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Talaromyces emersonii Thermostable Enzyme Systems and Their Applications in Wheat Baking Systems

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In this study, novel extracellular thermozymes were produced by the thermophilic fungus *Talaromyces emersonii* (IMI 392299) on low-cost carbon inducers. This paper reports the cocktail characterization, substrate hydrolysis studies, and their application in baking. Relevant enzymes were optimally active at pH 4.5−5.0 and 70 °C. Model studies confirmed production of significant levels of yeast monosaccharide sugars during cereal flour hydrolysis. The "thermozyme cocktails" are thermostable secreted *T. emersonii* enzyme blends. In baking trials, these thermozyme cocktails showed significant improvements in bread quality with respect to hardness, staling, and loaf volume ($p < 0.5$). Thermozyme cocktail B- treated loaf volume was 23.2% greater than the control and 49.5% softer. Staling analysis showed that bread treated with cocktail B was 41.7% softer than the control. This is the first report of *T. emersonii* thermozymes positively influencing bread quality.

KEYWORDS: Characterization; *Talaromyces emersonii*; thermophilic fungus; bread quality

INTRODUCTION

Plant cell wall modifying enzymes have widespread use in various baking applications. Shelf life, volume, textural characteristics, crust color, flavor, and nutritional quality of bread and other cereal products can be influenced by enzymes (*1*). Enzymes that function at higher temperatures have received increased attention in industry because of their innate stability (*2*). Amylolytic enzymes, α-amylase (EC 3.2.1.1), β-amylase (EC 3.2.1.2), α-glucosidase (EC 3.2.1.20), glucoamylase (EC 3.2.1.3), and pullulanase (EC 3.2.1.41), are the most widely used thermozymes in the starch and baking industries. Industrially, low endogenous α-amylase activity can result in a low bread volume when using some cereals. Consequently, α-amylase supplementation is required, usually with fungal enzymes from *Aspergillus* and *Rhizopus* species. These amylases positively influence dough viscosity, handling properties, bread volume, crumb structure, softness, and shelf life (*3*). Cellulolytic and hemicellulolytic hydrolases including cellobiohydrolase (EC 3.2.1.91), endo-β-1,4-glucanase (EC 3.2.1.4), β-glucosidase (EC 3.2.1.21), endo-β-1,3(4)-glucanases (EC 3.2.1.73 and EC 3.2.1.6), endo-1,4-β-D-xylanase (EC 3.2.1.8), α-L-arabinofuranosidase (EC 3.2.1.55), acetyl xylan esterases (EC 3.1.1.72), and feruloyl esterases (EC 3.1.1.73) act on nonstarch polysaccharides (NSP). They can improve dough handling properties and increase the baked product volume. NSP such as arabinoxylans and β-glucans play an important role in water binding and have been shown to markedly influence dough extensibility as well as bread crumb structure, even though the hemicellulose component of white wheat flour is only approximately 2.5−3% (*4*). It is widely accepted that endoxylanase positively affects bread oven-spring, volume, and textural properties and retards staling (*5*). Thermophilic fungi have attracted growing attention because of their potential as sources of thermostable xylanases (*6*−*10*).

Exogenous protein hydrolases (proteases) capable of gluten protein hydrolysis dramatically effect bread quality, having a deleterious effect at higher dosages. Lower proteolytic activity reduces dough viscosity and mixing time, improving extensibility and bread crumb by cleaving gluten peptide bonds and releasing water (*11*).

Talaromyces emersonii is a thermophilic filamentous fungus and natural saprophyte that inhabits soil and compost heaps. These environments offer humid and aerobic conditions, which, combined with elevated temperatures, provide an ideal environment for the growth of this eukaryote (*12*). *T. emersonii* produces highly specific thermozyme cocktails with efficient catalysis and excellent long-term storage properties (*7*, *13*). These thermozymes refer to thermostable fungal enzymes and work at temperatures 10−20 °C higher than commercially available *Trichoderma* sp. enzymes (*14*). Like these mesophilic fungi, *T. emersonii* also has GRAS (generally regarded as safe) status, making it safe for use in food processing. Adding to the cost effectiveness of using *T. emersonii* as an enzyme factory is its ability to produce the enzymes of interest extracellularly, in higher amounts than bacteria or yeast (*10*). Secretion into the surrounding growth

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medium makes downstream processing of enzymes relatively uncomplicated. Enzyme systems from this fungus have been previously described and characterized, and patents have been developed for key applications (*15*). Hydrolysis studies using thermozymes allow higher reaction temperatures, which decrease reaction time, reduce microbial contamination, and lower hydrolysate viscosity, all of which are desirable characteristics in many biotechnological and industrial applications (*16*).

The aim of this research is to compare the baking performances of five *T. emersonii* thermozyme cocktails produced on low-cost carbon sources. These cocktails were characterized, and enzyme-treated loaves were compared, with end point measurements of bread quality. Reaction products formed during cereal substrate hydrolysis by the *T. emersonii* thermozyme cocktails were also evaluated, with the findings complementing the baking results.

MATERIALS AND METHODS

Materials. All materials were obtained from Sigma Aldrich (Dublin, Ireland) unless otherwise stated. Commercial wheat flour (Baker's Flour, Odlums, Dublin, Ireland) and instant dried yeast (Puracor, Leuven Belgium) were incorporated in the recipe used for baking trials. The commercial wheat flour used is common "baker's flour"; its nutritional and analytical specifications per 100 g of flour are protein, 12.1 g; carbohydrate, 75.3 g; fat, 1.4 g (saturated fat, 0.2 g); sodium, 3 mg; and fiber, 3.1 g. It has a moisture content of 13.5−14.5%.

