INDUCTION OF ENZYME COCKTAILS BY LOW COST CARBON SOURCES FOR PRODUCTION OF MONOSACCHARIDE-RICH SYRUPS FROM PLANT MATERIALS

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The production of cellulases, hemicellulases, and starch-degrading enzymes by the thermophilic aerobic fungus *Talaromyces emersonii* under liquid state culture on various food wastes was investigated. A comprehensive enzyme screening was conducted, which resulted in the identification of spent tea leaves as a potential substrate for hydrolytic enzyme production. The potent, polysaccharide-degrading enzyme-rich cocktail produced when tea leaves were utilised as sole carbon source was analysed at a protein and mRNA level and shown to exhibit high level production of key cellulose and hemicellulose degrading enzymes. As presented in this paper, the crude enzyme preparation produced after 120 h growth of *Talaromyces emersonii* on used tea leaves is capable of hydrolysing other lignocellulosic materials into their component monosaccharides, generating high value sugar syrups with a host of industrial applications including conversion to fuels and chemicals.

Keywords: Lignocellulosic waste; Talaromyces emersonii; Saccharification; Cellulase; Hemicellulase

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INTRODUCTION

The quantity of municipal solid waste (MSW) generated in the European Union annually amounts to almost 200 million tonnes. Between 30 and 40% of this waste consists of biodegradable food and garden waste (Eurostat. 2001). Negative environmental effects such as damage to the atmosphere resulting from greenhouse gas emissions, potential pollution of water-courses, and obvious socio-economic problems do not make landfilling a feasible, long-term solution to this waste disposal crisis. However, many European countries continue to remain heavily reliant on a landfilling network with, for example, most recent figures revealing that approximately 62.5% of MSW generated in Ireland is disposed of in landfills, with 57% of organic waste that is disposed being landfilled (National Waste Report 2008).

The aim of this study is to divert waste away from landfills by developing a low-cost process for the utilisation of biodegradable food waste while simultaneously achieving the production of high, value-added products. With the European challenge for 2010 being the incorporation of 5.75% biofuel into conventional fuels, ethanol

production from biomass is attracting considerable attention.   The conversion of lignocellulosic biomass into fermentable sugars is an important part of this process, and for ethanol production to be economically feasible, utilisation of both the cellulose and hemicellulose fractions of the biomass is required (Ollson and Hahn-Hägerdal 1996). However, much research conducted to date has been focused on the use of commercially available cellulase enzymes, which are produced using expensive inducers and are not designed specifically for the hydrolysis of lignocellulose (Nikolov et al. 2000; van Wyk 1999). This paper represents the first detailed evaluation of an enzyme cocktail from a thermophilic source, produced economically using spent tea leaves as sole carbon source and capable of hydrolysing both the cellulose and hemicellulose fractions of a range of waste substrates.

Whilst a number of non-pathogenic, safe, microbial organisms are used as enzyme producers, *T. emersonii* was chosen because of its availability and a number of advantageous properties. Namely, the cost effectiveness of using this fungus as an enzyme factory derives from the fact that it produces the enzymes of interest in this study extracellularly, secreting them into the growth media, thus making substantial quantities of enzymes quite easily obtainable.   Furthermore, because *T. emersonii* is a thermophilic organism, enzymes can be produced with significantly higher temperature optima and stabilities. Conducting biotechnological processes at higher temperatures has considerable advantages, most notably an increased reaction rate and the reduced risk of pathogenic contamination (Haki and Rakshit 2003). In this study, unsupplemented waste materials including vegetable and fruit residues were used as a source of nutrients for the cultivation of *T. emersonii,* with the aim being to economically produce a wide variety of extracellular polysaccharide-degrading enzymes.   These enzymes can be employed to break down lignocellulosic materials to yield sugar syrups and lignin-rich residues. The sugar syrups can be subsequently anaerobically digested into methane, fermented to bio-ethanol and other chemical feedstocks by yeast and fungi, while the lignin-rich residues represent an important source of thermal energy and agricultural fertilizers.

EXPERIMENTAL

Materials

Unless otherwise stated, all general reagents and chemicals were purchased from Sigma Chemical Company, Dublin, Ireland.

Methods

Microorganism

Liquid cultures of *T. emersonii* (IMI 393751) were grown at 45°C, as described by Tuohy et al. (1990). Inocula for these studies were taken from laboratory stocks of the microorganism, that were routinely subcultured, at 45°C, on Sabouraud Dextrose Agar (SDA). Glucose 'starter' cultures were prepared by aseptically inoculating sterile medium containing 2.0% (w/v) glucose with 2-3, 1cm^2 pieces of mycelial mat taken from the outer edges of actively growing agar-plate cultures. Glucose starter cultures were grown as described for 48 h and used to inoculate larger volumes of medium containing 2% glucose (w/v), which were grown for a further 48 h. 10% (v/v) samples from these

cultures served as inocula for primary induction media. The inducing substrate (carbon source) was added at a concentration of 2% (w/v). Cultures were allowed to grow for 120 h and harvested after growth by centrifugation at 6,000 g for 20 min. The supernatant was filtered through sterile, fine-grade muslin to remove any particulate matter. Mycelia were frozen at minus 70°C, and the culture filtrate was used for enzyme assay and protein estimation.

