Diacetyl: identification and characterisation of molecular mechanisms for reduction in yeast and their application in a novel enzyme based assay for quantification in fermentation systems.

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Abstract

Diacetyl (2,3-butanedione) is an important flavour active, oxidative compound that has significant impact on cellular health as well as financial impact in industrial fermentations. The presence of diacetyl in certain fermented beverages, such as beer, results in an unpleasant butterscotch-like flavour and its concentration needs to be reduced by yeast to below the taste threshold prior to filtration and packaging. This results in significant process inefficiency. Furthermore, diacetyl negatively impacts cellular health and has been associated with neurodegenerative diseases and general cell aging amongst others. The reduction of this compound is therefore essential for cellular health.

Several yeast cell enzymatic mechanisms responsible for diacetyl reduction were identified and characterised, including Old Yellow Enzyme (OYE) isoforms and D-Arabinose Dehydrogenase (ARA1). OYE isoforms displayed different micromolar affinities and catalytic turnover rates for diacetyl and catalysed diacetyl reduction in a biphasic manner. ARA1 catalysed diacetyl reduction in a monophasic manner with a millimolar Michaelis constant.

Knowledge gained in these studies was applied in investigations of diacetyl production and reduction in industrial brewing operations and the enzymatic systems further exploited for the development of a novel enzyme based assay to determine diacetyl concentrations in beer samples. Concentrations as low as 0.2 μM were detectable with high repeatability.

Résumé

Le diacétyle (2,3-butanedione) est un important aromate, de nature oxydative, ayant un impact considérable sur la santé cellulaire, ainsi que des répercussions financières indéniables dans les procédés de fermentation à l'échelle industrielle. La présence du diacétyle dans certaines boissons fermentées, dont la bière, confère un arôme indésirable de caramel au beurre. Il est alors important de laisser la levure réduire la concentration de diacétyle en dessous de son seuil de perceptibilité avant de procéder à la filtration et l'empaquetage du produit final. Cette étape de maturation réduit largement l'efficacité du procédé. De plus, le diacétyle a une portée négative sur la viabilité cellulaire et a été associé, en autre, à certaines maladies neurodégénératives et au vieillissement prématuré des cellules.

Plusieurs mécanismes enzymatiques responsables de la réduction du diacétyle ont été identifiés et caractérisés chez la levure. Ceux-ci incluent les isoformes de Old Yellow Enzyme (OYE) et D-Arabinose déshydrogénase (ARA1). Les isoformes de OYE ont démontré différentes affinités et différents pouvoirs catalytiques en réponse à diverses concentrations micromolaires de diacétyle. Ces trois isoformes catalysent la réduction du diacétyle de façon biphasique. À l'opposé, ARA1 catalyse la réduction du diacétyle de façon monophasique, avec une constante de Michaelis de l'ordre de quelques millimoles.

Les connaissances acquises à travers ces études ont été appliquées à l'investigation de la production et de la réduction du diacétyle lors d'opérations brassicoles industrielles. Les systèmes enzymatiques ont d'ailleurs été exploités pour le développement d'une nouvelle méthode d'analyse permettant la détermination de la teneur de diacétyle dans des échantillons de bière. Des concentrations aussi faibles que 0.2 μM ont été détectées de façon reproductible.

Les résultats obtenus par ces études démontrent un clair potentiel pour des applications autant dans le domaine des sciences de la vie que dans le domaine des fermentations industrielles.

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Contributions of Authors

- Normand Cyr assisted with gas chromatographic analysis of enzyme assay samples in chapters 2 and 3 and amino acid analysis in Chapter 7.
- Maxime Blanchette assisted with vicinal diketone analysis in Chapters 7 and 8.
- Genevieve Brousseau assisted with kinetic characterisation in Chapter 3.
- Rona Strasser assisted with gene cloning experiments in Chapters 4 and 5 and gene activity quantification in Chapter 7.
- John Sheppard and Armando Jardim contributed in their positions as supervisors.

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Research objectives

The research documented herein resulted from a multidisciplinary investigation in the field of brewing and fermentation. The overall objective was to understand the mechanisms responsible for reducing diacetyl in Saccharomyces yeast and to apply the findings in a commercial device to quantify diacetyl in beer and brewing wort. The objectives can be further broken down into the sections given below.

1) Identify and characterize enzymes responsible for reducing diacetyl in *Saccharomyces* yeast.

   - Purify diacetyl reductase activity from anaerobically grown yeast harvested from beer fermentations and characterize recombinant form expressed in an *E. coli* host organism.
   - Generate gene deletion mutant yeast lacking the genes for the enzyme activity identified above.
   - Purify diacetyl reductase activity from deletion yeast grown aerobically in bioreactors and characterize recombinant form expressed in an *E. coli* host organism.
   - Analyze industrial beer fermentations for diacetyl production and reduction, in relation to free amino acid content of wort and α-acetohydroxyacid synthase, α-acetohydroxyacid reductase and diacetyl reductase gene activities.

2) Investigate characteristics of brewing wort and suitability for an enzyme based assay to determine the concentration of diacetyl in beer streams

   - Characterize properties of beer wort relevant to assay objectives.
   - Minimize background noise to improve assay sensitivity.
   - Investigate applicability of enzymes characterized in objective 1 for assay based quantification of taste-threshold values of diacetyl.
   - Characterize candidate enzyme specificity and kinetic parameters in optimized brewing wort environment.

3) Improve recombinant protein production yields for scale-up of enzyme production for use in a commercial bioassay system through the development of a high cell density culture technique.

Contribution to knowledge

Several mechanisms capable of reducing the toxic vicinal diketone (VDK) metabolic byproducts methylglyoxal, diacetyl and pentanedione in *Saccharomyces* yeast have been identified and characterised.

1) Old yellow enzyme (OYE) isoforms 1, 2, and 3 (oye1p, oye2p and oye3p) were all shown to catalyse VDK reduction. Genetic analysis confirmed that oye1p, oye2p and oye3p were all found in *S. pastorianus* (lager yeast), whereas only oye2p and oye3p were found in *S. cerevisiae* (ale yeast). Kinetic analysis of substrate conversion by OYE enzymes suggested that the catalytic equilibrium strongly favored the formation of keto-hydroxy products in a biphasic manner. oye2p displayed the lowest Michaelis constant (K_m) with diacetyl (2 μM) and pentanedione (224 μM). Further investigations suggested that the biphasic kinetics appeared caused by modification of protein arginine residues by the VDK's.

2) D-Arabinose dehydrogenase (ara1p) was also capable of catalysing VDK reduction, although, in contrast to OYE proteins, the reduction kinetics were monophasic. While it was not possible to observe reverse catalytic activity of keto-hydroxy substrates to diketones with OYE proteins, ara1p was able to catalyse reversible reactions at non-physiological alkaline pH values. Kinetic analysis of substrate conversion suggested

that the K_m was the lowest with pentanedione (4.2 mM) and highest with methylglyoxal (14.3 mM).

3) An additional enzyme of unknown identity was partially purified. Kinetic analysis suggested a low K_m of 69 μM with the substrate diacetyl and saturated kinetics with only 500 μM diacetyl, in contrast with both OYE proteins and ara1p which all required concentrations greater than 20 mM to reach saturation velocity.

4) An enzyme based assay was developed for determining diacetyl concentration in fermenting beer using the diacetyl reducing enzymes identified and characterized above. Beer sample background absorbance could be sufficiently reduced for monitoring NADPH dependant reaction velocity at 340 or 365 nm by sequential filtration through a size exclusion filter and a C-18 cartridge. Analysis of analyte retention during sample cleanup suggested that the diacetyl precursor, α-acetolactate, and diacetyl are not retained during filtration at regular beer pH (~4). Accurate analysis of total diacetyl concentration required the complete conversion of the diacetyl precursor α-acetolactate, which could be rapidly achieved by heating the sample at 80°C in the presence of $FeCl_3$ and $FeSO_4$.

1. Introduction

Yeast, in particular *Saccharomyces* species, has played an important role in human society for millennia. For more than 5000 years, mankind has consumed bread and alcohol in ever increasing quantities, with alcoholic fermentation being the oldest known example of mankind harnessing micro-organisms for our own use. Sumerians and Egyptians most likely did not understand why, or how, leaving a small portion of the previous "brew" in the container helped turn the next one into an alcoholic preparation, but this knowledge was faithfully passed from one generation to the next. By the 16th century, monks had become the keepers of these techniques and had refined the techniques considerably. During times of rampant disease, their brews were known to be safe for consumption, although people may not have been aware that it was because they were boiled as part of the brewing process. During these times fermentation was still very much an unknown. The chances of successfully making the sweet beverage turn into alcohol were greatly improved by placing them, once cooled, in a vessel that has previously been used to successfully make a good brew.

Science began to establish itself shortly thereafter and chemistry became a tool to describe many observations that had previously been a mystery. Alcohol was no exception, and several scientists attempted to describe the reactions that were occurring when alcohol was being produced. At the time it was suggested that alcohol was formed as the result of a chemical reaction taking place inside the container, which wasn't entirely incorrect. It was only after the microscope was invented in the 19th century that

scientists began to understand that it was a biochemical reaction occurring within micro-organisms and not chemical reaction in the surrounding liquid that was responsible for alcohol formation. By this stage, it was late in the 19th century and alcohol manufacture was already moving towards a large scale, but a turn of events would soon turn the industry into a giant one: pure cell culture.

In the late 1890's, Emile Hansen, working in his laboratory at Carlsberg brewery in Denmark, developed a technique that allowed him to isolate a single cell from a fermentation culture. Using this technique, the first pure lager yeast brewing culture, Carlsberg #1, was isolated and is still in use today. If the isolation plates were kept clean, that single cell would produce a colony of identical cells, which in turn could be used to inoculate a small quantity of sterile liquid. The cells would multiply in the liquid and this in turn could be used to inoculate a larger volume of liquid. The larger volume could be used to inoculate a larger volume and the process continued until a sufficiently large quantity of cells had been obtained to proceed with a rapid and vigorous fermentation. All of this with a pure culture, derived from a single cell. This technique, along with good hygiene practice, allowed breweries to achieve reproducible results. The beer brewed by a brewery using these techniques became more reliable, which lead to consumer confidence in the product. Less beer was spoilt and lost during production, meaning that efficiency increased. With increased consumer confidence, improved profitability, and the development of a good distribution and transport system, breweries began to expand rapidly.

Today beer is fermented in batch volumes as large a 1 million litres and some individual breweries produce in excess of 2 billion litres a year. Breweries were pioneers

in science and process development and have amassed a vast amount of knowledge about their operations. Quality control in raw material preparation is excellent and has allowed brewers to consistently produce branded products with almost identical profiles year after year, despite the fact that raw materials are grown in the earth and are subject to weather dependant variation. Although these materials, and yeast, being living organisms cannot be entirely controlled, good process practices have allowed brewers to contain their characteristics. An enormous amount of effort has been put into understanding how yeast function and how the brewing process affects yeast. As a result, yeast handing practices have improved and processes have been automated.

With current technology it is quite possible to produce in excess of 450 million litres annually in a brewery with less than 150 staff, including administration and ancillary services. In larger brewing operations, this is not a luxury, brought about by the presence of technology, but a necessity. The past ten years have seen unprecedented consolidation within the brewing industry, accelerated by globalisation, the need to remain stable and profitable and the prevailing macroeconomic model. Certain developed markets, such as North America and Western Europe are highly profitable, but are coming under increasing competition from wine (with its perceived health benefits), spirits and trendy alcopops. These markets are experiencing shrinkage in beer volumes, while other developing markets, such as Brazil, China and Russia are experiencing healthy organic growth. Despite the low profit margin in these markets, brewers are positioning themselves aggressively in the belief that retail prices will eventually rise and are buying up local brewers at a furious rate. As a result of this, the top ten breweries in the world now control more than 80% of the global volume, with the top five accounting

for more than 70% of the total. With tiny profit margins in many parts of the world, brewers are again striving to improve their efficiencies. One area coming to attention, which although consistent in performance, is fermentation, which for the most part has to be allowed to run its course.

Generally, a fermentation begins when yeast is added (pitched) into a fermenter containing sweet wort, produced from malted barley and hops. The yeast consumes sugars and amino acids present in the wort, along with some micronutrients (wort is highly nutritious) and produces ethanol, carbon dioxide and several hundred flavour compounds. The (primary) fermentation is normally finished within less than a week, depending on the temperature, after which a portion of the yeast is removed for reuse and the fermented beer allowed mature. This process will usually last for a period at least as long at the primary fermentation and is necessary in order to achieve a more balanced and desirable flavour profile. This is required because along with producing alcohol, the yeast produce a variety of metabolic byproducts, which enter the beer. Some of them, particularly 2,3-butanedione, commonly known as diacetyl, have undesirable flavours. Diacetyl can be described as smelling like butterscotch, and is actually used as a flavouring agent in butterscotch sweets and in "buttery" popcorns, but with taste threshold of approximately 100 parts per billion, or 100 micrograms per litre, its presence in beer can be problematical. Diacetyl reduction is, in fact, the single main reason for the aging process in beer fermentation and considerable effort has been put into understanding how and why yeast produce this compound. In actual fact, yeast don't produce diacetyl, but do produce the precursor compound (α-acetolactate) during amino acid synthesis. For reasons still unknown, this compound can leak from cells, or be

transported from the cells into the fermenting beer, where it slowly degrades to form the undesirable diacetyl. The yeast then consume this diacetyl and reduce it to form another compound (acetoin) which has a much higher taste threshold and does not smell like butterscotch. So although the product may not smell like diacetyl, if the brewers filter out the yeast and package the beer while acetolactate is still present, the product will eventually have the characteristic butterscotch smell. As a result, brewers have to wait until the acetolactate has been reduced to a sufficiently level that even if it does turn into diacetyl, will not be detectable.

Brewers still do not understand why acetolactate ends up in the beer. They know that if they alter the amino acid biosynthesis pathway that leads to acetolactate production, they can reduce the levels in wort, but genetic modification remains unacceptable in brewing. Brewers therefore have to simply accept that diacetyl will be produced and a maturation process will be required. This ties up fermentation vessels for long periods of time and leads to additional capital costs, as more fermentation vessels are required to achieve the same throughput, as well as additional process costs for vessel cooling. In order to further improve production efficiency, research needs to be conducted into the mechanisms available to the yeast cell to reduce diacetyl. The reasons for this are complex and are laid out in the discussions that will follow, but essentially, diacetyl is a compound that is toxic to the cell. It is capable of binding to certain amino acid residues in proteins and modifying them. This in turn alters the structure and function of the protein (see Chapter 3 for an example) causing severe problems for cellular function. Evidence has arisen that the acetolactate can be decarboxylated within the cell to form diacetyl. If this occurs, then cellular mechanisms required for regular

metabolic function may be impaired. The possibility exists that the pathway responsible for acetolactate production and reduction might be negatively affected, which in turn could lead to an accumulation of excess acetolactate, which is then removed from the cell, perhaps to avoid further formation of diacetyl. Although this remains speculative, the mechanisms for protein damage exist, but the mechanisms responsible for diacetyl reduction are not well known. In order to improve our understanding of the overall functioning within the cell, the mechanisms responsible for diacetyl reduction need to be characterised. Once these mechanisms are understood, alternative routes can be explored to control diacetyl formation in brewing systems and reduce overall fermentation time. The work contained herein has attempted to elucidate several mechanisms for diacetyl reduction in yeast and develop applications for this knowledge in brewing.

2. Literature Review

2.1 Introduction

The yeast *Saccharomyces cerevisiae* is one of the most important organisms known to man from both a scientific and economic perspective. Scientifically, it serves as an important model organism for higher eukaryotes and aids in our understanding of how our cells function. Its compositional similarity to cells found in higher organisms, ease of genetic and biochemical manipulation, rapid rate of growth as well as nutritional and environmental robustness make it a convenient and popular choice for the study of eukaryotic cellular behaviour. Yeast is also used industrially for the production very large quantities of fuel ethanol, wine, spirits and beer amongst others, contributing significantly to the economy.

S. cerevisiae research encompasses many fields and many aspects of yeast have been well characterized, such as cellular composition (Robinow and Johnson, 1991), gene regulation (Hinnebusch, 1986a), growth conditions (Watson, 1987), substrate transport (Fiechter *et al.*, 1987), enzyme characterization (e.g. (Schopfer and Massey, 1991; Zelenaya-Troitskaya *et al.*, 1995)) and genetic behaviour (e.g. (Alexandre *et al.*, 2001; James *et al.*, 2003)). With vast amounts of literature available it is now becoming possible to look beyond the individual genes, pathways, growth rates etc. in an attempt to understand the behaviour of the yeast in dynamic environmental conditions. This remains a challenge and requires understanding not only of the individual aspects of yeast behaviour, but how they function together and are influenced by external factors.

Saccharomyces species are capable of anaerobic fermentation, during which sugars are converted into alcohols (Hornsey, 1999), the most important being ethanol. Ethanol has significant economic interest, with applications in combustible fuels, solvents, pharmaceutical products and alcoholic beverages, amongst others. Alcoholic fermentation is complex. The differences between industrial ethanol manufacture, wine making and beer brewing are significant from both a process and metabolic perspective. An important area of concern in all ethanol production processes is metabolic by-product formation. In most products, particular metabolic by-products are desired and contribute to the fermented product's profile. In industrial ethanol manufacture, by-product formation is undesirable - the sole product of interest is distilled ethanol. Efficient conversion of substrate to ethanol is, therefore, the primary interest and research is ongoing in order to achieve better results. In wine and beer manufacture, certain metabolic by-products are desired, as they may impart flavor and complexity in the beverage. Well balanced products have added value, demand higher market prices and, therefore, make by-product formation a commercial interest.

In contrast, certain metabolic by-products impart undesirable flavours and aromas, for example 2,3-butanedione (diacetyl) in beer (Angelino, 1991). Product maturation following alcoholic fermentation serves to reduce the levels of these compounds and is critical to obtaining a product of acceptable quality (Hornsey, 1999). Significant research has been conducted on metabolic by-product production and reduction in an effort to either improve conditions or control them (Stewart and Russell, 1986). This remains a critical issue in the brewing industry as consumers expect a product to taste the same from batch to batch. With increased demand and product commercialization, beer has

become an engineered product, made from natural ingredients that are subject to significant variation. The research conducted in the chapters that follow contributes to the scientific knowledge pool of yeast metabolic function as well as to the brewing industry through improved understanding and control of the process. In a further development of the scientific knowledge obtained from the fundamental studies, a biosensor capable of monitoring μg/L diacetyl levels in beer fermentations is being developed. This objective has necessitated the use of research knowledge pooled from several different fields of study that include bioengineering, biochemistry and molecular biology, as reflected in the literature reviewed below.

2.2 Beer and Brewing

Breweries have a strong history of research (Stewart and Russell, 1986) and have made significant contributions to science of cell culturing (Hansen, 1883) and the concept of pH (Sörenson, 1909). Economically, brewing is one of the most important biotechnological industries (García *et al.*, 1993) and global beer production takes place on an unprecedented scale. For example, global production volumes exceeded 155 billion litres in 2004 (Sohn, 2005), an increase of 4.9% on 2003 in a climate of increased consolidation, aggressive competition and higher growth in wine and spirits-based beverages. Companies therefore have increasing pressure to optimize processes to ensure competitiveness in the global marketplace. The need for research and development remains principle, as breweries move towards automation and implementation of advanced fermentation control techniques.

Beer fermentation can broadly be split into two categories: ale and lager. Ales, mostly made with polyploid *Saccharomyces cerevisiae* strains, are generally produced at higher temperatures (18 to 20 °C), are characterised by shorter fermentation times (Hornsey, 1999) and have more "fruity" aromas, as a result of increased acetate by-product production at elevated temperatures. In addition, the yeast clusters in loose flocs, trapping gas and rises to the top of the liquid in the fermentation vessel. For this reason, traditional ale vessels are often in the shape of an open-top rectangular bin allowing easier yeast collection from the top of the fermented beer. Lagers, fermented with polyploid *Saccharomyces pastorianus* strains are characterised by lower fermentation temperatures (8 to 14 °C), longer fermentation times, more neutral aroma profiles and flocculation at the bottom of the vessel (the so-called bottom fermenting yeast). For this reason, lager fermentation vessels are commonly tall, closed top cylindroconical designs permitting easy yeast collection from the bottom. *S. pastorianus* and *S. cerevisiae* differ from each other genetically and efforts have been made to elucidate the ancestry of the lager yeast strain (Casaregola *et al.*, 2001). Genetic analysis has suggested that lager yeast is a hybrid of *S. cerevisiae* and most likely *S. bayanus*.

There are some obvious differences between ale and lager strains. While it is easy to observe characteristics such as a preference for a higher or lower temperature and the related time of fermentation, it is much more difficult to generalise about flavour production. Although we can generally characterise the ale strains as producers of more "fruity" aromas, such as iso-amyl-acetate, large differences in flavour and aroma profile can be observed between different strains. The conditions in the media also affect flavour production. For example, the levels of free amino acids in brewing wort can impact the

level of diacetyl production (Petersen *et al.*, 2004). Environmental conditions, such as wort pH, fermentation temperature, nutrient conditions, yeast handling, cell age and genetic behaviour can affect flocculation (Jin and Speers, 1998; Verstrepen *et al.*, 2003). Detailed information about yeast characteristics such aroma and flavour production in specific worts using specific strains is not readily available as this information is protected intellectual property and is not commonly released by breweries. Chapter 6 will provide further insight into some of the differences between ale and lager fermentations.

2,3-Butanedione (diacetyl), the decarboxylated product of α-acetolactate, is perhaps the best known quality parameter in brewing. This compound has an undesirable butterscotch-like aroma (Wainwright, 1973) and significant resources have been devoted to understanding its production and reduction in beer fermentations (e.g. (García *et al.*, 1993; Gjermansen *et al.*, 1988; Hardwick, 1994; Tolls *et al.*, 1970; Wainwright, 1973; Yonezawa and Fushiki, 2002)). With a flavour threshold as low as 0.1 parts per million (ppm) or 1.16 μM (Wainwright, 1973; Yonezawa and Fushiki, 2002), together with the fact that yeast can produce up to 10 ppm of diacetyl (Cyr *et al.* manuscript in preparation) it is important to reduce this concentration once fermentation is complete. Elimination of this metabolite remains one of the key reasons for prolonged product aging. Due to the traditional nature of the brewing industry, genetic modification of yeast remains undesirable and is likely to face consumer resistance. While extensive research into genetic expression and manipulation of genes encoding enzymes producing the diacetyl precursor α-acetolactate (2-Hydroxy-2-methyl-3-oxo-buteric acid, 2-acetolactate, acetolactate) has taken place (Dillemans *et al.*, 1987; Gjermansen *et al.*, 1988; Hansen and Kielland-Brandt, 1996; James *et al.*, 2003; Villa *et al.*, 1995), these findings have not

been implemented into beer production. Most research is oriented towards accurate diacetyl detection and quantification (Buckee and Mundy, 1994; García-Villanova and Estepa, 1993; Garza-Ulloa *et al.*, 1979; Hardwick, 1994; Izquierdo-Ferrero *et al.*, 1997; Mathis *et al.*, 1993; McCarthy, 1995; Zhang *et al.*, 1999) and prediction (García *et al.*, 1993). Most methods for diacetyl detection require laboratory analysis although some efforts have focused on online detection systems (Izquierdo-Ferrero *et al.*, 1997; Zhang *et al.*, 1999). These systems have not yet been implemented.

2.3 The yeast *Saccharomyces cerevisiae*

S. cerevisiae, schematically depicted in Fig. 2.1, is a heterotrophic eukaryotic organism (Hornsey, 1999). It possesses a rigid cell wall, which has multiple functions such as protection, shape retention and specialized enzyme activity (Russel, 1995). The exact composition of the wall varies with growth conditions, culture age and strain (Russel, 1995) but the general structure has been well studied chemically (Robinow and Johnson, 1991), structurally (Kollár *et al.*, 1995; Kollár *et al.*, 1997) and physically (Osumi, 1998). Genetic regulation of cell wall biogenesis has also been investigated and wall thickness can vary from 150 to 300 nm depending on strain and growth conditions (Smits *et al.*, 2001).

The plasma membrane, consisting mostly of lipids and proteins, is situated on the interior side of the cell wall and serves as a barrier between the external and internal aqueous solutions. The membrane serves the role of regulating the uptake of nutrients and excretion of metabolites (Russel, 1995)

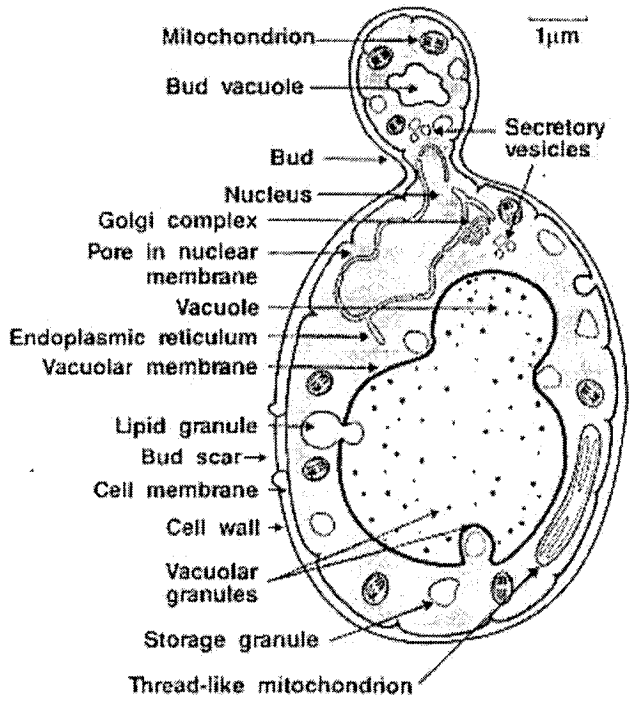


Figure 2.1 Main features of a typical budding yeast cell, from (Russel, 1995)

S. cerevisiae also contains membrane-bound organelles typical of other organisms such as a nucleus and mitochondria. In vegetative cells, the nucleus is usually situated adjacent to a prominent vacuole (Russel, 1995), which is the most conspicuous organelle within the cell (Hornsey, 1999). The nucleus is approximately 2μm in diameter and houses 16 very small and linear chromosomes. The perforated nuclear membrane remains intact throughout the cell cycle. The mitochondria are associated with the

tricarboxylic acid cycle, as well as electron transport, oxidative phosphorylation and amino acid biosynthesis. Cellular mitochondrial content varies according to growth conditions (Russel, 1995).

Viability of a cell population (the number of live cells) and vitality (a measure of cell fitness) are affected by numerous factors such as environmental stress and temperature fluctuations. These have been comprehensively reviewed (Heggart *et al.*, 2000) and rapid methods developed for use in breweries (Mochaba *et al.*, 1998).

2.3.1 Nutritional requirements of *S. cerevisiae*

The nutritional requirements of yeast cell growth were among the first properties to be described (cited in (Hornsey, 1999; Rose, 1987)). The overall requirements include; a carbon source, a nitrogen source, growth factors, inorganic ions, oxygen and water. There are certain limitations imposed on each of these. For example, many laboratory yeast strains do not possess the ability to transport and metabolize the tri-glucose maltotriose (Zastrow *et al.*, 2000; Zastrow *et al.*, 2001), while brewing yeast strains have retained this property. Furthermore, *S. cerevisiae* is unable to hydrolyze long chain carbohydrates, thus necessitating an adequate supply of mono-, di-, or tri-saccharides in the growth medium. Pentose sugars cannot be utilized by *S. cerevisiae* (Rose, 1987). Glucose repression in yeast is a global regulatory system that controls carbon source utilization, mitochondrial biogenesis, gluconeogenesis and other metabolic pathways (Hu *et al.*, 1995).

All strains are capable of utilizing ammonium ions as nitrogen source (Rose, 1987), but preference is given to amino acids and di- or tri-peptides. Amino acids are consumed by the yeast in a preferential order (García *et al.*, 1993) which can be roughly divided into four groups. Group A amino acids are taken up almost immediately and consist of arginine, asparagine, aspartate, glutamate, glutamine, lysine, serine and threonine. Group B amino acids are consumed gradually and consist of histidine, isoleucine, leucine, methionine and valine. Group C amino acids, consisting of alanine, glycine, phenylalanine, tryptophan and tyrosine are removed gradually, after an initial lag period. Group D consists of proline, which is only slightly taken up over the course of a 60 hour fermentation. Several endo- and exoproteases have been discovered in *S. cerevisiae*, but these do not catalyze the hydrolysis of extracellular proteins (Rose, 1987) and hence the availability of free amino nitrogen is of vital importance in industrial processes such as brewing. The uptake of amino acids impacts flavour production and is discussed below.

While *S. cerevisiae* possesses the ability to actively metabolize substrates in the absence of oxygen, this process is inefficient (Rose, 1987) with a significantly larger quantity of sugar being consumed compared to aerobic respiration. Oxygen is required for aerobic respiratory purposes as well as the synthesis of unsaturated fatty acids (Hornsey, 1999), which are found mostly in membranes. When all oxygen reserves have been depleted, yeast cells are unable to multiply. It is thus critically important to adequately aerate brewing wort when serial repitching is practiced, in order to ensure a healthy population that does not age (when harvested with the correct selective cropping

procedures). It is common brewing practice to provide sufficient oxygen for two cell doubling cycles - a four fold increase in cell concentration (Chapter 6).

2.3.2 Brewing Yeast metabolites

S. cerevisiae produces a vast array of metabolites during both aerobic and anaerobic metabolism, totaling approximately 600 to 800 compounds (Angelino, 1991). Broadly, yeast produces ethanol, carbon dioxide, higher (fusel) alcohols, acids, esters, carbonyl compounds, vicinal diketones and sulphur compounds. The extent of formation of these products depends on the overall metabolic balance (of metabolites, intermediates and other compounds within the cells) of the yeast culture (Stewart and Russell, 1986). There are many factors that can influence the balance and, therefore, the metabolic products, including flavours, produced. Product formation is primarily dictated by the raw materials, brewing process conditions, yeast strain employed and fermentation and maturation conditions (Angelino, 1991). Much research has been conducted in an effort to understand the impact of these and other factors on metabolite production (Pickerell *et al.*, 1991). Yeast handling procedures have subsequently been improved in brewing operations (O'Connor-Cox, 1998), for example reduction of alcoholic stress on harvested yeast through dilution with sterile water, reduced yeast stress through reduced intervals between cropping and repitching and ensuring sufficient aeration prior to repitching.

Ethanol is formed from sugars such as glucose, maltose and maltotriose. Using the Embden-Meyerhof-Parnas pathway, 1 g of glucose theoretically yields 0.51 g of ethanol and 0.49 g of CO_2 (Stewart and Russell, 1986), however, due to biomass

production as a result of wort oxygenation and cell division, these values are closer to 0.46 g ethanol and 0.44 g CO_2.

The principle acids that are formed in beer can be divided into two groups, namely organic acids and aliphatic acids. Organic acids, such as acetic, lactic and pyruvic acid are derived mainly from malt, carbohydrate metabolism in the tricarboxylic acid cycle and amino acid metabolism (Angelino, 1991). They influence flavour and pH. Short and medium chain (up to C_{12}) acids accumulate in the medium during fermentation and account for a large portion of the beer aliphatic acids. These acids are formed by aliphatic acid biosynthesis from acyl coenzyme A (acyl CoA) and the levels found in beer are directly related to the levels found in the yeast cell. Their soapy flavour effects are undesirable. Ale yeasts generally produce less medium chain aliphatic acids than lager yeasts.

Fusel alcohols are produced as bi-products of amino acid breakdown or biosynthesis and their production in beer has been successfully modeled (García *et al.*, 1994). In all cases keto-acids are produced, which are then decarboxylated to their corresponding aldehydes, which in turn are reduced to form higher alcohols.

Esters, such as ethyl acetate are important, flavour active compounds and add fruity flavours to beer. These compounds are produced intracellularly by enzymatic reactions between CoA derivatives of aliphatic acids and alcohols. Acetic acid is usually present in the highest concentration of all the acids and hence acetates of ethanol and higher alcohols are usually the most abundant (Angelino, 1991).

Sulphur compounds are generally flavour active constituents in beer and are mostly derived from malt and hops. Notable products produced by the cell include

sulphur dioxide and hydrogen sulphide (H_2S), both of which are intermediates in biosynthesis pathways for the production of the sulphur containing amino acids methionine and cysteine. Hydrogen sulphide can react with ethanol to form ethanethiol, a mercaptan. In regular vigorous fermentations, most H_2S is removed by means of CO_2 evolution (Angelino, 1991). Another product of concern is dimethyl sulphide (DMS) which is produced when dimethyl sulfoxide (DMSO) is reduced by yeast DMSO reductase. This enzyme is regulated by the nitrogen levels present in the wort (unfermented beer). Most worts contain sufficiently high nitrogen levels and hence only low levels of DMS are usually present (Angelino, 1991).

Aldehydes, such as acetaldehyde, and ketones, such as 2,3-butanedione play an important role in beer flavour. The majority of aldehydes result from raw materials, such as malt and hops, and from pre- and post-fermentation chemical reactions. Some products are formed when keto-acids leak from the cell and are oxidatively decarboxylated to form compounds such as 2,3-butanedione. Many aldehydes and ketones are reduced by the yeast during fermentation and consequently these highly flavour active compounds often have little impact on final product flavour (Angelino, 1991). Vicinal diketones (VDKs), such as 2,3-butanedione, commonly known as diacetyl, and 2,3-pentanedione are, however, rigorously monitored. These products impart butterscotch-like flavours in beer, and are highly undesirable in most products at concentrations above the taste threshold (Russel, 1995).

2.3.3 Diacetyl

Diacetyl concentration is one of the most important parameters in brewing as a result of a very low flavour threshold and undesirable aroma (Wainwright, 1973; Yonezawa and Fushiki, 2002). Extensive research has been conducted on this compound in recent years (Russel, 1995). Focal points have included reduction and regeneration in rapid maturation systems using immobilized yeast cells (Yamauchi *et al.*, 1995), online monitoring of VDKs and their precursors using spectrophotometric methods (Izquierdo-Ferrero *et al.*, 1997) and gas chromatography (Mathis *et al.*, 1993) as well as offline measurement with high performance liquid chromatography (HPLC) in conjunction with fluorescence detection (McCarthy, 1995), spectrophotometry (Garza-Ulloa *et al.*, 1979) and gas chromatography (GC) using various techniques and detectors (Buckee and Mundy, 1994; Convention, 1998; Hardwick, 1994; Landaud *et al.*, 1998). The brewing industry standard relies on the use of headspace sampling gas chromatography with an electron capture detector (ECD). One area that is, however, neglected is the issue of residual α-acetolactate, which will gradually decompose to yield diacetyl. This process can be sped up with sample heating, but full conversion requires the use of agents such as aniline hydrochloride (Hardwick, 1994) or a mixture of $FeSO_4$ and $FeCl_3$ (Gollop *et al.*, 1987; Landaud *et al.*, 1998). However, these two agents do not work with equal efficiency per unit time and temperature (see Chapter 7) and some methods do not use them at all. The time temperature interval employed for acetolactate decarboxylation is critical to accurate diacetyl quantification (Blanchette *et al.*, 2006). As a result of inconsistent analytical procedures and several different official methods, it may be assumed that analytical error is commonplace.

Diacetyl is the decarboxylated product of α-acetolactate (Wainwright, 1973), an intermediate in the isoleucine, leucine, valine (ILV) biosynthesis pathway (Dillemans *et al.*, 1987). Many of the biochemical considerations have been known for over a quarter century, such as the effect of limitation of the above branched chain amino acids on ILV pathway biosynthetic activity, and hence precursor production (Magee and Hereford, 1969). Diacetyl production has also been shown to be dependant on the yeast strain employed and its condition when pitched (inoculated) into the wort (Wainwright, 1973). Yet surprisingly, although diacetyl formation pathways have been well characterized (Cullin *et al.*, 1996; Dillemans *et al.*, 1987; Gjermansen *et al.*, 1988; Pang and Duggleby, 1999; Poulsen and Stougaard, 1989; Velaso *et al.*, 1993; Villa *et al.*, 1995; Xiao and Rank, 1990; Xie and Jiménez, 1994) (see yeast genetics below), and diacetyl production during fermentation modeled (García *et al.*, 1993), little is known about cellular mechanisms responsible for reduction.

These reductive mechanisms are of critical importance in yeast and other organisms for the maintenance of cellular health. α,β-Dicarbonyls such as diacetyl and methylglyoxal, are highly reactive compounds that preferentially react with arginine, lysine, and cysteine residues causing protein cross linking (Lundblad, 1995; Oya *et al.*, 1999). In mammalian cells, methylglyoxal and diacetyl have been implicated in a number of diseases that include diabetic vascular complications (Brownlee, 1995), neurodegenerative diseases (Kovacic and Cooksy, 2005), atherosclerosis, and general cellular deterioration and aging (Wondrak *et al.*, 2002). Several proteins that have been shown to be susceptible to chemical modification, include ovalbumin (Hipkiss and Chana, 1998), plasminogen (Lerant *et al.*, 2000), and glyceraldehyde-3-phosphate

dehydrogenase (Morgan *et al.*, 2002). Regulatory mechanisms have been documented in *Saccharomyces cerevisiae* exposed to methylglyoxal (Aguilera and Prieto, 2004), but little is known about the cellular responses to diacetyl.

2.3.4 Yeast Genetics

2.3.4.1 Introduction

The general concepts pertaining to yeast genetics are described elsewhere (Sherman, 1997) and gene expression, regulation and control thereof has been reviewed (McCarthy, 1998; Tucker and Parker, 2000). Below is a general discussion on the genetics in brewing yeasts and the differences between *S. cerevisiae* (ale yeast) and *S. pastorianus* (lager yeast) genetics. Many regard lager yeast to be a hybridization between *S. cerevisiae* and an other *Saccharomyces* species (Kielland-Brandt *et al.*, 1995), although debate continues regarding the other parent, with genetic analysis suggesting *S. monacensis* over *S. bayanus*.

S. cerevisiae has 16, small, straight chromosomes found within the nucleus. Individual chromosome sizes range from 200 to 2200 kilobases (kb), with a total size of 12 052 kb (Sherman, 1997). The yeast genome contains approximately 3.5 times more DNA than *Escherichia coli*. The entire genetic code has been mapped and sequenced and the function of many of genes is known (Giaever *et al.*, 2002). Yeast genes range in size from 40 to 4910 codons with an average of 1.45 kb or 483 codons (Sherman, 1997).

Most laboratory yeast strains are haploid, whereas brewing strains are diploid, polyploid or aneuploid (Kielland-Brandt *et al.*, 1995; Russel, 1995). Polyploidy has

retarded the study of industrial yeasts, as traditional methods for studying genetic behaviour, such as mutagenesis, are often not feasible with more than one set of chromosomes. Techniques, such as mutagenesis had previously been seen as promising for the study of industrial strains (Stewart and Russell, 1986), however, mutations often do not reveal themselves, owing to the presence of non-mutated alleles. Furthermore, the stability of industrial strains has been attributed to their characteristically low mating ability, poor sporulation and low spore viability (Kielland-Brandt *et al.*, 1995; Russel, 1995).

A feature that remains unique to yeast is the ability to carry out transformation directly with synthetic nucleotides. This allows for the production of numerous altered forms of proteins, which can be exploited in the analysis of gene regulation, chromosome structure and structure-function relationships of proteins, amongst others (Sherman, 1997). Transcription and proteins related to the process have been well studied in yeast (Burley, 1996; McCarthy, 1998; Tucker and Parker, 2000). Identification of brewing yeast genes often takes place by assaying their phenotype partially or fully in the background of laboratory strains (Kielland-Brandt *et al.*, 1995).

2.3.4.2 The ILV pathway and the diacetyl precursor α-acetolactate

The metabolic precursor for diacetyl can be found in the ILV pathway (Figures 2.2 and 2.3), which is an amino acid biosynthesis pathway leading to the synthesis of isoleucine leucine and valine (Dillemans *et al.*, 1987). Like other pathways, there are control systems in place that regulate the levels of gene expression (Holmberg and Petersen,

1988). There are, however, several levels of genetic control and some of the genes involved in this pathway are further regulated by a higher order system known as general amino acid control (Hinnebusch, 1986a). Evidence suggests that approximately 30 enzymes in seven amino acid pathways are controlled by this system (Hinnebusch, 1986b), with the protein product of the *GCN4* gene being the single positive transcription regulator. Ilv1p, which catalyses the deamination of threonine at the start of the isoleucine pathway (see Figure 2.2), is one of the genes that falls under this general control system and is designated as multi-functional due to its role in regulation of the pathway and in biosynthesis (Holmberg and Petersen, 1988).

As previously mentioned, diacetyl has received much attention from brewers and genetic events are without exception. Wainwright (1973) authored a comprehensive review detailing all the biochemical knowledge available at the time. With the advent of genetic studies, this knowledge has accelerated rapidly and the pathways involved in diacetyl precursor formation as well as their regulation are now well documented. α-Acetohydroxyacid synthase (Ilv2p), catalyzing the production of acetolactate from pyruvate has been purified and characterized in recombinant form (Poulsen and Stougaard, 1989) and shown to fall under branched chain amino acid regulation (Xiao and Rank, 1990) as well as have a regulatory subunit (Ilv6p) (Cullin *et al.*, 1996). The two subunits have both been expressed and characterized (Pang and Duggleby, 1999). Ilv2p catalyses the production of α-acetolactate while acetolactate reductoisomerase (Ilv5p) reduces it to di-hydroxy-isovaleric acid (Dillemans *et al.*, 1987). This enzyme may also perform a function in mitochondrial DNA stability as suggested by the instability of mtDNA in Δ*ilv5* cells (Zelenaya-Troitskaya *et al.*, 1995). Two similar isoforms of Ilv5p

genes have been cloned from a polyploid *S. cerevisiae* brewing strain (Xie and Jiménez, 1994) and multiple copies of *ILV5* have been successfully integrated in polyploid yeast, resulting in decreased diacetyl formation (Mithieux and Weiss, 1995). Dihydroxyacid dehydratase (Ilv3p) is located downstream of Ilv5p, catalyzing the dehydration of 2,3-dihydroxy-isovaleric acid to 2-keto-isovaleric acid and has been cloned. Although significant efforts have focused on Ilv2p and Ilv5p, which are responsible for acetolactate production and reduction respectively, research has expanded to include genes that influence this pathway, such as branched-chain amino acid permease (*BAP2*), involved in amino acid uptake (Grauslund *et al.*, 1995). The presence of certain amino acids in media induce expression of Bap2p (Didion *et al.*, 1996).

Alternative strategies have been tested to reduce diacetyl levels, including mutation of the *ILV2* gene – α-acetohydroxyacid synthase (Figure 2.2), catalyzing the formation of α-acetolactate (Gjermansen *et al.*, 1988) and over expression of the downstream α-acetolactate reduction genes *ILV5* and *ILV3* (Villa *et al.*, 1995).

The development of DNA microarrays have recently been exploited to study global gene expression patterns in response to various environmental conditions, e.g. (Alexandre *et al.*, 2001; Ferea *et al.*, 1999; James *et al.*, 2003; Mithieux and Weiss, 1995). The results of these studies are being integrated into the *Saccharomyces* Genome Database (SGD), at Stanford University (Ball *et al.*, 2000) and made available to the research community, speeding the rate of progress in functional analysis of the yeast genome.

