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β-Glucoside metabolism in *Oenococcus oeni*: Cloning and characterization of the phospho-β-glucosidase CelD

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ABSTRACT

In previous work, we reported characterization of a phospho-β-glucosidase gene *bglD* in a β-glucoside metabolizing operon in the oenologically important lactic acid bacterium *Oenococcus oeni*. Here we report a second phospho-β-glucosidase gene *celD* which has been cloned and expressed in *Escherichia coli*. This gene is found in a putative operon 6043 bp long encoding six genes designated *celA* to *celF*. Comparative sequence analyses of lactic acid bacteria suggest that the open reading frames of *celA*, *B* and *F* from the sequenced *O. oeni* PSU-1 encode phosphoenolpyruvate dependent phosphotransferase system (PEP-PTS) components IIB, IIA and IIC, respectively, which regulate the uptake and phosphorylation of β-glucosides across the cytoplasmic membrane. *celE* is speculated to have a regulatory function. *celD* was cloned and expressed in *E. coli* followed by purification of the gene product. The purified protein His-tagged CelD (485 residues, Mw = 55.8 kDa) has high homology to known phospho-β-glucosidases and has high activity towards the phosphorylated β-glucoside para-nitrophenol-β-D-glucopyranoside-6-phophate (pNPβG6P). CelD has an optimum pH between 4.0 and 5.0 and is most active at 40 °C. The gene *celC* was cloned, heterologously expressed and purified (481 residues, Mw = 55.7 kDa) but showed no significant activity towards pNPβG6P despite high sequence homology to *celD* and characterized phospho-β-glucosidases. Neither CelC nor CelD are active against non-phosphorylated β-glucosides.

1. Introduction

Lactic acid bacteria (LAB) are important in winemaking subsequent to the alcoholic fermentation. LAB deacidify wine in the malolactic fermentation (MLF) by converting malic acid to lactic acid. MLF is carried out principally by *Oenococcus oeni*, but also *Lactobacillus* spp. and *Pediococcus* spp. [1]. It is of interest to understand β-glucosidase metabolism in *O. oeni* as many aroma compounds in wine and must are found in the glycosidic form (i.e. linked to a sugar) yet are only perceivable in their non-glycosidic form [2]. Other glycosylated compounds found in wine, including many phenolics, are suggested to have a health effect, whilst the deglycosylated form of some may be even more beneficial [3,4]. Anthocyanins, which contribute to wine colour, are found in the glycosidic form in young wines, however, it is not clear what effect, if any, anthocyanin deglycosylation has on colour and its stability during the maturation and aging of wine. Studies on various fruit juices [5] and Sicilian blood oranges [6] show that there is a marked breakdown of anthocyanins with increasing β-glucosidase activity. However, Wightman and Wrolstad [7] demonstrated that there is no loss of pigmentation when commercial juice processing enzyme preparations of β-glucosidases are used in recommended doses using boysenberry juice as a substrate. Commercial enzymes for oenological use are mostly isolated from fungal rather than bacterial sources. Bacteria have a lower optimum pH for growth than fungi, and are able to grow in harsh post-alcoholic fermentation conditions with up to 15% ethanol (v/v) [8]. This suggests that enzymes originating from bacteria may be better suited to use in wine-like conditions.

The most common pathway for β-glucoside utilization in bacteria is dependent on extracellular or cell wall-associated β-glucosidases [9]. β-Glucosidase activity has been detected in whole cells [10,11] of 22 commercial wine strains of *O. oeni*. Spano et al. [12] identified a putative β-glucosidase gene from *O. oeni*, however, it bore low homology to the published *O. oeni* sequence [13]. Michelmyer identified a glycosyl hydrolase family (GHF) 3 β-glucosidase [14] from *O. oeni*. The presence of intracellular phospho-β-glucosidases and the phosphoenolpyruvate dependent phosphotransferase system (PEP-PTS) [15] can also be responsi-

Abbreviations: PEP-PTS, phosphoenolypyruvate-phospho transferase system; *p*NPβG6P, para-nitrophenol-β-D-glucopyranoside-6-phosphate; *p*NP, para-nitrophenol; LAB, lactic acid bacteria; MLF, malolactic fermentation; GHF, glycosyl hydrolase family; IPTG, isopropyl-D-thiogalactopyranoside.

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Table 1
Primers used for cloning and sequencing celD and celC.

Gene	Primer	Oligonucleotide sequence (5′ to 3′)	Restriction site
celD (Fwd)	AC003	CCGCTCGAGATGACTGAAACAACAAAAAGTGGATTA	XhoI
celD (Rev)	AC004	ATCGGGATCCTCAGTCTAAGTCTAATCCGTTCGAA	BamHI
celC (Fwd)	AC005	GGGAATTCCATATGAGTGAGGGAATTCAAATGCCCAAAGGT	NdeI
celC (Rev)	AC006	CCGCTCGAGCTATATGTCTAGATCTTTACCATTTGATTC	XhoI
celD (Fwd)	ORF2fwdse	GGTGATGAAACGGAACC	na[a]
celD (Rev)	ORF2bwds	CCATGGAGTGTAACCCA	na[a]
celC (Fwd)	ORF3fwdse	CAATGGTGATGAAACGG	na[a]
celC (Rev)	ORF3bwds	ACCCCATGGAGTGTAACC	na[a]

[a] Used for sequencing. No restriction site required.

ble for any whole-cell β-glucosidase activity. The PEP-PTS allows bacterial cells to grow on various carbon sources (including β-glucosides) by the simultaneous uptake and phosphorylation of the substrate. The PEP-PTS has two main cytoplasmic components, HPr and EI, which are utilized by all systems, and an EII complex specific to the substrate and normally localized in the membrane [15,16].

