A Metabolic Model of Exopolysaccharide Production in *Lactobacillus delbrueckii* subsp. *bulgaricus*

A thesis presented in partial fulfilment of the requirements
for the degree of

Doctor of Philosophy
In
Biotechnology

at Massey University, Palmerston North,
New Zealand

Alan David Welman
2002

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ABSTRACT

Exopolysaccharides (EPS) from lactic acid bacteria (LAB) play an important role in enhancing the rheology and texture of fermented dairy foods. Whilst LAB are attractive vehicles for the production of probiotics due to their GRAS (Generally Recognized as Safe) status, the economic production of EPS by LAB remains constrained by their metabolism. Rational metabolic engineering studies aimed at altering the production of EPS and lactic acid require an understanding of the contributions of whole biosynthetic networks, or segments thereof, to the end–products. *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 was confirmed to follow a homofermentative pattern of catabolism, and to export exopolysaccharide (EPS), lactate, and galactose as principal metabolites concurrently with cell growth. The EPS formed was found to diminish after a period of time, probably due to degradation of the polymer. Intracellular glucose resulting from the splitting of lactose taken up into the cell was the principal source of carbon for EPS, lactate and biomass. Kinetic models which were applied to describe the production of EPS, lactate and galactose in batch fermentation suggested, however, that a small percentage of carbon from galactose could have been diverted towards the formation of EPS, lactate, and biomass. Emphasis was placed upon developing a rational screening programme in order to generate a mutant of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 with a higher specific yield of EPS production than the parent strain, for the purposes of understanding the flux to sugar-nucleotides associated with raised levels of EPS production. This process resulted in the isolation of a chemically induced mutant of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 with a higher specific EPS yield than the parent strain. In addition, it was demonstrated that by changing the environmental conditions viz. by reducing the water activity of the growth medium, carbon flux to the EPS-synthesizing pathways could be raised in batch culture. Detailed information on the enzymatic activities and metabolite levels associated with EPS formation at steady state metabolic conditions was derived from continuous culture studies. The formation of EPS, lactate, and galactose, as well as the distribution of carbon fluxes through the glucose 6-phosphate and glucose-1-phosphate branch points in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 was measured at different growth rates in continuous culture. This investigation revealed an enhanced carbon flux to the EPS-synthesizing pathway being associated with higher growth rates, despite a limitation imposed by a "bottleneck" at the glucose-6-phosphate branch-point. Results of similar metabolic flux studies in continuous culture with *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483, grown under conditions of osmotic stress supported this deduction. Comparative metabolic flux investigations between the chemically induced mutant of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483, previously isolated, and the parent strain, provided further evidence of this constriction of carbon flow.

Collectively, these investigations suggested that any improvement in the flux of carbon towards EPS formation in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 could be effected by the enhancement of the activities of phosphoglucomutase, UDP-glucose pyrophosphorylase, and UDP-galactose 4-epimerase. In all of the metabolic studies undertaken in continuous culture, raised levels of carbon flux toward EPS sugar-nucleotides occurred in conjunction with raised levels of specific lactate production rates. In these instances, raised levels of ATP which were measured could be associated with raised levels of glycolysis and the biosynthesis of certain sugar-nucleotides. These findings implied that the cell dissipated excess cellular energy by the formation of sugar-nucleotides, however it may be that the raised flux toward the sugar nucleotides was a direct response to excess energy available in the cell. Any strategy aimed at enhancing carbon flow to EPS by the diversion of carbon away from glycolysis would need to be counterbalanced by the cell's requirement to generate ATP via this pathway, including its need to maintain its redox balance.

FOREWORD

Taking this dissertation to press represents a satisfying stage of what has been my goal for a number of years.

I would like to acknowledge the excellent contribution of Assoc. Prof. Ian Maddox, who has served as an excellent supervisor and remained a steadfast navigator throughout this period of research. In addition, Dr Vaughan Crow of the New Zealand Dairy Research Institute (Fonterra Research Centre) who, with his impressive wealth of knowledge, has served as a superb co–supervisor and consultant. I refer as well to Dr Richard Archer of Fonterra Cooperative Group Limited, whose co–supervisorship sponsored challenging intellectual excursions, which are so important for this process, and whose capital investment in an HPLC allowed for so much of the progress made in this study. I wish to thank my co–supervisor Dr Derek Haisman of the Institute of Food, Nutrition and Human Health, whose intellect and humour remained a constant beacon. Last, but by no means least, I wish to acknowledge Prof. Harjinder Singh of the Institute of Food, Nutrition, and Human Health who has co-supervised as an interested and knowledgeable party.

I wish to thank Massey University, which has funded the entire study through the "Technology for Business Growth" programme. The New Zealand Dairy Research Institute (Fonterra Research Centre) deserves special mention for allowing me access to use equipment and consumables. In particular, they have provided useful advice on analytical matters.

Throughout the tenure of this doctoral study, I have been assisted by a diverse cross–section of University staff members within the Institutes of Technology and Engineering, Food Nutrition and Human Health, and Molecular Biology. I would like to thank them as a team, whose combined efforts provided the services that I required for the execution of the research. The important contributions of individuals I have acknowledged separately. I have enjoyed, in particular, the interesting discussions with co–students and the many post-graduate students from around the world working in the same laboratories.

I am particularly grateful for being afforded the opportunity of travelling to Belgium in May of 2001, to present a paper at the First International Symposium of Exopolysaccharides from Lactic Acid Bacteria. This was a superb opportunity to share research findings with a variety of eminent researchers. This trip represented a highlight of this study, both academically and

culturally, and I am particularly pleased to have had the opportunity to visit the works of the Flemish grandmasters, such as the Breughels at the National Art Museum, admire the inner city architecture preserved from the 15th century, and take in the ambience of Brussels.

I wish to thank my sponsors for enabling my visit to Napier in New Zealand, in order to attend the "Molecules for Life" conference in December of 2001. It was opportune that I could render a paper to coincide with the completion of the experimental phase of the studies.

This thesis would not be complete without acknowledging the most important sponsors of my doctoral study, those being my wife Lorraine, and my family at home, whom are owed the greatest gratitude for allowing me to pursue this dream.

Palmerston North,
New Zealand
August, 2002

CONTENTS

CHAPTER 1

EXOPOLYSACCHARIDE PRODUCTION BY LACTIC ACID BACTERIA

Some lactic acid bacteria (LAB) have long been known to produce a polysaccharide polymer, which is secreted from the cell. This extracellular polysaccharide or "exopolysaccharide" (EPS) has an economic value in that it has the ability to impart functional effects on foods, in particular on fermented dairy products, as well as confer beneficial health effects. Bacteria such as *Xanthomonas campestris* and *Sphingomonas paucimobilis* have been used to produce reliable quantities of xanthan and gellan respectively, however their application does not suit all foodstuffs. A demand, therefore, has emerged for the production of functional polysaccharides which can impart improved rheological properties in foods. LAB occupy a unique position in that they have a GRAS (Generally Recognized As Safe) classification and serve as likely candidates as "cell factories" for the production of functional exopolysaccharides. Improvement of the productivity of EPS from LAB, as well as the formation of EPS with a structure and size which imparts a desired functionality has become a key goal for research work in this area. Enhancements of this nature will require a deep understanding of the production kinetics and biosynthetic metabolism of EPS in LAB; this review sets out to present a background to this knowledge.

1.1 Exopolysaccharides produced by lactic acid bacteria

Exopolysaccharides (EPS) from LAB have found their most valuable application in the improvement of the rheology, texture, and mouthfeel of fermented milk products such as yoghurt. EPS from LAB, whilst having no taste of their own, will increase the residence time of the milk product in the mouth, hence imparting an enhanced perception of taste (Duboc and Mollet, 2001). An additional physiological benefit that has been hypothesized is that the EPS will remain for longer periods in the gastrointestinal tract, thus enhancing the opportunity for probiotic bacteria to colonize (German *et al.*, 1999). LAB EPS has been claimed to have antitumoral effects (Kitazawa *et al.*,1991), immunostimulatory activity (Hosono *et al.*, 1997; Chabot *et al.*, 2001), and the ability to lower blood cholesterol (Nakajima *et al.*,1992).

Exopolysaccharides are long-chain polysaccharides consisting of branched repeating units of sugars or sugar derivatives. These sugar units are mainly glucose, galactose, and rhamnose, existing in different ratios (Cerning, 1990, 1995; de Vuyst and Degeest, 1999). They are generally secreted into their surroundings during growth, and are not permanently attached to

the surface of the microbial cell (Laws *et al.*, 2001). This feature distinguishes them from the capsular polysaccharides which are structurally similar, but remain permanently attached to the surface of the cell.

EPS from microbial sources can be classified into two groups viz. homopolysaccharides (e.g. cellulose, dextran, mutan, alternan, pullulan, levan, and curdlan) and heteropolysaccharides (e.g. gellan and xanthan) (Laws *et al.*, 2001). Homopolysaccharides consist of repeating units of only one type of monosaccharide (viz. D-glucose or D-fructose) and hence can be divided into two major groups viz. glucans and fructans. The fructans and glucans share a common feature in that they are synthesized by extracellular transglycosylases using sucrose as the glycosyl donor (Monsan *et al.*, 2001). Amongst the LAB, for example, the fructan, levan, is produced by the action of a levansucrase (E.C. 2.4.1.10) in *Leuconostoc mesenteroides* subsp. *mesenteroides* (NRRL B-512F) (Robyt and Walseth, 1979), and *Lactobacillus reuterii* (Van Geel-Schutten *et al.*, 1999; van Geel-Schutten, 2000), amongst others. The glucan, dextran, is produced by a dextransucrase found in a variety of LAB, for example the strains *Leuc. mesenteroides* subsp. *mesenteroides* NRRL B512-F (Monsan *et al.*, 2001) and *Leuc. mesenteroides* B-1355 (Cote and Robyt, 1982).

In contrast, heteropolysaccharides have repeating units which demonstrate very little structural similarity to one another (de Vuyst *et al.*, 2001). The molecular mass of these polymers ranges between 1.0×10^4 and 6.0×10^6 (Cerning, 1995). The heteropolysaccharides are constructed from multiple copies of oligosaccharides (Laws *et al.*, 2001) which can contain between three and eight residues. Two or more different monosaccharides are normally present in each repeating unit, and they exhibit a variety of different linkage patterns. These features have served as a means to classify heteropolysaccharides produced by LAB on the basis of the structure of the repeating unit and the monosaccharide composition thereof.

The heterogeneity of structure and size in heteropolysaccharides, and the differing effects on rheology of the polymer, has raised questions as to whether a structure-function relationship can be established for EPS. As the polysaccharides derived from different LAB show large variation in composition, charge, spatial arrangement, rigidity, and ability to interact with proteins, it is understandable that no defining correlation between EPS concentrations and viscosities has been established (Duboc and Mollet, 2001). However, Laws and Marshall (2001) cite, as a prerequisite for polymer solutions to have high viscosity, long chains of subunits and/or stiff chains are required. Even though a relationship between chain stiffness and EPS composition has not been established, it is claimed that evidence exists for this. Backbone linkages of the $\beta(1\rightarrow4)$ type, as found in *Lactococcus lactis* subsp. *cremoris* B40, for example,

(Tuinier *et al.*, 1999), result in stiffer chains than do β(1→2) or β(1→3) linkages, and α–linkages result in more flexible chains than β-linkages (Laws and Marshall, 2001). Looijesteijn *et al.* (2000) have also demonstrated an increase in viscosity being correlated with increasing molecular mass.

1.2 Metabolism of Lactic Acid Bacteria

As a background to the discussion on EPS–synthesizing pathways, the pathways related to lactose utilization in LAB are discussed below.

1.2.1 Categorization of Pathways Related to Lactose Utilization

Vedamuthu (1978) described four categories of enzymatic pathways which are generally recognized for use of lactose by microorganisms, three of which are applicable to dairy organisms *viz.*

1. A mechanism by which lactose is hydrolysed to glucose and galactose by β-galactosidase (β-gal). This system is coupled to an inducible galactoside permease, or alternatively an energy–requiring active transport system. β-Galactosidase is reported in species of *Lactobacillus* (Premi *et al.*, 1972) and *Streptococcus thermophilus* (McKay *et al.*, 1970).

2. A coupled phosphoenolpyruvate–phosphotransferase-β-D–phosphogalactoside galactohydrolase system, in which lactose is translocated simultaneously with phosphorylation to yield lactose–6-phosphate; lactose–6–phosphate is subsequently cleaved by β-D–phosphogalactoside galactohydrolase (P-β-gal) to yield galactose-6–phosphate and glucose (Thompson, 1987). This mechanism has been found to be present in lactic streptococci (McKay *et al.*, 1969; McKay *et al.*, 1970), and lactobacilli (Premi *et al.*, 1972).

3. A hypothetical mechanism by which lactose is phosphorylated directly at the first carbon position (Moustafa and Collins, 1968).

1.2.2 Central Metabolic Pathways of Lactic Acid Bacteria

The lactic acid bacteria depend mainly upon sugar fermentation for the generation of energy. Two main groups are recognized on the basis of their differential use of glucose. These groups are classified according to the end products derived from catabolism of glucose (Thompson, 1987).

In the homofermentative species, glucose is converted virtually quantitatively (in excess of 90 %) to lactic acid via the EMP pathway (Figure 1.1). Fructose-1, 6-bisphosphate aldolase (E.C. 4.1.2.13) is regarded as a key enzyme in the EMP pathway and is common to all homofermentative organisms (Thompson, 1987).

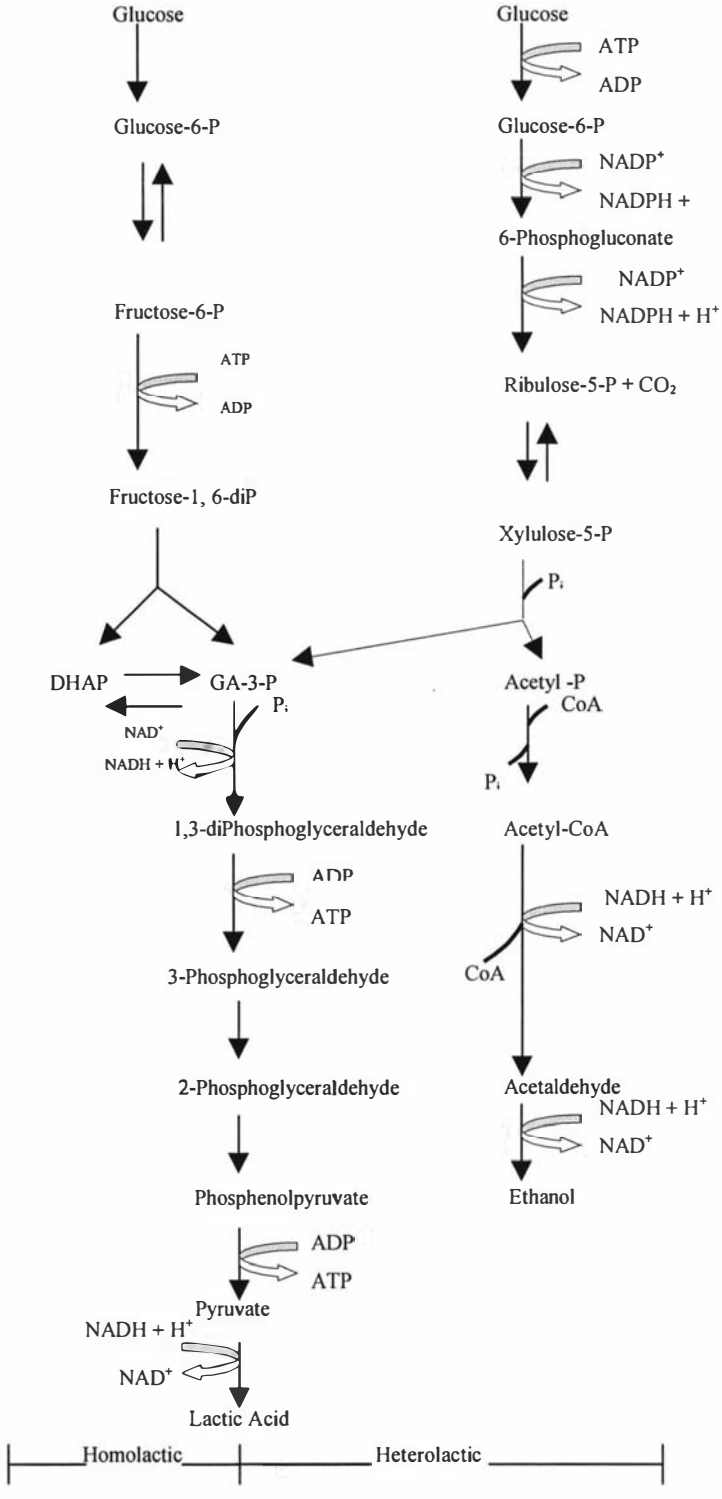

Fig. 1.1. Central metabolic pathways for the utilization of glucose by lactic acid bacteria (modified from Thompson, 1987)

The products of glucose metabolism in heterofermentative organisms are lactic acid, ethanol, and CO_2 (Figure 1.1). Glucose-6-phosphate is not converted to fructose-6-bisphosphate (FDP) by fructose-1,6-bisphosphate aldolase as in the homofermentative species, but to 6-phosphogluconate, utilizing the Pentose–Phosphoketolase Pathway (PPK) (Hexose Monophosphate Shunt / Pentose Phosphate Pathway / 6–Phosphogluconate Pathway) for the generation of acetyl–phosphate and glyceraldehyde-3-phosphate (Thompson, 1987).

The homolactic route will generate two moles of lactate from one mole of glucose utilized, and a net ATP gain of two. (In reality, some ATP will be diverted for the formation of biomass). The heterofermentative route will generate one mole of lactate, one mole of ethanol, and one mole of CO_2 for every mole of glucose consumed, with a concomitant gain of one mole of ATP. It is important to note that not all LAB are exclusively "homofermentative". Under certain conditions, glycolytic flux may divert to a heterolactic path, and some LAB utilize the PPK pathway depending upon the substrate they are metabolizing (Axelsson, 1993).

Dependent upon the isomeric nature of the LDH present, the isomer of lactic acid produced will vary being either exclusively L (+), D (-), or a racemic mixture, or with one form predominating (Garvie, 1980). Table 1.1 summarizes LAB on the basis of genus, according to major pathway, fermentation pattern, and the isomeric configuration of lactic acid produced.

Table 1.1 Principal Genera of lactic acid bacteria showing main fermentation pathways operative, and isomeric configuration of lactic acid produced (modified from Thompson, 1987)

Genus	Representative Species (re-classification by genus and / or species name in parentheses)	Major pathway used	Fermentation pattern	Lactic Acid Configuration
Streptococcus				
Group N (lactic)	*lactis* (*Lactococcus lactis* subsp. *lactis*)	EMP	Homo	L (+)
	cremoris (*Lactococcus lactis* subsp. *cremoris*)	EMP	Homo	L (+)
	diacetylactis (*Lactococcus lactis* subsp. *lactis*)	EMP	Homo	L (+)
Thermophilic	*thermophilus*	EMP	Homo	L (+)
Lactobacillus	*helveticus*	EMP	Homo	DL
	jugurti	EMP	Homo	DL
	acidophilus	EMP	Homo	DL
	bulgaricus	EMP	Homo	D (-)
	lactis	EMP	Homo	D (-)
	casei	EMP	Homo	L (+)
	plantarum	EMP	Homo	DL
	buchneri	PPK	Hetero	DL
	brevis	PPK	Hetero	DL
Pediococcus	*cerevisiae*	EMP	Homo	DL
Leuconostoc	*lactis*	PPK	Hetero	D (-)
	cremoris (*mesenteroides* subsp. *cremoris*)	PPK	Hetero	D (-)
	mesenteroides	PPK	Hetero	D (-)

Two additional key metabolic pathways involved in sugar metabolism, and which are found in LAB are the Tagatose-6-phosphate pathway (TPP) and the Leloir pathway. Both pathways form intermediates from galactose which are common to the EMP pathway (Figures 1.2 (a) and (b)).

In the case of the TPP (Bissett and Anderson, 1974), galactose-6-phosphate formed by the galactose phosphotransferase system (PTS $^{(gal)}$) is converted to dihydroxyacetone phosphate (DHAP), and glyceraldehyde-3-phosphate (G-3-P), which coincides with the EMP pathway (Figure1.2 (a)). The Leloir pathway is usually linked to a galactose permease transport system (Fig. 1.2 (b)) and generates glucose-6-phosphate, which is catabolized via the EMP pathway (Thomas *et al*, 1980).

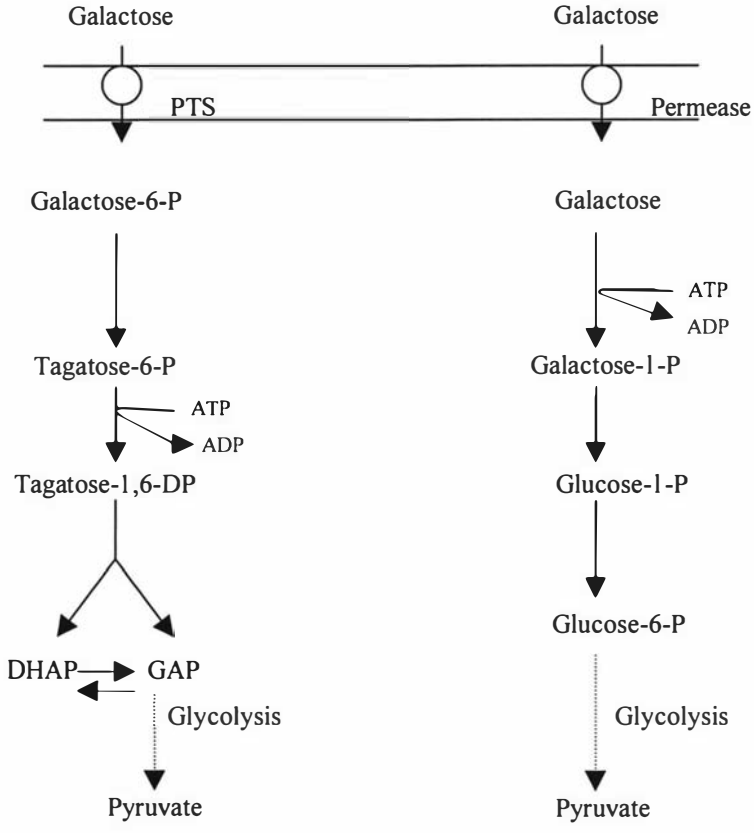

Fig. 1.2. (a) Tagatose-6-phosphate pathway, and (b) Leloir pathway, for the metabolism of galactose in lactic acid bacteria (Axelsson, 1993)

1.2.3 Lactose Transport and Metabolism in Lactic Acid Bacteria

Many LAB translocate lactose simultaneously with the phosphorylation of the sugar by the PEP–dependent lactose phosphotransferase system (PTS $^{(lac)}$), generating lactose-6-phosphate

(Thompson, 1979). Lactose-6-phosphate is split by β-D–phosphogalactoside galactohydrolase (P-β-gal) to form galactose-6-phosphate and glucose. These products subsequently enter the D-Tagatose-6-phosphate pathway and EMP pathway respectively (Bissett and Anderson, 1974), forming pyruvate and lactic acid. This transport system is prevalent amongst the commercial "starter" strains in the dairy industry viz. *Streptococcus lactis*, *Streptococcus cremoris*, and *Streptococcus. lactis* subsp. *diacetylactis* (Vedamuthu, 1978), which convert greater than 90 % of the lactose fermented to L-lactic acid (Thomas, 1976). These species are characterized by negligible or no β-gal activity (Farrow, 1980), and high $PTS^{(lac)}$ and P-β-gal activities (Thompson, 1979).

The genes encoding for the PTS systems are plasmid–located and are essential for rapid transport and homolactic fermentation of lactose (Lawrence *et al.*, 1976). These starter species are different from their wild–type counterparts, which convert lactose to lactate at a much slower rate, as well as producing fermentation products other than lactic acid (Farrow, 1980). High levels of β-gal are prevalent in these strains, as well as low levels of P-β-gal relative to the starter strains (Thompson, 1979). Table 1.2 summarizes the mode by which lactose is utilized amongst dairy starter bacteria.

Table 1.2 Mode of lactose utilization in dairy starter bacteria (modified from Vedamuthu, 1978)

Bacterial Species (re-classification by genus and species name in parentheses)	**Lactose Utilization Mechanism**
S. lactis (*Lactococcus lactis* subsp. *lactis*)	P-β-gal
S. cremoris (Lactococcus lactis subsp. *cremoris*)	P-β-gal
S. lactis subsp. *diacetylactis* (*Lactococcus lactis* subsp. *lactis*)	P-β-gal
S. thermophilus	β-gal and P-β-gal
Lactobacillus spp.	β-gal and / or P-β-gal
Propionibacterium shermanii - adjunct	β-gal and P-β-gal

A number of thermophilic LAB metabolize only the glucose after transport of lactose into the cell, and splitting of the lactose molecule by β-gal, with galactose being transported out of the cell into the medium (Hutkins and Morris, 1987). This essentially amounts to a net loss of carbon from the network. Premi *et al.* (1972) suggest that many LAB contain a dual uptake system in that they have both a lactose PTS and a lactose permease system which is operational. Fox *et al.* (1990), however, argue that low P-β-gal activity exhibited in conjunction with high β-gal activity may be questionable in that the artificial substrate used for P-β-gal (orthonitrophenylgalactose phosphate) may be hydrolysed by β-gal or by a phosphatase.

Smart *et al.* (1993) analysed β-gal and P-β-gal activities in 58 representative strains of 6 genera of LAB *viz. Lactococcus, Streptococcus, Lactobacillus, Leuconostoc, Pediococcus, and Bifidobacterium.* β-gal activity was found in all 6 genera represented and P-β-gal in the lactococci, *Lactobacillus casei*, *Lactobacillus brevis* and two strains of *Leuconostoc.* Some strains of *Lactococcus lactis*, *Lb. casei* and *Leuc.* spp. were demonstrated to have both enzymes.

1.3 Biosynthesis of Exopolysaccharides in Non–Dairy bacteria

Extensive investigations have been undertaken on the production of EPS by non-dairy bacteria. A preview of aspects of this work is useful as a background to the discussion on EPS production by LAB.

1.3.1 Intracellular Synthesis

Historically, the biosynthetic pathways of a number of glycoconjugates located within the cell envelope of bacterial cells have undergone in–depth study. The synthesis mechanisms of lipopolysaccharides have been studied, for example, in *Salmonella typhimurium* (Osborn *et al.*, 1972) and cell wall teichoic acid synthesis of *Bacillus coagulans* (Shimada *et al.*, 1989). These mechanisms involve sugar-nucleotides as sugar donors, with C55–lipid isoprenoids acting as the molecular support upon which the polysaccharide repeating units are assembled.

The biosynthesis of bacterial EPS bears close similarity to the process by which the bacterial wall components, peptidoglycan and lipopolysaccharide are produced (Sutherland, 1990). Most of the EPS biosynthetic precursors are manufactured intracellularly within the cytoplasm. This is regarded as a logical arrangement as they would be freely soluble and can be easily channelled to the biosynthetic process where they are needed at the cell membrane. The resulting polymer is exported to sites outside of the cytoplasmic membrane (Sutherland, 1990).

The subject as related to non–dairy organisms has been extensively reviewed by Sutherland (1972, 1990):

Exopolysaccharides that are synthesized at cell membrane sites need "activated" or energy - rich forms of the monosaccharides. These molecules are generally nucleoside diphosphate sugars, however a limited number of nucleoside monophosphate sugars also function in this way. The sugar nucleotides provide a variety of functions. Firstly, these activated sugar nucleotides are able to release 31.8 kJ / mole on hydrolysis, compared with the 20 kJ/mole from

a sugar phosphate. This is sufficient energy for the assembly of oligosaccharide sequences on the appropriate carrier molecules, as well as converting monosaccharides from one type to another. These assembly and interconversion reactions occur via different mechanisms viz. epimerisation, oxidation, decarboxylation, reduction, and rearrangement. Figure 1.3 shows the interconversion of the galactose, glucose, and mannose nucleotides.

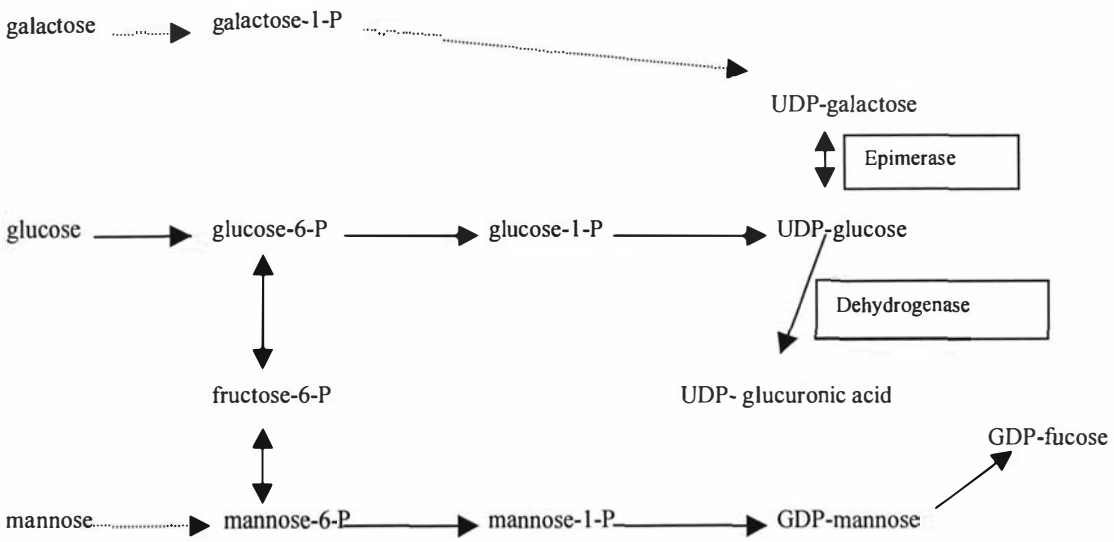

Fig. 1.3. Catabolic pathways for sugar-nucleotide synthesis and interconversions. Broken lines indicate substrate–specific catabolic systems (modified from Sutherland, 1990).

Similarities exist between the synthesis of EPS and other glycoconjugates, such as LPS (lipopolysaccharide) and CPS (capsular polysaccharide). A unique membrane isoprenoid–associated lipid was demonstrated by Osborn and Weiner (1968) and Wright *et al.* (1967) to be involved as an intermediate carrier of glycosyl residues in the biosynthesis of the O–antigen repeating sequence of *Salmonella*. Wright *et al.* identified this carrier as a C55 polyisoprenyl monophosphate (also referred to as "bactoprenyl phosphate" or "undecaprenyl phosphate") to which the O –antigen repeating unit oligosaccharides were bound.

Troy *et al.* (1971) were first to report the involvement of a lipid–linked oligosaccharide in CPS biosynthesis in *Aerobacter aerogenes*. Interestingly, in *A. aerogenes* type 8, out of a probable total of ten enzymes involved in EPS production, six are specific to capsule formation. Figure 1.4 illustrates the pathway for synthesis of polysaccharides in *A. aerogenes* A4.

Smith *et al.*(1959) studied the enzymes involved in capsule formation in *Diplococcus pneumoniae*, and determined that even in non–capsulate strains, high levels of sugar nucleotides were detected, suggesting that control was exerted at the initial stages of precursor formation, and not

at the sugar transferase level. Defects in, or the lack of enzymes responsible for sugar nucleotide formation led to the failure to produce polymer. If an alternative monosaccharide was incorporated into the culture medium, the synthesis of EPS can sometimes be restored. Thus, if galactose is a component of the slime or capsule, lack of the enzyme UDP-galactose-4-epimerase will result in no polymer formation. Growth in a galactose–containing medium can, in *Escherichia coli* and *K. aerogenes*, restore the slime production or capsulation (Sutherland, 1990).

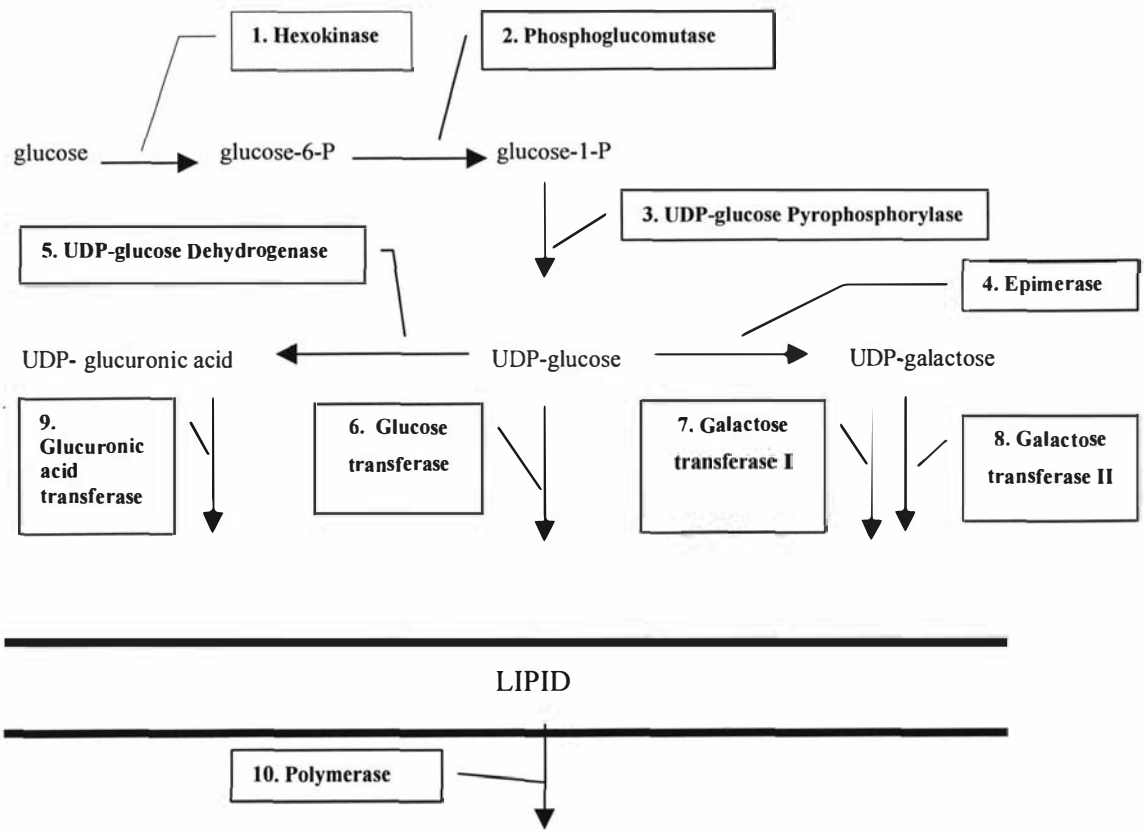

Fig. 1.4. Biosynthetic pathway for capsular polysaccharide of *Klebsiella aerogenes*. The enzymes numbered 1 – 4 are involved in precursor synthesis of several different polymers, and the remainder are involved in exopolysaccharide synthesis (modified from Sutherland, 1972).

It appears that the requirement for a lipid acceptor, on which the repeating units of the polysaccharides are assembled, is a common feature of all such polymers occurring external to the microbial cell membrane. The total isoprenoid lipid available in bacterial cells has been estimated to be 5.5 x 10^4 molecules, which is 0.02 % of the cell's dry mass. The availability of the lipid carrier may be controlled by phosphorylation and de-phoshorylation (Sutherland, 1990). Figure 1.5 illustrates the activation and deactivation of the isoprenoid lipid.

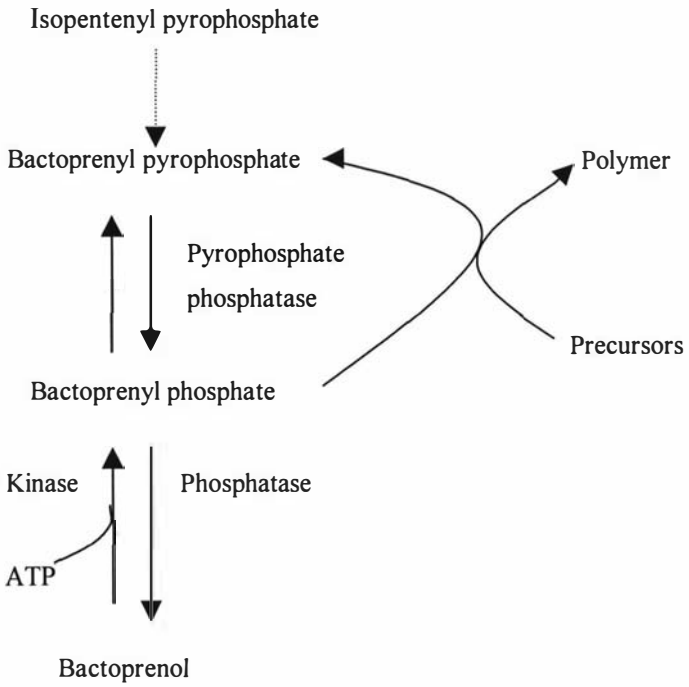

Fig. 1.5. The activation and deactivation of isoprenoid lipid (modified from Sutherland, 1990).

The structure of the metabolically active form of C55–isoprenyl phosphate is illustrated in Figure 1.6.

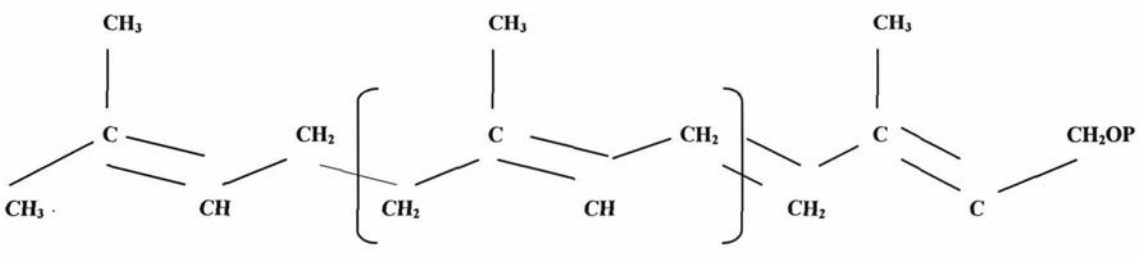

Fig. 1.6. Structure of the metabolically active form of C55–isoprenyl phosphate (from Sutherland, 1990)

Studies on the synthesis of *Xanthomonas campestris* (Ielpi *et al.* , 1981, 1983) revealed that a similar pathway was found in *Klebsiella* and other species (Sutherland, 1990) (see Figure 1.7). In this instance, experiments using isotopically-labelled precursors revealed that acetate and pyruvate were added sequentially to the lipid precursor, together with the monosaccharides.

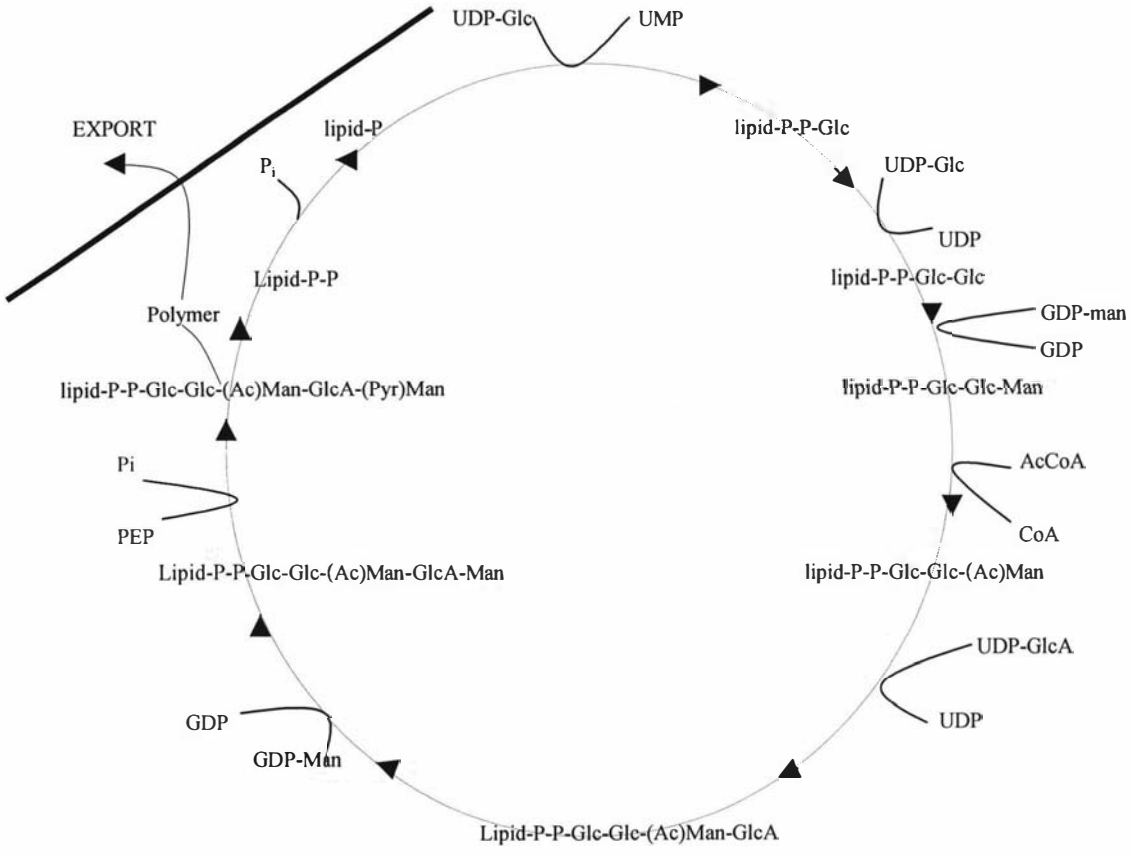

Fig. 1.7. Pathway assembly of Xanthan (modified from Sutherland, 1990 and Ielpi *et al.*, 1981; 1983)

The lipid precursor acts as a "scaffolding" upon which the polysaccharide is assembled by sequential addition of monosaccharides. In the case of xanthan synthesis, following assembly of the "repeat unit" on the isoprenoid lipid, polymerization occurs to generate acetylated and pyruvylated xanthan. The initial pentasaccharide has a linear conformation, and branching will only occur as the oligosaccharide repeat units are polymerized (Ielpi *et al.*, 1981). It is probable that polymerization occurs by addition to the non–reducing terminus, while attached to the isoprenoid lipid pyrophosphate molecule. The larger fragment is transferred to lipid carrying a single repeat unit; this process of attachment continues until the macromolecule is formed. In studies by Ielpi *et al.* (1993) on *X. campestris* and Semino and Dankert (1993) on *Acetobacter xylinum,* it was determined that the function of the sugar nucleotides and polyisoprenoids are exactly the same as those in lipopolysaccharide, peptidoglycan, and teichoic acid synthesis.

Knowledge of the final stages of EPS synthesis is incomplete. The site of assembly and polymerization of the repeat units is regarded as occurring at the cytoplasmic membrane (Osborn *et al.*, 1972). A postulated pathway for the terminal stages of EPS synthesis is depicted in Figure 1.8.

It has been speculated that the lipid pyrophosphate–repeating unit chain becomes increasingly hydrophilic with increasing chain length. The EPS must be released from the lipid pyrophosphate and passed into the extracellular environment. If capsular material is formed, it must be attached to a specific element on the cell surface (Sutherland, 1990).

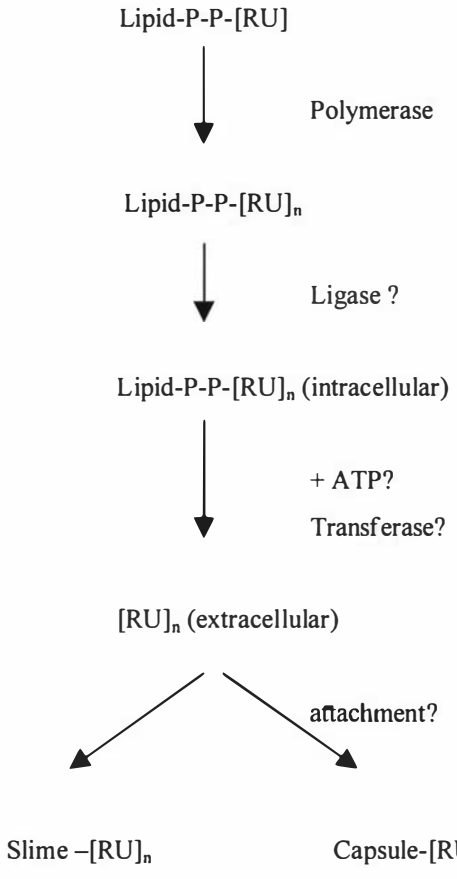

Fig. 1.8 The hypothesised final stages of exopolysaccharide synthesis and excretion. (RU = a repeat unit saccharide) (from Sutherland, 1990).

1.3.2 Extracellular Synthesis

According to Sutherland (1990), only one polymer of industrial importance is synthesized extracellularly viz. dextran, produced on a commercial scale by selected strains of *Leuc. mesenteroides*. The production of dextrans requires sucrose as a carbon substrate. Sucrose induces the enzyme dextransucrase (E.C.2.4.1.5) (1,6-α-glucan : D-glucose 2-glucosyltransferase). Sucrose is cleaved releasing free fructose. The glucosyl residue is transferred to the reducing end of a nascent dextran polymer. The final dextran product may be made up of 95 % 1,6-α-linked glucosyl residues, with 1,2- , 1,3- , and 1,4-α- linkages which may be present. A number of postulated mechanisms explaining the branching of the dextran molecule have been put forward. It is hypothesized that a C(3)-OH on the acceptor molecule

acts as a nucleophile on C(1) of the reducing terminal of a dextran–dextransucrase molecular complex. The polysaccharide becomes displaced from the enzyme, and a 1,3-α-glucosyl branch is formed (Sutherland, 1990).

1.4 Exopolysaccharide Biosynthesis in Lactic Acid Bacteria

Studies into EPS biosynthesis by LAB have concentrated on ropy strains of *L. lactis*, *Lb. delbrueckii subsp. bulgaricus*, and *S. thermophilus*. These studies have for the most part taken place within the past decade.

1.4.1 Genetics of EPS synthesis in lactic acid bacteria

The genes encoding EPS synthesis in *Lc. lactis* and *Lb. casei* are plasmid-located (Van Kranenburg *et al.*, 1997), and are regarded as the reason for EPS-producing instability in mesophilic LAB (Vedamuthu and Neville, 1986; Vescovo *et al.*, 1989). All the thermophilic LAB studied so far appear to have the *eps* genes located on the chromosome (de Vuyst *et al.*, 2001). The first description of genes controlling EPS biosynthesis was for *S. thermophilus* Sfi6 (Stingele *et al.*, 1996, 1999). Stingele *et al.* (1996) identified the eps genetic locus of *S. thermophilus* Sfi6, revealing a 15.25 kb region encoding 16 ORF's, within which a 14.52 kb region encodes 13 genes (*epsA* to *epsM*) capable of directing EPS synthesis. Homology searches of the predicted proteins showed a high level of homology (40 % - 68 % identity) for *eps A, B, C, D*, and *E* with the genes encoding CPS in *Streptococcus pneumoniae* and *Streptococcus agalactiae*. As with *Lb. delbrueckii* subsp. *bulgaricus*, the genes required for EPS biosynthesis in *S. thermophilus* appear to be located on the chromosome, and not on a plasmid (Cerning, 1990; Vescovo *et al.*, 1989). The EPS-synthesizing genes are however characterized by instability, possibly due to mobile genetic elements or to a generalized instability of the genome. These genes appeared to show homology with genes from other EPS-producing bacteria in respect of regulation, determination of chain-length, biosynthesis of the repeating unit, and polymerization and export of EPS (Stingele *et al.*, 1996). An 11.2 kb gene cluster (*cps A* to *cps L*) was identified in *S. thermophilus* NCFB 2393 (Griffin *et al.*, 1996; Almirón-Roig *et al.*, 2000). Homology was found between this cluster and that of *S. pneumoniae* (Griffin *et al.*, 1996). Van Kranenburg *et al.* (1997) determined that all the essential information needed for the biosynthesis of EPS by *L. lactis* NIZO B40 was encoded in a single 12 kb gene cluster located on a single 40 kb plasmid (*eps RXABCDEFGHIJKL*), driven by a promoter upstream of *eps R*. The predicted gene products of 11 of the 14 genes were shown to be homologous in sequence to gene products involved in EPS, CPS, LPS, or teichoic acid biosynthesis of other bacteria. The EPS gene cluster from *L. lactis* strain NIZO B40 was also found to be similar in organization to those encoding EPS biosynthesis in *Streptococcus*

thermophilus , *S. pneumoniae*, and *S. agalactiae* (Van Kranenburg *et al.*, 1997). Expression of the *eps D* gene in *E. coli* demonstrated that its product is a glucosyltransferase, linking the first sugar of the repeating unit to the lipid carrier (Van Kranenburg *et al.*, 1997).

This investigation by Van Kranenburg *et al.* (1997), is far reaching in that this is the first time that a comprehensive biosynthetic pathway has been proposed for *L. lactis*. It was proposed that the *L. lactis* proteins of *eps* E and *eps* F may act as one glycosyltransferase, performing the same reaction, in a similar fashion to the capsular polysaccharide gene products of *Cps14 F* and *Cps14 G* of *S. pneumoniae* which transfer the second sugar of the repeating unit to the first lipid–linked sugar in CPS biosynthesis (Kolkman *et al.*, 1997).

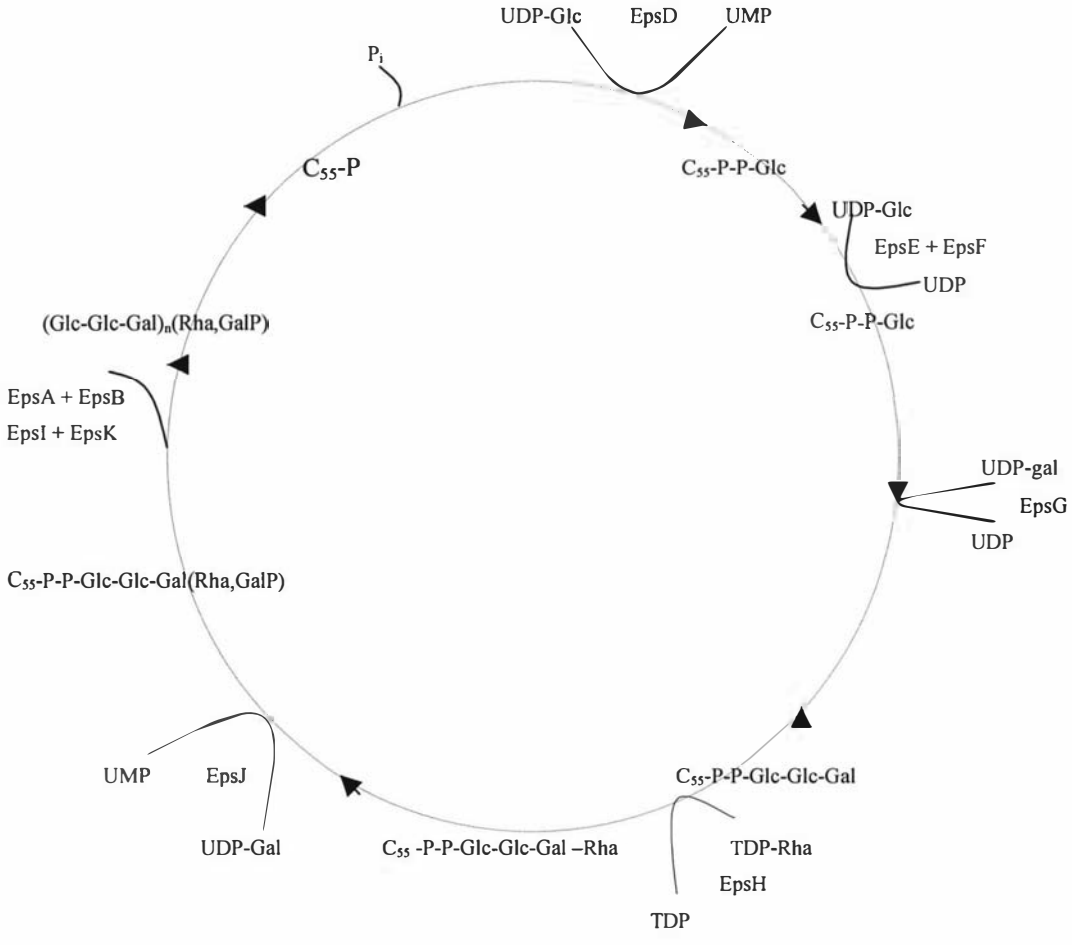

Fig. 1.9. Model for EPS biosynthesis in *Lactococcus lactis* NIZO B40 (modified from de Vos *et al.*, 1998 – ref. Van Kranenburg *et al.*, 1997).