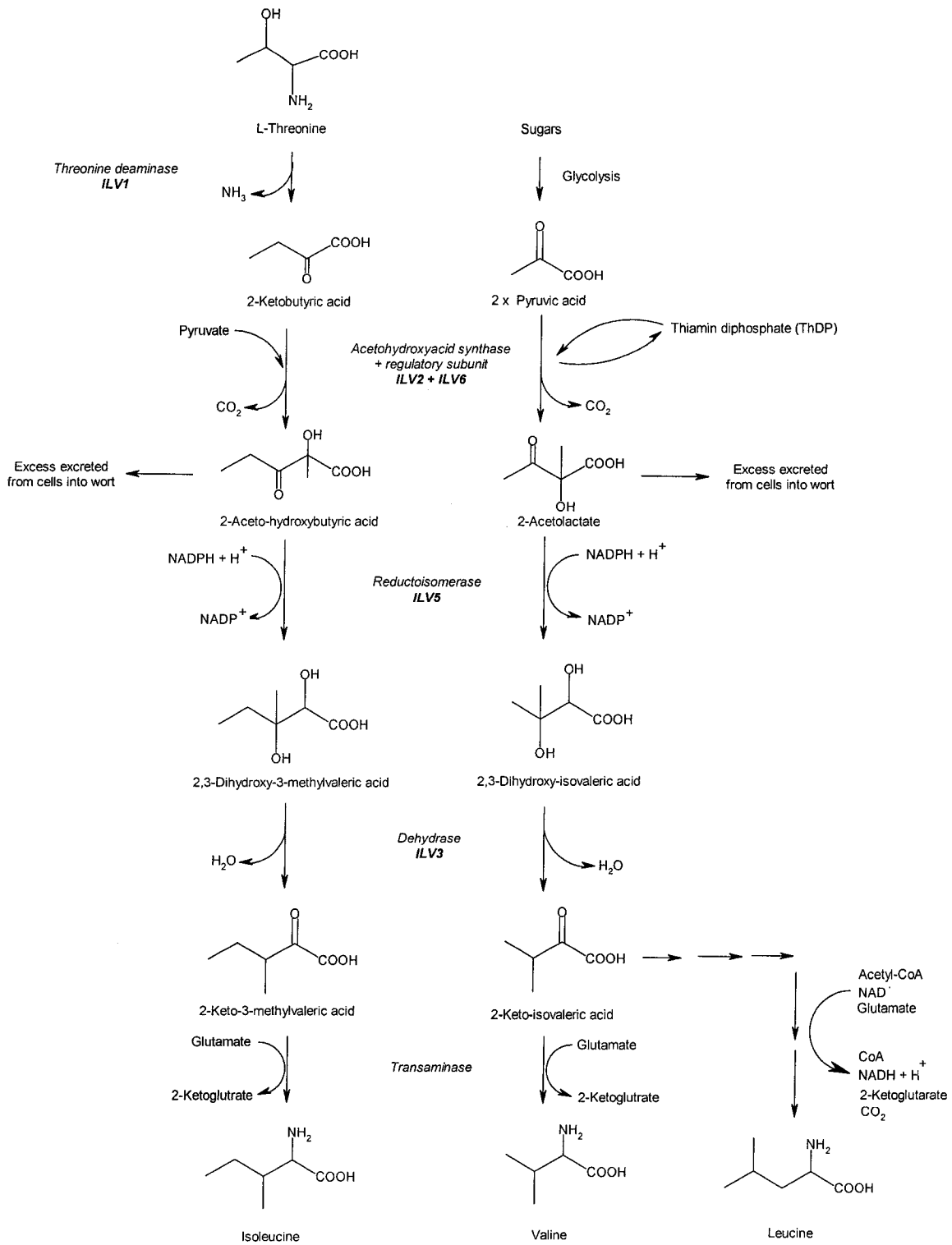
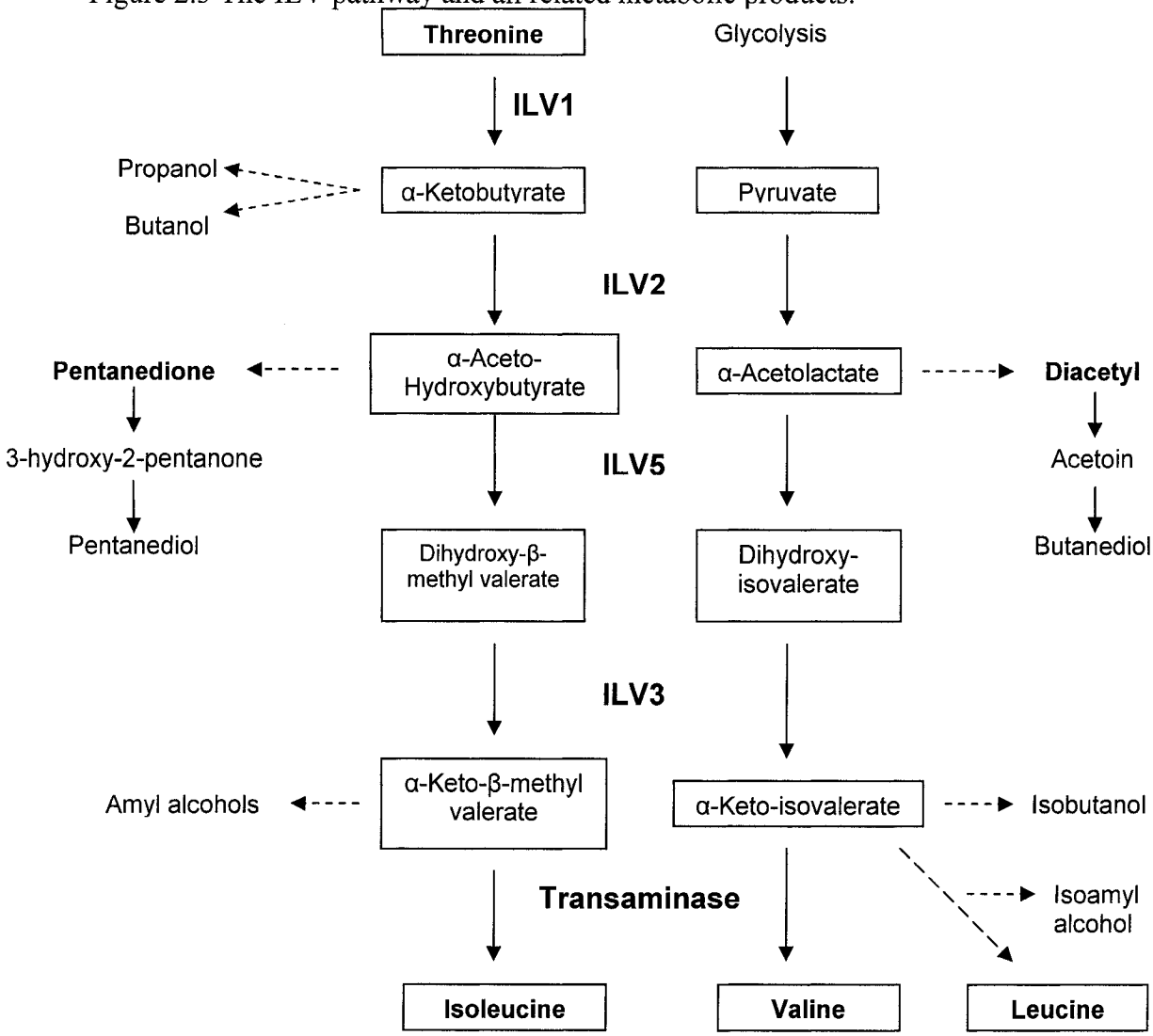


Figure 2.2 Detailed pathway for isoleucine, leucine and valine biosynthesis. Enzymes catalyzing specific reactions are in italic. Genes coding for those enzymes are in bold italic below the enzyme name.

Figure 2.3 The ILV pathway and all related metabolic products.



2.4 Enzymes

2.4.1 Introduction

Enzymes function as biocatalysts by influencing the rate at which specific chemical reactions take place. These protein catalysts mediate numerous reactions, ranging from fat and carbohydrate metabolism to more intricate reactions related to cell energetics (Kuby, 1991). Protein metabolism pathways, such as the ILV pathway (Figure 2.3) have numerous enzymes, with each responsible for catalysis of an intermediate. In 1902 it was first suggested that an enzyme-substrate complex was an obligate intermediate in these catalytic reactions (Kuby, 1991) which lead researchers to attempt explanations of the catalytic activity and progress of reactions using mathematical terms. Equations were developed to account for the effect of increased substrate concentration on velocity. Shortly after, the effect of pH on enzyme activity was observed, bringing to light the importance of environmental parameters to enzyme function.

During this time, however, it was uncertain whether enzymes were in fact proteins. By 1932 there was still little known about the structure of mechanisms of enzymes (Schopfer and Massey, 1991). It was in this year that Warburg and Christian first isolated an enzyme, which would occupy an important place in enzymology history, known as Old Yellow Enzyme (OYE) (NADPH oxidoreductase, EC 1.6.99.1) (Warburg and Christian, 1932). In 1934 OYE was purified to electrophoretic homogeneity (Schopfer and Massey, 1991) and found to be composed of a protein and a yellow co-factor in a one to one stoichiometry. Separation of these caused a complete loss of activity, while a complete return to activity was observed upon recombination. It was

thus demonstrated that the protein was an essential structural element, required for catalytic activity.

Knowledge of enzymes and enzyme classes has grown considerably since then, now encompassing several classes of enzymes including proteases, dehydrogenases, phosphotransferases, ATP-ases, hydrolases, metaloenzymes and flavoenzymes. With increased understanding, enzymes are now being used to catalyze stereoselective reactions (Rodríguez *et al.*, 2001) that would otherwise prove difficult to achieve using conventional chemical techniques.

2.4.2 Flavin Enzymology

Old Yellow Enzyme was the first enzyme to be shown to contain both a protein and a small organic co-factor. Following the demonstration of loss of activity upon separation, research was conducted to elucidate the identity of the yellow cofactor. Structural studies lead to the discovery that the cofactor was flavin mononucleotide (FMN), the phosphoric acid ester of riboflavin (vitamin B_2) (Warburg and Christian, 1938). This demonstrated a biochemical role for this essential vitamin growth factor. By 1955 it had been discovered that the use of vitamin cofactors was widely occurring in nature, with the vitamins thiamine (vitamin B_1), nicotinic acid amide (vitamin B_3) and pyridoxine (vitamin B_6) behaving in a similar manner (Schopfer and Massey, 1991). The class of enzymes is thus known as flavoenzymes, due to the presence of a flavin group in the cofactor, and includes Old Yellow Enzyme, D-amino acid oxidase as well as cytochrome c, quinone and cytochrome P-450 reductases.

Flavin enzymes catalyze a wide variety of reactions, all of which rely on the catalytically important isoalloxazine ring in the flavin (Schopfer and Massey, 1991). With all flavin enzymes containing the above ring structure, it can be deduced that the catalytic specificity of an enzyme must be due to the protein groups that surround the flavin. There appears to be broad agreement with this postulation (Schopfer and Massey, 1991) but the mechanisms have not been clarified yet.

2.4.3 Old Yellow Enzyme

OYE remains one of the most well studied enzymes, yet surprisingly, its functional role in cell metabolism is still not known (Fitzpatrick *et al.*, 2004) apart from the ability to utilize NADPH as a reductant (Schopfer and Massey, 1991). In the years since Warburg and Christian first isolated OYE (Warburg and Christian, 1932), much has become known about OYE and its flavin environment. The original enzyme was known as "Yellow enzyme" as a result of the color imparted by the flavin cofactor. The discovery of a new yellow enzyme in 1938 by Haas lead to a renaming of Warburg's enzyme as "Old Yellow Enzyme" (Williams and Bruce, 2002). The naming was later standardized, with the originally discovered enzyme named OYE1 and the "new" yellow enzyme OYE2. In recent years, OYE1 has been cloned (Saito *et al.*, 1991) and the structure resolved at 2 angstrom resolution using X-ray crystallography (Fox and Karplus, 1994). A third isoform (OYE3) has been discovered (Niino *et al.*, 1995) and several similar proteins have been found in other organisms (Fitzpatrick *et al.*, 2003; Stott *et al.*, 1993; Williams and Bruce, 2002). Despite all these advances, the physiological role of OYE has yet to be determined.

2.4.3.1 Physical properties

The open reading frame for OYE1 codes for a polypeptide of 400 amino acids with a M_r of 45,021 (Saito *et al.*, 1991). The initiation amino acid methionine is not found on the final protein, yielding an expressed product of 399 amino acids and a mass of 44,890 Daltons (Stott *et al.*, 1993). This correlates well with previous data obtained via SDS acrylamide gel electrophoresis (Schopfer and Massey, 1991). The protein exists as a dimer, with 4 sets of hydrogen bonds holding the units together. There is one FMN cofactor bound per subunit, each with a dissociation constant of approximately 10^{-10} M at 25°C in a 100 mM potassium phosphate buffer at pH 7 (Schopfer and Massey, 1991). It has previously been claimed that the enzyme is sensitive towards degradation, even when stored at -20 °C (Schopfer and Massey, 1991), however, recent finding suggest that the enzyme is stable for several months at 4 °C if filter sterilized (unpublished observations). In its purified form, OYE is free of carbohydrate and the metals calcium, copper, iron, magnesium, manganese and zinc.

OYE2 was identified and sequenced from *Saccharomyces cerevisiae* (Stott *et al.*, 1993) and found to be similar to OYE1 cloned from *Saccharomyces carlsbergensis* (now considered to be *S. pastorianus*). The expressed product has 399 amino acids and a calculated molecular mass, excluding the start codon, of 44,886 Daltons. There are 33 differences in the amino acid sequence of OYE1 and OYE2. Analysis reveals 92% identity and 95% homology (Stott *et al.*, 1993), where homology indicates substitution by an amino acid that has a similar function, for example substitution of leucine with isoleucine.

The discovery of a third OYE gene, the second from *S. cerevisiae* (Niino *et al.*, 1995) has allowed for the study of a family of similar proteins with slightly different catalytic abilities. OYE3 was determined to be 44,788 Daltons by electrospray mass spectrometry, which correlates well with a mass of 44,920 predicted from the DNA sequence. OYE1 and OYE3 have 80% identity and 87% homology, while OYE2 and OYE3 share 82% identity and 89% homology. Further investigations need to be performed to compare the characteristics of these highly homologous, yet catalytically different enzymes.

2.4.3.2 Enzyme structure

The crystal structure of OYE1 has been determined (Fox and Karplus, 1994) allowing for the determination of numerous structural details. The enzyme has been found to be a dimer in its native form, with each monomer having approximate dimensions of 60 Å by 45 Å (Karplus *et al.*, 1995). Each subunit has one catalytic pocket, with each arranged on opposite sides when in dimer form. In terms of function, the enzyme could, therefore, be described as a monomer although this was never tested until now (Chapter 3).

Amino acid side chains that interact with the FMN subunit have been found to be threonine^{37}, glycine^{72}, glutamine^{114}, arginine^{243}, glycine^{324}, asparagine^{325}, phenylalanine^{326}, glycine^{345}, glycine^{347} and arginine^{348} (Fox and Karplus, 1999). Most of these amino acid residues are conserved in all three *Saccharomyces sp.* OYE enzymes, except for phenylalanine^{326}, which is substituted by tyrosine in OYE3. Figure 2.4 shows the amino acid alignment of OYE1, 2 and 3. Residues that interact with FMN are shown

