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Expression of bacterial levanase in yeast enables simultaneous saccharification and fermentation of grass juice to bioethanol

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ABSTRACT

This study demonstrates use of recombinant yeast to simultaneously saccharify and ferment grass juice (GJ) to bioethanol. A modified *Bacillus subtilis* levanase gene (*sacC*) in which the native bacterial signal sequence was replaced with a yeast α-factor domain, was synthesised with yeast codon preferences and transformed into *Saccharomyces cerevisiae* (strain AH22) using the expression vector pMA91. AH22:psacC transformants secreted sacCp as an active, hyper-glycosylated (>180 kDa) protein allowing them to utilise inulin (β[2-1] linked fructose) and levan (β[2-6] linkages) as growth substrates. The control (AH22:pMA91) strain, transformed with empty plasmid DNA was not able to utilise inulin or levan. When cultured on untreated GJ levels of growth and bioethanol production were significantly higher in experiments with AH22:psacC than with AH22:pMA91. Bioethanol yields from AH22:psacC grown on GJ (32.7[±4] mg mL^{-1}) compared closely to those recently achieved (Martel et al., 2010) using enzymatically pre-hydrolysed GJ (36.8[±4] mg mL^{-1}).

1. Introduction

Residual plant biomass components from crop production and rotation systems have been identified as possible substrates for microbial fermentation of biofuels (Lin and Tanaka, 2006; Koga, 2008). Perennial herbaceous plants such as switchgrass (Schmer et al., 2008), Miscanthus (de Vrije et al., 2009) and perennial ryegrass (Martinez-Perez et al., 2007) constitute potential resources. The conversion of ryegrass (*Lolium perenne* L.) biomass to hydrogen using mixed microflora has recently been demonstrated (Kyazze et al., 2008) as has been microbial fermentation of ryegrass fructans to bioethanol (Martel et al., 2010). In the latter study, fructans in grass juice from perennial ryegrass *L. perenne* (cv. Aberdart) were hydrolysed to bioavailable fructose monomers using a novel core domain of bacterial (*Lactobacillus paracasei*) fructosidase and, with other carbohydrates, fermented to ethanol using Brewer's yeast. Bioethanol yields were higher in experiments with enzyme-pre-treated than untreated grass juice feedstock (36.5 and 28.2 mg ethanol mL^{-1}, respectively; Martel et al., 2010). However, while enzyme pre-treatment methodologies are well-documented in research literature and also used in the field (Hendriks and Zeeman, 2009 for review) they are costly and labour intensive. In the biofuel industry, there remains a need to develop cost-efficient, industrially viable biomass conversion strategies (Soetaert and Vandamme, 2005).

Microbial species that can simultaneously saccharify and ferment complex or recalcitrant substrates to biofuels (hereafter focusing on ethanol) represent a conversion technology. However, the task of isolating such species for large-scale industrial application is not straightforward (Alper and Stephanopoulos, 2009). Several of the bacteria that possess the enzymes required to hydrolyse plant polysaccharides (lignocellulose, lignin, fructans, etc.) do not ferment ethanol efficiently. For example, *Bacillus subtilis* produces both cellulase and inulinase enzymes; however, under fermentative conditions, *B. subtilis* produces lactate, acetate and butanediol, but only trace amounts of ethanol (Cruz-Ramos et al., 2000). Likewise, while ethanol yields from commercial strains of brewing yeast can be very high (Ingledew, 1999), *Saccharomyces cerevisiae* does not produce the enzymes needed to degrade recalcitrant plant biomass.