Van Kranenburg *et al.* (1997) postulated further that the two glycosyltransferases involved in subsequent steps of biosynthesis of the repeating unit are expected to be encoded by *eps G* and

eps H, on the basis of their homology with other putative glycosyltransferases. A model for EPS biosynthesis as described by van Kranenburg *et al.* (1997), is illustrated in Figure 1.9.

Eps I is thought to encode for the polysaccharide polymerase, and *eps K* involved in polysaccharide export from the cell, due to its moderate homology with *RfbX*, involved in export of the O–antigens in *Shigella flexneri* (Macpherson *et al.*, 1995), and the "flippase" of *Salmonella enterica* (Liu *et al.*, 1996).

A high level of similarity between the gene clusters of all the LAB studied appears evident. The genes in the clusters appear to be oriented in one direction, and are transcribed as a single mRNA (Stingele *et al.*, 1996; Van Kranenburg *et al.*, 1997; Jolly and Stingele, 2001). The functional sequences of these clusters in Gram-positive bacteria which synthesize polysaccharide at the cell surface all appear to follow a similar trend viz. regulation, chain-length determination, biosynthesis of the repeating unit, polymerization, and export (Jolly and Stingele, 2001).

1.4.2 Biosynthetic pathways leading to heteropolysaccharides

The biosynthesis of EPS in LAB can be divided into four groups of reaction sequences viz. reactions involved with sugar transport into the cell, biosynthesis of sugar-1-phosphates, activation and coupling of sugars, and mechanisms associated with the export of the EPS (Laws *et al.*, 2001).

A key intermediate linking the anabolic pathways of EPS production and the catabolic pathways of sugar degradation appears to be glucose-6-phosphate, where the flux of carbon bifurcates between the formation of fructose-6-phosphate toward the products of glycolysis, biomass, and ATP formation, and towards the biosynthesis of sugar-nucleotide precursors of EPS (Figure 1.10). The enzyme involved in the conversion of glucose-6-phosphate to glucose-1-phosphate is phosphoglucomutase (PGM), and has been cited as potentially playing an important role in the divergence of flux between these catabolic and anabolic pathways (Sjoberg and Hahn-Hagerdal, 1989, Hugenholtz and Kleerebezem, 1999, Degeest and de Vuyst, 2000).

The branching between EPS formation and glycolysis from glucose-6-phosphate is shown in Fig. 1.10. Glucose-1-phosphate serves as a branchpoint for the formation of the sugar-nucleotide precursors UDP-glucose and dTDP-glucose via the action of UDP-glucose pyrophosphorylase and dTDP-glucose pyrophosphorylase, respectively. UDP-galactose is formed from UDP-glucose by UDP-galactose 4-epimerase, and dTDP-rhamnose from dTDP-glucose by the rhamnose synthetic enzyme system. It is important to note that these sugar-

nucleotides are used to form a variety of polysaccharides in the cell, and hence the enzymes associated with their formation are shared, and often termed "housekeeping enzymes". Some involvement of the Leloir pathway is possible, if the system is present in the cell. Escalante *et al.* (1998) also commented that, on the basis of their results, it was unlikely that all the enzymes of the Leloir pathway are involved in energy metabolism, and could be involved in the formation of EPS precursors.

The subsequent stage of EPS synthesis, viz. assembly of the monosaccharide repeating unit is achieved by a number of EPS-specific enzymes, as identified by Stingele *et al.* (1996) in *S. thermophilus* Sfi6 and Van Kranenburg *et al.* (1997) in *L. lactis* NIZO B40. This repeat unit is assembled on a lipid carrier molecule which is attached to the cytoplasmic membrane of the cell (Van Kranenburg *et al.*, 1999).

The sugar nucleotides are linked to form the repeating unit by the action of a host of gene products on the EPS gene cluster. These gene products act as glycosyltransferases (Boels *et al.*, 2001), which were demonstrated in *L.lactis* NIZO B40 (Van Kranenburg *et al.*, 1997, 1999) (Figure 1.9). Assembly of the polysaccharide repeating unit commences with the linkage of UDP-glucose to a lipid carrier by a priming glycosyltransferase (*eps D*). *Eps E* and *eps F* work in tandem, joining a second glucose to the lipid-glucose structure. *Eps G* is responsible for the addition of galactose to the lipid-linked assembly, followed by the sequential addition of rhamnose (*eps H*) and galactose-phosphate (*eps J*). Oba *et al.* (1999) studied the biosynthetic intermediates of the EPS from *L. lactis* subsp. *cremoris* strain SBT0495 (a viilian polysaccharide), and determined the structure of the repeating unit to be β-D-glucosyl-(1→4)-(α-L-rhamnosyl-(1→2)-(α-D-galactose-1-phosphoryl-(→3)-β-galactosyl-(1→4)-β-D-glucose. This repeating unit of glucose, galactose, rhamnose and phosphate occurs in a molar ratio of 2:2:1:1. The structure corresponds to that determined by Nakajima *et al.* (1992) in the same strain. These saccharides are regarded as representing the first three steps of the sequential assembly of EPS on the C55-polyisoprenoid located in the cytoplasmic membrane.

The mechanism of polymerization in LAB of the repeat unit and its subsequent export from the cell is unclear. Due to the high level of homology between gram-positive and gram-negative organisms in respect of the repeat-unit synthesis, it is likely that a similar mechanism will occur at the level of EPS polymerization and export. Laws *et al.* (2001) have speculated that a simple model would involve firstly the action of a "flippase" to move the lipid-bound repeating units from the cytoplasmic face of the membrane to the periplasmic face, similar to that of O-antigen synthesis (Whitfield and Valvano, 1993). Based upon the same analogy, a polymerase would

catalyse the linking of the repeat units, followed by the action of an enzyme to uncouple the lipid-bound polymer and control chain length.

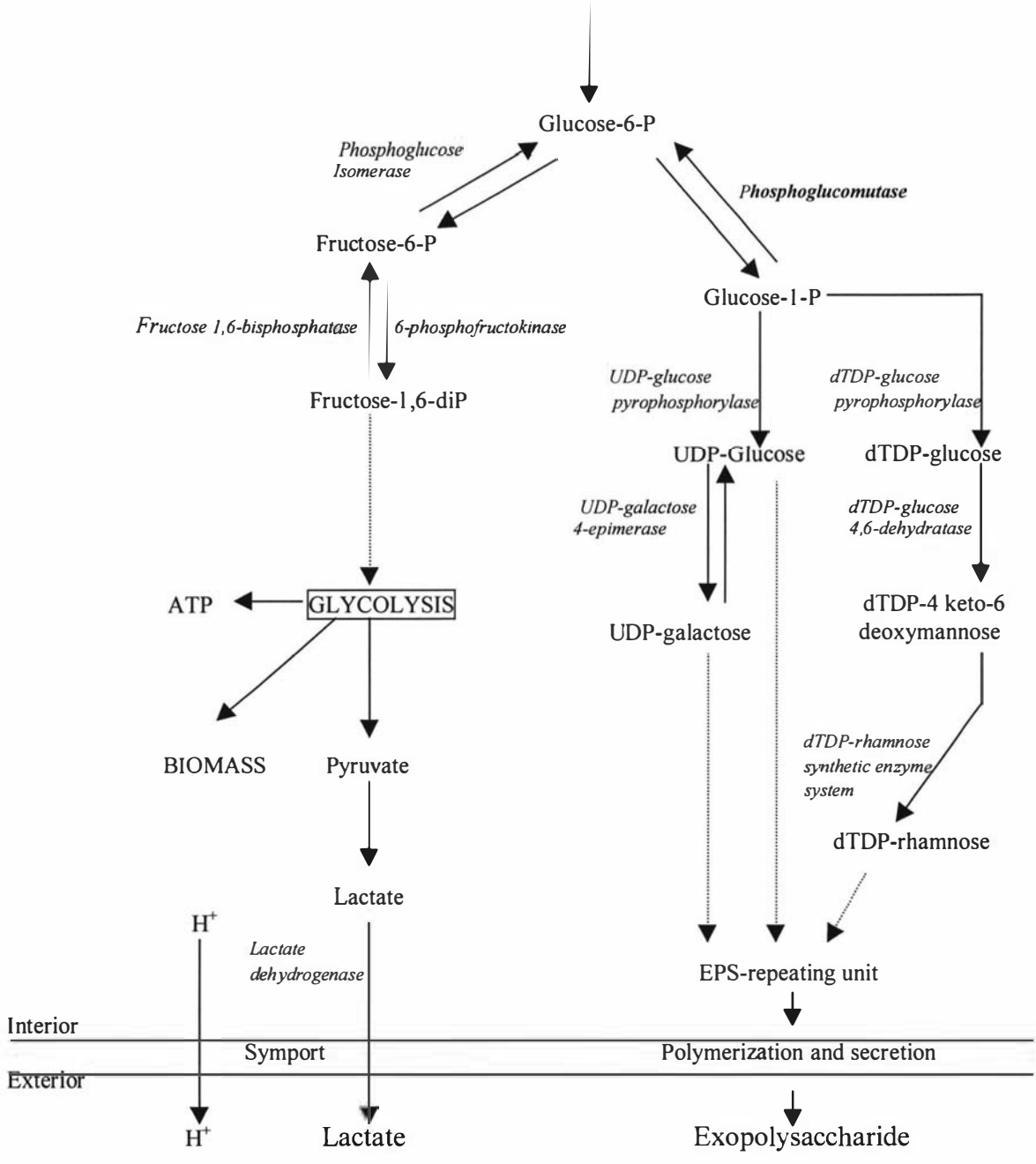

Fig. 1.10. Simplified diagram of the split of carbon to the EPS-synthesizing pathways, and to glycolysis.

1.4.3 Structural and compositional studies on EPS in LAB

Structural analyses on the EPS produced have been undertaken on ropy strains of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 (Grobben *et al.*, 1996) grown in continuous culture. The EPS was found to consist of repeating units of glucose and galactose (in the ratio 1 : 2.4) when grown on fructose, and glucose, galactose and rhamnose in a ratio of 1 : 7.0 : 0.8 when grown on a mixture of fructose and glucose. Compositional analysis of the EPS produced by *Lb. bulgaricus* strain CNRZ 416 (Cerning *et al.*, 1986), and strain rr (Gruter *et al.*, 1993), demonstrated the presence of glucose, galactose, and rhamnose in a ratio of 1 : 5 : 1. Grobben *et al.* (1995) found that the sugar composition of the EPS produced was unaffected by residual galactose in the medium. The EPS secreted by *S. thermophilus* Sfi6 was shown to consist of a tetrasaccharide repeating unit →3)-β-DGalp-(1→3)-[α- DGalp-(1→6)]- β - DGlcp-(1→3)- α-D-Galp-Nac-(1→ (Stingele *et al.*, 1996), corresponding to the structure reported for three other strains of *S. thermophilus* (Doco *et al.*, 1990). The monosaccharide ratio was reported to occur in a ratio of 1 : 2 : 1.

Lemoine *et al.* (1997) characterized the structures of the EPS produced by *S. thermophilus* Sfi39 and Sfi12. The EPS were found to have molecular masses of greater than 2 x 10^6 Da. Sfi39 EPS consisted of D-glucose and D-galactose in a molar ratio of 1 : 1, and Sfi12 EPS comprised D-galactose, L-rhamnose, and D-glucose in a molar ratio of 3 : 2 : 1. It was concluded in this study that the texturizing properties of "ropy" strains of *S. thermophilus* are based on the production of EPS showing chemical compositonal similarities, but structural differences. Escalente *et al.* (1998) investigated the role of the enzymes UDP-glucose pyrophosphorylase and UDP-galactose 4-epimerase in exopolysaccharide production of Gal^- ropy and non–ropy strains of *S. thermophilus.* The EPS polymer produced from both strains contained glucose, galactose, and rhamnose when grown on lactose, indicating that glucose was the sole precursor of EPS (as well as for bacterial growth). Structural studies have been undertaken on a variety of lactobacillus species, with structural and compositional differences being determined between the different species. In *Lactobacillus paracasei*, the EPS was determined to be a heteropolymer with repeating units composed of D-galactose, 2-acetamide-2-D-deoxy-D-galactose, and glycerol-3-phosphate in a molar ratio of 3 : 1 : 1 (Robijn *et al.*, 1996[a]). The exopolysaccharide produced by *Lactobacillus acidophilus* LMG 9433 was determined to be a charged heteropolymer consisting of repeating units with a composition of D-glucose, D-galactose, D-glucuronic acid and 2-acetamido-2-deoxy-D-glucose in molar ratios of 2 : 1 : 1 : 1 (Robijn *et al.*, 1996[b]). The viscous exopolysaccharide produced by *Lactobacillus sake* strain 0-1 was found to be a polymer of molecular weight 6 x 10^6 Da comprising polysaccharide repeating units in molar ratios of 3 : 2 : 1, with one of the repeating units being partially 2-O-

acetylated (Robijn *et al.*, 1996[c]). *Lactobacillus helveticus* strain 766 EPS was determined to consist of hexasaccharide repeating units composed of D-glucose and D-galactose in a molar ratio of 2 : 1 (Robijn *et al.*, 1995). EPS isolated from *Lb. helveticus* strain Lh59 was determined to have a molecular weight greater than or equal to 2 x 10^6 Da (Stingele *et al.*, 1997) and a hexasaccharide repeating unit arrangement, corroborating the findings of Robijn *et al.* (1995). The structure was found to be identical to that of *Lb. helveticus strain TN-4.* The structural composition of *Lactobacillus rhamnosus* (strain C83) EPS was determined to be a neutral heteropolysaccharide consisting mainly of glucose and galactose, with trace levels of another sugar, probably fructose (Gamar-Nourani *et al.*, 1998). The same authors determined that the composition of the EPS is independent of the nature of carbon source, medium used, pO_2, pH, temperature, and kinetic parameters. Grobben *et al.* (1995) demonstrated that the sugar composition of EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* was not influenced by carbon source, temperature, or pH. Van den Berg *et al.* (1995) demonstrated that the EPS composition of *Lactobacillus sake* strain 0-1 was independent of the type of carbon source used. A similar finding was reported by Manca de Nadra *et al.* (1985) relating to the composition of *Lb. bulgaricus* strain CRL 420. Contrary to these findings, Cerning *et al.* (1994) reported variances in the EPS sugar composition depending on the carbon source and type of medium used in *Lb. casei* strain CG11.

1.5 Heteropolysaccharide production in *Lactobacillus delbrueckii* subsp. *bulgaricus*

1.5.1 The Genus *Lactobacillus*

The genus *lactobacillus* consists of 65 recognized species that are gram–positive, non–spore–forming, catalase negative, non–motile and facultatively anaerobic, and are compiled in the list of Skerman *et al.* (1980), or validly published (Weiss *et al.*, 1983; Hammes and Vogel, 1995). Nine of these species have been subsequently re-allocated to alternative genera or species, retaining 56 species organized into three groups based upon evolutionary relationship viz. the *Lactobacillus delbrueckii* Group, the *Lactobacillus casei Pediococcus* Group, and the *Leuconostoc* Group (Hammes and Vogel, 1995) (Groups A, B, and C, respectively). This organization incorporates previous categorizations on the basis of phylogenetic relationships by Kandler and Weiss (1986), and correspond to the Groups I, II, and III defined by these authors. The criteria for allocation to Groups A, B, and C are defined as follows (Hammes and Vogel, 1995):

Group A lactobacilli: - obligately homofermentative. In greater than 85% of all cases, hexoses are fermented to lactic acid via the EMP. This group possesses fructose-1, 6-bisphosphate aldolase (FDP), but lacks phosphoketolase. The Group does hence not ferment gluconate or

pentose sugars.

Group B lactobacilli: - facultatively heterofermentative. Hexoses are converted to lactic acid via the EMP in virtually all cases. This group of organisms possesses both an aldolase and phosphoketolase, and is hence able to ferment both hexose and pentose. The enzymes of the Pentose Phosphate Pathway (PPP) are repressed in the presence of glucose.

Group C lactobacilli: - obligately heterofermentative. Hexoses are converted to lactic acid, ethanol, acetic acid, and CO_2 in equimolar quantities via the PPP. Pentose may also be fermented via this pathway.

The three groups are subdivided on the basis of their phylogenetic relationship. The letter "a" indicates a relationship to the *Lb. delbrueckii* Group, "b" to the *Lb. casei – Pediococcus* group, and "c" to the *Leuconostoc* Group (Hammes and Vogel, 1995). For example, *Lb. delbrueckii subsp. bulgaricus* would under this classification be designated as "Aa", defining the species as belonging to the obligately homofermentative lactobacilli within the *Lb. delbrueckii* group. *Lb. helveticus* would also be classified as "Aa" according to this system of classification.

1.5.2 Metabolism and EPS formation in *Lactobacillus delbrueckii* subsp. *bulgaricus*

Lb. delbrueckii subsp. *bulgaricus* can take up lactose via a permease system, and possesses intracellular β-galactosidase activity resulting in the formation of glucose and galactose (Hickey *et al.*, 1986, Leong-Morgenthaler *et al.*, 1991). Galactose is generally exported from the cell via a lactose-galactose antiport system (Hutkins and Ponne, 1991), however some yoghurt starter culture strains have been reported to utilize galactose (O'Leary and Woychik, 1976). The glucose is subsequently catabolized via the EMP to pyruvic acid, which is in turn, converted to D(-)-lactic acid by lactate dehydrogenase (Tamime and Deeth, 1980). The formation of D-lactate in *Lb. delbrueckii* subsp. *bulgaricus* is thought to be controlled in part by a "loop" mechanism through the activation of pyruvate kinase by fructose- 6-phosphate, and the inhibition of phosphofructokinase by phosphoenolpyruvate (Lebras *et al.*, 1998). Glucose is taken up into the cell via a phosphoenolpyruvate : glucose phosphotransferase system (Hickey *et al.*, 1986). A β–D-phosphogalactosidase which cleaves lactose-phosphate to yield glucose and galactose-6-phosphate has also been reported in *Lb. bulgaricus* (Premi *et al.*, 1972). *Lb. delbrueckii* subsp. *bulgaricus* is limited in its ability to ferment sugars; other sugars that can be fermented are fructose, and in some instances, mannose (Tamime and Deeth, 1980). *Lb. delbrueckii* subsp. *bulgaricus* has been shown to have a homofermentative metabolism of

glucose, fructose, and lactose (Kandler, 1983). It has not been clarified as to whether the Leloir enzymes are functional in *Lb. delbrueckii* subsp. *bulgaricus*.

A metabolic pathway for the synthesis of sugar nucleotides for EPS production has been proposed by Grobben *et al.* (1996). In this model, phosphoglucomutase converts glucose-6-phosphate to glucose-1-phosphate, which is the substrate for the formation of dTDP-glucose by the action of dTDP-D-glucose pyrophosphorylase, and UDP-glucose, catalysed by UDP-glucose pyrophosphorylase. The sugar-nucleotide UDP-galactose is formed from UDP-glucose by UDP-galactose 4-epimerase. The dTDP-glucose is subsequently converted to dTDP-rhamnose by the sequential action of dTDP-glucose 4,6-dehydratase and the dTDP-rhamnose synthetic enzyme system. UDP-glucose, UDP-galactose, and UDP-rhamnose serve as the principal sugar nucleotides for the assembly of the EPS repeating-units.

The most detailed work undertaken to date on the physiology of EPS production in *Lb. delbrueckii* subsp. *bulgaricus* has been on the strain NCFB 2772. This strain produced significantly more EPS when grown on glucose as opposed to fructose, with no rhamnose having been detected when grown on fructose (Grobben *et al.*, 1996). Indeed, no activities of the enzymes leading to the formation of rhamnose were detected when the organism was grown on fructose, however, activities of all the enzymes leading to UDP-glucose and UDP-galactose were found. In contrast, the enzymes associated with all three sugar-nucleotides showed activity in extracts of glucose-grown cells (Grobben *et al.*, 1996). These relationships between EPS production and sugar-nucleotide formation, when the cells were grown on glucose or fructose, were further investigated in relation to the molecular weight and structure of the polymers. *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772, when grown on glucose, formed two types of EPS with molecular weights of 1.4×10^6 Da and 4×10^4 Da, at the same time, and in virtually equivalent amounts. When grown on fructose as the source of carbohydrate, the NCFB 2772 strain produced mainly a polymer of molecular weight 4×10^4. The lower molecular weight fraction was deduced to be produced independently of carbohydrate source; both fractions contained differing compositional ratios of glucose, galactose, and rhamnose, with the low molecular weight component consisting of very low levels of rhamnose (Grobben *et al.*, 1997). The low levels of rhamnose in the low-molecular weight fraction did not correlate with the absence of the enzymes associated with the formation of this sugar (Grobben *et al.*, 1996) when *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 was grown on fructose; this was ascribed to the levels of these enzymes existing below the threshold limit of detection, when grown under these conditions (Grobben *et al.*, 1997).

1.5.3 Structure and composition of exopolysaccharide polymers produced by *Lactobacillus delbrueckii* subsp. *bulgaricus*.

Heteropolysaccharides produced by *Lb. delbrueckii* subsp. *bulgaricus* can be described as branched oligosaccharides of between 4 and 7 units, consisting of glucose, galactose, rhamnose, and N-acetylgalactosamine in the subunit (Marshall *et al.*, 2001). One exception has been noted in strain CRL 420, which was reported to have an EPS structure which was substantially different from all the rest, consisting of glucose and fructose in a ratio 1:2. (Manca de Nadra *et al.*, 1985). Gruter *et al.* (1993) characterized the structure of the EPS from *Lb. delbrueckii* subsp. *bulgaricus* rr grown in skimmed milk. The EPS was found to be a heteropolymer consisting of galactose, glucose, and rhamnose residues occurring in the molar ratio 5 : 1 : 1. (Figure 1.11).

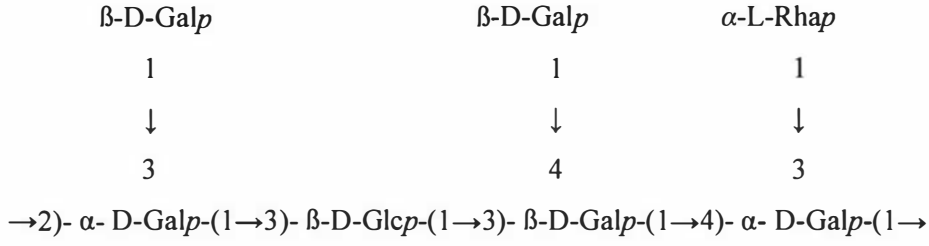

Fig. 1.11. Structure of the exopolysaccharide repeating unit of *Lb. delbrueckii* subsp. *bulgaricus* rr (Gruter *et al.*, 1993).

Marshall *et al.* (2001) reported identical EPS structures of *Lb. delbrueckii* subsp. *bulgaricus* LYO3, "24", and "25" to that of strain rr. The polysaccharide produced by *Lb. delbrueckii* subsp. *bulgaricus* 291 was determined by Faber *et al.* (2001) to consist of a branched pentasccharide (Figure 1.12).

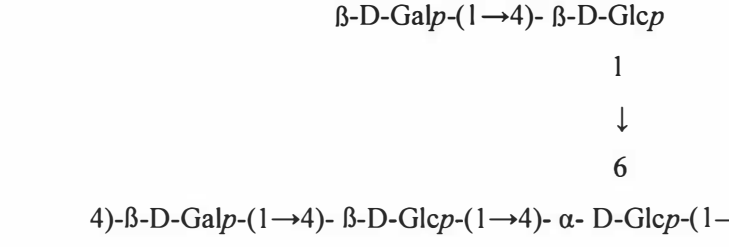

Fig. 1.12. Structure of the exopolysaccharide repeating unit of *Lactobacillus delbrueckii* subsp. *bulgaricus* 291 (Faber *et al.*, 2001).

The EPS of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 was determined to be a neutral polymer (Kitazawa *et al.*,1998), however a loose association of EPS from *Lb. delbrueckii*

subsp. *bulgaricus* with protein, has been suggested to occur (Garcia-Garibay and Marshall, 1991).

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CHAPTER 2

STRATEGIES FOR THE ENHANCEMENT OF EXOPOLYSACCHARIDE PRODUCTION IN LACTIC ACID BACTERIA

Part of this chapter was submitted for oral presentation at the Eighth International Pacific Rim Biotechnology Conference (November 17 – 20, 2002), Auckland, New Zealand. Abstract submitted on 31 July, 2002.

INTRODUCTION

No clearcut fundamental principles exist for the metabolic engineering of lactic acid bacteria (LAB) to overproduce exopolysaccharides (EPS). Much of the activity to date has been focused upon elucidating the genetic organization of EPS-encoding genes, as well identifying the enzymatic mechanisms associated with EPS formation. Attempts at improving production of EPS have involved, for example, the overexpression of genes associated with the production of the sugar nucleotide precursors of EPS (Kleerebezem *et al.*, 1999), and cloning the EPS gene cluster from *Streptococcus thermophilus* into a heterologous host, a strain of *Lactococcus lactis* (Stingele *et al.*, 1996). Thus far, no economically significant improvements in EPS production by LAB have been reported. Much more information is needed about the regulation of the EPS-synthesizing pathways even before a significant impact will be seen to be made upon the yield and specific yield of microbial EPS production. A variety of limiting steps in the lactose-utilizing pathways, the EPS–synthesis pathway, as well as the EPS export mechanisms, are likely to be operative in LAB, and will need to be taken into account in developing strategies for the enhancement of EPS production, or even the alteration of the EPS structure. These factors could include regulatory controls, energy availability, and competition for precursors and substrates (e.g. the C55-isoprenoid lipid carrier).

STRATEGIES FOR THE ENHANCEMENT OF EPS PRODUCTION

Metabolic engineering of catabolic pathways and lactate production

The genetic and metabolic capacity of LAB can be exploited to generate a variety of different products from lactose. Essentially, this involves diverting metabolic flux away from lactate to produce known or novel metabolites (de Vos *et al.*, 1998).

One of the advantages of using LAB for metabolic engineering arises from the virtually complete uncoupling of their basic catabolic and cellular biosynthetic pathways (Hols *et al.*, 1999). This means that these catabolic pathways are not used for synthesis reactions, leaving growth unaffected. In addition, the relative simplicity of their metabolism creates an advantage. Strategies that have been applied in order to achieve the rerouting of carbon flux to overproduce specific products include single, multiple and whole pathway engineering, the engineering of redox reactions, and engineering global control systems (Hols *et al.*, 1999).

A simplified representation of essential metabolic conversions is illustrated in Figure 2.1, and is used to illustrate the potential of single gene engineering.

Hols *et al.* (1999) categorized lactose catabolism as being split into three major "blocks" of reactions *viz.* (i) lactose transport and hydrolysis of the imported sugar into the constituent glucose and galactose moieties; (ii) a second reaction network comprising all the reactions that generate energy, NADH, and pyruvate; (iii) a third "block" being the reduction of pyruvate to lactate by LDH and utilization of NADH (to form NAD^+).

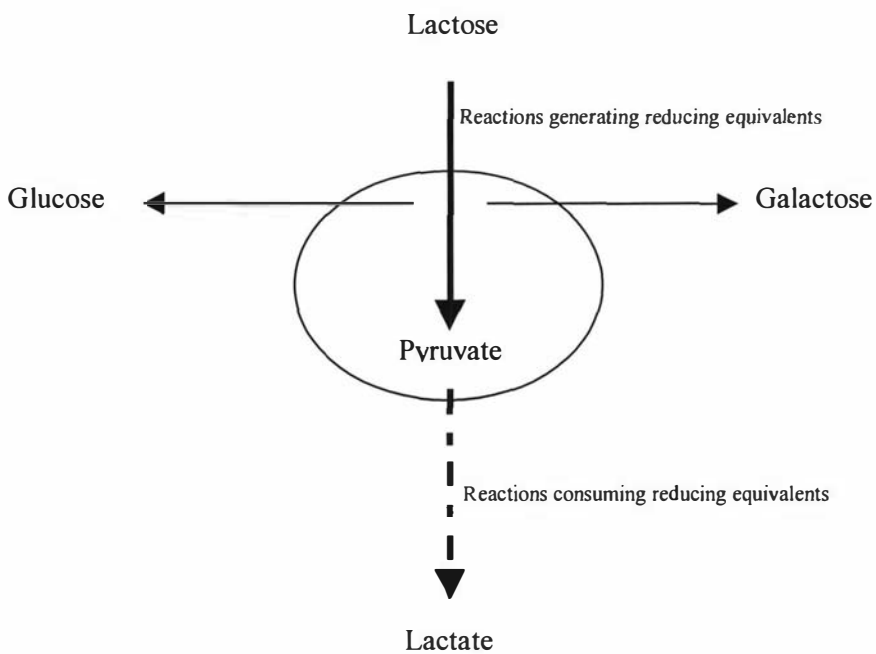

Fig. 2.1. Simplified pathway of essential metabolic conversions in lactic acid bacteria (modified from Hols *et al.*, 1999).

An economically viable titre of EPS produced in fermentation for use as a food additive would be in the range of 10-15 g/l (van den Berg *et al.*, 1995). If EPS production were coupled to growth of the cell, it has been proposed that a reduction in the formation of lactate, which is

known to inhibit growth, should result in the elevation of EPS formation (van den Berg *et al.*, 1995). Conceivably this reduction in lactate production could allow for more carbon to be diverted away from glycolysis towards EPS formation.

The first referenced LAB partially deficient in LDH activity was a *L. lactis* strain, characterized by McKay and Baldwin (1974). Subsequently, mutant LDH–negative strains of *L. lactis* which had a completely inactivated LDH gene were derived (Platteeuw *et al.*, 1995). The metabolic consequence of this is that high intracellular concentrations of pyruvate accumulate with the concomitant production of acetate and ethanol (Platteeuw *et al.*, 1995). This is a strategy that has been used in order to obtain overproduction of these metabolic end–products (e.g. ethanol), and is likely to be the consequence if LDH is reduced in activity, or abolished in LAB strains. In order to achieve higher EPS yields, additional regulatory alterations would be necessary such that excessive carbon is not diverted to unwanted metabolites via the pyruvate node.

For the effective abolition or reduction in LDH activity it may be necessary to remove or reduce the expression of two LDH isoenzymes, specific for each of the two stereoisomers of lactic acid, as has been necessary in other instances (de Vos *et al.*, 1998).

A number of products have been produced from pyruvate in LDH–deficient strains viz. ethanol, alanine, and diacetyl. Ethanol production was achieved by overexpression of the *Zymomonas genes* encoding for pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) (de Vos *et al*, 1998). Similarly, carbon flux has been diverted towards alanine by over–expression of the *Bacillus sphaericus alaD* gene for an alanine dehydrogenase in an LDH–deficient strain of *L. lactis* (Hols *et al.*, 1999).

Ferain *et al.* (1996) produced a "double knockout" strain of *Lactobacillus plantarum* (NCIMB 8826), in which the expression of L-LDH and D-LDH was muted. The mutated organism produced a variety of compounds not generated by the wild type, including ethanol, acetoin, mannitol, 2,3-butanediol, succinate, and pyruvate. ^{13}C–flux studies revealed that the larger proportion of carbon flux was diverted away from lactate to acetoin production. It has been suggested that in mutated LDH^- strains, high concentrations of intracellular pyruvate occur, as in the case of wild–type strains of *L. lactis* and *Leuconostoc spp.* which excrete pyruvate under conditions in which C4–compounds are formed (Hugenholtz and Starrenburg, 1992).

The flavour compound diacetyl is formed from the intermediate α-acetolactate by an oxidative decarboxylation step. The formation of this intermediate, which is formed from a condensation reaction of two pyruvate molecules, was enhanced by over-expression of the *L. lactis als* gene

for acetolactate synthase. These plasmids, when cloned into an LDH–deficient strain (NZ2700), generated a substantially enhanced diversion of the carbon flux to the α-acetolactate intermediate (Plateeuw *et al.*, 1995).

An important milestone in the development of whole-pathway engineering in LAB was the elucidation of the 12 kb gene–cluster of *L. lactis* NIZO B40 encoding for a phosphorylated EPS and the introduction of plasmids carrying this EPS operon into other *L.lactis* strains (Van Kranenburg *et al.*, 1997). This achievement demonstrated that the plasmid–encoded trait can be successfully transferred to another host. Wells *et al.* (1998) reported the expression of *Streptococcus pneumoniae* genes for capsular polysaccharide in *L. lactis.* This particular ability may be important in a future strategy, as an alternative organism may be needed in order to successfully generate the levels of flux to EPS production as a consequence of higher endogenous metabolic flux e.g. uptake systems. In this instance, the mucoidness trait located on a plasmid–vector would have to be transformed into the suitable host. Stingele *et al.* (1996) succeeded in expressing the *S. thermophilus* gene cluster in *L. lactis*. In this instance, the 14.5 kb SacI-BamHI fragment (which included the putative promoter upstream of epsA and the putative terminator downstream of epsM), was cloned into the multicopy vector pJIM 2279. The resultant recombinant plasmid pFS101 was retransformed with PJIM 2279 into a non–ropy *L. lactis* strain. EPS–secreting colonies were identified by rhuthenium–red screening.

A key consideration in generating LDH–deficient or LDH^- mutants is the impact that this would have on the redox balance in the cell, as LAB, under anaerobic conditions, have to transfer all reducing equivalents to the metabolic end–products, and for the generation of NAD^+ from NADH. In *Streptococcus mutans*, for example, abolition of LDH activity proved lethal to the organism as it cannot recycle NADH by alternative routes (Hugenholz and Kleerebezem, 1999). Engineering of a strain of *L. lactis* (LDH^- and ALR^-) to overproduce alanine (Hols *et al.*, 1999) did not result in an NAD^+ deficit, as alanine has a lower redox potential than pyruvate. An artificial reduction of NAD^+ regeneration could therefore potentially compromise normal metabolism in the cell, by alteration of the glycolytic flux. In this event, it could become necessary to retain a level of LDH activity such that a sufficient level of NAD^+ was maintained. Alternatively, restoration of this balance could theoretically be achieved by exploiting an NADH oxidase (NOX), in order to create the necessary NADH / NAD^+ ratio to support the metabolic status needed for EPS overproduction. Lopez de Felipe *et al.* (1998), for example, have succeeded in over–producing NADH oxidase by cloning the *S. mutans* NOX-2 gene on the plasmid vector pNZ 8020 into *L. lactis*, under control of the endogenous *nisA* promoter. This system facilitated a 150–fold overproduction of NADH–oxidase, and a resultant mixed–acid type of fermentation. Similarly, NOX over-expression was achieved by cloning the *nox* gene

under the control of the *nisA* promoter (NICE) in *L. lactis* (de Ruyter et al., 1996; Hugenholz and Kleerebezem, 1999).

A significant reduction in glycolytic flux, however, could exert a negative effect on EPS production, as the catabolism of glucose-6-phosphate in strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *S. thermophilus* yields two moles of ATP. As ATP is needed for the biosynthesis of sugar nucleotides and the C55-isoprenoid lipid carrier molecules, as well as being required for polymerization and export of the EPS, a reduction in available energy would be expected to compromise EPS formation. This viewpoint is supported by the results of Looijesteijn *et al.* (2000), who showed that the efficiency of EPS production in *L. lactis* was highest when ATP was in excess of the level required for cell growth.

Metabolic engineering of the EPS-producing pathway.

The relatively low levels of EPS produced by LAB could possibly be increased by increasing the metabolic flux toward the nucleotide sugar precursors (Figure 2.2) of the EPS repeating units (Kleerebezem *et al.*, 2000). Although laborious, and necessitating high-throughput screening techniques, classical mutagenesis has been used for the overproduction of metabolites in LAB, and some success could potentially be achieved in the area of EPS production. For example, ethyl methansulphonate (EMS) has been employed to obtain mutants constitutive for glucansucrase (Kim and Robyt, 1994), as well as N-Methyl-N'-nitro-N-nitrosoguanidine (NTG) (Kitaoka and Robyt, 1998), and random mutagenesis of EPS–producing *Lactobacillus sake* 0-1 (Breedveld *et al.*, 1998). UV mutagenesis has been applied, for example, on *Streptococcus diacetylactis* (Oberman *et al.*, 1982). It is more likely, however, that the most significant improvements in EPS production will be achieved using a targeted approach, and it is in this respect that an understanding of the metabolism associated with EPS production is necessary in order to devise these strategies. Glucose-1-phosphate is a common precursor of the sugar nucleotides UDP-glucose, UDP-galactose, and dTDP-rhamnose (Figure 2.2). The conversion of glucose-6–phosphate to glucose-1-phosphate by phosphoglucomutase and the subsequent formation of UDP-glucose catalysed by UDP-glucose pyrophosphorylase have been suggested as potential controlling points in EPS production (Hugenholtz and Kleerebezem, 1999). It has been demonstrated that over-expression of the *pgm* gene (for phosphoglucomutase) and the *GalU* gene (for UDP-glucose pyrophosphorylase) results in an accumulation of UDP-glucose and UDP-galactose respectively, in cells of *L. lactis* (Kleerebezem *et al.*, 1999) (See Figure 2.2). Anba *et al.* (2001) have claimed a five-fold improvement in EPS yield in a Gal^+ strain of *S. thermophilus* as a consequence of a directed mutation of the *pgm* gene.

More recently, over-expression of the *GalU* gene, in combination with the *pgm* gene into *S. thermophilus* LY03 (Gal^-) was reported to have resulted in an increase in EPS yield from 0.17 to 0.31 g/mol carbon from lactose (Levander *et al.*, 2002). Over-expression of the *GalU* gene alone did not improve EPS titre. Interestingly, a Gal^+ mutant of *S. thermophilus LY03* was reported in the same study, to generate even higher yields of EPS (0.31 g/mol carbon from lactose). This latter finding raises the possibility of utilizing the Leloir pathway enzymes for enhancing utilization of lactose for the production of EPS. De Vuyst and Degeest (1999) have speculated on the possibility of utilizing galactose exclusively for cell function in galactose-utilizing strains via the tagatose-6-phosphate pathway, while using glucose for EPS-synthesis. The Leloir enzyme, UDP-galactose 4-epimerase, could be a specific target as well for over-expression, as UDP-galactose has been demonstrated to play a critical role in EPS formation in *L. lactis* (Boels *et al.*, 2001).

Another approach to enhancing the production of EPS exists at the level of biosynthesis of the EPS polymer, and in particular, by raising the activity of glycosyltransferases associated with this process. Van Kranenburg *et al.* (1999) demonstrated a small increase in EPS production by over-expression of the priming glycosyl transferase *epsD* gene in *L. lactis*. Similar over-expressions have been claimed by Stingele *et al.* (1999) in *S. thermophilus*, *Lactobacillus helveticus*, and *Lb. delbrueckii* subsp. *bulgaricus*.

Consideration of the individual enhancements reported in the literature suggest that a potential strategy could involve cloning the entire *eps* gene cluster on a single plasmid with a high copy number. However, strain stability in a production setting would remain a key criterion in pursuing this approach.

Heterologous expression of the complete *S. thermophilus* Sfi6 EPS gene cluster into a strain of *L. lactis* (MG 1363) was achieved by Stingele *et al.* (1999). Although only a low level of EPS was produced in the host, the work demonstrated that this type of approach was achievable. The potential commercial value of employing an approach of cloning entire operons or clusters of genes associated with EPS production is reflected by recent patent publications in this field (e.g. Mollet and Stingele, 1996; Rallu *et al.*, 2001). The market application of such strains would however be subject to regulatory controls and public acceptance of these types of products.

Although not the subject of this review, apart from the importance of raising the production of EPS in LAB, it is necessary that the EPS produced imparts the desired texture. It has been

demonstrated in *L. lactis* subsp. *cremoris* B40 that the viscosity of EPS in solution depends not only upon the concentration of EPS, but the specific volume (Tuinier *et al.*, 1999); high specific volumes occur for EPS with large chain lengths and "stiff" linkages (Laws *et al.*, 2001). Research into generating "designer" polysaccharides from LAB is still in its infancy, and most of the work to date relating to the control of EPS structure in LAB has been in the area of glycosyltransferases. The potential exists for controlling the formation of EPS structure by introducing new or existing glycosyltransferases into LAB (Boels *et al.*, 2001).

In overview, the relatively inefficient conversion of energy from carbohydrates by LAB in comparison to aerobic producers of polysaccharides, presents a large technical barrier to economic production. The most valuable application from metabolic engineering of EPS production in LAB will most likely ensue from a combination of yield and structural enhancements which impart valuable functional or health-promoting characteristics in designer-type foods.

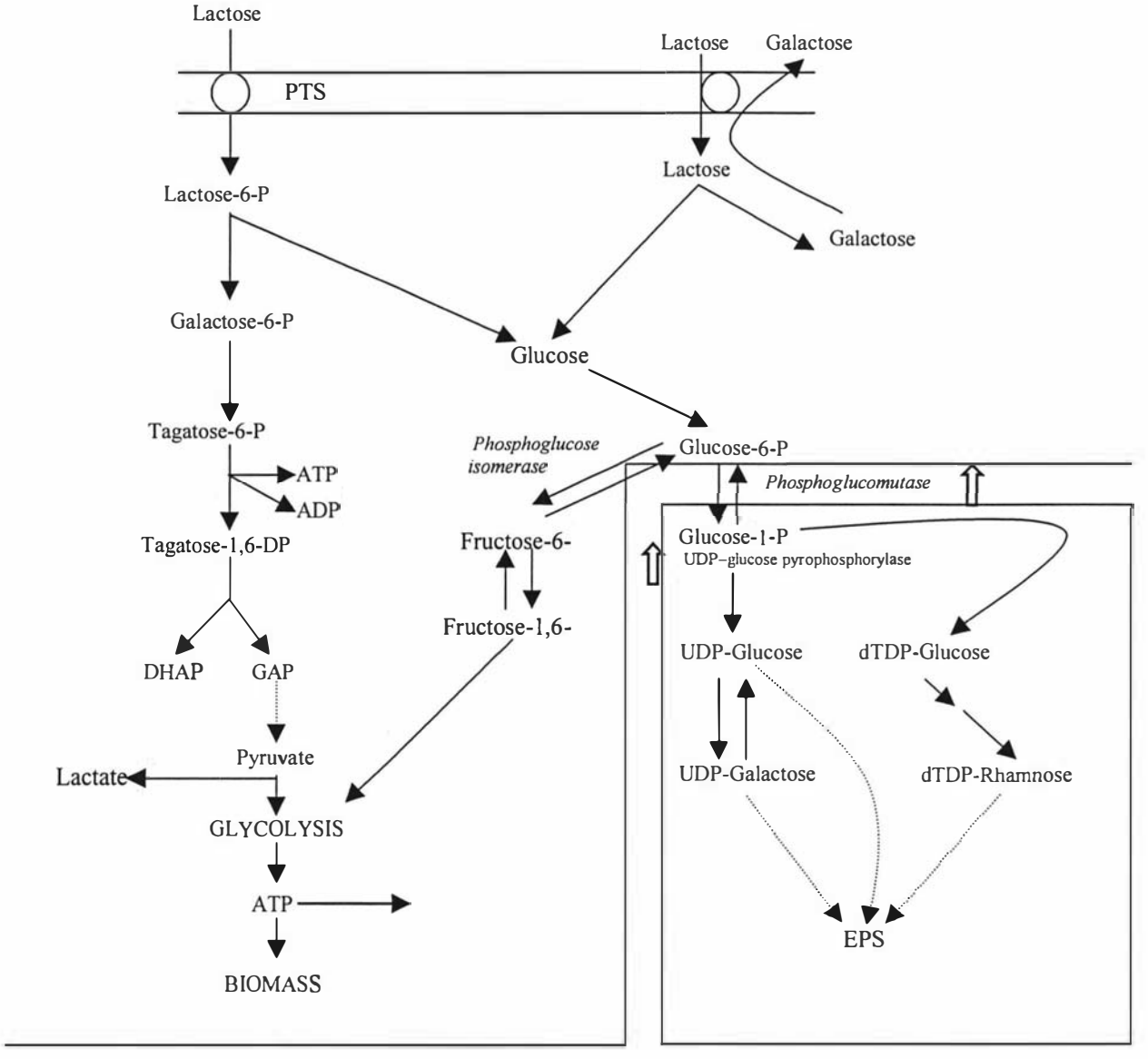

Fig. 2.2. Schematic bioreaction network for metabolically engineering the overproduction of EPS in Gal⁻ strains of *Lactococcus lactis* (PTS system), and *Lactobacillus delbrueckii subspecies bulgaricus* and *Streptococcus thermophilus*). (Modified from De Vuyst and Degeest, 1999). Metabolic enhancement: ⇧

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CHAPTER 3

SELECTION OF A GROWTH MEDIUM FOR SCREENING, FERMENTATION, AND METABOLIC STUDIES OF EXOPOLYSACCHARIDE-PRODUCING STRAINS OF *LACTOBACILLUS DELBRUECKII* SUBSP. *BULGARICUS*

ABSTRACT

Three media formulations which have been previously used for growth and studies of lactic acid bacteria (LAB) were modified, and compared for the purposes of selection of a suitable medium for the study of an exopolysaccharide-producing strain of *Lactobacillus delbrueckii* subsp. *bulgaricus*, using lactose as a carbon source. A modified version of MRS medium, a medium incorporating a casein hydrolysate as the sole amino acid source, and a modified version of a medium cited for its use in enumeration studies of LAB, were tested using two different ropy strains of *Lb. delbrueckii* subsp. *bulgaricus* which are known to produce different levels of exopolysaccharide (EPS). The modified MRS medium and the medium containing casein hydrolysate emerged as suitable for the purposes of growth of *Lb. delbrueckii* subsp. *bulgaricus* and the convenient and accurate measurement of key metabolites. Characteristics such as viscosity and ropiness, two key screening techniques for the initial identification of EPS-producing strains were enhanced when the medium containing casein hydrolysate was used.

INTRODUCTION

Studies involving EPS production by *Lb. delbrueckii* subsp. *bulgaricus* frequently employ complex media such as skimmed milk (Garcia–Garibay and Marshall, 1991), or whey ultrafiltrate. LAB, as a group, are nutritionally fastidious, and media should either contain a large number of non–defined nutrients such as meat extract, peptone and yeast extract (Vanos and Cox, 1986) or be a chemically defined medium such as cited by Chervaux *et al.* (2000). Measurement of EPS in a complex medium is interfered with by carbohydrate polymers contained in the medium formulation (Kimmel and Roberts, 1998). In addition, high levels of undissolved solids interfere with biomass analyses, as well as creating difficulties in respect of the analysis of extracellular metabolites such as lactate and galactose.

A medium formulation such as MRS (de Man *et al.*, 1960) is commonly used for the study of *Lb. delbrueckii* subsp. *bulgaricus* (Kimmel and Roberts, 1998), and has been employed in a modified form (with glucose substituted by lactose) (Garcia–Garibay and Marshall, 1991) for the study of EPS production by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772. Van Geel–Schutten *et al.* (1998) employed a modified MRS medium (MRS-l) (lactose at 100 g/l substituted for glucose) for the screening of *Lactobacillus* species producing large amounts of EPS.

Kimmel and Roberts (1998) developed a semi–defined medium, based upon the MRS medium formulation, and suitable for growth of *Lb. delbrueckii* subsp. *bulgaricus* (Strain RR) resulting in EPS levels similar to those which could be achieved using MRS medium, but which did not contain yeast extract, beef extract or proteose peptone, as these components were determined to account for 94% of the total background EPS equivalents in the uninoculated broth.

The present study was undertaken in order to obtain a semi-defined growth medium which was conducive to *Lb. delbrueckii* subsp. *bulgaricus* producing extracellular polysaccharide, using lactose as a carbon source. In addition, it was desired that the medium had the qualities of being able to be used in both liquid and solid form, be suitable for enumeration purposes, as well as the identification of colonial traits, such as mucoidy, or ropiness in EPS–producing LAB. The ability to be further adapted for screening for high EPS producers, was an additional desired characteristic, as well as serving as a "utility" substrate for further physiological and biochemical studies.

Comparative studies were undertaken on modified versions of three different semi–defined media *viz*. MRS (de Man *et al.*, 1960), a medium specifically designed for the growth of EPS–producing LAB (Kimmel and Roberts, 1998), but with an increased casein– hydrolysate content, and an elective medium (Vanos and Cox, 1986), adapted from a formulation of Chalmers (1955). Chalmers medium was originally designed as a solid medium for the enumeration of streptococci, and does not contain citrate or acetate, which are known to inhibit some LAB (Vanos and Cox, 1986; Shaw and Harding, 1984). Vanos and Cox (1986) further modified the medium by raising the concentration of lactose (from 10 g/l to 20 g/l) and introducing glucose (20 g/l). In addition, Evans peptone was replaced with soy peptone, rendering the medium richer in shorter–chain peptides and free amino acids which are necessary to stimulate growth in all LAB, and increased the $CaCO_3$ level in order to better distinguish high acid-producing colonies (Vanos and Cox, 1986). In the current study, the glucose was omitted from this medium, and cultures were undertaken in a liquid form of the medium, although a

solid form of the medium was envisioned for use in detecting different levels of acid production. Lactose was incorporated as the sole sugar source in all three media tested.

Initially, *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 was tested in tandem with a strain known to exhibit a relatively lower EPS–producing ability (CNRZ 737), in order to assess phenotypic characteristics associated with higher EPS production on solid media, and comparative levels of biomass formation, lactose consumption, and extracellular metabolite production by the two strains when grown in the different media.

MATERIALS AND METHODS

Media. MRS medium (de Man *et al.*, 1960) (MRS-l) was modified by the addition of lactose in place of glucose and consisted of lactose, 20.0 g/l; peptone, 10.0 g/l; beef extract, 8.0 g/l; yeast extract 4.0 g/l; sorbitan monoleate ("Tween 80"), 1.0 g/l; dipotassium phosphate (K_2HPO_4), 2.0 g/l; magnesium sulphate ($MgSO_4.7H_2O$), 0.20 g/l; manganese sulphate ($MnSO_4.4H_2O$), 0.05 g/l; ammonium citrate, 2.0 g/l; sodium acetate, 5.0 g/l. Modified Chalmer's medium (CM-l) consisted of lactose, 20.0 g/l; soy peptone, 3.0 g/l; beef extract, 3.0 g/l; yeast extract 3.0 g/l; calcium carbonate 2.0 g/l. The medium according to Kimmel and Roberts (1998) (KM-l) was modified by replacement of the glucose component with lactose and consisted of lactose, 20.0 g/l; yeast nitrogen base (without amino acids and ammonia), 5.0 g/l; bacto casitone (casein hydrolysate), 20.0 g/l; sorbitan monoleate ("Tween 80"), 1.0 g/l; dipotassium phosphate (K_2HPO_4), 2.0 g/l; magnesium sulphate ($MgSO_4.7H_2O$), 0.10 g/l; manganese sulphate ($MnSO_4.4H_2O$), 0.05g/l; ammonium citrate, 2.0 g/l; sodium acetate, 5.0 g/l. In all instances, the medium was prepared in separate, double–strength aliquots of lactose and the remainder of the nutrients as described above. After steam–sterilization, the aliquots were allowed to cool, and pooled, achieving the desired concentration of medium constituents. Where solid medium was prepared, 15.0 g/l of agar was used.