in bold. Figure 2.5 shows two views of the FMN group with the interacting amino acid residues that have been highlighted above and in Figure 2.4. Phe^{326}, the residue substituted with tyrosine in OYE3, is shown in dark grey. Amino acids 206 to 216, the (top loop in Figure 2.6), are conserved throughout the family of proteins (Williams and Bruce, 2002) and form part of the dimer interface in OYE (Fox and Karplus, 1994). Detailed studies on individual FMN interacting amino acids have been performed to elucidate their functions (Brown *et al.*, 1998; Brown *et al.*, 2002; Kohli and Massey, 1998). The flavin environment has also been closely examined to determine solvent accessibility and hydrogen bonding patterns with each flavin atom (Fox and Karplus, 1999).

```
OYE1     -SFVKDFKPQALGDTNLFKPIKIGNNELLHRAVIPPLTRMRALHPGNIPNRDWAVEYYTQ
OYE2     MPFVKDFKPQALGDTNLFKPIKIGNNELLHRAVIPPLTRMRAQHPGNIPNRDWAVEYYAQ
OYE3     MPFVKGFEPISLRDTNLFEPIKIGNTQLAHRAVMPPLTRMRATHPGNIPNKEWAAVYYGQ
          .***.*:* :* *****:******.:* ****:******** *******::**. ** *

OYE1     RAQRPGTMIITEGAFISPQAGGYDNAPGVWSEEQMVEWTKIFNAIHEKKSFVWVQLWVLG
OYE2     RAQRPGTLIITEGTFPSPQSGGYDNAPGIWSEEQIKEWTKIFKAIHENKSFAWVQLWVLG
OYE3     RAQRPGTMIITEGTFISPQAGGYDNAPGIWSDEQVAEWKNIFLAIHDCQSFAWVQLWSLG
         *******:*****:* ***:********:**:**: **.:** ***: :**.***** **

OYE1     WAAFPDNLARDGLRYDSASDNVFMDAEQEAKAKKANNPQHSLTKDEIKQYIKEYVQAAKN
OYE2     WAAFPDTLARDGLRYDSASDNVYMNAEQEEKAKKANNPQHSITKDEIKQYVKEYVQAAKN
OYE3     WASFPDVLARDGLRYDCASDRVYMNATLQEKAKDANNLEHSLTKDDIKQYIKDYIHAAKN
         **:*** *********.***.*:*:*  : ***.*** :**:***:****:*:*::****

OYE1     SIAAGADGVEIHSANGYLLNQFLDPHSNTRTDEYGGSIENRARFTLEVVDALVEAIGHEK
OYE2     SIAAGADGVEIHSANGYLLNQFLDPHSNNRTDEYGGSIENRARFTLEVVDAVVDAIGPEK
OYE3     SIAAGADGVEIHSANGYLLNQFLDPHSNKRTDEYGGTIENRARFTLEVVDALIETIGPER
         ****************************.*******:**************::::** *:

OYE1     VGLRLSPYGVFNSMSGGAETGIVAQYAYVAGELEKRAKAGKRLAFVHLVEPRVTNPFLTE
OYE2     VGLRLSPYGVFNSMSGGAETGIVAQYAYVLGELERRAKAGKRLAFVHLVEPRVTNPFLTE
OYE3     VGLRLSPYGTFNSMSGGAEPGIIAQYSYVLGELEKRAKAGKRLAFVHLVEPRVTDPSLVE
         *********.*********.**:***:** ****:*******************:* *.*

OYE1     GEGEYEGGSNDFVYSIWKGPVIRAGNFALHPEVVREEVKDKRTLIGYGRFFISNPDLVDR
OYE2     GEGEYNGGSNKFAYSIWKGPIIRAGNFALHPEVVREEVKDPRTLIGYGRFFISNPDLVDR
OYE3     GEGEYSEGTNDFAYSIWKGPIIRAGNYALHPEVVREQVKDPRTLIGYGRFFISNPDLVYR
         *****. *:*.*.*******:*****:*********:*** ***************** *


OYE1     LEKGLPLNKYDRDTFYQMSAHGYIDYPTYEEALKLGWDKK
OYE2     LEKGLPLNKYDRDTFYKMSAEGYIDYPTYEEALKLGWDKN
OYE3     LEEGLPLNKYDRSTFYTMSAEGYTDYPTYEEAVDLGWNKN
         **:*********.*** ***.** ********:.***:*:
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Figure 2.4 Alignment of OYE1, 2 and 3. Amino acid residues that interact with the cofactor FMN are shown in bold face print. Alignment performed with ClustalX.

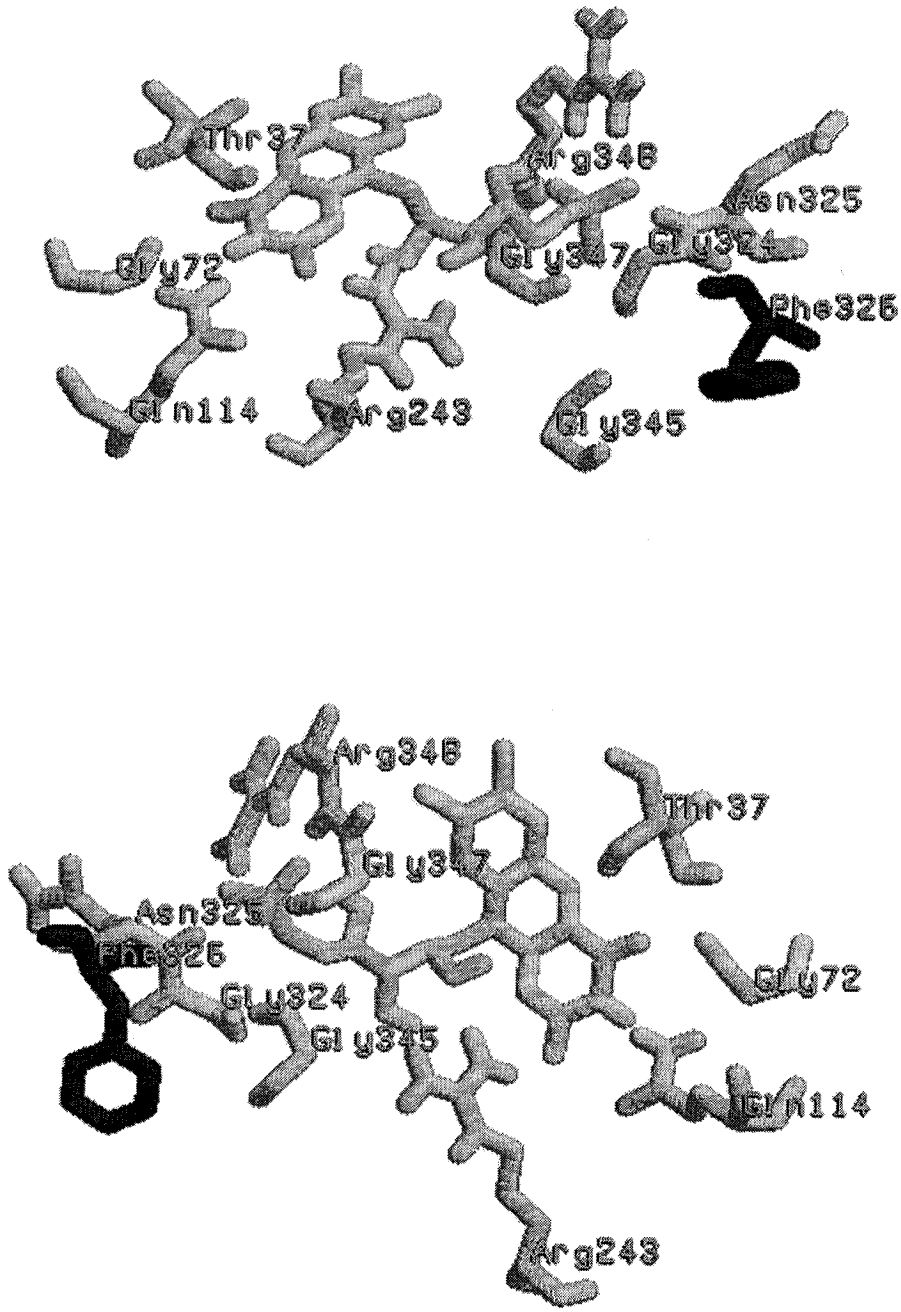

Figure 2.5 Flavin mononucleotide with interacting amino acid side chains shown. FMN is shown with conserved amino acids in light grey and Phe326 in dark. Top image displays a ¾ top view. Bottom image displays the FMN molecule from below.

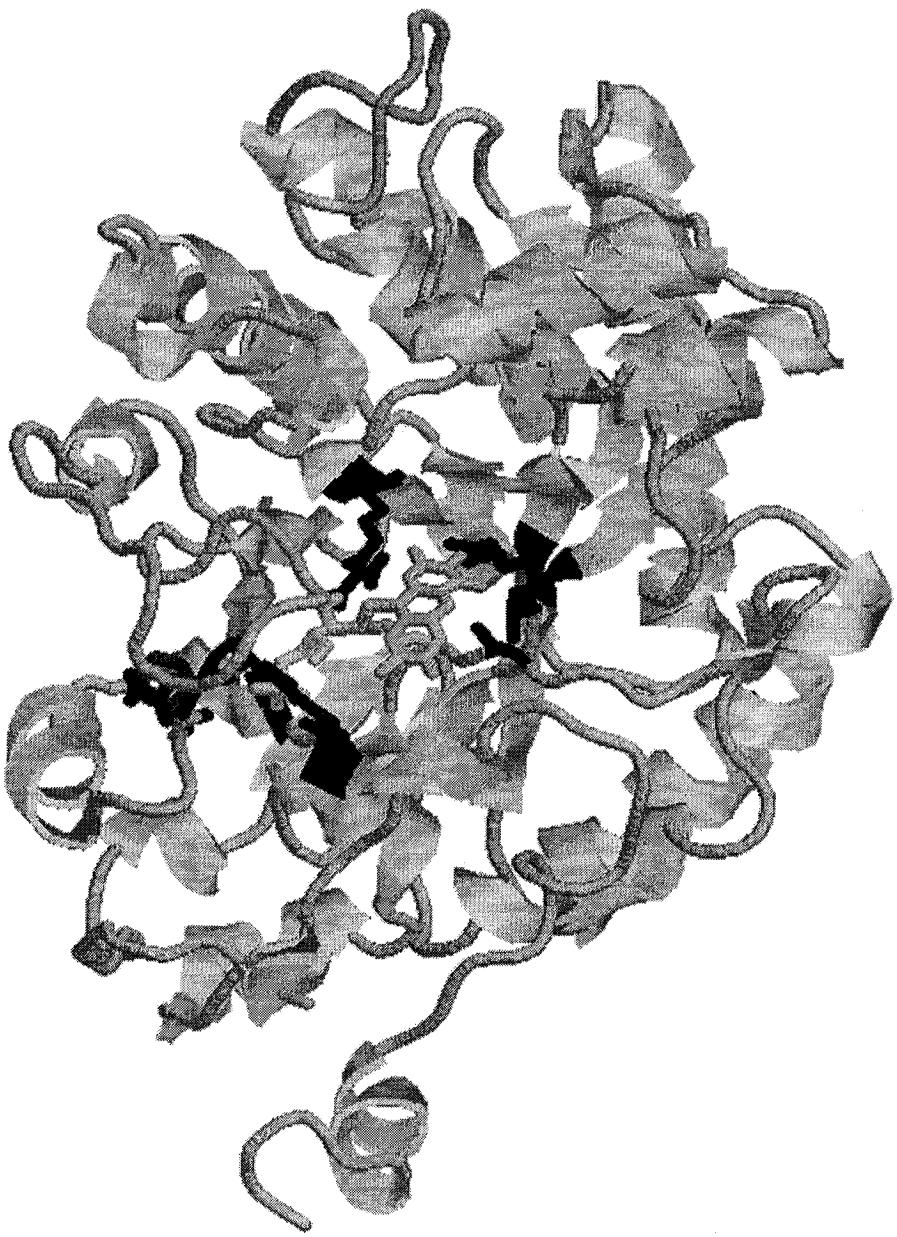

Figure 2.6 OYE1 enzyme showing looking into the catalytic pocket, with FMN and interacting amino acids shown (darker – see Figure 2.5 for further details) as well as the conserved domain forming part of the dimer interface (top loop).

2.4.3.3 Catalytic function and kinetics

OYE is readily reduced by NADPH in both the α- and β-forms (Massey and Schopfer, 1986; Schopfer and Massey, 1991). This is uncommon, as the majority of pyridine nucleotide dependant enzymes display a preference for β-NADPH several orders of magnitude higher than α-NADPH. All three yeast OYE isoforms show rapid equilibrium binding with NADPH and display K_d values in the region of 10 μM at pH 7.0 and 25 °C (Karplus *et al.*, 1995). Of the substrates that can be catalytically reduced, many have reaction rates that are faster than the NADPH oxidase activity, and are, therefore, limited in rate by the reduction of the enzyme by the NADPH (Karplus *et al.*, 1995). Under the conditions above, the development of a charge transfer band takes place in approximately 1000 s^{-1} to 500 s^{-1} for OYE2 and 200 s^{-1} for OYE3. This is followed by reduction of the enzyme bound flavin in 3.9 s^{-1} for OYE2 and 18 s^{-1} for OYE3 (Niino *et al.*, 1995). Rapid reaction kinetic studies with OYE1 were performed at 4°C making a direct comparison impossible. These studies were also performed before other isoforms had been discovered and suggested a biphasic reduction process. Later studies with recombinant proteins indicated monophasic reduction with rates of approximately 16 s^{-1} and 1.0 s^{-1} (Niino *et al.*, 1995). Other substances that can act as reductants include dithionite or EDTA/light/deazaflavin. Two electrons are required per flavin for complete reduction (Massey and Schopfer, 1986). Figure 2.7 depicts the catalytic cycle of OYE with NADPH and the oxidizing agent diacetyl or molecular oxygen.

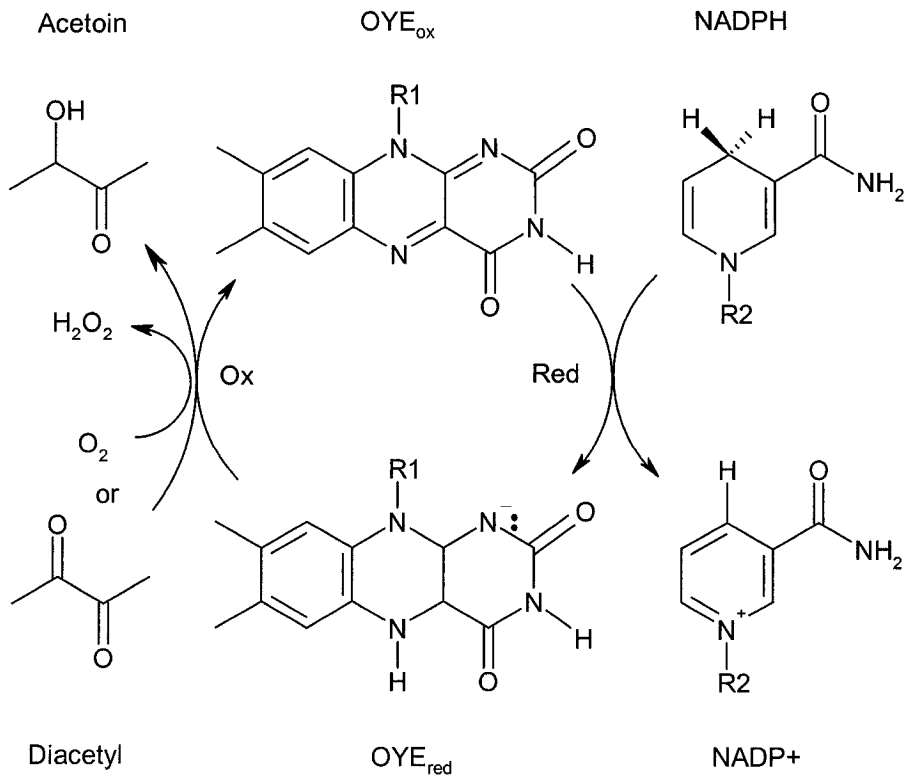

Figure 2.7 Schematic depiction of OYE catalytic cycle utilizing NADPH and an oxidant, adapted from (Kohli and Massey, 1998).

Reoxidation can be accomplished with a variety of substrates including α/β-unsaturated aldehydes and ketones, Fe^{3+}, methylene blue, quinones, cytochrome *c* and ferricyanide (Williams and Bruce, 2002). Studies have suggested that this takes place via a "ping-pong" mechanism which is consistent with structural studies that suggest that the NADPH and electron acceptors bind in the same pocket, meaning that they cannot bind simultaneously (Karplus *et al.*, 1995). The enzyme is, therefore, reduced by NADPH, following which the $NADP^+$ is expelled from the catalytic pocket and the oxidizing substrate binds. Molecular oxygen is capable of reoxidizing OYE, but is commonly viewed as an opportunistic electron acceptor (Stott *et al.*, 1993) with OYE reacting

preferentially with any available substrate that is capable of being reduced (Schopfer and Massey, 1991). Maximum reaction rates with oxygen are slow: 2400 M^{-1} s^{-1} with OYE2 and only 570 M^{-1} s^{-1} with OYE3 (Karplus *et al.*, 1995). Some α,β-unsaturated carbonyl-containing compounds, such as cyclohexenone, can be reduced by OYE. Reduction of cyclohexenone by reduced OYE has been found to be saturated with a k_{cat} of 250 ± 25 min^{-1} and a K_d of 10μM for OYE2. OYE3 on the other hand showed significantly different values with a K_{cat} of 385 ± 15 min^{-1} with much higher K_d of 500μM (Karplus *et al.*, 1995; Niino *et al.*, 1995). Recently OYE was found to react with nitroglycerin and propylene dinitrate (Meah *et al.*, 2001) suggesting possible interesting applications in explosive remediation amongst others (Williams and Bruce, 2002).

Acids, esters and amides do not react with OYE, while the majority of known inhibitors contain phenolic functional groups, for example para-hydroxybenzaldehyde. The incorporation of affinity tags for rapid purification has been shown to impair the structure and function of a bacterial OYE homologue (Fitzpatrick *et al.*, 2003; Fitzpatrick *et al.*, 2004) but the results may not be applicable given that the protein is both smaller than the yeast OYE homologues and forms a tetrameric structure.

It has recently been shown that the OYE2 protein interacts with actin (Haarer and Amberg, 2004) and may, therefore, play a role in protecting the actin cytoskeleton from oxidative stress.

Recent brewing research in beer flavour deterioration implicated 3-deoxy-2-hexosulose, the most abundant α-dicarbonyl in beer, as an important precursor to staling flavour compounds. Further investigation revealed that OYE is capable of reducing this compound (Sánchez *et al.*, 2003). Reactivity was also noted with other dicarbonyl

compounds such as methylglyoxal and 2,3-butanedione, The lack of assay information, however, raises important questions about the reactivity and specificity, which will be addressed in the coming chapters.

2.4.4 Yeast D-Arabinose dehydrogenase

Little is known about the cytosolic enzyme D-arabinose dehydrogenase (ARA1) in *S. cerevisiae*. First purified in 1998, the enzyme was shown to catalyse the oxidation of D-arabinose, L-xylose, L-fucose and L-galactose in the presence of $NADP^+$ (Kim *et al.*, 1998). However, the maximum oxidative activity with these substrates was observed at the non-physiological pH of 10.0. The requirement of Ara1p for D-arabinose catalysis was confirmed by gene disruption. Yeast knockouts of D-arabinose dehydrogenase displayed no D-arabinose catalytic activity (Kim *et al.*, 1998). Chapter 4 provides more analysis of Ara1p structure and function.

Gre1p and Gre3p are two proteins that display similar sequences to Ara1p and belong to the same Aldo-keto reductase family of proteins. All three proteins display similar up-regulation in the presence of stress inducing conditions, such as exposure to DTT (Travers *et al.*, 2000) and heat and osmotic shock (Gasch *et al.*, 2000). A similar, highly correlated expression is observed during a diauxic shift from anaerobic to aerobic conditions (DeRisi *et al.*, 1997) suggesting that these aldo-keto reductases may perform similar roles in protecting the cell from oxidative and other stresses.

2.4.5 Yeast mitochondrial peroxiredoxin (thioredoxin peroxidase)

S. cerevisiae have several mechanisms which confer protection against certain oxidative stresses. These mechanisms are required to protect the cell from reactive oxygen species (ROS) and other damaging compounds derived from incomplete reduction of molecular oxygen during respiration and aerobic metabolism (Park *et al.*, 2000). One of these protective mechanisms is known as the thioredoxin system, and is located in two distinct compartments - the cytosol and the mitochondria (Trotter and Grant, 2005). Both systems are comprised of similar proteins, coded for by separate genes, and include a thioredoxin and a thioredoxin reductase as part of a catalytic cascade. Thioredoxins (TRX) are small, highly conserved heat stable proteins (Garrido and Grant, 2002; Trotter and Grant, 2002) with masses of ~14 kDa while thioredoxin reductases (TRR) are ~37 kDa. The cytosolic mechanism is encoded by the genes *TRX1*, *TRX2* and *TRR1* (Trotter and Grant, 2005) while the mitochondrial system is comprised of *TRX3* and *TRR2* (Pedrajas *et al.*, 1999). Mutagenesis studies have suggested a role for the thioredoxin system in the protection against oxidative stresses induced by hydroperoxides (Garrido and Grant, 2002). In this system Trx2p has been highlighted as playing a dominant role in the degradation of these peroxides. It has also been suggested that the cytosolic system provides protection against certain reductive stresses brought about by the presence of reducing agents such as dithiothreitol (Trotter and Grant, 2002).

Antioxidant enzymes such as catalase and superoxide dismutase play an important role in the cellular defence systems against the ROS compounds hydrogen peroxide (H_2O_2), alkyl hydroperoxides, hydroxyl radicals and superoxide anion, which are capable of damaging proteins, DNA and lipids (Chae *et al.*, 1994; Park *et al.*, 2000). A novel

family of peroxidases, known as peroxiredoxins, have been discovered that catalyze the reduction of H_2O_2 (Chae *et al.*, 1994). Peroxiredoxins are ubiquitously expressed proteins found in organisms ranging from bacteria through eukaryotic cells and are thought to be active peroxidases supported by thioredoxin and possibly other electron donors (Chae *et al.*, 1994). Yeast peroxiredoxin knockout cell lines exhibit hypersensitivity to oxidative and nitrosative stresses associated with exposure to H_2O_2 or peroxinitrite (Wong *et al.*, 2002; Wong *et al.*, 2004). Purified peroxiredoxin enzymes lacked redox cofactors such as metals or prosthetic groups, which is in dramatic contrast to other peroxidases, thioredoxin reductase and OYE. Instead, peroxiredoxins reduce hydroperoxides using disulphur-reducing compounds as electron donors and are thus dependant on conserved cysteine residue for reductase activity. The peroxiredoxin superfamily comprises two subgroups which are classified according to the presence of one or two conserved cysteine residues and are named 1-Cys and 2-Cys peroxiredoxins accordingly.

Yeast have 5 peroxiredoxin isoforms (Wong *et al.*, 2002). Thiol-specific antioxidant protein (TSA) 1 was the first discovered and shown to be the major thioredoxin peroxidase in the cytosol (Chae *et al.*, 1994). It has been suggest that Tsa1p is required for the transcriptional induction of other components of the thioredoxin system in response to H_2O_2 (Ross *et al.*, 2000). More recently a 1-Cys type peroxiredoxin (Prx1p) with thioredoxin peroxidase activity was localized to the mitochondria (Pedrajas *et al.*, 2000). This ~29.5 kDa protein functions as part of the previously characterized mitochondrial thioredoxin system (Pedrajas *et al.*, 1999) that included thioredoxin Trx3p and thioredoxin reductase Trr2p. The reaction mechanism for this system is believed to

be comparable to the cytosolic cascade (Chae *et al.*, 1994) in which NADPH reduces thioredoxin reductase-bound FAD to $FADH_2$ which in turn reduces the conserved disulphide bond via an intraprotein hydride transfer. The reduced reductase then reduces a disulphide bond on thioredoxin. The reduced thioredoxin then interacts with and reduces a peroxiredoxin such as thioredoxin peroxidase. This is the first example of 1-Cys Prx that has thioredoxin peroxidase activity. Research conducted in our laboratory suggests that Prx1p may be responsible for reducing diacetyl within the mitochondria, which may be formed as a result of amino acid biosynthesis (Park *et al.*, 1995).

2.4.6 Other diacetyl reductase enzymes

There is currently no *Saccharomyces cerevisiae* enzyme designated as diacetyl reductase although several authors refer to unidentified enzymes possessing diacetyl reduction ability as being a diacetyl reductase (Gupta *et al.*, 1973; Tolls *et al.*, 1970). Several enzymes from a variety of organisms (Bernardo *et al.*, 1984; Blomqvist *et al.*, 1993; Branen and Keenan, 1970; González *et al.*, 1988; Ishikura *et al.*, 2001; Nakagawa *et al.*, 2002; Sarmiento and Burgos, 1982; Sawada *et al.*, 1985; Silber *et al.*, 1974; Strecker and Harary, 1954; Verduyn *et al.*, 1988a; Verduyn *et al.*, 1988b; Vidal *et al.*, 1988) have been implicated in catalyzing the reduction of diacetyl to 3-hydroxy-2-butanone (acetoin) or to 2,3-butanediol. The general reaction scheme is shown in Figure 2.8.

Two enzymes capable of catalyzing the reduction of diacetyl and other diketones have been purified from bakers yeast (Heidlas and Tressl, 1990). The purified proteins where not, however, molecularly identified. One enzyme appeared to be a monomer with

an apparent molecular mass of 36 kDa and displayed an apparent K_m of 2 mM for both diacetyl and pentanedione. Other substrates utilised by this enzyme included 2,3-hexanedione, 1,2-cyclohexanedione, 2-oxo aldehydes and short chain 2- and 3-oxo esters. The other enzyme was reported to consist of two subunits, with molecular masses of 48 kDa and 24 kDa and had an apparent K_m of 2.3 mM and 1.5 mM for diacetyl and pentanedione respectively. The 2 subunit enzyme was able to accept a broad range of substrates, including 2,3-, 2,4- and 2,5-diketones, 2-oxo aldehydes, 1,2-cyclohexanedione, methyl ketones as well as 3-, 4- and 5-oxo esters.

Purification of diacetyl reductase activity from polyploid brewing yeast has been attempted (Legeay *et al.*, 1989). The results contrast those from bakers yeast (Heidlas and Tressl, 1990) and suggested two proteins with molecular masses of 150kDa and 120kDa for the two different strains studied. Apparent K_m values with diacetyl as substrate were reported as 35 and 135 mM for the two proteins respectively. The enzymes are also reported to require NADH as cofactor rather than NADPH.

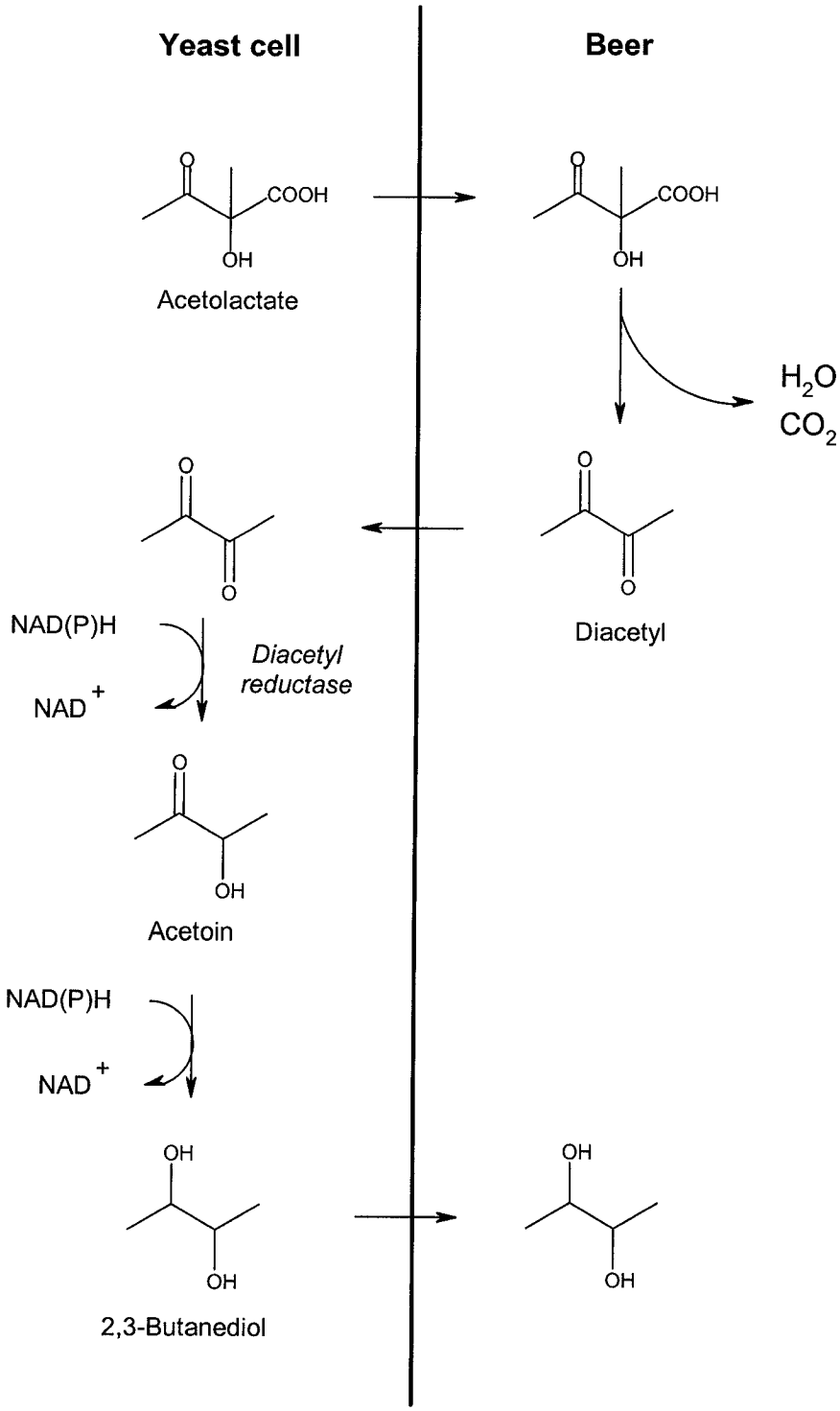

Figure 2.8 Schematic of proposed diacetyl reduction by yeast in beer fermentations.

It has previously been suggested that yeast alcohol dehydrogenase 1 (Adh1p) is capable of reducing diacetyl and pentanedione (Hardwick *et al.*, 1976) with an apparent K_m of 250 mM. This value is extraordinarily high and it is unlikely that Adh1p would catalyze this reduction efficiently at physiological concentrations. Diacetyl concentrations during peak evolution are reported to be in the low micromolar (mid parts per billion) range (García *et al.*, 1993; Landaud *et al.*, 1998; Petersen *et al.*, 2004; Trelea *et al.*, 2002). Yeast Adh1p was first cloned in 1982 (Bennetzen and Hall, 1982) after which definitive experimentation could be performed to determine substrate specificity. It has been reported that the commercially available Adh1p at the time (1976) differed in amino acid sequence from the cloned version (Bennetzen and Hall, 1982) which could affect catalytic ability (Reid and Fewson, 1994). Several other alcohol dehydrogenases have been discovered and characterized in various yeasts (Denis, 1984; Donoviel and Young, 1996; Dorsey *et al.*, 1993; Drewke and Ciriacy, 1988; Ganzhorn *et al.*, 1987; Karnitz *et al.*, 1992; Passoth *et al.*, 1998; Pessione *et al.*, 1990; Reid and Fewson, 1994; Shain *et al.*, 1992; Wales and Fewson, 1994). In a comparative study between bacterial and yeast ADH enzymes, it was found that the native yeast enzyme was a 43.5 kDa monomer (Wales and Fewson, 1994). Isoenzymes Adh1p, 2p and 3p in *Saccharomyces cerevisiae* require NADH as a cofactor (Reid and Fewson, 1994) as does Adh4p (Drewke and Ciriacy, 1988). Kinetic studies performed with Adh4p (Drewke and Ciriacy, 1988) showed catalytic activity with ethanol and *n*-propanol, but little activity for other alcohols. No data was presented for reactivity with diacetyl or other diketones.

A stereospecific (2R,3R)-2,3-butanediol dehydrogenase enzyme (BDH) has been identified (González *et al.*, 2001) that is capable of catalyzing the conversion of butanediol to acetoin. The native enzyme is predicted to be a homodimer with 41.5 kDa subunits, contains a zinc cofactor, specifically used NAD(H), and displays similarity to several yeast alcohol dehydrogenase enzymes. The BDH reverse reaction was reported to utilize action and NADH as the best substrates, followed by 2,3-butanedione (diacetyl) and 1-hydroxy-2-propanone. The enzyme was also able to accept hydroxyacetone, methylglyoxal, dihydroxyacetone and 2,3-pentanedione.

The possibility exists that other proteins may perform diacetyl reduction activities. Aldo-keto reductases are known to catalyze the NADPH dependant reduction of carbonyl containing compounds to their corresponding alcohols (Hur and Wilson, 2000; Petrash *et al.*, 2001; Rodríguez *et al.*, 2001). It was recently reported that YPR1, an aldo-keto reductase was capable of catalyzing diacetyl reduction (Ford and Ellis, 2002). The reaction rates were much lower than with nitrobenzaldehyde or the reported preferred physiological substrates hexanal, methylglyoxal and glyceraldehyde. The apparent K_m and k_{cat} values for diacetyl were reported as 6.1 ± 0.9 mM and 5.7 ± 0.5 s^{-1} respectively. Assays were performed at 25°C and pH 5.4. The apparent K_m is close to 5.7 mM that was previously reported for another yeast aldo-keto reductase, GRE3 (Nakamura *et al.*, 1997). A maximum velocity (V_{max}) of 10.7 U mg^{-1} was reported in 100 mM phosphate buffer at pH 7.0 and 30 °C, with 100 μM NADPH. Other aldo-keto reductases include GCY1 and ARA1 reviewed in section 2.4.4.

A small 10 kDa, NADPH specific enzyme purified from *E.coli* was reported to have apparent K_m values of 4.4 mM and 20 μM with diacetyl and NADPH respectively,

at 25°C and pH 7.0 (Silber *et al.*, 1974). The enzyme reportedly reduced diacetyl to acetoin but did not catalyze further reduction to butanediol. An enzyme possessing diacetyl reductase activity was recently purified from *Leuconostoc pseudomesenteroides* (Rattray *et al.*, 2000) and cloned (Rattray *et al.*, 2003). The enzyme had a molecular mass of 30 kDa and was found to catalyze the irreversible reduction of diacetyl to acetoin with specificity for NADH, while the reversible reduction of acetoin to 2,3-butanediol utilized either NADH or NADPH. The apparent K_m values for diacetyl and pentanedione were 5.1 and 5.6 mM respectively, with corresponding V_{max} values of 333 and 373 μmol min^{-1} mg^{-1}.

Recent attempts to study diacetyl reductase activity in ale (*S. cerevisiae*) and lager yeasts (*S. pastorianus*) suggested two major enzymes with activity in lager yeast and one in ale yeast (Bamforth and Kanauchi, 2004). 2,3-pentanedione activity was 3 times higher than diacetyl activity in lager yeast as opposed to ale yeasts where they were comparable. It was speculated that the additional pentanedione activity was due to alcohol dehydrogenase, although no protein identification was performed. Molecular masses were roughly 60 kDa and 20 kDa. Ale yeast enzyme kinetic studies yielded apparent K_m values of 79 mM and 45 mM for diacetyl and pentanedione respectively, while lager yeast values were 15 mM and 38 mM respectively.

It should be noted that apart from the plethora of potential diacetyl reductase enzymes, which are summarized in table 2.1, experimentation has been performed, in many cases, on crude cell lysate or partially purified fractions, as was the case with the results presented by (Bamforth and Kanauchi, 2004). In many cases, diacetyl reductase activity could, therefore, be considered the sum of activities of numerous reductase

enzymes. This makes it impossible to determine the activity in relation to specific protein concentration. It has been shown that diacetyl reductase activity can vary from strain to strain (Murphy *et al.*, 1996) but it is also likely that activity may vary during a fermentation as proteins are produced and degraded in response to environmental conditions. This issue has not been addressed.

Table 2.1 Summary of enzymes known to have diacetyl reductase activity.

Organism	Enzyme	Size (kDa)	Donor	Diacetyl		Pentanedione		Conditions		Reference
				K_m	V_{max}	K_m	V_{max}	Temp	pH	
S. cerevisiae	Diacetyl reductase	36	NADPH	2 mM		2 mM		25°C	6.9	(Heidlas and Tressl, 1990)
S. cerevisiae	Diacetyl reductase	48 + 24	NADPH	2.3 mM		1.5 mM		25°C	6.4	(Heidlas and Tressl, 1990)
S. carlsbergensis	Diacetyl reductase	150	NADH	35 mM				40°C	7.0	(Legeay et al., 1989)
S. carlsbergensis	Diacetyl reductase	120	NADH	135 mM				36°C	7.5	(Legeay et al., 1989)
S. cerevisiae	ADH1	37	NADH	250 mM					6.5	(Hardwick et al., 1976)
S. cerevisiae	BDH	2 x 41	NADH	Not given				25°C	7.0	(González et al., 2001)
S. cerevisiae	Diacetyl reductase	20	NADH	79 mM	0.4 mU/ min	45 mM	0.09 mU/ min	25°C	6.5	(Bamforth and Kanauchi, 2004)
S. pastorianus	Diacetyl reductase	60 + 20	NADH	15 mM	2.56 mU/ min	38 mM	1.43 mU/ min	25°C	6.5	(Bamforth and Kanauchi, 2004)
S. cerevisiae	GRE3	32	NADPH	5.7 mM				30°C	7.0	(Nakamura et al., 1997)
S. cerevisiae	YPR1	37	NADPH	6.13 mM				25°C	5.4	(Ford and Ellis, 2002)
S. cerevisiae	OYE2	44	NADPH	Not given				35°C	6.5	(Sánchez et al., 2003)
H. polymorpha	Dihydroxy-acetone reductase		NADH	5.4 mM				37°C	6.0	(Verduyn et al., 1988b)
H. polymorpha	Acetone reductase		NADH	4.3 mM				37°C	6.0	(Verduyn et al., 1988b)
C. utilis	Butanediol dehydrogenase		NADH	4.3 mM				30°C	6.0	(Verduyn et al., 1988a)
L. pseudo-mesenteroides	Diacetyl reductase	3 x 27	NADH	5.1 mM		5.6 mM		30°C	5.5	(Rattray et al., 2000)
S. aureus	α-diketone reductase	68	NADH	15 mM		6 mM		25°C	6.4	(Vidal et al., 1988)
Beef liver	Diacetyl reductase	76	NADH	40 μM				25°C	6.1	(Sarmiento and Burgos, 1982)
Pigeon liver	Diacetyl reductase	110	NADH	3.1 mM				25°C	6.1	(Sarmiento and Burgos, 1982)
E. coli	Diacetyl reductase	10	NADPH	4.4 mM				25°C	6.1	(Silber et al., 1974)

2.5 Recombinant protein manufacture

The development and implementation of a protein-based biosensor requires the production of recombinant proteins. Initially, small quantities are required for protein characterization and experimental development. Successful implementation of a mass-market device, however, requires the scaling up and optimization of protein production in order to produce sufficient quantities in a cost effective manner.

The Gram-negative *Escherichia coli* bacteria is the most widely used host for the production of recombinant proteins (Åkesson, 1998) in part because its genetics have been so well categorized (Lee, 1996). Other reasons for its widespread use include its ability to grow rapidly, using inexpensive substrates while achieving high cell densities (Baneyx, 1999). *E. coli* is not, however, suitable for the production of all proteins, particularly many from higher eukaryotic organisms, such as humans. This is mainly due to the bacteria's inability to perform many of the posttranslational modifications that may be required for activity, such as glycosylation, as well as limited ability to facilitate the formation of extensive disulphide bonds (Lee, 1996; Makrides, 1996). Proteins that retain their activity without posttranslational modification, or that do not undergo modification are suitable candidates for recombinant production. Many have been produced, such as human growth hormone (Chang *et al.*, 1987), bioadhesives (Wong *et al.*, 1998), biodegradable plastics (Wang and Lee, 1998), lycopene (Alper *et al.*, 2005) and proteins native to the parasitic organisms *Schistosoma mansoni* and *Trypanosoma brucei* (Craig *et al.*, 1991).

The production of recombinant proteins is often achieved with the aid of plasmid vectors (Makrides, 1996) which allow for the convenient insertion of a gene of interest as well as a gene to aid in selection of positively transformed clones. Selection is commonly achieved through the use of plasmid encoded antibiotic resistance (Baneyx, 1999) whereby growth media supplemented with the antibiotic of choice kills cells that do not harbour the plasmid. Positive selection pressure serves to ensure that only cells that maintain the plasmid will remain in the culture.

There are numerous strategies that can be used to increase protein production. These include manipulation of transcriptional regulation, translational regulation and targeting of protein production to the cytoplasm, periplasmic environment or secretion to the media (Makrides, 1996). Of particular importance is the selection of a suitable promoter for expression and compartment targeting whereby the protein is targeted to the cytoplasm or elsewhere. Overproduction of recombinant protein, which normally accounts for between 10 and 50% of the total cell protein (Makrides, 1996), is often accompanied by partially folded or misfolded protein, followed by segregation into insoluble inclusion bodies (Baneyx, 1999), which can reduce the protein yield (Hoffmann *et al.*, 2001). While these bodies contain recombinant protein at a high level of purity, efforts required to renature the protein can often overshadow the ease of purification. It is often desirable to ensure that the protein remains soluble. This can be achieved by slowing down the specific productivity (Lee, 1996) allowing more time for proper folding to take place (Baneyx, 1999).

Molecular strategies directed towards the achievement of high level expression have been reviewed elsewhere in great detail (Makrides, 1996) and have been effectively

used in practice for increasing product formation 8.5-fold relative to the wild-type strain used for modification (Alper *et al.*, 2005). Significant amounts of research effort have focused on high cell-density culture (HCDC) as a means of increasing the cell density, and hence product yield per unit volume (Lee, 1996; Riesenberg, 1991; Yamanè and Shimizu, 1984). HCDC is commonly achieved using fed-batch systems (Yamanè and Shimizu, 1984), during which concentrated nutrients are fed into a reactor in a controlled manner (Lee and Ramirez, 1994; Lee, 1996; Rocha and Ferreira, 2002; Wong *et al.*, 1998; Yang, 1992), in order to prevent the accumulation of metabolic by-products, such as acetate, which inhibit cell growth and product synthesis (Han *et al.*, 1992). The formation of acetate during aerobic growth in *E. coli* is known as glucose overflow metabolism and has been modeled (Xu *et al.*, 1999). It is a similar phenomenon to ethanol production observed in yeast during aerobic growth in high sugar concentrations. Glucose uptake and utilization has been well studied in *E. coli* systems (Lin and Neubauer, 2000; Lin *et al.*, 2001; Natarajan and Srienc, 2000; Picon *et al.*, 2005) and the production of excess acetate can be avoided or reduced through the use of proper feeding strategies (Åkesson, 1998) and genetic manipulation (Farmer and Liao, 1997). Some of the control strategies that have been successfully employed include dissolved oxygen control (Konstantinov *et al.*, 1990; Riesenberg *et al.*, 1991), pH-stat (Suzuki *et al.*, 1990), glucose limitation (Knorre *et al.*, 1991), glucose oscillations (Lin and Neubauer, 2000) or combinations of these factors (Kim *et al.*, 2004). With the use of these control strategies, 50 grams of dry cell weight (DCW) per litre are routinely obtained for *E. coli* cultures (Lee, 1996).

While many fermentations have been performed in complex media, there is a growing trend towards the use of chemically defined media for certain industrial fermentations (Zhang and Greasham, 1999). Defined medium is composed of pure chemicals in precisely known proportions and has been used for many years for microbial biochemical studies where it is necessary to achieve reproducible results, with minimal interactions with the medium (Neidhardt *et al.*, 1974; Zhang and Greasham, 1999). Advantages of using chemically defined media include enhanced process consistency, improved control, monitoring and scale up as well as the elimination of negative phosphate regulation, which commonly occurs in secondary metabolite fermentations using complex media (Zhang and Greasham, 1999). A disadvantage, however, is that acetate accumulation can occur at lower growth rates than in complex media (Meyer *et al.*, 1984). Chemically defined medium has been successfully used in numerous *E. coli* fed-batch fermentations for the production of recombinant products (Alper *et al.*, 2005; Riesenberg *et al.*, 1991; Wang and Lee, 1998; Wong *et al.*, 1998).

Quite often, protein expression techniques rely on the use of isopropyl-β-D-thiogalactopyranoside (IPTG) or lactose to bind to and inactivate the *lac* operon repressor, inducing *lacZ* gene expression, which is then coupled to expression of the recombinant product of interest. An interesting, but little used expression promoter is the bacterial alkaline phosphatase (*pho*A) promoter. While other high level expression promoters often lead to insoluble, inactive or toxic protein levels within the cells, vectors containing the *pho*A promoter have been successfully used to produce satisfactory quantities of active, soluble protein where other promoters have failed (Craig *et al.*, 1991). This promoter has been used by others (Jardim *et al.*, 2000) to successfully

produce several eukaryotic proteins. *pho*A forms part of a larger group of genes known as the Pho regulon, which are controlled by external inorganic phosphate (P_i) concentrations. During P_i starvation, the phosphate starvation response is invoked, triggering a phosphorylation cascade and leading to the secretion of alkaline phosphatase into the periplasmic space to release orthophosphate from organophosphate compounds. *pho*A codes for alkaline phosphatase and is induced after P_i drops below 10 μM (Van Dien and Keasling, 1998). Controlled expression of *pho*A promoter systems has been achieved using low phosphate induction (LPI) media, which is a modified formulation of the chemically defined MOPS salts based enterobacteria media previously developed (Neidhardt *et al.*, 1974), containing no potassium phosphate (Craig *et al.*, 1991). A problem, however, with LPI media is that despite an excellent active protein to total cell protein ratio, cultures only grow to a low cell density. The result is an overall low net protein yield. Given the attractiveness of the *pho*A promoter expression system, an effort to boost the cell yield in LPI media would likely result in a viable alternative HCDC system for commercial production of recombinant proteins (Chapter 5).

2.6 Using proteins for detecting and quantifying compounds

The issues of safety and quality control in food and beverages remain prominent concerns for both consumers and manufacturers. As a result, manufacturer testing for pathogenic organisms, toxic compounds, contaminants and flavour defects is necessary. Routine testing for these compounds often relies on complex and expensive instrumentation such as gas chromatography (GC) or high performance liquid chromatography (HPLC)

requiring highly skilled technical operators in order to ensure accuracy and reproducibility in results (Verma and Singh, 2003).

