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ORIGINAL PAPER

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Comparison of wild-type and UV-mutant β-glucanase-producing strains of *Talaromyces emersonii* with potential in brewing applications

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Abstract A screen of 46 UV-mutant strains of the moderately thermophilic fungus *Talaromyces emersonii* yielded two mutants (TC2, TC5) that displayed gross morphological differences to the parent strain and enhanced activity against mixed linkage cereal β-glucans. Activity against β-(1, 3)(1, 4)-D-glucan from barley (BBGase) was measured during growth of the mutant and wild-type strains on a variety of carbon sources, ranging from solka floc to crude cereal fractions. In liquid culture, TC2 and TC5 secreted 1.2- to 8.6-fold more BBGase than the parent strain and markedly less β-glucosidase (exo-activity); enzyme levels were dependent on the carbon source. Cellulose induced high BBGase. However, beet pulp, wheat bran, carob and tea-leaves were cheap and effective inducers. *T. emersonii* wild-type, TC2 and TC5 crude enzyme preparations achieved similar end-points during the hydrolysis of commercial barley β-glucan (13.0–16.9%), but were more active against crude β-glucan from barley (16.0–24.2% hydrolysis). The products of hydrolysis were quantified by high-performance anion-exchange chromatography. Mash trials indicated that enzyme preparations from all three organisms effected a significant reduction in wort viscosity and residual mash β-glucan. Finally, TC2 and TC5 produce more efficient β-glucan-depolymerizing enzymes; and wheat bran and solka floc can be used to provide inexpensive and potent enzyme cocktails with potential in brewing applications.

Keywords β-Glucanase · *Talaromyces emersonii* · UV-mutants · Brewing · Thermophilic fungus · Barley β-glucan

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Introduction

β-Glucan polysaccharides represent a large and diverse group of polysaccharides that play important structural, storage and protective functions in the cell walls of plants, fungi and algae [25]. In the main cereals of commerce, β-glucans comprised of glucose residues linked via β-1,3 and β-1,4 glycosidic bonds are the main non-starch polysaccharides and account for about 70% (w/w) of the endosperm cell wall in barley [3]. The exact molecular size of these polysaccharides and the proportion and distribution of β-1,3 and β-1,4 linkages varies considerably [1, 9, 14, 30]. Poor hydrogen bonding between cereal β-glucans chains accounts for their high water-binding capacity and tendency to yield highly viscous solutions. In the brewing industry, slow malting, prolonged run-off time of wort, lengthy beer-filtration time and formation of gummy precipitates and hazes are generally associated with high barley or malt β-glucan content [6, 11].

Enzymes are used to depolymerize cereal β-1,3;1,4-glucans (β-glucan hydrolases) in a reproducible and controlled manner at key stages in the brewing process, or are added to beer in cold storage, in order to overcome some of the problems outlined above [23]. Four main candidate enzymes with hydrolytic activity against mixed linkage cereal β-glucans have been identified: lichenase [EC 3.2.1.73, (1–3)(1–4)-β-D-glucan 4-glucanohydrolase], 1,3(4)-β-D-glucanase (EC 3.2.1.6), 1,3-β-D-glucanase [EC 3.2.1.39, (1–3)-β-D-glucan 3-glucanohydrolase] and 1,4-β-D-glucanase [EC 3.2.1.4, (1–4)-β-D-glucan 4-glucanohydrolase, or "cellulase"] [22]. Only enzymes belonging

to mechanistic class EC 3.2.1.73 are specific for 1,4-linkages adjacent to 1,3-linkages in cellotriosyl and cellotetraosyl repeat units present in the main water-soluble (1–3)(1–4)-β-D-glucan isolated from barley [2, 16]. To date, the majority of true ''lichenases'' have been isolated from *Bacillus* sp., with few reported examples from fungal species [21, 22]. Endogenous cereal enzymes are rapidly inactivated within 5 min at typical mashing and kilning temperatures. Therefore, enzymes from thermophiles that possess enhanced thermal stability in an acidic environment and retain substantial biological activity on exposure to elevated temperatures are particularly well suited to application in the critical early steps of the brewing process, to maximize brewhouse yield.

Selection of hyper-producing β-glucanase microbial strains secreting enzymes with the correct specificities, using non-chemical based mutagenesis techniques (e.g. UV irradiation), provides a strategy to reduce the costs associated with enzyme production. *Talaromyces emersonii*, a thermophilic aerobic fungus with generally regarded as safe (GRAS) status, produces an extensive array of extracellular polysaccharide hydrolases, including multi-component cellulase and xylanase enzyme systems [18, 26, 27]. Several of the enzymes from these systems have been purified and are characterized by noteworthy thermal stability [10, 28]. In earlier studies, hyper-cellulolytic mutants of the parent *T. emersonii* strain were isolated by a UV mutagenesis approach. In this work, we report on the production, screening and selection of 46 UV-mutants, referred to as TC1–TC46. Two of these mutants (TC2, TC5) were characterized by increased activity against barley β-glucan during growth on solka floc (SF; ball-milled spruce cellulose) and were selected for further study. β-Glucanase production by both mutants during growth on a variety of β-glucan-rich substrates was investigated and model mash trials were conducted to evaluate the ability of TC2, TC5 and wild-type enzyme preparations to reduce the mash viscosity and β-glucan content.