In the field of bacteriology the potential to develop ethanologenic bacteria continues to attract research attention (Barbosa and Ingram, 1994; Gold et al., 1996; Inui et al., 2004; Liu et al., 2006). In fact, the development of a laboratory *B. subtilis* strain that ferments ethanol has recently been reported (Romero et al., 2007). Bacteria possessing a native cellulosome are also of interest not least because of the potential to create and apply designer cellulosomes in the future (Bayer et al., 2007 for review). The scope of research in the area of yeast biotechnology also continues to

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expand with the identification of new industrial and commercial applications for genetically customised yeast (Aristidou and Penttila, 2000; Donalies et al., 2008; Pretorius et al., 2003). The technology to engineer yeast has been available for some time and several recombinant laboratory strains of *S. cerevisiae* have already been developed (Wanker et al., 1995b; Ho et al., 1998; Jin et al., 2005; Diaz et al., 2009).

The following study demonstrates the use of a recombinant *S. cerevisiae* strain expressing *B. subtilis* (*sacC*) levanase as an extracellular protein to simultaneously saccharify plant biomass to bioethanol. The *B. subtilis* protein was of interest because of its broad substrate spectrum (Wanker et al., 1995a) and specific activity on inulin- and levan-type fructans (β[2–1] and β[2–6] linked fructose, respectively). Both types of fructan are present in perennial ryegrass biomass (Bonnett et al., 1994; Bonnett et al., 1997; Sims et al., 1992). The main objective of this study was to determine if levels of bioethanol comparable to those previously produced using enzyme pre-treated grass juice and a commercial brewing strain (Martel et al., 2010) could be fermented using the recombinant strain.

2. Methods

2.1. Plasmid construction and yeast transformants

The *Bacillus subtilis* (*sacC*) levanase gene (Accession No. X05649) was engineered to encode a *S. cerevisiae* α-secretion signal (replacing the native *B. subtilis* secretion factor) and triplet sequences optimised for yeast protein expression (Fig. 1). The modified *sacC* gene was synthesised (GeneCust Europe) and supplied in a pUC57 shuttle vector with flanking *Bgl*II restriction sites allowing a single-digest and direct ligation into pMA91 for yeast expression driven by the phosphoglycerate kinase promoter (Mellor et al., 1983). Following gene ligation into pMA91, the resulting plasmid (psacC) was transformed into *Escherichia coli* strain DH5α (Novagen); *E. coli* transformants were cultured using Luria–Bertani media supplemented with sodium ampicillin (100 mg L^{-1}) for plasmid maintenance. Purified psacC was isolated from *E. coli* (Wizard Plus SV Minipreps, Promega) and transformed into *Saccharomyces cerevisiae* strain AH22 (MATa, *leu2-3,2-112, can1, his4-519*) using the lithium acetate method (Ito et al., 1983); this yielded the experimental construct AH22:psacC. A control construct (AH22:pMA91) was created by transforming AH22 with empty pMA91 vector. Enzymes used for DNA manipulation were purchased from Promega (Madison, Wisconsin, USA); all other media and reagents were purchased from Sigma (Poole, Dorset, UK).

2.2. Culture maintenance

Recombinant AH22 yeast transformants were selected and maintained using glucose-based yeast minimal (glucose YM) plates containing (w/v): 2% agarose, 1.34% yeast nitrogen base without amino acids, 2% glucose and L-histidine (100 mg L^{-1}). For all experimental work, single colonies were used to inoculate 20 mL volumes of glucose YM to achieve starter cultures (18 h, 30 °C, 200 rpm).

