β-GALACTOSIDASE PRODUCTION BY *Kluyveromyces lactis* IN BATCH AND CONTINUOUS CULTURE

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DECLARATION

I hereby declare that this dissertation represents my own work. It has not been submitted for any diploma/degree or examination at any other Technikon/University. It is being submitted for the Degree of Master of Technology: Biotechnology, in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, Durban, South Africa.

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This 24 day of June, 2011, at Durban University of Technology.

DEDICATION

To my mum, Jessie

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'…in all things we are more than conquerors through him who loved us.' Romans 8:37

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ABSTRACT

Kluyveromyces sp. have adapted to existence in milk due to the evolution of permeabilisation and hydrolytic systems that allow the utilisation of lactose, the sugar most abundant in milk. Lactose hydrolysis, to equimolar units of glucose and galactose, is facilitated by a glycoside hydrolase, i.e., β-galactosidase (EC 3.2.1.23). The versatility of this enzyme allows its application in numerous industrial processes, amongst the most significant of which, is its role in the alleviation of lactose intolerance, one of the most prevalent digestive ailments, globally. In this study, β-galactosidase production by *Kluyveromyces lactis* UOFS y-0939 was initially optimised in shake flask culture with lactose as the sole carbon source, and thereafter, production was scaled up to batch, fed-batch and continuous culture. Shake flask studies revealed optimum conditions of 30°C, pH 7 and a 10% inoculum ratio, to be most favourable for β-galactosidase synthesis, producing a maximum of 0.35 ± 0.05 $U.ml^{-1}$ when cell lysates were prepared by ultrasonication with glass beads. Batch cultivation in 28.2 and 40 $g.L^{-1}$ lactose revealed that elevated levels of the carbon source was not inhibitory to β-galactosidase production, as maximum enzyme activities of 1.58 and 4.08 $U.ml^{-1}$, respectively, were achieved. Cell lysates prepared by ultrasonication and homogenisation were compared and homogenised cell lysates were more than 3.5 fold higher that those prepared by ultrasonication, proving homogenisation to be the superior method for cell disruption. The lactose feed rate of 4 $g.L^{-1}.h^{-1}$ in fed-batch culture operated at ± 20.4% DO, appeared to be inhibitory to biomass production, as indicated by the lower biomass productivity in fed-batch (0.82 $g.L^{-1}.h^{-1}$) than batch culture (1.27 $g.L^{-1}.h^{-1}$). Enzyme titres, however, were favoured by the low DO levels as a maximum of 8.7 $U.ml^{-1}$, 5.5 fold more than that obtained in batch culture, was achieved, and would be expected to increase proportionally with the biomass. Continuous culture operated at a dilution rate of 0.2 h^{-1}, under strictly aerobic conditions, revealed these conditions to be inhibitory to the lactose consumption rate, however, the non-limiting lactose and high DO environment was favourable for β-galactosidase synthesis, achieving an average of 8 ± 0.9 $U.ml^{-1}$ in steady state.

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Humankind has, for centuries, been riddled with a myriad of illnesses that, although not life-threatening, nonetheless, adversely affect the quality of life of many. The progression of our species and consequent lifestyle changes over time, have attributed to these ailments. Diet is often considered the main contributor to a number of non-specific abdominal symptoms (Locke *et al*., 2000). The prevalence of perceived food intolerance in the general population is about 20-25% (Nanda *et al*., 1989). There are several non-allergenic mechanisms by which food can cause adverse symptoms, e.g. from pharmacological reactions to foods containing a drug (e.g. caffeine, salicylates and histamine), or enzyme deficiencies leading to a food intolerance (Mcdougall, 2009). One of the most common enzyme deficiencies is that of the intestinal enzyme, lactase, which decreases the ability to metabolise milk sugar (lactose).

1.2 LACTOSE: THE MILK SUGAR

Lactose (β-galactose-1,4-glucose) is a disaccharide composed of the simple sugars glucose and galactose, bonded through a β-1→ 4 glycosidic linkage. It is the key nutrient in mammalian milk, comprising the major carbohydrate source during the neonatal period. Lactose is, from both an evolutionary and a biological viewpoint, a unique sugar, since it is only found as a free molecule in milk. It is synthesised by lactose synthetase, found exclusively in the mammary glands of virtually all female mammals (Büller and Grand, 1990). Human milk contains the highest concentration of lactose (about 7%), while that of most common milk product animals, i.e., cows and goats, is below 5% (Schaafsma, 2008). Lactose is also present in a broad range of dairy products and processed foods such as ice cream, salad sauces and sausages (Ratzinger *et al*., 2010). It is even applied in the pharmaceutical industry where it is chemically oxidised to lactobionate, which is a strong chelator of calcium and is used in calcium supplement (Gerling, 1997).

In addition to its nutritional value, lactose improves the absorption of the minerals calcium, magnesium and zinc (Abrams *et al.*, 2002) and promotes the growth of *Bifidobacterium* (Anania *et al.*, 2008), a natural inhabitant of the gastrointestinal tract that aids in digestion. Furthermore, galactose, which is released upon the digestion of lactose, is an essential nutrient for the formation of cerebral galactolipids (Anania *et al.*, 2008).

1.2.1 Digestion and metabolism of lactose

Most dietary disaccharides are normally hydrolysed and absorbed in the proximal portion of the small intestine (Fox and Thomson, 2007). Lactose exits in two isomeric forms, alpha and beta, which are in equilibrium in aqueous solutions (Gänzle *et al.*, 2008). These forms differ in the orientation of the hydroxyl group in position one of the glucose moiety. In suckling mammals, milk lactose is largely hydrolysed into its monosaccharide components mainly by the enzyme lactase-phlorizin hydrolase (LPH), commonly known as lactase or β-galactosidase, which is bound to the mucosal membrane of the small intestine. Human LPH is encoded by the lactase gene located on the long arm of chromosome 2 (Kruse *et al.*, 1988). After hydrolysis, the monosaccharides are actively absorbed and transported to the liver via the portal vein. Both glucose and galactose share the same absorption pathway and are the only sugars that are actively absorbed. Lactase has a higher affinity for the beta form of lactose and this causes a shift in the equilibrium between both isomeric forms of the sugar in favour of the beta form which is referred to as mutarotation. After absorption, lactose is converted into glucose in the liver via the Leloir pathway, as described in the three equations below. The conversion requires three enzymes: galactokinase (E1), galactose-1-phosphate uridyltransferase (E2) and uridine-diphospho-galactose-4-epimerase (E3) (Schaafsma, 2008).

Eq. 1 $\quad \text{Gal + ATP} \xrightarrow{\text{E1}} \text{Gal-1-P + ADP}$

Eq. 2 $\quad \text{Gal-1-P + UDPG} \xrightarrow{\text{E2}} \text{UDPGal + G-1-P}$

Eq. 3 $\quad \text{UDPGal} \xrightarrow{\text{E3}} \text{UDPG}$

1.2.2 Lactose intolerance

Modern classifications have divided adverse reactions to food into those that are immune mediated, i.e., food allergy, and those that are also reproducible, but not immune-mediated, i.e., food intolerance. Non-immunological reactions to food can be further subdivided into those that are due to enzymatic deficiencies or pharmacological reactions, and those that do not fit into either categories (Khakoo *et al*., 2001).

The most common form of disaccharidase deficiency is primary adult hypolactasia, commonly known as lactose intolerance (Auricchio *et al*., 1963; Dahlqvist *et al*., 1963). Lactose intolerance is defined as the inability to digest lactose into the monosaccharides glucose and galactose, owing to insufficient levels of lactase (Kerber *et al*., 2007). There are three main types of lactase deficiency.

i. **Congenital lactase deficiency:** is the condition in which the lactase enzyme is absent or severely reduced at birth and remains abnormal throughout life. This is extremely rare and due to an error of metabolism (Sahi, 1994a). Individuals with this disorder would have to follow a lifelong lactose-free or extremely low lactose diet.
ii. **Secondary lactase deficiency:** is a temporary condition resulting from disease or injury that results in damage of the intestinal mucosa where lactase is normally active (Villako and Maaroos, 1994). Once healing has occurred, lactose digestion improves.
iii. **Primary lactase deficiency:** is the most common type and is also referred to as late onset or lactase nonpersistence. This type of deficiency is directly related to the loss of the majority of intestinal lactase activity, yet the loss is seldom total (McBean and Miller, 1998).

The age of presentation with symptoms of lactose intolerance varies between ethnic groups, but is dependent on the speed of decline in lactase and the amount of lactose digested. Classically, the onset is around the beginning of the second decade of life, approximately 10-14 years (Fox and Thomson, 2007). The timing of the onset of lactase decline may be genetically determined (Sahi, 1994b).

1.2.3 The global prevalence of lactose intolerance

It has been reported that people who are descendants of those who lived in areas where dairy herds could be raised, such as in Europe, have developed the ability to digest milk. Most adults whose ancestors lived in extreme climates that did not support dairy herding, or areas where deadly cattle diseases were prevalent prior to 1900, however, do not retain the ability to digest milk after infancy. These included the continent of Africa and many regions of Asia (Cornell University, 2005). Bloom and Sherman (2005) conducted a comprehensive lactose intolerance study spanning 91 populations in 39 countries ranging latitudinally from southern Africa to southern Greenland. The data generated included the mean adult lactose malabsorption frequencies and information on average latitude, temperature and number of deadly endemic cattle diseases prior to 1900 for each of these countries. It was found that the frequency of adult lactase malabsorption in populations from Eurasia and Africa decrease with increasing latitude and increase with increasing temperature, and especially with numbers of deadly cattle diseases that were present before 1900. The implication is that harsh climates and dangerous diseases negatively impacted dairy herding and geographically restricted the availability of milk, thereby causing humans to physiologically adapt to these circumstances.

About 75% of adults worldwide are reported to be lactose maldigesters or have low lactase levels (Suarez and Savaiano, 1997; Scrimshaw and Murray, 1988). The percentage of adult primary lactase deficiency in Europe, Africa, Asia, the Middle East and the Americas are shown in Table 1.1. It is evident from Table 1.1 that adult primary lactase deficiency is most prevalent in populations of the Far East, and significantly high in certain regions of Africa, particularly the north east and north west. In South Africa, the prevalence of lactose intolerance is approximately 11.03% of the total population (Dairy Allergies and Lactose Intolerance, 2011). An earlier study by Segal *et al.* (1983) reported 78% lactase deficiency among the different tribes of the South African Black population and also concluded lactase deficiency to be impartial to tribal origin. In northern Europe, however, lactase deficiency is at a minimum. Of the populations of North America, Blacks and Indians exhibited the highest percentage of lactase deficiency.

Table 1.1 Prevalence of adult primary lactase deficiency (percentage of adult population) (Alm, 2002).

Country	Percentage
France	30-40
Germany	15-20
Russia	20-30
Finland	15-20
Sweden	<5
Greece	70-80
Ethiopia	80-90
Nigeria	80-90
Nomadic Fulani	<10
Sudan	60-65
China	90-100
Japan	95-100
India	60-65
Jordan	20-25
Israel	70-80
Israel (Jemenites)	40-50
North America (Whites)	10-15
North America (Blacks)	65-70
North America (Indians)	85-90
Mexico	50-60
Uruguay	60-65
South America (Indians)	90-100
Greenland (Eskimos)	85-90
Australia (Aborigines)	80-85

1.2.4 Symptoms and diagnosis of lactose intolerance

Individuals with lactose maldigestion may experience symptoms of intolerance depending on the dose of lactose consumed (Lin *et al*., 1993), gastrointestinal transit (Lin *et al*., 1993; Martini and Savaiano, 1988), the nature of the dairy food consumed (Savaiano and Lewitt, 1987; Savaiano *et al*., 1984) and the ability of the colon to metabolize the lactose (Hertzler and Savaiano, 1996; Johnson *et al*., 1993). Since the intestinal digestion of lactose is performed by membrane bound lactase (located on the brush border of the small intestine), in the event that this hydrolysis is incomplete, lactose would be transported to the colon. Colonic bacteria ferment this sugar and produce short-chain fatty acids and the gases hydrogen, carbon dioxide and methane. Short-chain fatty acids are rapidly absorbed by the colonic mucosa. The development of diarrhoea or gaseous symptoms depends partly on the balance between the production and the removal of these fermentation products, e.g. diarrhoea occurs only when the rate of delivery of lactose to the colon exceeds the rate at which the bacteria ferment lactose (Suarez *et al*., 1995; Saunders and Wiggins, 1981). Further symptoms include the occurrence of abdominal pains, distension, bloating (Vernia *et al*., 2010), and nausea or vomiting (Anania *et al*., 2008). In some instances, gastrointestinal motility is decreased and subjects can present with constipation. Such systemic symptoms as headaches, light-headedness, muscle and joint pain, and heart arrhythmia have also been attributed to lactose malabsorption (Vernia *et al*., 2010; Matthews *et al*., 2005).

Several tests are currently used for the diagnosis of lactose intolerance. The direct measurement of lactase activity in biopsies from the small intestinal mucosa is the 'gold standard' (Newcomer *et al*., 1975). This method, however, is invasive, expensive and is not readily available (Kerber *et al*., 2007). It is also undesirable for the diagnosis of paediatrics due to the invasive nature of the procedure. Furthermore, the results of the biopsy can be influenced by the uneven distribution of lactase activity throughout the small intestinal mucosa (Usai *et al*., 2008). A low cost, and the most widely used procedure in clinical practice, is the hydrogen breath test (HBT) (Vernia *et al*., 2010). When lactose is digested to its component glucose and galactose, glucose is eventually oxidized to carbon dioxide which is expelled by breathing. Lactose that is not absorbed is converted by intestinal bacteria to hydrogen gas and short chain fatty acids. This hydrogen gas is also expelled by breathing. The HBT measures the hydrogen concentration in the exhaled air after the oral administration of lactose. A study conducted by Arola (1994) evaluated the validity of HBT to direct lactase analysis and determined its specificity and sensitivity to be in the range between 89-100% and 69-100%, respectively. Despite these statistics, there are certain limitations to this procedure that lead to false negative results (Vernia *et al*., 2010). Prior intake of oral antibiotics, active diarrhoea, the use of colonic enemas, a drop in colonic pH and hydrogen utilisation by methanogenic bacteria may result in a reduction, or even the elimination of the hydrogen response, resulting in false negative tests (Vernia *et al*., 2003; Solomons, 1983; Perman *et al*., 1981). Furthermore, the HBT is time-consuming and cumbersome for both the patient and the medical professional.

A more recent diagnostic tool for lactose intolerance utilises genotyping of two single nucleotide polymorphisms (SNPs) located about 14 kb and 22 kb upstream of the lactase gene (LCT-13910 C/T and LCT-22018 G/A) (Kuokkanen, 2003; Enattah *et al*., 2002). Enattah *et al.* (2002) reported that, in patients of European decent, the CC_{-13910} genotype was found to be almost completely associated with lactose intolerance, while the GG_{-22018} genotype was slightly less consistent with lactase deficiency. A later study by Kerber *et. al.*, (2007) compared the results of LCT genotyping with the results of HBT among Austrian patients presenting with symptoms of irritable bowel syndrome. The correlation between the CC_{-13910} and GG_{-22018} genotype and a positive HBT was almost perfect (97.4% and 100%, respectively). Ingram *et al.* (2007) conducted a study among 15 different groups living in Africa and the Middle East, and discovered that while the CC_{-13910} genotype was almost completely associated with lactose intolerance in European individuals, this was not so with people of East African or Arabian ancestory. Re-sequencing of the lactase gene resulted in the discovery of new SNPs that are frequent in East Africans and even some West Africans. The use of the CC_{-13910} genotype as a diagnostic tool for lactase intolerance in East Africans and Arabians, is therefore not advised.

1.2.5 Improving lactose intolerance

It has been reported that the appearance and severity of lactose intolerance is related to lactose dosage (Tamm, 1994; Villako and Maaroos, 1994; Martini and Savaiano, 1988). The larger the amount of lactose consumed, the greater the risk of more frequent and severe symptoms. It is now well accepted that lactase deficient individuals can consume up to 11 g of lactose per day without side effects, when this portion is distributed over the day and ingested with meals (Heyman, 2006; McBean and Miller, 1998). In order to determine the threshold for lactose, affected individuals should start by consuming small portions of lactose-containing foods frequently, and gradually increase the serving size until the individual begins to experience symptoms. Moreover, several investigators have demonstrated that consuming lactose with solid food, as opposed to in water after a 12 hour fast, improves tolerance to lactose. When lactose is consumed with solid food, gastric emptying and/or delivery of lactose to the colon is slowed, which allows more opportunity for any endogenous lactase enzyme present to hydrolyse the lactose (McBean and Miller, 1998).

The use of fermented dairy foods has also long been used as a strategy for overcoming lactose intolerance. Yoghurt, for example, has been demonstrated repeatedly to improve lactose digestion and intolerance. This is attributed to the release of lactase (β-galactosidase) from the bacterial cultures, *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, used in the preparation of yoghurt (Vesa *et al*., 1996). The lactase in these cultures survive passage through the acid environment of the stomach due to the excellent buffering capacity of yoghurt (Martini *et al*., 1987), and is active in the intestine, where it aids in the digestion of lactose (Martini *et al*., 1991; Pochart *et al*., 1989; Kolars *et al*., 1984). Hertzler and Clancy (2003) investigated whether plain and flavoured kefir, a fermented milk beverage prepared using kefir grains, improved lactose digestion and tolerance in adults with lactose maldigestion. The species of bacteria and yeasts used to make kefir vary, depending on geographic location. The kefir used in their study contained *S. lactis*, *L. plantarum*, *S. cremoris*, *L. casei*, *S. diacetylactis*, *Saccharomyces florentinus* and *Leuconostoc cremoris*. The investigation revealed that plain kefir improved lactose digestion just as well as plain yogurt. This was possibly due to the high level of lactase activity in kefir (approximately 60% higher than the plain yoghurt). Furthermore, the flavoured kefir was tolerated just as well as the plain kefir and plain yoghurt. Hertzler and Clancy (2003) thus demonstrated another potential strategy for overcoming lactose intolerance, although further investigations are necessary.

Lactose-hydrolysed milk and dairy products are also available for those lactose maldigesters who wish to consume large quantities of lactose without developing symptoms and for those exceptional cases where the patient isn't able to tolerate even small doses of lactose (McBean and Miller, 1998). Lactose hydrolysis in milk and whey, a by-product of the cheese manufacturing process, can be performed by acids or acid resins. This technology, employing extremely acidic conditions (pH < 1.5) at very high temperatures (up to 150°C), was known since the beginning of the 20th century (Whittier, 1925). The use of acid is, however, undesirable for the hydrolysis of lactose in milk due to the generation of off-flavours, odours, colours and reduction of nutritional value of the milk (Laredo *et al*., 2002). An alternative is the enzymatic treatment of milk. The interest of the dairy industry in this approach has peaked in recent years due to newly acquired knowledge of the extent of lactose malabsorption and the potential market for modified milk products (Harju, 2003; Jelen and Tossavainen, 2003; Zadow, 1993; Zadow, 1992). Lactose-reduced milk is prepared industrially by adding aqueous solutions of enzyme to previously pasteurized milk and allowing the reaction to occur for a specified period of time. Once the appropriate level of lactose reduction has been reached, the milk is re-pasteurised to stop lactose hydrolysis (McBean and Miller, 1998).

Alternatively, the lactose maldigester can, himself, reduce the lactose content of the milk he wishes to ingest, by incubating milk with an enzyme preparation overnight. This ‘miracle’ enzymatic ingredient, lactase, is readily available globally in both liquid (drop) and solid (capsule, tablet) form. The addition of 5 or 15 drops of the liquid enzyme to one cup of milk hydrolyses 70% or 100 % of the lactose, respectively (Suarez *et al*., 1995). Another option is the consumption of oral enzyme replacement tablets or capsules. When taken at the beginning of a meal, they have been proved to reduce lactose maldigestion.

1.3 β-GALACTOSIDASE: A GLYCOSIDE HYDROLASE

A hydrolase is defined as an enzyme that catalyses the hydrolysis of a chemical bond. Enzymes are classified by an Enzyme Commission (EC) Number based on the chemical reaction they catalyse (Webb, 1992), hydrolases being classified as EC 3. Furthermore, glycoside hydrolases, or glycosylases, classified as EC 3.2.1, hydrolyse the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety (Sarry and Günata, 2004). Carbohydrates are complex structures, since the number of possible combinations from small oligosaccharides results in a structural and functional diversity for these compounds far greater than that possible with peptides or nucleic acids of comparable size. Oligo- and polysaccharides, therefore, play a pivotal role in an array of biological processes. Glycoside hydrolases, the enzymes that hydrolyze these molecules, are, therefore, also implicated in a wide spectrum of biological processes. Several systems of classification exist for glycoside hydrolases, the simplest of which is based on their substrate specificities (Henrissat and Davies, 1997). This classification is on the basis of the recommendations of the International Union of Biochemistry and Molecular Biology (Webb, 1992) and is expressed in EC number. *O*-Glycoside hydrolases are given the code EC 3.2.1.x, where the substrate specificity is indicated by ‘x’. In some cases, ‘x’ may also represent the molecular mechanism or the type of linkage. The advantage of this system is its simplicity. Its limitation, however, becomes evident in the classification of enzymes that act on several substrates. Classification of glycoside hydrolases into families based on amino acid sequence similarities has thus been reported to be complimentary to other classifications and a powerful tool for modern scientists (Henrissat and Davies, 1997).

Hydrolases are among the group of enzymes of increasing industrial application of which β-galactosidase has received special interest (Furlan *et al*., 2000). According to the CAZy (carbohydrate active enzymes) database, it has been classified under the glycoside hydrolase 2 (GH 2) family of carbohydrate active enzymes (Panesar *et al*., 2006). β-Galactosidase (EC 3.2.1.23) occurs widely in nature and has been isolated from animals, plants and microorganisms. Microbial β-galactosidase is more technologically important (Gosová *et al*., 2008) due to its many advantages, including the easy handling, higher multiplication rate and high production yield of microorganisms (Panesar *et al*., 2006). A number of fungi, yeasts and bacteria have been assessed as potential sources of β-galactosidase (Table 1.2). These organisms are, however, only a few examples of the vast array of investigated microbial sources of β-galactosidase, several of which have been purified, sequenced and extensively characterised.

Table 1.2 Potential sources of β-galactosidase (Cho *et al*., 2003; El-Grindy, 2003; Hoyoux *et al*., 2001; Nagy *et al*., 2001; Mahoney, 1997; Berger *et al*., 1995; Adams *et al*., 1994; Brandao *et al*., 1987).

Fungi	**Yeasts**	**Bacteria**
Aspergillus foetidis	*Candida pseudotropicalis*	*Bacillus circulans*
A. fonsecaeus,	*S. anamenis*	*B. coagulans*
A. niger	*S. fragilis*	*B. stearothermophilus*
A. oryzae	*Kluyveromyces bulgaricus*	*Bifidobacterium bifidum*
A. carbonarius	*K. fragilis*	*B. infantis*
Fusarium moniliforme	*K. lactis*	*Corynebacterium murisepticum*
F. oxysporum	*K. marxianus*	*Escherichia coli*
Mucor meihei		*L. acidophilus*
M. pusillus		*L. bulgaricus*
Penicillium conescens		*L. lactis*
P. chrysogenum		*L. sporogenes*
Trichoderma reesei		*L. thermophilus*
		L. citrovorum
		Pseudomonas fluorescens
		S. cremoris
		S. lactis
		S. thermophilus

1.3.1 General description and characteristics of microbial β-galactosidase

The β-galactosidase most studied is that from *E. coli* (Panesar *et al*., 2006). β-galactosidase from *E. coli* has a molecular weight of 464 kDa (Panesar *et al*., 2006) and both Mg^{2+} and Na^{+} are required for maximal enzyme activity (Wallenfels and Wiel, 1972). The enzyme consists of a tetramer composed of four polypeptide chains, each consisting of 1023 amino acids. Each 1023 amino-acid-monomer is in turn made up of five domains, the third of which has an α/β or 'TIM' barrel structure with the active site located on the C-terminal end of the barrel. In the tetramer, the four monomers are grouped around three mutually-perpendicular two-fold axes of symmetry.

Amino acid sequences have been established for β-galactosidase from several other bacterial sources and display extensive homology with the enzyme from *E. coli* (Mahoney, 2003). Conversely, relatively little is known about the enzyme structure from eukaryotes, although the enzyme from *K. lactis* does show extended sequence homologies with the *E. coli* enzyme, thus suggesting a close evolutionary relationship (Panesar *et al*., 2006).

