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Thesis title:

Genes and mechanisms responsible for β-glucoside metabolism in the oenologically important lactic acid bacterium *Oenococcus oeni*

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Abstract

The lactic acid bacterium *Oenococcus oeni* plays a pivotal role in winemaking by carrying out malolactic fermentation (MLF), which results in the decarboxylation of L-malic acid to L-lactic acid. It is the species commonly inoculated for MLF but also it will often spontaneously develop after alcoholic fermentation because of its superior tolerance to wine conditions such as high alcohol (up to 16% v/v), low pH (from 3.0 to 4.0) and little or no residual sugar. A marked increase in aroma has been reported after the completion of MLF. This increase has been principally attributed to enzymatic modifications by lactic acid bacteria. In accordance with this *O. oeni* has been reported to possess β-glucosidase activity. The hydrolysis of β-glucosides in wine can have a significant impact on the sensory profile of a wine by conferring an increase in aroma. Many aroma compounds in wine and must are found in the glycosidic form (i.e. linked to a sugar) and are only perceivable in their non-glycosidic form. For this reason it is of interest to characterise such activities, particularly in *O. oeni*.

Comparative sequence analyses of lactic acid bacteria suggest that six open reading frames (AG1 and ORFs 1 to 5) from the sequenced *O. oeni* PSU-1 are involved in the hydrolysis of β-glucosides. The ORFs 1 to 3 demonstrated homology to glycosyl hydrolase family (GHF) 1 β-glucosidase/β-glucanase/phospho-β-glucosidase N-terminal and active site signature sequences, whilst AG1 and ORF 4 were lacking the N-terminal signature sequence. Glycosyl hydrolase family 3 β-glucosidase signature sequences

were identified in ORF 5. ORF 1 (subsequently designated *bglD*) was characterised as a GHF 1 phospho-β-glucosidase and found to be part of a phosphoenolpyruvate phosphotransferase system (PEP-PTS) β-glucoside metabolising operon, *bgl*. Site directed mutagenesis identified a single amino acid responsible for the affinity of BglD towards phosphorylated substrates, providing insight to the catalytic mechanism for all GHF 1 enzymes. ORF 2 and 3 (designated *celD* and *celC*) were also characterised as GHF 1 phospho-β-glucosidases and are components of a second PEP-PTS β-glucoside metabolising operon, *cel*. Neither AG1 nor ORF 4 could be expressed as soluble proteins and it is speculated that the lack of the GHF 1 N-terminal signature sequence is responsible for this. ORF 5 was found to be a GHF 3 β-glucosidase. Transcriptional analysis indicates that these β-glucosidase metabolising operons may be regulated by carbon catabolite repression and transcriptional anti-termination.

Given the potential impact of β-glycosidases on the sensory profile of wine, it is hoped that the characterization of β-glycosidase systems from *O. oeni* will provide information to aid winemakers in tailoring wine aroma, colour and overall complexity where grape quality may otherwise be compromised due to adverse weather conditions or poor viticultural practices.

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His support, love and encouragement have made this journey an awesome life experience.

Statement of Authorship

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to **Alana Capaldo** and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Alana Capaldo

Chapter 1

1. Literature Review

1.1. Introduction: Winemaking and enzymes

In recent years, winemaking has gone from an age old tradition to a scientifically enhanced production line. The sensory profile of a wine can be drawn from many aspects of the winemaking process. The grapes from which the wine is derived play an important role in defining the varietal characteristics (sensory properties associated with a particular type of grape) and their quality affects both the microbial and chemical stability of the end product. Vinification practices including oak treatment, maceration (grape skin/juice contact), and the use of inoculated or indigenous yeast and bacteria strains also impact on the structure, palate weight and aroma profile. Techniques can also be used to enhance the amount of aroma released from grapes, especially when these are of less than optimum quality due to inappropriate viticultural practices, disease and unfavourable weather conditions. Blending of more than one wine is common and may mask faults; however desired varietal characteristics might also be subdued.

The microbial population associated with winemaking has been of great interest as a source of enzymes with the ability to increase aroma and other attributes linked to wine quality. In an ever increasing eco-friendly society where 'natural is better', enzymes derived from wine-associated micro flora are regarded as a less invasive path than exogenous enzymes. Microbes are found in the vineyard and winery as well as new French oak barrels (Goddard

et al. 2010). During alcoholic fermentation, yeast (commonly *Saccharomyces cerevisae*) is either inoculated as pure cultures or forms part of the indigenous population of *S. cerevisiae* and non-*Saccharomyces* species eg *Kloeckera*, *Hanseniaspora*, *Brettanomyces*, *Debaryomyces*, *Candida*, *Metschnikowia*, *Pichia*, *Torulaspora* and *Zygosaccharomyces* (Howell et al. 2006). Both non-*Saccharomyces* and *S. cerevisiae* species are responsible for the conversion of sugar to ethanol, however the former are typically much less tolerant of ethanol (5 - 8% (v/v)), allowing *S. cerevisiae* to outcompete the non-*Saccharomyces* species. A secondary malolactic fermentation (MLF) conducted by lactic acid bacteria (LAB) decarboxylates L-malic acid to L-lactic acid with the production of carbon dioxide (Wibowo et al. 1985). *Oenococcus oeni* is the most commonly inoculated LAB for MLF but *Lactobacillus spp.* and *Pediococcus spp* may also be found in wine. LAB associated with winemaking have been reported to possess β-glucosidase activity (Grimaldi et al. 2005a, 2005b, 2000). Since many aroma compounds in wine and must are found in the glycosidic form (i.e. linked to a sugar) and are only perceivable in their non-glycosidic form (Maicas and Mateo 2005), it is of interest to characterise such activities, particularly in *O. oeni*.

1.1.1. LAB in wine

Lactic acid bacteria (LAB) are able to decarboxylate L-malic acid to L-lactic acid in malolactic fermentation (MLF) (**Figure 1**). Typically this occurs after the primary alcoholic fermentation has ceased and there is minimal residual sugar together with high alcohol levels. An operon in the PSU-1 genome designated *mle* has been identified as being responsible for the conversion of

malate to lactate (Denayrolles et al. 1994, Labarre et al. 1996). The *mle* operon is conserved among LAB (Denayrolles et al. 1994) and contains three genes, *mleA*, *mleP*, and mleR encoding malate decarboxylase, malate permease and a regulatory product respectively. As a consequence, species of *Pediococcus*, *Lactobacillus*, *Leuconostoc* and *Oenococcus* are able to carry out MLF (Wibowo et al. 1985, Lonvaud-Funel 1999, Mills et al. 2005, Salema et al. 1996). *Lactobacillus* spp. and *Pediococcus* spp. grow within 2 - 4 days in higher pH wine conditions (>pH 3.5) (Lerm et al. 2010) and are, therefore, more likely to be present in the initial stages of MLF in high pH wines. High pH conditions can be found in emerging riper, fruitier red wine styles in Australia, and because of this, strains of *Lactobacillus plantarum* are now of interest from a commercial point of view (du Toit et al. 2011). pH seems to be a determining factor in the natural selection of dominant species which carry out MLF. Interestingly it is *O. oeni* that typically outcompetes *Lactobacillus* spp and *Pediococcus* spp during MLF in wines with a pH below 3.5. Conversely, *O. oeni* has slightly less tolerance to sulphur dioxide than *Lactobacillus* (du Toit et al. 2011, Delfini and Morsiani 1992) and *Pediococcus* spp (Lerm et al. 2010), and takes longer to grow in deMan, Rogosa and Sharpe (MRS) broth under ideal laboratory conditions. Molecular techniques (Lonvaud-Funel et al. 1991, Reguant and Bordons 2003, Cocolin et al. 2011) have been extensively trialled and optimised to analyse the dynamics of the bacterial population that spontaneously develops to carry out MLF. All of these studies have concluded that *O. oeni* is able to withstand the adverse conditions found in wine with a pH below 3.5, proliferate and outcompete other LAB species.

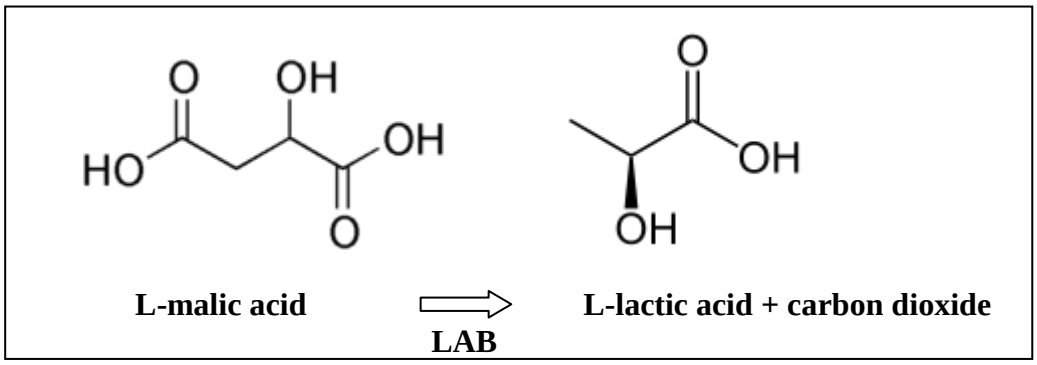


Figure 1. Schematic representation of the decarboxylation of dicarboxylic acid malic acid to mono-carboxylic acid lactic acid with the resultant deacidification of wine.

1.1.2. Sensory impact of MLF on wine

The conversion of malic acid to lactic acid by LAB contributes significantly to the organoleptic quality of wine. MLF is either purposefully inoculated or left to spontaneously commence in red wines and some white wines whereby a more complex and developed sensory profile, as opposed to a fresher style, is sought (Liu 2002). By undergoing a deacidification process during the secondary fermentation, there is a contribution to the palate weight and balance of the wine as well as its microbial stability. Besides the changes in sensory properties directly attributed to the conversion of malic acid to lactic acid, there are a number of aroma active compounds which are made more or less available due to bacterial interactions with the wine matrix, external influences such as oak, and other microflora present (Lerm et al. 2010).

1.2. Carbohydrate metabolism of LAB contributing to wine aroma

1.2.1. Citrate metabolism and diacetyl formation

One of the most important volatile compounds produced during MLF is diacetyl (Bartowsky et al. 2002). Formed as an intermediate in citrate metabolism by *O. oeni*, it is thought to be metabolized in the same way by *Lactobacillus* spp (du Toit et al. 2011). Diacetyl, which imparts a buttery or butterscotch flavour, is unstable and tends to be reduced to acetoin and further reduced to 2,3-butanediol by LAB (Martineau and Henick-Kling 1995). Acetoin and 2,3-butanediol have higher sensory thresholds 150 mg L^{-1} and 600 mg L^{-1}, respectively (Francis and Newton 2005, Bartowsky and Henschke 2004) than diacetyl, which can be detected in Chardonnay at 0.2 mg L^{-1} and in Cabernet Sauvignon at 2.8 mg L^{-1} (Martineau et al. 1995). The production of

diacetyl, acetoin and 2,3-butanediol can be advantageous for sparkling production and certain styles of Chardonnay, however the buttery character is not always desired in fresher styles of wine. Commercial strains are therefore now marketed as being citrate negative or positive, depending on the particular strain's ability to metabolise citric acid (Carminati et al. 2010).

1.2.2. Methionine metabolism

In wine, sulphur compounds and their derivatives can be important from a sensory point of view (Landaud et al. 2008). Strains of *Lactobacillus* and *O. oeni* can metabolise methionine in wine to form the volatile aroma compounds 3-(methylsulphanyl) propan-1-ol and 3-(methylsulphanyl) propionic acid (Pripis-Nicolau et al. 2004, Landaud et al. 2008). These compounds contribute significantly to the complexity of red wines by contributing chocolate and 'roasted' aromas, both of which occur in greater amounts following MLF (Pripis-Nicolau et al. 2004).

1.3. Enzymatic activities by LAB that contribute to wine aroma

During the growth and proliferation of LAB in wine, as well as citrate and methionine metabolism and the degradation of malic acid in MLF, there are a multitude of enzymatic modifications by LAB that contribute significantly to the sensory profile of a wine. The dairy industry has been the focus of many studies on the enzymatic activities of LAB and their role in flavour production (Liu et al. 2008, 2010). Fewer studies have investigated the impact of enzymatic activities from LAB on the sensory properties of wine (Matthews et

al. 2004, Sumby et al. 2010, Liu 2002). The principal enzymatic activities of LAB that affect the sensory profile of a wine are peptide degrading enzymes, polysaccharide degrading enzymes, phenoloxidases, esterases/lipases and glycosidases (**Table 1**). The proteolytic system in LAB is important in wine because of its ability to generate peptides and amino acids (Liu et al. 2010) both in red and white wines (Manca de Nadra et al. 1997, 1999). Amino acids can in turn be utilized for both bacterial growth and flavour formation via the production of aldehydes, alcohols and esters (Liu et al. 2008).

Polysaccharide degrading enzymes hydrolyse grape cell wall components such as cellulose (primarily β-glucans), hemicellulose (primarily xylans), and pectic substances (Whitaker 1990, Maier et al. 2008). This hydrolysis can have a direct impact on winemaking processes (i.e. clarification) as well as increasing the amount of phenolic compounds from grape skins, affecting both wine colour and taste (Pardo et al. 1999).

Phenoloxidases (such as laccases and tyrosinases), can oxidize phenolic compounds present in wine including hydroxybenzoic and cinnamic acids and their derivatives, catechins, anthocyanins, flavonols, flavanones, and tannins (Ribereau-Gayon et al. 2006). Strains of *Lactobacillus* spp. have been found to possess tannase (Rodríguez et al. 2008b, 2008a) and phenolic acid activity (de las Rivas et al. 2008).

Tannase activity in wine may lead to the degradation of tannins, phenolic compounds responsible for astringency and palate weight, and oak derived compounds (Vaquero et al. 2004). The hydrolysis of tannic acid yields gallic

Table 1. Enzymatic activities detected in LAB relevant to winemaking.

Enzymes in LAB	**Catalytic action**
Proteolytic enzymes	Degradation of proteins
Peptidolytic enzymes	Degradation of peptides
Polysaccharide degrading enzymes	Degradation of polysaccharides
Phenoloxidases	Transformation of phenolic compounds (includes tannases)
Esterases	Hydrolysis and synthesis of esters
Lipases	Hydrolysis of lipids
Glycosidases	Hydrolysis of β-glycosides

acid and glucose, of which gallic acid has been found to be stimulatory towards MLF and the growth of LAB in wine (Lekha and Lonsane 1997). Tannases hydrolyse the ester bonds in polyphenols forming protein-tannin interactions associated with haze. Hazes in wine arising from protein instabilities are ordinarily treated with the addition of bentonite, but this has the potential to cause a significant loss in aroma/colour compounds via a stripping effect (Aguilar and Gutierrez-Sanchez 2001). As such, tannases may represent a superior alternative treatment to address issues of protein stability.

Esterases and lipases contribute directly to the sensory properties of wine through the synthesis and hydrolysis of esters and lipids (Sumby et al. 2010). Esters of organic acids, ethyl esters of straight-chain fatty acids and acetates of higher alcohols constitute the majority of the fruity aroma in wine (Ebeler 2001). Esterases show a greater affinity towards 2-10 carbon atom substrates while lipases act on substrates with over 10 carbon atoms (Sumby et al. 2010). Strains of *O. oeni*, *Lactobacillus* and *Pediococcus* have been assayed and found to have widespread esterase activity (Matthews et al. 2006), suggesting a potential formation of esters as well as their hydrolysis. One esterase from an *O. oeni* strain isolated from wine has been characterised as a purified protein (Sumby et al. 2009). This esterase (EstB28) is active in wine conditions and shows enormous potential to be used as an additive in winemaking.

The above indicates that there are a number of enzymatic modifications in wine that may be carried out by LAB, which have important implications in wine quality. The final class of enzymes present in LAB, glycosidases, will be

discussed in the following sections. These enzymes may have a direct impact on the production of volatile aroma compounds by hydrolysing the glycosidic bond between glycoconjugated aroma/sensory compounds.

1.4. Wine aroma and β-glycosidases in wine

Wine aroma is composed of a vast matrix of terpenes, norisoprenoids, methoxypyrazines, volatile thiols, esters and higher alcohols. An aroma compound may be more or less sensorally detectable depending on the particular wine or juice matrix. Compounds which affect wine aroma can be attached to a sugar moiety as a non-volatile glycoconjugate. Many sugar linked compounds are no longer sensorally available in a glycosylated state (Maicas and Mateo 2005). In wine, monoterpenes, norisoprenoids, benzene derivatives, C6 alcohols, volatile phenols and lactones can be found in the glycosydic form (Ugliano 2009, Rusjan 2010). Glycosides can either be monosaccharides (β-D-glucopyranosides) or further conjugated with α-L-arabinopyranose, α-L-rhanmopyranose, β-D-xylopyranose, β-D-glucopyranose or β-D-apiofuranose to form disaccharides or trisaccharides (**Figure 2**) (Prosen et al. 2007, Gunata et al. 1985). The principal aglycones (which are commonly found in glycosydic form in wine) that have the potential to affect the sensory properties of wine are discussed.

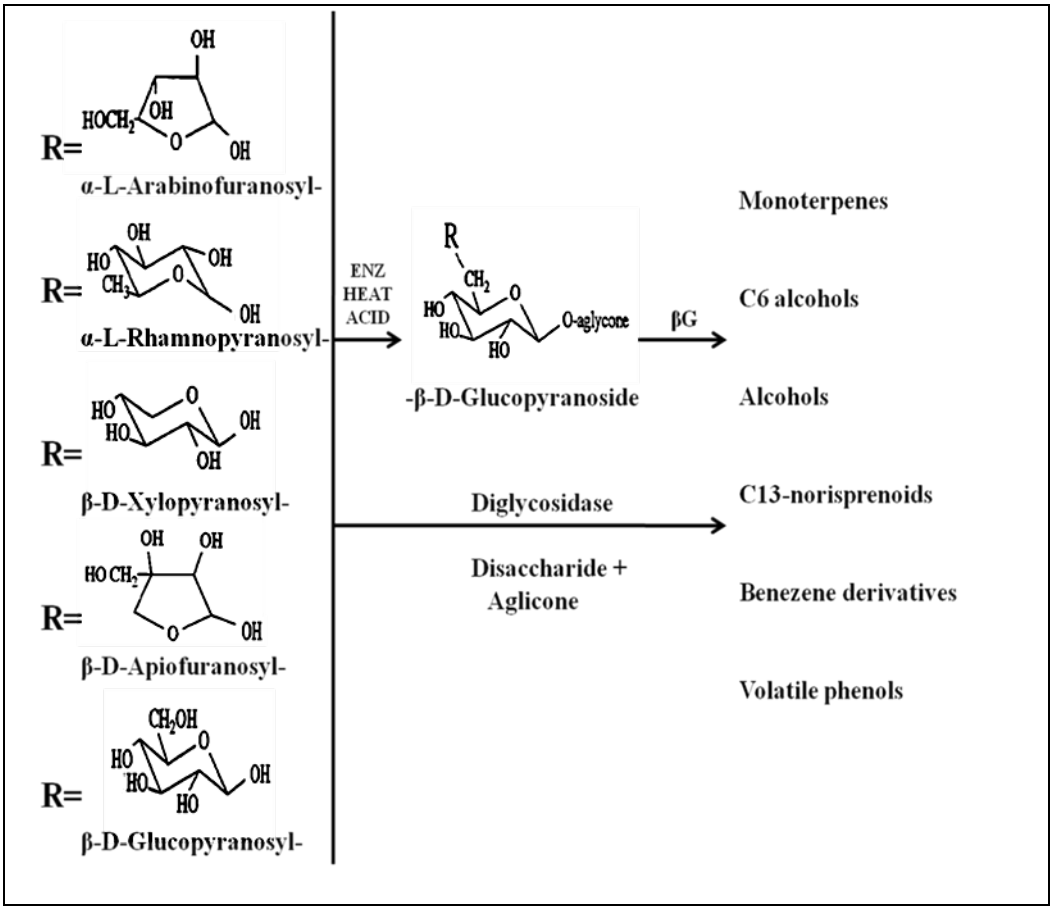


Figure 2. Glycoconjugate moieties and liberated aroma compounds in wine. The diagram demonstrates enzymatic hydrolysis in one and two step mechanisms. βG: β-glucosidase. ENZ: Enzymatic hydrolysis which cleaves the outermost sugar moiety, leaving a monoglucoside. ACID: Acid hydrolysis. HEAT: Heat hydrolysis. Adapted from Winterhalter & Skouroumounis (1997)

1.4.1. Monoterpenes

Grape-derived monoterpenes are a major component of the varietal aroma in wine and are formed in the early stages of berry maturation (Rusjan 2010) from the biosynthesis of acetyl coenzyme A (Mateo and Jimenez 2000). Mateo & Jimenez (2000) categorised monoterpenes in wine into three main groups; free aroma compounds; polyhydroxylated forms of monoterpenes, which are odourless but also highly reactive and able to form odorous compounds such as nerol oxide (Williams et al. 1980b); and lastly the non-volatile glycosylated form of monoterpenes. The free form of monoterpenes is volatile and plays an integral role in wine aroma. However, it is the non-odorous glycosylated state that is much more prevalent in grapes and juice than the free form. Monoterpenes impart a pleasant, desirable set of aromas varying from floral to citrus fruit (**Table 2**). Grape varietals can range from being very aromatic to neutral. White varietals such as Muscat and Gewürztraminer are typically some of the most aromatic, providing an excellent base for many studies which focus on aromatic profiles of wine (Williams et al. 1980a, Palomo et al. 2007). Typically red varietals fall into the less aromatic end of the spectrum along with whites such as Chardonnay and Semillon (Mateo and Jimenez 2000), where monoterpenes make a small contribution to the varietal aroma of these varieties. Gunata et al. (1985) identified the free and bound monoterpenes in several varieties ranging from very aromatic to almost neutral (**Table 3**). The ratio of free monterpenols to bound can vary from 1:5 in Muscat to 1:15 in Gewürtztraminer (Gunata et al. 1985, Williams et al. 1982a), while Syrah has only 4% of the total monoterpenes as volatile aroma contributing compounds (**Table 3**).

Table 2. Monoterpenes which have been identified in wine as being responsible for a significant part of aroma compounds

Monoterpene	**Aroma description**	**Aroma threshold µg L^{-1}**	**Reference**
Nerol	Rose	400	Ribereau-Gayon et al. 2006
Citronellol	Citrus, citron	100	Selli et al. 2004
Linalool	Floral, lavender	25	Ferreira et al. 2000
α-terpineol	Lily	400	Prosen et al. 2007
Geraniol	Rose, geranium	30	Escudero et al. 2004

Table 3. Levels of bound and free terpenols and aromatic alcohols in various grape varieties (Gunata et al. 1985), results are given as pg L^{-1} of juice. White varietals are coloured in yellow and red grape varietals are coloured in red.

Most aromatic

Grape variety	Total terpenols		Geraniol		Linalool		Nerol		α-Terpineol		Citronellol		2-Phenylethanol		Benzyl alcohol	
	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free
Muscat Ottonel	2873	1679	1291	172	722	1449	635	35	186	12	39	10.7	265	24	326	66
Muscat of Frontignan	1398	1640	396	107	207	1409	658	74	75	26	62	24	96	25	93	38
Muscat of Alexandria	4040	1513	1507	342	1839	1084	618	59	61	21	21	7.5	157	58	109	41
Muscat of Hamburg	1047	594	426	241	172	281	318	52	86	nd	45	19.7	48	18	158	42
Gewürztraminer	4325	282	3356	218	22.5	5.6	617	43	183	3.2	146	12	159	37	185	35
Riesling	276	58	65	26	87	19.4	10.3	5.4	114	7.4	nd	nd	249	49	312	64
Cinsaut	314	13	69	13	5.4	nd	6.9	nd	233	nd	nd	nd	177	9	135	8
Grenache	71	11.8	40	5.2	5.4	6.6	8.2	nd	17.4	nd	nd	nd	81	18	160	52
Carignane	81	7.4	40	4.8	26	2.6	14.8	nd	nd	nd	nd	nd	102	24	124	38
Cabernet Sauvignon	26	5.3	12	3.6	4.2	1.7	nd	nd	9.9	nd	nd	nd	88	16	144	38
Syrah	36	1.7	36	1.7	nd	nd	nd	nd	nd	nd	nd	nd	93	6	183	8

Least aromatic

1.4.2. C_{13} Norisoprenoids

Norisoprenoids are also grape-derived compounds formed via the oxidation of carotenoids (Baumes et al. 2002, Mathieu et al. 2005). Typically C_{13} norisoprenoids are found in the grape skin in amounts 2 – 3 times of that in the pulp (Razungles et al. 1987). The oxidation of C_{13} norisoprenoids is stimulated by sunshine and they are accumulated from véraison to grape maturity (Mathieu et al. 2005). The oxidised C_{13} norisoprenoid is subsequently glycosylated by a glycosyltransferase into a non-volatile form (Mathieu et al. 2005). C_{13} norisoprenoids typically have low sensory threshold values. This makes the desirable aroma spectrum by the two norisoprenoids β-damascenone and β-ionone (**Table 4**), important aroma precursors in wine, particularly in otherwise less aromatic varietals (Cabaroglu et al. 2003).

1.4.3. Phenolic compounds

Phenolic compounds such as anthocyanins, stilbenes and benzene derivatives including benzyl alcohol, 2-phenylethyl alcohol and vanillin, can be found in the glycosidic form. Their hydrolysis can affect both the aroma and visual profile of a wine. Anthocyanins affect wine colour and stilbene derivatives have documented health benefits (Meng et al. 2004, Williamson et al. 1996). The release of benzene derivatives such as the volatile phenol vanillin and benzene derivative 4-hydroxybenzoic acid by β-glucosidases from *O. oeni* also contributes to wine aroma (Bartowsky et al. 2004).

Table 4. C_{13} Norisoprenoids in wine that affect wine aroma when deglycosylated.

C_{13}-Norsoprenoid	**Aroma description**	**Aroma threshold µg L^{-1}**	**Reference**
β-damascenone	Apple, rose, honey	0.05	López et al. 2003
β-ionone	Seaweed, violet, flower, raspberry	0.09	Escudero et al. 2004

1.4.4. Resveratrol

Other glycosylated compounds found in wine, including many phenolics, and more specifically the antioxidant resveratrol, are suggested to have positive health effects, whilst the aglycone form of some may be even more beneficial (Meng et al. 2004, Williamson et al. 1996). It has been suggested that there is a negative correlation between the concentration in the berry of resveratrol and anthocyanins (Becker et al. 2003). Trans-resveratrol is a stilbene, and in grapes stilbene synthesis starts with phenylalanine utilizing the chalcone synthesis pathway. Chalcones are a flavonoid precursor, thus suggesting that the less resveratrol that is synthesized, the greater the production of flavonoids, and thus anthocyanins (**Figure 3**).

1.4.5. Wood interactions

LAB are able to interact with components of the wood in barrels and influence the concentration of volatile compounds released (de Revel et al. 2005). Noticeably, wood volatile compounds are found in higher amounts after MLF (de Revel et al. 1999). Compounds such as oak lactone, eugenol, isoeugenol and vanillin have been associated with distinctive woody, spicy and smokey flavours. These are generally held to be sensorally beneficial to the wine and are modified by *O. oeni* in the presence of oak. The increase in vanillin was attributed to three enzymes in LAB: *α*-L-arabinofuranoside, *β*-D-rhamnopyranoside, or *β*-D-xylopyranoside (Bloem et al. 2008). *α*-L-arabinofuranoside, *β*-D-xylopyranoside as well as α- and β-glucosidase activities have been previously observed in *O. oeni* (Grimaldi et al. 2005a, 2000), implying that the modifications of wood volatile compounds are significantly influenced by glycosidic activity in *O. oeni*.