2.3. Constitutively expressed extracellular proteins

Control (AH22:pMA91) and AH22:psacC starter cultures were used to inoculate 1.5 L volumes of glucose YM. These were grown to stationary phase (30 °C, 200 rpm, 36 h) and cooled to 4 °C prior

```
  1  MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYLDLEGDFDVAVLPFSNSTN
 61  NGLLFINTTIASIAAKEEGVSLEKREAEAADSSYYDEDYRPQYHFTPEANWMNDPNGMVY
121  YAGEYHLFYQYHPYGLQWGPMHWGHAVSKDLVTWEHLPVALYPDEKGTIFSGSAVVDKNN
181  TSGFQTGKEKPLVAIYTQDREGHQVQSIAYSNDKGRTWTKYAGNPVIPNPGKKDFRDPKV
241  FWYEKEKKWVMVLAAGDRILIYTSKNLKQWTYASEFGQDQGSHGGVWECPDLFELPVDGN
301  PNQKKWVMQVSVGNGAVSGGSGMQYFVGDFDGTHFKNENPPNKVLWTDYGRDFYAAVSWS
361  DIPSTDSRRLWLGWMSNWQYANDVPTSPWRSATSIPRELKLKAFTEGVRVVQTPVKELET
421  IRGTSKKWKNLTISPASHNVLAGQSGDAYEINAEFKVSPGSAAEFGFKVRTGENQFTKVG
481  YDRRNAKLFVDRSESGNDTFNPAFNTGKETAPLKPVNGKVKLRIFVDRSSVEVFGNDGKQ
541  VITDIILPDRSSKGLELYAANGGVKVKSLTIHPLKKVWGTTPFMSNMTGWTTVNGTWADT
601  IEGKQGRSDGDSFILSSASGSDFTYESDITIKDGNGRGAGALMFRSDKDAKNGYLANVDA
661  KHDLVKFFKFENGAASVIAEYKTPIDVNKKYHLKTEAEGDRFKIYLDDRLVIDAHDSVFS
721  EGQFGLNVWDATAVFQNVTKES*
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Fig. 1. Modified *Bacillus subtilis* levanase (*sacC*) protein sequence with *S. cerevisiae* α-factor (underlined). Emboldened type indicates peptides present in native *B. subtilis* sacC protein (Accession No. A27286) identified after trypsin digestion and nano-LC/MS/MS analysis of a 75 kDa protein secreted by AH22:psacC yeast transformants (Fig. 2b, lane F1).

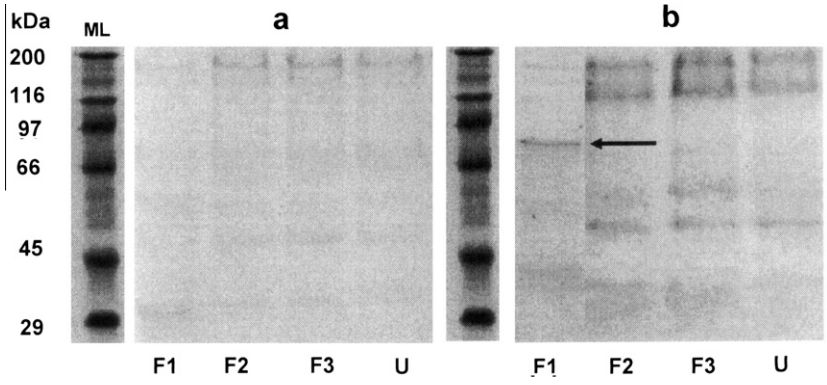

Fig. 2. Endoglycosidase treated (endo F1, F2 and F3) extracellular proteins in super-concentrated culture filtrate from (a) control (AH22:pMA91) and (b) *Bacillus subtilis* (AH22:psacC) yeast transformants. ML = molecular weight ladder; U = untreated. Arrow indicates 75 kDa deglycosylated sacC protein.

to protein work. Extracellular culture fluid and cells were first separated by centrifugation (10 min, 3000×*g*, 4 °C) and the culture fluid filter-sterilised. Cell-free fractions were then concentrated (from 1 L to 2 mL) using 15 mL Spin Concentrators (Agilent Technologies) with a MW cut off of 30 kDa. Super-concentrated culture fluid (CF) preparations ($_{A91}$CF and $_{sacC}$CF) were finally frozen (−80 °C). Extracellular proteins in 30 μL volumes of $_{A91}$CF and $_{sacC}$CF were visualised using SDS–polyacrylamide gel electrophoresis (Laemmli, 1970). Hyper-glycosylated proteins observed as diffuse bands (Fig. 2) were treated with an enzymatic de-glycosylation kit (NDEGLY, SIGMA) comprising three endoglycosidases (endo F1, F2, and F3). Endo F1 cleaves simple oligosaccharide

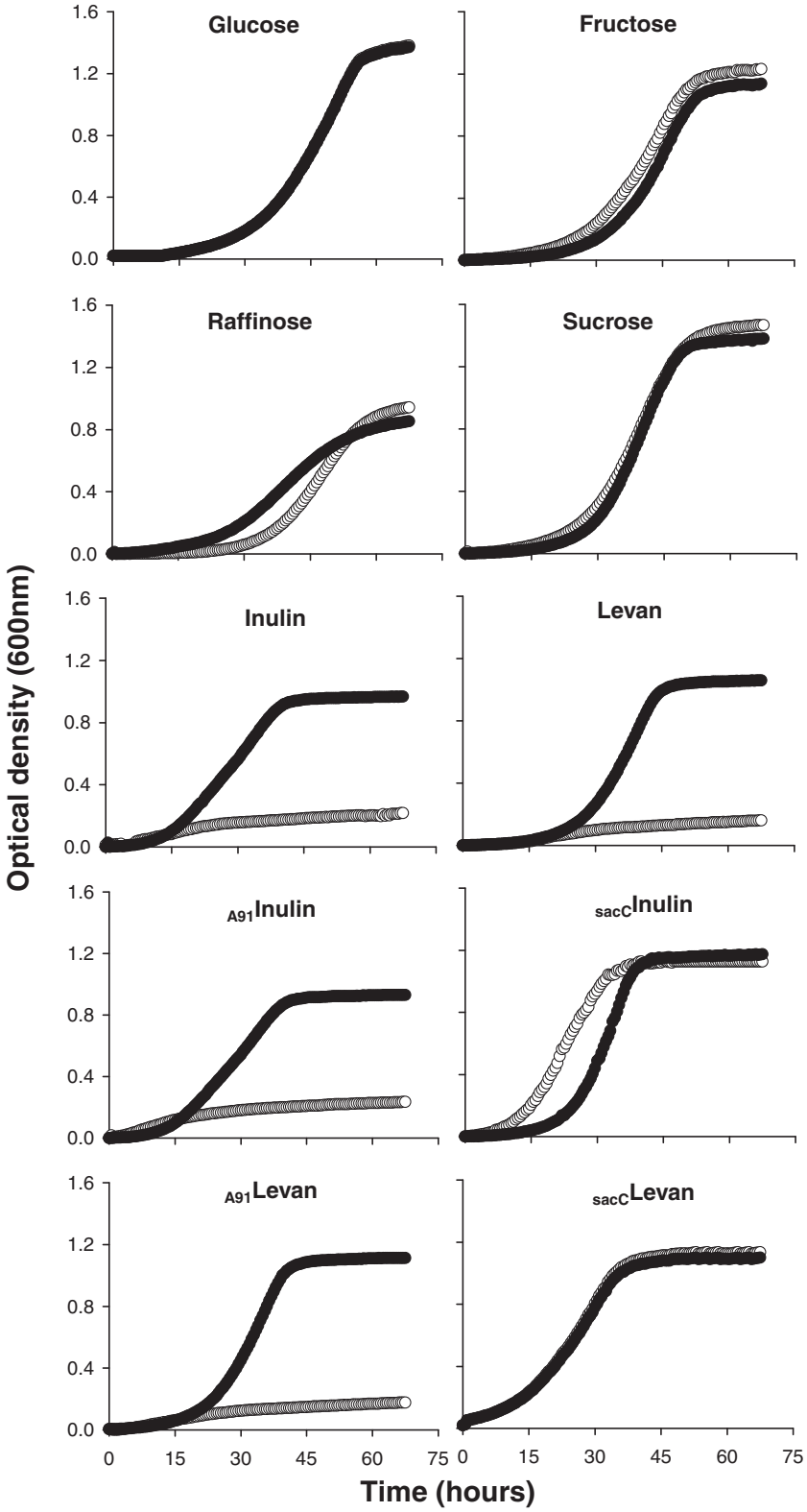

Fig. 3. Growth curves for AH22:pMA91 (○) and AH22:psacC (●) yeast transformants on untreated and pre-treated (subscript A91 and sacC) synthetic media.

