Increased production of bacST4SA by *Enterococcus mundtii* in an industrial-based medium with pH-control

by

Johannes Cornelius Jacobus Coetzee

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Dr. J.F. Görgens
Prof. L.M.T. Dicks

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature: ……..

Date: ……..

Summary

Lactic acid bacteria (LAB) are producers of bacteriocins, ribosomally synthesized antimicrobial peptides. Bacteriocins are secreted into the surrounding environment where they inhibit growth of other bacteria competing for the same nutrients in a particular environment, usually closely related strains. Some of the bacteriocin-sensitive bacteria include food spoilers and - pathogens, which makes bacteriocins potential natural food preservatives. The need for more natural preservation techniques in the food industry is high: Consumers prefer ready-to-eat, minimally processed foods containing no chemical preservatives, but at the same time food spoilage and food-related illnesses are areas of big concern. The antibacterial and antiviral properties of some bacteriocins have also made them suitable for controlling bacterial infections, e.g. as part of pharmaceutical ointments. The increasing rate of resistance against antibiotics by micro-organisms has created a market for alternative treatments for infections. Commercial bacteriocin manufacturing proceeds in controlled fermentations or by extraction from plant material.

Enterococcus mundtii ST4SA produces a bacteriocin, bacST4SA, with properties giving it potential for use as a food preservative or as part of a pharmaceutical product. In this study, production of bacST4SA by fermentation of low-cost food-grade growth media, sugarcane molasses, corn steep liquor (CSL) and cheese whey, was considered to increase the economic viability of production for food application. Furthermore, individual de Man Rogosa and Sharpe (MRS) medium components, pH and fed-batch fermentation were evaluated to improve bacST4SA activity.

Yeast extract (YE) was selected from MRS components for supplementation of CSL, based on ANOVA analyses of fractional factorial designs. Medium containing pure CSL (7.5 $g_{total\ sugars}.l^{-1}$), yeast extract (6.5 $g.l^{-1}$) and glucose (7.5 $g.l^{-1}$) yielded 102400 AU (Arbitrary Units).ml^{-1} during fermentations kept at pH 6.5 for 6 h and then adjusted to 5.5. Residual glucose accumulated in the growth medium during exponential fed-batch fermentations. CSL supplemented with YE and glucose yielded activity that represented a two-fold increase over activity obtained in MRS broth, an expensive commercial growth medium. BacST4SA was produced in a growth-associated manner and increased up to a certain maximum plateau value, whereafter improvements in growth environment and higher biomass levels had no

effect on bacST4SA production. This could be explained by a limited immunity of *E. mundtii* ST4SA cells to bacST4SA, or the absence of a specific nutrient needed for bacteriocin production. The use of pH-control increased the production rate of bacST4SA. Glucose was consumed slower by *E. mundtii* ST4SA in fed-batch fermentations compared to batch fermentations, probably due to lactic acid that was added to the growth medium as part of CSL during exponential feeding.

In future work it is recommended that more work should be done on fed-batch fermentations to eliminate growth inhibition and subsequently achieve high biomass levels. The effect of temperature and/or a stressful environment can also be evaluated for bacST4SA production. *E. mundtii* ST4SA can be stressed by aeration of the culture, or by including NaCl or ethanol in the growth medium. Finally, the use of membrane reactors to extract lactic acid can also be considered. This may prevent end-product inhibition whilst also providing a pure form of lactic acid for selling purposes.

Opsomming

Melksuurbakterieë produseer bakteriosiene, ribosomaal vervaardigde antimikrobiese peptiede. Bakteriosiene word in die onmiddellike omgewing van die produseerder uitgeskei waar hulle ander bakterieë inhibeer, gewoonlik dié met 'n na-verwantskap aan die produseerder. Bakteriosien-sensitiewe bakterieë sluit voedsel bederwers en - patogene in, wat bakteriosiene potensiële natuurlike preserveermiddels vir voedselprodukte maak. Daar is tans druk op die voedselindustrie om meer natuurlike preserverings metodes te gebruik: Verbruikers verkies natuurlike produkte wat gereed is om te eet, met 'n minimum of geen chemiese preserveermiddels, maar terselfdertyd is voedsel berderwing en voedsel-verwante siektes 'n area van groot bekommernis. Die antibakteriese en antivirale eienskappe van sommige bakteriosiene het hulle ook bruikbaar gemaak as potensiële farmaseutiese produkte. Dit kan 'n moontlike verligting bring aan die soeke na alternatiewe middels om infeksies te genees aangesien antibiotika besig is om effektiwiteit te verloor as gevolg van weerstandige mikro-organismes. Kommersiële bakteriosien vervaardiging word deur middel van beheerde fermentasies of ekstraksie uit plant materiaal gedoen.

Enterococcus mundtii ST4SA produseer 'n bakteriosien, bacST4SA, wat as 'n voedsel preserveermiddel of as deel van 'n farmaseutiese produk gebruik kan word. In hierdie studie is die produksie van bacST4SA in lae-koste groeimedia, suikerriet molasse, mielie week water en kaaswei poeier, ondersoek om die produksiekoste van die bakteriosien vir voedsel toepassing te verlaag. Verder was daar met medium komponente, pH en voer stuk fermentasies geëksperimenteer om bacST4SA opbrengste te verhoog. Die medium komponente oorweeg was gebasseer op dié van 'n kommersiële medium, de Man Rogosa and Sharpe (MRS).

Gisekstrak was geïdentifiseer uit MRS komponente vir toevoeging tot mielie week water deur middel van ANOVA analises van parsiële faktoriale ontwerpe. Medium bestaande uit suiwer mielie week water (7.5 $g_{totale\ suikers}.l^{-1}$), gisekstrak (6.5 $g.l^{-1}$) en glukose (7.5 $g.l^{-1}$) het bacST4SA aktitiwiteit gelewer van 102400 AE (Arbitrêre Eenhede).ml^{-1}. Gedurende fermentasie was die pH konstant gehou by 6.5 vir 6 ure waarna dit tot 5.5 verlaag is. Glukose in die groeimedium het geakkumuleer gedurende eksponensiële voer stuk fermentasies. Mielie week water, 'n industriële byproduk, kon suksesvol aangewend word as groeimedium vir produksie van

bacST4SA en verryk met gisekstrak en glukose het dit aktiwiteit gelewer wat twee keer meer was as in MRS verkryg. BacST4SA was geproduseer as 'n groei-verwante produk en het toegeneem tot 'n maksimum plato waarde, waarna verbeterings in groeimedium en verhoogde biomasse geen effek of produksie gehad het nie. Die plato aktiwiteit waarde kan toegeskryf word aan 'n beperkte immuniteit van *E. mundtii* ST4SA teen sy eie bakteriosien wat gevolglik produksie beperk. Die tempo van glukose opname deur *E. mundtii* ST4SA was laer in voer stuk fermentasies as in stuk fermentasies, moontlik as gevolg van melksuur wat as deel van mielie week water tot die groeimedium gevoer was.

Dit word aanbeveel dat meer werk aan voer stuk fermentasies gedoen word om groei inhibisie uit te skakel om sodoende hoë biomassa vlakke te bereik. Die effek van temperatuur en/of 'n stresvolle omgewing op bacST4SA produksie kan ook getoets word. 'n Stresvolle omgewing kan geskep word deur lug deur die kultuur te stuur of NaCl of etanol by die groeimedium te voeg. Membraan fermentors kan ook oorweeg word aangesien hulle voorkom dat melksuur opbou tot 'n inhiberende konsentrasie deur dit gedurende fermentasie te ekstraheer.

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Chapter 2. Lactic acid bacteria: Fermentation and bacteriocin production

CHAPTER 1

INTRODUCTION

1.1 Introduction

Lactic acid bacteria (LAB) produce lactic acid (LA) as a metabolic end-product (Axelsson, 2004) and play a substantial role in our daily lives. Dairy foods such as cheese and fermented milk products, e.g. yoghurt, are produced from milk by using LAB as starter cultures (O'Sullivan et al., 2002). Certain strains of LAB have probiotic properties and protect the gastrointestinal tract against infection by pathogenic bacteria (Mikelsaar, 2004; Salminen et al., 2004). This 'antimicrobial effect' of LAB can be traced back to a number of fermentation products that includes LA and peptides called bacteriocins.

LA is produced as an end-product of the metabolic pathway (hence the name LAB), while bacteriocins are ribosomally synthesized peptides (Cleveland et al., 2001). The antimicrobial ability of LA lies in its ability to reduce the surrounding pH (Alakomi et al., 2000). In addition to this LA can permeabilize membranes rendering the target bacteria more susceptible to other antimicrobial agents (Alakomi et al., 2000). Bacteriocins, on the other hand, are thought to be a defensive or survival mechanism and generally inhibit growth of closely related strains (Deegan et al., 2006). This may be to lower competition for nutrients, which is fiercest amongst similar strains (Deegan et al., 2006). Bacteriocin-like substances with antifungal properties have also been reported for some strains of LAB (Schnürer and Magnusson, 2005). Generally, the mode of action of bacteriocins is to interfere with the target organism's cell wall leading to cytoplasm leakage and subsequently death (Deegan et al., 2006).

In recent years there has been an upheaval of interest in bacteriocins for use in the medical-, veterinary- and especially the food industry (Cleveland et al., 2001; Nomoto et al., 2005). The fact that LAB have been used in food products for centuries has focused research on their potential use as natural food preservatives (Deegan et al., 2006; O'Sullivan et al., 2002). Demands for more effective food preservation techniques are high. In the United States alone, the food-borne pathogen *Listeria monocytogenes* causes 2500 cases of listeriosis that results in 500 deaths annually (Deegan et al., 2006). Bacteriocin-like substances with antifungal properties are of special importance for food preservation, as yeasts and moulds have developed increased resistance against traditional preservatives such as sorbic and benzoic acids (Schnürer et al., 2005). Currently, only two bacteriocins from LAB are commercially produced by fermentation for application in food, i.e. nisin and pediocin PA-1/AcH (Deegan et al., 2006). Nisin, probably the best known bacteriocin, is FDA-approved and used in more than 48 countries as a natural food preservative, especially in canned foods, dairy products

and processed cheese (Cleveland et al., 2001; Deegan et al., 2006; O'Sullivan et al., 2002). Commercially produced bacteriocins may increase in the near future.

Before bacteriocins can be marketed as a product, it is necessary to find a suitable production process. Being a fermentation end-product, a number of criteria will determine whether a bacteriocin can be produced commercially. The objective will always be to produce the highest amount of product at the lowest cost and in the shortest time. Bacteriocins are ribosomally synthesized and many of them display primary metabolite growth-associated kinetics (Franz et al., 1996; Herranz et al., 2001; Lv et al., 2005). Subsequently, the amount of bacteriocin produced is significantly affected by changes in growth conditions. Generally, changes in growth medium (Franz et al., 1996; Kim et al., 2006; Li et al., 2002; Zendo et al., 2005), pH (Franz et al., 1996; Herranz et al., 2001; Kim et al., 2006; Zendo et al., 2005) and temperature (Kim et al., 2006) are used to increase bacteriocin production. Moreover, the growth phase can be prolonged to increase the bacteriocin production period, e.g. fed-batch (Lv et al., 2005) and continuous fermentations (Parente et al., 1997). Other techniques, such as exposing bacteria to stressful conditions to stimulate an immune response and thereby increasing bacteriocin production, have also been used with reasonable success. Stressful environments can be created by inclusion of NaCl (Herranz et al., 2001; Leroy et al., 2003) or ethanol (Herranz et al., 2001) in growth medium or aerating cultures (Desjardins et al., 2001).

Media optimization is not only a means of improving bacteriocin production, but it is also a very important cost factor. Cultivation media can account for up to 30 % of production costs in commercial fermentations (Rivas et al., 2004). To lower media costs, food-based industrial by-products rich in carbon and/or nitrogen have been used with reasonable success for production of bacteriocins (e.g., cheese whey; Cladera-Olivera et al., 2004; Guerra et al., 2001) and LA (e.g. molasses; Wee et al., 2004). The high level of impurities and food origin of these low-cost media have made bacteriocins obtained from fermentation best suited for use in the food industry. Nisin, for example, is produced by fermentation of low-fat milk (Deegan et al., 2006).

Certain factors can inactivate bacteriocins during fermentation. The purpose, therefore, is not always to increase bacteriocin production, but to stabilize it. Medium components such as NaCl (Leroy et al., 2003), ethanol (Mortvedt-Abildgaard et al., 1995) and high carbon source concentrations (Leroy et al., 2003) have been used to stabilize bacteriocins. Medium pH has also been shown to significantly affect bacteriocin stability (Herranz et al., 2001; Zendo et al., 2005). It is therefore clear that finding an optimal production process is a complex problem, but essential to the economic viability of commercial bacteriocin production.

The genus *Enterococcus* belongs to the family of LAB. Enterococci are found in many fermented foods such as cheese, meat and olives where they play an important role in ripening to improve taste and flavour (Moreno et al., 2006). They are also producers of bacteriocins, generally referred to as enterocins. Enterocins are characterized by their strong activity against *Listeria* spp., which are natural food pathogens (Moreno et al., 2006). *Listeria monocytogenes* survive high salt concentrations and low temperatures rendering the species a big problem in the food industry (Deegan et al., 2006). This has made *Enterococci* and their enterocins very popular for studies concerning use as natural food preservatives.

The aim of this study was to find a suitable process for production of high amounts of a bacteriocin produced by a strain of *Enterococcus mundtii* in a low-cost growth medium. The bacteriocin displayed antagonistic behaviour against the potential food pathogens *Enterococcus faecalis* and *Staphylococcus aureus* (Todorov et al., 2005), which makes it suitable for use in the food industry. Moreover, the bacteriocin had antiviral properties (Todorov et al., 2005) and may be evaluated for use in the pharmaceutical industry.

This thesis is presented in the form of five chapters, chapter 1 being the introduction, chapter 2 the literature study, chapter 3 the research problem, chapter 4 the research chapter and chapter 5 the conclusions and recommendations. Chapter 4, the research chapter, is written according to the style of an article, since it is to be submitted for publication in 2007.

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CHAPTER 2

LACTIC ACID BACTERIA: FERMENTATION AND BACTERIOCIN PRODUCTION

2.1 Lactic acid bacteria (LAB)

2.1.1 Introduction

A certain group of bacteria produce mainly lactic acid (LA) as the main product after fermentation of carbohydrates (Axelsson, 2004; De Vuyst and Vandamme, 1994; Hofvendahl and Hahn-Hägerdahl, 2000). Not surprisingly, they are called lactic acid bacteria (LAB) and constitute one group of a whole family into which bacteria are divided. LAB are commonly found in nutrient rich environments, e.g. fermented meat, vegetables, fruit, beverages and dairy products, but they can also be found in the respiratory, genital and intestinal tracks of humans and animals (Axelsson, 2004; Schnürer and Magnusson, 2005). LAB belong to the *Clostridium* subdivision of Gram-positive bacteria (i.e. bacteria with a G + C DNA content < 55 %) and includes approximately 20 genera. The most important (with respect to food and -related fields) of these are the following (Axelsson, 2004; Hofvendahl et al., 2000): *Aerococcus (Aer.)*, *Carnobacterium (Car.)*, *Enterococcus (Ent.)*, *Lactobacillus (Lb.)*, *Lactococcus (Lc.)*, *Leuconostoc (Leu.)*, *Oenococcus (Oen.)*, *Pediococcus (Ped.)*, *Streptococcus (Str.)*, *Tetragenococcus (Tet.)*, *Vagococcus (Vag.)* and *Weissella (Wei.)*. They are all cocci except *Lb.* and *Car*. that are rods. Most strains of LAB can further be classified by the following characteristics (Hofvendahl et al., 2000):

- → unable to synthesize ATP by respiration,
- → micro-aerophylic,
- → catalase negative,
- → non-motile,
- → non-sporulating
- → able to grow in high saline conditions and
- → high acid tolerance (survive at pH 5.0 and lower)

LAB have a limited ability to synthesize B-vitamins, nucleic acids and amino acids causing them to have complex nutritional requirements (Callewaert and De Vuyst, 2000; Oh et al., 2005). Most of them have GRAS (Generally Regarded As Safe) status, but some strains are pathogenic, e.g. strains of *Streptococcus* (Fernandez et al., 2005; Hofvendahl et al., 2000).

LAB, especially *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus*, have long been used as starter cultures in the fermentation of food and beverages such as milk, yoghurt, cottage cheeses, wine, meat etc. (Fernandez et al., 2005; Schnürer et al., 2005).

Many LAB are also used in yoghurts and other dairy products because of their probiotic properties (Schillinger et al., 2005). As starter cultures LAB do not only contribute to the flavour and aroma of these products, but play an important role in food preservation (Deegan et al., 2006). It is, in fact, the ability of LAB to inhibit the growth of food-spoiling bacteria and pathogens that has made them a valuable part of the food- and feed industry today. Hence, LAB are sometimes also referred to as natural preservatives. The inhibitory substances produced by LAB include the following: Hydrogen peroxide, diacetyl, bacteriocins and organic acids (Deegan et al., 2006; Ouwehand and Vesterlund, 2004). Apart from these, secondary products may also form and cause inhibition. Hypothiocyanate is a secondary product formed during a lactoperoxidase catalyzed reaction between hydrogen peroxide and thiocyanate that occurs in raw milk (Ouwehand and Vesterlund, 2004). Organic acid(s) generated during fermentation is antimicrobial in their ability to lower the pH to a level where many food-spoilage and pathogenic bacteria cannot grow (Ouwehand et al., 2004). LA can also permeabilize membranes, thereby rendering the target bacteria more susceptible to other antimicrobial agents (Alakomi et al., 2000). Increased production of metabolic end-products is achieved by fermentation of these bacteria in a controlled environment. LAB have several trademarks making them suitable for industrial fermentations (De Vuyst et al., 1994):

- → They have been used in the food industry for years and are therefore well studied with abundant information available on large-scale production
- → Most have GRAS status
- → LAB produce useful end-products free from toxins
- → They grow at low pH values, decreasing chances of contamination
- → They are micro-aerophilic and aero-tolerant, thus requiring a simple fermentation process
- → Some LAB grow rapidly, shortening fermentation times
- → LAB can be fermented on cheap substrates such as whey, molasses, corn steep liquor etc.
- → They can secrete proteins

There are a few aspects of LAB that can hamper industrial fermentation processes, e.g. LAB frequently have low growth rates and require complex nutrients for growth (Yun et al., 2003).

2.1.2 Metabolism of LAB

2.1.2.1 Fermentation

LAB can be divided into two groups based on their ability to ferment glucose under stress-free conditions, i.e. conditions where growth factors are non-limiting and oxygen is limited. Group 1 (homofermentative) LAB, convert glucose almost exclusively to LA, while group 2 (heterofermentative) LAB, produces LA, ethanol or acetic acid and CO_2 from glucose fermentation (Axelsson, 2004; Hofvendahl et al., 2000). These two groups sprout from two different catabolic pathways that are followed during fermentation. The Embden-Meyerhof-Parnas (EMP) pathway, also referred to as glycolysis, results in either homolactic- or mixed acid fermentation. The pentose phosphoketolase (PKP) pathway, also referred to as the 6-phosphogluconate pathway, pentose phosphate pathway or the hexose monophosphate shunt, forms part of heterolactic fermentation.