The *bgl* operon has already been identified in *O. oeni* by this laboratory [17] by sequence analysis and cloning and characterization of the phospho-β-glucosidase BglD. Here we report on a second β-glucoside operon to be identified in *O. oeni* by sequence analysis [13,18] from the published genome of PSU-1 (CP000411) [13]. According to comparative sequence analyses of lactic acid bacteria [18] six open reading frames (ORF) between 329032 and 333480 (PSU-1 numbering) were putatively designated as encoding components of the PEP-PTS, IIB, IIA and IIC and two (phospho-)β-glycosidases. Components IIA–IIC regulate the uptake and phosphorylation of β-glycosides across the cytoplasmic membrane [19]. No function has been assigned to the sixth ORF in this operon, *celE*.

We propose that *O. oeni* are able to take up β-glucosides via a PEP-PTS involving phosphorylation and subsequent hydrolysis of the phosphorylated glucosides in the cytoplasm through the action of phospho-β-glucosidases. The objective of this study was to gain knowledge on β-glucoside metabolism in *O. oeni*. Accordingly the ORFs putatively encoding (phospho-)β-glycosidases, designated *celC* and *celD* were heterologously expressed in *E. coli* followed by purification and characterization. The genes *celC* and *celD* were sequenced from the wine strain Lalvin 4X (VL92) to identify any sequence changes from the published genome PSU-1. This report details findings for CelD relating to substrate specificity, pH and temperature optima, kinetic data and metal ion dependency. CelC was cloned and purified however it was not further characterized due to its low specific activity towards both phosphorylated and non-phosphorylated substrates. Only the cloning strategy and protein purification is therefore shown for CelC.

2. Materials and methods

2.1. Bacterial strains and growth

Previous work in this laboratory [10,11] demonstrated the presence of β-glucosidase activity in whole *O. oeni* cells. The strain with the highest activity, Lalvin 4X (VL92) was used for all subsequent DNA work. The strain was cultivated from a glycerol stock of the freeze dried commercial form in de Man, Rogosa and Sharpe broth (Amyl Media, Dandenong, VIC Australia) supplemented with 10% (v/v) filter sterilized preservative free apple juice and grown at 30 °C for approximately 4–5 days. The *E. coli* strain DH5α (Invitrogen Australia Pty Limited, Australia) [20] was used for cloning and sequencing (Sequencing by the Australian Genome Research Facility, Brisbane Node, QLD, Australia). The *E. coli* strain BL21 (DE3) pLysS (Novagen, USA) was used for protein expression. Both strains were maintained in 26% glycerol (v/v) at −80 °C and cultivated in Terrific Broth [21] at 37 °C overnight. When necessary the Terrific Broth was supplemented with 34 μg/mL chloramphenicol and/or 50 μg/mL of ampicillin sodium salt.

2.2. PCR amplification and cloning of celD and celC

celD, a 1458 bp fragment, was amplified by PCR using primers AC003 and AC004 (Table 1). The PCR fragment was cleaned with the Wizard® Plus PCR cleanup kit (Promega, Corporation, Sydney, Australia) digested with XhoI and BamHI (New England Biolabs, USA) in accordance with the recommended protocols and cloned into a previously digested pET 14.b plasmid (Novagen) to yield plasmid pET 14.b-*celD*. Pfu polymerase (Integrated Sciences, NSW, Australia) was used according to the manufacturer's instructions to amplify the ORF. *celC* was initially amplified by PCR with primers AC005 and AC004 (Table 1) to obtain a 2919 bp fragment and then cleaned using DNA PCR wizard clean up kit (Promega) according the manufacturer's instructions. This PCR fragment was used as template DNA and re-amplified by PCR using primers AC005 and AC006 to obtain a 1446 bp fragment, which encodes the *celC* gene. This fragment was digested with NdeI and XhoI and cloned into similarly digested plasmid pET 14.b. Plasmid DNA was extracted using the Wizard® Plus SV Minipreps DNA Purification System (Promega). Plasmid DNA was sequenced directly using primers listed in Table 1 three times for each gene to ensure PCR fidelity.

2.3. Expression of celD and celC in E. coli

Overnight cultures of pET 14.b-*celD* and pET 14.b-*celC* in BL21 (DE3) pLysS were inoculated (1/100 dilution) into Terrific Broth and grown at 37 °C until reaching an optical density of 0.6 at 600 nm. The temperature was then lowered to 15 °C for approximately 15 min and subsequently isopropyl-D-thiogalactopyranoside (IPTG) (Sigma–Aldrich, Australia) was added to a final concentration of 1 mM. Flasks were left on a shaker at 160 r.p.m. for 48 h at 10 °C. The cells were harvested by centrifugation (13,000 × *g* for 10 min at 5 °C) and lysed with amine free Bugbuster Mastermix (Novagen) according to the manufacturer's instructions.