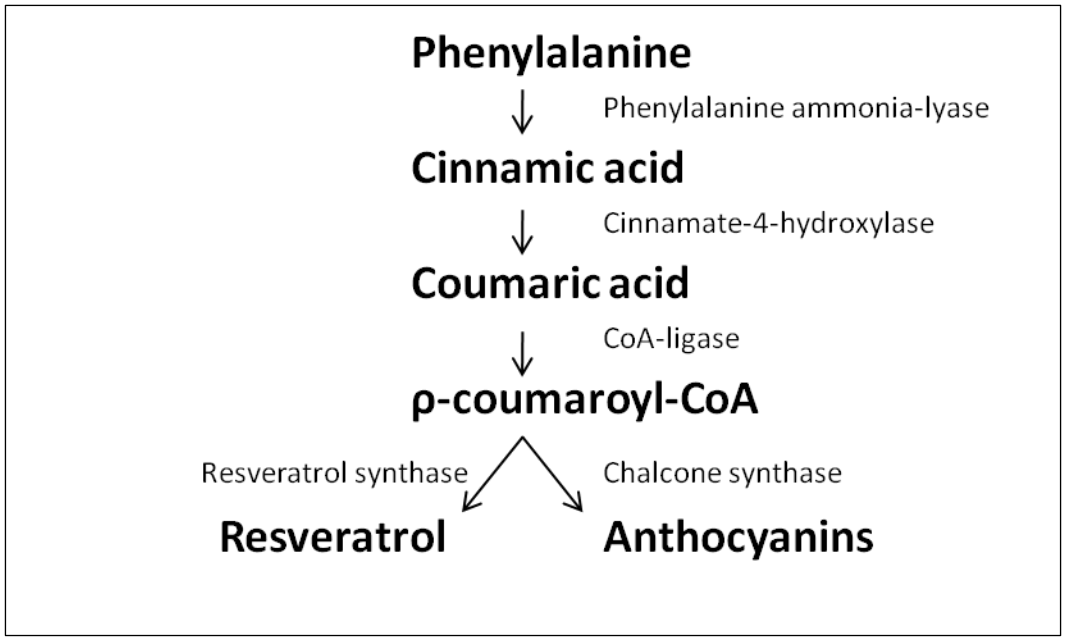


Figure 3. The formation of resveratrol, adapted from Becker et al.(2003), and anthocyanins, adapted from Stobiecki & Kachlicki (2006) via the chalcone synthesis pathway from phenylalanine.

1.5. Potential undesirable effects of β-glycosidic activity in wine

1.5.1. Anthocyanins

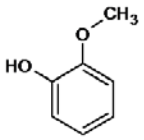
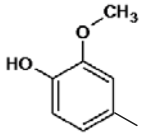
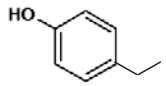
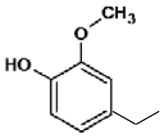
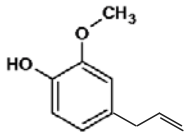
Anthocyanins, which contribute to wine colour, are found in the glycosidic form in young wines (Ribereau-Gayon et al. 2006). In their aglyconic form (anthocyanidin), they are unstable and can polymerise with other phenolic compounds such as tannins during wine maturation or simply degrade, conferring an undesirable instability of wine colour (Wightman et al. 1997). Anthocyanins found in wine are cyanidin, delphinidin, peonidin, petunidin and malvidin. There is a marked lack of information regarding the effects of β-glycosidase activity on anthocyanins when considering wine colour development and stability (Vernocchi et al. 2011). Studies on various fruit juices (Wightman and Wrolstad 1995), Sicilian blood oranges (Barbagallo et al. 2007) and the juices from Cabernet Sauvignon and Pinot Noir grapes (Wightman et al. 1997) demonstrate a significant breakdown of anthocyanins with increasing β-glucosidase activity. However, Wightman et al. (1996) established 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. Strain variation is an important factor when considering substrate specificity of microbe derived enzymes (Le Traon-Masson and Pellerin 1998, Vernocchi et al. 2011). Vast differences in β-glucosidase activity have been demonstrated between strains of LAB (Grimaldi et al. 2005b, 2005a) and yeast (Ugliano et al. 2006). Le Traon-Masson & Pellerin (1998) demonstrated that specificities varied greatly between two purified β-glucosidases from *Aspergillus* spp, whereby one had very high affinity for cellobiose, but degraded malvidin-3-glucoside at a much

slower rate. The second only demonstrated affinity towards anthocyanidin-3-glucosides (Le Traon-Masson and Pellerin 1998) suggesting that varying β-glucosidase specificities that can be selectively harnessed for either targeted hydrolysis of anthocyanins (in rose wines for colour reduction), or else a limited action on anthocyanins with a preferred substrate leading to the release of volatile aroma compounds.

1.5.2. Smoke taint

Following severe bushfires in 2009 in some of Australia's most renowned grape growing areas, there was an interest in understanding the compounds involved in 'smoke taint' in smoke-exposed grapes. Smoke taint has been attributed to the volatile phenols guaiacol, 4-methylguaiacol, 4-ethylguaiacol, 4-ethylphenol and eugenol (Kennison et al. 2007), and is characterized by 'burnt', 'dirty' and 'smoky' aromas, as well as a noticeable retro-nasal 'ash' component (**Table 5**). Sheppard et al. (2009) identified guaiacol and 4-methylguaiacol in grapes exposed to bushfire smoke at critical points in the growth phase of the berry. It was noted by Kennison et al. (2008) that while trace amounts of these compounds were present in free run juice, more significant levels of guaiacol, 4-methylguaiacol, 4-ethylguaiacol and 4-ethylphenol were detected after the wine had gone through alcoholic fermentation and MLF (Kennison et al. 2008). Glycoconjugates of guaiacol were identified in juice (Hayasaka et al. 2010b) along with several disaccharide precursors in grapes (Hayasaka et al. 2010a).

Table 5. Volatile aroma compounds attributed to smoke taint found in the glycosidic form in wine, and thought to be released during fermentation (Kennison et al. 2008).

Guaiacol		Smokey, phenol-like, aromatic sharp, sweet
4-methylguaicol		Smokey, toasted, ash, vanilla-like, sweet, phenol-like, fruity, sharp
4-ethylguaicol		Smokey, sweet, spicy, clove-like
4-ethylphenol		Horsey, leather, medicinal, smokey, barnyard, animal, stable, sweaty saddle
Eugenol		Clove, vanilla-like, phenol-like

The effect of β-glucosidase activity on wine is not advantageous in the case of volatilised smoke taint aromas and the loss of colour through the hydrolysis of anthocyanins to the less stable anthocyanidins. Trials carried out by Ristic & co-workers (2011) concluded that smoke taint could be minimised by avoiding winemaking treatments which prolong skin/juice contact, as the glycoconjugated smoke aromas are mostly localised in the skin of grapes (Dungey et al. 2011), as well as utilising various oak treatments. Rather than eliminate smoke taint, oak treatments add to the complexity of the wine and can mask these undesirable aromas (Ristic et al. 2011).

1.6. Yeast-LAB interactions and their effect on volatile compounds

Wine is often left to go through MLF whilst in the presence of dead yeast cells (lees) remaining from alcoholic fermentation. The purpose being enhanced flavour production (Palomero et al. 2009). One of the principal components of yeast cells is mannoprotein, which apart from growth stimulatory effects, has been shown to increase the activity of α-glucosidase, β-glucosidase, peptidases and N-acetyl β-glucosaminidase (Guilloux-Benatier and Feuillat 1993). Conversely, mannoproteins also have the ability to bind to aglycones subsequent to β-glucosidic activity, thereby diminishing the amount of free volatiles (Boido et al. 2002).

1.7. Acid and heat hydrolysis

Acid and heat hydrolysis have been investigated as a means of liberating potential aroma in juices and wine from the glycosylated fraction (Williams et al. 1982b). Acid hydrolysis at pH 3.2 (a typical wine pH) is evident, whereby linalool, α-terpineol and smaller amounts of nerol were detected from

synthetic neryl, geranyl and linalyl β-D-glucoypyranosides. However at the lower pH of 1.0, the same monoterpene glycosides produced 1,8-cineoles, isomers of 2,2-dimethyl-5-(1-methylprop-1-enyl) tetrahydrofuran and 2-(5,5-dimethyltetrahydrofuran-2-yl) butan-2-ol, 1- and α-terpineols, as well as lower amounts of 4-terpineols (Williams et al. 1982b). Prolonged heating of grape juice from *Vitis vinifera* at pH 3.0 was observed to alter the aroma composition by imparting a eucalyptus-like aroma, mainly attributed to the 1,8-cineoles liberated. Thus, it is clear that wine exposure to very low pH and prolonged heating will cause molecular rearrangement of compounds as well as deglycosylation. However, acid and heat hydrolysis are not common practice in winemaking given the likely detrimental effect on many sensorally important characteristics such as colour and taste, as well as compromising the aging potential by changing the acid profile.

1.8. Enzymatic hydrolysis

Enzymatic hydrolysis of glycosides is common practice in winemaking and does not cause the molecular rearrangement of the hydrolysates. Typically, aroma-releasing commercial preparations consist of part-purified pectolytic enzymes from *Aspergillus niger* with residual β-glycosidic activities. Currently available commercial aroma-releasing enzymes are listed in **Table 6**. Only one of these commercial preparations has β-glucosidic activity alone. The non-aromatic grape varietal, Emir, was subjected to the commercial enzyme preparation intended for aroma release, the AR-2000 pectinase enzyme preparation (5 g/hL), possessing β-apiosidase, β-glucosidase, α-arabinofuranosidase, and α-rhamnosidase activity (**Table 6**). The free

Table 6. Commercially available enzyme preparations recommended for aroma release

Preparation	**Recommended dose**	**Producer/ Distributor**	**Comments**
AR 2000	2–3 g/hL	DSM Food Specialties	Pectolytic enzyme with side activities derived from *Aspergillus niger*
Novarom blanc	5–10 g/hL	Lamothe Abiet/Novozymes	Polygalacturonase with β-glucosidase activity
Expression 20	3–5 g/hL	Oenofrance	Pectolytic activity with secondary activities such as β-glucosidases, rhamnosidases and apiodases
Depectil AR	5–10 g/hL	Martin Vialatte OEnologie	Endo and exo polygalacturonase >25 000 nkat/g, pectin-methyl-esterase > 6 000 nkat/g, pectinelyase > 500 nkat/g, β-glucosidases > 45 000 nkat/g
Lafazym arome	5–10 g/hL	Laffort Oenologie	β-glucosidase
Lyvarome A5	4 g/hL	Lyven	Pectinase from *Aspergillus niger* for increasing the aromatic intensity
Exarom	5–10 g/hL	Institut Oenologique de Champagne	Pectinolytic and glucosidic activities
Endozym cultivar	2–4 g/hL	Pascal Biotech	Pectinolytic with β-glucosidic activities

monoterpenic fraction remained under the respective thresholds after such treatment and several precursors of β-damascenone, an intensely aromatic C_{13}-norisoprenoid, increased significantly (Cabaroglu et al. 2003). The most dramatic increase was noted between the free and bound benzene derivatives, however this was in part attributed to the lack of specificity of the commercial preparation and possible cinnamate esterase activity. This indicates that there is a need for greater specificity when attempting to modify the aroma profile of a wine using commercial enzyme preparations.

1.8.1. Plant glycosidases

Studies have demonstrated that most terpene-based glycoconjugates in grape berries are predominantly diglycosides (Voirin et al. 1992). The hydrolysis of disaccharides can either occur sequentially or in a one-step procedure (**Figure 2**). Sequential hydrolysis involves cleavage of the second (outer) sugar before a β-glucosidase can hydrolyse the single glucose molecule to liberate the volatile aglycone. One step hydrolysis is driven by a diglycoside which directly cleaves the aglyconic linkage of diglycosides, directly liberating a disaccharide and an aglycone (Gunata et al. 1988).

Enzymes endogenous to yeast and bacteria such as β-glycosidases, α-arabinofuranosidases and α-rhamnopyranosidases may be better suited to juice and wine conditions than glycosidases originating from the grapevine. (Aryan et al. 1987, Lecas et al. 1991). β-glycosidases, α-arabinofuranosidases and α-rhamnopyranosidases demonstrate an increase in activity as berry ripening progresses (Aryan et al. 1987), however the study only measures activity at varying maturity levels and gives no indication of whether the enzymes were more active or if more enzyme was produced at later stages in berry

development. β-glucosidases represent an important step in flavour production in most of these studies and are also the most abundant glycosidase activity in the grape berry itself (Sarry and Gunata 2004). Gunata et al (1998) presented evidence of a grape diglycosidase which was able to hydrolyse disaccharide glycosides, releasing a diglycoside and aglycone. However, as with many other plant derived β-glycosdases, the activity is comparatively low in wine like conditions, approximately 40% relative maximum activity at pH 3.5 and less than 30% relative maximum activity at 20°C. It is for this reason that enzymes purified for commercial use have been sought from alternative sources such as of microbial origin.

1.8.2. Yeast and fungal β-glycosidases

Saccharomyces cerevisiae is the principal species in wine that metabolises glucose via alcoholic fermentation (Verstrepen et al. 2006). Strains of non-*Saccharomyces* yeast such as *Kloeckera*, *Hanseniaspora*, *Brettanomyces*, *Debaryomyces*, *Candida*, *Metschnikowia*, *Pichia*, *Torulaspora* and *Zygosaccharomyces* may also be associated with the initial stages of fermentation, whereby most have an ethanol tolerance ranging from 5 to 9 % (v/v) (Manzanares et al. 2000, Mendes Ferreira et al. 2001). Strains of *S. cerevisiae* exibited α-arabinofuranosidase, α-rhamnosidase and β-glucosidase activities (Delcroix et al. 1994). Non-*Saccharomyces* strains seem to have higher β-glucosidase activity than *S. cerevisiae* (Arévalo Villena et al. 2005). Rosi et al (1994) demonstrated that while all strains of *Debaryomyces castellii, Deb. hansenii, Deb. polymorphus, Kloeckera apiculata* and *Hansenula anomala* showed β-glucosidase activity, only one out of the 153 strains of *S. cerevisiae* tested (*S. cerevisiae* 1014) was able to hydrolyse β-glucosides,

albeit with permeabilized cells. No activity was found when the supernatant from whole cells of the same strain was assayed. This suggests that in *S. cerevisiae* the enzyme responsible for β-glucosidase metabolism is intracellular and not localized on the cell membrane or secreted, as occurs with many of the non-*Saccharomyces* strains (Rosi et al. 1994, Arévalo Villena et al. 2005). Given such species and strain variation, non-*Saccharomyces* yeast species in wine have been of much interest as a source of enzymes for oenological use. Strains of *Pichia* spp. and *Torulaspora* spp. are now being marketed as a solution to be used in conjunction with *S. cerevisiae* as a controlled fermentor with significant β-glucosidase activity at the beginning of fermentation; *S. cerevisae* is subsequently inoculated once the alcohol is too high for the non-*Saccharomyces* strains to survive (www.chr-ansen.com/products/product_areas/wine_ingredients/frootzen).

Exogenous fungal glycosidases (for example from *Aspergillus* spp.) are commonly used in commercial preparations due to their stability at wine pH and lack of inhibition by glucose, as opposed to β-glycosidases from plants or yeast (Cabaroglu et al. 2003). β-glycosidase, α-arabinofuranosidase and α-rhamnosidase activities have all been found to increase with the growth of the fungus *Botrytis cinerea,* typically present in mould contaminated grapes. One endo-β-glucosidase from *Aspergillus niger* has been identified and found able to hydrolyse geranyl-β-rutinoside (Shoseyov et al. 1988). Subsequent studies verified a noticeable increase in monoterpenes and flavour compounds when both free and immobilized forms of the enzyme were used to treat Muscat Roy wine and passionfruit juice at pH 2.45 (Shoseyov et al. 1990). Due to the high

levels of activity exhibited by fungal β-glycosidases in wine conditions, fungi are the most common source of such enzyme preparations (**Table 6**). However, given the variation across enzymes derived from plant, yeast and fungi, the potential of sourcing enzymes from bacteria is of interest as bacteria carry out MLF in wine and thereby spend anywhere from 10 days to 4 months actively metabolizing malic acid.

1.8.3. Bacterial β-glycosidases

While LAB are the principal bacteria associated with wine, through their role in MLF, acetic acid bacteria (AAB) can also be present. AAB are part of the gram positive Acetobacteraceae family, produce acetic acid, the principal component of vinegar, and can contribute this spoilage characteristic in wine. They are very well suited to high levels of alcohol and sugar (Bartowsky and Henschke 2008). One β-glucosidase has been characterized in *Acetobacter xylinum* ATCC 23769 (Gullo et al. 2006) which has not been pursued further for oenological use. Given that available commercial enzyme preparations are often crude extracts with multiple activities in it may be that an enzyme preparation from AAB may have undesirable activities. LAB are desired in wine to conduct MLF in wine, however most of the research on LAB has been associated with the dairy industry.

LAB are able to proliferate and function at wine pH (3.0 to 4.0), and high ethanol conditions of up to 16%. The very fact that wine-associated LAB are able to grow post-alcoholic fermentation is evidence of this. β-glucosidase activity has been observed in whole LAB cells (Barbagallo et al. 2004, Grimaldi et al. 2000, Guilloux-Benatier et al. 1993) of numerous commercial

preparations of *O. oeni*, as well as amongst *Lactobacilli spp*. and *Pediococci spp* (Grimaldi et al. 2005b). Whole *O. oeni* cells have also demonstrated activity against β-D- and α-D-glucopyranosides as well as β-D-xylopyranoside, α-L-rhamnopyranoside and α-L-arabinofuranoside substrates (Grimaldi et al. 2005a). Such activities exhibited strain dependence and a high degree of tolerance to wine-like conditions.

1.9. β-glucosidases: Characterisation and properties

Of th e β-glycosidases purified from grapes and microbes, the pH and temperature optima range approximately from 4.0 to 6.0 and 40°C to 50°C respectively (Sarry and Gunata 2004). Glycosidases from filamentous fungi are more heat-resistant than those from plants and yeasts (Sarry and Gunata 2004). *Aspergillus niger* has multiple forms of β-apiosidase, β-glucosidase, α-rhamnosidase and α-arabinofuransidase (Gunata et al. 1997). Glucose acts as an inhibitor to β-glycosidases, an important factor considering that grape juice typically starts with over 220 g L^{-1} of sugar (equimolar amounts of glucose and fructose). Most glycosidases display intolerance for glucose, however maximal tolerance may persist up until 18 to 54 g L^{-1} glucose (Shoseyov et al. 1988). The endo- β-glucosidase from *A. niger* is rendered completely inactive in the presence of 1M of glucose, equivalent to 180 g L^{-1}, and only retained 19% relative activity in 0.25 M (45 g L^{-1}) (Shoseyov et al. 1988). Conversely, fructose (the principal sugar which remains at the end of fermentation given that *S. cerevisiae* is glucophilic), and sucrose, had no effect on enzyme activity. Interestingly, the few strains of *S. cerevisiae* that have β-glucosidase activity are glucose insensitive, where β-glucosidases from other species are much less tolerant (Darriet et al. 1988). However, given that β-

glucosidase activity reported in *S. cerevisiae* is much lower than non-*Saccharomyces* species, the focus of many studies has been on the β-glucosidase activity from non-*Saccharomyces* yeast.

LAB typically grow to a cell population of 10^8 cells/mL once alcoholic fermentation has ceased or there are minimal amounts of residual sugar left, and therefore would be a suitable vehicle for any desired β-glucosidase activity of whole cell as glucose would not be an inhibiting factor. In order to understand why β-glucosidases from diverse sources have varying affinity towards different substrates, it is important to take into account their catalytic mechanism, substrate specificity and amino acid sequence. The classification seeks to explain and group glycosyl hydrolases (GH) based on comparative sequences, proposed by Henrissat et al (1991), within which the β-glucosidases fall into glycosyl hydrolase families 1 and 3.

1.10. Classification of glycosyl hydrolases

Glycosyl hydrolases have been classified into families by amino acid sequence similarity (Henrissat and Davies 1997, Henrissat and Bairoch 1993, Henrissat 1991) and have a broad spectrum of activities. The family of most interest for this research is the glycosyl hydrolase family (GHF) 1 (**Table 7**), although GHF 3 is also of some interest (**Table 8**).

1.10.1. Glycosyl hydrolase family 1: β-glucosidases and phospho-β-glucosidases

The GHF1 sub-categories, β-glucosidases and phospho-β-glucosidases, are two potential avenues for β-glucosidase metabolism in *O. oeni*. β-glucosidases can either be intracellular or extracellular (McHale and Coughlan 1981) and hydrolyze the β-glycosidic bonds between conjugated glucosides and disaccharides.

Phospho-β-glucosidases are intracellular enzymes, and in the case of bacteria, can function in conjunction with the phosphoenolpyruvate-phospho transferase system (PEP-PTS) (Deutscher et al. 2006). This system simultaneously phosphorylates and transports the β-glucoside into the cell where it can then be hydrolysed by an intracellular phospho-β-glucosidase.

Table 7. GHF 1 enzymes with varied specificities according to the CAZY website http://www.cazy.org/; (Cantarel et al. 2009).

Table 8. GHF 3 enzymes according to the CAZY website http://www.cazy.org/ (Cantarel et al. 2009).

Both β-glucosidases and phospho-β-glucosidases share high sequence similarity in conserved regions and use the same catalytic mechanism. However, phospho-β-glucosidases as opposed to β-glucosidases require a phosphate group attached to the C6 of the glucose moiety in order to bind to the substrate (Wiesmann et al. 1995).

1.11. GHF1 β-glucosidases (EC 3. 2. 1. 21)

1.11.1. Catalytic mechanism

To date, several GHF 1 β-glucosidases have been identified, purified and characterized from LAB (**Table 9)**. Despite the varying specificities of β-glucosidases the mechanism of catalysis has been well defined experimentally for glycosyl hydrolases. Mutagenesis, kinetic studies and crystal structures for GHF 1 have been used to confirm the catalytic mechanism (Isorna et al. 2007). The cleavage of β-1-4 glycosidic bonds occurs via a retaining double displacement mechanism which involves two catalytic residues: a proton donor (acid) and a nucleophile (base), typically represented by two glutamic acid residues in highly conserved regions. **Figure 4** depicts the proposed mechanism of action (Rye and Withers 2000, Ly and Withers 1999).

Table 9. Characterized GHF 1 β-glucosidases from lactic acid bacteria.

Gene	Source organism	Molecular size (kDa)	Accession number	Reference
bglA	*Bacillus circulans subsp. alkalophilus*	51.3	AAA22266	Paavilainen et al. 1993
βGA	*Bacillus sp.*	51	AB009410	Hashimoto et al. 1998
bglB	*Paenibacillus polymyxa (Bacillus polymyxa)*	51.6	P22505	González-Candelas et al. 1990)
bglA	*Paenibacillus polymyxa (Bacillus polymyxa)*	51.5	P22073	González-Candelas et al. 1990, Sanz-Aparicio et al. 1998b
bglA	*Paenibacillus* sp. HC1	51.4	Q2WGB4	Harada et al. 2005
βG	*Lactobacillus casei*	80	-	Coulon et al. 1998

Figure 4. Catalytic mechanism of retaining glycosyl hydrolases, adapted from Ly & Withers (1999), with the formation of a covalent intermediate. The retaining double displacement mechanism involves two catalytic residues: a proton donor (acid) and a nucleophile (base), typically represented by two glutamic acid residues in highly conserved regions. The catalytic nucleophile attacks the substrate at the anomeric centre forming a covalent intermediate (in square brackets). The proton donor first protonates the glycosidic oxygen and deprotonates the nucleophilic water molecule, resulting in the hydrolysis of the glycoside to give glucose and the aglycon (R).

1.12. GHF1 Phospho-β-glucosidases (EC 3. 2. 1. 86)

As mentioned previously, GHF1 phospho-β-glucosidases can function in conjunction with the PEP-PTS to hydrolyse β-glucosides. The PEP-PTS is a well characterized transport system unique to bacteria. It is composed of two generic cytoplasmic components (HPr and EI) common to all PEP-PTS, combined with a carbohydrate specific EII complex (Deutscher et al. 2006, Deutscher 2008) (**Figure 5**). The PEP-PTSs have been characterised in *Escherichia coli* and *Bacillus subtilis*, with each containing over 15 distinct EII complexes (Reizer et al. 1999, Deutscher et al. 2006). The EII complexes are composed of single proteins with multiple domains or up to four distinct proteins (Deutscher et al. 2006). It is thought that *O. oeni* contains upwards of 15 EII complexes based on sequence homology to a number of characterized EII proteins from other bacteria (Karp et al. 2005). A large number of phospho-β-glucosidases have been identified from bacteria (**Table 10**), and characterized as being part of the PEP-PTS for that particular organism.

The significance of the PEP-PTS in LAB has been demonstrated through the expression of the *Lactobacillus plantarum bglGPT* operon (Marasco et al. 2000) in *E. coli* cells. Whole recombinant *E. coli* cells with the *bglGPT* operon from *L. plantarum* inserted were able to hydrolyse ρ-nitrophenol-β-D-glucopyranoside. This result indicates that the *bglGPT* was able to function as a transport system within *E. coli*, an organism with the same PEP-PTS. As *E. coli* cannot ordinarily hydrolyse β-glucosides (Schnetz et al. 1987), whole cell β-glucosidase activity can be directly attributed to a GHF1 phospho-β-glucosidase functioning in conjunction with the PEP-PTS.