1.3.1.1 Mechanism of action of β-galactosidase

β-Galactosidase has two catalytic activities, the hydrolysis of the disaccharide lactose to glucose and galactose, and the conversion of lactose to another disaccharide, allolactose, which is the natural inducer for the *lac* operon (Matthews, 2005). Enzymatic hydrolysis of the glycosidic bond of lactose takes place via general acid catalysis that requires two critical residues, a proton donor and a nucleophile/base. Wallenfels and Malhotra (1960) first proposed that the cystine and histidine residues acted as proton donor and nucleophile/base, respectively. It has now been established, however, that β-galactosidase from a variety of microbial sources, has two glutamic acid residues (Glu^{482} and Glu^{551}) that act as both proton donor and nulceophile/base in the enzymatic reaction.

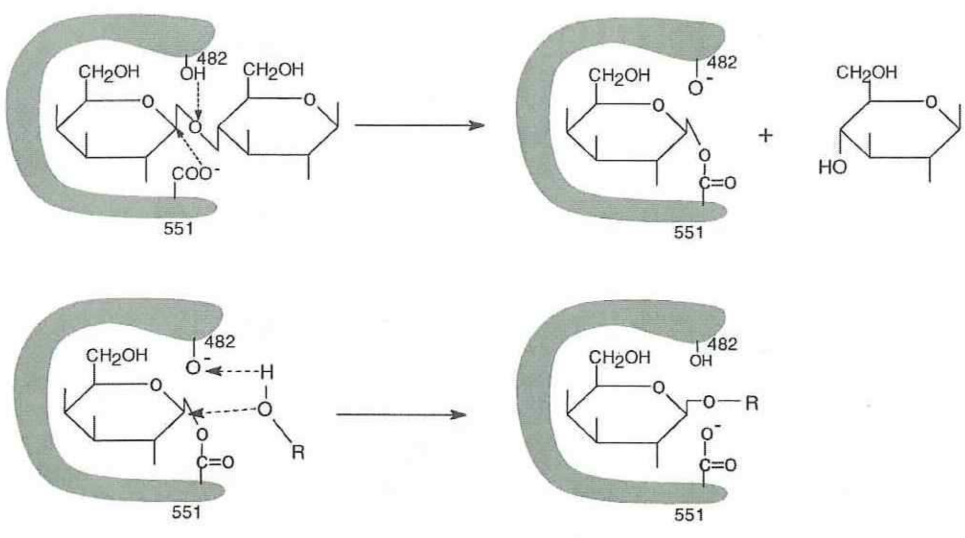


Fig. 1.1 Schematic mechanism of lactose hydrolysis by β-galactosidase (Zhou and Chen, 2001).

The reaction mechanism (Fig. 1.1) involves the initial formation of an enzyme-glycosyl complex with the liberation of a glucose molecule. The enzyme-glycosyl complex is transferred to an acceptor molecule that contains a hydroxyl group. In diluted lactose solutions, water rather than glucose and lactose, is more prevalent and thus acts as acceptor, so that galactose is formed and released from the active site. In a solution containing a high concentration of lactose, however, the lactose molecule has more of an opportunity to act as the acceptor, binding with the enzyme-galactose complex to form oligosaccharides (Zhou and Chen, 2001). This process is termed transgalactolysation.

1.3.2 Applications of β-galactosidase

One of the major applications of enzymes in industry is the preparation of lactose-hydrolysed milk and whey using β-galactosidase. In addition to the use of the enzyme in milk and fermented dairy products as mentioned in section 1.2.5, the enzymatic hydrolysis of lactose offers additional benefits in the areas of health, food technology and the environment. The cleavage of lactose under certain conditions, results in the simultaneous formation of galactooligosaccharides (GOS), which are used as prebiotic food ingredients, thereby promoting the growth of desirable intestinal microflora (Gosová *et al*., 2008; Mahoney, 1998). As these GOS are generally indigestible, they can also act as a dietary fibre source (Gosová *et al*., 2008). The temperature, concentration of substrate and origin of the enzyme play an important role in the enzymatic synthesis of GOS (Boon *et al*., 2000). The initial lactose concentration, however, has a more predominant effect. In general, higher quantities of larger sized GOS can be produced with higher initial lactose concentrations (Panesar *et al*., 2006).

In food technology, the high lactose content in milk products such as ice cream, frozen milks, whey spreads and condensed milk, can lead to excessive lactose crystallisation (Gosová *et al*., 2008). The most undesirable texture defect in dairy products is sandiness, particularly in ice cream and the Norwegian whey cheese, mysost. Sandiness is caused by lactose crystals which are large enough to be detectable in the mouth, but which do not dissolve readily, thus producing a rough or gritty sensation (Gänzle *et al*., 2008). Gelatine has been reported to be a crystalline inhibitor that reduces the rate of crystallisation by 25-60% but is ineffective in preventing sandiness in ice cream (Nickerson, 1962). The use of β-galactosidase to process ice cream could reduce lactose concentrations to acceptable values, thus preventing crystallisation and improving some qualities of dairy foods, e.g., increasing the digestibility, softness, creaminess, etc. (Zadow, 1993).

Milk whey (cheese whey), which is the most important by-product in cheese manufacturing and having a high lactose concentration, poses a major environmental problem. Over the past 50 years or so, half of the world's production has been wasted directly into aqueous habitats (González-Sizo, 1996), where the biological oxygen demand would have increased many times (Panesar *et al*., 2006). The enormity of the problem is evident in the data quoted by Yang and Silva (1995) that shows in the United States alone, 27 million tons of waste cheese whey and permeabilised whey are available as substrates for lactose conversion per year. Mawson (1994) showed that the bioconversion of milk whey lactose could reduce more than 75% of water pollution. The hydrolysis of whey using β-galactosidase is thus an important application of this enzyme. Concentrated hydrolysed whey is a very useful sweet syrup that can be used in the dairy, confectionary, baking and soft drink industries (Pivarnik *et al.*, 1995).

1.3.2.1 Immobilisation of β-galactosidase

Immobilised enzyme is defined as 'the enzyme physically confined or localised in a certain defined region of space with retention of its catalytic activity, which can be used repeatedly and continuously' (Chibata, 1978). β-Galactosidase is one of the most studied enzymes in terms of its immobilisation (Gosová *et al*., 2008). Discarding the enzyme after a single use in the applications as described in 1.3.2, would be far too costly. It must, therefore, be immobilised to allow reuse after batch reactions. Economic considerations indicate that use of the immobilised enzyme for lactose hydrolysis is economically feasible despite the cost of the immobilisation process (Aravind and Mulimani, 2008; Giacomini *et al*., 1998).

The selection of a suitable immobilisation method depends on the physical properties of the enzyme (molecular weight, protein chain length, and position of the active site), matrix, reaction conditions, reactor and so forth (Tanaka and Kawamoto, 1999). The three most popular methods of β-galactosidase immobilisation are:

i. Physical adsorption, the simplest and oldest method for immobilising an enzyme onto a water soluble carrier, has been demonstrated by numerous researchers for the immobilisation of yeast and fungal β-galactosidase (Carpio *et al*., 2000; Woudenberg-van-Oosterom *et al*., 1998; Papayannakos and Markas, 1993; Bakken *et al*., 1990).

ii. Covalent binding is the most frequently used method for β-galactosidase immobilisation. Enzymes are linked to the support using functional groups not essential for the catalytic activity. Szczodrak (2000) demonstrated the covalent binding of β-galactosidase from *K. fragilis* on porous silanised glass modified by glutaraldehyde. The coupling efficiency was very high since more the 90% of the enzyme was active and 87.5% of the protein was bound to the support.

iii. Gel entrapment is most popular for the immobilisation of whole cells, rather than enzymes. It is based on the localisation of an enzyme within the lattice of a polymer matrix or membrane. *K. fragilis* β-galactosidase was entrapped in alginate-carrageenan gels to form beads (Mammarella and Rubliolo, 2005). The presence of K-carrageenan had favourable influence on the enzymatic reaction as the gel was formed with K^+ ions, which increased the enzyme activity. Limited investigations involving the gel entrapment of yeast and fungal β-galactosidase, as compared to covalent binding, exist.

1.3.2.1.1 Industrial applications of immobilised β-galactosidase

Although numerous immobilisation systems for lactose hydrolysis have been investigated, few have been successfully scaled up and even less of these have been applied at an industrial or even pilot scale. This is mainly because the materials and procedures for immobilisation are either too costly, or difficult to implement at industrial scale (Albayrak and Yang, 2002a). In the 1970's, however, SnamProgetti (Italy) and Sumitomo Chemicals (Japan) pioneered the use of immobilised β-galactosidase in the food industry. SnamProgetti used β-galactosidase from *K. lactis*, entrapped in cellulose triacetate fibres, to hydrolyse lactose in milk (Marconi and Morisi, 1978). Sumitomo Chemicals had developed a highly pure immobilised β-galactosidase from *A. oryzae*, covalently bound to the macroporousamphoteric ion exchange resin of a phenol formaldehyde polymer. This was used for producing market milk and hydrolysed whey (Katchalski, 1993). Industrial scenarios where immobilised β-galactosidase had been successfully implemented have been detailed in Table 1.3.

Table 1.3 Successful examples of immobilised β-galactosidase applied in industry (Grosová *et al*., 2008).

Company and location	Source of enzyme	Immobilisation Technique	Application of enzyme
Specialist Dairy Ingredients (North Wales)	Unknown	Covalent binding to silica beads	Lactose hydrolysed whey
Gist Brocades (Holland); Centrale del Latte (Italy)	*K. lactis*	Entrapment in cellulose triacetate fibres	Lactose hydrolysis in milk
Rohm GmbH (Germany)	Fungal	Covalently bound to macroporous beads made of Plexiglas-like material	Processing of whole milk
Valio Laboratory (Finnland)	Fungal	Adsorption to phenol-formaldehyde resin Duolite ES-762	Whey processing

Industrially, the choice of suitable reactor system with immobilised biocatalyst depends mainly on the type of immobilisation and the type of process (Roy and Gupta, 2003). The packed bed reactor (PBR) is the most popular of all bioreactors for enzymatic lactose hydrolysis. In a PBR the immobilised enzyme is held in a column and substrate is pumped through in plug flow direction. This type of bioreactor permits the use of biocatalysts at a high density, resulting in high volumetric productivities. The PBR is preferred in the process involving product inhibition, which occurs in enzymatic hydrolysis of lactose, especially for fungal β-galactosidase (Grosová *et al*., 2008).

1.4 MICROBIAL ‘FACTORIES’ OF INDUSTRIAL β-GALACTOSIDASE

Although β-galactosidase has been isolated from many different biological sources, microorganisms are considered to be the most suitable source of this enzyme for industrial applications. The optimum conditions for β-galactosidase production by these organisms differ, thus also dictating the industrial processes in which they are applied. Microorganisms producing industrial enzymes should ideally have a higher production capacity and require cost effective enzyme extraction procedures (Panesar *et al*., 2006). Fungi and yeast, especially those of the genus *Aspergillus* and *Kluyveromyces*, respectively, are the organisms most commonly used in the β-galactosidase production industry.

1.4.1 *Aspergillus* sp.

The fungal species most frequently used for the production of β-galactosidase, has been the filamentous fungus, *A. niger* (Bailey and Linko, 1990). However, β-galactosidase preparations derived from *A. oryzae* have more recently appeared on the market, e.g. Röhm Lactase 2214 C (Germany), Biocon Biolactase (USA), MKC Takamine ® and EDC Enzecor ® fungal lactase (Bailey and Linko, 1990). Other products include Fungal lactase from the Enzyme Development Corporation (USA) and β-galactosidase from Sigma-Aldrich (UK). Fungal β-galactosidases usually have a pH optimum in the acidic range (2.5 to 5.4), rendering them suitable for the processing of acid whey and its ultrafiltration permeate (Panesar *et al*., 2006). A further advantage of the *A. oryzae* enzyme is its higher residual activity at the natural pH of milk, 6.7 (Van Griethuysen-Dilber *et al.*, 1988), which makes it a more versatile biocatalyst. The patented *A. oryzae* ATCC 20423 strain was reported to produce β-galactosidase activity of 500 $nkat.ml^{-1}$ in shake flask culture medium containing wheat bran and spent grain.

A further application of β-galactosidase from *Aspergillus* sp. is the formulation of enzyme tablets or capsules that can be ingested by lactose intolerant individuals prior to the consumption of a dairy product, to curb lactose maldigestion. In the stomach, gastric fluids provide strongly acidic conditions and β-galactosidase enzyme preparations that are effective at neutral, alkaline or slightly acidic conditions are thus either inactivated or destroyed. β-galactosidase preparations from *Aspergillus* sp., particularly *A. niger*, would be suitable for action in such conditions since they are pH stable and thus would not require enteric coatings to provide protection against the harsh gastric environment (Huff, 1973). Those currently on the market range from 14 000 to 65 000 lactase units per gram.

The attractiveness of *Aspergillus* β-galactosidase is that the enzyme is largely expressed extracellularly and cell disruption is, therefore, not required. Furthermore, immobilisation of β-galactosidase from *Aspergillus* sp. has been reported by several authors (Haider and Husain, 2009; Haider and Husain, 2007; Albayrak, 2002a, b, c) and its advantages over the free form of the enzyme are well known. As described in section 1.3.2.1.1, immobilised forms of the *Aspergillus* enzyme, are currently being implemented in industry.

1.4.2 *Kluyveromyces* sp.

Yeasts have been used in both traditional and modern biotechnology for the production of food, beverages, fine chemicals, pharmaceutical reagents and enzymes. There are a wide diversity of yeasts that have biotechnology applications, including the genus *Kluyveromyces* (Lane and Morrissey, 2010). The genus *Kluyveromyces* was named by van der Walt (1956), who transferred species from the genus *Saccharomyces* to this group. After numerous classifications and re-classifications, the genus *Kluyveromyces*, currently comprises only 6 species (Lachance, 2007), of which *K. lactis* and *K. marxianus* are the most studied.

1.4.2.1 *K. lactis*

K. lactis is described as a budding yeast whose natural habitat is dairy products (van der Walt, 1970). *K. lactis* research began in the early 1960s and was initially driven by a purely academic concern: the possible adaptive regulation of sugar metabolism in a lower eukaryote. Biotechnological interest in the species followed much later (Fukuhara, 2006). Since then, *K. lactis* has been used by the scientific community as the model organism in the *Kluyveromyces* genus, leading to a much better understanding of its physiology and to the full sequencing of its genome through the ‘Génolevures’ project (Fonseca *et al*., 2008; Dujon *et al*., 2004).

1.4.2.1.1 Properties of *K. lactis* β-galactosidase

The β-galactosidase from *Kluyveromyces* sp. has a neutral pH optima and is, therefore, suitable for the hydrolysis of milk that can be used for the preparation of flavoured milk, cheese and yogurt. Furthermore, the quality of ice cream can be significantly improved by the addition of lactozyme (β-galactosidase) as the enzyme would prevent crystallisation, thereby reducing sandiness (Panesar *et al*., 2006). Another desirable quality of this enzyme is that its GRAS (Generally Regarded As Safe) status has been affirmed by the United States Food and Drug Administration (FDA) as a direct ingredient of food (Randolph, 1984).

K. lactis is known to produce an inducible, intracellular β-galactosidase encoded for by the *LAC4* gene. Poch *et al*. (1992) cloned and sequenced the entire *LAC4* gene of a strain of *K. lactis* and found that the deduced amino acid sequence revealed extended sequence homologies with all the published prokaryotic β-galactosidase sequences, suggesting that the eukaryotic β-galactosidase is closely related, evolutionarily and structurally, to its prokaryotic counterparts.

Cavaille and Combes (1995) investigated the structural and kinetic properties of MAXILACT LX 5000, a commercial liquid preparation of β-galactosidase from *K. lactis*. The 200 kDa enzyme is a glycoprotein containing 45% (w/w) carbohydrate and is composed of two identical subunits. Galactose and glucose (the hydrolysis products of the enzyme) are competitive and non-competitive inhibitors, respectively. β-galactosidase has similar affinity for both lactose (the substrate) and galactose (the product) as both are capable of occupying the active site of the enzyme with equal probability (Jurado *et al.*, 2002). The Michaelis-Menten constant for *o*-nitrophenol-β-D-galactopyranoside (ONPG), the preferred substrate for the β-galactosidase assay, is 1.7 mM and that of lactose is 17.3 mM (Cavaille and Combes, 1995) and the optimum pH and temperature for β-galactosidase production in *K. fragilis* (*K. marxianus*) is pH 6.6 and 37°C and in *K. lactis* is pH 6.9 to 7.3 and 35°C (Jurado *et al.*, 2002).

1.4.2.1.2 Intracellular β-galactosidase

As mentioned previously, β-galactosidase is located intracellularly in *K. lactis*. Various methods have been investigated for the release of this enzyme. Disruption methods can be divided into those that produce cell leakage in response to the application of shear forces, and those that result in digestion of the cell envelope by the action of chemicals and/or exogenous and endogenous lytic enzymes (Numanoğlu and Sungur, 2004). Becerra *et al.* (2001) reported on chemical (chloroform, methanol, ethanol, isopropanol and *t*-butanol) and mechanical (vortexing, vortexing with glass beads and sonication) methods of *K. lactis* cell disruption, of which sonication proved to be the most effective. These methods may be suitable for use in the laboratory, but not so in large-scale industrial processes. Two methods commonly used in pharmaceutical or biotechnology industries for large-scale cell disruption are high-speed bead milling and high pressure homogenising (Bury *et al*., 2001).

Industrially, the application of intracellular β-galactosidase is impeded due to the difficulty and excessive cost of releasing active enzyme in good yields from cells and the expense of purification process. An interesting alternative could be the use of whole cells as a source of β-galactosidase. This poses a further problem due to the poor permeability of the cell membrane to lactose. The use of various agents has been investigated for the permeabilisation of *Kluyveromyces* yeast cells (Panesar *et al*., 2006). Detergents such as digitonin and cetyltrimethylammonium bromide (CTAB) have been reported to be successful, with the activity of the permeabilised cells being significantly greater than the untreated cells (Bachhawat *et al*., 1996; Bhat *et al*., 1993; Joshi *et al*., 1989; Gowda *et al*., 1988).

Numanoğlu and Sungur (2004) investigated permeabilisation of *K. lactis* whole cells using two chemical (toluene and ethanol-chloroform) and one mechanical (vortexing with glass beads) disruption method. The cells were immobilised into gelatin using glutaraldehyde as the crosslinker. The activity obtained for the immobilised cells was 30% higher than that obtained for the free, permeabilised cells, and the immobilised enzyme retained more than 80% of its original activity after 17 uses within 34 days. This seems to be a promising method to decrease the cost of the β-galactosidase biocatalyst in the food industry, by the elimination of costly purification processes.

1.4.2.2 *K. marxianus*

It is important to mention *K. marxianus*, formerly known as *K. fragilis* and *S. fragilis* (Cortés *et al*., 2005). The common trait shared with *K. lactis* is the ability to assimilate lactose and to use this sugar as a carbon source. In addition, both organisms have achieved GRAS and QPS (Qualified Presumption of Safety) status in the United States and European Union, respectively. The contrast between the two, however, is that, while *K. lactis* has been the recognised academic research species in the *Kluyveromyces* genus, *K. marxianus* has been preferred for use in industry. This is largely due to the numerous desirable characteristics exhibited by *K. marxianus*. It has been reported to have the fastest growth rate of any eukaryotic microbe (Lane and Morrissey, 2010) and is able to grow on an array of substrates at higher temperatures. Strains of *K. marxianus* have been isolated from a variety of habitats, therefore, metabolic diversity is broad and the potential for biotechnological application is varied (Fonseca *et al*., 2008). The most interesting characteristic, however, is the natural ability to excrete enzymes, e.g. extracellular inulinase (Hensing *et al*., 1994), thus eliminating the need for cell disruption.

Amongst its numerous biotechnological applications, including the production of ethanol from whey or lactose, the production of biomass, single cell proteins, aromatic compounds, and numerous environmental applications, such as the treatment of paper wastes and sludge, *K. marxianus* has the potential to produce endogenous enzymes, of which β-galactosidase is one (Lane and Morrissey, 2010). Numerous authors have reported on the effect of a variety of culture conditions on the production of β-galactosidase by *K. marxianus* (Barberis and Segovia, 2002; Schneider *et al*., 2001; Barberis and Gentina, 1998; Barberis and Segovia, 1997; Topete *et al*., 1997; Inchaurrondo *et al*., 1994; Ozilgen *et al*., 1988; García-Garibay *et al*., 1987; Mahoney *et al*., 1975). Furthermore, Furlan *et al*. (2000) also showed the potential of a *K. marxianus* strain to produce β-galactosidase in a lactose-free medium with sucrose as the main carbon source. These extensive investigations have led to two commercial products currently on the market that are derived from *K. marxianus*, Lactozyme (Novozymes A/S, in Bagsvaerd, Denmark) and β-galactosidase (Sigma-Aldrich in the UK) (Panesar *et al*., 2006).

1.5 CULTURE METHODS FOR β-GALACTOSDASE PRODUCTION BY *Kluyveromyces sp.*

The production of β-galactosidase in shake flask cultures has been extensively investigated. For example, β-galactosidase production by a strain of *K. lactis* was optimised in shake flask experiments, investigating agitation speed, pH, initial substrate concentration and incubation time (Dagbagli and Goksungur, 2008). In the shake flask scenario, however, growth parameters that affect sugar transport, such as pH and dissolved oxygen concentration (Weusthuis *et al*., 1994) cannot be regulated. Cultivation in a fermenter, where process parameters can be controlled, would seem a more feasible option for the production of β-galactosidase (Inchaurrondo *et al*., 1994; Moresi *et al*., 1989; Beausejour *et al*., 1981; Dickson and Markin, 1980).

There are two main types of feeding scenarios in a fermenter, batch and fed-batch. To minimise the Crabtree effect, a metabolic regulatory pathway that results in a metabolic shift leading to the undesirable fermentation of lactose to ethanol under fully aerobic conditions, the fed-batch feeding scenario is preferred. The advantage of fed-batch culture is that cell growth can be controlled by regulating the nutrient feeding rates and thus extend the duration of high volumetric enzyme productivity, as demonstrated by Nor *et al*. (2001).

The chemostat or continuous culture is another cultivation method that could be employed for the production of β-galactosidase. In such a fermentation, nutrients are continuously fed to the culture, and spent media containing biomass and product, is continuously removed at the same rate of feeding. Continuous cultivation offers unique possibilities to manipulate the rate of sugar transport in growing cells (Weusthuis *et al*., 1994), and, therefore, enzyme production. Limited data is, however, available regarding the continuous production of β-galactosidase, and more research into this area is required.

1.6 *K. lactis* UOFS y-0939

K. lactis UOFS y-0939, the organism under investigation in this study, has other designations, including, CBS 2359, NRRL Y-1140, ATCC 8585 and NCYC 1368, depending on the culture collection in which it has been deposited. It was first isolated by N. C. Laffer from a creamery in Illinois, USA and initially deposited in the culture collection of the Agricultural Research Service of the US Department of Agriculture, the NRRL, in 1971. The yeast is known to grow aerobically on lactose and to produce an intracellular β-galactosidase. Numerous studies have been conducted with this particular strain of *K. lactis*, including a physiological study of β-galactosidase induction in the organism (Dickson and Markin, 1980), characterisation of the transport of lactose in *K. lactis* (Dickson and Barr, 1983), sequencing of the *K. lactis* β-galactosidase (Poch *et al*., 1992), a study on different techniques for the purification of β-galactosidase (Becerra *et al*., 1998) and the investigation of various methods for the extraction of intracellular proteins (Becerra *et al*., 2001), among others. Furthermore, the production of β-galactosidase in aerobic continuous culture by this particular strain of *K. lactis*, has been investigated (Inchaurrondo *et al*., 1998), although, not extensively.

1.7 SCOPE OF THE STUDY

Kluyveromyces sp. have exhibited tremendous potential as industrially significant strains. Their application in the production of β-galactosidase, particularly for the alleviation of lactose intolerance in present day South Africa, is of primary importance. Since this *K. lactis* UOFS y-0939 strain shows great potential for large-scale commercial production of β-galactosidase, more information on the optimal growth under different feeding scenarios is required. It was, therefore, the purpose of this study to optimise cultivation conditions of the wild type *K. lactis* UOFS y-0939 strain for the production of the enzyme in shake flask cultures. Optimised shake flask cultures were scaled up to batch and fed-batch cultures. Finally, β-galactosidase production in continuous culture was investigated.