2.4. Purification of CelD and CelC

The pET 14.b-*celD* and pET 14.b-*celC* constructs both incorporated a polyhistidine-tag at the N terminus. The resulting fusion proteins were purified using immobilized metal-affinity chromatography with Talon® cobalt resin (Clontech, USA) according to the manufacturer's instructions. Protein fractions from the purifications were eluted with McIlvaine buffer [22] at pH 4.5 and analyzed by SDS-PAGE [23] and quantified by the method of Bradford [24]. Long term storage was achieved at −80 °C, and short term (2 months) at 4 °C in 10% (v/v) glycerol.

Table 2
Gene arrangement of the *cel* operon of *O. oeni*, derived from the published genome sequence PSU-1 [13] with proposed gene names.

ORF	Gene name	Function
OEOE 0338	*celA*	PTS system IIB component
OEOE 0339	*celB*	PTS system IIA component
OEOE 0340	*celC*	Putative phospho-β-glycosidase
OEOE 0341	*celD*	6-Phospho-β-glucosidase
OEOE 0342	*celE*	Putative transcriptional regulator
OEOE 0343	*celF*	PTS system IIC component

2.5. Assay of β-glucosidase and phospho-β-glucosidase activity

β-Glucosidase enzyme activity was quantitatively determined by measuring para-nitrophenol (*p*NP) released from *p*NP-β-D-glucopyranoside, *p*NP-α-D-glucopyranoside, ortho-nitrophenol-β-D-glucopyranoside, *p*NP-β-D-galactopyranoside, *p*NP-α-D-galactopyranoside, *p*NP-α-xylopyranoside, *p*NP-α-L-rhamnopyranoside and *p*NP-α-L-arabinofuranoside. Unless otherwise stated the standard assay consisted of incubation of the purified histidine tagged enzyme with a final substrate concentration of 2 mM in McIlvaine buffer at pH 5.5 for 30 min in a total volume of 50 μL at 37 °C. Phospho-β-glucosidase activity was measured in assay conditions identical to those described above, using the phosphorylated substrate *p*NP-β-D-glucopyranoside-6-phosphate (*p*NPβG6P). This substrate was prepared from the non-phosphorylated glycoside by the method of Wilson and Fox [25]. The amount of *p*NP released was determined by measuring the absorbance of the reaction mixture at 400 nm [26]. All assays were blanked against ultra pure water (18.2 MΩ cm) and had the control (substrate minus enzyme) taken away from the absorbances read at 400 nm. BglD, a phospho-β-glucosidase reported previously [17] and a β-glucosidase from *Aspergillus niger* (Sigma–Aldrich, Australia) were used as positive controls under standard assay conditions. Specific activity was expressed in μmole of *p*NP liberated per minute per mg of enzyme under the standard assay conditions.

The pH and temperature optima of the purified CelD were determined. Accordingly, pH values ranging from pH 3.0 to 7.5 were used in the standard assay, whilst the effect of temperature was determined by pre-incubating buffer and substrate for 10 min in a PCR machine in 0.2 mL PCR tubes at the chosen assay temperature prior to the addition of enzyme. Assays were incubated for 30 min at temperatures ranging from 4 °C to 70 °C, transferred to a 96-well microtitre plate and absorbance at 400 nm recorded. The kinetic constants, K' and V_{max} correspond to the activity determined at 25 °C. The effect of the metal ions Mn^{2+}, Mg^{2+}, Cu^{2+}, Co^{2+} and Ca^{2+} at a concentration of 10 mM was also investigated. All assays were performed in triplicate and were repeated at least three times.

3. Results and discussion

3.1. Sequence analysis of the cel operon

From the published *O. oeni* PSU-1 genome sequence (CP000411) [13] and comparative studies on genomes of lactic acid bacteria, six genes were identified that are transcribed in the same direction [18]. Sequence homology of the translated DNA in conserved regions to characterized bacterial genes indicates that they form components of a PEP-PTS operon in this organism (Table 2). This 6043 bp region we have designated *cel*, based on sequence similarity to *cel* genes from various organisms (Table 3) and to distinguish it from the *bgl* operon in *O. oeni* previously characterized in this laboratory [17]. The *cel* operon is speculated to function by phosphorylation and uptake of β-glycosides for use as alternative carbon sources.

celC and *celD* were both sequenced, demonstrating two amino acid changes in *celD* and one in *celC* (underlined in Fig. 1) from the published PSU-1 sequence, all of which are found in non-conserved and non-catalytic regions. *celD*, a putative (phospho-)β-glycosidase, is assigned to the glycosyl hydrolase family (GHF) 1 [27–29] based on sequence homology and highly conserved signature sequences (Fig. 1). *celD* is found on the *O. oeni* genome 17 bp from a putative (phospho-)β-glycosidase *celC*. There are few differences between GHF 1 phospho-β-glucosidases and GHF 1 β-glucosidases, which can cause confusion when attempting to infer enzyme function from the sequence alone. One principal difference appears to be the serine located in position 453 (numbers according to Fig. 1), which has been shown to be at least partially responsible for the affinity of GHF 1 phospho-β-glucosidases for phosphorylated substrates [30]. In characterized GHF 1 β-glucosidases the serine in position 453 is typically replaced by a glutamic acid residue. The one known exception being from *Pectobacterium carotovorum*, in which the characterized GHF 1 β-glucosidase/phospho-β-glucosidase AscB has an arginine in position 453 and demonstrates an affinity for both phosphorylated and non-phosphorylated substrates.