Materials and methods

Materials

Yeast extract and Sabouraud dextrose agar (SDA) medium were obtained from Oxoid (Basingstoke, UK). SF (ball-milled spruce cellulose) was purchased from Brown & Co. (Berlin, N.H., USA). Barley β-glucan [viscosity ca. 25 centipoise units (cps); 1 poise = 0.1 N s m^{-1}] and pachyman were obtained from Megazyme International (Bray, Ireland). Carboxymethylcellulose [CMC; viscosity of 2% solution (25°C) 10–20 cps, degree of substitution 0.65–0.85, degree of polymerisation (DP) 200], laminaran (*Laminaria digitata*), lichenan (*Usnea barbata*), 4-nitrophenylglucopyranoside and 2-deoxyglucose were from Sigma–Aldrich (Dublin, Ireland). Avicel was obtained from Merck Ltd. (Darmstadt, Germany). Sorghum and maize were supplied by Quest International (Ireland) Ltd. (Carragaline, Ireland) and unmolassed beet pulp (BP) was a kind gift from Mr. J. Conway (Irish Sugar Ltd., Carlow, Ireland), while all other crude organic inducers were sourced in large batches (to ensure inter-experiment reproducibility) from a local health-food store (Healthwise, Galway City, Ireland). The low-molecular-weight standard protein calibration kit for SDS-PAGE was purchased from Amersham Biosciences (Uppsala, Sweden). GelBond, an agarose support medium, was from FMC Bio-products (Rockland, Me., USA). Unless otherwise stated, all other research chemicals were purchased from Sigma–Aldrich and all solvents were from Alliedsignal Riedel de Haën (Seelze, Germany).

Micro-organism and cultivation techniques

T. emersonii IMI392299 was obtained from laboratory stocks and routinely sub-cultured on SDA medium. Liquid cultivation on the appropriate carbon sources was conducted at 45°C, pH 4.5 and 250 rpm in 250 mL (or 2 L) Erlenmeyer flasks containing sterilized mineral salts/inducing medium; culture filtrates were harvested periodically over 0–322 h of growth, as described previously [21].

Isolation of mutants

For the purpose of mutant isolation, 2.0 mL (0.1%) suspensions of mycelia, prepared from the parent or wild-type strain and washed with sterile H_2O, were subjected to UV irradiation (UV-C lamp, at 0.1 mJ m^{-2}) over an even mycelial surface area and minimum depth (2 cm vertical distance) for defined time-periods (0–60 min). Kill-off rates were typically 45–92%, depending on the exposure time. UV-treated mycelia were then used to inoculate the surface area of sterile agar plates containing 0.2% (w/v) of the catabolite repressor 2-deoxyglucose. Agar plate cultures were incubated at 45°C for 3–4 days, after which mutants were visible as large white colonies (fluffy in appearance). A total of 46 single colonies were selected and subjected to several cycles of rigorous purification. These cultures were referred to as TC1–TC46 and were morphologically distinct from the parent organism. Eleven of these mutants were chosen for further investigation on the basis of their stability and growth rate on β-glucan-containing agar plates.

Assay of enzyme activity and protein content

For convenience, all β-glucanase assays, unless otherwise stated, were carried out at 50°C for 15 min in 100 mM sodium acetate (NaOAc) buffer, pH 5.0, in a reaction volume of 600 µl, with a final substrate concentration of 0.66% (w/v), except for CMC, which was used at a final concentration of 1.98% (w/v). Enzyme-catalysed hydrolysis of individual polysaccha-

rides was determined by measuring, in independent reaction cocktails, the release of reducing sugar (glucose) equivalents from barley-β-glucan (BBG), CMC (β-1,4-glucan), pachyman (β-1,3-glucan), laminaran (β-1,3-glucan with some β-1,6 branch-points) and lichenan (β-1,3;1,4-glucan), using the dinitrosalicyclic acid reagent, as outlined previously [21]. β-Glucosidase activity was determined by monitoring the increase in absorbance at 410 nm (A_{410}) due to the release of the 4-nitrophenolate (4-NP) anion after incubation of suitably diluted enzyme with 1 mM 4-NP-glucopyranoside in a 110 μL reaction volume for 30 min at 50°C, in 100 mM sodium acetate buffer, pH 5.0 [27]. In all cases, enzyme activity was expressed in international units (IU) mL^{-1}, i.e. micromoles of reducing sugar equivalents or 4-nitrophenol released per minute of reaction time per millilitre of enzyme solution. Protein present in crude enzyme samples was determined as described elsewhere [8, 29].

Statistical analysis of enzyme production data

All cultures were grown in replicates of three, at two independent time-periods, and culture filtrates were assayed in triplicate (on two separate experimental occasions). An ANOVA analysis was conducted on the data obtained. Two independent estimates of the common population variance (σ^2) were made, one using the variance among sample means (mean square due to error; MSE) and the second based on the within treatments variance (mean square due to treatments; MSTR). The ratio MSTR/MSE was analysed to see whether it belonged to a F distribution and to test the null hypothesis. If significant differences existed between populations (i.e. mutant(s) vs wild type), the value MSTR/MSE was inflated. Consequently, if $F > F_\alpha$ (or if the P value $< \alpha$), where α was the level of significance (at 0.01 or 0.05), the null hypothesis was rejected, i.e. the mutants were significantly different from each other and the wild type. Sample mean, variance and standard deviation values were estimated for each data set obtained with individual strains at each growth time-point. MSTR, MSE, F value, numerator/ denominator degrees of freedom, F_α values and P values were calculated to conduct the ANOVA on data at each time-point [4]. Standard deviation values were calculated for each replicate set at each time-point.

Electrophoretic profiling of crude extracts

The profile of proteins present in selected wild-type, TC2 and TC5 time-course culture samples was evaluated by SDS-PAGE on 12.0% gels, according to the Laemmli method [15]. Protein bands and β-glucanase-active bands [0.2% (w/v) BBG as substrate] were detected as described earlier [21].