Figure 5. Schematic representation of the phosphoenolpyruvate phospho transferase system (PEP-PTS). The sugar is transported into the cell, where it is phosphorylated. The newly phosphorylated sugar is hydrolyzed by cytoplasmic phospho-β-glucosidases and the phosphate group phosphorylates components of the PEP-PTS (B). The phosphorylated carbohydrate feeds into glycolysis, either as glucose-6-phosphate or fructose-6-phosphate (A). One of the phosphoenolpyruvate molecules formed in glycolysis drives the transport and initial phosphorylation of the carbohydrate. The ratio of PEP, pyruvate and the concentration of extracellular sugars influence the phosphorylation state of the PEP-PTS. The figure was adapted from Deutscher et al. (2006)

Table 10. Phospho-β-glucosidases identified and characterized from bacteria. NP – not purified

Gene	Source organism	Molecular size (kDa)	Accession number	Reference
bglC (yckE)	*Bacillus subtilis* subsp. *subtilis* str. 168	NP	P42403	Setlow et al. 2004
bglA	*Bacillus subtilis* subsp. *subtilis* str. 168	NP	P42973	Setlow et al. 2004, Zhang and Aronson 1994
ydhP	*Bacillus subtilis* subsp. *subtilis* str. 168	NP	O05508	Setlow et al. 2004
bglH	*Bacillus subtilis* subsp. *subtilis* str. 168	NP	P40740	Le Coq et al. 1995
abgA	*Clostridium longisporum*	52	Q46130	Brown and Thomson 1998
bglB	*Enterococcus faecium (Streptococcus faecium)*	NP	Q9X564	Franz et al. 1999
arbB	*Erwinia chrysanthemi*	52	P26206	El Hassouni et al. 1992
bglB	*Escherichia coli* K-12 MG1655	NP	P11988	Mahadevan et al. 1987, Schnetz et al. 1987
ascB	*Escherichia coli* K-12 MG1655	NP	P24240	Hall and Xu 1992
bglA	*Escherichia coli* K-12 MG1655	NP	Q46829	Schnetz et al. 1987
pbgA	*Fusobacterium mortiferum*	53	P94793	Thompson et al. 1997
casB	*Klebsiella oxytoca P2*	52.2	Q48409	Lai et al. 1997
bglA	*Streptococcus mutans*	NP	Q9KJ76	Cote et al. 2000
celA	*Streptococcus mutans*	MA	Q8DT00	Old et al. 2006
ascB	*Pectobacterium carotovorum*	53	AY622309	An et al. 2005
celG	*Pectobacterium carotovorum*	54	DQ987482	Hong et al. 2007
bglB	*Pectobacterium carotovorum*	53	AY542524	An et al. 2004
bglA	*Pectobacterium carotovorum*	57.3	AY769096	Hong et al. 2006
bglH	*Lactobacillus plantarum*	NP	AJ250202	Marasco et al. 2000

The catalytic mechanism of GHF1 phospho-β-glucosidases is speculated to be the same double displacement mechanism as other GHF1 glycosidases. The one exception is the region where the phosphate group is received; in β-glucosidases there is a net negative charge from a glutamic acid, and in phospho-β-glucosidases, the glutamic acid is commonly replaced by a serine, which has no charge. The negatively charged phosphate group attached to C6 on the substrate, would ordinarily be repelled by a glutamic acid but not a serine (Wiesmann et al. 1995, Hill and Reilly 2008).

1.13. GHF3 β-glucosidases (EC 3. 2. 1. 21)

Much of the research on β-glucosidases has been dedicated to GHF1. The enzymes in GHF 3 can have the following functions: β-glucosidase (EC 3.2.1.21); xylan 1,4-β-xylosidase (EC 3.2.1.37); β-N-acetylhexosaminidase (EC 3.2.1.52); glucan 1,3-β-glucosidase (EC 3.2.1.58); glucan 1,4-β-glucosidase (EC 3.2.1.74); exo-1,3-1,4-glucanase (EC 3.2.1.-); alphα-L-arabinofuranosidase (EC 3.2.1.55). GHF 3 β-glucosidases have been purified and characterised (**Table 11**), but representative enzymes from only a few taxa have been crystallized: β-D-glucan exohydrolase from *Hordeum vulgare* (Varghese et al. 1999); a β-hexosaminidase from *Vibrio cholerae*; a β-hexosaminidase from *B. subtilis* and just recently a β-glucosidase from *Thermotoga neapolitana* (Pozzo et al. 2010). From the characterized GHF3 β-glucosidases, a clear distinction can be seen in the size of the protein in comparison to GHF1 β-glucosidases (**Table 11** in comparison to **Table 9**), whereby the former tend to be upwards of 70 kDa, and the latter around 50 kDa. The characterized GHF3 β-glucosidase from *B. subtilis,* BglB, has a pH optimum of 8.0 and codes for a monomeric protein of 82 kDa (Hashimoto et

al. 1998). The enzyme has 100% activity towards ρNP-β-D-glucopyranoside but is only slightly active (< 3%) towards the substrates ρNP-β-D-xylose, sophorose, cellobiose, gentiobiose and salicin (Hashimoto et al. 1998).

1.13.1. Catalytic mechanism

The β-glycosidase from barley is the most thoroughly characterized and indicates that the catalytic mechanism is much the same as the retaining double displacement mechanism for GHF1 β-glucosidases (**Figure 4**). The catalytic nucleophile and proton donor residues have been identified as Asp^{285} and Glu^{491} respectively (Varghese et al. 1999) and the enzyme has broad specificity for β-D-oligosaccharides with (1→2), (1→3), (1→4) or (1→6) linkages (Hrmova et al. 2002).

1.14. Concluding statement

This review of literature highlights how knowledge of microbial biotechnology and enzymatic applications has the potential to increase the levels of sensorally important compounds in wine. Given the core role of glycosylated grape-derived compounds in the aroma and colour of wine, a characterization of potentially impactful enzymes of commonly inoculated wine microorganisms, *O. oeni*, is warranted.

The aims of this PhD project are to identify the genes and mechanisms responsible for β-glucosidase metabolism in the oenologically important lactic acid bacterium *O. oeni*. It is expected that the outcomes from this research will aid winemakers tailor the sensory profile of wine and produce a superior 'value for money' product.

Table 11. Characterised and purified GHF3 β-glucosidases from microbial and plant origin

Purified protein	Source organism	Accession number	Protein size (kDa)	Reference
CelA	*Azospirillum irakense* (Bacteria)	AF213463	73	Faure et al. 2001
SalA	*Azospirillum. irakense* (Bacteria)	AF090429	78.5	Faure et al. 1999
SalB	*Azospirillum irakense* (Bacteria)	AF090429	64.6	Faure et al. 1999
BglB	*Bacillus subtillis* (Bacteria)	AB009411	82	Hashimoto et al. 1998
TnBgl3B	*Thermotoga neapolitana* (Bacteria)	DQ873691	81.1	Pozzo et al. 2010, Turner et al. 2007
ARA-I	*Hordeum vulgare* (Plant)	AY029259	79.2	Lee et al. 2003
XYL	*Hordeum vulgare* (Plant)	AY029260	80.5	Lee et al. 2003

Chapter 2

2. Bioinformatic analysis and cloning of genes encoding the enzymes responsible for β-glucoside metabolism in *O. oeni*

2.1. Bioinformatic analysis

This chapter outlines the approach taken to identify genes via bioinformatic analysis in the *O. oeni* genome which were likely to be responsible for β-glucoside metabolism. Six genes were identified as likely candidates, five of which shared homology to glycosyl hydrolase family (GHF) 1 β-glucosidase/β-galactosidase/phospho-β-glucosidase signature sequences. The sixth gene was identified as having high homology to GHF 3 β-glucosidases, in contrast to the other genes. In order to establish the function of these genes and their role in β-glucosidase metabolism, attempts were made to purify their respective gene products.

The PSU-1 *O. oeni* genome was fully sequenced in 2006 (Genbank accession number CP000411) (Mills et al. 2005). Since then two other *O. oeni* genomes ATCC BAA-1163 and AWRIB429 have been sequenced. *O. oeni* is a heterofermentative organism and can utilize pentoses and hexoses via the phosphoketolase pathway. Its DNA make-up is composed of a single circular chromosome of 1,780,517 nt, a G + C content of 38% and 1701 open reading frames (ORF) of which 75% have been functionally classified (Mills et al. 2005). As mentioned earlier, the aims of this project were to identify the genes and mechanisms responsible for β-glucoside metabolism in *O. oeni*. Six

genes were putatively identified (AG1, ORF 1, ORF 2, ORF 3, ORF 4 and ORF 5) based on comparative sequence analysis of the published PSU-1 *O. oeni* genome with other lactic acid bacteria (Makarova et al. 2006). Candidates were identified based on the presence of signature sequences important in GHF 1 and 3 β-glycosidases (**Table 1**). Two candidates ORF 1 and 5, have a leucine in the place of a methionine as a start codon and it is not known if this may affect the activity of the gene product.

2.2. Putative β-glycosidase genes in *O. oeni*

2.2.1. GHF 1 β-glycosidases

Sequence analysis of AG1 and ORF 1 – 4 from *O. oeni* strain PSU-1 indicate that these genes are GHF 1 β-glycosidases. They contain several signature sequences from this group of glycosyl hydrolases (**Figure 1**), namely the two glutamic acid residues important in catalysis and the GHF 1 N-terminal signature sequence. Interestingly, ORF 4 is missing half of the GHF N-terminal signature sequence and the first methionine does not fit with the consensus sequence **(Figure 1)**. The N-terminal sequence signature is conserved among GHF 1 glycosidases (F-x-[FYWM]- [GSTA]-x-[GSTA]-x-[GSTA](2)-[FYNH]-[NQ]-x-E-x-[GSTA] (PROSITE ID: PS00653)). Little functional data has been obtained for the role of this particular signature sequence despite 3804 unreviewed sequences entered into the database (UniProtKB/TrEMBL) and 189 recently reviewed (UniProtKB/Swiss-Prot) with the same highly conserved N-terminal signature sequence. Ito et al (2002) based on the protein sorting prediction program PSORT (Nakai and

Table 1. Putative genes identified in *O. oeni* PSU-1 which may be responsible for β-glucosidase metabolism.

Gene	Putative function	Gene length (bp)	Position in genome	Gene ID*
AG1	β-glucosidase/6-phospho-β-glucosidase/β-galactosidase	1332	Complement (211150..212482)	-
ORF 1	β-glucosidase/6-phospho-β-glucosidase/β-galactosidase	1443	Complement (211150..212592)	OEOE_0224
ORF 2	β-glucosidase/6-phospho-β-glucosidase/β-galactosidase	1458	(331260..332717)	OEOE_0341
ORF 3	β-glucosidase/6-phospho-β-glucosidase/β-galactosidase	1446	(329798..331243)	OEOE_0340
ORF 4	β-glucosidase/6-phospho-β-glucosidase/β-galactosidase	1323	(1143928..1145250)	OEOE_1210
ORF 5	β-glucosidase-related glycosidase	2214	Complement (1486489..1488702)	OEOE_1569

*NCBI Gene tag

Horton 1999), predicted that the motif was a signal sequence but they were not able to validate this experimentally. The only difference between AG1 and ORF 1 is the absence of the N-terminal signature sequence tag and it is not known how this may impact gene function and product.

2.2.2. GHF 3 β-glycosidases

ORF 5 has been identified by sequence analysis as a putative GHF 3 β-glycosidase and has homology to CelA, SalA and SalB from *Azospirillum irakense,* BglB from *Bacillus subtilis*, TnBgl3B from *Thermotoga neapolitana*, ARA-I and XYL from *Hordeum vulgare* (**Table 2**). The catalytic residues have been identified as Asp^{228} and Glu^{414}. An alignment of the amino acid sequences of characterized GHF 3 β-glucosidases TnBgl3BT from *Thermotoga neapolitana* and BglB *Thermotoga neapolitana* to ORF 5 indicates the high degree of homology and the highly conserved regions containing the catalytic residues are highlighted with arrows (**Figure 2**).

Table 2. Sequence alignment of ORF 5from the sequenced *O. oeni* genome PSU-1 (CP000411) to characterized GHF 3 β-glucosidases

Gene	Species	Accession number	Percentage %		Reference
			Identities	Positives	
celA	*Azospirillum irakense*	AF213463	26	44	Faure et al. 2001
SalA	*Azospirillum irakense*	AF090429	35	53	Faure et al. 1999
SalB	*Azospirillum irakense*	AF090429	25	40	Faure et al. 1999
BglB	*Bacillus subtillis*	AB009411	24	37	Hashimoto et al. 1998
TnBgl3B	*Thermotoga neapolitana*	DQ873691	36	55	Pozzo et al. 2010, Turner et al. 2007
ARA-I	*Hordeum vulgare*	AY029259	29	43	Lee et al. 2003
XYL	*Hordeum vulgare*	AY029260	27	43	Lee et al. 2003

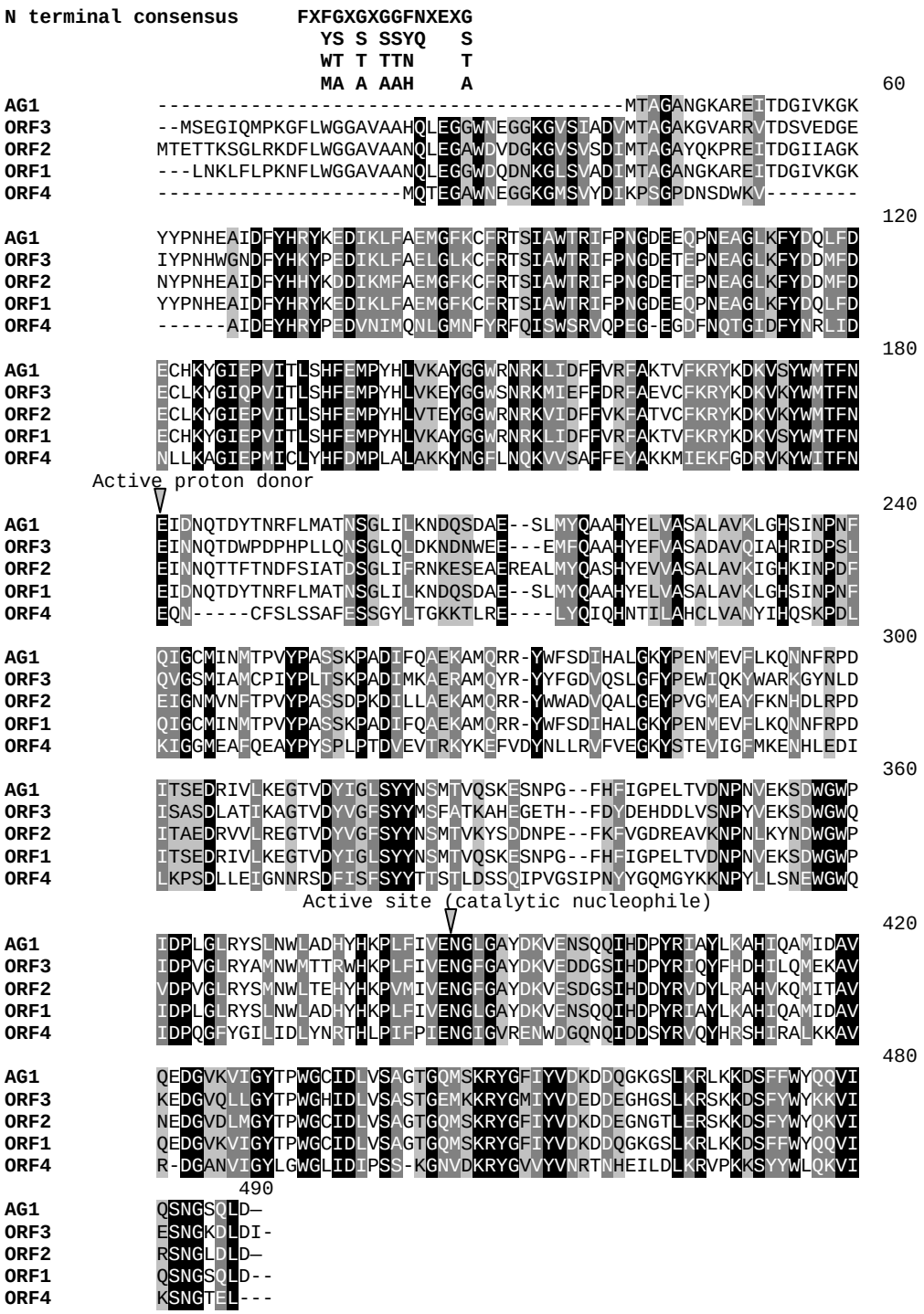
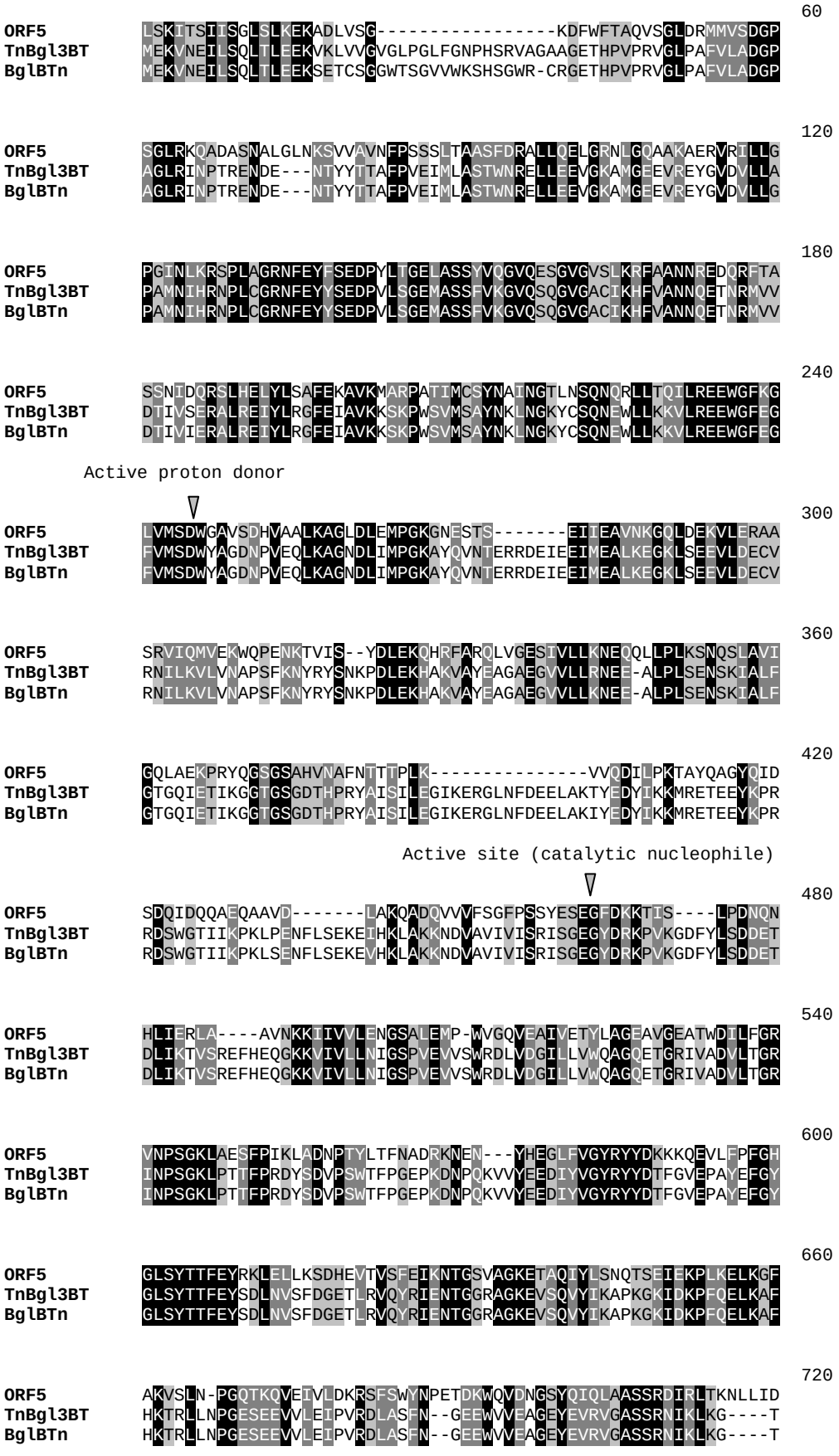


Figure 1. Based on sequence comparison the active site for GHF 1 is located in position 382 – 390 and comprises the residues FIVENGLGA with the glutamic acid (see indicator arrow) residue acting as the catalytic nucleophile (Sanz-Aparicio et al. 1998a, Hill and Reilly 2008). A second highly conserved region occurs at position 177-183 TFNEIN whereby a second glutamic acid residue (see indicator arrow) has been demonstrated in other species (Hill and Reilly 2008) to be the active proton donor residue. The N-terminal signature sequence (F-x-[FYWM]- [GSTA]-x-[GSTA]-x-[GSTA](2)-[FYNH]-[NQ]-x-E-x-[GSTA] (Prosite ID: PS00653) is indicated above the sequence alignment.


ORF5
TnBgl3BT
BglBTn
Active proton donor
Active site (catalytic nucleophile)

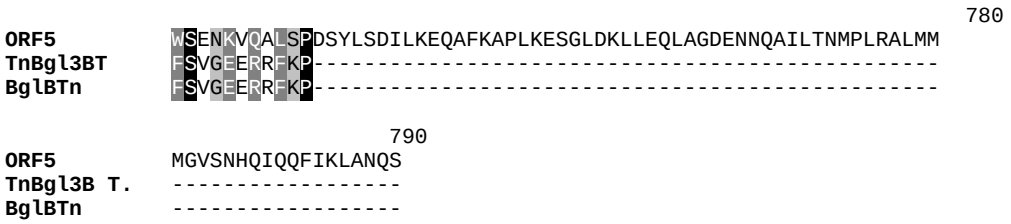


Figure 2. Alignment of the amino acid sequences of characterized GHF 3 β-glucosidases to ORF 5: TnBgl3BT, *T. neapolitana* strain DSM 4359; BglBTn, *T. neapolitana* strain Z2706-MC24. ORF 5, *O. oeni* strain PSU-1. Identical residues are highlighted white lettering on a black background, highly conserved residues are highlighted with white lettering on dark grey and conserved residues are represented by black lettering on light grey. The indicator arrows denote the conserved active sites and the numbers above the residues refer to alignment positions.

2.3.Materials and methods

2.3.1. Cloning of AG1, ORF4 and ORF5

The DNA encoding the putative β-glucosidase/6-phospho-β-glucosidase/β-galactosidase AG1, was cloned into the expression vectors pET 39.b, pET 14.b, pET 16.b, pET 43.1b (Novagen) for expression in *Escherichia coli*. The putative GHF1 β-glycosidase ORF 4 and the putative GHF 3 β-glycosidase ORF 5 were also cloned into pET 14.b ORF 4 was subsequently transformed into *E. coli* strain BL21 (DE3) for over-expression of the gene product. Furthur work on the expression and characterisation of the recombinant proteins encoded by ORFs 1-3 will be discussed in subsequent chapters.

2.3.2. Cloning and expression of AG1 in *E. coli*

2.3.2.1. Growth and Strains

DNA was extracted from the *O. oeni* wine strain Lalvin 4X (VL92) using the Ultraclean™ Microbial DNA Isolation kit (Mo Bio Laboratory) according to the manufacturer's instructions and verified on an 1% (w/v) agarose gel stained with GelRed™ (Biotium, distributed by Jomar Diagnostics). AG1, a 1332 bp fragment, was amplified by PCR using primers shown in **Table 3** depending on the vector of choice. The primers were sourced from Sigma-Aldrich, with restriction sites incorporated (**Table 3**). The restriction enzymes were sourced from New England Biolabs and utilized in accordance with the recommended protocol. The PCR fragment was digested and cloned into a previously digested plasmid. PCR reactions to amplify the target ORF utilized Pfu Ultra II fusion Hotstart DNA Polymerase (Integrated Sciences) with the

manufacturer's recommendations listed in **Table 4**. The plasmids (**Table 5**) harbouring AG1 were transformed into the *E. coli* strain DH5α. DNA was extracted using the Wizard® Plus SV Minipreps DNA Purification System (Promega) and sequenced by the Australian Genome Research Facility (Brisbane). Plasmid DNA was then transformed into the *E. coli* strain Rosetta (DE3) (Novagen) for overexpression (**Table 5**).

2.3.2.2. Cloning of ORF 4 & 5

The amplification and cloning of ORF 4 and ORF 5 (using the primers listed in **Table 6**) was carried out according to the methods mentioned for AG1. ORF 4 was cloned into pET 14.b and transformed into competent Rosetta *E. coli* cells. ORF 5 was amplified and sequenced but not cloned into a plasmid for over-expression.

2.3.3. Gene expression and product analysis

E. coli cells transformed with either the recombinant plasmids containing the cloned genes or expression vector alone, were grown in an overnight culture and then inoculated 1:100 dilution into 200 mL Terrific Broth, appropriately dosed with antibiotics. The cultures were grown to an optical density of 0.8 measured at 600 nm, chilled down to 15°C and induced with IPTG to a final concentration of 0.4 mM. The cultures were placed on a shaker set at 160 rpm in a 12°C temperature-controlled room for 24 hours before being analysed for gene expression. Cell cultures were centrifuged and the cell pellet was lysed with Bug Buster MasterMix (Novagen) according to the manufacturer's instructions to recover any soluble gene product, or further manipulated to identify insoluble product. Total protein was separated by SDS-PAGE (Laemmli 1970) on a 12 % gel (See **Appendix 1** for electrophoresis buffers).

Table 3. Primers for cloning the AG1 gene into the expression vectors listed.

Primer	Oligonucleotide sequence (5' - 3')	Tm°C*	RS†	Vector
ACbG2b	CCGCTCGAGTTAATCTAATTG ACTGCCGTTTGACTTAATAAC CT	78	*Xho*I	pET 16.b/14.b
ACbG2f	GGGAATTCCATATGACTGCCG GAGCCAATGGAAAAGCA	84	*Nde*I	pET 16.b/14.b
ACbG1f	TCCCCCGGGGCAGCATGACTG CCGGAGCCAATGGAAAAGCA	94	*Sma*I	pET 43.1b
ACbG1b	CGCGGATCCTTAATCTAATTG ACTGCCGTTTGACTTAATAAC CT	79	*Bam*HI	pET 43.1b
pET39.bB wdBg	CGCGATATCCCTAGGTTAATC TAATTGACTGCCGTTTG	77	*Sca*I	pET 39.b
pET39.bF WBg	GGATCCATGACTATGACTGCC GGAGCCAATGGA	83	*Bam*HI	pET 39.b

†RS, Restriction site.
*Calculated using the finnzymes Tm calculator (http://www.finnzymes.com/tm_determination.html)

Table 4. PCR conditions using PfuUltra™ fusion HS DNA polymerase

Segment	Number of cycles	Temperature	Duration
1	1	95°C	2 minutes
2	30	95°C	20 seconds
		Tm - 5°C	20 seconds
		72°C	15 seconds per kb
3	1	72°C	3 minutes

Table 5. *E. coli* vectors and strains used for the cloning of AG1, ORF4 and ORF 5, and the expression of AG1 and ORF4

Vector	Promotor	Antibiotic resistance	Features
pET 14.b*	T7	Ampicilin (100 μg mL^{-1})	N terminal His-tag
pET 16.b*	T7 lac	Ampicillin (100 μg mL^{-1})	N-terminal His-tag
pET 39.b*	T7 lac	Kanamycin (30 μg mL^{-1})	Dsb-tag (Signal sequence)
pET 43.1b*	T7 lac	Ampicillin (100 μg mL^{-1})	Nus-tag (Increase solubility)
Strains			
Rosetta	-	Chloramphenicol (34 μg mL^{-1})	general expression host; provides seven rare codon tRNAs
DH5α	-	No antibiotic resistance	cloning host

*Plasmids sourced from Novagen

Table 6. Primers used for the amplification of ORF 4 and 5.

Gene	Primer	Oligonucleotide sequence (5' - 3')	Tm°C *	RS†	Vector
ORF 4	ORF4FNDE	GGGAATTCCATATGCAAACTGAAGGTGCCTGG	79	*Nde*I	pET 14.b
ORF 4	ORF4BXHO	CCGCTCGAGCTACAATTCTGTTCCATTAGACTTG	76	*Xho*I	pET 14.b
ORF 5	ORF5F(L)NdeI	GCGCGGCATATGTTGTCTAAGATTACTTC	71	*Nde*I	pET 14.b
ORF 5	ORF5F(M)NdeI	GCGCGGCATATGTCTAAGATTACTTC	68	*Nde*I	pET 14.b
ORF 5	ORF5BBamHI	CCGCTCGAGTTAACTTTGATTGGC	70	*Xho*I	pET 14.b

*Calculated using the Finnzymes Tm calculator
†RS, Restriction site incorporated.