1.7.1 Objectives

i) To optimise temperature, pH and inoculum size in shake flask cultures for the production of β-galactosidase by *K. lactis* UOFS y-0939.

ii) To optimise the ultrasonication period required for maximum cell disruption and, therefore, enzyme yield.

iii) To scale up β-galactosidase production to 3 L batch fermentation by applying the optimised conditions as obtained from shake flask experiments.

iv) To investigate β-galactosidase production in a 10 L fed-batch fermentation.

v) To examine β-galactosidase production in a 3 L continuous fermentation.

CHAPTER TWO: OPTIMISATION OF β-GALACTOSIDASE PRODUCTION IN SHAKE FLASK EXPERIMENTS

2.1 INTRODUCTION

Knowledge of the effects that different environmental factors have on enzymatic activity would be highly useful to industrial applications. Such factors include temperature, pH, medium composition, shaking speed, and shear (Jurado *et al*., 2004). The effect of temperature on enzyme activity has been described by two thermal parameters: the Arrhenius activation energy, which describes the effect of temperature on the catalytic rate constant, and thermal stability, which describes the effect of temperature on the thermal inactivation rate constant. Irregularities in this model has recently been resolved by a new model (the Equilibrium Model), which more completely describes the effect of temperature on enzyme activity by including an additional mechanism by which enzyme activity decreases as the temperature is raised (Peterson *et al*., 2007).

The activities of many enzymes are also pH dependent due to ionogenic groups in the enzyme active site being able to provide for catalytic activity only with a certain state of protonation. In this case, catalysis depends on the concentration of the activated enzyme and thus on the pH of the medium (Tsygankov *et al*., 2007). Enzymes typically are most active in a pH range of 5 to 9 since most physiological environments reflect this pH. Individual enzymes usually have a narrow pH range, since a variety of amino acid residues, including the carboxyl and amide termini of proteins, have a pKa range in the range of intracellular pH. As a result, a change in pH can protonate or deprotonate a side group, thereby changing its chemical features. A more drastic pH change can denature the enzyme by altering the protein folding, thereby completely deactivating the enzyme or by causing irreversible proteolysis (Berg, 2007).

Several investigations have focused on the optimisation of a variety of culture conditions for the production of β-galactosidase by *Kluyveromyces* sp. (Dagbagli and Goksungur, 2008; Furlan *et al*., 2001; Furlan *et al*., 2000; Borjorge *et al*., 1999; Fiedurek and

Szczorak, 1994; Chen *et al*., 1992). Medium composition, in addition to environmental parameters, plays an essential role in enzyme activity. Since *K. lactis* produces an intracellular β-galactosidase, cell disruption is the first stage in the procedure for isolating the intracellular material (Keshavarz *et al*., 1987). Cell disruption thus has considerable influence on the total quantity of the desired product recovered, the biological activity of the product, its association with other cellular components, and the possible presence of proteolytic degradation and contaminants that may influence the subsequent purification steps (Millard and Charles, 1990). Ultrasonication, which is the cell disruption method used in the optimisation phase of this study, is widely used at laboratory scale. The structure and function of biological molecules can be altered by the ultrasound vibrations. The most common interaction mechanisms are acoustically induced cavitational activity, heat or chemical effects. In addition, inactivation of biomolecules by ultrasonication can also be caused by mechanical effects i.e., shear stress developed by eddies arising from the created shock waves. Thus, the level and intensities of ultrasound has a direct effect on the activity or inactivity of many enzymes (Şener *et al*., 2006).

This phase of the investigation focused on optimising temperature, pH and inoculum size for the production of β-galactosidase by *K. lactis* UOFS y-0939 using shake flask cultivation, in a growth medium containing lactose as the sole carbon source. The time required for maximum cell disruption using ultrasonication was also optimised.

2.2 MATERIALS AND METHODS

2.2.1 Strain and culture conditions

K. lactis UOFS y-0939 was obtained from the UNESCO MIRCEN Yeast Culture Collection, Department of Microbial, Biochemical & Food Biotechnology, University of the Free State. This strain was maintained on YPD agar plates, containing 3 $g.L^{-1}$ yeast extract (BioLab); 5 $g.L^{-1}$ peptone (BioLab); 10 $g.L^{-1}$ anhydrous glucose (Saarchem); 20 $g.L^{-1}$ bacteriological agar (BioLab), and stored at 4°C. For fermentation experiments the organism was sub-cultured onto YPD agar plates and incubated at 30°C for ± 48 h.

2.2.2 Work cell bank preparation

Two *K. lactis* UOFS y-0939 colonies from a freshly prepared plate culture, were inoculated into YPD broth (200 ml) in 500 ml conical flasks with the following medium composition: 3 $g.L^{-1}$ yeast extract; 5 $g.L^{-1}$ peptone; and 10 $g.L^{-1}$ anhydrous glucose. The experiments were done in duplicate and flasks were incubated in an orbital shaker (Infors HT Multitron, Switzerland) (30°C; 200 rpm). After approximately 25 h of growth, late exponential phase was reached. The monoseptic state of one of the flasks was verified microscopically (Olympus BX-41 phase contrast microscope, Japan) and this flask culture was used for the preparation of the work cell bank. An equal volume of monoseptic broth and 50% (v/v) glycerol were mixed [effective glycerol concentration, 25% (v/v)], and from this, aliquots (1 ml) were aseptically dispensed in 1.2 ml cryovials (Corning). The vials were placed in cryo-storage vessels (Mr Frosties) containing iso-propanol (Saarchem) pre-cooled at 4°C. These vessels were stored (-80°C; 24 h) after which, the vials were transferred to cryo-boxes for long-term storage.

2.2.3 Work cell bank validation

After 36 h of storage (-80°C), three random cryovials from the work cell bank were selected and used to inoculate three flasks containing YPD broth (200 ml). The monosepticity of the Log M and time zero samples were determined by microscopic evaluations and streak plating. The flasks were incubated (30°C, 200 rpm) and, thereafter, samples were taken at 2 h intervals. Growth was monitored by measuring optical density at 600 nm with a Biochrom Libra S12 spectrophotometer (United Kingdom). The pH (Cyberscan pH 510, Eutech Instruments, Indonesia), biomass (section 2.2.3.1) and glucose concentration (section 2.2.3.2) were determined for each sample.

2.2.3.1 Determination of biomass concentration

Sample broth (1 ml) was added to pre-dried and pre-weighed microcentrifuge tubes and centrifuged (Biofuge Fresca, Heraeus, Thermo Electron Corporation, Germany) (13 000 rpm, 5 min) to remove the culture media and suspended particles. The pellet was washed with 0.1 M HCl (Saarchem) (1 ml), followed by a second wash with distilled water (1 ml). The resultant pellet was dried in an oven (100°C, ± 24 h), then cooled in a dessicator (± 5 h). The tubes were then weighed and the final biomass in the sample was calculated. All biomass determinations were performed in triplicate.

2.2.3.2 Determination of glucose concentration

Aliquots of the sample broth (1 ml) were centrifuged (13 000 rpm, 5 min). The resultant supernatant was diluted and analysed for glucose concentration using the Biochemistry Analyser (YSI Inc., United States of America).

2.2.4 Optimisation studies: Inoculum medium

The inoculum medium used for the optimisation studies was as determined by Manera *et al*. (2008) to be optimum for β-galactosidase production by *K. marxianus* CCT 7082. Medium composition for liquid broth were as follows: 28.2 $g.L^{-1}$ lactose (Lactose Edible Grade 200 Mesh, CJ Petrow Chemicals); 17 $g.L^{-1}$ yeast extract; 8.8 $g.L^{-1}$ ammonium sulphate (Saarchem); 5 $g.L^{-1}$ potassium dihydrogen orthophosphate (Saarchem), and 0.4 $g.L^{-1}$ magnesium sulphate heptahydrate (Saarchem). The medium was prepared in 0.2 M potassium phosphate buffer and the pH adjusted. Lactose was prepared separately and added to the inoculum medium prior to inoculation.

2.2.4.1 Optimisation of the ultrasonication period

Aliquots (1.5 ml) of an exponential phase *K. lactis* culture were centrifuged (13 000 rpm, 5 min, 4°C). The pellet was washed once with ultrapure water (1 ml), and thereafter, resuspended in 0.2 M potassium phosphate buffer (1.5 ml). Borosilicate glass beads (0.2 g) of 1 mm diameter, were added to each tube. Negative control samples (without glass beads) were also prepared. Samples were ultrasonicated in an ultrasonic water bath (Ultrasonic Cleaner DC 400H, MRC, Israel) for 10, 20 and 30 min at 4°C. The control samples were not ultrasonicated. Microscopic observation, viable cell count (section 2.2.4.1.1) and β-galactosidase activity (section 2.2.4.1.2) was performed on all samples.

2.2.4.1.1 Viable cell count

Serial dilutions of each of the 10, 20 and 30 min samples, and the controls, were prepared in buffered peptone water (BioLab). Aliquots (100 µl) of each dilution were spread onto YPD agar plates (in triplicate) and incubated (30°C, ± 48 h). *K. lactis* colonies on each plate were enumerated and plates exhibiting > 30 and < 300 colonies were considered. The viable cell count per sample (cfu.ml^{-1}) was then calculated.

2.2.4.1.2 β-Galactosidase activity

β-Galactosidase activity was determined using ONPG (Sigma) as the substrate, according to the method described by the Food Chemicals Codex: General Tests and Apparatus (1981:491-492). Cell lysate (1 ml) was added to 8.3 mM ONPG (4 ml) prepared in [27.2 g.L^{-1} potassium dihydrogen orthophosphate, 37.2 mg.L^{-1} disodium ethylenediaminetetraacetic acid (EDTA) dehydrate (Saarchem) and 20.3 mg.L^{-1} magnesium chloride hexahydrate (Saarchem)] (PEM) buffer, pH 6.5, and incubated (37°C, 15 min). The reaction was stopped by adding 1 ml of the reaction mixture to 1 ml of 10% sodium carbonate (Saarchem). The mixture was diluted to 10 ml with ultrapure water. Liberated *o*-nitrophenol (ONP) was measured spectrophotometrically at 420 nm. The extinction coefficient (ε) was calculated from a standard curve prepared with ONP concentrations ranging from 0.0012 to 0.2 µmol.ml^{-1}. One β-galactosidase unit was defined as that quantity of enzyme that would liberate 1 µmol of ONP per min under the conditions of the assay and was calculated as follows:

β-Galactosidase activity ($U.ml^{-1}$) = $A \times 5 \times 10/(\varepsilon \times 15)$

A	=	average of the absorbance readings for the sample
5	=	volume in ml of the incubation mixture
10	=	final volume in ml of the diluted incubation mixture
ε	=	extinction coefficient of *o*-Nitrophenol
15	=	incubation time in min

2.2.4.2 Evaluation of optimum temperature for β-galactosidase activity

The seed culture was prepared by inoculating a cryovial culture of *K. lactis* UOFS y-0939 (1 ml) into YPD broth (200 ml) which was incubated in an orbital shaker (30°C, 180 rpm). At late exponential phase, the monoseptic seed culture (30 ml) was transferred to the inoculum medium (270 ml), with the effective inoculum size being 10%. The temperatures evaluated were 25, 30 and 35°C and all experiments were performed at pH 7. Flasks were incubated in an orbital shaker (180 rpm) and following Log M and the time zero sample, sampling was performed at 2 h intervals. The optical density at 600 nm, media pH, biomass, lactose concentration, β-galactosidase activity, specific β-galactosidase activity and specific β-galactosidase activity on biomass were determined as described in sections 2.2.3.1, 2.2.4.2.1, 2.2.4.1.2, 2.2.4.2.3 and 2.2.4.2.4, respectively.

2.2.4.2.1 Determination of lactose concentration

Lactose concentration was measured using the Biochemistry Analyser. Aliquots of the sample broth (1 ml) were centrifuged (13 000 rpm, 5 min) and the resultant supernatant used for analysis. A lactose standard curve was constructed with lactose standards ranging from 2.5 to 25 $g.L^{-1}$. All samples were diluted 10 times and analysed with the Biochemistry Analyser. Lactose concentration was determined using regression analysis of the standard curve.

2.2.4.2.2 *K. lactis* UOFS y-0939 cell lysate preparation

Aliquots (1.5 ml) of sample broth was dispensed into two microcentrifuge tubes, and centrifuged (13 000 rpm, 5 min, 4°C). The pellet was washed once with ultrapure water (1 ml), and thereafter, resuspended in 0.2 M potassium phosphate buffer, pH 7 (1.5 ml). Samples were kept cold at all times. Borosilicate glass beads (0.2 g) of 1 mm diameter, were added to each tube and the samples stored (-80°C). At 12 h, negative control samples (without glass beads) were similarly prepared. Samples were thawed on ice and ultrasonicated in an ultrasonic water bath for the optimum period (4°C). The samples were centrifuged (13 000 rpm, 30 min, 4°C) and the supernatant transferred to a clean microcentrifuge tube and stored (-80°C) until further analysis.

2.2.4.2.3 Specific β-galactosidase activity

Specific activity was determined as a function of the total protein concentration in the sample. Total protein was measured according to a minor modification of the method described by Bradford (1976). A standard curve was constructed using bovine gamma globulin (BGG) as the protein standard, in concentrations ranging from 0.025 to 1 $mg.ml^{-1}$, instead of bovine serum albumin (BSA). The cell lysate (50 µl) was added to Bradford dye (950 µl) and the reaction mixture incubated (room temperature, 5 min). Samples were then read spectrophotometrically at 595 nm. Protein concentration ($g.ml^{-1}$) was determined from regression analysis of the standard curve and specific β-galactosidase activity was calculated as follows:

$$\text{Specific } \beta\text{-galactosidase activity } (U.g^{-1}) = A \times 5 \times 10/(\varepsilon \times 15 \times W)$$

A	=	average of the absorbance readings for the sample
5	=	volume in ml of the incubation mixture
10	=	final volume in ml of the diluted incubation mixture
ε	=	extinction coefficient of *o*-Nitrophenol
15	=	incubation time in min
W	=	total protein concentration of the sample

2.2.4.2.4 Specific β-galactosidase activity on biomass

Specific β-galactosidase activity on biomass was determined as a function of the biomass concentration of the sample, and was calculated as follows:

Specific β-galactosidase activity on biomass ($U.g^{-1}$) = $A \times 5 \times 10/(\varepsilon \times 15 \times W)$

W = total biomass concentration of the sample

2.2.4.3 Evaluation of optimum media pH for β-galactosidase activity

The culture medium for the evaluation of the effect of media pH on enzyme activity was as described in section 2.2.4. *K. lactis* UOFS y-0930 was grown in culture flasks as described in section 2.2.4.2. The optimum growth temperature, as determined in section 2.2.4.2, was applied for all pH experiments. The different culture pHs evaluated were 5.5 and 7, and was maintained by adjusting the pH of the growth medium with 2 M potassium hydroxide (Saarchem) prior to sterilisation. Sampling and analyses were performed as described in section 2.2.4.2.

2.2.4.4 Evaluation of optimum inoculum size for β-galactosidase activity

K. lactis UOFS y-0930 was grown in culture flasks as described in section 2.2.4.2. The optimum temperature and pH, as determined in sections 2.2.4.2 and 2.2.4.3, was applied for all inoculum size experiments. The inoculum sizes investigated were 6, 8 and 10% (v/v). Sampling and analyses were performed as described in section 2.2.4.2.

2.3 RESULTS

2.3.1 Work cell bank validation

The *K. lactis* UOFS y-0939 work cell bank was validated in order to determine the growth kinetics of the test organism and, therefore, the time of transfer of the seed culture to the inoculum flasks for optimisation studies. This was determined to be 18 h. In the seed culture medium containing glucose, an 8 h lag phase at 30°C was observed and the maximum growth rate (μ_{max}) of the organism was 0.44 h^{-1} between 8 and 14 h (Fig. 2.1). The yield coefficient ($Y_{x/s}$) of biomass achieved on glucose was 0.67 $g.g^{-1}$.

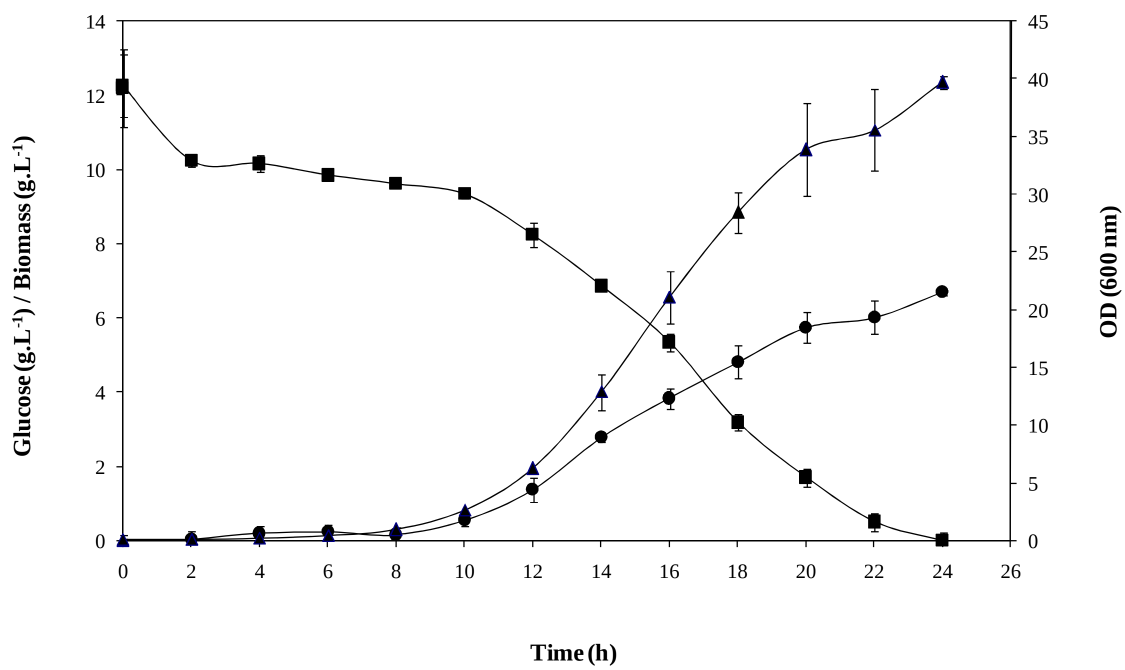


Fig. 2.1 Growth of *K. lactis* UOFS y-0939 at 30°C and 200 rpm using shake flask cultivation. Glucose concentration (■), biomass (●) and optical density at 600 nm (▲) were monitored for 24 h. Each point represents the mean of triplicate values with ± standard deviation (SD).

2.3.2 Optimisation of the ultrasonication period

Microscopic observation, viable cell count and β-galactosidase activity were performed to evaluate the effectiveness of ultrasonication in a water bath for 10, 20 and 30 min at 4°C. Microscopically, an increasing number of cells were disrupted with an increase in ultrasonication time (Fig. 2.2). The number of viable cells decreased as the ultrasonication period increased, with a 16% reduction in the number for viable cells achieved after 30 min as compared to the control (Fig. 2.3A). Consequently, volumetric β-galactosidase activity increased as a greater number of cells were disrupted, with the highest activity (0.42 ± 0.1 $U.ml^{-1}$) achieved after ultrasonication for 30 min (Fig. 2.3B).

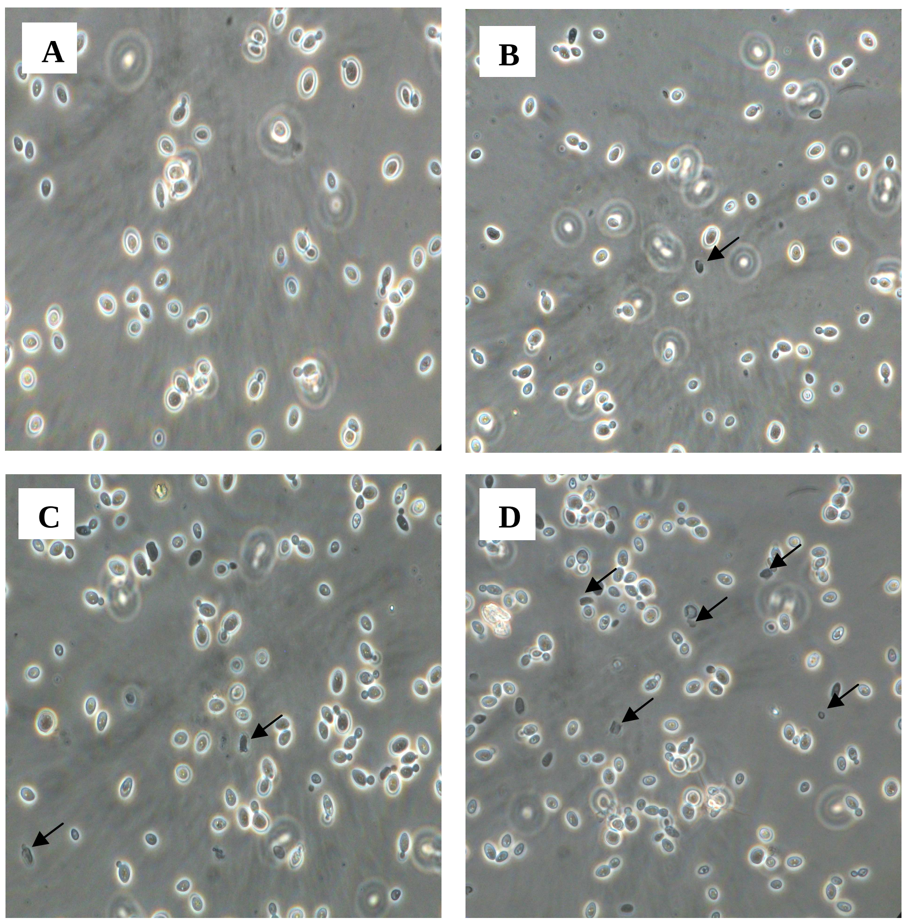
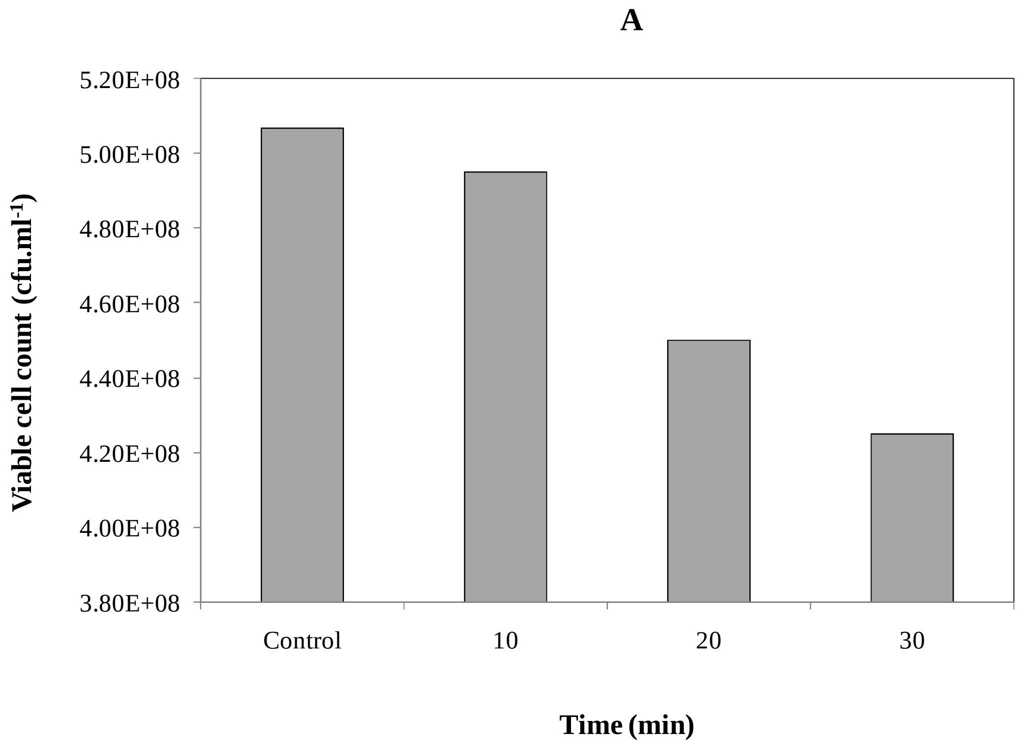


Fig. 2.2 Microscopic evaluation (100X magnification) showing the effect of ultrasonication on the disruption of *K. lactis* UOFS y-0939 exponential phase cells. Samples include the negative control (A) and samples sonicated for 10 (B), 20 (C) and 30 (D) min. Arrows indicate disrupted cells.