Lignocellulosic substrates

Banana peel, carob powder, carrots, coffee, dilisk, oatmeal biscuits, potatoes, tea bags, tea leaves, tomatoes, and wheat flour were utilised as growth (inducing) substrates in liquid state fermentation.

Measurement of enzyme activities and protein concentration

For convenience, all enzyme assays, unless otherwise stated, were carried out at 50°C in 100 mM sodium acetate buffer pH 5.0, and expressed as IU/mL and IU/g solid substrate in starting growing media (Moloney et al. 1983; Tuohy et al. 1989, 1990). The hydrolysis of CM-cellulose (6% (w/v), 10 min incubation), Avicel (1% (w/v); 90 min; Merck, Germany), β-glucan (from barley; 1% (w/v); 10 min; Megazyme Int., Bray, Co. Wicklow, Ireland), xylan (from oats spelt; 1% (w/v); 5 min), wheat arabinoxylan (1% (w/v); 10 min; Megazyme Int.), pustulan (from *Umbilicaria papullosa*; 1% (w/v); 10 min; Calbiochem), pullulan (1% (w/v); 10 min), soluble starch (2% (w/v); 20 min), dextrin (2% (w/v); 20 min), and raw starch (from wheat; 2% (w/v); 10 min) was measured as reducing sugars released by the dinitrosalicylic acid (DNS) method (using appropriate standards), following incubation with neat or diluted enzyme (Miller 1959). Total cellulase activity was determined (Mandels et al. 1976; Wood and Bhat 1988) using 1 x 6 cm strips of Whatman No. 1 filter paper as the assay substrate. Exo-glycosidase activities (e.g. β-glucosidase) were measured by monitoring the increase A_{410} following 10 min incubation of the appropriate 1mM 4-nitrophenyl α- or β-glycoside with enzyme (the reaction was stopped by the addition of 1M sodium carbonate). Cellobiohydrolase I (CBH I) activity was measured according to the method of Tuohy et al. (2002). All enzyme activities were presented in international units (μmoles.mL^{-1}.min^{-1}, where 1IU=16.67 ηkatals). Enzyme yields in each fermentation were represented and calculated as IU per gram of inducing substrate (IU/g IS). Protein concentration was quantified by a sodium deoxycholate-TCA modification of the Lowry method using bovine serum albumin as the standard (Bensadoun and Weinstein 1976; Lowry et al. 1951).

Fungal RNA extraction and Northern analysis

RNA from *T. emersonii* mycelia cultivated on tea leaves at timed intervals was isolated as described by Chomczynski and Sacchi (1987), separated electrophoretically on 1.2% formaldehyde-agarose gels, and blotted onto a nytra supercharge nitrocellulose membrane. Hybridisation was carried out overnight at 60°C in 7% SDS, 50% deionised formamide, 5X SSC, 50 mM sodium phosphate, pH 7.0, N-laurylsarcosine and 2% blocking reagent. Digoxygenin (DIG) labelled probes amplified from *T. emersonii* chromosomal DNA using degenerate primers designed against existing polysaccharide degrading enzyme gene sequences present in the National Centre Biotechnology

Information database, at a concentration of 20 ng/mL of hybridisation buffer, were used to analyse gene expression. Detection was performed after incubation of the membrane with the anti-DIG-AP antibody conjugate using CDP-Star as the chemiluminescent substrate according to the manufacturer's instructions (Roche Molecular Biochemicals).

Enzymatic hydrolysis

Enzymatic hydrolysis of various waste and conventional cellulose substrates by the crude enzyme cocktail, produced by *T. emersonii* after 120 h growth on tea leaves was investigated. For maximum efficiency, the hydrolysis and consequent production of reducing sugars was optimised through variations of the following parameters: duration of hydrolysis, pH, temperature, enzyme-substrate ratio and substrate pre-treatment. Standard enzymatic hydrolysis was carried out at 50^oC in a Thermo Hybaid oven on a rotating platform shaker at 37 rpm. A 1 mL aliquot of the 120 h enzyme cocktail, as described in Table 1, was added to 0.5 g food waste in 10 mL 100 mM sodium acetate buffer pH 5. Aliquots were removed at timed intervals and enzymatic action was terminated by boiling each reaction mixture (and controls) for 10 min. The degree of hydrolysis achieved was determined by measuring the reducing sugars released according to the DNS method (Miller 1959).

To determine the optimum pH for saccharification, the reaction mixture was incubated at different pH values ranging from 2.3-7.0. Samples were removed at various time intervals, and the reducing sugars released were measured. The temperature for optimum activity of the enzyme cocktail was evaluated by incubating the reaction mixture at various temperatures between $50\text{-}80^oC$ over time and measuring the reducing sugars released at various time intervals. The optimum enzyme dosage was determined by quantifying the reducing sugars released from oats spelt xylan treated with varying concentrations of enzyme, from 9.7-77.9 IU endo-xylanase activity/g substrate over time. To evaluate the effect of pre-treatment, substrates were autoclaved at 105^oC, 15 p.s.i. for 30 min and homogenized for 10 min.

Analysis of hydrolysis products by High Performance Liquid Chromatography

Products of hydrolysis generated during the time course degradation of carob powder, wheat arabinoxylan and rye arabinoxylan by the 120 h tea leaf induced enzyme cocktail were identified by HPLC analysis. The concentrations of glucose, mannose, xylose, and galactose were determined using a polymer column (Aminex HPX-87P: Bio-Rad, Munchen, Germany) at 85^oC, and the compounds of interest were detected with a refractive index detector (Waters 2410; Milford, MA), according to the method Rudolf et al. (2004).