chains from glycosylated proteins; endo F2 cleaves biantennary complex oligosaccharides; endo F3 cleaves triantennary complex oligosaccharides. The identity of a deglycosylated 75 kDa protein band, suspected to be extracellular sacCp (Fig. 2b), was verified by trypsin digestion and nano-LC/MS/MS analysis.

2.4. Growth experiments

Optical density (OD) measurements for AH22:pMA91 and AH22:psacC yeast transformants (Fig. 3) were made using a Bioscreen C (Oy Growth Curves Ab Ltd., Finland). For all experiments, uniform starting (t_0h) population densities (5×10^5 cells mL^{-1}) were achieved by diluting starter cultures to 1×10^7 cells mL^{-1} with filter-sterilised water and adding this cell dilution to the growth media of interest (50 μL cells + 950 μL media). The resulting 1 mL volumes were vortexed and aliquoted into microplate wells (4 × 200 μL replicates per treatment) and incubated without shaking in the Bioscreen at 20 °C. Optical density data were collected for 75 h, exported from the Bioscreen C in ASCII format and analysed using Excel (Microsoft Office 2003). In addition to manual (Neubauer haemocytometer) cell counts, ΔOD values (maximum OD − minimum OD) have been tabulated (Tables 1 and 2). Maximum doubling times (Table 1b) were estimated by dividing the natural logarithm of 2 by the fastest population growth rates (μ) derived using linear trend lines ($y = mx + c$, where y = vertical axis coordinate; m = gradient; x = horizontal axis coordinate and c = y axis intercept) fitted to log-transformed OD data.

2.5. Synthetic media

Experiments were first undertaken to compare the substrate utilisation (Fig. 3) and growth parameters (Table 1) of AH22:pMA91 and AH22:psacC transformants. Synthetic growth media were prepared as described for glucose YM excepting the use of fructose, sucrose, raffinose, chicory inulin (2% w/v) or bacterial levan (1% w/v) as alternative carbon sources. Pre-treated inulin and levan solutions (hereafter $_{A91}$Inulin, $_{sacC}$Inulin, $_{A91}$Levan and $_{sacC}$Levan) were prepared by supplementing 1.9 mL of inulin or levan with 100 μL of $_{A91}$CF or $_{sacC}$CF. The resulting 2 mL volumes were filter-sterilised and incubated at 37 °C for 18 h prior to use as substrates in growth experiments. All experiments using synthetic (and grass juice) media were conducted at 20 °C.

2.6. Grass juice media: extraction

The Bioscreen was used to monitor the growth of AH22:pMA91 and AH22:psacC transformants cultured on grass juice feedstock (hereafter GJ) extracted from the high-sugar perennial ryegrass *L. perenne* (cv. Aberdart); GJ was supplied by the Institute of Biological, Environmental Research and Rural Sciences (IBERS, UK). Briefly, stands of *L. perenne* were maintained in 20 × 1.25 m field plots under optimised management regimes (Wilkins et al., 2003). Vegetative ryegrass was harvested in mid-June using a Haldrop plot harvester and juiced using a Green-Star™ GS1000 twin screw juicer (Savant Distribution Ltd., UK). *L. perenne* biomass was processed at 4 °C within 2 h of harvest; all GJ extracts were stored and transported in a frozen state. Residual fibrous grass material was also frozen for use in alternative crop research trials.