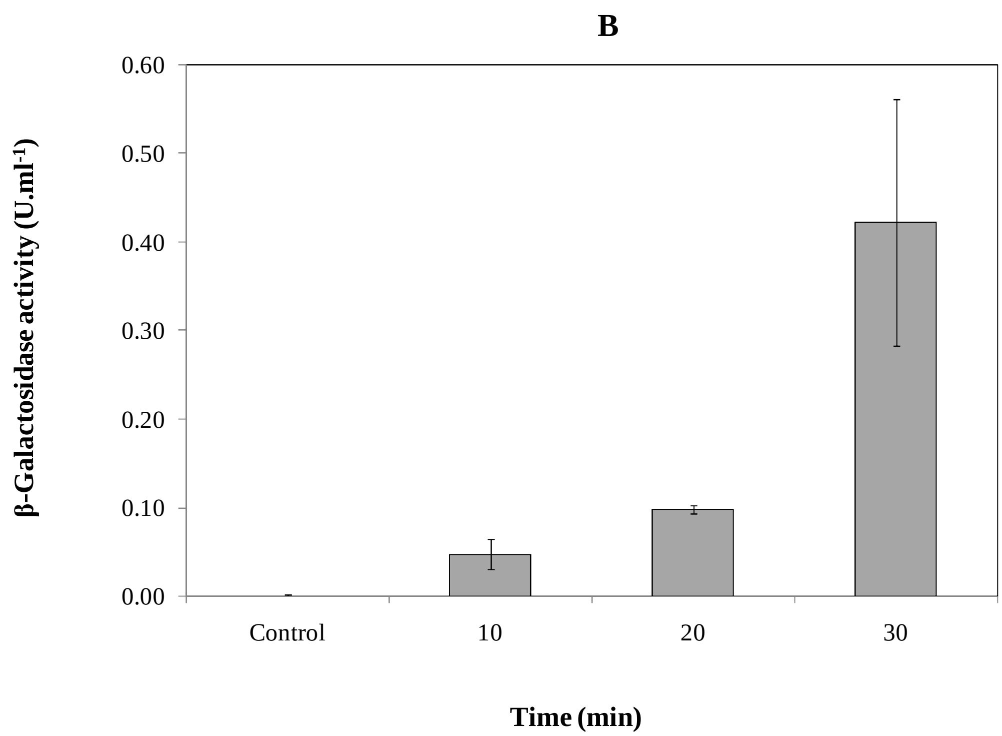


Fig. 2.3 Viable cell count (A) and β-galactosidase activity (B) as indicators of the effectiveness of 10, 20 and 30 min ultrasonication periods, on the disruption of *K. lactis* UOFS y-0939 exponential phase cells. Each bar represents the mean of triplicate determinations with ± SD. [Note: error bars are not visible in (A) due to the scale.]

2.3.3 Effect of temperature on growth and β-galactosidase activity

A 10% inoculum of an 18 h *K. lactis* UOFS y-0939 seed culture was transferred to the inoculum medium containing lactose as the sole carbon source. When the effect of temperature on growth and β-galactosidase activity by *K. lactis* UOFS y-0939 was investigated using this medium, the highest growth rates, μ_{max} of 0.31 h^{-1} and 0.30 h^{-1} was observed at incubation temperatures of 30°C and 35°C, respectively, while the lowest μ_{max} of 0.21 h^{-1} was observed at 25°C. The effect of temperature on substrate consumption by *K lactis* UOFS y-0939 under growth conditions was also evaluated. The lactose consumption rate was observed to be most efficient at an incubation temperature of 35°C, with an initial lactose of 28.2 $g.L^{-1}$ completely depleted within 10 h (Fig. 2.4). Conversely, when the same organism was incubated at 25°C, the initial lactose (28.2 $g.L^{-1}$) was completely depleted after 14 h of growth, implying a much slower lactose utilisation rate. Consequently, the highest biomass produced following exponential growth was at 30°C (7.25 ± 0.3 $g.L^{-1}$) with the lowest biomass production of 5.64 ± 0.3 $g.L^{-1}$ at 35°C. Even though growth at 25°C also led to an eventual biomass of 7.62 ± 0.2 $g.L^{-1}$ being produced after 14 h, the substrate consumption in this case was much slower in comparison to other evaluated temperatures. The yield coefficient of biomass on lactose, ($Y_{x/s}$) at 30°C was determined to be 0.26 $g.g^{-1}$. β-Galactosidase activity of 0.35 ± 0.05 $U.ml^{-1}$ and specific activity on biomass was observed to be the highest at 30°C at the late exponential phase (12 h) and thereafter decreased due to the complete depletion of lactose, as was the occurrence at 35°C (Fig. 2.5 and 2.6B). At 25°C, however, β-galactosidase activity continued to increase as the organism was still in the exponential phase of growth at 14 h. All negative control samples prepared at 12 h exhibited either zero, or negligible β-galactosidase activity and specific activity (data not shown).

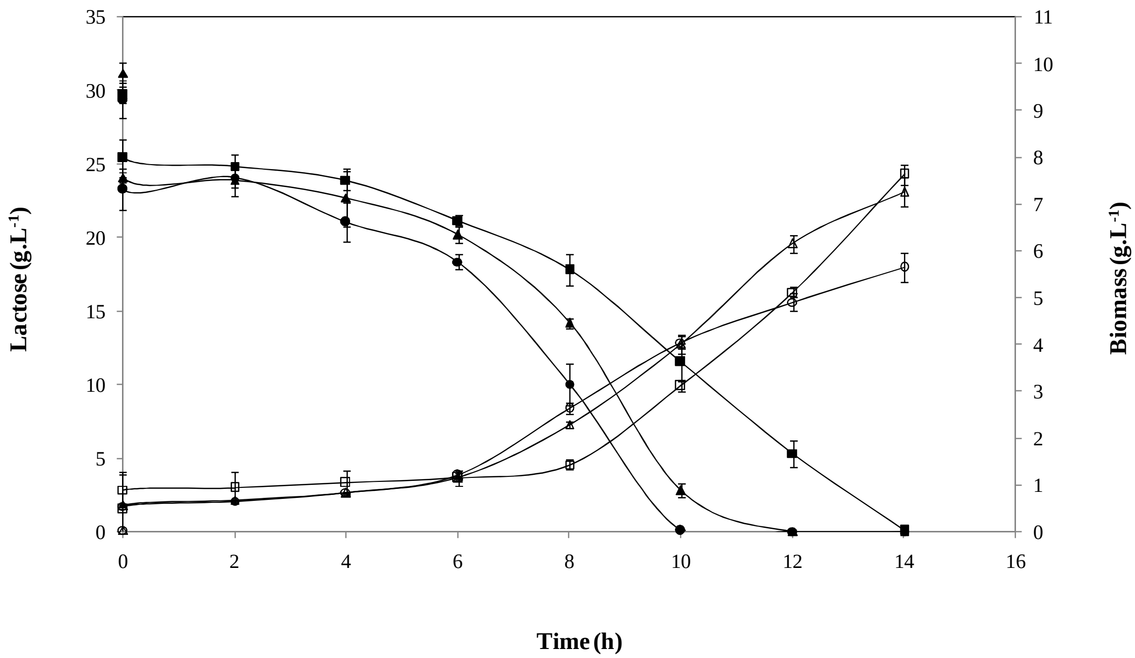


Fig. 2.4 Effect of incubation temperature on the growth of *K. lactis* UOFS y-0939 at pH 7 and 180 rpm, during shake flask cultivation, indicating lactose utilisation at 25°C (■); 30°C (▲); 35°C (●), and biomass at 25°C (□); 30°C (Δ); 35°C (○). Each point represents the mean of triplicate determinations with ± SD.

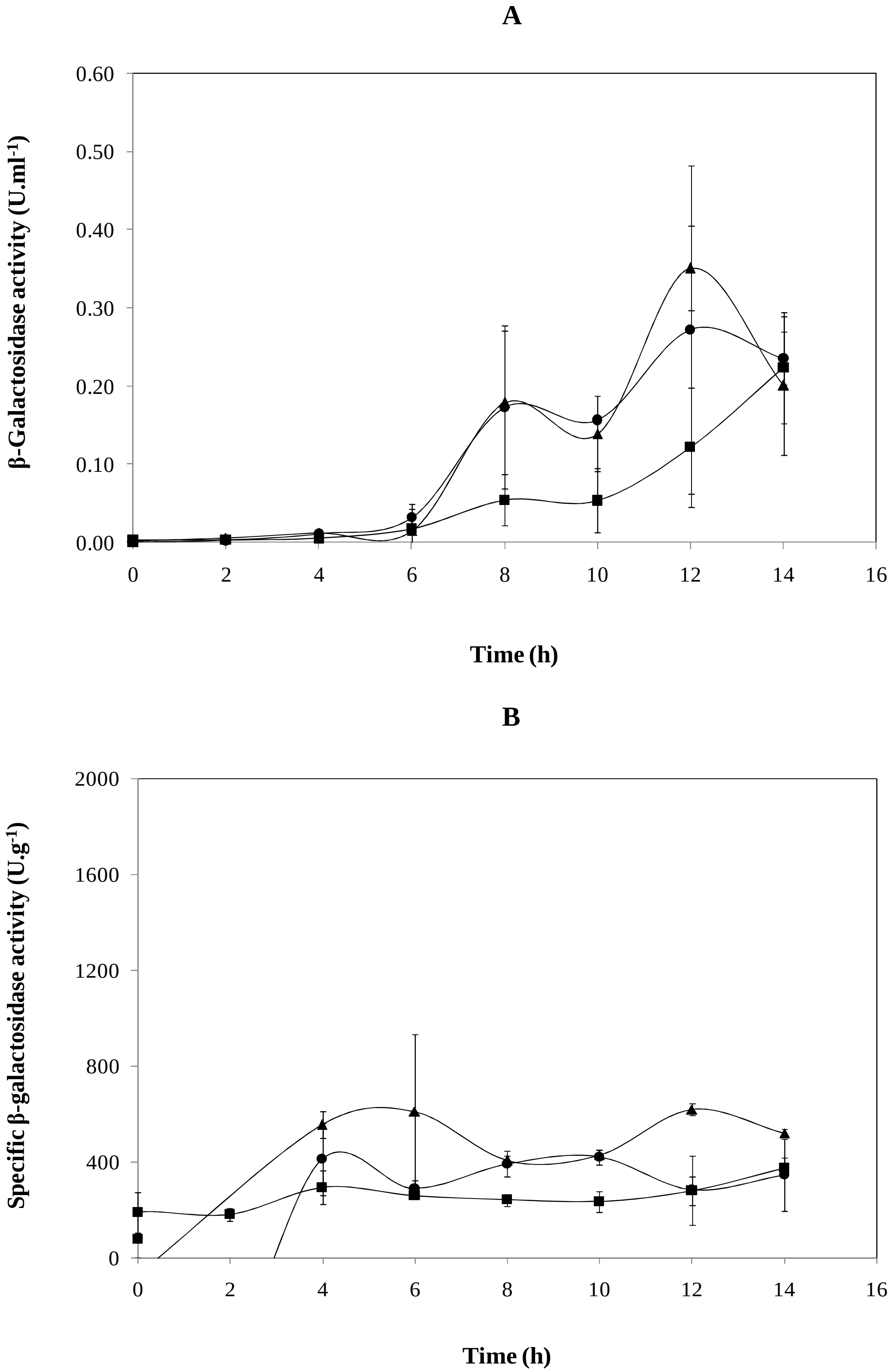


Fig. 2.5 Effect of incubation temperature, during shake flask cultivation of *K. lactis* UOFS y-0939 at pH 7 and 180 rpm, on β-galactosidase activity (A) and specific activity (B) at 25°C (■); 30°C (▲); 35°C (●). Each point represents the mean of triplicate determinations with ± SD.

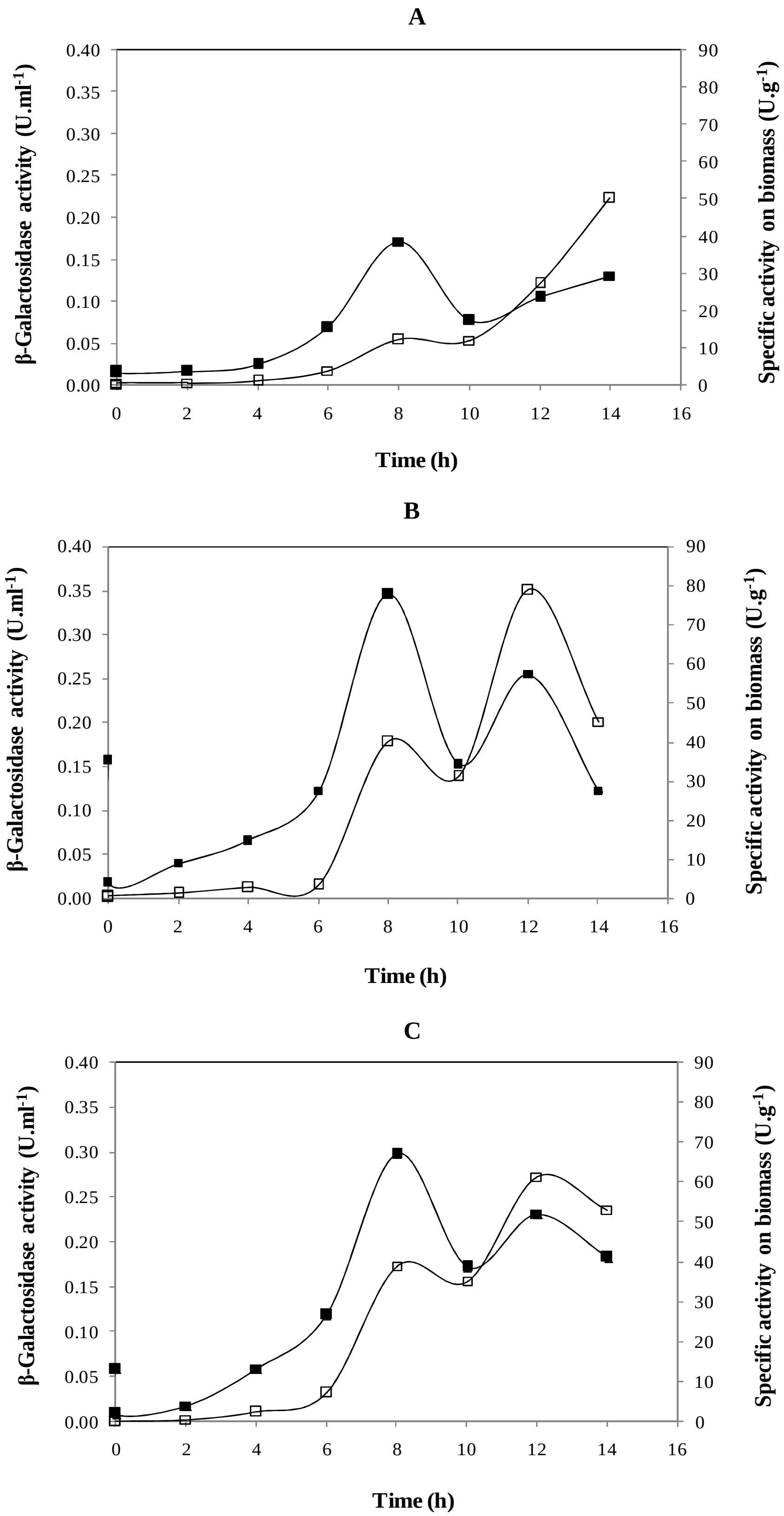


Fig. 2.6 Comparison between β-galactosidase activity (□) and specific activity on biomass (■) during shake flask cultivation of *K. lactis* UOFS y-0939 at pH 7 and 180 rpm, at 25°C (A), 30°C (B) and 35°C (C). Each point represents the mean of triplicate determinations. SD is not shown.

2.3.4 Effect of pH on growth and β-galactosidase activity

The effect of pH on growth and β-galactosidase activity at the optimum temperature of 30°C was evaluated for 14 h (Fig. 2.7 and 2.8A). Even though pH 5.5 was observed to be more favourable for growth, indicating the shortest lag phase (3 h) and highest biomass (9.39 ± 0.3 g.L^{-1}), this pH was not favourable for β-galactosidase activity since it resulted into the low activity of 0.28 ± 0.15 U.ml^{-1}. The pH of 7 was determined to be more favourable for both growth and β-galactosidase activity as lactose was completely consumed by 12 h, at which time the highest activity of 0.35 ± 0.05 U.ml^{-1} was observed. All negative control samples prepared at 12 h exhibited either zero, or negligible β-galactosidase activity and specific activity (data not shown).

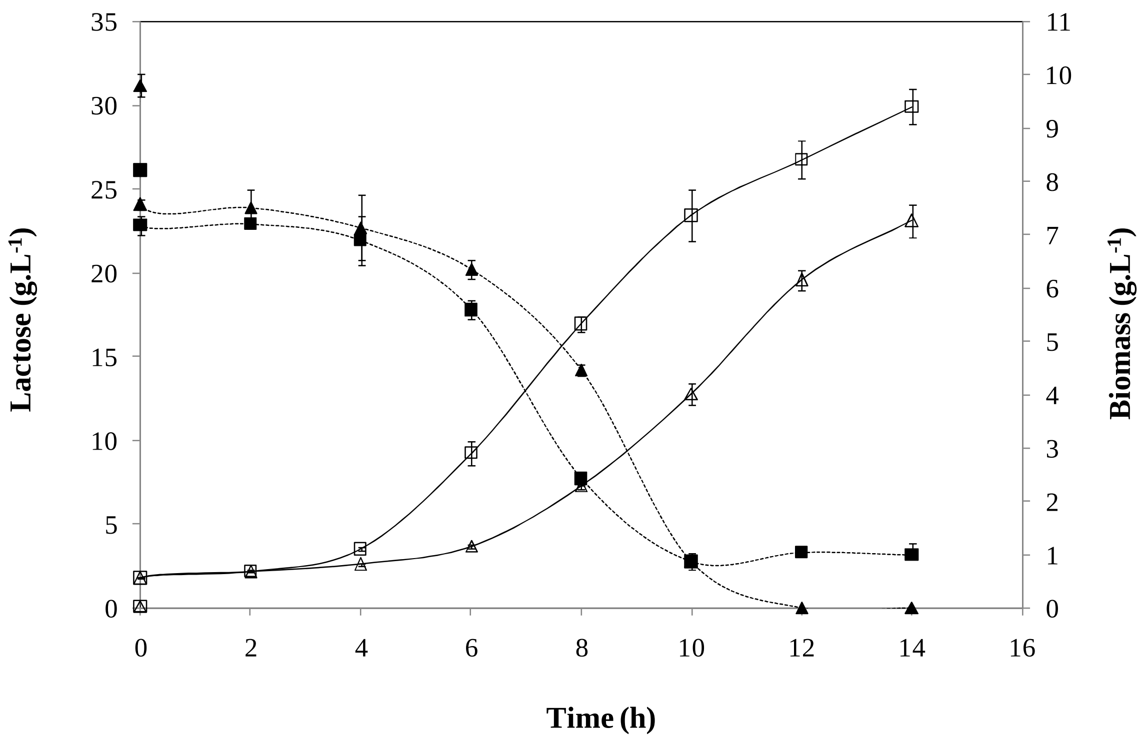


Fig. 2.7 Effect of pH on the growth of *K. lactis* UOFS y-0939 at 30°C and 180 rpm, during shake flask cultivation, indicating lactose utilisation at pH 5.5 (-■-); pH 7 (-▲-) and biomass at pH 5.5 (□); pH 7 (Δ). Each point represents the mean of triplicate determinations with ± SD.

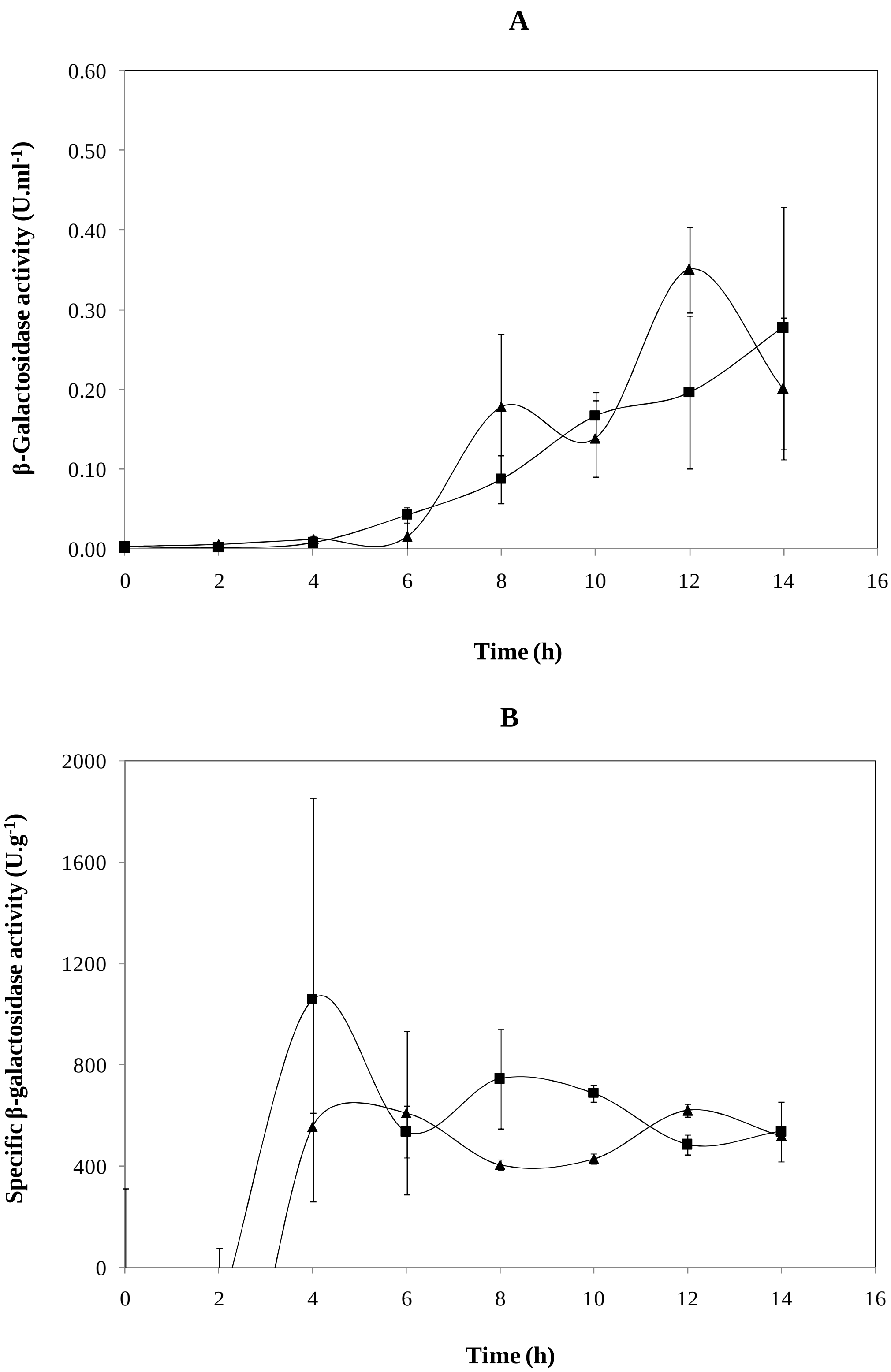


Fig.2.8 Effect of pH, during the shake flask cultivation of *K. lactis* UOFS y-0939 at 30°C and 180 rpm, on β-galactosidase activity (A) and specific activity (B) at pH 5.5 (■); pH 7 (▲). Each point represents the mean of triplicate determinations with ± SD.