RESULTS AND DISCUSSION

Enzyme Production

Enzymes, as they are produced by living systems, are expensive to obtain even in minute levels and thus add significantly to production costs in industry. *T. emersonii*, originally isolated from a compost heap, is a natural degrader of biomass-rich materials and as presented in this paper can utilise everyday waste as inducing substrates for

hydrolytic enzyme production. The choice of inducing substrate is very important for economical enzyme production on a large scale. The use of conventional purified cellulose and xylan inducing substrates elevate the cost of production significantly. A good substrate should provide all of the necessary nutrients and inducing components required by the fungus. In this study, several vegetable and fruit residues were identified and evaluated as potential substrates for hydrolytic enzyme production due to their abundance and low cost.

T. emersonii was found to produce a range of enzyme activities when grown in an unsupplemented medium utilising these vegetable and fruit residues, as carbon sources. The enzyme activities obtained after growth of *T. emersonii* for 120 h on various substrates are summarised in Table 1. Comparison of the inducing substrates shows that no single substrate was best for all of the enzyme activities analysed and that relative susceptibilities of the substrates to degradation, and consequently enzyme production patterns, were very much dependent on the composition of the inducing carbon source used in the growth media.

Table 1. Enzyme Production (IU/g inducing substrate), by Liquid Cultures of *T. emersonii* Grown on Various Substrates for 120 h

	Carob powder	Coffee	Dilisk	Potatoes	Tea bags	Tea leaves
Endo-cellulose	206.0	73.7	102.3	94.7	141.3	372.9
Avicelase	2.9	0.0	15.0	0.5	0.0	13.7
Barley-β-glucanase	106.1	75.9	148.5	161.2	172.2	50.5
CBH I	14.5	2.4	8.3	1.5	7.0	24.5
β-Glucosidase	50.5	38.7	46.7	46.5	20.9	36.9
β-Xylosidase	2.8	2.2	4.9	1.1	2.8	6.8
Endoxylanase	876.7	101.4	1038.9	119.4	444.4	1065.4
α-Arabinofuranosidase	9.8	1.1	3.4	5.5	4.7	5.3
Arabinoxylanase	1523.5	192.5	1067.0	160.0	891.0	3019.5
β-1,6-Glucanase	37.7	45.6	55.6	27.1	48.9	33.2
α-1,6-Glucanase	6.9	0.7	7.3	11.9	9.6	0.0
β-Amylase	27.0	0.0	22.4	18.8	18.7	33.9
α-Amylase	9.1	0.0	3.6	0.0	10.0	1.2
Glucoamylase	0.2	1.1	0.3	0.5	1.2	0.5

This is clearly illustrated by the different activity profiles exhibited during cultivation of *T. emersonii* on closely related substrates. For example, when the similar substrates, tea leaves and tea bags, were utilised as inducing substrates, contrasting enzyme activity profiles were produced, as shown in Figs. 1 and 2. Based on comparison of these enzyme activity profiles, it is clear that tea bags are more difficult for the fungus to metabolise, and higher levels of both cellulose and hemicellulose degrading activities are observed when tea leaves are utilised as sole carbon source. In addition, these differences may directly reflect the processing of tea during the manufacturing of tea bags. Tea bags are made from cellulosic or synthetic fibres that have been bound together and pre-treated with a wet strength binding agent, generally a pectin or a starch derivative. Correspondingly, higher levels of β-1,3-1,4-glucanase, β-1,6-glucanase, and β-amylase activities were exhibited when *T. emersonii* was cultured on tea bags (Fig. 2). Rhamnogalacturonans are the major constituents of pectic substances (Whitaker 1984).

The presence of rhamnogalacturonan degrading activity (11.55 IU/g inducer) when tea bags were utilised as sole carbon source suggests the presence of pectic substances or their derivatives.

There are many examples to illustrate the substrate-dependent nature and inducibility of the enzyme production system of *T. emersonii*. When *T. emersonii* was cultivated on substrates with a high hemicellulose content, for example, elevated levels of xylan degrading enzyme activity were observed. Carob powder, which is used as a cocoa substitute, and contains around 45% carbohydrate (Avallone et al. 1997), a large proportion of which is hemicellulose (Roukas 1999), induced very high levels of arabinoxylanase and oats spelt xylanase activities, while cellulase activity produced was lower. The cell walls of the seaweed dilisk (*Palmaria palmata*), found in abundance around the west coast of Ireland, are mainly composed of mix-linked β-(1,3)/β-(1,4)-D-xylans (Deniaud et al. 2003), and this structural feature influenced the high levels of endo-xylanase activity exhibited, when this substrate was utilised as sole carbon source.

