DISS. ETH NO. 18785

BIOTECHNOLOGICAL PRODUCTION OF ANTIMICROBIAL 3-HYDROXYPROPIONALDEHYDE FROM GLYCEROL USING FREE AND IMMOBILISED *LACTOBACILLUS REUTERI* CELLS AND ITS REACTIVE EXTRACTION

A dissertation submitted to

ETH ZURICH

for the degree of

Doctor of Sciences

presented by

DAVID PAUL RÜTTI

Dipl. LM-Ing. ETH

Born July 24, 1980

Citizen of Ersigen BE

accepted on the recommendation of

Prof. Dr. Christophe Lacroix, examiner

Dr. Sabine Vollenweider, co-examiner

Dr.-Ing. Jenny Weißbrodt, co-examiner

Zurich, 2010

Table of contents

Abbreviations

16S-rRNA	part of the ribosomal RNA
3-HPA	3-hydroxypropionaldehyde
ANOVA	analysis of variance
ATP	adenosin triphosphate
B_{12}	vitamin B_{12}
cfu	colony forming units
D	dilution rate: rate of flow of medium over the volume of culture in the bioreactor
df	degrees of freedom
DNA	deoxyribonucleic acid
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
EC	Enzyme Commission
EDTA	ethylenediaminetetraacetic acid
e.g.	*exempli gratia*
EMP	Embden-Meyerhof-Parnas pathway
EPS	exopolysaccharide
EtOH	ethanol
FC	free cells
G+C	guanine and cytosine
GRAS	general recognised as safe
GSH	tripeptide glutathione
HPLC	high performance liquid chromatography
IC	immobilised cells
IRA-400	AMBERLITE® IRA-400 anion exchange resin
IRA-Cl	IRA-400 chloride form
IRA-SO_3H	IRA-400 hydrogensulfite form
ISPR	*in situ* product removal
Kpi	potassium phosphate buffer (0.1 M, pH 7.0)

Kpi^+	potassium phosphate buffer Kpi (pH 7.0) with 0.1 M glucose, 1 mM glutathione, 1 mM $MgCl_2$
LAB	lactic acid bacteria
LB	lysogeny broth or Luria-Bertani medium
LD	lethal dose
LD_{50}	median lethal dose
log	common logarithm to base 10 (log_{10})
MANOVA	multi analysis of variance
MBC	minimum bactericidal concentration
MIC	minimum inhibitory concentration
MRS medium	de Man, Rogosa, and Sharpe broth, cultivation medium for lactic acid bacteria
MRS^+	MRS medium supplemented with 20 mM glycerol
MRS_G	MRS^+ medium supplemented with 40 g/l glucose
MSDS	material safety data sheet
NaBi	sodium bisulphite / sodium hydrogensulphite
NADH, NAD^+	nicotinamide adenine dinucleotide (reduced, oxidised form)
1,3-PDO	1,3-propanediol
PKP	phosphoketolase pathway
PTT	polytrimethylene terephthalate
RCB	repeated-cycle batch
RNA	ribonucleic acid
reuterin	3-hydroxypropionaldehyde dimer
rpm	revolutions per minute
SCFA	short chain fatty acids
sp.	species
subsp.	subspecies
v/v	volume per volume
w/v	weight per volume
% (w/v)	mass of the solute in g per 100 ml of the resulting solution
w/w	weight per weight

Coefficients and Variables

a_0	intercept
a_1-a_3	equation coefficients
ε	molar extinction coefficient [L mol^{-1} cm^{-1}]
n_D^{20}	refraction index at 20°C
ρ_{beads}	density of the beads as a function of cell load [g ml^{-1}]
$C_{3\text{-HPA}}$	maximum cumulative accumulated 3-HPA [mM]
C_v	volumetric biomass concentration in the reactor [cfu ml^{-1}]
C_w	viable cell counts in beads [cfu g^{-1}]
R^2	coefficient of determination
T	bioconversion temperature [°C]
t	bioconversion time [h]
Type	coded incubation type [-1: 1x3 h; 1: 3x1 h]
V_{beads}	volume of beads [ml]
V_{total}	total bioconversion volume [ml]

Summary

The efficient production of the antimicrobial agent 3-hydroxypropionaldehyde (3-HPA or reuterin), an interesting compound for the food and chemical industry, and for medical applications, still represents a major challenge especially at large scale. Thus 3-HPA is commercially not available. The most promising approach to its availability is the production from the renewable raw material glycerol using intact and live *Lactobacillus reuteri* ATCC 55730 cells in a two-step process. However, the conversion process requires various factors from coenzyme B_{12} to activating cations, reactivation proteins and energy as ATP, and high cell density. The conversion is limited by inhibitory and toxic effects of 3-HPA on the biocatalyst; in fact cells are required alive for bioconversion. Based on these requirements, three hypotheses were formulated for this study. First, 3-HPA production is increased by addition of cofactors of the glycerol dehydratase, glucose, and oxidative cell protectors to the bioconversion step and variation of pH. Secondly, cells immobilisation leads to high and efficient 3-HPA production because of high cell density and cell protection. Thirdly, *in situ* product removal decreases the toxicity of 3-HPA towards producing *L. reuteri* cells by instant removal of the accumulated 3-HPA leading to an increased production and eases purification of 3-HPA.

During this study, 3-HPA accumulation could not be enhanced by adding cofactors of the involved glycerol dehydratase, oxidative cell protector glutathione, or glucose to the bioconversion step. Addition of the tripeptide glutathione and glucose showed no influence on *L. reuteri* survival during bioconversion. Addition of glucose, however, increased significantly the production of by-products such as 1,3-propanediol and lactic acid and decreased overall glycerol bioconversion. pH fluctuation showed no effects on *L. reuteri* survival, but the phosphate-citrate buffer for pH control caused a significant two-fold decrease of 3-HPA accumulation. Unbuffered, unadjusted, and unsupplemented 400 mM glycerol solution was therefore shown to be best for highest 3-HPA production. On the other hand, 3-HPA accumulation was successfully enhanced by providing glucose/glutathione during washing step before bioconversion, while the by-product 1,3-propanediol increased only marginally.

3-HPA accumulation using immobilised *L. reuteri* cells was successfully achieved and showed more than 3.2-fold increased specific productivity (3-HPA production per cell) and 2.7-fold increased specific production per consumed MRS medium during biomass production step compared to free cell bioconversion. Furthermore, any centrifugation steps could be avoided to separate cells after conversion and reuse them in a second bioconversion step. Main drawbacks were observed in the 4.8-fold lower mean conversion rate of 99±16 mM h^{-1} and 1.4-fold lower maximum 3-HPA concentration of 182±14 mM compared to free cells. To test recycling of immobilised cells, beads were incubated in MRS medium for 16 h after glycerol bioconversion. *L. reuteri* cells were damaged during conversion under starvation even after short bioconversion time to 45 min, and no growth occurred during reactivation step.

Further efforts were done to develop a novel purification method suitable for in situ production removal. After testing 8 different strategies, the anion exchange resin IRA-400 in hydrogensulfite form was selected. 3-HPA was bound to the resin (hydrogensulfite form), released as 3-HPA adduct by elution with saturated NaCl solution, and extracted from eluent after water removal (lyophilisation) and ethanol extraction. This method is suitable for up-scaling but further studies are required. The overall yield of the purification ranged between 14.5 mol % and 45.3 mol %. Furthermore, we showed the potential of the system to be used for an *in situ* production removal process. However, the bioconversion was completely inhibited when the anion exchange resin in hydrogensulfite form was added in the reaction mixture and future work is therefore needed to overcome this limitation.

For the quantification of hydrogensulfite and 3-HPA in presence of hydrogensulfite, two methods were established based on photometrical methods previously published by Ellman (Ellman test, hydrogensulfite) and Dickinson (purpald assay, 3-HPA), respectively.

This research work demonstrated that unbuffered, unadjusted, and unsupplemented glycerol solution was best for highest 3-HPA production. Immobilised culture was successfully applied for 3-HPA accumulation and showed superior specific productivity without centrifugation steps. Finally, a novel purification method for 3-HPA was developed with a high potential to be successfully applied in an *in situ* product removal process at large scale.

Zusammenfassung

Die effiziente Produktion des antibiotisch wirksamen 3-Hydroxypropionaldehyds (3-HPA oder Reuterin) stellt noch immer eine grosse Herausforderung dar, erst recht im Grossmassstab. Die Eigenschaften von 3-HPA eröffnen unzählige potentielle Einsatzgebiete im Lebensmittelsektor, in der chemischen Industrie wie auch im Gesundheitswesen. 3-HPA ist allerdings bis heute nicht kommerziell erhältlich. Die biotechnologische Synthese aus dem erneuerbaren Rohstoff Glycerin mittels lebender *Lactobacillus reuteri* ATCC 55730 Zellen in einem Zwei-Stufen-Prozess ist ausserordentlich erfolgsversprechend. Dieser Zwei-Stufen-Prozess sieht eine Trennung zwischen Zellwachstum und Biokonversion vor. Die biotechnologische Synthese benötigt allerdings eine Reihe von Faktoren wie Koenzym B_{12}, Kationen, Aktivatorproteinen und Energie als ATP. Dessen Produktion hängt direkt von der Zelldichte ab und wird limitiert durch die Produkt-Toxizität, welche bis zum Absterben des Biokatalysators führen kann.

Im Rahmen dieser Dissertation wurden folgende drei Hypothesen getestet. Erstens, die 3-HPA Produktion wird durch pH-Variation und dem Zufügen von Kofaktoren des beteiligten Enzyms Glyzerindehydratase, Glukose und Zellantioxidant Glutathion erhöht. Zweitens führt die Zellimmobilisierung von *L. reuteri* in Polysaccharid-Beads zu einer effizienten 3-HPA Produktion aufgrund hoher Zelldichten und Schutzwirkung für den Katalysator. Drittens verringert ein *in situ* Produkt Entfernungsprozess die Anreicherung von toxischem 3-HPA, was zu einer erhöhten Produktion und erhöhten Viabilität von *L. reuteri* führt.

Während dieser Arbeit wurde die These gefestigt, dass native 400 mM Glyzerinlösung am geeignetsten für maximale 3-HPA-Produktion ist. Denn die 3-HPA-Produktion wurde weder durch die Zugabe von Enzymkofaktoren und Glukose, noch durch das Zellantioxidant Glutathion signifikant verbessert. Im Gegenteil: Die Zugabe von Glukose verminderte die 3-HPA Endkonzentration und erhöhte unerwünschte Nebenprodukte wie 1,3-Propandiol und Laktat. Die Viabilität von *L. reuteri* nach der Biokonversion wurde durch das Tripeptid Glutathion und Glukose nicht beeinflusst. Änderungen des pH-Wertes zwischen 4 und 7 zeigte keinen

Einfluss auf 3-HPA-Konzentration nach 2 h, aber durch den zugesetzten Phosphat-Citrat-Puffer wurde die maximale 3-HPA-Konzentration um fast die Hälfte reduziert. Hingegen konnte die Akkumulierung von 3-HPA durch Zugabe von Glukose und Glutathion während des Waschens vor der Biokonversion gesteigert werden, ohne signifikante Beeinflussung des Beiprodukts 1,3-Propanediol.

Die Produktion von 3-HPA mittels immobilisierter *L. reuteri* Zellen wurde erfolgreich etabliert und zeigte Vorteile gegenüber der bekannten Produktion mittels freier Zellen in einer 3.2-fach erhöhten spezifischen Produktivität (3-HPA Produktion pro Zelle) und einer 2.7-fach erhöhten spezifischen Produktivität pro verbrauchtes Volumen an MRS Medium. Zusätzlich konnten Zentrifugierungsschritte vermieden werden. Nachteile sind einzig in der dreifach geringeren Umwandlungs-geschwindigkeit von 99±16 mM h^{-1} und 1.4-fach geringeren Endkonzentration von 182±14 mM zu finden. In einem weiteren Schritt wurde die Möglichkeit zur Rezyklierung immobilisierter Zellen in MRS Medium nach der Biokonversion untersucht. Allerdings wurden die Zellen schon durch die Hungerperiode während Biokonversion so geschädigt, dass in frischen MRS Medium nach 16 Stunden kein Wachstum festgestellt werden konnte. Auch eine Verringerung der Hungerperiode von 3 h auf 45 min brachte nicht den gewünschten Erfolg.

Die Lösung dieser Einschränkung wurde in einer *in situ* Produktentfernung (ISPR) von 3-HPA gesucht. Das Testen von acht verschiedenen Reinigungsstrategien von 3-HPA identifizierte das Anionentauscherharz IRA-400 in der Hydrogensulfitform, 3-HPA selektiv zu binden und wieder abzugeben. Der daraus entwickelte Reinigungsprozess von 3-HPA besteht aus dessen Bindung an IRA-400 in der Hydrogensulfitform, dessen Eluierung als Sulfitaddukt mittels gesättigter Kochsalzlösung und dessen Ethanol-Extrahierung aus dem trockenen Salz nach der Lyophilisation. Die Ausbeute reinem 3-HPA war je nach 3-HPA-Anfangskonzentration zwischen 14.5 mol % und 45.3 mol %. Das Reinigungsverfahren zeigte grosses Potential für den Einsatz in einem ISPR Prozess. Dabei wurde 3-HPA wie erwartet aus der Glyzerinlösung entzogen. Allerdings wurde die Biokonversion komplett gehemmt, dass weitere Untersuchungen zur Lösung dieses Inhbitionsproblems nötig sind.

Zwei neue photometrische Methoden wurden zur Quantifizierung von Hydrogensulfit und 3-HPA in Verbindung mit Hydrogensulfite entwickelt, die auf den Ellman Test und respektive dem Purpald Test aufgebaut sind.

In der vorliegenden Arbeit konnte gezeigt werden, dass nicht supplementierte und ungepufferte Glyzerinlösung am geeignetsten für maximierte 3-HPA-Produktion ist. Immobilisierte *L. reuteri*-Kultur wurde erfolgreich für die 3-HPA Produktion eingesetzt und zeigte eine verbesserte spezifische Produktivität ohne Zentrifugierungsschritte gegenüber freien Zellkulturen. Zuletzt wurde im Rahmen dieser Arbeit eine neue Methode zur Reinigung von 3-HPA entwickelt und dessen hochpotentiellen Möglichkeiten im Einsatz in einer ISPR demonstriert. Die vorliegende Arbeit bietet also eine solide Basis für die erfolgreiche Entwicklung einer ISPR von 3-HPA aus der Glyzerinlösung im Grossmassstab.

1 Introduction

1.1 History of 3-HPA – From Rags to Riches

Spontaneous fermentation is a millennia-old food preservation method applied long before microorganisms were discovered (Hansen, 2002). However, such spontaneous processes involve risks of spoilage and malodour leading to losses of food. For example, French wine industry of the 19th century, the second economical sector with 500 million Francs turnover annually, was struggling with spoilage that resulted in a bitter tasting of the wine (Pasteur, 1866). Consequently, research on spoilage bloomed, eventually leading to identification of microbes causing spoilage and bitterness (Pasteur, 1866). It took some more time, however, to elucidate the exact mechanism of bitterness in wine (Voisenet, 1910). Small amounts of glycerol are produced during wine fermentation, which is desired because glycerol contributes to the sweetness and fullness of the wine (Pasteur, 1858b; Pasteur, 1858a). During wine ripening, lactic acid bacteria (LAB) contribute to wine flavour by degrading malic acid to the less acidic lactic acid. However, the capability of some of these bacteria to convert glycerol to 3-hydroxypropionaldehyde (3-HPA) (Voisenet, 1910; Voisenet, 1914b; Voisenet, 1914a) can have an undesired effect; the acidic conditions in wine enable the conversion of 3-HPA to acrolein (Nef, 1904), which reacts with phenols derivates leading to bitter tasting (Rentschler and Tanner, 1951).

Later, the potential of 3-HPA as a raw material for plastic production was discovered (Hall and Stern, 1950), followed by its identification as an antimicrobial, often referred to as reuterin today (Axelsson *et al.*, 1989; Talarico and Dobrogosz, 1989). Nowadays 3-HPA is still problematic in wine industry (Sponholz, 1993), but it is also regarded as a chemical with a wide range of potential applications in the food and chemical industries, as well as in health care (reviewed by Vollenweider and Lacroix, 2004). The probiotic lactic acid bacterium *Lactobacillus reuteri* used during this study is one bacterium among others capable of production 3-HPA from glycerol.

1.2 Lactic acid bacteria

1.2.1 Lactic Acid Bacteria

Lactic acid bacteria (LAB) constitute a heterogeneous group of Gram-positive bacteria that share the common trade of converting carbohydrates rapidly to lactic acid leading to an acidification of the environment. LAB are catalase-negative, carbohydrate fermenting, acid tolerant, anaerobic or aerotolerant, non-sporulating, and non-motile rods or cocci (Schleifer and Ludwig, 1995; Krämer, 1997). However, the discovery of LAB with a functional electron-transport chain for utilisation of molecular oxygen (Duwat *et al.*, 2001) and the identification of putative catalase genes in genome sequences of many LAB (Makarova *et al.*, 2006), suggests that this classical definition needs some reconsideration. LAB are adapted to nutrient-rich environments as e.g. milk, plant material, and the intestine of mammals (Schleifer and Ludwig, 1995; Krämer, 1997). In the past decades the genomic era has shed new light on the adaptation to nutrient-rich environments: most LAB possess high number of transporters, limited biosynthetic capacity, and extensive gene lost compared to other members of the phyla firmicutes (Makarova *et al.*, 2006). Due to their high growth and acidification rates and due to their high acid tolerance, LAB are usually the predominant flora in traditional food and feed fermentations where they contribute to the shelf-life, flavour, aroma, texture, and quality of the food. LAB prolong shelf-life of food by outcompeting spoilage organisms for nutrients, by lowering the pH, and by producing anti-microbial compounds like lactic acid and bacteriocins (Hansen, 2002). Many LAB produce vitamins as e.g. vitamin B_{12}, folate, riboflavine, and thereby enhancing the quality of the food (Kleerebezem and Hugenholtz, 2003; Sybesma *et al.*, 2003; Taranto *et al.*, 2003; Burgess *et al.*, 2004; Santos *et al.*, 2008). Furthermore, many LAB are natural inhabitants of the human gastrointestinal tract and have a potential beneficial effect on the host, the probiotic effect (Ahrné *et al.*, 1998). Food fermentations using LAB have been used for millennia and therefore LAB occurring in such fermentations are generally recognised as safe (GRAS) (Hammes, 1998). All these properties make LAB highly interesting for food industry and therefore LAB have been a research object since the dawn of microbiology.

1.2.2 Risks, safety, and problems

Although many LAB occurring in food fermentations process the GRAS status, some species have also been isolated from clinical samples as e.g. *L. casei*, *L. paracasei*, and *L. rhamnosus*, suggesting (opportunistic) pathogenic properties (Saxelin *et al.*, 1996; Hammes, 1998). However, among 3,317 clinical samples, lactobacilli were only detected in 8 samples, in spite of their common presence in the GI-tract. This infrequent abundance suggests strongly that the pathogenic potential of lactobacilli is very low (Saxelin *et al.*, 1996). Furthermore, consumption of the probiotic lactobacillus *L. reuteri* is generally regarded as safe, even in immune deficient individuals, indicating again a low pathogenic potential of lactobacilli (Wolf *et al.*, 1995; Wolf *et al.*, 1998; Valeur *et al.*, 2004; Savino *et al.*, 2007).

Apart from potential pathogenicity of LAB, also the presence of antibiotic resistance genes is a safety problem. Antibiotic resistance genes are widespread, not only in pathogenic bacteria but also among non-clinical isolates, probably due to the constant selective pressure caused by the extensive use of antibiotics (Teuber, 1999; Kastner *et al.*, 2006). The transfer of such resistance genes from intrinsic bacteria to e.g. pathogenic bacteria leads to spreading of antibiotic resistances in human, potentially impacting worldwide on human health (Teuber *et al.*, 1999). LAB carrying antibiotic resistance genes are therefore undesired for daily consumption.