2.3.5 Effect of inoculum size on growth and β-galactosidase activity

Inoculum variations of 6, 8 and 10% were investigated at the optimum growth temperature and pH. A lag phase of 6 h was observed for all inoculum ratios, however, the specific lactose utilisation rate at 8% inoculum size (2.82 $g.L^{-1}.h^{-1}$) was marginally higher than that at 6 and 10% (2.35 $g.L^{-1}.h^{-1}$) (Fig. 2.9). Biomass trends demonstrated the highest titre at 6% (9.28 ± 0.1 $g.L^{-1}$), followed by 8% (8.64 ± 0.5 $g.L^{-1}$), and 10% inoculum size (7.25 ± 0.3 $g.L^{-1}$) (Fig 2.9). β-galactosidase activity at 12 h corresponded to the biomass trend since the highest activity (0.41 ± 0.02 $U.ml^{-1}$) was observed at 6% inoculum size and the lowest (0.35 ± 0.05 $U.ml^{-1}$) at 10% (Fig. 2.10A). The activity at the investigated inoculum ratios, however, did not appear significantly different as the variation between 6 and 8%, and 8 and 10% was 0.03 $U.ml^{-1}$, and 0.06 $U.ml^{-1}$ between 6 and 10%, thereby suggesting that inoculum size did not considerably influence β-galactosidase activity. All negative control samples prepared at 12 h exhibited either zero, or negligible β-galactosidase activity and specific activity (data not shown).

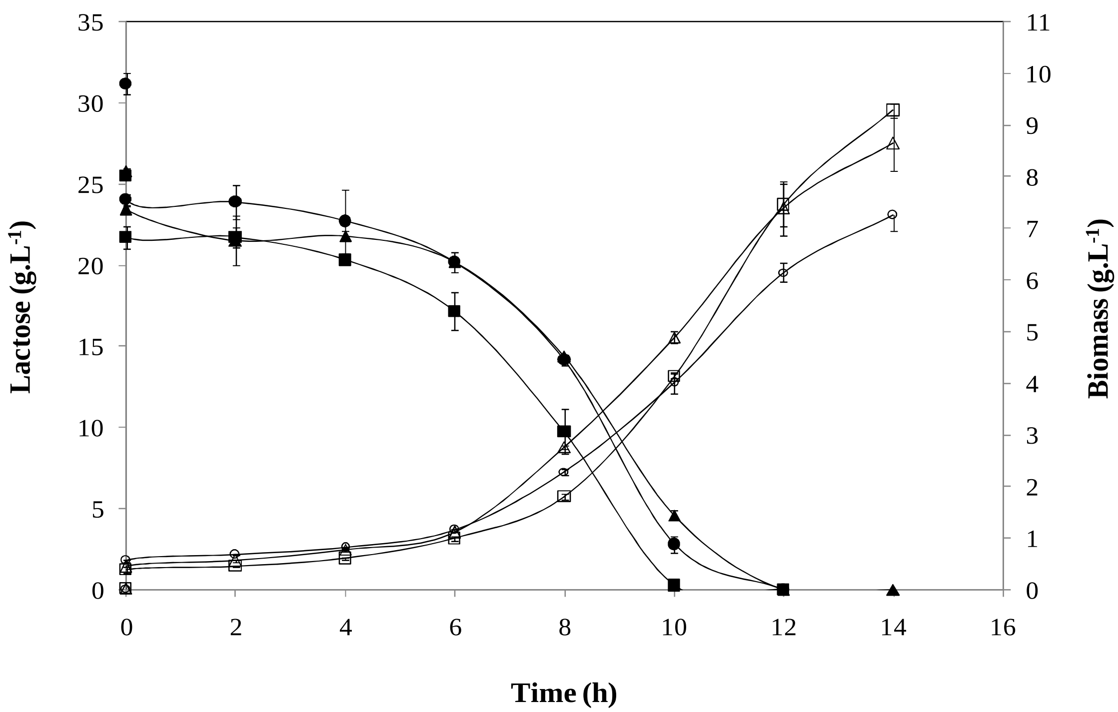


Fig. 2.9 Effect of inoculum size on the growth of *K. lactis* UOFS y-0939 at 30°C, pH 7 and 180 rpm, during shake flask cultivation, indicating lactose utilisation at 6% (■); 8% (▲); 10% (●), and biomass at 6% (□); 8% (Δ); 10% (○). Each point represents the mean of triplicate determinations with ± SD.

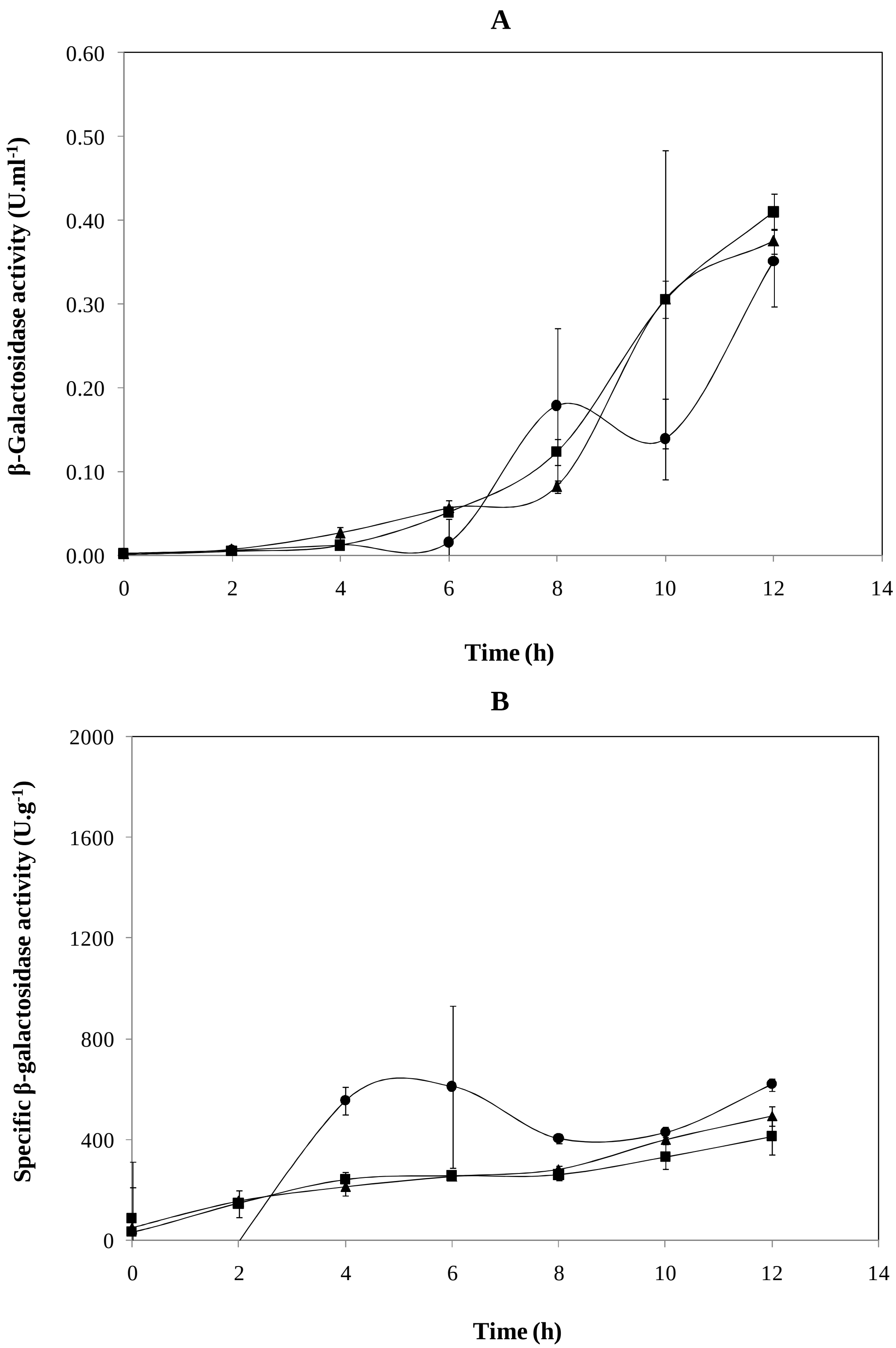


Fig. 2.10 Effect of inoculum size during the shake flask cultivation of *K. lactis* UOFS y-0939 at 30°C, pH 7 and 180 rpm, on β-galactosidase activity (A) and specific activity (B) at 6% (■); 8% (▲); 10% (●). Each point represents the mean of triplicate determinations with ± SD.

2.4 DISCUSSION

Lactose is one of many carbon compounds that can be metabolised by *K. lactis* (Wésolowski-Louvel *et al*., 1996). Two genes, *LAC4* and *LAC12* encoding β-galactosidase and lactose permease, respectively, are responsible for lactose utilisation (Breunig *et al*., 2000). β-galactosidase is an intracellular enzyme, the production of which is induced by the sugars lactose and its component, galactose (Poch *et al*., 1992), and repressed by glucose (Martins *et al*., 2002).

The growth dynamics of *K. lactis* UOFS y-0939 was observed in a medium containing 10 $g.L^{-1}$ glucose (Fig. 2.1). Complete glucose consumption was observed only after 24 h of growth and 0.67 g of biomass was produced per gram of glucose. Even though the μ_{max} of 0.44 h^{-1} between 8 and 14 h, was high, the 8 h long lag phase is an indication that glucose is not an optimal carbon source for the growth of *K. lactis* UOFS y-0939, though it is the preferred carbon source for numerous other organisms. Lactose, however, is the preferred carbon source for *K. lactis* (Dickson and Markin, 1980). Subsequent experiments were, therefore, conducted in a medium containing lactose as the sole carbon source.

The rate of chemical reactions generally increase as the temperature is elevated (Daniel *et al*., 2007). This is evident in Fig. 2.4 where lactose was consumed at the fastest rate at 35°C, and considerably slower at 25°C. A maximum growth rate (μ_{max}) of 0.31 h^{-1}, however, was observed at 30°C, with the highest β-galactosidase activity of 0.35 ± 0.05 $U.ml^{-1}$. A decline in β-galactosidase activity at 10 h at both, 30 and 35°C is depicted in Fig. 2.5A. As lactose is hydrolysed to glucose and galactose, the cumulative presence of glucose, a known repressor of β-galactosidase, may have resulted in the decline of β-galactosidase activity at that point of the growth phase. Glucose has been described to influence β-galactosidase synthesis in *E. coli* by: (i) inducer exclusion, the prevention of entry of lactose into the cell; (ii) carbon catabolite repression, the reduction of the differential rate of β-galactosidase synthesis by half, and; (iii) transient repression, the reduction in the initial rate of β-galactosidase production (Dickson and Markin, 1980).

At 30°C, after the exhaustion of glucose, the organism may have synthesised more β-galactosidase in an effort to utilise the residual lactose of 2.8 g.L^{-1}, resulting in the second activity peak. This phenomenon has been described by Dagbagli and Goksungur (2008) who investigated the production and optimisation of β-galactosidase by *K. lactis* NRRL Y-8279. A similar effect was observed at 35°C, however, as the residual lactose concentration at 10 h was 0.05 g.L^{-1}, the second activity peak resulted in a lower β-galactosidase activity (0.27 ± 0.2 U.ml^{-1}) than at 30°C (0.35 ± 0.05 U.ml^{-1}). In complete contrast, however, is the report by Dickson and Markin (1980) who investigated β-galactosidase induction in *K. lactis* NRRL Y-1140 (the organism under investigation in this study) and Y-1118. It was reported that glucose did not exclude lactose from *K. lactis*, nor cause permanent carbon catabolite repression of β-galactosidase, or prevent lactose utilisation. The decline in enzyme activity at 30 and 35°C after complete consumption of the carbon source (stationary phase) was also observed by Dagbagli and Goksungur (2008), and may have been as a result of the deficiency of inducer, the presence of proteases, or enzymatic inhibition of the hydrolysis products, in that high concentrations of galactose has been reported to inhibit β-galactosidase activity (Martins *et al*., 2002). Specific β-galactosidase activity per total protein (Fig. 2.5B) and specific activity on biomass (Fig. 2.6) was also reported. During lag phase of growth when minimal enzyme titres were produced, specific β-galactosidase activity values were low. As growth progressed, however, specific enzyme activity increased, and eventually plateaued during exponential and stationary phase, as an indication that β-galactosidase was neither being produced at excessively high levels, neither was there repression of enzyme synthesis. This trend was also observed in both the pH (Fig. 2.8B) and inoculum size (Fig. 2.10B) optimisation studies. The direct relationship between β-galactosidase activity and biomass production at all temperatures investigated, is demonstrated in Fig. 2.6, confirming the observation by Dagbagli and Goksungur (2008). Volumetric β-galactosidase activity is therefore, growth associated. This trend was also observed in all the shake flask (pH and inoculum size) experiments that followed (data not shown).

The ideal pH range for yeast growth is 5 to 6 (Rech *et al*., 1999). This was confirmed in the present investigation, as indicated in Fig. 2.7. At pH 5.5, the shortest lag phase (3 h) was observed and the highest biomass was attained (9.39 ± 0.3 $g.L^{-1}$). This result was in agreement with that of Rech *et al.* (1999), who confirmed that pH 5.5 resulted in a greater initial growth rate for two strains of *K. marxianus* (CBS 712 and 6556) and Furlan *et al.* (1995), cited in Rech *et al.* (1999), who reported that several *K. marxianus* strains showed optimum growth at pH 5.5. Growth rate may have been optimal at pH 5.5, however, maximum β-galactosidase activity (0.28 ± 0.15 $U.ml^{-1}$) was lower at this acidic pH than at neutral pH 7 (Fig. 2.8A). This was comparable to the work of Burin and Buera (2002) who reported that at pH values up to 5.6, β-galactosidase activity from *S. lactis* (*K. lactis*) was significantly lower than at pH 6. Guy and Bingham (1978) also observed that β-galactosidase activity from *S. lactis* (*K. lactis*) declined rapidly below pH 5.9 in fluid whey, as did Zhou and Chen (2001) who reported that a commercial β-galactosidase from *K. lactis* exhibited very low activity at around pH 5.5. This was mainly due to the fact that the *iso*-electric point (p*I*) of the enzyme was 5.42. At pH 7, the maximum activity of 0.35 ± 0.05 $U.ml^{-1}$ was observed at 12 h (Fig. 2.8A). Possible glucose repression of β-galactosidase activity was only observed at pH 7 at 10 h, and not at pH 5.5. Since a pH of ±7 has been demonstrated by several authors, to be optimal for β-galactosidase production by *Kluyveromyces* sp. (Dagbagli and Goksungur, 2008; Zhou and Chen, 2001; Gist-Brocades, 1998), as confirmed by the present study, pH 7 was, therefore, concluded to be optimum for enzyme production in subsequent studies. It must be noted that the effect of pH 8 was on *K. lactis* UOFS y-0939 growth and β-galactosidase production was also investigated. This data, however, has not been reported since enzyme titres at pH 8 were not significantly greater than at pH 7.

The size of the inocula is a well investigated process parameter in microbial fermentations (SreenivasRao *et al*., 2004; Papagianni and Moo-Young, 2002; Medina *et al*., 1997). The effect of inoculum size on the growth and β-galactosidase production by *Kluyveromyces* sp. has not been extensively reported, however, 10% inoculum size has been used in *K. marxianus* shake flask investigations (Manera *et al*., 2008) resulting in a β-galactosidase activity of 10.6 $U.ml^{-1}$ at optimum conditions. The present study demonstrated that a lag phase of 6 h was observed for all inoculum ratios (Fig. 2.9), an unusual result as a lower inoculum size, i.e., fewer yeast cells, should lead to an extended lag phase. Furthermore, biomass titres and β-galactosidase activity followed the similar trend, as detailed in section 2.3.3, however, β-galactosidase activity between the inoculum sizes, did not appear significantly different at 12 h after complete lactose depletion (Fig. 2.10A). Furlan *et al.* (2001) had investigated the optimisation of inoculum ratio for β-galactosidase production by *K. marxianus* in a lactose-free medium and reported that there was no significant difference between inoculum ratios of 1% (v/v) and 10% (v/v), thus validating the result of the present investigation. It was the aim of the investigation to determine the optimum inoculum ratio for β-galactosidase production and since 6, 8 and 10% did not result in significant differences in β-galactosidase activity, and as 10% inoculum size had been used in the study that optimised the inoculum medium employed in the present investigation (Manera *et al*., 2008), 10% inoculum ratio was used in the successive fermentations.

The effectiveness of ultrasonication with glass beads at various times, as a cell disruption method was also investigated. It was observed that a greater number of cells were disrupted as the ultrasonication period increased. The viable cell count revealed that a 16% reduction in the number for viable cells was achieved after ultrasonication for 30 min, as compared to the control, and β-galactosidase activity increased to 0.42 ± 0.1 U.ml^{-1}, a 99.5% improvement as compared to the control (0.002 ± 0.0001 U.ml^{-1}) (Fig. 2.3). Albeit the application of 30 min in subsequent shake flask investigations, ultrasonication in a water bath was not optimal for the disruption of *K. lactis* UOFS y-0939 cells, as compared to other disruption methods. In the study by Dagbagli and Goksungur (2008), isoamyl alcohol was most effective in the extraction of β-galactosidase from *K. lactis* NRRL Y-8279, and resulted in the maximum volumetric activity of 14.12 U.ml^{-1} in a medium with 30 g.L^{-1} lactose, pH 7 and growing at 30°C, conditions similar to those in the present study. Comparatively, the highest activity achieved in the present study under the optimum conditions, was 0.35 U.ml^{-1} (Fig. 2.5A), a considerable 40 fold less than that of Dagbagli and Goksungur (2008). It can be concluded that the use of an ultrasonic water bath had significantly underestimated the β-galactosidase activity. Becerra *et al.* (2001), however, reported sonication as the most effective method for β-galactosidase extraction in the strain of *K. lactis* as used in this study, compared to other mechanical extraction methods, and extraction by solvents and enzymes. It must be noted, though, that isoamyl alcohol was not among the solvent extraction methods tested by Becerra *et al.* (2001). It is of interest to note that the physiological state of cells and the conditions of growth at the time of extraction have a major effect on disruption kinetics. Cells harvested during the exponential phase of growth are more easily disrupted than those harvested during the stationary phase. This is due to cells grown at a higher specific growth rate being probably easier to disrupt than those grown at lower specific growth rates, since the cells would direct the available energy towards reproduction rather than strengthening of the cell wall (Keshavarz *et al*., 1987).

Shake flask optimisation studies, therefore, revealed that *K. lactis* UOFS y-0939 favoured a temperature and pH of 30°C and 7, respectively, for the optimal yield of β-galactosidase. Furthermore, an inoculum ratio of 10% was to be applied in subsequent investigations. Since cell disruption in an ultrasonic water bath was found to be ineffective, it was also necessary to apply an alternative disruption method in the scale-up investigations that follow.

CHAPTER THREE: BATCH, FED-BATCH AND CONTINUOUS CULTURE FOR THE PRODUCTION OF β-GALACTOSIDASE

3.1 INTRODUCTION

Since the primary goal of fermentation research is the cost effective production of biological products, it is important to develop a cultivation method that allows production of the desired product to a high concentration with high productivity and yield (Lee *et al*., 1999). Batch fermentation is still the most important process operation used in bioprocessing industries due to its simplicity (Busairi, 2008). The batch reactor is a closed system where all components, except gaseous substrates such as oxygen, pH-controlling substances and antifoaming agents, are placed in the reactor in the beginning of the fermentation. During the process, there are no input or output flows. The microbial growth phase in such a process follows the typical lag, exponential, stationary and death phase path. For the batch cultivation of an aerobic microorganism, aeration is an important design parameter in the bioreactor, and by its efficient control, the overall productivity of the process can be increased (Saarela *et al*., 2003). Despite its advantages, however, batch culture becomes less suitable when substrate inhibition on cell growth by product formation occurs (Hammes and Whiley, 1993). Some problems of batch culture can be overcome by fed-batch operation (Busairi, 2008).

In the fed-batch process, substrate nutrients are fed continuously or sequentially without the removal of fermentation broth (Busairi, 2008), and is widely used for the production of microbial biomass, ethanol, organic acids, antibiotics, vitamins and enzymes (Roukas and Kotzekidou, 1998). Fed-batch is generally superior to batch processing and is especially beneficial when changing nutrient concentrations affect the productivity and yield of the desired product (Yamane and Shimizu, 1984). Since both overfeeding and underfeeding of the limiting nutrient is detrimental to cell growth and product formation, development of a suitable feeding strategy is critical in fed-batch cultivation (Lee *et al*., 1999). Various strategies have been developed and include the following:

i. Simple indirect feedback method: couples nutrient feeding with the measurement of pH (pH-stat) or dissolved oxygen (DO-stat) (Lee, 1996).
ii. Exponential feeding strategy: allows exponential cell growth by feeding substrate in proportion to the exponentially growing cell mass (Nor *et al*., 2001).
iii. Corrected feedback control strategy: incorporates an online method to estimate μ to implement exponential feeding with compensation for changes in μ (Nor *et al*., 2001).
iv. Nutrient feeding according to nutrient uptake or demand: the carbon source in the culture medium can be controlled at a desired value if it can be measured online (Lee *et al*., 1999).

Batch and fed-batch production of β-galactosidase from *Kluyveromyces* sp. has been investigated (Cortés *et al*., 2005; Pinheiro *et al*., 2003; Nor *et al*., 2001; Barberis and Segovia, 1997). The enzyme is reported to be continuously synthesised in induced cultures, and in batch operations, the maximum yield being obtained at the beginning of the stationary phase of growth, after which the yield of the enzyme decreases (Ranzi *et al*., 1987). Pinheiro *et al*. (2003) investigated the effect of an increase in total air pressure on cell growth and intracellular β-galactosidase activity in batch cultures of *K. marxianus* CBS 7894 and discovered that specific β-galactosidase productivity increased from 5.8 to 17.0 U.gCD^{-1}.h^{-1} (units per gram cell dry weight per hour) using 6 bar air pressure instead of air at atmospheric pressure. β-galactosidase production is influenced by a variety of culture conditions and some studies have revealed the important role of dissolved oxygen tension (DOT) or oxygen transfer rate (OTR) (Schneider *et al*., 2001; Barberis and Gentina, 1998; García-Garibay *et al*., 1987). The influence of oscillating DOT on the batch production of β-galactosidase by *K. marxianus* NRRL-Y1109, in comparison to constant DOT, was investigated by Cortés *et al*. (2005) who demonstrated that maximum specific β-galactosidase activity (2 800 ± 250 U.g^{-1}) and final volumetric activity (32 700 ± 2000 U.L^{-1}) was achieved at the lowest oscillation period tested (300 seconds), while at a constant DOT of 20%, specific β-galactosidase activity and final volumetric activity were 2 500 U.g^{-1} and 31 100 U.L^{-1}, respectively.

In fed-batch operation, DO-stat is the most common strategy for industrial aerobic yeast cultures due to its simplicity and straightforward implementation (Yamane and Shimizu, 1984). Nor *et al*. (2001) reported maximum volumetric β-galactosidase activity of 26.2 $U.ml^{-1}$, and specific activity of 2 $U.mg^{-1}$dry weight, during the fed-batch cultivation of a wild-type mutant of *K. fragilis* (*K. marxianus*) under DO stat conditions (25-35% DO). Comparative maximum volumetric β-galactosidase activity and specific activity reported under batch conditions were 3.1 $U.ml^{-1}$ and 1 $U.mg^{-1}$dry weight, respectively.

Continuous culture offers the advantage of well defined growth conditions such as growth rate and nutrient limitation (Inchaurrondo *et al*., 1998). The nutrients are continuously fed to the culture while continuous removal of culture fluid containing biomass, products and non-depleted nutrients, ensures that the culture volume is kept constant. The media that is fed into the culture is designed such that one nutrient of choice (the limiting nutrient), for example, the carbon source, determines the biomass concentration in the culture, and is almost completely consumed so that its residual concentration in the culture is minimal. In steady state, when the growth rate and dilution rate are equal, the concentration of all nutrients, including the growth-limiting substrate, remains constant over time, and as a result of constant growth conditions, the physiology of the microorganism also remains constant (Weusthuis *et al*., 1994). It has been reported that in many yeasts, the biomass yield from the substrate is virtually constant over a wide range of dilution rates (Pirt, 1975), leading to a linear relationship between the dilution rate and the specific rate of substrate consumption. This linear relationship, however, only applies when the biomass yield does not change with the dilution rate. This phenomenon was observed when the dilution rate of aerobic, glucose-limited cultures of *C. utilis* CBS 621 was varied between 0.1 and 0.5 h^{-1} (Postma *et al*., 1988).