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 crude extract with a final substrate concentration of 2 mM in McIlvaine buffer at pH 5.5 (see **Appendix 1** for buffer composition) for 30 minutes in a total volume of 50 µL at 37°C. The amount of *p*NP released was determined by measuring the absorbance of the reaction mixture at 400 nm (An et al. 2005). All assays were blanked against ultra pure water and had the control (substrate minus enzyme) taken away from the absorbances read at 400 nm. A β-glucosidase from *Aspergillus niger* (Sigma-Aldrich, Australia) was used as a positive control under standard assay conditions. Specific activity was expressed in µmoles of *p*NP liberated per minute per mg of enzyme under the standard assay conditions.

2.4. Results

2.4.1. Cloning into *E. coli* expression vectors

The AG1 gene was amplified using the primers listed in **Table 3**. All fragments were all approximately 1300 bp in size. The fragments in lanes 2 and 3 amplified with primers for cloning into pET 14.b and pET 16.b (**Figure 3A**), whilst the fragments in lanes 4 and 5 had been amplified with primers for subsequent cloning into pET 43.1b. AG1 was amplified with primers for cloning into pET 39.b (**Figure 3B**).

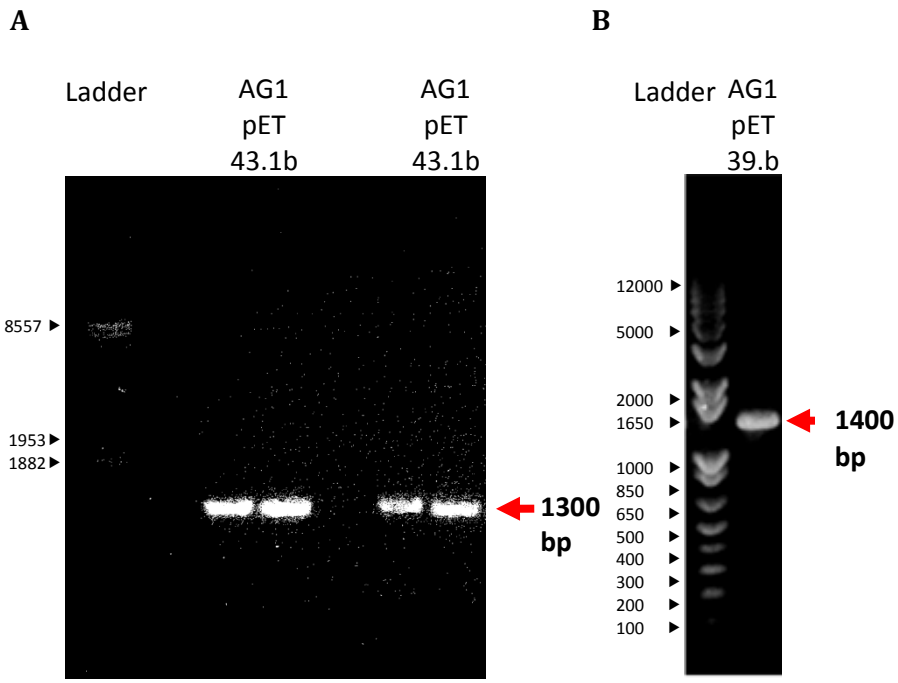


Figure 3. Gel electrophoresis showing left to right; A) Ladder, AG1 amplified with primers from **Table 3** for cloning into pET 14.b and 16.b (AG1 ACbg2b/ ACbG2f), pET 43.1b (ACbg1f/ ACbG1b). B) pET 39.b (pET39.bBwdBG/pET39.bFWBg)

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ORF 4 and ORF 5 were amplified as described in Materials and Methods using the primers listed in **Table 6** (**Figure 4**). The PCR products displayed single DNA bands of approximately 1,300 bp and 2,200 bp for ORF 4 and 5, respectively. Both products incorporated *Nde*I-*Xho*I restriction sites for cloning into pET 14.b. ORF 5 has a leucine codon TTG as start codon. Although not common in bacteria, leucine is able to function as a start codon as opposed to methionine. In *E. coli*, TTG is estimated to serve as initiator for about 3% of the bacterium's proteins (Blattner et al. 1997). It was decided to incorporate leucine as a start codon in one set of primers (**Table 6**) for amplification of ORF 5 and methionine as a start codon in a second set of primers (**Table 6**) for the amplification of ORF 5 (M). By incorporating leucine and methionine as start codons it was possible to determine the start codon's effect on solubility and consequent activity of the ORF 5 gene product once expressed.

2.4.2. Expression of AG1

AG1 was expressed in pET 14.b, pET 16.b and pET 43.1b as described in Materials and Methods. AG1 was expressed in pET 43.1b as a soluble protein with a NUS-tag attached as an 85 kDa band (**Figure 5**). The 85 kDa gene product appeared to increase in intensity with increasing inductions times. When the vector alone was induced, the NUS-tag was expressed as a soluble protein of approximately 33 kDa. Without induction, the vector alone did not produce a product.

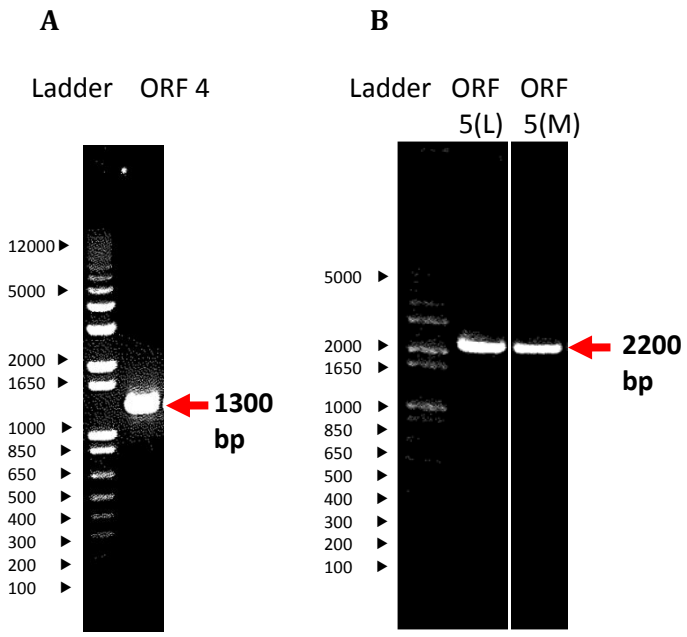

Figure 4. A) Gel electrophoresis showing left to right; Ladder, PCR amplification of ORF 4 with ORF4FNDE and ORF4BXHO (Table 4) B) Ladder, PCR amplification of ORF 5 (L) with ORF5F(L)NdeI and ORF5BBamHI; ORF 5 (M) with ORF5F(M)NdeI and ORF5BBamHI

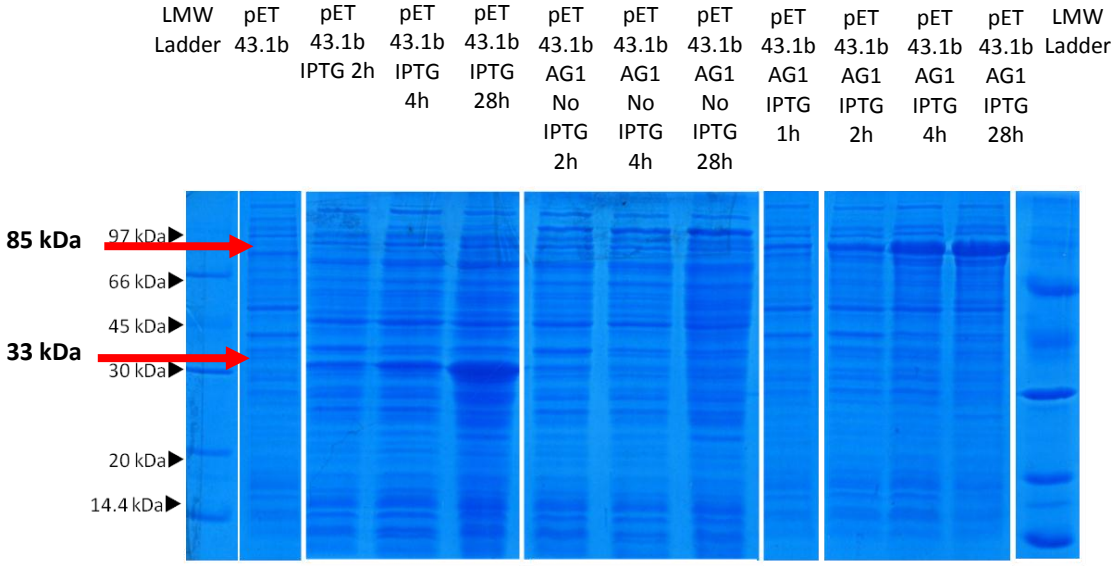

Figure 5. Electrophoretic analysis of the soluble fraction of AG1 expressed in pET 43.1b on a 12% (w/v) SDS-polyacrylamide gel. From left to right; low molecular weight (LMW) marker; Crude extract of pET 43.1b without IPTG, the same with IPTG after 2, 4 and 28 hours, crude extract of AG1 expressed in pET 43.1b without IPTG after 2 hours, 4 hours and 28 hours; crude extract of AG1 expressed in pET 43.1b with IPTG after 1 hour, 2 hours, 4 hours and 28 hours of induction; low molecular weight marker. The gel was stained with 0.05% Coomassie blue.

Figure 6 demonstrates that the over-expression of AG1 from pET14.b and pET16.b at 12°C did not produce a soluble protein of the expected size (approximately 50 kDa) after 2, 4 and 28 hours. No AG1 protein was visible with Coomassie Blue staining as shown by the comparison of total protein extracts from cells harbouring the expression vector or corresponding recombinant plasmid.

In order to determine whether over-expression resulted in the production of insoluble protein as inclusion bodies (Weisman et al. 2010), the cell pellet remaining from the cell lysis protocol was further manipulated. The insoluble extract was separated by gel electrophoresis and stained with Coomassie Blue as shown in **Figures 7** and **8**. pET 14.b AG1 and pET 16.b both produced a distinct band approximately 55 kDa in size which increased in amount with prolonged induction of 28 hours.

The AG1 gene was cloned into another vector, pET 39.b, which incorporates a DsbA tag of approximately 21 kDa (Yu and Kroll 1999) on the fusion protein, as well as a histidine tag. **Figure 9** demonstrates the inability of AG1 to be expressed in pET 39.b and to be present in the soluble fraction despite DsbA being soluble when produced from the vector without AG1 cloned into it (**Figure 9**).

The ORF 4 gene did not produce a soluble protein (**Figure 10**) when expressed in pET 14.b at low temperatures (10°C to 20°C, data not shown). A distinct additional protein band was evident in the insoluble fraction (**Figure 10**).

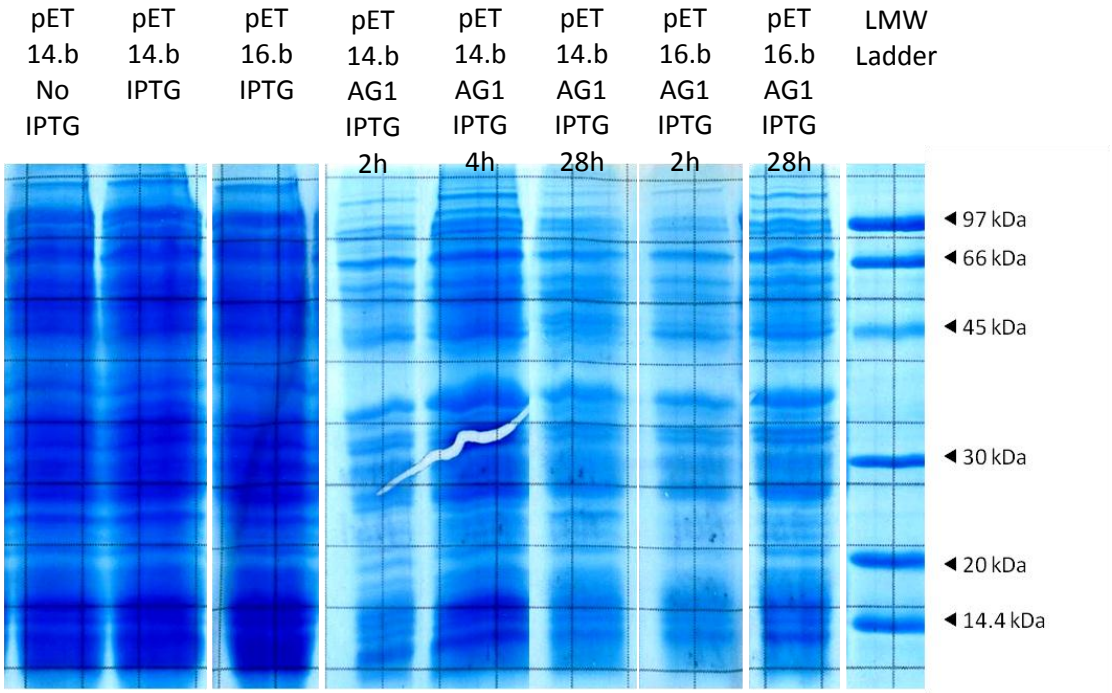

Figure 6. Crude extract of AG1 expressed in pET 14.b for 2 hours , 4 hours, 28 hours, no IPTG, plasmid only. Crude extract of AG1 expressed in pET 16.b for 2 hours, 4 hours, no IPTG. The gel was stained with 0.05% Coomassie blue.

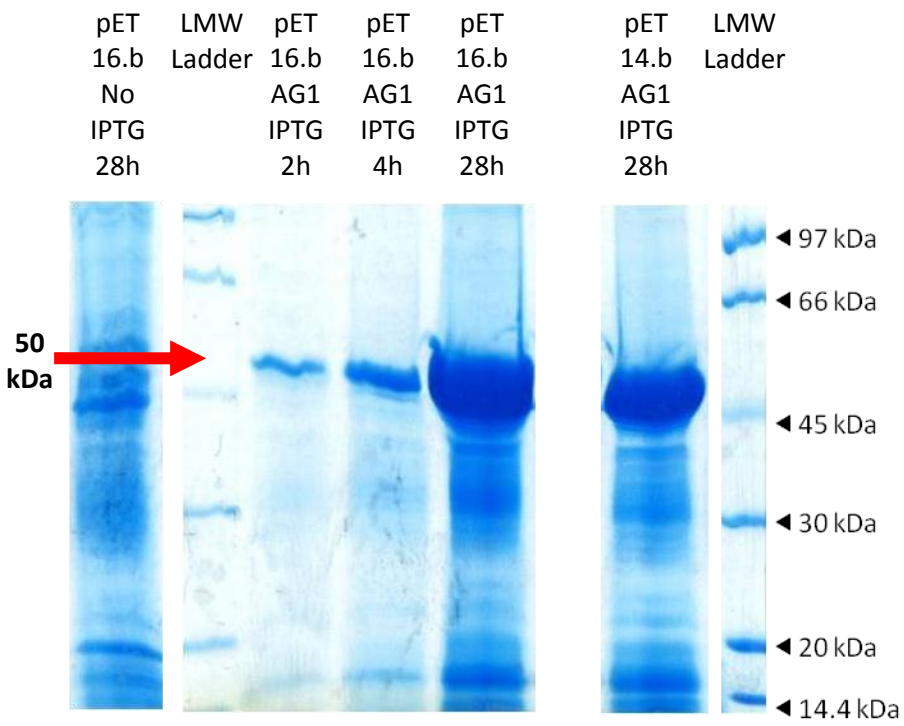

Figure 7. Electrophoretic analysis of the insoluble fraction of AG1 expressed in pET 16.b on a 12% (w/v) SDS-polyacrylamide gel. The low molecular weight markers are located in lanes 1 and 7; Crude extract of the AG1 gene expressed in pET 16.b after 2 hours of induction, 4 hours, 28 hours, 28 hours without IPTG and the pET 16.b vector expressed without the AG1 gene inserted. The gel was stained with 0.05% Coomassie blue.

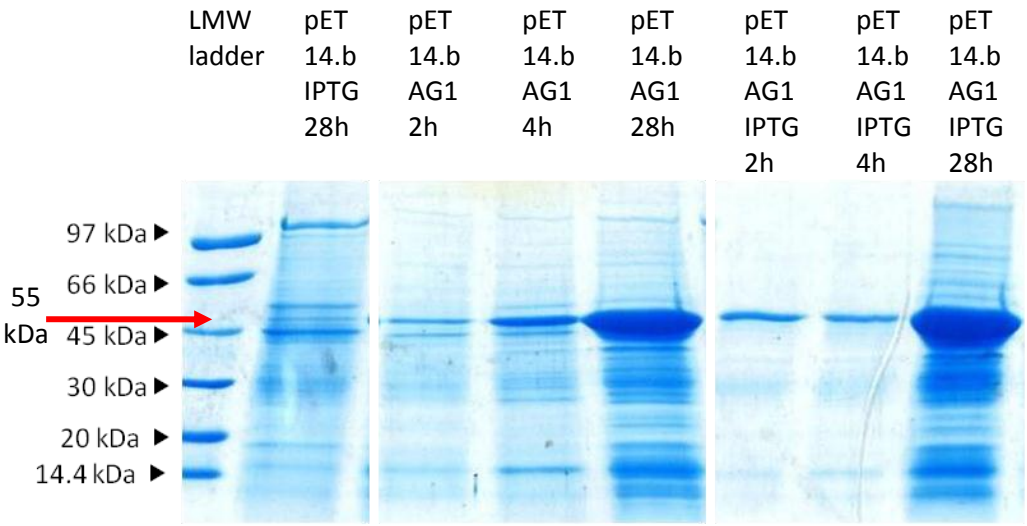

Figure 8. Electrophoretic analysis of the insoluble fraction of AG1 expressed in pET 14.b on a 12% (w/v) SDS-polyacrylamide gel. In order from left to right, low molecular weight marker (LMW ladder); Crude extract of the vector alone IPTG expressed after 28 hours of induction; AG1 in pET 14.b after 2 hours without IPTG induction, 4 hours and 28 hours; Crude extract of AG1 IPTG expressed in pET 14.b after 2 hours, 4 hours and 28 hours. The gel was stained with 0.05% Coomassie blue.

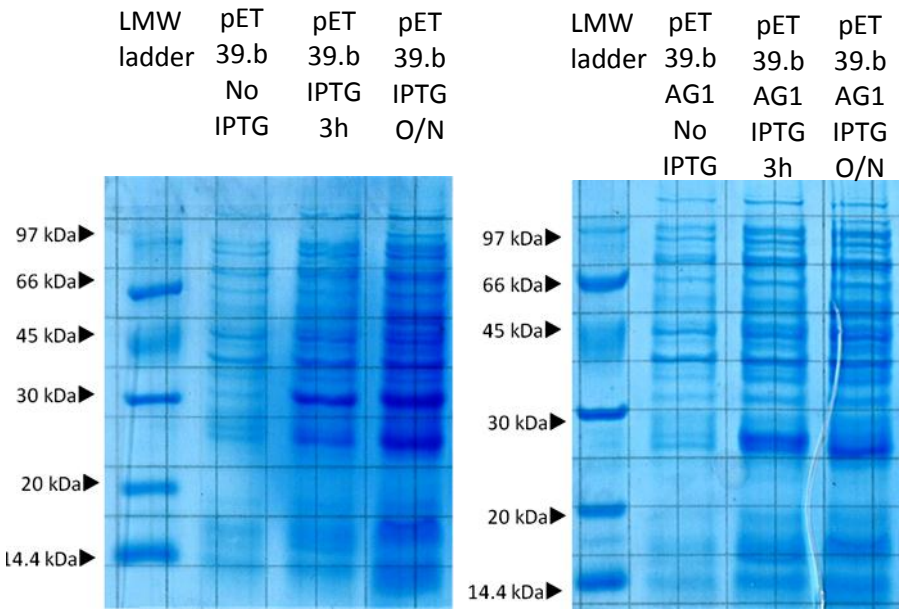

Figure 9. Electrophoretic analysis of AG1 expressed in pET 39.b on a 12% (w/v) SDS-polyacrylamide gel. From left to right; Low molecular weight (LMW) ladder, pET 39.b prior to IPTG induction, after 3 hours IPTG induction and overnight (O/N) IPTG induction; Low molecular weight (LMW) ladder, AG1 in pET 39.b prior to IPTG induction, after 3 hours of IPTG induction and overnight (O/N) IPTG induction.

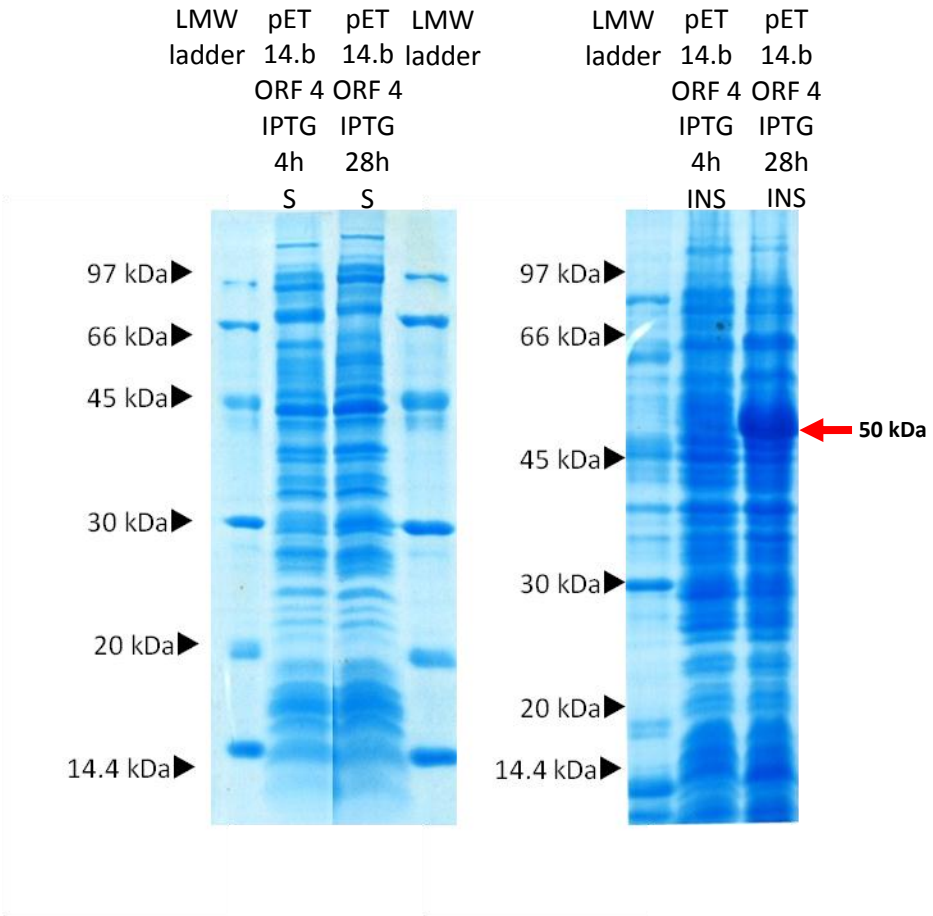

Figure 10. Electrophoretic analysis of ORF 4 expressed in pET 14.b on 12% (w/v) SDS-polyacrylamide gels. From left to right; Low molecular weight (LMW) ladder, soluble fraction of the cell extract from ORF 4 expressed in pET 14.b after 4 hours of IPTG induction and after 28 hours of induction, low molecular weight ladder. Low molecular weight ladder, insoluble fraction of the cell extract from ORF 4 expressed in pET 14.b after 4 hours of IPTG induction and after 28 hours of induction.

2.4.3. ORF 5

ORF 5 was amplified and sequenced from Lalvin 4X (VL92) but all work on ORF 5 ceased upon the publication elsewhere of work relating to this enzyme (Michlmayr et al. 2010). The β-glucosidase enzyme was demonstrated to be a GHF 3 β-glucosidase from the *Oenococcus oeni* strain ATCC BAA-1163 (Michlmayr et al. 2010).

2.5. Discussion

Six genes of the sequenced *O. oeni* genome PSU-1 were identified by homology to encode putative β-glycosidases. Five of these were categorised as putatitive GHF 1 β-glycosidases whilst the last was speculated to be a putative β-glycosidase of GHF 3. Their substrate specificities are not known, both GHF 1 and 3 enzymes have highly variable specificity. ORF 1 and 5 have a leucine in the place of a methionine start codon. AG1 was expressed as a soluble protein attached to a NUS-tag. The function of the NUS-tag is to aid protein solubility due to its ability to act as a highly soluble protein chaperone when co-expressed with another less soluble protein in *E. coli* (Turner et al. 2005) under appropriate promoter/inducer-control, such as the T7 (Yin et al. 2003).

No activity could be found when assaying the AG1-NUS-tag fusion protein against para-nitrophenol-β-D-glucopyranoside (data not shown), suggesting that either the protein needed to be cleaved from the NUS-fusion tag because the presence of the tag was interfering with enzyme catalytic mechanism or that the enzyme was not functional as a β-glucosidase despite high homology

to characterised β-glucosidases. A two-fold increase in activity of a recombinant cyclomaltodextrinase of thermophilic origin was reported after the NUS-tag was removed by digestion with enterokinase, when compared to the untreated fusion protein (Turner et al. 2005). However, due to the absolute lack of activity of AG1-NUS towards β-glucosides, it was decided to continue work expressing AG1 in other expression vectors. AG1 was subsequently cloned into the expression vectors pET 14.b, pET 16.b and pET 39.b. When expressed in pET 14.b and pET 16.b, AG1 was only found in the insoluble fraction. The DsbA tag on pET 39.b is intended to aid the solubilisation of fusion proteins expressed in *E. coli* by catalyzing the formation and isomerization of disulfide bonds as well as transporting it to the periplasm (Yu and Kroll 1999). AG1 was still unable to be expressed as a soluble fusion protein (**Figure 9**) despite the DsbA tag expressing as a soluble protein in the vector alone following IPTG induction.

ORF 4 was expressed as an insoluble protein in pET 14.b at low temperatures and no activity was evident when assaying the whole cells or cell extract against para-nitrophenol-β-D-glucopyranoside (data not shown).

In the publication of a characterizational study on ORF 5 from the *Oenococcus oeni* strain ATCC BAA-1163 (Michlmayr et al. 2010), the purified enzyme had an optimal pH of 5.0, a temperature of 50°C and demonstrated tolerance to both glucose and ethanol.

It was speculated that a fundamental part lacking in both AG1 and ORF 4 was the GHF1 N-terminal signature sequence, which could play an essential role in the enzyme's solubility and function. Further clarification was sought for AG1, and an attempt to incorporate an extended N-terminal sequence despite the lack of a start codon methionine is discussed in the following chapters. Characterisation needs to be undertaken to understand the specificities of these enzymes and their relevance to winemaking. ORF 1 – 3 will be refered to as *bglD*, *celD* and *celC* respectively in subsequent chapters.

Chapter 3

3. β-glucoside metabolism in *Oenococcus oeni*: Cloning and characterisation of the phospho-β-glucosidase BglD

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The GenBank accession numbers for the nucleotide sequence bglD is:

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Designed experiments, performed experimental work, analysed and interpreted data and wrote the manuscript.

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Michelle Walker (Co-supervisor)

Supervised work and helped in the preparation of the manuscript.

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Sign: Date:

Vladimir Jiranek (Principal supervisor)

Supervised work and helped in the preparation of the manuscript and acted as communicating author.