1.2.3 Probiotics

Certain LAB, in particular strains from the genera *Lactobacillus*, play an important role in the ecology of the gastrointestinal tract of mammals and a number of strains are shown to have probiotics effects on the host (Ahrné *et al.*, 1998). Probiotics are live-microorganisms which when administered in adequate amounts confer health benefits to the host (FAO/WHO, 2002). The activity of probiotic bacteria includes modulation of the intestinal flora via production of antimicrobial compounds and via competition with (pathogenic) microbes for nutrients and mucosal binding sites. Additionally, probiotics adhere to the mucosa and stimulate mucus production thereby reinforcing the mucosal and epithelial barrier. They also stimulate the

immune system and receptors in the enteric nervous system. Further, they modulate enzymatic activities in the colon and produce short chain fatty acids (SCFA) and other metabolic products from indigestible dietary compounds, thereby contributing significantly to the energy absorption out of food (Fuller, 1991; Holzapfel *et al.*, 2001).

Probiotic activity leads to several beneficial effects for the host as e.g. increased resistance to infectious diseases, particularly those of the gastrointestinal tract, and decreased duration of diarrhoea. Additionally, decreasing effects in blood pressure and serum cholesterol concentrations have been reported. Further, improvement of lactose intolerance, reduced prevalence of allergies in susceptible individuals, and reduced risk for certain cancers are beneficial effects assigned to the activity of probiotic bacteria (Reid, 1999; Tannock, 1999; Marteau *et al.*, 2001; Parvez *et al.*, 2006). The potent beneficial effects, however, might entice into advertising probiotics as universal remedy. Probiotics need to be seen as supplement to the diet and not as alternative to a well-balanced diet and conventional medical therapies. This has to be kept in mind especially concerning marketing strategies and advertising campaigns (Atlas, 1999).

Selection of probiotic strains for consumption is not only dependent on their probiotic activity. Probiotic strains should be safe for the host and therefore preferably have a human origin. Another safety aspect is that they should not carry virulence factors (Culligan *et al.*, 2009) and their potential to transfer antibiotic resistances should be minimal. Further, probiotic strains must be able to survive the acids in the stomach and the bile salts in the duodenum in order to reach their intestinal target side. Finally, from industrial point of view, they should be feasible for large-scale production, survive storage in the food, and have no adverse effects on the sensory properties of the food (Gibson and Fuller, 2000).

However, some exceptions to these guidelines are possible. For example the probiotic *Escherichia coli* Nissle strain shows no high resistance to acid or bile salts but its survival through stomach passage is ensured by encapsulation of the microbes (Guarner *et al.*, 2005).

1.2.4 *Lactobacillus reuteri*

Lactobacillus reuteri (*L. reuteri*) is an obligate heterofermentative lactic acid bacterium, inhabiting autochthonously the gastrointestinal tract of humans and animals (Valeur *et al.*, 2004). *L. reuteri* was originally isolated from human faeces and identified as *L. fermentum* Biotype II (Lerche and Reuter, 1962). The bacterium was reclassified based on molecular biological methods, namely determination of murein type, G+C content, and DNA homology and named *L. reuteri* after Gerhard Reuter (Kandler and Stetter, 1973; Kandler *et al.*, 1980). *L. reuteri* is found in a variety of ecological niches like sourdough, meat and dairy fermentations, and in the gastro-intestinal (GI) as well as the urogenital tract of humans and other animals (Lerche and Reuter, 1962; Reuter, 1965; Lindgren and Dobrogosz, 1990; Molin *et al.*, 1992; Vogel *et al.*, 1994; Naito *et al.*, 1995; Rodriguez *et al.*, 2003; Jin *et al.*, 2007; van Coillie *et al.*, 2007).

Cells of the type-strain *L. reuteri* DSM 20016 were described as slightly irregular bent rods of the size of 0.7-1.0 by 2.0-5.0 μm with rounded ends (Kandler *et al.*, 1980). Cells occur individually, in pairs, or in small clusters of 3 to 5. *L. reuteri* grows up to 45°C but not at 15°C, optimally at 35-38°C, and at initial pH values of 5.0-7.5, optimally at 6.0-6.8. *L. reuteri* is able to ferment a number of carbohydrates like glucose, fructose, arabinose, ribose, sucrose, lactose, maltose, melbiose, raffinose, and gluconate. The cell wall does not contain teichoic acid and the murein is of the lysine-D-iso-asparagine type. The G+C content of DNA is 41 mol % (Kandler *et al.*, 1980).

Fast growing homofermentative lactobacilli use the Embden-Meyerhof-Parnas pathway (EMP) for glucose fermentation and produce exclusively lactic acid. Heterofermentative lactobacilli, such as *L. reuteri*, utilise glucose via the phosphoketolase pathway (PKP). Glucose is activated to glucose-6-phosphate, which is cleaved via ribulose-5-phosphate to acetyl phosphate and glyceraldehyde-3-phosphate which are further converted to the fermentation end-products lactic acid, acetic acid, ethanol, and CO_2 (Gänzle *et al.*, 2007). The amount of ATP obtained by both pathways differs; one mol of glucose forms two mol of ATP via the EMP-pathway whereas only one mol ATP is formed via PKP. However, if an external NADH-acceptor is available, one additional ATP can be produced by the conversion

of acetyl-phosphate to acetate instead to ethanol. Addition of an NADH-acceptor leads to increased growth rate and biomass production (El-Ziney *et al.*, 1998; Arsköld *et al.*, 2008). Such NADH-acceptors are e.g. fructose, molecular oxygen, glycerol, and oxidised glutathione (Schütz and Radler, 1984 ; Condon, 1987 ; Gänzle *et al.*, 2007; Arsköld *et al.*, 2008).

Production of antimicrobial compounds by LAB, especially bacteriocins, is important for fermentation stability, probiotic activity, and food preservation (reviewed by Cotter *et al.*, 2005). Some *L. reuteri* strains isolated from sourdough produce reutericyclin, N-acylated tetramic acid, which shows anti-microbial activity against Gram-positive bacteria including the pathogens *Staphylococcus aureus* and *Listeria innocua*, and the opportunistic pathogen *Enterococcus faecium*. The minimum inhibitory concentration (MIC) of reutericyclin for Gram-positive bacteria is in the range of 0.06 to 6.5 mg l^{-1}, whereas the Gram-negative bacterium *Escherichia coli* has a MIC up to 100 mg l^{-1} and yeasts even above 100 mg l^{-1} (Gänzle *et al.*, 2000; Gänzle, 2004).

So far, one bacteriocin produced by *L. reuteri* has been identified and designated reutericin 6. Reutericin 6 is active against some related *Lactobacillus* spp., but not against *Escherichia coli* (*E. coli*), *Staphylococcus aureus*, nor *Bacillus subtilis* (Toba *et al.*, 1991). The plasmid encoding for reutericin 6 production is 100% homologous to the bacteriocin gassericin A plasmid from *Lactobacillus gasseri*, indicating that the plasmid conjugated from *L. gasseri* to *L. reuteri* (Ito *et al.*, 2009). Remarkably, gassericin A has a broader antimicrobial spectrum compared to reutericin 6, likely due to different posttranslational modifications (Kawai *et al.*, 2004).

Many *L. reuteri* strains, including strain ATCC 55730 used in this work, produce the broad-range antimicrobial substance 3-hydroypropionaldehyde (3-HPA or reuterin, see below). Reuterin is effective against Gram-negative and Gram-positive bacteria, yeasts, fungi, viruses, and protozoa (Axelsson *et al.*, 1989; Talarico and Dobrogosz, 1989; Yunmbam and Roberts, 1992; Rodriguez *et al.*, 2003; Cleusix *et al.*, 2007). Furthermore, 3-HPA is a precursor for the production of chemicals (e.g. acrylic acid and hydroxypropionic acid) and it is useful for cross linking biological tissues and hemoglobin polymerisation (Vollenweider and Lacroix, 2004).

1.2.5 *Lactobacillus reuteri* ATCC 55730 (SD2112)

In this study, *L. reuteri* ATCC 55730 (also known as SD2112 and previously referred to as *L. reuteri* ATCC 53608 by Lüthi-Peng *et al.*, 2002a and 2002b, and Doleyres *et al.*, 2005) isolated from breast milk was used (Casas and Mollstam, 1998). Strain ATCC 55730 has probiotic properties (Casas and Dobrogosz, 2000; Valeur *et al.*, 2004; Savino *et al.*, 2007) and is able to secrete reuterin (Casas and Mollstam, 1998). *L. reuteri* ATCC 55730 has been used as probiotic food supplement (tablets) or in milk products since 1991 but has been replaced now by its plasmid-cured daughter strain *L. reuteri* DSM 17938 (Rosander *et al.*, 2008).

The probiotic properties of strain *L. reuteri* ATCC 55730 has been confirmed by many randomised, double-blinded, and placebo-controlled clinical studies. It was shown that *L. reuteri* ATCC 55730 can interact with and adhere to mucosa cells, an important probiotic property (Valeur *et al.*, 2004). Consumption of *L. reuteri* ATCC 55730 reduced the incidence and severity of virus-type diarrhoea (Ruiz-Palacios *et al.*, 1996), decreased duration of acute gastroenteritis of 0.5 to 3 year old children (Shornikova *et al.*, 1997a; Shornikova *et al.*, 1997b), and showed superior activity against infantile colic compared to the antifoam agent Simethicone (Savino *et al.*, 2007). The strain also reduced incidence and duration of gastrointestinal and respiratory diseases of healthy adults, which resulted in a significant decrease of employees' sick-days (Tubelius *et al.*, 2005), and decreased incidence and duration of fever and diarrhoea in 7 months infants, thus reduced clinical visits and prescription of antibiotics (Weizman *et al.*, 2005). Ingestion of *L. reuteri* ATCC 55730 reduced frequency and intensity of antibiotic-associated side effects during eradication therapy for *Heliobacter pylori* of 12-year old boys (Lionetti *et al.*, 2006) and suppressed the development of peptic ulcer of *H. pylori*-positive adults (Saggioroa *et al.*, 2005; Imase *et al.*, 2007). Further, the number of *E. coli* was reduced an *in vitro* model after addition of *L. reuteri* ATCC 55730, which was likely due to 3-HPA production (Cleusix *et al.*, 2008). When administered unconventionally by sucking on a probiotic tablet or drinking through a straw, *L. reuteri* reduced the viable cell concentration of salivary *Streptococcus mutans* of young adults (Caglar *et al.*, 2006). *L. reuteri* ATCC 55730 had no negative effects when administered to children and to healthy or immuno-compromised adults at doses of up to 10^{11} cfu day^{-1} for 21 days,

indicating that the strain has no pathogenic properties and can be regarded as safe (Wolf *et al.*, 1995; Wolf *et al.*, 1998; Valeur *et al.*, 2004; Savino *et al.*, 2007).

L. reuteri ATCC 55730 strain is known to show resistance against a variety of antibiotics of which at least two are not intrinsic (Klein *et al.*, 2000; Kastner *et al.*, 2006). Two plasmids harbouring genes encoding tetracycline (*tet*(W)) and lincosamide (*lnu*(A)) resistance were detected, both potentially transferable to other bacteria in the gut (Kastner *et al.*, 2006). To eliminate the risk resistance proliferation strain ATCC 55730 was cured from these two plasmids, resulting in the daughter strain DSM 17938 with a tetracycline and lincosamide sensitive phenotype (Rosander *et al.*, 2008).

Strain ATCC 55730, and now strain DSM 17938, have high probiotic activity and are therefore marketed as such (Biogaia, Stockholm, Sweden). These two strains are only one example of successful marketing of probiotic by the food-industry.

1.3 3-Hydroxypropionaldehyde

1.3.1 3-Hydroxypropionaldehyde

3-Hydroxypropionaldehyde (3-HPA) is viscous and colourless liquid with a slightly acrid smell (Table 1.1). Studies on the structure of 3-hydroxypropionaldehyde (3-HPA) revealed that 3-HPA in water is part of a dynamic three component system (HPA system or HPA, Figure 1.1) (Hall and Stern, 1950; Talarico and Dobrogosz, 1989). 3-HPA can be reversibly dehydrated to acrolein (Nef, 1904), the compound responsible for the bitter taste in wine (Rentschler and Tanner, 1951). Nowadays, the name reuterin is used as a synonym for 3-HPA, especially in the field of food preservation and health care.

The 3-HPA hydrate form is predominant at concentrations below 1.4 M at weak acidic pH (Vollenweider *et al.*, 2003). With increasing 3-HPA concentrations, the mol fraction of the dimeric form increases up to 50 mol % at a concentration of 4.9 M. 3-HPA solution also contain minor fractions of hemiacetal, acetal, hemiacetal dimer, acetal trimer, oligomers, and polymers (Chen *et al.*, 2002; Sung *et al.*, 2003).

Besides, the composition of 3-HPA is pH-dependent (Sung *et al.*, 2003). Polymers, hemiacetal dimer, and acetal trimer occur only at basic conditions, whereas 3-HPA hydrate is formed at acidic pH (Sung *et al.*, 2003).

Table 1.1 Physical properties of 3-hydroxypropionaldehyde.

Attribute		**Reference**
Synonyms	3-hydroxypropanal, hydracrylaldehyde, beta-hydroxypropanal, beta-hydroxypropionaldehyde, 3-oxo-1-propanol, hydracrolein, reuterin	(3-Hydroxypropionaldehyde MSDS)
CAS No.	2134-29-4	(3-Hydroxypropionaldehyde MSDS)
Formula	$C_3H_6O_2$	(3-Hydroxypropionaldehyde MSDS)
Molecular weight	74.04 g mol^{-1}	(3-Hydroxypropionaldehyde MSDS)
Molecule charge	Uncharged	(Römpp Enzyklopädie)
Smell	Plastic-like and slightly acrid or tart	Laboratory of Food Biotechnology, ETH Zurich
Consistency	Viscous colourless liquid at 25°C	Laboratory of Food Biotechnology, ETH Zurich
Vapour pressure	72.0 Pa at 25°C (calculated)	(3-Hydroxypropionaldehyde MSDS)
Boiling point	40°C at 0.2 mmHg (26.7 Pa)	(Hall and Stern, 1950)
Refraction index n_D^{20}	1.4418 (3-HPA), 1.4783 (3-HPA dimer)	(Hall and Stern, 1950)
Water solubility	Fully miscible	Laboratory of Food Biotechnology, ETH Zurich
Stability	Stable at 4°C, but quick dehydration to acrolein at > 37°C in water	(Lüthi-Peng *et al.*, 2002b; Vollenweider *et al.*, 2003)

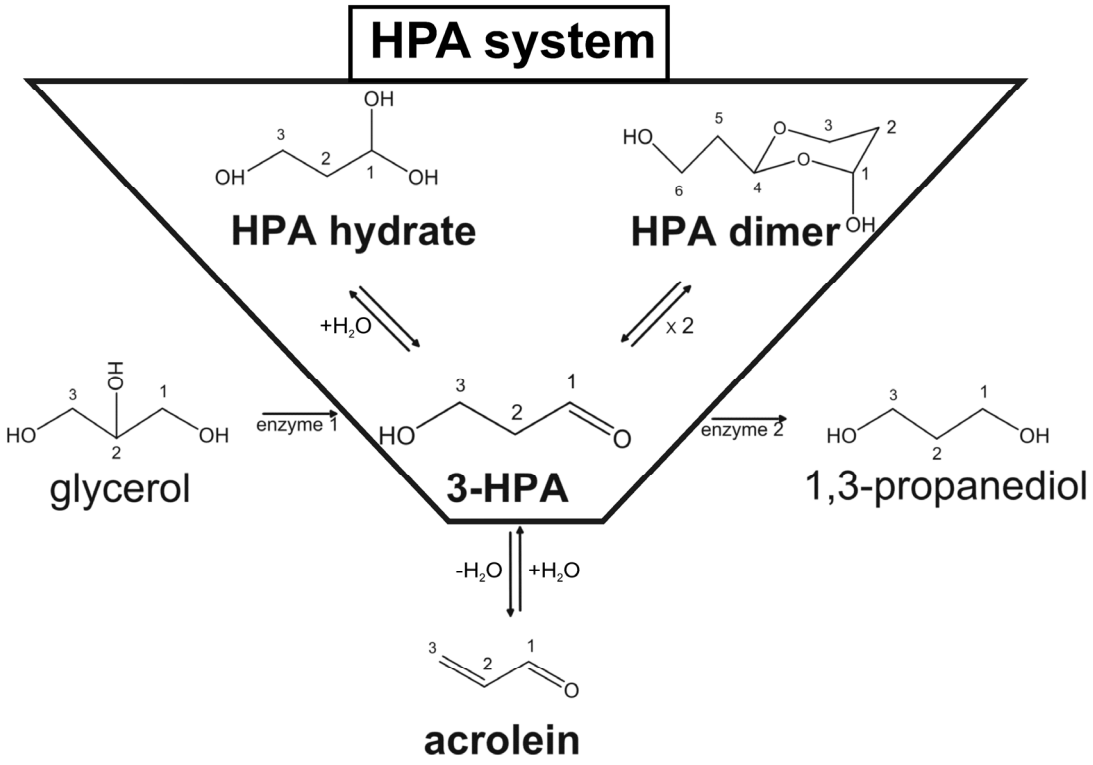

Figure 1.1 Formation of 3-hydroxypropionaldehyde, 1,3-propanediol, and acrolein. → Enzymatic reaction; ⇌ equilibrium reaction; *enzyme 1*: glycerol dehydratase; *enzyme 2*: 1,3 propanediol oxidoreductase; *HPA hydrate*: 1,1,3-trihydroxypropane, *HPA dimer*: 2-(2-hydroxyethyl)-4-hydroxy-1,3-dioxane (adapted from Vollenweider and Lacroix, 2004).

The aldehyde group of 3-HPA reacts with compounds like oxygen, nitrogen, sulphur, and carbon in a nucleophilic addition reaction (Schauenstein *et al.*, 1977; O'Brien *et al.*, 2005). However, the reactivity of aldehydes depends not only on the aldehyde group but also on the structure and presence of other functional groups in the molecule. Indeed, α,β unsaturated aldehydes, as e.g. acrolein, react rapidly with sulphydryl, amino, and hydroxyl groups at 25°C and neutral pH, whereas saturated aldehydes react only at elevated temperatures with amino groups in a slower and reversible reaction (Schauenstein *et al.*, 1977). Toxicity of aldehydes needs to be determined for each aldehyde separately as demonstrated on the two related three-carbon aldehydes acrolein and propanal. Oral median lethal doses (LD_{50}) for rats are 0.046 g kg^{-1} for acrolein (Acrolein MSDS) and 1.41 g kg^{-1} for propanal (Propanal MSDS), a 30-fold difference. For 3-HPA, only limited data are available. It is known that 3-HPA readily reacts with proteins (Lüthi-Peng *et al.*, 2002b; Sung *et al.*, 2003) and medium components such as lactose (Lüthi-Peng *et al.*, 2002b) under conditions

present in biological systems. It was shown that 3-HPA was significantly less toxic than glutaraldehyde after comparison of the 2 compounds on mouse fibroblasts (Chen *et al.*, 2002). However, such comparison provides only very limited information about the toxicity of reuterin. The lethal dose of 3-HPA in mice was estimated to be 7.5 mg which equals a LD_{50} of approximately be 260-320 mg kg^{-1}, which is 7 fold higher compared to LD_{50} of acrolein, but seven times lower compared to propanal (Yunmbam and Roberts, 1993).