Arabinoxylans constitute a high proportion of cereals such as barley, rye, and wheat. Consequently, arabinoxylanases have a range of applications, which include improving the digestability of cereal based animal feeds and increasing dough quality in the baking industry (Beg et al. 2001). In addition, the hydrolysis of arabinoxylan prior to the utilisation of wheat hemicellulose in the ethanol fermentation industry is a crucial step for efficient substrate conversion (Sorensen et al. 2003). *T. emersonii* was seen to produce high levels of arabinoxylanase activity on many of the carbon sources analysed, with arabinoxylanase activities of 27.7, 9.9, and 54.9 IU/mL exhibited on carob powder, oatmeal biscuits, and spent tea leaves, respectively. In fact, the best overall substrate for the production of enzyme activity was tea leaves, which induced high levels of cellulose- and hemicellulose-degrading enzymes. Levels of production of these extracellular polysaccharide-degrading enzymes peaked at the cultivation time point of 120 h, as evident in Fig. 1. The crude filtrate at this timepoint was harvested and this enzyme cocktail used in hydrolysis studies.

The maximal cellulose and hemicellulase activities produced by *T. emersonii* presented in this study compare favourably with similar studies and in many cases exceed the maximal levels reported for other cellulose and hemicellulose-producing organisms (Olsson et al. 2003; Thygesen et al. 2003; Jørgensen et al. 2005). These enzymes produced from *T. emersonii* have a range of potential biotechnological applications, including the production of foodstuffs, natural food additives, high value biochemicals, in animal feeds, in diagnostics, in the biofuel industry, and in paper processing and recycling, to reduce the amounts of chlorine chemicals required. In order to obtain more information regarding the observed induction of cellulose and hemicellulose degrading enzymes by tea leaves, Northern analysis studies were carried out.
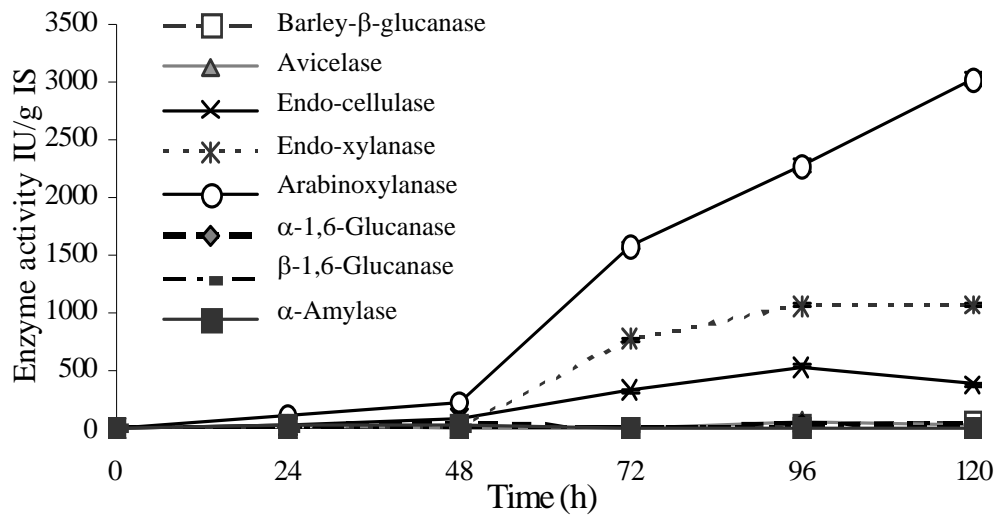
Figure 1 (A). Time-course production of endo-acting polysaccharide-degrading enzyme activity by *T. emersonii* cultivated on 2% tea leaves
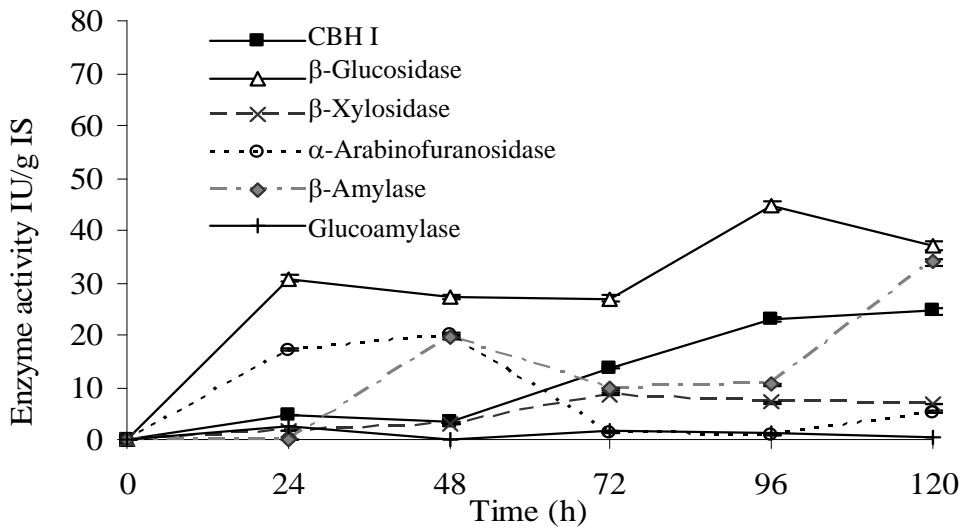