Both the putative (phospho-)β-glycosidase CelC and the (phospho-)β-glycosidase CelD characterized in this study show high homology to the characterized proteins: BglB in *E. coli*, BglA and BglH *in Bacillus subtillis*, ArbB in *Erwinia chrysanthemi*, AbgA in *Clostridium longisporum*, CelA in *Streptococcus mutans* and AscB, BglA, BglB in *P. carotovorum* (Table 3). The hydrolysis of a glucosidic bond involves two catalytic residues: a proton donor (acid) and a nucleophile (base) [31]. Based on sequence comparison with characterized phospho-β-glucosidases the active site for CelD is located in position 390–399 and comprises the residues FIVENGLGA with the glutamic acid (position 392) residue acting as the catalytic nucleophile [30,32]. CelD also shows a highly conserved region at position 183–188 containing TFNEIN whereby a second glutamic acid (position 186) has been demonstrated in other species [30] to be the active proton donor residue (Fig. 1).

BLAST analysis demonstrates that CelE has homology to the DNA binding HTH region of *Bacillus subtilis* GlvR and YebF from *Lactococcus lactis* regulatory gene. GlvR was found to be fundamental for the transcription of the *glv* operon [33], and the interruption of YebF demonstrated complete lack or severe inhibition of growth in cellobiose- and/or lactose-containing media, suggesting that YebF is involved in the metabolism of cellobiose and lactose through the PEP-PTS system [34]. To gain some insight into the biological function of CelC and CelD, the products of these gene products were expressed and characterized in *E. coli*.

3.2. Expression, purification and characterization

CelC showed no significant activity towards either phosphorylated or non-phosphorylated β-glycoside substrates (data not shown), and thus only data relating to the cloning and characterization of CelD are presented. No activity has since been defined (<0.27 μmole *p*NP/min/mg), referring to the greatest specific activity found for CelC (data not shown) whereby mg rather than pg of enzyme were used, thus lowering the specific activity to a point which was deemed not active. Only the cloning strategy and protein purification has been shown for CelC. pET 14.b-*celD* and pET 14.b-*celC* in BL21 (DE3) pLysS produced large amounts of soluble IPTG-inducible protein whose estimated molecular weight, 55.8 and 55.7 kDa, respectively, corresponded to that expected for the full length polypeptides encoded by CelD and CelC. Purification was achieved as described in Section 2. Protein fractions analyzed by SDS-PAGE revealed the presence of a single protein band for both CelD and CelC (~55 kDa, Fig. 2). Fractions that contain these proteins were pooled, quantified, aliquoted and stored at 4 °C and

Table 3
Degree of identity to known sequences of the genes in the cel operon.

Protein	Putative homologues		Percentage (%)		Accession number	Reference
			Positives	Identity		
CelA	*Pectobacterium carotovorum*	BglE	66	47	AY769096	[38]
		CelE	69	50	DQ987482	[39]
	O.oeni	BglC	84	73		[17]
CelB	*Streptococcus mutans*	CelD	72	43		[40,41]
	Pectobacterium carotovorum	BglI	62	36	AY769096	[38]
	O.oeni	BglB	82	64		[17]
CelC	*O.oeni*	BglD	77	63		[17]
	E.coli	BglA	72	58	Q46829	[42]
		AscB	66	47	M73326	[43]
		BglB	65	51	M16487	[42,44]
	Bacillus subtillis	BglA	73	58	P42973	[45]
		BglH	68	54	Z34526	[46]
	Erwinia chrysanthemi	ArbB	65	50	M81772	[47]
	Clostridium longisporum	AbgA	66	49	L49336	[48]
	Pectobacterium carotovorum	AscB	63	45	AY622309	[26]
		BglA	73	57	AY769096	[38]
		BglB	67	50	AY542524	[49]
		CelG	47	30	DQ987482	[39]
	Streptococcus mutans	CelA	81	66	NC_004350	[40,41]
	Lactobacillus plantarum	BglH	64	50	AJ250202	[50]
CelD	*O.oeni*	BglD	85	73		[17]
	E.coli	BglA	73	58	Q46829	[42]
		AscB	66	47	M73326	[43]
		BglB	64	50	M16487	[42,44]
	Bacillus subtillis	BglA	73	58	P42973	[45]
		BglH	67	53	Z34526	[46]
	Erwinia chrysanthemi	ArbB	65	50	M81772	[47]
	Clostridium longisporum	AbgA	66	49	L49336	[48]
	Pectobacterium carotovorum	AscB	65	48	AY622309	[26]
		BglA	73	59	AY769096	[38]
		CelG	43	29	DQ987482	[39]
		BglB	68	52	AY542524	[49]
	Streptococcus mutans	CelA	81	66	NC_004350	[40,41]
	Lactobacillus plantarum	BglH	65	51	AJ250202	[50]
CelE	*Bacillus subtilise*	GlvA[a]	54	34	P54717	[33]
	Lactoccocus lactis	YebF[a]	69	44	NP_266568	[51]
CelF	*Streptococcus mutans*	CelE	53	33		[40,41]
	Pectobacterium carotovorum	CelF	55	35	DQ987482	[39]
	Pectobacterium carotovorum	BglF	56	33	AY769096	[38]
	O.oeni	BglA	52	33		[17]

[a] Homologous to the HTH domain, rpiR family; pfam01418.