1.3.2 Application of 3-hydroxypropionaldehyde

3-HPA has some characteristics that are interesting for food and chemical industry, as well as in medical applications (Vollenweider and Lacroix, 2004). First, 3-HPA has a strong microbial activity against a whole range of microorganisms including Gram-positive and Gram-negative bacteria, yeasts, and moulds (Talarico *et al.*, 1988). Further it has interesting chemical properties enabling its use in cross-linking and fixation of biological materials (Chen *et al.*, 2002; Sung *et al.*, 2003; Chen *et al.*, 2004) and as a precursor for chemicals as e.g. acrolein, acrylic acid, 3-hydroxypropionic acid, and 1,3-PDO (Vollenweider and Lacroix, 2004). The following paragraphs will explain in detail the potential of 3-HPA in different fields.

1.3.2.1 3-Hydroxypropionaldehyde as an antimicrobial agent

An antimicrobial substance produced by *L. reuteri* was first described in 1989 and designated reuterin after its producer strain (Axelsson *et al.*, 1989). Reuterin was described as a neutral, water soluble, non-protein, low molecular weight (< 200 g mol^{-1}) derivate of glycerol (Axelsson *et al.*, 1989). Additional chemical characterisation revealed reuterin to be an equilibrium mixture of monomeric, hydrated monomeric, and cyclic dimeric forms of 3-hydroxypropionaldehyde (Axelsson *et al.*, 1989; Talarico and Dobrogosz, 1989).

3-HPA inhibits a wide range of Gram-negative (Table 1.2) and Gram-positive bacteria (Table 1.3), as well as protozoa and bacteriophages (Table 1.4). Among these

are notorious food pathogens like *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli* (*E. coli*) O157:H7, *Staphylococcus* spp., and *Clostridium* spp. (Mahapatra *et al.*, 2005). Therefore 3-HPA has a high potential as a food preservative. Indeed there are studies that have shown that 3-HPA inhibits growth of food spoilage organisms in milk, cottage cheese, and meat (Daeschel, 1989; El-Ziney and Debevere, 1998; El-Ziney *et al.*, 1999; Arqués *et al.*, 2004). However, 3-HPA can be transformed into the toxic acrolein (Nef, 1904), which is toxic and reacts readily with other compounds present in food, as already shown for wine and apple cider (Pasteur, 1866; Voisenet, 1910; Voisenet, 1914b; Voisenet, 1914a; Rentschler and Tanner, 1951; Garai-Ibabe *et al.*, 2008). Therefore the toxicity and stability of 3-HPA in food matrices have to be elucidated before 3-HPA can be applied in food industry.

The antimicrobial spectrum of 3-HPA against intestinal bacteria showed a minimal inhibitory concentration (MIC) below 7.5 mM for the majority of the tested bacteria (Table 1.2 and Table 1.3). Cleusix *et al.* (2007) showed that lactobacilli and *Clostridium clostridioforme* were more resistant with MIC ranging from 15 to 50 mM (Table 1.3), and the high MIC value for lactobacilli might enable the application of 3-HPA in food that is fermented by lactobacilli.

Additional preservative factors or hurdles (e.g. temperature, pH, water activity, competitive flora) sum up to the inhibitory effects of every preservative factor. Therefore, undesired or pathogenic bacteria, which are not inhibited by the amount of 3-HPA present in the food could be suppressed by the hurdle strategy (Leistner, 1978; Leistner, 2000).

The viability of *L. reuteri* after 2 h of 3-HPA production was significantly higher at 4°C than at 37°C, indicating higher toxicity of 3-HPA at elevated temperatures (Doleyres *et al.*, 2005). Similarly, *E. coli* was less sensitive to 3-HPA at lower temperatures (Rasch, 2002). A pH dependent effect of reuterin on growing *E. coli* cells could not be determined since pH itself showed an inhibiting effect on growth (Rasch, 2002). However, the reactivity of 3-HPA towards proteins was significantly lower at pH 4.0 compared to pH 7.5, indicating decreased toxicity at lower pH (Schauenstein *et al.*, 1977; Sung *et al.*, 2003).

3-HPA is also active against eukaryotic organisms, although there is only limited data available. 3-HPA is effective against the cause of the sleeping's disease *T. brucei brucei*. *In vitro*, 70 μM 3-HPA killed the microbe completely after incubation at 27°C

for 24 h. This concentration is known as minimal bacteriocidal concentration (MBC) in the bacterial field, thus *T. brucei brucei* show an increased sensitivity to 3-HPA than bacteria with MBCs between 0.4 and 120 mM (Table 1.2 and Table 1.3).

According to Chen *et al.* (2002), mammalian cells are less susceptible to 3-HPA exposure compared to glutaraldehyde (1,5-pentanedial). Cytotoxicity of 3-HPA and glutaraldehyde was assessed *in vitro* using a mouse-derived established cell line of 3T3 fibroblasts and the proportion of viable cells in time was measured in a test agent-treated culture (Sung *et al.*, 1999; Chen *et al.*, 2002). Similarly, the lethal dose of 3-HPA in mice was estimated to 260-320 mg kg^{-1} (1500 U or 7.5 mg, Yunmbam and Roberts, 1993), which is about 15 to 20 fold higher compared to LD_{50} of glutaraldehyde (LD_{50} rats intraperitoneal: 17.9 mg kg^{-1}; Glutaraldehyde MSDS). Irritation of e.g. mucosal or long term effects of reuterin on human health might already occur at lower concentrations after prolonged exposure, whereas the metabolic faith of reuterin in humans is completely unknown and still has to be elucidated (Stevens *et al.*, 2010). Therefore, additional studies on the toxicity of reuterin are still required before the compound can be used in food, agricultural, or medical applications.

3-HPA has also been shown to be a useful agent in sterilisation of biological tissues in health care or pharmaceutical applications (Chen *et al.*, 2002; Sung *et al.*, 2003). In-between 30 and 90 ppm (0.4-1.2 mM) 3-HPA was sufficient to decontaminate biological tissues containing *Pseudomonas aeruginosa*, *E. coli*, *S. aureus*, and *B. subtilis* within 4 h. Treatment of *Trypanosoma brucei brucei*-infected mice with 3-HPA for 7 days led to a 68% reduction in parasitemia and to an increased survival of the mice (Yunmbam and Roberts, 1993) (Table 1.4). As discussed in the previous paragraph, 3-HPA inhibits a large variety of food spoiling bacteria and food borne pathogens whereas it has also potential as disinfectant agent in medical applications. However, additional studies on the toxicity of reuterin are still required before the compound can be used in food, agricultural, or medical applications.

Table 1.2 Activity of reuterin against Gram-negative bacteria

Organism	Strain	Concentration	Reference
***In vitro* activity**			
Escherichia coli	DSM 5698	MIC: 7.5-15.0 mM; MBC: 15.0-30.0 mM	(Cleusix *et al.*, 2007)
Escherichia coli	ATCC 25922	MIC: 35.0±0.0 ppm; MBC: 43.0±2.9 ppm	(Chen *et al.*, 2002)
Escherichia coli	K12 (wt), 431, 73, P55, 263, P159, PII-C7	MIC: 20-25 AU (2.2-2.7 mM)[a]	(Axelsson *et al.*, 1989; Dobrogosz and Lindgren, 1994)
Salmonella typhimurium	-[b]	MIC: 20 AU[a] (2.2 mM)	(Axelsson *et al.*, 1989)
Pseudomonas aeruginosa	2 strains	MIC: 25 AU[a] (2.7 mM)	(Axelsson *et al.*, 1989)
Pseudomonas fluorescens	-[b] -	[b]; very sensitive	(Dobrogosz and Lindgren, 1994)
Pseudomonas aeruginosa	ATCC 27853	MIC: 33.0±2.9 ppm; MBC: 50.0±0.0 ppm	(Chen *et al.*, 2002)
Bacteroides vulgatus	DSM 1447	MIC: < 1.9 mM; MBC: 1.9-3.8 mM	(Cleusix *et al.*, 2007)
Bacteroides thetaiotaomicron	DSM 2079	MIC: 1.9-3.8 mM; MBC: 1.9-3.8 mM	(Cleusix *et al.*, 2007)
Bacteroides fragilis	LMG 10263	MIC: 3.8-7.5 mM; MBC: 3.8-7.5 mM	(Cleusix *et al.*, 2007)
Shigella species	-[b] -	[b]; very sensitive	(Dobrogosz and Lindgren, 1994)
Proteus species	-[b] -	[b]; very sensitive	(Dobrogosz and Lindgren, 1994)
Klebsiella pneumoniae	ATCC 25955	20 mM: no inhibition *et al.*	(Barbirato , 1996)
Citrobacter freundii	ATCC 8090	20 mM: no inhibition *et al.*	(Barbirato , 1996)
Enterobacter agglomerans	CNCM1210	30 mM: inhibition of growth	(Barbirato *et al.*, 1996)
Escherichia coli VTEC	LMG 8223	MIC: 4 U[c] (0.3 mM)	(El-Ziney and Debevere, 1998)
Escherichia coli K12	LMG 2578	MIC: 4 U[c] (0.3 mM)	(El-Ziney and Debevere, 1998)
Escherichia coli K12	LMG 2579	MIC: 4 U[c] (0.3 mM)	(El-Ziney and Debevere, 1998)
Escherichia coli ETEC	CIP 81.86	MIC: 4 U[c] (0.3 mM)	(El-Ziney and Debevere, 1998)
Escherichia coli O157:H7	932	MIC: 4 U[c] (0.3 mM)	(El-Ziney and Debevere, 1998)
Escherichia coli O157:H7	MRK 1542	MIC: 4 U[c] (0.3 mM)	(El-Ziney and Debevere, 1998)
Activity on biological tissue			
Escherichia coli	ATCC 25922	MBC: 90.0±0.0 ppm (1.2 mM)	(Chen *et al.*, 2002)
Pseudomonas aeruginosa	ATCC 27853	MBC: 50.0±0.0 ppm (0.7 mM)	(Chen *et al.*, 2002)

Table 1.2 Continued

UHT Milk and Cottage cheese			
Escherichia coli O157:H7	932	50-250 U[c] g^{-1} cheese and 150 U[c] milk; all concentrations bacteriocidal at 7°C	(El-Ziney and Debevere, 1998)
Ground beef			
Coliform bacteria		50-100 U g^{-1}: Bactericidal at 4°C, 6 days	(Daeschel, 1989)
Surface of pork meat			
Escherichia coli O157:H7	MRK 1542	500 U[c]: Reduction of log 2.8 after dipping for 15 s and storage 24 h at 7°C	(El-Ziney *et al.*, 1999)
Ground pork			
Escherichia coli O157:H7	MRK 1542	50-150 U[c] g^{-1}: all concentrations bacteriocidal at 7°C	(El-Ziney *et al.*, 1999)
Milk			
Escherichia coli O157:H7	ATCC 43894	8 U[c]: Reduction of log 1 after 24 h at 37°C	(Arqués *et al.*, 2004)
Salmonella choleraesuis subsp. *choleraesuis*	CECT 409	8 U[c]: Reduction of log 1.5 after 24 h at 37°C	(Arqués *et al.*, 2004)
Yersinia enterocolitica	CECT 559	8 U[c]: Reduction of log 1.7 after 24 h at 37°C	(Arqués *et al.*, 2004)
Aeromonas hydrophila subsp. *hydrophila*	CECT 398	8 U[c]: Elimination of log 4 after 24 h at 37°C	(Arqués *et al.*, 2004)
Campylobacter jejuni	LMG 6629	8 U[c]: Elimination of log 4 after 24 h at 37°C	(Arqués *et al.*, 2004)
***In vitro* model of colonic fermentation**			
Escherichia coli	-[b]	*Lactobacillus reuteri* ATCC 55730 produced 3-HPA that decreased *E. coli* population	(Cleusix *et al.*, 2008)

a) 1 AU equals 8 µg reuterin ml^{-1} (0.11 mM 3-HPA monomer) (Dobrogo sz and Lindgren, 1994; Cleusix *et al.*, 2007)
b) No data available, not stated in reference
c) 1 U equals 5 µg reuterin ml^{-1} (0.07 mM 3-HPA monomer) (Chung *et al.*, 1989; Yunmbam and Roberts, 1993)

Table 1.3 Activity of reuterin against Gram-positive bacteria

Organism	Strain	Concentration	Reference
***In vitro* activity**			
Bifidobacterium catenulatum	LMG 11043	MIC: 1.9-3.8 mM; MBC: 7.5-15.0 mM	(Cleusix *et al.*, 2007)
Bifidobacterium longum	DSM 20219	MIC: 1.9-3.8 mM; MBC: 3.8-7.5 mM	(Cleusix *et al.*, 2007)
Bifidobacterium longum infantis	DSM 20088	MIC: 1.9-3.8 mM; MBC: 1.9-3.8 mM	(Cleusix *et al.*, 2007)
Bifidobacterium adolescentis	DSM 20083	MIC: 3.8-7.5 mM	(Cleusix *et al.*, 2007)
Bifidobacterium bifidum	DSM 20456	MIC: 3.8-7.5 mM; MBC: 7.5-15.0 mM	(Cleusix *et al.*, 2007)
Bifidobacterium breve	DSM 20213	MIC: 7.5-15 mM; MBC: 7.5-15.0 mM	(Cleusix *et al.*, 2007)
Lactobacillus acidophilus	ATCC 4356	MIC: 15.0-40.0 mM; MBC: 15.0-40.0 mM	(Cleusix *et al.*, 2007)
Lactobacillus casei	ATCC 334	MIC: 15.0-40.0 mM; MBC: 40.0-80.0 mM	(Cleusix *et al.*, 2007)
Lactobacillus fermentum	ETH	MIC: 15.0-40.0 mM; MBC: 15.0-40.0 mM	(Cleusix *et al.*, 2007)
Lactobacillus salivarius	ETH	MIC: 15.0-40.0 mM; MBC: 40.0-80.0 mM	(Cleusix *et al.*, 2007)
Lactobacillus reuteri	DSM 20016	MIC: 30.0-50.0 mM; MBC: 60-120 mM	(Cleusix *et al.*, 2007)
Lactobacillus reuteri	ATCC 55730	MIC: 30.0-50.0 mM; MBC: 60-120 mM	(Cleusix *et al.*, 2007)
Lactobacillus acidophilus	6 strains	MIC: 2-5 AU[a] (0.2-0.5 mM)	(Axelsson *et al.*, 1989)
Lactobacillus plantarum	2 strains	MIC: 5 AU[a] (0.5 mM)	(Axelsson *et al.*, 1989)
Pediococcus cerevisiae	-[b]	MIC: 5 AU[a] (0.5 mM)	(Axelsson *et al.*, 1989)
Leuconostoc mesenteroides	-[b]	MIC: 4 AU[a] (0.4 mM)	(Axelsson *et al.*, 1989)
Enterococcus faecium	DSM 20477	MIC: 3.8-7.5 mM; MBC: 30.0-50.0 mM	(Cleusix *et al.*, 2007)
Eubacterium biforme	DSM 3989	MIC: 1.9-3.8 mM; MBC: 3.8-7.5 mM	(Cleusix *et al.*, 2007)
Eubacterium eligens	DSM 3376	MIC: 1.9-3.8 mM; MBC: 1.9-3.8 mM	(Cleusix *et al.*, 2007)
Colinsella aerofaciens	DSM 3979	MIC: 3.8-7.5 mM; MBC: 7.5-15.0 mM	(Cleusix *et al.*, 2007)
Streptococcus salivarius	DSM 20560	MIC: 3.8-7.5 mM; MBC: 15.0-30.0 mM	(Cleusix *et al.*, 2007)
Streptococcus lactis	2 strains	MIC: 5 AU[a] (0.5 mM)	(Axelsson *et al.*, 1989)
Streptococcus cremoris	-[b] -	[b]; very sensitive	(Dobrogosz and Lindgren, 1994)
Clostridium difficile	ETH	MIC: < 1.9 mM; MBC: 3.8-7.5 mM	(Cleusix *et al.*, 2007)
Clostridium clostridioforme	DSM 933	MIC: 15.0-30.0 mM; MBC: 15.0-30.0 mM	(Cleusix *et al.*, 2007)
Clostridium sporogenes	-[b] -	[b]; sensitive	(Dobrogosz and Lindgren, 1994)
Ruminococcus productus	DSM 2950	MIC: 7.5-15.0 mM; MBC: 3.8-7.5 mM	(Cleusix *et al.*, 2007)
Listeria innocua	HPB 13	MIC: 7.5-15.0 mM; MBC: 7.5-15.0 mM	(Cleusix *et al.*, 2007)
Listeria ivanovii	HPB 28	MIC: 3.8-7.5 mM; MBC: 7.5-15.0 mM	(Cleusix *et al.*, 2007)
Staphylococcus epidermidis	-[b]	MIC: 15 AU[a] (1.6 mM)	(Axelsson *et al.*, 1989)
Staphylococcus aureus	ATCC 25923	MIC: 23.0±2.9 ppm; MBC: 41.0±2.5 ppm	(Chen *et al.*, 2002)
Bacillus megaterium	-[b]	MIC: 20 AU[a] (2.2 mM)	(Axelsson *et al.*, 1989)

Table 1.3 Continued

Bacillus subtilis	ATCC 6633	MIC: 35.0±0.0 ppm; MBC: 30.0±0.0 ppm	(Chen *et al.*, 2002)
Listeria monocytogenes	LMG 10470, LMG 13305, Ohio serotype 4b, Scott A, V7, 121	MIC: 8 U^c (0.5 mM)	(El-Ziney and Debevere, 1998)
Listeria innocua	LMG 11387	MIC: 8 U^c (0.5 mM)	(El-Ziney and Debevere, 1998)
Listeria ivanovii	LMG 11388	MIC: 8 U^c (0.5 mM)	(El-Ziney and Debevere, 1998)
Listeria seeligeri	LMG 11383	MIC: 8 U^c (0.5 mM)	(El-Ziney and Debevere, 1998)
Listeria welshimeri	LMG 11389	MIC: 8 U^c (0.5 mM)	(El-Ziney and Debevere, 1998)
Activity on biological tissue			
Staphylococcus aureus	ATCC 25923	MBC: 90.0±0.0 ppm (1.2 mM)	(Chen *et al.*, 2002)
Bacillus subtilis	ATCC 6633	MBC: 30.0±0.0 ppm (0.4 mM)	(Chen *et al.*, 2002)
UHT Milk and Cottage cheese			
Listeria monocytogenes	Ohio serotype 4b	50-250 U^c g^{-1} cheese and 150 U^c milk; all concentrations bacteriocidal at 7°C	(El-Ziney and Debevere, 1998)
Surface of pork meat			
Listeria monocytogenes	Ohio serotype 4b	500 U^c: Reduction of log 0.63 after dipping for 15 s and storage 24 h at 7°C	(El-Ziney *et al.*, 1999)
Ground pork			
Listeria monocytogenes	Ohio serotype 4b	50-250 U^c g^{-1}: Bacteriocidal for >= 150 U g^{-1} at 7°C	(El-Ziney *et al.*, 1999)
Milk			
Listeria monocytogenes	Ohio serotype 4b	8 U^c (0.5 mM): Bacteriostatic for at least 24 h at 37°C	(Arqués *et al.*, 2004)
Staphylococcus aureus	CECT 4013	8 U^c (0.5 mM): Bacteriostatic for at least 24 h at 37°C	(Arqués *et al.*, 2004)
Cujada – Semisolid dairy product			
Listeria monocytogenes	Scott A	2 U^c (0.1 mM): No activity at 10°C	(Arqués *et al.*, 2004)
Staphylococcus aureus	CECT 976	2 U^c (0.1 mM): Slower growth at 10°C	(Arqués *et al.*, 2004)

a) 1 AU equals 8 µg reuterin ml^{-1} (0.11 mM 3-HPA monomer) (Dobrogo sz and Lindgren, 1994; Cleusix *et al.*, 2007)
b) No data available, not stated in reference
c) 1 U equals 5 µg reuterin ml^{-1} (0.07 mM 3-HPA monomer) (Chung *et al.*, 1989; Yunmbam and Roberts, 1993)

Table 1.4 Activity of reuterin against yeasts, protozoa, and bacteriophages.