Rye, bran, red sorghum flour, and wholemeal flours were obtained locally (Healthwise Ltd. and Evergreen Health Food Store, Galway, Ireland). Tea leaves (dried) and "retail flour" were sourced locally (Lyons Original Blend, Unilever Ireland; and Odlums strong flour milled from hard wheats, both purchased from Dunnes Stores Ltd., Galway, Ireland).

Fungal Culture Methods. The food grade GRAS thermophilic fungus *T. emersonii* (IMI 392299) was routinely subcultured on Sabouraud dextrose agar (SDA) at 45 °C.

Liquid cultures of the fungus were grown at 45 °C, 220 rpm, and pH 4.5, in mineral salts medium, as previously described (*13*). Glucose "starter" cultures were prepared by aseptically inoculating sterile mineral salts medium containing 2% glucose (w/v) as main carbon source, with small sections of mycelial mat taken from the newest filaments on an actively growing SDA plate. Glucose starter cultures (typically consisting of a final volume of 100 mL of nutrient medium in a 250 mL Erlenmeyer flask) were grown for 36 h under the specified conditions, and then 10% (v/v) inoculum was transferred to a second larger volume of medium containing 2% w/v glucose, which was incubated for a further 24 h. Inocula of 10% (v/v) from these cultures were then used to seed 2 L Erlenmeyer flasks containing a total of 400 mL of nutrient medium supplemented at 1% (w/v) with various inducers: thermozyme cocktail A, tea leaves; thermozyme cocktail B, bran; thermozyme cocktail C, Odlums retail wheat flour; thermozyme cocktail D, sorghum; and thermozyme cocktail E, glucose. The cultures were grown as described before for 120 h, after which time the culture was filtered through several layers of sterile muslin and centrifuged at 6000*g* for 30 min at 4 °C. These crude extracts were stored in aliquots at −20 °C until required for further use.

Enzyme Activity Assays. Enzyme assays were conducted on culture supernatants at 50 °C in 100 mM ammonium acetate buffer, pH 4.5, unless otherwise specified. Each reaction and its control were run in triplicate. The hydrolysis of soluble starch (1% w/v, 30 min), raw starch (1% w/v, 30 min), wheat straw xylan (WSX; 1% w/v, 15 min; Megazyme International Ireland Ltd., Bray, Co. Wicklow), oat spelts xylan (OSX; 1% w/v, 15 min), birch wood xylan (BWX; 1% w/v, 15 min), and β-glucan (from barley; 1% w/v, 15 min; Megazyme Intl. Ireland Ltd.) was determined as reducing sugars released by the dinitrosalicyclic acid (DNS) method (*17*) using appropriate standards. Absorbance values were measured at 550 nm using a Wallac VICTOR Multilabel Counter UV−vis spectrophotometer (Perkin-Elmer). Enzyme activity was determined by reference to a glucose standard curve (0−1 mg/mL) and expressed in international units per milligram of enzyme (i.e., μmol of product formed/mL of enzyme/min of reaction time).

A microassay was used for the detection and quantification of peptidase activity, namely, tripeptidyl peptidase and sedolisin. Activity was measured by incubating a 10 μL aliquot of suitably diluted enzyme with 90 μL of 1 mM Ala-Ala-Ala-*p*-nitroanilide (tripeptidyl peptidase activity) or, L-aspartic acid−*p*-nitroanilide (sedolisin activity), for a reaction period of 15 min, at 50 °C using a 30 mM Tris-HCl buffer, pH 7.8 (assay buffer and diluent for enzyme samples). A fixed volume (100 μL) of 1 M Na_2CO_3 was added to stop the reaction. The amount of *p*-nitroanilide released was determined by measuring the increase in absorbance at 405 nm, using a Wallac VICTOR Multilabel Counter UV−vis spectrophotometer. A standard curve of *p*-nitroanilide was prepared and used to calculate enzyme activity in international units (IU) per milligram (*18*).

Exoacting glycosyl hydrolase activities, including acetyl esterase (*p*-nitrophenyl acetate), β-glucosidase (*p*-nitrophenyl-β-D-glucopyranoside), α-glucosidase (*p*-nitrophenyl α-D-glucopyranoside), β-xylosidase (*p*-nitrophenyl β-D-xylopyranoside), and α-L-arabinofuranosidase (*p*-nitrophenyl-α-L-arabinofuranoside), were measured using the appropriate 1 mM 4-nitrophenyl α- or β-glycoside as substrate (*9*). The increase in absorbance at 405 nm on release of the nitrophenolate anion, following incubation of the enzyme with the appropriate substrate for 15−30 min at 50 °C (the stopping reagent was 1 M Na_2CO_3), was monitored spectrophotometrically.

The protein content of crude extracellular extracts was determined with the Bradford reagent using bovine serum albumin (BSA, fraction V) as a standard (*19*).

Model Substrate Hydrolysis Studies and Total Carbohydrate Content. Known quantities (1 g) of each of the following five polymeric substrates, rye flour, wholemeal flour, wheat flour, rye arabinoxylan (Megazyme Intl. Ireland Ltd.), and birch wood xylan, were incubated at 50 °C and pH 4.5 for 24 h in a shaking incubator, with 50 μL/mL of crude enzyme cocktail in a total reaction volume of 10 mL. Samples were removed at timed intervals, and the resulting hydrolysate sugar contents were measured using the DNS method. Reaction products formed were analyzed as described below. Suitable controls were included in all cases, and hydrolysis experiments were carried out in triplicate.