2.1.2.2 Base case: Glucose fermentation

Homofermenters

The fermentation process is started when a sugar, like glucose, is transported into the cell. During the transport process glucose is activated by the formation of a high-energy phosphate bond. Homofermenters use the phosphoenolpyruvate:phosphotransferase system (PEP:PTS) to import and phosphorylate glucose. Glucose is then subjected to a series of reactions which signals the start of the EMP pathway (Hofvendahl et al., 2000). The EMP pathway can be summarized by the following reaction (also see Fig. 1):

$$\text{Glucose} + 2\ \text{ADP} + 2\ PO_4^{3-} + 2\ NAD^+ \rightarrow 2\ \text{Pyruvic acid} + 2\ \text{ATP} + 2\ \text{NADH} + 2\ H^+ + H_2O \qquad [1]$$

The importance of the above reaction lies in the formation of ATP molecules (adenosine 5'-triphosphate) - for every molecule of glucose there is a net increase of 2 ATP molecules. ATP is the energy carrying compound in cells. Energy is released when ATP's terminal phosphate bond is broken transforming it to ADP (adenosine 5'-diphosphate). The reaction transforming ADP to ATP is called substrate level phosphorylation and can be simplified by the following reaction:

$$\text{X-P} + \text{ADP} \rightarrow \text{X-H} + \text{ATP} \qquad [2]$$

X-P is the phosphorylated sugar. ADP and ATP are therefore always present in the cell and are never depleted. In much the same way there exists a relationship between NAD and

NADH, NAD being the oxidised state of NADH. The final step of EMP requires the release of NAD from NADH for its re-use in reaction 1. NAD can be produced through different reactions, the simplest one being the reduction of pyruvic acid (pyruvate) to lactic acid (reaction 3) giving rise to homofermentation.

$$CH_3.CO.COOH + NADH + H^+ \rightarrow LA\ (CH_3CHOH.COOH) + NAD^+ \quad [3]$$

Reaction 3 is not always followed and two other fermentation types, mixed acid- and butanediol fermentation, are also possible. During mixed acid fermentation pyruvic acid is converted to one mole of lactic- and formic acid and half moles of acetic acid and ethanol while butanediol is the end-product of butanediol fermentation. In fermentations there can be no net change in oxidation state. In mixed acid fermentations, for example, glucose is converted to formic acid which is more oxidised than glucose. It is therefore necessary that ethanol, having a lower oxidation state than glucose, is also formed to balance the oxidation state. The metabolic end-products change as a result of different pyruvate pathways, and/or the presence of external electron acceptors, e.g. oxygen or organic substrates (Axelsson, 2004; Hofvendahl et al., 2000). The EMP pathway is used by all LAB except leuconostocs, the obligately heterofermentative lactobacilli, oenococci and some *Weissella* spp. (Axelsson, 2004).

In special cases homofermenters are known to produce mixed acids. This can happen during glucose limitation, growth on other sugars, a change in pH or at decreased temperatures (Guerra et al., 2005; Hofvendahl et al., 2000).

Heterofermenters

In the case of heterofermenters glucose is actively transported by means of an H^+-symport permease which is driven by an electrochemical proton gradient. Glucose is then phosphorylated by an ATP-dependent glucokinase (Hofvendahl et al., 2000). The PKP pathway can be summarized by the following net reaction:

$$Glucose + ATP + 6\ NADP^+ \rightarrow glyceraldehyde\ 3\text{-}P + ADP + 6\ NADPH \quad [4]$$

Fig. 1 depicts the basic outline of the PKP pathway. During heterofermentation equimolar amounts of LA, CO_2 and ethanol or acetic acid is formed (Hofvendahl et al., 2000).

There are two reasons why a cell follows the PKP pathway that leads to heterofermentation. Firstly, it provides the cell with C4 and C5 phosphates needed in growth related cell reactions,

and secondly, it generates NADPH for use during cell growth and maintenance (Ratledge, 2001). In many cases the EMP and PKP pathways function together. The ratio of the two pathways then depends on the cells function at that time. It is generally assumed that both pathways are used in a ratio EMP:PKP of 2:1 during the active growth phase. When growth slows down the need for products from the PKP pathway is decreased shifting the ratio to anywhere between 10:1 and even 20:1 (Ratledge, 2001).

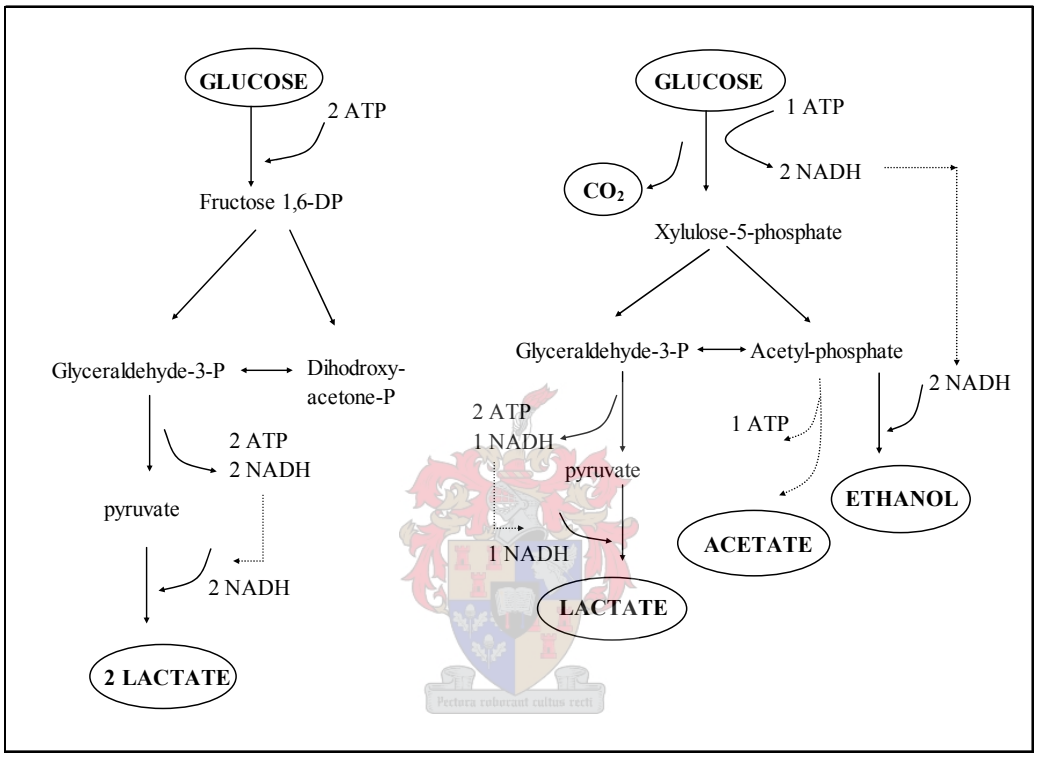

Fig. 1. Shortened versions of the Embden-Meyerhof-Parnas pathway (left) and phosphoketolase pathway (right). Adapted from Hofvendahl et al. (2000).

2.1.2.3 Other hexose sugars

Fermentation proceeds in the same way for almost all other hexose sugars (mannose, fructose, etc.), but the transport system vary for different strains (Hofvendahl et al, 2000). One exception of the hexose sugars is galactose. *Lactococcus lactis* and *Enterococcus faecalis* phosphorylate galactose through the PTS system and metabolism then proceeds via the tagatose-6-phosphate pathway. Most often strains of this category can also transport galactose with a permease and then convert it to glucose-6-phosphate using the Leloir pathway (Axelsson, 2004).

2.1.2.4 Disaccharides

Disaccharides can enter the cell either as free sugars or sugar phosphates. In the latter case the PTS system is used. Once in the cell free disaccharides are hydrolysed to monosaccharides while sugar phosphates are split equally into monosaccharides and monosaccharide phosphates. Monosaccharides are fermentable and are taken up in the catabolic pathway suited for each (Axelsson, 2004).

2.1.2.5 Pentose sugars

All LAB, except obligate homofermentative *lactobacilli* (Group 1), are pentose positive, i.e. able to ferment pentose. Pentose sugars are transported into the cell by specific permeases before they are phosphorylated and converted to ribulose-5-phosphate or xylulose-5-phosphate by epimerases or isomerases. These C5 phosphates are then taken up in the lower half of the PKP pathway (Axelsson, 2004).

Heterolactic fermentation of pentose delivers end-products that differ from those obtained from glucose. Since pentose enters the cycle as xylulose-5-phosphate, no dehydration reactions are necessary, ending CO_2 formation and the need to reduce acetyl-phosphate to ethanol (Fig. 1). In a new reaction acetyl-phosphate is converted to acetate in a substrate-level phosphorylation step with the release of an ATP molecule. Homofermenters usually use the EMP pathway, but the presence of substrates that are fermented by the PKP pathway induces the use of the PKP pathway.

2.1.3 The genus *Enterococcus*

The genus *Enterococcus* belongs to the family of LAB. Traditionally, *Enterococcus* species were identified by their ability to grow at 10 and 45 ºC, pH 9.6, and in the presence of 6.5 % NaCl and 40 % bile (Moreno et al., 2006). This system of classification has been revised as many of the more recently described enterococci do not have all of these characteristics. Enterococci are homofermenters and produce L(+) LA (Franz and Holzapfel, 2005). They occur in a wide variety of habitats and can be found in the environment (soil, surface waters, waste water and plants), gastrointestinal tracts of humans and warm-blooded animals and in food such as meat, cheese and fermented vegetables (Franz et al., 2005; Moreno et al., 2006).

Enterococci have properties of biotechnological importance, e.g. they are used as cheese starter cultures, produce biogenic amines and are probiotics (Franz et al., 2005). Strains of *Enterococcus faecalis* and *Enterococcus faecium* have been used in human and animal probiotics (Moreno et al., 2006). Contrarily, enterococci are also able to cause food spoilage

(mainly meat products) and human disease (infections). They are also known to have a resistance against antibiotics and contain virulence factors. Subsequently, the use of enterococci as probiotics is still an issue of controversy and remains a closely studied area. Each strain of *enterococcus* should be tested separately and is only allowed in food applications if it does not contain virulence factors and is not resistant against the antibiotic vancomycin (Leroy et al., 2003).

2.2 Fermentation end-products of LAB

Fermentation can lead to different end-products depending on the bacterial strain used and conditions during growth. The LAB fermentation end-products lactic acid and bacteriocins are discussed below.

2.2.1 Lactic acid

2.2.1.1 Background

Lactic acid (LA) can be produced either synthetically or biologically (Bai et al., 2004). Synthetic production proceeds by using petroleum substrates such as ethylene as reactants in chemical reactions (Wee et al., 2004). Biological production is accomplished by fermentation of LAB or the fungi *Rhizopus oryzae* (Ohkouchi and Inoue, 2006). LA ($CH_3CHOHCOOH$) has two optical isomers: D(-) and L(+) LA (Ohkouchi et al., 2006; Rivas et al., 2004). A racemic (optically inactive) D-L LA product is formed during synthetic production whereas fermentation has the benefit of yielding an optically pure form of LA, thus either D(-) or L(+) LA (Hofvendahl et al., 2000; Wee et al., 2004). It is for this reason that 70 to 80 % of LA production (120 000 tonnes/year) is done by fermentation (Kubicek, 2001; Schiraldi et al., 2003, Zhang et al., 2007). The optical orientation of LA produced by fermentation is dependent on the strain used and the fermentable carbon source present (Wee et al., 2004). The L(+) isomer is preferred above the D(-) isomer in food applications as large amounts of the latter can be harmful to humans. The recommended daily intake for D(-) LA is 100 $mg.kg^{-1}$ body weight for adults and zero for infants (Hofvendahl et al., 2000).

2.2.1.2 Production

LA is produced in reactors with volumes of up to 100 m^3. Fermentation conditions vary for different strains, but the following has been considered adequate for use as a guideline: pH is controlled in the range between 5.5 and 6.5, temperature is maintained at 45 ºC or higher and a low agitation rate is used to keep conditions anaerobic (Kubicek, 2001). Medium pH is controlled by the addition of calcium carbonate, ammonia or other bases, or by *in situ*

removal of LA (Hofvendahl et al., 2000). LA has a very low selling price and accordingly inexpensive carbon sources are used as growth media. Subsequently, LA production from organic wastes to lower production costs is still an extensively studied area (Oh et al., 2004; Ohkouchi and Inoue, 2006; Rivas et al., 2004; Wee et al., 2004). Generally, industrial media have to be supplemented to improve their performance. These supplements can be a source of carbon, nitrogen, minerals or vitamins and depends on the medium that is used and the requirements of the culture being fermented. Industrial media with supplements have the ability to perform just as well as complex media. A glucose medium supplemented with corn steep liquor and spent yeast cells gave LA yields and productivity similar to a medium containing glucose, yeast extract, peptone, sodium acetate, sodium citrate and K_2HPO_4 (Rivas et al., 2004). The carbon concentration in the batch media usually varies between 120.0 and 180.0 $g.l^{-1}$. Fermentation lasts 4 to 6 days with final conversion yields of between 85 and 95 % (Kubicek, 2001).

2.2.1.3 End-product inhibition

During fermentation of LAB, LA is produced causing medium pH to decrease and growth to go into stationary phase. Inhibition at low pH has been attributed to the presence of increased amounts of H^+-ions and undissociated LA (Vereecken and Van Impe, 2002) and can be prevented by use of pH-control. However, lactate salt is formed when LA is neutralized by a base during pH-controlled fermentations and accumulates in the medium. LAB are sensitive to lactate, and for each strain a concentration exists that will cause growth to go into stationary phase. Average values of maximum lactate concentration for LAB lie in the range 45.0 to 83.0 $g.l^{-1}$ (Boonmee et al., 2003). Inhibition caused by lactate salt can be avoided by using extraction methods for pH-control, e.g. electrodialysis, membrane separation, aqueous two-phase systems, and adsorption. (Boonmee et al., 2003; Hofvendahl et al., 2000; Schiraldi et al., 2003; Schnürer et al, 2005). This can significantly affect growth kinetics. In strains with lactate inhibition growth will continue until a maximum lactate concentration is reached. Thereafter, glucose will be converted to lactic acid in a non-growth related way. When *Lactococcus Lactis* NZ133 was fermented rapid uptake of lactose and production of lactate occurred in the stationary phase (Boonmee et al., 2003). Other acids, such as acetic acid, can also inhibit growth, but they are usually present in much lower concentrations in homofermentative LAB and therefore not a problem. *Lactococcus lactis* IO-1, known to produce considerable amounts of acetic acid, was not inhibited by acetate but by lactate during fermentation (Ishizaki and Ueda, 1995).

2.2.1.4 Purification and isolation

LA can be extracted during fermentation or it can be purified at the end of fermentation. Systems where LA is extracted during fermentation eliminate the possibility of end-product inhibition as a result of high lactate concentrations. Purifying lactate at the end of fermentation can be done with different methods, but precipitation with H_2SO_4 is generally used. At the end of fermentation the cells are removed (filters are usually used) and H_2SO_4 is added that causes a sulphate salt to precipitate, releasing the LA. The sulphate salt depends on the base that was used for pH-control. Controlling the pH with calcium hydroxide, for example, results in the solid salt calcium sulphate (gypsum) during regeneration (Hofvendahl et al., 2000). Ammonium hydroxide and calcium carbonate are preferred above calcium hydroxide as they form the fertilizer ammonium sulphate and carbon dioxide respectively which have better value than gypsum (Hofvendahl et al., 2000).

2.2.1.5 Application

There exists a wide range of possible applications of LA with the biggest fields being the leather, textile, food and pharmaceutical industries (Hofvendahl et al., 2000). LA can be used as a preservative (its ability to lower pH inhibits growth of possible pathogens), acidulant, flavourant in food and raw material in the formation of lactate ester, propylene glycol, 2,3-pentanedione, propanoic acid, acrylic acid, acetaldehyde and dilactide (Parente et al., 1997; Wee et al., 2004). The current demand for LA, preferably L(+), has increased since its application as a monomer for the production of polylactic acid (PLA), a biodegradable polymer (Yun et al., 2003). PLA serves as a substitute for the traditional synthetic polymers from petrochemical origin (Wee et al., 2004). Biodegradable polymers find application in the medical (high resorption thread, prosthesis), pharmaceutical (low diffusion drug) and food industries (packaging), as previously reported (Hofvendahl et al., 2000; Payot et al., 1999; Schiraldi et al., 2003).

2.2.2 Other end-products

During fermentation it is also possible that hydrogen peroxide, propionic acid, diacetyl (2,3-butanedione) and ethanol can be formed (Schnürer et al., 2005).

2.2.3 Bacteriocins

2.2.3.1 Background

Bacteriocins from LAB can be defined as small proteins or peptides that are ribosomally synthesized and secreted into the growth medium (Leroy et al., 2003; Ouwehand et al., 2004).

Bacteriocin production is considered a cell's defence or survival mechanism as bacteriocins exhibit an antagonistic activity towards other bacteria, usually strains closely related to the producer (Gollop et al., 2003; Leroy et al., 2002 a; Verellen et al., 1998). Most probable, this is because similar strains will inhabit the same ecological niche as the producing strain and will therefore compete for the same nutrients (Deegan et al., 2006). However, bacteriocins from LAB with activity against Gram-negative bacteria, moulds and yeasts have been reported (Gollop et al., 2003). The antimicrobial action of bacteriocins is facilitated by their interference with the cell wall or membrane of target organisms. This is done by either inhibiting cell wall formation or causing pores in the cell wall that leads to cytoplasm leakage and subsequently death (Cleveland et al., 2001; Deegan et al., 2006; O'Sullivan et al., 2002). The producer is protected from its own bacteriocins by immunity proteins (Deegan et al., 2006; Nes et al., 2002). The start of bacteriocin production depends on the bacterial strain. It can be produced as a growth related product (Callewaert et al., 2000; De Vuyst et al., 1994 b), while in some LAB bacteriocin production occurs in the stationary phase (Ekinci and Barefoot, 2006; Onda et al., 2003). Bacteriocins can be classified into four main classes as described by Nes et al. (1996) and are shown in Table 1.

Table 1

Classification of bacteriocins (Adapted from Ouwehand et al. (2004)).

Class	**Subclass**	**Description**
Class I		Also referred to as lantibiotics; small (< 5 kDa), post-translational modified peptides, identified by presence of modified thioether amino acids such as lanthionine, β-methyllanthionine and α, β unsaturated amino acids such as dehydroalanine and dehydrobutyrine; best known example is nisin, others include lacticin 481 and plantaricin C
	Ia(1)	Elongated, cationic, membrane active, slight net positive (+) or negative (-) charge
	Ia(2)	Elongated, cationic, membrane active, high net (-) charge

	Ib	Globular, inhibit enzyme activity
Class II		Small (< 10 kDa), heat-stable (100 - 121 ºC) and unmodified peptides that are membrane active and contain no lanthionine
	IIa	Peptides are pediocin-like with strong activity against *Listeria* species; contain a conserved YGNGVXC amino acid motif at the N-terminus
	IIb	Two-component peptides
	IIc	Bacteriocins are secreted via the *sec*-pathway or pre-protein translocase
Class III		Large (> 10 kDa), heat-labile proteins
Class IV		Complex bacteriocins consisting of a protein with a lipid and/or carbohydrate attached e.g. plantaricin S or lactocin 27

Gram-positive bacteria (this includes LAB) are producers of mainly class I and II bacteriocins (Zendo et al., 2005). *Enterococci* are producers of mainly Class II bacteriocins, also called the non-lantibiotics (Diep and Nes, 2002). Class II bacteriocins are, with a few exceptions, produced by a minimum of four genes (five for two-peptide bacteriocins). These genes are: (i) a structural gene to encode the pre-peptide (two needed for two-peptide bacteriocins); (ii) an immunity gene that is responsible for the immunity protein needed to protect the producer from its own bacteriocin; (iii) a gene for the membrane associated ABC-transporter that transfers the bacteriocin through the membrane and (iv) a gene that encodes a protein that helps with the secretion of the bacteriocin into the environment, but of which the function is not fully understood (Nes et al., 2002). The pre-peptide mentioned here is a pre-form of the bacteriocin and contains an N-terminal extension which is removed during interaction with the dedicated ABC-transporter (Diep et al., 2002; Nes et al., 2002). Some of the exceptions to this are found in the Class IIc section. These bacteriocins have sec-leaders and are secreted through the bacteria's secretory (sec) system (Diep et al., 2002; Nes et al., 2002). Other

bacteriocins again are synthesized without a leader and not much is known about their mode of transport (Diep et al., 2002).