Organism	Strain	Concentration	Reference
Yeasts			
Saccharomyces cerevisiae	-[a] -	[a]; sensitive	(Dobrogosz and Lindgren, 1994)
Protozoa			
Trypanosoma brucei brucei	367H	35 µM[b] (70 µM)[c]: No surviving cells after incubation for 24 h	(Yunmbam and Roberts, 1992)
Protozoa in vertebrata			
Trypanosoma brucei brucei	367H	500 U[e] (1.25 mg) applied intraperitoneal thrice a day for 3 days: Reduction of parasitemia by 56% in mice	(Yunmbam and Roberts, 1993)
Phages			
Lambda phage of *Escherichia coli* -	[a]	10 AU[d] (0.08 mg ml^{-1}) decreased phage production by 10 fold	(Dobrogosz and Lindgren, 1995)
Phage 8014-B2 of *Lactobacillus plantarum*	-[a]	25 AU[d] (0.2 mg ml^{-1}) decreased phage production by 10 fold	(Dobrogosz and Lindgren, 1995)

a) No data available, not stated in reference
b) Published concentration calculated from the molecular weight of the 3-HPA-dimer
c) Concentration calculated from the molecular weight of 3-HPA
d) 1 AU equals 8 µg reuterin ml^{-1} (0.11 mM 3-HPA monomer) (Dobrogo sz and Lindgren, 1994; Cleusix *et al.*, 2007)
e) 1 U equals 5 µg reuterin ml^{-1} (0.07 mM 3-HPA monomer); injected volume 0.5 ml (Chung *et al.*, 1989; Yunmbam and Roberts, 1993)

1.3.2.2 Mode of action of 3-HPA as antimicrobial agent

The mechanism for growth inhibition of reuterin is not known yet, especially because the chemistry of the HPA system is complicated (Vollenweider *et al.*, 2003). The first postulated structure for reuterin was a dimeric form and a three-dimensional molecular model of the hemiacetal revealed an analogue of D-ribose (Dobrogosz and Lindgren, 1988). From this model the observed inhibition of a DNA-building blocks enzyme, ribonucleotide reductase, could be explained by a model in which reuterin acts as an inhibitor by competing for the ribose recognition site of ribonucleotide reductase (Dobrogosz and Lindgren, 1988; Talarico and Dobrogosz, 1989; Dobrogosz and Lindgren, 1995). However, the observed inhibition of thioredoxin could not be explained by the carbohydrate-like structure theory (Talarico and Dobrogosz, 1989). Furthermore, it contradicts with the NMR-analysis on the composition of the reuterin system, which revealed predominantly monomers in physiological concentrations (Vollenweider *et al.*, 2003).

A different model postulates that the aldehyde group reacts towards sulfhydryl groups of ribonucleotide reductase and thioredoxin (Schauenstein *et al.*, 1977; Sung *et al.*, 2003; Vollenweider *et al.*, 2003; O'Brien *et al.*, 2005). Derivatised sulfhydryl groups in the active site of the reductase and modified S-S bounds in the thioredoxin lead to an altered structure of the enzyme and enzyme inactivation (Vollenweider *et al.*, 2010).

1.3.2.3 3-Hydroxypropionaldehyde as a precursor for bulk chemicals

The value of 3-HPA as a precursor for other chemicals was mentioned as already in the 1950s (Hall and Stern, 1950). 3-HPA can directly be dehydrated to acrolein, oxidised to 3-hydroxypropionic acid, and reduced to 1,3-PDO. Further, 3-HPA can be indirectly oxidised to acrylic acid with acrolein as intermediate. All these derivates are starting materials for polymers and therefore highly interesting for the chemical industry. As an example, acrylic acid is polymerised into viscosity modifiers and flocculants, and used in the paint industry. 1,3-PDO is a precursor for polytrimethyleneterephthalate (PTT) which has similar properties as Nylon. PTT is commercially available under the trade names Corterra and Sorona and designed for

carpets and special textile fibres (Vollenweider and Lacroix, 2004 ; Willke and Vorlop, 2004).

1.3.2.4 3-Hydroxypropionaldehyde as a cross-linking agent

Tissue fixation, for instance for bioprosthesis (transfer of non-human originated tissues to humans), is needed to reduce the antigenicity and increase the resistance against enzymatic degradation of bioprosthesis. The most commonly used agent for tissue fixation is glutaraldehyde (Chen *et al.*, 2002). Remarkably, 3-HPA is able to crosslink biological tissues and to polymerise hemoglobin (Chen *et al.*, 2002; Sung *et al.*, 2003; Chen *et al.*, 2004). 3-HPA fixated tissue showed comparable strength, degree of cross-linking, and resistance to degradation, but better biocompatibility than glutaraldehyde fixated tissues (Sung *et al.*, 2002; Sung *et al.*, 2003). 3-HPA has also superior sterilising properties of bacterially contaminated tissue compared to glutaraldehyde whereas it was less cytotoxic for mouse-derived 3T3 fibroblasts (Chen *et al.*, 2002). A decreased process speed was the only drawback, but fortunately this enabled accurate control of the cross-linking degree. In conclusion, 3-HPA shows a high potential in replacing glutaraldehyde for these applications.

Plasma-free hemoglobin solutions might act as a blood substitute for the treatment of temporary reduced oxygen-carrying capacity of patient's blood after a trauma or a surgical intervention. Haemoglobin solutions are very attractive because they are highly biocompatible with the human body, have a low risk of viral infections, and can be stored up to one year. However, haemoglobin requires chemical modifications by pyridoxylation with pyridoxal-5'-phosphate followed by polymerisation to reduce its high oxygen affinity and rapid renal excretion. 3-HPA polymerised haemoglobin solution showed excellent properties and may be a new option in the development of blood substitutes (Chen *et al.*, 2004).

1.4 Biological synthesis of 3-HPA

Glycerol dehydratase (glycerol hydrolyase EC 4.2.1.30) catalyses the conversion of glycerol, 1,2-propanediol, and 1,2-ethanediol to 3-HPA, propanal, and acetaldehyde, respectively, with glycerol as preferred substrate and vitamin B_{12} as a cofactor (Abeles *et al.*, 1960; Smiley and Sobolov, 1962). Bacteria possessing a glycerol dehydratase often possess functional very closely related 1,2-diol dehydratases (EC 4.2.1.28) (Daniel *et al.*, 1999). These enzymes catalyse the same reactions, with 1,2-propanediol and 1,2-ethanediol as preferred substrates, but will not be further discussed here.

The pH range of the glycerol dehydratase was determined to be pH 5.5-7.5 and pH 6.5-8 with highest activity at pH 6.0 and pH 7.2 for enzymes isolated from *Lactobacillus* sp. strain 208-A/NRRL B-1720 and *L. reuteri* sp., respectively (Talarico and Dobrogosz, 1990). The K_m values were 3.3 mM for glycerol and 0.3 µM for coenzyme B_{12} (Talarico and Dobrogosz, 1990). A temperature of 28°C was optimal for a glycerol dehydratase from *Citrobacter freundii* (Daniel and Gottschalk, 1992). The effect of temperature on *Lactobacillus*-originated dehydratase, was not described until now.

In *Citrobacter freundii*, glycerol dehydratase consists of three subunits (DhaB, DhaC, DhaD) whose corresponding genes (*dhaB*, *dhaC*, *dhaD*) are genetically linked in chromosome (*dha*-region) together with glycerol oxidising enzymes encoding genes and a 1,3-propanediol dehydrogenase encoding gene. The expression of the *dha* genes is induced under anaerobic conditions after addition of dihydroxyacetone or glycerol. The reported native molecular mass of the glycerol dehydratase is approximately 190 kDa (Schneider *et al.*, 1970; Stroinski *et al.*, 1974; Talarico and Dobrogosz, 1990; Daniel *et al.*, 1999), and SDS PAGE yielded a single major band at 52 kDa and two weak protein bands at 70 kDa and 40 kDa corresponding likely to the three subunits of the glycerol dehydratase (Talarico and Dobrogosz, 1990; Daniel *et al.*, 1999). Two additional proteins (DhaF and DhaG) encoded in the *dha* region were identified to be responsible for the reactivation of the dehydratase by exchanging inactive coenzyme with active in presence of Mg^{2+} under expense of ATP (Seifert *et al.*, 2001).

Conversion of glycerol to 3-HPA involves a radical mechanism with the unique covalent Co-C bond of the coenzyme B_{12}, which bond is stable in aqueous solution. Binding of the substrate and coenzyme to the apoenzyme in the initial step activates the Co-C bond of the apoenzyme forming a Co and an adenosyl radical. Then the adenosyl radical is transferred to the substrate glycerol. Substrate radical is rearranged to product radical by transfer a hydroxylgroup from C-2 to C-3 and subsequent regeneration of the enzyme/cofactor by transfer of a hydrogen atom from adenosyl group to the product (reviewed by Daniel *et al.*, 1999). Radical reactions are sensitive to side reactions leading to irreversible loss of activity due to cessesion of radical cycle (Toraya, 1998; Daniel *et al.*, 1999).

A variety of ions were identified which activate coenzyme B_{12}-dependent glycerol dehydratases (Table 1.5). The *Lactobacillus* glycerol dehydratase requires an univalent cation for activity, NH_4^+ is the most active (Smiley and Sobolov, 1962), whereas K^+ was identified as the most active cation for the *K. pneumoniae* enzyme (Schneider and Johnson, 1971). Mg^{2+} was required for stabilisation of the apoenzyme, and Mg^{2+} and SO_3^{2-} for exchange of the coenzyme with the active form (5,6-dimethylbenzimidazolylcobamide) in *K. pneumoniae* (Schneider *et al.*, 1970).

Table 1.5 Activators of glycerol dehydratase (adapted from Brenda).

Ion	Concentration[a]	Organism[b]	Reference
K^+	5x10^{-4}-2 M	*Lactobacillus* sp., *C. freundii*, *K. pneumoniae*	(Smiley and Sobolov, 1962; Schneider and Johnson, 1971; Toraya and Fukui, 1977)
Mg^{2+}	0-100 (50) mM	*K. pneumoniae*, *K. oxytoca*	(Schneider *et al.*, 1970)
Mn^{2+}	3 mM	*K. pneumoniae*	(Honda *et al.*, 1980)
Co^{2+}	3 mM	*K. pneumoniae*	(Honda *et al.*, 1980)
NH_4^+	0.5-100 mM	*Lactobacillus* sp., *K. pneumoniae*	(Smiley and Sobolov, 1962; Schneider and Johnson, 1971; Toraya and Fukui, 1977)
Rb^+	0-2 M	*Lactobacillus sp.*, *C. freundii*, *K. pneumoniae*	(Smiley and Sobolov, 1962; Schneider and Johnson, 1971)
SO_3^{2-}	0-200 (50) mM	*K. pneumoniae*	(Schneider *et al.*, 1970)

a) Concentration range of the enzyme assay. *Concentration in parenthesis* maximal enzyme activity
b) *Lactobacillus* sp: *Lactobacillus* sp. strain 208-A (NRRL B-1720); *K. pneumoniae*: *Klebsiella pneumoniae*; *K. oxytoca*: *Klebsiella oxytoca*; *C. freundii*: *Citrobacter freundii*

The enzyme of *K. pneumoniae* was inhibited by EDTA, salicylic acid, tetraethylammonium chloride, and the cations Ag^+, Li^+, Na^+ and Hg^{2+} (Smiley and Sobolov, 1962; Schneider *et al.*, 1970; Schneider and Johnson, 1971; Stroinski *et al.*, 1974; Johnson *et al.*, 1975; Honda *et al.*, 1980). (2,3-dihydroxypropyl)cobalamin,

cobeta-2,3-dihydroxypropyl-[1'-O-(4-tolyl)cobamide], and HO-$(CH_2)_n$-cobalamin (chain lengths of n=2-5) showed competition with the coenzyme B_{12} which leads to inhibition of the *K. pneumoniae* enzyme (Poppe *et al.*, 1999). The enzyme of *Lactobacillus* sp. was inhibited by cyanocobalamin and Na^+ (Smiley and Sobolov, 1962). Altogether, conversion of glycerol by a glycerol dehydrates is a complicated reaction with involvement of over 50 genes, a co-factor, and a number of activating or deactivating ions.

Glycerol dehydratases were detected in *Citrobacter freundii*, *Citrobacter pasteurianum*, *Clostridium butyricum*, *Clostridium pasteurianum*, *Enterobacter agglomerans*, *Escherichia blattae* (Sönke *et al.*, 2004), *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus leichmanni* (Stroinski *et al.*, 1974), and *L. reuteri* (Smiley and Sobolov, 1962). All characterised dehydratases from these microorganisms are coenzyme B_{12}-dependent and closely related (Daniel *et al.*, 1999), with exception of the glycerol dehydratase of *Clostridium butyricum* (O'Brien *et al.*, 2004) and the diol dehydratase of *Clostridium glycolicum* (Hartmanis and Stadtman, 1986), which are not coenzyme B_{12}-dependent. Glycerol dehydratases have been extensively studied in genera of Enterobacteriaceae such as *Klebsiella, Citrobacter*, and *Salmonella*. A homologue of the diol dehydratase coding *pdu* operon of *Salmonella enteritica* serovar Typhimurium was detected in *L. reuteri* DSM 20016 (Sriramulu *et al.*, 2008). No further isofunctional dehydratase could be detected in *L. reuteri* DSM 20016, therefore the responsible enzyme for glycerol conversion, the glycerol dehydratase, is encoded by *pduCDE* within this *pdu* operon (Sriramulu *et al.*, 2008). This was confirmed by mutational analyses of the *pduCDE* genes in strain JCM112 (epigenetic to strain DSM20016) (Morita *et al.*, 2008).

Bacteria harbouring dehydratases might have an advantage under strictly anaerobic conditions due to production of external electron acceptor (3-HPA) or the degradation of 1,2-propanediol in complex media (Honda *et al.*, 1980; Daniel *et al.*, 1999). Production of 1,3-PDO by *L. reuteri* under large colon mimicking conditions was demonstrated in an *in vitro* model of colonic fermentation with immobilised human faeces (Cleusix *et al.*, 2008) and production of 3-HPA and 1,3-PDO was demonstrated *in vivo* in mice (Morita *et al.*, 2008).

3-HPA is normally an intracellular intermediate that is not accumulated but reduced by an NAD^+-dependent oxidoreductase to 1,3-PDO, the end-product of reductive glycerol fermentation (Schütz and Radler, 1984). *L. reuteri* possesses the unique ability not only to produce 1,3-PDO but also to excrete high amounts of free 3-HPA (Doleyres *et al.*, 2005), which enables biotechnological production of 3-HPA using *L. reuteri*.

1.5 Biotechnological production of 3-HPA

1.5.1 Strategies for the production of 3-HPA

Production of organic compounds such as 3-HPA using biocatalysts has several advantages compared to chemical processes (Pérez, 2001). High regio- and stereoselectivity, mild reaction conditions, modest energy input, and reduction of by-products. Biocatalysis can be performed with crude and purified enzymes, mixtures of enzymes, and with intact pro-, eukaryotic, and plant cells.

Biotechnological production with the purified enzyme glycerol dehydratase is not applicable beyond laboratory scale due to rapid loss in activity of the enzyme (Smiley and Sobolov, 1962; Talarico and Dobrogosz, 1990) and the extensive and complex reactivation procedure (Seifert *et al.*, 2001). An excellent alternative for the purified enzyme is the bioconversion of glycerol to 3-HPA using live bacteria (Vollenweider and Lacroix, 2004). Among all tested bacteria, *L. reuteri* ATCC 55730 was shown to excrete high amounts of free 3-HPA (Doleyres *et al.*, 2005). Furthermore, this strain is fast growing (unpublished data), shows tolerance to oxygen (Kandler *et al.*, 1980), and is not pathogen (Valeur *et al.*, 2004), which are beneficial characteristics in the handling of fermentation processes and for the produced 3-HPA, especially when applied in food or medical applications. Therefore, the bioconversion process of glycerol to 3-HPA using live *L. reuteri* ATCC 55730 was addressed in this thesis.

1.5.2 Two-step process

Glycerol bioconversion of *L. reuteri* is dependent on the availability of fermentable carbohydrates (Veiga-da-Cunha and Foster, 1992b; El-Ziney *et al.*, 1998; Sauvageot *et al.*, 2000; Lüthi-Peng *et al.*, 2002a). NADH produced during glucose utilisation, can be reduced to NAD^+ during conversion of 3-HPA to 1,3-propanediol (1,3-PDO) by a NADH-dependent dehydrogenase (1,3-propanediol: NAD^+ oxidoreductase) (Schütz and Radler, 1984; Veiga-da-Cunha and Foster, 1992a). The accumulation of 3-HPA in glycerol solution is favoured when little (proximate glucose to glycerol ratio of 0.33) or no glucose is present (Mills *et al.*, 1954; Serjak *et al.*, 1954; Sobolov and Smiley, 1960; El-Ziney *et al.*, 1998; Sauvageot *et al.*, 2000; Lüthi-Peng *et al.*, 2002a). To minimise conversion of 3-HPA into 1,3-PDO, a two-step process was developed: biomass production in the first step and the subsequent conversion of glycerol by resting cells in glycerol containing media in a second step (Talarico and Dobrogosz, 1989; Lüthi-Peng *et al.*, 2002a). Such a two-step process does not only enable maximum yields of 3-HPA, but also reduces significantly the formation of side-products. This strategy also suppresses undesired fermentation-products such as lactic acid, acetic acid, ethanol produced by growing cells. Optimisation of glycerol bioconversion in the presented thesis targeted this promising two-step method since the highest reported accumulation of free 3-HPA (235 mM) so far was reached with this method (Doleyres *et al.*, 2005).

1.5.3 Free cell cultures

With one exception, studies on 3-HPA accumulation have been performed using free cell cultures (FC). Doleyres *et al.* (2005) and Lüthi-Peng *et al.* (2002b) emphasised the need for a high initial biomass concentration (up to 1.6×10^{10} cfu ml^{-1}) for optimal 3-HPA accumulation and therefore cells were concentrated for the bioconversion step after biomass production. In general, *L. reuteri* cells are cultivated in MRS broth supplemented with 20 mM glycerol. Addition of glycerol increases biomass production and growth rate of *L. reuteri* (Veiga-da-Cunha and Foster, 1992b; El-Ziney *et al.*, 1998). Supplementation with 20 mM glycerol seemed adequate for stimulation of biomass production and growth rate (data not published) as there is

apparently no accumulation of 3-HPA at toxic levels, as occurs at higher glycerol concentration (El-Ziney *et al.*, 1998). Cells were subsequently harvested by centrifugation, washed, and resuspended in a glycerol containing aqueous solution for 3-HPA production (Talarico *et al.*, 1988; Doleyres *et al.*, 2005).

Cell viability needs to be maintained during 3-HPA production to enable repetitive successive incubations (Doleyres *et al.*, 2005). Excreted 3-HPA is toxic for the producer and therefore methods to enhance cell viability need to be developed. Such methods can be based on decreasing 3-HPA toxicity (section 1.3.2.1), increasing tolerance of the producing strain, immediate arresting of 3-HPA in an *in situ* product removal (ISPR) process (section 1.5.4), or protection of the cells by immobilisation (section 1.5.5).

Reactivity and thus toxicity of aldehydes such as 3-HPA is pH dependent and decreases at lower pH (Schauenstein *et al.*, 1977). The effect of pH on 3-HPA accumulation was investigated so far by one study. A low maximal 3-HPA accumulation of 30 mM was obtained at pH 5 using 10^9 cfu ml^{-1} *L. reuteri* ATCC 55730 cells incubated in phosphate buffer at different pH between 4 to 7, however, survival after accumulation was not determined (Lüthi-Peng *et al.*, 2002b). The viability of *L. reuteri* after 2 h of 3-HPA production was significantly higher at 4°C than at 37°C, indicating lower toxicity of 3-HPA at lower temperatures (Doleyres *et al.*, 2005).