With the advent of molecular biology and the ability to produce recombinant, biologically active proteins and enzymes in abundant amounts, it is now possible to develop novel detection methods based on biosensors, which measure specific compounds based on molecular recognition or specific enzymatic reactions. Biosensors have an advantage in that proteins or enzymes are highly specific to certain compounds, a factor that eliminates many false positive results due to contaminating components. Many of the sensors that have been developed are based on immobilised, naturally occurring or recombinant enzymes (Dattelbaum *et al.*, 2005; Tsai and Doong, 2005; Zhang *et al.*, 1999), immunoassays (Shriver-Lake *et al.*, 2004), optical methods (Guenter, 2005) or even intact microbial cells (Dollard and Billard, 2003; Verma and Singh, 2003). These techniques often require the use of expensive instrumentation, such as specialised microscopes, or components that are difficult or very expensive to manufacture in large quantities, such as antibodies, thus limiting mass technology roll-outs. Problems that exist with protein based biosensors are often related to shelf life and life-span of the protein or probe, the later of which is often affected by fouling of the probe tip. Research is ongoing in attempts to resolve this issue in various systems (Bartlett *et al.*, 2002; Wilson and Rauh, 2004).

Flavour compounds and their quantification in food and beverage products are often of great importance in ensuring quality standards. Deviations from specification can result in pronounced off-flavours and consumer unacceptability. Examples of this are most often found in fermented foods where flavour development is directly related to

microbial action. An appropriate example is diacetyl formation by yeast during alcoholic fermentation and by bacteria during dairy product manufacture. In alcoholic beverages, such as beer, the presence of detectable amounts of diacetyl is often undesired (Petersen *et al.*, 2004), as opposed to dairy products, such as butter, buttermilk and yoghurt, where diacetyl is considered to be one of the most important flavour compounds (Curic *et al.*, 1999). The production of sufficient levels, or reduction to below threshold levels, is often the rate determining step in product manufacture, dictating how long a product remains in a fermentation vessel prior to downstream processing. Accurate quantification of diacetyl is therefore important to ensure efficient plant management. Currently, diacetyl is routinely measured using gas chromatography (Buckee and Mundy, 1994; Rankin, 2001). The instruments used for these purposes are sensitive and costly, leading to an exclusion of smaller manufacturers and the reliance on highly skilled technical staff for accurate and reproducible results. A more cost effective, reliable and accurate device that provides rapid results would benefit many manufacturers and lead to improved product quality.

Recently, OYE enzymes were identified, overexpressed in large amounts in a biologically active state and kinetically characterised with diacetyl as substrate (van Bergen *et al.*, 2006a). Three known isoforms have been identified (Niino *et al.*, 1995) and these enzymes are amongst the best characterized in yeast. Despite the fact that these enzymes have been studied for decades, only recently has a potential biological role been described (Haarer and Amberg, 2004; Williams and Bruce, 2002). Previous experiments with OYE isoforms have shown that this family of flavoenzymes is capable of reducing a variety of ketone compounds (Sánchez *et al.*, 2003; Vaz *et al.*, 1995). It has also been found that sterol compounds are capable of inhibiting catalytic activity at low

concentrations (Vaz *et al.*, 1995). These biophysical characteristics of the OYE isoforms may be exploited, for example, in the detection and quantification of VDKs in food and beverages but possibly for the detection of hormones in food products as well.

Introductory connecting text

When initial plans were laid out to model metabolic flux in diacetyl production and reduction pathways in brewing yeast, it became clear that the enzymes responsible for diacetyl reduction where unknown. Furthermore, the possibility existed that more than one enzyme was involved. It was therefore essential to identify these enzymes and characterise them with the vicinal diketone (VDK) substrates diacetyl and pentanedione. In order to proceed with characterisation, enzymes showing diacetyl reductase activity were initially purified from brewing yeast harvested at the end of primary fermentation. Yeast cells were lysed in a glass bead mill by resuspending cells with glass beads in cell lysis buffer. Lysates were centrifuged to remove beads, intact cells, and cell debris and DNA precipitated and removed by centrifugation. The diacetyl reductase activity was differentially precipitated by progressive addition of $(NH_4)_2SO_4$ and the protein precipitate harvested by centrifugation. Protein pellets were resuspended in buffer and assayed for activity.

The 80% $(NH_4)_2SO_4$ pellet, containing the bulk of the diacetyl reductase activity, was applied to an octyl sepharose column and developed using a linear $(NH_4)_2SO_4$ gradient (Fig. CT1A). Fractions containing diacetyl reductase activity were pooled, dialysed and then loaded onto an Active Red affinity column. The Active Red column was developed with a linear NaCl gradient (Fig. CT1B). Fractions containing diacetyl reductase activity were pooled, dialysed, applied to a DEAE Sepharose CL-6B column

and developed with a linear NaCl gradient (Fig. CT1C). Diacetyl reductase containing fractions were pooled and assessed for purity by silver stained SDS-PAGE.

The purified protein was identified as Old Yellow Enzyme (OYE) using Matrix Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometry. This was the first protein identified with diacetyl reductase activity in this project and formed the starting point for the papers that follow.

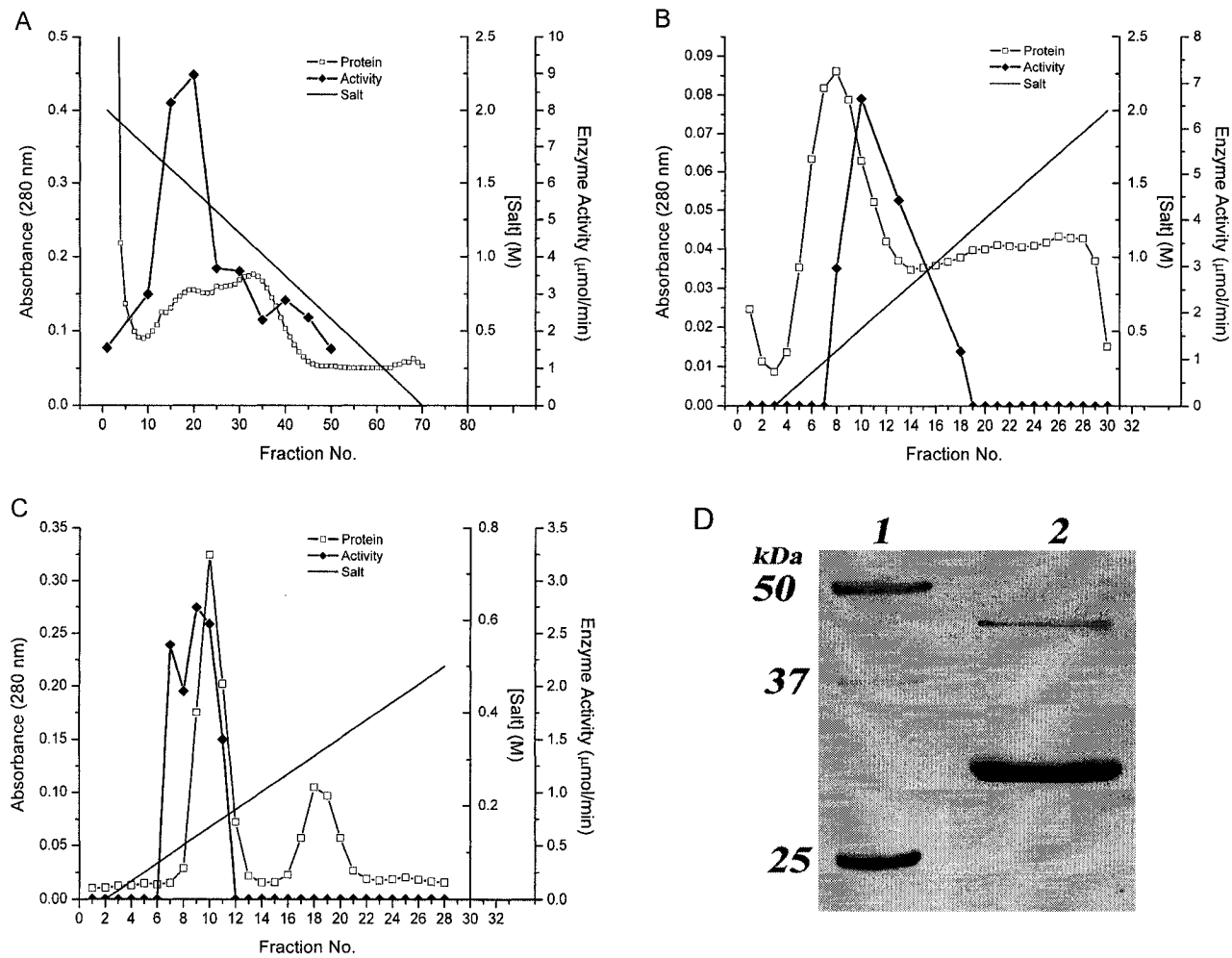

Figure CT1. Purification of diacetyl reductase activity from brewing yeast. (A) Protein elution and activity profiles of diacetyl reductase from brewers yeast eluted from an octyl sepharose hydrophobic interaction column using a 2 M to 0 M $(NH_4)_2SO_4$ linear gradient. (B) Chromatography of diacetyl reductase containing fractions from the octyl sepharose column on an Active Red affinity column. The column was developed with a linear 0 to 2 M NaCl gradient. Fractions were collected and assayed for protein content and diacetyl reductase activity. (C) Protein elution and activity from brewers yeast cell extract eluted from a DEAE sepharose CL-6B ion exchange column using a 0 to 0.5 M NaCl gradient.

(D) Silver stained SDS-PAGE analysis of native diacetyl reductase activity isolated from the DEAE sepharose column, (lane 1) molecular weight markers and (lane 2) diacetyl reductase active fraction.

3. Old Yellow Enzyme Catalyzes Diacetyl Reduction in *Saccharomyces* Yeast

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3.1 Abstract

A diacetyl (2,3-butanedione) reducing enzyme was purified from *Saccharomyces* brewing strains and identified by MALDI-TOF mass spectrometry to be Old Yellow Enzyme (OYE). Three OYE isoforms were expressed in *E. coli*, purified, and kinetically characterized using the substrates diacetyl, pentanedione, phenylglyoxal, and 1,2-cyclohexanedione. Of the three yeast isoforms (Oye1p, Oye2p and Oye3p), Oye1p was most efficient at reducing diacetyl, while Oye2p preferentially utilized pentanedione as a substrate. All OYE isoforms catalyzed the single step reduction of diacetyl and pentanedione to acetoin and 3-hydroxy-2-pentanone. Oye3p showed no detectable activity with low vicinal diketone concentrations. Of interest was the observation that OYE enzymes exhibited biphasic kinetic behaviour with α,β-dicarbonyl substrates, compounds known to modify proteins at arginine residues. At high dicarbonyl concentration OYE exhibited an increase in enzymatic activity, providing a mechanism by which cells can rapidly up regulate enzymatic activity during dicarbonyl stress.

3.2 Introduction

α,β-Dicarbonyls such as methylglyoxal and diacetyl are metabolic byproducts produced largely through non-enzymatic reactions that include the Maillard reaction, β-elimination of triose phosphate (Cooper, 1984), and oxidative decarboxylation of α-acetolactic acid, an intermediate in the isoleucine, leucine, valine (ILV) branched amino acid biosynthetic pathway (Holmberg and Petersen, 1988). In *Saccharomyces*, diacetyl production is tightly associated with branched amino acid biosynthetic activity (Dillemans *et al.*, 1987; Gjermansen *et al.*, 1988; Mithieux and Weiss, 1995; Petersen *et al.*, 2004) and the amount of free amino acids available to the cells (Hinnebusch, 1986a; Holmberg and Petersen, 1988; Magee and Hereford, 1969). These toxic dicarbonyl compounds react preferentially with proteins, modifying arginine, lysine, and cysteine residues, leading to protein damage that has been linked to a number of chronic and aging diseases (Lundblad, 1995; Oya *et al.*, 1999) that include diabetic vascular complications (Brownlee, 1995), neurodegenerative diseases (Kovacic and Cooksy, 2005), atherosclerosis, and general cellular deterioration and aging (Wondrak *et al.*, 2002). Several proteins that have been shown to be susceptible to chemical modification, include ovalbumin (Hipkiss and Chana, 1998), plasminogen (Lerant *et al.*, 2000), glyceraldehyde phosphate dehydrogenase (Morgan *et al.*, 2002), hsp27 (Schalkwijk *et al.*, 2006) and corepressor mSin3A (Yao *et al.*, 2006). Removal of these dicarbonyl compounds is therefore essential for maintenance of cellular health.

Glyoxalase I and II are a two enzyme system that utilize glutathione in a sequential reaction to detoxify methylglyoxal to lactate (Aguilera and Prieto, 2004). In contrast, little is known about the enzymatic mechanisms responsible for the elimination

of diacetyl. Diacetyl is thought to be sequentially reduced in a two step reaction to 3-hydroxy-2-butanone (acetoin) and then 2,3-butanediol by two separate enzymes that use either NADH or NADPH as a hydride donor (Gabriel *et al.*, 1971) (Fig. 3.1). This assertion is strengthened by the identification and characterization of the NADH requiring enzyme 2,3-butanediol dehydrogenase (González *et al.*, 2001) that catalyzes the conversion of acetoin to 2,3-butanediol. Gene disruption studies have suggested that in addition to butanediol dehydrogenase, it is likely that *Saccharomyces spp.* have other enzymes capable of catalyzing 2,3-butanediol production, as elimination of the butanediol dehydrogenase gene did not result in the accumulation of acetoin (González *et al.*, 2001).

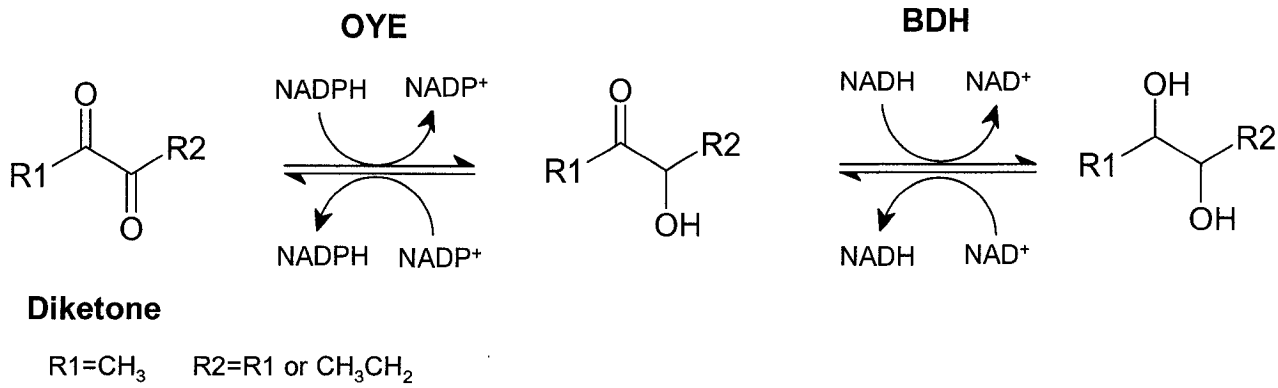


Figure 3.1. Reaction scheme for enzymatic reduction of vicinal diketones. The vicinal diketones diacetyl (2,3-butanedione) and 2,3-pentanedione are sequentially reduced in two separate NADPH/NADH dependent reactions to acetoin and 2,3-butanediol by Old Yellow Enzyme (OYE) and butanediol dehydrogenase (BDH).

In addition to being toxic to cells, vicinal dicarbonyls have a significant economic impact. In fermentation, for example, the production of diacetyl as a metabolic byproduct

negatively affects certain beverages, such as beer, by contributing an undesirable butterscotch-like aroma and flavor characteristic that can be detected to at very low concentrations. The levels of this compound are naturally reduced by the yeast during maturation or aging of the fermented beer. Extensive studies have focused on the characterisation of yeast metabolic pathways linked to diacetyl production and its subsequent reduction to 2, 3-hydroxybutanone (acetoin); a compound that has a much higher flavor threshold (Bamforth and Kanauchi, 2004; Dillemans *et al.*, 1987; Gjermansen *et al.*, 1988; Hardwick *et al.*, 1976; Legeay *et al.*, 1989; Mithieux and Weiss, 1995; Tolls *et al.*, 1970; Wainwright, 1973).

Yeast alcohol dehydrogenase (ADH) has been previously postulated to be the primary enzyme responsible for reducing diacetyl (Bamforth and Kanauchi, 2004; Hardwick *et al.*, 1976), but kinetic analysis has revealed that ADH has a K_m value of 250 mM for diacetyl, suggesting that ADH would be highly inefficient in reducing diacetyl under physiological conditions, as concentrations of diacetyl measured in fermentation streams range from ~ 7.5 to 12 μM (Petersen *et al.*, 2004; Trelea *et al.*, 2002). More recently, biochemical studies identified two diacetyl reductase activities with K_m values of 2.0 and 2.3 mM in bakers' yeast (Heidlas and Tressl, 1990), however these enzymes were not identified.

In this study we have purified enzymes displaying diacetyl reductase activity from *S. cerevisiae* and *S. pastorianus* yeasts. The enzymes were identified by MALDI-TOF and tandem mass spectrometry to be the Old Yellow Enzyme (OYE). The OYE enzymes were over expressed in *E. coli* and kinetically assayed for diacetyl and pentanedione reductase activity.

3.3 Experimental procedures

3.3.1 Chemicals and substrates

All chemicals, substrates and reagents were obtained from either Fisher Scientific (Ottawa, ON, Canada) or Sigma-Aldrich (Oakville, ON, Canada) unless otherwise specified. Oligonucleotide primers were obtained from Qiagen (Mississauga, ON, Canada). Restriction endonucleases and Platinum Pfx DNA polymerase were purchased from Invitrogen (Burlington, Canada). Acetoin was purchased from SigmaAldrich as the crystalline dimeric form, which was converted to the monomeric form by melting the crystals at 95 °C and diluting in assay buffers immediately prior to use.

3.3.2 Reductase activity

Diacetyl or pentanedione reductase activity was measured spectrophotometrically on a Beckman DU-640 spectrophotometer at 340 nm at 25 °C in 100 mM sodium phosphate at pH 7.0 containing 4 mM diacetyl and 100 μM NADPH or NADH in a 1.0 ml reaction. For kinetic analysis, reaction mixtures were supplemented with 10 mM glucose and 10U of glucose oxidase to deplete reactions of residual oxygen that could act as a non-specific hydride acceptor (Schopfer and Massey, 1991). The K_m values for diacetyl, pentanedione and phenylglyoxal were determined by varying the concentration of the vicinal diketones from 1 μM to 128 mM at a fixed NADPH concentration of 200 μM. The NADPH Km was determined by varying the concentration from 1 μM to 200 μM using diacetyl and pentanedione as the second substrate at a concentration of 34 or 200 μM. The effect of pH on enzymatic activity was assessed using a universal reaction buffer containing 50

mM acetic acid, 50 mM Na_2HPO_4 and 50 mM Tris base and the pH was adjusted from 3.5 to 10 using HCl or NaOH.

3.3.3 Old Yellow Enzyme purification

Diacetyl reductase activity was purified from ale and lager yeast cultures obtained from Molson Breweries at the end of fermentation. Yeast cells were lysed in a glass bead mill by resuspending 50 g of cells and 150 g of glass beads (400 to 600 μm) in 180 ml cell lysis buffer containing 50 mM Tris pH 7.2, 5 mM β-mercaptoethanol (β-ME) and 2 μg/ml phenylmethyl sulfonyl fluoride (PMSF). Lysates were centrifuged at 3000 rpm for 10 min to remove beads, intact cells, and cell debris. DNA was precipitated by adding 5 g of streptomycin sulphate and mixing for 6 h at 4°C, followed by centrifugation at 10,000 x g for 10 min. The diacetyl reductase activity was differentially precipitated by progressive addition of $(NH_4)_2SO_4$ to 20, 40, 60 and 80% saturation. At each concentration samples were stirred for 20 min at 4°C and the protein precipitate harvested by centrifugation at 10,000 x g for 15 min. Protein pellets were resuspended in 25 mM sodium phosphate with 2 mM β-ME at pH 7.0 (buffer A) and assayed for activity.

The 80% $(NH_4)_2SO_4$ pellet, containing the bulk of the diacetyl reductase activity, was adjusted to 2 M $(NH_4)_2SO_4$ and applied to an octyl sepharose column (5 mm x 62 mm) equilibrated with 2 M $(NH_4)_2SO_4$ in buffer A. The column was washed with 10 column volumes of 2 M $(NH_4)_2SO_4$ and developed using a linear 350 ml 2-0 M $(NH_4)_2SO_4$ gradient. Fractions containing diacetyl reductase activity were pooled, dialysed exhaustively against 2 l of buffer A and then loaded onto an Active Red affinity

column (5 mm x 62 mm). The Active Red column was washed with 10 column volumes of buffer A and developed with a linear 150 ml 0-2 M NaCl gradient. Fractions containing diacetyl reductase activity were pooled, dialysed overnight against 2 l of buffer A, applied to a DEAE Sepharose CL-6B column (8 mm x 50 mm) (Sigma) and developed with a linear 300 ml 0-0.5 M NaCl gradient. Diacetyl reductase containing fractions were pooled and assessed for purity by silver stained SDS-PAGE.

3.3.4 Identification of diacetyl reductase

Fractions containing diacetyl reductase activity were precipitated with trichloroacetic acid and digested with porcine trypsin (Promega, Madison, WI, USA). Digests were analyzed by Matrix Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometry on a Voyager DE-STR Biospectrometry Workstation (Applied Biosystems, Foster City, CA, USA). Protein identification was confirmed by de novo sequence analysis electrospray mass spectrometry on a QStar Pulsar 1 (Applied Biosciences, Foster City, CA, USA). The Saccharomyces genome database (www.yeastgenome.org) was searched using the Mascot search algorithm. Proteomic analysis was performed at the University of Victoria Genome BC Proteomics Centre.

3.3.5 Old Yellow Enzyme expression

Open reading frames encoding Old Yellow Enzyme (OYE) isoforms 1-3 were amplified from *S. cerevisiae* or *S. pastorianus* brewing yeast DNA by polymerase chain reaction using primer pairs 5'-CATGCCATGGCATTTGTAAAAGATTTTAAGCC-3' and 5'-

CGGAATTCTTACTTTTTGTCCCAGCCTAATTTG-3' for *OYE1*, 5'-CATGCCATGGCATTTGTTAAGGACTTTAAGCC-3' and 5'-CGGAATTCTTAATTTTTGTCCCAACCGAGTTTTA-3' for *OYE2*, and 5'-CATGCCATGGCATTTGTAAAAGGTTTTGAGCC-3' and 5'-CGGAATTCTCAGTTCTTGTTCCAACCTAAATC-3' for *OYE3* (endonuclease restriction sites are underlined). The *OYE1* and *OYE2* open reading frames were amplified with Platinum *Pfx* DNA polymerase (Invitrogen, Burlington, ON) for 30 cycles of denaturation at 95 ^{0}C for 45 s, annealing 58 ^{0}C for 30 s, and extension at 68 ^{0}C for 75 s. A similar program was used to amplify *OYE3*, however, the annealing temperature was decreased to 55 ^{0}C. PCR products were digested with *Nco*I and *EcoR*V and inserted into the corresponding sites of the pET30b(+) plasmid (Novagen, CA, USA) . The integrity of the OYE sequence was confirmed by automated DNA sequence analysis. *E. coli* ER2566 (*fhuA2 lacZ::T7 gene1 [lon] ompT gal sulA11 R(mcr-73::miniTn10*--Tet^S*)2 [dcm] R(zgb-210::Tn10*--Tet^S*) endA1 Δ(mcrC-mrr)114::IS10*) (New England Biolabs, MA, USA) was transformed with pET30b(+)-*OYE1*, pET30b(+)-*OYE2* or pET30b(+)-*OYE3* constructs and cultures grown to an OD_{600} of 0.7 in Luria broth containing 100 μg/ml kanamycin. OYE expression was induced by adding isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 0.5 mM and incubating the culture with vigorous agitation at 25 °C for 6 h. Cultures were harvested and the cell pellet was resuspended in 20 ml lysis buffer containing 50 mM sodium phosphate pH 7.5, 0.5 M NaCl and 20 mM imidazole and lysed with two passes through a French press. Cell lysates were clarified by centrifugation at 38,000 x g for 30 minutes and the recombinant OYE enzymes purified using Ni^{2+}-NTA affinity chromatography (Qiagen, Mississauga, ON). The supernatant

was loaded onto the column and washed with 20 column volumes of lysis buffer followed by 20 column volumes of 50 mM sodium phosphate pH 7.5 containing 0.5 M NaCl and 40 mM imidazole. Proteins were eluted using a step gradient of 80, 160 and 320 mM imidazole. Fractions containing OYE were concentrated and desalted using an Amicon filter concentrator (Millipore, ON, Canada). Protein concentrations were determined spectrophotometrically (Gill and von Hippel, 1989).

3.3.6 Gas chromatography analysis

Enzymatic reactions containing 10 mM diacetyl, pentanedione or acetoin plus 5 mM NADPH and ~2 nmol of OYE1 were incubated at 20 °C for 4 h and the formation of acetoin and 2,3-butanediol was monitored by gas chromatography (GC). Two µl aliquots of the reaction mixtures were injected onto a Hewlett Packard 5890 Series II GC equipped with a FID detector. An HP-Innowax column (30 m x 0.250 mm ID, 0.25 µm film) with a helium carrier gas flow rate of 1.2 ml/min and split ratio of 5:1 was used. The injector and detector temperatures were 220 °C and 280 °C, respectively. Following sample injection, the column temperature was held at 35 °C for 5 min then ramped at 5 °C/min to 150 °C and 20 °C/min to 250 °C.

3.4 Results

3.4.1 Purification of native diacetyl reductase

Enzymes capable of reducing diacetyl were purified from stationary phase *S. cerevisiae* brewer's yeast grown under anaerobic conditions using a modification of a previously reported strategy (Heidlas and Tressl, 1990) that employed a combination octyl sepharose, active red affinity, and DEAE sepharose. Silver-stained SDS PAGE analysis of the purified diacetyl reductase preparation following the final chromatographic step on a DEAE column revealed two bands with molecular weight of 45 kDa and 30 kDa (Fig. 3.2). This pattern was similar to the preparation reported by Heidlas and Tressl (Heidlas and Tressl, 1990). Mass fingerprint analysis using MALDI-TOF identified the 45 kDa protein as Old Yellow Enzyme (OYE) isoform 2; with a Mowse score of 59 and 22% coverage when the Saccharomyces database was searched using the MASCOT algorithm with an error of 150 ppm. These results were consistent with the biochemical data showing that the identified diacetyl reductase was an NADH/NADPH requiring enzyme. BLAST searches of the National Center for Biotechnology Information and *S. cerevisiae* yeast genome databases identified three OYE isoforms exhibiting a sequence identity of 92% for OYE1 and OYE2, 80% for OYE1 and OYE3 and 82% for OYE2 and OYE3 as previously reported (Niino *et al.*, 1995; Stott *et al.*, 1993). In addition to OYE2, the DEAE column chromatography preparation also contained a 30 kDa protein that was identified as alanine racemase, a non-NAD(P)H dependent enzyme containing a pyridoxal phosphate cofactor binding site responsible for catalyzing the conversion of L-alanine to D-alanine (Uo *et al.*, 2001). The peptide mass fingerprint identity of the OYE2

and alanine racemase was also confirmed by de novo sequencing of several peptides by electrospray tandem mass spectroscopy.

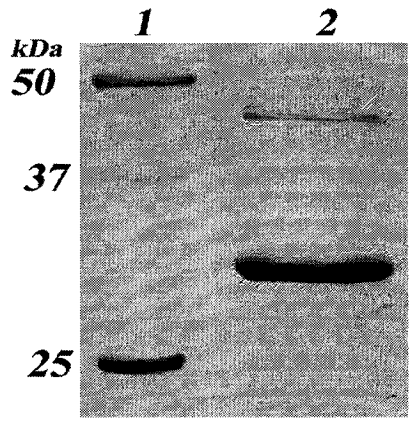


Figure 3.2. Purification of *S. cerevisiae* diacetyl reductase. Silver stained SDS-PAGE analysis of native diacetyl reductase activity isolated from the final DEAE sepharose chromatography column step used to purify diacetyl reductase activity from brewer's yeast; (lane 1) molecular weight markers and (lane 2) diacetyl reductase active fraction.

3.4.2 Diacetyl reduction by Oye1p

To validate the conjecture that the OYE enzymes were effective at reducing diacetyl, all three isoforms were overexpressed in *E. coli* and purified to homogeneity. Spectral analysis of the purified protein revealed a broad absorbance in the visible region with a λ_{max} at 462 and 380 nm, consistent with the recombinant enzymes having a bound oxidized FMN group in the active site, as previously reported (Schopfer and Massey, 1991).

Spectrophotometric assays showed that OYE enzymes were capable of oxidizing NADPH in a diacetyl dependent manner. However, since these enzymes are also known to utilize molecular oxygen as a hydride acceptor, albeit slowly (Brown *et al.*, 1998), it was necessary to demonstrate the reduction of the vicinal diketone utilizing an alternative approach. Gas chromatographic analysis of reaction mixtures showed that Oye1p, Oye2p, and Oye3p were capable of converting diacetyl to acetoin (3-hydroxy-2-butanone) in an NADPH dependent manner. In the absence of NADPH or NADH no reduction of diacetyl was detected (Fig. 3.3B-E). Protracted incubations of the reaction showed that the Oye1p and Oye2p isoforms were not capable of reducing diacetyl to 2,3-butanediol (Fig. 3.1 and Fig. 3.3) even in the presence of excess NADPH. Moreover, reaction mixtures containing acetoin (10 mM) as the sole substrate and an excess of Oye1p or Oye2p showed no detectable 2,3-butanediol formation. These data suggest that Oye1p and Oye2p are capable of catalysing a single reduction step, and that conversion of acetoin to 2,3-butanediol likely requires a separate enzymatic system (Fig. 3.3). Addition of Oye1p to a reaction mixture containing equal amounts of diacetyl and 2,3-pentanedione revealed that the former substrate was preferentially utilized by Oye1p, as shown by the appearance of a dominant peak corresponding to acetoin. Some conversion of pentanedione to 2-hydroxy-3-pentanone and the isomer 3-hydroxy-2-pentanone is indicated by the small doublet peak eluting ~16-17 min. (Fig. 3.3E), however the identity of these compounds was not definitively established since commercial standards for the hydroxypentanone isomers were not readily available.

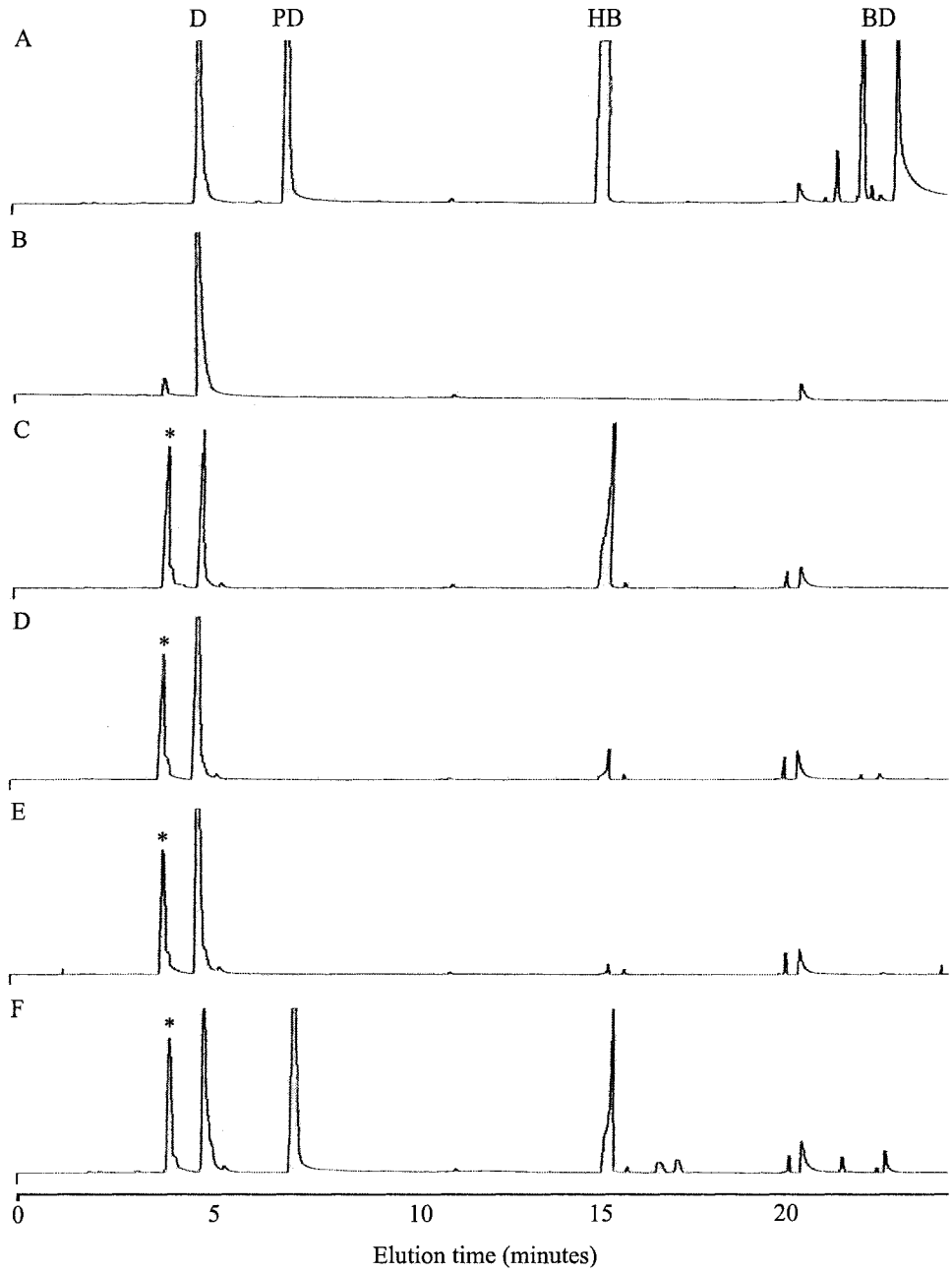


Figure 3.3: Analysis of OYE reaction products: The conversion of diacetyl (2,3-butadione (D) to acetoin (3-hydroxy-2-butanone) (HB) by OYE isoforms was analysed by gas chromatography. Two microliter aliquots were injected and analytes were eluted by ramping the column temperature to 250 °C and detected using a flame ionization detector. A species associated with the addition of NADPH to the reaction mixtures is marked with a *. (A) Elution profile for a standard mixture containing equal amounts of diacetyl (D),

2,3-pentanedione (PD), 3-hydroxy-2-butanone (HB) and 2,3-butanediol (BD). (B) Elution profile for reduction reaction containing 10 mM diacetyl, 10 μM Oye1p without NADPH. (C) Elution profile of reduction reaction containing 10 mM diacetyl, 10 mM NADPH and 10 μM Oye1p incubated at 20 0C for 4 h prior to analysis. Acetoin is formed as a reaction product with no apparent formation of 2,3-butanediol. (D) Elution profile for reduction reaction containing 10 mM diacetyl, 10 mM NADPH and 10 μM Oye2p. (E) Elution profile for reduction reaction containing 10 mM diacetyl, 10 mM NADPH and 10 μM Oye3p. (F) Elution profile for a reduction reaction containing 10mM 2,3-butanedione, 10 mM 2,3-pentanedione, 10 mM NADPH and 10 μM Oye1p.

Prior to kinetic analysis, the pH optimum for Oye1p and Oye2p catalyzed reduction of diacetyl was assessed using a universal buffer containing acetate, phosphate, and Tris. As shown in Figure 3.4, the maximal reductase activity of both enzymes occurred at pH 6.0. This pH optimum was comparable to the value reported by Heidlas and Tressl (Heidlas and Tressl, 1990) for the diacetyl reductase activity that preferentially produced the S enantiomer of acetoin.

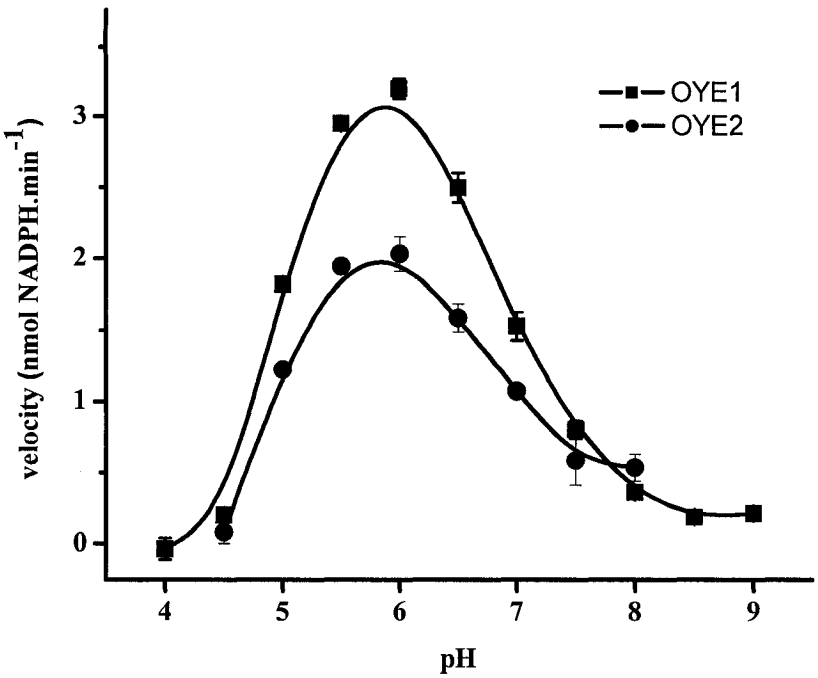


Figure 3.4. pH optimum for Oye1p and Oye2p activity: Diacetyl reduction was performed in a universal buffer composed of 50 mM each acetic acid, sodium phosphate, and Tris and the pH adjusted from 3.5 to 10 with HCl or NaOH. Reaction mixtures contained 4 mM diacetyl, 200 μM NADPH, 10 mM glucose, 10 U of glucose oxidase, and 4 μM Oye1p or Oye2p and the reaction was monitored at 340 nm. Control reaction mixtures lacking Oye1p or Oye2p were used to correct for absorbance changes associated with NADPH hydrolysis at acidic pH.

3.4.3 Kinetic analysis of OYE isoforms

Kinetic analysis of the purified recombinant Oye1p, Oye2p and Oye3p revealed that these enzymes were capable of reducing the α,β-dicarbonyl substrates diacetyl and pentanedione in a NADH or NADPH dependent reaction. The kinetic experiments were performed under anaerobic conditions by exhaustively sparging solutions with nitrogen

gas and by including glucose oxidase and glucose in the reaction mixture to scavenge residual traces of oxygen, an opportunistic electron acceptor (Schopfer and Massey, 1991). Although Michaelis-Menten plots (Fig. 3.5, inset), using diacetyl concentrations spanning 3 orders of magnitude, showed saturation kinetics for all three OYE isoforms, analysis of the kinetic data using either Eadie-Hofstee or Hanes-Woolf transformations clearly revealed an apparent bimodal kinetic behaviour. With diacetyl, Oye1p and Oye2p exhibited a negative cooperativity with respect to substrate affinity and positive cooperativity with respect to activity. This kinetic pattern was characterized by a high binding affinity (K_{m1}, micromolar concentration) but low activity (k_{cat1}) phase and low binding affinity (K_{m2}, millimolar) but high activity (k_{cat2}) phase (Fig. 3.5 A & B). Similar biphasic responses were also observed for pentanedione. However, not all OYE isoforms utilized these dicarbonyl substrates with comparable efficiency, particularly at low substrate concentrations. Linear fits for the two phases of the Hanes-Woolf plots were used to determine the Km and Vmax values for both phases of the curve (Fig. 3.6 & 3.7, Table 3.1). Oye1p and Oye2p exhibited comparable K_{m1} values of ~ 2 μM for diacetyl and turnover rates of 0.4 and 0.2 min^{-1}, respectively, with Oye1p having a marginally higher selectivity for diacetyl (Table 3.1). In contrast, Oye3p showed no measurable enzymatic activity with diacetyl at low micromolar concentrations. This was not surprising since previous kinetic studies showed that the reoxidation step of recombinant Oye3p, using O_2 as the second substrate, was notably slower than that of Oye2p (Niino *et al.*, 1995). With pentanedione, only Oye2p was capable of reducing this substrate at low concentrations. The K_{m1} value for pentanedione was ~100-fold higher than that of

diacetyl, suggesting that the additional methylene group dramatically alters the ability of Oye1p and Oye2p to bind pentanedione.

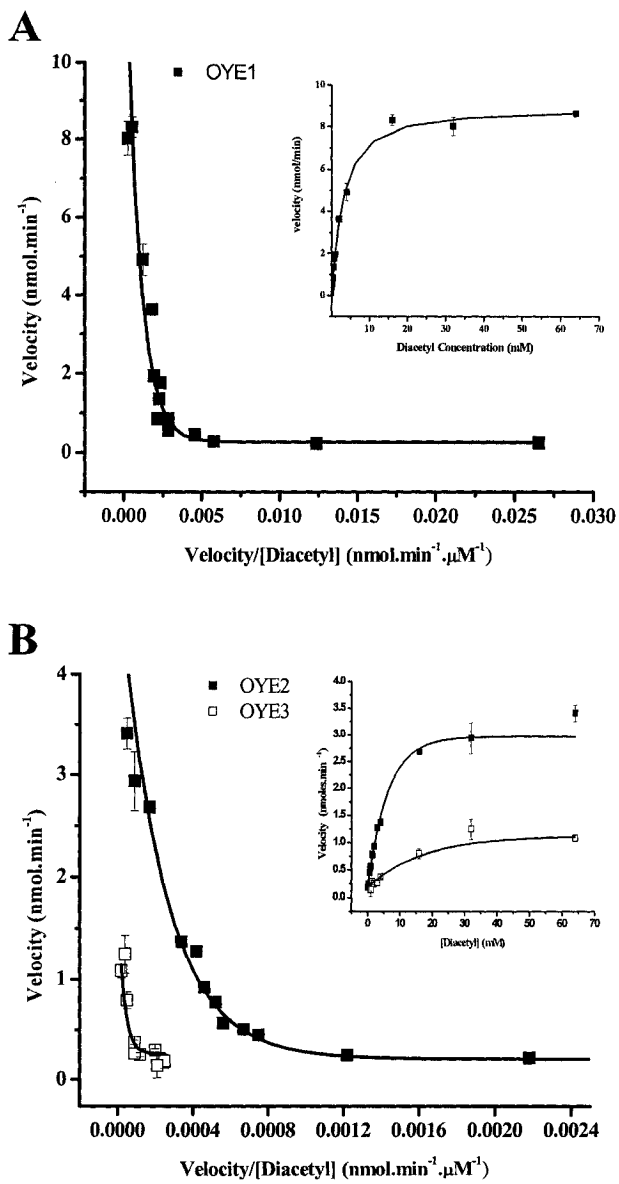


Figure 3.5. Diacetyl reduction by OYE isoforms. Steady state kinetic were performed with all three OYE isoforms using diacetyl concentrations ranging from 1 μM to 64 mM

and fitting the initial rate data to the Michaelis-Menten equation revealed that saturation kinetics were obtained (inset). Eadie-Hofstee transformation of the kinetic data revealed that reduction of diacetyl by OYE isoforms was more complex and exhibited a biphasic relationship, indicative of these enzymes having two binding affinities. (A) Oye1p and (B) Oye2p and Oye3p.

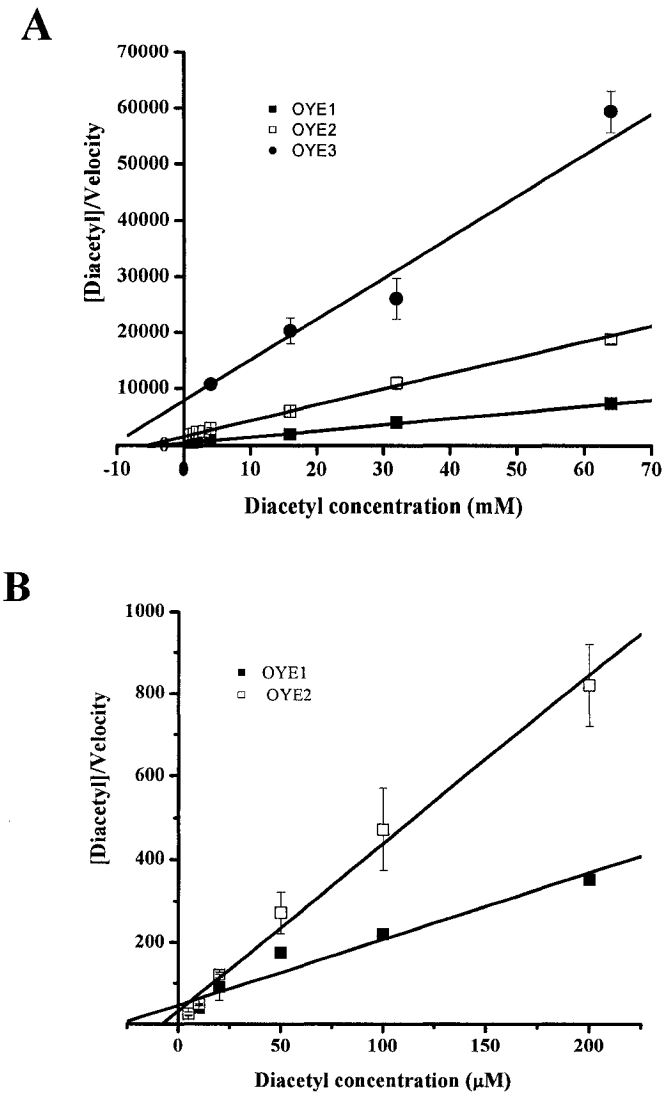


Figure 3.6. Hanes-Woolf plots for OYE. To determine the two apparent K_m values for diacetyl binding to OYE a linear regression analysis was performed using the Hanes–Woolf plots and linear fits were determined for the two kinetic phases. (A) Linear fits for high diacetyl concentrations and (B) linear fits for the low diacetyl concentration. Data analysis was performed using the Microcal Origin 7.0 software package.

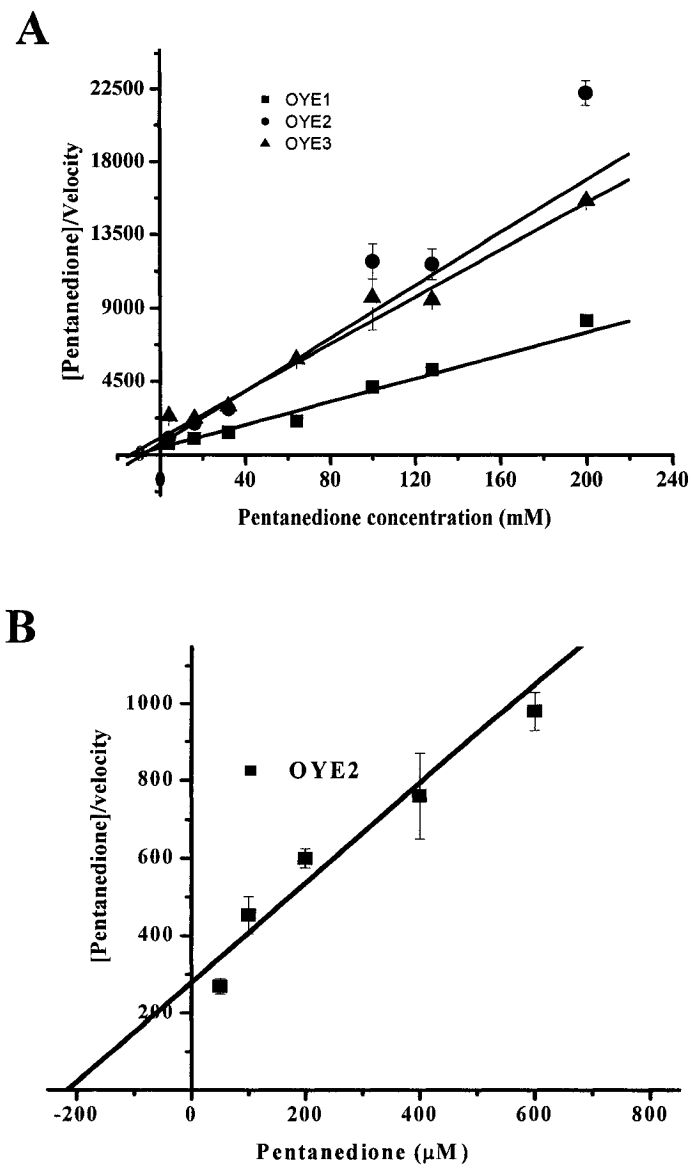


Figure 3.7. Hanes-Woolf plot for OYE. To determine the two apparent K_m values for 2,3-pentanedione binding to OYE a linear regression analysis was performed using the Hanes–Woolf plots and linear fits were determined for the two kinetic phases. (A) Linear fits for high concentrations of 2,3-pentanedione and (B) linear fits for low concentrations of 2,3-pentanedione. Data analysis was performed using the Microcal Origin 7.0 software package.

At millimolar concentrations of diacetyl and pentanedione all three isoforms of OYE exhibited vigorous activity, however the estimated K_{m2} values for these substrates range from ~0.5-14 mM. In all cases Oye1p had the highest turnover rates (k_{cat2}) ranging from ~27-81 min^{-1}, depending on the dicarbonyl substrate (Table 3.1), followed by Oye2p and Oye3p, respectively. It is interesting to note that at higher substrate concentrations the catalytic rates are ~10-100-fold more efficient than that observed with low substrate concentrations (Table 3.1). Whether the K_{m2} and k_{cat2} parameters are of biological significance is unclear since the concentration of diacetyl in fermentation streams or mammalian tissues seldom exceed 100 μM (Mattessich and Cooper, 1989; Petersen *et al.*, 2004; Trelea *et al.*, 2002).

Oye1p catalyzed reactions are known to proceed via an ordered ping-pong mechanism where NAD(P)H binds in the catalytic pocket, reduces the FMN cofactor, followed by the release of $NAD(P)^+$ prior to binding and reducing the second substrate (Massey and Schopfer, 1986). Steady-state analysis of NADPH and NADH turnover showed that under anaerobic conditions these substrates exhibited monophasic kinetics (Fig. 3.8). The K_m values for NADPH were 11.0 and 6.8 μM using fixed concentrations of 34 or 200 μM of diacetyl, respectively. These values are in good agreement with NADPH K_m values previously reported for Oye1p (Massey and Schopfer, 1986). The steady state turnover rates for the reduction of diacetyl obtained in these latter experiments were 0.2-0.5 min^{-1}. These values were comparable to the k_{cat1} observed with diacetyl (Table 3.1). Comparable K_m values of 10.6 μM were also obtained for NADH using 34 μM of diacetyl (Fig. 3.8).

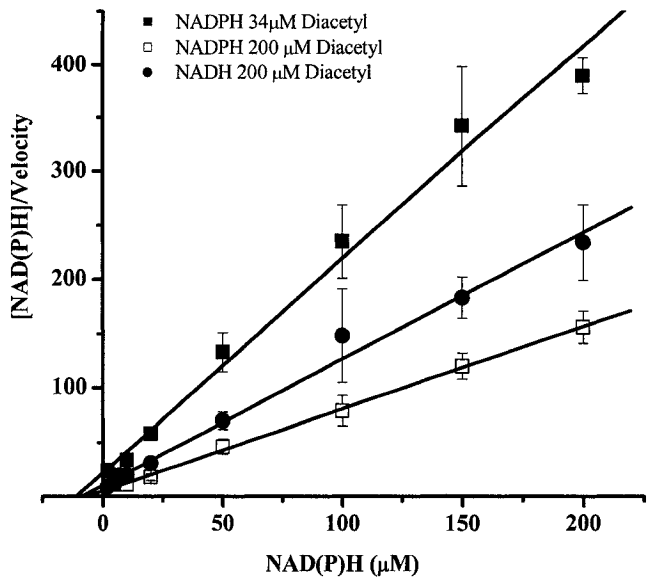


Figure 3.8. Hanes-Woolf kinetic plot for NADH and NADPH. The kinetic parameters for NADH and NADPH were determined using a fixed concentration of 34 or 200 μM of diacetyl and varying the concentration of NADH or NADPH from 1 to 200 μM. Reactions were initiated by adding Oye1p to a final concentration of 2.8 nM and the oxidation of NADH and NADPH was monitored at 340 nm.