The production of many Class II non-lantibiotics (sakacins, plantaricins, enterococcins and carnobacteriocins) proceeds via what is known as a three-component regulatory system consisting of a pheromone, a histidine protein kinase (HPK) and a response regulator (RR). The pheromone, present as a peptide in the medium, activates the membrane-located HPK. This causes a series of reactions, one of in which the RR receives a phosphate group. The phosphorylated RR then activates gene expression that includes expression of the pheromone itself. This leads to an auto-activation loop in which genes for bacteriocin production and regulation are transcribed. In the final step the ABC-transporters transfer the bacteriocin and pheromone through the cell membrane whereafter the cycle is continued (Diep et al., 2002; Nes et al., 2002).

2.2.3.2 Factors affecting bacteriocin production

Although bacteriocins are ribosomally synthesized, external factors such as pH, temperature and nutrients have the ability to affect production (Diep et al., 2002; Nes et al., 2002). Production of bacteriocins by LAB usually follows primary metabolite growth-associated kinetics, i.e. production occurs during the exponential growth phase and ceases once stationary phase is reached (Leroy et al., 2002 a; Leroy and De Vuyst., 2002 b; Parente et al., 1997). This is, however, not always the case and the relationship between bacteriocin production and growth depends on the strain used (Parente et al., 1997). In some cases a correlation between peptide- and biomass production is reported (Abriouel et al., 2003; Callewaert et al., 2002), while in other cases bacteriocin production only starts when stationary phase is reached, or production is regulated by external factors such as medium pH (Guerra et al., 2001; Leroy et al., 2002 b). Bacteriocin production (per volume and per viable cells) increased when *Enterococcus faecalis* subsp. *liquefaciens* A-48-32 was grown in high cell density cultures (Abriouel et al., 2003). Bavaricin MN production, on the other hand, was more affected by pH variations than growth promoters in batch and continuous cultures (Parente et al., 1997).

Conditions best for growth does not always increase bacteriocin production (Verellen et al., 1998; Zendo et al., 2005). Leroy et al. (2002 b) found that bacteriocin production by *Enterococcus faecium* RZS C5 was limited to the early growth phase. Improving biomass yields by addition of nutrients to the growth medium did not increase bacteriocin activity. Cell growth of *Enterococcus mundtii* QU 2 in APT medium was 1.5 times higher than in MRS broth, but volumetric bacteriocin activity ($AU.ml^{-1}$) in ATP medium was 3.1 % of that

obtained in MRS (Zendo et al., 2005). Generally, however, it can be assumed that factors that increase cell growth will also increase bacteriocin production (Callewaert et al., 2002; Parente et al., 1997).

Improving bacteriocin production by a specific strain usually involves optimization of media and other physical properties such as temperature and pH (Callewaert et al., 2002; Carolissen-Mackay et al., 1997), but other methods can also be employed. Since bacteriocin production is a defence mechanism of the cell, production may be increased when the cell is introduced to stressful conditions such as oxidative stress, salt stress, nutritional stress, temperature stress and pH stress (Leroy et al., 2002 a). In some studies oxidative stress decreased activity, while in others it showed no significant effect. Leroy et al. (2002 a) increased bacteriocin production of *Enterococcus faecium* RZS C5 by prolonging the production phase by including NaCl at concentrations of up to 40.0 $g.l^{-1}$ in the growth medium. This also seemed to keep the bacteriocin stable for a longer time. It is, however, not possible to assume a direct relationship between bacteriocin production and stress. The performance of these experiments depends on the strain used and the type of stress applied (Leroy et al., 2002 a). Additionally, aeration of normally anaerobic cultures has been shown to increase bacteriocin levels. A dissolved oxygen concentration of 60 % significantly increased nisin activity compared to anaerobic cultures of *Lactococcus lactis* (Amialli et al., 1998).

Another way in which bacteriocin production has been induced was when inactive, alien bacterial cells were added to growth media. Sip et al. (1998) improved divercin activity by 64 times when *Carnobacterium divergens* AS7 was cultivated in the presence of a bacteriocin sensitive strain. This interaction between cells and the environment is called quorum sensing. There are other cases of bacteriocin production that is regulated by quorum sensing (Kuipers et al., 1998). Examples of these are nisin production by *Lactococcus lactis* and sakacin P production in *Lactobacillus sake* (Kuipers et al., 1998).

Although there exists various ways to increase bacteriocin production, it is possible that activity will only increase until it reaches a certain maximum value. Leroy and De Vuyst (2001) studied the effect of nutrients on bacteriocin production by *Lactobacillus sakei* CTC 494. Initially, an increase in nutrients led to higher cell densities and also bacteriocin activity, but further increases in nutrient concentrations only resulted in higher cell densities. The activity did not exceed a certain plateau value, which resulted in a decrease in the bacteriocin production per cell. The authors suggested that the bacteriocin could become toxic to the producer cells if the concentration became too high (Leroy et al., 2001). It has also been suggested that bacteriocin production ceases when a certain cell density is reached, whereafter

the number of bacterial cells are high enough to ensure survival and defensive metabolites are not needed (Leroy et al., 2002 b).

2.2.3.3 Stability during fermentation

In a fermentation process there are many factors that can cause bacteriocins to become denatured and lose their antimicrobial nature. Activity can decrease during the stationary phase as a result of bacteriocin inactivation by specific or non-specific proteases released during cell lyses, aggregation and/or adsorption of the bacteriocin molecules to the surface of the producer cells (Callewaert et al., 2002; Herranz et al., 2001; Leroy et al., 2002 a; Parente et al., 1997). This has been observed for a number of bacteriocins, e.g. lactacin B, amylovorin L471, propionicin PLG-1 and jenseniin G (Ekinci et al., 2006). Generally, inactivation of bacteriocins are strongly dependent on pH and in several cases it has been found that inactivation is increased at higher pH values (Herranz et al., 2001). In some cases inactivation may also be caused by cells of a producing strain that has become sensitive to the bacteriocin. In several cases it was found that regulation and transcription of genes responsible for bacteriocin production and immunity happened simultaneously (Callewaert et al., 2002). When cells in culture ceased to produce bacteriocins they also lost their immunity against their own bacteriocins resulting in cell death and a decrease in bacteriocin activity (Callewaert et al., 2002). Medium components can also contribute to the stability of bacteriocins. In previous work by Leroy et al. (2003) bacteriocin titres was stabilized by high initial concentrations of NaCl (40.0 $g.l^{-1}$) and glucose (80.0 $g.l^{-1}$).

2.2.3.4 Purification of bacteriocins

Another important step to consider during bacteriocin production is purification. When considering a possible growth medium it is important to keep in mind that media containing a lot of impurities will complicate downstream separation processes (Carolissen-Mackay et al., 1997). Apart from the traditional separation process for proteins, i.e. ammonium sulphate precipitation, ion-exchange chromatography, hydrophobic interaction followed by reverse-phase chromatography, alternative methods such as Triton-X and chloroform have also been described for purification of bacteriocins (Carolissen-Mackay et al., 1997; Ouwehand et al., 2004). These methods rely on the hydrophobic or amphiphilic nature of bacteriocins (Ouwehand et al., 2004).

2.2.3.5 Measuring bacteriocin activity and concentration

Various methods are used to determine bacteriocin activity. It is therefore important to consider them, as different methods will result in different values for antimicrobial activity. One method used is critical dilution (Ekinci et al., 2006). The supernatant is serially diluted

with a phosphate buffer (sometimes replaced by distilled water) before it is spotted on indicator lawns containing the sensitive strain. One arbitrary unit (AU) per ml is defined as the reciprocal of the highest zone with no growth. Different indicator lawns will result in different inhibition zones depending on the sensitive strains susceptibility to the bacteriocin (Carolissen-Mackay et al., 1997). Another method that can be used is the photometric assay. A cell-free supernatant containing bacteriocins are serially diluted with distilled water, whereafter 2.5 ml of the supernatant is added to a sterile tube inoculated with 2.5 ml of the sensitive strain. In this case one bacteriocin unit is described as the amount of antimicrobial compound needed to result in a 50 % growth inhibition compared to control tubes (Guerra et al., 2005).

There is a lot of variability in the methods used to determine bacteriocin activity, but different bacteriocins can be compared by converting their activity from $AU.ml^{-1}$ to International Units $(IU).ml^{-1}$, using a correlation curve (Cheigh et al., 2002). $IU.ml^{-1}$ is based on activity observed for Nisaplin, a commercial product containing nisin (Cheigh et al., 2002).

Besides activity, the concentration of bacteriocins can also be determined with traditional protein assays. These include the following: Folin-Lowry, Biuret, Bradford and Bicinchoninic acid (BCA) assay (Carolissen-Mackay et al., 1997; Tari et al., 2006). The Lowry method or a modified version thereof is used most often e.g. lactacin B, pediocin AcH and leucocin A-UAL 187 (Carolissen-Mackay et al., 1997).

2.2.3.6 Applications

Food products

Some bacteriocins can, besides inhibiting closely related species, also inhibit food-borne pathogens and spoilage organisms (Carolissen-Mackay et al., 1997; Parente et al., 1997). Many of bacteriocin-producing LAB have long been used as starter cultures for the fermentative preparation of dairy, meat and vegetable products. Bacteriocins have most probably been consumed for decades along with starter cultures without causing illness (O'Sullivan et al., 2002). Furthermore, the majority of bacteriocins are heat-stable, non-toxic and degradable by enzymes in the gastrointestinal tract. This, along with LAB's close history with food, makes bacteriocins ideal candidates for use in the food industry as natural food preservatives (Abriouel et al., 2003; Callewaert et al., 2002; Gollop et al., 2003; O'Sullivan et al., 2002). Moreover, the food industry is experiencing increased pressure by consumer demands to develop natural, minimally processed food products with a long shelf life without the use of chemical preservatives (Ennahar et al., 1999). The reduced use of food preservation techniques and materials is not an easy task and holds many dangers. An estimated 5 to 10 %

of food worldwide production is lost to spoilage by fungi (Schnürer et al., 2005). Food pathogens such as *Listeria* spp., resistant against refrigeration temperatures and high salt concentrations, are responsible for 2500 illnesses of which 500 are fatal in the United States alone (Deegan at al., 2006). Subsequently, the need for natural preservatives such as bacteriocins is high. However, it is advised that bacteriocins should not be seen as the main control measure for preserving food, but rather as an extra obstacle for spoilage or pathogenic bacteria (Deegan et al., 2006).

Bacteriocins can be administered to food as (i) a separate product in the form of a purified or semi-purified bacteriocin, (ii) an ingredient that was prepared by fermentation of bacteriocin-producing strains or (iii) it can be produced *in situ* by a starter culture (Deegan et al., 2006; Herranz et al., 2001; Leroy et al., 2002 a; Parente et al., 1997). A new option currently being explored and that can be grouped under (i) is the use of bacteriocin activated polythene films for food packaging (Deegan et al., 2006; Mauriello et al., 2004). Starter cultures have several advantages over the over methods: They eliminate the commercial process needed for production of bacteriocins (mainly the fermentation and purification steps) and inclusion of starter bacteria in food enhances the flavour and aroma of the food. However, not all foods require a starter culture, which makes a purified or semi-purified bacteriocin product more applicable. In the food industry bacteriocins can be incorporated as a concentrated and not purified product (Deegan et al., 2006) whereas the pharmaceutical industry demands a product of high purity. A good example here is nisin, a bacteriocin used as a food preservative and sold under the name Nisaplin™. It is prepared by concentrating and spray drying a milk-based supernatant obtained after fermentation of *Lactococcus lactis* (Deegan et al., 2006).

The majority of bacteriocins are not active against Gram-negative bacteria. However, they can be used in combination with other stress-inducing processes, e.g. heating, freezing, acid treatment, chelating agents, high hydrostatic pressure and electroporation (Guerra et al., 2005), to make them effective against Gram-negative and resistant Gram-positive bacteria. Gram-negative bacteria can also be inhibited by using food-grade chelating agents such as ethylene diamine tetra-acetate (EDTA) and citrate in combination with bacteriocins. These agents bind to Mg^{2+}-ions in the outer lipopolysaccharide layer thus rendering Gram-negative bacteria sensitive to bacteriocins (Messens and De Vuyst, 2002).

There are certain pre-requisites to which bacteriocin producers from LAB should comply to before they can be considered for industrial food applications (O'Sullivan et al., 2002): (i) The producer strain should have GRAS status; (ii) The bacteriocin should inhibit growth of a broad spectrum of pathogens and/or food spoilage bacteria, including *Listeria monocytogenes*

and *Clostridium botulinum*, or it should be active against a specific pathogen; (iii) it should be heat stable; (iv) hold no health risks; (v) improve product value by adding to the safety, quality and flavour of the product and lastly (vi) it should have a high specific activity once incorporated in the product. Naturally, the bacteriocins should also not inhibit growth of other starter cultures if used.

Medicinal and pharmaceutical products

Recently bacteriocins have also been applied in the field of medicine. It is known that micro-organisms are becoming increasingly resistant against antibiotics (Schnürer et al., 2005). There is therefore reason to fear that existing treatments against bacterial infections will become ineffective. The discovery of bacteriocins and research on them have brought a possible solution to this problem. As they are able to inhibit growth of target bacteria, bacteriocins are being considered for the treatment of bacterial infections. It has certain advantages - the product is natural, made from 'good bacteria', and holds no danger of causing any illness or other problems. Typical treatment scenarios include topical skin infections or multiple drug-resistant systemic infections (Guerra et al., 2005). Probiotic strains and also bacteriocin producers have the ability to protect the gastrointestinal tract against colonization by pathogenic bacteria. These bacteriocins are produced *in situ* once the probiotic strain settles in the intestines (Avonts et al., 2004).

Bacteriocins can be confused by antibiotics, but they differ in four main areas: (i) They are ribosomally synthesized peptides, not metabolites; (ii) producer cells are immune to bacteriocin activity; (iii) bacteriocins have a different antimicrobial mode of action and (iv) they have a narrow spectrum of inhibition (Ouwehand et al., 2004).

2.2.3.7 Commercially produced bacteriocins

At present there are only two bacteriocins that are produced commercially, i.e. nisin produced by *Lactococcus lactis* and pediocin PA-1/AcH produced by *Pediococcus acidilactici*, and they are both used in the food industry. Nisin and pediocin PA-1/AcH are used in products called Nisaplin™ and ALTA™, respectively (Deegan et al., 2006). Nisin was first discovered in the 1920's when it was thought to be applicable in the therapeutic industry. On further investigation however, nisin proved to be unstable at physiological pH and was highly susceptible to enzyme degradation. This, together with its ability to inhibit foodborne pathogens (especially *Listeria monocytogenes*) made it better suited for the food industry (Deegan et al., 2006). Nisin was first marketed in England in 1953 (Deegan et al., 2006). Today it is a FDA-approved product and sold as a dried powder, which is prepared from a skim milk derived fermentate. Nisaplin is used in at least 48 countries as a food additive,

mostly in processed cheese, dairy products and canned foods (O'Sullivan et al., 2002). It is expected that commercial production of bacteriocins for use as food preservatives will increase in the future.

2.2.3.8 Enterocins: Bacteriocins from Enterococci

Bacteriocins produced by enterococci are generally referred to as enterocins. The majority of bacteriocins identified in this genus are from *Enterococcus faecium* and *Enterococcus faecalis* (Zendo et al., 2005). Enterocins can be grouped in all three main classes, but there are some which do not fall within this classification structure because of unusual structures or genetic characteristics. Studies have shown that enterocins usually have activity against the foodborne pathogens *Listeria* (Franz et al., 2005, Moreno et al., 2006) and *Clostridium* (Leroy et al., 2003). This, combined with the fact that many *enterococci* are natural food inhabitants, make them very attractive for use as biopreservatives. Naturally, research has been focused on enterococci isolated from food products, i.e. *E. faecium* and *E. faecalis*. Some of the foods include dairy products such as cheese and raw milk and meats such as Spanish-style dry fermented sausages (Franz et al., 2005).

2.3 Fermentation processes employed to optimize bacteriocin production

2.3.1 Media optimization

2.3.1.1 Introduction

Growth media is one of the key factors to consider during the optimization of a fermentation process. The importance of growth media becomes obvious when mentioning that it can contribute to as much as 30 % of the total cost of microbial fermentations (Rivas et al., 2004). Media proposed for industrial scale fermentations should therefore comply with a number of criteria: It should be cost-effective, result in high product yields, minimize fermentation time and ease downstream purification processes (Guerra et al., 2005). These factors frequently clash, e.g. media that gives the highest product yield will in most cases not be the most cost-effective. The choice of 'best' media therefore depends on the specific situation and will usually consist of a trade-off between the different factors.

Today's market has an abundant selection of complex media for cultivation of microbial strains: De Man Rogosa and Sharpe (MRS), Brain Heart Infusion (BHI), CM, NaLa, M17, Trypticase Soy Broth Yeast Extract (TSBYE) etc. (Guerra et al., 2001; Li et al., 2002). Most

of these media were designed for specific strains (e.g. M17 and MRS for lactobacilli) and provide very good growth environments, but they are expensive and therefore not suited for application in industrial-scale fermentations to produce bacteriocins. Being growth media for a broad range of bacteria they are also not optimal in composition and concentration for use in specialized processes. Growth of a bacteriocin producer, *Lactobacillus sakei* CTC 494, in MRS, for example, was inhibited mainly because of nutrient depletion, suggesting that MRS medium was not fully optimized for bacteriocin production (Leroy et al., 2001). Another example is CM media that yielded a two-fold increase in volumetric bacteriocin activity once it was optimized for bacteriocin production (Li et al., 2002).

The growth-associated nature of bacteriocin production suggests that optimization of growth media can be used to increase bacteriocin levels by improving cell growth (Mataragas et al., 2004). Media optimization therefore plays an important role with respect to bacteriocin production and cost reduction. Cost reduction is realized by using cheap industrial sources of carbon or nitrogen.

In most cases media is designed to be carbon limited. This means there are enough nitrogen, vitamins, minerals, and other trace elements present not to become limiting to the organism. Once media is carbon limited the growth of micro-organisms can be controlled. Adding more carbon, for example, will then allow growth to higher cell densities as long as the other components are sufficiently available.

2.3.1.2 Industrial media

Micro-organisms need carbon and/or nitrogen sources for good growth and product formation. Inexpensive sources of these can be found in the form of industrial waste- or by-products. Examples are molasses, soy, hydrolysed wheat flour, cheese whey and corn steep liquor (Guerra et al., 2001; Hofvendahl et al., 2000; Oh et al., 2005; Wee et al., 2004). A number of criteria should be considered when opting to use industrial media in fermentations. These include the carbon or nitrogen content of the media, price, availability and level of impurities. One of the most important factors to look for in an industrial carbon source is the type of carbon present in the media. The carbon source should preferably be in a fermentable form and also be present at a sufficient concentration. Most bacteria can ferment mono- and disaccharides, but only a limited amount possesses the ability to ferment complex carbohydrates such as starch. Examples of bacteria able to ferment complex sugars are amylolytic LAB such as *Lactobacillus amylophilus*, *Lactobacillus amylovorus* and *Lactobacillus plantarum* A6 (Ohkouchi et al., 2006). These LAB produce the enzyme amylase that breaks starch down to monomer fermentable sugars and can be used in a

fermentation process where simultaneous saccharification and fermentation takes place (Ohkouchi et al., 2006).

In the case of bacteria not able to ferment complex carbon sources it is necessary to have a pre-treatment step where the sugars are hydrolysed to produce fermentable sugars. Hydrolysis can be done by either adding commercial enzymes such as glucoamylase to the broth, or by using heat treatment at low pH values (Ohkouchi et al., 2006). The problem with additional treatments like these is the extra cost and complexity they bring to the production process. This renders the project less favourable from both an economical and production point of view. Consequently, it is much more favourable to use media that already contain fermentable sugars.