1.5.4 *In situ* product removal

In situ product removal (ISPR), often referred as extractive fermentation, is a technique to separate a product immediately from the producing cells. As a consequence the free product does not accumulate in the medium. ISPR aims for the following advantages: avoid inhibition or inactivation of the biocatalyst, achieve product stability by preventing degradation and uncontrolled losses, and facilitate the downstream process (Freeman *et al.*, 1993). Consequently, increased product concentration, increased conversion yield, and increased volumetric productivity can be expected.

Methodology of ISPR can be divided into 5 groups (Stark and von Stockar (2003): evaporation, extraction, permeation, immobilisation, and precipitation (Table 1.6).

Due to the properties of 3-HPA only reactive extraction seems suitable for appropriate ISPR (sections 1.3.1). Chromatography and membrane separation are difficult to scale up, and reduced pressure distillation, gas stripping and pervaporation cannot be applied because of the low volatility of 3-HPA. 3-HPA cannot be extracted from aqueous phase as it is too hydrophilic (solvent or solid phase extraction), nor absorbed on an ion exchanger because it is uncharged. Recrystallisation, a further common purification method, starts with a hot saturated solution, which is cooled until crystallisation of the substance in pure form. This cannot be applied for purification of 3-HPA, because 3-HPA is heat sensitive (Lüthi-Peng *et al.*, 2002b).
3-HPA was successfully derivatised to its less toxic semicarbazone using semicarbazide, resulting in very high production of 621 mM 3-HPA-semicarbazone from 761 mM glycerol in small scale experiments with *Klebsiella pneumoniae* (Vancauwenberge *et al.*, 1990). Although these results are promising, the recovery of 3-HPA from the semicarbazone was neither reported (Slininger and Bothast, 1985; Slininger *et al.*, 1990; Vancauwenberge *et al.*, 1990), nor successful performed in our laboratory (unpublished data). However, the capture of 3-HPA with semicarbazide during bioconversion indicates that high production levels are possible using ISPR technology.

Table 1.6 Overview of *in situ* product removal techniques (Stark and von Stockar, 2003).

ISPR Technique	Needed product characteristics	Examples
Evaporation		
stripping, (vacuum-) distillation, pervaporation, transmembrane distillation	volatile, low molecular weight	ethanol
Extraction		
organic solvent (inclusive perstraction)	hydrophobic	ethanol, flavours
supercritical fluid	hydrophobic, low molecular weight	flavours
reactive (inclusive perstraction)	reactive, charged	organic acids, ethanol
aqueous two-phase	hydrophobic, high molecular weight	enzymes
Permeation		
dialysis	low molecular weight	lactic acid, ethanol
electrodialysis	charged, low molecular weight	lactic acid
reverse osmosis	low molecular weight	ethanol
Immobilisation		
hydrophobic adsorption	hydrophobic	butanol, flavours
ion-exchange	charged	organic acids
affinity adsorption	hydrophobic or charged	proteins
Precipitation	hydrophobic and charged	lactic acid, citric acid

1.5.5 Immobilised cell cultures

Compared to controlled LAB and bifidobacteria free cell cultures, immobilised cell (IC) cultures have been shown to enable growth of high cell density populations, improve resistance to bacterial contamination and bacteriophage attack, enhance plasmid stability, prevent cell washing out during continuous fermentation, and protect cells physically and chemically (Champagne *et al.*, 1994; Doleyres and Lacroix, 2005; Lacroix *et al.*, 2005).

Trauth *et al.* (2001) tentatively explained the phenomenon of increased cell tolerance by a possible modification of the cell membrane and physiology, and by cell proximity in a saturated matrix, which gives protection to the cell membrane. In contrast, the increased tolerance to various environmental stresses reported by Doleyres *et al.* (Doleyres *et al.*, 2004b) when cells were immobilised was not associated with cell or strain specific mechanisms or physical protection by cell contact and high density in the gel matrix, since in this study tolerance was measured with released cells and for different stresses and strains. This result suggests that the non-specific increase in cell resistance during long-term continuous IC fermentations might be partly due to a reversible increase in thickness, structural and compositorial changes of the cell membrane, as proposed by Trauth *et al.* (2001) to account for an increased tolerance to quaternary ammonium sanitizers of IC.

Improved tolerance to freeze-drying, oxygen peroxide, simulated gastro-intestinal conditions, nisin, and antibiotics of free cells produced in the effluent medium of continuous IC cultures might be explained by non-specific stress adaptation (Lacroix *et al.*, 2005). Growing IC are exposed to steep inhibitory product, pH and biomass gradients in colonised beads (Masson *et al.*, 1994; Cachon *et al.*, 1998; Schepers *et al.*, 2002a; Schepers *et al.*, 2002b; Doleyres *et al.*, 2004a) due to diffusional limitations of both substrates and inhibitory products, in this case lactic and acetic acids, which can induce non-specific stress adaptation (Lacroix *et al.*, 2005).

IC technology seems well suited for high 3-HPA production since 3-HPA accumulation is dependent on the biomass concentration and cell viability, which is negatively influenced by accumulated 3-HPA. Furthermore, no centrifugation steps are required for medium exchange.

Maximal 3-HPA accumulation of approximately 85 mM after 4-8 h and a maximum initial conversion rate of 50 mM h^{-1} during 1 h were reported with alginate immobilised *L. reuteri* NRRL B-14171 (DSM 20016) cells. These values were significantly higher than values obtained with free cells (maximal 3-HPA accumulation of 80 mM and initial conversion rate of 28 mM h^{-1}, respectively) (Zamudio-Jaramillo *et al.*, 2009). Unfortunately, cell viability was only determined at the beginning of glycerol bioconversion. However, this study suggests that immobilised cell technology has a high potential for biotechnological 3-HPA production at increased rate.

1.6 Aims and Objectives

3-HPA has some characteristics that are interesting for food and chemical industry, as well as in medical applications (Vollenweider and Lacroix, 2004). 3-HPA is commercially not available, but a promising approach to its availability seems the biotechnological production from the renewable raw material glycerol under mild conditions at a high yield. However, biological synthesis of 3-HPA from glycerol requires various factors from coenzyme B_{12} to cations, reactivation proteins and energy as ATP (Talarico and Dobrogosz, 1990; Seifert *et al.*, 2001), thus it is not feasible to perform the synthesis with the purified enzyme glycerol dehydratase. The problem was solved by intact and live *L. reuteri* ATCC 55730 cells in a two-step process (Lüthi-Peng *et al.*, 2002b; Doleyres *et al.*, 2005), but the optimisation of this process is tricky. It is known that biotechnological production of antimicrobial agents such as 3-HPA is often limited by the inhibitory or toxic effects of the product on the biocatalyst. However, cell viability needs to be maintained during 3-HPA production to enable repetitive successive incubations (Doleyres *et al.*, 2005). This thesis addresses the potential of decreasing 3-HPA toxicity, increasing tolerance of the producing strain, immediate arresting of 3-HPA in an *in situ* product removal (ISPR) process, and protection of the cells by immobilisation.

1.6.1 Hypotheses

Three hypotheses were formulated for this study:

1. 3-HPA production is increased by addition of cofactors of the glycerol dehydratase, glucose, and oxidative cell protectors to the bioconversion step as well as variation of pH.
2. Cell immobilisation leads to high and efficient 3-HPA production because of high cell density and cell protection.
3. *In situ* product removal decreases the toxicity of 3-HPA towards producing *L. reuteri* cells leading to an increased 3-HPA production and eases purification of 3-HPA.

1.6.2 Aim of this study

The general objective of this work is to optimise 3-HPA production process based on the current two-step bioconversion of glycerol using the food-grade lactic acid bacterium *L. reuteri* ATCC 55730 and to develop a novel purification of 3-HPA based on reactive extraction.

1.6.3 Specific objectives

The specific objectives of the work are:

1. Optimisation of fermentation conditions for optimal 3-HPA production using the two-step process with free cells by studying effects of pH, glucose concentration, cofactors of the enzyme glycerol dehydratase, biomass concentration, and antioxidant supplement (chapter 2).

2. Development of a novel biotechnological production process for 3-HPA using immobilised *Lactobacillus reuteri* cells with exhibiting high resistance to 3-HPA toxicity based on the two-step process with free cells. Therefore, different fermentation conditions for biomass production and glycerol conversion

(temperature, incubation time, cyclic incubations, glycerol concentration) will be studied (chapter 3).

3. Development of an efficient affinity like purification step for 3-HPA
   Different ligands will be tested for their ability to selectively trap and release 3-HPA. A downstream process to release and separate 3-HPA from the ligand will be developed (chapter 4).

4. Combination of the optimised fermentation process parameters with the affinity-like purification process for developing an efficient *in situ* product removal process for 3-HPA. The affinity-like purification step will be combined with the fermentation process in order to decrease the toxicity of 3-HPA and increase the product yield (chapter 4).

2 Effects of pH, glucose, and cofactors on 3-hydroxypropionaldehyde production in a two-step process using high biomass concentration of *Lactobacillus reuteri*

Data presented in this chapter were submitted for publication with the following authors: D. P. Rütti, S. Vollenweider, X. Mauler, A. Kunert, C. Lacroix.

2.1 Abstract

3-Hydroxypropionaldehyde (3-HPA, reuterin) is a broad-spectrum antimicrobial substance produced by probiotic *Lactobacillus reuteri* from glycerol. The involved enzyme glycerol dehydratase needs a complex environment for activity: vitamin B_{12} as coenzyme, a univalent cation, activating proteins, and energy as ATP. Highest 3-HPA accumulation was previously achieved in a two-step process using whole cells as biocatalyst. The aim was to optimise 3-HPA production at high biomass concentration of *L. reuteri* ATCC 55730. Effects of the ions Na^+, K^+, NH_4^+, Mg^{2+}, Mn^{2+}, Ca^{2+} and corresponding anions (Cl^-, HPO_4^{2-}, SO_4^{2-}), vitamin B_{12}, the detoxification peptide glutathione, physiological solution, glucose, and pH were tested on glycerol bioconversion and *L. reuteri* viability.

We showed that native glycerol solution was optimal for 3-HPA production at 20°C using high initial biomass concentration of *L. reuteri* (about $1x10^{10}$ cfu ml^{-1}) under anaerobic conditions. Supplements, added buffer, and glucose did either significantly decrease maximum 3-HPA concentration or increase the amount of undesired by products such as 1,3-propanediol and lactic acid. 3-HPA accumulation could be further optimised by providing glucose and glutathione during the usual wash step prior bioconversion.

2.2 Introduction

3-hydroxypropionaldehyde (3-HPA), also referred as reuterin, shows a broad inhibition range towards microorganisms including bacteria, yeasts, moulds, and protozoa (Dobrogosz *et al.*, 1989; El-Ziney and Debevere, 1998; Cleusix *et al.*, 2007). Among these are human pathogenic and food spoilage organisms such as *Listeria*, *Staphylococcus*, or *Salmonella*, making 3-HPA interesting for health care and food industry (Vollenweider and Lacroix, 2004). Its potential as precursor for bioplastics was described as early as 1950 (Hall and Stern, 1950). 3-HPA is currently not commercially available.

A promising approach is the biotechnological production from glycerol catalysed by a cobamide-dependent dehydratase under anaerobic conditions. Related glycerol dehydratases of *Lactobacillus reuteri* (*L. reuteri*) are dependent on coenzyme B_{12} such as 5,6-dimethylbenzimidazolylcobamide (Smiley and Sobolov, 1962; Daniel *et al.*, 1999) and are activated by the following cations: Na^+, K^+, NH_4^+, Mg^{2+}, Mn^{2+}, Ca^{2+} (Smiley and Sobolov, 1962; Schneider *et al.*, 1970; Schneider and Johnson, 1971; Toraya and Fukui, 1977; Honda *et al.*, 1980). These dehydratases are furthermore quickly deactivated during bioconversion and reactivated by two specific proteins in presence of Mg^{2+} under expense of ATP (Seifert *et al.*, 2001). Highest 3-HPA accumulation (235±3 mM) was achieved in a two-step process with whole bacteria of *L. reuteri* ATCC 55730 as biocatalyst (Doleyres *et al.*, 2005).

The influence of activating ions was not yet investigated and the effect of energy/glucose and pH on 3-HPA accumulation was determined at low biomass concentration and low 3-HPA accumulation (< 80 mM) (Lüthi-Peng *et al.*, 2002a; Lüthi-Peng *et al.*, 2002b). According to Lüthi-Peng *et al.* (2002b), different bioconversion media showed different effects on 3-HPA accumulation at different biomass concentrations. The influence of glucose and pH on 3-HPA production, therefore, has to be tested using high biomass concentration of *L. reuteri*.

Effects of the ions Na^+, K^+, NH_4^+, Mg^{2+}, Mn^{2+}, Ca^{2+} and corresponding anions (Cl^-, HPO_4^{2-}, SO_4^{2-}) were tested together with vitamin B_{12}, glucose, physiological solution (Baumgart and Becker, 2004), and the tripeptide glutathione (GSH) which plays a

role in detoxification processes in cells (Nunoshiba and Yamamoto, 1999) on 3-HPA accumulation and *L. reuteri* survival during bioconversion.

The present study focused on the impact of enzyme cofactors, pH, glycerol and glucose concentration, physiological solution, and GSH on 3-HPA accumulation at high biomass concentration of resting *L. reuteri* cells (about 10^{10} cfu ml^{-1}) for high 3-HPA production.

2.3 Material and Methods

2.3.1 Bacterial strain and inoculum preparation

The stock culture of *Lactobacillus reuteri* (*L. reuteri*) ATCC 55730 (SD2112), obtained from Biogaia (Stockholm, Sweden), was kept at -80°C in a solution containing 10% (w/v) glycerol and 6% (w/v) skim milk powder (Emmi, Lucerne, Switzerland). Bacteria were grown in MRS medium (Biolife, Milan, Italy) at 37°C without pH control and shaking. For inoculum preparation, 1% (v/v) of the stock culture was propagated in 10 ml MRS medium and incubated for 15 h.

2.3.2 Reagents and materials

All reagents were purchased from Sigma-Aldrich (Buchs, Switzerland) at ≥ 98.5% purity unless otherwise specified. Glycerol had ≥ 98.0% purity. All solutions were autoclaved at 121°C for 20 min.

2.3.3 Biomass production

Biomass was produced according to Vollenweider *et al.* (2003). The activated stock culture inoculated (1% (v/v)) 40 ml MRS medium containing 20 mM glycerol (MRS^+) and was incubated at 37°C. This 40 ml culture was added to 1 litre of fresh MRS^+ after 8 h and incubated for further 16 h to obtain the preculture. Cells of the preculture were then harvested by centrifugation (3000 *g*, 10 min, 20°C), washed with

1 litre potassium phosphate buffer (Kpi 0.1 M, pH 7.0, 20°C), centrifuged, and used immediately for 3-HPA production.

2.3.4 Bioconversion of glycerol to 3-HPA

3-HPA was produced according to Doleyres *et al.* (2005). The cell pellet from biomass production step (1 litre) was suspended in total 100 ml glycerol solution for bioconversion to 3-HPA. Bioconversions (2 ml) were performed in 24-well tissue culture test plates (Orange Scientific, Braine-l'Alleud, Belgium) in triplicates at 20°C. After 2 h, 3-HPA was quantified using a tryptophan test adapted for small volumes as described below. Bioconversion was carried out anaerobically (AnaeroGen, Oxoid, Pratteln, Switzerland). Viable cells were determined at the beginning and the end of bioconversion.

2.3.5 Glycerol bioconversion after washing with glucose-supplemented buffer

Cells from two combined 1-litre precultures (section 2.3.3) were split equally into four groups and harvested by centrifugation. Cells of two groups were suspended in 500 ml Kpi^+ each (Kpi supplemented with 100 mM glucose, 1 mM glutathione, 1 mM $MgCl_2$) and were incubated for 30 min at 20°C (washing). Cells of the other two groups were suspended in 500 ml Kpi and incubated accordingly. After, the cells of each group were harvested, resuspended in 50 ml 250 mM glycerol solution, and incubated for 45 min at 20°C (production) within its group. After the first production, cells of each group were harvested, washed accordingly in either Kpi or Kpi^+, and finally reincubated in fresh 50 ml 250 mM glycerol solution for 45 min. 3-HPA was quantified by HPLC after each production step and viable cells were determined at the beginning of the first and at the end of each 3-HPA production step.

2.3.6 Specific 3-HPA productivity per viable cell

Cells from a 1-litre preculture (section 2.3.3) were split into four groups in duplicate (250 ml, 125 ml, 75 ml, and 50 ml) and harvested by centrifugation. Cells of each

group were suspended in the same amount of Kpi^+ and were incubated for 30 min at 20°C (washing). After incubation, cells of the first duplicate were harvested, resuspended in 25 ml 250 mM glycerol solution, and incubated for 2 h at 20°C (production). Cells of the second duplicate were harvested, resuspended in 25 ml 500 mM glycerol solution, and incubated for 2 h accordingly. 3-HPA was quantified by HPLC after 2 h of incubation and viable cells were determined at the beginning of the production step. Specific productivity of 3-HPA was calculated by dividing the accumulated 3-HPA by the initial viable cell concentration of *L. reuteri*.

2.3.7 Influence of added compounds, glucose and glycerol concentrations, and pH

Different compounds were tested separately for their influence on 3-HPA production from 400 mM glycerol. These were potassium chloride (100 mM, KCl), sodium chloride (100 mM, NaCl; Mallinckrodt Baker, Phillipsburg, NJ), dipotassium phosphate (50 mM, K_2HPO_4), disodium phosphate (50 mM, Na_2HPO_4 (½) and 100 mM, Na_2HPO_4; Merck, Darmstadt, Germany), dipotassium sulfate (50 mM, K_2SO_4), magnesium sulfate (100 mM, $MgSO_4$), magnesium chloride (100 mM, $MgCl_2$), manganese sulfate (100 mM, $MnSO_4$), calcium chloride (100 mM, $CaCl_2$), diammonium phosphate (50 mM, $(NH_4)_2HPO_4$), diammonium sulfate (50 mM, $(NH_4)_2SO_4$), glutathione (100 µM, GSH), vitamin B_{12} (100 µM, B_{12}), B_{12}+GSH (100 µM B_{12} supplemented with 100 µM GSH), physiological solution (100 or 145 mM NaCl with 1 g l^{-1} peptone from casein; Merck; Baumgart and Becker, 2004). The concentrations were assumed according to the concentration of washing buffer prior glycerol bioconversion (0.1 M Kpi; Lüthi-Peng *et al.*, 2002a; Lüthi-Peng *et al.*, 2002b; Doleyres *et al.*, 2005) because only limited data was available for enzyme assays with the glycerol dehydratase of *L. reuteri* (Smiley and Sobolov, 1962; Talarico and Dobrogosz, 1990).

Vitamin B_{12} concentration was adjusted according to the coenzyme concentration used in glycerol dehydratase enzyme assay (Talarico and Dobrogosz, 1990; Seifert *et al.*, 2001) and GSH was added in the same amount. In this experiment, bioconversion was carried out under aerobiosis. A mixture of physiological solution (100 mM

NaCl), glucose (40 mM), vitamin B_{12} (100 µM), and $MgCl_2$ (100 mM) was added to bioconversion to test synergism.

Glycerol (0, 200, 400 mM; Doleyres *et al.* 2005) and glucose (0, 20, 40 mM; Lüthi-Peng *et al.* 2002a) were tested in a full factorial design for their effects on 3-HPA production. The influence of pH (4, 5, 6, 7) and glucose (0, 20, 40 mM) was tested in a full factorial design in a citrate-phosphate buffer (stock solutions of 0.2 M citrate and 0.4 M phosphate, McIlvaine, 1921) in 400 mM glycerol solution. The control bioconversion without buffer was adjusted to pH 4 with 5 M NaOH prior incubation.