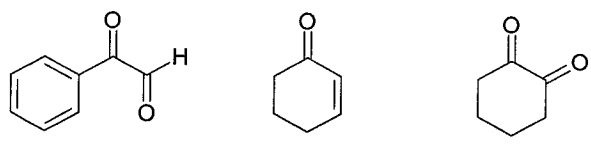
3.4.4 Analysis of biphasic kinetics

An unusual feature of diacetyl and pentanedione reduction by OYE enzymes was the biphasic kinetic behaviour. To ascertain if the biphasic kinetics were associated with acetoin formation, product inhibition kinetics were performed using Oye1p. Gas chromatography analysis confirmed that Oye1p cannot convert acetoin to 2,3-butanediol, thus the possibility that the biphasic kinetics were due to Oye1p catalyzing two reduction

reactions could be discounted. In addition, reaction mixtures containing either 34 or 200 μM diacetyl as the substrate in the presence of 2.0 mM acetoin showed no inhibition in diacetyl reduction confirming that acetoin was not tightly bound by Oye1p. These data further discount the possibility that the biphasic behaviour was due to the slow release of the reaction product acetoin.

To investigate the possibility that the OYE biphasic kinetics were due to chemical modification of arginine or lysine residues at high diacetyl or pentanedione concentration, kinetic analysis of carbonyl reduction was performed with the non-physiological substrates phenylglyoxal, 1,2-cyclohexanedione, and 2-cyclohexen-1-one (Fig. 3.9A). The former two substrates, like diacetyl, are known to readily modify arginine residues (Lundblad, 1995). Steady state kinetics with phenylglyoxal and 1,2-cyclohexanedione showed a similar biphasic behaviour with the K_{m1} being in the micromolar range and K_{m2} being in the high micromolar to millimolar range (Fig. 9B & Table II). As with diacetyl and pentanedione, a significant increase of 5-9-fold in the turnover rate was observed with the higher substrate concentrations (Table 3.2). In contrast, reduction of 2-cyclohexen-1-one, a substrate that is structurally very similar to 1,2-cyclohexanedione, by Oye1p showed monophasic kinetics as shown by Eadie-Hofstee and Hanes-Woolf plots (Fig. 3.9C&D). The K_m values and monophasic kinetics were comparable to values previously reported for 2-cyclohexen-1-one (Brown *et al.*, 1998).

A



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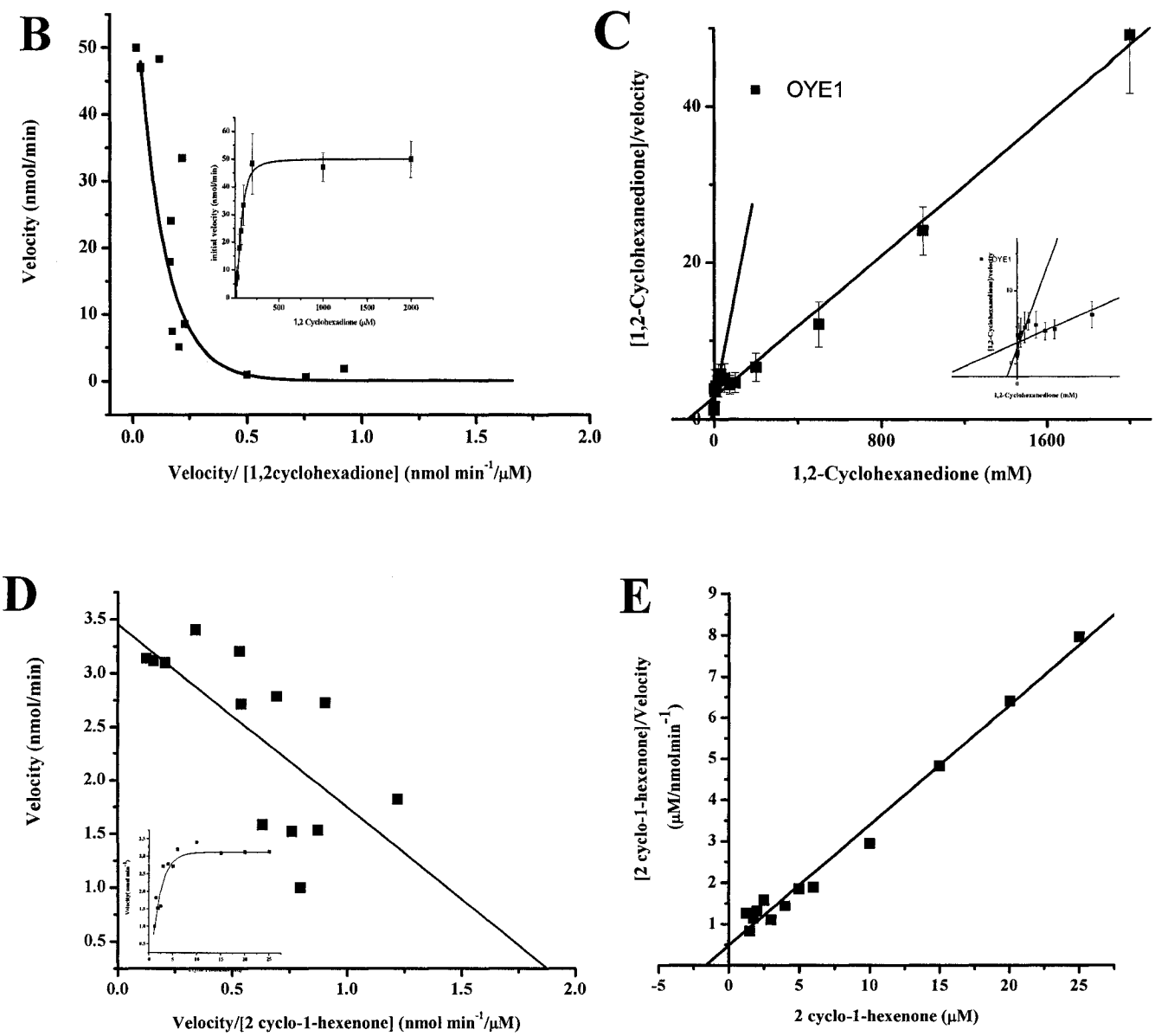

Figure 3.9. Oye1p reduction of non-physiological substrates. (A) Chemical structures of the substrates reduced by OYE, (1) phenylglyoxal, (2) 2-cyclohexen-1-one, (3) 1,2-cyclohexanedione. (B) Eadie-Hofstee plot of kinetic data for Oye1p reduction of 1,2-cyclohexanedione, the inset figure is a Michaelis-Menten plot showing substrate saturation. K_m and k_{cat} values were determined by a two phase linear fit of the data replotted as a Hanes-Woolf plot (C). (D) Eadie-Hofstee plot of kinetic data for Oye1p

reduction of 2-cyclohexen-1-one, the inset figure is a Michaelis-Menten plot showing substrate saturation. K_m and k_{cat} values were determined by fitting the data to the Hanes-Woolf equation (E).

Table 3.1 Kinetic properties of Old Yellow Enzyme isoforms with vicinal dicarbonyls

	Diacetyl				Pentanedione			
Enzyme	K_{m1} (μM)	k_{cat1} (min^{-1})	K_{m2} (mM)	k_{cat2} (min^{-1})	K_{m1} (μM)	k_{cat1} (min^{-1})	K_{m2} (mM)	k_{cat2} (min^{-1})
OYE1	2.40	0.88	3.20	27.5	n/a	n/a	12.4	81.2
OYE2	2.00	0.42	5.40	7.10	224	2.20	8.40	33.7
OYE3	n/a	n/a	10.8	2.1	n/a	n/a	14.3	21.5

n/a no activity

Table 3.2 Kinetic properties of Old Yellow Enzyme isoforms with non-physiological vicinal dicarbonyls

	Phenylglyoxal				1,2-Cyclohexanedione				2-Cyclohexen-1-one	
Enzyme	K_{m1} (μM)	k_{cat1} (min^{-1})	K_{m2} (mM)	k_{cat2} (min^{-1})	K_{m1} (μM)	k_{cat1} (min^{-1})	K_{m2} (μM)	k_{cat2} (min^{-1})	K_{m1} (μM)	k_{cat1} (min^{-1})
OYE1	68	8.50	1.12	76.4	15.2	3.6	126	22	3.4	3.44
OYE2	n/a	n/a	0.52	25.4	ND	ND	ND	ND	ND	ND
OYE3	~59	2.30	2.66	40.0	ND	ND	ND	ND	ND	ND

ND not determined

3.5 Discussion

Old Yellow Enzyme 1 was one of the first enzymes to be purified and biochemically characterized (Warburg and Christian, 1933). The reaction mechanism and the three dimensional structure of this oxidoreductase have been extensively studied by Massey and colleagues (Brown *et al.*, 1998; Fox and Karplus, 1994; Fox and Karplus, 1999; Karplus *et al.*, 1995; Kohli and Massey, 1998; Schopfer and Massey, 1991). However, many of these studies utilized a series of synthetic substrates that included aromatic aldehydes and ketones, cyclic ketones or alkenes, as the physiological function and substrates for OYE were unknown. In these studies we employed conventional chromatography techniques to isolate the reductases involved in metabolism of diacetyl and pentanedione, toxic dicarbonyl compounds formed as byproducts of the Maillard reaction and branched amino acid biosynthetic pathways. Using this approach, a highly enriched fraction containing two proteins, alanine racemase and Oye2p was obtained (Fig. 3.2). Three OYE isoforms have been described (Niino *et al.*, 1995; Schopfer and Massey, 1991; Stott *et al.*, 1993), however, not all yeast strains possess all three isoforms. For example, *S. cerevisiae* has genes encoding Oye2p and Oye3p, whereas bottom fermenting brewing yeast strains (*S. pastorianus*/*S. carlsbergensis*) encode all three OYE isoforms.

Under steady state conditions, in the absence of oxygen, all three OYE isoforms exhibited a biphasic kinetic behaviour for the reduction of diacetyl, 2,3-pentanedione, phenylglyoxal, and 1,2-cyclohexanedione when analyzed by the Eadie-Hofstee or Hanes-Woolf methods. Deconvolution of these plots yielded a high affinity K_{m1} (μM) with a

low turnover rate (k_{cat1}) component and a low affinity (K_{m2}) with high turnover rate (k_{cat2}) for α,β-dicarbonyl substrates (Table 3.1 & 3.2).

Previous studies with *Saccharomyces* OYE enzymes have reported a biphasic kinetic behaviour (Brown *et al.*, 1998; Niino *et al.*, 1995; Schopfer and Massey, 1991). However, in these studies the complex kinetics observed were attributed to micro heterogeneities in the native enzyme preparation containing OYE homo and heterodimers (Miura *et al.*, 1986; Saito *et al.*, 1991; Stott *et al.*, 1993) or due to the presence of two substrates, such O_2 or $O_2^{\cdot-}$, which exhibit different reactivities with the reduced form of OYE (Brown *et al.*, 2002). However, we can suggest that these factors did not account for the bimodal kinetics since; first, recombinant OYE which forms homodimers (data not shown) were used in these studies. Second, molecular oxygen or an $O_2^{\cdot-}$ reactive anion is not involved as all experiments were performed under anaerobic condition using glucose oxidase as an O_2 scavenger. Moreover, if O_2 or $O_2^{\cdot-}$ reactive anion was involved in the biphasic kinetic behaviour, similar K_m and k_{cat} values should have been obtained for an OYE isoform, regardless of the dicarbonyl used in the steady state experiments.

The biphasic kinetics observed with the OYE enzyme reduction of α,β-dicarbonyl substrates may be due to: first, dicarbonyl substrates exhibiting different binding affinities for the oxidized and reduced forms of OYE, which would give rise to non-Michaelis-Menten kinetics, as previously proposed for the flavoprotein monoamine oxidase and Oye1p (Ramsay *et al.*, 1993; Vaz *et al.*, 1995). However, under steady state conditions, in the presence of saturating concentrations of NADPH, it is anticipated that the levels of oxidized OYE would be negligible (Schopfer and Massey, 1991). Second, it is possible that the reduction rates for *cis* and *trans* isomers of the α,β-dicarbonyls may differ.

Thermodynamic studies of diacetyl and phenylglyoxal have suggested that the *trans* isomer is more stable and thus more abundant at 25 °C (Rochefort and McBreen, 2001; Strom *et al.*, 2003), however the reduction rate of the *trans* isomer may be slower than that of the *cis* isomer. The effect of stereoselectivity on OYE reaction rates has been reported for the substrate 3-oxodecalin-4-ene (Heidlas and Tressl, 1990; Vaz *et al.*, 1995). If the *cis-trans* isomeration was implicated in the biphasic kinetics then monophasic kinetic should have been observed with the constrained dicarbonyl 1,2-cyclohexanedione. Third, it is possible that in the presence of high α,β-dicarbonyls, these compounds modify the OYE enzymes and alter protein structure (Seidler and Kowalewski, 2003) resulting in biphasic kinetics. Finally, OYE enzymes exist as functional dimers (Niino *et al.*, 1995; Stott *et al.*, 1993; Vaz *et al.*, 1995). Binding of substrate molecule to one subunit may induce a conformational change in the second subunit that influences the binding affinity and turnover rate of the second subunit. This possibility is unlikely, as construction of Oye1p mutants that do not dimerize also exhibit biphasic kinetics with diacetyl (manuscript in preparation). Finally, the most plausible explanation of the biphasic kinetics is modification of OYE enzymes by diacetyl, pentanedione, phenylglyoxal, and 1,2-cyclohexanedione - reagents that have been extensively used for selective arginine modification (Lundblad, 1995). This is supported by the finding that 2-cyclohexen-1-one, a substrate that has a comparable structure to 1,2-cyclohexanedione, but does not react with arginine residues, exhibited monophasic kinetics.

Modulation of enzymatic activity by reactive compounds such as α,β-dicarbonyls would be a unique mechanism, for rapidly responding to increased levels of these toxic metabolites, which would be independent of transcription and translation. It is of interest

that vicinal dicarbonyls preferentially modify a select group of proteins such as Hsp27, which is involved in repair or degradation of proteins in stressed cells (Schalkwijk *et al.*, 2006), or mSin3A a corepressor that links glycolysis to gene transcription (Yao *et al.*, 2006).

OYE enzymes have been extensively exploited as a model system for the study of flavoproteins, however, the biological function and substrates of these enzymes are largely unknown, even though they are known to reduce a wide variety of non-physiological substrates including enones, phenols, sterols, and trinitrotoluenes (Meah *et al.*, 2001; Sánchez *et al.*, 2003; Vaz *et al.*, 1995). Recently, Oye2p has been shown to associate with actin and protect the yeast cytoskeleton from oxidative stress (Haarer and Amberg, 2004). Here, based on *in vitro* enzymatic data we propose that Oye1p and Oye2p are possibly involved in protecting *Saccharomyces* from reactive dicarbonyl stress by degrading diacetyl and pentanedione byproducts. These enzymes are not solely responsible for this process since *S. cerevisiae* Δoye2oye3 double knockout cell lines are viable (unpublished data). DNA microarray experiments have suggested that *S. cerevisiae* Oye2p may be an important factor for countering oxidative stress, since expression of this gene is significantly up regulated in yeast cells treated with H_2O_2 or menadione (Gasch *et al.*, 2000). This response to oxidative stress is consistent with the dicarbonyl detoxifying function we propose for Oye1p and Oye2p, since oxidative stress is known to increase the levels of diacetyl and methylglyoxal (Wondrak *et al.*, 2002).

3.6 Acknowledgements

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Connecting text

Having identified Old Yellow Enzyme as being capable of reducing the physiological substrates diacetyl and pentanedione, and OYE having an unknown function within the cell, we attempted to elucidate the function of the protein within the yeast cell. *S. cerevisiae* BY4742Δoye2oye3 knockout strains (Brachmann *et al.*, 1998) were constructed and grown in parallel with strains containing the OYE isoforms. Fermentations were performed, but yielded no significant differences in diacetyl evolution. Diacetyl, spiked into stationary phase fermentations, was removed by the yeast with similar efficiency. Cultures were then grown aerobically, in triplicate, in the presence of various concentrations of diacetyl and although the presence of diacetyl affected the doubling time markedly, there was no significant difference between wild type and knockout strains (Fig. CT2). It became evident that another mechanism was available within the yeast cells to effectively reduce diacetyl. It was unknown if this mechanism was a compensatory mechanism or otherwise, but it needed to be identified and characterised before progressing any further.

Cells were grown aerobically in bioreactors and harvested at late exponential growth, as opposed to yeast grown with limited oxygen and harvested at late stationary phase for the OYE purifications in Chapter 3. Cells harvested during late exponential phase appeared to have significantly higher protein levels and enzyme activity and yielded two activity peaks during preliminary purifications. The first peak was further purified and these results are reported in the following chapter.

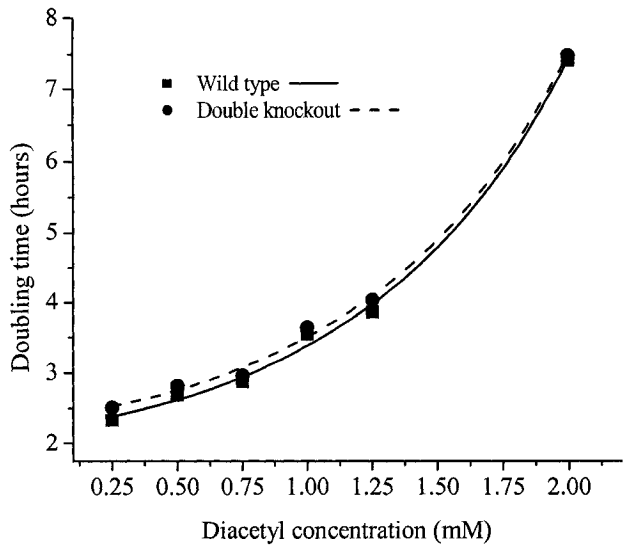

Figure CT2. The impact of external diacetyl concentration on cell doubling time in wild type BY4742 and Δoye2oye3 double knockout haploid yeast strains.

4. α,β-Dicarbonyl Reduction by *Saccharomyces* D-Arabinose Dehydrogenase

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4.1 Abstract

An α,β-dicarbonyl reductase activity was purified from *Saccharomyces cerevisiae* and identified as the cytosolic enzyme D-Arabinose dehydrogenase (ARA1) by MALDI-TOF/TOF. Size exclusion chromatography analysis of recombinant ARA1 revealed that this protein formed a homodimer. ARA1 catalyzed the reduction of the reactive α,β-dicarbonyl compounds methylglyoxal, diacetyl, and pentanedione in a NADPH dependant manner. ARA1 had apparent Km values of ~14 mM, 7 mM and 4 mM for methylglyoxal, diacetyl and pentanedione respectively, with corresponding turnover rates of 4.4, 6.9 and 5.9 s^{-1} at pH 7.0. pH profiling showed that ARA1 had a pH optimum of 4.5 for the diacetyl reduction reaction. ARA1 also catalyzed the $NADP^+$ dependant oxidation of acetoin, however this reverse reaction only occurred at alkaline pH values.

4.2 Introduction

Metabolic processes in the yeast *Saccharomyces cerevisiae* produce a variety of intermediate byproducts that may be toxic or mutagenic to the cell. For example, biosynthesis of the branched amino acids isoleucine, leucine, valine via the regulated ILV pathway (Holmberg and Petersen, 1988) can lead to the accumulation of the precursor metabolite α-acetolactate. This compound can undergo non-enzymatic decarboxylation (Gjermansen *et al.*, 1988; Park *et al.*, 1995) to produce diacetyl (2,3-butanedione). α,β-dicarbonyls such as diacetyl and methylglyoxal, are highly reactive compounds that can react with arginine, lysine, and cysteine residues resulting in damage and cross linking of proteins (Lundblad, 1995). In mammalian cells methylglyoxal and diacetyl have been implicated in a number of diseases that include diabetic vascular complications (Brownlee, 1995), neurodegenerative diseases (Kovacic and Cooksy, 2005), atherosclerosis, and general cellular deterioration and aging (Wondrak *et al.*, 2002). Several proteins have been shown to be susceptible to chemical modification, including ovalbumin (Hipkiss and Chana, 1998), plasminogen (Lerant *et al.*, 2000), and glyceraldehyde phosphate dehydrogenase (Morgan *et al.*, 2002). Removal of these dicarbonyl compounds (Fig. 4.1) is therefore essential for maintenance of cellular health. While *Saccharomyces cerevisiae* displays a regulatory mechanism when exposed to methylglyoxal (Aguilera and Prieto, 2004), little is known about the cellular responses to diacetyl produced during amino acid metabolism.

Aside from being an excellent model system for eukaryotic cell studies, *Saccharomyces spp.* have significant commercial application in alcoholic beverage

production. During the fermentation process yeast release the metabolite diacetyl, an undesirable compound that imparts a butterscotch-like aroma and unpleasant flavor that can be detected down to ~ 1 μM concentrations (Yonezawa and Fushiki, 2002). The presence of diacetyl in beer requires a lengthy maturation period during which yeast enzymatically reduce diacetyl to acetoin (2-hydroxy-3-butanone), a more flavor-neutral compound with a significantly increased aroma threshold (Fig. 4.1).

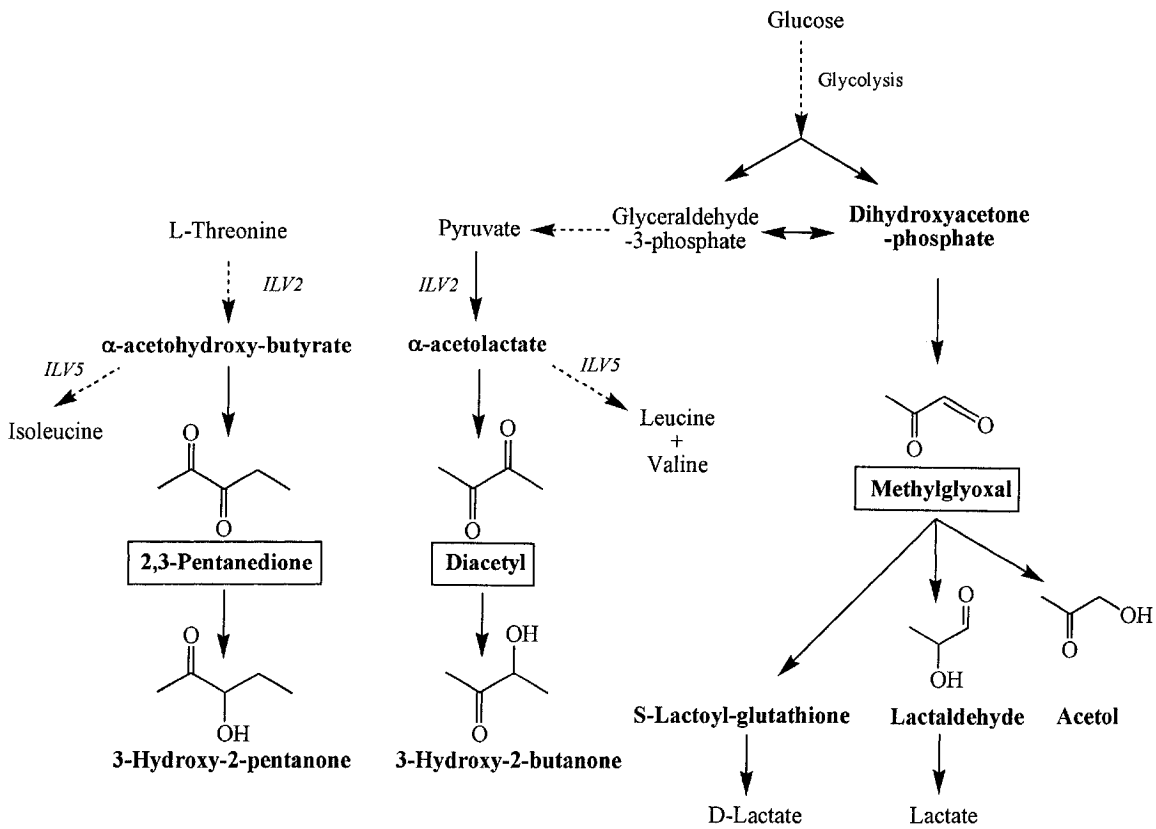

Figure 4.1 Reaction scheme showing pathways for the formation and reduction of methylglyoxal, diacetyl and 2,3-pentanedione in yeast.

Despite the physiological and industrial significance of this compound, little is known about the yeast enzymes involved in reduction. Historically, yeast alcohol dehydrogenase was shown to catalyze diacetyl reduction (Hardwick *et al.*, 1976), but the

high Michaelis constant (K_m) of 250 mM suggests that this enzyme would exhibit an extremely slow catalytic efficiency since diacetyl concentrations seldom exceed 100 μM under physiological concentrations. Heidlas and Tressl (Heidlas and Tressl, 1990) purified two enzymes of 36 and 75 kDa capable of catalyzing diacetyl reduction in *S. cerevisiae* with a K_m of 2 and 2.3 mM respectively for diacetyl. Both enzymes also catalyzed the reduction of 2,3-pentanedione and displayed K_m values of 2 and 1.5 mM, respectively. However the protein sequence of these was not determined. Ypr1p, a 36.4 kDa aldo-keto reductase from bakers yeast, was shown to catalyze diacetyl reduction and yielded a K_m of 5.7 mM (Nakamura *et al.*, 1997). Partially purified diacetyl reductase activities from ale (*S. cerevisiae*) and lager (*S. pastorianus*) yeast displayed K_m values 79 mM and 15 mM for diacetyl respectively (Bamforth and Kanauchi, 2004). More recently a diacetyl reductase activity purified from anaerobic *S. cerevisiae* cultures was shown to be Old Yellow enzyme (OYE). Kinetic characterization of the OYE2 isoform show that this enzyme reduced diacetyl and pentanedione with biphasic kinetics (van Bergen et al. submitted for publication). K_m values of ~2 and ~180 μM for diacetyl and pentanedione were determined for OYE2.

In this study we partially purified a diacetyl reductase activity from *S. cerevisiae* grown aerobically. Mass spectrometry analysis identified this protein as D-arabinose dehydrogenase (*ARA1*). The enzymatic activity of this enzyme was validated by over expression and kinetic analysis of recombinant Ara1p with the substrates methylglyoxal, diacetyl and pentanedione.

4.3 Materials and Methods

4.3.1 Materials

All reagents and substrates were purchased from Sigma-Aldrich or Fisher Scientific Canada unless otherwise specified. Molecular weight standards were purchased from Bio-Rad.

4.3.2 Growth of Yeast

Starter cultures (100 ml) of *S. cerevisiae* BY4742 (Brachmann *et al.*, 1998) were grown in a shaker flask to stationary phase at 30 °C in YPD media and used to inoculate 3.7 L BioEngineering (Switzerland) bioreactors containing 2.25 l of YPD. Cultures were grown aerobically at 30 °C to early stationary phase, cooled and harvested.

4.3.3 Enzyme purification

Yeast cell pellets were washed in 50 mM phosphate pH 7.0 buffer, resuspended in 50 mM Tris pH 7.2, 5 mM β-mercaptoethanol (β-ME) and 2 μg/ml phenylmethyl sulfonyl fluoride (PMSF) lysis buffer, ruptured with four passes through a French press and the lysate clarified by centrifugation at 38,000 x g for 30 min.

The diacetyl reductase activity was differentially precipitated by progressive addition of $(NH_4)_2SO_4$ to 20, 40, 60 and 80% saturation. At each concentration, samples were stirred for 20 min at 4 °C and the protein precipitate harvested by centrifugation at

10,000 x g for 15 min. Protein pellets were resuspended in 25 mM sodium phosphate with 2 mM β-ME at pH 7.0 (buffer A) and assayed for activity.

The 80% $(NH_4)_2SO_4$ pellet, containing the bulk of the diacetyl reductase activity, was adjusted to 2 M $(NH_4)_2SO_4$ and applied to an octyl sepharose column (5 mm x 62 mm) equilibrated with 2 M $(NH_4)_2SO_4$ in buffer A. The column was washed with 10 column volumes of 2 M $(NH_4)_2SO_4$ and developed using a linear 150 ml 2-0 M $(NH_4)_2SO_4$ gradient. Fractions containing diacetyl reductase activity were pooled, dialysed exhaustively against 2 l of buffer A and then loaded onto an Active Red affinity column (5 mm x 62 mm). The Active Red column was washed with 10 column volumes of buffer A and developed with a linear 150 ml 0-2 M NaCl gradient. Fractions containing diacetyl reductase activity were pooled, dialysed overnight against 2 l of buffer A, applied to a DEAE Sepharose CL-6B column (5 mm x 62 mm) and developed with a linear 150 ml 0-0.5 M NaCl gradient. Diacetyl reductase containing fractions were precipitated with trichloroacetic acid, washed with acetone and analyzed by Coomassie-blue stained SDS-PAGE.

4.3.4 Identification of diacetyl reductase

A protein band on SDS-PAGE correlating with peak enzymatic activity was excised, digested with porcine trypsin (Promega, Madison, WI, USA) and analyzed by tandem time-of-flight on a Matrix Assisted Laser Desorption/Ionization 4800 MALDI-TOF/TOF (Applied Biosystems, Foster City, CA, USA). The protein mass fingerprints analysis in conjunction with de novo sequence data confirmed that the isolated enzyme was D-

arabinose dehydrogenase. The *Saccharomyces* genome database was searched using the Mascot search algorithm. Proteomic analysis was performed at the University of Victoria Genome BC Proteomics Centre.

4.3.5 Expression of D-arabinose dehydrogenase

The open reading frame encoding D-Arabinose dehydrogenase (*ARA1*) was amplified from *S. cerevisiae* cDNA by polymerase chain reaction using primer pairs 5'-GGAATTCCATATGTCTTCTTCAGTAGCCTC-3' and 5'-CGGGATCCTTAATACTTTAAATTGTCCAAG-3' containing the restriction sites *NdeI* and *BamHI* respectively (endonuclease restriction sites are underlined). The open reading frame was amplified using Platinum *Pfx* DNA polymerase (Invitrogen, Burlington, ON) with 32 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, and extension at 68 °C for 75 s. PCR products were digested with *NdeI* and *BamHI* and inserted into the corresponding sites of the pET15b(+) plasmid (Novagen, CA, USA). The integrity of the ARA1 sequence was confirmed by automated DNA sequence analysis. *E. coli* ER2566 (New England Biolabs, MA, USA) was transformed with pET15b(+)-*ARA1* and grown to an OD_{600} of 0.7 in LB broth containing 100 μg/ml ampicillin. Aralp expression was induced by adding isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 0.5 mM and incubating the culture with vigorous agitation at 25 °C for 6 h. Cultures were harvested and the cell pellet resuspended in 20 ml lysis buffer containing 50 mM sodium phosphate pH 7.5, 0.5 M NaCl and 20 mM imidazole then lysed with two passes through a French press. Cell lysates were clarified by centrifugation at 38,000 x g for 30 min and the supernatant loaded onto a Ni2+-NTA

column. The column was washed with 20 column volumes of lysis buffer followed by 20 column volumes of 50 mM sodium phosphate at pH 7.5 containing 0.5 M NaCl and 40 mM imidazole. Proteins were eluted using a step gradient of 80, 160 and 320 mM imidazole. Fractions containing Ara1p were concentrated and desalted using an Amicon filter concentrator (Millipore, ON, Canada). Protein concentrations were determined spectrophotometrically according to the method of Gill and von Hippel (Gill and von Hippel, 1989) using an extinction coefficient of 42,985 M-1cm-1.

4.3.6 Size exclusion chromatography analysis of ARA1

The quaternary structure of recombinant Ara1p was analyzed using size exclusion chromatography on a Beckman-Coulter high performance liquid chromatography system equipped with a Bio-Sil SEC-250 column (Bio-Rad) equilibrated in 50 mM potassium phosphate at pH 7.5 containing 150 mM NaCl. 25 μg of Ara1p was injected and the column developed at a flow rate of 0.5 ml/min. The SEC column was calibrated with a standard protein mixture (Bio-Rad) containing thyroglobulin, Bovine gamma-globulin, chicken ovalbumin, Equine myoglobin and Vitamin B12. Proteins were detected with a UV detector at 280 nm.

4.3.7 Reductase and oxidase activities

α,β-Dicarbonyl reductase activity was measured using a Beckman DU-640 spectrophotometer at 340 nm to monitor NADPH oxidation. To determine the pH optimum for the Ara1p reductase and oxidase reactions, assays were performed using a

universal reaction buffer containing 50 mM acetic acid, 50 mM Na_2HPO_4 and 50 mM Tris base and the pH was adjusted from 3.5 to 10 using HCl or NaOH. Forward reactions were performed at 25 ^{0}C with 1.0 mM diacetyl and 200 μM NADPH or 200 μM NADH and 0.5 μM Ara1p. Oxidase reactions were performed using 10 mM acetoin, 400 mM glycerol, or 20 mM D-arabinose with 200 μM $NADP^+$.

4.3.8 Gas chromatography analysis

Enzymatic reactions containing 10 mM diacetyl or acetoin plus 10 mM NADPH and ~0.25 nmol of ARA1 were incubated at 20 °C for 4 h and the formation of acetoin or 2,3-butanediol was monitored by gas chromatography (GC). Two μl aliquots of the reaction mixtures were injected onto a Hewlett Packard 5890 Series II GC equipped with a FID detector. A Zebron ZB-Wax column (30 m x 0.250 mm ID, 0.25 μm film) (Phenomenex, Torrance, CA) was used, with a helium carrier gas flow rate of 1.2 ml/min and a split ratio of 30:1. The injector and detector temperatures were 220 °C and 280 °C, respectively. Samples were injected onto the column at 45 °C, immediately ramped at 10 °C/min to 245 °C and held for 2 minutes.

4.3.9 Enzyme kinetics

Kinetic analysis of Ara1p with methylglyoxal, diacetyl, 2,3-pentanedione were performed in 100 mM phosphate buffer at pH 7.0 to approximate physiological conditions. The K_m values for the dicarbonyl substrates were determined using saturating concentration of NADPH (200 μM) and varying the concentration of methylglyoxal, diacetyl, or 2,3-

pentanedione from 0.1-20 mM. The reduction reactions were started by the addition of 250 pmoles of Aralp into a 1.0 ml reaction volume. Reaction velocities were monitored spectrophotometrically at 340 nm, on a Beckman DU-640 spectrophotometer equipped with a Pelletier temperature control unit, to determine the rate of $NADP^+$ production. To determine the NADPH K_m value, a fixed diacetyl concentration of 10 mM was used and the NADPH concentration was varied from 5-200 μM. For these reactions only 5 pmoles of Aralp were added to a 1.0 ml reaction volume to prevent rapid consumption of the NADPH at low substrate concentrations. All kinetic assays were performed at 25 ^{0}C and data analysis was performed using the MicroCal Origin 7.0 graphing software package (Northampton, MA).

4.4 Results

4.4.1 Purification of diacetyl reductase activity

An enzyme capable of reducing methylglyoxal, diacetyl, and pentanedione was purified from *S. cerevisiae* employing a combination of octyl sepharose, active red affinity, and DEAE sepharose chromatography. Previous studies focused on diacetyl reducing enzymes in brewers yeast harvested under anaerobic conditions (van Bergen *et al.* submitted for publication). Here we investigated diacetyl reductase enzymes expressed by *S. cerevisiae* BY4742 grown under aerobic conditions. The bulk of the diacetyl reductase activity was recovered in the 80% $(NH4)_2SO_4$ protein pellet from crude yeast lysates. The diacetyl reductase activity was further fractionated using hydrophobic interaction chromatography. This chromatographic step produced two peaks of enzyme activity (fractions 9-14 and 24-29) (Fig. 4.2A). Application of the first peak of activity (fractions 9-14) onto an active red dye affinity column, which preferentially captures enzymes that contain an NADH or NADPH binding pocket, and development with a linear NaCl gradient yielded a single peak of diacetyl reductase activity in fractions 16-26 (Fig. 4.2B). An additional chromatographic step on DEAE sepharose column resulted in a single peak of enzymatic activity (fractions 10-14) (Fig. 4.2C). The second peak of diacetyl reductase activity obtained from the hydrophobic interaction column, based on the chromatographic behaviour, is similar to the OYE (van Bergen *et al.* submitted for publication). To confirm this hypothesis this activity is being further purified for mass spectrometry characterization.

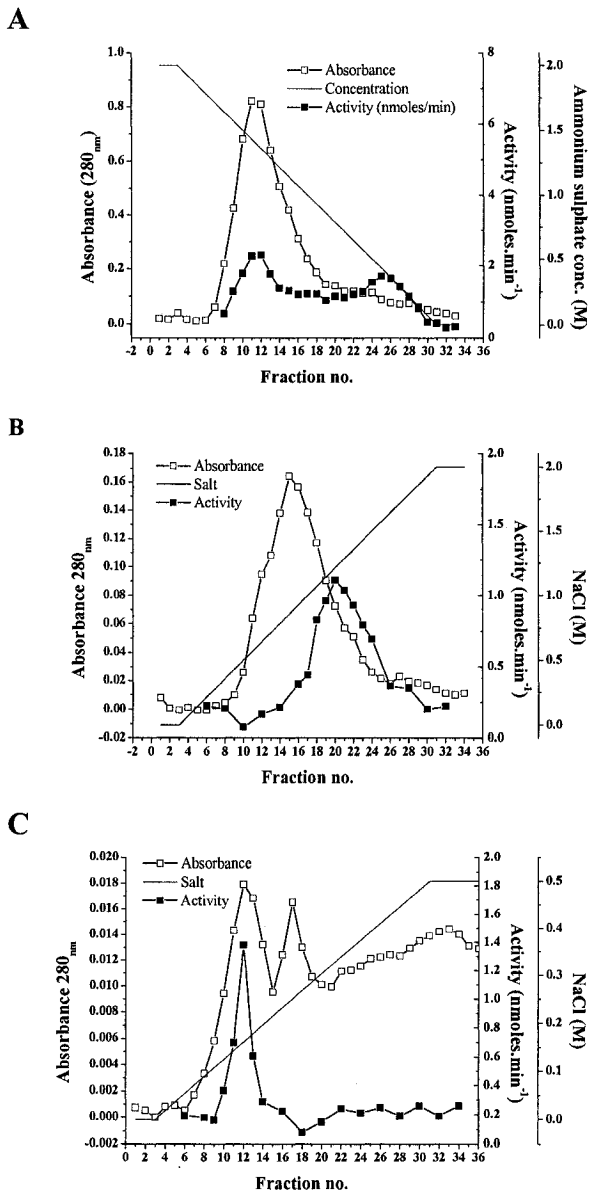

Figure 4.2 Purification of diacetyl reductase activity from aerobically grown cultures of *Saccharomyces cerevisiae*. (A) Protein elution and activity profiles of diacetyl reductase eluted from an octyl sepharose hydrophobic interaction column using a 2 M to 0 M $(NH_4)_2SO_4$ linear gradient. (B) Chromatography of diacetyl reductase containing fractions from the octyl sepharose column on an Active Red affinity column. The column was developed with a linear 0 to 2 M NaCl gradient. Fractions were collected and

assayed for protein content and diacetyl reductase activity. (C) Protein elution and activity from brewers yeast cell extract eluted from a DEAE sepharose CL-6B ion exchange column using a 0 to 0.5 M NaCl gradient.

4.4.2 Identification of D-Arabinose dehydrogenase

Analysis of the dicarbonyl reductase fractions, obtained from the DEAE column, by Coomassie-blue stained SDS PAGE revealed a ~40 kDa band which correlated with the diacetyl reductase activity. Mass fingerprint analysis of this protein using MALDI-TOF/TOF identified the putative diacetyl reductase as D-Arabinose dehydrogenase (*ARA1*) with a Mascot score of 1040 that corresponded to 59% sequence coverage. The *de novo* sequencing of 10 peptides ranging in mass from 883 to 2353 Da further confirmed the identity of the diacetyl reductase as Ara1p.

4.4.3 Expression and characterization of Ara1p

To definitively establish that Ara1p was capable of reducing α,β-dicarbonyl substrates, the Ara1p was overexpressed in *E. coli* as a hexahistidine tagged construct and purified to homogeneity by Ni^{2+}-NTA metal affinity chromatography. SDS-PAGE analysis of the purified recombinant enzyme showed that it migrated as a single band with a Mr of ~40 kDa (Fig. 4.3A). In contrast, previous studies with purified native Ara1p suggested that this enzyme may be a heterodimer composed of 39 and 40 kDa subunits, that under native conditions had a mass of 74 kDa (Kim *et al.*, 1998). Size exclusion chromatography

indicated that Aralp migrated with a mass of ~75-80 kDa (Fig. 4.3B), consistent with Aralp forming a homodimeric structure.

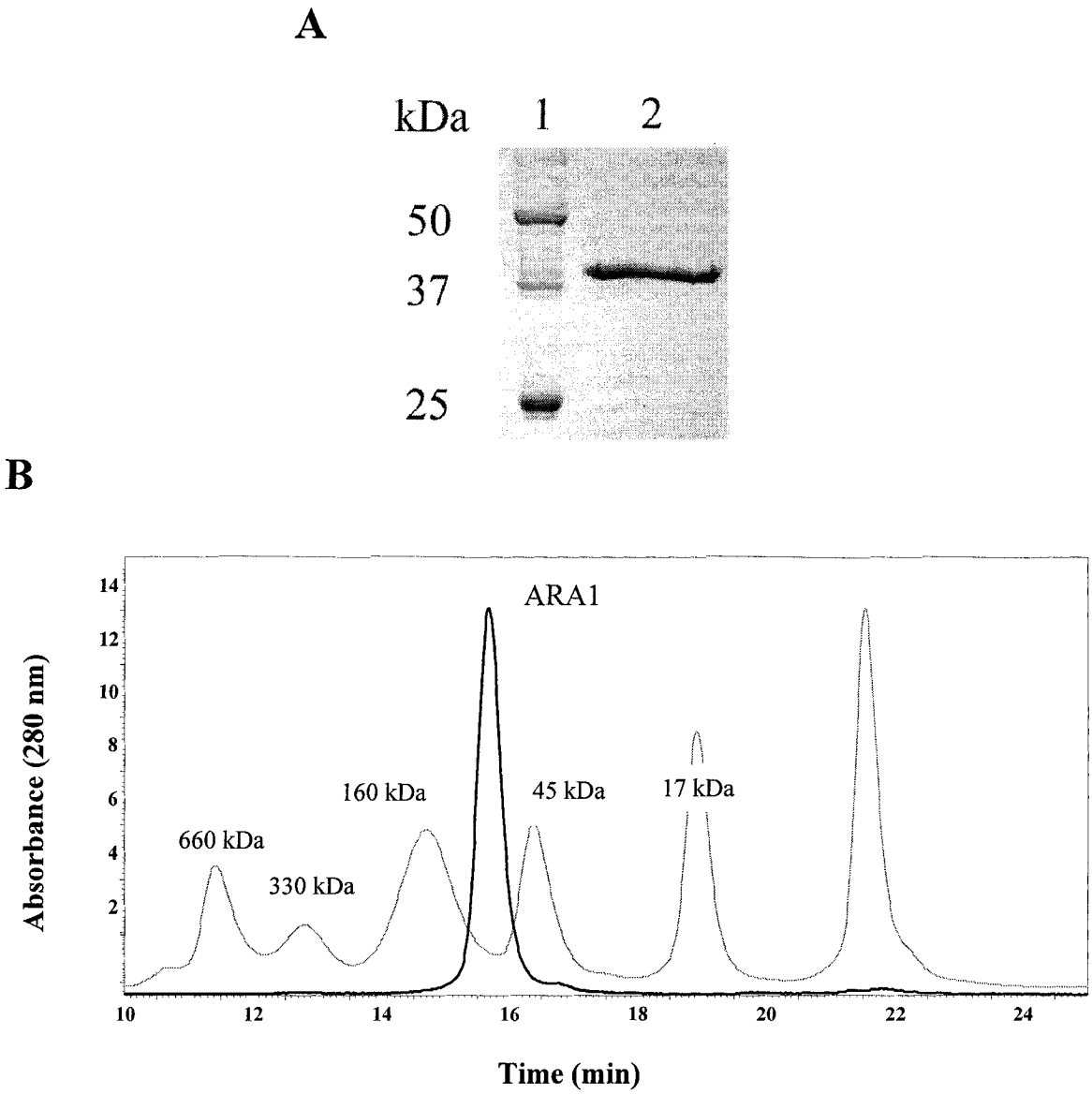

Figure 4.3 Molecular mass analysis of recombinant Ara1p. SDS-PAGE of recombinant Ara1p and (B) HPLC size exclusion chromatography analysis.

4.4.4 ARA1 activity and reaction products

The optimum pH for the reduction of diacetyl by Ara1p was determined by performing assays in a universal buffer in the presence of 1.0 mM diacetyl and 200 μM NADPH. The maximal forward reaction for the conversion of diacetyl to acetoin was observed at a pH of 4.5-5.5 with the activity decreasing sharply towards neutral pH (Fig. 4.4A). Similar assays using NADH as the hydride source showed no significant activity over this pH range, confirming that Ara1p specifically utilizes NADPH. Previous studies have shown that a function of Ara1p is to catalyze the oxidation of D-arabinose to form D-arbinono-1,5-lactone (Kim *et al.*, 1998). The ability of Ara1p to catalyze the reverse reaction using 10 mM acetoin and 200 μM $NADP^+$ showed that below pH 8.0 no detectable activity was observed at 25 °C (Fig. 4.4B). The reverse reaction rates increased to 1.0 nmol/min/nmol of protein at alkaline pH. A similar pH affect was observed for D-arabinose oxidation were optimal activity was observed pH 10 (Kim *et al.*, 1998). BLASTP searches of the *Saccharomyces* genome database revealed that Ara1p shared ~60% sequence similarity with Gcy1p a putative $NADP^+$ glycerol dehydrogenase, however, oxidase experiments using 400 mM glycerol showed that Ara1p was not capable of catalyzing the formation of glyceraldehyde.

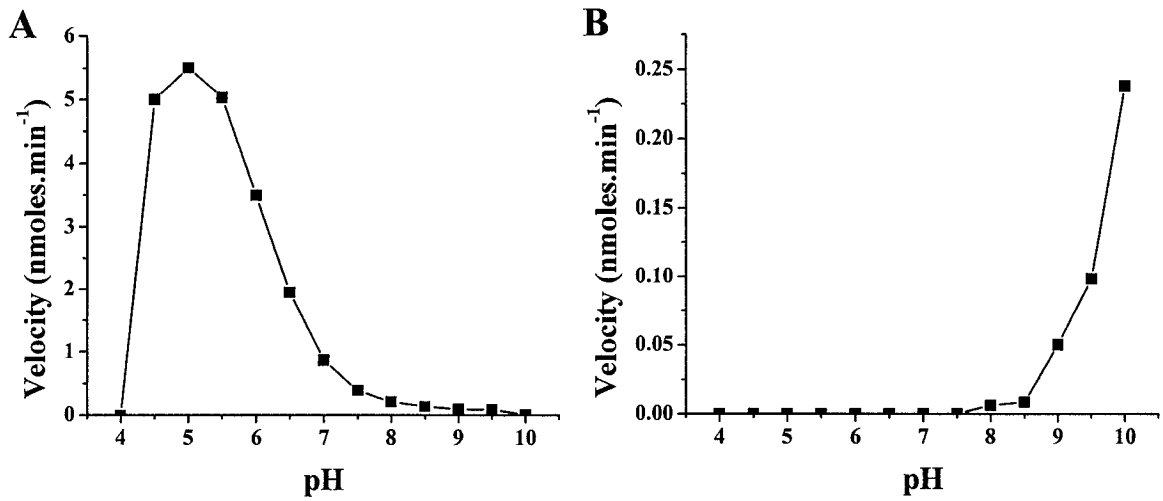

Figure 4.4 The effect of pH on D-Arabinose dehydrogenase activity. Forward reactions (A) were performed using 1 mM diacetyl, 200 μM NADPH and 0.25 nmoles Aralp in universal buffer at 25 °C. Investigation of reverse reactions (B) was performed using 10 mM acetoin, 200 μM NADP+ and 0.25 nmoles Aralp under identical conditions as forward reactions.

To directly assess the Aralp reduction products of vicinal diketones in the presence of NADPH, the reactions were analyzed by gas chromatography. Gas chromatogram traces confirm that in the absence of NADPH at pH 7.0 no diacetyl is converted to acetoin or 2,3-butanediol (Fig. 4.5 B and C). In contrast, with the addition of NADPH, Aralp was capable of converting diacetyl to acetoin almost quantitatively (Fig. 4.5 C). To investigate if Aralp could fully reduce diacetyl to 2,3-butanediol, the intermediate substrate acetoin was incubated with Aralp. Analysis of this reaction mixture showed no significant decrease in the level of acetoin even after prolonged incubations (Fig. 4.5 D). This chromatogram however, contains a tiny peak eluting at