The total carbohydrate content of polymeric substrate used in the model hydrolysis studies was evaluated by using the phenol−sulfuric acid method of Dubois et al. after total acid hydrolysis of the starting material (*20*).

Separation and Analysis of Sugar Reaction Products. Standard sugars (glucose, xylose, arabinose, cellobiose, xylobiose, and xylotriose) were used to identify sugars released from the respective cereal and commercial xylan substrates during hydrolysis experiments with the thermozyme cocktails. Hydrolysis was calculated according to the formula (grams of hydrolysis products) × 100/grams of polysaccharides in the substrate. Enzyme hydrolysates were analyzed by high-performance anion exchange chromatography (HPAEC) on an ICS-3000 Ion Chromatography System for Dionex Corp. (Sunnyvale CA). Products of hydrolysis were fractionated on a CarboPac PA-100 column using a decreasing 200−18 mM NaOH gradient at a flow rate of 1 mL/min at room temperature. An ED40 electrochemical detector, in the integrated pulsed amperometry mode, allowed detection of eluting product peaks using the carbohydrate (standard quad) waveform. Saccharide concentrations in the identified peaks were determined using Chromeleon version 6.70 software with reference to individual saccharide standards treated in an identical manner.

Baking Recipe. The recipe was prepared as a baker's percentage (100% flour basis), that is, 100% baker's flour, 60% water (this volume included *T. emersonii* thermozyme cocktail when applicable), 2% salt, and 1.5% yeast. The *T. emersonii* thermozyme cocktails were included on the basis of the total protein content of the enzyme mix per 100 g of flour (1 mg of protein/100 g of flour). Cocktails A, B, C, D, and E contained 0.26, 0.35, 0.10, 0.21, and 0.10 mg/mL of protein, respectively. Bread made without exogenous enzymes and bread treated with cocktail E (glucose substrate) served as controls.

Bread-Baking Procedure. The ingredients, with a total weight of 1635 g (1000 g of flour), were mixed in a Stephan mixer (Stephan Söhne, Hameln, Germany) at ambient temperature for 30 s at level 1 and for 60 s at level 2. After mixing, the dough was kneaded and rested in a proofer (Koma, Koeltechnische, The Netherlands) at 30 °C and 85% relative humidity for 15 min before it was divided into 450 g portions, molded

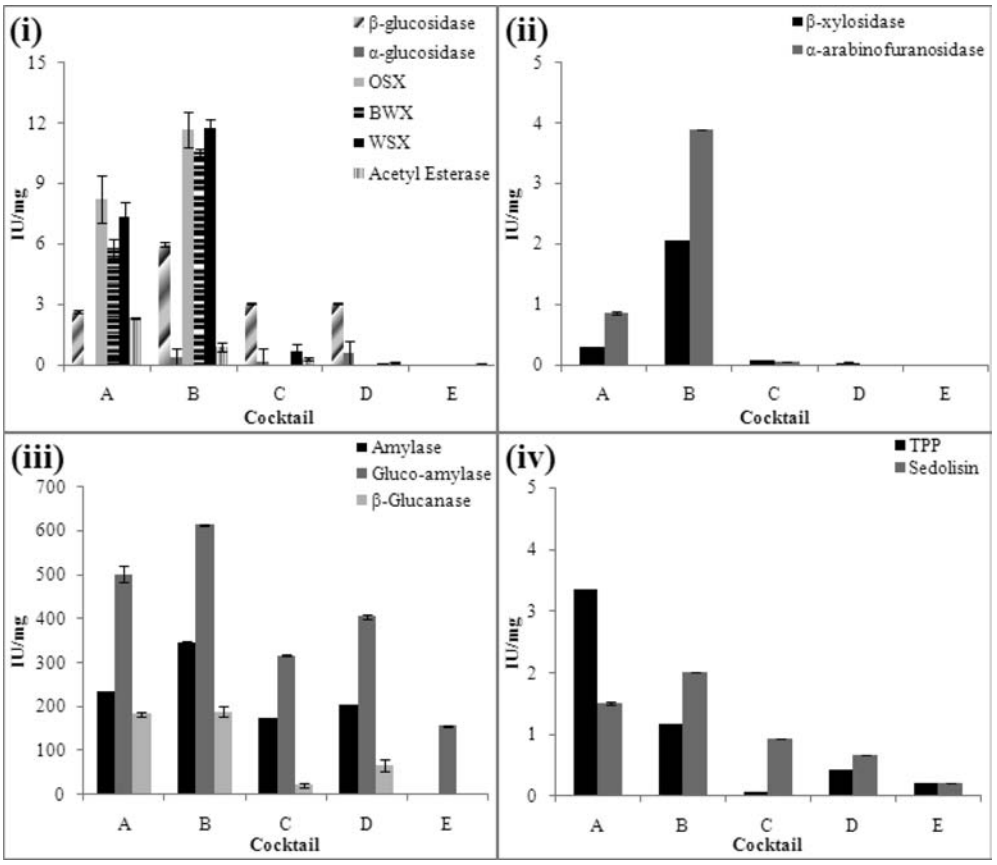

Figure 1. *T. emersonii* thermozyme cocktails A−E hydrolytic enzyme activities: (i) endoacting enzymes; (ii) exoacting enzymes; (iii) amylolytic enzymes; (iv) proteolytic activities. OSX, oat spelt xylan; BWX, birch wood xylan; WSX, wheat straw xylan; TPP, tripeptidyl peptidase.

using a bread molder (Holtkamp, Holland), and placed in nonstick baking tins (180 mm ×120 mm × 60 mm, Sasa U.K., Middex, U.K.). The dough was then proofed for 70 min under the same conditions and baked immediately in a deck oven (MIWE condo oven, MIWE Michael Wenz GmbH, Arnstein, Germany) at 230 °C top and bottom heat for 30 min. The oven was presteamed (300 mL) before loading, and on loading the bread, the oven was steamed by injecting 700 mL of water. For staling experiments, loaves were subjected to modified atmosphere packaging 120 min after baking, when adequately cooled, with 60% N_2/40% CO_2 and were stored at room temperature for 2 and 5 days.