−80 °C. CelD maintained 100% activity when stored at 4 °C for up to two months or at −80 °C in 10% (v/v) glycerol for up to 2 years (data not shown).

No activity was detected when assaying CelD against non-phosphorylated β-glucosides (data not shown), where the enzyme, buffer and substrate were added did not show any variation from the control (substrate plus buffer minus enzyme). The effect of pH on the activity of CelD against *p*NPβG6P was determined at 37 °C in buffers ranging in pH from 3.0 to 7.5 (Fig. 3A).

CelD had an optimum pH of 4.0–5.0, and retained high levels of activity (above 78%) at pH values of up to 6.0. Typically wines range from pH 2.9 to 3.4 for whites and pH 3.4 to 3.6 for reds [35]. The application of CelD in the modification of wine aroma is not necessarily precluded by such pH values as the enzyme requirement for phosphorylated substrates dictates that it be applied in whole cell form where higher pH values are likely.

The temperature dependence of CelD was determined using *p*NPβG6P at an assay pH of 5.5. This pH was selected because CelD demonstrated high activity at this value (above 80%) and absorbance readings could be taken without prior dilution or pH adjustment. CelD exhibited a temperature optimum of 40 °C, below which activity was largely retained such that, as much as 74% was seen at 4 °C (Fig. 3B). By comparison, at values above 40 °C, activity declined rapidly so that only 2% was retained at 60 °C and none at 70 °C. Winemaking conditions classically vary from approximately 10 °C for pre-fermentation cold maceration and up to maxima around 33 °C in red wine fermentations in the middle of the cap [36], a temperature range across which CelD has high activity.

The activity of CelD was measured in the presence of various divalent metal ions (Fig. 3C). In a winemaking context, calcium and magnesium cations are present in the greatest amounts (up to 150 mg/L; thus 3.75 mM Ca^{2+} and 6.25 mM Mg^{2+}) whilst cobalt, manganese and copper are found in trace amounts [35]. Typical concentrations of copper in wine are 0.3–0.4 mg/L [35] which equates to approximately 6 nM. Calcium is derived from vineyard and deacidification treatments, but forms insoluble salts which tend to precipitate out of the wine. Magnesium is present in the grape and remains in wine in the form of soluble salts, whilst copper can originate from copper fining treatments. In the absence of enzyme, no chemical hydrolysis of pNP was evident when incubated in the presence of 10 mM metal ions for 1 hour at 37 °C. The phospho-β-glucosidase activity seen in the presence of enzyme was significantly increased by the addition of 10 mM Ca^{2+} or Mg^{2+}. By comparison 10 mM Mn^{2+} or Co^{2+} resulted in activities 20% lower than seen in the absence of these ions, both of which were not statistically significant, ($p > 0.05$) to the control. Near complete inhibition was seen with 10 mM Cu^{2+}. Whilst the latter three metal ions were inhibitory in our assay this might not necessarily be the case at the lower concentrations typically found in wine. Some support for this possibility comes from the fact that the highly structurally