2.7. Grass juice media: preparation

Aliquots of GJ were screened to remove large particulates, filter-sterilised (Ministart, Sartorius, 0.2 μm) and frozen (−80 °C) prior to use in 6 growth and fermentation media (Table 2) that were prepared as previously described (Martel et al., 2010). In summary, media 1 consisted of untreated GJ, media 2 comprised GJ supplemented with a novel truncated *L. paracasei* β fructosidase protein ($_t$fosEp) expressed in *E. coli* (Martel et al., 2010), media 3 comprised GJ supplemented with heat-denatured $_t$fosEp. Media 4, 5 and 6 comprised GJ supplemented with commercial exo-inulinase, commercial endo-inulinase or an exo- and endo-inulinase mixture, respectively. *L. paracasei* $_t$fosEp (our laboratory) or commercial

Table 1
Growth parameters for control (AH22:pMA91) and *Bacillus subtilis* (AH22:psacC) yeast transformants on synthetic media: a = ΔOD readings (t_{70}h); b = fastest doubling times derived from population growth curves; subscripts (A91 and sacC) indicate pre-treatment of inulin and levan solutions with extracellular fluid from AH22:pMA91 and AH22:psacC cultures, respectively.

	Growth substrate									
	Glucose	Fructose	Sucrose	Raffinose	Inulin	$_{A91}$Inulin	$_{sacC}$Inulin	Levan	$_{A91}$Levan	$_{sacC}$Levan
(a) *ΔOD*										
AH22:pMA91	1.36	1.23	1.47	0.94	0.21	0.23	1.13	0.16	0.18	1.11
AH22:psacC	1.37	1.14	1.38	0.85	0.97	0.93	1.17	1.06	1.11	1.08
(b) *Doubling time* (*h*)										
AH22:pMA91	10.5	9.6	6.3	6.4	63.6	48.5	11.0	76.2	69.3	11.1
AH22:psacC	10.5	7.4	6.4	9.8	11.7	11.8	9.9	7.4	8.0	11.1

Table 2
Growth and fermentation experiments with control (AH22:pMA91) and *Bacillus subtilis* (AH22:psacC) yeast transformants on grass juice (GJ) feedstock. Final (t_{75}h) ethanol concentrations, ΔOD and population density measurements with significantly lower values ($P < 0.05$) emboldened.

	Media					
	1 GJ	2 GJ + $_t$fosEp	3 GJ + $^{hd}_t$fosEp	4 GJ + Exo-I	5 GJ + Endo-I	6 GJ + Exo/Endo-I
Ethanol (mg mL^{-1})						
AH22:pMA91	**26.6 [4.2]**	31.8 [2.9]	**26.5 [3.4]**	31.0 [3.8]	**26.3 [1.7]**	31.0 [1.7]
AH22:psacC	32.7 [4.0]	33.3 [2.8]	32.1 [0.8]	32.9 [2.4]	33.1 [2.6]	32.7 [2.0]
ΔOptical density						
AH22:pMA91	**1.32**	1.42	**1.32**	1.45	**1.32**	1.46
AH22:psacC	1.41	1.49	1.44	1.48	1.43	1.43
Density (cells mL^{-1})						
AH22:pMA91	**1.81×10^8**	2.16×10^8	**1.68×10^8**	2.18×10^8	**1.66×10^8**	2.13×10^8