Figure 1 (B). Time-course production of exo-acting polysaccharide-degrading enzyme activity by *T. emersonii* cultivated on 2% tea leaves
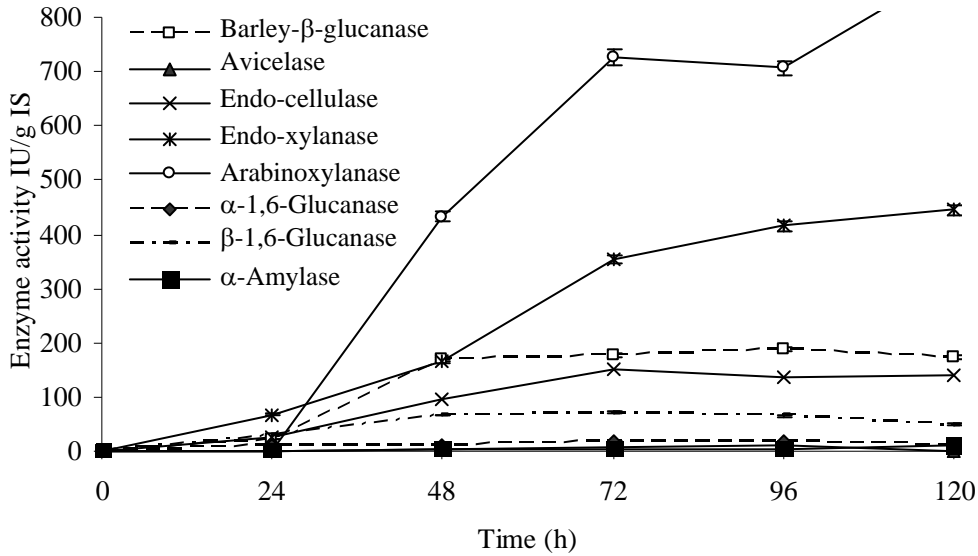
Figure 2 (A). Time-course production of endo-acting polysaccharide-degrading enzyme activity by *T. emersonii* cultivated on 2% tea bags
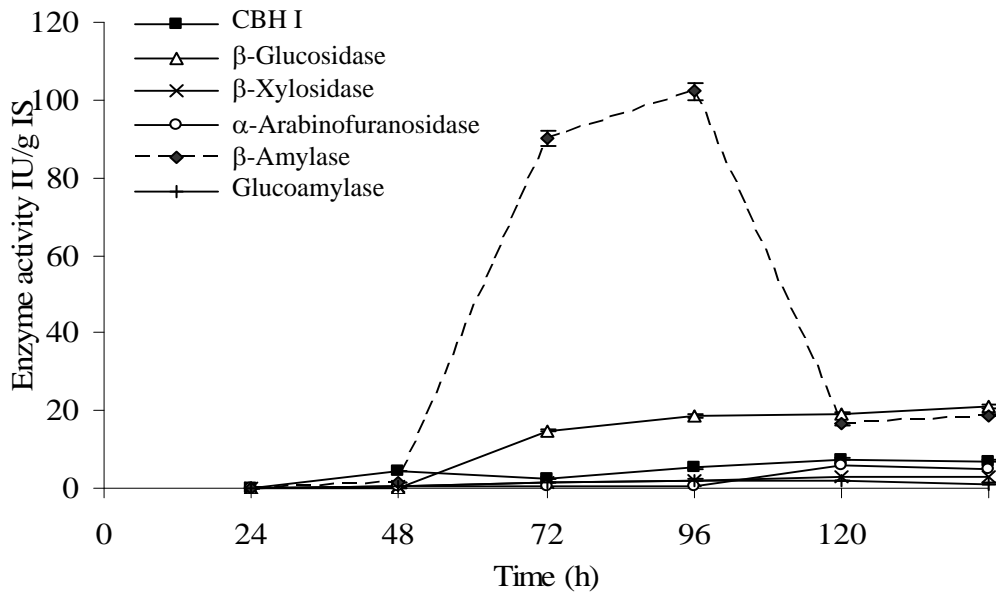

Figure 2 (B). Time-course production of exo-acting polysaccharide-degrading enzyme activity by *T. emersonii* cultivated on 2% tea bags

Northern Analysis

The extent to which the expression pattern of polysaccharide-degrading enzyme activity observed above is mediated at a molecular level was investigated. The expression of five key cellulase-encoding genes (*cbh1, cbh2, bg1, cel3a*, and *eg1*) and one key xylanase encoding-gene (β-*xyl 1*) during cultivation of *T. emersonii* on tea leaves were

therefore analysed by Northern analysis. As evident in Fig. 3, tea leaves were observed to induce expression of all of the genes analysed, with the expression of certain cellulases and the expression of β-xylosidase being especially marked.

β-xylosidase transcripts were evident after 48 h and increased to a maximum at 120 h growth, indicating that tea leaves are an excellent inducer of xylanase expression. This correlates with the enzyme production activity profile seen in Fig. 1, where little or no β-xylosidase activity was observed after 24 h growth, with activity increasing steadily from after 48 h.

A potent cellulolytic enzyme system was also induced by tea leaves with all of the components necessary for complete cellulose hydrolysis, including CBH I, CBH II, β-glucosidase I, Cel3a (β-glucosidase III), and high levels of endo-glucanase expression observed to be induced (Fig. 3). The genes *cbh1* and *cbh2* encoding the key cellulose degrading enzymes CBH I and CBH II showed similar patterns of expression; initial transcript levels were low with little or no expression evident at 24 h, then levels increased to a maximum at 72 h growth, decreasing again and returns to a maximum at 120 h. Tea leaves induced expression of both β-glucosidase genes, *bg1* and *cel3a*, which correlates with the potent cellulolytic system known to be produced and the profile of β-glucosidase activity seen in Fig. 1(B). Expression of *bg1* and *cel3a* was temporal, with transcript levels seen to increase and decrease. The endoglucanase gene, *eg1*, appears to be induced more quickly with low level expression observed at 24 h, increasing rapidly to maximal levels after 48 h growth with transcription seen to decrease again after 72 h. This expression pattern may indicate the physiological role of the endoglucanase enzyme, suggesting a role in the initial attack of amorphous regions in the cellulose molecule, releasing glucooligosaccharides (de Vries and Visser 2001).