2.3.8 Metabolite analyses

2.3.8.1 High throughput 3-HPA quantification

3-HPA was quantified using colorimetric method tryptophan test (Circle *et al.*, 1945; Lüthi-Peng *et al.*, 2002b) adapted for high sample throughput and small sample volume. A 100 µl sample was mixed with 76 µl tryptophan solution (Sigma-Aldrich, 10 mM in 0.05 M HCl stabilized with 2.5 ml l^{-1} toluene; Mallinckrodt Baker) and 300 µl concentrated hydrochloric acid (37% (w/w)) in a 96-deepwell microplate (2 ml, Life Systems Design, Merenschwand, Switzerland). 3-HPA containing samples were diluted with distilled water in the concentration range of the standard curve prior mixing with reagents. The microtiter plate was incubated in a water bath for optimal heat transfer for 20 min at 37°C. Extinction was read at 560 nm (PowerWave SX, Biotek Vermont, USA) in a 96-well tissue culture test plate (Orange Scientific) after additional five minutes of incubation at 37°C in the photometer. Release of HCl vapour was avoided by sealing the microplate covers with Parafilm (Pechiney Plastic Packaging, Chicago IL). Standard curves were recorded with known amounts (0-5 mM) of freshly distilled acrolein. Analyses were performed in quadruplicates.

2.3.8.2 Quantification of glycerol, 1,3-propanediol, glucose, lactic acid, ethanol, and 3-HPA

Glycerol, 1,3-propanediol, glucose, lactic acid, ethanol, and 3-HPA were determined by HPLC (LaChrom HPLC system, Merck Hitachi, Dietikon, Switzerland).

Separation was performed on a Biorad Aminex HPX-87H column (300 x 7.8 mm; Bio-Rad Laboratories AG, Reinach BL, Switzerland) with 10 mM sulphuric acid (HPLC grade) at a flow rate of 0.6 ml min^{-1} and temperature of 40°C. Lactic acid was measured at 214 nm (diode array detector, DAD), all other compounds by refractive index detector (RI). Glycerol has the same retention time than lactic acid, but the lactic acid contribution to glycerol peak could be subtracted using lactic acid concentration quantified with DAD at 214 nm. Quantification was performed with external standards of known amounts of commercially pure substances except 3-HPA which was purified in our laboratory (Vollenweider *et al.*, 2003). Samples were centrifuged immediately at 14000 *g* for 5 min after sampling and stored at -20°C. Analyses were performed in duplicates.

2.3.9 Viable cell determination

Viable cell counts of *L. reuteri* were determined by plating serially diluted samples with physiological solution on MRS agar (Biolife MRS with 15 g l^{-1} agar; Becton Dickinson AG, Allschwil, Switzerland), followed by an anaerobic incubation in jars using AnaeroGen (Oxoid, Pratteln, Switzerland) for 48 h at 37°C. Reported data are means of duplicate analyses.

2.3.10 Statistical analyses

ANOVA was performed using the general linear model of SPSS. A full factorial design with multiple independent and dependent variables was analysed using MANOVA. If the null hypothesis of equal population means was rejected, multiple pairwise comparisons were performed on the group means by Tukey-HSD-tests. Multicollinearity was detected by Spearman's correlation analysis. P-values lower than 0.05 were considered as significant. All error indicators are standard errors of at least duplicate independent repetitions. Outliers were identified by box plots. All statistical analyses were calculated by SPSS (Version 16.0, SPSS, Chicago IL, USA).

2.4 Results

2.4.1 Effects of enzyme cofactors on 3-HPA production

The influence of salts, physiological solution, vitamin B_{12}, and GSH on the production of 3-HPA by *L. reuteri* in 400 mM glycerol solution on 3-HPA production was tested in microtiter plates (2 ml) with $2.4x10^{10}$ cfu ml^{-1} *L. reuteri* cells at 20°C during 2 h under aerobiosis. None of the supplements increased significantly ($P > 0.05$) 3-HPA production at the tested concentrations compared to the control (61±8 mM, Figure 2.1).

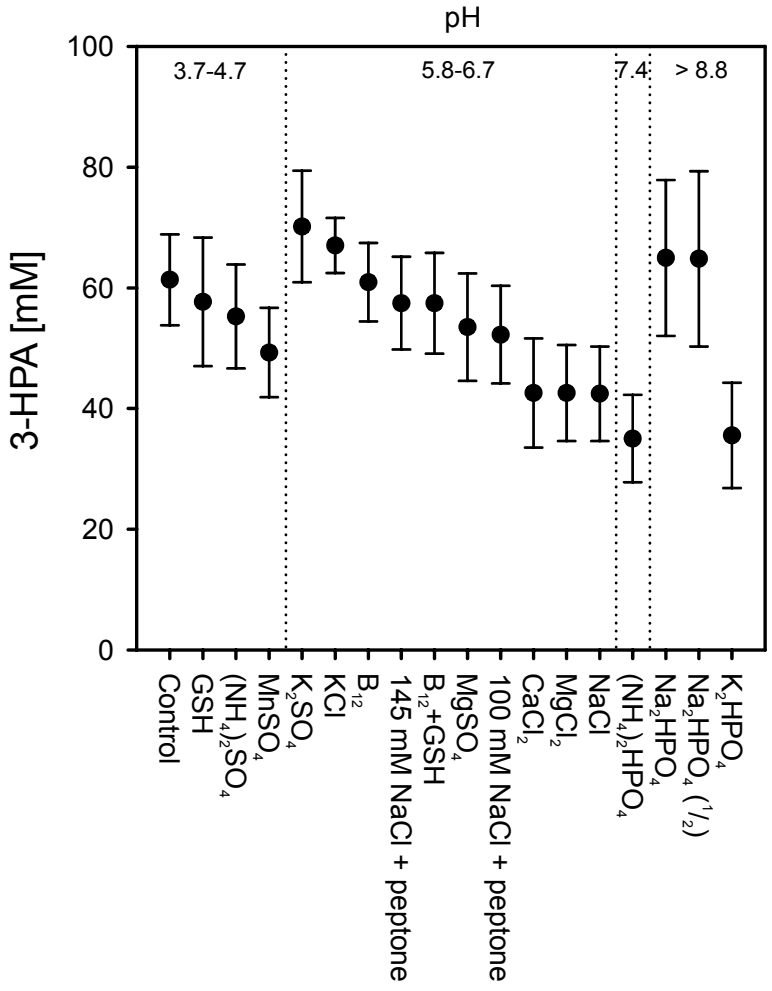

Figure 2.1 3-HPA production using $2.4x10^{10}$ cfu ml^{-1} *L. reuteri* cells after 2 h incubation at 20°C in the presence of supplements in 2 ml of 400 mM glycerol solution. Supplements are grouped by the initial pH. Bioconversion was carried out under aerobiosis. *Error bars* show standard errors of four experiments.

2.4.2 Influence of anaerobiosis on 3-HPA production

The low 3-HPA concentration of the control of 61±8 mM in the previous experiment in 2 ml under aerobiosis (Figure 2.1) could be increased to 191±8 mM (Figure 2.2) by incubation in a jar under low oxygen atmosphere using AnaeroGen.

2.4.3 Effect of glucose concentration and pH on 3-HPA accumulation

Bioconversion of 400 mM glycerol was studied in presence of glucose (0, 20, and 40 mM) at different pH (4-7) in citrate-phosphate buffer during 2 h production at 20°C and initial biomass concentration of $4.6\pm1.1x10^{9}$ cfu ml^{-1}. Two controls without buffer were included in the design. The first control (no glucose) was adjusted to pH 4 with NaOH prior incubation, and the second control contained glucose (0, 20, and 40 mM) without pH adjustment at all. No effect ($P > 0.05$) of pH and glucose on 3-HPA production in buffered glycerol solution was observed (Figure 2.2). A significant lower 3-HPA production ($P < 0.01$) was found for buffered glycerol conversions (mean 97±5 mM) in relation to the adjusted control (pH 4, 187±3 mM). Highest 3-HPA production (191±8 mM, $P < 0.05$) was achieved without buffer or glucose. Any addition of glucose decreased the 3-HPA yield to mean equivalent productions of 163±2 and 152±6 mM 3-HPA with 20 and 40 mM glucose, respectively. 3-HPA productions in unbuffered and not pH-adjusted glycerol solution showed after 2 h an end-pH of 4.1±0.1 and 3.3±0.1 without glucose and in presence of glucose, respectively, independently ($P > 0.05$) of initial pH. Bioconversion of 200 mM unbuffered glycerol solution showed no significant influence ($P > 0.05$) on 3-HPA accumulation in presence of glucose compared to 400 mM unbuffered glycerol solution. The two controls without glucose were not significantly ($P > 0.05$) different.

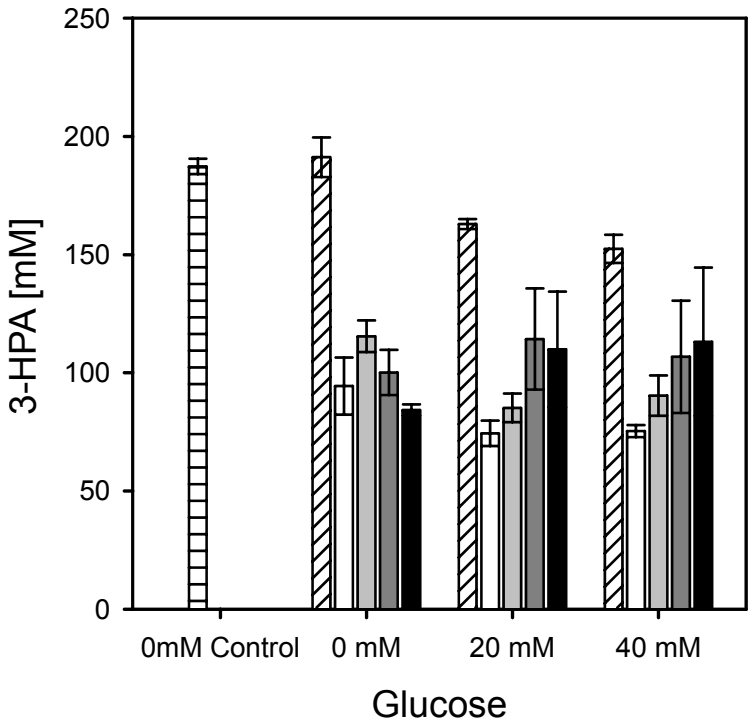

Figure 2.2 3-HPA production at different pH and glucose concentrations after incubation of $4.6\pm1.1x10^9$ cfu ml^{-1} for 2 h in 400 mM glycerol at 20°C. pH was controlled by a citrate-phosphate buffer (0.2 M citrate, 0.4 M phosphate). *Empty* pH 4, *light grey* pH 5, *grey* pH 6, and *black bars* pH 7. Unbuffered controls: *Horizontally shaded* pH adjusted to pH 4 prior production, *diagonally shaded* no pH control. *Error bars* are standard errors of three experiments.

L. reuteri viability was not affected significantly ($P > 0.05$) by glucose and pH in 400 mM glycerol at 20°C. Cell concentration of unadjusted control without glucose after 2 h incubation was determined to $3.0\pm1.0x10^9$ cfu ml^{-1}. This corresponds to a survival of 82±27%, which was not significantly ($P > 0.05$) higher to those in presence of 20 and 40 mM glucose, namely 28±2% and 53±19%, respectively. In buffered glycerol solution, the viability after bioconversion of all pH was determined to $2.2\pm1.1x10^9$ cfu ml^{-1} not differing ($P > 0.05$) from initial biomass concentration due to low accumulated 3-HPA concentration.

1,3-propanediol (1,3-PDO) and ethanol productions were significantly ($P < 0.01$) influenced by glycerol and glucose concentrations during bioconversion (Table 2.1). Lactic acid production was only produced when at least 20 mM glucose was present ($P < 0.01$) and was independent of initial glycerol ($P > 0.05$) concentration. Mean lactic acid production from 20 or 40 mM glucose was determined to be 14.0±0.9 mM. 1,3-PDO was not detected in absence of glycerol, in contrast, 16.4±0.8 mM 1,3-PDO

was produced in glycerol solution (200 and 400 mM) without glucose. Highest 1,3-PDO (26±2 mM) accumulation was observed when both glucose and glycerol were present, independently of their amount ($P > 0.05$). Ethanol (12.9±3.1 mM calculated for the two occurrences) was only produced in presence of glucose without glycerol (Table 2.1).

The molar sum of products (3-HPA and 1,3-PDO) should theoretically equal the amount of used glycerol. Table 2.1 displays molar product sums exceeding used glycerol. This might be explained by different quantification methods of glycerol and 1,3-PDO (HPLC), and 3-HPA (tryptophan test).

Table 2.1 Use of glycerol and glucose, and yields of 3-HPA, 1,3-propanediol, lactic acid, and ethanol during glycerol bioconversion at 20°C with $4.6\pm1.6\times10^{9}$ cfu ml^{-1} *L. reuteri* cells. *Error indicators* are standard errors of duplicate experiments.

Initial glycerol [mM]	Initial glucose [mM]	Used glycerol [mM]	3-HPA (trp test) [mM]	1,3-propanediol [mM]	Yield HPA/glycerol [mol %]	Sum of products [mM]	Used glucose [mM]	Lactic acid (214 nm) [mM]	Ethanol [mM]
0	0	0±1	1±1[a]	< 1.4[1a]	-	1±1	0.0±0	< 2.2[1a]	< 1.3[2]
200	0	160±19	182±1[b]	16.4±0.7[b]	114±16	198±10	0.0±0	< 2.2[1a]	< 1.3[2]
400	0	208±61	191±8[b]	16.4±0.8[b]	92±33	207±20	0.0±0	< 2.2[1a]	< 1.3[2]
0	20	0±0	0±1[a]	< 1.4[1a]	-	1±0	16.4±3.6	15.7±4.3[b]	12.2±4.6
200	20	137±1	145±11[c]	26.2±1.8[c]	106±6	171±10	13.5±1.0	12.6±1.6[b]	< 1.3[2]
400	20	151±1	163±2[bc]	26.0±1.8[c]	108±9	189±25	13.8±0.9	12.4±1.7[b]	< 1.3[2]
0	40	0±0	1±1[a]	< 1.4[1a]	-	2±0	16.4±0.2	17.1±5.6[b]	13.6±5.8
200	40	138±2	154±6[bc]	26.6±1.8[c]	112±5	181±16	12.2±0.8	12.7±1.7[b]	< 1.3[2]
400	40	154±2	152±6[c]	26.0±2.2[c]	99±6	178±17	11.9±1.2	12.5±1.8[b]	< 1.3[2]

1) Below quantification limit of this method; 2) Below detection limit of this method

a), b), c) Values in a row followed by the same letter are not significantly different (MANOVA)

2.4.4 Influence of glucose, B_{12}, physiological solution, and $MgCl_2$ on 3-HPA production

A mixture (2 ml) containing 40 mM glucose, 100 mM $MgCl_2$, 100 µM vitamin B_{12}, physiological solution (100 mM NaCl containing peptone), and 400 mM glycerol was tested anaerobically for 3-HPA production during 2 h at 20°C with an initial biomass concentration of $4.6x10^9$ cfu ml^{-1}. This mixture reduced significantly ($P < 0.05$) the amount of accumulated 3-HPA (121±23 mM) compared to the control in 400 mM glycerol without supplements (209±31 mM 3-HPA). No influence ($P > 0.05$) of the mixture was found on *L. reuteri* viability at 20°C. Cell viability of the control after 2 h incubation was determined to be $3.0±1.0x10^9$ cfu ml^{-1} corresponding to a survival of 82±27%. *L. reuteri* survival in the mixture was determined to 61±15%.

2.4.5 Effect of glucose during washing

Glucose supplementation during bioconversion of glycerol was found to have a negative effect on 3-HPA accumulation. Further investigations were performed to test the influence of added glucose during the washing step on 3-HPA production and cell viability (section 2.3.5) using a cell concentration of $1.9±0.4x10^{10}$ cfu ml^{-1} (Figure 2.3). Washing with Kpi^+ (Kpi with 100 mM glucose, 1 mM GSH, 1 mM $MgCl_2$) enhanced significantly ($P < 0.05$) both 3-HPA production steps compared with the control (Kpi and Kpi/Kpi, respectively) (Figure 2.3). Similarly, 1,3-PDO productions were significantly ($P < 0.05$) increased from 12.7±0.4 to 16.8±0.8 mM and 4.3±0.3 to 9.2±0.5 mM in the first production and in the mean of the second, respectively, by washing with Kpi^+ compared to washing with the control (Kpi and Kpi/Kpi, respectively).

L. reuteri cell viability remained very high after the first and second incubation of Kpi washed cells ($1.3±0.7x10^{10}$ cfu ml^{-1} and $6.3±2.3x10^9$ cfu ml^{-1}, respectively), whereas the viability after two incubations with at least once Kpi^+ washed cells decreased clearly by 1.2 to 2.0 log_{10} (Figure 2.3).

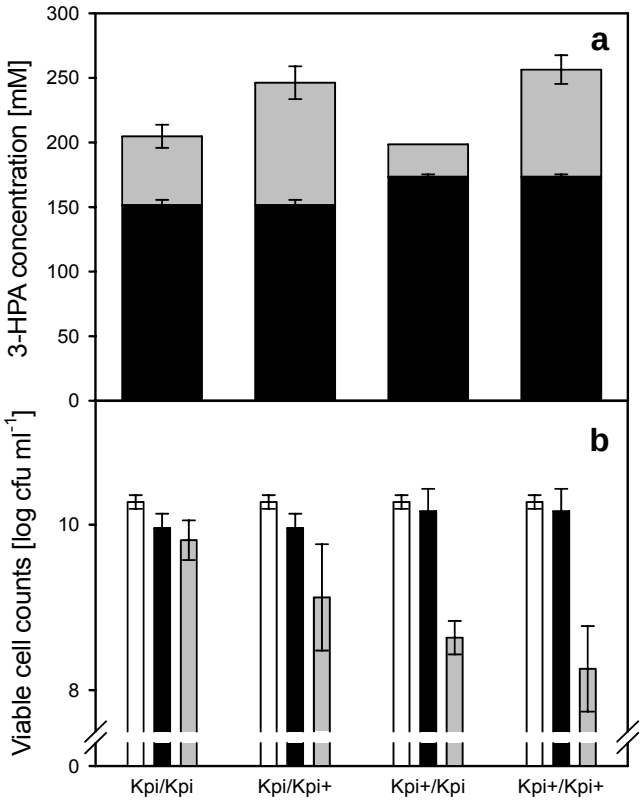

Figure 2.3 3-HPA production (a) during two successive cell incubations (45 min each) at 20°C in 250 mM glycerol after washing (30 min) with either Kpi or Kpi^+ (supplemented with 0.1 M glucose, 1 mM $MgCl_2$, 1 mM glutathione). *Empty* initial biomass concentration: $1.9\pm0.4 \times 10^{10}$ cfu ml^{-1} (b), *filled* after first production (a,b), *grey bars* after second production (a,b). *Error bars* are standard errors of two experiments.

2.4.6 Specific 3-HPA productivity per viable cell

In order to find the optimal cell concentration for 3-HPA production, the specific productivity was determined at different initial biomass concentrations (2.2×10^9, 3.7×10^9, 5.3×10^9, and 1.3×10^{10} cfu ml^{-1}) in 250 and 500 mM glycerol solution at 20°C incubated for 2 h. In 250 mM glycerol, 3-HPA concentration increased with increasing initial biomass concentration (47, 78, 117, and 158 mM, respectively) leading to specific productivities of 21.2, 21.3, 22.4, and 12.1 $\times 10^{-12}$ mol per cell, respectively. In 500 mM glycerol, 3-HPA concentrations showed similar effects, with values of 51, 89, 124, and 191 mM, respectively, leading to slightly higher specific productivities of 22.6, 24.3, 23.6, and 14.6 $\times 10^{-12}$ mol per cell, respectively, compared to 250 mM glycerol.