AH22:psacC	2.16×10^8	2.18×10^8	2.15×10^8	2.21×10^8	2.19×10^8	2.15×10^8

Aspergillus niger inulinases (Megazyme International Ireland Ltd) were used at a final concentration of 15 μg protein mL^{-1} (n.b. media 6 = 7.5 μg mL^{-1} of each enzyme). Previous methodology (Martel et al., 2010) was used to determine concentrations of glucose, fructose and ethanol in all growth and fermentation studies. The concentration of monosaccharides in media 1–6 at t_0h was estimated to be: (i) glucose, 7.9 [±0.4], 8.3 [±0.5], 8.0 [±0.4], 8.0 [±0.5], 8.5 [±0.5] and 8.8 [±0.5] mg ml^{-1}, respectively; (ii) fructose, 21.5 [±2.7], 36.8 [±4.1], 20.6 [±1.6], 30.5 [±1.6], 21.5 [±2.7], 27.8 [±1.6] mg mL^{-1}, respectively. Note that enzymatic hydrolysis of GJ with $_t$fosEp (media 2) yielded > 15 mg mL^{-1} more fructose than was measured in untreated GJ (media 1). The typical total sugar, fructan and protein composition of *L. perenne* (cv. Aberdart) has been reported elsewhere (Udén, 2006; Conaghan et al., 2008). Student's *t*-tests were used to determine any levels of growth and ethanol production recorded in experiments with *B. subtilis* (AH22:psacC) yeast transformants that were significantly higher ($P < 0.05$) than those seen under the same experimental conditions with control (AH22:pMA91) transformants (Table 2). Statistical analyses were performed using SPSS (Version 13.0).

3. Results and discussion

The phenotype of a yeast transformant putatively expressing sacC protein (AH22:psacC) was investigated in parallel with a control transformant (AH22:pMA91) that lacked a gene insertion. Experiments were conducted to compare the growth of both yeast transformants on several synthetic growth media, including inulin and levan; these plant fructans are not part of the natural substrate range of the *S. cerevisiae* (AH22) host. SDS–polyacrylamide gel electrophoresis and nano-LC/MS/MS analysis revealed that AH22:psacC yeast transformants expressed sacCp as an active, hyper-glycosylated protein. Growth and bioethanol experiments using grass juice feedstock (Martel et al., 2010) and both yeast transformants were also performed.

3.1. Identification of *Bacillus subtilis sacC* protein (*sacCp*)

SDS–polyacrylamide gel electrophoresis (Fig. 2) identified several native extracellular proteins in culture fluid (CF) from AH22:pMA91 and AH22:psacC yeast transformants ($_{A91}$CF and $_{sacC}$CF, respectively). Hyper-glycosylated proteins were observed as diffuse 150–200 kDa bands in untreated $_{A91}$CF and $_{sacC}$CF. Two more distinct 50 and 60 kDa protein bands were also seen in both $_{A91}$CF and $_{sacC}$CF (Fig. 2, untreated lanes).