~13 min, whether this peak is due to 2,3-butanediol is unclear since the current chromatographic system cannot easily resolve this compound efficiently (Fig. 4.5 D). Similarly, reactions containing pentanedione, showed that Ara1p was also able to reduce pentanedione also near quantitatively to hydroxypentanone. However whether the preferred product is 2-hydroxy-3-pentanone or 3-hydroxy-2-pentanone is unclear since the standards required for calibration of the gas chromatograph were not available commercially. It is clear from the doublet eluting at ~8.0 min that production of one of these isomers is markedly favored (Fig.4.5 E). In addition to the substrate and product peaks, these traces also contain two additional peaks that were not identified. Peak A is present in all reactions containing Ara1p and peak B appears to be associated with reaction mixtures containing NADPH.

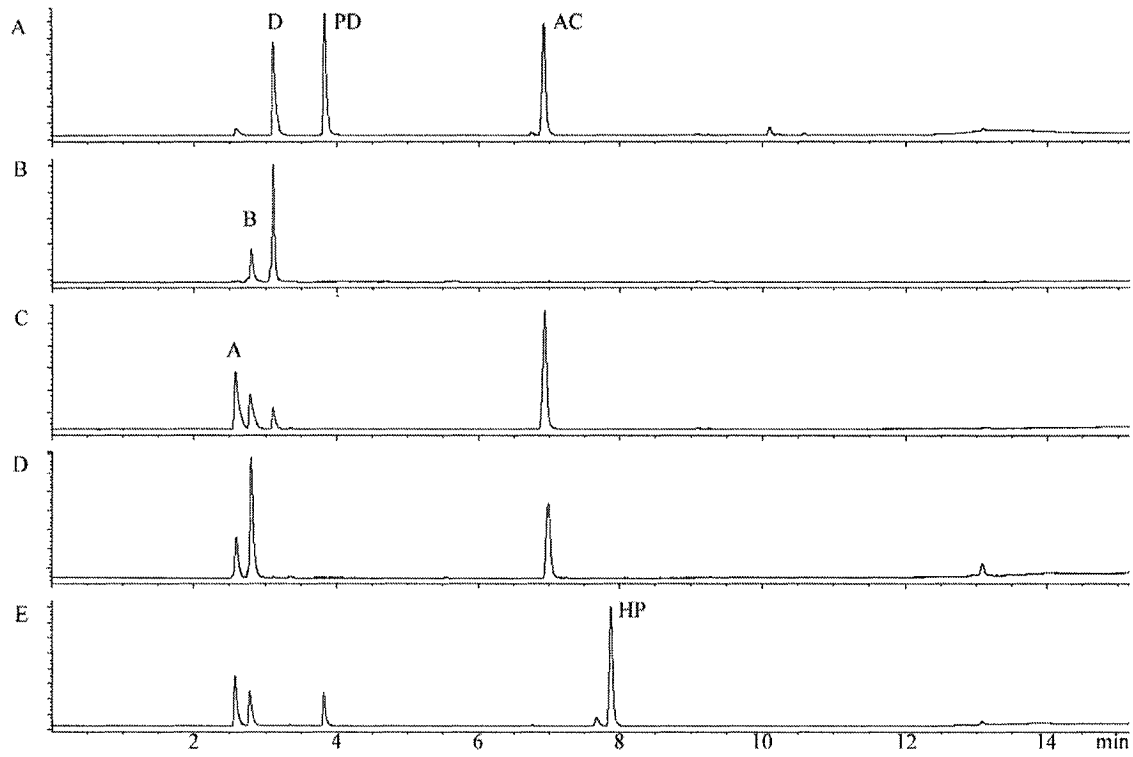

Figure 4.5 Gas chromatographic analysis of Aralp reaction products. The conversion of diacetyl (D) to acetoin (AC) by Aralp was confirmed by gas chromatography analysis of the reaction mixture. (A) Elution profile for a standard mixture containing equal amounts of diacetyl (D), pentanedione (PD), acetoin (HB) and butanediol. (B) Elution profile for reduction reaction containing 10 mM diacetyl, 0.25 nmole Aralp without NADPH. (C) Elution profile of reduction reaction containing 10 mM diacetyl, 10 mM NADPH and 0.25 nmole Aralp. Acetoin is formed as a reaction product with no apparent formation of butanediol. (D) Elution profile for prolonged reduction reaction containing 10mM acetoin, 10 mM NADPH and 0.25 nmole Aralp. A small amount of butanediol was formed. (E) Elution profile for reduction reaction with 10 mM 2,3-pentanedione (PD), 10 mM NADPH and 0.25 nmole Aralp. 3-hydroxy-2-pentanone (HP) was formed.

4.4.5 Kinetic analysis of ARA1

To asses the efficiency with which Ara1p utilized α,β–dicarbonyl substrates, kinetic analysis was performed using methylglyoxal, diacetyl, and 2,3-pentanedione in the presence of a saturating concentration of NADPH, to approximate pseudo-first order kinetics. Michaelis-Menten plots of the initial velocities *versus* substrate concentration revealed that saturation was observed with diacetyl concentration of 20 mM. Fitting the initial rate data to the Lineweaver-Burke equation, K_m values of 14.3 mM, 5.0 mM and 4.2 mM were determined for methylglyoxal, diacetyl and pentanedione respectively, suggesting that Ara1p could catalyze the reduction of these substrates in physiological conditions. Turnover rates for these reactions were 4.4, 6.9 and 5.9 s^{-1} for methylglyoxal, diacetyl and 2,3-pentanedione, respectively. The K_m value measured for NADPH using diacetyl as the second substrate was 14 μM and the k_{cat} for the reaction was 3.7 s^{-1}.

Specificity constants (k_{cat}/K_m) for the three dicarbonyl substrates (Table 4.1) suggest that Ara1p exhibits a slight preference for 2,3-pentanedione, which is largely driven by the increased binding affinity of this larger vicinal diketone. It is interesting to note that the binding affinity for these substrates is enhanced ~two-fold as the substrate length is increased by a methyl or methylene group.

Table 4.1 Kinetic Parameters for D-Arabinose Dehydrogenase

	Km (mM)	k_{cat} (s^{-1})	k_{cat}/Km
Methylglyoxal	14.3	4.4	0.30
Diacetyl	7.7	6.9	0.9
Pentanedione	4.2	5.9	1.41
NADPH	0.014	3.7	257

Attempts to determine the K_m and k_{cat} for acetoin using 200 μM NADPH at pH 7.0 and D-arabinose reduction using 200 μM $NADP^+$ at pH 7.0 were not possible since the reaction rate were extremely slow and could not be reliably measured using a spectrophotometric assay, although with prolonged incubation periods some reduction of $NADP^+$ was detected with both substrates.

4.5 Discussion

α,β-Dicarbonyls are toxic metabolic byproducts that are known to chemically react with proteins and nucleic acids, leading to protein damage and inducing mutagenesis and cellular aging (Kovacic and Cooksy, 2005; Wondrak *et al.*, 2002). Commercially, this metabolite has significant financial implications as its presence in beverages such as beer can impart an unpleasant flavor. While considerable attention has recently been focused on the deleterious effects of methylglyoxal (Morgan *et al.*, 2002; Seidler and Kowalewski, 2003; Thornalley, 1996) and the protective mechanisms used to mitigate this reactive compound (Aguilera and Prieto, 2001; Aguilera and Prieto, 2004; Hipkiss

and Chana, 1998; Wondrak *et al.*, 2002), little is known about the effect of diacetyl on cellular health and the enzymatic machinery involved in reducing this toxic compound.

The ability of Aralp to utilize diacetyl as substrate was confirmed using recombinant Aralp expressed in *E.coli*. Size exclusion chromatography indicated that recombinant Aralp formed a homodimer. Previous studies with native Aralp suggested that this protein was a heterodimer due to the appearance of 39 and 40 kDa bands on SDS-PAGE (Kim *et al.*, 1998). The possibility exists that the 39 kDa band could be a result of partial protein degradation.

Recombinant Aralp displayed vigorous reductase activity with methylglyoxal, diacetyl and pentanedione at physiological pH values. Although the concentration of diacetyl within cells is not known and likely varies as a function of the isoleucine-leucine-valine biosynthetic pathway, the low millimolar K_m values for diacetyl and pentanedione suggest that Aralp could function to eliminate these metabolites in cells grown under aerobic conditions. Aralp was found to only catalyze a single reduction step effectively, converting diacetyl to acetoin. At physiological pH the forward reaction was favored, as notable back conversion of acetoin to diacetyl was only detected at pH 10. Therefore it is unlikely that Aralp would catalyze the formation of diacetyl from acetoin within the cell. Previously Aralp was reported to catalyze the oxidation of D-arabinose, L-xylose L-fucose, and L-galactose in an $NADP^+$ dependent manner. In that study, native Aralp was shown to have high millimolar K_m values for these carbohydrates substrates with maximal turnover rates of ~160-241 s^{-1} occurring at pH 10 (Kim *et al.*, 1998). Experiments with recombinant Aralp showed that this enzyme was capable of oxidizing D-arabinose, however, the reaction rates were extremely slow at pH 7.0.

Although the apparent K_m values for diacetyl and 2,3- pentanedione are 7.7 and 4.2 mM respectively, the turnover rates for these substrates at physiological pH are sufficiently fast (~6-7 s^{-1}) to be able to catalyze the degradation of the compounds.

To elucidate the mechanism(s) responsible for diacetyl reduction, we recently purified Old Yellow Enzyme (OYE) isoforms as a principal enzyme exhibiting vicinal diketone reductase activity with both diacetyl and 2,3-pentanedione, expressed in stationary phase brewers yeast cultured under anaerobic conditions (van Bergen *et al.* submitted for publication). In this study we examined the impact of growing yeast aerobically to determine if the culture conditions alter the enzymatic mechanism by which dicarbonyl metabolites may be detoxified. In these yeasts, two major peaks of diacetyl activity were detected. The first peak eluting on a hydrophobic interaction was identified as D-arabinose dehydrogenase; while second peak, which has not yet been molecularly characterized, exhibited a chromatographic behavior analogous to Oyep. The ability of yeast to alter the enzymatic machinery involved in the metabolism of α,β-dicarbonyls in response to a variety of environmental stresses has been previously reported (Aguilera and Prieto, 2001). Aguilera & Prieto (Aguilera and Prieto, 2001) showed that addition of NaCl or H_2O_2 to logarithmic phase *S. cerevisiae*, conditions that induce an increase in methylglyoxal levels, resulted in an upregulation in *GLOI* (glyoxalase I) and *GRE3* (aldolase reductase); enzymes known to degrade this metabolite. Analysis of *ARA1* expression patterns, from DNA microarray experiments (db.yeastgenome.org/cgi-bin/expression), revealed that the *ARA1* transcript was markedly up regulated in yeast cells subjected to a variety of environmental stress that included heat shock, oxidative stress, stationary phase, diauxic shift (Gasch *et al.*, 2000), and unfolded protein response

(Travers *et al.*, 2000). A comparison of *ARA1* expression patterns with *GRE3* and *GLOI*, genes known to be up regulated during cell stress, showed a Pearson expression correlation of 0.8 suggesting that expression of *ARA1*, *GRE3*, and *GLOI* are co-regulated. Moreover, the finding that Aralp can catalyze the reduction of α,β-dicarbonyl compounds is consistent with the conjecture of Aralp having a function in adapting to cellular stress.

4.6 Acknowledgements

Anne-Marie Sdicu (McGill) is kindly acknowledged for her assistance and to Dr. Howard Bussey (McGill) for providing the BY4741 and BY4742 strains. This research is supported by a grant from the Natural Sciences and Engineering Research Council (NSERC) of Canada (JS & AJ), Canada Foundation for Innovation, New Opportunities (AJ), and Le Fonds Québécois de la recherche sur la nature et les technologies, Regroupement Strategique to the Centre for Host-Parasite Interactions.

Connecting text

Having observed two clear activity peaks in both the wild type and Δoye2oye3 knockout BY4742 yeast (see Fig. 4.2A) strains employed for enzyme purification it was necessary to identify both. In the previous chapter, the identity of the first activity peak was resolved as D-Arabinose dehydrogenase. The protein comprising the second peak displayed robust activity with diacetyl at low substrate concentrations. Kinetic analysis showed that the second, unknown protein reached saturation kinetics with 500 μM diacetyl (Fig. CT3). This was the lowest saturation concentration observed for all of the proteins that were characterised and suggested activity with low diacetyl concentrations.

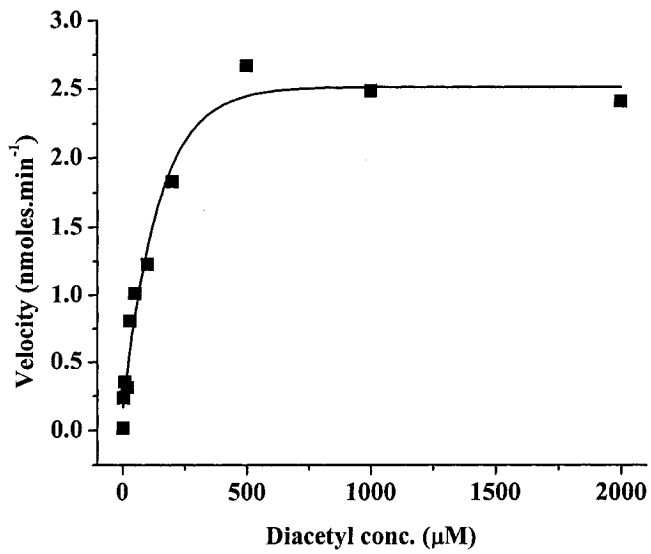

Figure CT3 Michaelis-Menten plots of the initial velocities versus substrate concentration for purified native yeast enzyme with diacetyl as substrate.

Further kinetic analysis with diacetyl as substrate yielded a Michaelis constant of 69 μM, as determined by Hanes-Woolf plot analysis (Fig. CT4). This suggests that protein X may catalyse the reduction of diacetyl at low, physiological concentrations.

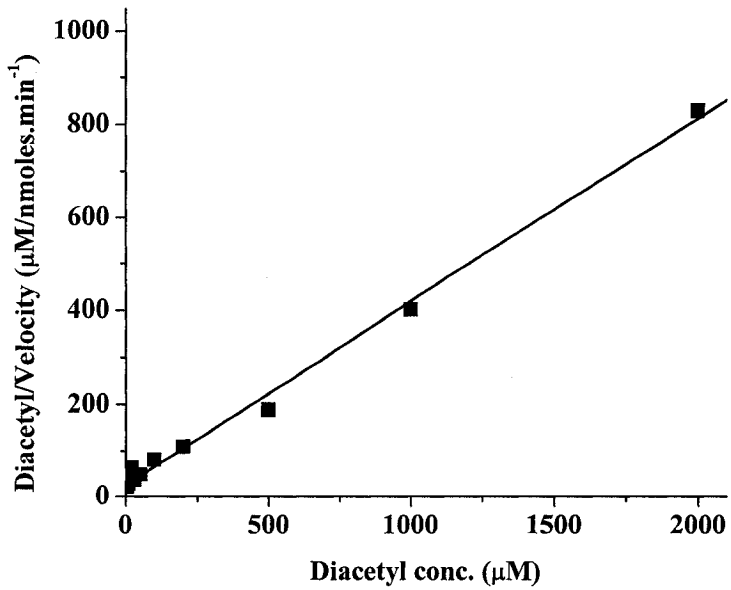

Figure CT4 Hanes-Woolf plot of kinetic results for purified native yeast protein with diacetyl as substrate.

We have now identified and characterised 2 separate mechanisms in yeast that are capable of reducing diacetyl and pentanedione (OYE and Ara1p) as well as located a significant third activity. In early experimentation these enzymes have displayed potential for application in a novel enzyme based assay for quantifying diacetyl in fermenting beer. In order to scale up the investigations and move towards commercialisation, larger amounts of recombinant proteins need to be produced.

However, most recombinant expression systems are covered by intellectual property rights and large royalties need to be paid in addition to a substantial licensing fee if the expression systems are used for commercial applications. In anticipation of an eventual scale-up in protein production we have investigated the use of an alternative, low phosphate induction mechanism as a means of circumventing these substantial charges

5. Investigations into scale-up and process development of recombinant protein production using low phosphate induction

5.1 Introduction

The Gram-negative *Escherichia coli* bacteria is the most widely used host for the production of recombinant proteins (Åkesson, 1998). Factors favouring its use include well characterised genetics (Lee, 1996), rapid growth, inexpensive growth media and the capacity to achieve high cell densities (Baneyx, 1999). Proteins that retain their activity without posttranslational modification, or that do not undergo modification are suitable candidates for recombinant production in the *E. coli* system (Lee, 1996; Makrides, 1996). Many products have been produced in *E. coli*, such as human growth hormone (Chang *et al.*, 1987), bioadhesives (Wong *et al.*, 1998), biodegradable plastics (Wang and Lee, 1998), lycopene (Alper *et al.*, 2005) and proteins native to the parasitic organisms *Schistosoma mansoni* and *Trypanosoma brucei* (Craig *et al.*, 1991).

There are numerous strategies that can be used to increase protein production. However, overproduction of recombinant protein, which normally accounts for 10 to 50% of the total cell protein (Makrides, 1996), is often accompanied by protein misfolding, followed by segregation into insoluble inclusion bodies (Baneyx, 1999), which can reduce the protein yield (Hoffmann *et al.*, 2001). While these inclusion bodies contain recombinant protein at a high level of purity, efforts required to renature the protein can often overshadow the ease of purification. It is often desirable to ensure that the protein

remains soluble. This can be achieved by slowing down the protein translation productivity (Lee, 1996) allowing more time for proper folding to take place (Baneyx, 1999) and reducing the level of unfolded protein that can aggregate.

Significant research effort has focused on high cell-density culture (HCDC) as a means of increasing the cell density, and hence product yield per unit volume (Lee, 1996; Riesenberg, 1991; Yamanè and Shimizu, 1984). HCDC is commonly achieved using fed-batch systems (Yamanè and Shimizu, 1984), during which concentrated nutrients are fed into a reactor in a controlled manner (Lee and Ramirez, 1994; Lee, 1996; Rocha and Ferreira, 2002; Wong et al., 1998; Yang, 1992), in order to prevent the accumulation of metabolic by-products, such as acetate, which inhibit cell growth and product synthesis (Farmer and Liao, 1997). Glucose uptake and utilization has been well studied in *E. coli* systems (Lin and Neubauer, 2000; Lin et al., 2001; Natarajan and Srienc, 2000; Picon et al., 2005) and the production of excess acetate can be avoided or reduced through the use of proper feeding strategies (Åkesson, 1998) and genetic manipulation (Farmer and Liao, 1997). Some of the control strategies that have been successfully employed include dissolved oxygen control (Konstantinov *et al.*, 1990; Riesenberg *et al.*, 1991), pH-stat (Suzuki *et al.*, 1990), glucose limitation (Knorre *et al.*, 1991), glucose oscillations (Lin and Neubauer, 2000) or combinations of the above (Kim *et al.*, 2004).

While many fermentations have been performed in complex media, there is a growing trend towards the use of chemically defined media for certain industrial fermentations (Zhang and Greasham, 1999). Defined medium is composed of pure chemicals in precisely known proportions and has been used for many years for microbial biochemical studies where it is necessary to achieve reproducible results, with minimal

interactions with the medium (Neidhardt *et al.*, 1974; Zhang and Greasham, 1999). Advantages of using chemically defined media include enhanced process consistency, improved control, monitoring and scale up as well as the elimination of negative phosphate regulation, which commonly occurs in secondary metabolite fermentations using complex media (Zhang and Greasham, 1999). A disadvantage, however, is that acetate accumulation can occur at lower growth rates than in complex media (Meyer *et al.*, 1984). Chemically defined medium has been successfully used in numerous *E. coli* fed-batch fermentations for the production of recombinant products (Alper *et al.*, 2005; Riesenberg *et al.*, 1991; Wang and Lee, 1998; Wong *et al.*, 1998).

Often, protein expression techniques rely on the use of isopropyl-β-D-thiogalactopyranoside (IPTG) to induce *lacZ* gene expression, which is then coupled to expression of the recombinant product of interest. An interesting, but little used expression promoter is the bacterial alkaline phosphatase (*pho*A) promoter. While other high level expression promoters often lead to insoluble, inactive or toxic protein levels within the cells, vectors containing the *phoA* promoter have been successfully used to produce satisfactory quantities of active, soluble protein where other promoters have failed (Craig *et al.*, 1991). This promoter has been used by others (Jardim *et al.*, 2000) to successfully produce several eukaryotic proteins. *phoA* codes for alkaline phosphatase and expression of this enzyme is induced when intracellular phosphate levels drop below 10 μM (Van Dien and Keasling, 1998). This enzyme is under the control of the *phoA* promoter, which blocks alkaline phosphatase expression when high phosphate concentrations are present. Controlled expression of heterologous proteins under the control of the *phoA* promoter system has been achieved using low phosphate induction

(LPI) media, which is a modified formulation of the chemically defined MOPS salts based enterobacteria media previously developed (Neidhardt *et al.*, 1974). The LPI media lacks potassium phosphate (Craig *et al.*, 1991). A problem, however, with LPI media is that despite an excellent yield of active protein, cultures only grow to a very low cell density. The result is an overall low net protein yield per litre of bacterial culture. Given the attractiveness of the *pho*A promoter expression system, an effort to boost the cell yield in LPI media would likely result in a viable alternative HCDC system for commercial production of recombinant proteins.

5.2 Methods and Materials

5.2.1 Recombinant *E.coli* strain construction

The open reading frame encoding Old Yellow Enzyme (OYE) isoform 1 was amplified *S. pastorianus* yeast cDNA by polymerase chain reaction using primer pairs 5'-CATGCCATGGCATTTGTAAAAGATTTTAAGCC-3' and 5'-CGGAATTCTTACTTTTTGTCCCAGCCTAATTTG-3' (endonuclease restriction sites are underlined). The *OYE1* open reading frame was amplified with Platinum *Pfx* DNA polymerase (Invitrogen, Burlington, ON) for 30 cycles of denaturation at 95 °C for 45 s, annealing 58 °C for 30 s, and extension at 68 °C for 75 s. PCR products were digested with *Nco*I and *EcoR*V and inserted into the corresponding sites of the pET30b(+) plasmid (Novagen, CA, USA). *E.coli* strain XL1-blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F'*proAB lacI*^{q}*ZΔM15 Tn10* (Tet^{r})]) was transformed with the plasmid and cultures grown in Luria broth containing 100 μg/ml kanamycin. Plasmid DNA was

harvested and the *NcoI*/*SalI* fragment containing the *OYE1* without the hexahistidine affinity tag cloned from the pET30b(+)-*OYE1* into the corresponding site of the pBAce expression vector (Craig *et al.*, 1991; Jardim *et al.*, 2000). *E. coli* XL1-Blue cells were then transformed with pBAce-*OYE1*.

5.2.2 Chemically defined low phosphate growth media

Batch cultures were grown using low phosphate induction (LPI) media; a modified formulation of the chemically defined MOPS salts based enterobacteria media previously developed (Neidhardt *et al.*, 1974), containing no potassium phosphate (Craig *et al.*, 1991). The composition of the modified MOPS salts and LPI media is provided in Table 5.1. 10X stock solutions were prepared with the constituents added in sequence to prevent precipitation. All media was filter sterilized and stored at 4 °C until needed.

5.2.3 Batch scale-up and standard bioreactor operating conditions

E. coli XL1-Blue cells transformed with pBAce-*OYE1* were inoculated into 100 ml of LPI media and grown at 37 °C in a shaker incubator overnight. 3.7 L BioEngineering bioreactors (Wald, Switzerland) with pH and dissolved oxygen control were prepared and sterilized in place with 900 ml of distilled water. 100 ml of 10X LPI was added after cooling to 37 °C and the media aerated prior to inoculation with 20 ml of starter culture. The culture temperature of 37 °C was monitored with a Pt100 RTD and maintained with a PID controller throughout all experimentation. Agitation was

maintained at 500 rpm with a non-enriched air flow rate of 50 L/hr delivered through a diffuser stone. Samples were collected at 1 hour intervals for analysis.

Table 5.1 MOPS salts and LPI composition

MOPS salts	Working concentration
MOPS (pH 7.4)	40 mM
Tricine (pH 7.4)	4 mM
$FeSO_4 \cdot 7H_2O$	10 μM
NH_4Cl	9.5 mM
K_2SO_4	276 μM
$CaCl_2 \cdot 2H_2O$	0.5 μM
$MgCl_2$	528 μM
NaCl	50 mM
H_3BO_3	0.4 μM
$MnCl_2 \cdot 4H_2O$	80 nM
$CoCl_2$	30 nM
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	3 nM
$CuSO_4$	10 nM
$ZnSO_4$	6.3 nM
LPI media	
MOPS salts	
Glucose	2 g/L (22 mM)
Casamino acids	2 g/L (~0.109 mM phosphate)
Thiamine	1.5 μM
Adenine	150 μM
Ampicillin	100 mg/L

5.2.4 Fed-batch growth and optimization

To optimize growth and protein production conditions, four optimisation approaches were systematically employed. Cells were initially grown in batch growth in

standard LPI in order to characterise the performance of a standard LPI system. Next, investigations were performed in order to asses the total cellular support capacity provided by nutrients in the LPI media. Batch growth experiments were performed in LPI enriched with previously identified growth limiting nutrients (Shin and Seo, 1990) carbon (glucose) and/or phosphate. Previously described HCDC feed stocks (Kim *et al.*, 2004; Lee, 1996; Wang and Lee, 1998) were used as a starting point to develop a LPI feed stock for fed-batch culturing. These feed stocks were adapted for the LPI system using insight gained from the systematic investigations above.

In order to optimise feed rates and feed stock composition in fed batch growth, a third set of investigations was performed. Starter cultures were used to inoculate media pre-enriched with glucose and non-growth limiting concentrations of phosphate and grown in batch mode to late exponential phase. Feed stock was then introduced at predetermined feed rates in order to further optimise the composition and determine the maximum linear feed rate that would balance oxygen demand with supply capability.

With the feed stock optimised, a set of investigations was conducted in fed-batch LPI media with additional glucose added prior to inoculation but with phosphate introduced with the feedstock. This allowed the determination of the maximum phosphate levels in the reactor that would balance a satisfactory growth rate with recombinant protein production. These methods are described in the following sections.

5.2.4.1 Determination of LPI support capacity and maximum growth rate in batch growth

Experiments were performed to determine the maximum supported cell concentration of *E. coli* XL1-pBAce-*OYE1* in LPI media. Literature described MOPS salts buffer to be capable of supporting up to 7 optical density unit (OD) cell cultures (at 600 nm) (Neidhardt *et al.*, 1974), yet shaker flask cultures in LPI had consistently yielded OD_{600} values of less than 1. Glucose and phosphate were identified as critical nutrients through literature surveys (Shin and Seo, 1990; Van Dien and Keasling, 1998) and were the focal point of characterisation and optimisation. Bioreactors were prepared with 900 ml of distilled water as described above. 100 ml of 10X LPI was added and inoculated with 20 ml of starter culture. Cells were grown in batch mode to early stationary phase before the addition of either glucose or monobasic ammonium phosphate in controlled amounts. LPI medium was supplemented with 10 grams per litre glucose and/or monobasic ammonium phosphate at various concentrations and the reactors inoculated with 20 ml of starter culture. Agitation was maintained between 500 and 900 rpm and non-enriched air at a flow rate of 50 to 300 l/hr, at atmospheric pressure, to maintain dissolved oxygen above 10% saturation. The pH was controlled at 6.9 for the duration of growth experiments using a proportional controller and automated addition of 0.5 M ammonium hydroxide solution. The pH controller proportional band was set at ±0.5 pH units with a dead-band of ±0.05 pH units and a feeding pulse of 0.5 s followed by a 20 s equilibration interval before any further addition of base. Samples were collected at 1 hour intervals for analysis.

5.2.4.2 Feed-stock composition and feed-rate optimisation for fed-batch growth

Glucose concentration and consumption rates remain one of the most critical factors in HCDC of *E. coli* cultures due to the so-called Crabtree effect and acetate accumulation, as a result of overflow metabolism (Åkesson, 1998; Farmer and Liao, 1997; Han *et al.*, 1992; Lee, 1996; Lin *et al.*, 2001; Natarajan and Srienc, 2000; Xu *et al.*, 1999). *E. coli* XL1-Blue cells have previously been used to manufacture poly-β-hydroxybutyrate in chemically defined HCDC (Wang and Lee, 1998). The feed stock was used as reference and adjusted to a final composition (per litre) of 500 g glucose, 20 g $MgSO_4$, 5 g proline, 250 mg thiamine and 150 mg ampicillin. Proline was added to the feed stock to accommodate overproduction of the OYE protein (which comprises 5% proline) in the proline auxotroph XL1-blue cell line. Feeding rate was optimised based on changes in dissolved oxygen uptake rates in accordance with previously developed principles (Sheppard and Cooper, 1990). Feed stock was added to the cultures while maintaining a constant oxygen supply rate. With the addition of nutrients the culture would respire more rapidly, leading to a drop in dissolved oxygen concentration. When all the nutrients were consumed, respiration would slow down and the culture would approach stationary phase. During this time the oxygen demand would decrease and the dissolved oxygen would increase. A sudden increase in DO was assumed to indicate the approach of stationary phase

5.2.4.3 Optimisation of phosphate feed rate for protein production

To investigate the effect of phosphate on cell growth and protein production, monobasic ammonium phosphate was added either to the reactors prior to inoculation, or introduced in the feed stream at rates of 1 μmole to 1 mmole per litre per hour. Nutrients were fed at a linear feed rate of 12 ml/hr of the above feed stock.

5.2.5 Sample analysis

Culture aliquots (5 ml) were withdrawn from the reactor at defined intervals and discarded prior to the collection of a further 5 ml sample for analysis.

Optical density was measured at 600 nm using an Amersham Biopharma 1000 spectrophotometer (GE healthcare, NJ, USA). Samples above 0.9 OD were diluted in 50 mM Phosphate buffer at pH 7.0 containing 100 mM NaCl.

Dry mass was determined by centrifuging an additional 20 ml sample at 10 000 x g for 20 minutes. The supernatant was discarded and the cells washed in distilled water once. After centrifugation, samples were resuspended in distilled water and quantitatively transferred to pre-weighed dishes and dried in an oven at 90 °C overnight. Samples were weighed and the dry mass per litre determined.

For protein production analysis, 50 μl of culture was centrifuged at 10,000 x g for 1 minute, the supernatant removed and the cells resuspended in 100 μl of 2X SDS sample buffer. Samples were boiled for 5 min and stored at 4 °C for later analysis by SDS-PAGE. Samples were loaded on an 8 % acrylamide gel and run at 20 milliamps for 40 min. Proteins were stained with Coomassie brilliant blue R250 and analysed.

5.3 Results

5.3.1 Batch growth in LPI

Batch growth in a bioreactor resulted in ~double the cell density when compared to shaker flask growth, reaching 1 OD in 20 h (Fig. 5.1A). Although overall growth was only slightly increased, recombinant protein produced accounted for a large portion of total cell protein (Fig. 5.1B). The long lag phase observed was likely a result of high initial dissolved oxygen concentrations providing an unfavourable environment for the microaerophilic culture (Neidhardt *et al.*, 1974). A rapid increase in growth rate was observed when DO fell below ~40% saturation (data not shown) and the lag phase could be significantly reduced through the use of a higher inoculum concentration. This is complicated with LPI, as starter cultures did not extend far beyond 0.5 OD. Alternative solutions to reduce the extended lag would be to add 10 mM $NaHCO_3$ to the inoculated reactor (Neidhardt *et al.*, 1974), or to grow a larger starter culture and concentrate prior to inoculation, or to repitch a portion of a previous high density culture. Although the later approach was tested, and reduced lag times to minutes instead of hours (see Fig. 5.2), the continual repitching of cultures would have limited reapplication to reduce the possibility of plasmid insert loss. It would therefore be essential to maintain selective pressure through the addition of a selection agent such as an antibiotic and to periodically prepare a fresh starter culture from a stabilate. The use of alternative media for starter cultures to achieve higher densities was not pursued due to the likely introduction of phosphate to the LPI system when scaling up.

Growth in LPI media is limited both by phosphate and glucose. Although the addition of phosphate leads to slight increases in cell density, these gains are severely limited by a lack of additional glucose to support further cell growth. The addition of 10 mM phosphate lead to slight increases in cell density (reaching an OD_{600nm} of ~2.0) which could be increased to ~5 OD with 5 g/l glucose or a maximum of ~8 OD with 10 g/l glucose (data not shown). Growth to any higher densities needed to be supported by additional nutrient feed, however, the addition of 10 g/l glucose and phosphate to the initial batch growth media allowed the culture to reach a significantly higher cell density prior to the start of nutrient feed. This reduced the possibility of overfeeding glucose and hence acetate accumulation.

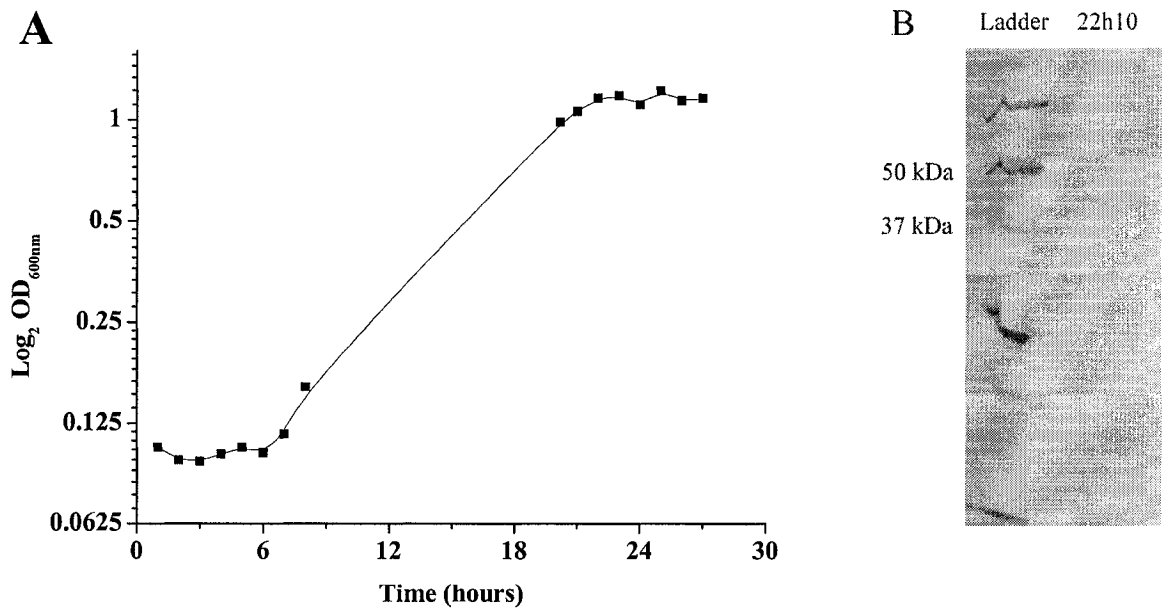

Figure 5.1 Growth and induction in standard batch LPI culture (A) growth and (B) induction at 22 hours.

5.3.2 Feed optimisation and maximum growth rate

Several feeding strategies were attempted during optimisation trials. To determine the growth ratio per unit glucose, trials were conducted with a pulsed feeding strategy, using dissolved oxygen (DO) as an indicator of metabolic activity (Sheppard and Cooper, 1990), following which optical density was checked and more feed stock added. Initial feeds comprising MOPS salts and glucose did not yield any significant increase in density and resulted in rapid acetate accumulation (data not shown). This was thought to be because the cells could not utilise the carbon quickly enough, due to the lack of other essential nutrients and hence the glucose rapidly accumulated above 2 g/l, resulting in acetate production.

Through sequential addition of various nutrients, it was determined that phosphate was the growth limiting nutrient at low cell density. The addition of optimised feed stock (500 g glucose, 20 g $MgSO_4$, 5 g proline, 250 mg thiamine and 150 mg ampicillin per litre) did not yield any density increases without the presence of phosphate. With phosphate required for growth, a set of experiments was designed to determine the cell density supported by various phosphate concentrations and sources. Monobasic ammonium phosphate supported the most rapid proliferation of cells with a doubling rate of 1 h^{-1} being obtained, with 10 g/l of glucose and 10 mM monobasic ammonium phosphate present at inoculation and a linear feed rate of 12 ml/hr. The maximum cell density obtained was 67 OD_{600} in 10 hours (Fig. 5.2), with a corresponding dry mass of 17.1 g/l. Although this represented more than a 100 fold increase in cell yield over standard LPI shaker flask cultures, the presence of such high phosphate concentrations,

however, inhibited the *phoA* promoter, as expected (Van Dien and Keasling, 1998), and the production of recombinant protein. An initial strategy had been to grow the cells in the presence of excess phosphate to a sufficiently high cell density, following which phosphate would become depleted and induction would commence. It became evident, however, that a minimal amount of phosphate was required for cellular function and protein production. The high cell density present in the reactor likely led to a rapid drop in phosphate levels and phosphate exhaustion. Once exhaustion occurred, protein production would not be able to take place, and the transient low phosphate conditions did not occur for long enough to support substantial protein production. A modified strategy was therefore developed in which phosphate would be fed into the reactor continuously, in order to maintain the low concentration of phosphate required for protein production.

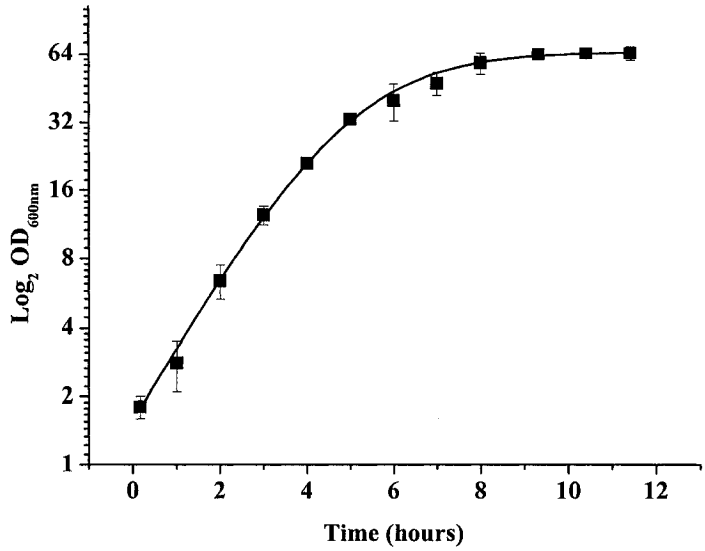

Figure 5.2 Growth in fed-batch LPI culture inoculated with 10 mM monobasic ammonium phosphate and 12 ml/hr linear feed rate. No induction was observable.

With excess phosphate present in the media cells were able to double at the maximum rate and it was therefore possible to optimise the linear feed rate to 12 ml/hr or 6 g of glucose per hour. Given the limitations of the oxygen supply system (non-enriched air), a linear feed of 12 ml/hr yielded the optimum controlled growth rate when higher cell densities were approached, allowing sufficient dissolved oxygen to be supplied to the cells without the need to switch to enriched air. For optimisation of protein production, the feed rate was therefore set at 12 ml/hr and the phosphate feed rate altered in an attempt to maintain growth while still obtaining induction using the *phoA* promoter. In order to achieve this, the phosphate levels had to remain very low to encourage induction (Van Dien and Keasling, 1998), but sufficiently high to allow satisfactory cell growth.