Infusion-mashing model study

A programmed infusion-mashing procedure was employed for the model study conducted in this work, which involved a series of stepwise increases in temperature adopted to: (a) reduce the total process time taken for wort production and (b) permit the use of poorer quality malts. A known quantity (30 g) of mash consisting of 25% barley and 75% malt in 150 mL of H_2O was heated to 65°C (strike temperature). The crude enzyme sample (at 50 IU, with respect to activity against BBG) was added and mixed for 75 min at 65°C. The temperature was then increased to 68°C for 55 min and finally raised to 78°C for a further 15 min before the mashing process was terminated.

Measurement of run-off, specific gravity and viscosity

Each mash was subsequently filtered through Whatman No. 1 filter paper into a graduated cylinder. Run-off volumes after 10 min, 20 min and at the end of filtration were noted and compared with values for a control (no β-glucanase added) included in the same mashing sequence. Specific gravity was measured using a calculating densitometer (DMA 55, PAAR), while viscosity values (1.0 mL extracts) were determined using a Carrimed ESL 100 Rheometer.

Extraction and quantification of residual β-glucan

The residual (unhydrolysed) β-glucan content of run-off extracts was determined following overnight incubation of 5.0 mL of each extract with 2.5 g (50% w/v) ammonium sulphate at 4°C to precipitate the β-glucan. The precipitated sample was centrifuged at 3,000 g for 15 min. The pellet fraction was redissolved by vortexing in 50% (v/v) ethanol and then recentrifuged at 3,000 g for 10 min to remove free glucose and low-molecular-weight β-glucooligosaccharides (repeated twice). The final pellet was dissolved in a known volume of 100 mM NaOAc buffer, pH 5.0, and residual β-glucan quantified [17].

Preparation of enzyme–substrate digests

Incubation mixtures, containing 0.75 mL of enzyme and 0.75 mL of 1.0% (w/v) or 10 mg mL^{-1} substrate (crude or pure BBG) in 100 mM NaOAc buffer, pH 5.0, were incubated at 50°C, with shaking at 150 rpm. The appropriate control (blank) samples were prepared in a similar manner. Aliquots were removed at fixed time-intervals, boiled for 10 min in sealed reaction tubes to inactivate the enzyme and analysed for reducing sugars and glucose released. Products of hydrolysis formed by selected enzyme preparations were separated, identified

and quantified by high-performance anion-exchange chromatography (HPAEC).

Separation of reaction products by HPAEC

The products of enzyme–substrate hydrolysis reactions were fractionated, identified and quantified by HPAEC with pulsed amperometric detection (PAD). Chromatography was carried out on a DX500 system (Dionex Corp., Sunnyvale, Calif., USA) [21]. Separation was achieved by means of a CarboPac PA-1 anion exchange column equipped with a guard column and attached to a GP40 gradient pump. Sample volumes of 5 μL were applied to the column, and reaction products were eluted using a gradient of 0–390 mM NaOAc in 100 mM NaOH over 20 min at a flow-rate of 1 mL min^{-1}. The column was re-equilibrated between successive injections, using 10 min washes in 100 mM NaOH. Detection was by pulsed amperometry (ED40 electrochemical detector) and data were analysed using PeakNet software (Dionex Corp.), on a Gateway 2000 PC. Standard curves were prepared by dissolving a known weight of individual cellooligosaccharides (glucose, cellobiose, cellotriose, cellotetraose, cellopentaose) in deionised water (18.2 mΩ). Peak–area ratios were calculated and standard curves were obtained by plotting carbohydrate concentration versus peak–area ratio, using Fig.P software. Where possible, the concentrations of oligosaccharides present in each sample were calculated using these standard curves.

Results and discussion

UV irradiation yielded 46 mutant colonies of the parent *T. emersonii* strain. Although other types of mutagens were investigated (e.g. the chemical nitrosoguanidine), UV irradiation yielded better results with regard to the number of stable mutants obtained and higher levels of BBGase. These observations are based on data collected over a period of 19 years, using different mutagenesis techniques (chemical, UV) to generate β-glucanase mutants of *T. emersonii*. The data were derived from repeated propagation of mutants from 20% (v/v) glycerol stocks stored at −80°C and from agar cultures without loss of viability, from microscopic and morphological analyses and from statistical analysis of biochemical data derived from repeated enzyme-production studies. The mutants reported here were significantly different from mutant strains of *T. emersonii* isolated in previous work [19], such as mutant UCG.208, which were selected for enhanced cellulolytic (filter paper, CMCase) activity [27]. The morphological and visual appearance of the mutant strains reported here were quite distinct from the parent organism.

In initial studies, mutant and wild-type strains were grown individually in liquid culture (three replicate cultures at two different experimental time-periods, i.e. $n = 6$), under pH-uncontrolled conditions, on 2% (w/v) SF and 2% barley flour as sole carbon sources. Higher yields of BBG-hydrolyzing activity were obtained for all strains with SF as inducer; activity levels (IU g^{-1} inducer) in time-course samples of the parent (wild-type) and 11 of the mutant strains are given in Fig. 1a–c. From these data, it can be seen that more than one peak of enzyme activity occurs in the time-course profile obtained for each organism and the time-point of maximum production varies between the strains, especially for some of the mutants (e.g. TC1, TC2, TC3, TC11). This phenomenon was observed previously, under similar culture conditions, with regard to the production of cellulase, xylanase, galactanase, various exo-glycosidase and lytic activities by the parent strain [26, 27]. Tight error bars for all data points reflect the accuracy of the replicate assays and support the observed periodicity of enzyme production. Several of the mutants secrete higher levels of BBGase than the wild type, with TC2–TC5 and TC10 being the most noteworthy. Observations were validated by an ANOVA of the data, where for both 1% and 5% probability of type I errors, $F > F_{\alpha}$ in all cases (and P values were < 0.01 or 0.001), thus supporting, with 95–99% confidence in the data, the conclusion that the mutant strains were different from the wild type and each other (population scores were not equal). In the ANOVA, BBGase activity was the dependent or response variable, while the organism (wild type or mutant) was the independent variable. MSTR, MSE and F values for the population of mutants and wild type over the 0–322 h of growth were determined.