Sign: Date:

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Food Chemistry, v. 125 (2), pp. 476-482

Chapter 4

4. Site directed mutagenesis of *O. oeni* GHF-1 BglD

4.1. Introduction

Three-dimensional structures are available for many glycosyl hydrolases, among them GHF 1 phospho-β-galctosidase (Wiesmann et al. 1995) and GHF 1 β-glucosidase (Sanz-Aparicio et al. 1998a). These structures can be used to gain insights into the catalytic residues of the GHF 1 phospho-β-glucosidase BglD, isolated from *O. oeni*, for which no directly comparable structures have been determined. GHF 1 glycosidases can have a broad range of specificities and it is almost impossible to deduce function from sequence analysis alone (Esen 1992). The GHF 1 enzyme BglD (Please see Chapter 3) exhibited affinity towards phosphorylated substrates, but has a primary sequence that shows high homology with GHF 1 β-glucosidases. Sequence analysis of *bglD* suggested that one residue in particular, S^{432}, may be responsible for the affinity of BglD towards phosphorylated substrates. This chapter described experiments designed and undertaken to validate the importance of the serine residue in position 432 (BglD numbering) in the affinity of the characterized phospho-β-glucosidase BglD towards phosphorylated substrates.

4.2. Importance of active site residues

The crystal structure of the 6-phospho-β-galactosidase from *Lactococcus lactis* indicates that the distance between the two well characterized catalytic glutamic acid residues (E^{186} and E^{394}) would form a cavity likely to accommodate the phosphate group (Wiesmann et al. 1995). The consensus

sequence for the GHF 1 phospho-β-galactosidase phosphate binding site is S^{428}-x-S^{430}-N^{431}-x-x-x-K^{435}-x-Y^{437}. This is in keeping with characterised GHF 1 phospho-glycosidases, whereby S^{428}, K^{435} and Y^{437} (equivalent to S^{432} , K^{439} and Y^{441} in *bglD*) are highly conserved whilst S^{430} and N^{431} (equivalent to G^{434} and T^{435} in *bglD*) vary. S^{428} and Y^{437} (S^{432} and Y^{441} in *bglD*) are normally replaced with a glutamic acid (E) and a phenylalanine (F), respectively, in other characterised GHF 1 enzymes (Wiesmann et al. 1995). It has been suggested that the net negative charge of a glutamic acid in place of S^{428} (S^{432} equivalent in *bglD*) would repel the similarly charged phosphate group and thereby make S^{428} unique to phospho-β-glycosidases (Marques et al. 2003, Sanz-Aparicio et al. 1998a, Wiesmann et al. 1995).

In an attempt to alter the specificity of GHF 1 β-glucosidase CelB from *Pyrococcus furiosus*, several mutations were introduced. The most interesting of these was the E417S (S^{432} BglD numbering), as it conferred an increase of up to 5-fold in the efficiency of hydrolysis of ortho-nitrophenol-β-D-galactopyranoside-6-phosphate and a significantly decreased (30- to 300- fold) affinity towards non-phosphorylated sugars (Kaper et al. 2000). The phospho-β-glucosidase AscB from *Pectobacterium c arotovorum* LY34 exhibited affinity towards both phosphorylated and non-phosphorylated substrates, however in this case the serine residue, the S^{432} equivalent (BglD numbering) contained an alanine (An et al. 2005).

The corresponding glutamic acid (S^{432} equivalent, BglD numbering) in structurally characterized β-glucosidases has been demonstrated to be

influential in catalytic properties, pH range (influencing a shift in pKa (Kaper et al. 2000)) and important for the stabilization of the glycosyl-enzyme intermediate during hydrolysis (Namchuk and Withers 1995). It is also thought to play a role in the broad specificity of GHF 1 β-glucosidases for both glucosides and galactosides (Sanz-Aparicio et al. 1998a).

In order to verify the importance of S^{432} (BglD numbering) as speculated in Chapter 3 and in accordance with experimental data for a phospho-β-galactosidase (Wiesmann et al. 1995), a β-glucosidase (Kaper et al. 2000) and a phospho-β-glucosidase (An et al. 2005), S^{432} in *bglD* , a characterized GHF 1 phospho-β-glucosidase, was mutated to a glutamic acid and expressed as a fusion protein in *E. coli* and designated BglD_S-E.

4.3. Materials and Methods

4.3.1. Gene synthesis

The gene *bglD* was synthesised by Gene Oracle, Inc (Mountain View, CA 94043) with a mutation to incorporate a glutamic acid (E) residue in the place of S^{432} and *Nde*I and *Xho*I restriction sites at the 5' and 3' ends of the sequence. This mutation was selected because of its speculated importance in the affinity of GHF 1 phospho-β-glucosidases towards phosphorylated substrates. The gene was supplied cloned into a pUC based vector, pGOV4, devoid of most common restriction sites (All rights of this vector are licenced to Gene Oracle, https://www.geneoracle.com/services/pGOV4.pdf.)

4.3.2. Bacterial strains and growth

The synthesized gene incorporating the S432E mutation, BglD_S-E, in pGOV4 was transformed into *E. coli* stain DH5α and grown overnight at 37°C in Luria Bertani broth (Sambrook and Russell 2001). Plasmid DNA was extracted as previously described (Chapter 3) and digested with *Nde*I and *Xho*I, along with the pET 14.b vector. All restriction enzymes were obtained from New England Biolabs and used according to the manufacturer's instructions. The resulting 1,446 bp fragment and digested pET 14.b vector were purified on a 1% (w/v) agarose gel stained with GelRed™ (Biotium, distributed by Jomar Diagnostics) as described previously (Chapter 3), and ligated using T4 DNA ligase (New England Biolabs). The resulting plasmid was then transformed into *E. coli* strain BL21 (DE3) for overexpression. All standard DNA procedures were carried out according to Sambrook & Russell (2001). Media were supplemented with 100 μg mL^{-1} of ampicillin sodium salt when necessary. Cultures of pET 14.b_BglD_S-E in BL21 (DE3) were grown in Terrific Broth at 37°C (Sambrook and Russell 2001) and the gene products were over-expressed as described in Chapter 3.

4.3.3. Purification

The gene product of pET 14.b BglD_S-E was purified as described in Chapter 3. The construct pET 14.b BglD_S-E encoded a polyhistidine-tag at the N terminus. The resulting fusion protein was therefore purified using immobilized metal-affinity chromatography with Talon® cobalt metal affinity resin (Clontech, distributed by Scientifix Pty. Ltd., Australia) according to the manufacturer's instructions. Twenty protein fractions of 0.5 mL were eluted

with buffer containing 250 mM imidazole and 10 mM of β-mercaptoethanol at pH 7.0. Fractions containing pure recombinant protein, based on a visualization via SDS-PAGE as described by Laemmli (1970), were pooled, quantified using NanoDrop technology (Thermo Scientific), aliquoted and stored at 4°C and -80°C in 10% (v/v) glycerol.

4.3.4. Assay of (phospho)β-glycosidase activity

β-glycosidase activity was determined quantitatively by measuring para-nitrophenol (*p*NP) and ortho-nitrophenol (*o*NP) release from *p*NP-β-D-galactopyranoside, *p*NP-α-L-arabinofuranoside, *p*NP-α-D-galactopyranoside, *o*NP-β-D-glucopyranoside, *p*NP-β-L-arabinopyranoside, *p*NP-α-L-arabinopyranoside, *p*NP-α-D-glucopyranoside, *p*NP-β-D-galactopyranoside, *p*NP-β-D-glucopyranoside and *p*NP-β-D-glucopyranoside-6-phosphate. The non-phosphorylated substrates were purchased from Sigma-Aldrich Australia and the phosphorylated substrate was synthesized according to the method of Wilson and Fox (1974). A crude extract of the histidine-tagged purified protein was incubated for 1 hour in 1 mL of McIlvaine buffer at pH 5.5 with 1 mM substrate and the reaction was centrifuged for 1 minute at 14000 x*g*. The amount of *p*NP or *o*NP released was calculated from the increase in absorbance at 400 nm relative to the control (buffer plus substrate). Crude extract from pET 14.b-BglD expressed in BL21 (refer to Chapter 3), purified BglD (refer to Chapter 3) and a commercial β-glycosidase from *Aspergillus niger* (Sigma-Aldrich, Australia) were used as positive controls.

4.4. Results and discussion

4.4.1. Sequence analysis of the phosphate binding site

When comparing the predicted phosphorylation site to known characterised phospho-β-glucosidases, β-glucosidases and 6-phospho-β-galactosidases there are several key points to consider. While the 6-phospho-β-galactosidase PBGAL seems quite similar to the β-glucosidases listed, it also bears strong sequence similarity to the phospho-β-glucosidases (**Figure 1**). Of the phospho-β-glucosidases (**Figure 1**), there is an extra amino acid (position 437, BglD numbering), also noted by Wiesmann et al (1995), which is not present in β-glucosidases or the 6-phospho-β-galactosidase. With the exception of *bglD* from *O. oeni*, all of the phospho-β-glucosidases listed have a glutamic acid in this location, which implies it is far enough away from S^{432} (BglD numbering) to not repel a phosphate group. *bglD*, the sole exception, has a glutamine, with a polar uncharged side chain as opposed to a negatively charged glutamic acid. It is not surprising that an alanine in the place of S^{432} (BglD numbering) in the case of AscB from *P. carotovorum* (An et al. 2005) still allows the hydrolysis of phosphorylated β-glucosides as alanine and serine are similarly structured and uncharged. This points to the notion that more than one residue might be important in the affinity of a β-glycosidase towards non-phosphorlyated substrates as AscB is able to hydrolyse both phosphorylated and non-phosphorlyated substrates.

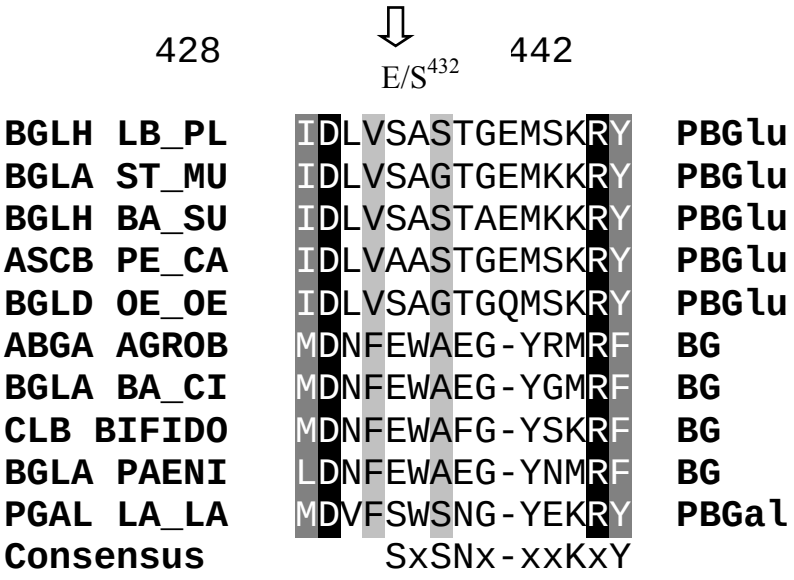

Figure 1. Sequence similarity of glycosyl hydrolase family 1 6-phospho/β-glucosidases/galactosidases. The arrows depict the amino acid residue where either a glutamic acid, serine or an alanine has been shown to be important for substrate specificity in GHF1 (6-phospho)/β-glycosidases, equivalent to E^{432} (BglD numbering, refer to Chapter 3). PBGlu, 6-phospho-β-glucosidase; PBGal, 6-phospho-β-galactosidase; BG, β-glucosidase. BGLH LB_PL, *bglH* from *Lactobacillus plantarum* (Marasco et al. 1998); BGLA ST_MU, *bglA* from *Streptococcus mutans* (Cote and Honeyman 2002); BGLH BA_SU, *bglH* from *Bacillus subtilis* (Le Coq et al. 1995); ASCB PE_CA, *ascB* from *Pectobacterium carotovorum* (An et al. 2005); BGLD OE_OE, *bglD* from *O. oeni* (Chapter 3); ABGA AGROB, *abgA* from *Agrobacterium* spp (Namchuk and Withers 1995); BGLA BA_CI, *bglA* from *Bacillus circulans* (Paavilainen et al. 1993); CLB BIFIDO, *clb* from *Bifidobacterium breve* (Nunoura et al. 1995); BGLA PAENI, *bglA* from *Paenibacillus spp* (Isorna et al. 2007). PGAL LA_LA, *pgal* from *Lactococcus lactis* (Wiesmann et al. 1997). The consensus sequence SxSNx-xxKxY proposed by Wiesmann et al. (1995) is aligned below the sequences.

The glutamate (E^{405}) corresponding to S^{432} (BglD numbering) in *Bacillus polymyxa*, a GHF 1 β-glucosidase, interacts with the hydroxyl groups of C4 and C6 substrates (**Figure 2**) (Sanz-Aparicio et al. 1998a). These interactions are depicted by dashed lines. The formation of the hydrogen bonds with E^{405} provides evidence substantiating the broad specificity of GHF 1 enzymes as these ligands allow for multiple substrates such as glucose (hydroxyl group C4 equatorial position) and galactose (hydroxyl group C4 axial position). This work supports the hypothesis that a phosphorylated C6 cannot be accommodated in this model (Sanz-Aparicio et al. 1998a, Wiesmann et al. 1995).

4.4.2. Synthetic gene verification and cloning

The sequence of BglD_S-E was synthesized and sequenced (**Figure 3**) to verify the introduction of the gene mutation (GAA in positions 1305-1307) whereby the S^{432} would be substituted with a glutamic acid. The synthesized gene was digested with *Nde*I and *Xho*I restriction enzymes along with the vector pET 14.b (**Figure 4**).