Rheofermentometer Tests. The Chopin rheofermentometer (Villeneuve-la-Garenne, France) was used to measure dough volume and gas production and loss, thereby allowing the calculation of the dough gas retention capabilities. Dough was prepared as for the baking studies. Gas production and dough height were measured using 300 g quantities of dough. Displacement of a 1500 g weight by the rising dough was measured over a 3 h period and was directly related to the volume of gas produced.

Bread Analyses. A Chromameter (Minolta CR-300, Osaka, Japan) was used to measure crust color (CIE *Lab** values). CIE *L** values (brightness), *a** values (red-green), and *b** values (yellow-blue) were measured. Five readings on the top crust of three loaves in three independent batches (i.e., average of 45 readings for each result reported) were recorded. Texture analysis was performed 60 min, 2 days, and 5 days after baking using texture profile analysis (TPA) tests with a TA-XT2*i* texture analyzer (Stable Micro Systems, Surrey, U.K.) equipped with a 25 kg load cell and a 35 mm aluminum cylindrical probe. The settings used were a test speed of 2.0 mm/s with a trigger force of 20 g to compress the middle of the breadcrumb to 40% of its original height. Results were analyzed using Texture Expert 1.17 software (Stable Micro Systems). Values for hardness, springiness, cohesiveness, and chewiness were calculated using the software.

Statistical Analysis of Data. Analysis of variance (ANOVA) and correlation analysis were applied to the data obtained for baking, texture analysis, and *Lab** color measurements using the statistical analysis software package PASW Statistics 17 (formerly SPSS, Chicago, IL). Tukey's HSD (honestly significant differences) test analysis was used with a confidence interval of 95%. Data mean values and standard deviations were calculated from at least three replicates in all cases.

RESULTS AND DISCUSSION

Enzyme Production. *T. emersonii* thermozyme cocktails were induced on several different carbon source substrates for 120 h. The thermozyme cocktail inducers used, tea leaves (cocktail A), wheat bran (cocktail B), wheat flour (cocktail C), sorghum (cocktail D), and glucose (cocktail E), were chosen on the basis of their chemical constituents and previous performance as enzyme inducers. The five enzyme mixtures (thermozyme cocktails) were subjected to biochemical and functional characterization to gain insight into their potential as novel baking enzymes. The results shown in **Figure 1** reveal the complex array of hydrolytic activities present in the thermozyme cocktails.

Amylolytic, xylanolytic, and proteolytic activities were detected in relatively high levels in the tea leaf induced enzyme cocktail, reflecting the typical carbohydrate (18−40% dry weight) and protein (18−30%) contents of the substrate. It is a cheap carbon source, and good enzyme induction has previously been reported when *T. emersonii* was cultured in spent tea leaves (*21*). Wheat bran is a well-documented inexpensive inducer of a broad range of hydrolases for *T. emersonii* and other filamentous fungi (*22*, *23*) and has been used as a fungal substrate for decades. The bran used in this study has been analyzed by the suppliers and consists of 37.5% fiber, 15.4% carbohydrate (14.1% starch), 13.3% protein, and 4.5% fat. Wheat bran thermozyme cocktails showed the best overall induction of the amylolytic, hemicellulolytic, and proteolytic activities tested, emulating the presence of complex xylan- and amylase-based