Treatment of $_{A91}$CF and $_{sacC}$CF with endoglycosidases F2 and F3 did not alter the appearance of the protein fractions observed in each (Fig. 2, samples F2 and F3). Conversely, treatment of $_{sacC}$CF with endoglycosidase F1 yielded a distinct 75 kDa deglycosylated protein band (Fig. 2b); no comparable protein was seen in $_{A91}$CF. Trypsin digestion and nano-LC/MS/MS analysis of this 75 kDa band identified 27 peptides (Fig. 1, emboldened amino acids) that matched the deposited protein sequence for *B. subtilis sacC* (Accession No. A27286). These accounted for 45% of the full-length peptide composition for *B. subtilis* sacC protein; a Mascot score of 1430 (scores > 1000 being largely unequivocal) confirmed the identity of sacCp. That no remnant of the α-factor polypeptide was detected (Fig. 1) suggests that efficient secretion was achieved with cleavage of this signal.

3.2. Growth phenotypes of recombinant yeast

That there were no significant differences between the growth of AH22:pMA91 and AH22:psacC yeast transformants on glucose, fructose, sucrose or raffinose (Table 1 and Fig. 3) indicates that the genetic manipulations documented herein neither enhanced nor adversely affected the metabolic capabilities ordinarily seen in the AH22 host strain. By contrast, AH22:psacC (but not AH22:pMA91) yeast transformants could utilise chicory inulin (β[2–1] linked fructose) and levan (β[2–6] linkages) for growth demonstrating that heterologous expression of the *sacC* gene conferred additional fructan-degrading abilities on AH22:psacC transformants and that the extracellular sacCp protein detected was active. Pre-treatment of inulin and levan solutions with $_{sacC}$CF ($_{sacC}$inulin and $_{sacC}$levan, respectively) enabled growth of the AH22:pMA91 strain to levels comparable to those observed with AH22:psacC transformants (Fig. 3). Moreover, AH22:psacC transformants grew to higher ΔOD values and displayed faster population doubling times on $_{sacC}$inulin and $_{sacC}$levan than on untreated fructan solutions (Table 1). Conversely, as would be expected for *S. cerevisiae*, AH22:pMA91 transformants were unable to utilise pre-treated $_{A91}$inulin or $_{A91}$levan solutions for growth.

3.3. Growth and bioethanol production on grass juice (GJ)

Despite the variation in final cell densities achieved in growth experiments with AH22:pMA91 and AH22:psacC transformants (Table 2), both displayed uniform maximum [±standard deviation] population doubling times (5.6 [±0.6] h) on all GJ media. The growth ($t_{75}h$ ΔOD values and cell density) and ethanol concentrations produced by AH22:pMA91 cultured using enzyme pre-treated GJ (Table 2; media 2, 4 and 6) did not differ significantly from those recorded when AH22:psacC was cultured using identical media. Conversely, the population growth and ethanol production of AH22:pMA91 transformants cultured using untreated GJ and GJ supplemented with an endo-inulinase or heat-denatured fructosidase (Table 2; media 