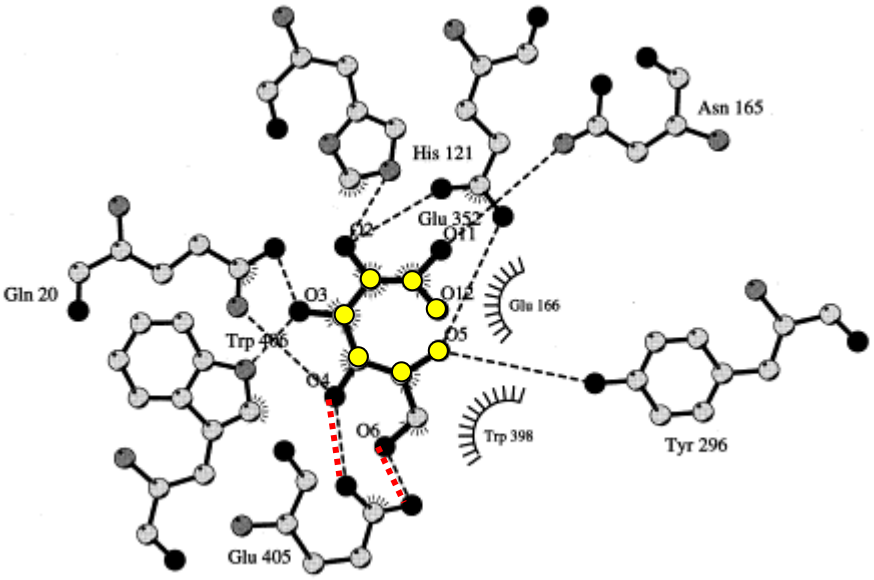

Figure 2. Schematic diagram of the possible enzyme-ligand hydrogen bonds between substrate (gluconate) and enzyme from *Bacillus pol ymyxa* (BglA) (Sanz-Aparicio et al. 1998a). Red dotted lines depict the hydrogen bonds associated with E^{405} (*bglD* S^{432} equivalent) which are speculated to be unable to form in the presence of a C6 phosphate group for reasons of conflicting charge and spatial availability. The carbon atoms of the substrate are depicted in yellow and the molecular structures surrounding it are the amino acids which Sanz-Aparicio, et al (1998a) speculate to interact with the substrate.

```
GCGCGACATATGCTGAACAAACTGTTTCTGCCGAAAAACTTTCTGTGGGGCGGCGCGGTG        60
          L  N  K  L  F  L  P  K  N  F  L  W  G  G  A  V          16
GCGGCGAACCAGCTGGAAGGCGGCTGGGATCAGGATAACAAAGGCCTGAGCGTGGCGGAT        120
A  A  N  Q  L  E  G  G  W  D  Q  D  N  K  G  L  S  V  A  D        36
ATTATGACCGCGGGCGCGAACGGCAAAGCGCGTGAAATTACCGATGGCATTGTGAAAGGC        180
I  M  T  A  G  A  N  G  K  A  R  E  I  T  D  G  I  V  K  G        56
AAATATTATCCGAACCATGAAGCGATTGATTTTTATCATCGTTATAAAGAAGATATTAAA        240
K  Y  Y  P  N  H  E  A  I  D  F  Y  H  R  Y  K  E  D  I  K        76
CTGTTTGCGGAAATGGGCTTTAAATGCTTTCGTACCAGCATTGCGTGGACCCGTATTTTT        300
L  F  A  E  M  G  F  K  C  F  R  T  S  I  A  W  T  R  I  F        96
CCGAACGGCGATGAAGAACAGCCGAACGAAGCGGGCCTGAAATTTTATGATCAGCTGTTT        360
P  N  G  D  E  E  Q  P  N  E  A  G  L  K  F  Y  D  Q  L  F        116
GATGAATGCCATAAATATGGCATTGAACCGGTGATTACCCTGAGCCATTTTGAAATGCCG        420
D  E  C  H  K  Y  G  I  E  P  V  I  T  L  S  H  F  E  M  P        136
TATCATCTGGTGAAAGTGTATGGCGGCTGGCGTAACCGTAAACTGATTGATTTTTTTGTG        480
Y  H  L  V  K  V  Y  G  G  W  R  N  R  K  L  I  D  F  F  V        156
CGTTTTGCGAAAACCGTGTTTAAACGTTATAAAGATAAAGTGAGCTATTGGATGACCTTT        540
R  F  A  K  T  V  F  K  R  Y  K  D  K  V  S  Y  W  M  T  F        176
AACGAAATTGATAACCAGACCGATTATACCAACCGTTTTCTGATGGCGACCAACAGCGGC        600
N  E  I  D  N  Q  T  D  Y  T  N  R  F  L  M  A  T  N  S  G        196
CTGATTCTGAAAAACGATCAGAGCGATGCGGAAAGCCTGATGTATCAGGCGGCGCATTAT        660
L  I  L  K  N  D  Q  S  D  A  E  S  L  M  Y  Q  A  A  H  Y        216
GAACTGGTGGCGAGCGCGCTGGCGGTGAAACTGGGCCATAGCATTAACCCGAACTTTCAG        720
E  L  V  A  S  A  L  A  V  K  L  G  H  S  I  N  P  N  F  Q        236
ATTGGCTGCATGATTAACATGACCCCGGTGTATCCGGCGAGCAGCAAACCGGCGGATATT        780
I  G  C  M  I  N  M  T  P  V  Y  P  A  S  S  K  P  A  D  I        256
TTTCAGGCGGAAAAAGCGATGCAGCGTCGTTATTGGTTTAGCGATATTCATGCGCTGGGC        840
F  Q  A  E  K  A  M  Q  R  R  Y  W  F  S  D  I  H  A  L  G        276
AAATATCCGGAAAACATGGAAGTGTTTCTGAAACAGAACAACTTTCGTCCGGATATTACC        900
K  Y  P  E  N  M  E  V  F  L  K  Q  N  N  F  R  P  D  I  T        296
AGCGAAGATCGTATTGTGCTGAAAGAAGGCACCGTGGATTATATTGGCCTGAGCTATTAT        960
S  E  D  R  I  V  L  K  E  G  T  V  D  Y  I  G  L  S  Y  Y        316
AACAGCATGACCGTGCAGAGCAAAGAAAGCAACCCGGGCTTTCATTTTATTGGCCCGGAA        1020
N  S  M  T  V  Q  S  K  E  S  N  P  G  F  H  F  I  G  P  E        336
CTGACCGTGGATAACCCGAACGTGGAAAAAAGCGATTGGGGCTGGCCGATTGATCCGCTG        1080
L  T  V  D  N  P  N  V  E  K  S  D  W  G  W  P  I  D  P  L        356
GGCCTGCGTTATAGCCTGAACTGGCTGGCGGATCATTATCATAAACCGCTGTTTATTGTG        1140
G  L  R  Y  S  L  N  W  L  A  D  H  Y  H  K  P  L  F  I  V        376
GAAAACGGCCTGGGCGCGTATGATAAAGTGGAAAACAGCCAGCAGATTCATGATCCGTAT        1200
E  N  G  L  G  A  Y  D  K  V  E  N  S  Q  Q  I  H  D  P  Y        396
CGTATTGCGTATCTGAAAGCGCATATTCAGGCGATGATTGATGCGGTGCAGGAAGATGGC        1260
R  I  A  Y  L  K  A  H  I  Q  A  M  I  D  A  V  Q  E  D  G        416
                                        ♦♦♦
GTGAAAGTGATTGGCTATACCCCGTGGGGCTGCATTGATCTGGTGGAAGCGGGCACCGGC        1320
V  K  V  I  G  Y  T  P  W  G  C  I  D  L  V  E  A  G  T  G        436
CAGATGAGCAAACGTTATGGCTTTATTTATGTGGATAAAGATGATCAGGGCAAAGGCAGC        1380
Q  M  S  K  R  Y  G  F  I  Y  V  D  K  D  D  Q  G  K  G  S        456
CTGAAACGTCTGAAAAAAGATAGCTTTTTTTGGTATCAGCAGGTGATTAAAAGCAACGGC        1440
L  K  R  L  K  K  D  S  F  F  W  Y  Q  Q  V  I  K  S  N  G        476
AGCCAGCTGGATCTCGAGGCGCGA                                            1460
S  Q  L  D  L  E  A  R                                              484
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Figure 3. The synthsised gene sequence with the S432E mutation. BglD_S-E had S^{432} (AGC) substituted for a glutamic acid (GAA) in position 1305-1307. The mutation is highlighted with arrows.

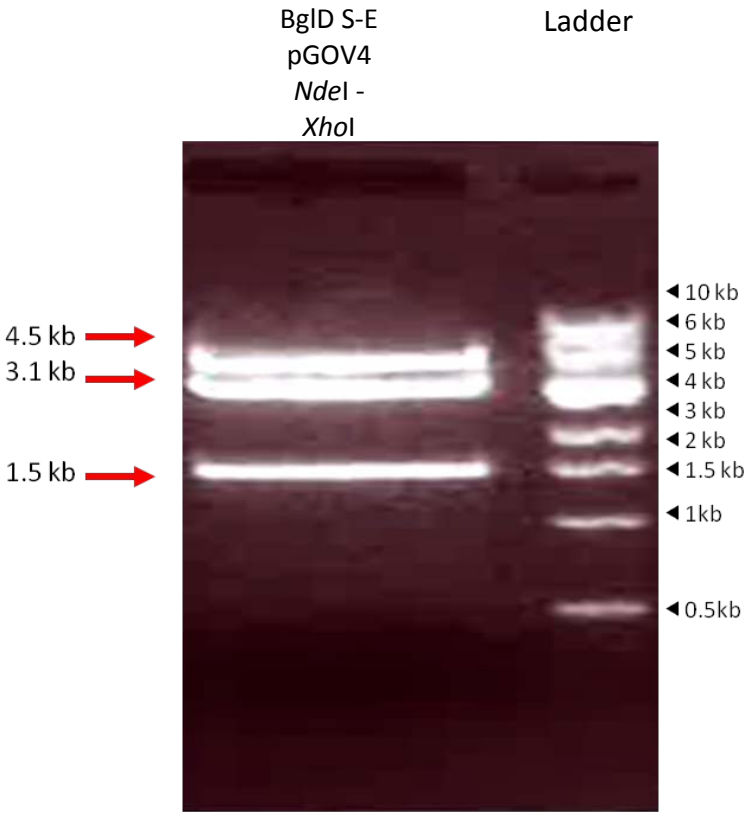


Figure 4. Agarose gel verification of synthesized gene (BglD S-E) in the vector pGOV4, digested with *Nde*I and *Xho*I. The gene was synthesized to incorporate an *Nde*I restriction site at the 5' end and an *Xho*I restriction site at the 3' end. The 4.5 kb fragment represents the pGOV4 BglD S-E construct cut once (partial digestion). The 3.1 kb fragment is the pGOV4 vector without the *bglD* S-E gene (double *Nde*I-*Xho*I cut). The 1.5 kb fragment is the *bglD*_S-E gene, which was excised, purified and used for all subsequent cloning work.

4.4.3. Expression, purification & characterisation of BglD_S-E

The pET 14.b-BglD S-E construct was transformed into *E. coli* strain BL21 (DE3) for over-expression of the gene product. A soluble IPTG-inducible protein of approximately 55 kDa (**Figure 5**) was observed. The yield of protein (approx 1 mg mL^{-1}) was similar to that obtained during purification of the unmodified BglD (Chapter 3).

A lysate of *E. coli* bearing the plasmid pET 14.b-BglD_S-E (the characterized GHF 1 phospho-β-glucosidase described in Chapter 3 with the serine in position 432 mutated into a glutamic acid) was assayed against pNP-β-D-glucopyranoside-6-phosphate (pNPβD6P) and pNP-β-D-glucopyranoside (pNPβG) (**Figure 6A**). Lysates had similar minimal activity towards pNPβD6P to those from cells with only the pET 14.b vector. Lysate from the *E. coli* cells harboring pET14.b_BglD (i.e. the non-mutated gene) had high levels of activity towards pNPβD6P. Furthermore, when assayed the purified protein BglD with a glutamic acid in the place of S^{432} (i.e. the mutated gene) had trace activity against non-phosphorylated and greatly reduced activity towards phosphorlyated substrates (**Figure 6A**). The wild-type BglD protein was assayed alongside the mutant BglD S-E and possessed high levels of activity against pNPβD6P (**Figure 6B**) as previously demonstrated (Chapter 3). The purified proteins BglD S-E and BglD were assayed against the substrates listed in **Table 2**. BglD S-E was demonstrated to have lost all ability to hydrolyse pNPβD6P as opposed to the non-mutated BglD, which retained activity towards the same substrate.

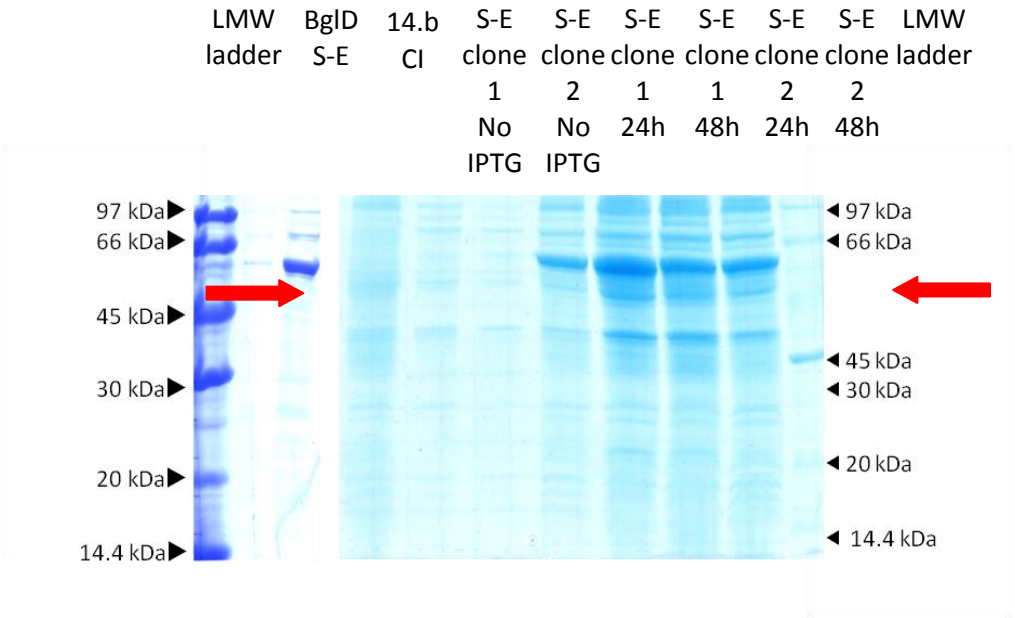
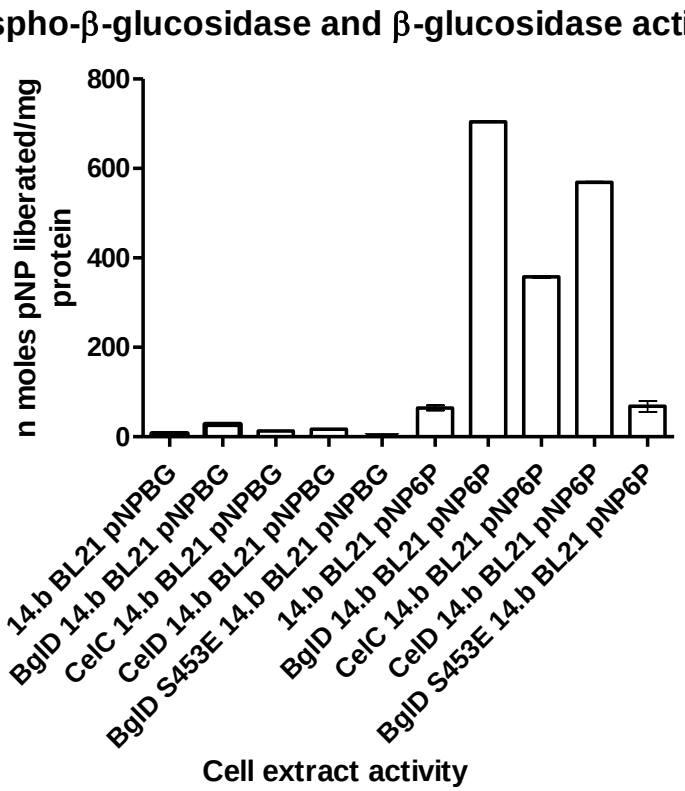


Figure 5. Electrophoretic analysis of the expressed phospho-β-glucosidase pET 14.b BglD_S-E clone 1 and 2 on a 12% (w/v) SDS-polyacrylamide gel pre and post IPTG induction for either 24 or 48 hrs). **LMW Marker**, Low molecular weight marker; **14.b cell extract,** crude extract of cells bearing vector pET 14.b without the bglD gene inserted, **BglD_S-E**, purified bglD_S-E. The gel was stained with 0.025% (w/v) Coomassie blue. The red arrows indicate the presence of a soluble BglD S-E protein.

A



B

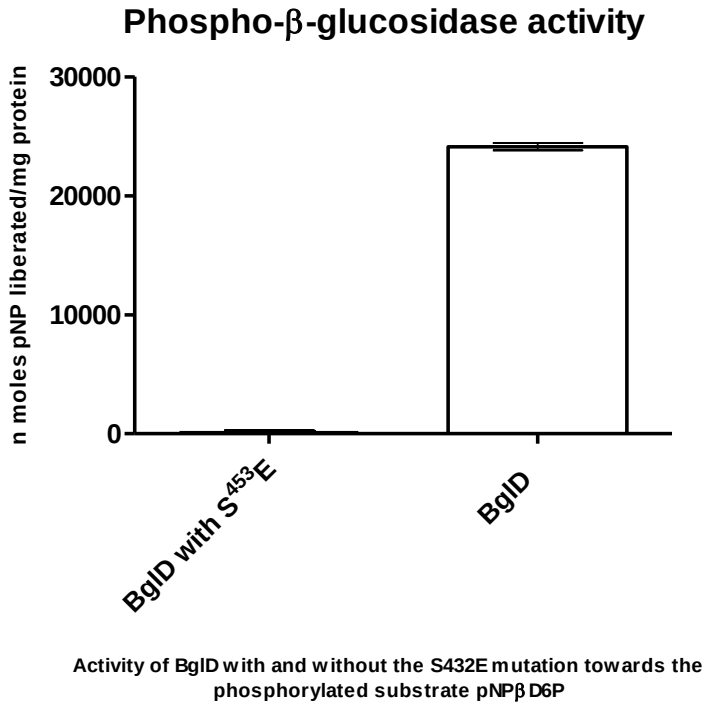


Figure 6. A) Activity of crude extract against pNPβD6P and pNPβG. Enzyme activity was assayed at 37°C for one hour in McIlvaine buffer pH 5.5 **B)** Activity of purified BglD_S-E and purified BglD (Chapter 3) against pNPβD6P in the same conditions mentioned above.

Table 2. Detection of activity of the purified BglD S-E and BglD.

Substrates tested	Enzyme	
	BglD_S-E	**BglD**
pNP-β-D-galactopyranoside	-	-
pNP-α-L-arabinofuranoside	-	-
pNP-α-D-galactopyranoside	-	-
oNP-β-D-glucopyranoside	-	-
pNP-β-L-arabinopyranoside	-	-
pNP-α-L-arabinopyranoside	-	-
pNP-α-D-glucopyranoside	-	-
pNP-β-D-galactopyranoside	-	-
pNP-β-D-glucopyranoside	-	-
pNP-β-D-glucopyranoside-6-phosphate	-	+

4.5. Conclusions

BglD, a characterised GHF 1 phospho-β-glucosidase BglD (Chapter 3), successfully had its specificity altered towards phosphorylated β-glucosides. BglD lost 100% affinity towards *p*-Nitrophenol-β-D-glucopyranoside-6-phosphate, and had no affinity towards non phosphorylated β-glucosides. An important residue was mutated in a speculated GHF 1 phosphate/substrate specific binding site, confirming that this residue in this site was important for the enzyme's specificity. This chapter also demonstrates that more than one residue in the same region is important for substrate specificity.

The characterised GHF 1 phospho-β-glucosidase BglD (Chapter 3) was mutated in an attempt to alter its specificity towards non-phosphorylated substrates. Mutations were introduced into GHF 1 β-glucosidases to target the proposed phosphate group recognition site and thereby change the specificity towards phosphorylated β-glucosides (Kaper et al. 2000) and broaden specificity towards β-glycosides (Hancock et al. 2005, Corbett et al. 2001). No information (in reported literature) is available about mutating the phosphorylation site of GHF 1 phospho-β-glucosidases towards non-phosphorylated substrates.

Elements of GHF 1 enzymes such as the N-terminal signature sequence and the catalytic sites are well characterized. In the instance of the active sites, two glutamic acid residues in positions E^{178} and E^{377} are highly conserved in GHF 1 enzymes and have been experimentally validated many times (An et al. 2004, An et al. 2005, Hong et al. 2007, Hong et al. 2006, Wiesmann et al.

1997). A third site, located inside the cavity that the two catalytic glutamic acids form, is important for the broad substrate specificity demonstrated by most GHF 1 enzymes and the phosphate group-enzyme interaction of GHF 1 phospho-β-glucosidases (Wiesmann et al. 1995, Hill and Reilly 2008). The single point mutation E417S in CelB demonstrated not only an increase in affinity towards phosphorylated substrates but a shift in pH optimum from 4.0 to 5.0 (Kaper et al. 2000). It is interesting to note that the wild type β-glucosidase CelB was able to hydrolyse oNP-β-D-galactopyranoside-6-phosphate (oNPβD6P) whereby BglD_S-E had lost the ability to hydrolyse pNPβD6P (Kaper et al. 2000). This reinforces the hypothesis that it is likely that more than one residue is important in the interaction of the phosphate group from the substrate with the phosphate receival site in GHF 1 phospho-β-glucosidase/galactosidases. However, in accordance with the conclusions made by Kaper et al. (2000), the equivalent BglD S^{432} seems to be the determining factor for the difference in substrate specificity between the two types of family 1 glycosidases.

BglD was successfully modified and observed a predicted lack of activity towards phosphorylated substrates. It would be of interest to also mutate the residues around S^{432} (BglD numbering) to provide insight into substrate specificities of GHF 1 enzymes and crystallize the wild type enzyme as the first crystallized GHF 1 phospho-β-glucosidase with associated characterisation data.

Chapter 5

5. β-glucoside metabolism in *Oenococcus oeni*: Cloning and characterisation of the phospho-β-glucosidase CelD

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The GenBank accession numbers for the nucleotide sequences are:

celC: JQ002657

celD: JQ002656

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Sign: Date:

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Chapter 6

6. *O. oeni* growth, gene expression and proposed transcriptional regulation

6.1. Introduction

β-glucosidases in *O. oeni* have a fundamental role in winemaking because of their potential to liberate desirable aromas which otherwise would not be in a free volatile form. Such aroma compounds usually exist as glycosides (Maicas and Mateo 2005). Several genes in *O. oeni* have been identified as important in the hydrolysis of β-glucosides in conjunction with the phosphoenolpyruvate dependent phosphotransferase system (PEP-PTS). Gene products from the PEP-PTS are responsible for the uptake and phosphorylation of β-glucosidases as ahead of their use as an alternative carbon source to glucose (Deutscher et al. 2006). The identification of two PEP-PTS β-glucoside metabolising operons in *O. oeni* using the sequenced genome PSU-1 is reported in Chapters 3 and 5.

The β-glucoside metabolizing operons in *E. coli* have received the greatest attention (bgl operon (Mahadevan et al. 1987), cel operon (Kricker and Hall 1987), and the *sac* operon (Parker and Hall 1988). Analogous operons have also been identified in Gram-positive bacteria including *Bacillus subtilis* (Le Coq et al. 1995), *Lactococcus lactis* (Bardowski et al. 1994), *Lactobacillus plantarum* (Marasco et al. 2000) and the gram-negative bacterium *Erwinia chrysanthemi* (El Hassouni et al. 1992). The regulation of such operons is

considered to be at a transcriptional level (Rutberg 1997) through the interaction of terminator and anti-terminator secondary DNA structures.

The PEP-PTS β-glucoside metabolizing operon from *E. coli, bglGFB,* codes for an anti-terminator (BglG), a PEP-PTS permease (BglF) and a phospho-β-glucosidase (BglB) (Mahadevan et al. 1987). This operon is directly responsible for the hydrolysis of aryl-β-glucosides such as salicin and arbutin. In *E. coli*, this operon is only functional subsequent to a series of spontaneous mutations (Reynolds et al. 1981, Reynolds et al. 1986). Once rendered functional, the *bglGFB* operon is inducible by β-glucosides. In the absence of β-glucosides, transcription has been shown to terminate due to a ρ-independent terminator situated within the operon which prevents the RNA polymerase from continuing transcription. In the presence of β-glucosides, BglG acts as a transcriptional anti-terminator, binding to the ρ-independent terminator thus allowing transcription to continue (Houman et al. 1990, Mahadevan and Wright 1987). BglG is able to function as a transcriptional anti-terminator in the presence of β-glucosides because the PEP-PTS component EII phosphorylates the β-glucoside, which is subsequently hydrolyzed inside the cell. However, in the absence of β-glucosides, BglG is phosphorylated and is no longer able to function as a transcriptional anti-terminator (Amster-Choder and Wright 1992).

The *bgl* and *ce*l operons in *O.oeni* were identified by sequence comparison of the sequenced PSU-1 genome to the characterised operons in other gram-positive bacteria (Refer to chapters 3 and 5). Further sequence analysis was

undertaken to identify the possible regulatory mechanisms in the *bgl* and *cel* operons in *O. oeni*. It is hypothesized, based on sequence similarity data to know transcriptional signals and functional data from characterised operons from other species, that both the *cel* and *bgl* operons have a number of regulatory mechanisms such as transcriptional anti-termination and carbon catabolite repression.

Previous work focused on the characterization and purification of the phospho-β-glucosidases BglD, CelC and CelD (Chapter 3 and 5). The following chapter seeks to confirm the hypothesis that these phospho-β-glucosidases function in conjunction with the PEP-PTS in *O. oe ni* by analysing gene expression when cells are grown in the presence and absence of specific carbon sources. Insights into the regulatory mechanisms in this chapter were executed by sequence analysis on the *bgl* and *cel* operon.

6.2. Materials and methods

6.2.1. Growth and strains

The *O. oeni* wine strain Lalvin 4X (VL92) was cultured for approximately five days in de Man, Rogosa and Sharpe (MRS) broth supplemented with 10% (v/v) filter sterilized preservative-free apple juice (commercially available). This culture was used to inoculate 50 mL flasks of MRS (1:100 dilution, i.e. 0.5 mL in 49.5 mL of media) made up without meat extract or dextrose, supplemented with 1% (w/v) of arbutin, cellobiose, salicin, arbutin/glucose, cellobiose/glucose or salicin/glucose which was subsequently filter sterilized.

Growth was monitored by measuring the optical density at 600 nm and visual confirmation by observing the cells at a 1000 x magnification.

6.2.2. Sequencing of the *bgl* and *cel* operons

A culture of *O. oeni* wine strain Lalvin 4X (VL92) was grown as described for 5 days. The culture was centrifuged at 14000 x g for 10 minutes and the cell pellet used to extract genomic DNA using the Ultraclean™ Microbial DNA Isolation kit (Mo Bio Laboratory) according to the manufacturer's instructions. The DNA was verified on a 1% (w/v) agarose gel in 1x Tris acetate EDTA (TAE) buffer pH 8.0 (data not shown) stained with GelRed™ (Biotium, distributed by Jomar Diagnostics). PCR amplification was performed using the purified genomic DNA as a template and primers listed in **Table 1,** and subsequently sequenced as described in Chapters 3 and 5.

Table 1. Primers used to amplify fragments of the *bgl* and *cel* operons.

Primer	Dir*	Sequence (5'- 3')	Length (bp)	Tm**	Amplicon length (bp)
bglseq1	F	CGGTTGCTTTCGGCTTATTCATGG	24	57.19	1550
bglseq2	R	ATTCCAGGATTCGCGGCGCT	20	59.21	
bglseq3	F	TCAACAGGGCGTTCATTGGAACAAC	25	58.44	1579
bglseq4	R	TGGGGATGGCCGATCGATCCG	21	60.51	
bglseq5	F	GACTTTGTCATAGGCTCCCAGACCG	25	58.88	1652
bglseq6	R	TGATCGCTCTGGCCATACACCT	22	57.52	
bglseq7	F	ACGATTCACGTTTCTCCTGCTTCTG	25	57.62	844
bglseq8	R	CGACTGACATCGAATTCCGCCA	22	57.43	
C1	F	GTGTAGCAACCATGATTAGGCGAG	24	55.95	882
C2	R	AGCGTCAACTAAGAACTTGTCAGC	24	55.72	
C3	F	AAAGGTCACTTTACAGTTGCTGACC	25	55.7	832
C4	R	GCAAGCCGGAGTTTTGAAGC	20	55	
C5	F	CGCTTTGCTGAAGTTTGTTTCAAGC	25	56.9	1057
C6	R	GTTTGCAGCAACGGCTCCTC	20	56.78	
C7	F	AGATGACGAAGGTCATGGTAGC	22	54.03	946
C8	R	ACATCAGCCCACCAGTAACGTC	22	56.67	
C9	F	TGGCTGAAAAAGCTATGCAAAGACG	25	56.89	930
C10	R	GCGGCATTAGCCATCTCACG	20	56.18	
C11	F	ATCAGAAAGTTCATGAGTTGAATCG	25	52.07	910
C12	R	CCATGAGCAAAACCCCCTCA	20	54.32	
C13	F	TCGACGAAAGGGTTTACAATGGT	23	54.58	979
C14	R	TTGTGGCACCAGAACCACCC	20	56.99	
C15	F	GCATTCACTGCTGGTAAAGCTC	22	54.39	1000
C16	R	AGTCTTCCCCCAAACCAGTATATC	24	53.53	

*Dir – direction; F (forward), R (reverse)

**Tm – melting temperature °C

6.2.3. Transcription analysis

Samples (1 mL) were taken from each culture at an OD_{600} of 0.5 and 1.0, and centrifuged at 14000 x g for 10 minutes. After the supernatant was removed, RNA was extracted using the High Pure RNA Isolation Kit (Roche), reverse-transcribed using iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions and quantified using a NanoDrop 1000 (Thermo Scientific). Primers were synthesized by Sigma-Aldrich Australia (**Table 2**). PCR amplification was carried out using DyNAzyme Ext (Finnzymes) using the cDNA as a template. The reference genes *ldhD* and *gyrA* were included as they have been validated for *O. oeni* in response to stress conditions as a useful tool for relative transcript quantification. The resulting cDNA was validated on a 2% (w/v) agarose gel stained with GelRed™ (Biotium, distributed by Jomar Diagnostics) run in 1% TAE buffer (**Appendix 2**) at 100 V. The reaction conditions are described in **Tables 3** and **4**.

Table 2. Primers used for transcriptional analysis of the *cel* and *bgl* operon.

Gene	Primer	5' – 3'	Tm*	Amplicon length (bp)
ldhD	ldhDprobe155Fwd	GGACGTTTATGAAAATGAGATTGG	64.5	73
ldhD	ldhDprobe155Rev	CAAACGAGCATCAGGGAACT	64.55	
gyrA	gyrAprobe148Fwd	CCAAAGGAATCCCGGTTATTA	63.79	70
gyrA	gyrAprobe148Rev	ACATTTATAACGGCCTGAACG	62.92	
celD	celDprobe11Fwd	AGCGGCCTGCGTAAAGAT	65.16	83
celD	celDprobe11Rev	CCTTTGCCATCCACATCC	64.52	
celC	celCprobe44Fwd	CAGATTGCGCATCGTATTGA	65.11	67
celC	celCprobe44Fwd	CGGATAAATCGGGCACAT	63.72	
bglD	bglDprobe61Fwd	CAGCGTCGTTATTGGTTTAGC	63.71	91
bglD	bglDprobe61Rev	CCGGACGAAAGTTGTTCTGT	64.43	

*TM, primer melting temperature

Table 3. PCR reaction utilized to amplify cDNA

PCR components	Stock solution	Final concentration	Volume (µL)
Forward primer	10 µM	0.5 µM	2.5
Reverse primer	10 µM	0.5 µM	2.5
Template cDNA	~1 ng/µL	1 pg – 10 ng	0.5
Buffer with $MgCl_2$	10 X	1 X	5
dNTPs	10 mM	200 µM	1
MQ	-	-	38
DyNAzyme ext	1 unit/µl	1 unit	1
Total volume	**-**	**-**	**50 µL**

Table 4. Conditions for PCR amplification of cDNA

Number of cycles	Cycle step	Temperature (°C)	Duration
x 1	Initial denaturation	94	2 minutes
x 25	Denaturation	94	30 seconds
	Annealing	58	30 seconds
	Extension	72	40 seconds
x 1	Final extension	72	10 minutes
		4	Hold

6.3. Results and discussion

6.3.1. Sequence analysis

6.3.1.1. Regulation by transcriptional anti-terminators

Fundamental components in the regulation of PTS β-glucoside metabolising operons are the presence of stem-loop terminator and anti-terminator structures. There appear to be numerous potential candidates for stem-loop structures both up and downstream of *celE* (data not shown). Rho-independent termination involves a stem loop structure of approximately 20 nucleotides which terminates transcription. By comparison, anti-termination of catabolic operons involves two elements: a stem-loop ribonucleic anti-terminator structure (RAT), which precedes the terminator structure, and a terminator protein, which binds to the RAT structure (Rutberg 1997). It has been demonstrated that in most cases the two overlap (Aymerich and Steinmetz 1992). However, the RAT is less stable than the terminator and becomes more stable when the terminator protein binds to it. This action does not allow for the terminator to form and permits transcription to continue. Rho-independent anti-termination of catabolic operons is substrate-induced in the case of the *E. chrysanthemi arb* operon (El Hassouni et al. 1992), and *bgl* operons from *E. coli* and *B. subtillis* and partially characterized *L. lactis bgl* operon (Bardowski et al. 1994). In the absence of β-glucosides, *E. coli* BglG is monomeric and phosphorylated and is not able to bind the RAT sequence, preventing the formation of a transcription terminating stem-loop structure. In the presence of β-glucosides, BglG is dimeric and binds to the RAT sequence, allowing transcription to continue (Amster-Choder and Wright 1992). The

RAT sequence from *E. chrysanthemi* has high homology to a portion of DNA from *O. oeni* between *celE* and *celF* (**Figure 1**) and is speculated to be a site of transcription anti-termination, whereby the product of *celE* may bind. Sequence comparison of *celE* to the NCBI database using NCBI BLAST programme (http://www.blast.ncbi.nlm.gov/Blast.cgi), showed homology to RpiR/YebK/YfhH family members, and more specifically to the HTH region of GlvR, a regulatory component of the *glv* operon in *B. subtilis* (Yamamoto et al. 2001) and *yebF* from *L. lactis*. Although the RpiR/YebK/YfhH family of regulatory genes has not been characterized, GlvR was shown to be essential for transcription of the maltose metabolising *glv* operon in *B. subtilis*. GlvR, the product of the *glvR* gene, has been validated as a positive regulator of the *glv* operon. Similarly to the *bgl* and *cel* operon in *O. oeni*, the *glv* operon also contains a 6-phospho-β-glucosidase *glvA*, and a PEP-PTS component *glvC* an EIICB transport protein (Yamamoto et al. 2001). The HTH DNA binding motif is found in many regulatory proteins (including *glvR* and *celE*), and is thought to play a role in *cel* operon regulation.

6.3.1.2. Carbon Catabolite Repression of carbon metabolizing operons in gram positive bacteria

A hierarchical usage of diverse carbon sources in bacteria is regulated by carbon catabolite repression (CCR). CcpA, a regulator of carbon metabolism in gram positive bacteria, binds to a *cre* sequence which can be located upstream or in promoter regions, or even in open reading frames (Lulko et al. 2007). CcpA represses the transcription of genes necessary for the catabolism of secondary carbon sources (Tina 1996). The activity of CCR has been extremely well documented in the gram positive *B. subtilis* and the gram

negative *E. coli*. Miwa et al (2000) proposed the catabolic responsive element *cre* consensus sequence WTGNAARCGNWWWCAW (N = any base; W = T or A; R = A or G) (Miwa et al. 2000). The *glv* operon in *B. subtilis* (previously discussed), is regulated by glucose, which exerts its effects via carbon catabolite repression requiring both CcpA and a *cre* sequence upstream of glvA, a 6-phospho-β-glucosidase and the first gene of the *glv* operon (Yamamoto et al. 2001). The presence of a putative *cre* sequence 51 bases upstream of the transcriptional start point of celA in *O. oeni* (**Figure 2**) suggests that if the *cre* sequence is operational, the *cel* operon may also be regulated by CCR. As mentioned it may be repressed in the presence of preferred carbon sources.

6.3.1.3. Growth of *O. oeni* on alternative carbon sources

O. oeni was able to grow on the naturally occurring plant β-glucosides salicin and arbutin and disaccharide cellobiose (with two glucose moieties) as sole carbon source (**Figure 3**). MRS media lacking a carbon source was also inoculated as control. MRS media without any carbon source supported minimal growth (OD_{600} < 0.1), thus demonstrating the inability of *O. oeni* to grow in the absence of a carbon source. The three cultures, one each containing supplementation from salicin, arbutin or cellobiose, had a greater initial lag time *O. oeni* (approximately 10 days) than salicin and cellobiose with added glucose (approximately 5 days). In the presence of arbutin plus glucose, the cells had a 10 day lag phase before increasing exponentially on day 10. Glucose was added at 1% (w/v) to three growth assays (AG, SG and CG) to establish the impact on growth patterns, and also to determine whether glucose repressed

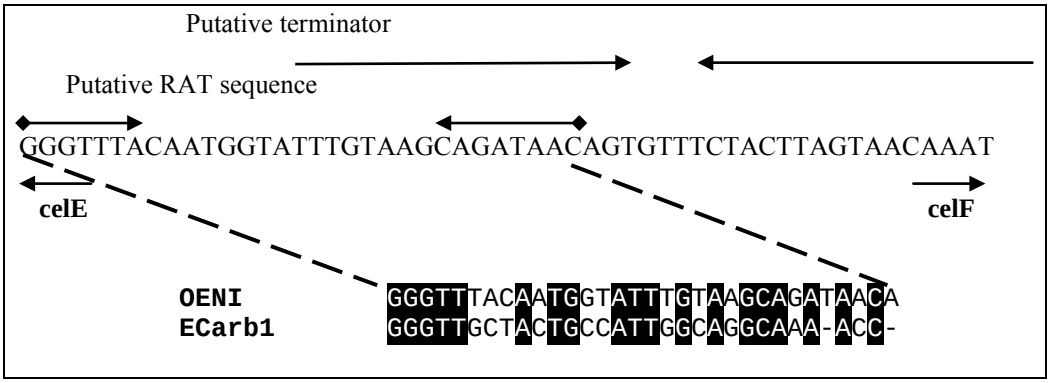

Figure 1. Alignment of a potential RAT sequence upstream of *O. oeni celE* to *E. chrysanthemi* ECarb1. OENI; speculated *cre* sequence in *O. oeni* PSU-1. ECarb1, *cre* sequence in *E. chrysanthemi*. The identical bases are highlighted in black with white lettering.

(A)

Consensus	WTGNAARCGNWWWCAW
O. OENI	ATGAAAGCGTTTTAAG
sdhCAB	ATGTACGCGTTTTCTT
glpFK	TTGACACCGCTTTCAT
kdgA	ATGGAAGCGCTGACAT
glpTQ	AAGAAAGCGCTATCAT
ydh	ATGTAACGCTTTTAAT
ydhT	TTGGAAGCGGTATCAT
phoPR	ATGAAAGCGCTATCAT
rocG	TTTAAAGCGCTTACAT
bglP	ATGAAAGCGTTGACAT

(B)

328959

cre -35

ATGAAAGCGTTTTAAGTAAACATTTTAAATAAAAAACAAATAAATATTATATTCAAA

-10 TS

GGGGTATTTTATTATG

Figure 2. Identification of a putative cre sequence found upstream of the *celA* gene in *O. oeni.* **Panel A**: Comparison of the putative *O. oeni cre* sequence to the consensus *cre* sequence (N = any base; W = T or A; R = A or G) and CcpA repressed genes from *B. subtilis*; *sdhCAB*, succinate dehydrogenase; *glpFK*, glycerol-3-phosphate permease glycerol kinase; *kdgA*, 2-keto-3-deoxygluconate-6-phosphate aldolase; *glpTQ*, glycerophosphoryl diester phosphodiester; *ydh*, hypothetical protein; *ydhT*, hypothetical protein; *phoPR*, two-component response regulator/two-component sensor histidine kinase; *rocG*, glutamate deydrogenase; *bglP*, (PTS) β-glucoside specific enzyme IIBCA component. The letters shaded in grey depict the amino acids that comply with the consensus sequence by Miwa et al (2000). **Panel B:** Schematic diagram of the location of a putative *cre* sequence upstream of the *celA* gene in *O. oeni*. TS: Transcriptional start point of *celA*. The initiation codon for Methionine (ATG) is boxed in red. The boxed numerals represent the position in the genome based on the PSU-1 sequence.

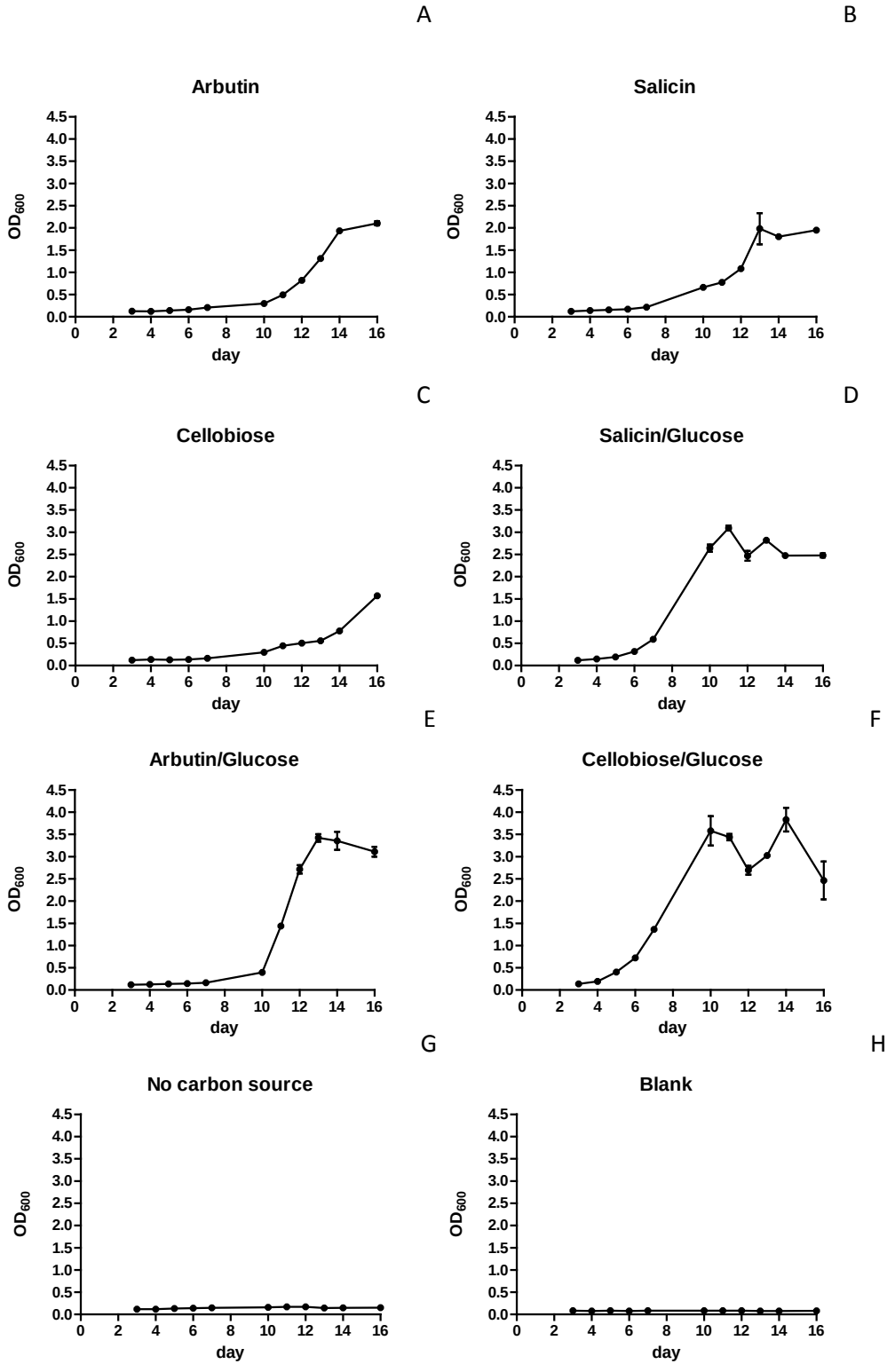

Figure 3. Growth of *O. oeni* strain Lalvin 4X (VL92) in MRS broth lacking meat extract and dextrose at 30°C under anaerobic conditions. The MRS broth was supplemented with 1% (w/v) of A) Arbutin, B) Salicin, C) Cellobiose, D) Salicin/Glucose E) Arbutin/Glucose F) Cellobiose/Glucose G) No carbon source H) Inoculated (Blank)

the phospho-β-glucosidase genes previously identified (refer to Chapters 3 and 5). The cultures were examined daily under the light microscope (at 1000 x magnification). **Figure 4** shows images taken of cells 16 days post inoculation. The cells grown in the presence of glucose (**Figure 4**: images AG, CG and SG) occurred as short chains, whilst those grown on arbutin, salicin and cellobiose as a sole carbon source grew in clusters with much longer angular shaped chains (**Figure 4**: images A, C and S).

RNA was extracted from the cultures at an OD_{600} of 0.5 and 1.0, reverse-transcribed as described in Materials and Methods, and used as a template for PCR. Five gene products were amplified: *celC*, *celD*, bglD and *ldhD* and *gyrA*. The PCR products were visualised under UV following gel electrophoresis on a 2% agarose gel. The cDNA from cultures sampled at OD_{600} 0.5 only produced a product for the reference genes *ldhD* and *gyrA*, and no product was observed for *bglD*, *celC* and *celD* at this time point (data not shown). **Figure 5** depicts electrophoretic analysis of the cultures sampled at OD_{600} 1.0. No PCR products were visible following amplification with *celC* primers. However, bands of approximately 80 bp corresponding to the reference genes *ldhD* and *gyrA* were observed under UV illumination when the same cDNA template was used (refer to **Table 1** for expected amplicon sizes). Different amounts of product could be identified between the different templates when amplified with the reference gene primers. This may be due to an inconsistent RNA extraction or simply varied amounts of cDNA.

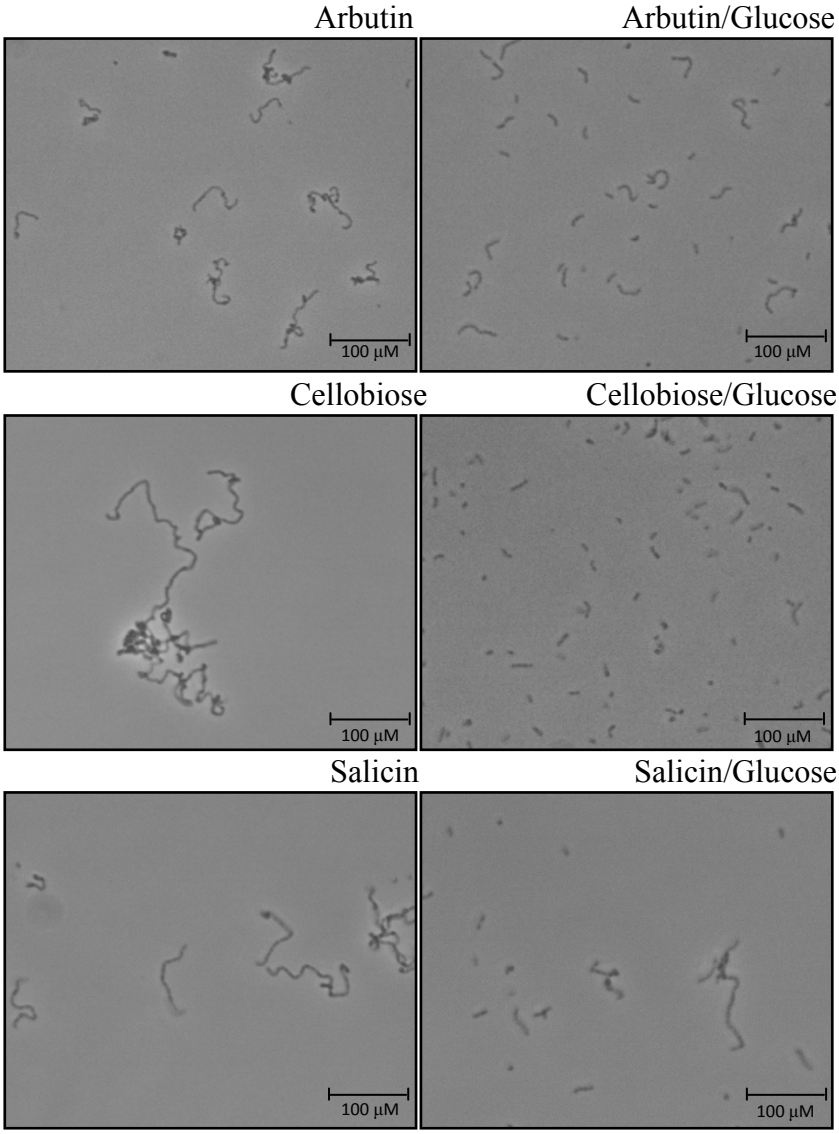

Figure 4. Images recorded on day 16 under 1000 x magnification. **Arbutin:** growth on 1% (w/v) Arbutin; **Arbutin/Glucose:** growth on 1% Arbutin and 1% Glucose; **Cellobiose:** growth on 1% Cellobiose; **Cellobiose/Glucose:** growth on 1% Cellobiose and 1% Glucose; **Salicin:** growth on 1% Salicin; **Salicin/Glucose:** Growth on 1% Salicin and 1% Glucose.

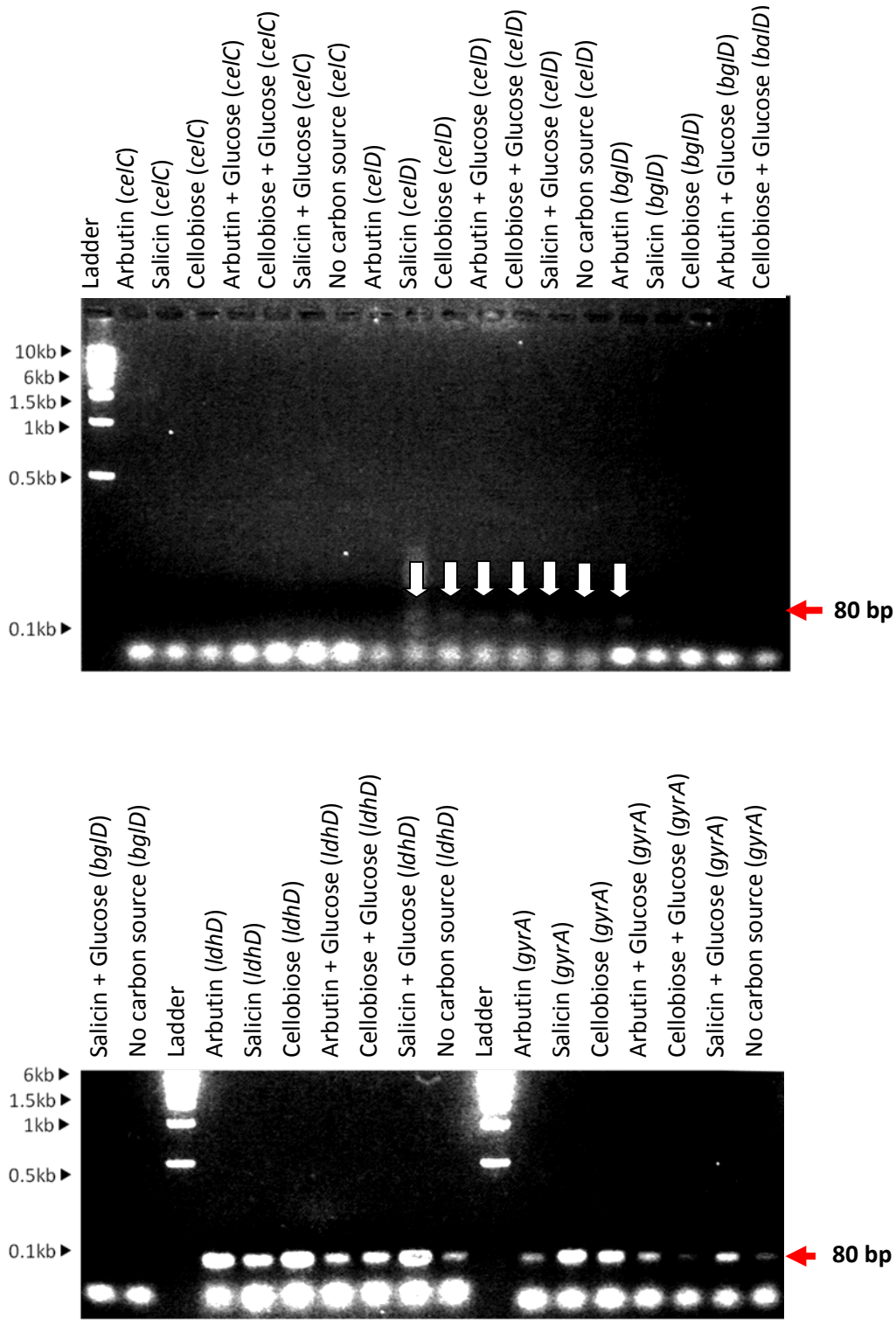

Figure 5. Electrophoesis of amplification products derived from cDNA (as template) and gene specific primers for *celC*, *celD* and *bglD*. *ldhD* and *gyrA* genes were used as controls for quantification purposes. DNA was visualised under UV, following separation on a 2% agarose gel in the presence of GelRed.

The cDNA was quantified as described in Materials and Methods, and 20 μL (which equated to over 1100 ng μL^{-1} of cDNA for all the samples) was loaded into each well of the agarose gel, to allow for maximum product visibility.

Amplification with *celD* primers yielded a faint product approximately 100 bp in size for all assays with the exception of arbutin (**Figure 5**). Amplification using *bglD* primers produced a faint product (just discernible, see arrow) when cells were grown in arbutin as the sole carbon source. This implies that the expression of the *bglD* and *celD* genes may be induced by the carbon source which the cell metabolizes.

6.3.1.4. Sequencing of the *cel* and *bgl* operons

The *bgl* operon was sequenced and found to be identical to the *bgl* operon in PSU-1 in terms of operon configuration (**Figure 6**); there were some nucleotide differences that have been highlighted in **Figure 7**. The *cel* operon was not able to be sequenced following several attempts to amplify 1 kb fragments based on the cel operon configuration in PSU-1 (**Figure 8**). When the *cel* operon is compared to the equivalent operons in the two other *O. oeni* genomes sequenced (AWRIB429 and ATCC BAA-1163), distinct differences in operon configuration could be determined so it is likely that the *cel* operon is actually set up quite differently in the wine strain Lalvin 4X (VL92) (**Figure 8**).

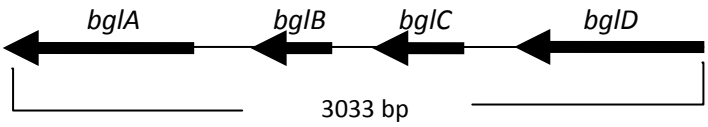

Figure 6. Spacial organization of the *bgl* operon in Lalvin 4X (VL92)