Two mutants, TC2 and TC5, were selected for further study following analysis of other β-glucan-depolymerizing activities present in the extracellular time-course filtrates obtained during growth on SF (see Fig. 2). The wild-type strain produces similar levels of BBGase and CMCase, especially from 168 h onwards.The final activity levels are virtually identical and only at the earlier growth times (48–144 h) are the levels of BBGase higher than the corresponding CMCase levels (e.g. 2.7-fold at 72 h). Laminarinase (β-1,3-glucanase) activity levels are very low at 48–120 h and only increase moderately thereafter. Likewise, activity against pachyman (β-1,3-glucan) is very low. Therefore, it is unlikely that β-1,3-glucanase activity contributes to the observed higher levels of activity against β-1,3;1,4-glucan. The profiles of extracellular BBGase versus CMCase obtained for TC2 and TC5 are markedly different to that of the wild type. TC2 produces the highest levels of BBGase, which are higher than the corresponding levels of CMCase. Laminarinase (and pachymanase) activity are lower than noted for the wild-type filtrates, except for the final time-point which could be associated with autolytic events. Likewise TC5 displays higher BBGase than CMCase or laminarinase throughout the growth time-course. The profile of production obtained with TC5 differed from the profiles for either TC2 or wild-type strains. Electrophoretic and zymogram analysis of the wild-type, TC2 and TC5 culture filtrates (identical

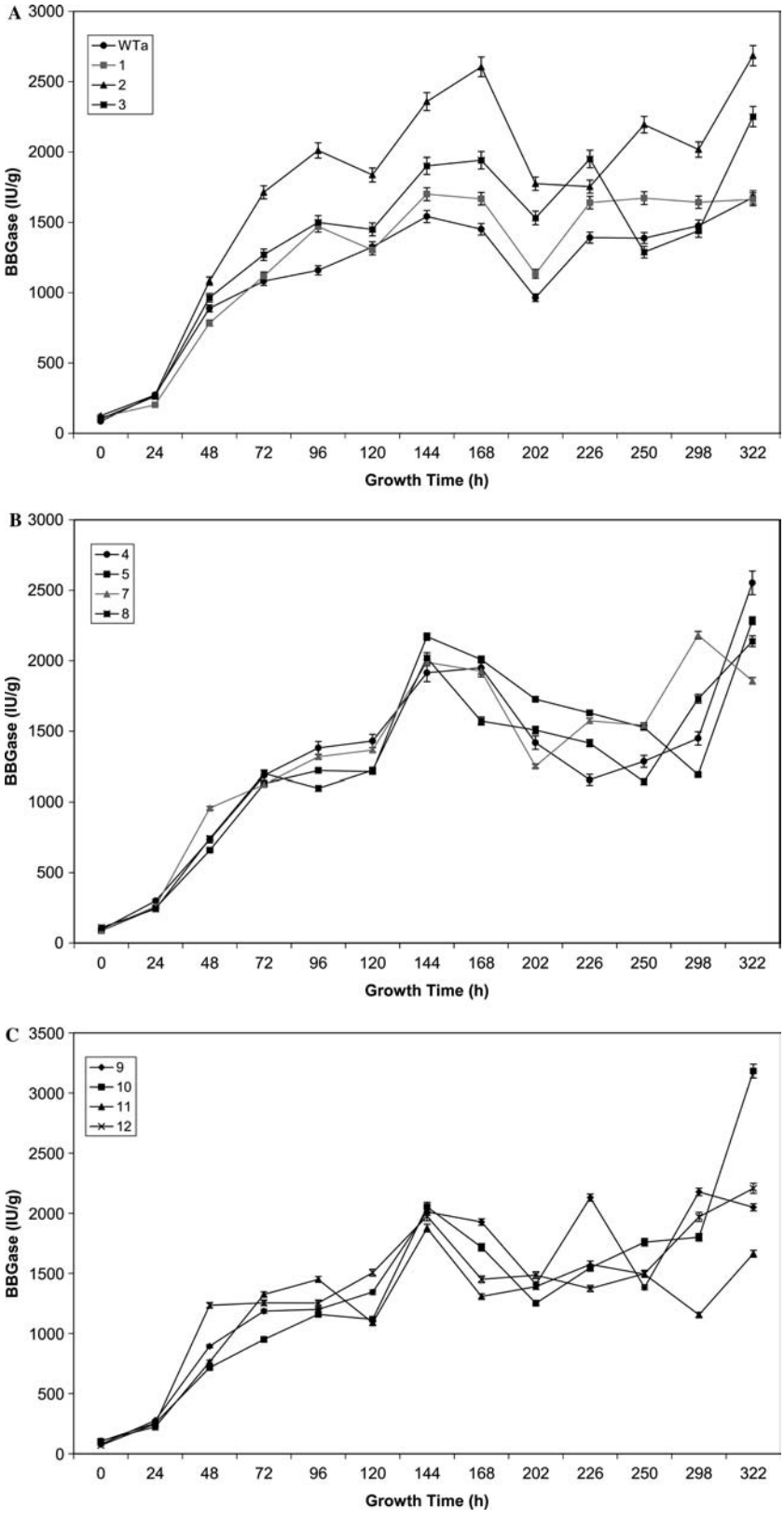

Fig. 1 **a**–**c** Mean sample activity values ($n = 6$), during time-course production of β-glucanase activity (barley β-glucan) by wild-type (*WTa*) and 12 mutant *T. emersonii* strains (IU g^{-1} = IU mL^{-1} ×55). **a** WTa and mutants 1–3, **b** mutants 4, 5, 7 and 8, **c** mutants 9–12

protein concentrations applied for all samples) suggests that production of different β-glucanase components may be induced in the mutants.

