevii*	Green Bean	~5.5 to 6.5	-	-	-	*Clavispora lusitaniae*	Pepper	~3.6 to 7	-	-	-
Saccharomyces cerevisiae	Peach	~3.5 to 5.5	-	-	-	*Candida tropicalis*	Peach	~3.5	+	-	+	*Pichia fermentans*	Green Bean	~6.2 to 7.4	+	-	-	*Geotrichum candidum*	Pepper	~3.6 to 7	-	-	-
Kazachstania gamospora	Peach	~3.5 to 5.5	-	-	-	*Kazachstania gamospora*	Peach	~3.5 to 5.5	-	-	-	*Pichia norvegensis*	Green Bean	~6.2 to 7.4	+	-	-	*Candida tropicalis*	Pepper	~3.6 to 7	-	-	-
Saccharomyces cerevisiae	Peach	~3.5 to 5.5	+	+	+	*Pichia manshurica*	Peach		-	-	-	*Pichia fermentans*	Green Bean	~6.2 to 7.4	+	-	-						
Pichia fermentans	Apple		+	-	-	*Candida humilis*	Peach	~3.5 to 5.5	-	-	-	*Pichia orientalis*	Green Bean		+	-	-						
Hanseniaspora uvarum	Apple		+	-	-	*Candida humilis*	Peach	~3.5 to 5.5	-	-	-	*Pichia fermentans*	Green Bean	~6.2 to 7.4	+	-	+						
Wickerhamomyces anomalus	Apple		+	-	-	*Candida humilis*	Peach	~3.5 to 5.5	-	-	+	*Wickerhamomyces anomalus*	Green Bean	~5.5 to 7.4	-	-	-						
Wickerhamomyces anomalus	Apple		+	+	-	*Kazachstania gamospora*	Peach	~3.5 to 5.5	-	+	+	*Saccharomyces cerevisiae*	Pear	~2 to 6.5	-	-	-						
Cyberlindnera jadinii	Mix of salad	~6.5	+	-	-	*Candida stellimalicola*	Peach	~5.5	-	-	+	*Candida tropicalis*	Pear	~2 to 6.5	-	+	-						
Barnettozyma californica	Mix of salad	~6.5 to 7	-	-	-	*Pichia kudriavzevii*	Apple		-	-	-	*Geotrichum candidum*	Pear	~2 to 6.5	-	-	-						
Cyberlindnera jadinii	Mix of salad	~6.5	-	-	-	*Rhodotorula mucilaginosa*	Apple		-	+	-	*Metschnikowia pulcherrima*	Pear	~2 to 6.5	-	-	-						
Candida tropicalis	Mix of salad	~6.5	+	+	-	*Rhodotorula mucilaginosa*	Apple		-	-	-	*Geotrichum candidum*	Pear	~2 to 6.5	-	-	-						
Suhomyces xylopsoci	Mix of salad		-	-	-	*Pichia kudriavzevii*	Apple		-	+	-	*Wickerhamomyces anomalus*	Pear	~2.5 to 6.5	+	-	-						
Candida solani	Mix of salad	~6.5 to 7	+	-	-	*Hanseniaspora uvarum*	Apple		-	-	-	*Candida stellimalicola*	Pear	~2.5 to 6.5	+	+	-						
Candida solani	Mix of salad	~6.5 to 7	+	+	-	*Pichia orientalis*	Apple		-	+	-	*Hanseniaspora uvarum*	Pear	~2 to 6.5	+	-	-						
Pichia norvegensis	Mix of salad	~6.5	-	-	-	*Pichia kudriavzevii*	Apple		-	-	-	*Geotrichum candidum*	Chard F.	~5.5 to 6.8	-	-	-						
Candida solani	Mix of salad	~6.5 to 7	-	-	-	*Hanseniaspora uvarum*	Apple		+	+	+	*Clavispora lusitaniae*	Chard F.	~5.5 to 6.8	-	-	-						
Pichia kudriavzevii	Green Bean	~5.5 to 6.5	+	-	-	*Hanseniaspora uvarum*	Apple		+	-	+	*Geotrichum candidum*	Chard F.	~5.5 to 6.8	-	-	+						

Labels: Yellow cells: Filamentous Fungi; Orange Cells with a "+": Positives; Cells with a "–": Negatives. Gray cells: No Data