The continuous cultivation of *Kluyveromyces* sp. has also been well investigated. For example, *K. fragilis* (*K. marxianus*) biomass production was investigated in a chemostat using a medium containing 2.5% fructose. The maximum biomass productivity obtained was 4.81 $g.L^{-1}.h^{-1}$ (Kim *et al*., 1998). Furthermore, the continuous flow aerobic process has been used successfully for the production of single cell protein from cheese whey using *K. fragilis* (Ghaly and Ben Hassan, 1995; Ghaly *et al*., 1992; Moresi *et al*., 1990; Mickle *et al*., 1974). The metabolic behaviour of *K. marxianus* in aerobic chemostat cultures of deproteinised whey was also studied to determine the characteristics of energy metabolism (Castrillo and Ugalde, 1992).

Continuous production of β-galactosidase by *Kluyveromyces* sp. has been investigated previously, however, there have been limited reports. The consequence of dilution rate on β-galactosidase activity in *K. lactis* grown in cheese whey permeate, was investigated (Ornelas *et al*., 2008) and 0.24 h^{-1} was found to be optimum. Barberis and Gentina (1998) examined the effect of dissolved oxygen concentration on continuous β-galactosidase production by a *K. fragilis* NRRL Y-1109 in a bioreactor with a defined medium (1 L) and reported 10% to enable both a higher specific β-galactosidase activity and higher volumetric productivity. Moreover, Inchaurrondo *et al*. (1998) reported on the growth and β-galactosidase synthesis of *K. lactis* in aerobic continuous culture in 900 ml medium, under carbon, nitrogen and phosphate limitation in a bioreactor. The induced β-galactosidase expression was repressed when cells were grown under nitrogen or phosphate limitation. In lactose or galactose-limited cultures, however, the enzyme accumulated in amounts equivalent to 10-12% of the total cell protein. Finally, the simultaneous production of β-galactosidase and inulase was investigated in batch and 3 L continuous cultures of *K. fragilis*. The highest specific β-galactosidase activity in continuous culture (0.78 $U.mg^{-1}$ dry cell weight) was obtained in a medium with an equal mixture of fructose and galactose (Hewitt and GrootWassink, 1984).

This chapter focused on assessing the production of β-galactosidase using batch, fed-batch and continuous processes on a scaled-up level, under optimised conditions.

3.2 MATERIALS AND METHODS

3.2.1 Seed culture and Bioreactor inoculum

K. lactis UOFS y-0939 seed culture and bioreactor inoculum (10%) was prepared as described in section 2.2.4.2. The inoculum was prepared (30°C, pH 7, 10% seed culture inoculum ratio) and incubated for 12 h, prior to transfer into the bioreactor.

3.2.2 Batch cultivation of *K. lactis* UOFS y-0939

Two Biostat® B-DCU Bioreactors (B. Braun Biotech International, Germany) of 6.6 L total volume were used for the cultivation of *K. lactis* UOFS y-0939 in batch culture in concurrent experiments. These were equipped with baffles and temperature, pH and dissolved oxygen (DO) controllers. The growth medium (3 L) was the same as described in section 2.2.4. Yeast extract and the salts were dissolved in tap water (1.7 L) and transferred to the bioreactor prior to sterilisation. The pH probe (Mettler Toledo) was calibrated prior to sterilisation. The pH was maintained using 25% ammonia (Saarchem) and 25% sulphuric acid (Saarchem). Antifoam (Durapol) was sterilised separately and used to control foaming during the fermentation process. The bioreactor was sterilised (121°C, 60 min). Following sterilisation, the dissolved oxygen probe (Mettler Toledo) was allowed to polarise for a minimum of 6 h, prior to calibration. Lactose was prepared separately and aseptically transferred to the bioreactor using a peristaltic pump (Watson Marlow 101 U/R, United Kingdom) prior to inoculation, after which the LogM sample was taken. The monosepticity and OD (600 nm) of the inoculum was determined and the inoculum aseptically transferred to the bioreactor, following which, the time zero sample was taken. Operating parameters were controlled and monitored online by the MFCS Shell software. Growth parameters were maintained at 30°C and pH 7 with 1.5 vvm airflow (4.5 slpm) and a stirrer speed of 800 rpm. Samples (30 ml) were withdrawn from both bioreactors at 2 h intervals. Growth was measured spectrophotometrically (at 600 nm), and by the determination of biomass (as described in section 2.2.3.1). The pH, lactose concentration, β-galactosidase activity and specific activity were also measured as described in sections 2.2.4.2.1, 2.2.4.1.2 and 2.2.4.2.3, respectively.

A second batch cultivation was also run under identical conditions, with the exception of an increase in lactose concentration to 40 $g.L^{-1}$.

3.2.2.1 *K. lactis* UOFS y-0939 cell lysate preparation

Two methods were compared to extract the enzyme. Firstly, each cell lysate was prepared by homogenisation of the fermentation broth using the Microfluidizer® M110S (Microfluidics, USA), operated by compressed air (13 000 psi). Each sample (20 ml), which was stored on ice, was homogenised (7 min). Aliquots (2 ml) of each sample were centrifuged (13 000 rpm, 4°C, 5 min). The resultant supernatant was transferred to clean microcentrifuge tubes and stored (-80°C) until further analysis. Secondly, in the batch fermentation cultivated with 28.2 $g.L^{-1}$ lactose, the sample at 12 h was also prepared by ultrasonication (as described in section 2.2.4.2.2) so as to compare the two extraction methods.

3.2.2.2 β-Galactosidase activity and specific activity

β-Galactosidase activity and specific activity were determined as described in sections 2.2.4.1.2 and 2.2.4.2.3, respectively.

3.2.3 Fed-batch cultivation of *K. lactis* UOFS y-0939

The fed-batch production of *K. lactis* UOFS y-0939 was performed in 30 L Biostat® C-DCU Bioreactor using a 10 L working volume. The bioreactor was sterilised *in situ*. The medium and operating conditions were the same as for the batch fermentation with the exception of the airflow which was reduced to 1 vvm (10 slpm). In addition, once DO had reached approximately 20%, airflow and stirrer speed were manually manipulated to maintain the DO. The lactose feed (167 $g.L^{-1}$) was started once the residual lactose in the initial charge had reached approximately 1.9 $g.L^{-1}$, and was fed at a rate of 4 $g.L^{-1}.h^{-1}$, which was the lactose consumption rate of *K. lactis* UOFS y-0939 as determined in preceding batch investigations. Sampling and analyses were performed as described in section 3.2.2, however, β-galactosidase samples were only prepared by homogenisation as described in section 3.2.2.1.

3.2.4 Continuous cultivation of *K. lactis* UOFS y-0939

The continuous production of β-galactosidase was performed in 6.6 L Biostat® B-DCU Bioreactors with 3 L working volume. The bioreactor medium and the feed composition was the same as that used for the batch fermentations (section 2.2.4.2). The bioreactor preparation was performed as described in section 3.2.2, however, further provisions were made for the feed inlet and the product outlet tubings. The feed (15 L) was prepared in Nalgene bottles (20 L) that were pressure tested prior to sterilisation (121°C, 2 h). Lactose (28.2 $g.L^{-1}$) was prepared separately and aseptically transferred to the feed after sterilisation.

The inoculation of the bioreactor, growth parameters, sampling and analyses were performed as described in section 3.2.2, however, sampling was performed at approximately 4 h intervals in steady state. The mass of the fermentation broth was also monitored throughout the run. The feed was started once the initial lactose in the medium was reduced to approximately 2 $g.L^{-1}$. The peristaltic pumps regulating the feed supply and product removal were calibrated to maintain a dilution rate of 0.2 h^{-1}, i.e., feeding and product removal were maintained at 0.2 L per volume of medium per hour.

In the first continuous fermentation, the extraction of β-galactosidase from *K. lactis* UOFS y-0939 biomass was performed by ultrasonication as described in section 2.2.4.2.2. The second continuous fermentation was also run under identical conditions, with the exception that the cell lysates were prepared by homogenisation as described in section 3.2.2.1.

3.3 RESULTS

3.3.1 Batch cultivation of *K. lactis* UOFS y-0939

The production of β-galactosidase was evaluated in batch culture with 28.2 and 40 $g.L^{-1}$ lactose as carbon source. It was observed that after 14 h, lactose in both fermentations had declined to an average of 1.6 $g.L^{-1}$ and did not proceed to complete depletion (Fig. 3.1). The μ_{max} at 28.2 $g.L^{-1}$ lactose (0.37 h^{-1}) was marginally higher than at 40 $g.L^{-1}$ lactose (0.32 h^{-1}). Although a final biomass of 20.07 $g.L^{-1}$ and 17.77 $g.L^{-1}$ was produced from 40 $g.L^{-1}$ and 28.2 $g.L^{-1}$ lactose, respectively (Fig. 3.1), the conversion of lactose to biomass was 20.7% greater at the lower lactose concentration level ($Y_{x/s}$ 40 $g.L^{-1}$ = 0.5 $g.g^{-1}$ and $Y_{x/s}$ 28.2 $g.L^{-1}$ = 0.63 $g.g^{-1}$). The most significant difference at both 28.2 and 40 $g.L^{-1}$ lactose concentrations is the β-galactosidase activity (Fig. 3.2). The final activity at 40 $g.L^{-1}$ lactose (4.08 $U.ml^{-1}$) is 2.6 fold higher than at 28.2 $g.L^{-1}$ (1.58 $U.ml^{-1}$). Furthermore, β-galactosidase activity and specific activity on biomass of homogenised samples were more than 3.5 fold greater than that achieved with ultrasonication (Table 3.1).

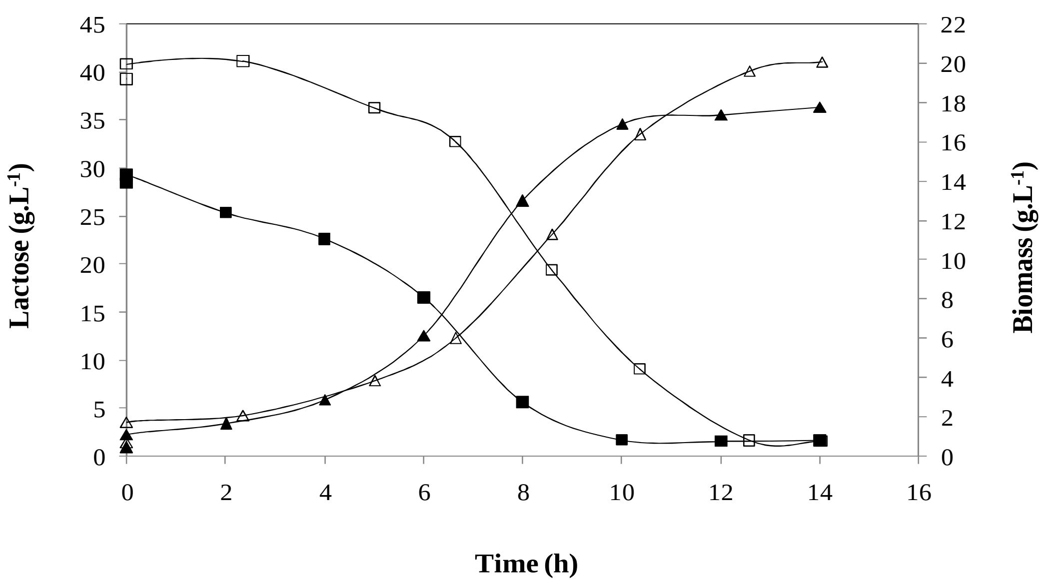


Fig. 3.1 Batch cultivation of *K. lactis* UOFS y-0930 at 30°C, pH 7, and 4.5 slpm airflow with a stirrer speed of 800 rpm, indicating lactose utilisation at an initial concentration of 28.2 $g.L^{-1}$ lactose (■); 40 $g.L^{-1}$ lactose (□), and biomass with 28. $g.L^{-1}$ lactose (▲); 40 $g.L^{-1}$ lactose (Δ).

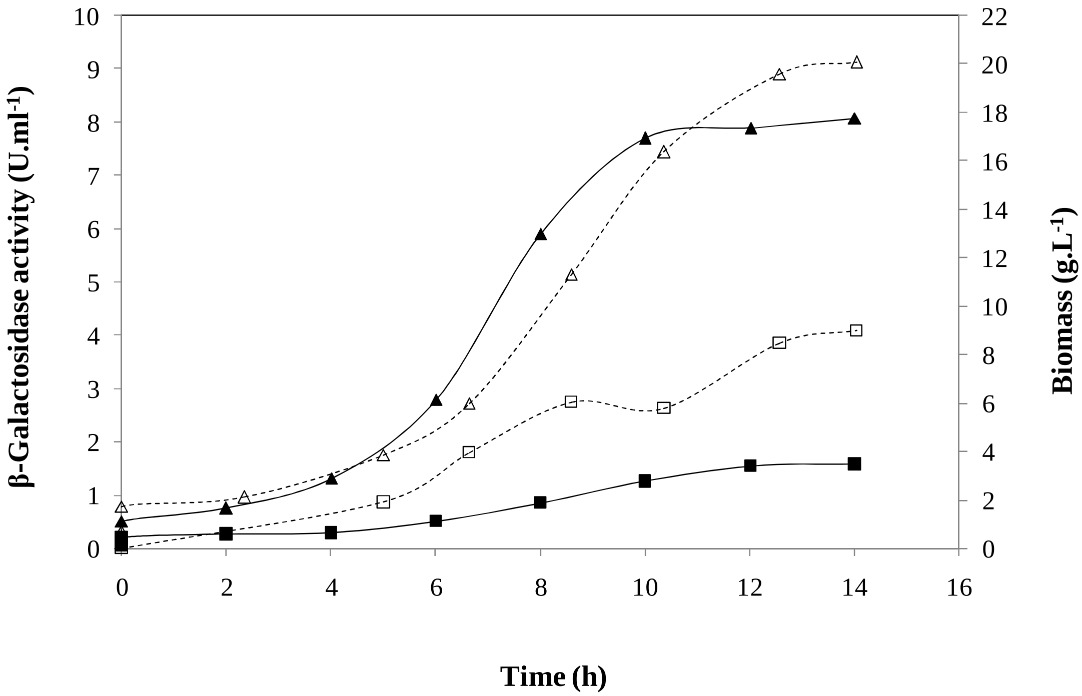


Fig. 3.2 Batch cultivation of *K. lactis* UOFS y-0939 at 30°C, pH 7 and 4.5 slpm airflow with a stirrer speed of 800 rpm, indicating β-galactosidase activity at 28.2 g.L^{-1} lactose (■); 40 g.L^{-1} lactose (□), and biomass at 28.2 g.L^{-1} lactose (▲); 40 g.L^{-1} lactose (Δ).

Table 3.1 Comparison of ultrasonication and homogenisation on β-galactosidase activity and specific activity on biomass during the batch production of *K. lactis* UOFS y-0939 in 28.2 g.L^{-1} lactose. Samples were taken at 12 h and each value represents the mean of duplicate determinations.

Sample	β-Galactosidase activity (U.ml^{-1})	Specific activity (U.g^{-1})
Ultrasonicated	0.43	24.57
Homogenised	1.55	91.02

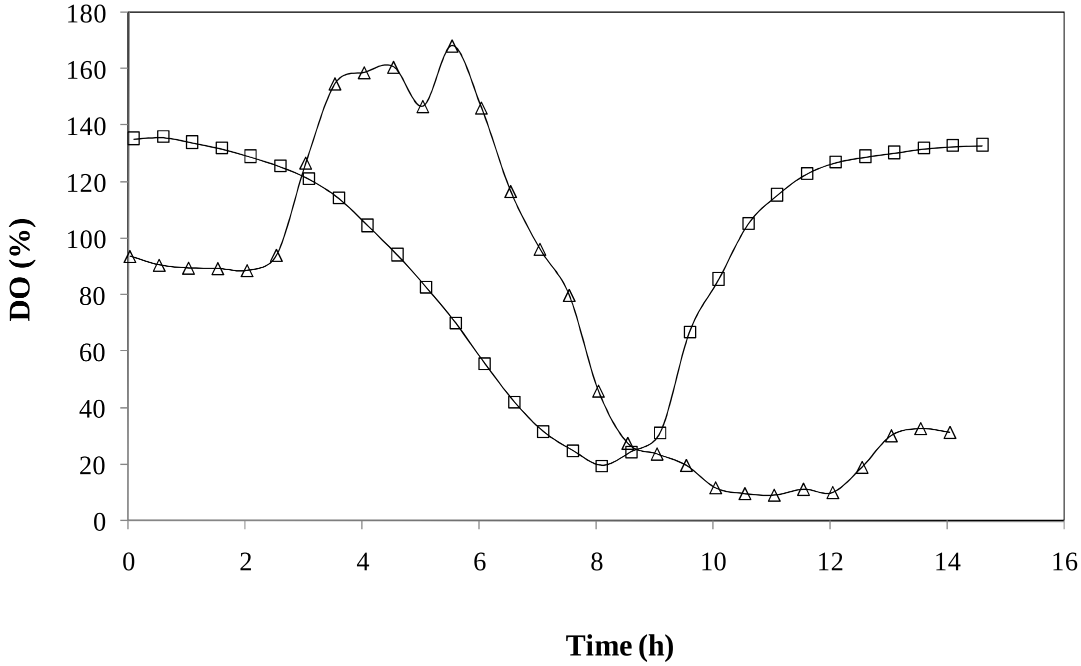


Fig. 3.3 Batch cultivation of *K. lactis* UOFS y-0939 at 30°C, pH 7 and 4.5 slpm airflow with a stirrer speed of 800 rpm, indicating dissolved oxygen at 28.2 g.L^{-1} (□) and 40 g.L^{-1} (Δ) lactose.

3.3.2 Fed-batch cultivation of *K. lactis* UOFS y-0939

The fed-batch cultivation of *K. lactis* UOFS y-0939 in 10 L fermentation medium was investigated with an initial lactose concentration of 28.2 g.L^{-1}. Lactose feed commenced once the initial lactose concentration had reached approximately 1.9 g.L^{-1} (15.33 h) and was fed at a rate of 4 g.L^{-1}.h^{-1}, at which time the biomass concentration and β-galactosidase activity was 12.5 g.L^{-1} and 1.7 U.ml^{-1}, respectively (Fig. 3.4). Biomass increased exponentially, until 20 h and the final biomass titre (34.47 g.L^{-1}) increased 2.8 fold at a rate of 0.8 g.L^{-1}.h^{-1} after the feed start. β-Galactosidase activity increased at a rate of 0.18 U.ml^{-1}.h^{-1} after the feed start, reaching a maximum of 8.7 U.ml^{-1} at 37.31 h with a corresponding biomass titre of 32 g.L^{-1}. An average DO of ± 20.4% was manually maintained from 20 h onwards, during which period β-galactosidase activity increased and reached a maximum (Fig. 3.5).

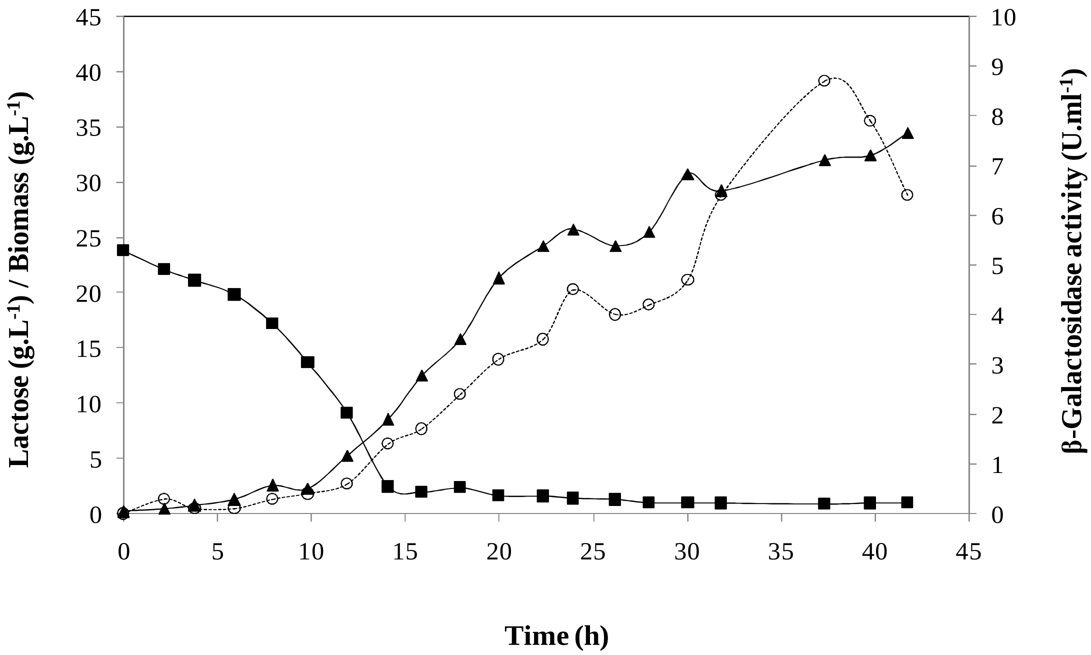


Fig. 3.4 Fed-batch cultivation of *K. lactis* UOFS y-0939 at 30°C, pH 7, with initial 10 slpm airflow and stirrer speed of 800 rpm, indicating lactose utilisation (■), biomass (▲) and β-galactosidase activity (-○-).

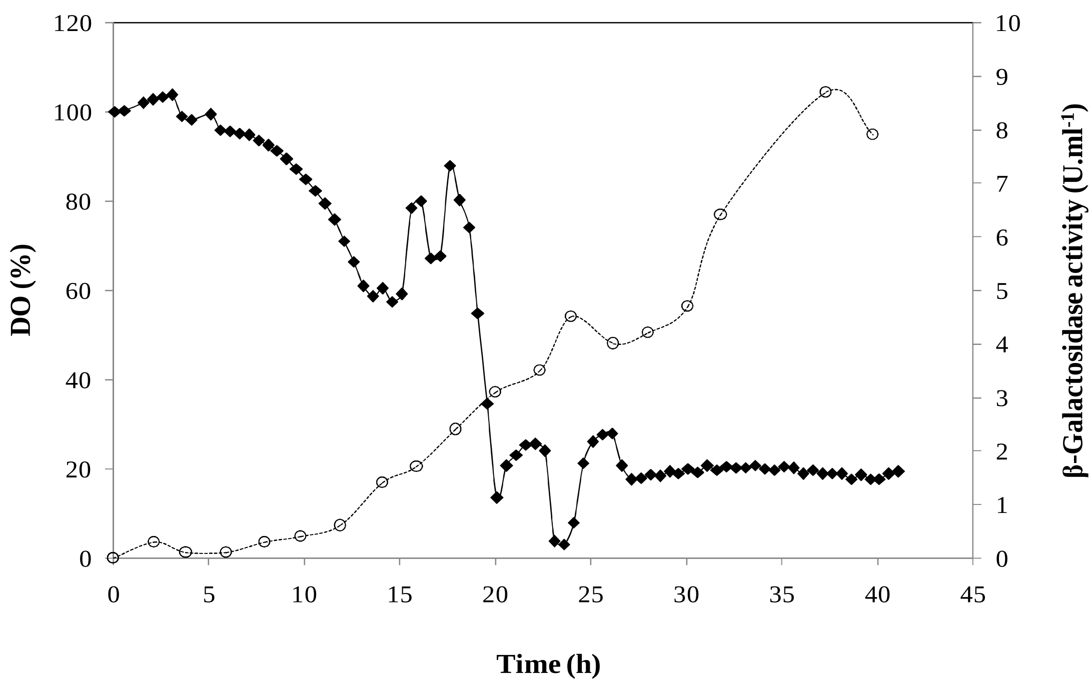


Fig. 3.5 Fed-batch cultivation of *K. lactis* UOFS y-0939 at 30°C, pH 7, with initial 10 slpm airflow and stirrer speed of 800 rpm, indicating DO (♦) and β-galactosidase activity (-○-).