Molasses, corn steep liquor and cheese whey are examples of industrial media that are commonly used. All of these media are readily available in South Africa. A brief discussion of these media and their origin are given below.

Molasses

Molasses, a by-product of the sugarcane industry, contains approximately 50.0 % (w/w) sugars (sucrose being the main sugar), some heavy metals (e.g. calcium, iron, copper, zinc, etc.), ash and other suspended solids (Hofvendahl et al., 2000; Wee et al., 2004). It is used as a carbon source in production of ethanol, yeast, and LA, and it is applied as animal feed (Hofvendahl et al, 2000; Payot et al., 1999). In some cases the heavy metals may interfere with the fermentation process, making pre-treatment necessary.

Corn steep liquor (CSL)

Corn steep liquor (CSL) is a by-product formed during the milling of corn. It is mainly a nitrogen source (Djekrif-Dakhmouche et al., 2005; Kulozik and Wilde, 1999; Rivas et al., 2004; Tari et al., 2006), but also contains sugars (mainly sucrose), vitamins and minerals. The value of CSL was evident in work by Lee et al. (2005) where 15.0 $g.l^{-1}$ yeast extract (YE) in a 10.0 % glucose solution could effectively be replaced by a 3.1 $g.l^{-1}$ YE and 5.0 % CSL solution. Bustos et al. (2004) used CSL supplemented with yeast extract and peptone for the optimization of D-lactic acid production by *Lactobacillus coryniformis*.

Cheese whey

Cheese whey (CW) is a liquid by-product formed during the cheese manufacturing process and usually contains high levels of lactose (40.0 $g.l^{-1}$), proteins and fat (Guerra et al., 2005;

Hofvendahl et al., 2000). It also contains small amounts of vitamins and minerals (Guerra et al., 2005) and can be found in different forms such as powder, ultrafiltrate etc. CW is used as a carbon source and is the most popular substrate for LA production by LAB (Hofvendahl et al, 2000). In most cases CW has to be supplemented with nitrogen, vitamins and trace elements to obtain good growth of LAB (Kulozik et al., 1999). Cladera-Olivera et al. (2004) used CW as medium for bacteriocin production by *Bacillus licheniformis* strain P40.

The disadvantage of using media such as molasses or CSL is their contribution to impurities in the fermentation media thus complicating separation processes, especially if bacteriocins are to be separated (Rivas et al., 2004). Media with high nitrogen content, especially proteins and peptides, can also cause downstream problems when the bacteriocin needs to be purified (Guerra et al., 2001; Li et al., 2002).

2.3.1.3 Statistical designs

Industrial media are often enriched with supplements to improve fermentation performance. Various techniques are used to identify the components and/or concentrations needed for optimal production. They range from the old fashioned, traditional 'one-factor-at-a-time method' to advanced statistical designs such as factorial- and fractional factorial designs (Djekrif-Dakhmouche et al., 2005; Guerra et al., 2001). Knowledge of statistical techniques is therefore strongly advised before attempting media optimization. This can be explained by referring to the 'one-factor-at-a-time method' that was predominantly used in the past. This method becomes very complex, time consuming and expensive when dealing with a wide range of possible components. It also does not take the possibility of interactions between components and/or factors such as pH or temperature into account and can thus give rise to wrong conclusions (Li et al., 2002; Tari et al., 2006).

These problems have been solved by the incorporation of statistical designs in experimental design. Employing statistical designs such as fractional factorial designs can significantly decrease the workload, detect interactions, save time and money and provide statistically accurate results (Cladera-Olivera et al., 2004; Djekrif-Dakhmouche et al., 2005). An example of this is the Plackett-Burman design where N-1 variables can be studied in N experiments to identify those significant for product formation (Djekrif-Dakhmouche et al., 2005). These factors can include individual medium components such as yeast extract, $CaCl_2$, glucose etc. or factors such as pH, temperature or salts (Djekrif-Dakhmouche et al., 2005). Response surface methodology (RSM), based on factorial designs, is another widely used technique and has been implemented in many studies to optimize a medium for bacteriocin production (Cladera-Olivera et al., 2004; Li et al., 2002; Liew et al., 2005). Li et al. (2001) used RSM to

optimize the medium composition for nisin production by *Lactococcus lactis* ATCC 11454. By using a 2^{6-2} fractional factorial design (FrFD) the number of runs was effectively reduced with a factor of 4. The validity of RSM was confirmed by an increase in nisin activity from 1074 $IU.ml^{-1}$ to 2150 $IU.ml^{-1}$.

Statistical models obtained from experimental designs enable the construction of equations that can describe metabolite production kinetics (Kulozik et al., 1999). Every component/factor from the design is fitted into an equation and their effect (positive or negative) is described by a constant preceding them. These models can be linear, quadratic, exponential etc. As early as 1959 *Luedeking and Piret* published a model describing the fermentation of simple substrates such as glucose to LA (Kulozik et al., 1999).

2.3.1.4 Improving media for bacteriocin production

LAB are known for their fastidious nutritional requirements (Liew et al., 2005). The carbon source is one of the most important components in growth media, but in many cases it will not be sufficient for successful cultivation of LAB. Additional nutrients such as nitrogen, minerals and vitamins are usually required to obtain good growth and metabolite production. *Lactobacillus johnsonii* La1 and *Lactobacillus gasseri* K7 could only grow in milk medium if yeast extract was added (Avonts et al., 2004). Successful cultivation is necessary as bacteriocin production is usually a growth-associated product (Avonts et al., 2004). However, this does not mean that factors affecting growth will also affect bacteriocin production (Kim et al., 2006; Li et al., 2002). Growth of *Lactococcus lactis* ATCC 11454 was promoted by sucrose, soybean peptone, YE and KH_2PO_4, but only soybean peptone and KH_2PO_4 had a significant effect on bacteriocin production (Li et al., 2002).

The type of carbon source used has a definite effect on bacteriocin production (Audisio et al., 2001; Guerra et al., 2005; Kim et al., 2006). This was demonstrated by Audisio et al. (2001) when a bacteriocin produced by *Enterococcus faecium* CRL1385 had activities of 11636, 1600 and 1454 $AU.ml^{-1}$ when brown sugar, glucose and crude complex carbohydrate samples were used, respectively. Lactose or sucrose was preferred above glucose as carbon source during micrococcin GO5 production by *Micrococcus* sp. GO5 (Kim et al., 2006). The concentration of carbon source at the start of and/or during fermentation also plays an important role in bacteriocin production (De Rojas et al., 2004; Papagianni et al., 2006). Nisin activity increased with increases in initial glucose concentrations up to 25.0 $g.l^{-1}$ during fermentation with *Lactococcus lactis*. Higher glucose concentrations did not further increase nisin production. It was also found that by keeping the residual glucose concentration at 10.0 $g.l^{-1}$ (glucostat), nisin levels increased even more. At 10.0 $g.l^{-1}$ it seemed that glucose

transport inside the cells was saturated and higher glucose concentrations did not result in higher nisin activity (Papagianni et al., 2006).

In most cases the presence of a nitrogen source is crucial for bacteriocin production (Zendo et al., 2005). Nitrogen source used as supplements include yeast extract, beef extract, bacteriological peptone, malt sprouts, soybean etc. (Kulozik et al., 1999; Zendo et al., 2005). Of all the available nitrogen sources, yeast extract has the most significant impact on bacteriocin production (Bustos et al., 2004; Kulozik et al., 1999; Rivas et al., 2004). Yeast extract is the water soluble content of autolyzed yeast cells (Rivas et al., 2004) and has proved its value as a nutrient by improving cell growth and metabolite production (Avonts et al., 2004; Kulozik et al., 1999; Rivas et al., 2004). It is assumed that the presence of purine and pyrimidine bases and also B-vitamins in yeast extract makes it so efficient (Rivas et al., 2004). Bacteriocin production by *Enterococcus mundtii* QU 2 in MRS broth containing yeast extract, peptone and beef extract decreased with 50 % when one of the nitrogen sources were omitted (Zendo et al., 2005). Kulozik et al. (1999) found that biomass levels increased from 3.0 to 10.0 $g.l^{-1}$ (approximate values) with a shift in yeast extract concentration from 5.0 to 25.0 $g.l^{-1}$.

Unfortunately, yeast extract is very expensive. In the search for alternative nitrogen sources, Rivas et al. (2004) used spent yeast cells of *Debaryomyces hansenii* as a nitrogen supplement in a CSL-glucose medium. Addition of the spent yeast cells resulted in a definite increase in bioconversion when compared to CSL without supplements. Lactic acid production was very similar to that found when normal yeast extract was added to CSL. Guerra et al. (2001) studied bacteriocin production of *Lactococcus lactis* and *Pediococcus acidilactici* when grown in whey. Diluted whey supplemented with yeast extract was used for fed-batch pediocin production by *Pedioccoccus acidilactici*. In the same work, diluted whey and glucose gave lower activity. Other supplements used in whey are peptone, milk powder, soy flour and CSL (Hofvendahl et al., 2000).

There are, however, many examples where nitrogen does not have a significant effect on bacteriocin concentration. Tween 80 was the most important medium component for optimizing bacteriocin production by *E. mundtii* QU 2 (Zendo et al., 2005). It has been proposed that Tween 80, a surfactant, stimulates secretion of peptides through its influence on membrane fluidity (Franz et al., 1996). Still, the use of Tween 80 is not recommended as it may interfere with the ammonium sulphate precipitation when bacteriocins are purified (Franz et al., 1996). Phosphates frequently increase bacteriocin production (Guerra et al., 2001; Li et al., 2001; Kim et al., 2006). Guerra et al (2001) used the four factors total sugars

(lactose), nitrogen (glycine), phosphorous (KH_2PO_4) and buffer concentration (potassium hydrogen phthalate-NaOH) when they experimented with cheese whey as medium for bacteriocin production. These four factors are generally considered to be significant for bacteriocin production. In their work they used the two strains *Lactococcus lactis* and *Pediococcus acidilactici*. It was found that nisin production by *Lactococcus lactis* was stimulated by an increase in phosphorous, but increases in nitrogen, buffer and sugar inhibited production. Li et al. (2001) had similar results also for nisin production with *Lactococcus lactis* where KH_2PO_4 increased nisin production. K_2HPO_4 caused an eight-fold increase in micrococcin GO5 concentration during fermentation of *Micrococcus* sp. GO5 (Kim et al., 2006). Moreover, ions also have an effect on bacteriocin production. $MgSO_4$ improved micrococcin GO5 production (Kim et al., 2006) and $MnSO_4$ resulted in a two-fold increase in pediocin PD-1, while Ca^{2+} ions (added as $CaCO_3$ or $CaCl_2$) suppressed bacteriocin production by *Enterococcus mundtii* QU 2 (Zendo et al., 2005).

There are also medium components that can improve activity levels by inducing a stressful environment and thus stimulating bacteriocin production, by stabilizing the bacteriocin or by preventing aggregation of the bacteriocin molecules (Herranz et al., 2001). NaCl and ethanol are two components that have been suggested to have the above mentioned properties and have been used with variable results. The presence of NaCl and ethanol were inhibitory for enterocin production by *Enterococcus faecium* P13 (Herranz et al., 2001) and *Enterococcus faecium* CTC492 (Nilsen et al., 1998), while they had a stimulatory effect on bacteriocin production by *Lactococcus lactis* subsp. *lactis* ADRIA 85LO30 (Uguen et al., 1999) and *Lactobacillus amylovorus* (Herranz et al., 2001), respectively.

2.3.1.5 Substrate inhibition

The concentrations of media components play an important role in bacteriocin production. Diluted whey yielded higher amounts of biomass and bacteriocin than concentrated whey. This indicated some form of substrate inhibition, or that bacteriocin production was influenced by the sugar concentration (Guerra et al., 2001). On numerous occasions it has been shown that initial carbohydrate concentrations in batch fermentations can affect bacteriocin production. Enterocin 1146 production rate decreased when initial glucose concentrations were raised from 5.0 to 30.0 $g.l^{-1}$ (Parente et al., 1997). Maximal activity was recorded at 20.0 $g.l^{-1}$. Biomass concentrations increased when the initial glucose concentration was raised from 5.0 to 20.0 $g.l^{-1}$ during batch fermentation of *Enterococcus faecium* DPC1146 (Parente et al., 1997). The same trend for bacteriocin production was observed for nisin that reached a maximum at 40.0 $g.l^{-1}$ sucrose, but with a decrease in yield

(Parente et al., 1997). This led to the authors to suggest that nisin production was carbon-source regulated.

Although growth media is normally improved by the addition of supplements, it can easily be oversupplied with nutrients, which can lead to growth and product inhibition. Callewaert et al. (1999) reported the inhibition of bacteriocin production due to high concentrations of the complex nitrogen source that were used. Moreover, a high nutrient concentration is expensive and will also complicate the downstream purification processes.

2.3.2 Effect of pH

During fermentation of LAB the pH decreases due to the formation of organic acids such as LA, acetic acid and formic acid. LAB can survive in low pH environments (pH 4 or less), but low pH can cause growth to slow down or completely stop (Kulozik et al., 1999; Parente et al., 1997). Generally, this is not desired in fermentations and can be prevented by controlling the pH by use of suitable buffers or bases, e.g. citrate buffer, NaOH, KOH, NH_4OH etc. pH-control can also be applied by extracting acids using methods such as membrane adsorption, electrodialysis and aqueous two-phase systems (Hofvendahl et al, 2000).

Bacteriocin production is dependent on the pH during cultivation. The use of pH-controlled fermentations can lead to higher growth rates and subsequently also increased production of primary metabolic products (Herranz et al., 2001; Kulozik et al., 1999). Growth of LAB is usually optimal in a medium pH range (pH 6.0 to 7.0) with higher and lower values causing a decrease in specific growth rate (Bai et al., 2004). Biomass- and LA production by *Lactobacillus lactis* BME5-18M was significantly lower at a pH maintained at 5.5, in comparison to constant pH values of 6.5 and 7.0 (Bai et al., 2004). Maximal bacteriocin production generally occurs when the pH is controlled in some way (Herranz et al., 2001; Parente et al., 1997). When *Enterococcus faecium* P13 was fermented at constant pH 6.0, a four-fold increase in enterocin P activity was obtained compared to fermentations without pH-control (Herranz et al., 2001). Jenseniin G production by *Propionibacterium thoenii (jensenii)* P126 was increased by 7.5 times during pH-controlled fermentations (Ekinci et al., 2006). Bacteriocin production usually increases when pH-control is performed at a pH lower than that needed for optimal growth (Herranz et al., 2001). Onda et al. (2003) found that bacteriocin activity increased eight-fold for *Lactococcus* sp. strain GM005 when the pH was controlled during fermentation. The activity showed an additional four-fold increase when pH-control was performed at pH 6.0 instead of 7.0. Bacteriocin production under pH-controlled conditions occurred during the stationary phase. It is, however, not always true that

bacteriocin production is higher at a lower pH. Enterocin production followed primary metabolite kinetics at pH 7.5 to 8.0, but production was limited to the early growth phase when 5.5 < pH < 6.5 (Herranz et al., 2001).

Control of growth medium pH can also influence bacteriocin stability. Bacteriocin activity can decrease as a result of adsorption to producer cells, inactivation by proteolysis, degradation and aggregation (Herranz et al., 2001; Kim et al., 2006; Parente et al., 1997). Previous reports indicate that adsorption of bacteriocins to producer cells increases at higher pH values (Messens et al., 2002; Zendo et al., 2005). Activity levels of a bacteriocin produced by *Enterococcus mundtii* QU 2 at pH 7.0 decreased to zero after stationary phase was reached, most probably as a result of adsorption to producer cells. Even before stationary phase activity was low compared to fermentations conducted at pH 6.0 (Zendo et al., 2005).

In recent work by Vazquez et al. (2005) repeated alkalization of batch cultures increased biomass- and bacteriocin production in four out of seven LAB. These four also had more effective nutrient consumption and longer productive periods. The effect was even more pronounced when the re-alkalization was combined with glucose addition. This caused the authors to question the general consensus that bacteriocin production is optimal at constant pH fermentations, but rather proportional to the pH gradient. This was also mentioned by Guerra et al. (2005). Pediocin production by *Pediococcus acidilactici* NRRL B-5627 proved to be a pH-dependent metabolite with an increase in drop of pH promoting pediocin production (Guerra et al., 2005). Re-alkalization induced a component of secondary bacteriocin metabolism (primary metabolite kinetics were followed in natural fermentations). During these re-alkalized fed-batch fermentations homofermentation switched to heterofermentation (Guerra et al., 2005; Vazquez et al., 2005).

2.3.3 Effect of temperature

Temperature is an important parameter in fermentations as it can significantly improve bacterial growth and shorten fermentation times. It can also add considerably to fermentation costs if heating requirements become too high. Whereas pH affects bacteriocin production and stability, temperature mainly affects bacteriocin production and to a lesser extent inactivation (Kim et al., 2006). *Enterococcus faecium* RZS C5 had better bacteriocin production between 25 and 35 ºC than at 20 ºC (Leroy and De Vuyst, 1999). Jenseniin G activity was higher when *Propionibacterium thoenii* P126 was cultivated at 32 ºC (21.3 $AU.ml^{-1}$) compared to that obtained at 21 ºC (4.0 $AU.ml^{-1}$), as reported by Ekinci et al. (2006). Production of micrococcin GO5 by *Micrococcus* sp. GO5 increased when the

temperature was increased from 25 to 37 ºC (Kim et al., 2006). In these cases maximal bacteriocin production coincided with temperatures that were optimum for cell growth. As is the case with pH, however, the optimal temperature for growth will not necessarily coincide with the one needed for bacteriocin production: *Propionibacterium thoenii* LMG2792 produces no bacteriocins at 30 ºC, but well at 22 ºC (Faye et al., 2000). In the production of bavaricin A highest activity was achieved at 4 ºC, which was far from the optimal growth temperature (Messens et al, 2002).

2.3.4 Cultivation modes

2.3.4.1 Batch fermentation

In batch fermentations all the media is present at the start of fermentation. As this is the simplest mode of fermentation it is usually used to optimize growth media and conditions such as temperature, pH, dissolved oxygen level etc. (Bai et al., 2004; Callewaert et al., 2002).

Batch fermentations have a number of limitations. One of the more important limitations is the lack of ability to control the specific growth rate of the microbial population. Fermentation normally ends when one of the growth factors is depleted (usually the carbon source). Biomass production is limited in batch fermentations as the amount of carbon source present at the start of fermentation can only be increased to a certain concentration before it will start suppressing growth and/or metabolite production (Bai et al., 2004; Kadam et al., 2006; Parente et al., 1997; Yun et al., 2003).

2.3.4.2 Fed-batch fermentation

Introduction

Substrate inhibition in batch fermentations can be avoided by using fed-batch fermentations (Lv et al., 2005), which leads to high cell densities (Bai et al., 2004; Callewaert et al., 2002). Ultimately, fed-batch fermentation allows control of the growth limiting factor of a culture and subsequently the growth rate. Fed-batch fermentation is therefore a popular method for production of growth-related products. Bai et al. (2004) decreased fermentation time with 28 %, increased cell dry weight with 37.8 %, productivity with 50.4 % and ammonium lactate with 7.3 % when fed-batch fermentation replaced batch fermentation. In a study evaluating different fed-batch feeding strategies, biomass, LA and productivity (expressed in g $LA.l^{-1}.h^{-1}$) was lowest in batch fermentations for each feeding strategy (Ding and Tan, 2006).

Bacteriocin production is often associated with bacterial growth and increased biomass can yield higher volumetric bacteriocin production, as was demonstrated for enterocin AS-48 (Abriouel et al., 2003) and amylovorin L471 (Callewaert et al., 2000). Fed-batch fermentation therefore offers another way of achieving higher bacteriocin yields (Ekinci et al., 2006; Lv et al., 2004, 2005). However, few reports describing bacteriocin production by fed-batch fermentation could be found. Subsequently, the effectiveness of fed-batch fermentation will sometimes be illustrated by its ability to improve biomass or lactic acid production, and not bacteriocin activity.