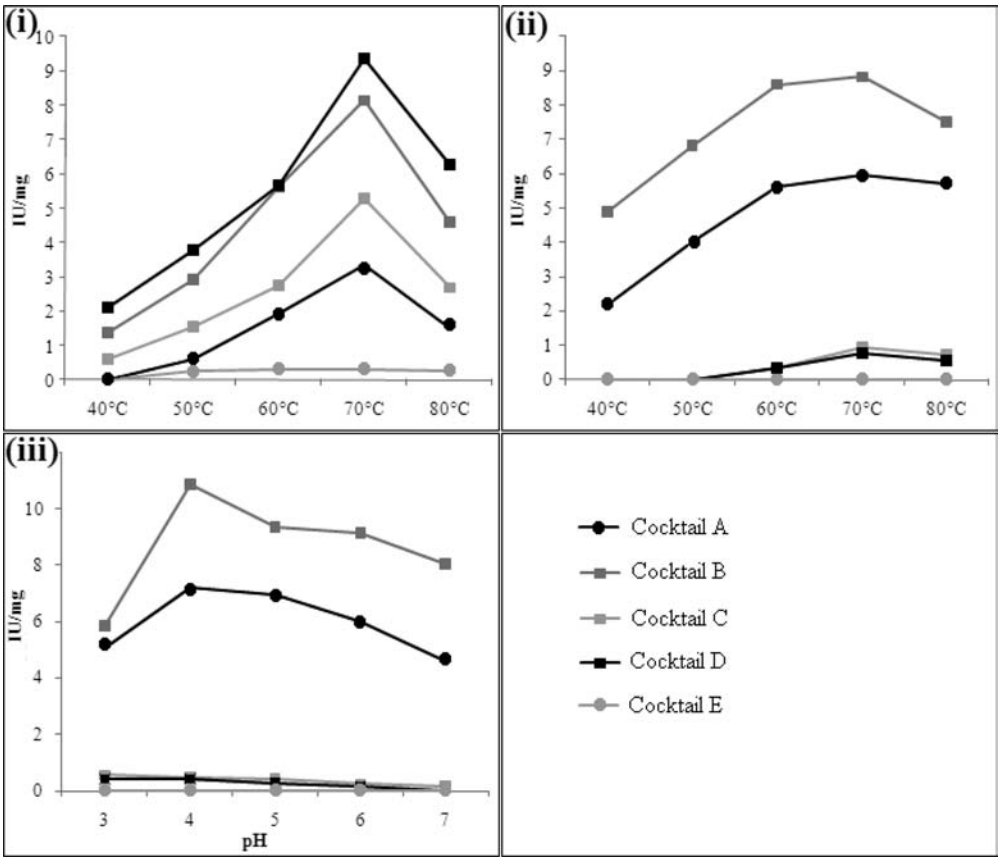

Figure 2. Temperature (i, ii) and pH (iii) optima (at 65 °C) of *T. emersonii* thermozyme cocktails A−E with respect to two substrates relevant to the baking industry: (i, iii) wheat arabinoxylan (xylanase activity); (ii) soluble starch (amylase activity). The average standard deviation value was ±0.6 IU/mL.

carbohydrates. The xylanase and amylase activities observed here correlate well with the antistaling properties of thermozyme cocktail B in baking trials.

Wheat flour was an interesting substrate to use for thermozyme cocktail induction given that the ultimate application of the fungal enzymes was in baking studies. The substrate was used to induce xylanolytic, amylolytic, and proteolytic enzymes relevant to baking. It has been characterized by the manufacturers and is primarily composed of approximately 75% carbohydrate, 12% protein, 1.4% fat, and 3% fiber as such; the dominant enzyme expressed was pertinent to starch degradation. Sorghum was ground before being used as a thermozyme cocktail inducer. It is composed of 79.5% carbohydrates, 10.6% protein (53% of which is prolamin, 34% glutelins, 5.6% albumins, and 7.1% globulins), 11.6% fiber, and 2.2% ash. It has previously been used in our laboratory as an inducer of *T. emersonii* thermozyme cocktails (unpublished data). The high carbohydrate levels stimulated production of amylolytic enzymes primarily. The various proportions of starch, xylan, and protein hydrolyzing enzymes present in thermozyme cocktails A−D are illustrated in the enzyme assay data and the baking results reported. Glucose was used as an inducer for "base-line" enzyme production. It also acted as a control for hydrolysis studies and in baking studies shows the fungal media residue effect on bread.

Previous studies investigating hemicellulase production by *Thermomyces lanuginosus* and *Chaetomium* sp. have shown that *T. emersonii* produces higher exoxylanase activity but lower endoxylanase activity (*24*−*26*). *Aureobasidium pullulan* (N13d), a mesophilic yeast, produces 58.5 IU/mg amylase activity (*27*), > 5-fold less than *T. emersonii*, under optimized conditions. The thermophilic fungus *Th. lanuginosus* produced up to 125 IU/mg amylase activity when optimally induced (*2*). This is 2.8-fold less than *T. emersonii* amylase production in this study. Induction of proteolytic activity in *T. emersonii* is similar to previously reviewed thermophilic fungal general peptidase induction including *Achaetomium macrosporum*, *Chaetomium thermophile*, *Sporotrichum thermophile*, *Humicola lanuginosa*, and *Torula thermophila* (*6*). The five thermozyme cocktails display different thermozyme cocktail hydrolytic activities, as discussed above. The further characterization of *T. emersonii* thermozyme cocktails was undertaken with specific emphasis on their potential application in the baking industry.

Cocktail Characterization. The five thermozyme cocktails displayed different hydrolytic enzyme profiles, as shown in **Figure 1** and discussed above. Further characterization of *T. emersonii* thermozyme cocktails was conducted to determine the performance of the thermozymes under reaction conditions relevant to baking applications. The efficiency of enzyme activity is dependent on the optimal temperature, pH, and enzyme dosage. Because xylan and starch are very important components of baking flour, these were the substrates chosen to evaluate temperature and pH optima; results are shown in **Figure 2**. Optimal hydrolytic activity using WAX was found in thermozyme cocktail D with best activity in all samples at 70 °C. Glucoamylase temperature optima occurred between 60 and 70 °C, with thermozyme cocktail B performing best. The optimum pH for enzyme activity was between 4.5 and 5.0, as expected (*12*). A dough proofing temperature of 30 °C and optimal enzyme working temperaturesof 65−70 °C were used in subsequent experiments. Optimal enzyme dosage was 100 μg/mL. Enzyme dosages for hydrolysis studies and in baking trials was based on these data.