Bacterial strains. The strains of *Lb. delbrueckii* subsp. *bulgaricus* (NCFB 2483 / NCIMB 702483 and CNRZ 737) were originally obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland, and Institut National de la Recherche Agronomique, France, respectively. Stock cultures were subsequently obtained from the culture collection of IFNHH (Massey University) as cultures preserved at –80°C on microbeads.

Culture conditions. The bacterial strains, coated onto microbeads, were inoculated directly into 250 ml medium in shake bottles (Duran, Schott). The cultures were incubated in an orbital

incubator shaker at a slow rotational speed (100 rpm) which was sufficient to keep the culture homogenous, and at a temperature of 37°C for a period of 60 h. Six replicate cultures were undertaken for each medium tested. The cultures were allowed to grow until lactose utilization had ceased. Separate fermentation experiments were undertaken for the determination of microscopic and viable cell counts over a period of 32 h. Incubation of cultures on solid medium was undertaken at 37°C for a period of 48 h.

Sampling. Sample aliquots (15 ml) were withdrawn aseptically at 8 hourly intervals for the determination of biomass, microscopic and viable cell counts, relative viscosity, and sugar conversion to extracellular metabolites. In instances where analyses could not be immediately undertaken, samples were stored in pre-sterilized bottles at –20°C. Samples for the determination of microscopic and viable cell counts were taken at 8 hourly intervals.

Analyses. Growth was monitored by absorbance measurement at 650 nm. Biomass was determined from a standard curve relating absorbance at 650 nm to washed dry cell weights. Relative viscosity was used as a means to assess the functional effect of the two strains on the different media types, as a logical consequence of EPS production. Microscopic cell counts were undertaken using an improved Neubauer counting chamber (Weber, England). Viable cell counts were determined by serial dilution plate counting. The solid media used corresponded to the liquid medium type, from whence the subculture was derived. Viscosities were measured on samples adjusted to the final pH of the respective broth cultures, using a Cannon–Fenske Routine Viscometer (PA, USA). Mucoidy of colonies was determined by visual appearance, and the ropiness of colonies and liquid broth were determined by testing with a sterile toothpick. Lactose depletion, and galactose and lactate formation was measured by HPLC (Waters model 590 - manual injection, coupled to a Waters R401 Differential Refractometer, and R440 UV spectrophotometer). The compounds were detected using an Aminex HPX-87H, 300 x 7.8 mm (Biorad, Richmond, CA), according to the method as described in Ross and Chapital (1987). The column temperature was maintained at 40°C, and the flow rate at 0.6 ml. $min.^{-1}$. Cell-free supernatant fractions of the broth were diluted in distilled and filtered water (MilliQ) prior to analysis. External standards were prepared for lactose, galactose, and lactic acid. All analyses were undertaken in duplicate.

RESULTS

Initial experiments showed that *Lb. delbrueckii* subsp. *bulgaricus* strains NCFB 2483 and CNRZ 737, when cultivated in the modified version of the medium according to Chalmers

(CM-l) showed poor growth (Figures 3.1. and 3.2.), lactose utilization, and conversion to lactate (pH change) (Figures 3.3 and 3.4) relative to those achieved with the other modified media under comparison. On the strength of these results, use of the CM-l medium was rejected as an option and all subsequent comparisons were undertaken on the modified MRS (MRS-l) and modified medium according to Kimmel and Roberts (1998) (KM-l).

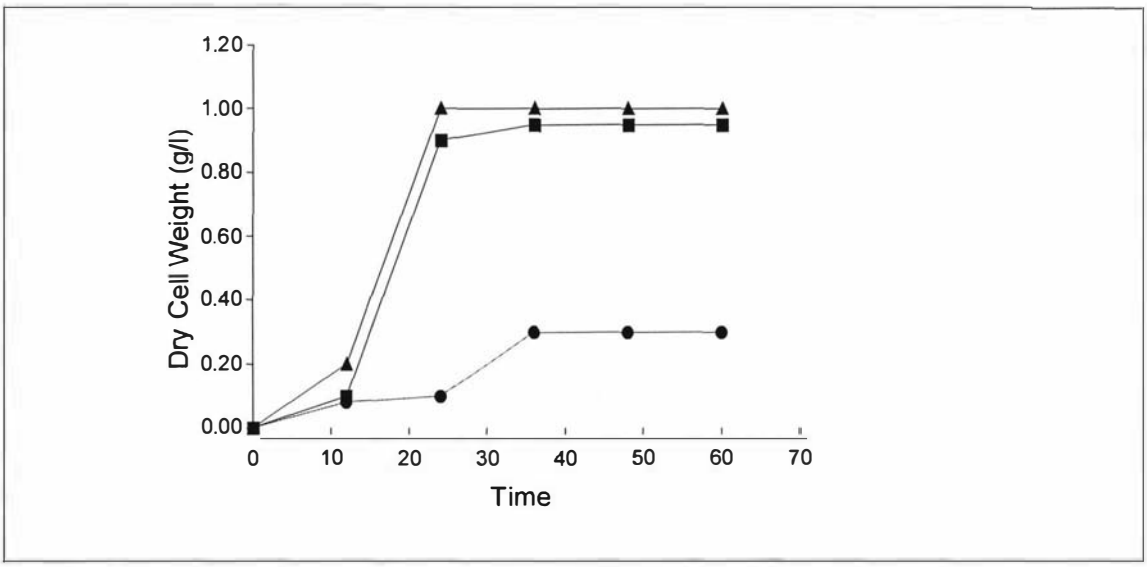

Fig. 3.1. Comparison of biomass (dry cell weight) concentrations of *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 grown in MRS-l medium (■), KM-l medium (▲), and CM-l medium (●).

No major differences in biomass were observed between the NCFB 2483 strain growing on MRS-l and KM-l medium, although marginally raised biomass levels on KM-l were evident throughout the time–course of the fermentation (Figure 3.1). Lactose consumption was higher until 36 h into the fermentation on KM-l medium relative to MRS-l medium, however the difference became negligible after this period (Figure 3.3). *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 was found to produce galactose on uptake of lactose. Comparison of galactose and lactate produced to lactose consumed revealed a similar pattern of production when both KM-l and MRS-l were used (Figure 3.5).

In contrast, the CNRZ 737 strain, however, produced significantly less biomass in MRS-l than KM-l medium (Figure 3.2), and correspondingly consumed less lactose (Figure 3.4). When grown on MRS-l medium, the CNRZ 737 strain attained a maximum biomass (X_{max}) later in the fermentation (48 h) than the maximum recorded (36 h) when growing on KM-l medium (Figure 3.2).

Functional changes to the medium as a result of EPS production were measured by means of the viscosifying effect which it had on the medium. Relative viscosities (RV) of the higher EPS-producing strain of *Lb. delbrueckii* subsp. *bulgaricus* (Strain NCFB 2483) demonstrated higher

maximum values when grown in the KM-l medium in comparison to those when grown on MRS-l medium (Figure 3.6). Over the time course of the fermentations, the viscosifying effects differed between the two media. In MRS-l medium, relative viscosities increased between 24 h and 48 h, whilst in KM-l medium, a maximum RV value was measured at 24 h into the fermentation, followed by a steady decrease during the remaining course of the fermentation (Figure 3.6).

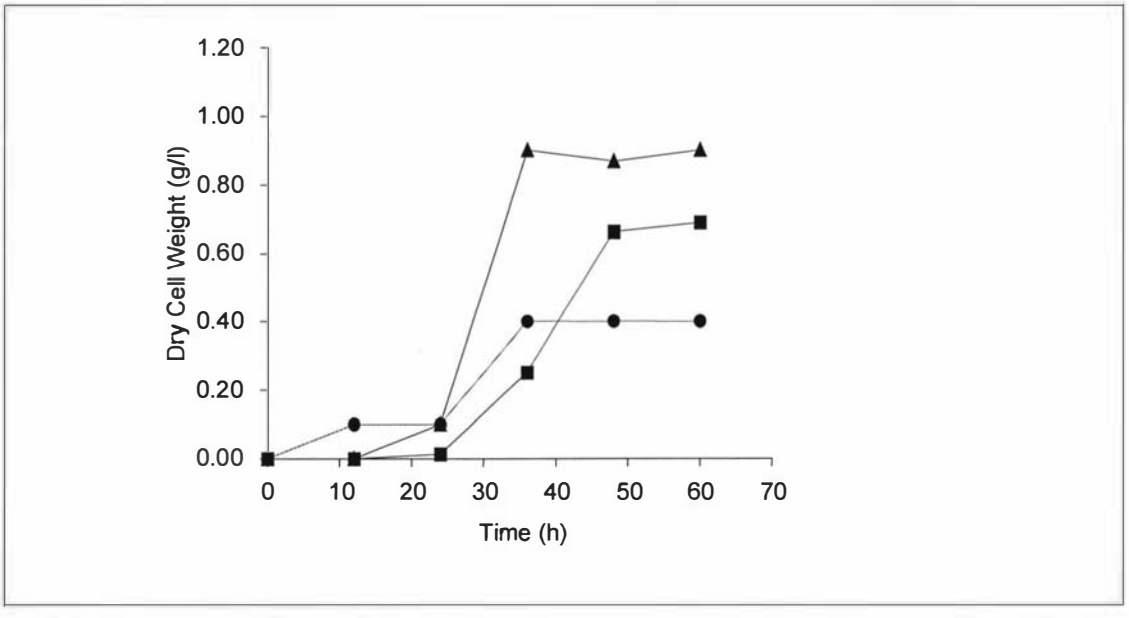

Fig. 3.2. Comparison of biomass (dry cell weight concentrations) of *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 737 grown in MRS-l medium (■), KM-l medium (▲), and CM-l medium (●).

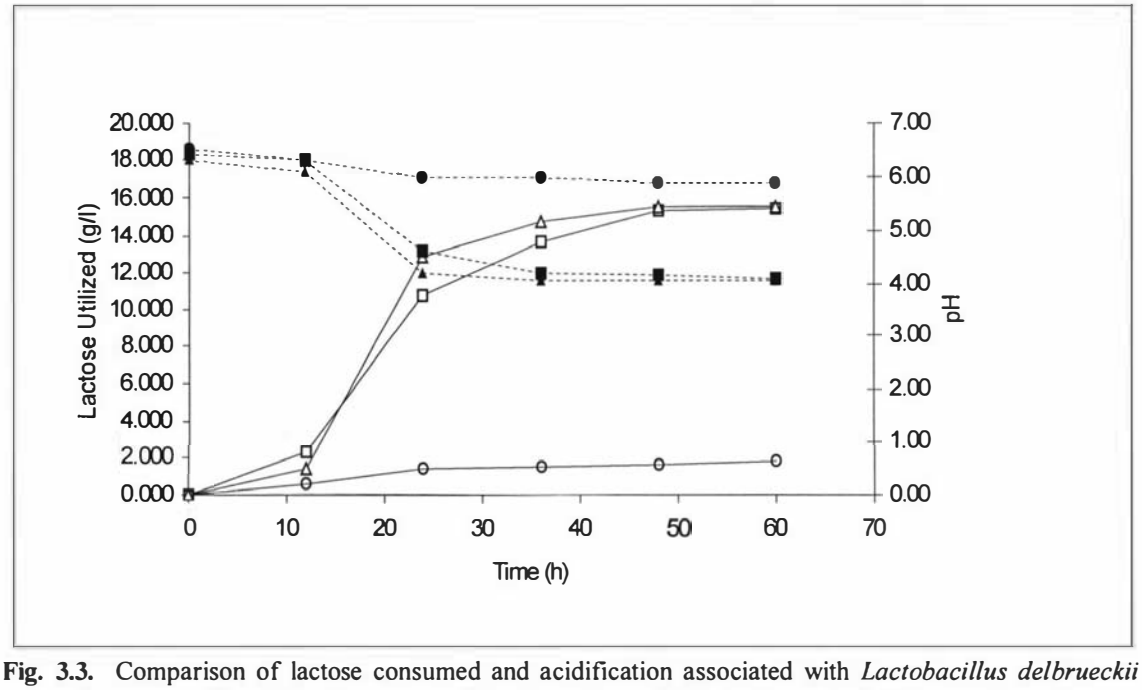

Fig. 3.3. Comparison of lactose consumed and acidification associated with *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 grown in MRS-l medium, KM-l medium, and CM-l medium; Lactose consumption in: MRS-l medium (□), KM-l medium, (Δ), and CM-l medium (○); pH: MRS-l (■), KM-l medium (▲), and CM-l medium(●).

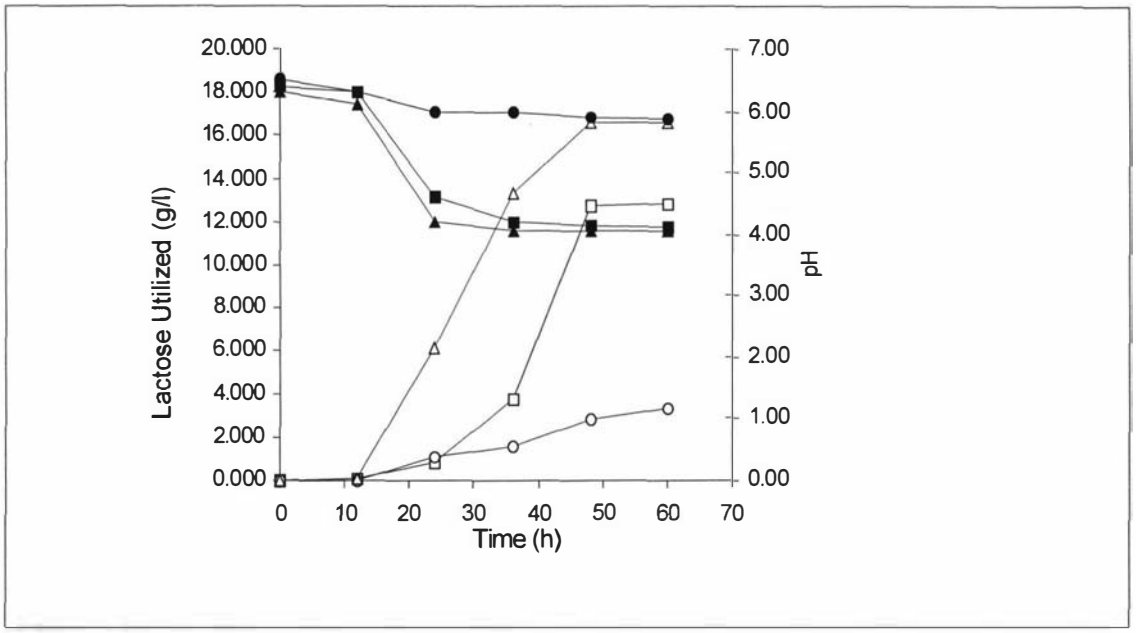

Fig. 3.4. Comparison of lactose consumed and acidification associated with *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 737 grown in MRS-l, KM-l, and CM-l medium; Lactose consumption in: MRS l medium (□), KM-l medium, (Δ), and CM-l medium (○); pH: MRS-l medium (■), KM-l medium (▲), and CM-l medium (●).

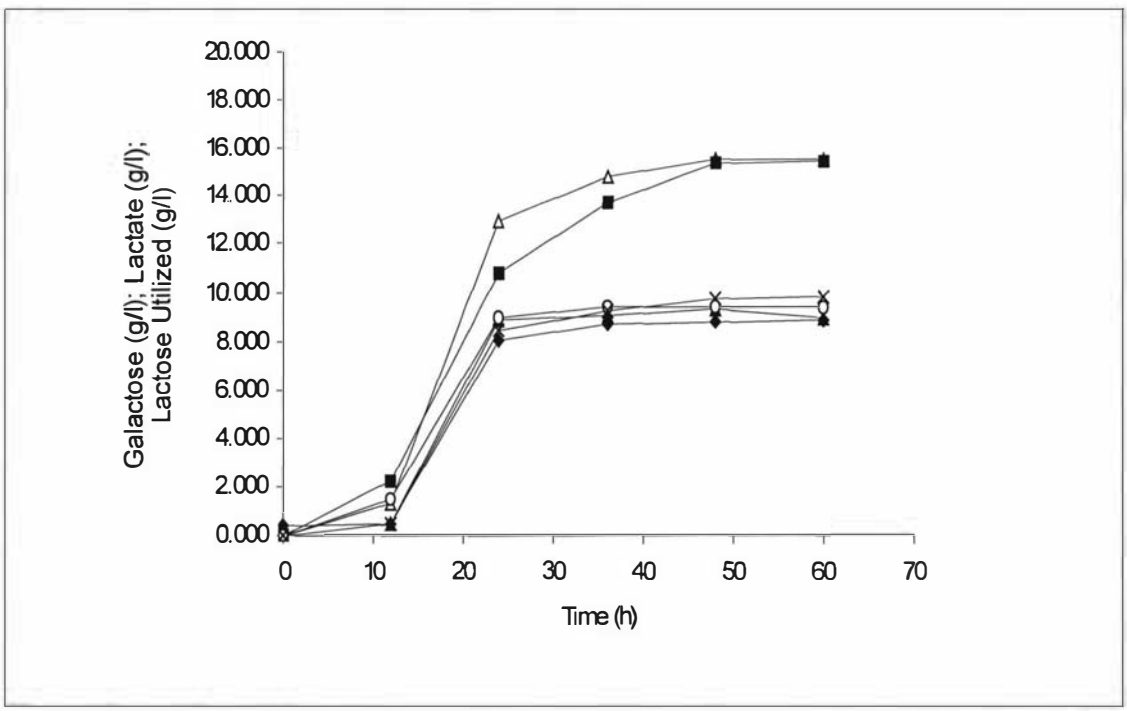

Fig. 3.5. Comparison of lactose consumed and galactose and lactate produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 grown in MRS-l and KM-l medium. Lactose consumption in: MRS-l medium (■), KM-l medium (Δ); lactate produced in: MRS-l medium (6), KM-l medium (▲); galactose production in: MRS-l medium (x), KM-l medium (○).

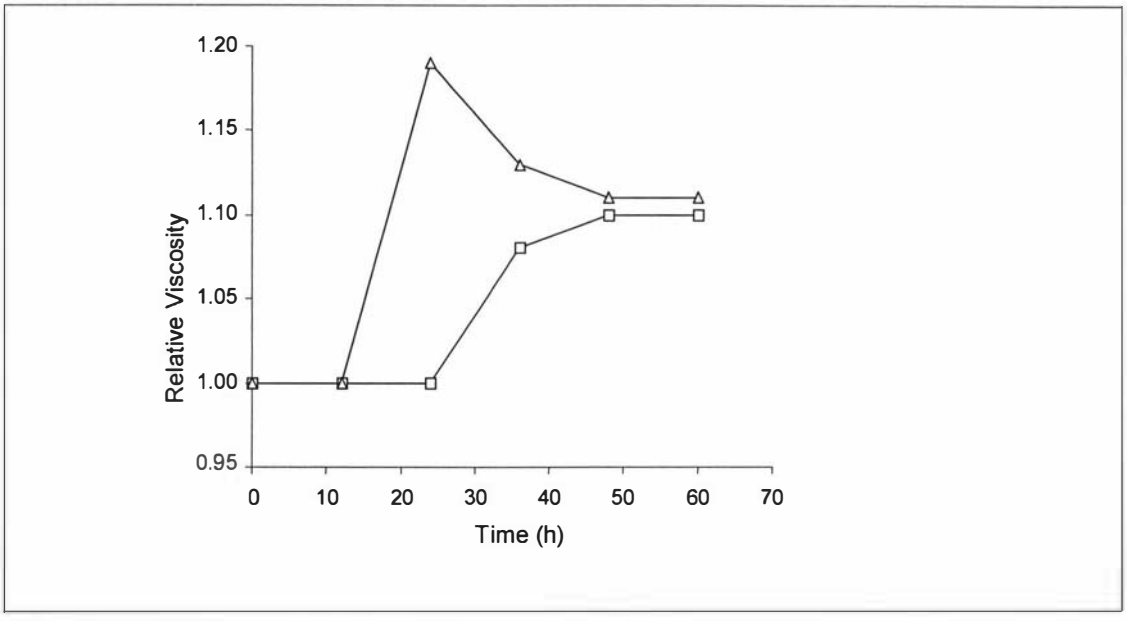

Fig. 3.6. Comparison of relative viscosities associated with *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 grown in modified MRS-l medium, and KM-l medium. Relative viscosity in: MRS-l (□), and KM-l medium (Δ).

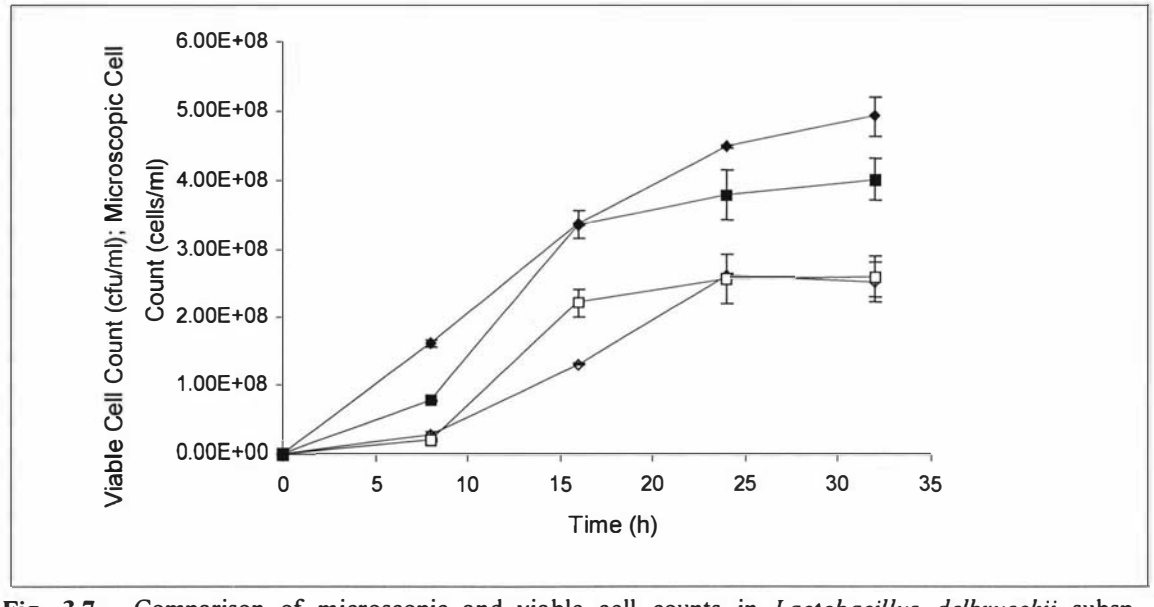

Fig. 3.7. Comparison of microscopic and viable cell counts in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 grown in modified MRS-l medium and KM-l medium; microscopic cell count in: MRS-l medium (■), KM-l medium (♦); viable cell count in: MRS-l medium (□), KM-l medium (◊). Error bars represent standard deviations.

Microscopic and viable cell counts for the two media showed similar trends over the time course of fermentation (Figure 3.7), with microscopic cell counts generally higher in KM-l – grown cultures, reflecting the differences in biomass (Figure 3.1). Generally, the colonies cultured on solid KM-l medium appeared more mucoid than when grown on the corresponding solid MRS-l medium. The strains, when grown in KM-l liquid culture medium, produced a broth with greater ropiness than when grown on MRS-l medium, up until 36 h of growth.

DISCUSSION

The modified medium as described by Vanos and Cox (1986), and incorporating lactose in place of glucose is not a suitable medium for use with the strains of *Lb. delbrueckii* subsp. *bulgaricus* tested for the purposes of screening and general studies. The relatively poor fermentation performance associated with growth of the strains on this medium could be ascribed in part to the lower levels of organic nitrogen in the form of proteose peptone, yeast extract, and beef extract, as formulated in the MRS-l medium, and possibly the requirement for additional minerals. In Grobben's development of a simplified defined medium for the purposes of enhancing EPS production by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 (Grobben *et al.*, 1998), more amino acids were reported to be necessary for growth than for *Lb. plantarum*, *Lactococcus lactis,* and *Streptococcus thermophilus*. The lack of citrate in CM-l could also have contributed to the poor growth, as it has been determined as a requirement for *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 by Grobben *et al.* (1998).

The medium as described by Kimmel and Roberts (1998), and modified by the replacement of the carbohydrate source (lactose instead of glucose) (KM-l) represents a viable alternative to MRS medium, which has been widely used as a semi–defined medium for studies involving LAB. An additional benefit of the KM-l medium is that the amino acid profile and carbohydrate used are similar to those which would be found in an industrial whey medium, but without the complications of the requirement to clarify the medium prior to undertaking biomass measurements and analysis of extracellular metabolites.

The enhanced viscosity exhibited during growth in KM-l medium in comparison to that achieved in MRS-l medium is advantageous for the purposes of screening on this basis. The viscosity measurements, to a certain extent, represent both EPS titre and functional effects. A relationship, albeit unclear, has been shown by other investigators to exist between the viscosity of broth and the amount of EPS produced, and that interactions between polysaccharide and milk protein constituents such as casein occur (Cerning *et al.*, 1986; 1992). The diminution in RV values (Figure 3.6) after approximately 20 h fermentation time, however, limits the experimental time–span over which experiments may be run. The reductions in RV values are possibly due to degradation of the EPS polymeric structure by glycohydrolases as has been previously reported by Gassem *et al.* (1997) and Petry *et al.* (2000) in *Lb. delbrueckii* subsp. *bulgaricus*.

microscopic cell counts associated with the use of KM-l medium relative to MRS-l medium (Figure 3.7) generally mimicked the relative biomass levels obtained (Figure 3.1), although it should be noted that more biomass data over the critical growth phase would be needed in order to make a detailed comparison in this respect. The solid agar–based media prepared using the respective formulations resulted in distinct colonies on both media which were easily enumerated after a period of 48 h incubation. The enhanced colonial mucoidy and visual ropiness in liquid KM-l medium as opposed to MRS-l medium renders the former the more suitable option for primary screening purposes

CONCLUSION

The medium as formulated by Kimmel and Roberts (1998) and MRS medium (de Man *et al.*, 1960), both modified by the replacement of glucose with lactose, proved to be suitable for the purposes of evaluation of growth and metabolite formation in EPS-producing strains of *Lb. delbrueckii* subsp. *bulgaricus*. The modified version of the medium according to Kimmel and Roberts (1998), however, produced cultures which were more viscous and ropy in liquid medium, and colonies which were more mucoid in appearance than when grown in the modified MRS medium. For the purposes of overall evaluation of screening and evaluation of EPS-producing strains, the modified medium of Kimmel and Roberts (1998) emerged as the preferred choice.

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CHAPTER 4

FERMENTATION PERFORMANCE RELATED TO EXOPOLYSACCHARIDE PRODUCTION BY *LACTOBACILLUS DELBRUECKII* SUBSP. *BULGARICUS* NCFB 2483 IN BATCH CULTURE

ABSTRACT

Lactobacillus delbrueckii subsp. *bulgaricus* NCFB 2483, which is known to produce exopolysaccharide (EPS), was grown in a semi-defined medium incorporating casein hydrolysate, in order to assess fermentation performance under simple batch culture conditions. Lactose was the sole source of sugar and was split intracellularly, with the major portion of the resultant galactose being exported from the cell, and the glucose being metabolized further. *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 demonstrates a mainly homofermentative pattern of carbon utilization, with lactate being the only organic acid produced. EPS production was closely growth–associated, following a pattern of primary kinetics with respect to the production of its metabolites. Performance parameters were derived from the fermentation data and kinetic models were applied in order to best describe the production of biomass and the extracellular metabolites produced.

INTRODUCTION

The production of EPS by lactic acid bacteria (LAB) represents an attractive alternative to the microbial EPS which currently occupy the biopolymer market viz. Xanthan from *Xanthomonas campestris*, the gellans from *Sphingomonas paucimobilis* (Sutherland, 1998), acetan from *Acetobacter xylinum* (Van Kranenburg *et al.*, 1999), and dextran from *Leuconostoc mesenteroides* (De Vuyst *et al.*, 2001). The reason for this is that many of the current biopolymers are produced by microbes which do not have a GRAS (Generally Recognized As Safe) status, hence limiting their use in foods. EPS from LAB currently play a role in the manufacture of fermented milk products such as yoghurt, imparting an improved rheology, texture and mouthfeel. In addition, these biopolymers have been strongly suggested to elicit health benefits (Duboc and Mollet, 2001). The production of these polymers is however limited by the productive capacity of the LAB, which are chiefly anaerobic organisms.

Some strains of *Lb. delbrueckii* subsp. *bulgaricus* serve as typical examples of the LAB which can produce excess exopolysaccharide. The compositional structures determined to the present time of EPS from *Lb. delbrueckii* subsp. *bulgaricus* have varied, however all have been found to be heteropolysaccharides, consisting of repeating units of monomers such as glucose, galactose, arabinose, mannose, fructose, and rhamnose. Gruter *et al.* (1993) determined the structural composition of EPS from *Lb. delbrueckii* subsp. *bulgaricus* strain rr, when grown in skim milk, to consist of branched heptameric repeating units with glucose, galactose, and rhamnose as the monomeric constituents. The compositional structure of EPS repeating units from *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 was determined by Grobben *et al.* (1995) to consist of glucose, galactose and rhamnose. Similar compositional structures (i.e. with the repeating unit composition consisting of glucose, galactose, and rhamnose), but with differing ratios of the sugars, were found in *Lactobacillus bulgaricus* CNRZ 737 and CNRZ 416a by Cerning *et al.* (1986). A similar compositional structure of the repeating unit has been determined for *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 (Goh, 2002).

In this study, the fermentation performance of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 was assessed in simple batch culture. The formation of EPS and other extracellular metabolites were monitored throughout the time-course of the fermentation, and performance characteristics were derived from the data. An attempt was made to simulate the production of these metabolites through the application of various models previously used to describe metabolite production in LAB. The development of these models is useful, as it enables the prediction of EPS and metabolite formation through the measurement of a minimum number of parameters e.g. biomass and lactose consumption.

The medium used in this study was that described by Kimmel and Roberts (1998), modified by the replacement of glucose with lactose as the sole sugar source (termed "KM-l"). This medium is based upon the MRS medium of de Man *et al.* (1960), which has found wide utility in general studies on LAB. The distinction between the two media is that in the medium of Kimmel and Roberts (1998), the protein source is derived entirely from hydrolysed casein, whilst in the MRS medium, the corresponding protein source is derived from peptone, and yeast and beef extracts. The hydrolysed casein possesses an amino acid profile more consistent with that which would be found in milk protein.

MATERIALS AND METHODS

Media. The medium according to Kimmel and Roberts (1998) (KM-l) was modified by replacement of the glucose component with lactose and consisted of lactose, 20.0 g/l; yeast nitrogen base (without amino acids and ammonia), 5.0 g/l; bacto casitone, 20.0 g/l; sorbitan monoleate ("Tween 80"), 1.0 g/l; dipotassium phosphate (K_2HPO_4), 2.0 g/l; magnesium sulphate ($MgSO_4.7H_2O$), 0.10 g/l; manganese sulphate ($MnSO_4.4H_2O$), 0.05 g/l; ammonium citrate, 2.0 g/l; sodium acetate, 5.0 g/l. In all instances, the medium was prepared in separate, double–strength aliquots of lactose and the remainder of the nutrients as described above. After steam- sterilization, the aliquots were allowed to cool, and pooled, achieving the desired concentration of medium constituents.

Bacterial strain and preparation of a working cell bank. The strain of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 (NCIMB 702483) was obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland. The culture was grown at 37°C from a freeze–dried powder to late-log phase in "medium 284" (NCIMB), modified by the deletion of glucose and incorporation of 20% glycerol, and adjusted to pH 6.2. The broth supernatant was removed by sterile centrifugation at 12 000 g for 15 minutes. The pellet was reconstituted with modified medium 284, as described above and preserved in 1 ml aliquots at -80°C. (Step-wise cooling: sequentially from ambient temperature to -4°C, -20°C, and -80°C).

Culture conditions. Aliquots (1 ml) of the culture strain were inoculated directly into 225 ml volumes into 250 ml medium in shake bottles (Duran, Schott). The cultures were incubated without pH control in an orbital incubator shaker at a slow rotational speed (100 rpm) which was sufficient to keep the culture homogenous, and at a temperature of 37°C for a period of 24 h. Trial experiments demonstrated that lactose consumption and metabolite production had ceased after 24 h had elapsed. Six replicate cultures were undertaken for each medium tested. The cultures were allowed to grow until lactose utilization had ceased.

Sampling. Sample aliquots (15 ml) were withdrawn aseptically at 4 hourly intervals for the determination of biomass, microscopic cell counts, and sugar conversion to extracellular metabolites. In instances where analyses could not be immediately undertaken, samples were stored in pre-sterilized bottles at –20°C.

Analyses. Growth was monitored by absorbance measurement at 650 nm. Biomass was determined from a standard curve relating absorbance at 650 nm to washed dry cell weights. Relative viscosity was used as a means to assess the functional effect of the strain on the different media types, as a logical consequence of EPS production. Microscopic cell counts were undertaken using an improved Neubauer counting chamber (Weber, England).

Extracellular polysaccharides were subjected to a crude isolation prior to analysis. Aliquots (100 µl) of whole broth underwent a two–fold precipitation with chilled ethanol (2.9 ml distilled H_2O and 7.0 ml 99.7 % ethanol) for 24 h periods at 4°C. The precipitate was recovered by centrifugation (35850 g, 40 min., 4°C) (Sorvall RC5C – SS34 rotor) and resuspended in 1.0 ml distilled water. The total sugar concentration in the resuspended sample was measured according to the method of Dubois *et al.* (1956), with dextran as the standard.

Lactose, galactose and lactic acid concentrations were determined in duplicate by HPLC (Waters Alliance 2690 Separations module). The HPLC system was coupled with a refractive index detector (Waters 2410) and UV spectrophotometer (Waters 2487). The compounds were detected using a single column (Aminex HPX-87H, 300 x 7.8 mm, Biorad, Richmond, CA), according to the method described in Ross and Chapital (1987). The column temperature was maintained at 40°C, and the flow rate at 0.6 ml. min^{-1}. Cell–free supernatant fractions were diluted with distilled and filtered water (MilliQ) prior to column analysis. Instrument, data accession, and processing methods were controlled using a Millenium® software system. External standards were prepared for lactose, galactose, and lactate determination (Sigma Chemical Co., St. Louis, MO).

RESULTS

EPS production was growth–associated, with a maximum value (0.161 g/l) measured at 20 h elapsed time (Figure 4.1).

Lactate and galactose formation followed the growth trend (Figure 4.1), with maxima measured at 24 h. The acidification of the broth corresponds to lactate formation with pH values achieving minimum values from 20 h onwards. Microscopic cell counts and biomass (dry cell weight) measurements over the 24 h fermentation period are illustrated in Figure 4.2. A plot correlating values of the two variables obtained over the 24 h time period yielded a RSQ value of 0.9998.

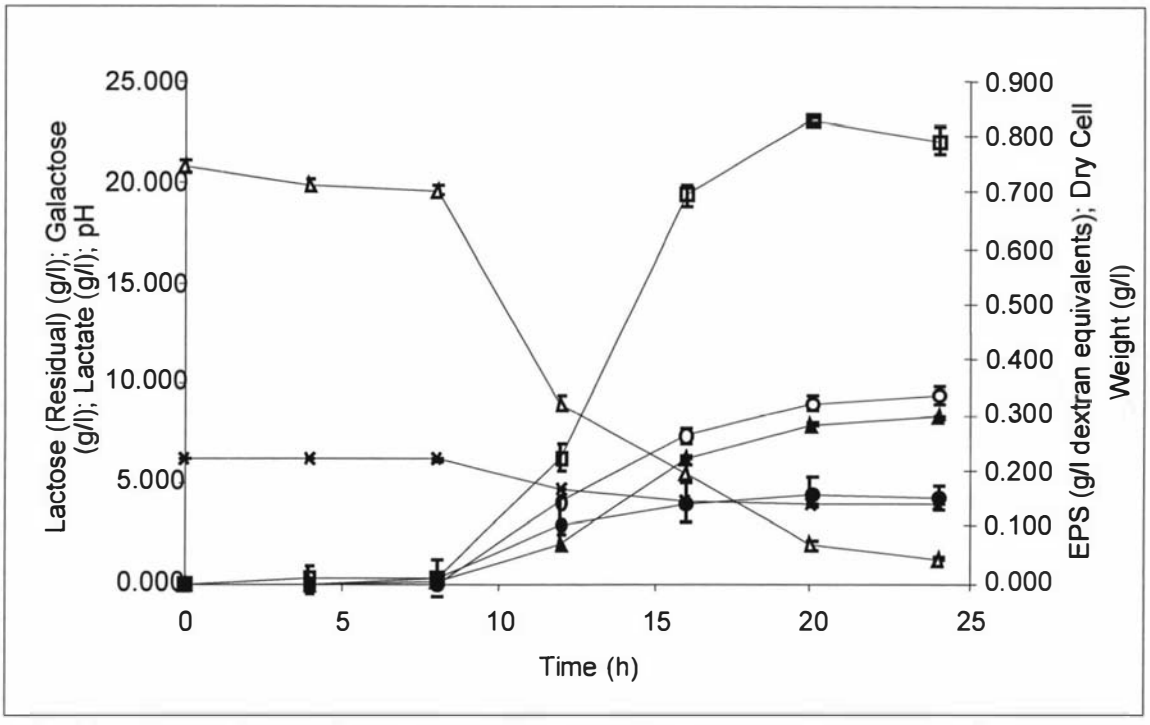

Fig. 4.1. Lactose consumed and extracellular metabolite production associated with *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 grown in KM-l medium at 37°C; Biomass (dry cell weight) titre (□); Residual lactose (Δ); Galactose (o); pH (x); Lactate (▲); EPS (●). All values the mean of 6 experiments. Error bars represent standard deviations.

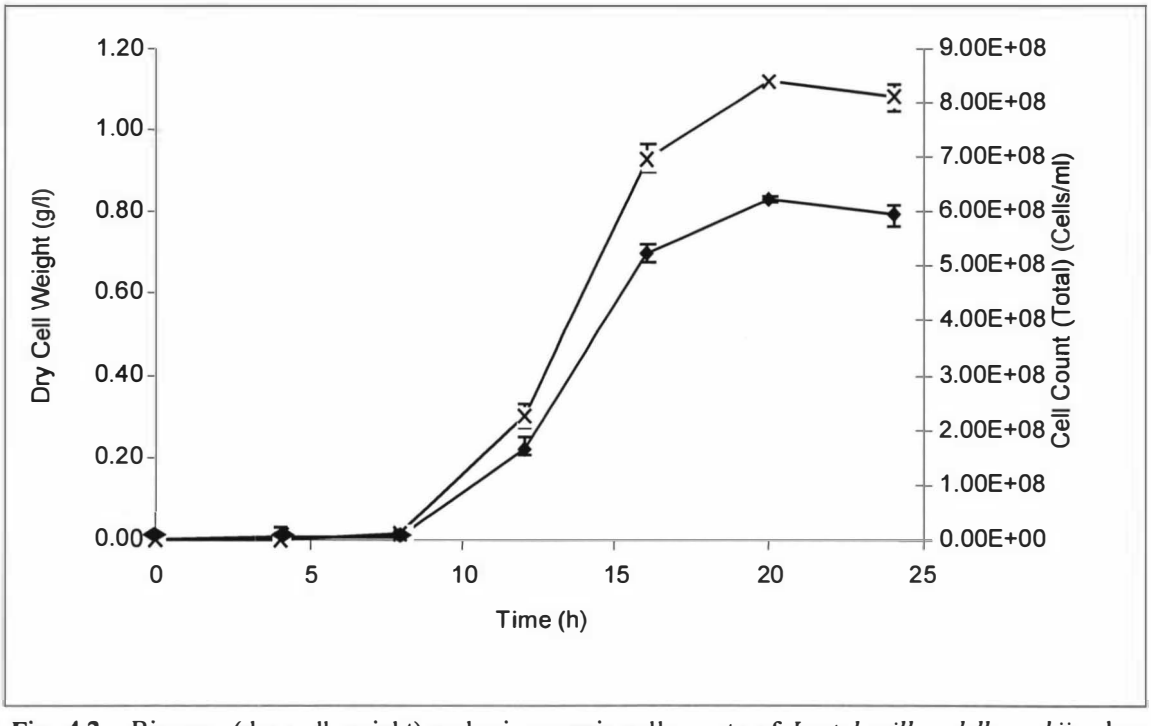

Fig. 4.2. Biomass (dry cell weight) and microscopic cell counts of *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 grown in KM-l medium at 37 ^{0}C; Biomass (dry cell weight) titre (♦); microscopic cell count (x). All values the mean of 6 experiments. Error bars represent standard deviations.

In order to generate a reasonable kinetic model describing the behaviour of biomass (X), extracellular products (P), and lactose utilization (S), in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 under the applied fermentation conditions, a variety of existing models were applied.

Biomass production. The logistic equation has been applied to describe the growth of microorganisms (Berkman, *et al.*, 1990; Weiss and Ollis, 1980; Klimek and Ollis, 1980):

$$\frac{dX}{dt} = \mu X\left(1 - \frac{X}{X_{max}}\right)$$

where X is the biomass concentration, X_{max} is the maximum biomass concentration, t is the elapsed time, and μ is the initial maximum specific growth rate. The above equation may be integrated and rearranged to give:

$$X(t) = \frac{X_o\, e^{\mu t}}{1.0 - (X_0 / X_{max})(1.0 - e^{\mu t})}$$

A plot of $\ln[\underline{X} / (1.0 - \underline{X}]$ against time yielded μ (the slope) (0.5 h^{-1}) and X_o, the initial minimum viable inoculum size (0.0012 g/l) from the y intercept (= - ln $[(X_{max} / X_o) - 1.0]$, where $\underline{X} = X(t)/ X_{max}$. The value X_{max} was 0.828 g/l (Table 4.1). A simulation of biomass formation in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 could then be undertaken (Figure 4.3).

Table 4.1. Experimentally derived parameters related to biomass, yield of biomass on lactose consumed, maximum growth rate, and rate of biomass production in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 grown in KM-l medium at 37°C. X_{max}: Maximum biomass; X': Maximum biomass prior to EPS degradation; X_o: Initial minimum inoculum biomass.

Biomass	X_{max} (g/l)	X' (g/l)	X_o (g/l)	$Y_{X/S}$ (g DCW/g lactose consumed)	μ_{max} (h^{-1})	r_X (g X/l/h)
	0.828 (at 20 h)	0.828 (at 20 h)	0.0012	0.04 (at 20 h) 0.04 (at 24 h)	0.5	0.04 (at 20 h) 0.03 (at 24 h)

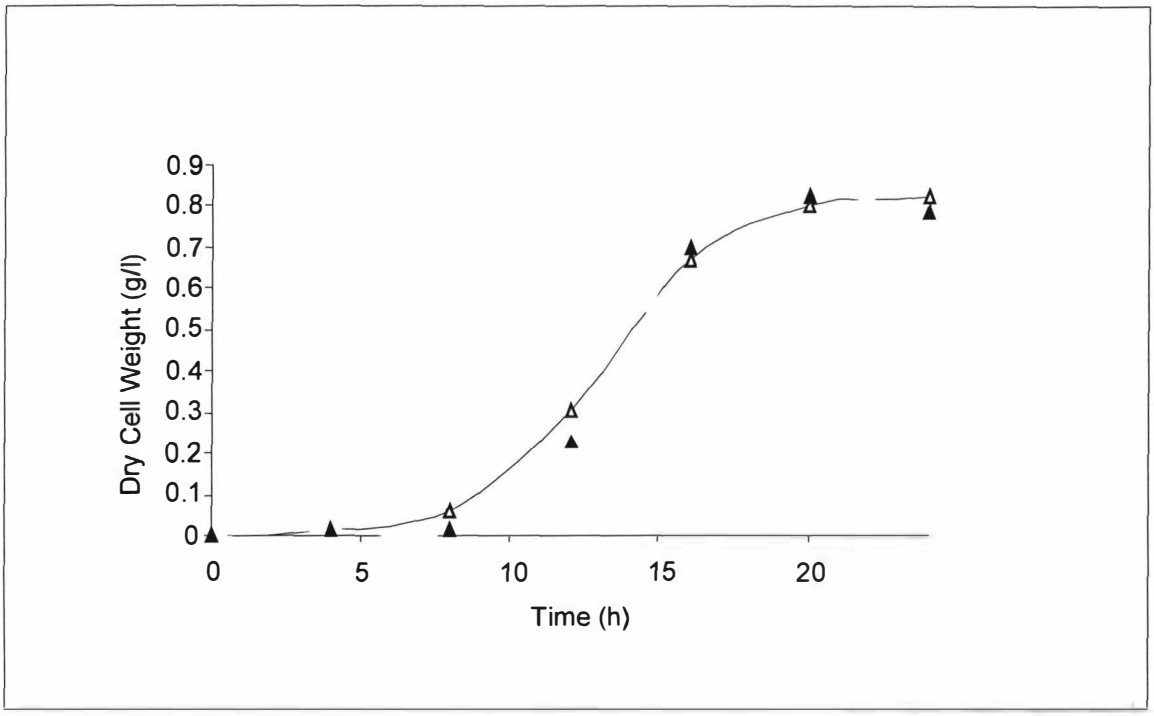

Fig 4.3. Simulation of biomass (dry cell weight) formation in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483. Actual biomass data (▲); simulated data (Δ).

For product formation, Luedeking and Piret (1959) developed a model for lactic acid production in *Lactobacillus delbrueckii, viz.*

$$\frac{dP}{dt} = nX + m\frac{dX}{dt}$$

The equation is split into a non–growth associated term "nX", and a growth–associated term "m dX/dt" describing product formation. Integration of the Luedeking–Piret equation describes the way in which product evolves with time (Klimek and Ollis, 1980):

$$P(t) = P_o + m\, X_o\{e^{\mu t}\,[1.0 - (\underline{X_o})\,.\,(1.0 - e^{\mu t})] - 1.0\} + n\,(X_{max} / \mu)\,\ln\,[1.0 - \underline{X_o}\,.\,(1.0 - e^{\mu t})]$$

In which P_o is the product titre at time t = 0. The factor "n" is calculated from:

$$n = \frac{(dP/dt)_{stat}}{X_{max}}$$

Product formation in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 is growth–associated (Figure 4.1), and the calculation of "n" is thus not included in the model simulation.

The factor "m" (mg P / mg X) was obtained from the slope of a plot of the integrated form of the Luedeking–Piret equation against $X - X_o$. In the case of EPS production, m was calculated to be 203 mg EPS (dextran equivalents) / g biomass.

EPS production. EPS production in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 closely follows the trend of biomass formation (growth-associated), reaching a measured maximum at 20 h fermentation time (Figure 4.4). A low level of lactose consumption however continues until the end of the growth period (Figure 4.1).

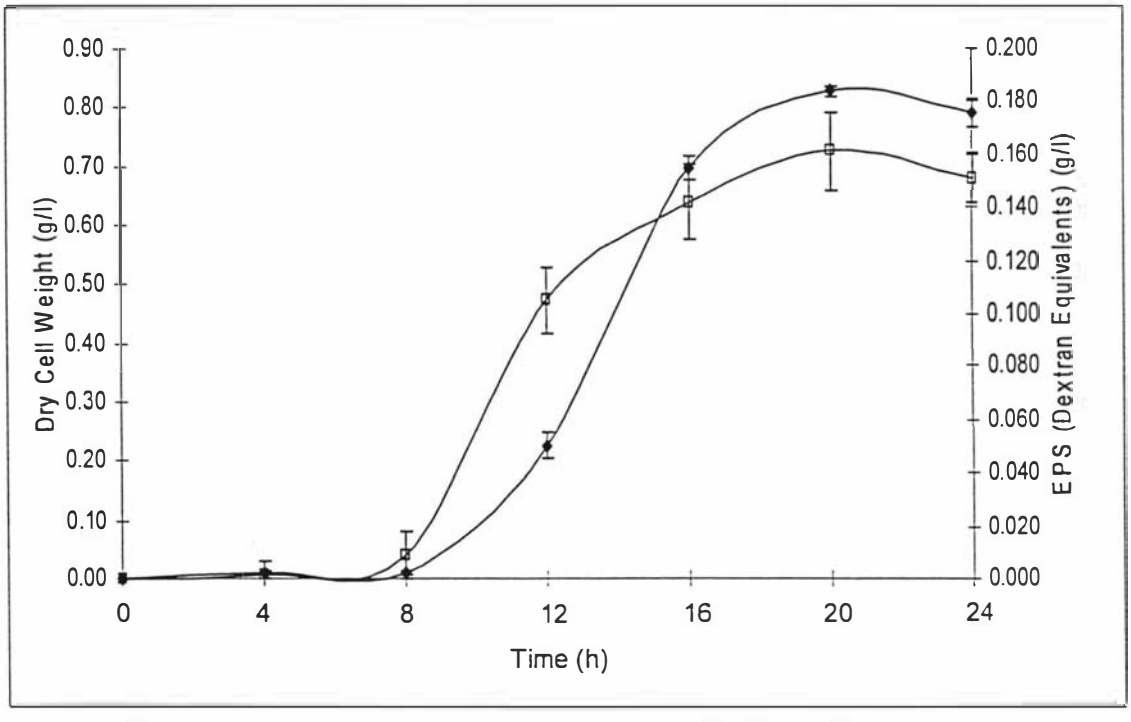

Fig. 4.4. EPS and biomass (dry cell weight) formation in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 grown in KM-l medium at 37°C. EPS titre (□); Biomass (dry cell weight titre) (•). All values the mean of 6 experiments. Error bars represent standard deviations.

A simulation of EPS formation by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 using the Luedeking-Piret equation is shown in Figure 4.5. The simulation was not attempted beyond the 20 h fermentation time, as the EPS titre decreased after this time, possibly due to degradation of the polymer.

Galactose production. *Lb. delbrueckii* subsp. *bulgaricus* strains which are galactose-negative, import lactose by a lactose/galactose antiport transport system (de Vos and Vaughan, 1994), as has been found in *S. thermophilus* LY03 (Hutkins and Ponne, 1991). Using sets of data from the 24h fermentation profiles of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483, a linear

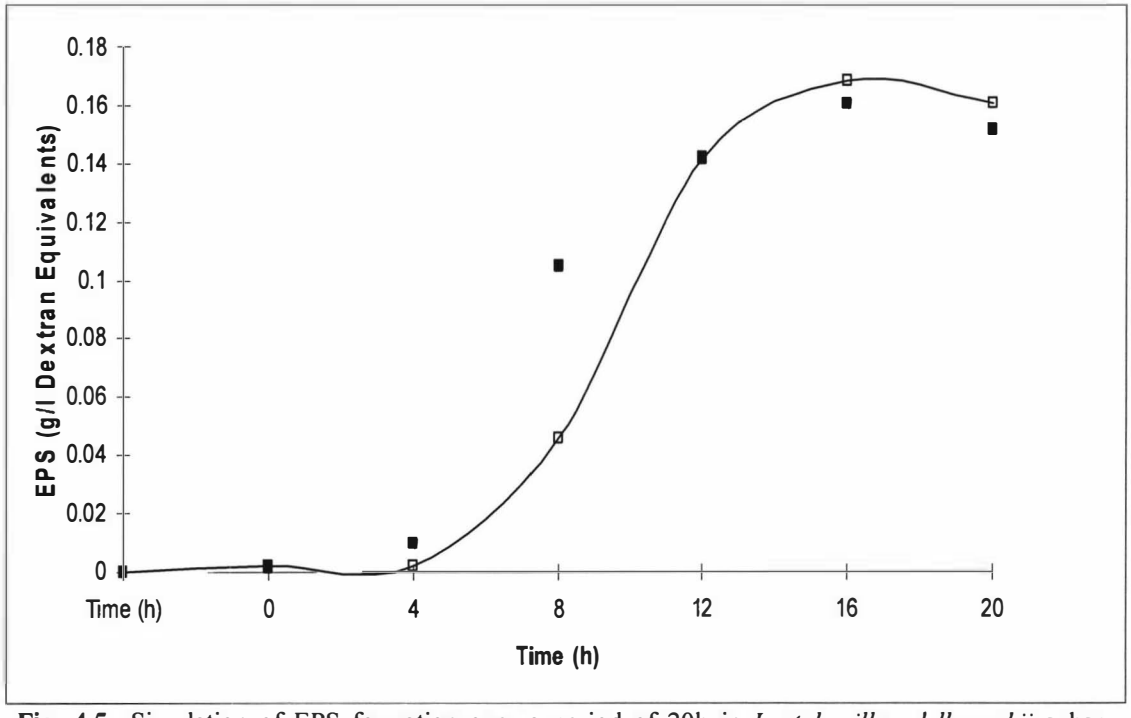

Fig. 4.5 Simulation of EPS formation over a period of 20h in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 grown in KM-l medium at 37°C; Actual EPS titre (■); Simulated EPS titre (□).