Feeding strategies

There are various feeding strategies that can be used in fed-batch fermentations, e.g. pH-stat, DO-stat, constant feeding, exponential feeding etc. (Bai et al., 2004; Ding et al., 2006; Lv et al., 2005; Oh et al., 2002; Rodrigues et al., 1998). Generally, the different feeding strategies can be grouped into two main categories: (i) a constant volumetric growth rate and (ii) a constant concentration of the limiting substrate (Nielsen, 2001). A constant volumetric growth rate can be used in systems where growth becomes dependent on external factors such as oxygen or heat removal. A constant substrate concentration is usually applied to conditions where growth can become inhibited by high concentrations of the substrate (Korz et al., 1995; Nielsen, 2001; Rodrigues et al., 1998), or where a nutrient is growth limiting. By controlling the substrate concentration the specific growth rate of the cells is also controlled (Nielsen, 2001).

Bai et al. (2004) used a constant feed rate in their fed-batch fermentations that started when the residual glucose concentration was between 5.0 and 10.0 $g.l^{-1}$. By increasing the feeding rate from 15.0 to 25.0 $ml.h^{-1}$ there was a slight increase in growth rate, ammonium lactate production and yield, but higher flow rates had a negative effect. Callewaert et al (2002) compared feeding strategies in their attempts to increase bacteriocin production by *Lactobacillus amylovorus* DCE 471. In constant fed-batch they evaluated the effects of limiting components by feeding with different media components. Apparently, it did not matter whether glucose, complex nitrogen source (CNS) or a mixture of both were fed to the reactor as they all led to the same level of bacteriocin activity. During this time bacteriocin production was growth associated. The effect of nutrient availability was investigated by slow and fast addition of a glucose and CNS mixture. Here, bacteriocin production was favoured by fast addition or, stated differently, a surplus of nutrients. This indicated the growth associated behaviour of bacteriocin production. In a final step glucose and CNS addition were controlled by the acidification rate of the growth medium. Nutrients could therefore be replaced as they were consumed. This method of fermentation led to the highest bacteriocin

activity (25.6 MAU.l^{-1} compared to previous peak of 12.8 MAU.l^{-1} obtained from constant feed fed-batch experiments).

To accurately control specific growth rate the conditions in the fermentor need to be kept constant. This can be done by keeping the dilution rate, a ratio of feed rate over volume, constant.

The dilution rate is given by equation 5

$$D = \frac{1}{V}\frac{dV}{dt} = \frac{1}{V} * F \qquad [5]$$

with D the dilution ratio in h^{-1}, V the volume in l, t the time in h and F the feed rate in l.h^{-1}.

Since the volume increases as feed enters the fermentor, the feed rate should increase exponentially to keep D constant. This is called exponential fed-batch and may be used to control the specific growth rate. The feed rate needed to maintain a constant substrate concentration can be easily derived by performing a cell mass balance on the system (Nielsen, 2001). The general mass balance is given by equation 6.

Accumulated = Formed + In - Out [6]

Since there is no biomass in the outlet and inlet equation 6 can be rewritten in terms of biomass:

$$\frac{d(XV)}{dt} = \mu_0 XV \qquad [7]$$

where X represents biomass in g.l^{-1} and μ the specific growth rate in h^{-1}. Integrated, this gives:

$$XV = X_0 V_0 e^{\mu 0 t} \qquad [8]$$

A substrate balance gives the following equation:

$$D\left(C_S^F - C_S\right) - Y_{X/S}\mu_0 X = 0 \qquad [9]$$

C denotes the concentration of the substrate in $g.l^{-1}$ and Y the ratio of substrate on biomass yield in g substrate.g $biomass^{-1}$. Equation 10 can now be formulated by combining equations 5, 8 and 9:

$$F = \frac{X_0 V_0}{Y_{X/S}(C_{S0} - C_S)} e^{\mu 0 t} \qquad [10]$$

Equation 10 can be used to control the specific growth rate of micro-organisms by keeping the substrate concentration constant.

Besides deciding on the manner in which the feed is to be added, it is also necessary to determine which feed- or dilution rate is optimal through experimental work. This was illustrated when nisin production by *Lactococcus lactis* was increased by 58 % to a final activity of 4185 $IU.ml^{-1}$ during fed-batch fermentation. The feed stream (400.0 $g.l^{-1}$ sucrose) was fed at different constant rates (6.0, 7.0, 8.0 and 10.0 $g.l^{-1}.h^{-1}$). Highest activity was obtained at a feed rate of 7.0 $g.l^{-1}.h^{-1}$. Feed rates higher than this caused an increase in the residual sucrose concentration and a decrease in nisin activity (Lv et al., 2005). The authors suggested that nisin biosynthesis was affected by residual sucrose concentration.

Another type of fed-batch fermentation being explored is membrane fermentors. These fermentors are especially suited for LAB fermentation. Here, membranes permeable to fermentation broth, but not bacterial cells, are used to keep the LA concentration below a toxic level. Extracted fermentation broth is replaced with the same volume of new media, often at a concentrated level to replenish nutrients. This type of fermentation then also leads to high cell densities (Schiraldi et al., 2003). However, fouling of the membranes has thus far been a limiting factor hampering the use of membrane fermentors.

Feed stream composition

LAB are known for their complex nutritional requirements and it may happen that feeding of a pure carbon source during fed-batch fermentation will not lead to higher biomass as the cells need other components for cell synthesis (Callewaert et al., 2002). Besides the main growth limiting component used to control the growth rate, the feed stream should contain enough other components needed in fermentation to ensure they do not become growth limiting. These components can be a source of carbon, nitrogen, vitamins, salt ions (e.g. Mg^{2+}, Ca^{2+}, Mn^{2+}) or any combination of them (Guerra et al., 2005). This way the depleted nutrients can be replenished allowing growth to higher biomass concentrations, and subsequently better

product formation than obtained with batch fermentations (Callewaert et al., 2002; Ekinci et al, 2006; Guerra et al., 2005). In exponential feed fed-batch fermentation of *Bacillus subtilis spoIIG* mutant a feed medium containing 200.0 $g.l^{-1}$ glucose needed a minimum of 400.0 $g.l^{-1}$ peptone before cell growth was carbon limited (Oh et al., 2002). When yeast extract was added to an exponential fed feed stream biomass, LA concentration and productivity increased during fed-batch fermentation of *Lactobacillus casei* (Ding et al, 2006). In high cell density cultivation of *Escherichia coli* a shortage of phosphates caused cell growth to stop (Korz et al., 1995). Lv et al. (2004) improved nisin activity obtained in batch fermentation by 64 % and 74 % when sucrose and a nitrogen source were fed, respectively.

Problems involving fed-batch fermentation

During changes in fermentation modes it may be that a culture changes its metabolic pathway. This may result in the formation of metabolic products that can inhibit growth, e.g. ethanol, acetic acid, formic acid etc. (Guerra et al., 2005; Parente et al., 1997). It is also possible that the 'normal' metabolic products can increase to a level that inhibits further biomass formation. In pH-controlled fermentations, for example, acid reacts with the base and forms a salt that can become inhibiting to growth. This happened during the fermentation of *Lactobacillus lactis* BME5-18M. Growth stopped even though there was still residual glucose left, the most probable reason being the accumulation of the salt ammonium lactate (Bai et al., 2004).

2.3.4.3 Continuous/chemostat fermentation

Continuous and exponential fed-batch fermentations work on the same principle, i.e. keeping conditions in the fermentor constant by holding a constant dilution rate. In the case of continuous fermentation, however, the feed/inlet stream can be constant as the volume will stay the same (see equation 5). When steady state is reached in continuous fermentations the specific growth rate equals the dilution rate (Gonzalez-Vara et al., 1996). Changing the dilution rate therefore also changes the specific growth rate. Hence a continuous culture is usually optimized by varying the dilution rate (Gonzalez-Vara et al., 1996).

Continuous production has the advantage of enabling cell recycle to the fermentor while bacteriocins from the permeate stream are removed by adsorption to resins (Parente et al., 1997). This may result in a more economic process, and higher cell densities can also be obtained. One of the main drawbacks of continuous fermentations is the increased risk of contamination as these fermentations can usually run for long time spans. In continuous fermentation of *Enterococcus faecium* DPC1146, the specific enterocin production rate ($MBU.g^{-1}.h^{-1}$) increased linearly with an increase in dilution rate from 0.0 to 0.6 h^{-1} (Parente et al., 1997). The same trend was observed for bavaricin MN activity for increases in dilution

rate (Parente et al., 1997). Nisin production, however, increased non-linearly to a peak value (dilution rate between 0.2 and 0.3 h^{-1}). It was reasoned that nisin production was influenced by carbon source regulation and cell growth rate (Parente et al., 1997). Lactococcin 972 yields were increased by ten times in continuous cultures compared to batch fermentations (De Rojas et al., 2004).

2.4 Concluding remarks

Since it was discovered that LAB are producers of antimicrobial peptides or bacteriocins, they have been an intensely studied subject. Today it is clear that bacteriocins can be applied in the food-, medical-, and veterinary industries. However, the fact that LAB have been used for centuries as starter cultures in various food products have focused research on using bacteriocins as natural food preservatives.

Basically, there are two ways in which bacteriocins can be applied. Firstly, they can be incorporated via an indirect method into food stuffs by using a bacteriocin producing starter culture. Secondly, they can be administered as a purified or semi-purified bacteriocin product in products where a starter culture is not allowed. For successful use in both cases it is required that factors affecting bacteriocin production and stability are well studied. This is especially true when the bacteriocin itself is the product. Commercial production of bacteriocins faces many obstacles as there are many factors that affect bacteriocin production and stability. Some of these include medium components, pH, temperature and stress inducing components e.g. NaCl. In addition, the effect of these factors varies amongst different strains making it impossible to fix a standard set of rules for bacteriocin production. Although certain guidelines can be used e.g. most bacteriocins are produced in a growth-associated manner and bacteriocin inactivation increases at higher pH values, there are no clear-cut rules to follow. It is therefore clear that in order to produce large scale quantities of a bacteriocin for commercial application, a suitable fermentation process has to be developed.

2.5 References

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CHAPTER 3

RESEARCH PROBLEM

3.1 Research problem

Bacteriocins from lactic acid bacteria (LAB) offer a valuable source of potential food preservatives. Although the antibacterial action of bacteriocins has also made them applicable in other fields, e.g. the medical industry, the safe use of LAB starter cultures in fermented food products for centuries has focused research on the food industry. Bacteriocins can be applied to food products either in the form of a bacteriocin producing starter culture or as a purified or semi-purified product.

Fermentations with bacteriocins as end-product aim to produce high levels at a low cost. In the food industry only a semi-purified product is required and low-cost, food-related industrial by-products can be used as growth media, thereby reducing production costs. Bacteriocin production, usually growth-associated, can be increased by optimizing factors affecting bacterial growth such as medium components, pH and temperature. Bacteriocin production has also been stimulated by exposing the producing strain to a stressful environment. This agrees with the natural function of bacteriocins, i.e. to ensure survival.

Enterococcus mundtii ST4SA, isolated from soybeans, produces a peptide with antibacterial and antiviral properties (Todorov et al., 2005). This peptide, therefore, has potential for application in the food industry as a natural food preservative, or in the pharmaceutical industry to control infections. However, before bacST4SA can be produced commercially, it is necessary to find a process that yields high levels, but is also cost-effective. Subsequently, as a first step in finding a commercial bacST4SA production process for food application, growth medium composition, pH-control and fed-batch fermentation were considered.

3.1.1 Media optimization

Molasses, corn steep liquor and cheese whey powder were evaluated as individual low-cost media for bacST4SA production. The choice of these components was based on previous reports, which indicated their successful use as growth media or supplementary components during growth of LAB. In order to increase bacST4SA production, medium components from MRS medium were considered for supplementation, based on high bacST4SA levels previously obtained in MRS.

BacST4SA production displayed primary metabolite, growth-associated kinetics during experiments in media optimization. This suggested that bacST4SA production would most likely be increased by growth to higher biomass, rather than exposing the producing strain to

a stressful environment. Subsequently, glucose was added to the growth medium under pH-controlled conditions to determine the effect of an increased biomass on bacteriocin production.

3.1.2 Effect of pH-control on bacteriocin production

Medium pH has a substantial effect on bacteriocin production and stability. The effect of pH on bacST4SA activity was investigated in pH-controlled fermentations with the optimum medium from the previous section.

3.1.3 Exponential fed-batch fermentation

Exponential fed-batch fermentation offers a means to control the specific growth rate of a micro-organism without high initial substrate concentrations, which may inhibit growth or bacteriocin synthesis, or both. Fed-batch fermentation of *E. mundtii* ST4SA was considered to improve volumetric bacST4SA activity obtained in batch fermentations by increasing biomass without substrate inhibition, which was observed at higher glucose levels.

3.2 References

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CHAPTER 4

Increased production of bacST4SA by *Enterococcus mundtii* in an industrial-based medium with pH-control

Introduction

Lactic acid bacteria (LAB) produce antimicrobial peptides, also known as bacteriocins, with application as natural food preservatives (Deegan et al., 2006; O'Sullivan et al., 2002). Bacteriocins are ribosomally synthesized peptides that inhibit growth of closely related strains (Leroy et al., 2003; Onda et al., 2003; O'Sullivan et al., 2002). Since many LAB have GRAS (Generally Regarded As Safe) status, their bacteriocins are considered safe (Callewaert et al., 2000 b; Eraso and Ines, 2004; Onda et al., 2003). The replacement of chemical preservatives such as sodium benzoate with natural preservatives is supported by increasing consumer preferences for healthy foods (Deegan et al., 2006, Schnürer and Magnusson, 2005). Moreover, food-related illnesses and losses due to food spoilage is still a concern and have created a demand for more efficient food preservation techniques. Nisin and pediocin PA-1 (AcH), produced by *Lactococcus lactis* and *Pediococcus acidilactici*, respectively, are the only bacteriocins produced commercially by fermentations for use as food preservatives (Deegan et al., 2006). Nisin is produced in pH-controlled fermentations of a milk-based medium (De Vuyst and Vandamme, 1994).

Commercial production of bacteriocins by LAB requires a cost-effective production process with high yields. High production yields are achieved by optimization of the growth medium (Kim et al., 2006; Li et al., 2001). Nisin production increased from 1074 $IU.ml^{-1}$ to 2150 $IU.ml^{-1}$ when CM medium (De Vuyst and Vandamme, 1992) was optimized (Li et al., 2001) and micrococcin GO5 production increased sixteen-fold when MRS medium (De Man et al., 1960) was modified (Kim et al., 2006). Growth media can account for up to 30 % of production costs (Rivas et al., 2004). Production of bacteriocins at commercially viable levels requires careful formulation of low-cost medium components. Cost-effective growth media can be obtained by using industrial food-grade by-products as carbon and/or nitrogen sources. Corn steep liquor, cheese whey and molasses have long been used as components in growth media for the cultivation of LAB (Hofvendahl and Hahn-Hägerdal, 2000; Lee, 2005; Wee et al., 2004). Corn steep liquor (CSL) is a by-product from the corn mill industry and is mainly used as a nitrogen source, although it also contains sugars (mainly sucrose), vitamins and minerals (Hofvendahl et al., 2000; Lee, 2005; Rivas et al., 2004). Cheese whey, a by-product of the dairy industry, is commonly used as medium for production of lactic acid (Hofvendahl et al., 2000). Whey is available in liquid or powder form and contains mainly lactose, proteins and salts (Hofvendahl et al., 2000; Kadam et al., 2006). Molasses originate from the sugarcane industry and contains mainly sucrose with traces of other minerals (Hofvendahl et al., 2000; Wee et al., 2004). The composition of growth medium and concentration of individual components for bacteriocin production can be optimized by means of statistical

response surface methodology (RSM), as described previously (Li et al., 2002; Liew et al., 2005; Zendo et al., 2005). In comparison to the traditional 'one-factor-at-a-time' method, RSM significantly reduces the number of experiments needed for selection of medium components and determination of their optimal concentrations. Moreover, RSM quantifies interaction between different components, thereby providing statistically reliable results (Cladera-Olivera et al., 2004; Li et al., 2002; Liew et al., 2005).

Bacteriocin production is also substantially affected by medium pH, giving preference to pH-control to increase production yields. Production of bacteriocins by *Enterococcus mundtii* QU2 (Zendo at al., 2005) and *Enterococcus faecium* P13 (Herranz et al., 2001) increased two-fold and four-fold, respectively, under pH-controlled conditions. Maximal bacteriocin production often occurs at a pH below levels needed for optimal growth (Parente and Ricciardi, 1999). Moreover, pH can stabilize bacteriocins, usually acidic pH values (Abriouel et al., 2003; Parente et al., 1999).

Many bacteriocins from LAB display primary metabolite growth-associated kinetics (Lv et al., 2005), suggesting that an increase in biomass may lead to higher bacteriocin yields (Callewaert and De Vuyst, 2000 a). Fed-batch fermentation is a means to increase biomass levels and bacteriocin production without using high initial substrate concentrations that may lead to inhibition of growth (Bai et al., 2004) and bacteriocin production, as reported for production of nisin at high sucrose levels (Lv et al., 2005). Fed-batch fermentations have been used to increase the production of bacteriocins jenseniin G (Ekinci and Barefoot, 2006), pediocin (Guerra et al., 2005) and amylovorin L471 (Callewaert et al., 2000 a).

Enterococcus mundtii ST4SA produces a bacteriocin (bacST4SA) active against Gram-positive and Gram-negative bacteria. Amongst the Gram-positive strains, the food pathogen *Staphylococcus aureus* is also inhibited (Todorov et al., 2005). This renders bacST4SA a potential natural food preservative, providing for interest in industrial manufacturing. In the present study medium components, CSL, cheese whey and molasses, were evaluated for cost-effective fermentative production of bacST4SA. In addition, medium supplements, pH-control and batch and fed-batch fermentations were evaluated to increase production of bacST4SA.

Materials and methods

Bacterial strains and growth media

Enterococcus mundtii ST4SA, previously isolated from soybeans (Todorov et al., 2005), was used as bacteriocin producer and stored at -80 ºC in MRS broth (Biolab, Biolab Diagnostics, Midrand, South Africa) supplemented with 20 % (v/v) glycerol. The bacteriocin produced by *E. mundtii* ST4SA, bacST4SA, was purified by Todorov et al. (2005) and was referred to as peptide ST4V. Inoculum for fermentation purposes was prepared by propagating strain ST4SA twice in MRS broth, starting from freeze culture. MRS (Biolab), corn steep liquor (African Products, Kuilsriver, South Africa), sugarcane molasses and cheese whey powder (Parmalat, Stellenbosch, South Africa) were used as individual growth media.

Test-tube experiments

Industrial by-products were evaluated individually for bacST4SA production. CSL at 2.4, 5.0 and 10.0 $g_{total\ sugars}.l^{-1}$, molasses at 2.3, 4.7 and 11.7 $g_{total\ sugars}.l^{-1}$ and cheese whey powder (CW_{powder}) at 5.0, 10.0 and 50.0 $g_{total\ sugars}.l^{-1}$. CSL and CW_{powder}, each at 5.0 and 10.0 $g_{total\ sugars}.l^{-1}$, were selected for combined use and fermented at all possible combinations of their concentrations. CSL at 3.0 and 6.0 $g_{total\ sugars}.l^{-1}$ was also supplemented with MRS components according to Tables 1 and 2. Fermentations were performed in test tubes containing 10 ml medium at 30 ºC without pH-control. Medium pH was adjusted to between 6.5 and 7.0 with NaOH before sterilization (121 ºC, 15 min). Inoculum consisted of a 2 % (v/v) 24-h-old culture of *E. mundtii* ST4SA. Bacteriocin activity was determined after 16 h.