For example, two protein bands (approx. 30 kDa and 36 kDa) corresponding to enzyme-active components in the zymogram analysis were noted in wild-type filtrates

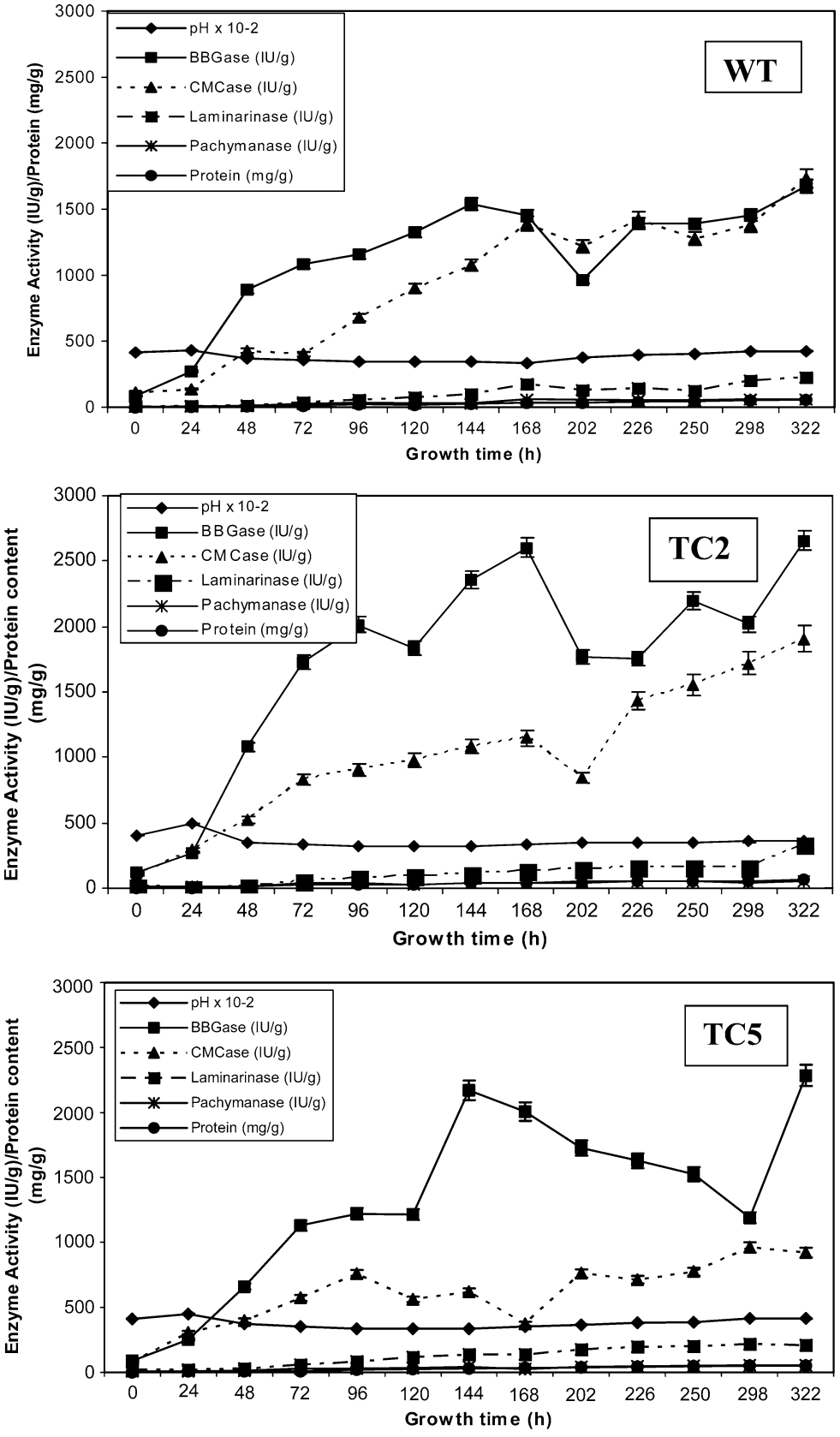

Fig. 2 Time-course production of extracellular β-glucan-degrading enzyme activities by *T. emersonii* IMI392299 parent strain (*WT*) and mutants TC2 and TC5 during liquid cultivation in unbuffered media containing SF as sole inducer. Extracellular protein production and change in pH of the culture medium over the 322 h growth period are illustrated. SD values were as follows: *WT* ±3.1–9.4, *TC2* ±3.0–5.6, *TC5* ±3.3–7.1

at 0–96 h (at 120 h only the 36-kDa component was present). In TC2 filtrates, a third, new enzyme-active band (31 kDa) was evident at 48 h. The intensity of this band increased throughout the growth time-course, while the intensities of the other two bands decreased. This result contrasts with findings reported by Morrison

and coworkers [20] for wild-type and mutant (UV7) strains of *T. emersonii*, which revealed an identical endoglucanase band in each culture filtrate that was markedly enhanced in UV7 filtrates, i.e. hyperexpression of the same gene accounted for the enhanced cellulase activity of mutant UV7 [20].