There are several possible reasons for this observed pattern of expression, which corresponds to the peaking and troughing of enzyme production levels seen in Fig. 1 (B). As the growth media used for *T. emersonii* cultivation was unbuffered, pH values at different stages of growth characteristically fluctuated. There are mechanisms by which fungi control the expression of enzymes according to the pH range in which they are active. One example of this was observed in *Aspergillus nidulans*, where the zinc finger transcription factor PacC was identified as a major factor involved in pH-dependent regulation (Caddick *et al*. 1986; Tilburn *et al*. 1995), it is likely that a similar mechanism is employed by *T. emersonii*.

Another explanation for the observed expression pattern is that cellulases are known to be subject to carbon catabolite repression (Strauss *et al*. 1995; Ilmen *et al*. 1996; Takashima *et al*. 1996), whereby glucose and other easily metabolisable sugars act as potent repressors of cellulase genes. It is likely that the observed drop off in expression after 48 h is a result of the accumulation of glucose in the culture medium, and as this glucose is utilized by the fungus, at 120 h repression is observed.
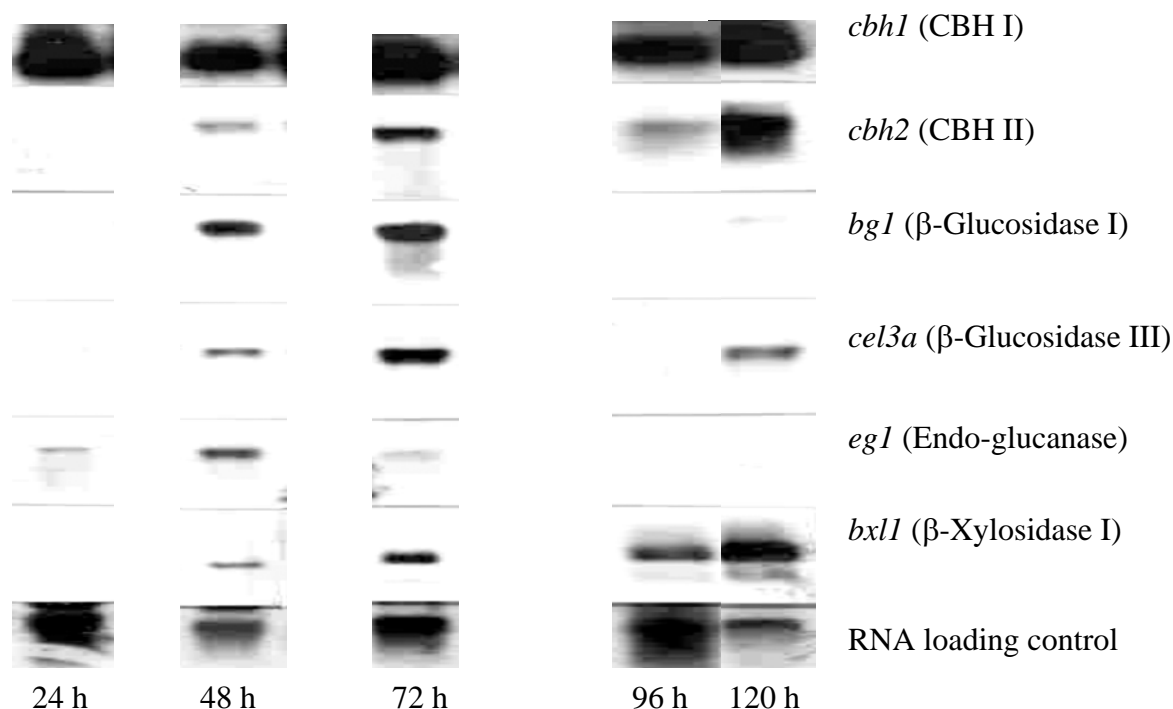
Figure 3. Northern analysis of CBH I expression, CBH II expression, β-Glucosidase I, β-Glucosidase III, Endo-glucanase expression and β-Xylosidase I expression following 1% (w/v) induction with tea leaves at 24 h, 48 h, 72 h, 96 h and 120 h. All inducers were at 10 mg/mL and the 18 s Ribosomal RNA loading control is shown