5.3.3 Effect of phosphate feed on cell growth and induction

Various phosphate feed rates were tested, ranging from 1 μmole/l/hr to 1 mmole/l/hr. A low feed rate of 1 μmole/l/hr did not increase cell yield in comparison to a standard LPI batch (Fig. 5.3 A), and although recombinant protein was produced, the production was negatively affected compared to the control LPI system as seen through increased non-recombinant protein production. As expected, a higher growth rate and overall cell yield is observable with a higher phosphate feed rate. Figures 5.3 B, C and D show protein production at various time points. It can be clearly seen in Figures 5.3 B and C that induction begins at ~9 to 10 hours and the recombinant protein comprises a major band by 11 hours. This production pattern is also seen at higher phosphate feed rates (Fig 5.3 D), although production onset occurred later than in lower feed rates. The protein production correlates well with a reduced growth rate and suggests that induction only occurs towards the end of growth, when phosphate concentrations are at low levels. However, compared to the control (Fig. 5.1 B) it can clearly be seen that there is a higher level of non-recombinant cellular protein manufacture in the presence of the phosphate and nutrient feed.

Protein induction was observed at later stages of growth in low phosphate feed rate cultures, associated with a lower growth rate. Although non-specific protein production increased with increased phosphate feed, a higher cell density could possibly compensate for the decrease in specific productivity. In order to test this hypothesis, the phosphate feed rate was increased, yielding higher initial growth rates and overall cell yields. Figures 5.3 through 5.5 display growth curves and cellular protein production with increasing phosphate feed rates. It can clearly be seen that increasing the rate of

phosphate addition impacts the overall cell yield and recombinant protein manufacture. Interestingly, higher feed rates of 400 μmol/l/hr and 1mmol/l/hr still yielded some recombinant protein, although initial growth was unbridled. Despite recombinant protein production, the relative yield was very negatively affected by general cellular protein production. This was similar to results obtained for growth with high initial concentrations of phosphate, for example 10 mM ammonium phosphate monobasic. While all fed-batch cultures lead to increased amounts of non-recombinant protein manufacture within the cell, a feed rate of 200 μmol/l/hr yielded satisfactory recombinant protein induction (Fig. 5.5 B). While a density of only ~7 OD was obtained – an order of magnitude lower than that obtained for growth with 10 mM phosphate – recombinant protein production was satisfactory and comprised the dominant band when analysed by SDS-PAGE. Figures 5.5 C and D show that although increased feed rates beyond 200 μmol/l/hr yielded higher cell numbers, recombinant protein production was negatively affected.

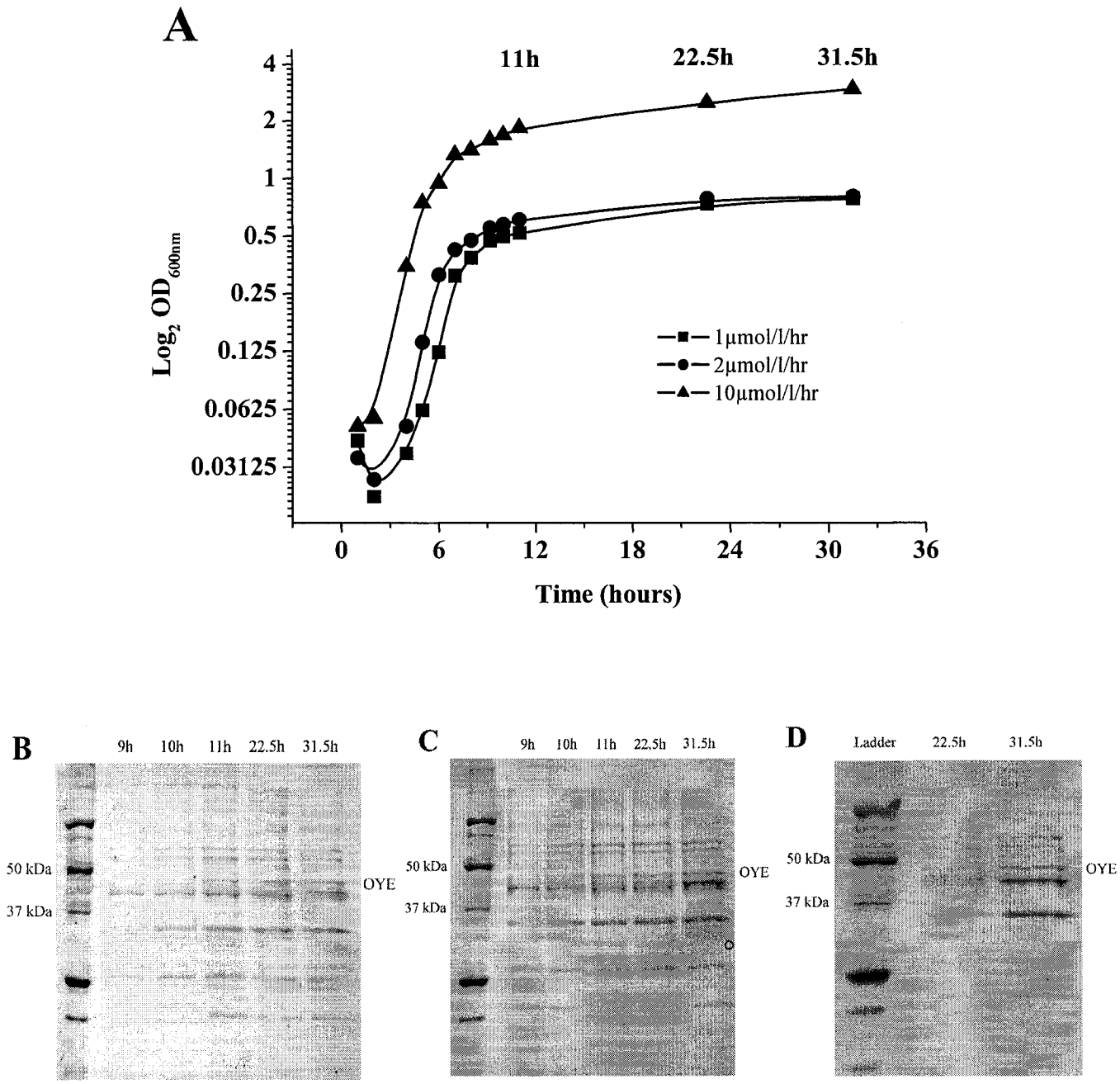

Figure 5.3 Growth and induction in LPI media with constant phosphate feed rate of 1, 2 and 10 µmol/l/hr. (A) Growth curves, (B) recombinant protein production with 1 µmol/l/hr, (C) recombinant protein production with 2 µmol/l/hr and (D) production with 10 µmol/l/hr.

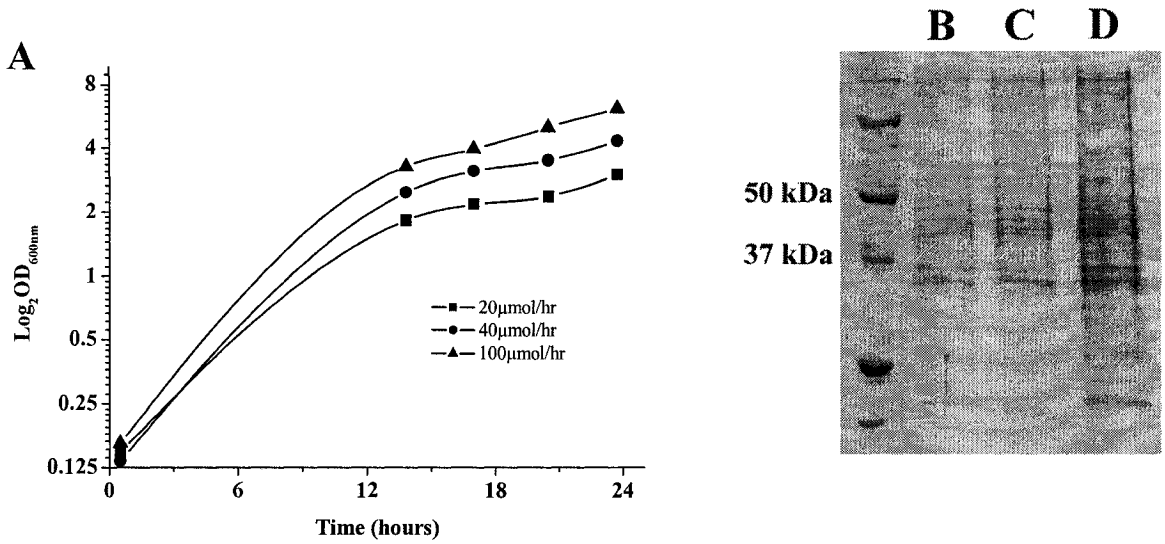

Figure 5.4 Growth and induction in LPI media with constant phosphate feed rate of 20, 40 and 100 µmol/l/hr. (A) Growth curves, (B) recombinant protein production with 20 µmol/l/hr, (C) recombinant protein production with 40 µmol/l/hr and (D) production with 100 µmol/l/hr, all at 20hr.

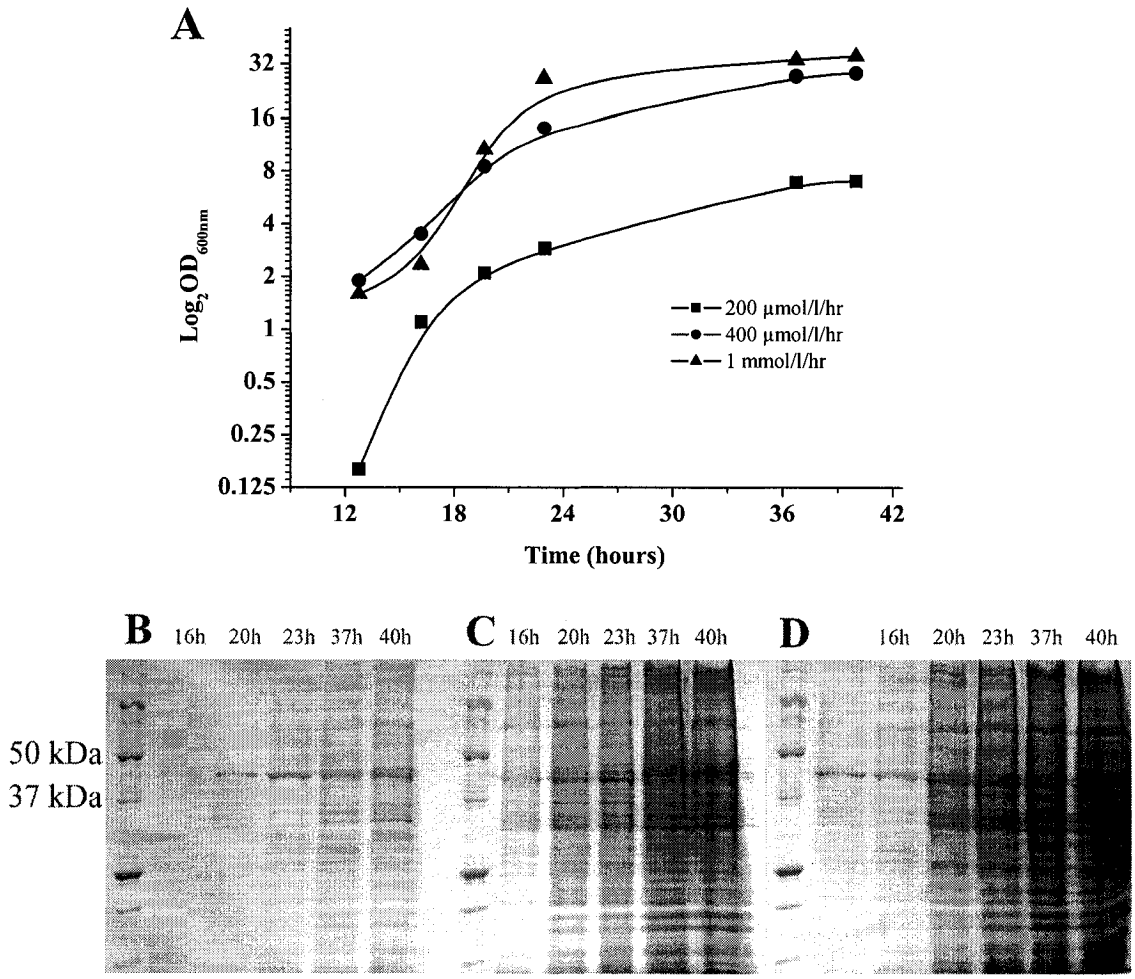

Figure 5.5 Growth and induction in LPI media with constant phosphate feed rate of 200, 400 and 1000 µmol/l/hr. (A) Growth curves, (B) recombinant protein production with 200 µmol/l/hr, (C) recombinant protein production with 400 µmol/l/hr and (D) production with 1 mmol/l/hr, all at 40hr.

Fermentations were terminated a period of time after a change in growth rate and the approach of stationary phase. An analysis of the final optical density obtained versus phosphate feed rate (Fig. 5.6) yielded an interesting observation that correlated well with protein production. When feed rate was plotted on a Log_{10} scale, an almost linear correlation is observed between overall cell yield and feed up to 200 µmol/l/hr. At higher

feed rates a significantly higher cell yield was obtained, but without a relative increase in recombinant protein yield. This increased cell yield is partly attributable to a higher cell growth rate, which is directly related to the amount of phosphate present in the feed. It would appear, therefore, that phosphate feed rates higher than 200 µmol/l/hr positively affect growth rate at the expense of protein production. This is likely due to the amount of phosphate being fed into the reactor being consumed sufficiently rapidly to allow an earlier onset of induction and the maintenance thereof for a longer period of time.

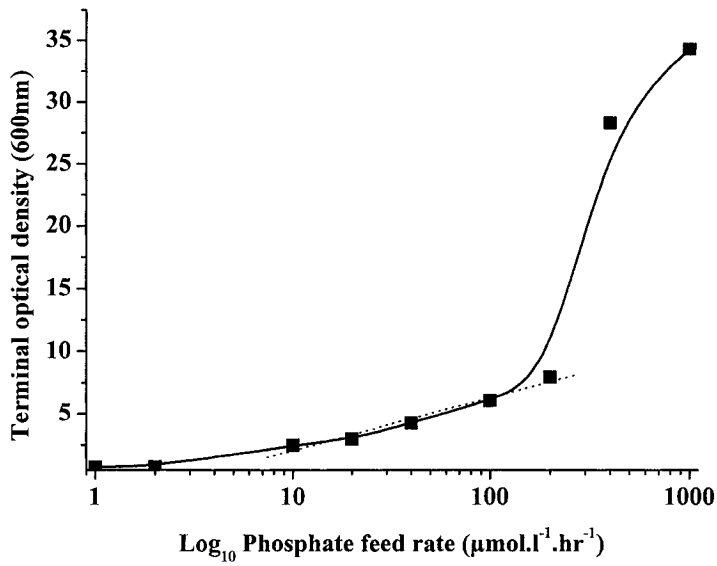

Figure 5.6 Analysis of final cell yield versus phosphate feed rate. A dramatic change in yield is observed for feed rates higher than 200 µmol/l/hr.

5.4 Discussion and Conclusion

The low phosphate induction system is a promising alternative to other more commonly used induction systems such as the T7 promoter. Relative recombinant protein production obtained in standard batch LPI cultures is excellent when compared with other promoter systems, but total recombinant protein yield is negatively affected by low cell yields. Clearly then, increasing the cell yield, while still maintaining similar levels of recombinant protein production, would make recombinant protein manufacture using the *phoA* promoter system more competitive and viable for larger scale operations. Here we have explored various strategies in an attempt to improve overall yields in LPI media.

~100 fold increases in cell density were obtained in LPI, with millimolar concentrations of phosphate added and operated in fed batch relative to standard LPI batch fermentations. Little or no recombinant protein was, however, produced during these fed batch operations as a result of high promoter-suppressing concentrations of phosphate during the majority of the growth phase and a short transient low phosphate concentration just prior to exhaustion. It is therefore essential to maintain a minimum phosphate level within the reactor in order for cellular mechanisms to function correctly, but to ensure that this level does not exceed the *phoA* promoter suppressing concentration.

The maintenance of phosphate above critical levels could be achieved by introducing phosphate into the reactor feed stream. The maintenance of phosphate feed rates at or below 200 μmol/l/hr lead to satisfactory recombinant protein expression as well as approximately a six to ten fold increase in cell density. A higher cell density with

protein expression was achievable using high feed rates, but was negatively affected by the production of large amounts of general cellular proteins. It would appear from the various trials that the critical factor in recombinant protein production is the establishment and maintenance of a second, slower growth rate, limited by the concentration of phosphate present in the reactor vessel. A longer maintenance of this slower, phosphate limited growth rate resulted in increased recombinant protein production.

While prolonged maintenance of slower, phosphate controlled growth rates resulted in increased recombinant protein production, the adoption of this recombinant protein production system on an industrial scale will require further analysis of complexity, time and cost factors. Batch LPI cultures are commonly inoculated, grown overnight and harvested the next morning, for a total growth time of approximately 12 to 15 hours. This system, if performed at volumes of 500 ml or less requires no feed pumps, bioreactors or modified aeration systems as cell growth rates and hence oxygen demand are low. This significantly reduces the operating costs and complexity compared to high cell density cultures but also reduces the yield per unit time and volume. At a feed rate of 200 μmol/l/hr, it was possible to increase the cell density six to ten fold in double the time and still maintain a comparable recombinant protein to overall cellular protein ratio.

To further optimise the yields and efficiency of the LPI recombinant protein production systems, further strategies need to be explored. An alternative feed strategy to further optimise the system would be the use of an exponential fed-batch mechanism to maintain optimised phosphate levels within the reactor in the presence of higher cell densities. This technique has been applied elsewhere to maintain cell doubling rates at higher densities (Lee, 1996) and could possibly be adapted to a phosphate controlled

growth system. Another mechanism would be to inoculate and grow recombinant cells in a batch mechanism with a high initial phosphate level to suppress recombinant protein production but encourage maximum cell growth. As stationary phase is approached, phosphate could be added to the feed stream to encourage further, controlled growth. These requirements as well as a satisfactory residence time could be provided with a continuous chemostat-type culture system. These mechanisms are however complex and require further lengthy investigation, optimisation and the use of advanced controllers and feed-back systems which were not available for the LPI studies conducted above. These additional systems could significantly add to the cost of manufacture, but further investigation would be required to determine the pay-back time of these systems when increased yields per unit time are considered. The advantages of using a chemostat would include predictable, continuous protein yields per unit time as well as a reduction in maintenance and cleaning costs as the system could be run for longer periods of time between shut-downs.

An important factor that should possibly be considered when determining overall benefits of the LPI system is that virtually all other induction and expression systems are protected, and royalties need to be paid on all products manufactured for commercial purposes using these systems. This can add several thousand dollars as a base price to any manufacture plus a percentage of operating profits and should be weighed in to any manufacturing decision.

Connecting text

In addition to the diacetyl reductase enzymes having a potential application in a novel enzyme based assay, we also wanted to return to the breweries and investigate diacetyl production and reduction in industrial fermentations. We approached a large commercial operation with annual production in excess of 400 million litres and established cooperation. Samples were collected from both ale and lager fermentations and the profiles of various parameters and metabolites were monitored. The ale and lager fermentations were dramatically different and the results are reported in the following chapter.

6. Diacetyl metabolism in brewing yeast

6.1 Introduction

The vicinal diketones (VDKs) diacetyl (2,3-butanedione) and 2,3-pentanedione are well known (Haukeli and Lie, 1972; Wainwright, 1973) flavour active compounds in beer. Diacetyl is of particular importance due to the undesirable butterscotch-like aroma it imparts in beer and a very low taste threshold of < 100 parts per billion (ppb) (Yonezawa and Fushiki, 2002). 2,3-Pentanedione possesses similar aroma properties, but a significantly higher flavour threshold and demands less attention from brewers.

The diacetyl and pentanedione precursor metabolites, α-acetolactate and α-acetohydroxybutyrate respectively, are produced during fermentation by yeast (Haukeli and Lie, 1972) as intermediates during biosynthesis of the branched amino acids isoleucine, leucine, valine via the regulated ILV pathway (Fig. 6.1) (Holmberg and Petersen, 1988). The overall pathway activity, and hence precursor and VDK formation, is partly affected by the concentration of isoleucine, leucine and valine present in the wort (Holmberg and Petersen, 1988; Petersen *et al.*, 2004; Xiao and Rank, 1990).

α-Acetolactate and α-acetohydroxybutyrate can undergo non-enzymatic decarboxylation within the cell (Park *et al.*, 1995) or are exported into the fermenting wort (Gjermansen *et al.*, 1988) where decarboxylation occurs, producing diacetyl and pentanedione. The mechanisms driving the extracellular excretion of α-acetolactate are not well understood. Several studies have shown that up-regulation or overexpression of acetohydroxyacid reductoisomerase (ILV5), the enzyme responsible for forward catalysis

of acetolactate to 2,3-dihydroxy-isovalerate reduces the levels of α-acetolactate detectable in wort (Dillemans *et al.*, 1987; Gjermansen *et al.*, 1988; Mithieux and Weiss, 1995; Villa *et al.*, 1995). Since genetic manipulation of yeast strains remains unacceptable in most markets, further research to determine environmental factors that regulate ILV5 activity, and thus diacetyl production, is required.

In wort, α-acetolactate decarboxylation is a relatively slow reaction which is influenced by factors such as alcohol concentration, pH and temperature (Kobayashi *et al.*, 2005). It is generally assumed that the precursors are decarboxylated in the wort and the VDK products re-enter the yeast where they are enzymatically reduced. The role of yeast in VDK elimination is strengthened by the observation that reduction ceases following yeast removal from beer. It is unknown whether diacetyl (or α-acetolactate) uptake is an active or passive mechanism. It is generally accepted that enzymatic reduction proceeds via a sequential mechanism that converts diacetyl to acetoin (3-hydroxy-2-butanone) - a more flavour-neutral compound with a significantly higher flavour threshold - and then 2,3-butanediol. Similarly, 2,3-pentanedione is reduced to 3-hydroxy-2-pentanone and then 2,3-pentanediol. Several enzymes may be responsible for this process, catalyzing either a single or both reduction steps (Bamforth and Kanauchi, 2004; Hardwick *et al.*, 1976; Tolls *et al.*, 1970; van Bergen *et al.*, 2006a; Van Bergen *et al.*, 2006b).

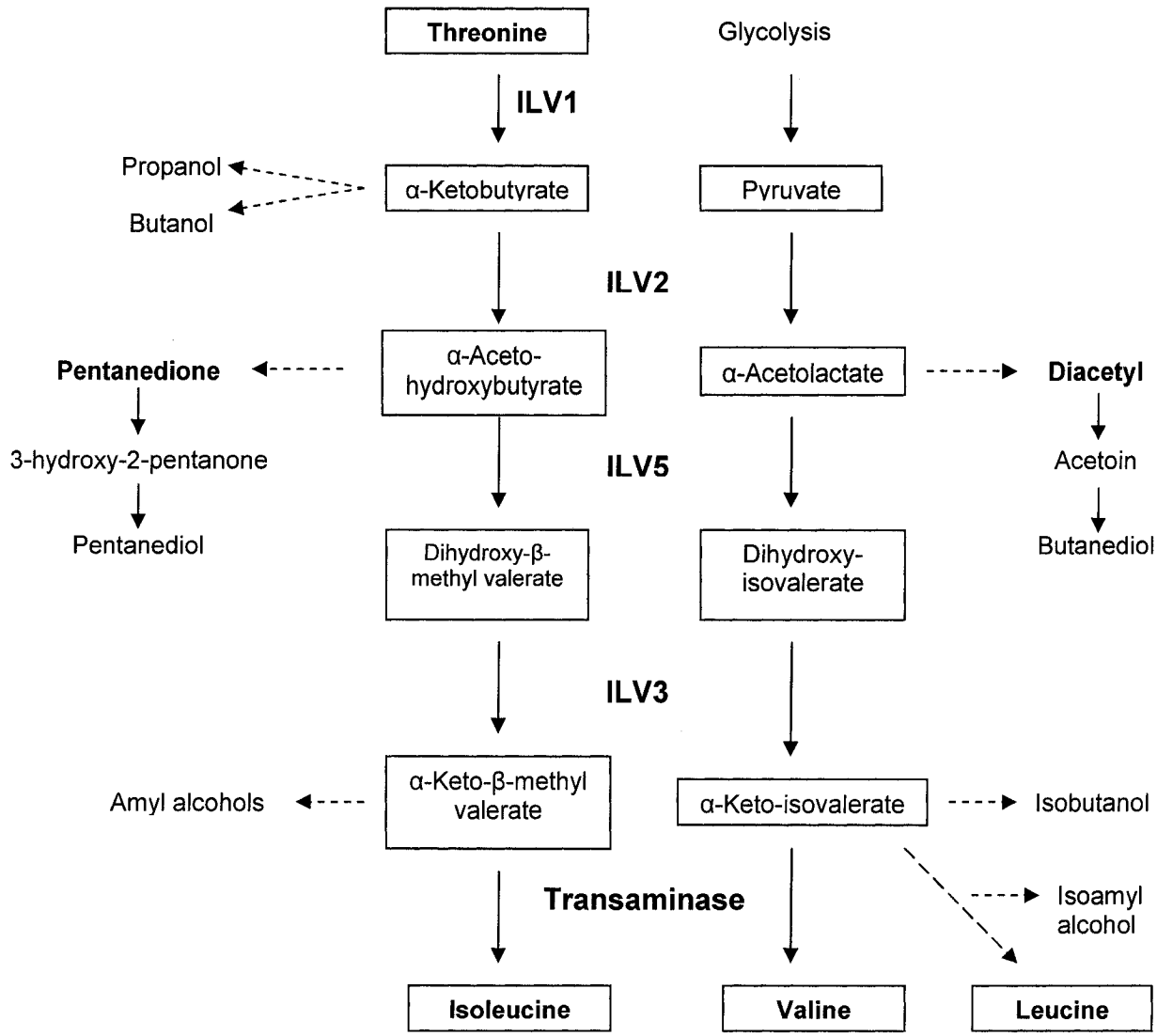

Figure 6.1 The ILV pathway and all related metabolic products

As a result of a rapid VDK uptake by yeast cells and slow precursor degradation, only low levels of free diacetyl are detected in maturing product. Significantly higher

levels of α-acetolactate and α-acetohydroxybutyrate are detectable following fermentation (Landaud *et al.*, 1998). To ensure that diacetyl remains below the flavour threshold throughout the product shelf life, it is essential that the VDKs and their precursors be reduced to below taste threshold values prior to filtration. The levels of these compounds are typically determined using headspace analysis gas chromatography with an electron capture detector, although this technique has displayed high result variance in collaborative trials (Convention, 1998). Commonly, the specified final diacetyl concentration in matured beer is 20-50 ppb. Accurate determination of diacetyl below threshold values remains difficult due to the complex nature of the medium being analyzed.

α,β-Dicarbonyls such as diacetyl, 2,3-pentanedione and methylglyoxal, are highly reactive compounds that can react with arginine, lysine, and cysteine protein residues resulting in damage and cross linking of proteins (Lundblad, 1995). In higher eukaryotic organisms methylglyoxal and diacetyl have been implicated in a number of diseases (Brownlee, 1995; Kovacic and Cooksy, 2005) including general cellular deterioration and aging (Wondrak *et al.*, 2002). Several proteins have been shown to be susceptible to chemical modification (Hipkiss and Chana, 1998; Lerant *et al.*, 2000), including glyceraldehyde phosphate dehydrogenase (Morgan *et al.*, 2002) – an essential enzyme in glycolysis. Removal of these dicarbonyl compounds is therefore essential for maintenance of cellular health.

Despite the industrial and physiological significance of diacetyl, little is known about the yeast enzymes involved in reduction. Historically, yeast alcohol dehydrogenase was shown to catalyze diacetyl reduction (Hardwick *et al.*, 1976), but the high Michaelis

constant (K_m) of 250 mM (~21.5 parts per thousand) suggests that this enzyme would exhibit an extremely slow catalytic efficiency with physiological concentrations. Other enzymes have been purified exhibiting significantly lower K_m values of 2 mM (~172 ppm) and 2.3 mM (~200 ppm) with diacetyl as substrate (Heidlas and Tressl, 1990). However the identity of these proteins remains unknown. Ypr1p, an aldo-keto reductase from bakers yeast, was shown to catalyze diacetyl reduction (Nakamura *et al.*, 1997) and others have investigated diacetyl reductase activity in partially purified enzyme fractions from ale (*S. cerevisiae*) and lager (*S. pastorianus*) yeast (Bamforth and Kanauchi, 2004). Recently two diacetyl reductase activities exhibiting low K_m were purified from *S. cerevisiae* cultures and shown to be Old Yellow Enzyme (OYE) (van Bergen *et al.* submitted for publication) and D-arabinose dehydrogenase (Ara1p) (Van Bergen *et al.*, 2006b).

In an effort to gain further understanding of diacetyl production and reduction in brewing systems, we have analyzed industrial ale and lager fermentations, producing different diacetyl and pentanedione profiles. Samples were collected and the amino acid consumption and diacetyl production profiles determined, along with the activity of several genes of interest.

6.2 Materials and methods

6.2.1 Fermentations and sample collection

Commercially available wort was prepared at 16.2 °P and inoculated with ale yeast at a pitching rate of 1×10^7 cells/ml. The wort was fermented at 20 °C in 700 hl

horizontal fermenters. Standard commercial lager wort was prepared at 17.8 °P, yeast pitched at 2×10^7 cells/ml and fermented at 14 °C in 5000 hl cylindroconical vessels. Samples were collected from all fermenters at 12 hour intervals. Initial lager samples were collected when the vessel was completely filled (six brews). Samples were immediately transported to the laboratory and fractionated. 50 ml was transferred to a falcon tube and immediately frozen in dry ice before storage at -80 °C until further analysis was performed. The remainder of the sample was immediately used to determine specific gravity, pH and viable cell concentration.

6.2.2 Vicinal diketone analysis

Samples stored at -80 °C were thawed, fractionated and immediately filtered through a 0.45 μm filter. α-Acetolactate and α-acetohydroxybutyrate were decarboxylated to form diacetyl and pentanedione respectively by adding 0.15 mM $FeSO_4$ and 0.15 mM $FeCl_3$, vigorously agitating and heating the sample at 80 °C for 10 min. Samples were cooled, derivatized with o-phenylenediamine dihydrochloride, loaded onto a Gemini C-18 column (150 x 4.6 mm, 5 μm) (Phenomenex, Torrance, CA) and eluted using a two step isocratic gradient. Diacetyl and pentanedione were quantified using high performance liquid chromatography coupled with electro-spray mass spectrometry (LCMS) (Agilent, Palo Alto, CA), as described elsewhere (Blanchette *et al.*, 2006).

6.2.3 Amino acid analysis

Free amino acids in beer filtrates were determined by derivatizing with o-pthalaldehyde according to product instructions (Pierce Biotechnology Inc., Rockford, IL) (Roth, 1971), loaded onto an Eclipse XDB-C18 column (150 × 4.6mm, 5 μm) (Agilent Technologies, Palo Alto, CA) equilibrated with 40% methanol in phosphate buffer (pH 6.6) and resolved at a flow rate of 1 ml/min. in 24 min. while ramping the organic solvent concentration to 62%. Isoleucine, leucine and valine were quantified using an 1100 series HPLC (Agilent, Palo Alto, CA), coupled with fluorescence detection (Gardner and Miller, 1980; Lindroth and Mopper, 1979).

Elution position of the derivatized amino acids was compared to a standard mixture containing isoleucine, leucine and valine.

6.2.4 RNA preparation

Cell pellets from a 5 ml sample were washed twice with 1 ml of ice cold sterile distilled water then resuspended in 1 ml Trizol reagent (Invitrogen, Burlington, ON) with 200 μl of 425-600 μm glass beads (SigmaAldrich, Mississauga, ON) and vortexed for 3 min prior to 5 min incubation at 65 °C. This procedure was repeated 3 times. Samples were cooled on ice for 5 min then centrifuged at 14,000 x g for 5 min at 4 °C. The aqueous phase was transferred to a clean microcentrifuge tube and extracted twice with 200 μl of chloroform with vigorous agitation, incubation at 20 °C for 3 minutes and then centrifuged at 12 000 x g for 15 min at 4 °C. RNA was precipitated from the aqueous phase with 0.6 volumes of isopropyl alcohol. Following a 10 minute incubation at 20 °C,

samples were centrifuged at 12 000 x g for 10 min at 4 °C and the pellet washed in ice cold 70% ethanol. Pellets were resuspended in 50 µl sterile distilled water, quantified, treated with RNAse free DNAse and stored at -80 °C until needed. RNA samples were reverse transcribed to cDNA using Omniscript RT (Qiagen, Mississauga, ON) prior to analysis.

6.2.5 Real-time PCR analysis

RNA levels of *ILV2*, *ILV5*, *OYE1* (in lager samples only), *OYE2*, *ARA1* and *PRX1* in the time course samples were normalised to the housekeeping gene ribosomal RNA (*RDN18*). Primer sets for each gene (Table 6.1) were purchase from Operon Biotechnologies (Huntsville, AL) and Sybr Green was purchase from Qiagen (Mississauga, ON). Fragments were denatured at 95 °C for 90 s and amplified with 45 cycles of denaturation at 95 °C for 15 s, annealing at 50 °C for 30 s, and extension at 72 °C for 30 s. Amplification, quantification and analysis were performed using an ABI 7500 (Applied Biosystems, Foster City, CA).

Table 6.1 Oligonucleotide primer sequences for quantitative real time PCR analysis

Primer	Primer sequence
RDN18 – forward	5'-GTTGGTGGAGTGATTTGTCT-3'
RDN18 – reverse	5'-AGACCTGTTATTGCCTCAAA-3'
ILV2 – forward	5'-AGCTGGTACTCCAGTGAAGA-3'
ILV2- reverse	5'-TAGCCTCCGCTAGTTTTATG-3'
ILV5 – forward	5'-ACGGTGAAAGAGGTTGTTTA-3'
ILV5 – reverse	5'-CCGATCAATGGGTATAGAGA-3'
OYE1 – forward	5'-TGATCGGTTACGGTAGATTC-3'
OYE1 – reverse	5'-CTTCATAGGTGGGGTAGTCA-3'
OYE2 – forward	5'-TGAATACTACGCTCAACGTG-3'
OYE2 – reverse	5'-TTGAAAATCTTGGTCCATTC-3'
ARA1 – forward	5'-CCGACGGTGACTATTTAGAA-3'
ARA1 – reverse	5'-GTTGGCTTAACTCTGCATTC-3'
PRX1 – forward	5'-GCAAAGGTTAAAAATGTTGG-3'
PRX1 – reverse	5'-ATGACGAAAACAGACCTCAC-3'

6.3 Results

6.3.1 Fermentations

Ale (*S. cerevisiae*) fermentations proceeded rapidly at 20 °C and reached a terminal gravity of 4 Plato by 72 h with a corresponding drop in pH from 4.6 to 3.6 (Fig. 6.2 A). The vigorous fermentation was accompanied by a rapid drop in group B free amino acids, with all available isoleucine, leucine and valine being consumed within 24 h

(Fig. 6.2 B). Analysis of the wort showed rapidly increasing diacetyl and pentanedione concentrations peaking at ~1300 ppb and ~600 ppb respectively by 24 h (Fig. 6.2 C) when all the group B amino acids had been consumed. Both diacetyl and pentanedione were reduced to taste threshold values within ~72 h. The rate of change in diacetyl (α-acetolactate) production corresponded with a similar change in valine consumption rate (Fig. 6.2 D).

In contrast, lager (*S. pastorianus*) fermentations proceeded much more slowly, reaching terminal gravity within ~120 h at 14 °C. The change in pH was similar to that observed in ale fermentations, dropping more rapidly than the gravity, to below pH 4, and then gradually returning to a less acidic value during maturation as organic acids were consumed (Fig. 6.3 A). Following an initial increase in wort valine concentration, free group B amino acids were consumed within ~72 h, which was significantly slower (~3 fold) than in the ale fermentations (Fig. 6.3 B) and is likely due to slower cellular metabolic and proliferation rates associated with the lower temperature. The evolution of diacetyl and pentanedione corresponded to the loss of free amino acids in the wort, at ~72 hours (Fig. 6.3 C). In contrast to the ale fermentations, the VDK concentration profiles were similar, reaching a similar peak concentration of ~750 ppb and displayed a slight shoulder during early the early stage of production, suggesting a change in the metabolic processes responsible for producing and reducing these compounds. This shoulder is clearly evident when the data is replotted as a function of the rate of change of α-acetolactate (potential diacetyl) released into the wort (Fig. 6.3 D). In these plots, a characteristic double peak was also observed for valine consumption rate.

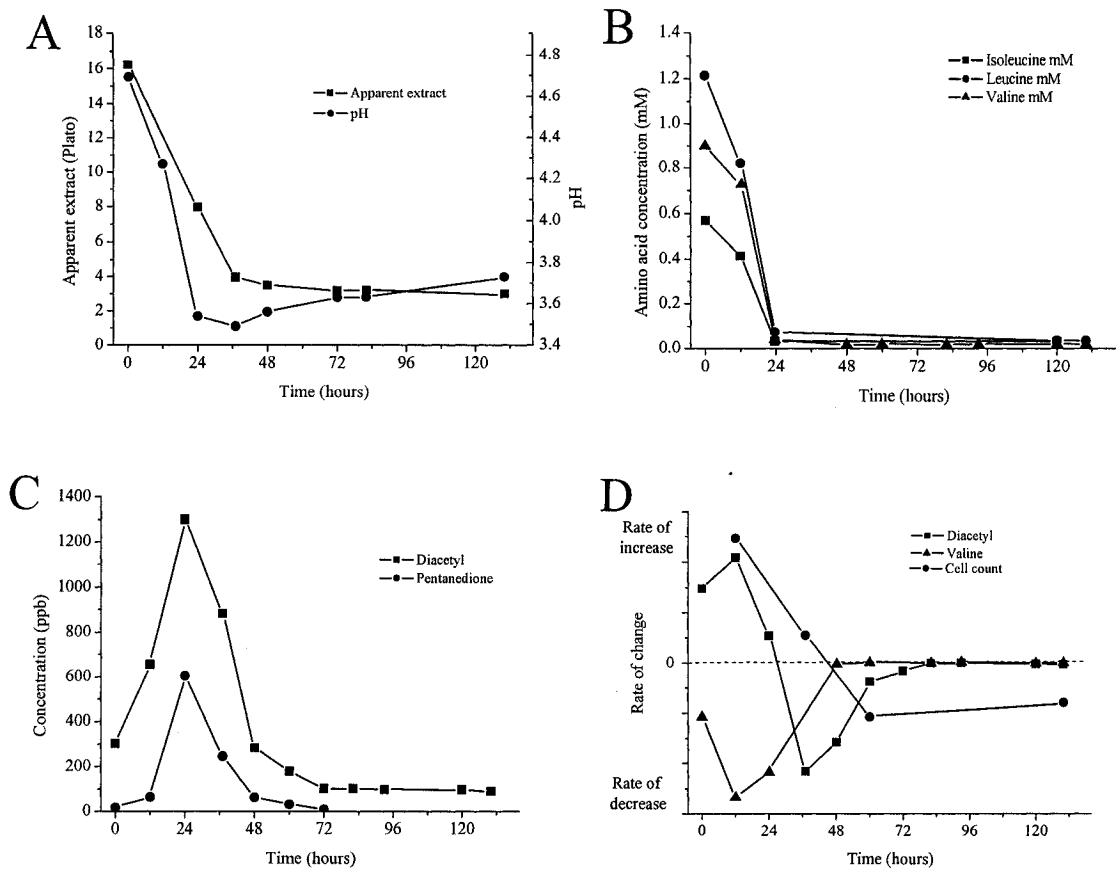

Figure 6.2. Ale yeast fermentation profile at 20 °C. (A) Apparent extract and pH profiles, (B) isoleucine, leucine and valine profiles, (C) diacetyl and pentanedione profiles and (D) rates of change in valine consumption, diacetyl production and cell concentration.

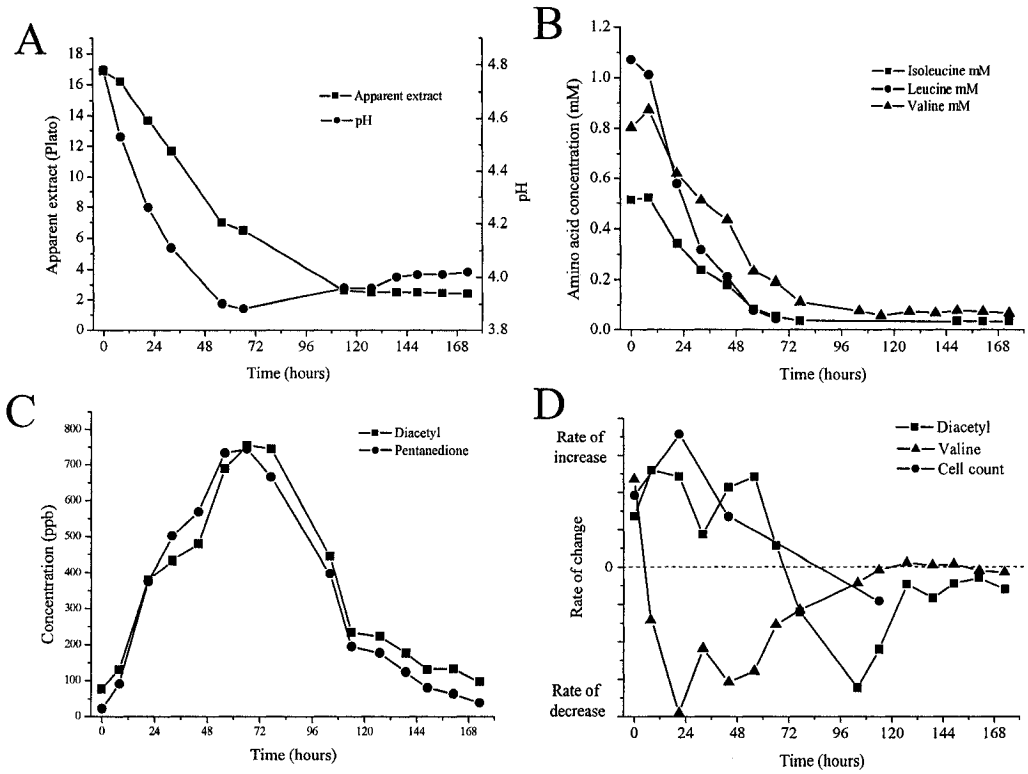

Figure 6.3. Lager yeast fermentation profile at 14 °C. (A) Apparent extract and pH profiles, (B) isoleucine, leucine and valine profiles, (C) diacetyl and pentanedione profiles and (D) rates of change in valine consumption, diacetyl production and cell concentration.

6.3.2 RNA expression analysis

To biochemically understand the metabolic pathways involved in the production and detoxification of the vicinal diketone metabolites, real-time PCR was employed to examine the levels of mRNA transcripts for the genes *ILV2*, *ILV5*, *OYE1*, *OYE2*, *ARA1* and *PRX1* as a function of fermentation age. In these studies the levels of the transcripts for these genes were normalised to 18S ribosomal RNA. The levels of each transcript of yeast culture sampled at time zero was set to an arbitrary value of 1 and changes in expression were compared to this zero point.

In Ale yeast, RNA's encoding enzymes involved in amino acid biosynthetic activity (*ILV2* & *ILV5*) displayed maximal expression at the time of pitching but rapidly decreased 3-fold and 10-fold by 12 and 48 h respectively (Fig. 6.4 A and B) when all of the extracellular levels of group B amino acids were depleted. *ILV2* and *ILV5* both displayed similar expression patterns across the fermentation which correlated well with the termination of primary fermentation activity. Although the ale fermentation was rapid, it suggests that synthesis of the precursors for group B amino acids occurred throughout the uptake of isoleucine, leucine and valine from the wort. This augments the observation that diacetyl and pentanedione were produced within the first 24 hours, despite the uptake of amino acids during that period. After 48 h, when no significant increase in cell biomass was detected, the ILV genes were down-regulated since the need for amino acids was diminished as cells entered stationary phase. However, ILV genes remained active at a diminished level and low levels of α-acetolactate remained present in the fermented beer throughout later stages of maturation (Fig. 6.2 C). *OYE2* gene activity appeared to behave in a similar manner to the amino acid biosynthesis genes, with maximal activity during initial fermentation and cell growth and a 10-fold down-regulation within 24 h (Fig. 6.4 C). *OYE2* remained active at a low level of expression for the duration of the fermentation, in contrast to *ARA1* and mitochondrial peroxiredoxin *PRX1*, both of which displayed up-regulation within 24 h of pitching (Figs. 6.4 D and E). The up-regulation of *ARA1* and *PRX1* correlated with the level of α-acetolactate present within the wort, with both genes displaying reduced activity as α-acetolactate levels decreased.

The colder and slower lager fermentations yielded different gene activity patterns as compared to the warmer and more rapid ale fermentations. As opposed to the ale fermentations, peak wort α-acetolactate concentration was reached in slightly less than 3 days (Fig 6.3 C). A distinct shoulder is visible during acetolactate evolution, suggesting a temporary slowing of production by the isoleucine-leucine-valine biosynthesis pathway (Fig. 6.3 D). This observation correlated well with the apparent changes in *ILV2* and *ILV5* activity (Figs. 6.5 A and B). Both amino acid biosynthesis genes displayed a slight down-regulation in activity within 24 h followed by a subsequent up-regulation, peaking at ~48 h. This pattern of metabolic events appeared to correlate with a change in acetolactate evolution pattern. However, these events were offset by ~12 h lag period. Both *ILV2* and *ILV5* remained active with a lower level of gene expression throughout maturation although expression patterns were different compared to the Ale yeast. In Lager yeast, *ILV5* displayed a higher level of expression than Lager *ILV2* during fermentation and maturation. Ale yeast *ILV5* displayed similar expression levels to Ale *ILV2*, and produced higher wort acetolactate concentrations.

Although diacetyl reductase enzymes displayed similar up-regulation, down-regulation, up-regulation, down-regulation cycles, these patterns were notably different from those of both the ILV pathway genes and the Ale yeast diacetyl reductases. Both *OYE1* and *OYE2* displayed up-regulation within 24 hours (Figs. 6.5 C and D) with *OYE1* displaying maximal expression activity at 48 hours and maximum *OYE2* expression occurring at 24 hours. Although *OYE2* displayed a greater up-regulation at its maximum, *OYE1* expression levels remained higher than *OYE2* for the duration of the fermentation. As opposed to *ARA1* and *PRX1* activity in Ale yeast fermentations, which displayed ~4.5

and ~6.5 fold up-regulation (Figs. 6.4 D and E), Lager yeast fermentations displayed only a slight *ARA1* up-regulation (Fig. 6.5 E) and down-regulation of *PRX1* during fermentation.

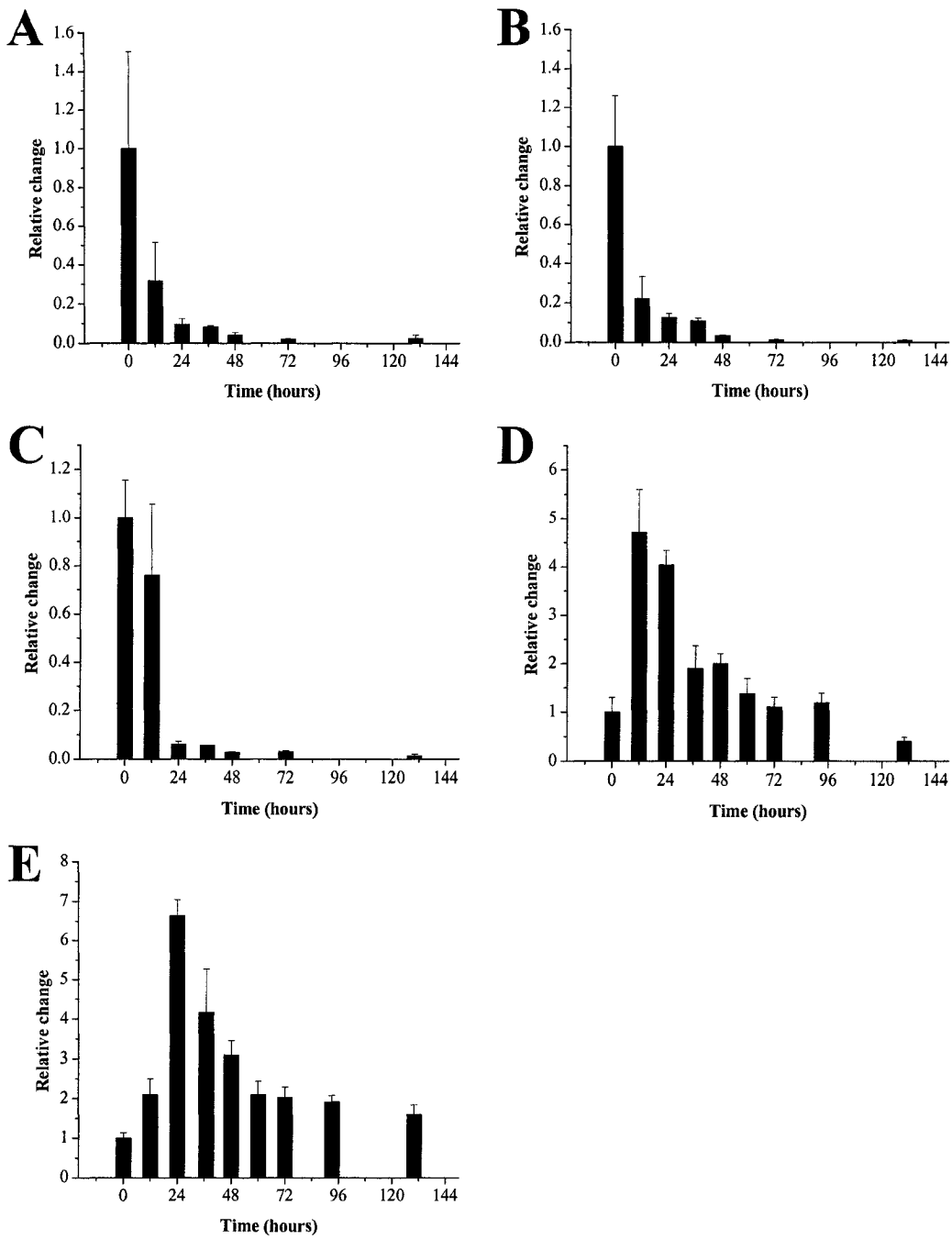

Figure 6.4. Analysis of RNA transcripts for genes involved in diacetyl production and reduction in ale yeast fermentations. (A) Acetohydroxyacid synthase (*ILV2*), (B) Acetohydroxyacid reductoisomerase (*ILV5*), (C) Old Yellow Enzyme isoform 2 (*OYE2*), (D) D-Arabinose dehydrogenase (*ARA1*) and (E) Mitochondrial peroxiredoxin (*PRX1*).

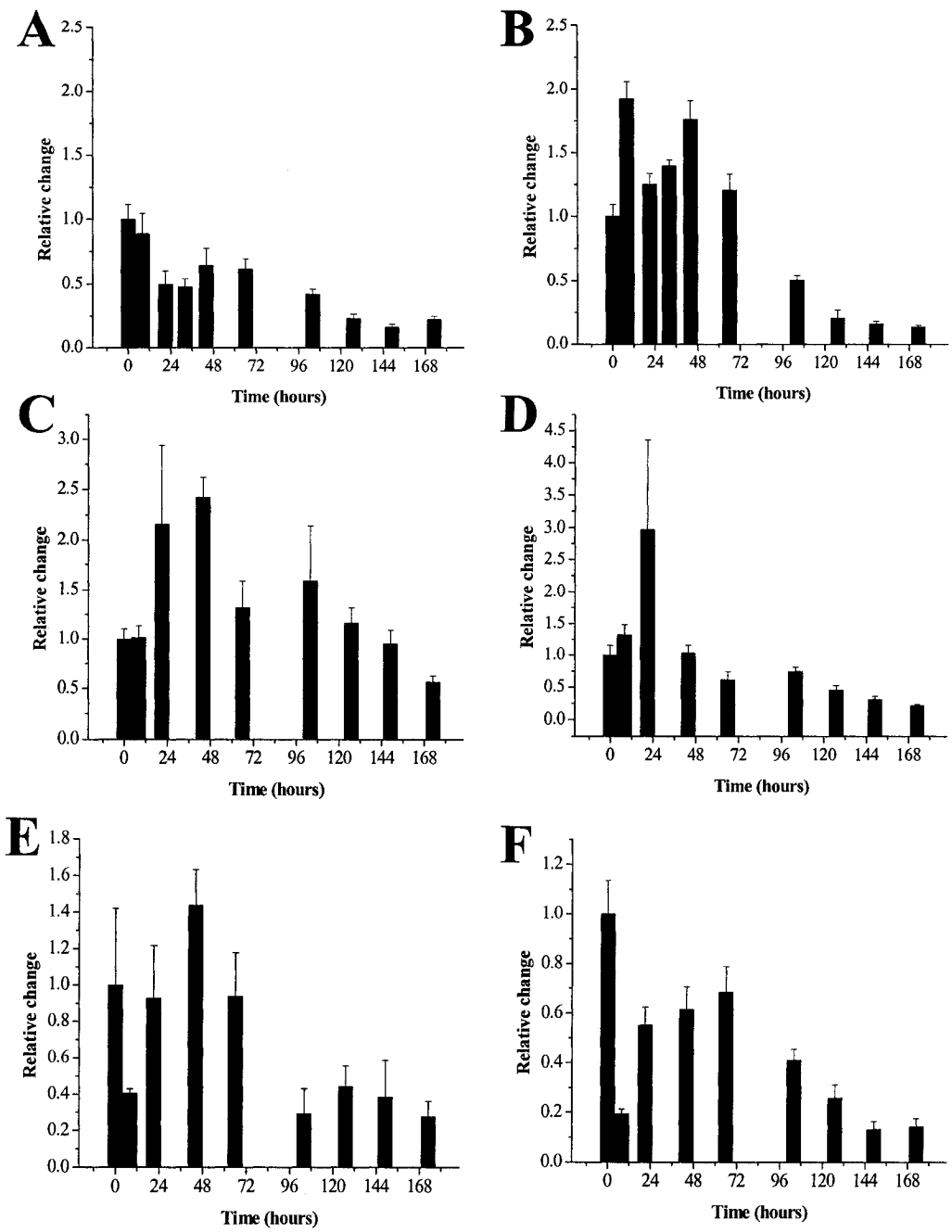

Figure 6.5. Analysis of RNA transcripts for genes involved in diacetyl production and reduction in lager yeast fermentations. (A) Acetohydroxyacid synthase (*ILV2*), (B) Acetohydroxyacid reductoisomerase (*ILV5*), (C) Old Yellow Enzyme isoform 1 (*OYE1*), (D) Old Yellow Enzyme isoform 2 (*OYE2*), (E) D-Arabinose dehydrogenase (*ARA1*) and (F) Mitochondrial peroxiredoxin (*PRX1*). mRNA was normalised to 18S rRNA.

6.4 Discussion and conclusion

Fermentation kinetics of *S. pastorianus* and *S. cerevisiae* yeast exhibit significant different behaviours and physical characteristics that include optimal brewing temperature and the accumulation of yeast at the top of the vessel (ale) or bottom of the vessel (lager) during flocculation. However, little is known about the differences in genetic behaviour of these polyploid yeast during this process. Pathways responsible for the production of flavour active compounds, such as diacetyl, have been described and it is well established that different strains excrete different quantities of α-acetolactate into the fermenting wort. Elegant experiments performed by Dillemans *et al.* (1987) suggest that an increase in *ILV5* activity may greatly reduce the levels of α-acetolactate in wort, however, the mechanisms that regulate *ILV5* expression have not been described.

Ale and Lager yeast require significantly different environmental conditions with respect to temperature and hence fermentation rate, which give ale and lager beers distinctive flavour profiles. It is therefore interesting to note the differences in ILV pathway behaviour between the Ale and Lager yeasts. Certain observations cannot be made as a result of the rapidity of the Ale fermentations, and indeed, the acetolactate evolution shoulder that is visible in the lager fermentation cannot be seen in the rapid ale fermentation, most likely due to lack of sample resolution. However, some general trends are observable. Ale yeast displayed a progressive down-regulation of *ILV2* and *ILV5* during fermentation, whereas Lager yeast generally displayed a down-regulation of *ILV2* and an initial ~2 fold up-regulation of *ILV5* followed by progressive down-regulation. While this up-regulated activity did not result in the elimination of α-acetolactate evolution, peak concentrations were ~750 ppb in lager and 1300 ppb in ale – an almost 2-

fold difference, reflected in *ILV5* activity. Furthermore, Ale yeast ILV pathway activity was almost completely shut-down after 72 h, which coincided with a near complete reduction of α-acetolactate in wort samples. In contrast, the Lager yeast ILV pathway remained active throughout the fermentation and displayed a more gradual down-regulation. This pathway behaviour correlated with a significantly shallower acetolactate reduction curve. These patterns suggest that wort α-acetolactate concentration is related at all times to ILV pathway activity, with slower reduction curves indicative of residual pathway activity.