Costs associated with the production of enzymes are often considered prohibitive to their application in an industrial context. While SF is a relatively cheap and readily available carbon source, other cereal residues rich in non-starch polysaccharides (including mixed-linkage β-glucans) are often generated in vast quantities as by-products or waste residues of food and agri-feed processing industries and thus represent cheap carbon sources. Production of BBGase by the wild type, TC2 and TC5 strains was investigated following growth for 120 h on a variety of cost-effective carbon sources, including a range of cereals/cereal fractions, BP and tea leaves (data not shown). In general, the mutant strains yielded higher levels of BBGase (15- to 30-fold) than the wild type on wheat bran (WB), BP, SF and Avicel. Subtle differences were noted in enzyme production and response to the various inducers between the wild-type and mutant strains, e.g. with TC2, Avicel was the best inducer, while SF included the highest levels of BBGase by TC5. The marked contrast in the inducing potential of WB versus the other cereal fractions, for all three strains, may be explained by a possible inaccessibility or difficulty in releasing β-glucan from the complex structural network of cereals [12].

Culture filtrates produced on selected inducers were investigated for the presence of other β-glucan-degrading enzymes (Table 1). TC2 and TC5 yielded significantly higher β-1,3;1,4-glucan-degrading activities than the wild type. Marked differences were noted with respect to BBGase versus CMCase and lichenase. BP and SF were the best inducers for the wild type and lichenase activity was higher than BBGase, while CMCase was lower. Avicel, SF, WB and BP all induced high levels of BBGase and lichenase in TC2 and TC5. The highest lichenase activity (5,615.5 IU g^{-1}) for all three strains was produced by TC5; high lichenase suggests significant potential in brewing applications. Mutant filtrates had low CMCase activity and even lower laminarinase. Overall, both mutants, especially TC5, secreted significantly less β-glucosidase activity (exo-activity) than the parent strain.

The ability of selected crude wild-type and mutant enzyme samples, i.e. WB- and SF-induced samples, to depolymerize purified commercial BBG and a crude mixed-DP BBG extracted from barley was investigated. On the basis of reducing sugar analysis, all of the enzyme preparations released higher levels of reducing sugar over a 24 h reaction period from the purified BBG, although the difference between the final end-point achieved with the TC5 enzyme preparations on both BBG substrates was marginal (Fig. 3). From the reducing sugar profiles, the SF-induced samples from all three organisms appeared to be marginally more effective than their WB-induced counterparts. The complexity of the crude BBG and the likely presence of interfering substances (phenolic compounds, extractives or even low-DP oligosaccharides) that potentially co-extract with the substrate, may influence enzyme activity. Reducing sugar analysis provides no information on the mode of action of these enzyme preparations or on potential differences that exist between samples with respect to the types of products formed. This type of

Table 1 Profile of β-glucan-depolymerizing activities and β-glucosidase in crude 120 h culture filtrates from wild type, TC2 and TC5 grown on β-glucan-rich substrates. *OB* Oat bran. Activities ($n = 6$) are given as IU g^{-1} inducing carbon source. BBGase, CMCase and lichenase SD: wild type 1.2–7.3, TC2 0.4–6.6, TC5 1.2–10.8; laminaranase and β-glucosidase SD: wild type 0.3–4.5, TC2 1.2–8.3, TC5 2.1–7.9

Organism	Inducer	BBGase	CMCase	Lichenase	Laminaranase	β-Glucosidase
Wild type	WB	648.5	203.5	1022.5	149.1	91.9
	BP	1444.3	681.5	2251.2	143.0	113.1
	WB/BP (1:1)	1036.4	343.8	1590.1	110.0	92.4
	OB	124.9	0.0	326.7	134.8	92.4
	Avicel	1089.6	661.7	2306.2	209.0	54.5
	SF	1414.1	1034.0	2834.2	127.1	91.3
	Sorghum	142.5	2.2	341.6	121.0	100.1
	Maize	25.9	0.0	0.0	123.8	82.0
TC2	WB	1706.7	192.5	1211.7	36.9	14.0
	BP	1945.9	761.2	2405.7	96.8	14.2
	WB/BP (1:1)	997.7	302.5	1670.9	0.0	11.0
	OB	92.4	13.2	253.0	88.6	9.3
	Avicel	2797.3	1197.9	3727.4	124.9	26.4
	SF	2542.7	1137.4	3158.1	159.5	42.0
	Sorghum	234.9	49.5	575.3	90.8	11.7
	Maize	14.9	0.0	66.6	56.1	12.1
TC5	WB	855.8	270.6	476.9	36.9	8.3
	BP	939.4	393.8	2900.2	62.7	9.5
	WB/BP (1:1)	700.2	164.5	407.0	39.1	6.9
	OB	193.1	4.4	90.8	107.8	6.6
	Avicel	875.1	371.8	3440.8	42.9	5.3
	SF	2246.8	1037.9	5615.5	68.8	7.2
	Sorghum	161.7	0.0	90.8	42.9	8.1
	Maize	127.1	4.4	63.3	73.2	6.4