Hydrolysis Studies

Food wastes are rich in cellulose, hemicellulose, and other carbohydrates, and a large spectrum of polysaccharide-degrading enzymes are required for their complete bioconversion into fermentable sugars. In this paper, the hydrolytic capacity of the enzyme cocktail produced by *T. emersonii* after 120 h cultivation on tea leaves to release simple sugars from commercial and lignocellulosic waste substrates, which have not been chemically pre-treated, was assessed. It was observed that the enzyme cocktail released reducing sugars from all substrates analysed, which were increasingly degraded over time. The commercial substrates rye arabinoxylan, wheat arabinoxylan, birchwood xylan, beechwood xylan, and oats spelt xylan were the most susceptible to biodegradation, yielding the highest levels of reducing sugars. The maximum sugar yield achieved after 72 h hydrolysis, corresponded to 40.3 mg reducing sugars, 18.0 mg xylose, and 3.8 mg mannose/g rye arabinoxylan. Substrates containing high arabinose to xylose ratios, such as wheat arabinoxylan and rye arabinoxylan, were the most susceptible to degradation. This is due to the high levels of arabinoxylanase activity present in the enzyme cocktail. HPLC analysis revealed xylose as the main product of wheat arabinoxylan hydrolysis. Smaller amounts of glucose, mannose, and cellobiose were also identified.
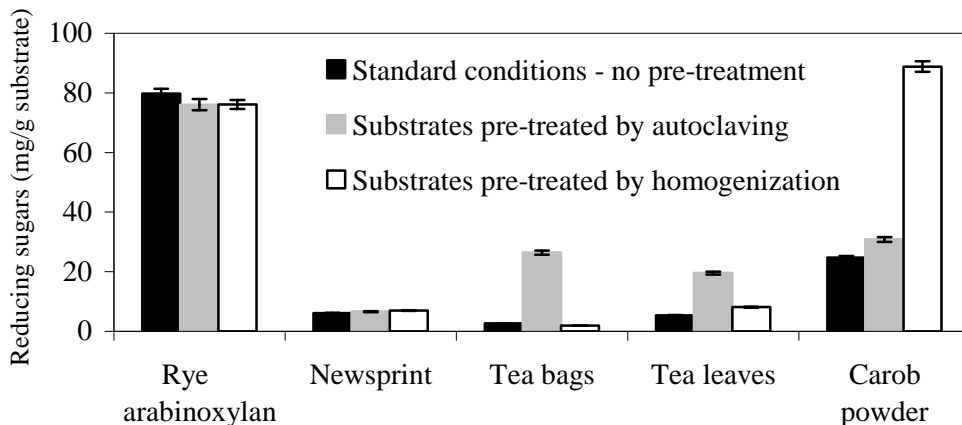
Figure 4. Hydrolysis of various substrates with the 120 h tea leaf induced enzyme cocktail from *T. emersonii*
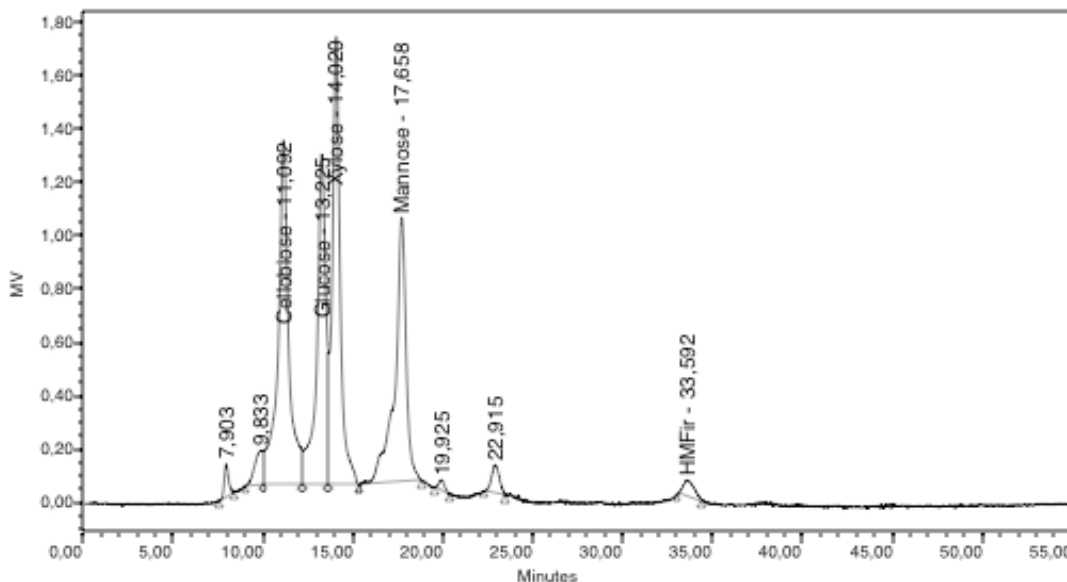

Figure 5. HPLC chromatogram of carob hydrolysis after 72 h treatment with the 120 h tea leaf induced enzyme cocktail

As evident in Fig. 4, homogenization and heat treatment (by autoclaving) enhanced waste substrate conversion to sugar, while having little or no effect on the soluble commercial substrates. The combination of heat treatment and homogenization of carob powder caused a 3.6-fold increase in sugar formation. The results of HPLC analyses of the hydrolysis products show maximum bioconversion after 72 h treatment of pre-treated carob powder with the 120 h tea leaf induced enzyme cocktail. Again xylose (29.6%) was the main hydrolysis product. Glucose (17.2%), mannose (23.22%), and cellobiose (22.7%) were also present, as shown in Fig. 5. To recover the maximum amount of sugars from both the cellulose and hemicellulose fractions of the waste substrates, different pre-treatment steps need to be investigated.