3.3.3 Continuous cultivation of *K. lactis* UOFS y-0939

In the first continuous fermentation, feeding commenced at 10.05 h, when residual lactose concentration was 2.72 g.L^{-1} (Fig. 3.6). The μ_{max} observed during the batch portion of the run was 0.43 h^{-1}. Steady state was maintained from 14.66 h onwards and an average residual lactose concentration of 1.22 ± 0.3 g.L^{-1} was evident during this time. The biomass value reached a maximum of 17 g.L^{-1} during late exponential phase and an average biomass of 19.02 ± 1.2 g.L^{-1} was maintained throughout the steady state. Ultrasonication of *K. lactis* UOFS y-0939 biomass confirmed maximum β-galactosidase activity of 1.6 U.ml^{-1} at the late exponential phase (Fig. 3.6) followed by a decrease in activity during steady state. A peak in β-galactosidase activity was also observed after 26.7 h. An average of 45.5 ± 8.9% DO was maintained throughout steady state even though DO was not controlled during the investigation (Fig. 3.7).

In the second continuous fermentation, albeit identical conditions, the lactose consumption rate was reduced when compared to the first continuous fermentation, as evident by the slower μ_{max} of 0.32 h^{-1}. Feeding was initiated at 15.77 h, when the residual lactose concentration was 1.57 g.L^{-1} (Fig. 3.8). Steady state was maintained from 20.15 h onwards and the average residual lactose concentration and biomass production during this time was 0.92 ± 0.2 g.L^{-1} and 18 ± 0.9 g.L^{-1}, respectively. β-galactosidase activity in the homogenised *K. lactis* UOFS y-0939 biomass was significantly higher (Fig. 3.8) in comparison to the ultrasonicated samples (Fig. 3.6). In contrast to the first continous fermentation, β-galactosidase activity continued to increase after feeding commenced and maintained an average of 8 ± 0.9 U.ml^{-1} in steady state with a maximum activity (10 U.ml^{-1}) observed after 32 h. An average DO of 34 ± 1.9% was maintained throughout the steady state (Fig. 3.9).

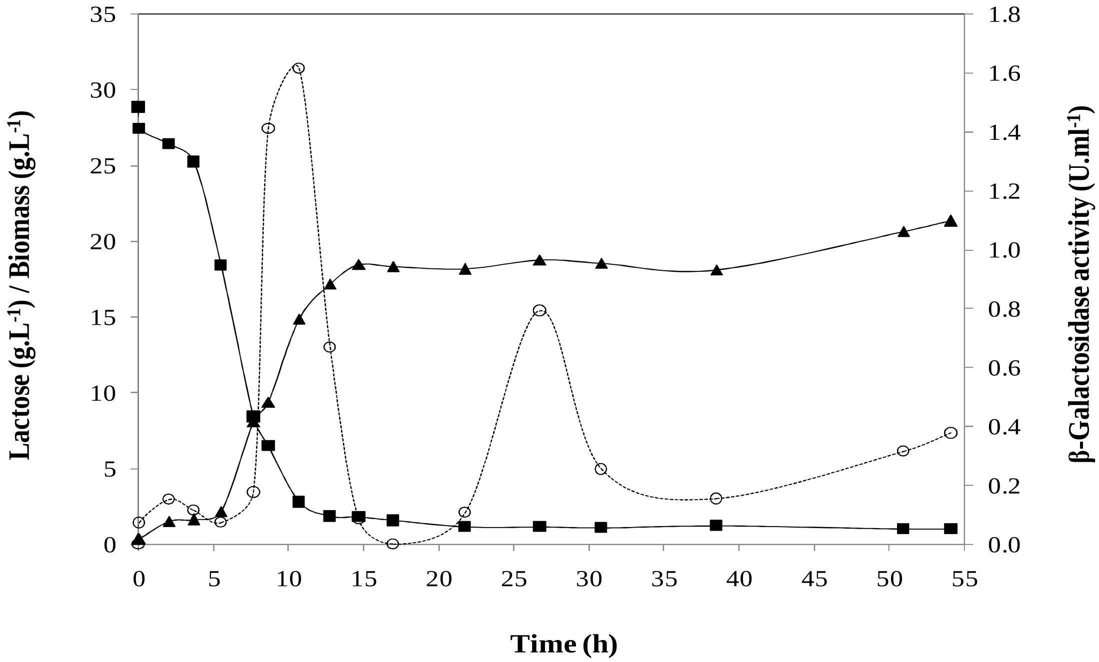


Fig. 3.6 Continuous cultivation of *K. lactis* UOFS y-0939 at 30°C, pH 7, and a stirrer speed of 800 rpm at a dilution rate of 0.2 h^{-1}, indicating lactose utilisation (■), biomass (▲), and β-galactosidase activity (-○-). Cell lysates were prepared by ultrasonication.

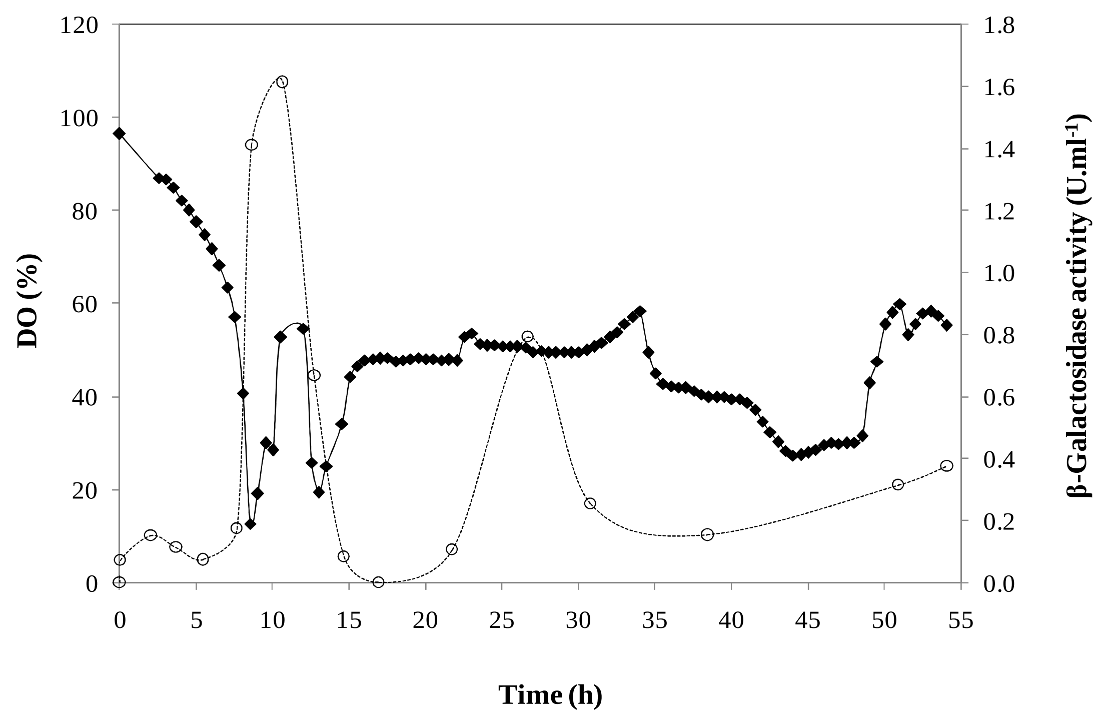


Fig. 3.7 Continuous cultivation of *K. lactis* UOFS y-0939 at 30°C, pH 7, and a stirrer speed of 800 rpm at a dilution rate of 0.2 h^{-1}, indicating DO (♦) and β-galactosidase activity (-○-). Cell lysates were prepared by ultrasonication.

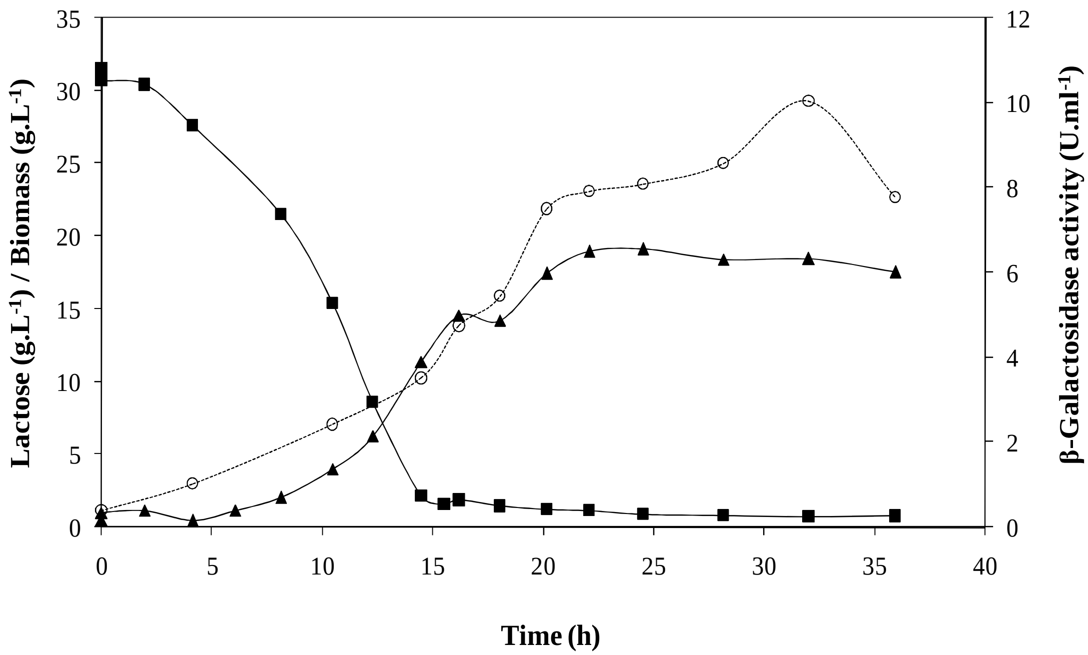


Fig. 3.8 Continuous cultivation of *K. lactis* UOFS y-0939 at 30°C, pH 7 and 4.5 slpm airflow with stirrer speed of 800 rpm, at a dilution rate of 0.2 h^{-1}, indicating lactose utilisation (■), biomass (▲), and β-galactosidase activity (-○-). Cell lysates were prepared by homogenisation.

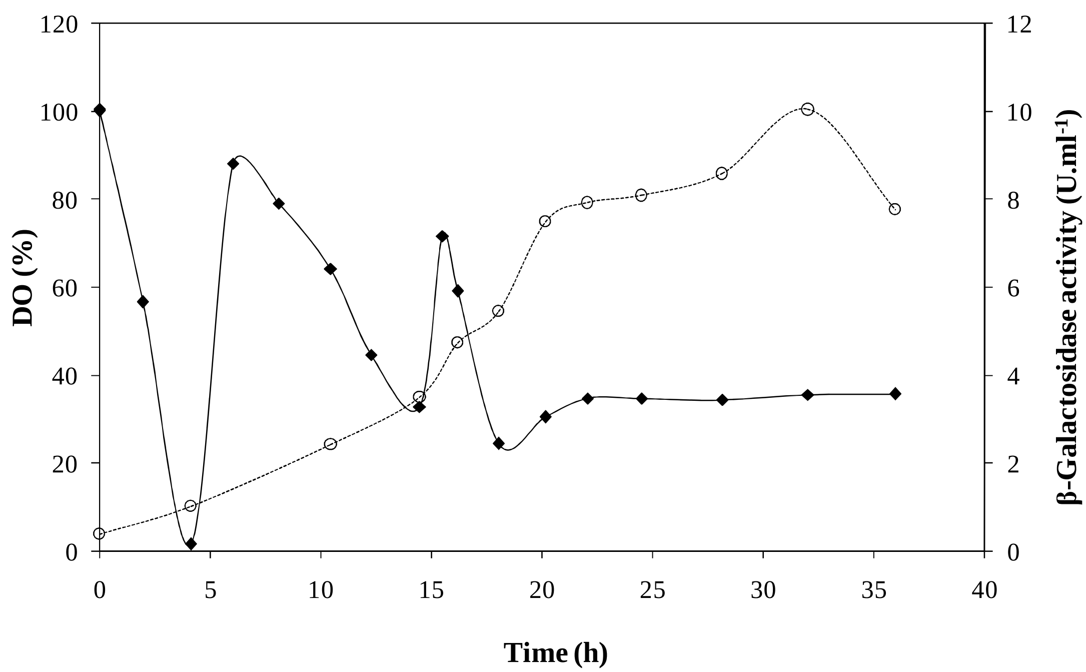


Fig. 3.9 Continuous cultivation of *K. lactis* UOFS y-0939 at 30°C, pH 7 and 4.5 slpm airflow with stirrer speed of 800 rpm, at a dilution rate of 0.2 h^{-1}, indicating DO (♦) and β-galactosidase activity (-○-). Cell lysates were prepared by homogenisation.

3.4 DISCUSSION

Controlled cultivation conditions are essential for the study of enzyme production. Shake flask cultures do not meet this requirement, since pH and dissolved oxygen concentration cannot be regulated, and both these parameters exert a strong influence on the rate of sugar transport (Van Leeuwen *et al*., 1992; Van den Broek, 1986; Van den Broek and Van Steveninck, 1982; Höfer and Misra, 1978), and thus, enzyme production. Batch cultivation in bioreactors equipped with such controllers would be more suitable.

β-Galactosidase production in *K. lactis* UOFS y-0939 batch culture was investigated using 28.2 and 40 $g.L^{-1}$ lactose as carbon source. After 14 h, biomass produced from 28.2 $g.L^{-1}$ lactose was 17.77 $g.L^{-1}$, and marginally higher at 40 $g.L^{-1}$ (20.07 $g.L^{-1}$) (Fig. 3.1). The point of interest is the yield of biomass on lactose ($Y_{x/s}$), in that more lactose had been converted to biomass at the lower sugar concentration, 28.2 $g.L^{-1}$ ($Y_{x/s}$ = 0.63 $g.g^{-1}$), than at 40 $g.L^{-1}$ ($Y_{x/s}$ = 0.5 $g.g^{-1}$). Lukondeh *et al*. (2005) also reported decreasing biomass yields with increasing lactose concentrations. Particularly, at 20 and 40 $g.L^{-1}$ lactose, biomass yield coefficients of 0.45 and 0.37 $g.g^{-1}$ was reported, respectively. This phenomenon has been described by Belem and Lee (1999), who reported that increases in lactose concentrations can lead to the accumulation of pyruvate resulting from the greater glycolytic flux in *Kluyveromyces* strains, thereby causing a reduction in final biomass yields. The DO profiles for both fermentations declined during lactose utilisation and displayed an increase once the initial lactose concentration neared depletion, thus following the usual trend of those in batch culture (Fig. 3.3).

The maximum β-galactosidase activity in batch culture of *K. lactis* UOFS y-0939 at both 28.2 and 40 $g.L^{-1}$ lactose was observed at stationary phase (Fig. 3.2). This was also demonstrated by Inchaurrondo *et al*. (1994) who investigated growth and β-galactosidase production by *Kluyveromyces* strains and *C. pseudotropicalis* in aerobic batch culture in lactose-limited medium. Maximum β-galactosidase levels were reached at the onset of stationary phase in all investigations, and remained stable thereafter. Pinheiro *et al*. (2003) also reported maximum β-galactosidase activity at early stationary phase, however, the activity subsequently exhibited a slight decrease, and as reported by Ranzi *et al*. (1987), activity decreased continuously in stationary phase.

In the present study, the highest β-galactosidase activity produced by *K. lactis* UOFS y-0939 at 28.2 $g.L^{-1}$ was 1.58 $U.ml^{-1}$, considerably lower than the activity achieved at 40 $g.L^{-1}$ (4.08 $U.ml^{-1}$) (Fig. 3.2). *K. lactis* has been chosen as a model, Crabtree negative, lactose utilising organism (Fukuhara, 2006; Lachance, 1998). According to the criteria of Alexander and Jeffries (1990) Crabtree negative yeasts show aerobic fermentation, but no detectable respiration repression, and are unable to grow in the absence of oxygen. In contrast, González-Siso *et al*. (1996) demonstrated that *K. lactis* produced the same quantity of ethanol as *S. cerevisiae*, the Crabtree positive prototype, when grown in discontinuous culture, oxygen-limited aerobic conditions, complete media, and 2% glucose. It was reported that even in well-aerated cultures, a slight fermentation was detectable if the substrate (sugar) levels were high enough. It has long been established that high sugar concentrations in batch cultured yeasts can result in Crabtree repression, which inhibits respiratory enzymes and increases ethanol production (Fiechter *et al*., 1987; De Deken, 1966), and has been known to result in a reduction in enzyme yields. In the present investigation, however, the elevated levels of lactose did not result in Crabtree repression, and were, therefore, non-inhibitory to β-galactosidase production.

Since an alternative cell disruption method, i.e., homogenisation, was employed as ultrasonication was proven to be ineffective, it was important to draw a comparison between the two methods. During the batch cultivation of *K. lactis* UOFS y-0939 in 28.2 $g.L^{-1}$ lactose, the cell lysate at 12 h was prepared by both homogenisation and ultrasonication. The β-galactosidase activity (1.55 $U.ml^{-1}$) and specific activity on biomass (91.02 $U.g^{-1}$) obtained after homogenisation was more than 3.5 fold higher those obtained after ultrasonication (Table 3.1), thus demonstrating the supremacy of homogenisation over ultrasonication. In the extraction of intracellular enzymes, the cell disruption method employed should be robust enough to disrupt cell envelopes efficiently, yet gentle enough to preserve enzyme activity (Numanǒglu and Sungur, 2004). The Microfluidizer® processor operates at elevated air pressures, and it is estimated that every 1 000 psi of pressure applied to water in the system, results in a temperature rise by 1.0 to 1.7ºC (temperature increase would vary depending on the material). Elevated temperature is known to denature proteins and would therefore compromise enzyme activity (it is for this reason that the Interaction Chamber is kept cold with ice during processing). In spite of these operational factors, the Microfluidizer® processor was efficient in the disruption of *K. lactis* UOFS y-0939 cells (microscopically, approximately 90% of cells were disrupted-data not shown) and the preservation of enzyme integrity.

Fed-batch culture has been successfully used to achieve high culture productivity due to its ability to overcome the Crabtree effect (Nor *et al*., 2001) by incremental feeding of essential nutrients into the bioreactor, and is thus, advantageous over the batch process. In practise, fed-batch cultivations are not operated at a constant growth rate, but involve a feed profile that leads to a continuous decrease in growth rate. Industrially, this profile is governed by a variety of factors, including oxygen transfer and the cooling capacities of industrial bioreactors (De Hollander, 1993; Beudeker *et al*., 1990).

The present study investigated β-galactosidase production by *K. lactis* UOFS y-0939 in fed-batch culture, employing a constant lactose feed. Due to the possibility of elevated lactose levels being inhibitory to biomass production, the study was conducted with 28.2 $g.L^{-1}$ lactose, instead of 40 $g.L^{-1}$. At the lactose feed rate of 4 $g.L^{-1}.h^{-1}$, an average residual lactose concentration of 1.34 $g.L^{-1}$ was observed throughout the fed-batch portion of the run. Residual lactose should, preferably, have been maintained at a lower concentration. The μ_{max} observed during the batch portion of the fermentation was 0.22 h^{-1}, and μ declined to 0.1 h^{-1} after feed start (between 15.88 and 23.95 h), which is typical of fed-batch operations.

K. lactis UOFS y-0939 was cultivated in fed-batch culture to maximise the production of biomass, and consequently, β-galactosidase activity. The fed-batch was successful in achieving higher biomass titres (34.47 $g.L^{-1}$ in 41.73 h) than batch fermentation, and consequent β-galactosidase activity (Fig. 3.4). β-galactosidase activity reached a maximum at 37.31 h (8.7 U.ml^{-1}) and then exhibited a decline (Fig. 3.4). The proportionate increase in biomass and β-galactosidase activity was also demonstrated by Nor *et al*. (2001) who reported that in the fed-batch production of β-galactosidase by a strain of *K. fragilis* (*K. marxianus*), high cell density (69 g dry wt.L^{-1}) resulted in increased volumetric enzyme activity (2 U.mg^{-1} cell dry wt). In contrast, the complexity of fed-batch operations has also been demonstrated (Weusthuis *et al*., 1994) where, as a result of an increase in biomass, a change in growth conditions is inevitable. It has been reported that the formation of many toxic by-products is linearly proportional to the amount of biomass produced which may contribute to reduced enzyme titres. This phenomenon may have been the cause of the decline in enzyme activity after 37.31 h. Furthermore, continuous lactose feeding would have resulted in increased measures of the hydrolytic products of lactose, glucose and galactose. As high concentrations of galactose has been reported to inhibit β-galactosidase activity (Martins *et al*., 2002), this too, may have contributed to the reduced enzyme activity.

The relationship between volumetric oxygen mass transfer (k_La), or OTR, and β-galactosidase specific activity has been shown (Acevedo *et al*., 1996; García-Garibay *et al*., 1987; Mahoney *et al*., 1974). Furthermore, it has been reported that β-galactosidase production is strongly dependent on dissolved oxygen concentration in the fermentation broth of *K. fragilis* (*K. marxianus*) grown on lactose as a sole source of carbon and energy (Barberis and Gentina, 1998). Two different studies investigating *K. fragilis* (*K. marxianus*) NRRL Y-1109, have reported the lower dissolved oxygen concentrations of 10% and 30% as being most suitable for β-galactosidase production (Barberis and Gentina, 1998; Barberis and Segovia, 1997). Barberis and Gentina (1998) also reported that even though the biomass concentration was 15% less at 10% DO, than at 60% DO, the increase in enzyme activity was greater than the reduced biomass concentration. In the present fed-batch study, the DO profile displayed an increase once the initial lactose concentration neared depletion and proceeded to decline at the onset of feeding (Fig. 3.5). The effect of 20% DO was investigated, and was achieved by manual manipulation of the airflow and stirrer controllers. It was observed that β-galactosidase activity continued to increase at 20% DO up to 8.7 U.ml^{-1}, thus validating the reports of Barberis and Gentina,

(1998) and Barberis and Segovia (1997). In addition, as lower DO concentrations have been reported to reduce biomass production, it may be observed that the maximum biomass produced (34.47 g.L^{-1}) may have been improved upon at a higher DO concentration.

Following fed-batch cultivation, the continuous production of β-galactosidase by *K. lactis* UOFS y-0939 was investigated in two separate bioreactor cultivations under identical conditions. Cell lysates from the first bioreactor cultivation were prepared by ultrasonication, while those from the second were prepared by homogenisation. Albeit indentical conditions, differences were evident in the batch portion of both continuous fermentations. While a μ_{max} of 0.43 h^{-1} was observed in the first study, and feeding commenced at 10.05 h (Fig. 3.6), feeding in the second study was initiated 5.72 h later (at 15.77 h) due to the slower growth rate (μ_{max} = 0.32 h^{-1}) (Fig. 3.8). The most probable cause for the difference in the second chemostat is that, due to an obstruction in the exhaust gas line causing pressure in the bioreactor, dissolved oxygen levels were drastically reduced between 0 and 6 h reaching a minimum of 1%. After the exhaust line had been detached, normal DO levels resumed. It can, thus, be deduced that the reduced dissolved oxygen available to the yeast cells during the first 6 h after inoculation, inhibited respiration thereby resulting in a slower growth rate and causing a delay in the feed start. Despite the initial discrepancy in the second continuous fermentation, the average biomass titres produced during steady state in both investigations, were similar [19.02 ± 1.2 g.L^{-1} (Fig. 3.6) and 18 ± 0.9 g.L^{-1} (Fig. 3.8)].

The rate of substrate consumption can be manipulated by varying the dilution rate (Weusthuis *et al*., 1994) and it has been reported that at very low dilution rates, energy requirements for maintenance processes affect the biomass yield (Pirt, 1975). Inchaurrondo *et al*. (1998) demonstrated that during the aerobic cultivation of *K. lactis* in continuous culture at a dilution rate of 0.5 h^{-1}, the biomass yield ($Y_{x/s}$) on lactose was 180 g.mol^{-1}, which was reduced to approximately 164 g.mol^{-1} at 0.1 h^{-1}. Furthermore, residual lactose that remained at approximately 0.1 mM from 0.05 to 0.25 h^{-1}, increased to approximately 0.5 mM at a dilution rate of 0.5 h^{-1}. The linear increase in oxygen uptake rate with an increase in dilution rate by *K. lactis* NRRL Y-1140 has also been reported (González-Siso *et al*., 1996). As it is known that an increase in dissolved oxygen in the medium increases biomass concentration (Barberis and Gentina, 1998), these reports substantiate that biomass yields are enhanced with an increase in dilution rate. In the present study, at the intermediate dilution rate of 0.2 h^{-1}, the biomass yield on lactose throughout steady state was 0.67 $g.g^{-1}$ and 0.63 $g.g^{-1}$ in the first and second, continuous fermentations, respectively.