Model Substrate Hydrolysis Studies. The efficacy of cereal biopolymer hydrolysis or modification depends on the enzymes

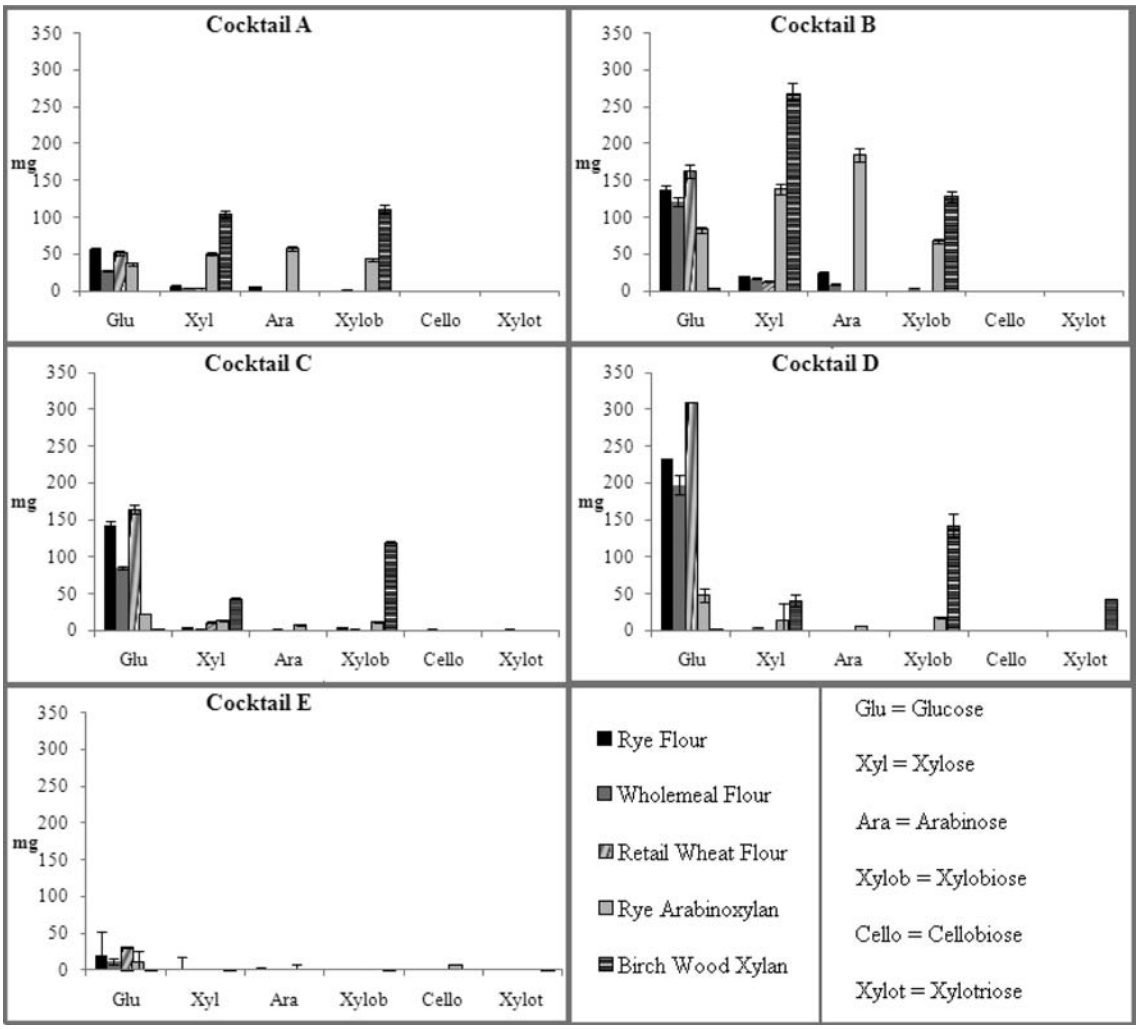

Figure 3. Monosaccharide, disaccharide, and trisaccharide yields (mg) from hydrolysis of five substrates with *T. emersonii* thermozyme cocktails A−E. Hydrolysis experiments consisted of 1 g each of rye flour, wholemeal flour, retail wheat flour, rye arabinoxylan, and birch wood xylan, which were incubated at 50 °C, pH 4.5, for 24 h in a 210 rpm incubator with 50 μL/mL crude thermozyme cocktail in a total reaction volume of 10 mL.

present in a cocktail and the substrate being hydrolyzed, as well as the reaction conditions used, such as temperature, pH, and enzyme dosage. The potential of the *T. emersonii* thermozyme cocktails was assessed by measuring the hydrolysis of flour substrates and xylans, to ascertain the potential of the cocktails for application in the modification of cereal constituents in baking. Optimal hydrolysis conditions for baking were employed, as using preferred conditions for the *T. emersonii* enzymes would result in superfluous hydrolysis of carbohydrates.

After incubation, thermozyme cocktail D hydrolysis of rye flour, wholemeal flour, and wheat flour were 31.4, 31.5, and 40.7%, respectively. Under these conditions, the enzymatic hydrolysis reported in this research is significant when compared to other studies. Koutinas et al. (*28*) used an *Aspergillus awamori* cocktail submerged fermentation on gelatinized wheat flour starch, achieving 57.5% hydrolysis in a saccharification experiment. Without pregelatinization, and after 78 h of hydrolysis, *A. awamori* enzymes led to 60% conversion from starch to glucose (*29*). Thus, *T. emersonii* thermozyme cocktails B and D with up to 47.6 and 40.7% carbohydrate hydrolysis, respectively, produce comparable end-point results for the substrates investigated in this study and are possible candidates for bioconversion and baking.

Incubation of non-pre-treated rye arabinoxylan and birch wood xylan with thermozyme cocktail B resulted in 47.6 and 40.1% hydrolysis, respectively. **Figure 3** summarizes the results obtained following HPAEC analysis of the hydrolysates. Reaction products were analyzed after treatment of 1 g of the different flours and xylans with each thermozyme cocktail for 24 h at 50 °C. Hydrolysates had high levels of the monomeric sugars glucose, xylose, and arabinose and smaller levels of galactose, xylobiose, and other unidentified disaccharides, trisaccharides, and sugar alcohols.