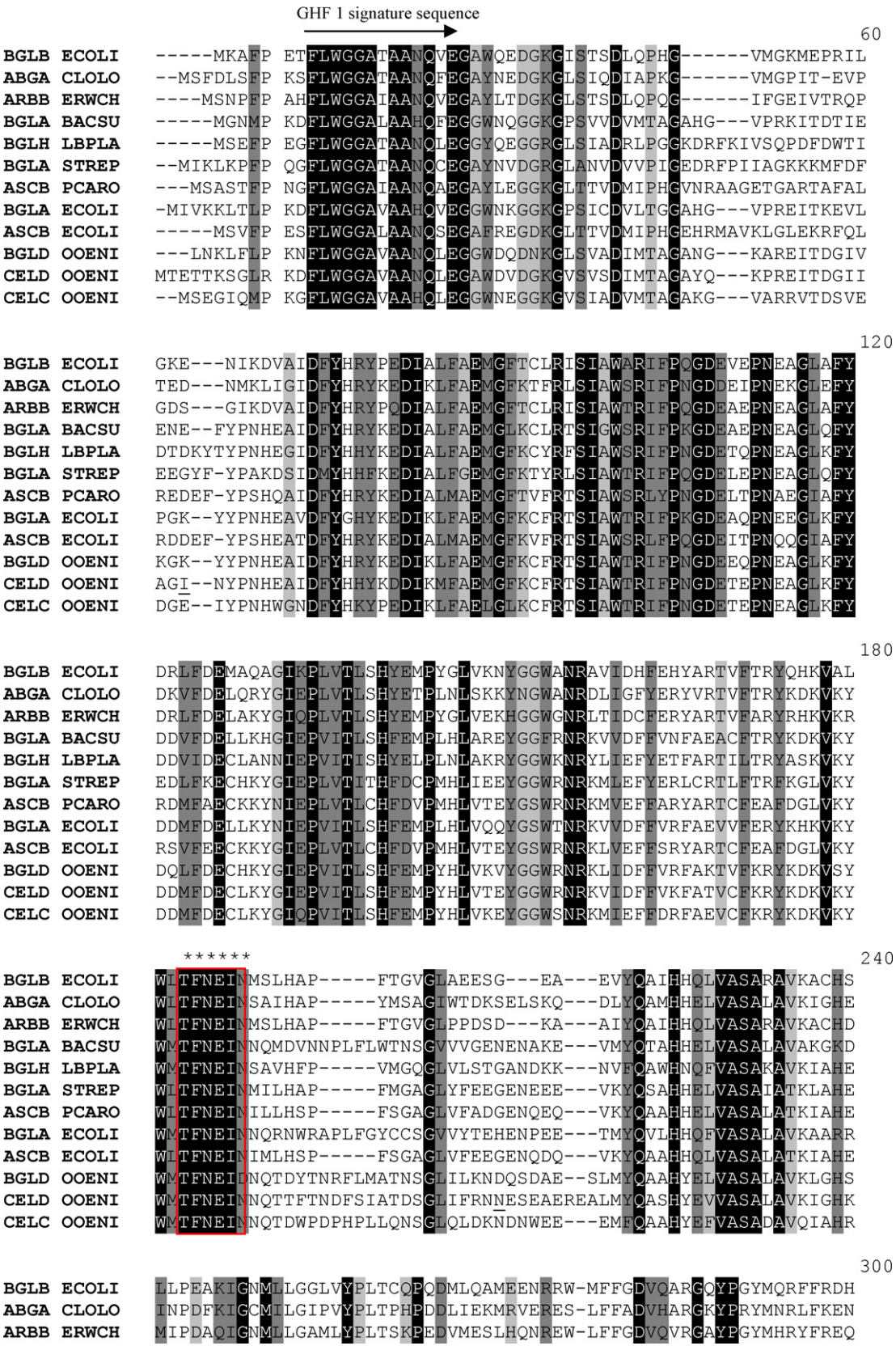

Fig. 1. Alignment of the amino acid sequences of phospho-β-glucosidases from characterized GHF 1: BGLB ECOLI, *E. coli* BglB. ABGA CLOLO, *Clostridium longisporum* AbgA. ARBB ERWCH, *Erwinia chrisanthemi* ArbB. BGLA BACSU, *Bacillus subtilis* BglA. BGLH LBPLA, *Lactobacillus plantarum* BglH. BGLA STREP, *Streptococcus mutans* BglA. BGLH BACSU, *Bacillus subtilis* BglH. ASCB PCARO, *Pectobacterium carovatorum* AscB. BGLA ECOLI, *E. coli* BglA. ASCB ECOLI, *E. coli* AscB. CELD OOENI, *O. oeni* CelD. CELC OOENI, *O. oeni* celC. BGLD OOENI, *O. oeni* bglD. CELA STREP, S*treptococcus mutans* celA. Identical residues are highlighted white lettering on a black background, highly conserved residues are highlighted with black lettering on dark grey and conserved residues are represented by black lettering on light grey. The numbers to the right denote residue positions, asterisks denote the conserved active sites and the numbers above the residues refer to alignment positions. Amino acid changes in celC and celD from the sequenced genomic strain PSU-1 are underlined.