2.5 Discussion

The aim of the study was to evaluate the effects of enzyme cofactors, bacterial cell protectors, glucose, pH, glucose during washing, *L. reuteri* cell density, and anaerobic conditions on 3-HPA and side-products accumulation, and cell viability. 3-HPA production in a two-step process (Talarico *et al.*, 1988; Lüthi-Peng *et al.*, 2002a) was chosen to minimise carry over from biomass production step to bioconversion and to reduce the complexity of the bioconversion medium. A two-step process was previously used for highest 3-HPA accumulation (Doleyres *et al.*, 2005).

A variety of enzyme cofactors (Na^+, K^+, NH_4^+, Mg^{2+}, Mn^{2+}, Ca^{2+}, and vitamin B_{12}) and survival supporting supplements (physiological solution and GSH) were tested on 3-HPA accumulation. For the glycerol dehydratase from *L. reuteri*, only limited data is available on the requirements of cofactors, pH, and glucose (Smiley and Sobolov, 1962; Talarico and Dobrogosz, 1990; Lüthi-Peng *et al.*, 2002a; Lüthi-Peng *et al.*, 2002b). Concentrations of substances were therefore assumed from 0.1 M Kpi washing buffer. However, the chosen concentrations of $MgCl_2$, $MgSO_4$, and $MnSO_4$ seem far too high when compared to enzyme assays performed with other glycerol dehydratases (Schneider *et al.*, 1970; Schneider and Johnson, 1971; Toraya and Fukui, 1977; Honda *et al.*, 1980) and MRS medium (de Man *et al.*, 1960). A concentration range between 1 and 3 mM would have been sensible. Concentration of NH_4^+ on the other hand seems fine as 50 to 100 mM had been previously used in different enzyme assays (Smiley and Sobolov, 1962; Schneider *et al.*, 1970; Schneider and Johnson, 1971; Toraya and Fukui, 1977).

The amount of accumulated 3-HPA in unsupplemented glycerol solution (Figure 2.1) was low in comparison to concentrations measured in larger volume but otherwise comparable conditions (235 mM, Doleyres *et al.*, 2005). The most obvious factors seem osmotic stress of the high salt and glycerol concentration and limitation production by oxygen.

A too high osmotic stress is unlikely responsible for the low 3-HPA accumulation in this experiment. Lactobacilli can still grow at 4% NaCl content (Gänzle *et al.*, 1998; Neysens *et al.*, 2003; Prasad *et al.*, 2003; Koch *et al.*, 2007) corresponding to an osmolarity of 1.4 Osm l^{-1}. The bioconversion medium containing 400 mM glycerol

and 100 mM salt has an osmolarity of only 0.6 Osm l^{-1}, which is far below the osmotic stress of 4% NaCl.

It is known that the bioconversion of glycerol to 3-HPA requires anaerobiosis (Doleyres *et al.*, 2005). The small test volume (2 ml) and its large surface did not allow anaerobic conditions when incubated under aerobiosis. A high 3-HPA production could be restored when bioconversion was performed under a low oxygen atmosphere using AnaeroGen in successive incubations.

None of the tested substances showed significant enhancing effects on 3-HPA production. Glycerol dehydratase isolated from *Lactobacillus* sp. required an univalent cation for activity, NH_4^+ was the most active (Smiley and Sobolov, 1962), whereas K^+ was identified as the most active cation for an enzyme isolated from *K. pneumoniae* (Schneider and Johnson, 1971). Mg^{2+} was required for stabilisation of the apoenzyme, and Mg^{2+} and SO_3^{2-} for exchange of the coenzyme with the active form in *K. pneumoniae* (Schneider *et al.*, 1970). Vitamin B_{12} is required as coenzyme of the glycerol dehydratase for glycerol conversion (Daniel *et al.*, 1999).

Supplements are therefore dispensable and cofactors are supplied in sufficient amount by the biomass production step in complex MRS medium, or the cells could not use them in starvation. Native glycerol solution is therefore optimal for high 3-HPA production. Reduction in bioconversion medium complexity have also positive effects on raw material costs, and in the adjunct down-stream process and purification of 3-HPA.

Glucose is an important C-source for lactic acid bacteria and used for energy generation (Schlegel, 1972). Speaking in terms of 3-HPA production, energy is needed in the reactivation of the involved enzyme glycerol dehydratase (Seifert *et al.*, 2001). Adding glucose will activate the biocatalyst's metabolism and might support *L. reuteri* to cope with 3-HPA toxicity. This effect was tested indirectly by glycerol bioconversion in presence of glucose.

Data presented in this study showed a significant 18% decrease in 3-HPA production to 157±4 mM and a 27% decrease of used glycerol when glycerol was converted in presence of glucose using high initial biomass concentration (4.6×10^9 cfu ml^{-1}). These findings are in accordance with data published by Lüthi-Peng *et al.* (2002a) that show a 83% reduction from 15 to 2.5 mM 3-HPA in 200 mM glycerol solution containing 20 mM glucose using 4 mg ml^{-1} cell dry weight (corresponding to about 10^9 cfu ml^{-1}

L. reuteri cells) at 37°C. NADH originated from glycolysis activated the dehydrogenase (reviewed by Vollenweider and Lacroix, 2004) and reduced 3-HPA to 1,3-PDO as discussed below. Synergism between single factors in the 3-HPA production was not observed in a glycerol bioconversion medium containing glucose (40 mM), physiological solution, vitamin B_{12} (100 µM), and $MgCl_2$ (100 mM) at 20°C.

In presence of buffer, no significant effects were detected, only a trend towards increased 3-HPA accumulation in presence of glucose at pH 7 was observed. It seems that stimulation of 3-HPA by glucose only occurs under conditions *L. reuteri* is able to grow. Lüthi-Peng *et al.* (2002a) reported a stimulation of glycerol utilisation and 3-HPA accumulation in presence of glucose in MRS medium containing 200 mM glycerol and modified glucose concentrations at 37°C. Glucose also showed a stimulation of 3-HPA accumulation by growing *Lactobacillus collinoides* cells (modified Carr medium, pH 4.8, 28 °C), however, overall 3-HPA production was low with approximately 6 mM (Garai-Ibabe *et al.*, 2008). When pH or temperature was decreased from 4.8 to 3.3 or 28 to 15°C a reduction of *L. collinoides* growth along with a decrease of sugar and glycerol consumption and maximal 3-HPA accumulation was observed. Thus, missing stimulatory effect of glucose on glycerol bioconversion at 20°C in glycerol solution presented in this work might be explained by unfavourable conditions for growth of *L. reuteri*, namely too low temperature and missing growth factors (de Man *et al.*, 1960; Kandler *et al.*, 1980).

Glucose supplement increased significantly the side-products lactic acid and 1,3-PDO, which are not desired for maximum 3-HPA yields and the adjacent down-stream process and purification. Production of lactic acid and ethanol, and their relation to used glucose during glycerol bioconversion is in accordance to phosphoketolase pathway (PKP) of heterofermentative lactobacilli (Arsköld *et al.*, 2008). Energy generation by the PKP pathway yields 1 mol lactic acid and 1 mol acetyl phosphate from 1 mol glucose. Ethanol and acetic acid is then formed from acetyl phosphate dependent on the intracellular NADH level. Thus, ethanol is only formed when no other NADH acceptor such as e.g. glycerol is present (Arsköld *et al.*, 2008). 3-HPA production with starved cells in mere glycerol solution is therefore favoured concerning high 3-HPA accumulation, minimising complexity of bioconversion medium, and minimising by-products.

The limitations of decreased 3-HPA production and increased side-product formation could be overcome by washing the cells with Kpi^+ (Kpi supplemented with 100 mM glucose, 1 mM glutathione, 1 mM $MgCl_2$) prior bioconversion. It was clearly shown that Kpi^+ increased 3-HPA production compared to washing with Kpi, especially in the second successive production. 1,3-PDO production was fairly increased from 3.8 mM to 8.9 mM and is therefore about 3-fold lower than in glycerol-glucose bioconversions. Viability of *L. reuteri* decreased remarkably by 1-2 $\log_{10}$ when the cells were washed at least once in Kpi^+. It is known that bacteria in the stationary phase are more stress resistant than during growth (van de Guchte *et al.*, 2002). This led to the assumption that glucose in the buffer activated *L. reuteri*'s metabolism leading to a decreased tolerance to 3-HPA.

Glutathione (GSH) is a small thiol-related antioxidant molecule that has been suggested to protect cells (Nunoshiba and Yamamoto, 1999). *Escherichia coli* mutants deficient in GSH biosynthesis showed hypersensitivity to the aldehydes acrolein (Nunoshiba and Yamamoto, 1999) and methylglyoxal (Ferguson and Booth, 1998) compared to the wild-type strain. GSH is also involved in lactic acid bacteria in maintaining a reduced intracellular environment and is accumulated from the medium in the cell (Wiederholt and Steele, 1994). The effect of adding GSH to glycerol solution for supporting *L. reuteri* to cope with 3-HPA toxicity was tested indirectly by 3-HPA accumulation. No conclusion could be drawn of directly supplied GSH because it was unintentionally oxidised by presence of air. Results of increased 3-HPA production after washing with Kpi^+, however, suggested that GSH in combination with glucose could detoxify 3-HPA by reaction of the SH group of GSM with the aldehyde.

An important factor related to bacterial growth, survival, and enzyme activity is the pH. Toxicity of aldehydes such as 3-HPA are pH dependent and decrease at lower pH (Schauenstein *et al.*, 1977; Sung *et al.*, 2003). In previous studies the effect of pH in the second step was either not investigated (Vollenweider *et al.*, 2003; Doleyres *et al.*, 2005) or in the range of 4-9 under unoptimised biomass concentrations (Lüthi-Peng *et al.*, 2002a) making a new test design necessary.

No significant pH effects on 3-HPA production using about 10^{10} cfu ml^{-1} *L. reuteri* cells in 400 mM glycerol solution at 20°C was detected, only a trend towards highest activity at pH 5 without glucose and pH 7 with glucose was visible. The presence of

buffer, however, decreased significantly 3-HPA production by 38 mol % in mean and therefore bioconversion is preferably performed in unbuffered glycerol solution.

3-HPA accumulation increased with increasing biomass production in the range from $2.2x10^9$ to $1.3x10^{10}$ cfu ml^{-1} agreeing with published data in 200 mM glycerol at 30°C (Doleyres *et al.*, 2005). Specific productivity of 3-HPA in both glycerol concentrations (250 and 500 mM) as an economic indicator was highest at $4\text{-}5x10^9$ cfu ml^{-1} with $22.4x10^{-12}$ mol per cell in 250 mM glycerol agreeing with published data in 200 mM glycerol using the same biomass concentration ($22.4x10^{-12}$ mol per cell) (Doleyres *et al.*, 2005). Doleyres *et al.* (2005) determined maximum specific 3-HPA productivity at biomass concentrations of $7.0x10^8$ and $2.3x10^9$ cfu ml^{-1} with each 37.4 $x10^{-12}$ mol per cell in 200 mM glycerol. This specific productivity is therefore about 2-fold increased compared to maximum specific productivity presented in this publication, leading to the assumption that a lower biomass concentration would be advantageous. This is explained by the lower accumulation of 86 mM 3-HPA, which has a decreased toxicity. However, this low 3-HPA concentration might decrease the efficacy of the adjacent 3-HPA purification.

To conclude, according to our data, unbuffered, unadjusted, and unsupplemented 400 mM glycerol solution is optimal for highest 3-HPA production. Highest specific productivity of 3-HPA was achieved with a *L. reuteri* concentration of $4\text{-}5x10^9$ cfu ml^{-1}. Current research is ongoing to bind the free 3-HPA *in situ* in order to reduce its toxicity and therefore increase its production.

3 Bioconversion of glycerol to 3-hydroxy-propionaldehyde using immobilised *Lacto-bacillus reuteri* ATCC 55730 cells in a two-step process

Data presented in this chapter were submitted for publication with the following authors: D. P. Rütti, S. Vollenweider, C. Lacroix.

3.1 Abstract

3-Hydroxypropionaldehyde or reuterin is a broad spectrum antibiotic, with cross-linking and polymerisation properties. A two-step biotechnological process for 3-HPA production from glycerol was developed with immobilised food-grade *Lactobacillus reuteri* ATCC 55730 cells. Biomass concentration after continuous colonisation step (mean of D = 0.5 h^{-1} and D = 1.5 h^{-1}) in MRS medium for 24 h was determined to 7.5±2.1x10^{10} cfu g^{-1}.

3-HPA accumulation using immobilised cells showed advantages over free cell productions in a 3.2-fold increased specific productivity of 3-HPA during a 1.5 h incubation at 30°C. Evidence suggests an enhanced survival. Mean maximal 3-HPA production at 30°C after 1.5 h was 182±14 mM 3-HPA using 10% (v/v) bead inoculum colonised with 6.8±0.5x10^{10} cfu g^{-1}. Immobilised cells showed, however, a 4.8-fold lower mean glycerol conversion rate compared to free cells. Using immobilised cells, maximal cumulated 3-HPA amount (316 mM) occurred at 20°C in three 1 h successive incubations with 1.8x10^{11} cfu g^{-1} cells in 360 mM glycerol. 3-HPA accumulation was influenced by initial biomass concentration and temperature, *L. reuteri* viability by accumulated 3-HPA and temperature.

Attempts to recycle beads after bioconversion were unsuccessful. Starvation lead to a reduction in *L. reuteri* viability and diminished growth in successive re-growth step in fresh MRS during 16 h.

3.2 Introduction

Lactobacillus reuteri (*L. reuteri*) is a probiotic lactic acid bacteria (LAB) inhabiting the gastrointestinal tract of humans and some animals (Dobrogosz *et al.*, 1989). In glycerol containing media, it has been shown to produce high amounts of the glycerol derivate 3-hydroxypropionaldehyde (3-HPA) also known as reuterin (Axelsson *et al.*, 1989; Doleyres *et al.*, 2005). This potent antimicrobial has a broad inhibition spectrum towards bacteria, moulds, yeasts, and protozoa, including human pathogenic and food spoilage organisms (Dobrogosz *et al.*, 1989; El-Ziney and Debevere, 1998; Cleusix *et al.*, 2007; Arqués *et al.*, 2008). 3-HPA is furthermore a precursor for the chemical industry, as reviewed by Vollenweider and Lacroix (2004).

3-HPA can be biotechnologically produced from glycerol using *L. reuteri* under mild conditions in a two-step process (Talarico and Dobrogosz, 1989; Lüthi-Peng *et al.*, 2002a; Doleyres *et al.*, 2005). The most important factors influencing free cell (FC) 3-HPA synthesis were initial biomass concentration, temperature, glycerol and glucose concentrations (Lüthi-Peng *et al.*, 2002a; Doleyres *et al.*, 2005). Maximal reported 3-HPA production was 235±3 mM 3-HPA in 400 mM glycerol solution using $1.6x10^{10}$ cfu ml^{-1} *L. reuteri* cells at 30°C after 45 min conversion (Doleyres *et al.*, 2005).

Doleyres *et al*. (2005) emphasised the need for high initial biomass concentration combined with good survival during bioconversion for high 3-HPA accumulation during successive bioconversions. A sharp reduction of biocatalyst's viability, however, was observed during bioconversion with FC, especially above a bioconversion temperature of 23°C (Doleyres *et al.*, 2005). Immobilised cells (IC) technology seems well suited for high 3-HPA production since IC grow to at high cell density and are physically protected. Further reported advantages are cell reutilisation, improved resistance to contamination and bacteriophage attack, enhanced plasmid stability, and prevention from cell wash out during continuous fermentation (Champagne *et al.*, 1994; Doleyres and Lacroix, 2005; Lacroix *et al.*, 2005). Furthermore, no centrifugation steps are required to harvest cells between two conversion steps due to discrete form of biocatalyst.

The aim of this study was to test the potential of IC for 3-HPA production to overcome the limitations of quick loss in viability, centrifugation, and cells not reusable. Therefore, different parameters of the IC conversion were tested on 3-HPA production and productivity, and *L. reuteri* viability. 3-HPA production carried out with FC served thereby as control.

3.3 Material and Methods

3.3.1 Bacterial strain and inoculum preparation

The stock culture of *Lactobacillus reuteri* (*L. reuteri*) ATCC 55730 (SD2112), obtained from Biogaia (Stockholm, Sweden), was kept at -80°C in a solution containing 10% (w/v) glycerol and 6% (w/v) skim milk powder (Emmi, Lucerne, Switzerland). For inoculum preparation, 1% (v/v) of the frozen *L. reuteri* was incubated in 10 ml MRS medium (Biolife, Milan, Italy) at 37°C for 15 h.

3.3.2 Reagents and materials

All reagents were purchased from Sigma-Aldrich (Buchs, Switzerland) at ≥ 98.5% purity unless otherwise specified. Sterile solutions and equipment were autoclaved at 121°C for 20 min.

3.3.3 Immobilisation

L. reuteri cells were immobilised in gellan/xanthan gum polysaccharide beads using a two phase dispersion process (Cinquin *et al.*, 2004). The autoclaved polymer solution was cooled to 45°C, inoculated with a 15 h MRS culture (2% (v/v)), poured into sterile oil (45°C), cooled down to induce gelation. The beads were hardened by soaking in cold (4°C) 0.1 M $CaCl_2$ solution for 30 min. Beads with diameters in the 1.0-2.0 mm range were selected by wet sieving with 4 l sterile 0.1 M $CaCl_2$. Immobilisation was completed in aseptic conditions within 1 h.

3.3.4 Immobilised cell fermentation – Bead colonisation

Bead colonisation was performed in a 500 ml bioreactor (Sixfors, Infors-HT, Bottmingen, Switzerland) containing 210 ml MRS medium with 20 mM glycerol (MRS^+) inoculated with 90 ml freshly prepared beads, and stirred with inclined blade impeller at 150 rpm. The pH was adjusted with 5 M NaOH to 5.5 and the temperature was set to 37°C. Headspace was flushed with N_2 (4 l h^{-1} at 20°C and 1 atm) to ensure anaerobic conditions. Repeated batch and continuous cultures were tested for bead colonisation. After colonisation, beads were washed with 1 litre 0.1 M $CaCl_2$ solution (20°C) and used immediately for bioconversion.

3.3.4.1 Continuous bead colonisation

Immobilised cells (IC) were continuously grown for 24 h in MRS_G (MRS^+ supplemented with 40 g l^{-1} glucose to avoid glucose limitation) after an initial 16 h batch fermentation. Two peristaltic pumps provided a continuous flow of MRS_G medium of 450 ml h^{-1} giving a dilution rate (D) of 1.5 h^{-1} calculated on the total fermentation volume. The effects of the dilution rate (6-h batch followed by 16-h continuous at D = 0.5 h^{-1}), time (24 or 48 h, D = 1.5 h^{-1}), and growth medium (MRS^+ or MRS_G) were tested for optimised bead colonisation.

3.3.4.2 Repeated-cycle batch culture biomass production

Repeated-cycle batch (RCB) fermentations were tested for their potential to reduce medium consumption for bead colonisation. Five RCB fermentations were performed in 350 ml MRS_G medium inoculated with 150 ml beads at pH 5.5 and 37°C. Medium was exchanged aseptically with fresh MRS_G (37°C) using a peristaltic pump within 2-3 min, when no base had been added for 30 min.

3.3.5 Free cell fermentation

Free cell (FC) pH controlled batch cultures were performed according to Doleyres *et al.* (2005). *L. reuteri* cells were grown for 15 h in 500 ml MRS^+ (500 ml bioreactor, Sixfors) at pH 5.5 and 37°C with headspace flushed with N_2. A 1% (v/v) inoculum of

the activated stock culture was used. Cells were then harvested by centrifugation (10 min, 20°C, 3000 *g*), washed with potassium phosphate buffer (0.1 M, pH 7.0, 20°C), and used immediately for glycerol bioconversion.