Shake-flask experiments

Two-hundred ml of a medium containing CSL (7.5 $g_{total\ sugars}.l^{-1}$) and YE (6.5 $g.l^{-1}$), referred to as CSL-YE medium, was fermented in shake-flasks at 30 ºC without pH-control (Fig. 1). The pH was adjusted to 7.0 before sterilization (121 ºC, 15 min). Samples were withdrawn aseptically at regular intervals and analyzed for cell growth (OD_{600}), pH and bacteriocin activity.

Batch fermentations with pH control

Batch fermentations were performed in Bioflo 110 fermentors (New Brunswick, Scientific Co. Inc., New Jersey, USA) containing 800 ml CSL-YE medium. Temperature and agitation rate were kept constant at 30 ºC and 75 rpm, respectively. Anaerobic conditions were achieved by sparging with N_2 gas at 0.5 volume air per volume vessel per min (vvm). Medium pH was adjusted after sterilization (121 ºC, 15 min). In constant pH fermentations,

the pH was maintained at 5.5, 6.5 and 7.5, respectively. Fermentations with combined pH was maintained at pH 6.5 for 6 hours, and then adjusted to 5.5 with 50 % (v/v) lactic acid. Medium pH was controlled by automatic addition of 3 M KOH. Glucose was added to CSL-YE medium at 7.5, 20.0 and 40.0 $g.l^{-1}$, respectively. Fermentations of CSL-YE media supplemented with glucose were maintained at combined pH. Samples were withdrawn aseptically at regular intervals and analyzed for cell growth (OD_{600} and cell dry mass) and bacteriocin activity.

Fed-batch fermentations

Fed-batch fermentations were performed in a Bioflo 110 fermentor (New Brunswick, Scientific Co. Inc., New Jersey, USA) with a working volume of 10 l. Sterilization was performed at 121 ºC for 15 min. Fed-batch fermentations were preceded by batch fermentation of a medium containing CSL (7.5 $g_{total\ sugars}.l^{-1}$), YE (6.5 $g.l^{-1}$) and glucose (7.5 $g.l^{-1}$). The feed stream was introduced after 15 h, just before stationary phase, and had the same composition as the batch medium with the exception of glucose, which was present at 100.0 $g.l^{-1}$. Each component of the feed stream was sterilized separately. CSL and glucose were sterilized at 121 ºC for 15 min, while YE was filter-sterilized. The start and end volumes of fed-batch fermentation were 3 and 9 l, respectively. The pH was controlled at 6.5 for the first 6 hours of batch fermentation and then adjusted to 5.5 for the remainder of batch and fed-batch fermentation. Temperature, agitation and sparging rate of N_2 were the same as in batch fermentations with pH-control. The feed rate (F), shown by equation 1, was derived from a mass balance assuming a constant specific growth rate and biomass yield on substrate.

$$F = \frac{X_0 V_0}{Y_{X/S}\left(C_{S0} - C_S\right)} e^{\mu 0 t} \qquad [1]$$

X_0 and V_0 are the cell mass and volume at the start of fed-batch, $Y_{X/S}$ is the cell yield on substrate, μ is the specific growth rate, C_{S0} and C_S are the substrate concentrations in the feed stream and fermentor, respectively, and t is the time (Nielsen, 2001). The feed rate was calculated to maintain the residual glucose concentration at 0.0 $g.l^{-1}$ and the specific growth rate at 0.063.h^{-1}. Software written in Python (Python, version 2.3, www.python.org) was used to control the feed rate according to equation 1. Samples were withdrawn aseptically at regular intervals and analyzed for cell growth (cell dry mass), bacteriocin activity and concentrations of lactic- and acetic acid, ethanol and glucose.

Bacteriocin activity

Bacteriocin activity was determined by using the agar-spot method (Ivanova et al., 1998). Cell-free supernatants were adjusted to pH between 5 and 6 and then heated at 80 ºC for 10 min. Serial dilutions were made with sterile phosphate buffer (pH 6.5) and spotted on agar plates seeded with 0.05 % (v/v) *Enterococcus faecium* HKLHS. Antimicrobial activity was defined as arbitrary units (AU) per ml. One AU is equal to the reciprocal of the highest dilution showing no growth. A sample calculation is given in the Appendix.

Analytical methods

Growth of *E. mundtii* ST4SA was monitored using OD measurements at 600 nm (OD_{600}) (SmartSpeck Plus, Biorad, USA) and cell dry mass (CDM, expressed in $g.l^{-1}$). To determine CDM, 20 ml of the culture was centrifuged (17400 x g, 4 ºC, 10 min), the cell-free supernatant decanted and the pellet washed in 20 ml sterile physiological water. A linear coefficient (0.432 $g.l^{-1}$ per OD) correlating OD_{600} values to CDM was determined and used to convert OD readings to CDM.

Total sugar concentration was determined by means of the phenol-sulphuric acid assay (Wee et al., 2004) with glucose as standard.

Levels of glucose, lactic acid, acetic acid and ethanol were determined by using a High Performance Liquid Chromatograph (Thermo, USA), equipped with an Aminex HPX-87H column (Biorad, Pretoria, South Africa). Samples of 10 µl were injected. The eluent was a 5 mM H_2SO_4 solution at a flow rate of 0.6 $ml.min^{-1}$ and at 45 ºC.

Experimental design with CSL and individual MRS components

CSL and individual components from MRS, except glucose, were screened in a fractional factorial design (FrFD) experiment (Tables 1 and 2). The high and low values of the components were their concentration in MRS and 0 $g.l^{-1}$, respectively, while for CSL the high and low values were 6 and 3 $g_{total\ sugars}.l^{-1}$, respectively (Table 1). The 2^{10-5} FrFD is shown by Table 2.

Table 1

Concentrations of MRS components and corn steep liquor as used in the 2^{10-5} fractional factorial design. The concentration of corn steep liquor is in $g_{total\ sugars}.l^{-1}$.

		$g.l^{-1}$		
			Concentration in FrFD	
	Component	Concentration in MRS	High (1)	Low (-1)
A	Special peptone	10	10	0
B	Beef extract	5	5	0
C	Yeast extract	5	5	0
D	CSL	---	6	3
E	Potassium phosphate	2	2	0
F	Tween 80	1	1	0
G	tri-Ammonium citrate	2	2	0
H	$MgSO_4$	0.1	0.1	0
J	$MnSO_4$	0.05	0.05	0
K	Sodium acetate	5	5	0

Table 2

2^{10-5} fractional factorial design matrix with MRS components and corn steep liquor

Run	A	B	C	D	E	F = ABCD	G = ABCE	H = ABDE	J = ACDE	K = BCDE
1	-1	-1	-1	-1	-1	1	1	1	1	1
2	1	-1	-1	-1	-1	-1	-1	-1	-1	1
3	-1	1	-1	-1	-1	-1	-1	-1	1	-1
4	1	1	-1	-1	-1	1	1	1	-1	-1
5	-1	-1	1	-1	-1	-1	-1	1	-1	-1
6	1	-1	1	-1	-1	1	1	-1	1	-1
7	-1	1	1	-1	-1	1	1	-1	-1	1
8	1	1	1	-1	-1	-1	-1	1	1	1
9	-1	-1	-1	1	-1	-1	1	-1	-1	-1
10	1	-1	-1	1	-1	1	-1	1	1	-1
11	-1	1	-1	1	-1	1	-1	1	-1	1
12	1	1	-1	1	-1	-1	1	-1	1	1
13	-1	-1	1	1	-1	1	-1	-1	1	1
14	1	-1	1	1	-1	-1	1	1	-1	1
15	-1	1	1	1	-1	-1	1	1	1	-1
16	1	1	1	1	-1	1	-1	-1	-1	-1
17	-1	-1	-1	-1	1	1	-1	-1	-1	-1
18	1	-1	-1	-1	1	-1	1	1	1	-1
19	-1	1	-1	-1	1	-1	1	1	-1	1
20	1	1	-1	-1	1	1	-1	-1	1	1
21	-1	-1	1	-1	1	-1	1	-1	1	1
22	1	-1	1	-1	1	1	-1	1	-1	1
23	-1	1	1	-1	1	1	-1	1	1	-1
24	1	1	1	-1	1	-1	1	-1	-1	-1
25	-1	-1	-1	1	1	-1	-1	1	1	1
26	1	-1	-1	1	1	1	1	-1	-1	1
27	-1	1	-1	1	1	1	1	-1	1	-1
28	1	1	-1	1	1	-1	-1	1	-1	-1
29	-1	-1	1	1	1	1	1	1	-1	-1
30	1	-1	1	1	1	-1	-1	-1	1	-1
31	-1	1	1	1	1	-1	-1	-1	-1	1
32	1	1	1	1	1	1	1	1	1	1

Significant components were identified by means of analysis of variance (ANOVA) with activity ($AU.ml^{-1}$) as the response variable. The effect of components (positive or negative) was determined by using the coefficients of a linear regression model. An α value of less than 0.1 was considered significant. Statistical analyses were conducted with R software package (The R Foundation for Statistical Computing, version 2.0.1, 2004, http://cran.r-project.org).

Results

Enterococcus mundtii ST4SA was cultivated in test-tubes and pH-controlled fermentations to optimize the medium composition and method of pH-control for production of bacteriocin bacST4SA. Industrial, food-grade medium components were used to replace MRS medium, to provide a cost-effect medium and improve bacteriocin production levels. Fed-batch fermentation was also evaluated.

Medium optimization

Bacteriocin production in industrial media

Bacteriocin production was quantified in molasses, corn steep liquor and cheese whey powder and compared to MRS control. Growth of *E. mundtii* ST4SA in MRS yielded bacST4SA levels of 51200 $AU.ml^{-1}$ (Fig. 1 and Table 3). Production followed primary metabolite growth-associated kinetics and reached maximum levels (51200 $AU.ml^{-1}$) after 9 h (Fig. 1). Stationary phase was reached after 12 h at pH 4.7 (Fig. 1). Only 25 to 50 % of the glucose in MRS (20.0 $g.l^{-1}$) was metabolized before growth ended (Appendix). No bacST4SA activity could be detected in molasses, while an activity of up to 1600 $AU.ml^{-1}$ was recorded in CW_{powder} at 50 $g_{total\ sugars}.l^{-1}$ (Table 3). Growth in CSL (5.0 and 10.0 $g_{total\ sugars}.l^{-1}$) yielded 12800 $AU.ml^{-1}$, which was eight-fold higher than obtained with CW_{powder}. BacST4SA activity improved from 3200 to 12800 $AU.ml^{-1}$ when the concentration of CSL was increased from 2.4 to 5.0 $g_{total\ sugars}.l^{-1}$, whereafter it stayed constant for higher CSL concentrations (Table 3).

Table 3

BacST4SA activity recorded in corn steep liquor, molasses and cheese whey powder

Run	Medium	Sugar content ($g_{total\ sugars}.l^{-1}$)	pH		BacST4SA activity ($AU.ml^{-1}$)
			Initial	End	
1	MRS		6.50	4.65	51200
2		2.4	6.50	4.56	3200
3	**CSL**	5.0	6.50	4.38	12800
4		10.0	6.50	4.41	12800
5		2.3	6.50	4.82	0
6	**Molasses**	4.7	6.50	5.01	0
7		11.7	6.50	4.97	0
8		5.0	6.27	4.14	800
9	**CW_{powder}**	10.0	6.55	4.44	800
10		50.0	6.47	4.82	1600

Combining corn steep liquor and cheese whey powder

A combination of CSL and CW_{powder} according to the 2^2 full factorial design (FFD) in Table 4 was evaluated for bacST4SA production. CSL supplemented with CW_{powder} did not yield higher bacteriocin levels and bacST4SA activity remained constant at 12800 $AU.ml^{-1}$ (Table 4), which was equal to activity obtained in pure CSL (Table 3).

Table 4

A 2^2 full factorial design with corn steep liquor and cheese whey powder used as individual components.

Run	CSL	CW_{powder}	pH		BacST4SA activity ($AU.ml^{-1}$)
			Initial	End	
1	1	1	6.49	5.51	12800
2	-1	1	6.49	5.10	12800
3	1	-1	6.45	5.43	12800
4	-1	-1	6.48	5.09	12800
MRS			6.86	4.53	51200

* Coded levels: (-1) equals 5 $g_{total\ sugars}.l^{-1}$ and (1) equals 10 $g_{total\ sugars}.l^{-1}$.

Screening of MRS components for improved bacST4SA production

Individual medium components from MRS that substantially affected bacST4SA production were identified in ANOVA analysis of a 2^{10-5} FrFD, for supplementation of CSL. Production of bacST4SA improved when CSL was supplemented, though some differences were observed between two batches of CSL. In batch 1 ANOVA analysis indicated that yeast extract (C) and CSL (D) were the only significant components with a positive effect on bacST4SA production (Table 5). For batch 2, Tween 80 (F) was identified as significant component for increasing bacST4SA levels in addition to yeast extract (YE) and CSL (Table 5). In both batches sodium acetate (K) had a substantial negative effect on bacST4SA

production (Table 5). The optimal concentration of CSL from batch 1 (7.5 $g_{total\ sugars}.l^{-1}$) and YE (6.5 $g.l^{-1}$) was determined by using the results from a 2^2 FFD with centre runs (Appendix). Inclusion of YE in CSL medium resulted in a four-fold increase in bacST4SA production (51200 $AU.ml^{-1}$), shown in Fig. 1. The selected CSL-YE medium displayed growth kinetics and bacteriocin production levels similar to those obtained in MRS (Fig. 1). At the end of fermentation only 8.8 % of the sugar initially present remained (Appendix).

Table 5

Significance of MRS components (indicated by *) on bacteriocin production and their estimated coefficients obtained from ANOVA analysis of a 2^{10-5} fractional factorial design. Data for two batches of corn steep liquor are shown.

	Batch 1			Batch 2		
Component	**Pr (> F)**		**Coefficient estimate**	**Pr (> F)**		**Coefficient estimate**
A Special peptone	0.33419		468.75	0.67754		233.30
B Beef extract	0.00442	*	-1431.25	0.37379		-500.00
C Yeast extract	0.05203	*	956.25	0.06816	*	1033.30
D Corn steep liquor	1.44E-10	*	3831.25	7.10E-04	*	1966.70
E Potassium phosphate	4.22E-07	*	-2781.25	0.76639		-166.70
F Tween 80	0.68867		193.75	3.87E-06	*	2766.70
G tri-Ammonium citrate	2.19E-05	*	-2243.75	0.51380		366.70
H $MgSO_4$	0.65108		-218.75	0.26062		633.30
J $MnSO_4$	0.92786		43.75	0.06816	*	-1033.30
K Sodium acetate	1.15E-04	*	-2006.25	0.00221	*	-1766.70
Reproducibility	0.25268			0.02088	*	

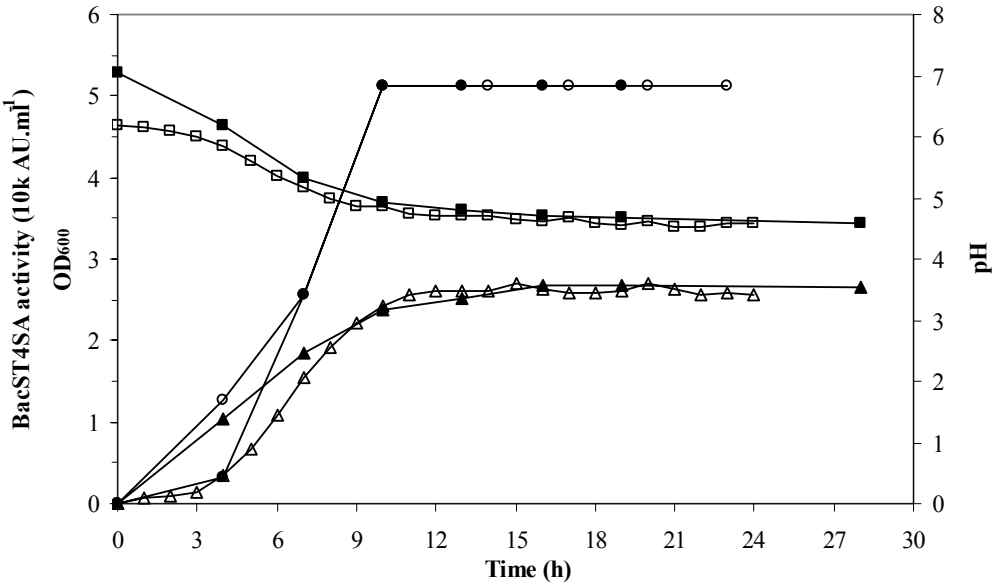

Fig. 1. Growth and bacST4SA production in MRS and CSL-YE medium. Fermentations were performed in static shake-flasks containing 200 ml media at 30 ºC. Growth (OD_{600}, ▲, Δ), pH (■, □) and bacST4SA production (10k $AU.ml^{-1}$, ●, ○) were monitored in MRS (open symbols) and CSL-YE medium (closed symbols).

Batch fermentations

Effect of pH on bacST4SA activity

Batch fermentations were maintained at constant pH 5.5, 6.5 and 7.5, respectively, and at a combined pH of 6.5 and then 5.5. The use of pH-control in batch fermentations improved the rate of bacteriocin production, had no effect on bacteriocin activity and some pH levels had a negative effect on bacteriocin stability. The production rate of bacST4SA, for pH-controlled fermentations and shake-flasks without pH-control, was highest at constant pH 6.5. Bacteriocin production and cell growth was lowest in medium maintained at pH 5.5, shown by Fig. 2 and 3, respectively. At constant pH 5.5 and 6.5, activity equal to that in uncontrolled shake-flask fermentations were recorded (51200 $AU.ml^{-1}$), but at pH 7.5 maximum activity decreased to 25600 $AU.ml^{-1}$ (Fig. 2). At pH 7.5 bacST4SA was almost immediately inactivated (Fig. 2) in comparison to pH 5.5 where inactivation occurred after 14 h (data not shown). A bacST4SA production rate equal to that in constant pH 6.5, but with increased stability was achieved by maintaining the pH at 6.5 for 6 hours and then at 5.5 (Fig. 2). In the absence of *E. mundtii* ST4SA cells, bacteriocin activity in a cell-free supernatant with high activity did not decrease during 24 h of incubation at pH 6.5 and 7.5 and 30 ºC (Appendix).

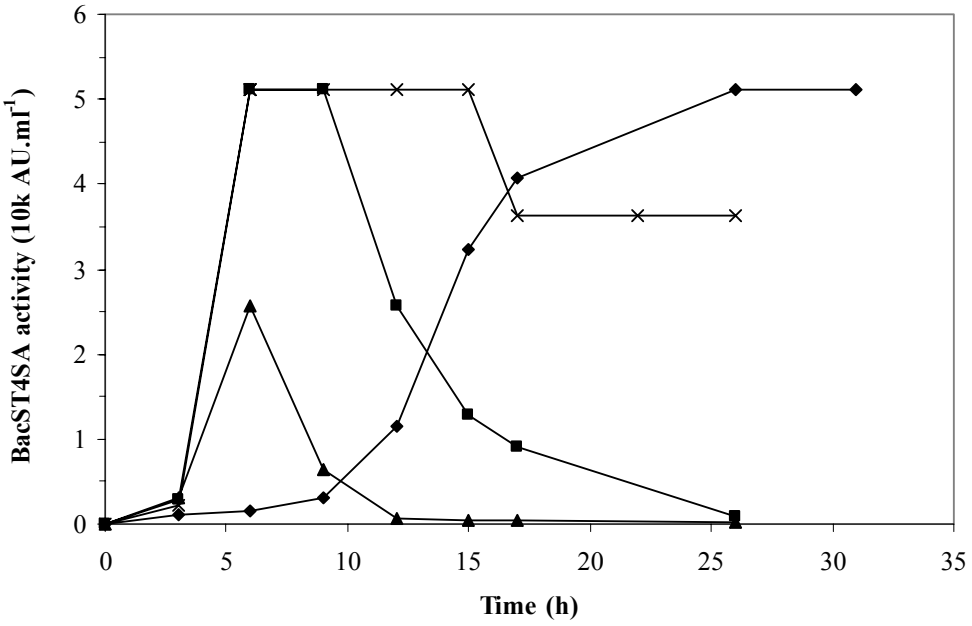

Fig. 2. BacST4SA production and stability under pH-controlled conditions. Fermentations were performed in a 1 l fermentor containing 800 ml CSL-YE medium at 30 ºC and 75 rpm. The pH was controlled at 5.5 (♦), 6.5 (■), 7.5 (▲) and a combination of pH 6.5 to pH 5.5 (×).