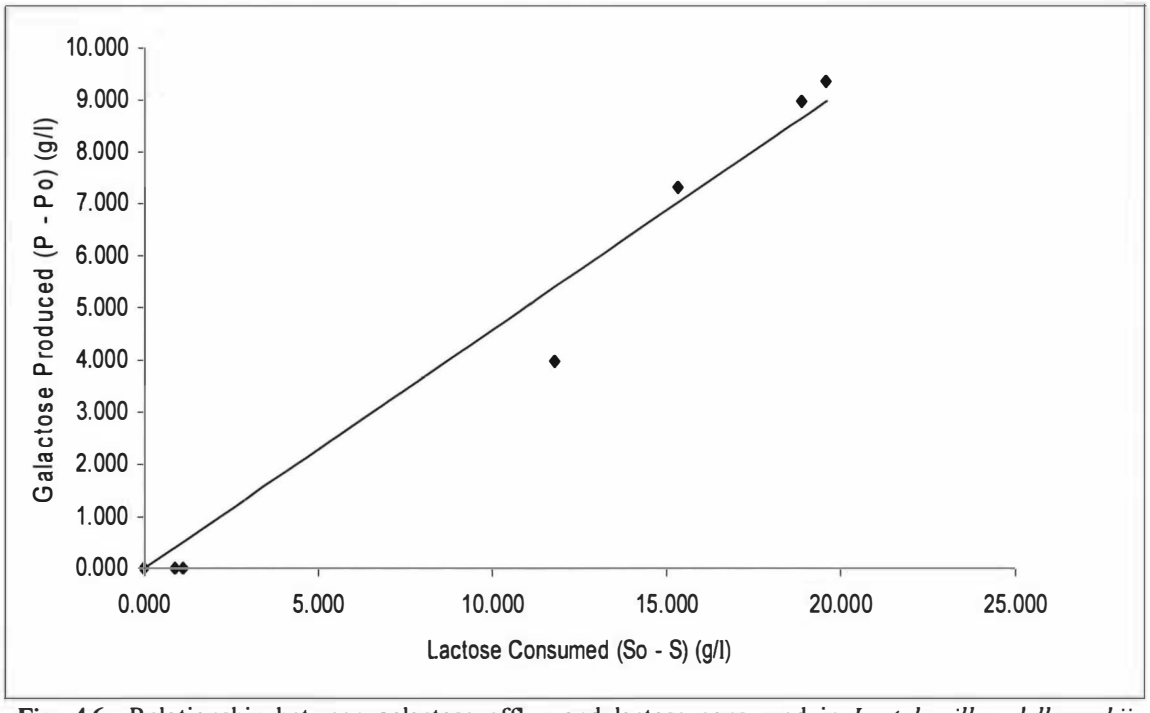

Fig. 4.6 Relationship between galactose efflux and lactose consumed in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 grown in KM-l medium at 37°C.

relationship can be shown to exist between galactose produced and lactose consumed (Figure 4.6). During growth and maintenance of the bacteria, galactose efflux may be described by the following equation (Degeest and De Vuyst, 1999):

$$\frac{dGal}{dt} = \frac{-1}{Y_{S/gal}} \frac{dS}{dt}$$

where $Y_{S/gal}$ represents the yield coefficient for galactose (a value of 2 g lactose consumed / g galactose produced was used). A simulation of galactose efflux over a 24 h fermentation period using the above relationship is shown in Figure 4.7.

Lactate production. *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 is homofermentative as no other organic acids apart from lactate were detected in the fermentation samples. Lactic acid formation can similarly be related to lactose utilization by the yield coefficient for lactic acid, *viz.* $Y_{S/lactate}$ which represents the lactose consumed (g) / lactate produced (g). The equation for lactate production is (Degeest and De Vuyst, 1999):

$$\frac{dLactate}{dt} = \frac{-1}{Y_{S/lactate}} \frac{dS}{dt}$$

A simulation of lactate production using the above relationship is shown in Figure 4.8. The value of $Y_{S/lactate}$ used was 2 g lactose consumed / g lactate produced.

Fermentation performance. Parameters associated with fermentation performance were calculated from experimental data, as well as from the simulated results derived from the applied models for EPS, lactate and galactose formation (Table 4.2) (maximum product titre, yield of product on biomass, and volumetric and specific rates of product formation). Specific yields and rates of production were calculated at 20 h for EPS production, and at 24 h for lacatate and galactose, as these times corresponded to the point at which the respective products were at a maximum during the time course of the fermentations.

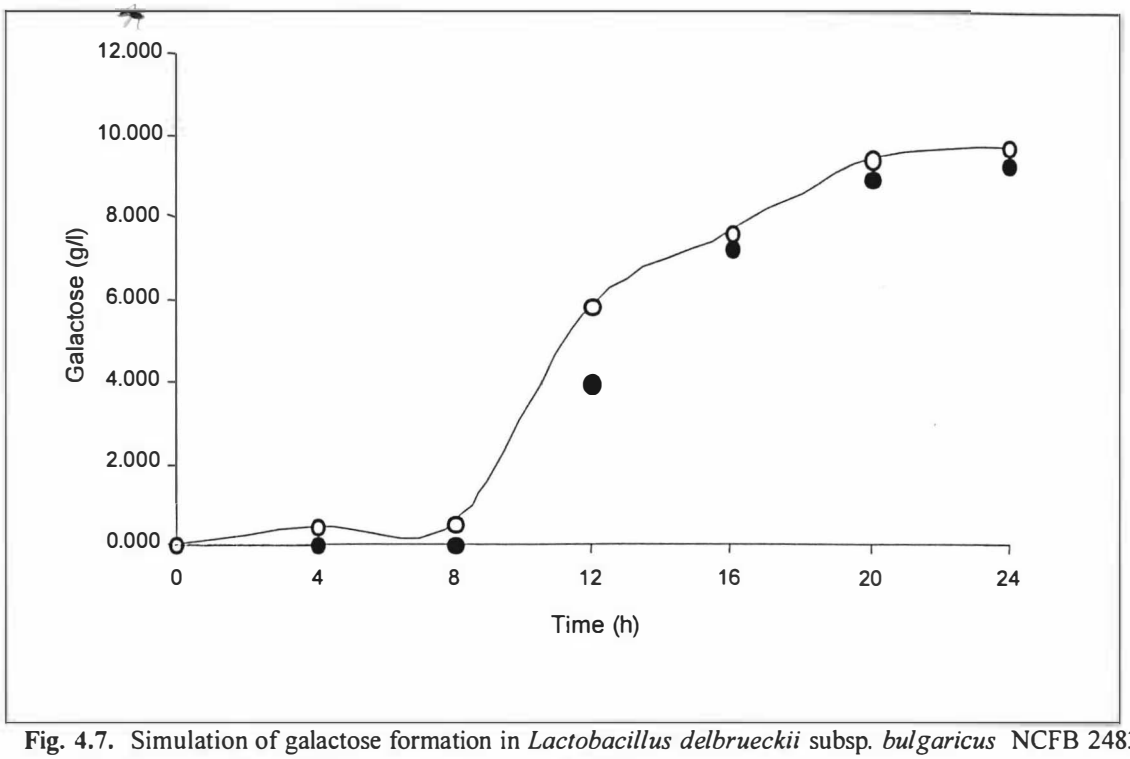

Fig. 4.7. Simulation of galactose formation in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 grown in KM-l medium at 37°C; Actual galactose titre (•); Simulated galactose titre (o).

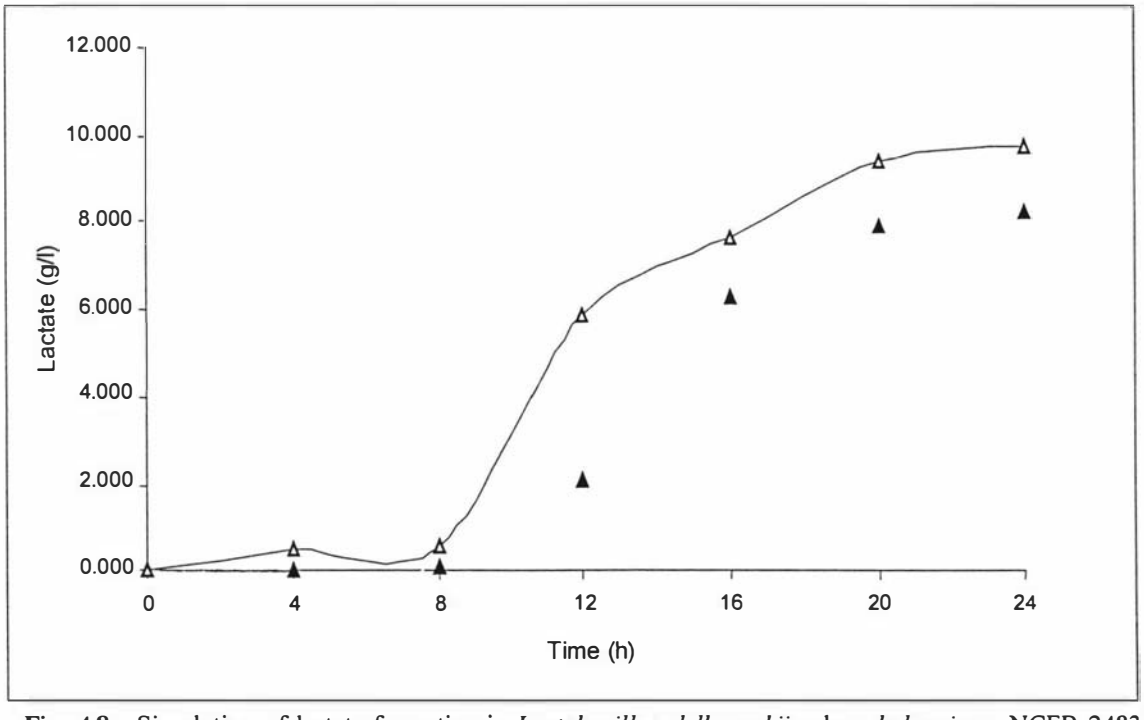

Fig. 4.8. Simulation of lactate formation in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 grown in KM-l medium at 37°C; Actual lactate titre (▲); Simulated lactate titre (Δ).

Table 4.2 Experimentally derived and simulated yield coefficients and rates of production of EPS, lactate, and galactose produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 grown in KM-l medium at 37°C.

EPS	EPS_{max} (g/l dextran equivalents)	$Y_{EPS/X}$ (g EPS/g DCW)	r_{EPS} (g/l/h)	$r_{EPS\ (specific)}$ (g/gX/h)
Experimental	0.16	0.19	0.01	0.01
Simulated	0.17	0.21	0.01	0.01
Lactate	$Lactate_{max}$ (g/l)	$Y_{Lactate/X}$ (g Lactate/g DCW)	$r_{lactate}$ (g/l/h)	$r_{lactate\ (specific)}$ (g/gX/h)
Experimental	8.227	10.39	0.34	0.43
Simulated	9.783	11.87	0.41	0.49
Galactose	$Galactose_{max}$ (g/l)	$Y_{Galactose/X}$ (g Galactose/g DCW)	$r_{galactose}$ (g/l/h)	$r_{galactose\ (specific)}$ (g/gX/h)
Experimental	9.342	11.80	0.39	0.49
Simulated	9.783	11.90	0.41	0.50

DISCUSSION

EPS production in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 is growth-associated (Figures 4.1 and 4.4), corresponding with previous findings in *Lb. delbrueckii* subsp. *bulgaricus* (Grobben *et al.*, 1995), and is consistent with the nature of EPS production in thermophilic LAB in general (De Vuyst *et al.*, 1998). The maximum titre of EPS produced (160 mg/l) was higher in comparison to the 140 mg/l (approximate) determined by Toba *et al.* (1992) for *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 grown in skim milk, but using ultrafiltration to separate the polysaccharide prior to analysis by the phenol-sulphuric acid method. In the present study, the reduction in EPS titre after achieving a maximum level at 20 h fermentation time can most probably be ascribed to the action of glycohydrolases, as was found by Gassem *et*

al. (1997) and Petry *et al.* (2000) in *Lb. delbrueckii* subsp. *bulgaricus*, and the cessation in biomass production. Biomass measurements correlated well with microscopic cell counts (Figure 4.2), reflecting the inhibitory effect on growth of lactic acid accumulation, and nutrient limitation (Figure 4.1). Carbon flux after the 20 h period is therefore almost exclusively diverted to galactose, lactate formation (Figure 4.1) and cell maintenance.

Metabolite production in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 follows a pattern of primary kinetics, characterized by biosynthesis of the metabolites commencing almost simultaneously with growth, a maximum rate of production during the exponential phase of growth, approaching a maximum near the end of this growth period (Figure 4.1). This pattern is described for *Lactobacillus bulgaricus* CRL 420 by Manca de Nadra *et al.* (1985), for *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 by Grobben *et al.* (1995), and in *Lb. delbrueckii* subsp. *bulgaricus* RR by Kimmel and Roberts (1998).

Lb. delbrueckii subsp. *bulgaricus* NCFB 2483 can be described as possessing a homofermentative metabolism, as no heterofermentative metabolites were detected upon analysis (*viz.* acetate, acetaldehyde). Small quantities (< 0.500 g/l) of ethanol were detected by column chromatography in the early growth phase, however this finding remains unconfirmed.

Use of the logistic equation provided a satisfactory model to describe the kinetics of biomass formation in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 (Figure 4.3). This equation has been used successfully to model the individual patterns of growth in a mixed culture of *S. thermophilus* and *L. bulgaricus* (Berkman *et al.*, 1990) in a medium consisting of lactose, peptone, and yeast extract, and for *S. thermophilus* LY03 by Degeest and De Vuyst (1999). Indeed, this equation is well known, and has been used to describe the growth of LAB by Mercier *et al.* (1992), Parente *et al.* (1994) and Lejeune *et al.* (1998).

The Luedeking-Piret equation, as was developed originally for the formation of lactic acid by *Lb. delbrueckii* (Luedeking and Piret, 1959), and used by Weiss and Ollis (1980) to describe the kinetics of polysaccharide production by *Xanthomonas campestris* B-1459, was applied to simulate EPS production by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 (Figure 4.5). The simulation was undertaken over the first 20 h of fermentation, as it was evident that the EPS became degraded after this time. Different kinetics would hence apply after this point. Modelling of this phase was not attempted due to the lack of sufficient data points during the period of degradation. The greatest point of diversion between the experimentally derived values for EPS production and the simulated values are at 8 h into the fermentation period

(Figure 4.5), where it appears that EPS production and biomass formation depart from the defined relationship. The model predicts higher EPS_{max} and $Y_{EPS/X}$ values than those obtained experimentally (Table 4.2). This could be ascribed to the inhibitory effects of lactic acid formation on biomass production and EPS–producing ability during the latter part of the fermentation. Predicted and experimental volumetric and specific rates of EPS production are however similar (Table 4.2).

Lactose, once taken up by the cell, is split intracellularly in an equimolar ratio by a ß-galactosidase to glucose and galactose. Glucose is metabolized by the Embden-Meyerhof Pathway and galactose is excreted from the cell (Kandler, 1983; Zourari *et al.*, 1992). A linear relationship between galactose efflux and lactose consumed is shown in Figure 4.6. The yield coefficient for galactose, $Y_{S/gal}$ (theoretically, 2 g lactose consumed / g galactose produced) may be used to model galactose production (Figure 4.7). The simulated profile of galactose formation is similar to that obtained experimentally. This is consistent with the nature of the mechanism by which lactose is split into equimolar quantities of glucose and galactose, with galactose being exported from the cell via an antiport system. The marginally lower galactose levels relative to the modelled levels throughout most of the time course of the fermentation are suggestive of the possibility that not all galactose is exported form the cell, and that a low level of flux to other metabolites might exist. Deviation from the theoretical value of $Y_{S/gal}$ has been reported by Degeest and De Vuyst (1999) in *S. thermophilus* LY03. This was explained by the hypothesis that not all the galactose was exported from the cell, but that some was converted to lactic acid. It is hence not inconceivable that a similar partial utilization of galactose might exist in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483. Marshall *et al.* (2001) have reported assimilation of galactose into EPS in galactose–negative strains of *Lb. delbrueckii* subsp. *bulgaricus*. Higher predicted specific yields and rates of galactose production relative to the experimental results (Table 4.2) lend credence to this proposition, however nutrient limitation and inhibitory stress by lactate accumulation may also afford a reason for differences between the experimental and modelled results.

In silico levels of lactic acid production (Figure 4.8) were consistently higher than the experimentally derived values. This can principally be ascribed to the fact that, whilst lactose is split on an equimolar basis to galactose which is exported, and glucose, the amount of lactose consumed per lactate produced is in reality higher than the theoretical value of 2 g/g. Apart from producing lactate, carbon from glucose in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 is diverted for use in building cell mass and for producing exopolysaccharide. The model hence does not take into account this additional carbon requirement, and assumes that the metabolic

destination of all glucose carbon is lactate. The corresponding differences between experimentally derived and simulated specific yields and rates of lactate production (Table 4.2) support this hypothesis, however, it is also likely that lactate accumulation exerted an inhibitory effect on the cell metabolism.

CONCLUSION

Lb. delbrueckii subsp. *bulgaricus* NCFB 2483 is a homofermentative organism following a primary kinetic pattern with respect to the production of the metabolites, lactate, galactose and EPS. After cessation of the growth period, EPS titres diminish, possibly as a consequence of degradation by glycohydrolases. Kinetic models which were applied to describe the formation of these metabolites suggest that whilst the bulk of galactose is exported unutilized via a lactose/galactose antiport, there exists the possibility that some galactose may be diverted for utilization intracellularly for lactate and EPS production. Intracellular glucose, emanating from the splitting of lactose, remains however, the main source of carbon for lactate, EPS, and biomass formation.

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CHAPTER 5

SCREENING AND SELECTION OF EXOPOLYSACCHARIDE-PRODUCING STRAINS OF *LACTOBACILLUS DELBRUECKII* SUBSP. *BULGARICUS*

ABSTRACT

An improved exopolysaccharide-overproducing strain of *Lactobacillus delbrueckii* subsp. *bulgaricus* was derived by chemical mutation and selection, for the purpose of selecting a strain suitable for metabolic investigation. Initial screening of the chemically-induced mutant pool relied primarily on the selection of strains with raised levels of lactic acid and reduced biomass formation. Supporting selection criteria used were ropiness and colonial mucoidy. Final screening of candidate strains was undertaken in batch culture, with evaluation based upon measurement of EPS produced in a semi-defined medium. The derived mutant was demonstrated to produce a 35 % improvement in specific EPS yield relative to the parent strain.

INTRODUCTION

Exopolysaccharides (EPS) produced by lactic acid bacteria (LAB) possess the possibility of replacing stabilizers and thickeners currently produced commercially by non-food-grade bacteria. Over the past number of years, various studies have concentrated upon understanding the biochemistry and genetics of EPS production in LAB, so that rational strategies can be developed for the improvement of EPS yield and the design of "tailor-made" polysaccharides. Metabolic engineering strategies that target increasing metabolic flux to EPS should include EPS formation pathways (de Vos, 1996). It has been suggested that a potential controlling factor in EPS biosynthesis is the availability of sugar nucleotides which are necessary for the construction of the polymers (Boels *et al.*, 2001).

In the present investigation, a stable, chemically-induced mutant of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 was isolated which had an enhanced specific yield of EPS, relative to the parent strain. This mutant was generated for future studies in order to compare the anabolic processes of sugar-nucleotide synthesis between the two strains. Primary screening was based upon the rationale, that any enhancements in the EPS-synthesizing pathway would be needed to be supported by a strain which produced sufficient ATP for the synthesis of sugar nucleotides, and the ability to adequately control the redox balance of the cell. The strains obtained for

further screening on the basis of enhancement of EPS yield, would exhibit a global enhancement in glycolysis, and demonstrate a mucoidy and/or ropiness.

Chemical mutagenesis of LAB has been undertaken in a number of instances using N-Methyl-N'-nitro-N-nitrosoguanidine (NTG). Kuila *et al.* (1971) used NTG to induce an increase in the formation of diacetyl and acetaldehyde in *Streptococcus diacetylactis*. Similarly, Piatkiewicz *et al.* (1981) effected an increase in acetoin and diacetyl in *Streptococcus lactis* subsp. *diacetylactis*. Miyamoto *et al.* (1983) obtained genetic variants of *Lactobacillus casei* subsp. *alactosus* which produced increased quantities of flavour compounds and lactic acid in soya milk. Renault and Heslot (1987) generated mutants which were defective in the structural gene of malolactic enzyme catalysing the conversion of L-malate to L-lactate and carbon dioxide in *Streptococcus lactis*, and stable galactose-fermenting strains of *Streptococcus thermophilus* were obtained by Benateya *et al.* (1990). β-Galactosidase-overproducing strains of *Lb. delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, *Bifidobacterium breve*, and *Bifidobacterium longum* were obtained by Ibrahim and O'Sullivan (2000). Few reported cases exist, however, of attempts to induce EPS overproduction in LAB using chemical mutagenesis. This paper describes a rational strategy to chemically induce an increase in specific EPS yield in *Lb. delbrueckii* subsp. *bulgaricus*.

MATERIALS AND METHODS

Media. The medium used was according to Kimmel and Roberts (1998) which was modified by replacement of the glucose component with lactose and consisted of lactose, 20.0 g/l; yeast nitrogen base (without amino acids and ammonia), 5.0 g/l; bacto casitone, 20.0 g/l; sorbitan monoleate ("Tween 80"), 1.0 g/l; dipotassium phosphate (K_2HPO_4), 2.0 g/l; magnesium sulphate ($MgSO_4$.7H2O), 0.10 g/l; manganese sulphate ($MnSO_4$.4H2O), 0.05g/l; ammonium citrate, 2.0 g/l; sodium acetate, 5.0 g/l. In all instances, the medium was prepared in separate, double–strength aliquots of lactose and the remainder of the nutrients as described above. After steam–sterilization, the aliquots were allowed to cool, and pooled, achieving the desired concentration of medium constituents. Where solid medium was prepared, 15.0 g/l of agar was used.

Bacterial strains. The strain of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 (NCIMB 702483) was obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland, and preserved as a cell bank in 1.0 ml aliquots at –80°C. Mutants selected for batch culture testing were preserved in the same fashion.

Chemical Mutation. The method used was a modification of that described by Monnet and Corrieu (1998). A mid-log phase culture of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483, washed and suspended in 0.1 M McIlvaine's Buffer (sodium citrate) (pH 6.2), was treated with N-Methyl-N'-nitro-N-nitrosoguanidine (250 μg/ml), and incubated at 37°C for 60 minutes. A 2-3 log reduction in cell number was desired. The mutated cells were subsequently washed three times in 0.1 M McIlvaine's Buffer, and resuspended in the modified medium of Kimmel (see above) for a further 3 h period of incubation at 37°C to resuscitate the cells prior to storage in 4 ml aliquots at –80°C. The individual mutants were isolated for colony-picking by serial dilution.

Culture conditions. For the culture of the parent (NCFB 2483) and mutant strains in 96-well format microtitre plates, individual colonies were inoculated into wells containing 250 μl medium, and the plates incubated under slow agitation (100 rpm) for a period of 24 h under anaerobic conditions. This was achieved by incubating the microtitre plates in sealed plastic bags, inflated with nitrogen gas.

Batch-culture testing was undertaken in 250 ml volumes in shake bottles (Duran, Schott), inoculated from the working cell banks, as described above. The cultures were incubated in an orbital incubator shaker at a slow rotational speed (100 rpm) which was sufficient to keep the culture homogenous, and at a temperature of 37°C for a period of 24 h. Sample aliquots (15 ml) were withdrawn aseptically at 4 hourly intervals for the determination of biomass and sugar conversion to extracellular metabolites. In instances where analyses could not be immediately undertaken, samples were stored in pre-sterilized bottles at –20°C.

Incubation of cultures on solid medium was undertaken at 37°C under anaerobic conditions for a period of 48 h.

Screening techniques and analyses. Growth was monitored by absorbance measurement at 650 nm. Biomass was determined from a standard curve relating absorbance at 650nm to washed dry cell weights. Plates for colony-picking were obtained by serial dilution.

Colonies were picked from plates with a spread of 90–100 colonies. Replica-plating was achieved using a sterilizable 96-pin inoculation tool, custom manufactured to transfer droplets of cell-suspension medium from the microtitre plates to 15 cm-diameter agar plates and to additional microtitre plates for the testing of the presence of acetoin.

Primary screening for EPS formation was attempted by the incorporation of 80 mg/l ruthenium red dye into agar medium (Bouzar *et al.*, 1996).

Acidifiability tests were undertaken according to a modification of the method described by Venus *et al.* (1991), using an OPTImax tunable Microplate reader (SOFTmax Pro software). After measurement of the absorbance at 650 nm after 24 h growth in the microtitre plate wells, 5 µl of a mixture of 0.5 % aqueous bromocresol purple and 0.5 % aqueous bromocresol green solutions was added to each well. After agitation, the absorbance was measured at 600 nm. The acidification was expressed as a ratio between the absorbance at 600nm relative to the absorbance at 650 nm.

The presence of acetoin, was tested using the Voges-Proskauer reaction according to the method of O'Meara (1931) and as described by Eddy (1961). O'Meara's reagent (5 µl) was added to 250 µl cultures grown for 24 h in microtitre plates.

Mucoidy of colonies was determined by visual appearance, and the ropiness of colonies and liquid broth were determined by testing with a sterile toothpick.

For the measurement of extracellular polysaccharides, aliquots (100 µl) of whole broth were subjected to a two–fold precipitation with chilled ethanol (2.9 ml distilled H_2O and 7 ml 99.7 % ethanol) for 24 h periods at 4°C. The precipitate was obtained by centrifugation (35 850 g, 40 min., 4°C) (Sorvall RC5C–SS34 rotor) and resuspended in 1.0 ml distilled water. The total sugar concentration in the resuspended sample was measured according to the method of Dubois *et al.* (1956), using dextran as the standard.

Lactose depletion, and galactose and lactate formation in shake-bottle cultures was measured by HPLC (Waters Alliance 2690, coupled to a Waters 2410 Differential Refractometer, and Waters 2487 UV spectrophotometer). The compounds were detected using an Aminex HPX-87H, 300 x 7.8 mm column (Biorad, Richmond, CA), according to the method as described in Ross and Chapital (1987). The column temperature was maintained at 40°C, and the flow rate at 0.6 ml. min^{-1}. Broth samples were clarified by centrifugation at 16 000 g for 15 minutes prior to dilution in distilled and filtered water (MilliQ), prior to analysis. External standards were prepared for lactose, galactose, and lactic acid. All analyses were undertaken in duplicate.

RESULTS

Chemical mutation. A mutant "pot" generated by treatment of a stock of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 with N-Methyl-N'-nitro-N-nitrosoguanidine was used for assessment of traits associated with raised EPS formation. A 10^{-7} dilution derived from a serial dilution of the mutant mixture yielded approximately 90 colonies per agar plate, after incubation at 37°C for 24 h under anaerobic conditions. These colonies were toothpicked into wells of medium in microtitre plates, as well as cross-inoculated onto replica agar plates.

Screening tests for EPS, mucoidy, ropiness, and acetoin production. The discriminatory value of the methods to test for mucoidy, and ropiness of the bacterial colonies or broth cultures was limited, as any differences in EPS production (or alternatively structure) were relatively small. The methods served to confirm these characteristics in strains selected on the basis of biomass and acid production.

Neither the NCFB 2483 parent strain nor mutant strains demonstrated acetoin production when tested with O'Meara's reagent.

In order to test the effectiveness of the rhuthenium red dye in the medium used, a strain of *Lb. delbrueckii* subsp. *bulgaricus* which was known to produce significantly less EPS than the NCFB 2483 strain was cultured on the solid medium. The strain producing negligible EPS did not take up the dye, whereas the NCFB 2483 strain showed a light pink colour. These results concur with those of Bouzar *et al.* (1996) in screening EPS production in *Lb. delbrueckii* subsp. *bulgaricus* CNRZ 1187 and two colonial variants, but differ, however, from the claims of Gancel *et al.* (1988) in respect of a ropy *S. thermophilus* strain. Identical testing using higher- and lower- EPS-producing strains of *S. thermophilus* and *Lactococcus lactis* did not show any conclusive differences in colour of the colonies. Due to the apparent insensitivity of this method as a screening test, its use was not pursued.

Analysis of biomass and acidification in microtitre plates. For comparative purposes, acidification and absorbance analyses were undertaken on the *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 parent strain. A 250 ml culture of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 was prepared from the stock culture in modified Kimmel's medium, as described in the Materials and Methods section. Colonies (64) cultured on solid agar medium from a dilution of the culture harvested at 16 h were toothpicked into 250 µl volumes of medium in the microtitre wells, and incubated under anaerobic conditions and gentle agitation at 37°C for 24 h. A

distribution of the acidifying ability and absorbance values of the NCFB 2483 strain is depicted in Figure 5.1.

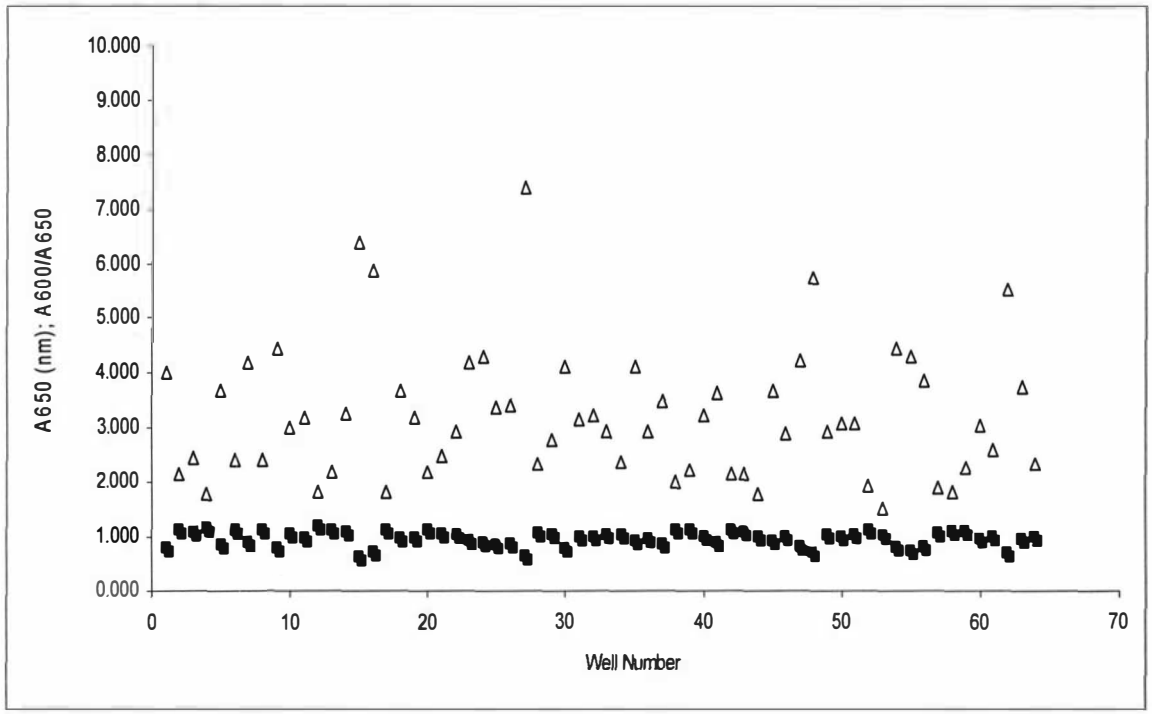

Fig. 5.1. Distribution of absorbance readings related to cell concentration and acidifying ability of *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 (A_{600} / A_{650}) cultured for 24 h in microtitre plate wells. The absorbance corresponding to cell concentration (■) was measured at 650 nm. Acidifying ability (Δ) was quantified by the ratio of A_{600} of the broth with bromocresol purple and bromocresol green added, to the A_{650} measurement.

Whilst a small number of colonies of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 had a low acidifying ability over the 24 h time period (i.e. high A_{600} / A_{650} values) (Figure 5.1), the mean value and standard deviation derived from the 64 colonies was 3.2 ± 1.2 at a 95 % level of confidence. In the same manner, 1 200 individual mutants were screened within a relatively short space of time. Strains were sought which formed less biomass than the parent strain, but which produced more acid per cell mass. As the A_{600} value diminishes with increasing acid production, mutants with an A_{600} / A_{650} ratio which was significantly lower than that of the parent strain (3.2 ± 1.2) were selected for further screening. Typical results of a spread of A_{650} values and A_{600} / A_{650} ratios for mutants from individual colonies are depicted in Figure 5.2.

From the 1 200 mutants screened, 9 were selected on the basis of their lower biomass and higher acidifying ability relative to the parent strain, mucoid appearance of colonies, and ropiness in broth. Upon re-culturing of these strains, seven retained their viability.

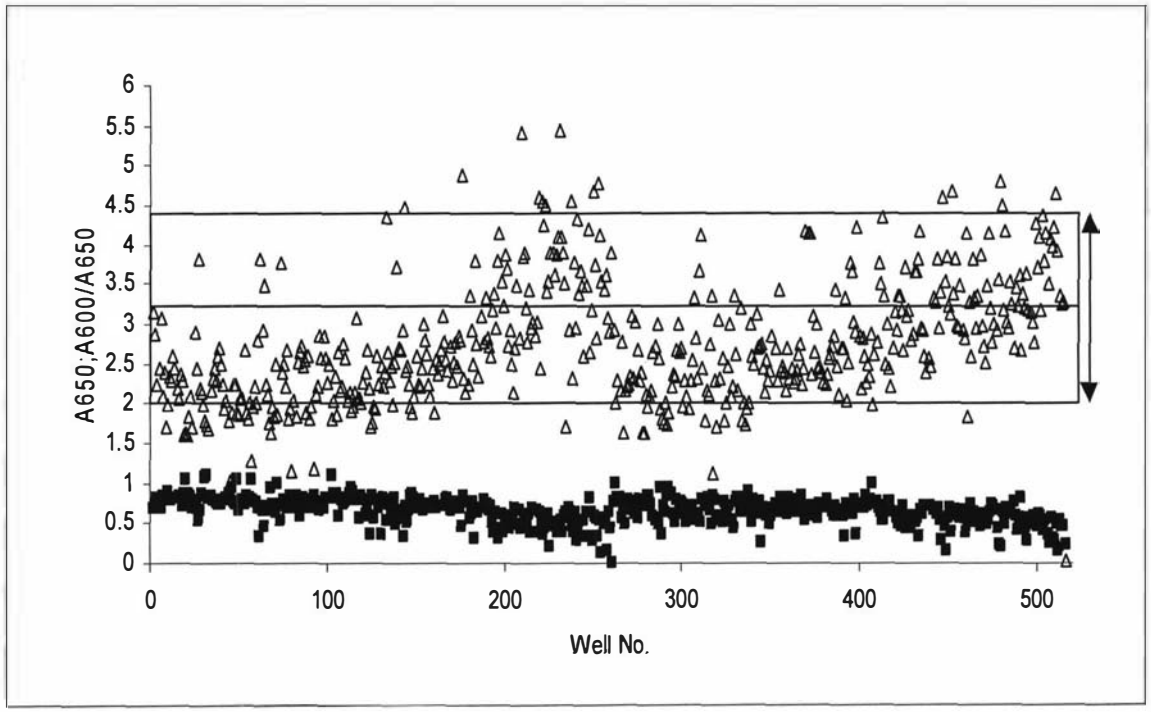

Fig. 5.2. Distribution of absorbance readings related to cell concentration and acidifying ability of *Lactobacillus delbrueckii* subsp. *bulgaricus* mutants cultured for 24 h in microtitre plate wells. The absorbance corresponding to cell concentration (■) was measured at 650 nm. Acidifying ability (Δ) was quantified by the ratio of A_{600} of the broth with bromocresol purple and bromocresol green added, to the A_{650} measurement. The mean ± SD of the A_{600} / A_{650} ratio for the parent strain is shown for comparison.

Batch fermentation testing. Six candidate strains were selected from the initial screening of 1 200 mutants for further evaluation. The strains were cultured in 250 ml medium for a period of 24 h, and analysed for EPS and lactate production. Biomass levels were all significantly lower, and specific lactate yields of all the mutant strains significantly higher than that of the parent (NCFB 2483) (Figure 5.3). Strains A2483M, B2483M, E2483M, and F2483M exhibited significantly higher specific EPS yields ($Y_{p/x}$) after 24 h incubation, than that of the parent strain ($Y_{p/x}$ = 0.20 ± 0.01). The E2483M strain was selected for further evaluation on the basis of having the highest $Y_{p/x}$ value for EPS (0.26 ± 0.003) amongst the selection of mutants tested. The performance of the E2483M mutant was compared directly with the parent NCFB 2483 parent strain over the time course of 24 h batch fermentations. The comparisons were performed in triplicate (Figures 5.4 and 5.5).

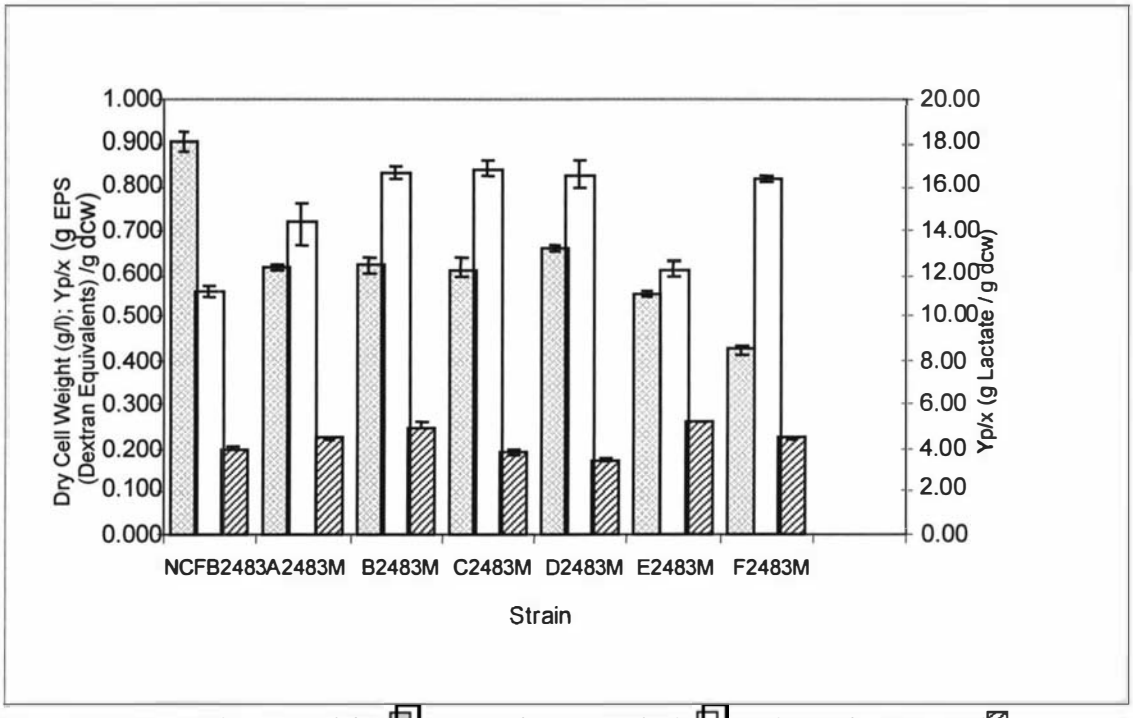

Fig. 5.3. Biomass (dry cell weight) ▣, specific lactate yield ▣, and specific EPS yield ▨ of mutants derived from *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483. All values the mean of three fermentations. Error bars represent standard deviations.

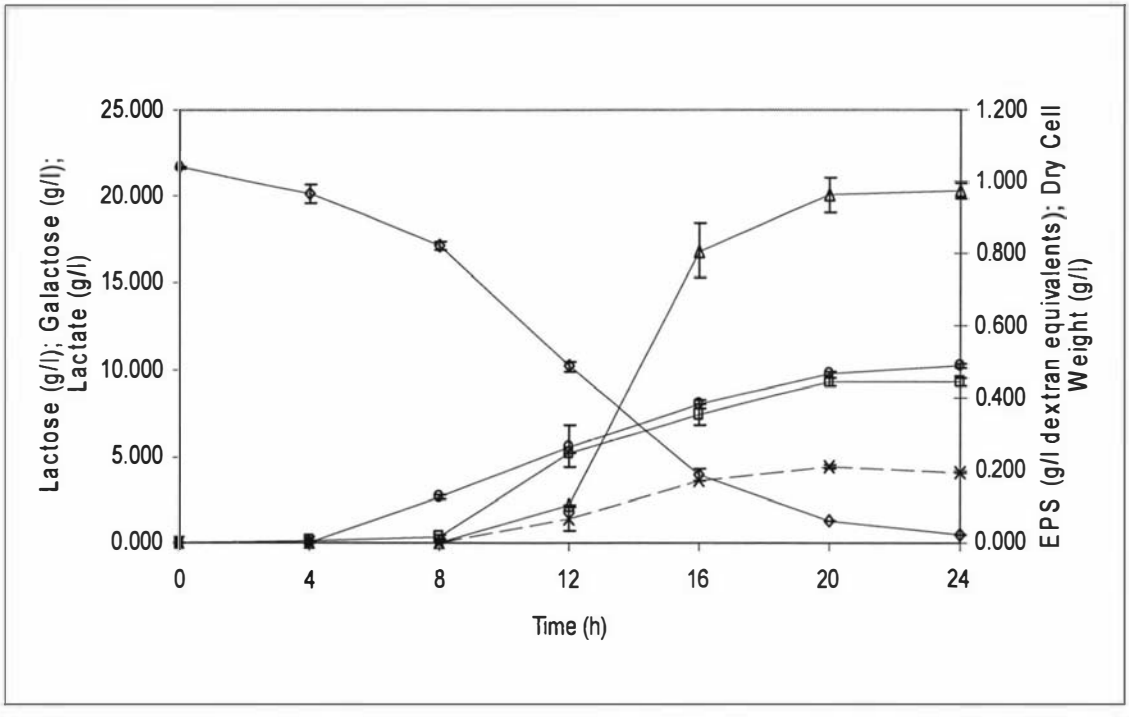

Fig. 5.4. Batch fermentation profile of *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 (parent strain). Residual lactose ◊; Biomass (Dry cell weight) Δ; Galactose ○; Lactate □; EPS x. (Mean values). Error bars represent standard deviations.

Part of Chapter 2 and general aspects of this thesis were submitted for oral presentation at the 8th International Pacific Rim Biotechnology Conference (November 17-20, 2002), Auckland, New Zealand.

EXOPOLYSACCHARIDE PRODUCTION IN LACTIC ACID BACTERIA

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Certain lactic acid bacteria (LAB) produce exopolysaccharides (EPS) which beneficiate fermented dairy products such as yoghurt by improving the rheology and mouthfeel of these foodstuffs, as well as providing health benefits. These effects depend upon the amount and compositional structure of the polysaccharides produced. No clear-cut fundamental principles exist for the metabolic engineering of LAB to overproduce EPS. Conventional methods of strain selection are likely to prove insufficient to redirect carbon flux toward the EPS-synthesizing pathways. Internationally, much of the research activity undertaken to date has been focused upon elucidating the genetic organization of EPS-encoding genes, as well identifying the enzymatic mechanisms associated with EPS formation. Much more information is needed about the regulation of the EPS-synthesizing pathways before a significant impact will be made upon productivity. A variety of limiting steps in the EPS–synthesis pathway, as well as the EPS export mechanisms have to be taken into account in developing strategies for the enhancement of EPS production, as has been demonstrated in a strain of *Lactobacillus delbrueckii* subsp. *bulgaricus*. In general, due to the inefficient conversion of carbohydrates by LAB, it is unlikely that EPS can be produced in sufficient quantities for use as food additives in the manner, for example, of xanthan production by *Xanthomonas campestris*. The most significant areas of improvement will most likely be achieved in their *in situ* production in fermented dairy products, and functional enhancements by structural modifications.

* Presenting Author

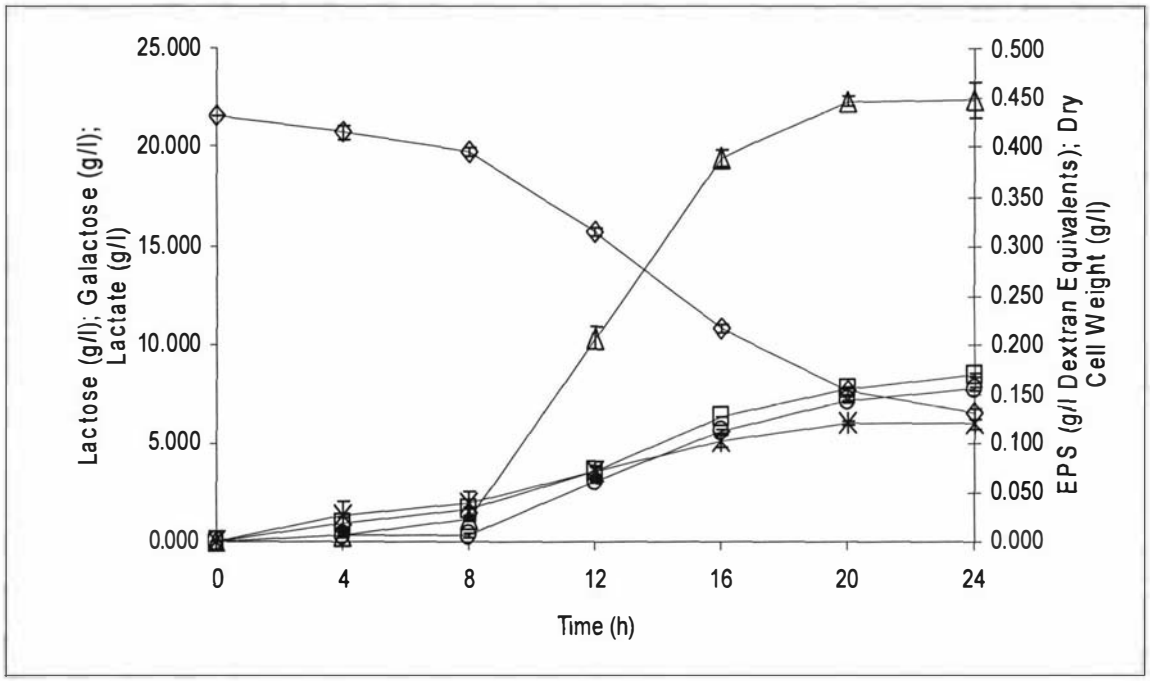

Fig. 5.5. Batch fermentation profile of *Lactobacillus delbrueckii* subsp. *bulgaricus* mutant strain E2483M. Residual lactose ◊; Biomass (Dry cell weight) Δ; Galactose ○; Lactate □; EPS *. (Mean values). Error bars represent standard deviations.

Specific yields of EPS relative to biomass of the parent and E2483M strains at 24 h were similar to those achieved in the preliminary batch-screening (a $Y_{p/x}$ value of 0.20 g/g for the parent strain vs. 0.27 g/g for the E2483M strain, representing a 35% improvement in specific EPS yield). The E2483M strain thus proved to be suitable for further investigation into the metabolism of EPS production.

DISCUSSION

The microtitre plate-based system with automatic analysis afforded a relatively rapid means of selecting mutant strains of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 which had a higher acidifying ability than the parent. The system was similar to a method described by Venus *et al.* (1991), differing in respect of the medium used. Similar small-scale fermentations in microtitre plates have been used to measure growth and lactate production by *Lactobacillus* LB-WT (Patnaik *et al.*, 2002). The variation in acidification levels of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 (Figure 5.1) can be partially explained by the influence of biomass, the tendency of the cells to form pellets in the microtitre wells, and natural variation amongst the population of cells. It was thus important to establish the range of variation in acidification of the parent strain in order for significant differences in mutant strains to be identified.

The medium used was effective for purposes of incorporation of dyes. The frequently-used tests for mucoidy and ropiness were limited in their ability to discriminate smaller differences in EPS production, and were hence used as a confirmatory test. The use of O'Meara's reagent was used to detect if any carbon in the mutants was diverted towards acetoin, diacetyl or 2,3-butanediol.

It has been proposed that mutants which are defective in LDH would allow the production of increased amounts of alternative catabolites in LAB (Montville *et al.*, 1988). These cell products would however most likely be confined to products produced from the breakdown of pyruvate, such as diacetyl and acetoin. Kuila and Ranganathan (1978) induced mutations in *Streptococcus lactis* subsp. *diacetylactis* with raised levels of diacetyl and acetaldehyde. LDH-deficient mutants of *Streptococcus mutans* were generated which produced elevated levels of acetoin (Hillman *et al.*, 1987). Whilst this strategy is useful for the diversion of carbon flux to catabolic products, a rerouting of carbon away from glycolysis towards the production of anabolic products such as EPS would, in the case of *Lb. delbrueckii* subsp. *bulgaricus*, be hampered by the associated reduction in the formation of ATP necessary for the formation of the sugar-nucleotide precursors of EPS. In the present study, it was proposed that selection of mutants which produced an excess supply of ATP via glycolysis while retaining their ability to control the redox potential via the pyruvate-lactate reaction would be more suited to producing EPS. Some support for this approach was to be found in the findings of Bouzar *et al.* (1996) who determined that colonial variants of *Lb. delbrueckii* subsp. *bulgaricus* CNRZ 1187 which formed more EPS, produced slightly more lactate than those that produced less EPS over 24 h of fermentation. A lower biomass than the parent strain and hence less diversion of sugar nucleotides for cell wall synthesis was seen to be an additional desirable characteristic. As expected, none of the mutants selected in this study produced products other than lactate from the pyruvate branch-point, as the cells would not need to meet any ATP shortfall via a heterofermentative route (Stephanopoulos *et al.*, 1998).

The second tier of fermentation-screening confirmed the effectiveness of the microplate screen in the selection of mutants with raised acid production and reduced flux of carbon to biomass formation (Figure 5.3). The mutant "E2483M" proved to have stable characteristics in terms of enhanced EPS production (Figure 5.5).

The screening procedure described above, proved to be an effective strategy for isolating chemically induced mutants with a raised carbon flux to EPS, for the purposes of metabolic studies of EPS-overproduction.

CONCLUSION

Mutants of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 with a presumptively enhanced EPS production than the parent strain were selected in the first instance on the basis of enhanced glycolytic activity and the formation of lactate, and reduced biomass. A second tier of fermentation-screening of mutants with these characteristics, yielded four mutants with a raised level of specific EPS production than the parent strain. Final fermentation testing of a selected mutant confirmed a stable trait of enhanced specific EPS production, suitable for further study of EPS metabolism.

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CHAPTER 6

STRESS-INDUCED METABOLIC SHIFT IN *LACTOBACILLUS DELBRUECKII* SUBSP. *BULGARICUS*: REDUCTION IN WATER ACTIVITY ALTERS PRODUCTION OF EXPORTED METABOLITES

These results were presented as an oral presentation at the First International Symposium on Exopolysaccharides from Lactic Acid Bacteria: from Fundamentals to Applications (May 16 – 19, 2001), Brussels, Belgium.