1, 5 and 3, respectively) were significantly lower ($P < 0.05$) than those measured in the same experiments with AH22:psacC (Table 2, emboldened values). The ethanol yields fermented by AH22:psacC transformants were always comparable to one-another (regardless of growth media) and typically higher than those measured in experiments with the AH22:pMA91 strain. The minimum and maximum [±standard deviation] ethanol concentrations recorded in experiments with AH22:psacC transformants were 32.1[±0.8] and 33.3[±2.8] mg mL^{-1} (media 3 and 2, respectively); minimum and maximum ethanol concentrations for AH22:pMA91 transformants were 26.3[±1.7] and 31.8[±2.9] mg mL^{-1} (media 5 and 2, respectively). Overall the release of fructose from fructan can be assumed to be responsible for the enhanced growth and ethanol yields of the AH22:psacC transformants compared to AH22:pMA91 transformants. The total concentrations of ethanol produced in these experiments are due to additional bioavailable carbohydrate (and non-carbohydrate) substrates present in grass biomass (Udén, 2006; Conaghan et al., 2008).

3.4. Development and application of yeast biotechnology

In an industrial setting, use of yeast that can simultaneously saccharify and ferment recalcitrant substrates would increase the overall efficiency of product (e.g. bioethanol) generation by (i) reducing the number of steps required for substrate conversion, (ii) favouring total substrate conversion and (iii) minimising losses of substrate to contaminating organisms (e.g. lactic acid bacteria). It is significant that the bioethanol yields fermented by the AH22:psacC yeast transformant in this study are comparable to ethanol concentrations (30–36.5 mg mL^{-1}) recently achieved using enzyme pre-treated grass juice and a commercial brewing strain (Martel et al., 2010). The construction of designer yeast strains that can hydrolyse and ferment alternative feedstocks to bioethanol (or

other products) constitutes an avenue for future research and development.

4. Conclusions

Grass biomass represents a potential non-food plant feedstock that could be used for the production of biofuels or other commercially relevant compounds. The use of genetically engineered yeast for bioconversion of grass feedstock is an attractive alternative to processes requiring enzyme addition. The AH22:psacC construct developed for this study constitutes a yeast expression system that could provide a route to the production of bioethanol (or other products) from grass. The phenotype observed in growth experiments with defined fructan-containing media offers a dominant marker for transformant selection where other genetic markers are not available and could have further applications in yeast biotechnology.

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