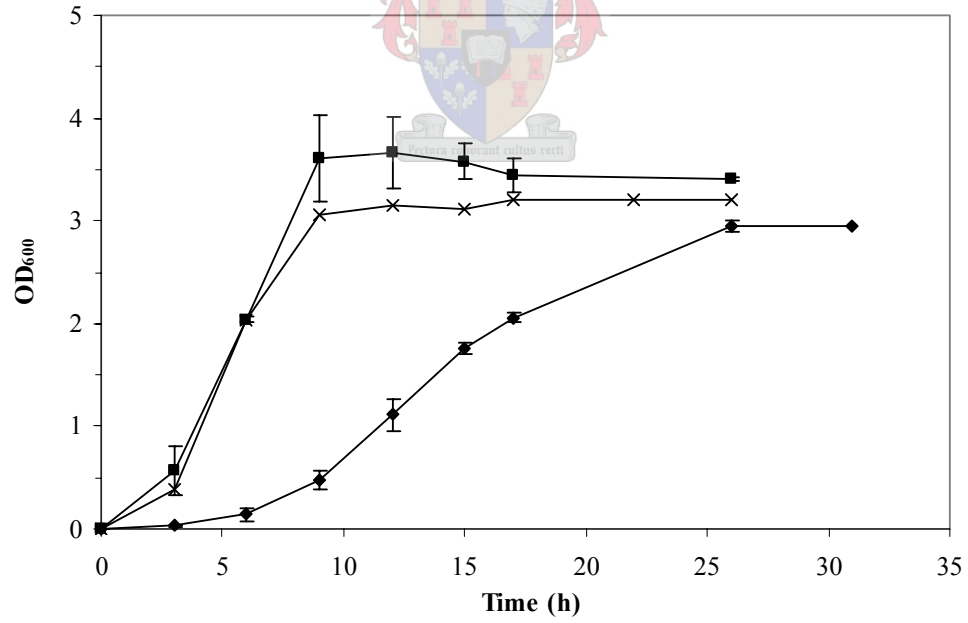

Fig. 3. The effect of pH-control on growth rate (OD_{600}). Fermentations were performed in a 1 l fermentor containing 800 ml CSL-YE medium at 30 ºC and 75 rpm. The pH was controlled at 5.5 (♦), 6.5 (■), and a combination of pH 6.5 to pH 5.5 (×).

Effect of glucose addition at start-up on biomass and bacST4SA production

Glucose was added to 7.5, 20.0 and 40.0 g.l^{-1} in pH-controlled batch fermentations containing CSL-YE medium, prior to inoculation. The inclusion of glucose in CSL-YE medium increased bacteriocin production, but negatively affected bacteriocin production rate at high concentrations. Supplementation of CSL-YE medium with 7.5 g.l^{-1} glucose increased bacteriocin activity to 102400 AU.ml^{-1} (Fig. 4 a) and maximum biomass levels improved with more than 80 % (Fig. 4 b). For higher glucose supplementations (20.0 and 40.0 g.l^{-1}), there was no increase in activity (Fig. 4 a), although biomass levels did increase (Fig. 4 b). In medium containing 40.0 g.l^{-1} glucose the production rate of bacST4SA decreased substantially (Fig. 4 a) and cell growth was slower (Fig. 4 b). Maximum bacteriocin activity was obtained well before growth ended (Figs. 4 a and b).

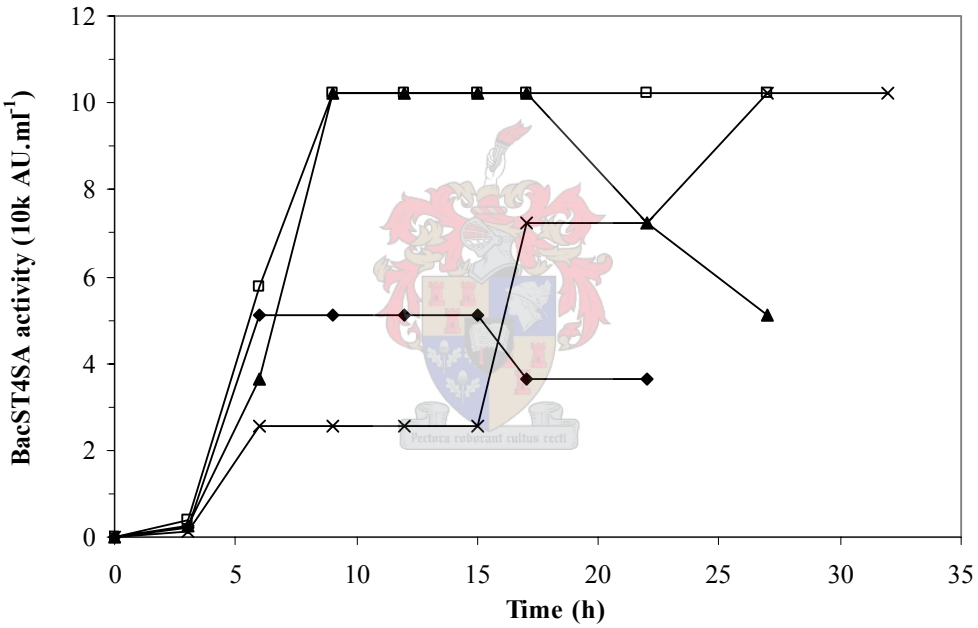

Fig. 4 a. The effect of glucose on bacST4SA production. Fermentations were performed in a 1 l fermentor containing 800 ml CSL-YE medium at 30 ºC and 75 rpm. The pH was controlled at 6.5 for the first 6 hours, and then adjusted to 5.5. Glucose was added prior to sterilization at 7.5 (□), 20.0 (▲) and 40.0 (×) g.l^{-1}. Fermentation of CSL-YE medium without glucose is also shown (♦).

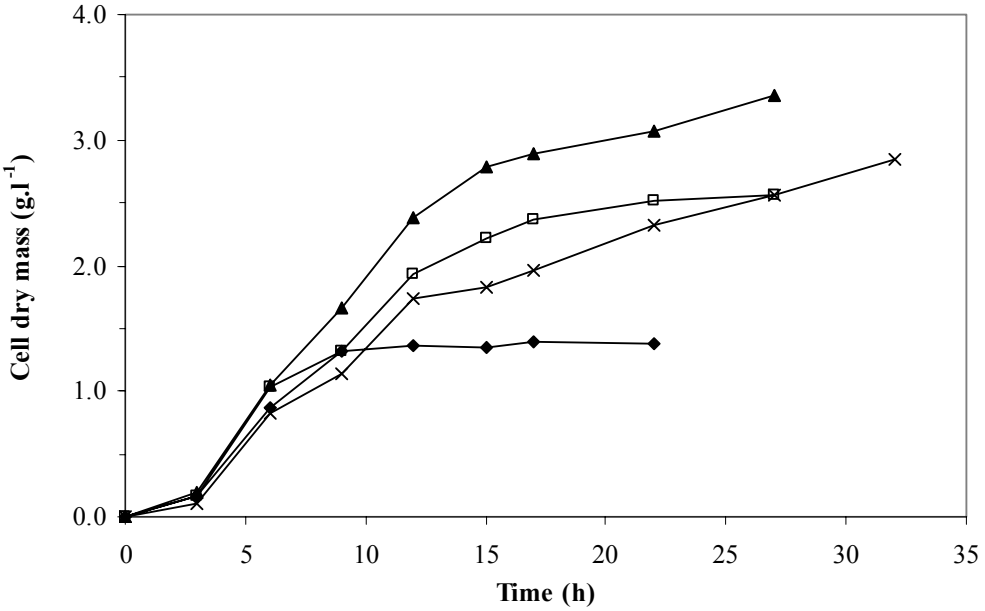

Fig. 4 b. The effect of glucose on biomass production. Fermentations were performed in a 1 l fermentor containing 800 ml CSL-YE medium at 30 ºC and 75 rpm. The pH was controlled at 6.5 for the first 6 hours, and then adjusted to 5.5. Glucose was added prior to sterilization at 7.5 (□), 20.0 (▲) and 40.0 (×) $g.l^{-1}$. Fermentation of CSL-YE medium without glucose is also shown (♦).

Fed-batch fermentation

Fed-batch fermentation of *E. mundtii* ST4SA with an exponential feed at 0.7 μ_{max} dilution rates resulted in an accumulation of glucose and a substantial increase in residual glucose concentration. Cell growth and lactate production occurred at a much lower rate than in batch fermentations. Although the final volume at the end of fed-batch fermentation represented a three-fold dilution of batch medium, lactic acid and cell dry mass did not show an equivalent decrease in concentration (Fig. 5). No ethanol or acetic acid was detected in the growth medium. To determine whether growth was inhibited by a lack of nutrients, nitrogen and phosphate were evaluated. Cell-free supernatants, taken after 4, 8 and 16 hours, supplemented with YE and K_2HPO_4 did not sustain growth of *E. mundtii* ST4SA (Appendix).

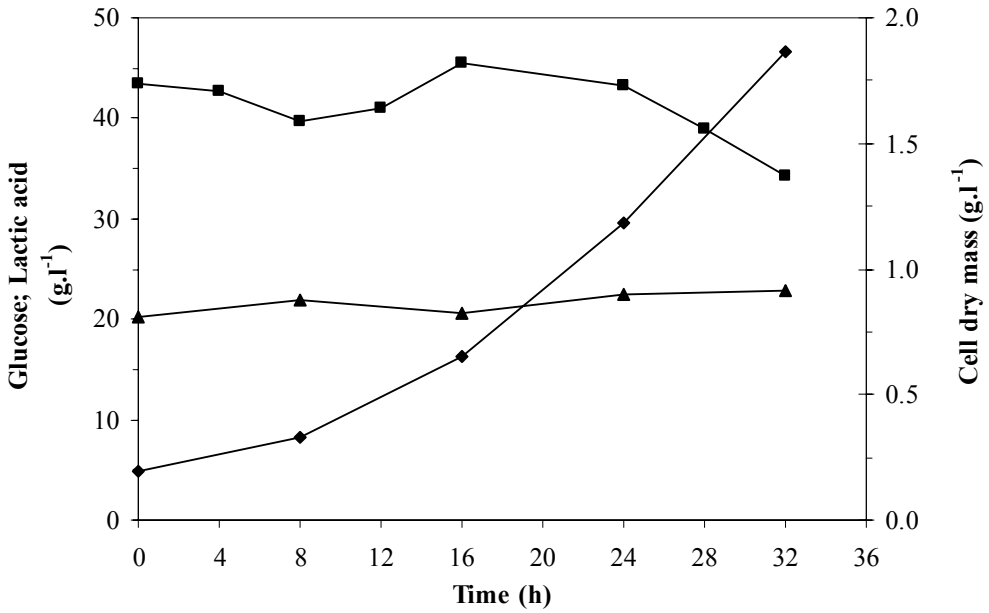

Fig. 5. Exponential fed-batch cultivation of *E. mundtii* ST4SA in a 10 l fermentor. The feed rate was calculated to control the growth rate at 0.063.h^{-1} while maintaining the residual glucose concentration at 0.0 g.l^{-1}. Residual glucose (♦), lactic acid (▲) and cell dry mass (■) were monitored.

Discussion

The utilization of low-cost, food-grade industrial by-products, corn steep liquor (CSL), cheese whey powder (CW_{powder}) and sugarcane molasses, as medium components for the production of bacST4SA was investigated, using a response surface methodology approach. In the present study, *E. mundtii* ST4SA grew well and produced high levels of bacST4SA (51200 AU.ml^{-1}) in uncontrolled fermentations in MRS. However, MRS is too expensive (€ 42.kg^{-1}, Biolab) for use in commercial fermentations. CSL, molasses and CW_{powder} are examples of low-cost, industrial by-products that have been used in bacteriocin production (Audisio et al., 2001; Cladera-Olivera et al., 2004; Guerra et al., 2001; Liu et al., 2005). Production of bacST4SA improved two-fold by using CSL, yeast extract and glucose at optimized concentrations as growth medium, in comparison to MRS medium, under conditions of pH-control.

Molasses, corn steep liquor and cheese whey powder as growth media

Of the three low-cost components considered, the highest production level of bacST4SA was obtained in CSL, which could be related to the availability of complex nitrogen and other growth factors in individual components. A bacteriocin activity of 12800 AU.ml^{-1} was

recorded in CSL, compared to 1600 AU.ml^{-1} for CW_{powder} (Table 3). No bacST4SA activity was detected in molasses (Table 3). Whereas CSL is often used as a complex nitrogen source (Kadam et al., 2006; Tari et al., 2006), the availability of nitrogen in CW_{powder} and molasses is more limited. LAB are fastidious organisms with a limited ability to synthesize B-vitamins, nucleic- and amino acids (Hofvendahl and Hahn-Hägerdal, 2000). Growth without these components is therefore limited, and could explain the lack of bacteriocin production in molasses, which is mainly a carbon source (Wee et al., 2004). The positive effect of a nitrogen source on bacteriocin production has been well documented (Franz et al., 1996; Tari et al., 2006; Zendo et al., 2005). Bacteriocin production improved four-fold to 12800 AU.ml^{-1} when the concentration of CSL increased from 2.4 $g_{total\ sugars}.l^{-1}$ to 5.0 $g_{total\ sugars}.l^{-1}$ (Table 3). The stimulatory effect of CSL on bacST4SA production could also have been due to residues of LAB that are naturally present in CSL, which could have triggered an immune response from *E. mundtii* ST4SA. Previous reports have suggested that LAB produce bacteriocins as a survival mechanism, based on the ability of bacteriocins to lower competition for nutrients by killing other bacteria (Deegan et al., 2006; Sip et al., 1998). In this regard it has been found that alien bacterial cells can stimulate bacteriocin production (Sip et al., 1998). BacST4SA production did not increase above 12800 AU.ml^{-1} when CSL and CW_{powder} were combined at different concentrations, indicating CSL was the only significant component for bacteriocin production (Table 4). Subsequently, CSL was used as basal medium for growth and bacteriocin production by *E. mundtii* ST4SA.

Supplementing corn steep liquor with yeast extract

Yeast extract (YE) was selected for supplementation of CSL, based on the screening of MRS components in a fractional factorial experimental design and ANOVA analysis, which was repeated for two batches of CSL. In both cases YE had a significantly positive effect on bacteriocin production, while in one case Tween 80 was an additional significant component (Table 5). Supplementation of CSL with YE resulted in a four-fold increase in activity, to 51200 AU.ml^{-1}, which agrees with the best production levels obtained in MRS (Fig. 1). Yeast extract has often proved to provide better bacteriocin production levels than other nitrogen sources (Kadam et al., 2006; Liew et al., 2005; Rivas et al., 2004; Wee et al., 2004). This may be explained by the high amount of free amino acids and short peptides (two and three amino acids) in yeast extract, which is easily used by bacteria and saves energy. The increased energy may lead to higher bacteriocin production (Cheigh et al., 2002; Mataragas et al., 2004). The positive effect of Tween 80 in one of the analyzed cases may be related to its emulsifying properties that may assist in solubilising antimicrobial peptides and increasing activity levels (Tari et al., 2006). Tween 80 was identified as the most important MRS component for bacteriocin production using *E. mundtii* QU2 (Zendo et al., 2005). Tween 80

was not included in the present medium formulation as it is not allowed in food applications, and it may interfere with bacteriocin purification processes making use of ammonium sulphate precipitation (Franz et al., 1996).

Supplementing corn steep liquor with glucose

Further supplementation of CSL-YE medium with 7.5, 20.0 or 40.0 $g.l^{-1}$ glucose resulted in a two-fold increase in bacST4SA activity (102400 $AU.ml^{-1}$) in comparison to levels obtained in MRS, due to a positive effect on biomass production (Fig. 4 b). Bacteriocin production by LAB often follows primary metabolite, growth-associated kinetics (Cheigh et al., 2002; Leroy et al., 2002, 2003), as in the present study (Fig. 1). The addition of glucose at 7.5 $g.l^{-1}$ improved biomass formation with more than 80 % (Fig. 4 b), which directly benefited the production of growth-associated bacST4SA (Figs. 4 a and b), as reported previously (De Rojas et al., 2004; Kim et al., 2006; Leroy et al., 2002). Glucose addition beyond 7.5 $g.l^{-1}$ did not stimulate further bacteriocin production, even though biomass levels increased (Figs. 4 a and b). A similar limited improvement in volumetric bacteriocin production by *Enterococcus faecium* DPC1146 (Parente et al., 1997) and CTC492 (Nilsen et al., 1998) at higher initial carbon concentrations, has been reported. At 40.0 $g.l^{-1}$ glucose supplementation, bacST4SA production was delayed (Fig. 4 b). LAB cultivation may be limited by a nutrient different than carbon at higher glucose concentrations, in which case glucose can still be converted to LA in a non-growth associated way (Zendo et al., 2005). Furthermore, inhibition of bacteriocin production at high substrate concentrations, as observed at 40.0 $g.l^{-1}$ glucose (Fig. 4 b), has also been reported for bacteriocins produced by *Enterococcus faecium* DPC1146 (Parente et al., 1997) and *Lactococcus lactis* subsp. *lactis* (Lv et al., 2005).

Limited production of bacST4SA

It was observed that *E. mundtii* ST4SA produced bacteriocins to a plateau activity of 102400 $AU.ml^{-1}$ whereafter production became limited, possibly as a result of a restricted immunity in protein biosynthesis (Callewaert et al., 2000 a, Leroy et al., 2001). Attempts to improve the growth environment by addition of K_2HPO_4 and more YE did not further increase bacST4SA activity. Moreover, bacteriocin production ended well before growth reached stationary phase (Figs. 4 a and b). This may indicate that recombinant organisms such as *Pichia pastoris* should rather be used for commercial bacteriocin manufacturing as they are not inhibited by most bacteriocins. Recombinant strains have been previously used for bacteriocin production. Gutiérrez et al. (2005) reported a 3.7-fold increase in enterocin P (EntP) production (28.2 $\mu g.ml^{-1}$) and a 16-fold increase in EntP activity (10240 $BU.ml^{-1}$) when *Pichia pastoris* was used to replace *Enterococcus faecium* P13 as bacterion producer.

Effect of pH-control on bacST4SA production

In pH-controlled fermentations the production rate of bacST4SA was positively affected by pH-control although maximum activity did not increase, while bacteriocin stability was reduced when control was at higher pH values (Fig. 2). The production rate of bacST4SA increased at higher cell growth rates, as was observed for pH-control at 6.5. However, at constant high pH values bacST4SA was rapidly inactivated. The loss of activity at higher pH values has been reported previously (Leroy et al., 2002; Zendo et al., 2005). The effect of high pH in the present study was either due to proteolytic degradation or adsorption to producer cells (Kim et al., 2006; Leroy et al., 2002; Zendo et al., 2005), as bacST4SA was stable in cell-free supernatant at pH 6.5 and 7.5 (data not shown). Although pH-control is regularly used to increase bacteriocin activity (Abriouel et al., 2003), bacST4SA activity was not improved by pH-control in the present study, but remained constant at 51200 $AU.ml^{-1}$. Controlling the pH at 6.5 for 6 hours and then at 5.5 resulted in a bacST4SA production rate equivalent to that obtained at pH 6.5, but with the improved stability observed at pH 5.5 (Fig. 2).