```
Lal4X(VL92)  ----------AGGATTTATAAGCGGGGTGCGCCGTTTC-ATAGTTGGTCTTGTGCTTTTG
PSU-1        TTCATGGTTGGCAATTTATTAGCGGGTGCCGCCGTTTCCATAGTTGGTCTTGTGCTTTTG
                                                                       180
Lal4X(VL92)  GTATATTTAATCCGTCCAACGGTTAAGGAAAAAAATAGCAATAAGTAAGTTATTTTCAAG
PSU-1        GTATATTTAATCCGTCCGACGGTTAAGGAAAAAAATAGCAATAAGTAAGTTATTTTCAAG
                                                                       240
Lal4X(VL92)  GGAAACTGATGGAAAAATAAAAAGGTCTTCCAAATTTGGAAGGCCTTTTTATTGAATTAT
PSU-1        GGAAACTGATGGAAAAATAAAAAGGTCTTCTAAATTTGGAAGGCCTTTTTATTAAATTAT
                                                                       300
Lal4X(VL92)  CAACCAGGAAGTTAGAATATAAAGGAATAAGGTTAAAGGAATTATGAAACGGCATATTAT
PSU-1        CAATCGGGAAGTTAGAATATAAAGGAATAAGGAATAAGGAATAATGAAACGGCATATTAC
                                                                       360
Lal4X(VL92)  TACTTAATTTTCAGTACATTTAAAAAA--AGACCCGCTCTTAGTATCAACTAAAGGTAGG
PSU-1        TACTTAATTTCCAGGACATTTTAAAAATAAGACCCGCTCTTAGTATCAACTAAAAGTAGG
                                                                       420
Lal4X(VL92)  CCTTGAATTAGGTAATTTTTTCCAATATTTTTTCTTTGAAAAATGAGAATGCTAAATTTT
PSU-1        CCTTTAATTAGATAATTTTTTCTAATATTTTTTCTTTGAAAAATGAGAATGCTAAATTTT
                                                                       480
Lal4X(VL92)  AGCTGCTTTTTCTTTTTCTTGTTTTAGCAATACATTATCGTAGTGTTTTATAAATGGATA
PSU-1        AGCTGCTTTTTCTTTTTCTTGTTTGAGCAATACATTATCGTAGTGTTTTATAAATGGATA
                                                                       540
Lal4X(VL92)  CCATACAAGAAAGGCGACAATAGCATCGACAATTGAAAGAACAGCTCCTTTCCAACTAGC
PSU-1        CCATACAAGAAAGGCGACAATAGCATCGACAATTGAAAGAACAGCTCCTTTCCAACTAGC
                                                                       600
Lal4X(VL92)  TGTTGCAATGAAACCACTTAAACCAACCGGAGTAGGCCAAGGCTGTTGAAGAATAACTTT
PSU-1        TGTTGCAATGAAACCACTTAAACCAACCGGAGTAGGCCAAGGCTGTTGAAGAATAACTTT
                                                                       660
Lal4X(VL92)  CGGCACTAATTTGGATGTTATCGCGACGTAGCTGACAATACCTGAGGCTAGAGGGGCACA
PSU-1        CGGCACTAATTTGGATGTTATCGCGACGTAGCTGACAATACCTGAGGCTAGAGGGGCACA
                                                                       720
Lal4X(VL92)  GATAAATGGAATAATTAGATTAATATTATAGACAATCGGCAAACCAAATAAGATTGGCTC
PSU-1        GATAAATGGAATAATTAGATTAATATTATAGACAATCGGCAAACCAAATAACATTGGCTC
                                                                       780
Lal4X(VL92)  ATTGATATTGAAAATAGCAGGGACAATTTCTACTTTTCCAATTTCTCTTAATTGGGTTGA
PSU-1        ATTGATATTGAAAATAGCAGGGACAATTTCTACTTTTCCAATTTCTCTTAATTGGGTTGA
                                                                       840
Lal4X(VL92)  ACGGGATCGGAATAACAGCCAGATTGCCAAACCCAAAGTTGCCCCGGATCCTCCGATAAT
PSU-1        ACGGGATCGGAATAACAGCCAGATTGCCAAACCCAAAGTTGCCCCGGATCCTCCGATAAT
                                                                       900
Lal4X(VL92)  AACGAAAGCGTTCAATGGATCACCGGCAAAGAAGTGAAAACCCCCTTTGGCATTCGATGC
PSU-1        AACGAAAGCGTTCAATGGATCACCGGCAAAGAAGTGAAAACCTCCTTTGGCATTCGATGC
                                                                       960
Lal4X(VL92)  CATGTTGGCCAAAACGATTGGTGTGTAAAAGGAACTCATAATAGTTGCTCCATGGACACC
PSU-1        CATGTTGGCCAAGACGATTGGTGTGTAAAAGGAACTCATAATAGTTGCTCCATGGACACC
                                                                      1020
Lal4X(VL92)  AAACCACCATAGGAAGTGGATCAGAAAGACGATAATCAAGAATCCCCACCATGTATCAGC
PSU-1        AAACCACCATAGGAAGTGAATCAGAAAGACGATAATCAAGAATCCCCACCATGTATCAGC
                                                                      1080
Lal4X(VL92)  GATGTTGCTGACAAATGAAAATGGAATGTAAAGAATTTTGAATATGTCGGTTCCCATAAT
PSU-1        GATGTTGCTAACAAATGAAAATGGAATATAAAGAATTTTGAATATGTCGGTTCCCATAAT
                                                                      1140
Lal4X(VL92)  GATCAAAATGAGATCGATTGCCGCGATCACGAAAGCGACACAAAAACCTGGAATTAGTGC
PSU-1        GATCAAAATGAGATCGATTGCCGCGATCACGAAAGCGACACAAAAACCTGGAATTAATGC
                                                                      1200
Lal4X(VL92)  GCTAAAGGAGTTAGACACACCAGCTGGTACTGAAGCTGGCATTTTTATCTGCCAATTGTG
PSU-1        GCTAAAGGAGTTAGACACGCCAGCCGGTACTGAAGCTGGCATTTTTATCTGCCAATTGTG
                                                                      1260
Lal4X(VL92)  TTTTATGGTAAAACGATAGATCTGAACCGTAATCCAGCCAACAATTAGTCCGGTAAAAAT
PSU-1        TTTTATGGTAAAACGATAGACCTGAACCGTAATCCAGCCAACAATTAGTCCGGTAAAAAT
                                                                      1320
Lal4X(VL92)  ACCCACGGCAGCAATTCTTGTAATTCCTGACGTTGAAACGGCGTAGCCACCACCAATAAT
PSU-1        ACCCACGGCAGCAATTCTTGTAATTCCTGAAGTTGAAACGGCGTAGCCACCACCAATAAT
                                                                      1380
Lal4X(VL92)  ATTAGCGGCTTTTAGGGACTGAACAAATTGGATACTACCGTTCTTCCAAACTAATTGTGG
PSU-1        ATTAACGGCTTTTAGAGACTGAACAAATTGGATACTACCGTTCTTCCAAACTAATTGTGG
                                                                      1440
Lal4X(VL92)  AACAGTGATGAAGAATGCCATTAAAAACATCAACAGGGCGTTCATTGGAACAACATTTAT
PSU-1        AACAGTGATGAAGAATGCCATTAAAAACATCAACAGGGCGTTCATTGGAACAACATTTAT
                                                                      1500
Lal4X(VL92)  ATGTTCTTCCTCTTGATATATTTTGGTGTACGAATAAGTAAATGTACCAGCAAAAACCAA
PSU-1        ATGTTCTTCCTCTTGATATATTTTGGTGTACGAATAAGTAAATGTACCAGCAAAAACCAA
                                                                      1560
Lal4X(VL92)  TGCTAAAATACCCATTGTCGAATTATAGACAACCTG-AACAGAT-GATAAAGCAACCAAG
PSU-1        TGCTAAAATACCCATTGTCGAATTATAGACAACCTGGAACAGATTGATAAATCGACCAAG
                                                                      1620
Lal4X(VL92)  AGTATCAGCGTAAAGCGCCGCGAATCCTGGAATTGGAAAAGCCTGCGGCAACACTGTCAA
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PSU-1         AGTATCAGCATAAAGCGCCGCGAATCCTGGAATTGGAAAAGCCTGCGGCAACACTGTCAA
                                                                      1680
Lal4X(VL92)   AATTAAAAAAAATTGCTCCTATGATTGTAAACGGGATAACACTAAAACCAGCCTGCATAAT
PSU-1         AATTAAAAAAAATTGCTCCTATGATTGTAAACGGGATAACACTAAAACCAGCCTGCATAAT
                                                                      1740
Lal4X(VL92)   TGCTCGGACAAATCGCGACCCAGCAAACTTACCAGCTGCTTTTGTCATATTATCTTTAAA
PSU-1         TGCTCGGACAAATCGCGACCCAGCAAACTTACCAGCTGCTTTTGTCATATTATCTTTAAA
                                                                      1800
Lal4X(VL92)   GGAGCTTGTTTGTTTATTATCCATGTTATTTCTCCTCAAACTTTGAACTTTCTGTCAAAA
PSU-1         GGAGCTTGTTTGTTTATTATCCATGTTATTTCTCCTCAAACTTTGAACTTTCTGTCAAAA
                                                                      1860
Lal4X(VL92)   TAGGACAAATATATTAATAACAAAGAAGCATTCAATCTATAATTTATCCCAATAAATTTA
PSU-1         TAGGACAAATATATTAATAACAAAGAAGCATTCAATCTATAATTTATCCCAATAAATTTA
                                                                      1920
Lal4X(VL92)   TTTTTATCTCAGGATCAGAAAACTAGCGAGTTTATTTTTATCAATTCATTTTATTTCAAT
PSU-1         TTTTTATTTCAGGATCGGAAAACTAGCGAGTTTATTTTTATCAATTCATTTTATTTCAAT
                                                                      1980
Lal4X(VL92)   CTGTATATCTGCTCGTTATCATTTATCCTCGATCCTTTTATACAGCTCAACTATCTCACC
PSU-1         CTGTATATCTGCTCGTTATCATTTATCCTCGATCCTTTTATACAGCTCAACTATCTCACC
                                                                      2040
Lal4X(VL92)   GGCCAGATCACGAAAGGTAATCGCGGTCATGACATGGTCCTGGGCATGGACCATTAACAG
PSU-1         GGCCAGATCACGAAAGGTAATCGCGGTCATGACATGGTCCTGGGCATGGACCATTAACAG
                                                                      2100
Lal4X(VL92)   ACTCATTTGGGCGTGTTCTCCGTTGGCTTCTTTAGTCAACATATCAGTCTGGGCGTTATG
PSU-1         ACACATTTGGGCGTGTTCTCCGTTTGCTTCTTTAGTCAACATATCAGTCTGGGCGTTATG
                                                                      2160
Lal4X(VL92)   GGCCTGGGCTAGAAAACGATCGGCTTCTGTTAGTTTTAGCTCGGCTTCTTTGAAGTCTTT
PSU-1         GGCCTGGGCTAGAAAACGATCGGCTTCTGTTAGTTTTAGTTCGGCTTCTTTGAAGTCTTT
                                                                      2220
Lal4X(VL92)   TTCTTTTGCGGCCTTAATGGCTAAGAAGGCTGCTCCTTTGGCATTGCCGCCGGCGATGAT
PSU-1         TTCTTTTGCGGCCTTAATGGCTAAGAAGGATGCTCCTTTGGCATTGCCGCCGGCGATGAT
                                                                      2280
Lal4X(VL92)   CAGGCTCATGATGACCTTTTGGTTTTCAATCTCTTCACTCATCTTAGCCGATCTCTTTTA
PSU-1         CAGGCTCATGATGACCTTTTGGTTTTCAATCTCTTCACTCATCTTAGCCGATCTCTTTTA
                                                                      2340
Lal4X(VL92)   AAGCATGGTTTAAAACTTTTTCTCCGTTCATCAGCCCATAGTCCTGCATGTTAATTACTT
PSU-1         AAGCCTGGTTTAAAACTTTTTCTCCGTTCATCAGCCCATAGTCCTGCATGTTAATTACTT
                                                                      2400
Lal4X(VL92)   CAACCGGGATTTTGACCTTTTCTTTAAACTGGGAGAGCATATAGCTGACCTGGGGTCCGA
PSU-1         CAACTGGGATTTTGACCTTTTCTTTAAACTGGGAGAGCATATAGCTGACCTGGGGTCCGA
                                                                      2460
Lal4X(VL92)   GCATTAAAATATCCGGCTGTTCGCTTTCTAGTTTGTTATCGGCGTCGGAAGCGGCCGTGG
PSU-1         GCATTAAAATATCCGGCTGTTCGCTTTCTAGTTTGTTATCGGCATCGGAAGCAGCCGTGG
                                                                      2520
Lal4X(VL92)   CAAAAATATCAACATCCTCGCCCTGTGCTTTAGCCGCCTTTTGCATCTTGCTTACCAGTA
PSU-1         CAAAGATATCAACATCTTCTCCCTGCTCTTTAGCCGCCTTTTGCATCTTGCTTACCAGTA
                                                                      2580
Lal4X(VL92)   GGCTGGTCGACATCCCGGCCGCACAAACCAACATTACTTTTTTCTCTGCCATTATTGTTC
PSU-1         GGCTGGTCGACATCCCGGCCGCACAAACCAACATTACTTTTTTCTCTGCCATTATTGTTC
                                                                      2640
Lal4X(VL92)   CTTTCACTATTAATCTAATTGACTGCCGTTTGACTTAATAACCTGTTGATACCAGAAAAA
PSU-1         CTTTCACTATTAATCTAATTGACTGCCGTTTGACTGAATAACCTGTTGATACCAGAAAAA
                                                                      2700
Lal4X(VL92)   GGAATCCTTTTTCAGTCTTTTTAAGCTACCTTTGCCCTGGTCGTCTTTATCGACATAGAT
PSU-1         GGAATCCTTTTTCAGTCTTTTTAAGCTGCCTTTGCCCTGGTCGTCTTTATCGACATAGAT
                                                                      2760
Lal4X(VL92)   AAAACCGTACCTTTTGGACATCTGTCCGGTTCCGGCGGAAACCAGATCGATACAACCCCA
PSU-1         AAAACCGTACCTTTTGGACATCTGTCCGGTTCCGGCGGAAACCAGATCGATACAACCCCA
                                                                      2820
Lal4X(VL92)   GGGCGTATAACCAATGACCTTAACCCCGTCTTCTTGAACTGCATCGATCATTGCCTGGAT
PSU-1         GGGCGTATAACCAATGACCTTAACCCCGTCTTCTTGAACCGCATCGATCATTGCCTGGAT
                                                                      2880
Lal4X(VL92)   ATGAGCTTTTAGATAAGCGATTCGATAAGGGTCATGGATCTGTTGGTTATTTTCGACTTT
PSU-1         ATGAGCTTTTAGATAAGCGATTCGATAAGGGTCATGGATCTGTTGGCTATTTTCGACTTT
                                                                      2940
Lal4X(VL92)   GTCATAGGCTCCCAGACCGTTTTCAACAATGAACAAGGGCTTGTGATAGTGGTCGGCCAG
PSU-1         GTCATAGGCTCCCAGACCGTTTTCAACAATGAACAAGGGCTTGTGATAGTGGTCGGCCAG
                                                                      3000
Lal4X(VL92)   CCAGTTTAA-GAATACCTAAGTCCCAACGGATCGATCGGCCATCCCCAATCGCTTTTTTC
PSU-1         CCAGTTTAAAGAATACCTAAGTCCCAACGGATCGATCGGCCATCCCCAATCGCTTTTTTC
                                                                      3060
Lal4X(VL92)   AACATTTGGATTATCAACGGTCAGTTCGGGACCAATGAAATGAAAACCCGGGTTGCTTTC
PSU-1         AACATTTGGATTATCAACGGTCAGTTCCGGACCGATGAAATGAAAGCCCGGGTTGCTTTC
                                                                      3120
Lal4X(VL92)   TTTTGATTGAACGGTCATTGAATTGTAATAACTCAATCCAATATAGTCGACAGTTCCTTC
PSU-1         TTTTGATTGAACGGTCATTGAATTGTAATAACTCAATCCAATATAGTCGACAGTTCCTTC
                                                                      3180
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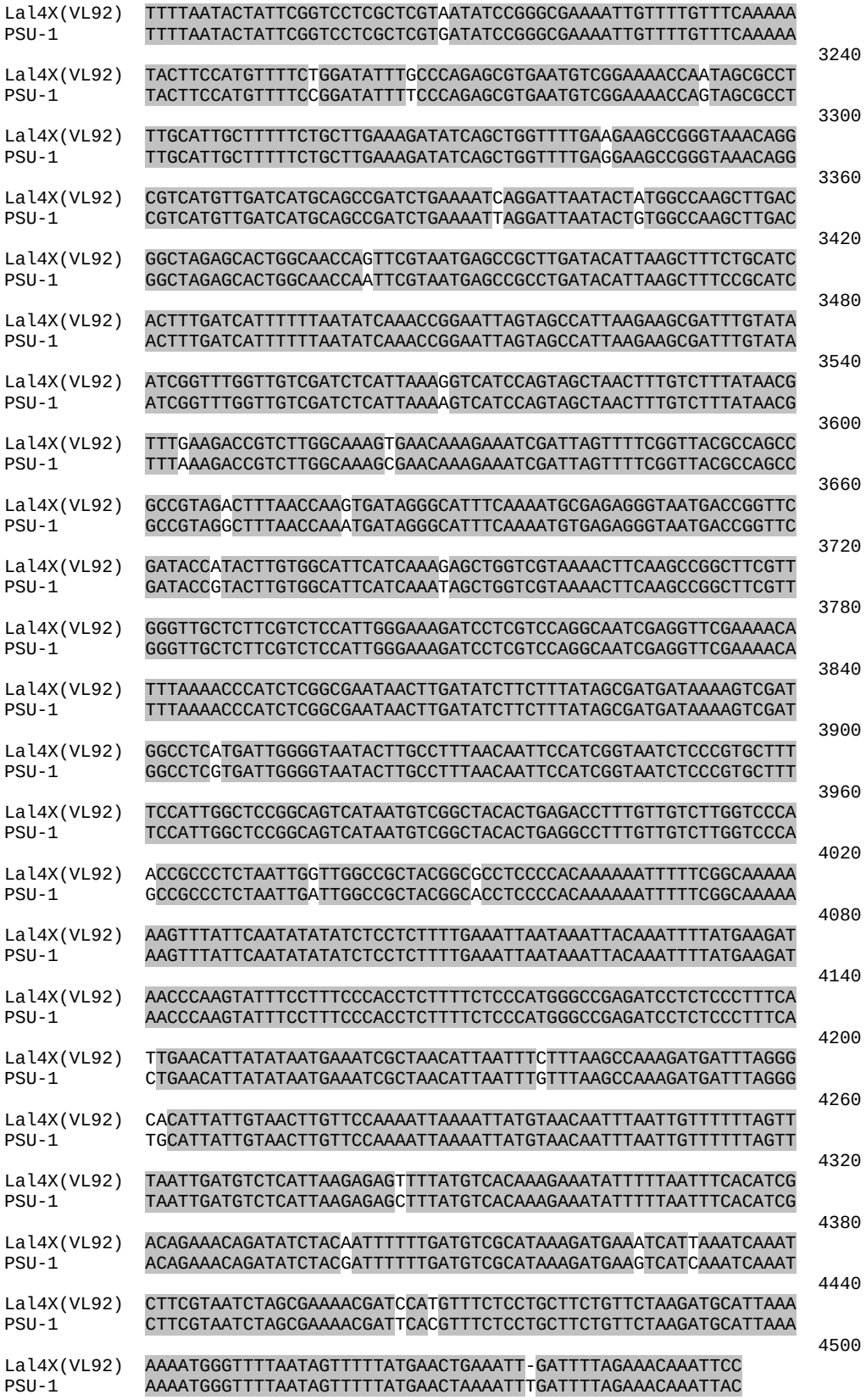