The reasons for α-acetolactate excretion from yeast cells during fermentation are currently unknown. However, α-acetolactate can be non-enzymatically oxidised within the cell to form diacetyl (Park *et al.*, 1995), which in turn has been shown to modify various proteins (Lundblad, 1995; Morgan *et al.*, 2002; Schalkwijk *et al.*, 2006) and negatively affect cellular health. Excretion of excess α-acetolactate may therefore be a mechanism to protect cells from carbonyl stress. This necessity can be further validated by the knowledge that α-acetolactate biosynthesis takes place within the mitochondria, the correct function of which is essential for energy generation and cellular survival (Zelenaya-Troitskaya *et al.*, 1995).

It is generally accepted that α-acetolactate is excreted from the cells, following which it is oxidatively decarboxylated to form diacetyl in the fermenting wort and then taken up by the yeast cells (Dillemans *et al.*, 1987; Gjermansen *et al.*, 1988). The decarboxylation is a slow, while diacetyl uptake is rapid as suggested by the absence of diacetyl in wort remaining in contact with yeast (Landaud *et al.*, 1998). It is also known that removal of yeast from fermented beer that still contains significant levels of

acetolactate results in the slow formation of diacetyl and hence the need for a maturation step that allows yeast to enzymatically degrade diacetyl and pentanedione. However, oxidative decarboxylation of α-acetolactate under an anaerobic cool environment, as encountered in fermentation and maturation vessels, is likely to be very slow at best. It may therefore be possible that α-acetolactate may be excreted from the yeast cells as a protective mechanism and reabsorbed at a later stage, when required for amino acid biosynthesis. Mechanisms responsible for α-acetolactate transport across the cell membrane have not been characterised or this hypothesis validated.

Observations of increased *ARA1* and *PRX1* activity in Ale yeast fermentations correlate well with previously observed up-regulation in haploid *S. cerevisiae* laboratory strains when subjected to diauxic shift (DeRisi *et al.*, 1997). In these experiments *ARA1* and *PRX1* displayed a Pearson correlation coefficient of greater than 0.8 (*Saccharomyces* Genome Database), suggesting that these genes behave in a similar manner to protect the cell against certain stresses. The co-up-regulation observed during ale maturation further suggests that these genes are involved in stress protection. *ARA1* has also previously been shown to up-regulate during stationary phase (Gasch *et al.*, 2000) although this was not observed with *PRX1*. The correlation in gene activity between *OYE2* and the ILV genes suggests that *OYE2* may play a protective role during either growth or peak metabolic events. This pattern of expression is also visible in Lager yeast fermentations but differs in *OYE1*, which is present exclusively in Lager yeast. The continual activity of *OYE1* suggests that oye1p may be involved in reduction of vicinal diketones at all times within the cell, as previously proposed (van Bergen *et al.*, submitted for publication). Likewise, the similar patterns of up and down regulation of *ARA1* and

PRX1 as compared to *ILV2* and *ILV5* activity suggest that they may play a role in vicinal diketone detoxification. Further investigation is required in order to elucidate the mechanisms responsible for up-regulation of both *ILV5* and the genes responsible for dicarbonyl detoxification.

Connecting text

Diacetyl clearly remains a very important consideration in brewing. As was visible in figures 6.1 and 6.2, diacetyl reduction occupies more than 50% of total fermentation time. While more research needs to be conducted into methods for controlling precursor evolution, the need remains strong and current for accurate quantification of this compound. The current standard method of analysis is head space gas chromatography. This requires expensive instrumentation and highly trained operators in order to reduce the analytical variance. This official method of analysis has displayed low repeatability in collaborative trials, indicating that many breweries are either over or under estimating their diacetyl levels. This can have significant financial impact on their operations, through longer-than-necessary maturation periods, or out of specification products. An accurate, yet cost effective, less labour intensive analytical procedure would benefit the entire industry and would bring diacetyl concentration assessment within reach of smaller breweries who simply cannot afford the instrumentation and labour costs. A simplified analytical system that could be operated at an analytical station within the fermentation cellar, as opposed to in a laboratory with additional personnel, would simplify operations and reduce costs through faster turnover of fermentation vessels and reduced labour demands.

The opportunity therefore exists for a simplified analytical system. An accurate analytical mechanism delivered to the market at a competitive price could capture significant market share for reasons laid out in chapter 1.

In an effort to develop an accessible commercial product we have evaluated the characteristics of beer that could be exploited, or controlled, in order to successfully quantify diacetyl. As will be seen in the following chapter, this is by no means a simple process, as beer contains more than 800 different compounds, many of which interfere with standard assay conditions.

7. A novel enzyme based assay for quantifying diacetyl in fermenting wort

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7.1 Abstract

The concentration of diacetyl is a key indicator of beer maturation. The taste threshold for diacetyl is ~0.1 parts per million (ppm). An accurate analysis at these concentrations is difficult to achieve and requires advanced instrumentation and highly skilled operators. At present no rapid real-time methodology for monitoring diacetyl levels during beer production is available. Here we present the development of an enzymatic strategy that can be automated for the development of an instrument providing a rapid measurement of diacetyl concentrations in beer and fermenter samples. The system consists of an enzymatic reaction cell coupled with an optical system that monitors the change in absorbance of NADPH at 365 nm. The detection limit of free diacetyl was found to be less than 50 parts per billion. The instrument could ideally be used by process operators or technicians to determine when the diacetyl threshold concentration has been reached and hence, the end of fermentation.

7.2 Introduction

The vicinal diketone (VDK) Diacetyl (2,3-butanedione) is a well known (Haukeli and Lie, 1972; Wainwright, 1973) flavor active compound in beer with an undesirable butterscotch-like aroma and very low taste threshold of less than 100 parts per billion (ppb) in beer (Yonezawa and Fushiki, 2002). 2,3-Pentanedione, another VDK with one additional methyl group, possesses similar aroma properties, but a significantly higher flavor threshold and is produced in a similar manner to diacetyl. The diacetyl and pentanedione precursor metabolites (α-acetolactate and α-acetohydroxybuterate respectively) are produced during fermentation by yeast (Haukeli and Lie, 1972) as intermediates in the biosynthesis of the branched amino acids isoleucine, leucine, valine via the regulated ILV pathway (Holmberg and Petersen, 1988). The overall pathway activity, and hence precursor and VDK formation, is partly affected by the concentration of isoleucine, leucine and valine present in the wort (Holmberg and Petersen, 1988; Petersen *et al.*, 2004).

α-Acetolactate and α-acetohydroxybuterate can undergo non-enzymatic decarboxylation within the cell (Park *et al.*, 1995) or be exported from the cell to the fermenting wort (Gjermansen *et al.*, 1988) where decarboxylation occurs, producing diacetyl. Once present in wort, the decarboxylation process is relatively slow and occurs as a function of alcohol concentration, pH and temperature (Kobayashi *et al.*, 2005). It is assumed that the precursors are decarboxylated in the wort and the VDK products enter the yeast cells where they are enzymatically reduced. The generally accepted mechanism is a sequential enzymatic reduction of diacetyl to acetoin (3-hydroxy-2-butanone) and then 2,3-butanediol, while 2,3-pentanedione is reduced to 3-hydroxy-2-pentanone and

then 2,3-pentanediol. Several enzymes may be responsible for this process and some only catalyze a single reduction step (Bamforth and Kanauchi, 2004; Hardwick *et al.*, 1976; Tolls *et al.*, 1970; van Bergen *et al.*, 2006a; Van Bergen *et al.*, 2006b).

As a result of rapid uptake of the VDKs by yeast cells and slow precursor degradation, it is common to find low levels of free diacetyl in the maturing product, but significantly higher levels of potential diacetyl and pentanedione – the precursors α-acetolactate and α-acetohydroxybuterate (Landaud *et al.*, 1998). VDK reduction ceases to occur when yeast is removed from the beer. It is therefore essential that both the VDKs and their precursors be reduced to below specification values before filtration occurs, in order to ensure that diacetyl remains below the flavor threshold throughout the product shelf life.

Several methods have been developed for quantifying VDKs and their precursors in beer, with precursors most often requiring decarboxylation to their respective VDKs prior to analysis. This is commonly achieved by heating samples in the presence of agents and oxygen to encourage decarboxylation (Gollop *et al.*, 1987; Hardwick, 1994). The resulting VDKs are then analyzed using methods that include fluorometric detection using high performance liquid chromatography (McCarthy, 1995), mass spectrometry combined with gas chromatography (Landaud *et al.*, 1998), mass spectrometry combined with high performance liquid chromatography (Blanchette *et al.*, 2006) headspace analysis gas chromatography (Chemists, 2004) and several spectrophotometric techniques that determine total VDKs (Chemists, 2004). Spectrophotometric techniques commonly rely on distillation followed by colorimetric determination of the reaction product of diacetyl with creatine and α-naphthol in an alkaline medium and this technique has been

applied in analysis of diacetyl in samples other than beer (Mattessich and Cooper, 1989). The standard analytical technique in brewing is headspace analysis gas chromatography using an electron capture detector, despite this method of analysis displaying poor precision in collaborative trials (Convention, 1998). Complex and expensive instrumentation such as GC or HPLC, used for routine analyses of these compounds, requires highly skilled technical operators in order to ensure accuracy and reproducibility in results (Verma and Singh, 2003).

Commonly, the specified final diacetyl concentration in matured beer is less than 50 ppb, with some products specifying values below 20 ppb. Accurate determination of diacetyl below threshold values remains difficult as a result of the low concentrations of analyte and the complex nature of the medium being analyzed. Here we describe the development of a sensitive enzyme-based assay, requiring minimal sample preparation, capable of determining total diacetyl levels below the taste threshold.

7.3 Methods and Materials

7.3.1 Spectrophotometric characterization of beer matrix

Beer, and fermenting wort were spectrophotometrically characterized in order to asses the potential impact on, or masking of, standard enzyme kinetic monitoring techniques. 6 locally sourced, leading commercial beers and samples from McGill University's pilot brewery were obtained and filtered (0.45 μm) prior to analysis. Absorbance in the UV-visible wavelengths was characterized using a DU-640 spectrophotometer (Beckman-Coulter, Fullerton, CA) with sweeps from 200 to 800 nm. The fluorescence properties of

the beer were characterized using a Cary fluorescence spectrophotometer (Varian, Palo Alto, CA) with excitation from 250 to 450 nm at 5 nm intervals and emission monitoring with a 5nm slit width from 250 to 600 nm for each interval.

7.3.2 Determination of α-Acetolactate and diacetyl concentrations

Acetolactate and diacetyl concentrations in samples were determined using high performance liquid chromatography coupled with electro-spray mass spectrometry (LCMS)(Agilent, Palo Alto, CA). α-Acetolactate was decarboxylated to diacetyl by adding 0.15 mM $FeSO_4$ and 0.15 mM $FeCl_3$ and heating the sample at 80 °C for 10 min after vigorous agitation (Fig. 7.1). Samples were cooled, derivatized with o-phenylenediamine, loaded onto Gemini C-18 column (150 x 4.6 mm, 5 μm) (Phenomenex, Torrance, CA), eluted using a two step isocratic gradient and quantified as described elsewhere (Blanchette *et al.*, 2006).

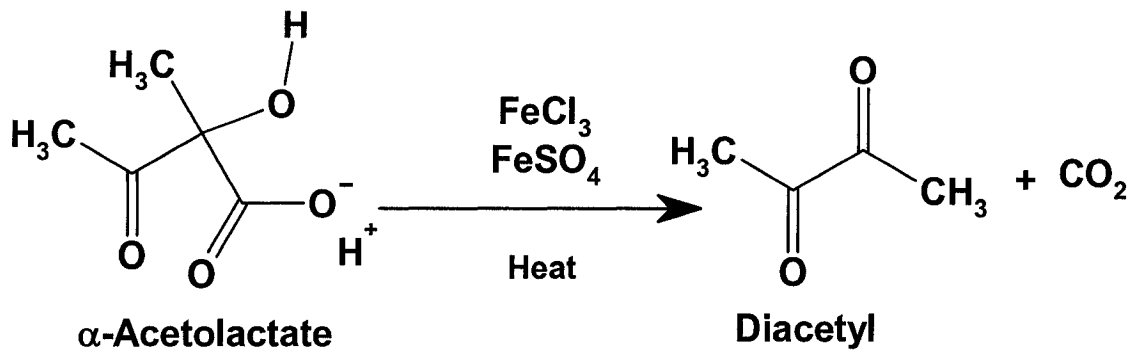

Figure 7.1. Reaction mechanism for conversion of acetolactate to diacetyl in the presence of $FeCl_3$ and $FeSO_4$

7.3.3 Sample pretreatment

Background noise in samples, that could interfere with standard biochemical assay techniques, was reduced by sequential filtration through a 0.45 μm filter in series with a Sepak C-18 cartridge (Waters, Milford, MA). Following sample cleanup, fluorescence and absorbance characteristics were evaluated to determine changes in beer properties, as described above.

Sample pH was adjusted to 7.0, when required, through the addition of concentrated sodium phosphate buffer at pH 7.0 to one fifth of the total volume.

7.3.4 Determination of column retention properties

In order to evaluate the potential for diacetyl and α-acetolactate retention during sample preparation procedures, samples with a known concentration of acetolactate or diacetyl were analyzed before and after column treatments, at both normal pH (~4) and at pH 7.0. Total diacetyl in beer samples was determined by LCMS prior to addition of acetolactate or diacetyl.

α-Acetolactate was synthesized by reacting ethyl-2-acetoxy-2-methyl-acetoacetate with 4 equivalents of sodium hydroxide as described elsewhere (Krampitz, 1948) and the concentration determined. Beer samples where spiked with known concentrations of α-acetolactate to simulate the presence of potential diacetyl during fermentations or with diacetyl and processed through the column. Fractions were collected and analyzed by LCMS to determine diacetyl and/or α-acetolactate retention.

7.3.5 Enzyme assays

Assays were performed in a Beckman Coulter DU-640 spectrophotometer equipped with a Peltier temperature control module. Samples were analyzed in either their native retail state or spiked with acetolactate and/or diacetyl prior to processing. Samples were sequentially filtered, the acetolactate converted to diacetyl and cooled to assay temperature. The pH was adjusted to 7.0 with the addition of concentrated phosphate buffer. A defined quantity of previously characterized recombinant diacetyl reductase enzyme or commercially obtained preparation (Sigma) was added and the spectrophotometer zeroed. 200 μM of NADPH was titrated into the solution and the reaction monitored at 340 or 365 nm. The initial reaction velocity of samples with known diacetyl concentrations was calculated and used to develop a calibration table. The concentration of unknown samples was determined by comparing velocities to a calibration table.

7.4 Results

7.4.1 Spectrophotometric characterization of beer matrix

All beer samples typically displayed very high absorbance values at 280, 340 and 365 nm. Light beers (beers 1 and 2) displayed slightly lower absorbance values than standard adjunct, or all malt beers (beers 3 to 6) above 350 nm. Absorbance values were above detector saturation below 350 nm (Fig. 7.2), effectively masking any capability of monitoring NADPH reduction without additional sample treatment.

Fluorescence intensity and localizations were similar for all 6 beers tested. Maximum emission was in the region of 430 to 450 nm using an excitation wavelength of 350 nm (Fig 7.3). This is similar to previously obtained results (Sikorska *et al.*, 2004) and is possibly as a result of high flavin content in beer (Andrés-Lacueva *et al.*, 1998).

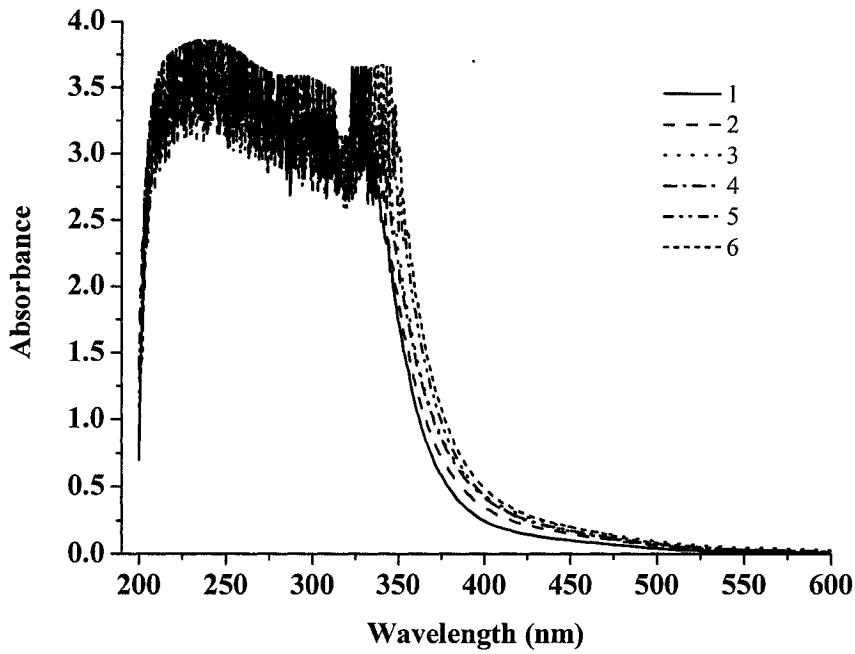

Figure 7.2. Absorbance characterisation of 6 leading commercial beers. (1 and 2) Domestic adjunct light beers. (3) Domestic premium adjunct lager. (4) Imported premium adjunct lager. (5 and 6) Imported super premium all malt lagers.

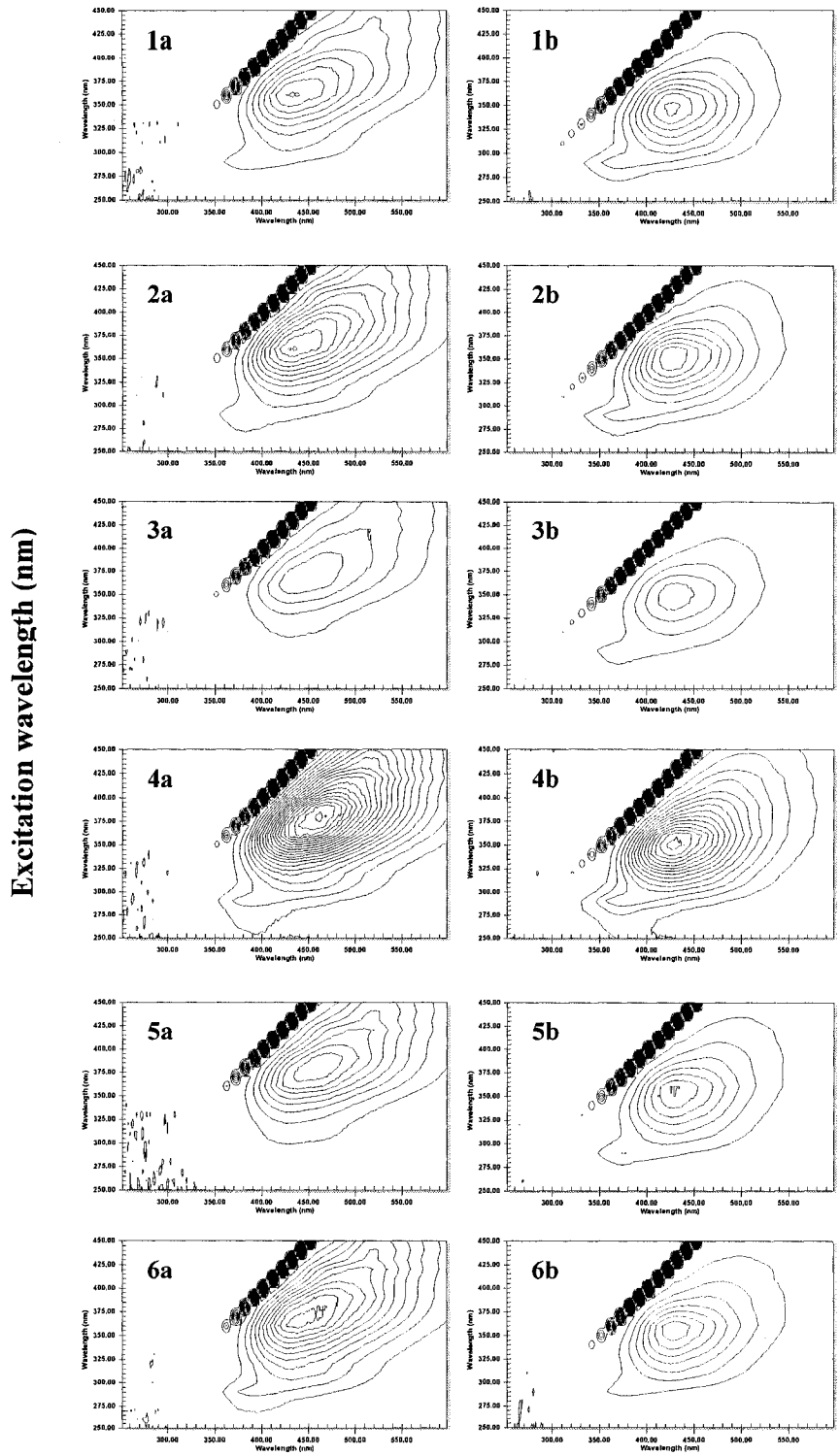

Figure 7.3. Fluorescence characterisation of six leading commercial beers (a) before and

(b) after C-18 filtration. (1 and 2) Domestic adjunct light beers, (3) domestic premium adjunct lager, (4) imported premium adjunct lager, (5 and 6) imported super premium all malt lagers.

7.4.2 Sample pre-treatment and column retention of analytes

Sample pretreatment with size exclusion filtration followed by C-18 filtration reduced absorbance values sufficiently to allow the monitoring of NADPH reduction by enzymatic assay. Samples were passed through the column and 1ml fractions collected. Sample absorbance in each fraction was measured with a focus on 340 and 365 nm as two wavelengths suitable for monitoring NADPH reduction. At both wavelengths, a significant drop in absorbance was observed in the first fraction as compared to the raw sample, followed by an increase in subsequent fractions (Figs. 7.4 and 7.5). In order to successfully quantify the levels of diacetyl in the product, however, it was necessary to assess the retention of the analytes of interest by the C-18 column.

Samples were spiked with known quantities of diacetyl or α-acetolactate and either passed through the column directly, or adjusted to the assay pH (pH 7) prior to passing them through. 1 ml fractions were collected and the analyte content quantified by LCMS and compared to the raw samples in order to determine retention characteristics. Analysis of beer fractions processed through the solid phase extraction columns at pH 4 showed that both α-acetolactate and diacetyl did not bind appreciably, as >90 and >98% of each of these compounds, respectively was recovered in the filtrates(Fig. 7.6). However, fractions eluted at pH 7 displayed significant retention of analytes (Fig. 7.7).

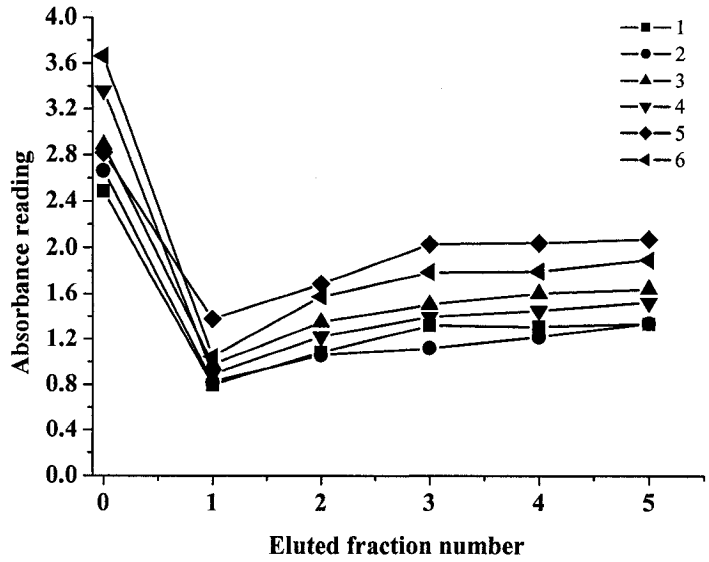

Figure 7.4. Absorbance changes at 340 nm in beer samples sequentially filtered through a 0.45 μm filter and a Sepak C-18 mini column.

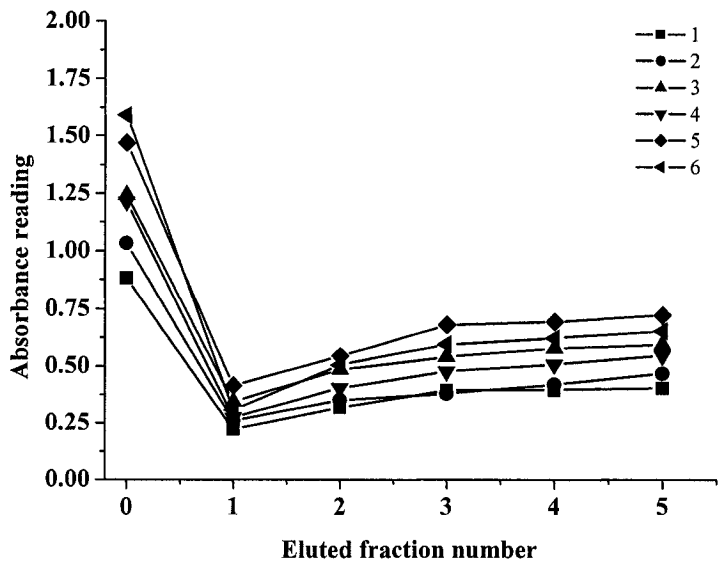

Figure 7.5. Absorbance changes at 340 nm in beer samples sequentially filtered through a 0.45 μm filter and a Sepak C-18 mini column.

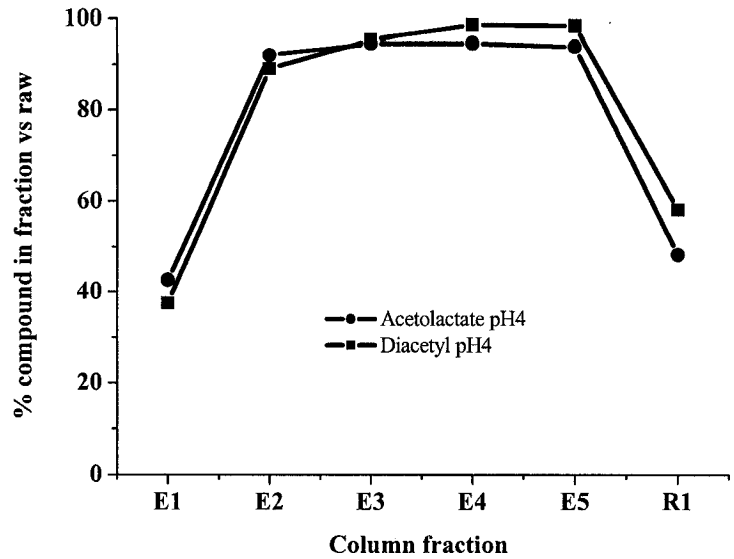

Figure 7.6. Analysis of α-acetolactate and diacetyl retention by a Sepak C-18 mini column at pH 4.0. 1ml fractions were eluted (E) prior to column rinsing (R).

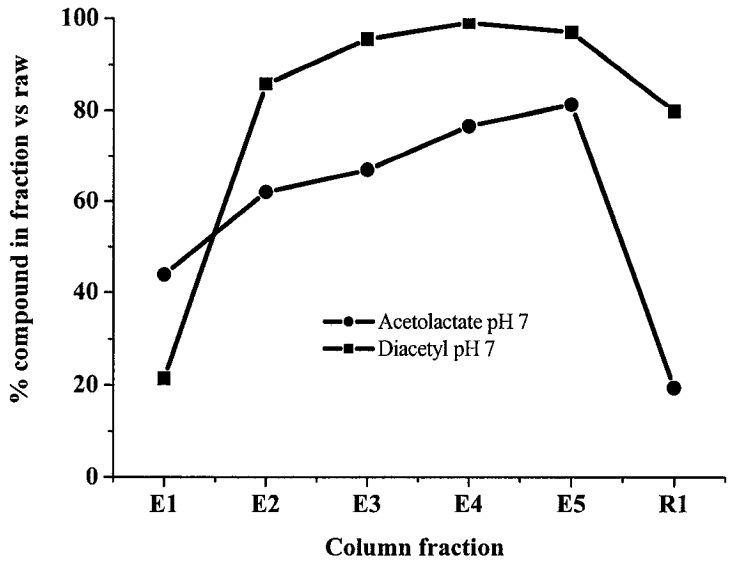

Figure 7.7. Analysis of α-acetolactate and diacetyl retention by a Sepak C-18 mini column at pH 7.0. 1ml fractions were eluted (E) prior to column rinsing (R).

8.4.3 Enzyme assays

A key step in the enzymatic detection of diacetyl in beer streams involves the quantitative conversion of α-acetolactate to diacetyl which can then be measured enzymatically. Using a mixture of ferrous and ferric iron in an oxidative environment it was possible to obtain >95% conversion in 10 min (data not shown) allowing for a significant process time saving.

In the development of an enzymatic assay that was sensitive enough to detect diacetyl down to the low ppb levels, three enzymes derived from *Saccharomyces* yeast were investigated: Old yellow enzyme (OYE) (van Bergen *et al.* submitted for publication) (van Bergen *et al.*, 2005), D-Arabinose dehydrogenase (ARA1) (Van Bergen *et al.*, 2006b) and a new enzyme X that has recently been purified from yeast. A commercial preparation purified from *Saccharomyces* yeast (Sigma) according to methods described elsewhere (Heidlas and Tressl, 1990) was also evaluated. Extensive analysis with Oyep and Aralp showed that these enzymes were capable of converting diacetyl to acetoin in an NADH or NADPH dependent manner, in both a buffered solution or in a beer matrix. Oyep, Aralp and the commercial preparation showed a good response at higher concentrations of diacetyl in beer, but did not, however, show sufficient specificity in beer matrix to reliably detect diacetyl below 320 ppb.

In contrast, enzyme X, which was recently purified from a yeast mutant lacking OYE activity, showed vigorous catalytic activity with diacetyl. Kinetic analysis revealed that this enzyme was capable of binding diacetyl tightly, as suggested by a diacetyl Km value of 38 μM (data not shown). More importantly however, was the finding that this enzyme displayed a good response and differentiation of diacetyl at concentrations that

were below the taste threshold. Enzyme X showed a high specificity for diacetyl in beer matrix, as no reductase activity was detected in the absence of the diacetyl substrate. Figure 7.8 shows enzyme X assay activity as a function of diacetyl concentration in all malt beer matrix. Activity was specific to low concentrations of diacetyl with reaction velocity reaching a plateau at higher concentrations (data not shown).

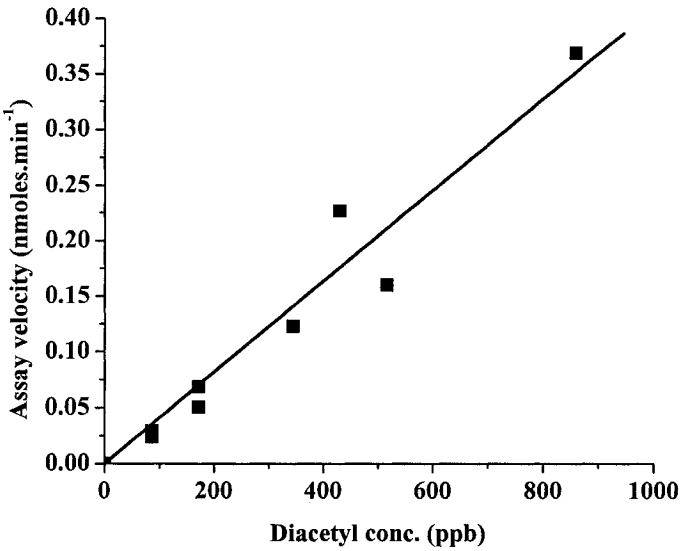

Figure 7.8. Diacetyl reductase activity trials of enzyme X in all malt beer matrix with low concentrations of diacetyl.

With initial trials suggesting that enzyme X could be suitable for measuring diacetyl at low physiological concentrations, further trials were conducted with different beers spiked with diacetyl at known concentrations. The addition of diacetyl to a light beer (beer #2) and subsequent enzymatic reduction with monitoring at 365 nm showed an acceptable correlation between concentration and reaction velocity (Fig. 7.9A). Tests

conducted using an all malt beer also proved satisfactory (Fig. 7.9B) although detection below 40 ppb was inconclusive, likely as a result of higher residual diacetyl present in the product compared to the light beer and the possibility of competing substrates.

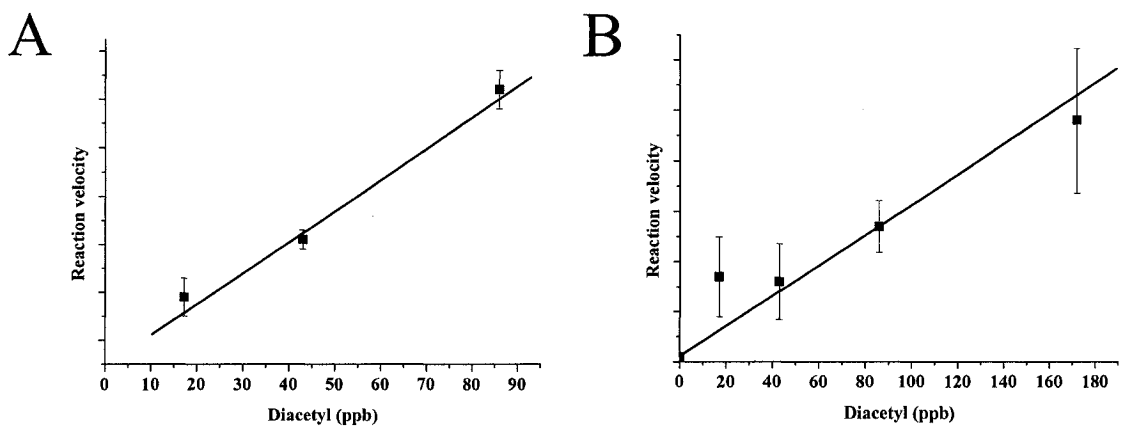

Figure 7.9. Reaction velocity profiles with enzyme X in beer spiked with diacetyl. (A) enzyme reaction velocity profile in commercial light beer, (B) enzyme reaction velocity profile in commercial all malt beer.

8.5 Discussion and conclusions

An enzymatic assay has been developed with sufficient sensitivity to detect low levels of diacetyl in beer matrix. With the use of a single, rapid, preprocessing step it was possible to reduce background noise in beer samples to sufficiently low levels for spectrophotometric monitoring of NADPH reduction by absorbance. It was not, however, possible to reduce the fluorescence background to sufficiently low levels to monitor the reaction by fluorescence spectrophotometry as the peak coincided with the fluorescence

optimum for NADPH. While absorbance values were effectively reduced to resolve NADPH consumption, it was essential to assess any possible analyte retention during processing. Passing beer through the C-18 column at normal beer pH values resulted in minimal retention of α-acetolactate and diacetyl, suggesting that this technique may be suitable for use in in-plant analysis of total diacetyl levels in maturing beer.

Three enzyme preparations purified in our laboratory as well as one commercial preparation were analyzed for use in enzymatic detection of low levels of diacetyl in beer. All 4 systems displayed good catalytic activity with low-ppb diacetyl concentrations in buffer. Three of the systems, however, displayed low level non-specific activity in beer matrices. Oyep showed strong activity with high levels of diacetyl and was capable of reliably determining diacetyl levels in beer above the taste threshold. Although OYE was capable of catalyzing the reduction of diacetyl at lower levels in buffer, background activity in beer made it difficult to reliably quantify total diacetyl concentrations below the taste threshold. Similarly, Aralp displayed poor resolution of diacetyl concentrations below 500 ppb in beer matrix as did the commercial enzyme preparation. In contrast, enzyme X displayed excellent sensitivity and specificity at low diacetyl concentrations. Reaction velocity appeared to plateau at lower diacetyl concentrations than the other enzyme systems (data not shown) but remained linear and responsive in the important taste threshold region.

Total sample process time was acceptable. Sample pretreatment was achieved in less than 60 seconds with acetolactate conversion lasting 20 minutes in total. pH correction, assay preparation and kinetic measurement lasted less than 15 minutes and is currently being further refined. Total sample treatment and analysis time is currently

being optimized in order to reduce processing time to less than 30 minutes. A prototype is currently being developed to pursue the objective of creating an alternative, sensitive and easy to perform enzymatic assay. Should this development prove successful, a viable and cost effective alternative to current gas chromatography methods could be provided to brewing operations of all size to improve product quality control and reduce costs.

8. General conclusions and summary

Diacetyl, a small, four carbon diketone, has significant biological and financial impact. Evidence has been uncovered suggesting that diacetyl damages certain cellular proteins and contributes to several diseases and general cellular aging in higher eukaryotic organisms. The presence of diacetyl and potential diacetyl in fermentation vessels likely costs the brewing industry millions of dollars annually through impeded productivity. A clearer understanding of the mechanisms responsible for reducing diacetyl in cellular systems could lead to advancements in treatment for diabetic vascular complications, neurodegenerative diseases, atherosclerosis, and at a more general level, cellular deterioration and aging. With an increased understanding of diacetyl production and reduction systems in yeast, brewers may be able to reduce overall fermentation times, thereby increasing product throughput and operational efficiency.

Several questions have arisen during the research conducted herein and remain unanswered. In chapter 3, we characterised a biphasic diacetyl reduction system, in contrast to the other monophasic reduction system in chapters 4. Of great interest was the apparent up-regulation of enzyme activity in the presence of higher diacetyl concentrations, with a decreased specificity. Although it has yet to be established if diacetyl is, in fact, binding to OYE proteins and modifying their structure, this would prove to be a novel mechanism for metabolic regulation. It is generally accepted that regulation of activity can occur at the level of transcription, via feedback mechanisms and at the translation level, through RNA lifetime and transcription activity. However, once a

protein has been created, the only way to increase the overall amount of activity would be to produce more of that protein and/or increase the interval between production and digestion. Here we have seen an up-regulation in the activity of the protein itself, which has not been previously documented. This hypothesis is being further investigated.

Another interesting observation is that the yeast cell is in possession of several distinct mechanisms capable of reducing diacetyl. Furthermore, these mechanisms are capable of catalyzing an array of reduction reactions with other vicinal diketons, such as pentanedione and methylglyoxal. This suggests that these compounds do have a significant impact on cellular mechanisms and hence need to be reduced. There is a need for further research to determine the deleterious effects of diacetyl on cellular mechanisms. Although several proteins have been shown to be modified by vicinal diketone compounds, the results in chapter 3 suggest that there are more that need to be resolved and attempts are currently underway to address this question.

Brewers may suggest that it is not diacetyl that is the problem in fermentation vessels, but the precursor α-acetolactate, which degrades slowly to form diacetyl, which in turn is rapidly taken up by the yeast. It has, however, been suggested that acetolactate can be non-enzymatically decarboxylated within the cell in the presence of coenzymes such as nicotinamide and flavins. If this occurs, then diacetyl will likely be present within the cell. Although this has not been quantified, it may be speculated that acetolactate is expelled from the cell as a protective mechanism, to prevent the formation of further diacetyl and subsequent cellular damage. Questions arise as to why there is an excess of acetolactate within the cells to begin with. Perhaps diacetyl, or another compound, damages acetohydroxyacid reductoisomerase (Ilv5p), and in doing so, slows

the shuttle of acetolactate towards valine and leucine. Acetolactate would then pool within the mitochondria (where most of the ILV pathway is located), the cellular "powerhouse", rich in NAD, NADP and FAD and most importantly, oxygen. These factors could possibly combine to encourage rapid diacetyl formation and subsequent damage of mitochondrial proteins, which are essential for cellular survival. A resulting expulsion of excess acetolactate may then occur to prevent further damage. This postulation has not been validated and in order to do so, the susceptibility of Ilv5p to modificational damage or inhibition needs to be assessed. Furthermore, mechanisms for acetolactate transport within the cell and across the cell membrane need to identified and characterised.

The identification of these diacetyl related mechanisms could possibly lead to improved brewing practices and operational efficiencies. It has been shown that the concentration of free amino acids present in brewing wort affects the amount of diacetyl (acetolactate) present in the wort. Interestingly, in chapter 6, we observed that diacetyl production and valine consumption rates were correlated, with both showing similar production/consumption rate change trends. This suggested that the two were somehow linked to the same general series of metabolic events. Despite the presence of abundant valine in the wort, and its active uptake, the ILV pathway remained active, producing acetolactate and hence diacetyl. This contrasts previous work that suggested that the presence of isoleucine, leucine or valine inhibited the ILV pathway through a feedback mechanism with Ilv2p. Theoretically then, while there is still valine present in the wort, no diacetyl production should be observable, but this is not the case. Further investigations need to be conducted to elucidate the mechanisms at play and the reasons

for pathway activity, despite the active uptake of valine from the fermentation media. It may be possible that the rate of valine consumption does not satisfy the demand for incorporation during protein production, but this is not yet known.

There are many questions that have arisen as a result of this research. These questions need to be answered in order to advance our understanding of cellular behaviour and in doing so solutions may be found to industrial problems. An example of this has been the development of a sensitive enzyme based assay for quantifying diacetyl in a complex medium. Brewing wort is complex, with over 800 different compounds present within the final product. Resolving a single compound at micrograms per litre (sub-μM concentrations) within this milieu remains a considerable challenge. Enzymes can be inhibited by other compounds or these compounds may compete with the substrate of choice. Through characterization of the enzymes present in yeast that are capable of reducing diacetyl, we were able to identify an enzyme displaying high specificity for dicarbonyl compounds in wort. This allowed the development of an assay with sufficient sensitivity to quantify the diacetyl at sub-μM concentrations. The further development of this assay based system will likely lead to the development of an automated diacetyl quantification system for use in breweries. The development of this unit will likely reduce the dependence of breweries on either high cost analytical equipment or overly laborious inaccurate techniques, leading to increased operational savings. Furthermore, the instrument would likely make diacetyl monitoring accessible to smaller facilities as well thereby enhancing quality control across a broader spectrum of the market and improving operational efficiencies.

Diacetyl remains an important compound in biology and industry. Here we have addressed several important questions surrounding diacetyl reduction in biological systems and applied the knowledge in the development of an assay that already will likely benefit industry, but will likely lead to further advances in this technology, and application in a broader spectrum of scientific investigations and industrial applications.

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