Exponential fed-batch fermentation

Exponential fed-batch fermentation of *E. mundtii* ST4SA at 0.7 μ_{max} dilution rates resulted in accumulation of residual glucose (Fig. 5), because glucose was consumed slower than in batch fermentations. Cell growth was limited and could not be improved by addition of K_2HPO_4 or YE (Guerra et al., 2005), indicating the presence of a metabolic inhibitor. Lactic acid, naturally present in CSL, may have suppressed growth when medium containing CSL was fed to the growth medium. The inhibitory effect of lactic acid on cell growth is well documented (Honda et al., 1995; Schepers et al., 2002). Another possibility is that growth of *E. mundtii* ST4SA is controlled by the amount of glucose initially present, and that strain ST4SA losses its ability to grow once it is close to reaching stationary phase. Although previous reports indicate the successful use of fed-batch fermentations for bacteriocin production, the fastidious nature of *E.mundtii* ST4SA proved to be limiting factor in fed-batch fermentations where growth was inhibited. As a result, the exponential fed-batch fermentations performed in this study were seen as an ineffective method for bacteriocin production and bacST4SA activity was not included in Fig. 5.

Conclusions

Production levels of bacST4SA in batch culture improved two-fold by the inclusion of CSL, yeast extract and glucose at optimized concentrations in the growth medium, in comparison to expensive MRS medium, when using pH-control. The use of a medium consisting mostly of

CSL, a food based industrial by-product, will significantly decrease costs of cultivation media for the production of bacST4SA as a natural preservative for the food industry.

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CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The following conclusions could be drawn from the results obtained in this study.

5.1.1 Media optimization

The food-grade industrial by-products corn steep liquor (CSL), cheese whey powder (CW_{powder}) and molasses could be used as individual growth media, although bacteriocin production by *Enterococcus mundtii* ST4SA was limited to CSL and CW_{powder}. The highest activity (12800 $AU.ml^{-1}$), probably related to the complex nitrogen content, was recorded in CSL. The stimulatory effect of CSL on bacST4SA production could also have been due to an immune response from strain ST4SA when it was exposed to residues of other LAB, which are naturally present in CSL.

The positive effect of complex nitrogen source on bacteriocin production was further observed when yeast extract (YE) was included in CSL, which resulted in a four-fold increase in bacST4SA activity to 51200 $AU.ml^{-1}$. YE was selected from individual MRS components, based on fractional factorial experimental screening experiments and ANOVA analyses, which was repeated for two batches of CSL. Differences were observed between two different batches of CSL. While YE was identified significant for bacST4SA production in CSL for both batches, Tween 80 was identified as an additional component for one of the batches.

BacST4SA production displayed primary metabolite, growth-associated kinetics and increased when biomass levels increased, but only to a maximum value (102400 $AU.ml^{-1}$) whereafter it was not affected by biomass. Higher initial glucose concentrations (40.0 $g.l^{-1}$) delayed *E. mundtii* ST4SA specific growth - and bacteriocin production rate, indicating substrate inhibition.

A ceiling-effect was observed for bacST4SA production. Once a maximum activity (102400 $AU.ml^{-1}$) was reached, improvements in the growth environment by addition of yeast extract and K_2HPO_4 and increases in biomass levels had no effect on bacteriocin production. This indicates that *E. mundtii* ST4SA may have a limited immunity to its own bacteriocins, thus restricting production. The implication for commercial production is that recombinant strains, which are not sensitive to the produced bacteriocin, should be used to produce bacteriocins.

5.1.2 Effect of pH-control on bacteriocin production

BacST4SA activity levels did not increase under pH-controlled conditions, but production rate and stability was substantially affected. At constant pH 6.5, bacteriocin production rate was highest, which coincided with the highest specific growth rate. However, bacST4SA was inactivated more rapidly at higher pH values. Applying pH-control at pH 6.5 for the first 6 hours of fermentation and then at 5.5 for the remainder of fermentation led to a bacST4SA production rate equal to that at constant pH 6.5, but with improved stability.

5.1.3 Exponential fed-batch fermentation

E. mundtii ST4SA consumed glucose far slower in fed-batch fermentations in comparison to batch fermentations and the residual glucose concentration increased during exponential feeding. The lower consumption rate, indicating growth inhibition, was most probably due to the presence of a metabolic inhibitor as YE and K_2HPO_4, components needed for growth of LAB, could not increase growth. The lack of growth made this an unsuitable method for bacST4SA production.

As a final concluding remark it can be said that fermentation optimization led to a two-fold increase in bacST4SA activity compared to expensive MRS medium, while production costs will significantly be reduced by using CSL, a low-cost industrial by-product.

5.2 Recommendations

Based on the results and conclusions, the following recommendations are made for improving bacST4SA yields.

5.2.1 Growth-associated production

Besides pH, other physical conditions such as temperature and dissolved oxygen concentration (DOC) can also be controlled in a fermentor for increased bacteriocin production. Generally, temperature affects the specific growth rate of cells and subsequently also bacteriocin production (Kim et al., 2006), whereas DOC may induce a stress-related condition that stimulates bacteriocin production, as previously reported by Parente et al. (1997).

Fermentations with lactic acid bacteria (LAB) are inhibited by formation of organic acids, mainly lactic acid (LA). Although glucose can still be converted to LA in a non-growth

associated way during inhibition (Boonmee et al., 2003), this is not favourable for bacteriocin production that proceeds predominantly in a growth-associated manner (Callewaert and De Vuyst, 2000; Mataragas et al., 2004). Continuous removal of LA during fermentation prevents end-product inhibition and improves growth and subsequently biomass formation (Honda et al., 1995), which may be beneficial for bacteriocin production. LA can be removed by using membranes (Jeantet et al., 1996), electrodialysis (Min-tian et al., 2005) or extractive fermentation (Honda et al., 1995). Methods such as aqueous two-phase extraction or electrodialysis produce lactic acid instead of lactate salt, which eliminates regeneration of lactate and may reduce purification costs (Hofvendahl and Hahn-Hägerdal, 2000). This in turn may render LA a potential secondary product in addition to bacST4SA. Moreover, the demand for LA has increased since their use in biodegradable polymers. This would add to the economic viability of bacteriocin production. Currently problems are still experienced with fouling of membranes. More work on fed-batch fermentations is required to prevent growth inhibition as observed in this study. A successful fed-batch technique will lead to higher biomass levels and this may result in increased bacST4SA production.

5.2.2 Stress inducing medium components

Growth medium is not always optimized for bacteriocin production by improving growth, but also by inclusion of potential stress inducing components such as NaCl or ethanol (Herranz et al., 2001; Mortvedt-Abildgaard et al., 1995). This may stimulate a defensive response from bacteria resulting in increased bacteriocin production. The effect of a stressful environment on bacST4SA production may be evaluated.

5.2.3 Recombinant expression

On a different note, bacteriocin production can also be improved by using a recombinant micro-organism such as the yeast *Pichia pastoris*, which is capable of producing high levels of foreign proteins or peptides (Gutiérrez et al., 2005 b). Cloning and expression of enterocin P, a bacteriocin from *Enterococcus faecium* P13, in *Escherichia coli* and *P. pastoris* has been reported by Gutiérrez et al. (2005 a, b). For *P. pastoris*, enterocin P production and antagonistic activity increased in comparison to values obtained in *E. faecium* P13 (Gutiérrez et al., 2005 b).

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Appendix

1. Calculation of bacteriocin activity ($AU.ml^{-1}$)

Example: Highest dilution zone without growth: 7

$$AU.ml^{-1} = 2^{(Dilution\_no\_growth+1)} \times 100$$
$$= 2^{(7+1)} \times 100$$
$$= 25600$$

2. Decrease in glucose during a pH-uncontrolled fermentation (24 h) of MRS medium

		OD (490 nm)			$g_{glucose}.l^{-1}$			
MRS	**Dilution**	#1	#2	#3	#1	#2	#3	**Average**
Before	100	1.968	1.923	1.971	19.70	19.25	19.73	19.53
	200	0.963	0.984	0.944	19.48	19.89	19.10	
After	100	1.423	1.467	1.504	14.30	14.73	15.10	14.99
	200	0.762	0.747	0.744	15.49	15.19	15.13	

→ Only about 5 of an available 20 $g.l^{-1}$ glucose was used by *E. mundtii* ST4SA

3. Two batches of corn steep liquor supplemented with MRS components in a 2^{10-5} fractional factorial design

3.1 Corn steep liquor batch 1

Repl.1				Repl.2				Repl.3			
pH		BacST4SA activity		pH		BacST4SA activity		pH		BacST4SA activity	
Initial	End	Dilution	AU.ml^{-1}	Initial	End	Dilution	AU.ml^{-1}	Initial	End	Dilution	AU.ml^{-1}
6.48	5.58	4	3200	7.56	5.48	3	1600	7.51	5.19	3	1600
6.48	5.33	5	6400	7.50	5.59	4	3200	7.58	5.20	4	3200
6.53	5.56	5	6400	7.53	5.02	5	6400	7.52	4.44	5	6400
6.52	5.52	5	6400	7.60	6.01	---	0	7.51	4.97	4	3200
6.49	5.25	5	6400	7.52	4.85	5	6400	7.61	4.47	6	12800
6.48	5.57	6	12800	7.47	5.19	5	6400	7.47	4.95	5	6400
6.57	5.40	3	1600	7.48	5.68	---	0	7.56	5.18	3	1600
6.49	5.50	5	6400	7.50	6.10	1	400	7.52	5.52	5	6400
6.46	5.30	6	12800	7.48	4.98	5	6400	7.56	4.94	6	12800
6.54	5.60	7	25600	7.49	5.35	---	0	7.42	4.93	7	25600
6.55	5.34	6	12800	7.50	5.43	6	12800	7.49	5.33	6	12800
6.51	5.59	5	6400	7.53	5.61	5	6400	7.51	5.50	5	6400
6.50	5.49	6	12800	7.55	5.44	5	6400	7.55	5.02	7	25600
6.46	5.38	6	12800	7.52	5.61	5	6400	7.49	5.11	6	12800
6.50	5.53	6	12800	7.55	5.16	6	12800	7.50	4.75	6	12800
6.53	5.39	6	12800	7.57	5.45	5	6400	7.53	5.12	7	25600
6.51	5.51	6	12800	7.57	5.56	3	1600	7.49	5.32	4	3200
6.48	5.77	3	1600	7.45	6.09	---	0	7.56	5.60	3	1600
6.53	5.65	1	400	7.49	6.23	---	0	7.43	5.80	1	400
6.48	5.71	2	800	7.52	6.48	---	0	7.50	6.00	1	400
6.55	5.67	2	800	7.55	6.15	---	0	7.50	5.87	2	800
6.47	5.52	3	1600	7.55	6.31	---	0	7.48	6.03	3	1600
6.56	5.61	3	1600	7.50	5.96	---	0	7.52	5.45	2	800
6.51	5.61	3	1600	7.51	5.82	3	1600	7.56	5.97	3	1600
6.47	5.57	5	6400	7.60	5.62	5	6400	7.50	5.40	4	3200
6.50	5.47	4	3200	7.45	5.76	4	3200	7.54	5.59	4	3200
6.47	5.63	4	3200	7.49	5.65	---	0	7.47	5.35	4	3200
6.48	5.49	5	6400	7.49	5.68	5	6400	7.52	5.45	6	12800
6.54	5.45	5	6400	7.47	5.21	6	12800	7.49	5.45	6	12800
6.55	5.69	6	12800	7.54	5.61	6	12800	7.54	5.54	7	25600
6.55	5.45	5	6400	7.43	5.61	4	3200	7.56	5.33	6	12800
6.52	5.63	4	3200	7.48	5.96	---	0	7.46	5.45	4	3200

3.2 Corn steep liquor batch 2

Repl.1				Repl.2				Repl.3			
pH		BacST4SA activity		pH		BacST4SA activity		pH		BacST4SA activity	
Initial	End	Dilution	AU.ml^{-1}	Initial	End	Dilution	AU.ml^{-1}	Initial	End	Dilution	AU.ml^{-1}
6.48	5.58	6	12800	6.47	5.54	6	12800	6.49	5.56	6	12800
6.48	5.33	6	12800	6.52	5.63	5	6400	6.54	5.58	5	6400
6.53	5.56	5	6400	6.47	5.38	5	6400	6.47	5.28	5	6400
6.52	5.52	7	25600	6.48	5.54	5	6400	6.49	5.40	7	25600
6.49	5.25	6	12800	6.53	5.35	6	12800	6.47	5.21	6	12800
6.48	5.57	7	25600	6.50	5.53	5	6400	6.54	5.35	7	25600
6.57	5.40	6	12800	6.48	5.64	5	6400	6.55	5.55	6	12800
6.49	5.50	4	3200	6.45	5.63	5	6400	6.52	5.54	6	12800
6.46	5.30	7	25600	6.52	5.36	6	12800	6.56	5.35	6	12800
6.54	5.60	6	12800	6.51	5.48	6	12800	6.52	5.35	6	12800
6.55	5.34	7	25600	6.53	5.60	5	6400	6.48	5.48	7	25600
6.51	5.59	6	12800	6.47	5.57	5	6400	6.45	5.50	6	12800
6.50	5.49	6	12800	6.48	5.56	6	12800	6.47	5.53	6	12800
6.46	5.38	6	12800	6.49	5.61	6	12800	6.55	5.54	6	12800
6.50	5.53	6	12800	6.48	5.33	6	12800	6.49	5.35	7	25600
6.53	5.39	7	25600	6.50	5.41	7	25600	6.51	5.44	6	12800
6.51	5.51	7	25600	6.45	5.52	6	12800	6.53	5.42	5	6400
6.48	5.77	5	6400	6.48	5.60	5	6400	6.52	5.53	6	12800
6.53	5.65	5	6400	6.47	5.71	4	3200	6.48	5.66	5	6400
6.48	5.71	5	6400	6.52	5.76	6	12800	6.51	5.67	6	12800
6.55	5.67	5	6400	6.54	5.75	5	6400	6.50	5.55	5	6400
6.47	5.52	7	25600	6.56	5.78	6	12800	6.51	5.56	6	12800
6.56	5.61	6	12800	6.47	5.47	7	25600	6.49	5.34	6	12800
6.51	5.61	6	12800	6.47	5.62	4	3200	6.54	5.54	6	12800
6.47	5.57	6	12800	6.48	5.56	5	6400	6.48	5.53	6	12800
6.50	5.47	6	12800	6.49	5.66	6	12800	6.51	5.58	7	25600
6.47	5.63	5	6400	6.57	5.56	7	25600	6.50	5.42	6	12800
6.48	5.49	6	12800	6.53	5.51	6	12800	6.52	5.54	6	12800
6.54	5.45	7	25600	6.48	5.46	7	25600	6.49	5.43	7	25600
6.55	5.69	6	12800	6.45	5.47	6	12800	6.53	5.49	7	25600
6.55	5.45	6	12800	6.49	5.57	5	6400	6.46	5.52	6	12800
6.52	5.63	6	12800	6.54	5.74	6	12800	6.52	5.65	7	25600
6.84	4.48	7	25600	6.84	4.52	7	25600	6.84	4.48	7	25600

4. Determination of optimal corn steep liquor (CSL) and yeast extract (YE) concentrations

			pH						BacST4SA activity (AU.ml^{-1})	
			Repl.1		Repl.1		STDEV			
Run	CSL	YE	Initial	End	Initial	End	Initial	End	Repl.1	Repl.2
1	-1	-1	7.53	5.04	7.50	5.06	0.04	0.03	25600	25600
2	-1	1	7.05	4.97	7.04	4.98	0.01	0.01	25600	25600
3	1	-1	7.49	5.17	7.51	5.18	0.04	0.02	36204	12800
4	1	1	7.02	5.04	7.03	5.06	0.01	0.03	25600	36204
5	0	0	7.28	5.11	7.31	5.07	0.04	0.05	25600	51200
6	0	0	7.27	5.09	7.29	5.09	0.04	0.00	25600	36204
7	0	0	7.25	5.02	7.25	4.98	0.01	0.05	51200	25600

* Coded levels:

Yeast extract (YE): (-1) equals 3 g.l^{-1}, (1) equals 10 g.l^{-1} and (0) equals 6.5 g.l^{-1}

Corn steep liquor (CSL): (-1) equals 5 $g_{total\ sugars}$.l^{-1}, (1) equals 10 $g_{total\ sugars}$.l^{-1} and (0) equals 7.5 $g_{total\ sugars}$.l^{-1}

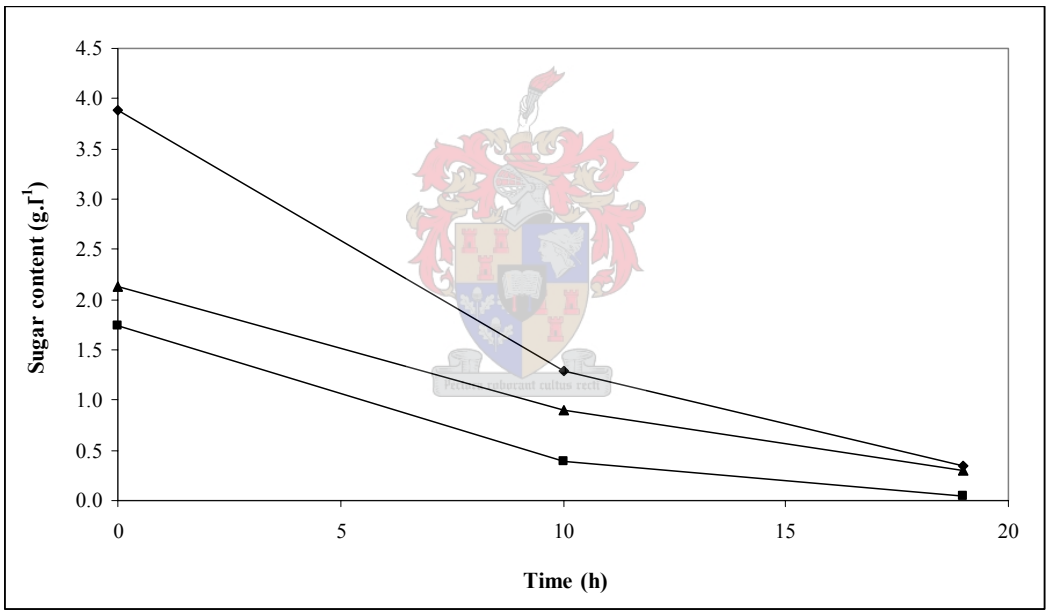
The combination CSL (0) and YE (0) were chosen as:

→ It gave the best result (51200 AU.ml^{-1})

→ The 'middle' value was seen as an optimal choice - not too high to unnecessarily increase production and purification costs, but not too low to risk a lack in nutrients

5. Decrease in total sugar concentration in medium containing corn steep liquor (7.5 $g_{total\ sugars}$.l^{-1}) and yeast extract (6.5 g.l^{-1})

Time (h)	Glucose (g.l^{-1})	Fructose (g.l^{-1})	Total sugar (g.l^{-1})	% Sugar fermented
0	1.747	2.136	3.883	0.0
10	0.391	0.899	1.290	66.8
19	0.041	0.301	0.342	91.2



6. Cell-free supernatants from fed-batch fermentations supplemented and re-inoculated

→ Concentrations of supplementary yeast extract and K_2HPO_4 are the same as in MRS

Supplement	Sample hour	Change in initial pH (pH 5.5)	Change in OD_{600}
None	4	None	None
	8	None	None
	16	None	None
Yeast extract (5 g.l^{-1})	4	None	None
	8	None	None
	16	None	None
K_2HPO_4 (2 g.l^{-1})	4	None	None
	8	None	None
	16	None	None