Figure 7. The sequenced *bgl* operon with highlighted SNP's (differences to PSU-1). The identical nucleotides are highlighted in grey. The SNP's have been left on a white background.

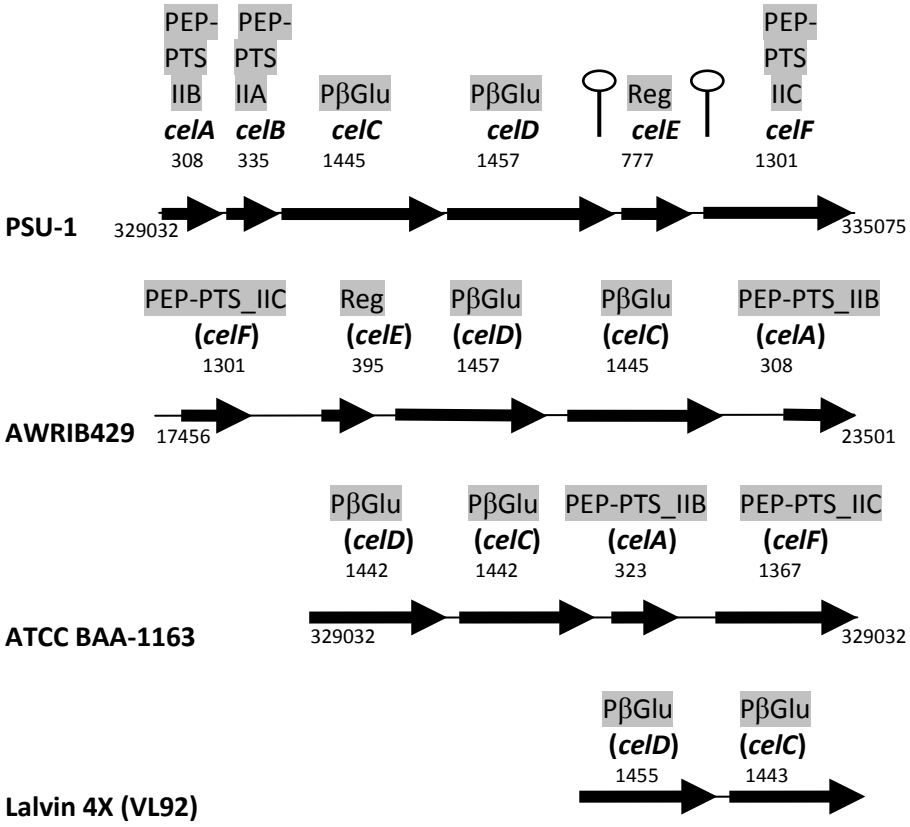

Figure 8. Genetic organization of the *cel* operon in four strains of *O. oeni*: PSU-1, AWRIB429, ATCC BAA-1163 and Lalvin 4X (VL92). The putative gene functions are highlighted in grey; the gene nomenclature for PSU-1 and the respective analogues for AWRIB429, ATCC BAA-1163 and Lalvin 4X (VL92) are in bold. The numbers above the gene represent the number of nucleotides in the gene. PEP-PTS-IIB, phosphoenolypyruvate phospho-transferase component IIB; PEP-PTS-IIC, phosphoenolypyruvate phospho-transferase component IIC; PEP-PTS-IIA, phosphoenolypyruvate phospho-transferase component IIA, PβGlu; phospho-β-glucosidase, Reg; putative transcriptional regulator. The numbers at either end of the operons represent the position in the genome.

6.4. Conclusions

Sequence analysis of the regulatory components in the *cel* operon from *O. oeni* strain PSU-1 (CP000411) indicated that it was most likely regulated by CCR and transcriptional anti-termination. The *bgl* operon from the same genome was analysed and was found lacking any of these regulatory structures. Attempts were made to sequence these operons, and whilst the *bgl* operon in Lalvin 4X (VL92) was found to be analogous to PSU-1, the sequence of the *cel* operon could not be determined. In Chapters 3 and 5, two genes from within the speculated *cel* operon of *O. oeni* PSU-1 were amplified together and found to be located 17 bp from each other. Two more *O. oeni* genomes, strain ATCC BAA-1163 (AAUV00000000) and AWRIB428 (ACSE00000000) were recently sequenced. The spatial arrangements of the genes in the *cel* operon in these strains were different to PSU-1, specifically in respect to the location of *celC* and *celD* genes. This suggests that there is a high amount of variability and gene movement within the species. This is further supported by the fact that the *O. oeni* genome in some strains has been found missing *mutS* and *mutL*, mismatch repair genes responsible for repairing any mutations which may occur (Marcobal et al. 2008). The absence of these genes in *Staphylococcus aureus* resulted in the accumulation of spontaneous DNA replication errors and high levels of polymorphism (Bon et al. 2009). It is speculated that the lack of these genes in *O. oeni* may be responsible for diversity between strains and specifically the differences in the genetic organization of the *cel* operon.

Genes of the *cel* and *bgl* operon are speculated to be involved in growth of *O. oeni* on certain carbon sources. *O. oeni* was able to grow on salicin, arbutin and cellobiose as sole carbon source. In the added presence of the preferred carbon source glucose, the bacterial cells grew more quickly and were morphologically found as shorter chains.

In the presence of salicin, arbutin or cellobiose as the sole carbon source, the cells had a greater lag phase and appeared to form longer chains and clusters with distinct angular features. It was hypothesized that in the presence of glucose, *bglD*, *celC* and *celD* would not be expressed due to carbon catabolite repression. No expression of *celC* was evident regardless of the presence of glucose. It may be that only *bglD* and *celD* are involved in the metabolism of β-glucosides in *O. oeni*, and *celC*, whose function is still unclear, may not play such a role.

Phospho-β-glucosidase activity was not sought throughout the experiment as a glycosyl hydrolase family 3 β-glucosidase has been identified from *O. oeni* (Michlmayr et al. 2010) which is not dependent on the PEP-PTS, and would obscure any β-glucosidase activity relevant to the PEP-PTS. Further work (including optimization of methods) needs to be undertaken in order to understand the regulation of the *cel* and *bgl* operon in *O. oeni*. Specifically, a broader analysis of the sample time points across the growth curve may indicate that phospho-β-glucosidase genes are only expressed at a specific point and the expression of *celC* may have been completely missed due to this.

Amplification of the remaining genes in the operon, speculated to be involved in the uptake and phosphorylation of β-glucosides in the cell, would give a greater understanding of how the PEP-PTS functions in *O. oeni*. However, the work presented in this chapter does highlight that CCR is unlikely to be the only regulating factor for β-glucoside metabolizing PEP-PTS operons identified in *O. oeni*.

Wild type strains of *E. coli* K-12 are not able to metabolize β-glucosides. Mutations in three cryptic loci enable the expression of PEP-PTS genes which allow the organism to take up and phosphorylate arbutin, salicin and cellobiose. It is possible that the *bgl* and *cel* PEP-PTS operons in *O. oeni* are in fact cryptic, as per the four operons in *E. coli*, requiring mutation for functionality (Parker and Hall 1988). Michlmayr et al. (2010) demonstrated that whole *O. oeni* cells are able to grow on cellobiose and release glucose from the hydrolysis of cellobiose. Growth on cellobiose was not specifically attributed to the identified glycosyl hydrolase family 3 β-glucosidase, which is independent of PEP-PTS (Michlmayr et al. 2010). These findings highlight the possibility of *O. oeni* possessing a variety of cellular mechanisms which enable growth and carbon sources to be utilized under stressful conditions such as the wine environment, where other organisms can not.

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7. Conclusions and future directions

Six putative genes were identified in *O. oeni* from the sequenced PSU-1 genome that were hypothesized to be involved in the metabolism of β-glucosides (AG1, ORF 1, ORF 2, ORF 3, ORF 4 and ORF 5' see **Table 1** below). AG1 and ORF 4, were cloned into various expression vectors but were not able to be expressed as soluble proteins (Chapter 2). Both genes lacked a fundamental part of the highly conserved glycosyl hydrolase family 1 N-terminal signature sequence. To date, the N-terminal signature sequence has not had a function attributed to it. It has been suggested, but not validated, that it may serve as a signal sequence required for the protein to be secreted outside the cell (Dharmawardhana et al. 1995). It has also been linked to polymerization (dimerization and hexamerization) of the monomeric form of glycosyl hydrolases (Sue et al. 2006).

ORF 1 (later labelled as *bglD*), speculated to be a GHF 1 β-glucosidase/β-galactosidase/phospho-β-glucosidase, has the same sequence as AG1, with an additional 34 bases on the 5-prime end, forming a complete GHF 1 N-terminal signature sequence (37 amino acids). ORF 1 (*bglD*) was expressed as a soluble protein in *E. coli*. The substrate activity towards the phosphorylated β-glucoside para-nitrophenol-β-D-glucopyranoside-6-phosphate and sequence identity to characterised genes was characteristic of a GHF1 phospho-β-glucosidase (Chapter 3).

Table 1. Putative genes identified in *O. oeni* PSU-1 which may be responsible for β-glucosidase metabolism.

Gene	Soluble protein	Function	Temp optima (°C)	N-terminal	Specificities	pH optima	Chapter
AG1	N	-	-	N	-	-	2
ORF 1 (*bglD*)	Y	6-phospho-β-glucosidase	40	Y	*p*-nitrophenol-β-D-glucopyranoside-6-phosphate	5.5	3
ORF 2 (*celC*)	Y	6-phospho-β-glucosidase	ND	Y	-	-	5
ORF 3 (*celD*)	Y	6-phospho-β-glucosidase	40	Y	*p*-nitrophenol-β-D-glucopyranoside-6-phosphate	4.0 – 5.0	5
ORF 4	N	-	ND	N	-	-	2
ORF 5	Y	β-glucosidase†	40 – 50	-	*p*-nitrophenol-β-D-glucopyranoside	5.0 – 5.5	2

*NCBI Gene tag
†ORF 5 characterised by Michlmayr et al 2010
ND = Not deterimined
N = No, Y = Yes, Temp = Temperature

AG1 and ORF 1 (*bglD*) were assayed for activity in whole cells as well as cell lysates, revealing that only the latter had significant phospho-β-glucosidase activity in the case of ORF 1 (BglD) (data not shown). It can be concluded therefore that the GHF 1 N-terminal signature sequence is important for protein solubility but not necessarily cell wall localization. Despite not being transported outside the cell, ORF 1 (BglD) was only able to be expressed as a soluble enzyme in a hydrophobic environment, suggesting that it may be localized close to the cell membrane in the cytoplasm. The purified ORF 1 (BglD) protein has 480 amino acid residues and a predicted molecular mass of 55.5 kDa. The enzyme exhibited high activity towards the phosphorylated β-glucoside para-nitrophenol-β-D-glucopyranoside-6-phosphate with a pH optimum of 5.5, and maintained similar levels of activity between temperatures of 4°C and 40°C. ORF 1 (BglD) was not active against non-phosphorylated β-glucosides (Chapter 3). The *bglD* gene is one of four genes (*bglA* to *bglD*) which form a putative β-glucosidase operon of 2178 base pairs. *bglA*, *B* and *C* are homologous to the characterized genes encoding the phosphoenolpyruvate dependent phosphotransferase system (PEP-PTS) components IIC, IIA and IIB which regulate the uptake, phosphorylation and translocation of β-glucosides across the cytoplasmic membrane. The PEP-PTS is a highly characterized bacterial transport system which enables the cell to metabolise alternative carbon sources in the absence of a preferred carbon source. Via this system, β-glucosides are able to be simultaneously taken up by the cell and phosphorylated, and subsequently broken down by cytoplasmic phospho-β-glucosidases. The glucose molecule released is likely to feed directly into glycolysis (Deutscher et al. 2006).

One of the principal realizations of this project was that the identification of high sequence similarity between GHF 1 β-glucosidases, β-galactosidases and phospho-β-glucosidases does not preclude the need for determination of enzyme function. Experimental work with the purified protein was necessary to establish the function of ORF 1/BglD, ORF 2 and ORF 3, the three proteins which were able to be expressed as soluble proteins. A single serine residue (Ser^{432} in ORF 1 (BglD)), present in characterized phospho-β-glucosidases as well as ORF1 (BglD), ORF 2 and ORF 3, was speculated to be important for affinity towards phosphorylated substrates (Wiesmann et al. 1995). In characterized GHF 1 β-glucosidases, the serine in position 432 (ORF 1 (BglD) numbering) is replaced with a glutamic acid. In order to determine whether substrate specificity was related to this specific amino acid substitution, ORF 1 (BglD) was mutated to replace the serine in position 432 with a glutamic acid (Chapter 4). The mutated enzyme was expressed under the same conditions as ORF 1 (bglD) and also required a hydrophobic environment in order to be expressed as a soluble protein. The mutated enzyme, however, lost all activity towards phosphorylated substrates and did not demonstrate any activity towards non-phosphorylated substrates as had been hypothesized. It can therefore be concluded that the serine in position 432 (ORF 1/BglD numbering) does play a significant role in the affinity of phospho-β-glucosidases towards phosphorylated substrates, but also that there is more than one residue important in the affinity of GHF 1 β-glucosidases towards β-glucosides.

ORF 2, referred to as *celD*, was able to be expressed in *E. coli* as a His-tagged protein (485 residues, Mw = 55.8 kDa) which was soluble. The purified protein did not have any affinity towards the non-phosphorylated β-glucosides assayed (Chapter 5), but demonstrated high activity towards para-nitrophenol-β-D-glucopyranoside-6-phosphate. Maximal activity was detected at 40°C and at a pH range of 4.0 to 5.0. Further, the protein showed high homology to characterised phospho-β-glucosidases.

ORF 3, subsequently named *celC*, is found 17 bases upstream from ORF 2/*celD*. The gene *celC* was cloned, heterologously expressed and purified (481 residues, Mw = 55.7 kDa) but showed no significant activity towards para-nitrophenol-β-D-glucopyranoside-6-phosphate despite high sequence homology to *celD* and characterized phospho-β-glucosidases. Both ORF 2/(*celD*) and ORF 3/(*celC*)were 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 (Chapter 6). ORF 3/(*celC*) was not able to be characterized as a glycosyl hydrolase family (GHF) 1 phospho-β-glucosidase or a β-glucosidase, despite its sequence homology to both. It complete lacked activity towards both phosphorylated and non-phosphorylated substrates. It may be that ORF 3/(*celC*) is important for forming glycosidic bonds via transglycosylation

rather than hydrolysis of glycosidic bonds as many glycosyl hydrolases can have this function (Henrissat et al. 2008). This enzyme warrants further research as any possible transglycosylation activity in *O. oe ni* could be important for emerging issues in the Australian wine industry such as smoke taint. Smoke taint (See Chapter 1) is masked when the contributing compounds are in the glycosidic form. An isolated glycosyltransferase for commercial use could therefore have an important role for reducing the perception of aroma compounds by glycosylation in a wine where a less aromatic style is sought or to glycosylate compounds regarded as a negative contribution to the wine, such as those responsible for smoke taint. It may also contribute to wine colour stability in wines which are made to be consumed very quickly by ensuring that anthocyanins remain in the more stable glycosylated form.

ORF 5 was identified as having high homology to GHF 3 β-glucosidases, in contrast to the other genes identified which belonged to the GHF 1 hydrolase family. Like ORF 1 (*bglD*), ORF 5 also had a leucine as a start codon. Before ORF 5 could be cloned and over-expressed, the β-glucosidase gene (ORF 5) was cloned and expressed in *E. coli* by Michlmayr et al (2010). The enzyme had an optimum pH of 5.0-5.5 and was most active between 45 and 50°C. Interestingly, this gene product also demonstrated some transglycosylation (synthesis of glycosidic linkages) activity in the presence of 5% (w/v) ethanol.

O. oe ni was able to utilize salicin, arbutin and cellobiose as sole carbon sources (Chapter 6). In the added presence of glucose (preferred carbon

source), the bacterial cells grew more quickly and occurred in shorter chains. Whilst in the presence of either salicin, arbutin or cellobiose alone, the cells had a longer lag phase and formed longer chains and clusters with distinct angular features. Preliminary transcriptional analysis of the PSU-1 genome indicates that carbon catabolite repression and transcriptional anti-termination may be involved in the regulation of the *bgl* and *cel* operons; however, further work is necessary to completely understand the regulatory mechanisms of the PEP-PTS in *O. oeni*.

This is currently the most in-depth study of the genes and mechanisms responsible for β-glucosidase metabolism in the oenologically important lactic acid bacteria *O. oeni*. Other studies have focused on whole cell activity or in the case of Michlmayr et al (2010), a specific gene. When this PhD project was initiated, strains of *O. oeni* were shown to have variable glycosidic activity which could have a potential effect on the sensory profile of wine (Grimaldi et al. 2005a, 2000). It is now known, which genes in the *O. oeni* genome are likely to be responsible for the metabolism of β-glucosides in wine. This information can be exploited by the Australian wine industry to increase the quality of the end product, when labour and production costs are constantly on the rise and fruit quality may be compromised due to adverse weather conditions. Strain selection for *O. oeni* can now be specifically targeted based on enzymatic activities and tailored specifically to suit the needs of a wine lacking in flavour. Conversely the use of strains wherein the β-glucoside metabolism is low because of non functional genes, limited gene expression or down-regulated expression, may be beneficial to wines which

are not in need of flavour/aroma production or have been exposed to external factors such as smoke, thus minimising the liberation of smoke taint compounds. Further work needs to focus on whole genome sequencing of different *O. oeni* strains combined with biochemical characterization, so as to match the genotype with phenotype. In this way, much like yeast strains that have been commercialized to produce a style of wine, more work can focus on bacterial strain variation for tailored MLF. If indeed the *bgl* and *cel* operons are repressed by glucose via carbon catabolite repression, it may be that inoculation times (i.e. concurrent with alcoholic fermentation or after primary fermentation) can also be selected to suit the desired aroma outcome and hence wine style. The information from this PhD has given the Australian wine industry and researchers everywhere a greater understanding of a key organism in winemaking, how it can interact with the wine matrix and how we can harness that knowledge and better suit wine styles to both domestic and export markets.

8. Appendix 1

5x electrophoresis buffer (1L)		**Final concentratration**
60.6 g	Tris base	0.5 M
144.1 g	Glycine	1.92 M
5 g	SDS	0.5%
Make up to 1L	Water	

5x SDS-PAGE sample buffer (10 ml)		
2.25 ml	Tris·Cl, pH 6.8 1 M	0.225 M
5 ml	Glycerol	50%
0.5 g	SDS	5%
5 mg	Bromophenol blue	0.05%
200 µL	β-mercaptoethanol	2%
Make up to 10 mL	Water	

Coomassie staining solution (1L)		
500 mg	Coomassie Brilliant Blue R-250	0.05% (w/v)
400 mL	Ethanol	40% (v/v)
100 mL	Glacial acetic acid	10% (v/v)
500 mL	Water	50% (v/v)
Filter before use		

Destaining solution (1L)		
400 ml	Ethanol	40% (v/v)
100 ml	Glacial acetic acid	10% (v/v)
500 ml	Water	50% (v/v)

McIlvainebuffer at pH 5.5
Adjust to pH 5.5 with the following solutions:

27 mL	0.2 M Na_2HPO_4	0.08 M
43 mL	0.1 M citric acid	0.06 M

9. Appendix 2

50 X Tris-Acetate-EDTA (TAE) buffer (1L)		**Final concentratration**
242 g	Trizma base	40 mM
57.2 mL	Glacial acetic acid	20 mM
100 mL	EDTA pH 8.0	1 mM

1X TAE buffer (1L)		
20 mL	50 X TAE buffer	2% (v/v)
980 mL	water	98% (v/v)

1% agarose gel in TAE buffer (1L)		
10 g	Agarose (Astral Scientific)	1% (w/v)
1000 mL	1 X TAE buffer	approx 99% (v/v)
40 µL	GelRed™	approx 0.004% (v/v)

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