ABSTRACT

The addition of humectants to a semi–defined fermentation medium was demonstrated to induce changes in the production of exported metabolites, suggesting an altered metabolic flux distribution through contributing pathways in an exopolysaccharide-producing strain of *Lactobacillus delbrueckii* subsp. *bulgaricus*. Reduction of the water activity from a value of 0.99 to 0.98 at mid–exponential growth phase by the addition of NaCl resulted in raised specific yields ($Y_{p/x}$) and ($Y_{p/s}$) of exopolysaccharides (EPS) and altered levels of lactate and galactose produced in the fermentation medium. A reduction in water activity to 0.96 did not significantly improve specific yields of the polysaccharides, but $Y_{p/x}$ for lactate was raised relative to the control fermentations. Dry cell weight titres were diminished at both elevated salt levels. The agent of metabolic change was assigned primarily to that of osmotic stress, an effect that was confirmed by the addition of sorbitol to the fermentation medium. The metabolic response to alterations in water activity in this strain of *Lb. delbrueckii* subsp. *bulgaricus* provides a potential means of creating conditions under which the contributions of enzyme activities and metabolite levels to whole–pathway fluxes may be assessed. These effects will be investigated in conjunction with growth of the organism in continuous culture. This information is valuable in directing metabolic engineering work aimed at altering the production of EPS and lactic acid, products that can influence the textural and flavour quality of a fermented milk product such as yoghurt.

INTRODUCTION

Certain strains of *Lb. delbrueckii* subsp. *bulgaricus* possess a ropy characteristic by virtue of an EPS which is produced. The use of such strains in milk products such as yoghurt imparts a thicker body and higher viscosity, enhances texture, and prevents syneresis (Escalante *et al.*, 1998; Wacher-Rodarte *et al.*, 1993; Cerning *et al.*, 1986). In addition, acidification is an important characteristic of the organism. These strains of *Lb. delbrueckii* subsp. *bulgaricus,*

being members of the lactic acid bacteria (LAB), could thus prove valuable vehicles for the generation of high quantities of these polysaccharides, especially as they are GRAS (Generally Recognized As Safe). In addition, the metabolism of LAB is relatively uncomplicated, and there is virtually no overlap between carbon (energy) and nitrogen metabolism (Hugenholtz and Kleerebezem, 1999). These features render these organisms as potentially useful for metabolic engineering, as the possibility exists that they can be modified without influencing growth (de Vos, 1998). At the present time, however, production levels of EPS in LAB are very low, and are a barrier to commercial exploitation of such strains (Van Kranenburg *et al.*, 1999). Overproduction of EPS through metabolic engineering requires a rational strategy involving an understanding of pathways to be manipulated, their fluxes, and control factors (Kleerebezem *et al.*, 2000).

In this study an attempt was made to induce a higher productive capacity for EPS production by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 by inducing stress conditions. Milk, the natural habitat for *Lb. delbrueckii* subsp. *bulgaricus* has a naturally high a_w and the organism has adapted to this condition; low water activities represent a highly stressful condition for yoghurt cultures (Fajardo-Lira *et al.*, 1997). If enhanced EPS production could be achieved by reducing water activity (a_w) in a semi-defined fermentation medium, further work can be undertaken to identify metabolic changes associated with the overproduction of the polymer in a perturbed metabolic state. Sodium chloride (NaCl) and sorbitol were employed as humectants in order to elicit the desired changes.

MATERIALS AND METHODS

Bacterial strain, culture medium, and growth measurement. *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 (NCIMB 702483) was obtained from the National Collection of Industrial and Marine Bacteria (Aberdeen, Scotland). A working cell bank consisting of 1.0 ml aliquots was prepared from the lyophilized culture, harvested at late log phase, and stored at -70°C. For all experiments, the culture medium used was that described by Kimmel and Roberts (1998), with lactose substituted for glucose as the carbon source. The medium was adjusted to pH 6 prior to sterilization at 121°C for 15 minutes. The lactose component was sterilized separately from the remainder of the medium. Batch cultures (250 ml working volume) were performed in bottles (Duran, Schott), and incubated at 37°C with agitation at 100 rpm in an incubator shaker (Gallenkamp INR-250, 32 mm diameter of orbit). Inoculation was performed with a 1.0 ml seed culture from the working cell bank. All fermentations (three replicate fermentations per condition) were undertaken for a total period of 24h, with 15 ml samples removed aseptically in a sterile cabinet, at 4 hourly intervals. Growth was monitored

by absorbance measurement at 650nm, using a Pharmacia Biotech Ultrospec 2000 spectrophotometer. Dry cell weight (X) was determined from a standard curve relating absorbance at 650 nm to washed dry cell weights.

Analyses. Extracellular polysaccharides were subjected to a crude isolation procedure prior to analysis. Aliquots (100 µl) of whole broth underwent a two–fold precipitation with chilled ethanol (2.9 ml distilled H_2O and 7 ml 99.7 % ethanol) for 24 h periods at 4°C. The precipitate was recovered by centrifugation (35 850 g, 40 min., 4°C) (Sorvall RC5C–SS34 rotor) and resuspended in 1.0 ml distilled water. The total sugar concentration in the resuspended sample was measured according to the method of Dubois *et al.* (1956), with dextran as the standard. Lactose, galactose and lactic acid concentrations were determined in duplicate by HPLC (Waters Alliance 2690 Separations module). The HPLC system was coupled with a refractive index detector (Waters 2410) and UV spectrophotometer (Waters 2487). The compounds were detected using a single column (Aminex HPX-87H, 300 x 7.8 mm, Biorad, Richmond, CA), according to the method described in Ross and Chapital (1987). The column temperature was maintained at 40°C, and the flow rate at 0.6 ml. min^{-1}. Cell–free supernatant fractions were diluted with distilled and filtered water (MilliQ) prior to column analysis. Instrument, data accession, and processing methods were controlled using a Millenium® software system. External standards were prepared for lactose, galactose, and lactate determination (Sigma Chemical Co., St. Louis, MO).

Experimental conditions – adjustment of water activity (a_w). Quantities of sterilized NaCl solution were added aseptically to the fermentations after 12 h into the fermentation, thereby providing the culture adequate time to become established. Sufficient NaCl was added to achieve a_w values of 0.98 and 0.96, in comparison to the ambient a_w value of the medium used (0.99). Fermentations were performed in triplicate corresponding to each condition. Water activity was measured using a DECAGON (CX-2 upgrade) water activity meter (Decagon Devices Inc., Pullman, WA), which has an accuracy of ± 0.003, and thus able to distinguish between the water activities tested. The instrument was standardized with a saturated solution of NaCl.

In separate experiments, quantities of sterilized sorbitol were added such that identical a_w values were achieved in the fermentation broth as when the NaCl was added. A calibration curve relating the a_w of sorbitol supplemented fermentation medium to sorbitol concentration was prepared as a means to predict the necessary quantity of sorbitol to add. (A linear relationship was obtained relating "y" (a_w) to "x" (concentration of sorbitol as % w/v): $y = -0.0012x + 0.992$).

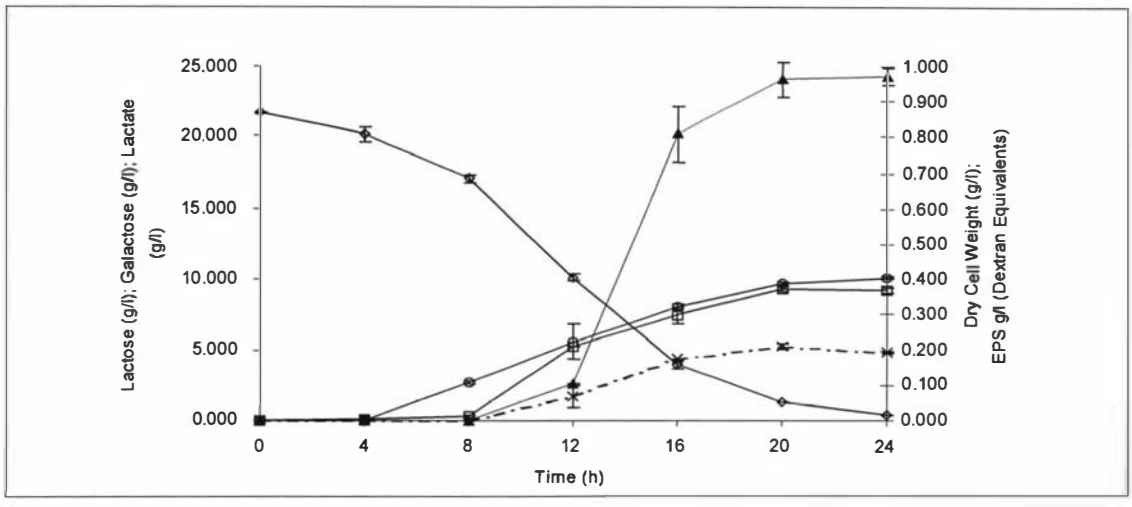
RESULTS

Reduction of a_w in the fermentation medium from the control value of 0.99 to 0.98 using NaCl resulted in a reduction of EPS titre over 20h fermentation time (Figures 6.1(a) and (b)). A concomitant reduction in dry cell weight concentration was observed in fermentations at the lower a_w. Fermentations run at the a_w of 0.96 (Figure 6.1(c)) showed a further reduction in EPS titre and dry cell weight relative to the control fermentations and those run at a_w 0.98, at the corresponding time.

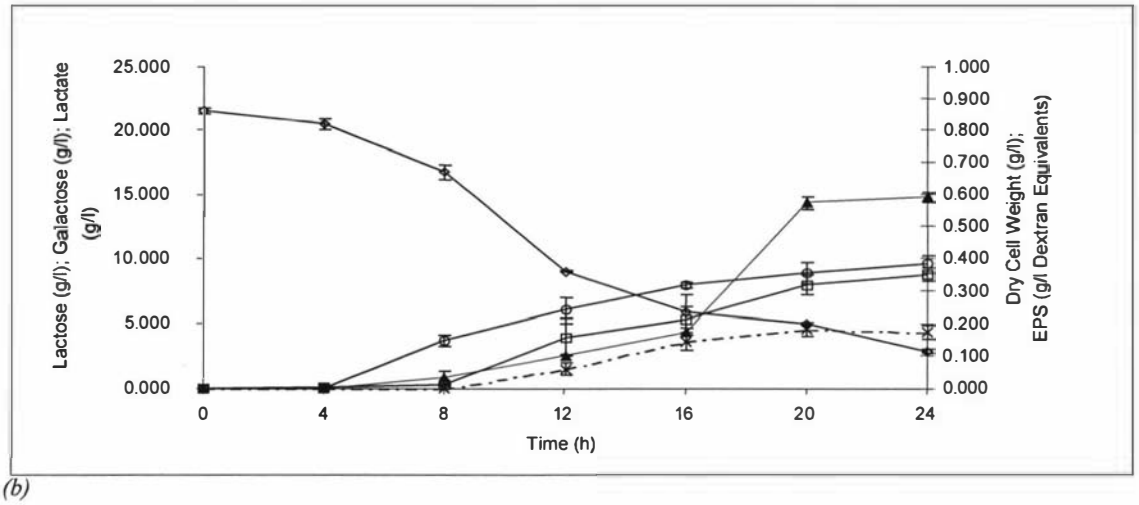
An overall decrease in lactose metabolized to lactate and galactose was observed in fermentations performed at a_w 0.98, relative to the control fermentations. A further reduction in lactose conversion was associated with an a_w of 0.96. Addition of sorbitol to the fermentation resulted in a reduction in EPS titre at a_w 0.98 (Figure 6.2(b)) relative to the control fermentations (Figure 6.2(a)), and a further reduction at a_w 0.96 (Figure 6.2(c)). As with the instances involving NaCl addition, reductions in dry cell weight titre, and lactose conversion to lactate and galactose were observed.

Yield values of EPS and lactate on dry cell weight (Yp/x) (Table 6.1), and lactose consumed (Yp/s) (Table 6.2) were calculated for the control fermentations and those conducted at reduced water activities. All calculations excluded any lag phase of the fermentation (i.e. up to 4 h). Adjustment of the water activity to 0.98 by addition of NaCl or sorbitol resulted in enhanced yields of EPS and lactate on dry cell weight. Addition of NaCl was observed to cause the greater effect of the two humectants. Further enhancement of the EPS and lactate yield on dry cell weight was not observed with reduction of the a_w to 0.96 with NaCl addition, however a further increase in Yp/x of lactate on dry cell weight was observed in the instance of sorbitol addition.

Raised levels of Yp/s of EPS and lactate on lactose consumed were observed in the fermentations adjusted to a_w 0.98 with both NaCl and sorbitol, except in the case of the lactate yield on lactose associated with sorbitol addition, in which a reduced yield was observed. A reduction in Yp/s values for both metabolites relative to the control occurred at a_w 0.96.



(a)



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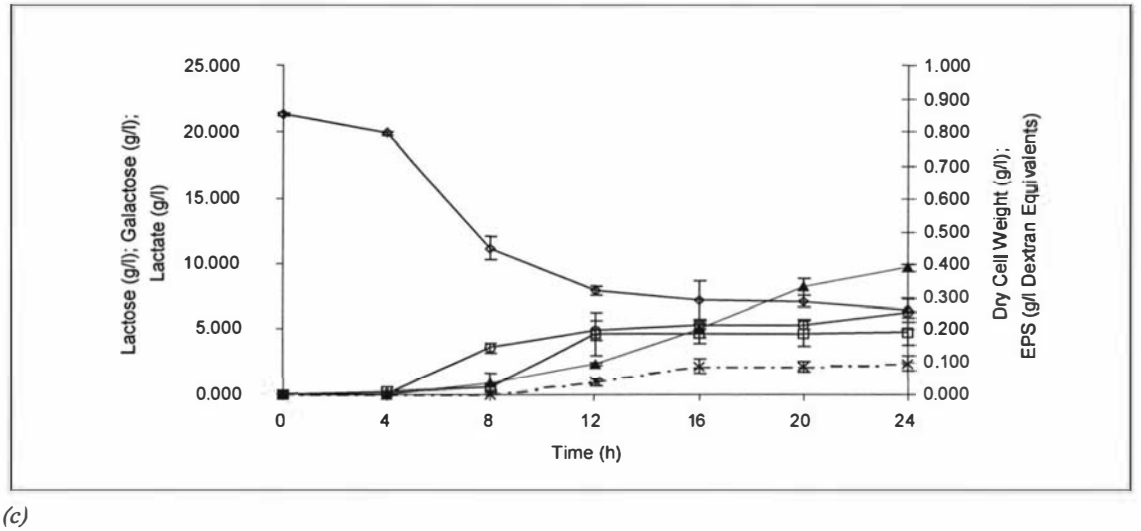
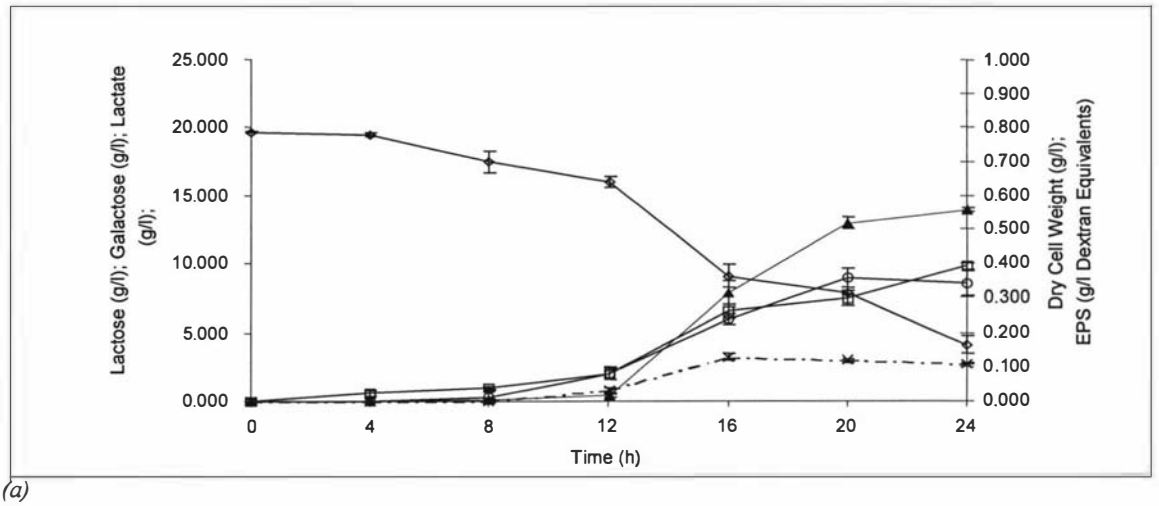


(c)

Fig. 6.1. Batch fermentation profiles of *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 at varying water activity levels using NaCl. Control fermentation (no NaCl addition), a_w 0.99 (a), a_w 0.99 →0.98 at 12h by NaCl addition (b), a_w 0.99 →0.96 at 12h by NaCl addition (c). Lactose ◊, galactose ○, lactate □, EPS ×, Biomass (Dry cell weight)▲. (Mean values). Error bars represent standard deviations.



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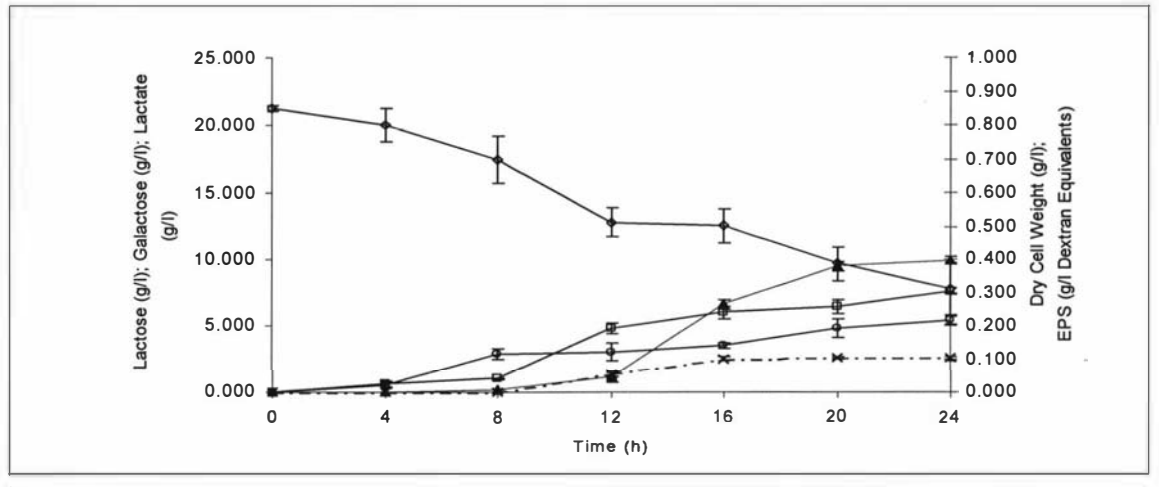

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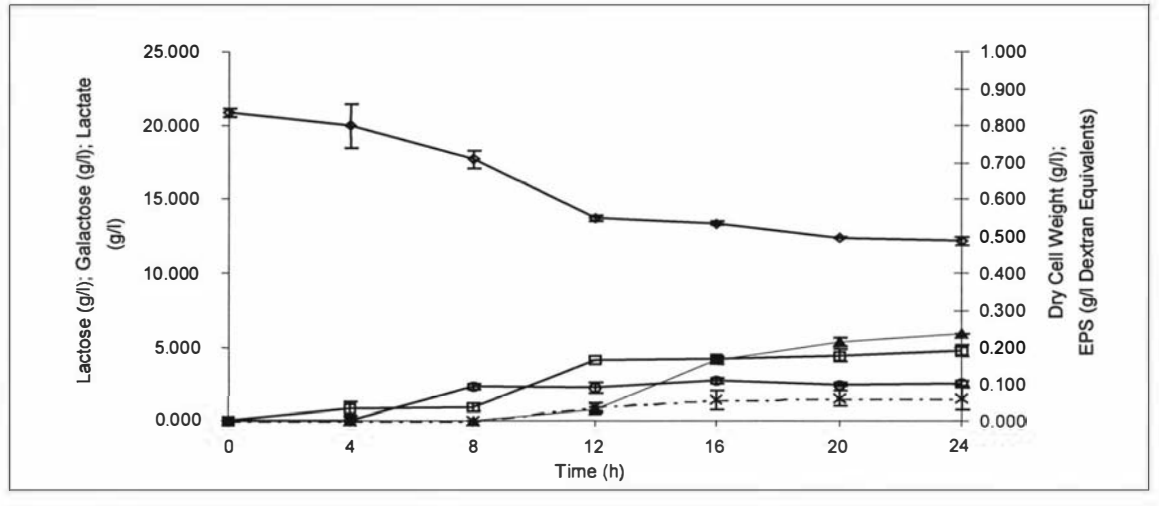

(c)

Fig. 6.2. Batch fermentation profiles of *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 at varying water activity levels using sorbitol. Control fermentation (no sorbitol addition), a_w 0.99 (a), a_w 0.99 →0.98 at 12h by sorbitol addition (b), a_w 0.99 →0.96 at 12h by sorbitol addition (c). Lactose ◊, galactose ○, lactate □, EPS ×, biomass (dry cell weight) ▲. (Mean values). Error bars represent standard deviations.

Table 6.1 Specific yields (Yp/x) of EPS and lactate production on dry cell weight by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 in response to a reduction in a_w (no humectant added to control).

Fermentation	Yp/x (EPS/dry cell weight) (g/g) NaCl addition	Yp/x (lactate/dry cell weight) (g/g) NaCl addition
Control a_w 0.99	0.22	9.55
a_w 0.98	0.32	13.76
a_w 0.96	0.24	13.63
Fermentation	Yp/x (EPS/dry cell weight) (g/g) Sorbitol addition	Yp/x (lactate/dry cell weight) (g/g) Sorbitol addition
Control a_w 0.99	0.25	13.24
a_w 0.98	0.27	15.36
a_w 0.96	0.26	16.74

Table 6.2 Specific yields (Yp/s) of EPS and lactate production on lactose consumed by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 in response to a reduction in a_w (no humectant added to control).

Fermentation	Yp/s (EPS/lactose) (g/g) NaCl addition	Yp/s (lactate/lactose) (g/g) NaCl addition
Control a_w 0.99	0.010	0.488
A_w 0.98	0.012	0.508
A_w 0.96	0.007	0.358
Fermentation	Yp/s (EPS/lactose) (g/g) Sorbitol addition	Yp/s (lactate/lactose) (g/g) Sorbitol addition
Control a_w 0.99	0.009	0.589
a_w 0.98	0.010	0.512
a_w 0.96	0.008	0.478

DISCUSSION

The sequential reduction in water activity of the culture medium from a_w 0.98 to 0.96 (Figures 6.1 and 6.2) results in physiological impairment of *Lb. delbrueckii* subsp. *bulgaricus* and a relative reduction in biomass, lactate, galactose and EPS titres (Figures 6.1 and 6.2). The trend

in which cell growth is reduced with decreasing water activity by *Lb. delbrueckii* subsp. *bulgaricus* has been reported previously by Shah and Ravula (2000). Larsen and Añón (1989) reported a sequential decrease in acid production by *Lactobacillus bulgaricus* ATCC 11842 and *Streptococcus thermophilus* ATCC 19258 in milks adjusted to a range of a_w values between 0.992 and 0.943 with glycerol. Fajardo-Lira *et al.* (1997) demonstrated that a low a_w amounts to a highly stressful condition for *Lb. delbrueckii* subsp. *bulgaricus*. Reduction of the a_w of the milk culture medium using either glycerol or sucrose reduced lactic acid production in both ropy and non–ropy strains of *Lb. delbrueckii* subsp. *bulgaricus* and *S. thermophilus;* sucrose, however, had the greater inhibitory effect over a range of a_w levels. A decreased acidity development as a consequence of reduced a_w has also been shown by Zourari *et al.* (1992). Liu *et al.* (1998) studied the effect of reduced water activity on lactose metabolism in *Lactococcus lactis* subsp. *cremoris* at different pH values, and noted a decrease in lactic acid levels upon reduction of a_w levels to below 0.99. Missing lactose carbon in the form of lactate was partially accounted for by an accumulation of galactose, which occurred under conditions of reduced pH or a_w, or both.

Previous reported attempts to induce EPS formation by a stress response in LAB have produced mixed results. Pham *et al.* (2001) induced stress conditions in *Lactobacillus rhamnosus* R by applying anaerobiosis and aeration, stimulating the production of EPS which had higher intrinsic viscosities. Looijesteijn and Hugenholtz (1999), however, did not identify EPS production to be a stress response in *L. lactis* subsp. *cremoris* NIZO B40 at elevated concentrations of NaCl, sucrose or glycerol in the culture medium. An increase in the lag phase, and decreases in the growth rate, final cell density, EPS level and specific EPS yield were reported to be associated with diminished a_w levels. Aeration (80 % saturation) did not elicit an enhancement in EPS formation either (Looijesteijn and Hugenholtz, 1999). In the present study, however, *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483, despite the general reduction in growth and extracellular metabolite titre associated with reduced a_w levels with both NaCl and sorbitol addition, showed an enhanced $Y_{p/x}$ of EPS at a_w 0.98 relative to the control at a_w 0.99 (Table 6.1), and less significantly, a small increase in $Y_{p/s}$ of EPS at a_w 0.98 (Table 6.2). The enhancements in specific EPS yield on biomass (Table 6.1) can be explained in general terms as being the consequence of a stress response. *Lb. delbrueckii* subsp. *bulgaricus* has been shown to produce a stress response to acid conditions, by forming the heat shock proteins, GroES, GroEL, and DnaK (Lim *et al.*, 2000). Exposure of the cell to a high osmolarity results in an efflux of water from the cell , a decrease in the turgor pressure, and a reduction in cytoplasmic volume (Csonka, 1989). Intracellular metabolites increase in concentration, hence causing a reduction in intracellular a_w and a change in activity of enzymes. In addition, conformational changes in substrates can be effected by a_w (Monsan and Combes, 1984). The cell responds by accumulating specific solutes such as potassium, amino acids

(glutamate and proline), amino acid derivatives (peptides and N-acetylated amino acids), quaternary amines (glycine betaine, carnitine), sugars (sucrose and trehalose), and tetrahydropyrimidines (Csonka, 1989; Poolman and Glaasker, 1998). These solutes do not interfere with cellular processes, and cause the cell turgor to be restored to pre–stress conditions.

In the instance of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483, the reduction in a_w from 0.99 to 0.98 was evidently sufficient to retard growth, however the productive capacity of the cell in respect of the formation of lactate and EPS was enhanced (Table 6.1), suggesting that the conditions stimulated enzymes associated with the production of these metabolites. In *E. coli*, for instance, exposure of the cell to osmotic stress has been reported to result in a ten-fold increase in activity of enzymes associated with trehalose degradation (Boos *et al.*, 1987; Csonka, 1989).

The observation that the $Y_{p/x}$ values of EPS to biomass at a_w 0.96 are higher relative to those of the control, when NaCl or sorbitol are used as humectants (Table 6.1), but lower than the corresponding values measured at a_w 0.98, suggests a similar internal effect having occurred at both water activity levels, but limited by the conditions applied at a_w 0.96.

Lactate yield on biomass shows a similar trend to EPS yield on biomass, however the pathway associated with the formation of lactate does not appear to be compromised by conditions at a_w 0.96 as is EPS yield on biomass.

The general similarity in trend of yields of EPS and lactate on lactose (Table 6.2) to the yields of these metabolites on biomass (Table 6.1), with the exception of the yield of lactate on lactose associated with sorbitol addition to the medium, further support the explanation that osmotic stress has influenced the internal metabolism of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 to the extent that EPS and lactate specific yields have become enhanced. The anomalous results associated with the yields of lactate on lactose associated with the reduction of a_w using sorbitol are not clear, however different effects on lactate production using different sugars as humectants have been reported in LAB (Troller and Stinson, 1981; Larsen and Añón, 1989).

The general similarity in effects on $Y_{p/x}$ of EPS and lactate by the addition of NaCl and sorbitol suggest that osmotic pressure was the prime causative agent of the perturbations. The observation that $Y_{p/x}$ changes effected by NaCl addition on EPS and lactate formation appear to be greater than those caused by sorbitol addition imply an additional action, possibly that of a change in ionic potential caused by NaCl, though this remains unclear. The growth inhibition

may have been caused in part by high cytoplasmic concentrations of salt, a condition which has been postulated in *Lactobacillus plantarum* to become inhibitory to the cell due to the binding of salt to intracellular macromolecules (Glaasker *et al.*, 1998).

The enhancement in specific yield of EPS by osmotic stress provides a potential means by which altered metabolic states associated with enhanced EPS production in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 may be examined. Further work is being undertaken in order to generate osmotically-stressed conditions in continuous culture, such that the metabolism can be studied under steady state conditions.

CONCLUSION

Athough dry cell weight, lactate, and EPS titres were negatively influenced by the reduction in a_w using NaCl, yields of EPS and lactate on dry cell weight were enhanced at a_w 0.98 suggesting that conditions have been identified by which the comparative levels of enzymes and metabolites may be studied. A similar result achieved by the addition of sorbitol confirms that the prime agent of metabolic change was that of osmotic pressure.

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CHAPTER 7

THE INFLUENCE OF GROWTH RATE ON EXOPOLYSACCHARIDE AND EXTRACELLULAR METABOLITE PRODUCTION BY *LACTOBACILLUS DELBRUECKII* SUBSP. *BULGARICUS* NCFB 2483

ABSTRACT

The production of exopolysaccharide (EPS) by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 is a strictly growth–associated phenomenon. The present study was undertaken in order to investigate the influence of varying growth rates on the production of EPS and extracellular metabolites by the organism when grown in a semi–defined medium incorporating lactose. In general, until the point at which the dilution rate exceeded the growth rate near a dilution rate of 0.50 h^{-1}, a pattern of increasing specific EPS yields and volumetric productivities was associated with increasing dilution rate. The highest EPS titres were recorded at dilution rates between 0.20 h^{-1} and 0.40 h^{-1}, with a maximum titre achieved at a dilution rate of 0.30 h^{-1}. Significantly lower EPS titres were achieved at dilution rates below 0.20 h^{-1}. The corresponding specific and volumetric productivities of lactate and galactose increased in response to the incremental changes in dilution rate up until 0.40 h^{-1}, despite a gradual reduction in titre and specific yield ($Y_{p/x}$) ocurring over this range of dilution rates. Elevated $Y_{p/s}$ values for EPS at the higher dilution rates suggest a diversion of carbon flux towards EPS being associated with the higher rates of growth.

INTRODUCTION

Studies undertaken on lactic acid bacteria (LAB) in continuous culture are limited as most fermentations using LAB involve batch processes. It has been observed that EPS production by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 is closely associated with growth (Welman, Chapter 4, PhD Thesis), as has been found in the thermophilic LAB strains such as *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 (Grobben *et al.*, 1995) and *S. thermophilus* LY03 (Degeest *et al.*, 2001). The purpose of this study was to investigate the relationship between growth rate and EPS production in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483, as well as the formation of lactate and galactose. These studies were intended to provide a basis for further investigation into the flux distribution from lactose to EPS, lactate, and galactose and the responses of key enzymes in associated metabolic pathways leading to these products. Previous

studies undertaken on *Lactobacillus delbrueckii* NRRL B-445 in continuous culture have demonstrated a close relationship between growth rate and lactate production (Major and Bull, 1985). Substrate limitation and product inhibition kinetics have been demonstrated on the same organism by Goncalves *et al.* (1991). Dilution rate has been shown to influence EPS production in *Lb. delbrueckii* subsp. *bulgaricus* RR in continuous culture (Gassem *et al.*,1997). The influence of different substrate limitations (glucose, leucine, phosphate) on EPS production by *Lactococcus lactis* subsp. *cremoris* NIZO B40 and NIZO B891 in continuous culture was studied by Looijesteijn *et al.* (2000). In this investigation it was concluded that under glucose limitation, the $Y_{p/s}$ for EPS was the highest, and the molecular mass lowest for all the limiting conditions applied.

MATERIALS AND METHODS

Medium. The medium used was that described by Kimmel and Roberts (1998) and modified by replacement of the glucose component with lactose. The medium consisted of lactose, 20.0 g/l; yeast nitrogen base (without amino acids and ammonia), 5.0 g/l; bacto casitone, 20.0 g/l; sorbitan monoleate ("Tween 80"), 1.0 g/l; dipotassium phosphate (K_2HPO_4), 2.0 g/l; magnesium sulphate ($MgSO_4.7H_2O$), 0.10 g/l; manganese sulphate ($MnSO_4.4H_2O$), 0.05g/l; ammonium citrate, 2.0 g/l; sodium acetate, 5.0 g/l. For the inoculum the medium was prepared in separate, double–strength volumes of lactose and the remainder of the nutrients as described above, adjusted to pH 6.0. After steam–sterilization, the aliquots were allowed to cool, and pooled, achieving the desired concentration of medium constituents. Where solid medium was prepared, 15.0 g/l of agar was used. Fermentation medium (15 l) was prepared by sterilizing separately, 1.5 l of a solution of lactose to make a final concentration of 20 g/l, and 13.5 l of a solution of the remainder of the nutrients combined with antifoam (Bevaloid, 15 ml) in distilled and deionized water in a 20 l carboy (sterilization by steam for 90 min at 121°C). The medium components were allowed to cool prior to being pooled. Any loss of feed volume through venting of the vessel after sterilization was restored by the aseptic addition of sterile water.

Bacterial strains and inoculum. The strain of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 (NCIMB 702483) was obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland. The fermentation inoculum (approx. 10% of the fermentor working volume) was prepared by inoculating duplicate inoculation flasks containing 150 ml of medium with a 1.0 ml aliquot from a working cell bank, preserved at –80°C, and incubating at 37°C for 24 h on an orbital shaker at 100 rpm.

Fermentation conditions. Continuous culture experiments utilized a Bioflo I fermentor (New Brunswick Scientific, NJ) equipped with pH and temperature control, and a magnetically coupled agitation system. A glass fermentation vessel (1400 ml working volume) was used with level control being maintained with an overflow weir. The fermentation feed medium was fed to the fermentation vessel by a peristaltic pump calibrated to deliver the required flow rates. A continuous head pressure of nitrogen was maintained for all experiments. The fermentor was operated initially in a batch mode in order for sufficient cell mass to accumulate prior to commencing the continuous studies. Temperature was maintained at a constant 37°C by a heating probe and circulation of cooling water through internal veins submerged in the fermentor. Agitation was set at 200 rpm and pH was maintained at 6.0 by titration with 2N KOH. Fermentations at each dilution rate were run until constant biomass levels were obtained, corresponding to steady state conditions; this was usually achieved after four to five fermentor volumes had been replaced.

Sampling and sterility. Sample aliquots (15 ml) were withdrawn aseptically at regular intervals for the determination of biomass, and sugar conversion to extracellular metabolites. In instances where analyses could not be immediately undertaken, samples were stored in pre-sterilized bottles at – 20°C. As is the requirement for the operation of continuous fermentation systems, a high level of aseptic control was applied in order to maintain monoseptic conditions in the fermentations. Fermentation medium and broth samples were examined microscopically, and plated onto solid culture medium for the detection of contaminants. The inoculated plates were incubated aerobically and anaerobically for a period of 60 h.

Analyses. Growth was monitored by absorbance measurement at 650 nm. Biomass was determined from a standard curve relating absorbance at 650nm to washed dry cell weights. Lactose utilization, and galactose and lactate formation, were measured by HPLC (Waters Alliance 2690, coupled to a Waters 2410 Differential Refractometer, and Waters 2487 UV spectrophotometer). The compounds were detected using an Aminex HPX-87H, 300 x 7.8 mm column (Biorad, Richmond, CA), according to the method as described in Ross and Chapital (1987). The column temperature was maintained at 40°C, and the flow rate at 0.6 ml. min^{-1}. Broth samples were clarified by centrifugation at 16 000 g for 15 minutes prior to dilution in distilled and filtered water (MilliQ) and analysis. External standards were prepared for lactose, galactose, and lactic acid and all analyses were undertaken in duplicate. Extracellular polysaccharides were subjected to a crude isolation prior to analysis. Aliquots (100µl) of whole broth underwent a two–fold precipitation with chilled ethanol (2.9 ml distilled H_2O and 7.0 ml 99.7% ethanol) for 24h periods at 4°C. The precipitate was recovered by centrifugation (35 850

g, 40 min., 4°C) (Sorvall RC5C – SS34 rotor) and resuspended in 1.0 ml distilled water. The total sugar concentration in the resuspended sample was measured according to the method of Dubois *et al.* (1956), using dextran as the standard.

RESULTS

Under steady state conditions, levels of biomass, residual lactose, EPS, lactate and galactose remained relatively constant at all the dilutions rates applied. The changes in these parameters over time as the culture at a dilution rate of 0.05 h^{-1} approached steady state conditions are shown in Figure 7.1.

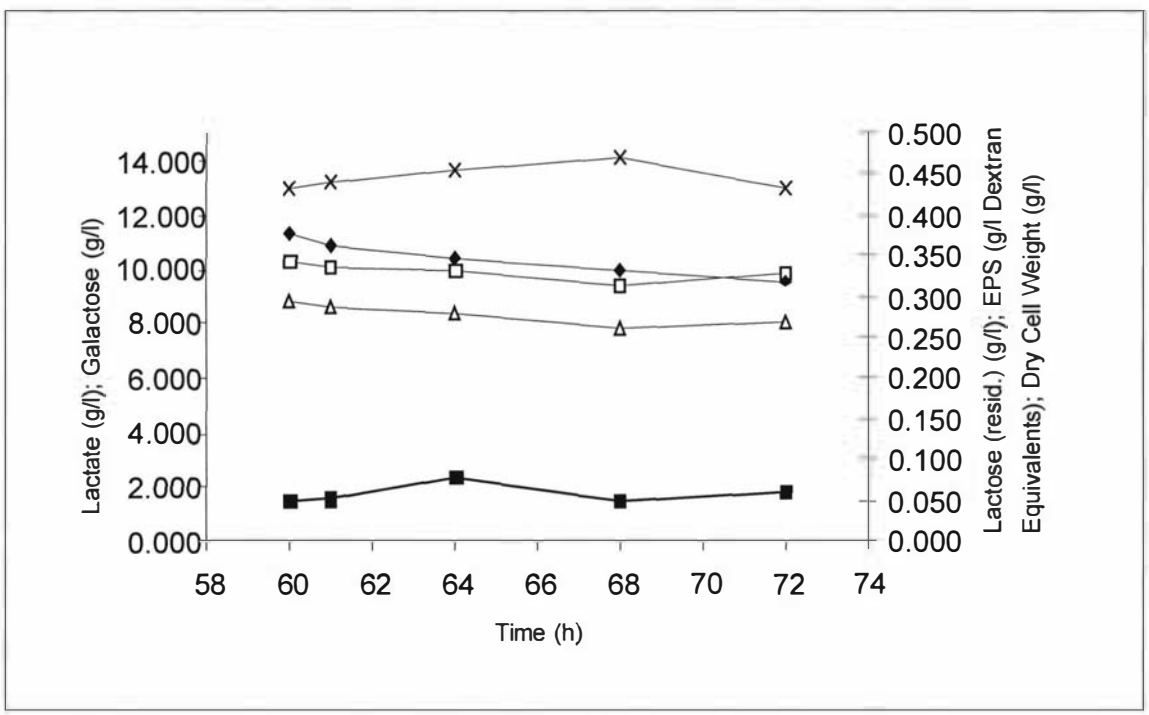

Fig. 7.1. Time course of changes in biomass, residual lactose, EPS, lactate, and galactose during continuous culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 at a dilution rate of 0.05 h^{-1} using a modified version of a medium according to Kimmel and Roberts (1998). Residual lactose ♦, biomass (dry cell weight) ×, EPS ■, galactose □, lactate Δ.

Dilution rate exerted a significant effect on extracellular metabolite levels produced by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483. Biomass levels remained relatively constant between the dilution rates 0.05 h^{-1} and 0.40 h^{-1}, following which a sharp decrease was recorded at the dilution rate of 0.50 h^{-1} (Figure 7.2). Accordingly, similar decreases in titre of lactate and galactose, and a corresponding increase in residual lactose was observed at this rate of growth.

The maximum EPS titre was recorded at a dilution rate of 0.30 h^{-1} (0.20 g/l), with titres generally significantly higher at the dilution rates 0.20 h^{-1} (0.18 g/l) and 0.40 h^{-1} (0.19 g/l) in

comparison to those at the dilution rates of 0.05 h^{-1} (0.07 g/l), 0.10 h^{-1} (0.08 g/l), and 0.50 h^{-1} (0.120 g/l) (Figure 7.2). A trend of increasing EPS titre with increasing growth rate was evident between the dilution rates of 0.05 $h^{-1}$and 0.30 h^{-1}.

Small increments in lactate formation occurred in relation to increasing dilution rate up until the dilution rate of 0.20 h^{-1} . A small reduction in lactate titre was measured at the dilution rates of 0.30 h^{-1} and 0.40 h^{-1}; a substantial reduction in lactate titre was measured at a dilution rate of 0.50 h^{-1} (Figure 7.2). Galactose production followed a similar decreasing trend from a dilution rate of 0.20 h^{-1} to that of 0.40 h^{-1}, with a substantial reduction in titre measured at 0.50 h^{-1} (Figure 7.2). The reductions in formation of these metabolites were mirrored by corresponding increases in residual (unutilized) lactose in the fermentation broth (Figure 7.2).

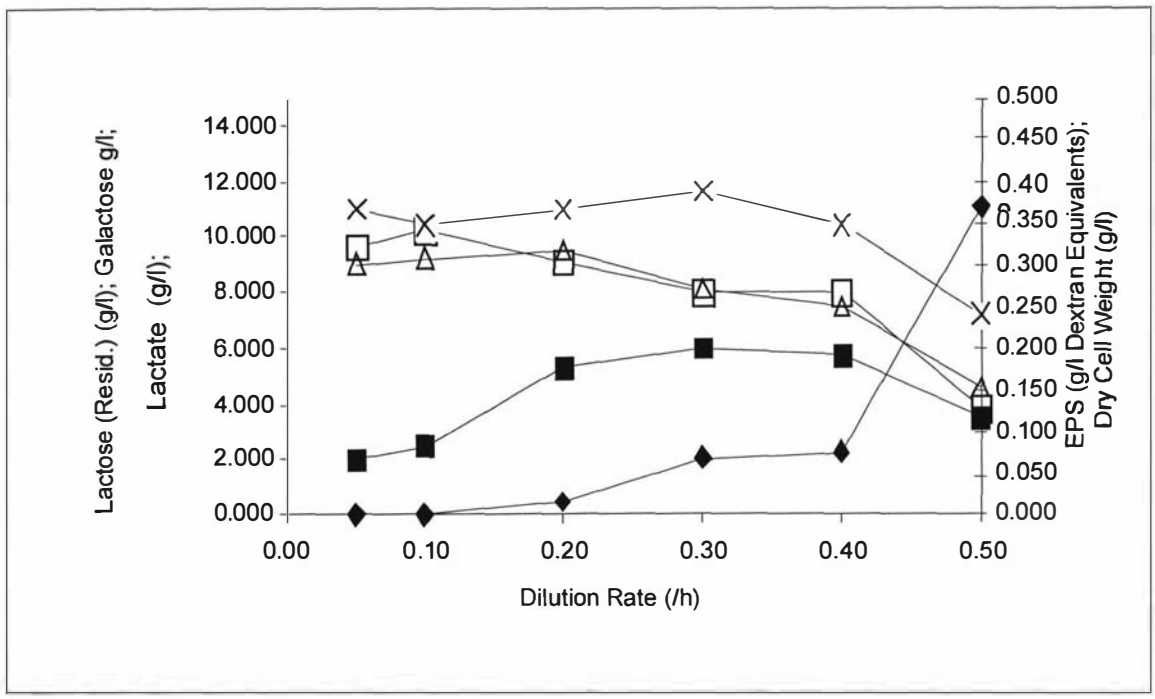

Fig. 7.2. Effect of dilution rate on biomass, residual lactose, EPS, lactate, and galactose during continuous culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 using a modified version of a medium according to Kimmel and Roberts (1998). Residual lactose ♦; biomass (dry cell weight) ×; EPS ■; galactose □; lactate Δ.

The influence of dilution rate on cellular performance in respect of EPS production rate was profound (Figure 7.3). The values calculated for $Y_{p/x}$ and volumetric productivity of EPS production showed a general increasing trend with increasing dilution rate, achieving their maxima at 0.40 h^{-1}, following which the values diminished markedly (Figure 7.3). Specific EPS production rate (relative to biomass) demonstrated a similar general trend with the increasing pattern continuing up to a dilution rate of 0.50 h^{-1}. The values of $Y_{p/s}$ (EPS) increased until a dilution rate of 0.30 h^{-1}, following which they remained relatively constant (Figure 7.3).

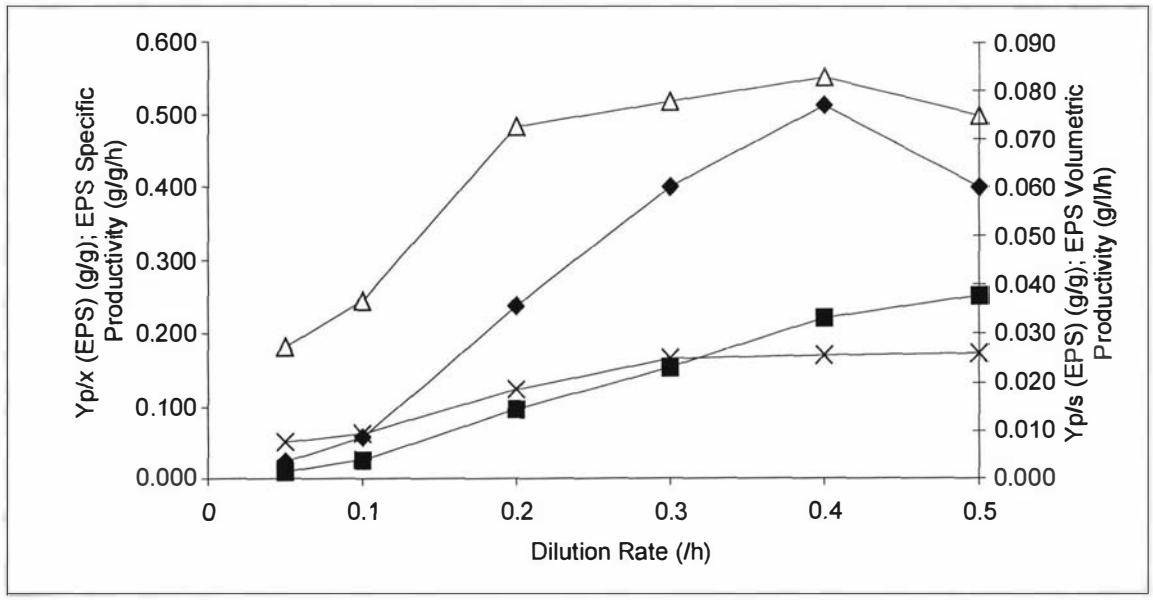

Fig. 7.3. Effect of dilution rate on $Y_{p/x}$, $Y_{p/s}$, volumetric productivity and specific production rate of EPS during continuous culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 using a modified version of a medium according to Kimmel and Roberts (1998). Volumetric productivity ♦; $Y_{p/s}$ ×; specific production rate ■; $Y_{p/x}$ Δ.

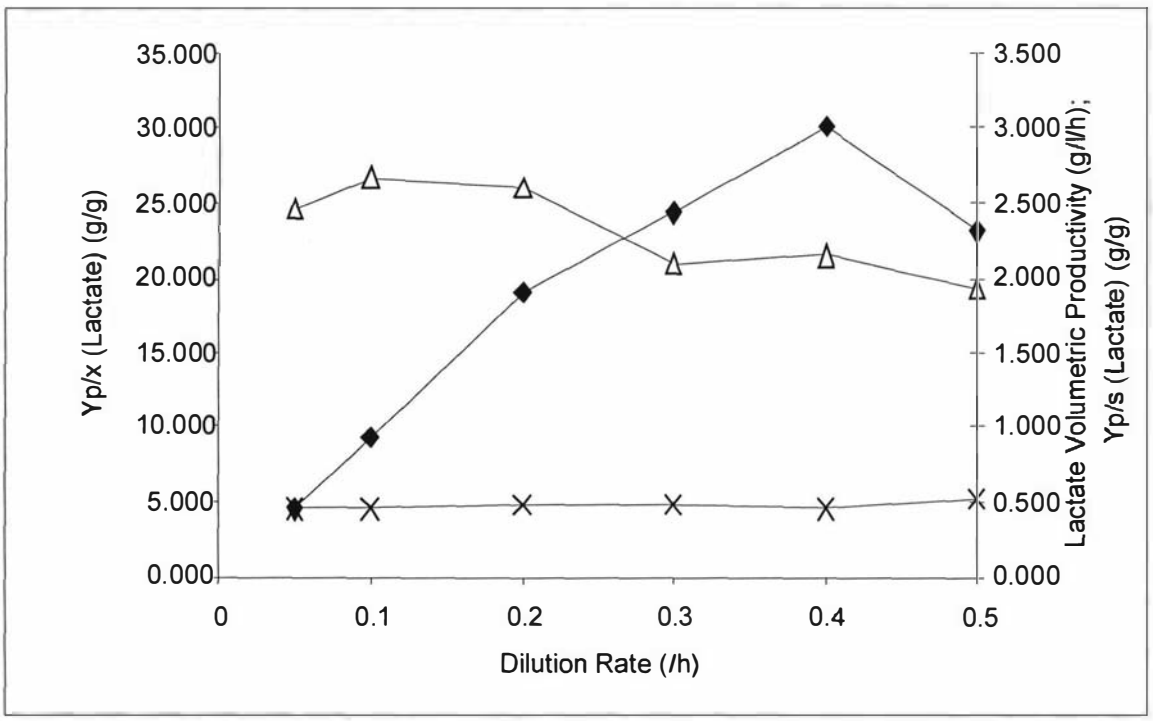

Fig. 7.4. Effect of dilution rate on $Y_{p/x}$, $Y_{p/s}$, and volumetric productivity of lactate during continuous culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 using a modified version of a medium according to Kimmel and Roberts (1998). Volumetric productivity ♦; $Y_{p/s}$ ×; $Y_{p/x}$ Δ.

The trends of volumetric production rates for the formation of lactate and galactose (Figures 7.4 and 7.5) followed similar patterns to that of EPS productivity (Figure 7.3). In all instances a maximum productivity was achieved at a dilution rate of 0.40 h^{-1}. Lactate $Y_{p/x}$ values appeared

higher at dilution rates of 0.20 h^{-1} and below, and slightly diminished at dilution rates of 0.30 h^{-1} and above (Figure 7.4). Slightly lower $Y_{p/x}$ values for galactose formation were associated with dilution rates between 0.20 h^{-1} and 0.40 h^{-1}; a substantial reduction was measured at a dilution rate of 0.50 h^{-1} (Figure 7.5). The trends for the increases in specific productivities of galactose, lactate, and EPS as a function of dilution rate were demonstrated to increase linearly relative to dilution rate up to 0.40 h^{-1} (Figure 7.6).