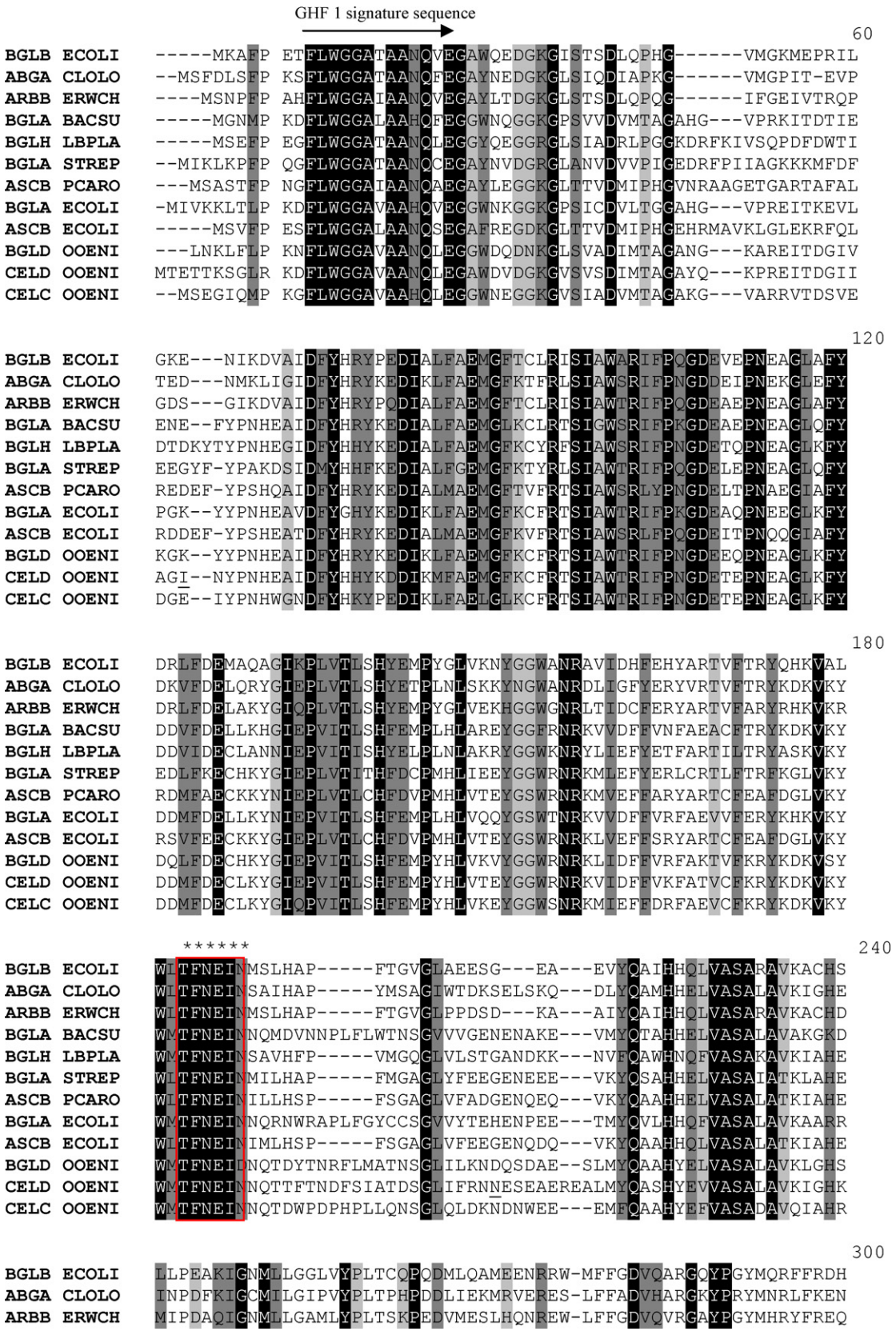

Fig. 1. (*Continued*).

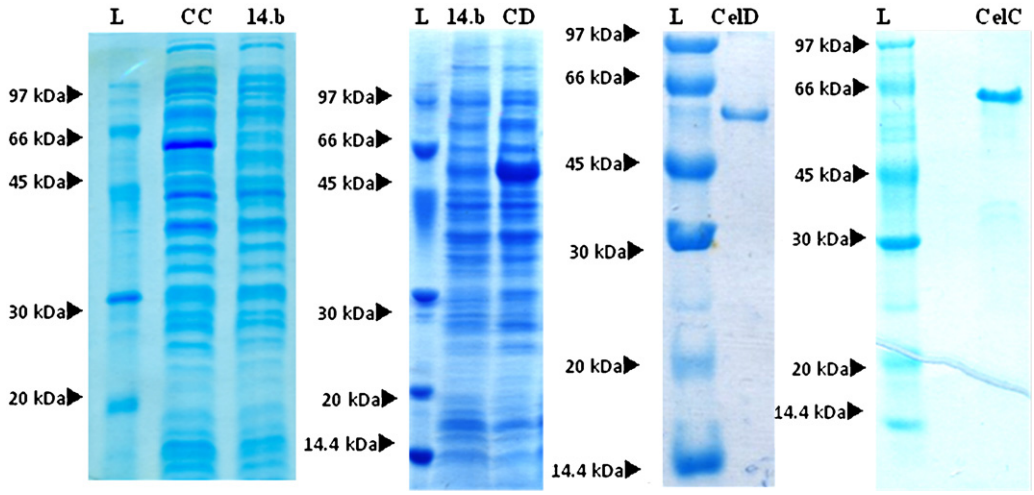

Fig. 2. Electrophoretic analysis of the purified phospho-β-glucosidase CelD and CelC. **L**, Low molecular weight marker. **14.b**, Crude extract of vector pET 14.b without CelD or CelC inserted. **CD**; Crude extract of vector pET 14.b with the *celD* gene inserted. **CC**; Crude extract of vector pET 14.b with the *celC* gene inserted. **celD**; purified CelD. **celC**; purified CelC. Separation was performed on a 12% (w/v) SDS-polyacrylamide gel. The gel was stained with 0.025% (w/v) Coomassie blue.

similar phospho-β-glucosidase isolated from *P. carovotorum*, *ascB*, (Table 3) exhibits losses of only about 10% when assayed with 5 mM Cu^{2+}, Co^{2+} and Mn^{2+}, as well as comparable increases in the presence of Mg^{2+} and Ca^{2+} [26]. Of course, further experimental work under wine conditions at appropriate cation conditions is required to confirm this.