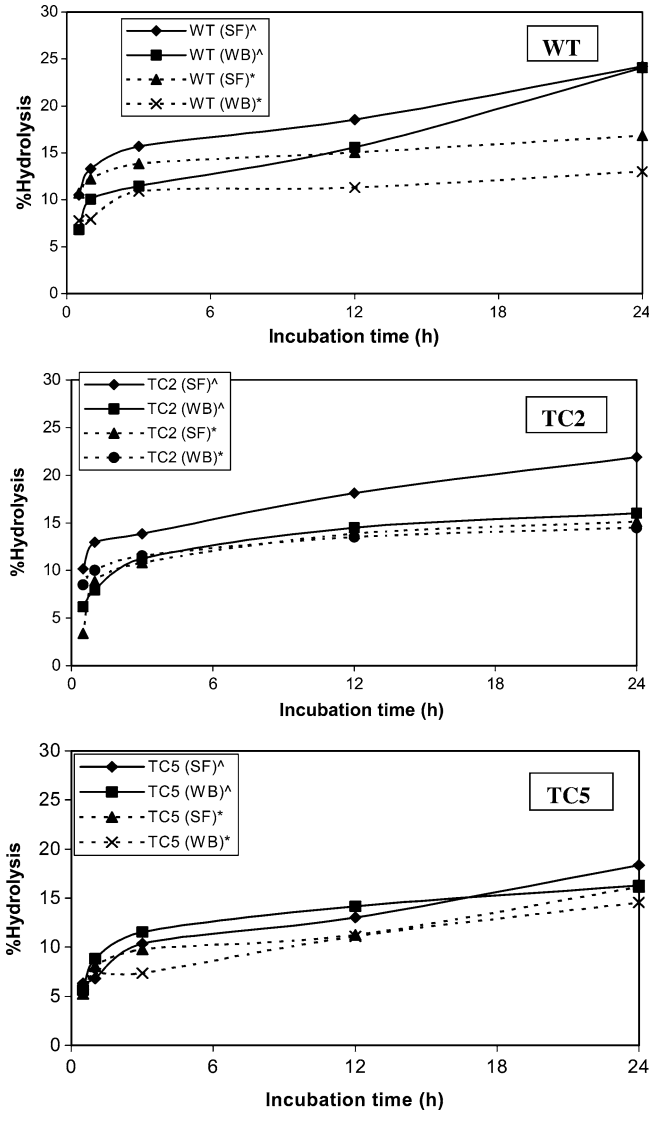

Fig. 3 Time-course hydrolysis of commercial purified BBG (∧) and crude barley BBG (*) catalysed by SF- and WB-induced WT, TC2 and TC5 enzyme samples during a 24 h incubation period at 50°C and pH 5.0. Hydrolysis was estimated on the basis of reducing sugars released at each time-point by WT, TC2 and TC5 enzyme preparations, relative to the total amount of reducing sugars that would be released if all of the glycosidic bonds in both BBGs were hydrolysed

information is essential for potential applications in brewing, as the natural target substrate in mash resembles the crude BBG preparation used here with respect to composition. In this study, HPAEC was employed to fractionate the oligosaccharide products released by the action of SF- and WB-induced wild-type, TC2 and TC5 enzyme samples from crude BBG, at early (30 min) and later (24 h) reaction times (Fig. 4a–d). At the initial reaction time-point (30 min), with SF-induced extracts, both mutant enzyme preparations were significantly more active than the wild-type enzyme sample, while at 24 h the wild type and TC2 enzyme preparations released higher (and comparable) levels of a number of

Fig. 4 a–d Comparison of the hydrolysis products formed by SF-induced (**a,b**) and WB-induced (**c,d**) WT, TC2 and TC5 enzyme samples at initial (30 min) and final (24 h) incubation time-points with crude BBG as substrate. The elution of cello-oligosaccharides, G to G_5, is indicated. Suitable standard oligosaccharides were not available for unambiguous quantification of mixed-linkage oligosaccharide products

products (DP 1, 2, 3) than the TC5 enzyme preparation (Fig. 4a,b). WB-induced TC2 and TC5 enzyme samples were much more active at the initial reaction time-point and released different and a greater variety of oligosaccharide products than the corresponding wild-type enzyme sample (Fig. 4c,d). At 24 h, the differences were even more apparent. This result confirms the importance of comparing the mode of action of the enzyme preparations, but also indicates that the mutant enzyme preparations would be much better candidates for application in brewing because of their behaviour on a BBG preparation very similar in composition to the in vivo substrate.

The potential of wild-type and mutant crude extracts as processing aids in brewing was investigated in mini-mash trials, using 75% malt, 25% barley and 50 IU of enzyme (in 150 mL of H_2O). Various parameters were analysed, such as changes in viscosity, mash β-glucan content and run-off times. Selections of crude enzyme extracts were investigated and the data are summarized in Table 2. Overall, the mini-mash trials illustrate the superiority of TC2 and TC5 enzymes relative to the wild type counterpart as agents to reduce viscosity and β-glucan content. Although the WB-induced extracts performed well, the most potent decrease in the BBG content of the mash residue was achieved by the SF-induced extracts. A plausible explanation for this result may be the potential cell-wall-loosening effect of cellulases, induced by SF, on whole and unprocessed kernels present in crude barley and malt, which would have the result of releasing wall-bound BBG. Lack of an apparent correlation between run-off times and decrease in residual BBG content is often observed in mash trials and is a common occurrence in the brewing industry; in practice, a low BBG content is preferred to good run-off times. Thus, mutants with enhanced ability to decrease residual BBG are of particular interest to brewers in their quest for enzymes to reduce mash viscosity.