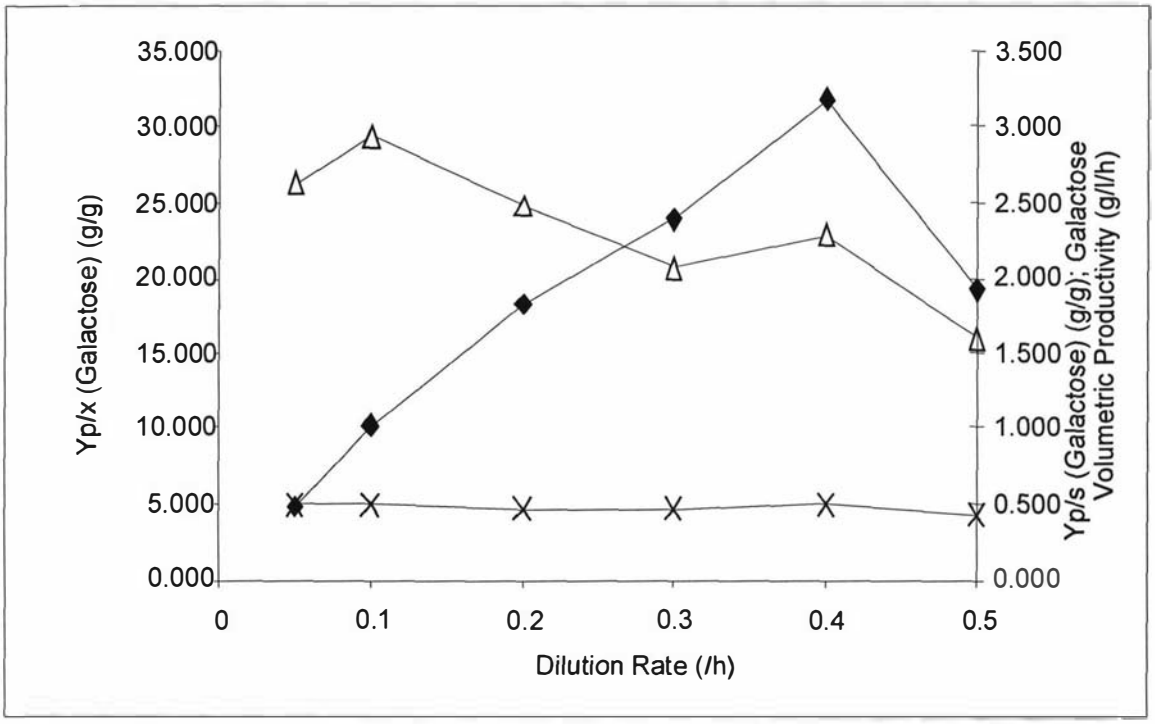

Fig. 7.5. Effect of dilution rate on $Y_{p/x}$, $Y_{p/s}$,and volumetric productivity of galactose during continuous culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 using a modified version of a medium according to Kimmel and Roberts (1998). Volumetric productivity ♦; $Y_{p/s}$ ×; $Y_{p/x}$ Δ.

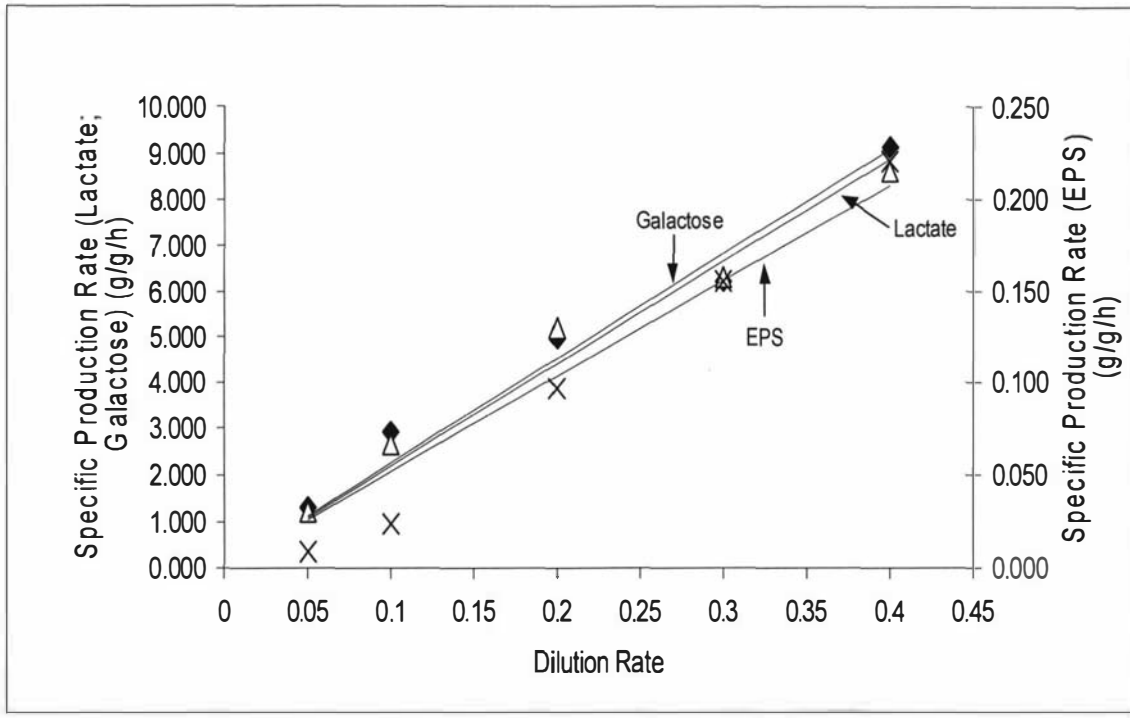

Fig. 7.6. Effect of dilution rate on specific productivity of EPS, lactate, and galactose during continuous culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 using a modified version of a medium according to Kimmel and Roberts (1998). Specific productivities for galactose ♦; EPS ×; lactate Δ.

DISCUSSION

EPS titres, productivities and specific yields were elevated at higher growth rates (Figures 7.2, 7.3) concurring with the results of Gassem *et al.* (1996) for *Lb. delbrueckii* subsp. *bulgaricus* RR. Gancel and Novel (1994), however, found that EPS production in *Stretococcus salivarius* subsp. *thermophilus* was high at low and high growth rates, suggesting activation of EPS formation by alternative regulatory pathways.

The maximum specific growth rate of the cell appeared to be between 0.40 h^{-1} and 0.50 h^{-1}, as washout of the fermentor biomass commenced within this range of dilution rates. This approximation accords with the previous determination (i.e. 0.50 h^{-1}) (Chapter 4, PhD Thesis – Welman, 2002) and is close to the value determined by Major and Bull (1985) for pH-controlled batch cultures of *Lb. delbrueckii.* The culture was lactose-limited at dilution rates below 0.20 h^{-1} (Figure 7.2), following which the precise nature of limitation became unclear at the higher dilution rates. The nutritional requirements of *Lactobacillus* are extremely complex (Major and Bull, 1985; Peters and Snell, 1954), and it is hence not apparent as to the potential nature of the limitation above these dilution rates. Keller and Gerhardt (1975) reported a similar

observation, and explained the limitation in terms of product inhibition, as opposed to substrate limitation. It is possible that lactic acid accumulation at higher growth growth rates served to limit cellular productivity in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 grown in continuous culture.

Increasing cell turnover caused an increase in the overall rate of production of lactate and galactose, as is evidenced by the increasing volumetric productivities, achieving a maximum at a dilution rate of 0.40 h^{-1} (Figures 7.4 and 7.5). Increasing specific production rates of these metabolites further support this assertion (Figure 7.6). Major and Bull (1985) determined the volumetric productivity of lactate production in *Lb. delbrueckii* NRRL-B445 grown on a glucose- and yeast extract-based medium to increase with increasing dilution rate, achieving a maximum value between the dilution rates 0.35 h^{-1} and 0.40 h^{-1} . The newly synthesized cells of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483, however, demonstrated slightly lower yields of of lactate and galactose per cell mass at growth rates between 0.20 h^{-1} and 0.40 h^{-1} (Figures 7.4 and 7.5).

The trend of increasing volumetric and specific productivity of EPS production (Figure 7.3) with cell growth rate could be ascribed in part to the fact that the supply of sugar nucleotides and ATP are available in greater excess to fuel the assembly of EPS polymers in more rapidly growing cells than slower ones. Higher $Y_{p/s}$ values for EPS production at raised growth rates (Figure 7.3) suggests that at increasing growth rates, carbon flux responds by shifting towards EPS formation at the minor expense of the formation of other metabolites. Due to the relatively low levels of EPS formed in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483, it could not be deduced, using the analytical methods applied, whether carbon was diverted from the formation of lactate or from galactose towards the production of EPS at the higher growth rates.

The linear relationship between EPS, lactate, and galactose specific production and dilution rate up until a dilution rate of 0.40 h^{-1} (Figure 7.6) demonstrates that these cellular products in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 are primary metabolites and are linked to growth. Major and Bull (1985) reported a similar finding for lactate production in *Lactobacillus delbrueckii* NRRL-B445.

CONCLUSION

Lb. delbrueckii subsp. *bulgaricus* NCFB 2483, when grown in continuous culture in a semi–defined medium, demonstrated a pattern of increasing EPS, lactate, and galactose productivities

with increasing growth rate , despite diminished lactate and galactose specific yields at dilution rates greater than 0.20 h^{-1}. Elevated $Y_{p/s}$ values for EPS production at the higher dilution rates point toward a redistribution of carbon flux in favour of EPS formation at the higher growth rates; this re-routing of carbon occurs potentially at the expense of lactate or galactose formation, although the analytical techniques applied were unable to provide confirmation of this. The study confirmed that the production of all three metabolites is growth-related.

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CHAPTER 8

METABOLIC CHANGES ASSOCIATED WITH REDUCED WATER ACTIVITY IN *LACTOBACILLUS DELBRUECKII* SUBSP. *BULGARICUS* NCFB 2483 GROWN IN CONTINUOUS CULTURE

ABSTRACT

An exopolysaccharide-producing strain of *Lactobacillus delbrueckii* subsp. *bulgaricus* (NCFB 2483) was subjected to conditions of reduced water activity (a_w) when grown in a semidefined medium in continuous culture. When the a_w of a steady state culture of the organism under lactose limitation was reduced from 0.99 to 0.98 by the addition of salt ("upshock"), the culture adjusted to a new equilibrium characterised by diminished levels of biomass, lactate, galactose, and exopolysaccharide (EPS). The value of $Y_{p/x}$ for EPS at the new equilibrium was reduced relative to the condition of the unperturbed state, whilst $Y_{p/x}$ values for lactate and galactose were substantially enhanced. At the same time, the yield of EPS on lactose consumed was reduced. The results suggest that lactate and galactose production become uncoupled from cell growth under conditions of hyperosmotic stress. Under the conditions applied, EPS production remained linked to cell biosynthesis. Under continuing conditions of reduced a_w, the culture appeared to recover, producing similar levels of biomass to that of the original culture at an a_w of 0.99. EPS and galactose levels were concurrently partially restored.

INTRODUCTION

Certain strains of *Lb. delbrueckii* subsp. *bulgaricus* have the ability to produce EPS, which together with the formation of lactate, play an important role in influencing the texture, stabilization, and prevention of syneresis of fermented dairy products such as yoghurt (Wacher-Rodarte *et al.*, 1993; Grobben *et al.*, 1997). The use of traditional dairy strains to produce industrially viable titres of highly functional EPS polymers is limited by the anaerobic nature of this group of bacteria. Due to energy limitation in lactic acid bacteria (LAB), a limitation is exerted upon the amount of EPS which the cell can produce. This situation is exacerbated if the production of EPS is coupled to growth. Improvements in the yields and structural configuration of these heteropolymers by LAB will rely principally upon genetic modifications to suitable strains. For this to occur rationally, it is of importance to develop an understanding of the metabolism associated with the formation of EPS in relation to global metabolism of the

cell. In the present study, an attempt was made to perturb the metabolism of the cell in order to induce a change in EPS formation, such that metabolic alterations could be measured.

As a means to perturb the metabolic network, salt-stress conditions were applied. Little has been reported about the effects of salt-stress on metabolic changes in *Lb. delbrueckii* subsp. *bulgaricus*. In a previous investigation (Welman, Chapter 6, PhD Thesis), it was found that for this organism, reduction of the water activity (a_w) of the growth medium from a value of 0.99 to 0.98 lowered the titres of biomass, EPS, lactate, and galactose formed in batch culture. The specific yields ($Y_{p/x}$ and $Y_{p/s}$) of EPS formation in batch culture were, however, elevated. Looijesteijn *et al.* (1999) concluded that EPS production in *Lactococcus lactis* subsp. *cremoris* NIZO B40 was not produced as a stress response. As EPS, lactate, and galactose formation by *Lb. delbrueckii* subsp. *bulgaricus* NCFB2483 are growth-related (Welman, PhD thesis, 2002, Chapters 4 and 7), the production of these extracellular metabolites under salt-stressed conditions were examined at steady state in continuous culture.

MATERIALS AND METHODS

Medium. The medium used was that described by Kimmel and Roberts (1998) and modified by replacement of the glucose component with lactose. The medium consisted of lactose, 20.0 g/l; yeast nitrogen base (without amino acids and ammonia), 5.0 g/l; bacto casitone, 20.0 g/l; sorbitan monoleate ("Tween 80"), 1.0 g/l; dipotassium phosphate (K_2HPO_4), 2.0 g/l; magnesium sulphate ($MgSO_4.7H_2O$), 0.10 g/l; manganese sulphate ($MnSO_4.4H_2O$), 0.05g/l; ammonium citrate, 2.0 g/l; sodium acetate, 5.0 g/l. For the inoculum the medium was prepared in separate, double–strength volumes of lactose and the remainder of the nutrients as described above, adjusted to pH 6.0. After steam–sterilization, the aliquots were allowed to cool, and pooled, achieving the desired concentration of medium constituents. Where solid medium was prepared, 15.0 g/l of agar was used.

Fermentation medium was prepared by sterilizing separately 1.5 l of a solution of lactose to create a final concentration of 20.0 g/l, and 18.0 l of a solution of the remainder of the nutrients combined with antifoam (Bevaloid, 15 ml) in distilled and deionized water, in a 20 l carboy (sterilization by steam at for 90 min. at 121°C). The medium components were allowed to cool prior to being pooled in the 20 l feed vessel. Under constant mixing in the feed vessel, a volume of the feed medium was pumped aseptically into a separately sterilized vessel containing NaCl dissolved in a small quantity of water. Sterile water was subsequently added to the individual feed vessels, such that the two newly reconstituted media volumes had a_w values of 0.99 and

0.98 respectively, and identical concentrations of the other medium components. Any loss of feed volume through venting of the vessel after sterilization was restored by the aseptic addition of the sterile water. The amount of NaCl necessary to add to the medium so as to achieve an a_w of 0.98 (25.3 g/l), was based upon a set of standards of NaCl dissolved in the fermentation medium.

Bacterial strains and inoculum. The strain of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 (NCIMB 702483) was obtained from the National Collection of Industrial and Marine Bacteria, Scotland, England. The fermentation inoculum (approx. 10% of the fermentor working volume) was prepared by inoculating duplicate inoculation flasks containing 150 ml of medium with a 1.0 ml aliquot from a working cell bank, preserved at –80°C, and incubating at 37°C for 24 h on an orbital shaker at 100 rpm.

Fermentation conditions. Continuous culture experiments utilized a Bioflo I fermentor (New Brunswick Scientific, NJ) equipped with pH and temperature control, and a magnetically coupled agitation system. A glass fermentation vessel (1400 ml working volume) was used with level control being maintained with an overflow weir. The fermentation feed medium was fed to the fermentation vessel by a peristaltic pump calibrated to deliver the required flow rates. A continuous head pressure of nitrogen was maintained for all experiments. The fermentor was operated initially in a batch mode in order for sufficient cell mass to accumulate prior to commencing the continuous culture studies. Temperature was maintained at a constant 37°C by a heating probe and circulation of cooling water through internal veins submerged in the fermentor. Agitation was set at 200 rpm and pH was maintained at 6.0 by titration with 2 M KOH. Fermentations at each dilution rate were run until constant biomass levels were obtained, corresponding to steady state conditions; this was usually achieved after four to five fermentor volumes had been replaced. Medium feed vessels corresponding to the different a_w levels (0.99 and 0.98) were connected and sterilized such that switching between media (upshock) could be effected instantaneously without making new sterile connections during operation of the fermentor.

Sampling and sterility. Sample aliquots (15 ml) were withdrawn aseptically at regular intervals for the determination of biomass, and sugar conversion to extracellular metabolites. In instances where analyses could not be immediately undertaken, samples were stored in pre-sterilized bottles at –20°C. As is the requirement for the operation of continuous fermentation systems, a high level of aseptic control was applied in order to maintain monoseptic conditions in the fermentations. Fermentation medium and broth samples were examined microscopically,

and plated onto solid culture medium for the detection of contaminants. The inoculated plates were incubated aerobically and anaerobically for a period of 60 h.

Analyses. Growth was monitored by absorbance measurement at 650 nm. Biomass was determined from a standard curve relating absorbance at 650 nm to washed dry cell weights. Lactose utilization, and galactose and lactate formation was measured by HPLC (Waters Alliance 2690, coupled to a Waters 2410 Differential Refractometer, and Waters 2487 UV spectrophotometer). The compounds were detected using an Aminex HPX-87H, 300 x 7.8 mm column (Biorad, Richmond, CA), according to the method as described in Ross and Chapital (1987). The column temperature was maintained at 40°C, and the flow rate at 0.6 ml. min^{-1}. Broth samples were clarified by centrifugation at 16 000 g for 15 minutes prior to dilution in distilled and filtered water (MilliQ), prior to analysis. External standards were prepared for lactose, galactose, and lactic acid. All analyses were undertaken in duplicate. Extracellular polysaccharides were subjected to a crude isolation prior to analysis. Aliquots (100 µl) of whole broth underwent a two–fold precipitation with chilled ethanol (2.9 ml distilled H_2O and 7.0 ml 99.7% ethanol) for 24h periods at 4°C. The precipitate was recovered by centrifugation (35 850 g, 40 min., 4°C) (Sorvall RC5C – SS34 rotor) and resuspended in 1.0 ml distilled water. The total sugar concentration in the resuspended sample was measured according to the method of Dubois *et al.* (1956), with dextran as the standard. Water activity was measured using a DECAGON (CX-2 upgrade) water activity meter (Decagon Devices Inc., Pullman, WA) which has an accuracy of ± 0.003, thereby providing an accurate distinction between the water activities tested. The instrument was standardized with a saturated solution of NaCl.

RESULTS

A dilution rate of 0.10 h^{-1} was selected for testing the effect of upshock on the culture of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 in continuous culture. Initial attempts at introducing the medium and humectant to the fermentation at a dilution rate of 0.05 h^{-1} did not appear to elicit any changes in biomass, and it was deduced that the culture had adjusted to the new conditions before a new steady state could be reached.

At a dilution rate of 0.10, biomass decreased substantially between a time period of 12 to 22 hours after initiation of the feed incorporating NaCl. A new equilibrium was established within three volume changes of the medium at an a_w of 0.98 (Figure 8.1). This state was characterised by reduced levels of biomass and EPS, and marginally reduced titres of lactate and galactose produced. The lactose-limiting conditions at an a_w of 0.99 were replaced by low residual

concentrations of lactose in the fermentation broth. Within a period of 48 h after the perturbation had commenced, biomass and galactose levels had returned to levels similar to those recorded prior to the introduction of the second medium feed, whilst the EPS titre was partially restored to a level above that of the intermediate equilibrium (Figure 8.1).

Values for $Y_{p/x}$, $Y_{p/s}$, volumetric productivity, and specific productivity were calculated for each of EPS, lactate, and galactose formation at the steady state condition at an a_w of 0.99, at the new equilibrium at an a_w of 0.98, and for the corresponding titres measured 26 h later, when the culture had adjusted in response to the new set of conditions (Table 8.1).

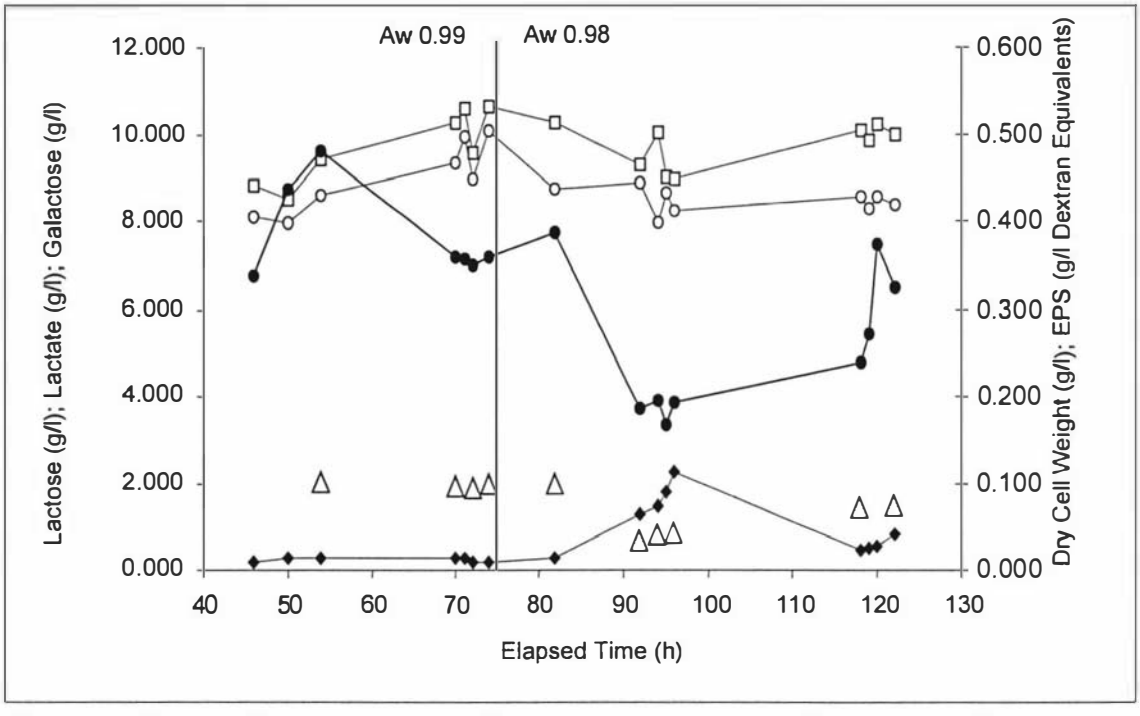

Fig. 8.1. Influence of salt upshock (reduction in a_w) on biomass, residual lactose, EPS, lactate, and galactose titre during continuous culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 using a modified version of a medium according to Kimmel and Roberts (1998). Biomass (dry cell weight) •; Residual lactose ♦; EPS Δ; Lactate o; Galactose □. All values the average of two steady state samples.

EPS specific yields and productivities became diminished under conditions of reduced water activity (a_w of 0.98), however increased again after prolonged exposure to the new environment, with the exception of specific productivity (Table 8.1). The restored yields and productivities did not rise to the levels of those calculated at the unperturbed state.

Although values calculated for $Y_{p/x}$ in respect of lactate and galactose production were significantly raised after upshock, they were restored to equivalent levels after longer exposure to the condition of lower water activity (Table 8.1). A similar trend was apparent for specific productivities of these two metabolites. No significant changes could be ascertained in respect

of $Y_{p/s}$ values for lactate and galactose over the time course of the fermentation. The volumetric productivity of lactate diminished in response to the humectant, remaining at a reduced level throughout the fermentation period. Similarly, galactose volumetric productivity was reduced, however this increased after extended exposure to the humectant in the medium (Table 8.1).

Table 8.1. Influence of salt upshock (reduction in a_w) on $Y_{p/x}$, $Y_{p/s}$, volumetric productivity and specific production rate of EPS, lactate, and galactose by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 in continuous culture, using a modified version of a medium according to Kimmel and Roberts (1998).

EPS	**a_w 0.99 (70 h)**	**a_w 0.98 (96 h)**	**a_w 0.98 (122 h)**
$Y_{p/x}$ (g/g)	0.27	0.21	0.23
$Y_{p/s}$ (g/g)	0.005	0.002	0.004
Vol. Productivity (g/l/h)	0.010	0.004	0.007
Spp. Productivity (g/g/h)	0.03	0.02	0.02
Lactate	**a_w 0.99 (70 h)**	**a_w 0.98 (96 h)**	**a_w 0.98 (122 h)**
$Y_{p/x}$ (g/g)	26.02	43.02	25.89
$Y_{p/s}$ (g/g)	0.49	0.47	0.44
Vol. Productivity (g/l/h)	0.94	0.82	0.84
Spp. Productivity (g/g/h)	2.60	4.30	2.59
Galactose	**a_w 0.99 (70 h)**	**a_w 0.98 (96 h)**	**a_w 0.98 (122 h)**
$Y_{p/x}$ (g/g)	26.68	46.82	30.91
$Y_{p/s}$ (g/g)	0.54	0.52	0.54
Vol. Productivity (g/l/h)	1.03	0.90	1.00
Spp. Productivity (g/g/h)	2.87	4.68	3.09

DISCUSSION

The reduction in biomass titres of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 in response to salt stress (Figure 8.1) implies that the change in osmotic pressure of the medium interfered with the biosynthetic pathways of the cell. Reduction in cell growth in response to decreasing water activity by *Lb. delbrueckii* subsp. *bulgaricus* has been reported previously (Shah and Ravula, 2000; Welman *et al.*, 2001). In anaerobically-grown *Lactobacillus plantarum*, the addition of 6% or 8% NaCl resulted in reduced biomass (Bobillo and Marshall, 1991). *Lb. plantarum* strain H4, when grown aerobically, at a dilution rate of 0.10 in continuous culture at different pH values, produced reduced levels of biomass in response to 6% salt addition to the medium, relative to the corresponding conditions without salt added (Bobillo and Marshall, 1992). Roy (1991) reported the inhibition of growth by salt stress in *Lactobacillus helveticus* strain L89.

EPS formation is directly coupled to growth in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 (Welman, PhD thesis, 2002, Chapters 4 and 7) and the titres correspondingly decreased in response to the condition of reduced a_w (Figure 8.1). The growth-relatedness of EPS formation in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 was retained after prolonged exposure to the conditions of the lower a_w. This was evident as the EPS titre increased or decreased with the respective increase or decrease in biomass titre as the cell and metabolism adjusted to the changed environment (Figure 8.1). The reduction in EPS titre associated with the lower a_w (0.98) accords with results obtained for *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 grown in batch culture (Welman, *et al.*, 2001). EPS yield values ($Y_{p/x}$, $Y_{p/s}$), however, were diminished in response to the alteration in water activity (Table 8.1), in contrast to the batch culture results in which $Y_{p/x}$ values for EPS were significantly raised, and $Y_{p/s}$ values were marginally higher. It is possible that lower pH values in combination with salt stress may have elicited the EPS yield enhancements in the batch culture experiments. Bobillo and Marshall (1992) have shown that changes in environmental pH can influence the fermentation end-products of *Lb. plantarum*, with the addition of salt to the medium influencing the extent of these changes. The influence of salt on intracellular metabolite levels associated with EPS production remains unclear and requires further investigation.

The reduction in lactate and galactose titres after exposure to the salt-stress accords with previous batch results (Welman *et al.*, 2001). The $Y_{p/x}$ for lactate, as was the finding in batch culture, was elevated after exposure to an a_w of 0.98 (Table 8.1). The substantial increases in $Y_{p/x}$ and specific productivity levels for lactate upon exposure to a reduced a_w (Table 8.1 at 96 h) point to an uncoupling of growth-associated biosynthesis under conditions of salt-stress. This condition appears to normalise after longer exposure to the increased osmotic pressure, and it is hence likely that *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 possesses mechanisms by which the production of lactate is protected from environmental stresses. Similar patterns for $Y_{p/x}$ and specific productivity of galactose production suggest its uncoupling from cellular biosynthesis in response to raised osmotic pressure. The uncoupling of lactate production from cell growth as a result of salt-induced stress was first reported by Turner and Thomas (1975); salt-stressed batch cultures of *Streptococcus cremoris* (*Lactococcus lactis* subsp. *cremoris*) inhibited growth whilst lactate formation continued. Similar uncoupling of lactate production and growth in response to salt-stress has been reported to occur in *Lb. helveticus* by Roy (1991). The distribution of carbon between lactose and galactose remains relatively unchanged, as is evident from the lack of any significant change in $Y_{p/s}$ values between the unperturbed steady state condition, and the new equilibrium corresponding to a lower a_w.

The apparent restoration of the production of biomass, EPS and lactate after extended exposure to a raised salt concentration (Figure 8.1), is indicative of the ability of *Lb. delbrueckii* subsp. *bulgaricus* to adapt to conditions of reduced a_w. The values calculated for $Y_{p/x}$ and specific productivity of lactate and galactose at this time further support this observation (Table 8.1). The results of this study confirm that *Lb. delbrueckii* subsp. *bulgaricus* can modify its metabolic pathways in response to salt-stress conditions, and subsequently re-adjust the bioreaction network under the new environmental conditions such that the level and distribution of carbon flux to principal metabolites are restored to levels similar to those prior to the perturbation. The mechanism of action of this adaptation can possibly be assigned to the cell restoring, to a certain extent, the turgor pressure by uptake of compatible solutes (osmoprotectants) in the medium (Csonka, 1989, Glaasker *et al.*, 1998; Poolman and Glaasker, 1998).

The results from this study have demonstrated that hyperosmotic stress caused the reduction in titres of biomass and principal metabolites of *Lb. delbrueckii* subsp. *bulgaricus*. The resultant increases in specific yields and productivities of lactate and galactose production can be ascribed to the adverse effect on biomass synthesis being greater than that on lactate and galactose production. The formation of these metabolites continued under hyperosmotic conditions, and became uncoupled from cell biosynthesis. EPS formation, which was adversely affected by the conditions of salt-stress, remained linked to growth. Per cell mass, hence, the catabolic pathways of lactose were enhanced, whilst EPS formation, in remaining linked to cell biosynthesis, was diminished. The continued metabolism of lactose and the formation of lactate during inhibition of cell biosynthesis would cause an increase in the intracellular concentration of adenosine triphosphate (ATP) due to its reduced employment in cell biosynthesis (Turner and Thomas, 1975). It would hence be expected that an excess of ATP would be available for the synthesis of the nucleotide precursors needed for EPS production, creating metabolic conditions which would favour the efficiency of EPS formation (Looijesteijn *et al.*, 2000), rather than diminish it. Cell growth and EPS formation share the utilization of substrate, ATP supply, lipid precursors (Looijesteijn and Hugenholz, 1999; Sutherland, 1982 and 1985), and sugar nucleotides such as UDP-glucose and UDP-galactose (De Vuyst *et al.*, 2001). In the present study, as the supply of lactose and the ATP pool were not limiting factors affecting cell growth or EPS production, under the hyperosmotic conditions applied, it is possible that the pathways or elements thereof leading to the assembly and supply of EPS-sugar nucleotides or EPS-lipid precursors, were unable to accommodate an increment in carbon flux through glucose-6-phosphate under the growth conditions used. The metabolic response to hyperosmotic stress at higher growth rates is unknown; higher growth rates are associated with higher EPS specific yields in *Lb. delbrueckii* subsp. *bulgaricus* (Welman, PhD thesis, 2002, Chapter 7) and in *Lactococcus lactis* subsp. *cremoris* NIZO B40 (Looijesteijn and Hugenholtz, 1999). Further

investigations are necessary in order to understand the relationship between carbon flux through the anabolic pathways of EPS formation, the catabolic pathways stemming from lactose breakdown, and the control that the pathways may have on one another. This work would incorporate studies of both intracellular metabolites and associated enzyme activities.

CONCLUSION

Salt upshock, when applied to *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 in continuous culture, resulted in reduced titres of biomass, EPS, lactate, and galactose, but raised specific yields and productivities of lactate and galactose, pointing to an uncoupling from growth of these two metabolites. The EPS specific yield diminished in response to the reduction in water activity. Prolonged exposure to the salt-stress led to a near normalization of elements of the metabolism. The stress condition did not cause EPS to become uncoupled from cell biosynthesis. Further investigation is necessary in order to elucidate the pathways leading to EPS formation in response to this perturbation

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CHAPTER 9

METABOLIC FLUX DISTRIBUTION AT THE GLUCOSE-6-PHOSPHATE AND GLUCOSE-1-PHOSPHATE NODES IN *LACTOBACILLUS DELBRUECKII* SUBSP. *BULGARICUS* NCFB 2483 GROWN IN CONTINUOUS CULTURE

ABSTRACT

Metabolic intermediates and enzymes activities associated with exopolysaccharide (EPS) production were determined at different dilution rates (0.05 h^{-1}, 0.10 h^{-1}, and 0.35 h^{-1}) in a strain of *Lactobacillus delbrueckii* subsp. *bulgaricus* (NCFB 2483) grown in continuous culture. Increasing specific productivities of EPS formation were associated with increasing dilution rates. The distribution of fluxes through the glucose-6-phosphate and glucose-1-phosphate branch-points was determined under steady state conditions over the range of dilution rates applied. Enzyme activities associated with these segments of biochemical pathways were determined simultaneously. An elevated level of flux to EPS at a dilution rate of 0.10 h^{-1} relative to a dilution rate of 0.05 h^{-1} was associated with diminished pools of glucose-1-phosphate and UDP-galactose, and a significantly raised activity of UDP-galactose 4-epimerase. At a dilution rate of 0.35 h^{-1} (raised EPS formation), UDP-galactose 4-epimerase was further raised, however an accumulation of glucose-6-phosphate became evident, pointing to a limitation in the flow of carbon towards fructose-6-phosphate or glucose-1-phosphate. Raised levels of ATP and ADP were measured at the dilution rates of 0.10 h^{-1}, and 0.35 h^{-1}, suggesting that the flow of carbon away from the catabolic pathway of glucose breakdown occurred in response to an excess supply of energy within the cell. UDP-galactose 4-epimerase appeared to play a key role in the generation of higher fluxes to EPS.

INTRODUCTION

Exopolysaccharides (EPS) produced by bacterial fermentation are commonly used as biothickeners in the food industry. Xanthan is a typical example, but is limited in its application by the fact that it is produced by *Xanthomonas campestris*, an organism that does not have GRAS (Generally Recognized As Safe) status (de Vuyst *et al.*, 1998). EPS from lactic acid bacteria (LAB), which are GRAS organisms, represent a group of polymers which have potential for application as food additives or functional food ingredients, and may confer health benefits. EPS from LAB are presently most commonly used in the dairy industry, and are used

to improve the rheology and textural quality of fermented products such as yoghurt. Engineering changes to the compositional structure of EPS as well as the economic production of these polymers by LAB for food applications will rely upon an understanding of the associated biosynthetic pathways. Significant progress in designing metabolic strategies for these purposes has already been made; apart from the need to optimize sugar uptake, assembly, and synthesis of EPS, a possible controlling mechanism in the production of EPS is the availability of the sugar nucleotides required for synthesis of the polymer (Boels *et al.*, 2001).

Lb. delbrueckii subsp. *bulgaricus* NCFB 2483, an EPS-producing strain, is homolactic in nature, utilizing the glucose moiety of lactose, and excreting the residual galactose from the cell. This pattern of utilization is consistent with most of the dairy strains of *Lb. delbrueckii* subsp. *bulgaricus* (Marshall *et al.*, 2001). Lactose uptake in strains that are galactose-negative occurs mostly via a lactose/galactose antiport system (de Vos and Vaughan, 1994). The lactose is split via a β-galactosidase into glucose, which is converted to lactate by the Embden-Meyerhoff pathway, and into galactose which is exported from the cell.

Lb. delbrueckii subsp. *bulgaricus* NCFB 2483 produces EPS in a growth-associated manner such that EPS formation increases with increasing growth rate (Welman, Chapters 4 and 7, PhD Thesis). Carbon flux to EPS production in LAB via the glucose-6-phosphate and glucose-1-phosphate branch-points was investigated in order to assess the flux distribution of carbon through these nodes in response to raised growth rates. The glucose-6-phosphate node closely links the catabolic pathways of glucose breakdown, and the anabolic network of EPS production, whilst the glucose-1-phosphate branchpoint links the distribution of carbon between the production of sugar nucleotides for glucose and galactose, and rhamnose incorporation into EPS. The conversion of glucose-6-phosphate to glucose-1-phosphate, catalysed by phosphoglucomutase (PGM), is possibly a key step in controlling the synthesis of sugar nucleotides (Boels *et al.*, 2001).

MATERIALS AND METHODS

Medium. The medium used was that described by Kimmel and Roberts (1998) and modified by replacement of the glucose component with lactose. The medium consisted of lactose, 20.0 g/l; yeast nitrogen base (without amino acids and ammonia), 5.0 g/l; bacto casitone, 20.0 g/l; sorbitan monoleate ("Tween 80"), 1.0 g/l; dipotassium phosphate (K_2HPO_4), 2.0 g/l; magnesium sulphate ($MgSO_4.7H_2O$), 0.10 g/l; manganese sulphate ($MnSO_4.4H_2O$), 0.05g/l; ammonium citrate, 2.0 g/l; sodium acetate, 5.0 g/l. For the inoculum the medium was prepared in separate,

double–strength volumes of lactose and the remainder of the nutrients as described above, adjusted to pH 6.0. After steam–sterilization, the aliquots were allowed to cool, and pooled, achieving the desired concentration of medium constituents. Where solid medium was prepared, 15.0 g/l of agar was used.

Fermentation medium (20 l) was prepared by sterilizing separately 1.5 l of a solution of lactose.H_2O (421.0 g) , and 18.5 l of a solution of the remainder of the nutrients combined with antifoam (Bevaloid, 15 ml) in distilled and deionized water in a 20 l carboy (sterilization by steam for 90 min. at 121°C). The medium components were allowed to cool prior to being pooled in the 20 l feed vessel. Any loss of feed volume through venting of the vessel after sterilization was restored by the aseptic addition of the sterile water.

Bacterial strains and inoculum. The strain of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 (NCIMB 702483) was obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland. The fermentation inoculum (approx. 10% of the fermentor working volume) was prepared by inoculating duplicate inoculation flasks containing 150 ml of medium with a 1.0 ml aliquot from a working cell bank, preserved at –80°C, and incubating at 37°C for 24 h on an orbital shaker at 100 rpm.

Fermentation conditions. Continuous culture experiments utilized a Bioflo I fermentor (New Brunswick Scientific, NJ) equipped with pH and temperature control, and a magnetically coupled agitation system. A glass fermentation vessel (1400 ml working volume) was used with level control being maintained with an overflow weir. The fermentation feed medium was fed to the fermentation vessel by a peristaltic pump calibrated to deliver the required flow rates. A continuous head pressure of nitrogen was maintained for all experiments. The fermentor was operated initially in a batch mode in order for sufficient cell mass to accumulate prior to commencing the continuous studies. Temperature was maintained at a constant 37°C by a heating probe and circulation of cooling water through internal vanes submerged in the fermentor. Agitation was set at 200 rpm and pH was maintained at 6.0 by titration with 2 M KOH. Fermentations at each dilution rate were run until constant biomass levels were obtained, corresponding to steady state conditions; this was usually achieved after four to five fermentor volumes had been replaced.

Sampling. Sample aliquots (15 ml) were withdrawn aseptically at regular intervals for the determination of biomass, and sugar conversion to metabolites. In instances where analyses could not be immediately undertaken, samples were stored in pre-sterilized bottles at -20°C. As

is the requirement for the operation of continuous fermentation systems, a high level of aseptic control was applied in order to maintain monoseptic conditions in the fermentations. Fermentation medium and broth samples were examined microscopically, and plated onto solid culture medium for the detection of contaminants. The inoculated plates were incubated aerobically and anaerobically for a period of 60 h.

For the enzymatic assays, a 300 ml aliquot of the fermentor volume (at steady state) was rapidly decanted via the overflow weir into a pre-chilled centrifuge bottle on ice. Samples for assay of intracellular metabolites in extracts were removed from the fermentor and extracted using a modification of a rapid system previously described by Guedon *et al.* (1999), Fordyce *et al.* (1984) and Thomas *et al.* (1979). A sterile syringe containing 1.5 ml of $HClO_4$, sealed at the exit with a silicone tube and stopcock, and pre-drawn to a volume of 15.0 ml under vacuum, was attached to the fermentor sampling port. Fermentation broth under steady state conditions was rapidly withdrawn from the fermentor vessel upon release of the stopcock; the final $HClO_4$ concentration in the syringe was 0.6 M.

Analyses. Growth was monitored by absorbance measurement at 650 nm. Biomass was determined from a standard curve relating absorbance at 650nm to washed dry cell weights. Lactose utilization, and galactose and lactate formation were measured by HPLC (Waters Alliance 2690, coupled to a Waters 2410 Differential Refractometer, and Waters 2487 UV spectrophotometer). The compounds were detected using an Aminex HPX-87H, 300 x 7.8 mm column (Biorad, Richmond, CA), according to the method as described in Ross and Chapital (1987). The column temperature was maintained at 40°C, and the flow rate at 0.6 ml. min^{-1}. Broth samples were clarified by centrifugation at 16 000 g for 15 minutes prior to dilution in distilled and filtered water (MilliQ), prior to analysis. External standards were prepared for lactose, galactose, and lactic acid. All analyses were undertaken in duplicate. Extracellular polysaccharides were subjected to a crude isolation prior to analysis. Aliquots (100 µl) of whole broth underwent a two–fold precipitation with chilled ethanol (2.9 ml distilled H_2O and 7.0 ml 99.7% ethanol) for 24h periods at 4°C. The broth precipitate was recovered by centrifugation (35 850 g, 40 min., 4°C) (Sorvall RC5C–SS34 rotor) and resuspended in 1.0 ml distilled water. The total sugar concentration in the resuspended sample was measured according to the method of Dubois *et al.* (1956), with dextran as the standard.

Assay of intracellular metabolites. Further treatment of the sample was undertaken according to the method described by Guedon *et al.* (1999) and Fordyce *et al.* (1984). The sample syringe containing extracted broth was held for 2 minutes at room temperature after the sampling procedure. The metabolite extracts were subsequently placed on ice for 10 minutes under N_2

gas, following which 0.69 g of K_2CO_3 was slowly added. The samples were neutralized with 3M KOH, such that the final pH was between 7.0 and 7.5. The extracts were centrifuged (10 000 G) for 10 minutes at 4°C, and the supernatants stored at -70°C until assayed.

Assays of the intracellular metabolites glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), and glucose-1-phosphate (G1P) were undertaken by a modification of the method described by Garrigues *et al.* (1997) using fluorimetric determination of NADPH formed in enzyme assays. Emission was measured at 460 nm after excitation at 350 nm using a Perkin Elmer LS 50B Luminescence Spectrometer. The concentration of NADPH formed was related to fluorimetric intensity by creation of a calibration curve (R^2=0.99) (Cook *et al.*, 1976). To 1150 µl of a mixture of 500 mM triethanolamine buffer (pH 7.6) containing 15 mM $MgSO_4$ and 4 mM EDTA, 40 µl 10 mM NADP, 760 µl H_2O, and 50 µl metabolite extract, was added 20 µl glucose-6-phosphate dehydrogenase (200 U/ml) in order to commence conversion of G6P. After the reaction had proceeded to completion, 20 µl of phosphoglucose isomerase (200 U/ml) was added in order to measure the concentration of F6P. Upon completion of this reaction, 20 µl phosphoglucomutase (200 U/ml) was added for the measurement of G1P. All assays were undertaken in triplicate.

Measurements of uridine-5'-diphosphoglucose (UDP-glucose) and uridine-5'-diphosphogalactose (UDP-galactose) were undertaken by an enzymatic method (Keppler and Decker, 1985) in which the formation of NADH was measured at A_{339} at 25°C. The absorbance (A_1) of 200 µl of sample extract in 500 µl of a mixture of NAD (20 mg), EDTA-$Na_2H_2.2H_2O$ (30 mg), and Na_2CO_3 (23 mg) in 10 ml 0.5 M glycine buffer at pH 7.0 was measured prior to the addition of 20 µl of UDP-glucose dehydrogenase (3 kU/l). The absorbance (A_2) was recorded after completion of the reaction. The absorbance (A_3) was measured after completion of further reaction following the addition of UDP-glucose 4-epimerase (10 kU/l).

The assay of deoxythymidine-5'-diphosphoglucose (dTDP-glucose) was undertaken according to a modification of the method of Bevill (1974). An extract (50 µl) of dTDP-glucose dehydrase (prepared from *E. coli.* Strain B56 according to the methods of Gilbert *et al.* (1965), and Wang and Gabriel (1969)) was incubated at 37°C for 1 minute in 1320 µl Tris buffer (50 mM pH 8.6; 2 mM EDTA) prior to the addition of 150 µl metabolite extract. The mixture was incubated at 37°C for 30 minutes following which 1480 µl 0.2 M NaOH was added. The assay mixture was subjected to a further incubation period of 15 minutes at 37°C, following which the absorbance was measured at 318 nm against a blank which was prepared identically, with the exception that the aliquot of 0.2 M NaOH was added before addition of the metabolite extract.

Preparation of cell-free extracts. Crude enzyme extracts were prepared according to a modification of the method of Escalante *et al.* (1998). Fermentation broth (280 ml) was centrifuged at 35 850 g for 40 minutes at 4°C, and the supernatant fraction discarded. The cell pellet was washed with 0.01M cold phosphate buffer followed by centrifugation at 35 850 g for 40 minutes at 4°C. The pellet was resuspended to a total volume of 28 ml (10 X concentration) in 0.01M phosphate buffer and kept on ice. Glass beads (0.13 mm diameter) were added in a ratio of 5.0 g glass to 1.0 g of cell mass. The cell suspension, was homogenized in a Braun MSK homogenizer (3 x 20 s at 400rpm) with concurrent cooling using a jet of dry CO_2 flowing over the homogenization chamber so as to prevent loss of enzyme activity. The homogenization procedure was pre-optimized in preliminary experiments. The glass beads were removed using a sintered glass funnel on ice. The supernatant fraction was further centrifuged at 35 850 g for 10 minutes at 4°C prior to assay. Where the extract could not be assayed immediately, it was stored at -20°C, and checked for loss of activity.

Enzyme assays. The enzyme assays were undertaken at 37°C, and the formation or disappearance of NAD(P)H was measured at 340 nm (ε_{340} = 6220 $M^{-1}.cm^{-1}$), unless stated otherwise. Protein concentration of the enzyme extracts was determined by the method of Lowry *et al.* (1951). Enzyme activities were expressed as nanomoles of substrate converted to product over 1 minute, per mg of protein in the extract. Measurements were reported as means with standard deviations.

The α-phosphoglucomutase reaction (Qian *et al.*, 1994; Grobben *et al.*, 1996) contained (per ml), 50 μmol triethanolamine, 5 μmol $MgCl_2$, 0.4 μmol NADP, 0.01 ml of glucose-6-phosphate dehydrogenase (180 $U.ml^{-1}$), and cell-free extract). The reaction was initiated by the addition of 2.5 μmol fructose-6-phosphate.

The dTDP-glucose pyrophosphorylase reaction mixture (Bernstein, 1965; Looijesteijn *et al.*, 1999) contained (per ml), 50 μmol Tris-HCl buffer (pH 7.8), 8 μmol $MgCl_2$, 0.3 μmol $NADP^+$, 0.1 μmol TDP-glucose, 2.1 U of α-phosphoglucomutase, 4 U of glucose-6-phosphate dehydrogenase, and cell-free extract. Inorganic pyrophosphate (4.7 μmol) was used to start the reaction.

The UDP-glucose pyrophosphorylase reaction assay (Bernstein, 1965; Looijesteijn *et al.*, 1999) contained (per ml), 50 μmol Tris-HCl buffer (pH 7.8), 14 μmol $MgCl_2$, 0.3 μmol $NADP^+$, 0.1 μmol UDP-glucose, 2.1 U of α-phosphoglucomutase, 4 U of glucose-6-phosphate

dehydrogenase, and cell-free extract. Inorganic pyrophosphate (4 μmol) was used to initiate the reaction.

The UDP-galactose 4-epimerase reaction mixture (Grobben *et al.*, 1996; Looijesteijn *et al.*, 1999; Degeest and de Vuyst, 2000) contained (per ml), 50 μmol Tris-HCl buffer (pH 8.5), 5 μmol $MgCl_2$, 0.5 μmol NAD^+, 0.015 U UDP-glucose dehydrogenase, and cell-free extract. UDP-galactose (0.2 μmol) was used to start the reaction.

The phosphoglucose isomerase assay (reverse reaction) (Schreyer and Böck, 1980; Grobben *et al.*, 1996) contained (per ml), 50 μmol potassium phosphate (pH 6.8), 0.4 μmol NADP, 10 μl glucose-6-phosphate dehydrogenase (200 $U.ml^{-1}$), and cell-free extract. Fructose-6-phosphate (2.5 μmol) was used to initiate the reaction.

The dTDP-glucose 4,6-dehydratase reaction (Zarkowsky and Glaser, 1969; Grobben *et al.*, 1996; De Geest and de Vuyst, 2000) contained (per ml), 50 μmol Tris-HCl buffer and cell-free extract. The reaction was started with 0.3 μmol dTDP-glucose and incubated for 10 minutes at 37°C. Samples were taken at 60 s intervals and added to 0.1 M NaOH, and incubated for a further 15 minutes at 37°C. The formation of dTDP 6-deoxy-D-xylo-4-hexulose (ε_{320} = 4600 $M^{-1}.cm^{-1}$) was determined spectrophometrically at 320 nm.

The dTDP-rhamnose synthetic enzyme system (Martins and Sá-Correia, 1993) was assayed in an identical fashion to that for the dTDP-glucose 4,6-dehydratase reaction, with the exception that NADPH (0.5 μmol) was added to the initial incubation mixture. The formation of dTDP-rhamnose was measured at an absorbance of 340 nm.

NADH oxidase was measured by the method of Lopez de Felipe and Hugenholtz (2001). The reaction mixture contained (per ml), 50 μmol potassium phosphate (pH 7.0), 0.3 μmol EDTA, 0.050 μmol FAD, and 0.3 μmol NADH. The reaction was started with the cell-free extract.

Assay of nucleotide pools. Adenosine 5'-triphosphate in the perchloric acid extracts was assayed using an assay kit (Sigma Diagnostics ATP kit, Catalog No. 366). Adenosine 5'-diphosphate was measured by the method of Guedon *et al.* (2000). The ADP in the sample extracts were converted to ATP using 7mM phosphocreatine in 0.1 M Tris-HCl buffer (pH 7.4), 0.4 mM $MgSO_4$, and 4U of creatine phosphokinase. The reaction mixture was incubated for 20 minutes at 30°C, following which the reaction was stopped by heating at 100°C for a period of 3 minutes. The samples were centrifuged at 8000 g for 15 minutes at 4°C.

RESULTS

Lactate and EPS titres increased as the dilution rate was shifted between 0.05 h^{-1} and 0.10 h^{-1} (Figure 9.1). At a dilution rate of 0.35 h^{-1}, the EPS titre was further raised; the lactate titre was diminished relative to the 0.10 h^{-1} value. Incremental changes occurred with increasing dilution rate of EPS specific yields relative to biomass and lactose consumed, and EPS volumetric and specific productivities (Figure 9.2). The results collectively demonstrated an enhanced flux of carbon towards EPS at the higher dilution rates. The specific production rates of the principal metabolites of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 (EPS, lactate and galactose) displayed similar general trends in response to the increasing dilution rates, and appear to match the corresponding pattern of specific rate of utilization of lactose in the cell (Figure 9.3). The values calculated for $Y_{p/s}$ of EPS revealed a pattern of increasing conversion of lactose carbon to EPS at the raised dilution rates (Figure 9.4). Calculation of the ratios of EPS to lactate produced, revealed no change in distribution of carbon toward EPS and lactate between the dilution rates of 0.05 h^{-1} and 0.10 h^{-1} (0.008 for both). An increased ratio (0.021) at the dilution rate of 0.35 h^{-1}, however, suggested a redistribution of carbon away from lactate production in favour of EPS formation at this dilution rate. The distribution of carbon between lactose and galactose did not appear to obey a fixed stoichiometry as was evident from the respective increment and decline in $Y_{p/s}$ between the dilution rates of 0.05 h^{-1} and 0.10 h^{-1}.

The levels of glucose-6-phosphate pools increased significantly between the dilution rates of 0.10 h^{-1} and 0.35 h^{-1}, whilst its metabolic product pools diminished with increasing dilution rate (Figure 9.5). The pool of dTDP-glucose was significantly raised at the higher dilution rates, however no significant changes could be observed for the UDP-glucose metabolite pools (Figure 9.6). UDP-galactose levels were lower at a dilution rate of 0.10 h^{-1} than 0.05 h^{-1}, however subsequently remained unchanged at a dilution rate of 0.35 h^{-1}.

Enzyme activities associated with the glucose-6-phosphate and glucose-1-phosphate branch points are shown in Table 9.1. The activities of dTDP-glucose-4,6-dehydratase (TGD) and the dTDP-rhamnose synthetic enzyme system were detected at levels too low for comparison.