Both continuous fermentations were operated at a dilution rate of 0.2 h^{-1} as it has also been found to be optimal for β-galactosidase production (Barberis and Gentina, 1998**)**. Furthermore, Inchaurrondo *et al*. (1998) reported the highest specific enzyme activity to correspond to *K. lactis* NRRL Y-1118 cells grown at dilution rates between 0.1 and 0.2 h^{-1}, and Ornelas *et al*. (2008) reported 0.24 h^{-1} to be optimum for *K. lactis* specific β-galactosidase activity. Inchaurrondo *et al*. (1998) further demonstrated that the specific rate of enzyme synthesis was shown to increase in an approximately linear manner to a maximum at an intermediate dilution rate of 0.25-0.35 h^{-1}, and declined above 0.4 h^{-1}. The present investigation compared β-galactosidase activity in cell lysates from *K. lactis* UOFS y-0939 continuous cultures prepared by ultrasonication and homogenisation. The maximum activity achieved by ultrasonication (1.6 $U.ml^{-1}$) occurred at late exponential phase (10.68 h) and thereafter declined during feeding, exhibiting a peak at 26.7 h (Fig. 3.7). As β-galactosidase is a growth associated product (Nor *et al*., 2001), enzyme activity during the steady state was expected to remain constant, similar to biomass concentrations. The discrepancy in the enzyme activity trend may possibly be due to the inefficiency of ultrasonication to disrupt the high titres of biomass (± 19 $g.L^{-1}$) (Fig. 3.6) achieved during continuous fermentation, as compared to the lower titres achieved in shake flask cultures (~ 7-9 $g.L^{-1}$) (section 2.3.5, Fig. 2.9). Comparatively, maximum β-galactosidase activity of the homogenised cells under identical conditions (10 $U.ml^{-1}$) (Fig. 3.8), exhibited greater than a 6 fold increase than that achieved by ultrasonication, thus confirming the efficacy of homogenisation as a cell disruption method, over ultrasonication. The production of β-galactosidase followed that of biomass and an average of 8 ± 0.9 $U.ml^{-1}$ was achieved in steady state (Fig. 3.8).

The current investigation was operated under non-sugar-limiting conditions as lactose was continually fed to the growing culture, resulting in a residual concentration of 0.92 ± 0.2 g.L^{-1} in the second continuous fermentation (Fig. 3.8). It was observed that under such conditions, β-galactosidase synthesis was uninhibited (Fig. 3.8). Inchaurrondo *et al*. (1998), however, reported that the maximum specific rate of β-galactosidase synthesis was reached in lactose- (and galactose-) limited *K. lactis* cultures at intermediate growth rates. β-galactosidase accumulated in amounts equivalent to 10% of the cell protein. When *K. lactis* cells were grown in a lactose-sufficient environment with nitrogen or phosphate limitation, however, β-galactosidase repression was observed. Enzyme levels were reduced 4 to 8 times compared to those in carbon-limited cultures and this was attributed to increased concentration of internally released glucose. Furthermore, when a lactose-limited chemostat was pulsed with an excess of lactose, growth ceased immediately and β-galactosidase was progressively inactivated. This phenomenon, known as substrate-accelerated death, was explained in terms of uncontrolled sugar uptake into the cell after the pulse, leading to osmotic stress. This, in combination with a possible decrease in internal pH due to rapid proton influx, could have resulted in cellular death (Inchaurrondo *et al*., 1998). It has been demonstrated that enzyme inactivation under conditions of stress, is mediated by mechanisms of phosphorylation and subsequent proteolytic degradation (Hilt and Wolf, 1992; Gancedo and Serrano, 1989).

In typical *Kluyveromyces* cultivation, high cell densities are reached and oxygen is usually the major limiting factor (Onken and Liefke, 1990). In the present study, steady state conditions were strictly aerobic as dissolved oxygen levels were maintained at 45.5 ± 8.9% and 34 ± 1.9% in the first and second continuous fermentations, respectively (Fig 3.7 and 3.9). These conditions appeared to be significantly favourable for the production of β-galactosidase, contributing to an average β-galactosidase activity of 8 ± 0.9 U.ml^{-1} in the second continuous fermentation (Fig. 3.9). Albeit the studies of Barberis and Gentina (1998) and Barberis and Segovia (1997) report the dissolved oxygen levels of 10 and 30% to be most suitable for β-galactosidase production, this present study indicates that greater than 30%, up to ± 45% DO is not inhibitory to β-galactosidase synthesis. This finding is comparable with that of García-Garibay *et al*. (1987), who theorised that the fermentation process should necessarily be operated at high k_La where the cell concentration is significantly increased, thus increasing volumetric enzyme productivity, while recognising that the enzyme activity will be slightly reduced.

It can be deduced from the scale-up investigations that, a high concentration of 40 $g.L^{-1}$ lactose is not inhibitory to β-galactosidase production by *K. lactis* UOFS y-0939 in batch culture, due to Crabtree repression. Furthermore, fed-batch cultivation of *K. lactis* UOFS y-0939 is more favourable for the production of higher biomass yields and equivalent β-galactosidase titres, than batch cultivations. It was also confirmed that homogenisation was a more effective cell disruption method than ultrasonication. Finally, continuous fermentation under non-lactose-limiting, fully aerobic conditions, is a favourable cultivation method for the production of β-galactosidase by *K. lactis* UOFS y-0939.

CHAPTER FOUR: GENERAL DISCUSSION AND RECOMMENDATIONS

Most microorganisms adapted to life in milk owe their ability to thrive in this habitat to the evolution of permeabilisation and hydrolytic systems for the use of the most abundant sugar present in milk as a carbon source, lactose (Rubio-Texeira, 2006). *Kluyveromyces*, the lactose-assimilating yeast, has long been used in industrial processes for the elimination of this sugar by the application of β-galactosidase, an enzyme abundant in nature, as acid hydrolysis is unacceptable for use in food (Bury *et al*., 2001). Bacteria, fungi and yeast are all good suppliers of this enzyme. Although bacteria could offer more versatility, the confirmed GRAS status of the yeasts *K. lactis* and *K. marxianus*, and the fungi *A. niger* and *A. oryzae*, still render these as the preferred sources of β-galactosidase for food biotechnology and the pharmaceutical industry (Bonekamp and Oosterom, 1994). Depending on their source, these enzymes differ in their optimum pH, thermostability and intracellular or secretary nature.

In the present study, shake flask cultivation served as a preliminary tool to determine optimum parameters for the growth of, and intracellular β-galactosidase production by *K. lactis* UOFS y-0939. The optimum temperature, pH and inoculum size, were to be applied in scaled up batch, fed-batch and continuous fermentations. It must be noted, though, that the current optimisation method employed, i.e., the variation of one parameter while maintaining the others at a constant level, is limited in that it does not reflect interaction effects among the variables employed and does not depict the net effect of various factors on enzyme activity (Dagbagli and Goksungur, 2008). A range of mathematical and statistical techniques have been developed, however, including response surface technology, that are designed to determine the effects of several variables and optimise different biotechnological processes (He and Tan, 2006).

In an optimisation study conducted by Atkaş *et al*. (2006), medium temperature and pH were found to be the most significant factors to influence lactose consumption by *K. marxianus* Y-8281. Present shake flask studies revealed that 30°C promoted the growth and β-galactosidase production by *K. lactis* UOFS y-0939. At 35°C, the lactose consumption rate was the most efficient; however, biomass production and β-galactosidase activity was not favoured. A pH of 7 was optimum for β-galactosidase synthesis, even though pH 5.5 was most suitable for growth and biomass production. The reduced β-galactosidase activity at pH 5.5 was supported by the reports of several researchers (Burin and Buera, 2002; Zhou and Chen, 2001; Guy and Bingham, 1978). Inoculum ratio also plays a crucial role in microbial fermentations, having a significant influence on the lag phase duration, growth rate, biomass and product formation. No significant difference was evident in β-galactosidase activity at 6, 8 and 10% inoculum ratios, and 10% was applied in subsequent fermentations.

Glucose repression of β-galactosidase was evident in the shake flask investigations. *Kluyveromyces* strains are reported to have variable degrees of sensitivity to glucose repression (Rubio-Texeira, 2006), with *K. lactis* UOFS y-0939 (also designated CBS 2359) reported to be weakly repressible (Breunig *et al*., 2000). In the *GAL/LAC* regulon there exists a delicate balance between activation and repression (Breunig *et al*., 2000). When external increases in the levels of glucose and its uptake occur in a *Kluyveromyces* cell, glucose repression of genes involved in the use of alternative carbon sources, are triggered. Among other effects, glucose inhibits the central regulator kinase, KlSnf1p, which results in increased levels of active KlMig1p in the nucleus. KlMig1p, most likely in combination with a yet undefined co-repressor complex, binds to an upstream repressor sequence in the *KlGAL1* promoter, inhibiting its expression. This impairs KlGal1p-dependent release of KlGal4p from KlGal80p repression, resulting in the inactivation of the galactose/lactose (*GAL/LAC*) regulon (Rubio-Texeira, 2006) (Fig. 4.1). Furthermore, Martins *et al*. (2002) also reported on the repressive effects of high concentrations of galactose on specific β-galactosidase activity by *K. marxianus* CBS 6556. In the current study, glucose and galactose levels were not measured. It is recommended that, in further investigations, these sugars should be quantified to possibly determine their inhibitory concentrations for β-galactosidase production by *K. lactis* UOFS y-0939.

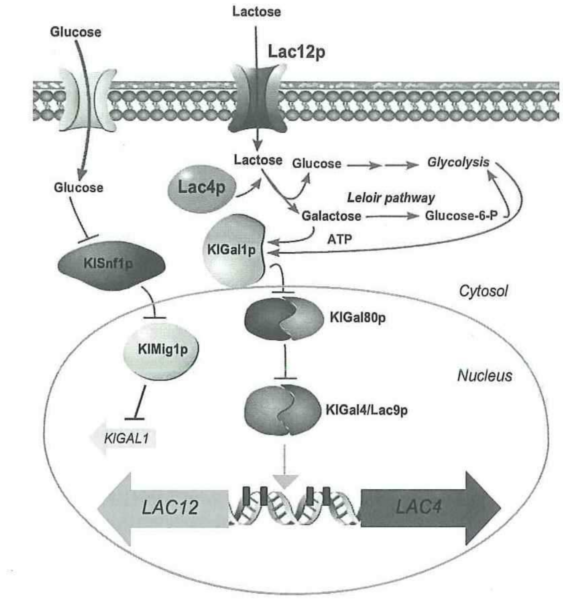


Fig. 4.1 Model for the regulation of lactose permeabilisation and hydrolysis, and glucose repression in *Kluyveromyces* (Rubio-Texeira, 2006).

The effectiveness of ultrasonication with glass beads as a cell disruption method was investigated in the present study. It is essential, when isolating intracellular microbial products, to use methods that are capable of breaking down the cell wall, yet at the same time, not cause inactivation of the biological substances (Özbek and Ülgen, 2000). The mechanism of ultrsonication is associated with cavitation, which is a combination of formation, growth and collapse of the vapour-filled bubbles created by high-intensity (ultrasound above 20 kHz) sound waves (Choonia and Lele, 2010). The efficacy of the cell disruption process is not only dependent on the acoustic power of the sound waves, but is also dependant on the physical strength of the cell wall of the microorganisms (Sauer *et al*., 1989). Furthermore, the rate of release of an intracellular enzyme is dependent on its location within the cell (Clementi, 1995). It is, therefore, important to note that β-galactosidase is located within the cytosol of the *Kluyveromyces* cell (Rubio-Texeira, 2006), and enzymes situated in this region, for example, alcohol dehydrogenase, are reported to be released instantaneously during cell disruption (Melendres *et al*., 1993; Wagner *et al*., 1992). While ultrasonication is widely used at laboratory scale, the high speed bead-mill and high pressure homogeniser are commonly used for large-scale applications, and have been reported to be more effective for the disruption of microbial cells and the release of β-galactosidase than ultrasonication, with the homogeniser being the most efficient method (Bury *et al*., 2001). This is confirmed in the present study where cell disruption was performed in an ultrasonic water bath at 4°C. After exposure of the *K lactis* UOFS y-0939 cells for 30 min, only a 16% reduction of viable cells was evident in shake flask culture. In batch culture, the comparison of ultrasonication and homogenisation on a stationary phase culture of *K. lactis* UOFS y-0939 revealed that homogenisation resulted in greater than a 3.5 fold increase in β-galctosidase activity and specific activity on biomass. Furthermore, in continuous culture, the highest β-galctosidase activity produced by homogenised cells (10 $U.ml^{-1}$) was 6 fold higher than that produced by ultrasonicated cells (1.6 $U.ml^{-1}$).

The inconsistency of ultrasonication as a cell disruption method was also proposed due to incomparable enzyme activities at similar periods in batch and continuous culture. Maximum β-galactosidase activity in continuous culture at late exponential phase (1.61 $U.ml^{-1}$) was 3.5 fold higher than that achieved in batch culture at this approximate period (0.43 $U.ml^{-1}$). Moreover, it has been reported that ultrasonication poses serious disadvantages such as non-specific cell wall disruption, high heat generation and prolonged operation time leading to the generation of harmful free radicals (Choonia and Lele, 2010), thus proving it to be an inefficient method for cell disruption. Alternatively, homogenisation offers the advantage of continuous operation, short disruption times to minimise product degradation, contained operation, and low operating costs (Middelberg, 1995; Keshavarz *et al*., 1987). It must be noted, though, that there were no reports on the utilisation of an ultrasonic water bath, as used in this study, for direct comparison.

Batch and fed-batch fermentation have been widely used for the production of numerous industrially important enzymes, e.g., inulinase, cellulase, amylase and xylanase, amongst others. The large scale batch production of xylanase, an enzyme employed in the animal feed, confectionary and pulp and paper industry, for example, has been extensively demonstrated (Hoq *et al*., 1994; Bailey *et al*., 1993; Gamerith *et al*., 1992; John and

Schmidt, 1988; Warzywoda *et al*., 1983), up to a 15 000 L scale (Gomes *et al*., 1993). Furthermore, Primalco Ltd Biotec (Finland) produce a commercial xylanase, Ecopulp X-200, from the fungus, *Trichoderma reesei*, in submerged fed-batch culture (Haltrich *et al*., 1996). These fermentation processes are, therefore, critical in the industrial scenario on a global scale.

In the present study, following shake flask investigations, scaled up β-galactosidase production by *K. lactis* UOFS y-0939 in batch, fed-batch and continuous culture under optimum conditions, was investigated. Batch cultivation at 28.2 and 40 $g.L^{-1}$ lactose concentration revealed that a higher biomass yield on lactose at 28.2 $g.L^{-1}$ ($Y_{x/s}$ = 0.63 $g.g^{-1}$) was observed than at 40 $g.L^{-1}$ ($Y_{x/s}$ = 0.5 $g.g^{-1}$). Furthermore, higher β-galactosidase activity was achieved at the elevated sugar concentration. At this point, it is important to note the metabolic mechanism of *K. lactis*. In terms of energy-yielding metabolism, yeasts do not form a homogenous group, however, the fate of pyruvate, a glycolytic intermediate, determines the type of energy metabolism. Oxidative metabolism takes place when all the pyruvate produced is converted by the tricarboxylic-acids' pathway (respiration). Alternatively, oxidoreductive metabolism occurs when pyruvate is reduced to ethanol or other compounds (fermentation). Interestingly, both mechanisms can co-exist simultaneously, leading to a mixed (respirofermentative) pattern of energy metabolism (Gancedo and Serrano, 1989). *K. lactis* is commonly known to be an aerobic, respiratory yeast and has been reported to be Crabtree negative in fully oxidative conditions (Kiers *et al*., 1998; González-Siso *et al*., 1996). Several researchers have, however, demonstrated *K. lactis* to exhibit a respirofermentative metabolism (González-Siso *et al*., 2000; González-Siso *et al*., 1996), and González-Siso *et al*. (1996) in particular, challenged the proposed classification of Alexander and Jeffries (1990), as described in section 3.4, and confirmed the importance of culture conditions, for example, aeration, carbon source, culture phase and nutrient limitations, in classifying a yeast as Crabtree positive or negative. The above, therefore, establishes that the *K. lactis* UOFS y-0939 strain under investigation, could have the potential of Crabtree repression in batch culture as a consequence of the higher lactose concentration which would inhibit respiratory enzymes, increase ethanol production and reduce enzyme synthesis.

In the present study, the lactose concentration of 40 $g.L^{-1}$ did appear to reduce biomass yields as compared to 28.2 $g.L^{-1}$ lactose, however, a non-inhibitory effect was evident on β-galactosidase production. This is due to β-galactosidase activity at 40 $g.L^{-1}$ lactose, (4.08 $U.ml^{-1}$) being 2.5 fold higher than at 28.2 $g.L^{-1}$ lactose (1.58 $U.ml^{-1}$), thereby implying the absence of Crabtree repression. Ethanol titres were, however, not investigated and it, therefore, cannot be concluded that fermentation did not occur. It is, thus, recommended that future investigations would include the measurement of ethanol to possibly demonstrate the fermentative ability of *K. lactis* UOFS y-0939 under the conditions of this study, i.e., 28.2 and 40 $g.L^{-1}$ lactose, 30°C, pH 7, 4.5 slpm airflow and a stirrer speed of 800 rpm.

Since high sugar concentrations can cause undesirable effects in batch culture, fed-batch cultivation offers the potential to maintain growth conditions while maintaining relatively low substrate (lactose) concentrations and associated salts and by-products early in the fermentation (Lukondeh *et al*., 2005). The fed-batch cultivation of *K. lactis* UOFS y-0939 where lactose was fed at a rate of 4 $g.L^{-1}.h^{-1}$, revealed an average residual lactose of 1.34 $g.L^{-1}$ throughout the feeding period and the maximum biomass achieved was 34.47 $g.L^{-1}$ in 41.73 h. This titre was 1.9 fold higher than that achieved in batch culture (17.77 $g.L^{-1}$ in 14 h). It must be noted, however, that the fed-batch cultivation was performed in an initial working volume of 10 L to which a total of 6.2 L of lactose feed was added, while the batch cultivation was performed in 3 L. It would, therefore, be expected that the increase in biomass would most likely be more than 1.9 fold greater in the fed-batch investigation. Furthermore, the biomass productivity in batch culture was 1.27 $g.L^{-1}.h^{-1}$ while that in fed-batch culture was 0.82 $g.L^{-1}.h^{-1}$. This result is in contrast with numerous investigations that report higher biomass titres and productivity in the fed-batch cultivation of *Kluyveromyces* sp. than in batch culture (Lukondeh *et al*., 2005; Nor *et al*., 2001; Barberis and Segovia, 1997). An indicator of the likely cause of the reduced biomass levels in fed-batch, is the 1.34 $g.L^{-1}$ average residual lactose during the feeding portion of the run. This concentration of residual lactose may, essentially, have been too high, implying that the feed rate of 4 $g.L^{-1}.h^{-1}$ contributed to elevated lactose concentrations that caused a reduction in final biomass titres, as described by Belem and

Lee (1999). It is, therefore, recommended that the lactose feed rate be optimised in future investigations.

The β-galactosidase production, however, did not appear to be inhibited as the enzyme activity of 8.7 $U.ml^{-1}$ in fed-batch was 5.5 fold higher than that obtained in batch culture (1.58 $U.ml^{-1}$). It can be deduced, however, that if biomass titres were increased, enzyme activity would have increased proportionally. Furthermore, Barberis and Segovia (1997) attributed an improvement in volumetric β-galactosidase production rates by a *K. fragilis* (*K. marxianus*) strain from 2 390 $UI.L^{-1}.h^{-1}$ in batch culture to 7 190 $UI.L^{-1}.h^{-1}$ in fed-batch culture, to good control of dissolved oxygen. It was observed that while ± 20.4 % DO during the fed-batch period of the present study, did not inhibit β-galactosidase production, increasing DO may have contributed to elevated biomass titres.

During the cultivation of *K. lactis* UOFS y-0939 in continuous culture at a dilution rate of 0.2 h^{-1}, average biomass titres of 19.02 ± 1.2 $g.L^{-1}$ and 18 ± 0.9 $g.L^{-1}$ in the first and second continuous fermentations, respectively, were comparable to those obtained in batch culture under similar conditions. Fed-batch cultivation, however, resulted in an approximately two fold higher biomass titre (34.47 $g.L^{-1}$) and had the potential of improving upon this value, provided that the lactose feed concentration was not inhibitory. In continuous cultures of *K. fragilis* (*K. marxianus*) NRRL Y-1190, Barberis and Gentina (1998) demonstrated that the specific rate of carbon source consumption varies with different DO saturation percentages. The results showed that the lower the DO saturation, the higher the specific rate of carbon source consumption, and 10% DO was found to be optimal. It was reported that at a lower aeration rate, the metabolism is more reductive, and as a consequence, produces both higher specific rate of carbon source consumption and higher enzyme expression. Furthermore, high levels of oxygen may have toxic effects on aerobic organisms, a phenomenon called oxidative stress. During the reduction of molecular oxygen to water through acceptance of four electrons, active oxygen species, such as superoxide anion radical ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\cdot}$) are generated. These reactive oxygen species cause damage to enzymes, nucleic acids, or lipids (Izawa *et al.*, 1995).

In the present study, steady state conditions were strictly aerobic and maintained at 45.5 ± 8.9% and 34 ± 1.9% DO in the first and second continuous fermentations, respectively. It is important to note that, although the specific rate of carbon source consumption was not measured, average residual lactose during steady state was 1.22 ± 0.3 $g.L^{-1}$ and 0.92 ± 0.2 $g.L^{-1}$ in the first and second continuous studies, respectively. It can therefore be deduced that, at a dilution rate of 0.2 h^{-1} and 45.5 ± 8.9% and 34 ± 1.9% DO, lactose consumption was not optimal, thereby supporting the report of Barberis and Gentina (1998). β-galactosidase activity, however, was not inhibited by the fully aerobic conditions since an average of 8 ± 0.9 $U.ml^{-1}$ was achieved in steady state in the second continuous fermentation, values which were comparable to that achieved in fed-batch fermentation at ± 20.4% DO during the feeding period (8.7 $U.ml^{-1}$). Furthermore, the non-limiting lactose conditions were not inhibitory to β-galactosidase synthesis. It is interesting to note that Barberis and Gentina (1998) also reported that *K. fragilis* (*K. marxianus*) NRRL Y-1190 metabolism was oxidoreductive and ethanol was always present in the culture, even when aeration conditions were close to 100% DO saturation, meaning that the respiratory capacity of the cells were saturated and that lactose was partially degraded down to ethanol. Ethanol concentration, however, was not evaluated in the present study but is recommended in further continuous investigations under the present conditions to demonstrate the relationship between ethanol production and DO levels.

This study, therefore, demonstrates that β-galactosidase production by *K. lactis* UOFS y-0939 grown under the present conditions, were not inhibited, in batch, fed-batch and continuous culture. Furthermore, fed-batch would be the best suited cultivation method to achieve high biomass titres, however, continuous cultures would ensure constant production of both biomass and enzyme, and thus would ultimately provide larger yields.

Globally, there is an increasing industrial demand for β-galactosidase and cost effective production methods are required to meet this demand. Furthermore, β-galactosidase has tremendous significance in the health sector, as a treatment for the alleviation of lactose intolerance. *K. lactis*, one of the favoured sources of the enzyme, is the solution to the global β-galactosidase need, amongst various others. Due to its versatility, *K. lactis* continually demonstrates industrial potential, many of which have yet to be unravelled.

4.1 FUTURE WORK

Based on the results obtained in this study, the following should be addressed in future investigations:

(i) Galactose and glucose concentrations should be measured during all bioreactor investigations to determine and prevent their inhibitory concentrations for β-galactosidase production by *K. lactis* UOFS y-0939.

(ii) Fed-batch and continuous cultures should be operated under lactose-limiting conditions, thereby possibly maximising β-galactosidase activity in these scenarios. In fed-batch and continuous fermentations, the lactose feed rate (4 $g.L^{-1}.h^{-1}$) and dilution rate (0.2 h^{-1}), respectively, should be lowered to determine their influence on biomass and β-galactosidase productivity.

(iii) Ethanol levels should be quantified to determine the possible occurrence of anaerobic respiration.

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