The kinetic data from CelD produced a sigmoidal curve, with a V_{max} of 176.7 μmole pNP liberated/min/mg protein, K' of 1211 μmole pNPβG6P, a Hill slope of 2.831 and an R^2 coefficient of 0.987 (Fig. 3D), indicating positive cooperativity [37]. This suggests a potential regulatory role for CelD, in which its activity is modulated by interaction with an allosteric regulator namely the

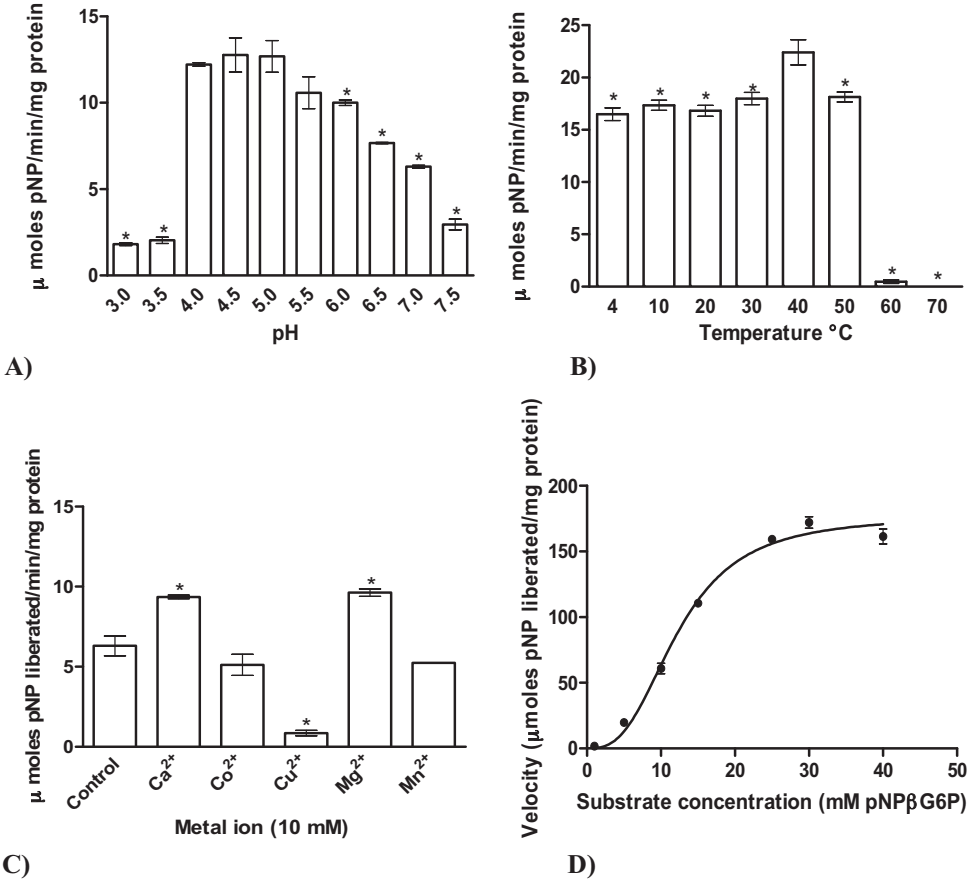

Fig. 3. Effect of pH, temperature and metal ion on the activity of CelD. (A) Effect of pH on the specific activity of CelD. Enzyme activity was assayed at 37 °C for 30 min. in McIlvaine buffers of indicated pH. (B) Effect of temperature on the specific activity of CelD. Enzyme activity was assayed at pH 5.5 for 30 min at the indicated temperature. The buffer was brought to temperature 10 min prior to commencement of the assay in a PCR machine. (C) Effect of 10 mM addition of Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+} on the specific activity of CelD. Enzyme activity was assayed at pH 5.5 for 30 min. (D) Effect of substrate concentration on enzyme kinetics (CelD), analyzed with allosteric sigmoidal non linear regression. *Significant differences compared to control on each panel (one-way ANOVA with Tukey's multiple comparison post test, $p < 0.05$).

substrate. It is of interest to note however, that the product of CelD, glucose-6-phosphate, is a direct product of the first enzyme of glycolysis and therefore it may be that factors regulating glycolysis are active also in the regulation of CelD activity [15].

We have characterized the phospho-β-glucosidase CelD in a putative PEP-PTS operon from a wine strain of *O. oeni*. The enzyme has a molecular weight of 55.8 kDa, exhibits positive cooperativity, pH and temperature optima of 4.0–5.0 and 40 °C respectively. Calcium and magnesium ions increased activity. Based on the characterization of other PEP-PTSs and their components in other bacteria, CelD is proposed to be an intracellular enzyme that acts on phosphorylated β-glucosides. Further experimental work needs to be undertaken in order to understand the specific role of CelD, a second putative phospho-β-glycosidase CelC and the other components of the *cel* operon involved in β-glucoside metabolism in *O. oeni*. Given the potential impact of β-glycosidases on the sensory profile of wine, it is hoped that the characterization of β-glycosidase systems from LAB, will provide information to aid winemakers in tailoring wine aroma, colour and overall complexity.

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