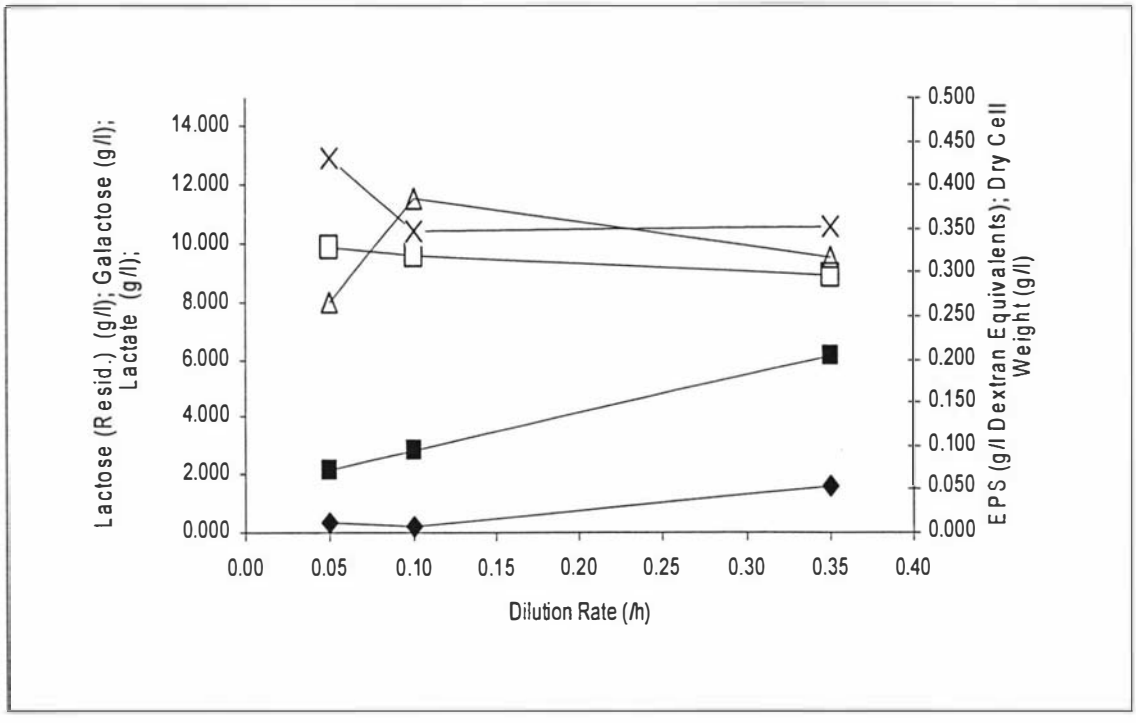

Fig. 9.1. Volumetric titres of EPS, lactate, galactose, biomass (dry cell weight), and residual lactose at different dilution rates (0.05 h^{-1}, 0.10 h^{-1}, and 0.35 h^{-1}) of *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 grown in continuous culture in a semi-defined medium. Residual lactose ♦; EPS ■; Lactate Δ; Galactose □; Biomass x. All values the average of two steady state samples

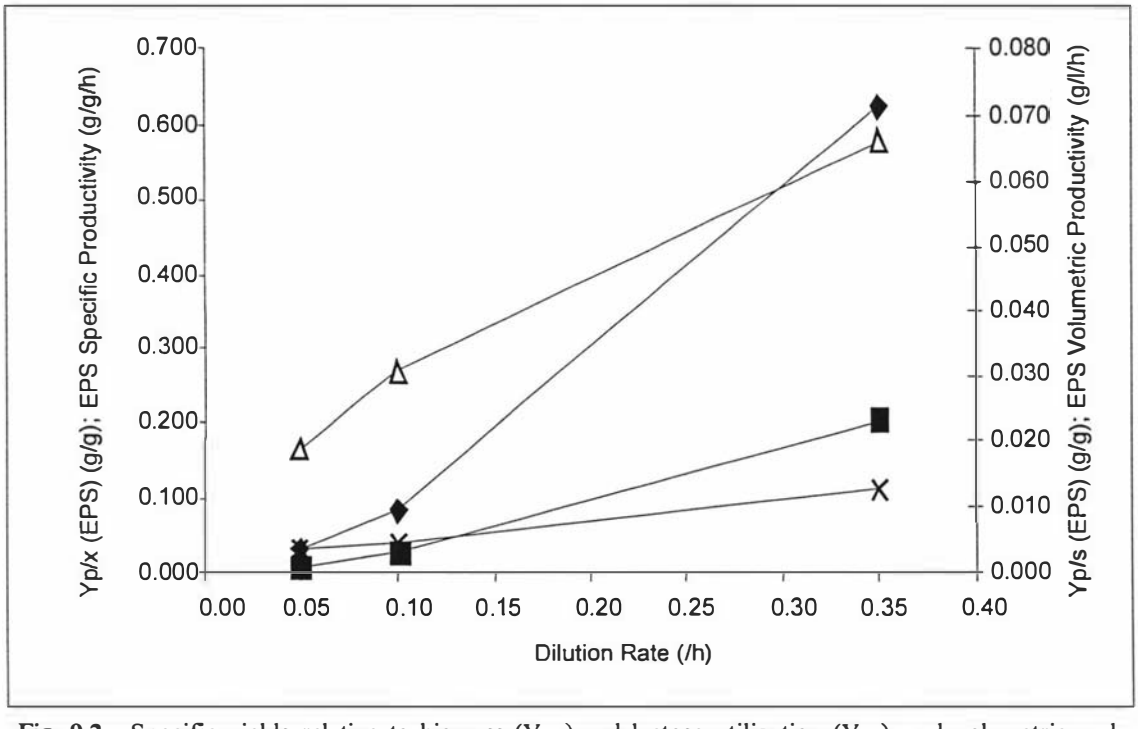

Fig. 9.2. Specific yields relative to biomass ($Y_{p/x}$) and lactose utilization ($Y_{p/s}$), and volumetric and specific productivities of EPS formation in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 grown in continuous culture in a semi-defined medium. Volumetric productivity (EPS) ♦; Specific EPS productivity ■; Yp/x (EPS) Δ; Yp/s (EPS) x. All values based on the average of two steady state samples.

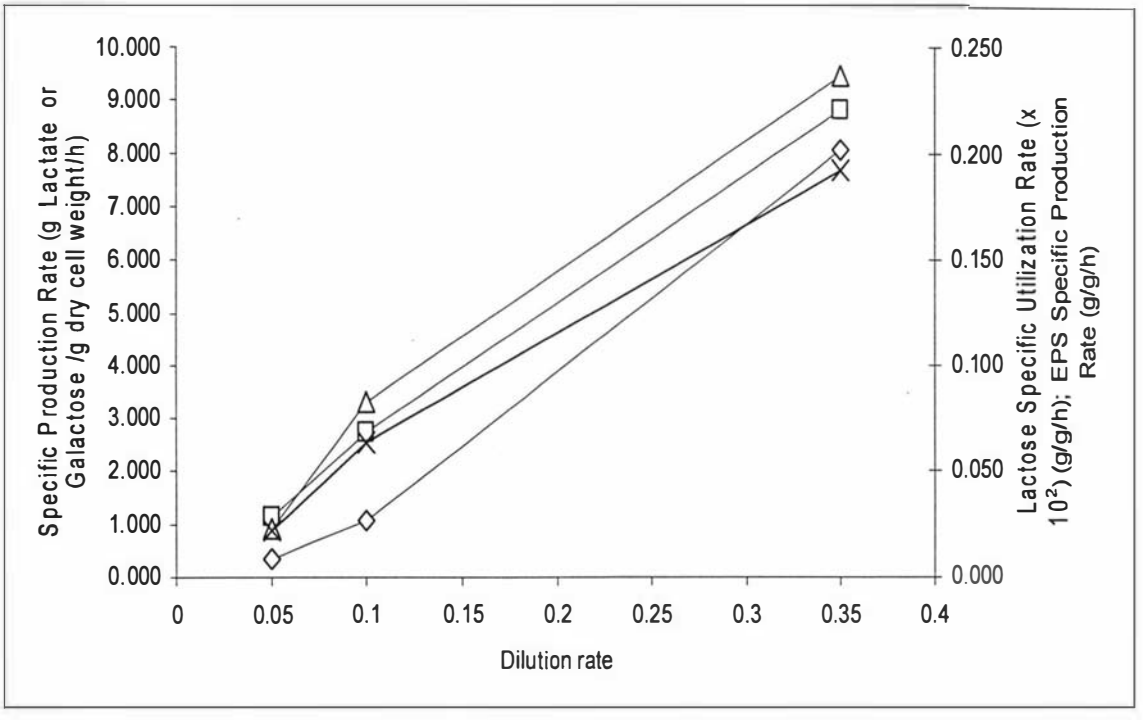

Fig. 9.3. Specific productivities of EPS, lactate, and galactose formation, and lactose utilization in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 grown in continuous culture in a semi-defined medium. EPS ◊; Lactate Δ; Galactose □; lactose x. All values based on the average of two steady state samples.

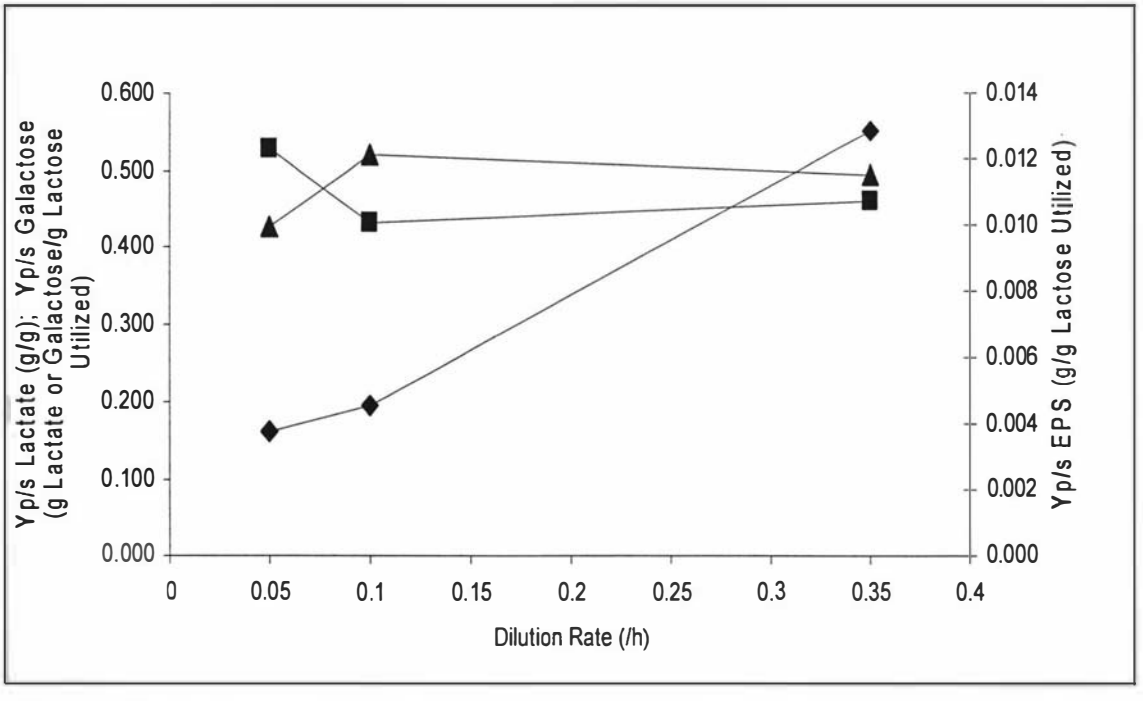

Fig. 9.4. Specific yields relative to lactose utilization ($Y_{p/s}$) of EPS, lactate, and galactose formation in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 grown in continuous culture in a semi-defined medium. EPS ●; Lactate ■; Galactose ▲. All values based on the average of two steady state samples.

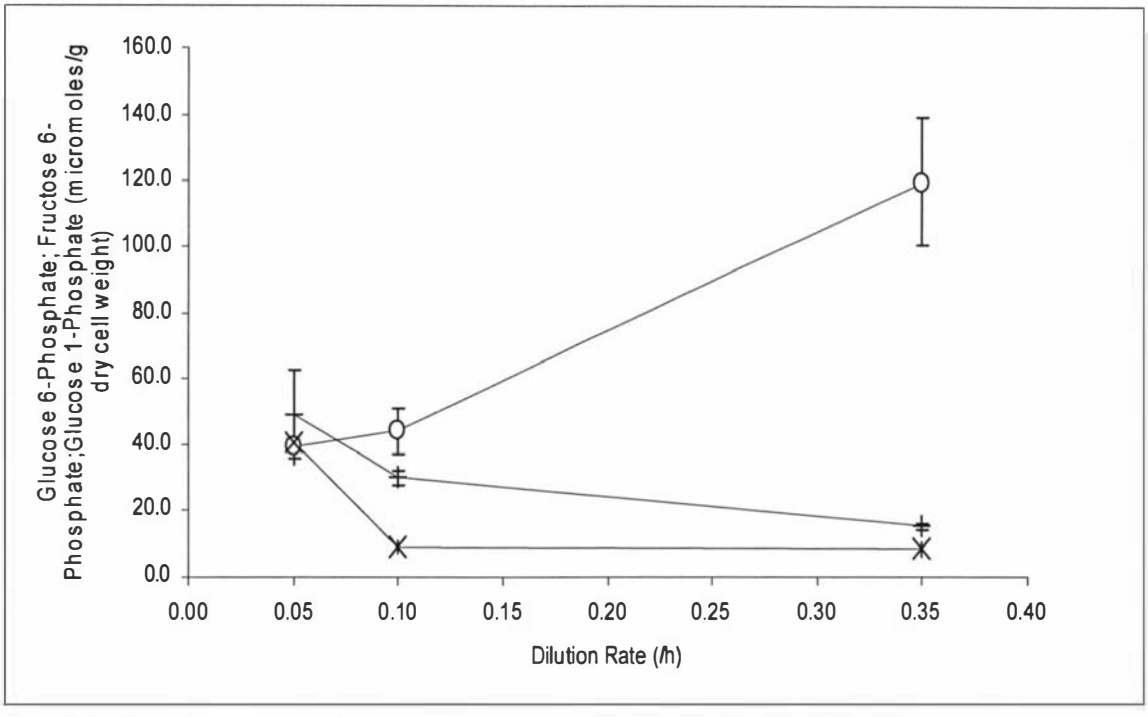

Fig. 9.5. Intracellular pools of glucose-6-phosphate, fructose-6-phosphate, and glucose-1-phosphate in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 grown in continuous culture in a semi-defined medium. Glucose-6-phosphate O; Fructose-6-phosphate +; Glucose 1-phosphate x. All values the average of three measurements. Error bars represent standard deviations.

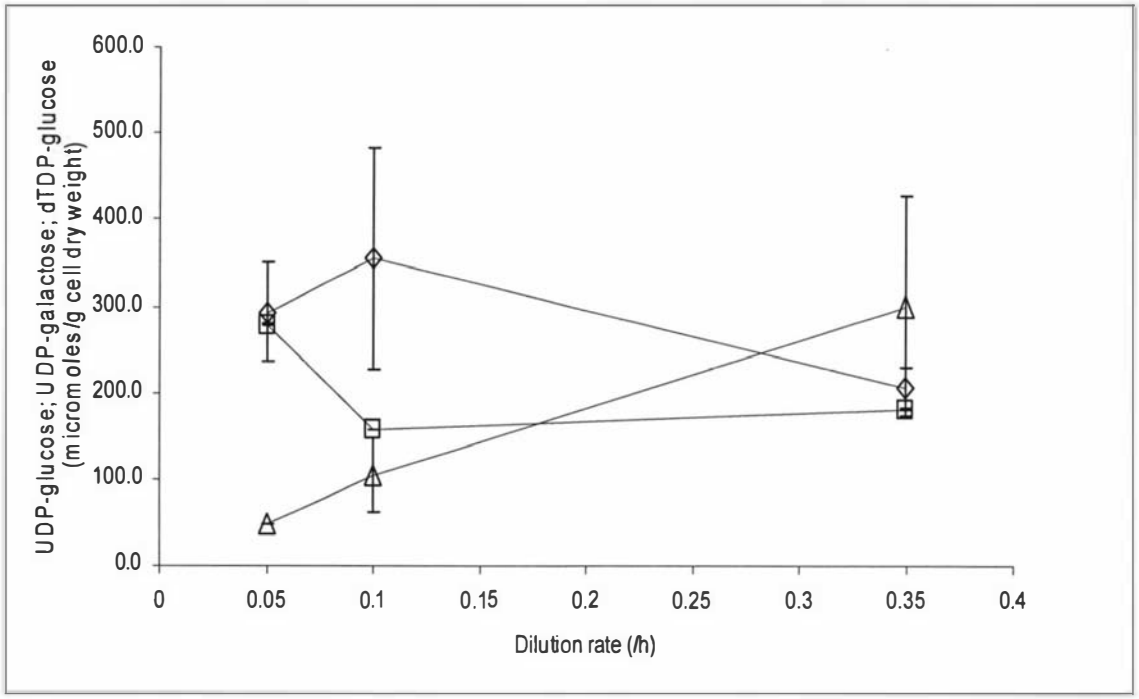

Fig. 9.6. Intracellular pools of UDP-glucose, UDP-galactose, and dTDP-glucose in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 grown in continuous culture in a semi-defined medium. UDP-glucose ◊; UDP-galactose □; dTDP-glucose Δ. All values the average of three measurements. Error bars represent standard deviations.

Table 9.1. Activities (nmol.mg cell protein^{-1}.min^{-1}) ± SD of enzymes associated with the glucose-6-phosphate and glucose-1-phosphate branch points in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 at different dilution rates in continuous culture, using a semi-defined medium.

Enzyme	Average activity (nmol.mg cell protein^{-1}.min^{-1}) at D=0.05 h^{-1}	Average activity (nmol.mg cell protein^{-1}.min^{-1}) at D=0.10 h^{-1}	Average activity (nmol.mg cell protein^{-1}.min^{-1}) at D=0.35 h^{-1}
α-Phosphoglucomutase (PGM)	10 ± 2	15 ± 1	11 ± 1
Phosphoglucose isomerase (PGI)	92 ± 10	66 ± 4	76 ± 6
UDP-glucose pyro - phosphorylase (UGP)	10 ± 2	14 ± 4	15 ± 2
UDP-galactose 4-epimerase (UGE)	13 ± 1	20 ± 1	30 ± 1
dTDP-glucose pyro – phosphorylase (TGP)	94 ± 2	69 ± 31	62 ± 2

Note: dTDP-glucose-4,6-dehydratase (TGD) and the dTDP-rhamnose synthetic enzyme system were detected in negligible quantities.

An attempt was made to relate EPS specific productivities (associated with the different dilution rates) and the specific activities of the enzymes associated with the formation of the sugar nucleotide precursors of EPS. UDP-galactose 4-epimerase emerged as bearing a marked association with raised levels of EPS production rates (Fig. 9.7).

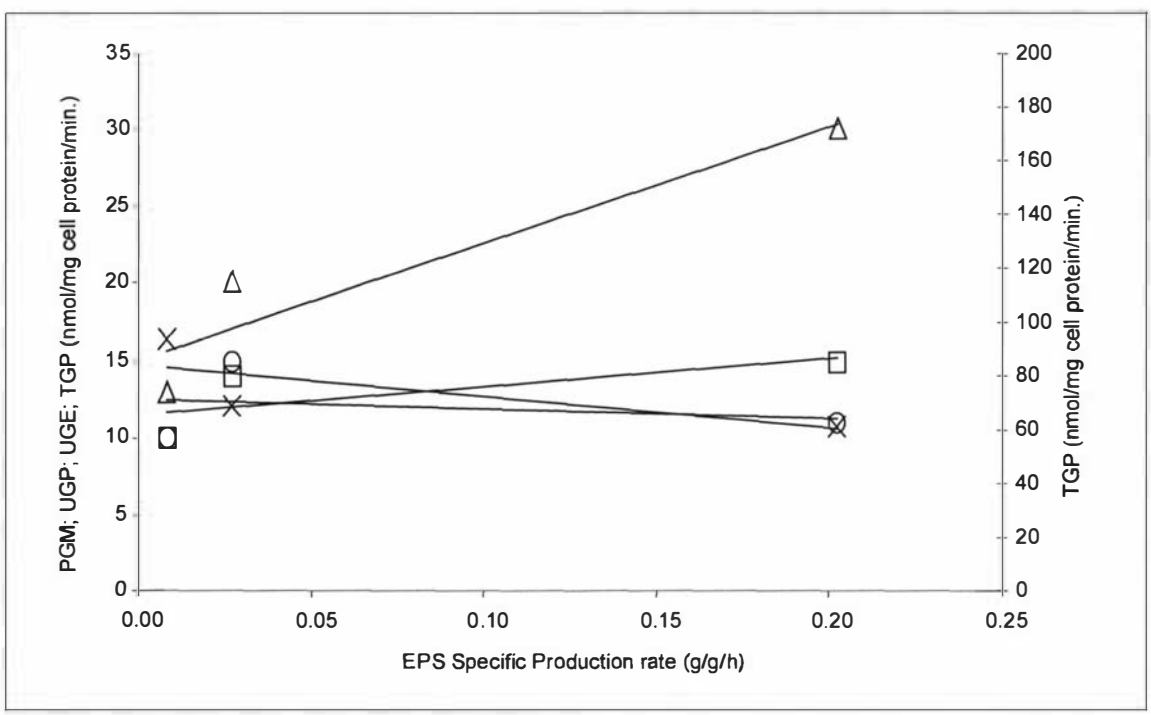

Fig 9.7. Activities (nmol.mg cell protein^{-1}.min^{-1}) of the enzymes α-Phosphoglucomutase (PGM) (O), UDP-glucose pyro –phosphorylase (UGP) (□), UDP-galactose 4-epimerase (UGE) (Δ), and dTDP-glucose pyro--phosphorylase (TGP) (x) in relation to EPS specific productivity in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483, grown in a semi-defined medium.

Additionally, a comparison was made between EPS titre (associated with increasing dilution rate), and ATP and ADP levels in the cell determined at the respective dilution rates. An increasing trend of the level of the energy-rich nucleotides was associated with increasing EPS formation (Figure 9.8).

Cellular NADH oxidase activities (regeneration of NAD^+ from NADH) were generally raised at the higher dilution rates, though no particular trend was clear in this respect (Table 9.2).

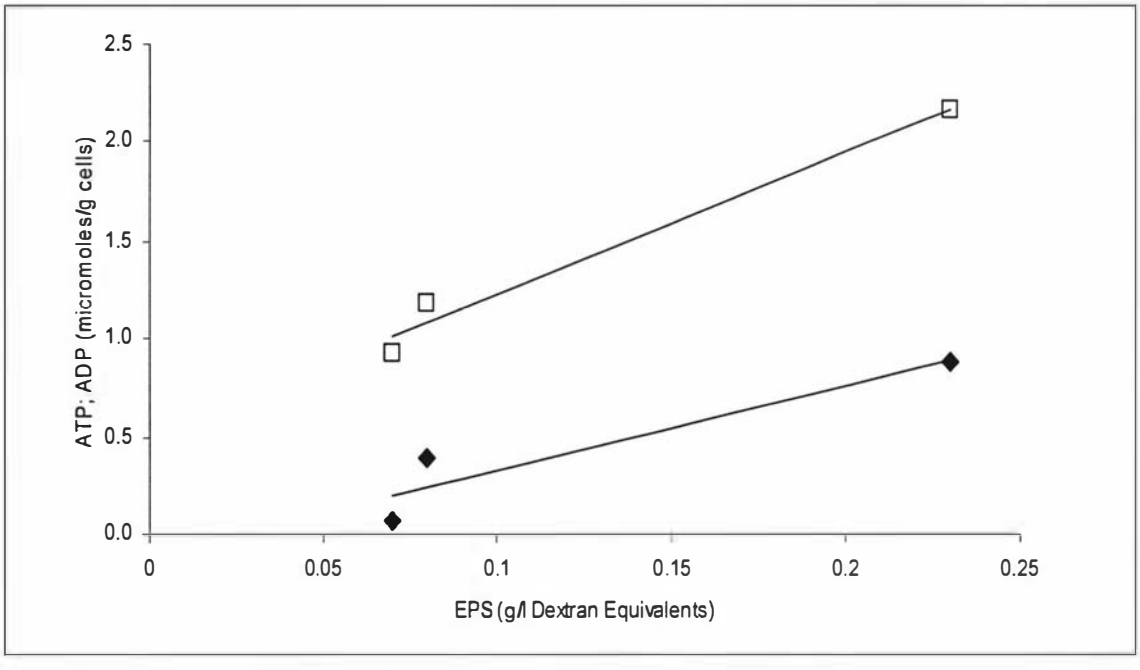

Fig 9.8. Correlation between ADP and ATP levels, and EPS formation in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483, grown in a semi-defined medium at steady state in continuous culture. ATP □; ADP ◆

Table 9.2. Activities (nmol.mg cell protein^{-1}.min^{-1}) ± SD of the NADH oxidase (NOX) enzyme in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 at different dilution rates in continuous culture, using a semi-defined medium.

Enzyme	**Average activity (nmol.mg cell protein^{-1}.min^{-1}) at D=0.05 h^{-1}**	**Average activity (nmol.mg cell protein^{-1}.min^{-1}) at D=0.10 h^{-1}**	**Average activity (nmol.mg cell protein^{-1}.min^{-1}) at D=0.35 h^{-1}**
NADH oxidase (NOX)	26 ± 1	48 ± 2	34 ± 1

DISCUSSION

In shifting the dilution rate of the culture of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 from 0.05 h^{-1} to 0.10 h^{-1}, the flux of lactose carbon to EPS increased; a subsequent elevation of the dilution rate to 0.35 h^{-1} raised the flux to EPS further (Figures 9.1 and 9.2). The metabolic flux to EPS remained growth-associated, as was the formation of lactate and galactose (Figure 9.3). These results contrast with those of Looijesteijn *et al.* (2000), in which the amount of EPS produced by *L. lactis* subsp. *cremoris* per A_{600} when grown in chemically defined medium in continuous culture (undefined limiting factor) increased with decreasing dilution rate, a consequence of energy production via glycolysis (production of lactic acid) being completely uncoupled from biomass formation. In the current investigation, lactose utilization rates followed the same trends as EPS formation. The elevation of $Y_{p/s}$ values of EPS formation with increasing dilution rate (Figures 9.4) demonstrates the diversion of carbon from the formation of other metabolites to EPS formation in response to a changed metabolic status of the cell at higher growth rates. Whether carbon was diverted from the formation of galactose or lactate is unclear, due to the low levels of EPS formed by the organism. The substantial elevation in the ratio of EPS to lactate at 0.35 h^{-1}, relative to the lower dilution rates, however, suggests that more carbon was diverted to EPS relative to lactate at the higher dilution rate. It is possible that the formation of lactate was diminished in favour of EPS production, as the anabolic pathways of EPS formation are linked to the catabolism of glucose (from lactose) by the bifurcation of carbon flux at the glucose-6-phosphate branch-point.

At the higher growth rates, corresponding to raised levels of formation of lactate and galactose, the evidence suggests that excess glucose carbon in the cell which could not be processed by glycolysis, was diverted at the glucose-6-phosphate branch point to EPS. The growth-related increase in flux of carbon to EPS and lactate is demonstrated by the increasing levels of intracellular pools of glucose-6-phosphate, and the diminished pools of its metabolites, fructose-6-phosphate (towards lactate formation) and glucose-1-phosphate (towards EPS formation) (Figure 9.5). The progressive accumulation of glucose-6-phosphate with increasing dilution rate (growth rate) was likely due to a limitation in the abilities of the enzymes phosphoglucomutase and phosphoglucoisomerase to convert their substrate. The corresponding specific activities of these enzymes at the different dilution rates support this position (Table 9.1). A small, but significant increase in the activity of phosphoglucomutase between a dilution rate of 0.05 h^{-1} and 0.10 h^{-1} , however, points to an enhancement of flux towards glucose-1-phosphate. Whilst no significant changes were observed at different dilution rates for UDP-glucose, the levels of UDP-galactose were reduced between a dilution rate of 0.05 h^{-1} and 0.10 h^{-1} (Figure 9.6). Ramos *et al.* (2001) determined that UDP-galactose and UDP-glucose were

significantly lower in a *Lactococcus lactis* EPS-producing strain than a non-producing one. In the present study, the pools of dTDP-glucose were progressively raised with increasing dilution rate, implying an inability of the cell to cope with accumulating levels of this metabolite. This fact, coupled with the negligible activities of the dTDP-glucose 6-hydratase enzyme and the dTDP-rhamnose synthetic enzyme system suggest strongly that the main avenue of carbon flux to EPS was via the formation of the precursors UDP-glucose and UDP-galactose. The composition of EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 would hence be expected to contain lower levels of rhamnose than glucose or galactose.

In terms of sugar-nucleotide generation in the EPS-producing pathway, the principal enzyme associated with raised levels of EPS was UDP-galactose 4-epimerase (Table 9.1), which demonstrated a trend of progressively increasing activity with increasing dilution rate. The association between EPS formation rate and UDP-galactose 4-epimerase activity was demonstrated by a simple correlation with EPS specific productivities (Figure 9.7). Although it is apparent that galactose produced by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 from lactose is exported (as in most of the dairy strains of *Lb. delbrueckii* subsp. *bulgaricus* - Marshall *et al.*, 2001), it is not inconceivable that UDP-galactose 4-epimerase, which serves the Leloir pathway in LAB, is involved in the utilization of very small amounts of galactose in the synthesis of EPS. The Leloir pathway has been firmly established for some strains of *Streptococcus thermophilus* (Thomas and Crow, 1984), however this route of galactose utilization remains to be proved in this strain of *Lb. delbrueckii* subsp. *bulgaricus.* Associations between the activity of UDP-galactose 4-epimerase and EPS formation have been reported in *L.lactis* (Forsén and Häivä, 1981), and in *S. thermophilus* strains LY03 (Degeest and De Vuyst, 2000) and Sfi 20 (Degeest *et al.*, 2001). In contrast, the UDP-galactose 4-epimerase activity of *Lb. delbrueckii* subsp. *bulgaricus* NCFB2772 was not shown to be closely associated with the sugar composition of EPS produced by this strain (Grobben *et al.*, 1996). Escalante *et al.* (1998) did not find an association between UDP-galactose 4-epimerase activity and EPS production in ropy or non-ropy Gal $^{-}$ strains of *S. thermophilus*. However, they did establish an association between UDP-glucose pyrophosphorylase and EPS production in the ropy strain during the first 12 h in a 20 h culture. In the present study, similar correlations could not be established for the other enzymes associated with the glucose 6-phosphate and glucose-1-phosphate nodes viz. α-phosphoglucomutase, UDP-glucose pyrophosphorylase, and dTDP-glucose pyrophosphorylase (Figure 9.7). Sjöberg and Hahn-Hägerdal (1989) reported a relationship between β-phosphoglucomutase and EPS production in *L. lactis*. Degeest and De Vuyst (2000) found (apart from the association between UDP-galactose 4-epimerase activity and EPS production) a correlation between α-phosphoglucomutase and UDP-glucose pyrophosphorylase, and EPS formation in *S. thermophilus* LY03. Mozzi *et al.* (2001) correlated

higher EPS synthesis in galactose-grown *Lactobacillus casei* CRL 87 with higher UDP-glucose pyrophosphorylase, dTDP-glucose pyrophosphorylase, and dTDP-rhamnose synthetic enzyme system activities. No coherent relationship between the activities of enzymes associated with the above nodes and raised EPS production can hence be established across various strains of LAB. Sutherland (1972) held that the possibilities for controlling polysaccharide synthesis can exist at any of the enzymes associated with EPS biosynthesis.

Enhanced EPS production related to raised growth rates in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 appeared to be associated as well with a raised energy status of the cell. A correlation was evident between EPS titres and ATP and ADP (Figure 9.8). It is hence possible that raised EPS production in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 occurs in response to a carbon and energy surplus in the cell, employing the pathways of nucleotide-sugar production usually dedicated to cell wall production as a means to consume excess ATP and carbon. The fully assembled carbon-rich polymers (EPS) are then able to be easily exported from the cell. It may be worthwhile to pursue further work in order to determine whether ATP levels in the cell can influence the molecular mass of the EPS produced, as has been speculated by Looijesteijn *et al.* (2000). The mechanism proposed involves an *eps* gene product (*EpsB*) which contains an ATP-binding domain similar to the ExoP-like proteins which have been found to determine EPS chain length in *L.lactis* (Van Kranenburg *et al.*, 1997).

The anabolic pathways of EPS production would hence be dependent upon glycolysis for the generation of sufficient ATP for the formation sugar-nucleotides dedicated to cell wall and EPS production. At the higher growth rates, a surplus of ATP would suppress glycolysis, thus limiting the regeneration of NAD^+ produced via pyruvate reduction. (Tuesink *et al.*, 1998; Neves *et al.*, 1999). It was of interest to see whether an alternative to this mode of NAD^+ regeneration was present viz. by conversion via NADH oxidase (NOX). The presence of NADH oxidase activity has been reported by Marty-Teysset *et al.* (2000) to occur in aerated and unaerated cultures of *Lb. delbrueckii* subsp. *bulgaricus* B107. In their investigation, the specific activity of the NADH-oxidase was the same in aerated and unaerated cultures, suggesting that the enzyme was not directly regulated by oxygen. In the present investigation, despite the anaerobic head-pressure applied to the fermentation culture in the present study, low levels of dissolved oxygen would have been introduced into the medium with the feed and constant agitation. The elevated levels of NOX which were associated with raised growth rates and raised levels of EPS formation may be an indication that *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 accommodates a higher NAD^+ requirement at higher growth rates by use of this regeneration route. It remains to be demonstrated whether increasing the aeration in the culture would promote the regeneration capacity of the cell for NAD^+ using NADH-oxidase in

preference to the reduction of pyruvate. Lopez de Filipe *et al.* (1998) have employed this system as a strategy to regulate the cofactor balance ($NADH/NAD^+$) in *L. lactis*, thereby diverting carbon flux away from lactate production in favour of other metabolites.

CONCLUSION

Lb. delbrueckii subsp. *bulgaricus* NCFB 2483 exhibited increasing levels of flux to EPS, lactate, and galactose in response to increasing growth rates, when grown on lactose in a semi-defined medium in continuous culture. An increasing proportion of carbon from lactose was diverted to EPS formation at the higher growth rates, with most of the flux being channeled via a route of formation involving glucose-1-phosphate, and the sugar nucleotides UDP-glucose and UDP-galactose, and controlled by raised levels of UDP-galactose 4-epimerase activity. An association between raised EPS titres and a surplus of energy availability in the cell is possible.

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CHAPTER 10

ENZYMES ASSOCIATED WITH RAISED METABOLIC FLUX TO EXOPOLYSACCHARIDE SUGAR NUCLEOTIDE PRECURSORS IN *LACTOBACILLUS DELBRUECKII* SUBSP. *BULGARICUS*

ABSTRACT

Exopolysaccharide (EPS) formation in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 was compared with that in a mutant strain of the organism, as a means to elucidate mechanisms associated with raised carbon flux to formation of the polymer. The same comparisons were undertaken on *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 under a perturbed metabolic state resulting from conditions of salt-stress. Under continuous culture conditions ($D=0.10\ h^{-1}$), the higher metabolic flux towards EPS formation in the mutant strain relative to the parent appeared to be mediated by raised levels of UDP-glucose pyrophosphorylase (UGP). Marginally raised UDP-galactose 4-epimerase (UGE) activities suggest a possible role for this enzyme in EPS overproduction. Although production of polymer by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 grown in continuous culture ($D=0.10\ h^{-1}$) was diminished under conditions of reduced water activity (adjustment from an a_w of 0.99 to 0.98), the level of flux towards sugar-nucleotide synthesis was raised, suggesting a metabolic "bottleneck" at the level of EPS assembly or export at this growth rate. In this instance the levels of activity of α-phosphoglucomutase (PGM), UDP-glucose pyrophosphorylase (UGP), UDP-galactose 4-epimerase (UGE), and dTDP-glucose pyrophosphorylase (TGP) were raised compared to the non-perturbed state. A higher accumulation of intracellular glucose-6-phosphate in the mutant, and in the salt-stressed culture are suggestive of a metabolic constriction at this branchpoint. In both instances, a raised flux towards sugar-nucleotide formation was associated with higher levels of ATP in the cell, and reduced levels of biomass at the dilution rate applied.

INTRODUCTION

Exopolysaccharides produced by lactic acid bacteria (LAB) are attracting increased interest, not only for their ability to improve the textural quality of dairy foods, but for their promise of conferring health benefits upon the consumer (De Vuyst *et al.*,2001). Investigations involving mouse models have suggested that EPS's possess immunostimulatory, antitumoral, and cholesterol-lowering activity (Kitazawa *et al.*, 1991, 1996, 1998; Nakajima *et al.*, 1992). A clear need therefore exists to improve the productive capacity of LAB for EPS formation, as

well as induce structural changes, which would demonstrably improve the functional characteristics of the polymers. Knowledge of intracellular concentrations of metabolites complements the measurement of enzymatic activities, and is a valuable tool in promoting targeted improvements of metabolites (Jensen and Hammer, 1998).

The biosynthetic network of EPS production can be broken down into four main groups of reactions *viz.* sugar transport into the cell, the synthesis of sugar-1-phosphates, the synthesis of sugar nucleotides and conversion into the EPS repeating unit, and EPS polymerization and export from the cell (Laws *et al.*, 2001) (Figure 10.1). The potential for exerting control over enzymes associated with EPS formation exists at any of these levels, and mutants lacking enzymes of any group fail to synthesize EPS (Looijesteijn *et al*, 1999; Sutherland, 1972). A number of studies undertaken to date have demonstrated an association between the activities of enzymes of sugar catabolism and sugar nucleotide formation with EPS production. Mozzi *et al.* (2001) determined that larger polymer synthesis by *Lactobacillus casei* on galactose-grown cells was correlated with higher UDP–glucose pyrophosphorylase, dTDP-glucose-pyrophosphorylase, and dTDP-rhamnose-synthetic enzyme system activities. Grobben *et al.* (1996) found a positive relationship between UDP-glucose pyrophosphorylase and EPS production by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772. The same correlation was found by Escalante *et al.* (1998) in *Streptococcus thermophilus*. A correlation between α-phosphoglucomutase, UDP-glucose pyrophosphorylase, UDP-galactose 4-epimerase and EPS production was reported by Degeest and De Vuyst (2000) in *St. thermophilus* LY03.

In a previous study, it was found that UDP-galactose 4-epimerase played a key role in mediating increased levels of EPS synthesis associated with increasing growth rates in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 (Welman, Chapter 9 – PhD Thesis). In the present work, the effects of a chemically induced mutation, and a perturbation by salt-stress on enzymes and metabolites involved in the formation of sugar-1-phosphates and sugar nucleotides in the EPS synthetic pathway were investigated.

MATERIALS AND METHODS

Medium. Preparation of the nutrient medium was undertaken according to a method described by Kimmel and Roberts (1998), but with the glucose component replaced with lactose. The medium consisted of lactose, 20.0 g/l; yeast nitrogen base (without amino acids and ammonia), 5.0 g/l; bacto casitone, 20.0 g/l; sorbitan monoleate ("Tween 80"), 1.0 g/l; dipotassium phosphate (K_2HPO_4), 2.0 g/l; magnesium sulphate ($MgSO_4.7H_2O$), 0.10 g/l; manganese sulphate

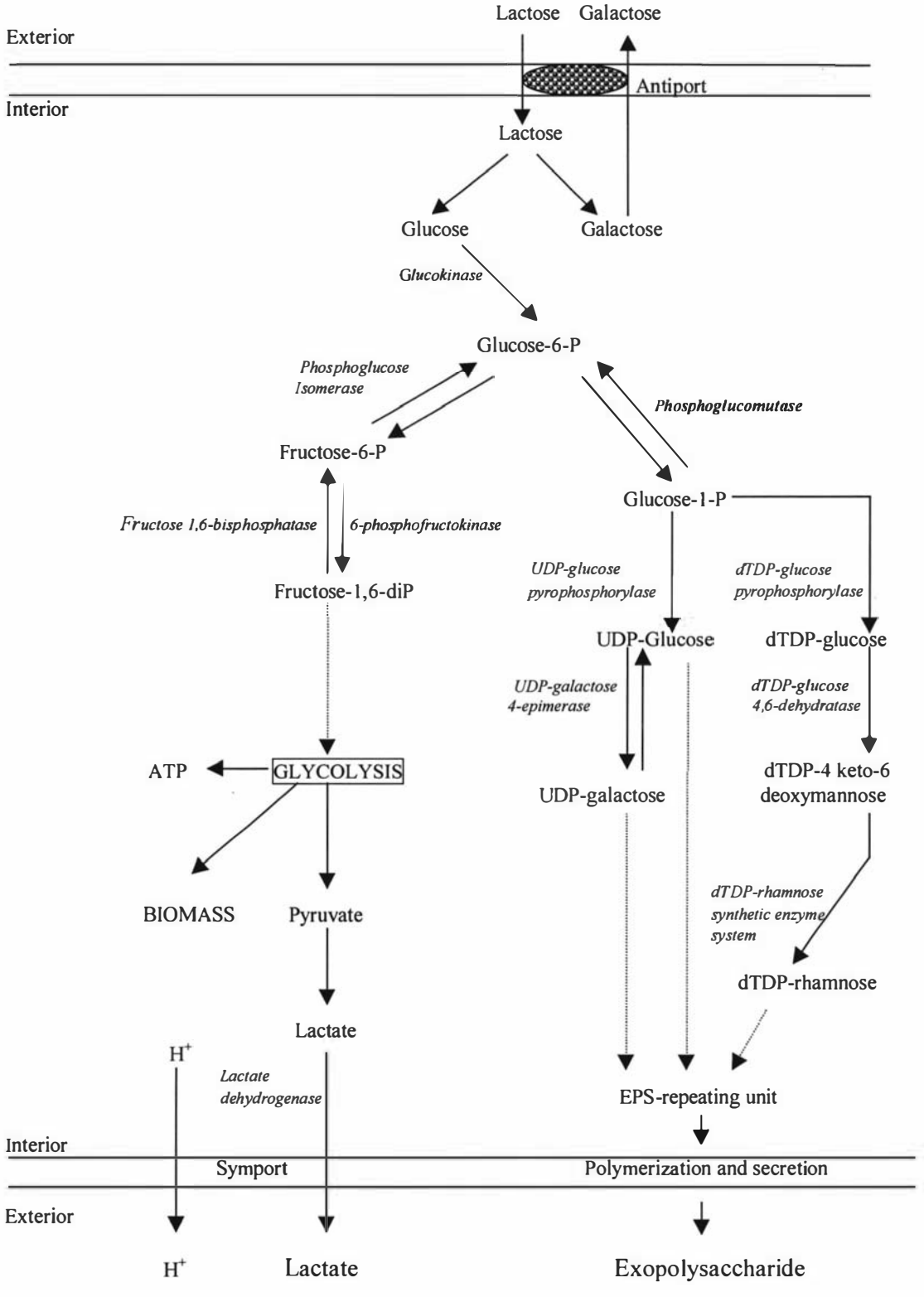

Fig. 10.1. Simplified scheme of the bioreaction network leading to exopolysaccharide synthesis in *Lactobacillus delbrueckii* subsp. *bulgaricus*.

($MnSO_4.4H_2O$), 0.05g/l; ammonium citrate, 2.0 g/l; sodium acetate, 5.0 g/l. The inoculation medium was prepared in separate, double–strength volumes of lactose and the remainder of the nutrients as described above, adjusted to pH 6.0. After steam–sterilization, the aliquots were allowed to cool, and pooled, achieving the desired concentration of medium constituents. Where solid medium was prepared, 15.0 g/l of agar was used.

The fermentation medium (20 l) was prepared by sterilizing separately 1.5 l of a solution of lactose.H_2O (421.0 g) , and 18.5 l of a solution of the remainder of the nutrients combined with antifoam (Bevaloid, 15 ml) in distilled and deionized water in a 20 l carboy (sterilization by steam at for 90 min. at 121°C). The medium components were allowed to cool prior to being pooled in the 20 l feed vessel. Loss of feed volume through venting of the vessel after sterilization was restored by the aseptic addition of the sterile water.

Preparation of the medium with reduced water activity (a_w of 0.98) was undertaken as described in Chapter 8 (Welman, PhD Thesis).

Bacterial strains and inoculum. *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 (NCIMB 702483) was obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland. The fermentation inoculum (approx. 10% by volume of the fermentor working volume) was prepared by inoculating duplicate inoculation flasks containing 150 ml of medium with a 1.0 ml aliquot from a working cell bank, preserved at –80°C, and incubating at 37°C for 24 h on an orbital shaker at 100 rpm. The mutant strain (E2483M) was obtained by screening of mutagenized strains for acidifying ability, mucoidy, ropiness, and EPS production. Chemical mutagenesis was undertaken using N-Methyl-N'-nitro-N-nitrosoguanidine (NTG) (Welman – Chapter 5, PhD Thesis).

Fermentation conditions. All of the continuous culture experiments were undertaken at a dilution rate of 0.10 h^{-1} in a Bioflo I fermentor (New Brunswick Scientific, NJ) equipped with pH and temperature control, and a magnetically coupled agitation system. A glass fermentation vessel (1400 ml working volume) was used with the liquid level being maintained with an overflow weir. The fermentation feed medium was fed to the fermentation vessel by peristaltic pump calibrated to deliver the required flow rates. A continuous head pressure of nitrogen was maintained for all experiments. The fermentor was operated initially in a batch mode in order for sufficient cell mass to accumulate prior to commencing the continuous studies. Temperature was maintained at a constant 37°C by a heating probe and circulation of cooling water through internal veins submerged in the fermentor. Agitation was set at 200 rpm and pH was maintained at 6.0 by titration with 2 M KOH. Fermentations at each dilution rate were run until

constant biomass levels were obtained, corresponding to steady state conditions; this was usually achieved after four to five fermentor volumes had been replaced. Fermentation conditions in respect of the adjustment of the medium a_w to 0.98 was undertaken as described in Chapter 8 (Welman - PhD Thesis).

Sampling. Sample aliquots (15 ml) were withdrawn aseptically at regular intervals for the determination of biomass, and sugar conversion to metabolites. In instances where analyses could not be immediately undertaken, samples were stored in pre-sterilized bottles at -20°C. As is the requirement for the operation of continuous fermentation systems, a high level of aseptic control was applied in order to maintain monoseptic conditions in the fermentations. Fermentation medium and broth samples were examined microscopically, and plated onto solid culture medium for the detection of contaminants. The inoculated plates were incubated aerobically and anaerobically for a period of 60 h.

A 300ml aliquot of broth was used for enzyme extraction; the broth (at steady state) was rapidly decanted via the overflow weir into a pre-chilled centrifuge bottle on ice. Samples for assay of intracellular metabolites in extracts were removed from the fermentor and extracted using a modification of a rapid system previously described by Guedon *et al.* (1999), Fordyce *et al.* (1984) and Thomas *et al.* (1979). A sterile syringe containing 1.5 ml of $HClO_4$, sealed at the exit with a silicon tube and stopcock, and pre-drawn to a volume of 15.0 ml under vacuum, was attached to the fermentor sampling port. Fermentation broth under steady state conditions was rapidly withdrawn from the fermentor vessel upon release of the stopcock; the final $HClO_4$ concentration in the syringe was 0.6M.

Analyses. Bacterial growth was monitored by absorbance measurement at 650 nm. Biomass was determined from a standard curve relating absorbance at 650nm to washed dry cell weights. Lactose utilization, and galactose and lactate formation was measured by HPLC (Waters Alliance 2690, coupled to a Waters 2410 Differential Refractometer, and a Waters 2487 UV spectrophotometer). The compounds were detected using an Aminex HPX-87H, 300 x 7.8 mm column (Biorad, Richmond, CA), according to the method as described in Ross and Chapital (1987). The column temperature was maintained at 40°C, and the flow rate at 0.6 ml. min^{-1}. Broth samples were clarified by centrifugation at 16 000 g for 15 minutes prior to dilution in distilled and filtered water (MilliQ), prior to analysis. External standards were prepared for lactose, galactose, and lactic acid. All analyses were undertaken in duplicate. Extracellular polysaccharides were subjected to a crude isolation prior to analysis. Aliquots (100 µl) of whole broth underwent a two–fold precipitation with chilled ethanol (2.9 ml distilled H_2O and 7.0 ml 99.7% ethanol) for 24h periods at 4°C. The broth precipitate was recovered by

centrifugation (35 850 g, 40 min., 4°C) (Sorvall RC5C – SS34 rotor) and resuspended in 1.0 ml distilled water. The total sugar concentration in the resuspended sample was measured according to the method of Dubois *et al.* (1956), with dextran as the standard.

Assay of intracellular metabolites. Treatment of the sample was undertaken according to the method described by Guedon *et al.* (1999) and Fordyce *et al.* (1984). The sample syringe containing extracted broth was maintained for 2 minutes at room temperature after the sampling procedure. The extracts of the metabolites were subsequently placed on ice for 10 minutes under N_2 gas, following which 0.69g of K_2CO_3 was slowly added. Neutralization of the samples was undertaken using 3M KOH, such that the final pH was between 7.0 and 7.5. The neutralized extracts were subsequently centrifuged at 10 000 g for 10 minutes at 4°C, and the supernatants stored at -70°C until assayed.

Assays of glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), and glucose-1-phosphate (G1P) were undertaken by a modification of the method described by Garrigues *et al.* (1997) using fluorimetric determination of NADPH formed in enzyme assays. Emission was measured at 460 nm after excitation at 350 nm using a Perkin Elmer LS 50B Luminescence Spectrometer. NADPH formed was related to fluorimetric intensity by means of a calibration curve (R^2=0.99) (Cook *et al.*, 1976). To 1150 µl of a mixture of 500 mM triethanolamine buffer (pH 7.6) containing 15 mM $MgSO_4$ and 4mM EDTA, 40 µl 10 mM NADP, 760 µl H_2O, and 50 µl metabolite extract, was added 20 µl glucose-6-phosphate dehydrogenase (200 U/ml) in order to commence conversion of G6P. After the reaction had proceeded to completion, 20 µl of phosphoglucose isomerase (200 U/ml) was added in order to measure the concentration of F6P. After this reaction had completed, 20 µl phosphoglucomutase (200 U/ml) was added for the measurement of G1P. Triplicate assays were undertaken for each metabolite.

Uridine-5'-diphosphoglucose (UDP-glucose) and uridine-5'-diphosphogalactose (UDP-galactose) were measured by an enzymatic method (Keppler and Decker, 1985) in which the formation of NADH is measured at A_{339} at 25°C. The absorbance (A_1) of 200 µl of sample extract in 500 µl of a mixture of NAD (20 mg), EDTA-Na_2H_2.2 H_2O (30 mg), and Na_2CO_3 (23 mg) in 10 ml 0.5 M glycine buffer at pH 7.0 was measured prior to the addition of 20 µl of UDP-glucose dehydrogenase (3 kU/l). The absorbance (A_2) was recorded after completion of the reaction. The absorbance (A_3) was again measured after completion of the subsequent reaction with UDP-glucose 4-epimerase (10 kU/l).

Deoxythymidine-5'-diphosphoglucose (dTDP-glucose) was assayed according to a modification of the method of Bevill (1974). An extract (50 µl) of dTDP-glucose dehydrase (prepared from

E. coli Strain B56 according to the methods of Gilbert *et al.* (1965), and Wang and Gabriel (1969)) was incubated at 37°C for 1 minute in 1320 µl Tris buffer (50 mM pH 8.6; 2 mM EDTA) prior to the addition of 150 µl metabolite extract. This mixture was subsequently incubated at 37°C for 30 minutes following which 1480 µl 0.2N NaOH was added. A further incubation period of 15 minutes at 37°C followed, after which the absorbance was measured at 318 nm against a blank which was prepared identically, with the exception that the aliquot of 0.2N NaOH was added before addition of the metabolite extract.