Rheofermentometer Results. The rheofermentometer was used to measure gas expansion and retention capabilities of dough during fermentation. **Table 1** shows the effects of the enzyme cocktails on dough properties. The maximum height of dough was observed with addition of the *T. emersonii* enzyme mixes, with cocktails D and E performing best. Doughs treated with cocktail D or E produced 2761 and 2722 mL of gas, which are 67 and 64% more than the control dough, respectively. This observation is a combination of yeast fermenting the substrates present and the gas retention capacity of the dough. In wheat dough, it has been previously shown that the sugars present are not a limiting factor for yeast activity (*30*). Despite the fact that thermozyme cocktails D and E had the highest gas production levels, the baked bread results show that cocktail B has the highest volume. The hydrolysis of soluble pentosans, by endoxylanases, is known to increase gas retention (*31*). Hydrolyzing enzymes in the cocktails can degrade network-forming compounds in the dough

matrix, which we see in dough treated with cocktail B, thus altering the gas retention capacity of the viscoelastic dough matrix. It is interesting to note that the high levels of simple hexose sugars and low levels of pentose sugars produced by cocktail D, and to a lesser extent by cocktail C, support good gas production during yeast fermentation. Cocktail B, on the other hand, yields high levels of pentose and hexose sugars. As baker's yeast cannot utilize pentose sugars, gas production may not be as good with this cocktail. Also, because cocktails D and E have considerable amylase and protease (and β-glucanase; cocktail D) levels, these enzymes may allow the dough network to expand to produce and hold high volumes of gas.

Baking Results. The impact of *T. emersonii* thermozyme cocktails (1 mg/100 g) on bread quality was assessed in comparison to non-enzyme-treated control loaves and thermozyme cocktail E treated loaves. Thermozyme cocktail E was induced on glucose, a baseline *T. emersonii* enzyme production substrate. It functions as a measure for the influence of growth media residue on bread characteristics. The flour used for baking is standard Irish commercial baking flour, which is weaker than typical European wheat flours. Farinograph experiments were previously performed (results not reported here) to determine optimal water level for the control, which was found to be 60%, giving an indication of the dough weakness. The results are summarized for all of the thermozyme cocktail treated breads and controls in **Table 2**.

Bread treated with thermozyme cocktail B was significantly ($p < 0.05$) larger and softer than all other enzyme-treated breads and the control (**Table 2**) and was 23.2% larger in volume than the control. This increase in volume can be attributed to the action of GH family 11 endoxylanases. Baking research thus far has realized the potential of this GH family as additives in the baking industry to improve handling and loaf quality. GH family 10 enzymes were deemed to be unsuitable as bread improvers. The benefit of employing xylanases as baking aides depends on their substrate specificity. They can enhance the final product by increasing dough flexibility and machinability, resulting in a larger, softer loaf volume with improved crumb characteristics (*32*). The enzymatic mechanism of xylanases in dough is not fully understood; however, water redistribution from arabinoxylan to wheat starch and gluten is involved (*33*).

The combination of cellulases and amylases present in cocktail B, combined with xylanase, contribute positively to the oven-spring of the loaves. This has been reported previously after the addition of commercial baking enzymes (*34*). The addition of xylanases weakens the dough by breaking down soluble pentosans, leading to improved oven-spring, as well as bread volume, shape, and texture (*25*). Soluble pentosans contribute to loaf volume by aiding in the extension of gluten necessary for loaf volume (*35*).

Cocktails B and C were significantly ($p < 0.05$) softer than all other crumb hardness values, whereas *T. emersonii* thermozyme cocktail B treated bread was 49.5% softer than the control. Cocktail B also performed best in staling analysis, being 41.7% softer than the control on day 5. With regard to chewiness, cohesiveness, and springiness, some statistical differences were noted between the control and cocktails B and D in particular. However, the variations are not convincing enough to indicate a substantial effect of these thermozyme cocktails on baking performance. As expected, thermozyme cocktail E treated controls had no relevant significant ($p > 0.05$) contribution to bread quality (**Table 2**).

Xylanase supplementation has previously been shown to facilitate bread volume increases, to improve crumb structure, and to decrease firmness in other studies, in addition to increased dough stickiness (*11*). Greater loaf volumes generally have a positive effect on crumb hardness. In this analysis, all loaves were softer than the control. However, only cocktails B and C were significantly ($p < 0.05$) softer than the average crumb hardness values, and thermozyme cocktail B was 49.5% softer than the control. Treatment with amylase is known to break down starch, making bread softer. Slow starch retrogradation is believed to be a major factor in bread staling, and so this process is partially counteracted by amylase (*1*). The exact mechanism of staling has not yet been elucidated; water redistribution, water evaporation, nongluten protein, nonstarch polysaccharide, and lipid alterations over time have also been investigated as potential contributing factors (*36*, *37*). Cocktail B also performed best in staling analysis as bread was 41.7% softer than the control on day 5. This result was expected from hardness levels on day 0 and characterization results, specifically, amylase, hemicellulase, cellulase, and protease activities present in the *T. emersonii* thermozyme cocktails. Bread treated with thermozyme cocktail B staled more slowly than all other treated and control breads.

Table 1. Gas Levels Produced and Retained by Dough in a Rheofermentometer for a 3 h Fermentation at 30 °C, When Untreated (Control) or Pretreated with *T. emersonii* Enzyme Cocktails (A−E)[a]

enzyme treatment	volume of gas produced (mL)	% of gas retained
control	1656.00 ± 14.14	98.65 ± 0.35
cocktail A	2114.50 ± 10.96	98.85 ± 0.40
cocktail B	2251.00 ± 19.92 a	99.05 ± 0.36
cocktail C	2279.50 ± 34.53 a	98.45 ± 0.39
cocktail D	2761.00 ± 38.18 b	99.20 ± 0.00
cocktail E	2722.00 ± 14.14 b	99.15 ± 0.07

[a] The results are significant with a significance level of 95% ($p < 0.05$). Values reported are mean values of at least three replicates ± standard deviations. Mean values followed by the same letter are not significantly different from each other and are in decreasing numerical order.