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Of the waste substrates analysed, carob powder was the most efficiently degraded, and after only 24 h hydrolysis the total sugar yield was 3.22 mg/mL reducing sugars. When a similar study was carried out to assess the saccharification of agro-waste materials by cellulases and hemicellulases produced by *Sporotrichium pruinosum* and *Arthrograohis sp.*, maximum hydrolysis was achieved utilising commercial xylan as substrate. After 24 h treatment with *Sporotrichium* enzymes the total sugar yield was 2.45 mg/mL. When the enzyme cocktail produced by *Arthrograohis* was employed, maximum sugar production was 2.34 mg/mL (Okeke and Obi 1995).

To maximise the efficiency of substrate degradation and sugar production it is necessary to optimise a number of parameters, including temperature, pH and enzyme-substrate ratio. The optimum temperature for reducing sugar release of the enzyme cocktail produced after 120 h growth on tea leaves was determined to be 60°C under standard assay conditions at pH 5.0. As evident in Fig. 6, the enzyme cocktail was observed to be very thermoactive, retaining 78% of its maximum enzyme activity at 80°C. This makes these enzymes relevant to high temperature industrial processes and their action at these elevated temperatures reduces the risk of contamination by mesophiles (Haki and Rakshit 2003). At temperatures above 80°C a rapid drop in enzyme activity was observed due to thermal inactivation of the enzyme cocktail, with almost total loss of activity observed above 90°C. The 120 h tea leaf induced enzyme cocktail was active in a broad pH range of 3.0-7.0, with a pH optimum of 5.5 at 50°C (Fig. 7). A rapid decline in enzyme stability was observed at pH 2.5 due to the acidic nature of this environment. The observed stability of this xylanase rich cocktail over such a broad pH range adds to its suitability for use in many industrial applications, e.g. the pulp and paper industry, where enzymes active at pH $\geq$ 7 are required. The enzyme concentration of 77.88 units xylanase activity/g substrate was observed to be the most efficient, releasing the maximum amount of reducing sugars from the substrate. Decreased production of reducing sugars was observed at enzyme concentrations other than the optimum.
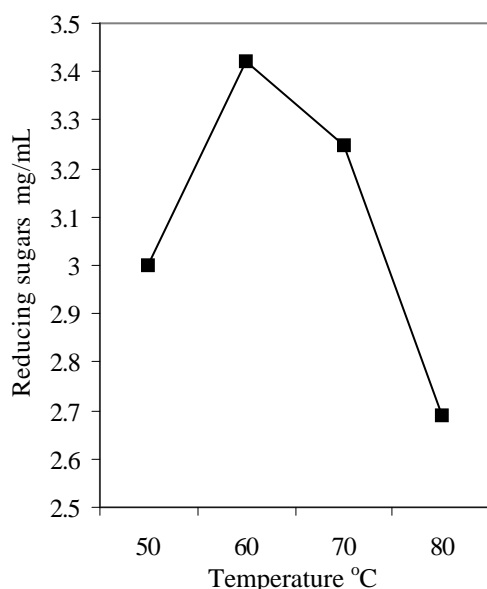

Figure 6. The temperature optima of the 120 h tea leaf induced enzyme cocktail from *T. emersonii*
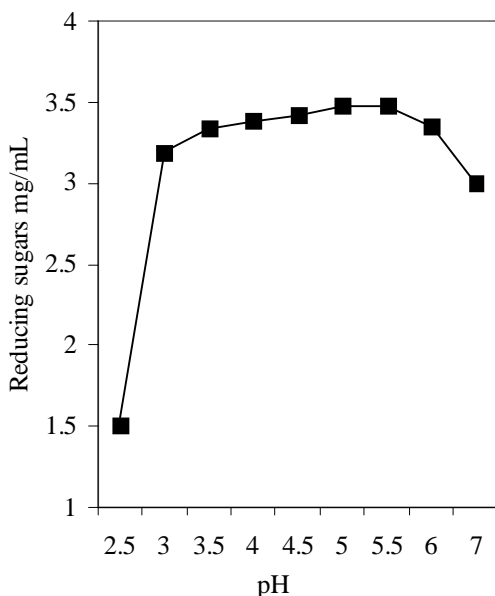
Figure 7. The pH optima of the 120 h tea leaf induced enzyme cocktail from *T. emersonii*

CONCLUSIONS

1. The results represented in this paper illustrate the high hydrolytic potential of a crude enzyme cocktail produced economically, utilising spent tea leaves as a carbon source, reducing the need for harsh pre-treatment methods.

2. Analysis of the enzyme cocktail composition on both a protein and a molecular level clearly shows that all of the components necessary for complete hydrolysis of cellulose and hemicellulose are present.

3. The ability of the hydrolytic cocktail produced to release reducing sugars from conventional hemicellulose and waste substrates that have not been chemically pre-treated is demonstrated and the hydrolysis products analysed, showing complete breakdown of the substrates. The ability of the enzyme preparation to release D-xylose units from rye and wheat arabinoxylans is of significant interest, leading to many potential industrial applications for this hydrolytic enzyme cocktail.

4. The enzyme cocktail was observed to work optimally at a temperature of $60^{\circ}C$ and at a pH of 5.5. The high temperature stability of the cocktail adds to its potential use in industry and especially in waste treatment, as it works at temperatures fatal to many pathogens and contaminants.

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