Preparation of cell-free extracts. Crude enzyme extracts were prepared according to a modification of the method of Escalante *et al.* (1998). Fermentation broth (280 ml) was centrifuged at 35 850 g for 40 minutes at 4°C, and the supernatant fraction discarded. The cell pellet was washed with 0.01M cold phosphate buffer by centrifugation at 35 850 g for 40 minutes at 4°C. The pellet was resuspended to a total volume of 28 ml (10 X concentration) in 0.01M phosphate buffer and kept on ice. Glass beads (0.13 mm diameter) were added in a ratio of 5.0 g glass to 1.0 g of cell mass. Homogenization of the cell suspension was undertaken in a Braun MSK homogenizer (3 x 20 s at 400 rpm) with concurrent cooling using a jet of dry CO_2 flowing over the homogenization chamber so as to prevent loss of enzyme activity. The homogenization procedure was pre-optimized in preliminary experiments. The glass beads were removed using a scintered glass funnel on ice. The supernatant fraction was further centrifuged at 35 850 g for 10 minutes at 4°C prior to assay. Where the extract could not be assayed immediately, it was stored at -20°C, and assayed for loss of enzymatic activity.

Enzyme assays. All of the enzyme assays were undertaken at 37°C, and the formation or disappearance of NAD(P)H was measured at 340 nm (ε_{340} = 6220 $M^{-1}.cm^{-1}$), unless stated otherwise. Protein concentration of the enzyme extracts was determined by the method of Lowry *et al.* (1951). Enzyme activities were expressed as nanomoles of substrate converted to product over 1 minute, per mg. of protein in the extract. Measurements were reported as means.

The α-phosphoglucomutase reaction (Qian *et al.*, 1994; Grobben *et al.*,1996) contained (per ml), 50 µmol triethanolamine, 5 µmol $MgCl_2$, 0.4 µmol NADP, 0.01 ml of glucose-6-phosphate dehydrogenase (180 $U.ml^{-1}$), and cell-free extract). The reaction was initiated by the addition of 2.5 µmol fructose-6-phosphate.

The dTDP-glucose pyrophosphorylase reaction mixture (Bernstein, 1965; Looijesteijn *et al.*, 1999) contained (per ml), 50 µmol Tris-HCL buffer (pH 7.8), 8 µmol $MgCl_2$, 0.3 µmol $NADP^+$, 0.1 µmol TDP-glucose, 2.1 U of α-phosphoglucomutase, 4U of glucose-6-phosphate

dehydrogenase, and cell-free extract. Inorganic pyrophosphate (4.7 μmol) was used to start the reaction.

The UDP-glucose pyrophosphorylase reaction assay (Bernstein, 1965; Looijesteijn *et al.*, 1999) contained (per ml), 50 μmol Tris-HCL buffer (pH 7.8), 14 μmol $MgCl_2$, 0.3 μmol $NADP^+$, 0.1 μmol UDP-glucose, 2.1 U of α-phosphoglucomutase, 4U of glucose-6-phosphate dehydrogenase, and cell-free extract. Inorganic pyrophosphate (4 μmol) was used to initiate the reaction.

The UDP-galactose 4-epimerase reaction mixture (Grobben *et al.*, 1996; Looijesteijn *et al.*, 1999; Degeest and de Vuyst, 2000) contained (per ml), 50 μmol Tris-HCL buffer (pH 8.5), 5 μmol $MgCl_2$, 0.5 μmol NAD^+, 0.015 U UDP-glucose dehydrogenase, and cell-free extract. UDP-galactose (0.2 μmol) was used to start the reaction.

The phosphoglucose isomerase assay (reverse reaction) (Schreyer and Böck, 1980; Grobben *et al.*, 1996) contained (per ml), 50 μmol potassium phosphate (pH 6.8), 0.4 μmol NADP, 10 μl glucose-6-phosphate dehydrogenase (200 $U.ml^{-1}$), and cell-free extract. Fructose-6-phosphate (2.5 μmol) was used to initiate the reaction.

The dTDP-glucose 4,6-dehydratase reaction (Zarkowsky and Glaser, 1969; Grobben *et al.*, 1996; De Geest and de Vuyst, 2000) contained (per ml), 50 μmol Tris-HCL buffer and cell-free extract. The reaction was started with 0.3 μmol dTDP-glucose and incubated for 10 minutes at 37°C. Samples were taken at 60 s intervals and added to 0.1 N NaOH, and incubated for a further 15 minutes at 37°C. The formation of dTDP-6-deoxy-D-xylo-4-hexulose (ε_{320} = 4600 $M^{-1}.cm^{-1}$) was determined spectrophometrically at 320 nm.

The dTDP-rhamnose synthetic enzyme system (Martins and Sá-Correia, 1993) was assayed in an identical fashion to that for the dTDP-glucose 4,6-dehydratase reaction, with the exception that NADPH (0.5 μmol) was added to the initial incubation mixture. The formation of dTDP-rhamnose was measured at an absorbance of 340 nm.

NADH oxidase was measured by the method of Lopez de Felipe and Hugenholtz (2001). The reaction mixture contained (per ml), 50 μmol potassium phosphate (pH 7.0), 0.3 μmol EDTA, 0.050 μmol FAD, and 0.3 μmol NADH. The reaction was started with the cell-free extract.

Assay of nucleotide pools. The assay of adenosine-5'-triphosphate in the perchloric acid extracts was undertaken using an assay kit (Sigma Diagnostics ATP kit, Catalog No. 366).

Adenosine-5'-diphosphate was assayed by the method of Guedon *et al.* (2000). ADP in the sample extracts were converted to ATP using 7mM phosphocreatine in 0.1 M Tris-HCl buffer (pH 7.4), 0.4 mM $MgSO_4$, and 4U of creatine phosphokinase. This reaction mixture was subsequently incubated for 20 minutes at 30°C, following which the reaction was stopped by heating at 100°C for a period of 3 minutes. The samples were centrifuged at 8000 g for 15 minutes at 4°C.

RESULTS

The mutant of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483, E2483M, proved to be stable when grown in continuous culture (Figure 10.2). The relevant titres and performance characteristics of the mutant strain relative to the parent are shown in Table 10.1.

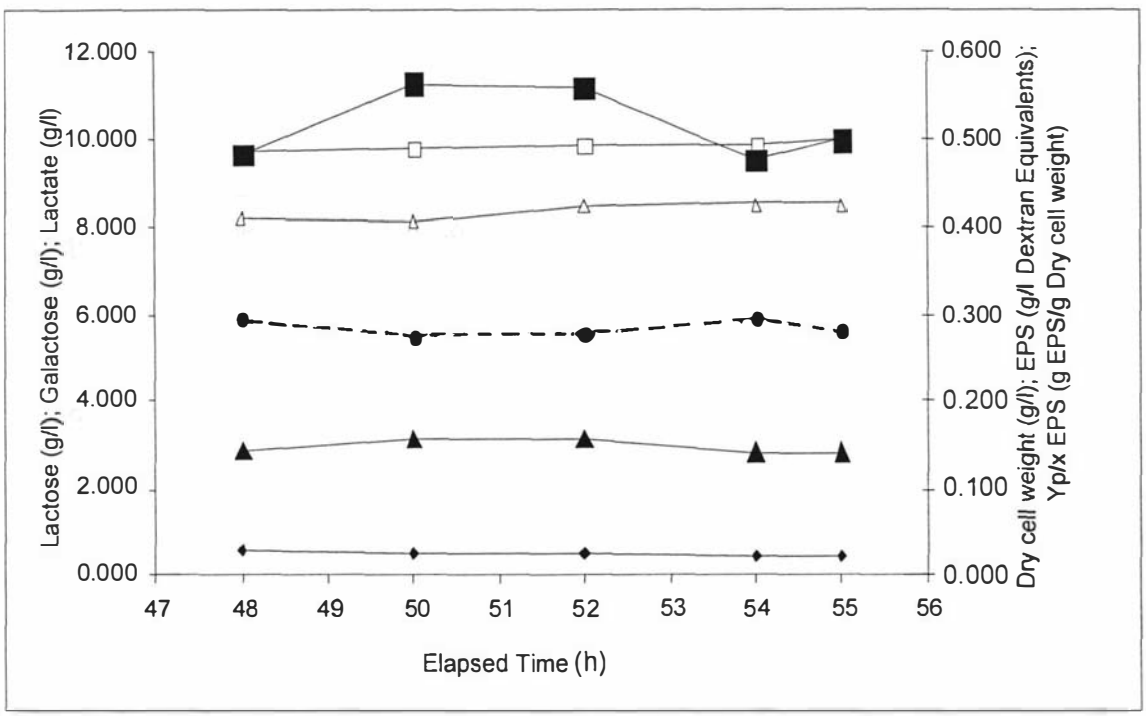

Fig. 10.2. Volumetric titres of EPS, biomass, galactose, lactate, and residual lactose, and specific yield of EPS relative to biomass of a mutant strain of *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 (E2483M) grown in continuous culture in a semi-defined medium. Residual lactose ♦; EPS ▲; Lactate Δ; Galactose □; Biomass (dry cell weight) ●, $Y_{p/s}$ ■. All values the average of two steady state samples.

The EPS titre of the mutant (E2483M) was substantially raised relative to the parent strain (Table 10.1). In addition, the values determined for $Y_{p/x}$ (EPS) and EPS specific productivity were elevated in comparison to the parent. The mutant exhibited a similar pattern of enhancement relative to the parent in respect of $Y_{p/x}$ and specific productivity of lactate and galactose (Table 10.1). No yield improvements on lactose consumed were apparent.

When *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 was grown in continuous culture at a dilution rate of 0.1 h^{-1} under conditions of reduced water activity (0.98) the EPS $Y_{p/x}$ and specific productivity values were marginally reduced relative to the corresponding values when the culture was grown at an a_w of 0.99, whilst substantial enhancements of these parameters were evident in the case of lactate and galactose production (Table 10.1) (see also Chapter 8 PhD Thesis – Welman). The $Y_{p/s}$ value for EPS production was substantively less than when grown in the medium at an a_w of 0.98, than when grown at an a_w of 0.99 (Table 10.1).

Larger intracellular pools of glucose-6-phosphate were present in the E2483M mutant strain than in the parent, however no obvious differences in the pools of fructose- 6-phosphate or glucose-1-phosphate were evident (Figure 10.3).

Table 10.1 Fermentation titres and performance characteristics of *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483, a mutant strain thereof (E2483M), and the NCFB 2483 strain at a reduced a_w (0.98), grown in continuous culture at a dilution rate of 0.10 h^{-1}. All values based on the average of two measurements.

	Parent Strain (NCFB 2483)	Mutant Strain (E2483M)	Strain NCFB 2483 under conditions of reduced a_w (0.98)
Dry Cell Weight (g/l)	0.359	0.297	0.191
Lactose (Residual) (g/l)	0.272	0.405	2.808
Galactose (g/l)	10.305	9.883	8.974
Lactate (g/l)	9.351	8.500	8.250
EPS (g/l)	0.104	0.141	0.041
EPS Vol. Productivity (g/l/h)	0.010	0.014	0.004
$Y_{p/x}$ (EPS) (g/g)	0.29	0.48	0.22
$Y_{p/s}$ (EPS) (g/g)	0.01	0.01	0.002
EPS Specific Productivity (g/g/h)	0.03	0.05	0.02
Lactate Vol. Productivity (g/l/h)	0.94	0.85	0.82
$Y_{p/x}$ (Lactate) (g/g)	26.02	28.62	43.17
$Y_{p/s}$ (Lactate) (g/g)	0.45	0.44	0.46
Lactate Specific Productivity (g/g/h)	2.60	2.86	4.32
Galactose Vol. Productivity (g/l/h)	1.03	0.99	0.90
$Y_{p/x}$ (Galactose) (g/g)	28.68	33.28	46.99
$Y_{p/s}$ (Galactose) (g/g)	0.50	0.51	0.50
Galactose Specific Productivity (g/g/h)	2.87	3.33	4.70

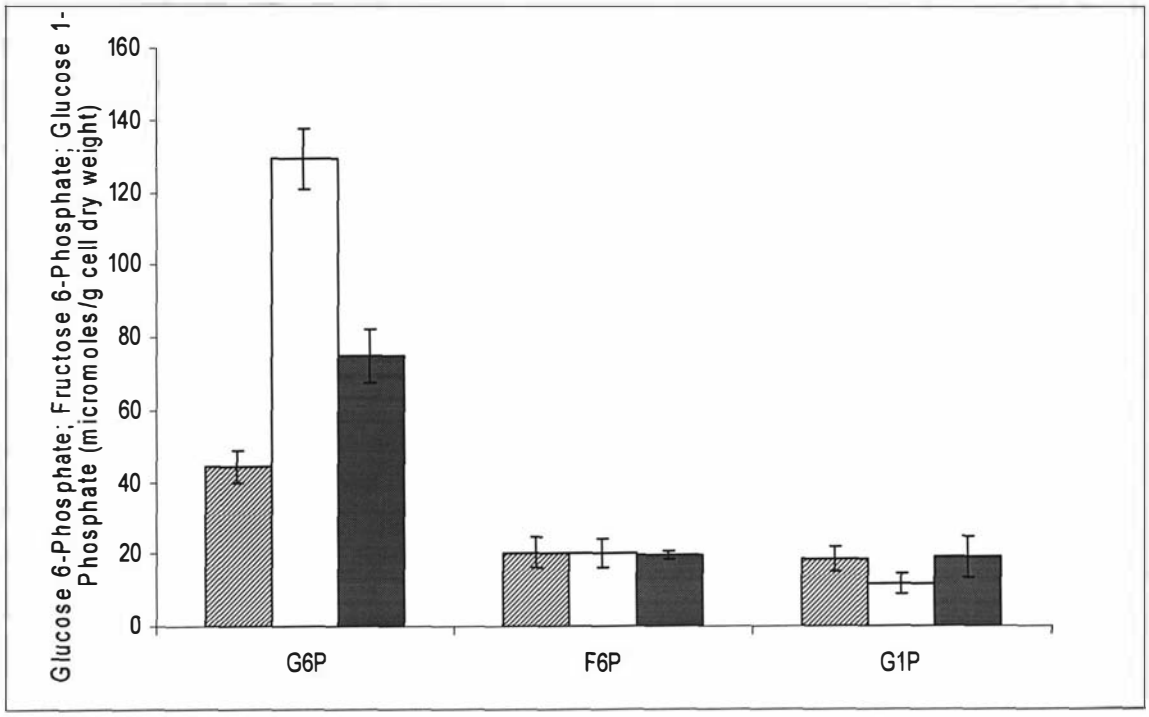

Fig. 10.3. Intracellular pools of glucose-6-phosphate, fructose-6-phosphate, and glucose-1-phosphate in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483 ▨, E2483M □, and the NCFB 2483 strain (grown under conditions of reduced a_w ▩, in continuous culture at a dilution rate of 0.10 h^{-1} in a semi-defined medium. Glucose-6-phosphate (G6P); Fructose-6-phosphate (F6P);Glucose-1-phosphate (G1P). All values the average of three measurements. Error bars represent standard deviations.

Intracellular pools of the EPS sugar-nucleotides UDP-glucose and UDP-galactose of the E2483M mutant were substantially lower than that of the parent strain. No dTDP-glucose was detected in the mutant strain (Figure 10.4).

Glucose-6-phosphate levels were raised considerably when the NCFB 2483 strain was exposed to conditions of reduced water activity; no major changes in the levels of fructose-6-phosphate or glucose-1-phosphate were observed (Figure 10.3). Intracellular pools of UDP-glucose, UDP-galactose, and dTDP-glucose were notably enhanced under conditions of reduced water activity (Figure 10.4).

The activities of enzymes associated with the glucose-1-phosphate and glucose-6-phosphate nodes are presented in Figure 10.5. The UDP-glucose pyrophosphorylase was substantially higher in the E2483M strain than in the parent. No marked differences in α-phosphoglucomutase and UDP-galactose 4-epimerase occurred, however the activity of the latter was marginally raised relative to the parent strain. Activity levels of dTDP-glucose pyrophosphorylase were markedly lower in the mutant strain than the parent. NADH-oxidase levels of the E2483M strain were raised relative to that of the parent (Figure 10.5).

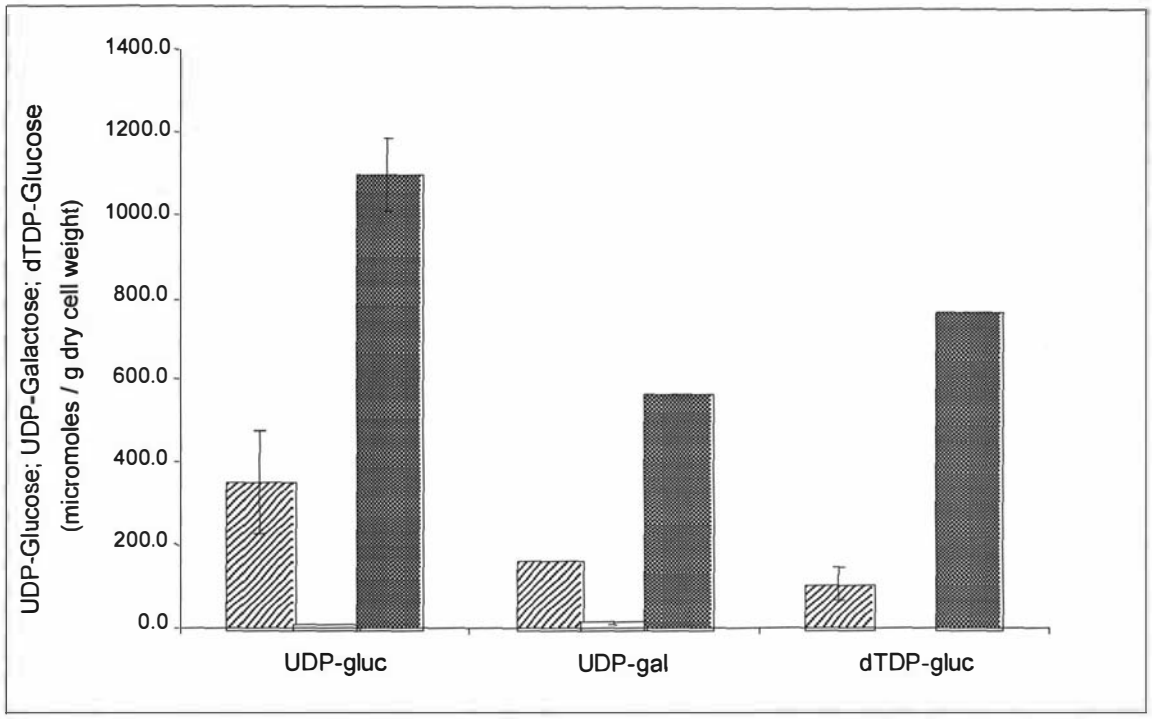

Fig. 10.4. Intracellular pools of UDP-glucose, UDP-galactose, and dTDP-glucose in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483▨ , E2483M□ , and the NCFB 2483 strain (grown under conditions of reduced a_w) ▩ , in continuous culture at a dilution rate of 0.10 h^{-1} in a semi-defined medium. UDP-glucose (UDP-gluc); UDP-galactose (UDP-gal); dTDP-glucose (dTDP-gluc). All values the average of three measurements. Error bars represent standard deviations.

The activity levels of phosphoglucoisomerase, α-phosphoglucomutase, UDP-glucose pyrophosphorylase, and dTDP-glucose pyrophosphorylase of the NCFB 2483 strain were substantially higher under conditions of reduced water activity; a marginal increase in the activity of UDP-galactose 4-epimerase was apparent under these conditions (Figure 10.5). dTDP-glucose-4,6-dehydratase (TGD) and the dTDP-rhamnose synthetic enzyme system were detected in negligible quantities. NADH-oxidase activity levels were lower under the conditions of reduced water activity (Figure 10.5).

Levels of ATP and ADP were higher in the E2483M mutant strain relative to the parent strain (Figure 10.6). When exposed to the reduced water activity in the semi-defined medium, *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 ATP levels were enhanced in comparison to the ambient level, and ADP levels were negligible (Figure 10.6).

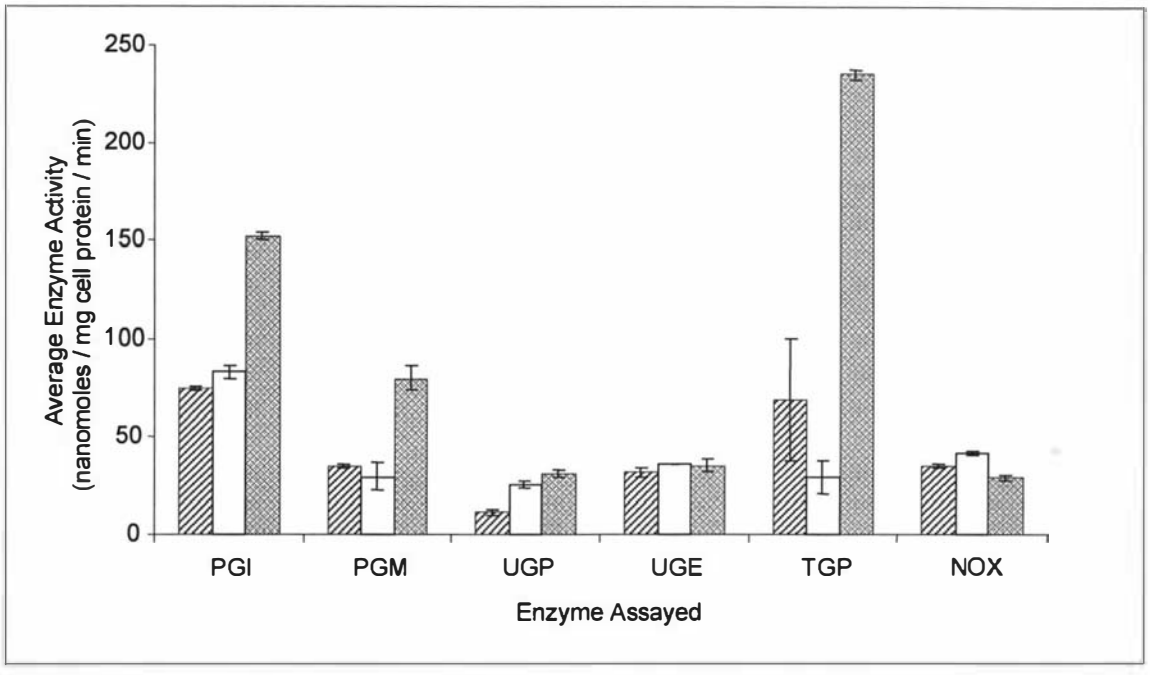

Fig. 10.5. Activities (nmol.mg cell protein^{-1}.min^{-1}) of enzymes associated with the glucose-6-phosphate and glucose-1-phosphate branch points, and NADH-oxidase in the *Lactobacillus delbrueckii* subsp. *bulgaricus* strains NCFB 2483▨ , E2483M□ , and the NCFB 2483 strain (grown under conditions of reduced a_w) ▩ , in continuous culture at a dilution rate of 0.10 h^{-1} in a semi-defined medium. Phosphoglucose isomerase (PGI); α-Phosphoglucomutase (PGM); UDP-glucose pyrophosphorylase (UGP); UDP-galactose 4-epimerase (UGE); dTDP-glucose pyrophosphorylase (TGP); NADH-oxidase (NOX). All values the average of three measurements. Error bars represent standard deviations.

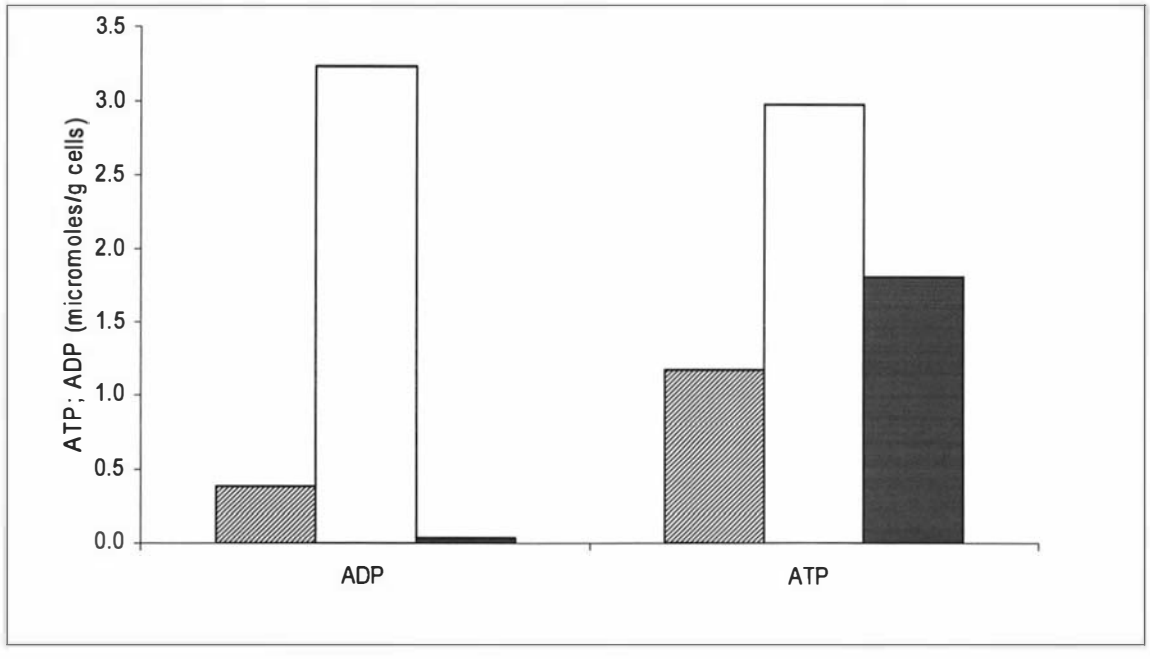

Fig. 10.6. Distribution of intracellular ADP and ATP levels in *Lactobacillus delbrueckii* subsp. *bulgaricus* strains NCFB 2483▨ , E2483M□ , and the NCFB 2483 strain (grown under conditions of reduced a_w) ▩, in continuous culture at a dilution rate of 0.10 h^{-1} in a semi-defined medium.

DISCUSSION

EPS formation in the mutant strain (E2483M) remained growth-associated, as in the parent strain of *Lb. delbrueckii* subsp. *bulgaricus* (NCFB 2483). The E2483M mutant strain, although producing a lower biomass than the parent strain, exhibited higher levels of carbon flux to EPS at a dilution rate of 0.10 h^{-1} as shown by the enhanced titre, $Y_{p/x}$ value, and specific productivity of the polymer (Table 10.1). No substantive difference between the distribution of lactose carbon between the strains was evident, as the corresponding $Y_{p/s}$ values for EPS, lactate, and galactose remained similar. Marginally raised $Y_{p/x}$ and specific productivity levels of lactate and galactose reflected an overall increase in flux to these metabolites as well (Table 10.1). Exposure of the NCFB 2483 strain to stress conditions (a reduction in a_w from 0.99 to 0.98) resulted in a reduction in biomass formation, lactose-carbon distribution to EPS, and a substantial increase in flux to lactate and galactose (Table 10.1).

Raised levels of glucose-6-phosphate in the E2483M strain indicate an accumulation of this metabolite and an increase in flux of carbon towards this branch-point; the stress perturbation reflects a similar pattern. No accumulation of the metabolites of this branch-point (fructose-6-phosphate and glucose-1-phosphate) were apparent in the case of the E2483M mutant, or the 2483 strain under conditions of a reduced a_w (Figure 10.3). The higher EPS titre and specific yields and productivities of the E2483M mutant, coupled with the low levels of the pools of UDP-glucose and UDP-galactose points to the fact that a raised level of turnover of these metabolites occurs relative to the parent strain (Figure 10.4). This is supported by the elevated levels of UDP-glucose pyrophosphorylase (UGP) and marginally raised UDP-galactose 4-epimerase (UGE) activities found in the mutant strain, relative to the parent strain (Figure 10.5). This observation does not support the concept that raised levels of sugar-nucleotides result in increased EPS formation, but rather that the enhancement of flux to EPS requires the solution of "multiple bottlenecks" (Kleerebezem *et al.*, 2002). A correlation between UGP levels and EPS synthesis was found in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 by Grobben *et al.* (1996). The lower level of activity of dTDP-glucose pyrophosphorylase (TGP) enzyme in the mutant strain (Figure 10.5) implies that the low level of dTDP-glucose is a result of a reduced formation of the sugar nucleotide in the E2483M mutant in comparison to the parent. The activities of the enzymes dTDP-glucose-4,6-dehydratase (TGD) and the dTDP-rhamnose synthetic enzyme system which were found to be negligible in both strains, were evidence of a low turnover of dTDP-glucose. No enhancement in the activity of α-phosphogucomutase (α-PGM) was evident in the mutant (Figure 10.5). Higher phosphoglucose isomerase (PGI) levels

in the mutant strain (Figure 10.5) correlate with a raised flux to lactate. Of interest is the fact that NADH oxidase (NOX) activity was substantially higher in the mutant than in the parent strain (Figure 10.5), possibly suggesting that it may be linked to higher EPS formation, albeit indirectly. Higher levels of ATP and ADP (Figure 10.6) demonstrate a higher energy availability for EPS production in the E2483M mutant compared to the parent strain, with higher levels available for production of the sugar-nucleotide precursors of EPS production. The higher flux to EPS in the E2483M mutant strain appears to be mediated by higher levels of UDP-glucose pyrophosphorylase and possibly by marginally higher levels of UDP-galactose 4-epimerase. A correlation between the activity of UDP-galactose 4-epimerase and EPS formation was reported in *Lactococcus lactis* (Forsén and Häivä, 1981), and in *S. thermophilus* strains LY03 (Degeest and De Vuyst, 2000) and Sfi 20 (Degeest *et al.*, 2001). The UDP-galactose 4-epimerase activity of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 was not demonstrated to be closely associated with the sugar composition of EPS produced by this strain (Grobben *et al.*, 1996). Escalante *et al.* (1998) did not find an association between UDP-galactose 4-epimerase activity and EPS production in ropy or non-ropy Gal$^-$ strains of *S. thermophilus*. The elevated level of EPS formation in the E2483M mutant was possibly also associated with raised levels of energy, which requires to be dissipated through an additional pathway. Relatively lower biomass levels in the E2483M mutant than in the parent strain would potentially have the effect that there was less competition for sugar-nucleotides for cell wall synthesis, than in the parent.

An enhanced ATP level in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 in response to osmotic stress (Figure 10.5) is the logical consequence of a raised glycolytic flux (Table 10.1). A larger accumulation of the pool of glucose-6-phosphate than under the unstressed condition (Figure 10.3), implies a higher flux toward the EPS–producing pathways. The accumulation of pools of UDP-glucose, UDP-galactose, and dTDP-glucose (Figure 10.4), and raised levels of α-phosphoglucomutase, UDP-glucose pyrophosphorylase, UDP-galactose 4-epimerase and dTDP-glucose pyrophosphorylase (Figure 10.5) under the stress-perturbation support this observation. Degeest and De Vuyst (2000) demonstrated a correlation between α-phosphoglucomutase, UDP-glucose pyrophosphorylase, UDP-galactose 4-epimerase and EPS production. Hugenholtz and Kleerebezem (1999) have raised the possibilty of the conversion of glucose-6-phosphate to glucose-1-phosphate (catalysed by α-phosphoglucomutase), and the synthesis of UDP-glucose from glucose-1-phosphate (catalysed by UDP-glucose pyrophosphorylase) being controlling factors in EPS production. Overexpression of the genes encoding for these two enzymes (*pgm* and *galU*, respectively), have been shown to effect an increased accumulation of glucose-1-phosphate and UDP-glucose (Kleerebezem *et al.*, 1999).

The lower EPS yield and specific productivity at a dilution rate of 0.10 h^{-1} and an a_w of 0.98 (Table 10.1) (see also Chapter 8, Welman, A.D. – PhD thesis) contradict the expected outcome of higher EPS formation under the conditions of reduced a_w, as was determined in batch culture (Chapter 6, Welman, A.D.- PhD thesis). A partial explanation for this that at the dilution rate applied (0.10 h^{-1}), assembly of EPS from the sugar-nucleotides, and export thereof was hindered by the conditions of reduced water activity. Negligible levels of activities of the enzymes dTDP-glucose-4,6-dehydratase and the dTDP-rhamnose synthetic enzyme system, and enhanced levels of dTDP-glucose pyrophosphorylase would have contributed to the accumulation of dTDP-glucose. Similarly, UDP-glucose and UDP-galactose accumulated under these conditions. The stress-conditions simultaneously elicited large increases in the flux of lactose-carbon to lactate and galactose. Under these conditions, thus, in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483, the accumulation of glucose-6-phosphate and EPS sugar-nucleotide precursors could have been the result of a carbon overflow at this branch-point, and fuelled by excess ATP present in the cell. Metabolic "bottlenecks", however, occurring downstream of the formation of the sugar-nucleotides restricted flux via this pathway to EPS

Future work aimed at improving the flux of carbon to EPS synthesis at the level of sugar-nucleotide synthesis in *Lb. delbrueckii* subsp. *bulgaricus,* would potentially yield the most significant results by overexpression of α-phosphoglucomutase and UDP-glucose pyrophosphorylase. UDP-galactose 4-epimerase would possibly be a target for improvement as well. It is important to note that relatively high levels of ATP are needed for the anabolic processes of EPS synthesis, and in particular, for the generation of sugar-nucleotide precursors essential for synthesis of the EPS repeating units. A strategy, hence, of reducing carbon flux via glycolysis to lactate as a means of "re-routing" carbon flow to EPS formation would become problematic by the nature of this requirement. This finding seems to be supported conceptually by the *in silico* metabolic control analysis of the effects of lactate dehydrogenase-knockout in *L. lactis* (Hoefnagel *et al.,* 2002), in which an accumulation of NADH and pyruvate occurred, and glycolysis was inhibited. An alternative strategy which would potentially augment the levels of UDP-galactose and UDP-glucose in *Lb. delbrueckii* subsp. *bulgaricus*, would be to insert genes encoding for the Leloir pathway enzymes such that the galactose could be utilized, and not exported from the cell.

CONCLUSION

Enhanced flux towards EPS synthesis in a mutant of *Lb. delbrueckii* subsp. *bulgaricus* grown in continuous culture at a dilution rate of 0.10 h^{-1}, was effected principally by a raised level of

activity of UDP-glucose pyrophosphorylase and possibly by a marginally elevated level of UDP-galactose 4-epimerase. The activities of these enzymes were also increased in a salt-stressed culture of the parent strain, which exhibited, in response to these conditions, an elevated flux to sugar nucleotide synthesis, but no increase in polymer production. The enzymes α-phosphoglucomutase and dTDP-glucose pyrophosphorylase were also raised in the salt-stressed culture in comparison to the non-perturbed state. Raised levels of ATP appeared to be associated with a higher flux toward sugar nucleotides in both instances. A strategy of enhancement of metabolic flux towards EPS production would rely not only upon the alleviation of specific "bottlenecks" in the EPS-synthetic pathway, but by the need to mediate pathways which control the redox potential of the cell, as well as generate ATP.

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CHAPTER 11

OVERVIEW

The purpose of this investigation was to develop an in-depth understanding of the metabolism of exopolysaccharide (EPS) synthesis in *Lactobacillus delbrueckii* subsp. *bulgaricus*, and to explain the main controlling factors in the reaction network. This information is of value in designing a strategy aimed at raising the flux of carbon towards EPS production. In approaching this study, the bioreaction network has been visualized as consisting of two groups of pathways, viz. the catabolic, ATP-generating reactions of glycolysis leading to lactate, and the energy-consuming reactions leading to EPS.

The undertaking of this investigation required the employment of a growth medium which had the properties of being formulated in both solid and liquid form, highly reproducible in its composition, and possessing utility in fermentation, metabolic, physiological, and screening studies. A semi-defined medium containing lactose and casein hydrolysate was selected as being suitable for the purposes of fermentation, metabolic, and screening studies for the selection of ropy and mucoid strains. Use of this medium resulted in a slightly enhanced fermentation performance to that achieved using standard MRS medium, with the exception of colonial mucoidy and the visual appearance of ropiness, which were significantly enhanced. The exclusion of yeast extract, beef extract, and proteose peptone was subsequently found to remove a significant component of the total background EPS equivalents in uninoculated broth. A high level of consistency of metabolite analyses was achieved using this medium. During these investigations, it was observed that the fermentation broth containing *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 underwent a diminution in viscosity after the growth period, suggesting a breakdown in EPS, possibly by glycohydrolases. This observation was confirmed in subsequent studies of EPS production.

In characterization studies in batch culture, *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 was verified to follow a homofermentative pattern of catabolism when grown on lactose, which split intracellularly after uptake into the cell, with at least the major portion of the resultant galactose being exported from the cell, and the glucose being metabolized further. The metabolism followed a primary kinetic pattern with respect to the production of the metabolites lactate, galactose and EPS, i.e. the formation of these metabolites are all growth-associated. Kinetic models which were applied to describe the formation of these metabolites suggested that whilst the major portion of galactose was exported unutilized via a lactose/galactose antiport, there

exists the possibility that some galactose may be diverted for utilization intracellularly for the formation of EPS. However, intracellular glucose, emanating from the splitting of lactose, remains, the main source of carbon for lactate, EPS, and biomass.

Effort placed into creating a mutant of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483, with a higher specific yield of EPS than the parent, was successful. The goal of this exercise was to compare the metabolism of the two strains such that key reactions associated with controlling EPS production could be identified. Using chemical mutagenesis of the parent strain, a mutant "pot" was obtained, and subjected to a screening process. In excess of 1000 mutant colonies were screened using a hierarchy of screening tests developed for this purpose. The screening strategy sought mutants which, amongst other traits such as ropiness and mucoidy, produced higher yields of lactate per biomass relative to the parent strain. In addition, relatively lower levels of biomass were a desirable characteristic. The rationale underpinning this approach, was that higher yields of lactate would necessitate a higher flux of carbon via glycolysis and the resultant generation of excess ATP for EPS biosynthetic mechanisms. Raised levels of conversion of pyruvate to lactate would have the supplementary effect of maintaining the cell's redox balance under conditions of raised glycolytic activity, bearing in mind that a lack of feedback regulation by lactate would compromise the cell's metabolism. This approach contrasts with strategies which divert carbon flux away from lactate formation for the purposes of overproduction of metabolites such as diacetyl. Indeed, the strains that were selected in the present study on the basis of their enhanced acidifying ability, tended to have higher specific EPS yields than the parent strain. No diversion of carbon towards diacetyl was detected. A second level of fermentation-screening of mutants with these characteristics, generated four mutants with a raised level of specific EPS production in comparison to the parent strain. A stable mutant strain, "E2483M", was selected for the comparison of its metabolism with the NCFB 2483 strain on the basis of its performance in batch culture.

Applying an environmental perturbation to an organism is a useful technique to measure changes in metabolism. It was determined, that in batch culture, the addition of the humectants NaCl and sorbitol induced changes in the production of exported metabolites, suggesting an altered metabolic flux distribution through contributing pathways in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483. Reduction of the water activity from a value of 0.99 to 0.98 at mid–exponential growth phase by the addition of NaCl resulted in raised specific yields ($Y_{p/x}$) and ($Y_{p/s}$) of EPS and lactate produced in the fermentation medium. The cause of the perturbation was osmotic stress. A reduction in water activity to 0.96 did not improve the specific yield of the EPS produced. These results suggested that conditions had been identified by which the contribution of enzymes to whole-pathway fluxes, particularly EPS, could be identified.

As the production of EPS by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 is a strictly growth–associated phenomenon, the influence of varying growth rates on the production of EPS and extracellular metabolites by the organism was investigated in continuous culture. It became evident that until the point at which dilution rate commenced exceeding growth rate near a dilution rate of 0.50 h^{-1}, a pattern of increasing specific EPS yields and volumetric productivities was associated with increasing dilution rate. The highest EPS titres were found to occur at dilution rates between 0.20 h^{-1} and 0.40 h^{-1}, with the maximum titre measured at a dilution rate of 0.30 h^{-1}. At dilution rates below 0.20 h^{-1}, much lower EPS titres were found. Lactate and galactose volumetric and specific productivities followed an increasing trend with increasing dilution rate up until a dilution rate of 0.40 h^{-1}, despite the gradually diminishing trend of corresponding titres and $Y_{p/x}$ values for these metabolites occurring over this range of dilution rates. A more significant decrease in productivity and yields were evident at a dilution rate of 0.50 h^{-1}, corresponding to the commencement of cell "washout" from the fermentor. Interestingly, elevated $Y_{p/s}$ values for EPS at the dilution rates greater than 0.30 h^{-1} suggested a diversion of carbon flux towards EPS being associated with the higher rates of growth, however due to the low titres of EPS produced, it was not possible to deduce whether carbon was directed away from lactate or galactose formation towards polymer synthesis. In these investigations, the growth-associated nature of EPS, lactate, and galactose production was confirmed. The maximum growth rate determined from the continuous culture studies correlated closely with those determined from the kinetic studies in batch culture.

Salt "upshock" (by the reduction of water activity from 0.99 to 0.98 using NaCl), when applied to *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 in continuous culture, resulted in reduced titres of biomass, EPS, lactate, and galactose. The specific yield per cell mass of EPS was reduced in response to the reduction in water activity, a result which contrasted with previous batch fermentation results. Specific yields and productivities for lactate and galactose were however raised, pointing to an uncoupling from growth of these two metabolites. Prolonged exposure to the conditions of reduced water activity led to a near normalization of elements of the metabolism. The stress condition did not cause EPS to become uncoupled from cell biosynthesis. It was concluded that further investigation was necessary in order to elucidate the pathways leading to EPS formation in response to this perturbation.

Knowledge of enzyme activities and intracellular metabolites is a valuable means of defining a directed strategy for improvement of the amount and structure of EPS produced, and hence functionality of the polysaccharide. Due to the growth-relatedness of EPS production in *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483, and the finding that EPS production increased with

increasing growth rate, it was decided to explore changes in metabolic intermediates and enzymes associated with the formation of sugar nucleotides, in response to varying growth rates. Chemostat continuous culture is an effective tool as it allows for the assessment of metabolic intermediates and enzymes under steady-state conditions at different growth rates. The distribution of fluxes through the glucose 6-phosphate and glucose 1-phosphate branch-points were determined under steady state conditions at the dilution rates 0.05 h^{-1}, 0.10 h^{-1}, and 0.35 h^{-1}. This was achieved by the measurement of enzyme activities and intracellular metabolites associated with these segments of biochemical pathways. An elevated level of flux to EPS at a dilution rate of 0.10 h^{-1} relative to a dilution rate of 0.05 h^{-1} could be correlated with diminished pools of glucose 1-phosphate and UDP-galactose, and a significantly raised activity of UDP galactose 4-epimerase. At a dilution rate of 0.35 h^{-1} (raised EPS formation), UDP galactose 4-epimerase was determined to be elevated further, however an accumulation of glucose 6-phosphate was strongly suggestive of a limitation in the flow of carbon towards fructose 6-phosphate or glucose 1-phosphate. At the dilution rates of 0.10 h^{-1}, and 0.35 h^{-1}, elevated levels of ADP and ATP were measured. An increasing amount of EPS produced could be correlated with an increase in ADP and ATP, raising the postulate that the flow of carbon away from the catabolic pathway of glucose breakdown towards EPS formation occurred in response to an excess supply of energy within the cell. In addition, ATP is utilized in the formation of sugar-nucleotide precursors for EPS and cell wall biosynthesis; raised levels of ATP in the cell would reduce competition for the nucleotide between cell wall and EPS biosynthesis. UDP galactose 4-epimerase appeared to play a sponsoring role in the generation of higher fluxes to EPS, in response to increasing growth rates. Interestingly, NAD^+ is not only regenerated in *Lb. delbrueckii* subsp. *bulgaricus* by the pyruvate to lactate reaction, but by NADH-oxidase as well. No clear trend in the activity of this enzyme was found, however the activity was elevated at higher growth rates. As the culture was not aerated, the enzyme would have been limited by the dissolved oxygen in the feed medium. As NAD^+ regeneration is necessary for cell biosynthesis, any strategy which was aimed at diminishing lactate production as a means to re-route carbon to EPS would possibly need to rely on NADH oxidase to regenerate the required NAD^+ for the cell.

In an attempt to further elucidate the mechanisms of raised carbon flux to EPS, and more specifically, the formation of the sugar nucleotide precursors of the polymer and associated enzyme activities in *Lb. delbrueckii* subsp. *bulgaricus*, the E2483M mutant was compared with the parent strain (NCFB 2483). A higher metabolic flux towards EPS in the E2483M mutant in comparison to the parent strain, in continuous culture (D=0.10 h^{-1}) appeared to be mediated by raised levels of UDP-glucose-pyrophosphorylase, and possibly by UDP-galactose-4-epimerase as well. Significantly elevated levels of glucose 6-phosphate pools in the mutant strain pointed

to a metabolic constriction at this branch-point, preventing potentially higher fluxes to glucose 1-phosphate, and sugar-nucleotide precursors. Raised levels of ATP were found in the mutant strain in comparison to the parent, thus fortifying the concept that EPS overproduction occurs as an "overflow" response to surplus energy present in the cell. Alternatively, the higher levels of EPS could be directly associated with the raised levels of ATP. Any strategy aimed at the improvement of EPS productive capacity by re-directing carbon flux away from lactate production would become problematic, as the ATP generated by glycolysis is vital for the anabolic pathways of EPS production.

As was observed in the mutant strain, a stress imposed upon a steady-state continuous culture ($D=0.10\ h^{-1}$) of *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483, induced by a reduction in water activity from 0.99 to 0.98, resulted in raised levels of ATP. Unlike the results in batch culture, the formation of EPS in response to the stress was reduced. It was discovered, however, that the flux to sugar nucleotide synthesis was raised, indicating that a constriction downstream of this level of biosynthesis occurred at this growth rate, possibly at the level of assembly or export of the EPS. This factor offered evidence that higher ATP levels can be associated with raised levels of sugar-nucleotide biosynthesis, either through their greater availability in the cell or because the synthesis of sugar nucleotides and polysaccharides provides a means by which *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2483 can dissipate excess energy, when required. A strategy which is aimed at reducing the flux of carbon to lactate as a means to enhance EPS production, would thus be flawed by the requirement for elevated levels of ATP for EPS overproduction. Under the salt-stress conditions applied, the levels of activity of α-phosphoglucomutase, UDP-glucose pyro–phosphorylase, UDP-galactose 4-epimerase, and dTDP-glucose pyro–phosphorylase were elevated compared to the non-perturbed state. As in the instance of the E2483M mutant, an accumulation of intracellular glucose 6-phosphate in the salt-stressed culture is indicative of a metabolic constriction at this branchpoint.

In overview, targeted improvements in *Lb. delbrueckii* subsp. *bulgaricus* would potentially have most effect if α-phosphoglucomutase, UDP-glucose pyro–phosphorylase, and UDP-galactose 4-epimerase activities were enhanced. It is likely that requirements for metabolic enhancements at the level of subunit assembly, polymerisation, and export of the EPS might ensue. The proposed strategy of substantially redirecting carbon flux away from lactate production towards EPS anabolism is not feasible in *Lb. delbrueckii* subsp. *bulgaricus* due to the substantial requirement for ATP for this purpose. Although NADH-oxidase activity is present, regeneration of NAD^+ would be compromised by reduced glycolytic activity. An alternative strategy might be to "switch on" or clone the Leloir enzymes, and utilize the galactose product of lactose for sugar nucleotide synthesis. In general, it should be noted that the anaerobic

character of *Lb. delbrueckii* subsp. *bulgaricus* restricts the productive capacity for EPS production, a limiting factor in the case of the development of all LAB for this purpose.

SPECIAL ACKNOWLEDGEMENTS

I would like to thank those staff of the Institute of Technology and Engineering and Food, Nutrition and Human Health at Massey University, who assisted me with the everyday requirements for execution of this study. In particular, technicians Suzanne Bassett and Michelle Curry deserve mention for the EPS analyses that they undertook. In addition, Suzanne Bassett was responsible for the timeous ordering of consumables. Kelvin T.Goh deserves special mention for his contribution to the application of the EPS analysis method. I would like to thank Dr Pak-Lam Yu for the *E. coli* B56 strain used for extraction of dTDP-glucose dehydrase, as well as his words of encouragement. Mr Grant Platt, the University glassblower, who found time to modify and repair fermentor vessels at very short notice, deserves mention as well. Finally, I would like to thank Mrs Maureen Oemcke for the final formatting of the entire text.

CONFERENCE ABSTRACTS

The results from Chapter 6 were presented as an oral presentation at the First International Symposium on Exopolysaccharides from Lactic Acid Bacteria: from Fundamentals to Applications (May 16-19, 2001), Brussels, Belgium.

Stress-Induced Metabolic Shift in *Lactobacillus delbrueckii* subsp. *bulgaricus*: Reduction in Water Activity Alters Production of Exported Metabolites

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Keywords: *Lactobacillus*; water activity; exopolysaccharides; metabolites

Abstract

The addition of humectants to a semi–defined fermentation medium was demonstrated to induce changes in the production of exported metabolites, suggesting an altered metabolic flux distribution through contributing pathways in an exopolysaccharide-producing strain of *Lactobacillus delbrueckii* subsp. *bulgaricus*.

Reduction of the water activity from a value of 0.99 to 0.98 at mid–exponential growth phase by the addition of NaCl resulted in raised specific yields Yp/x and Yp/s of exopolysaccharides and altered levels of lactate and galactose produced in the fermentation medium. A reduction in water activity to 0.96 did not significantly improve specific yields of the polysaccharides, but Yp/x for lactate was raised relative to the control fermentations. Dry cell weight titres were diminished at both elevated salt levels. The agent of metabolic change was assigned primarily to that of osmotic stress, an effect that was confirmed by the addition of sorbitol to the fermentation medium.

The metabolic response to alterations in water activity in this strain of *Lb. delbrueckii* subsp. *bulgaricus* provides a means of creating conditions under which the contributions of enzyme activities and metabolite levels to whole–pathway fluxes may be assessed. These effects are currently being investigated in conjunction with growth of the organism in continuous culture. Significant differences were observed to occur in EPS, galactose, and lactate production between different growth rates, pointing toward a metabolic redistribution of carbon under these

conditions. This information is valuable in directing metabolic engineering work aimed at altering the production of exopolysaccharides and lactic acid, products that can influence the textural and flavour quality of a fermented milk product such as yoghurt.

Aspects from this thesis were presented as an oral presentation at the "Molecules for Life" Conference (December 4-7, 2001), Napier, New Zealand.

METABOLIC MODEL OF EXOPOLYSACCHARIDE PRODUCTION IN *LACTOBACILLUS DELBRUECKII* SUBSP. *BULGARICUS*

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Exopolysaccharides (EPS) from lactic acid bacteria (LAB) play an important role in enhancing the texture and quality of fermented dairy foods such as yoghurt. Whilst LAB are attractive vehicles for the production of probiotics due to their GRAS status, the economic production of EPS by LAB remains constrained by their metabolism. Metabolic engineering aimed at altering the production of EPS and lactic acid requires an understanding of the contributions of whole pathways or steps in these pathways to the end–products. A strain of *Lactobacillus delbrueckii* subsp. *bulgaricus* was shown to export exopolysaccharide (EPS), lactate, and galactose as principal metabolites concurrently with cell growth. Measurement of both extracellular and key intracellular metabolites in the organism, grown on lactose in continuous culture at different growth rates, revealed that glucose 6-phospate is a key branch–point in the partitioning of carbon flux between glycolysis and the EPS–synthesizing pathway. In addition, a dual increase in lactate and EPS production was observed at raised growth rates. Perturbation of the EPS and lactate-synthesizing pathways by the application of an environmental stress (reduction of the water activity from a value of 0.99 to 0.98) resulted in an enhanced flux to both EPS and lactate. Energy for the formation of the sugar nucleotide precursors, essential for the assembly of cell wall and EPS, is derived mainly from glycolysis. Under sugar–limitation, a competition would exist between cell wall and EPS synthesis for the availability of sugar nucleotide precursors. Potential enhancement of EPS production by limiting lactate production is obstructed by the organism's requirement to control its redox potential primarily via the conversion of pyruvate to lactate. Analyses of perturbed metabolic states in the parent strain and that of a mutant support this viewpoint. The contribution of key enzymes associated with EPS synthesis are discussed.