Table 2. Properties of Bread Produced When Untreated (Control) or Pretreated with *T. emersonii* Enzyme Cocktails (A−E)[a]

parameter	control	A	B	C	D	E
loaf volume (mL)	1138.89 ± 0.05 b	1255.56 ± 83.82 b	1402.78 ± 82.90 c	1253.33 ± 35.28 b	1226.67 ± 30.55 b	1067.78 ± 121.58 b
*Lab** crust color	47.87 ± 0.90	49.47 ± 0.78 c	48.33 ± 2.15 bc	48.80 ± 1.45 c	46.72 ± 0.61	46.82 ± 3.27
hardness (day 1) (N)	6.00 ± 0.74 e	4.95 ± 0.81 d	3.03 ± 0.44 bc	3.74 ± 0.50 b	4.16 ± 0.64 c	5.22 ± 0.49 d
hardness (day 2) (N)	13.24 ± 1.47 e	10.18 ± 0.74 cd	8.90 ± 1.11 bc	11.38 ± 1.20 d	10.51 ± 1.5 d	13.02 ± 1.54 e
hardness (day 5) (N)	20.69 ± 0.16 e	16.08 ± 0.34 d	12.07 ± 2.45 bc	15.97 ± 1.48 d	14.36 ± 1.48 cd	19.21 ± 1.53 e
chewiness (N)	10.65 ± 0.72 c	10.30 ± 1.30 bc	8.23 ± 0.22 c	10.78 ± 2.60 c	8.36 ± 0.22 bc	12.16 ± 1.79 c
cohesiveness (N)	0.77 ± 0.10 b	0.78 ± 0.11 b	0.79 ± 0.11 c	0.76 ± 0.12 bc	0.78 ± 0.10 bc	0.76 ± 0.10 b
springiness (N)	1.24 ± 0.40	1.19 ± 0.40 ab	1.64 ± 1.00 d	1.35 ± 0.71 bc	1.43 ± 0.71 cd	1.27 ± 0.54

[a] The effect of the enzyme treatment is significant with a significance level of 95% ($p < 0.05$). Values reported are mean values of 5 samples from three independent replicate batches of loaves ± standard deviations. Mean values followed by the same letter in the same row are not significantly different from each other. Letters are indicative of values, where a < b < c < d < e < f, for all groups. When a mean value fits more than two letter groupings, it is omitted from all groups. Comparisons are made according to each line.

Wheat xylan contains 10−15% L-arabinofuranosyl units at the C-2 and C-3 positions of the D-xylose backbone (*38*, *39*). Thus, the high level of α-L-arabinofuranosidase (EC 3.2.1.55) activity present in thermozyme cocktail B can contribute to the degradation of xylan in synergy with endoxylanase by the cleavage of these side chains (*40*). Interaction between endoxylanase and α-L-arabinofuranosidase has previously been documented in cereal modification research (*41*). Certain *p*NP-arabinofuranoside-degrading enzymes are active on other polymers such as galactans and arabinans. However, given the superior baking and hydrolysis performance of thermozyme cocktail B, it seems likely that a proportion of the *p*NP-arabinofuranoside-degrading activity detected in this study is relevant to arabinoxylan degradation. Despite additional enzyme contribution, xylan depolymerization still relies on endo-1,4-*β*-xylanase (EC 3.2.1.8) and *β*-xylosidase (EC 3.2.1.37) activities, both of which are detected in highest levels in cocktail B.

Arabinoxylan is an extremely important component of the dough as it accounts for almost a third of the water-binding capacity of wheat flour, having an important impact on dough and bread quality (*34*). GH family 10 xylanases, which primarily or only hydrolyze water-unextractable (75−80% of total arabinoxylan content) arabinoxylans, are preferable baking additives. They cause the water-unextractable arabinoxylan fraction to be solubilized, resulting in a loss of water and increased viscosity and hence the formation of a strong viscoelastic dough, freeing the gluten network (*32*, *33*). The increased dough stability is associated with a decrease in stickiness and increased flexibility and causes increased oven-spring and a finer, softer crumb in the baked product (*42*).

It is important to note that the thermozyme cocktails are crude unoptimized enzyme extracts. Further fractionation of specific relevant activities from these thermozyme cocktails could reveal further potential compared to mesophilic enzymes in relation to thermostability and functional properties. Low dosages of the crude thermozyme cocktails are sufficient as their thermostability allows them to function longer during baking. This adds to the efficiency of the baking process.

In conclusion, *T. emersonii* thermozyme cocktails used as baking enzymes have a positive effect on the final product. Enzyme screening studies revealed the presence in all cocktails of hemicellulases, amylolytic enzymes, and proteases needed for the manipulation of dough, albeit in various relative amounts. The results in this study show that small quantities of crude enzyme cocktails have the ability to positively affect bread volume, softness, and antistaling properties, thus generally improving baking performance. This makes *T. emersonii* a novel source of efficient thermozymes for the baking industry.

ABBREVIATIONS USED

BWX, birchwood xylan; OSX, oat spelts xylan; RAX, rye arabinoxylan; TPA, texture profile analysis; WSX, wheat spelts xylan.

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