In conclusion, although the production of overproducing cellulase mutants to obtain more efficient enzyme systems for the bioconversion of nature's richest and renewable energy source, cellulose, is well documented [7, 13, 19, 20], to the authors' knowledge, no studies to date have focused on the isolation of overproducing 1,3;1,4-β-glucanase mutants from a thermophilic GRAS fungal source for application in the brewing industry. Complete or substantial loss of endogenous cereal BBGase has been reported within 5 min at a typical conversion temperature of 65°C [5, 24] in the brewing industry. Therefore, a real potential exists for the use of exogenous thermostable enzymes (or thermozymes),

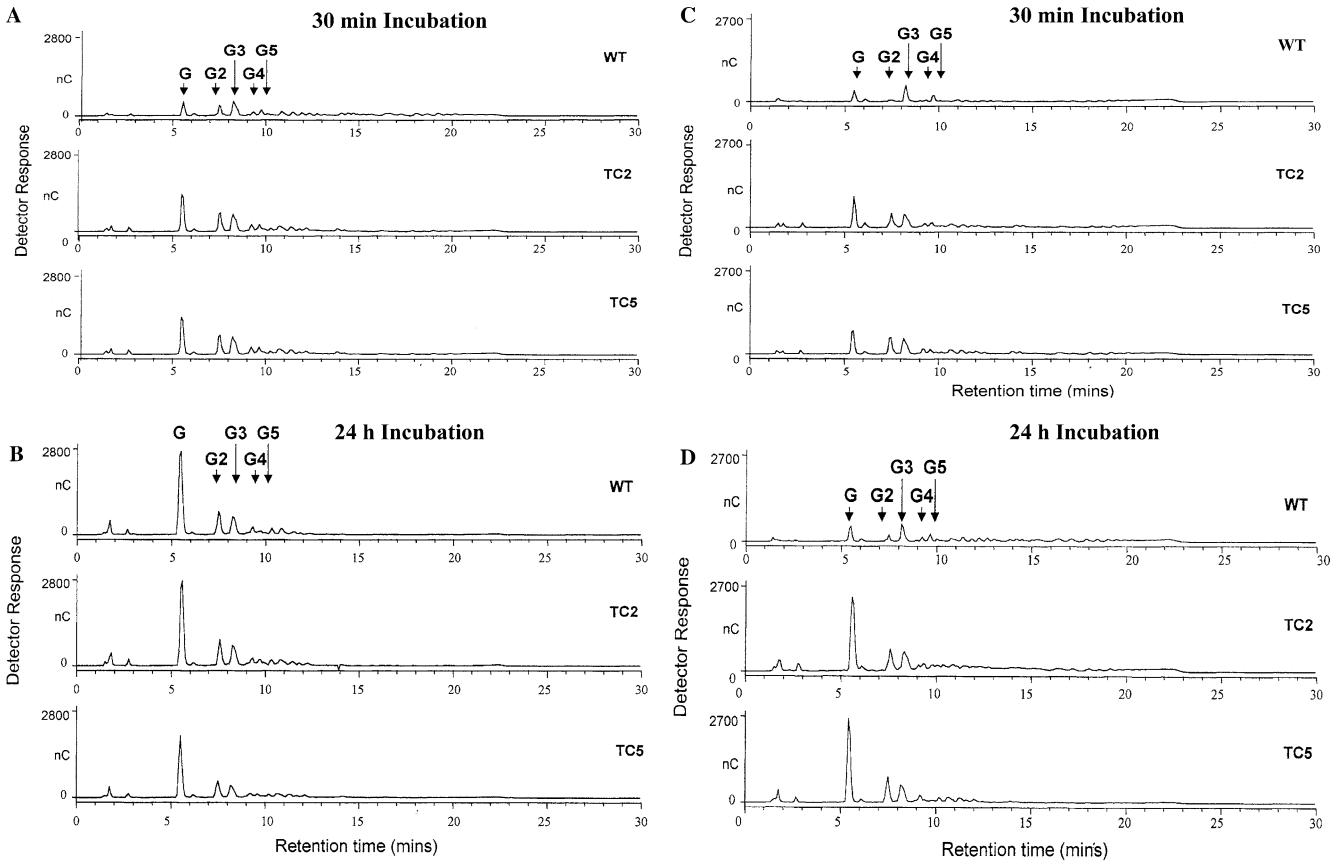

Table 2 Model mash trials conducted with selected *T. emersonii* wild-type and mutant enzyme samples at a dosage of 50 IU with 30 g of grist per 150 mL of water in a standard mash programme (see text). Run-off SD 1.5–7.4, viscosity SD 4.3–12.1, β-glucan SD 1.3–14.9

Strain	Inducing carbon source	Run-off (mL)	Viscosity (Pa $S^{-1} \times 10^{-3}$)	β-Glucan (mg mL^{-1})
Control[a]	None	100	3.680	1,451
Wilf type (WT)	SF	115	1.867	48
WT	WB/BP[b]	112	1.810	260
WT	Rice flour	110	1.864	144
WT	Brown rice flakes	115	1.852	1,385
WT	Wheat flakes	100	1.836	332
WT	Rye flakes	100	3.086	1,200
TC2	SF	115	1.673	20
TC2	WB	105	1.587	950
TC5	SF	100	1.698	140
TC5	WB	110	1.750	305
TC5	Barley flour	115	1.689	554

[a]Control = no enzyme treatment
[b]WB/BP = 1:1 mixture of wheat bran and beet pulp

especially if these enzyme preparations can be produced in a cost-effective manner and using a waste residue (e.g. WB) as the inducing carbon source. This work reports on the isolation of two such mutants (TC2, TC5) from *T. emersonii*. Through a combination of enzyme production/profiling and hydrolysis studies (including analysis of the products of hydrolysis), coupled with mini-mash trials, the results show that these mutants have greater activity against commercial BBG and crude, mixed-DP BBG (Table 1; Fig. 3). Further biochemical and application studies are underway to explore the full biotechnological potential of these mutants.

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