3.3.6 Viable cell determination

All samples were stored on ice and analysed within 1 h. Samples were serially diluted 10-fold with physiological solution (1 g l^{-1} peptone from casein and 8.5 g l^{-1} NaCl). Viable cell counts of *L. reuteri* were determined by plating out 100 µl of each dilution on MRS agar plates in duplicate followed by an anaerobic incubation for 48 h at 37°C in jars (AnaeroGen, Oxoid, Pratteln, Switzerland). Reported data are means of duplicate analyses. Viable cell count determination of IC in beads (C_w) was performed by dissolving approximately 0.5 g accurately weighed beads in 20 ml EDTA solution (1% (w/v), pH 7.0) using a stomacher (Lab Blender 400, Bury St Edmunds, England) for 5 min, followed by serial dilutions as described before (Cinquin *et al.*, 2004). Cell counts were expressed as colony-forming units (cfu) per ml of fermentation medium or per g (wet weight) gel beads. The volumetric cell concentration of IC was calculated using Equation 3.1:

$$C_v = \rho_{beads} \cdot C_w \cdot \varphi_{beads} \quad (3.1)$$

where C_v is volumetric cell concentration in the reactor (cfu ml^{-1}), C_w the viable cell concentration in beads (cfu g^{-1}), ρ_{beads} the density of the beads (g ml^{-1}) as a function of cell load, φ_{beads} the volume fraction of beads (ml ml^{-1}). The density of colonised beads was calculated by linear equation (Equation 3.2) experimentally established by Arnaud *et al.* (1992b):

$$\rho_{beads} = C_w \cdot 8.37 \cdot 10^{-13} + 1. \quad (3.2)$$

To detect bacterial contamination, a sample was observed under the microscope scanning for cocci-shaped bacteria and plated out on LB agar (37°C, 72 h; BD, Allschwil, Switzerland) on which *L. reuteri* is growing very poorly.

3.3.7 Production of 3-HPA

3.3.7.1 IC bioconversion

3-HPA production with IC after bead colonisation was performed in a 500 ml bioreactor (Sixfors) containing 10% (v/v) beads (wet volume) in 360 mM sterile glycerol solution. Headspace was flushed with N_2 during 3-HPA production. Beads volume was determined in a sterile graduated cylinder by adding 25 ml beads to 100 ml glycerol solution to a total volume of 125 ml. Initial rate of 3-HPA production was calculated from the first data point of the bioconversion.

The influence of temperature (20 and 30°C), biomass concentration (from $6.2x10^8$ to $1.8x10^{11}$ cfu g^{-1}), colonisation method (successive batch and continuous culture), and conversion step (one 3h incubation, 1x3 h; three successive 1 h incubations, 3x1 h) were tested on 3-HPA accumulation and *L. reuteri* viability in 360 mM glycerol solution. 3-HPA samples (2 ml) were withdrawn every half hour (1x3 h) or initially after 5 min and at the end of each successive production step (3x1 h). Approx 0.7 g beads were taken at the beginning and every hour for viable cell determinations. Bead samples were withdrawn aseptically using a sterile 5 ml glass pipette, which was introduced upside down into the fermenter. Experiments were performed in duplicate.

3.3.7.2 Control production with FC

Control 3-HPA productions were carried out in duplicate in a 100 ml Erlenmeyer flask stirred with a magnetic bar according to Doleyres *et al.* (2005). The cell pellet from biomass production step was suspended in either 50 ml 300 mM or 400 mM glycerol solution for its bioconversion to 3-HPA carried out for 2 h at 30°C, with headspace flushed with N_2. The initial biomass concentration was $3.1\pm0.7x10^{10}$ cfu ml^{-1}. The initial rate of 3-HPA production was calculated from the first data point of the bioconversion.

3.3.8 Metabolite analyses

Culture samples were immediately centrifuged at 14000 *g* for 5 min (4°C) after sampling, the supernatant filtered through a 0.45 µm filter, and stored at -20°C.
3-HPA, glycerol, 1,3-propanediol (1,3-PDO), glucose, acetic acid, lactic acid, and ethanol were determined by HPLC (LaChrom HPLC system, Merck Hitachi, Dietikon, Switzerland). Separation was performed on a Biorad Aminex HPX-87H column (300 x 7.8 mm; Bio-Rad Laboratories AG, Reinach BL, Switzerland) with 10 mM sulphuric acid (HPLC grade) at a flow rate of 0.6 ml min^{-1} and temperature of 40°C. Acetic and lactic acid were quantified at 214 nm (diode array detector, DAD), all other compounds by refractive index detector. Acetic acid and 3-HPA have the same retention time with this method. Consequently, 3-HPA could only be quantified in the bioconversion without added glucose, therefore without acetic acid production. Glycerol has the same retention time as lactic acid, but the lactic acid contribution to glycerol peak could be subtracted using lactic acid concentration quantified with DAD at 214 nm. Quantification was performed with external standards of known amounts of commercially pure substances, except for 3-HPA, which was produced and purified on site (Vollenweider *et al.*, 2003). Reported data are means of duplicate independent analyses.

3.3.9 Statistical analyses

ANOVA was performed using general linear model of SPSS. If the null hypothesis of equal population means was rejected, multiple pair wise comparisons were performed on the group means by Tukey-HSD-tests. Multicollinearity was detected by Spearman's correlation analysis. In this study P-values lower than 0.05 were considered as significant. Standard errors of at least duplicate independent repetitions are reported. All statistical analyses were carried out with SPSS (Version 16.0, SPSS, Chicago IL, USA).

The influence of temperature, incubation type, and initial biomass concentration on 3-HPA production was modelled by a linear regression model (Equation 3.3):

$$C_{3\text{-HPA}} = a_0 + a_1 \cdot T + a_2 \cdot Type + a_3 \cdot \log(C_w) \quad (3.3)$$

where $C_{3\text{-HPA}}$ is maximum cumulative 3-HPA production [mM]; a_0, intercept; T, temperature [°C]; *Type*, coded incubation type [-1: 1x3 h; 1: 3x1 h]; log(C_w), $\log_{10}$ of the initial biomass concentration in beads [$\log_{10}$ (cfu g^{-1})]; a_1-a_3, equation coefficients. All non-significant terms ($P > 0.05$) were excluded using stepwise elimination procedure.

3.4 Results

3.4.1 Influence of colonisation step on 3-HPA production

The effect of the biomass production step was tested on 3-HPA production using IC in 360 mM glycerol in three successive bioconversions (3x1 h) using 10% (v/v) beads at 20°C. Mean biomass concentration after continuous bead colonisation was determined to 7.5±0.5x10^{10} cfu g^{-1} beads. Variation of the dilution rate (6-h batch followed by 16-h continuous D = 0.5 h^{-1}), continuous fermentation time (24 or 48 h, D = 1.5 h^{-1}), and growth medium (MRS^+ or MRS_G) showed no effects ($P > 0.05$) on biomass concentration in beads, fermentation profile of the out flow (glucose, lactic acid, acetic acid, ethanol, and 1,3-PDO), and 3-HPA production. Only differences in medium consumption to colonise 90 ml beads were detected: 2.6 litre at a flow rate of D = 0.5 h^{-1} during 16 h, and 11.0 litre MRS medium at D = 1.5 h^{-1} during 24 h.
Colonisation in repeated batch fermentations led to 4.7±0.9x10^{10} cfu g^{-1} beads viable *L. reuteri* cells. A significant lower 3-HPA production ($P < 0.05$, n = 2) was measured with beads colonised in repetitive batches, with 50±9, 34±8, and 8±6 mM 3-HPA for each of the three successive bioconversion steps, than after continuous fermentation with 76±9, 51±4, and 37±5 mM 3-HPA, respectively (Figure 3.1, *circles*). Viable cell counts decreased by 0.4±0.2 $\log_{10}$ and 0.5±0.3 $\log_{10}$ after third bioconversion compared to initial cell concentration using batch and continuous colonised beads, respectively (Figure 3.1, *triangles*). 1,3-PDO accumulation was same ($P > 0.05$) for the different colonisation methods and remained low at levels between 2 and 7 mM.

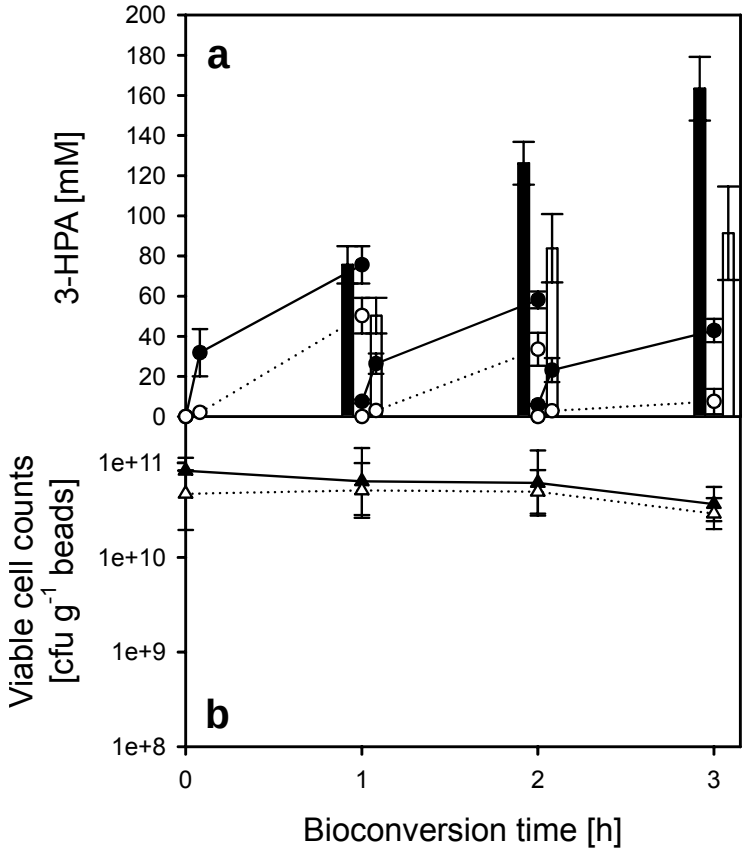

Figure 3.1 Influence of biomass production step on 3-HPA production (a, *circles*) and *L. reuteri* viability (b, *triangles*) during three successive 1 h bioconversions from immobilised cells at 20°C in 360 mM glycerol solution. *Filled symbols*/*solid line* continuous colonisation (mean of D = 0.5 h^{-1} and D = 1.5 h^{-1}), 7.5±0.5x10^{10} cfu g^{-1} *L. reuteri* cells. *Open symbols*/*dotted line* repeated batch colonisation, 4.7±0.9x10^{10} cfu g^{-1}. *Bars* cumulative volumetric 3-HPA production measured for three bioconversion steps. *Error bars* show standard errors of two experiments.

3.4.2 3-HPA accumulation using free and immobilised cells

3-HPA production with FC (3.1±0.7x10^{10} cfu ml^{-1}) at 30°C during 2 h reached after 1 h a maximum concentration of 216±4 and 247±12 mM 3-HPA (n = 2) in 300 and 400 mM glycerol, respectively, and remained unchanged afterwards (Figure 3.2a). Cell viability remained very high for 1 h, but dropped drastically thereafter, and after 2 h no viable cells were detected in 300 mM (detection limit 50 cfu ml^{-1}) and only 1.6x10^{2} cfu ml^{-1} were found in 400 mM glycerol (Figure 3.2b).

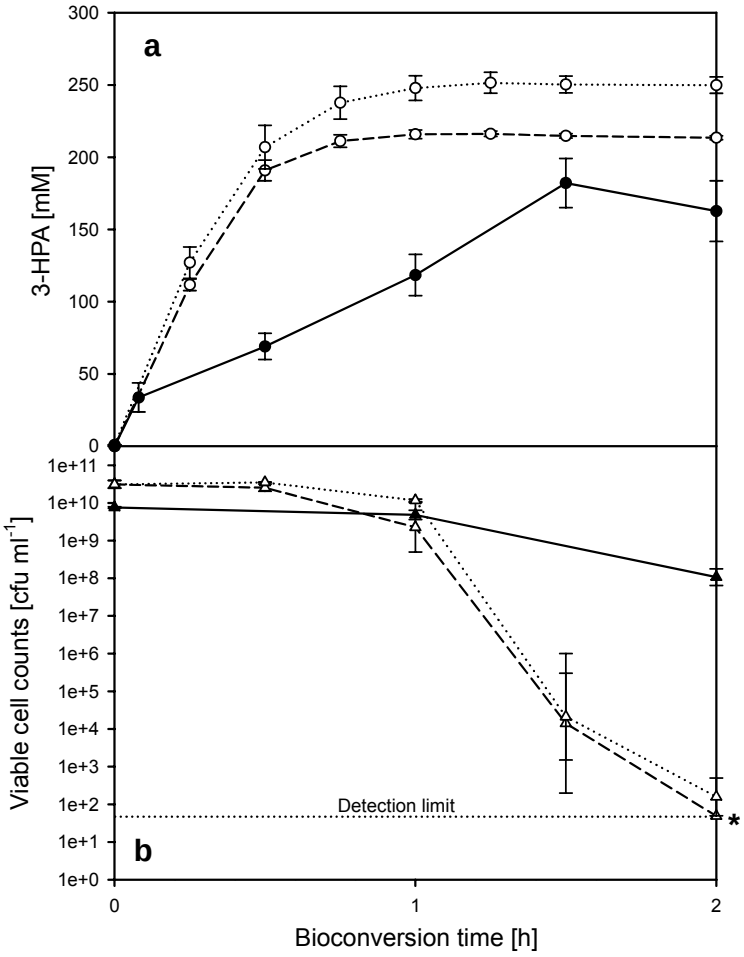

Figure 3.2 3-HPA production (a, *circles*) and cell viability (b, *triangles*) with free (*open symbols*) and immobilised (*filled symbols*) *L. reuteri* cells carried out at 30°C. Initial biomass concentration in beads was 6.8±0.5x10^{10} cfu g^{-1} corresponding to 7.2±0.6x10^{9} cfu ml^{-1} reactor, and FC concentration was 3.1±0.7x10^{10} cfu ml^{-1}. *Dashed line* 300 mM glycerol, *dotted line* 400 mM glycerol, *solid line* 360 mM glycerol. *Asterisk* cell counts are below detection limit. *Error bars* are standard deviations of duplicate experiments.

Immobilised *L. reuteri* cells at a lower concentration of 7.2±0.6x10^{9} cfu ml^{-1} (6.8±0.5x10^{10} cfu g^{-1}) compared to FC in 360 mM glycerol at 30°C produced maximal 3-HPA levels of 182±14 mM 3-HPA after 1.5 h (Figure 3.2a). 3-HPA production rate of FC was maximal at the beginning of the conversion step with 478±26 mM h^{-1} and decreased after 15 min conversion. A similar maximal rate was observed for IC (405±121 mM h^{-1}) although the 3-HPA production rate decreased dramatically after 5 min and remained more or less constant until 1.5 h at 99±16 mM h^{-1}. Specific productivity of 3-HPA using IC is 3.2-fold increased compared to FC with 25.3 and 8.0x10^{-12} mol per cell, respectively.

Cell viability remained stable with IC after 1 h bioconversion and decreased to $1.1x10^{8}$ cfu ml^{-1} after 2 h (Figure 3.2b).

3.4.3 Effect of temperature on 3-HPA production and cell viability

The effect of the temperature on 3-HPA accumulation and *L. reuteri* viability in 360 mM glycerol solution is illustrated for an extended bioconversion (1x3 h, Figure 3.3) and successive bioconversions (3x1 h, Figure 3.4) using 10% (v/v) beads colonised with $6.3\pm2.0x10^{10}$ cfu g^{-1} and $5.6\pm3.2x10^{10}$ cfu g^{-1}, respectively.

During the 3 h bioconversion at 30°C, 3-HPA concentration increased steadily reaching a maximum of 182±14 mM after 1.5 h (Figure 3.3a) and remained stable afterwards (3 h, $P > 0.05$). Cell viability remained stable for 1 h and decreased drastically to $2.5\pm1.6x10^{6}$ cfu g^{-1} after 3 h (Figure 3.3b). At 20°C, 3-HPA was steadily produced during the 3 h production reaching 116±1 mM and cell viability remained high, with only a slight decrease after a bioconversion time of 3 h (0.3 log_{10}).

During the successive 1 h production cycles, 3-HPA production decreased after each incubation cycle, with 3-HPA concentrations of 70±12, 45±3, and 31±6 mM corresponding to cumulated productions of 115±14 and 147±20 mM determined at the end of the second and third cycle, respectively at 20°C (Figure 3.4a). At 30°C, the corresponding 3-HPA productions were increased (first and second cycle) or decreased (third cycle) compared with 20°C, with 115±18, 49±6 mM, and 23±10 mM, respectively. The cumulated productions at 30°C (164±12 and 187±2 mM after the second and third cycle, respectively) were higher than at 20°C. Viable cell counts remained very high during the three incubations at 20°C with a non-significant 0.3 log_{10} reduction in viability, whereas a significant ($P < 0.05$) decrease of 1.8 log_{10} at 30°C was observed (Figure 3.4b).

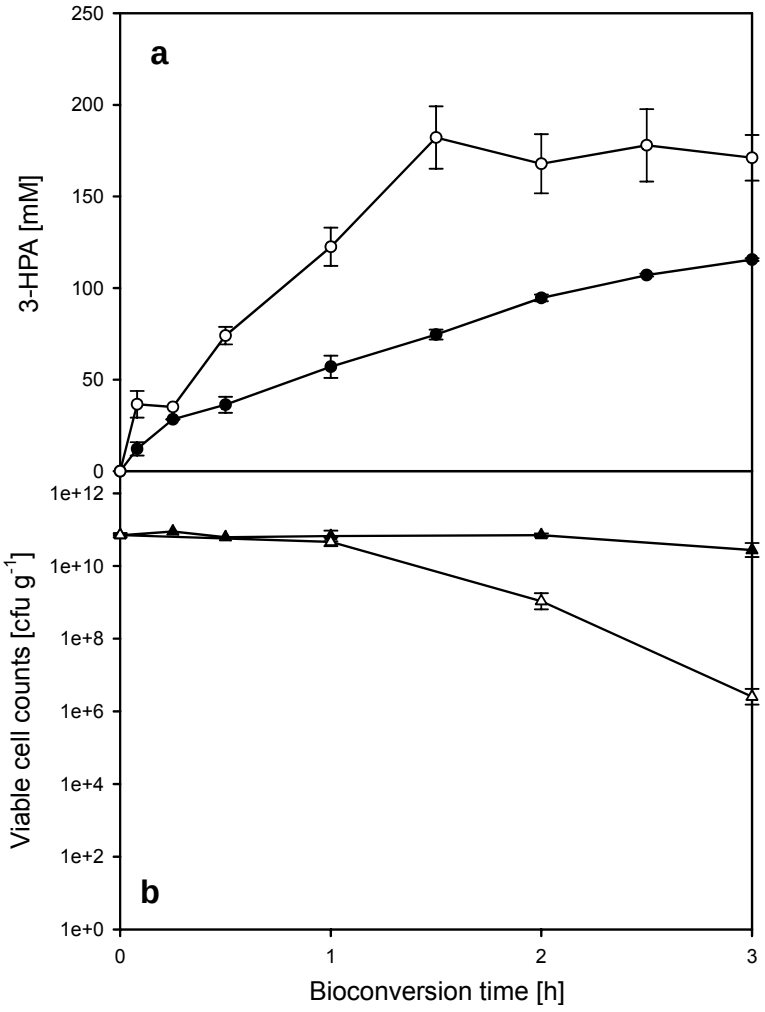

Figure 3.3 Effect of temperature on 3-HPA production (a, *circles*) and cell viability (b, *triangles*) using IC with 10% (v/v) beads colonised with 6.3±2.0x10^{10} cfu g^{-1} (6.6±1.9x10^{9} cfu ml^{-1}) *L. reuteri* cells in 360 mM glycerol at 20°C (*filled*) and 30°C (*open*). *Error bars* are standard deviations of triplicate experiments.

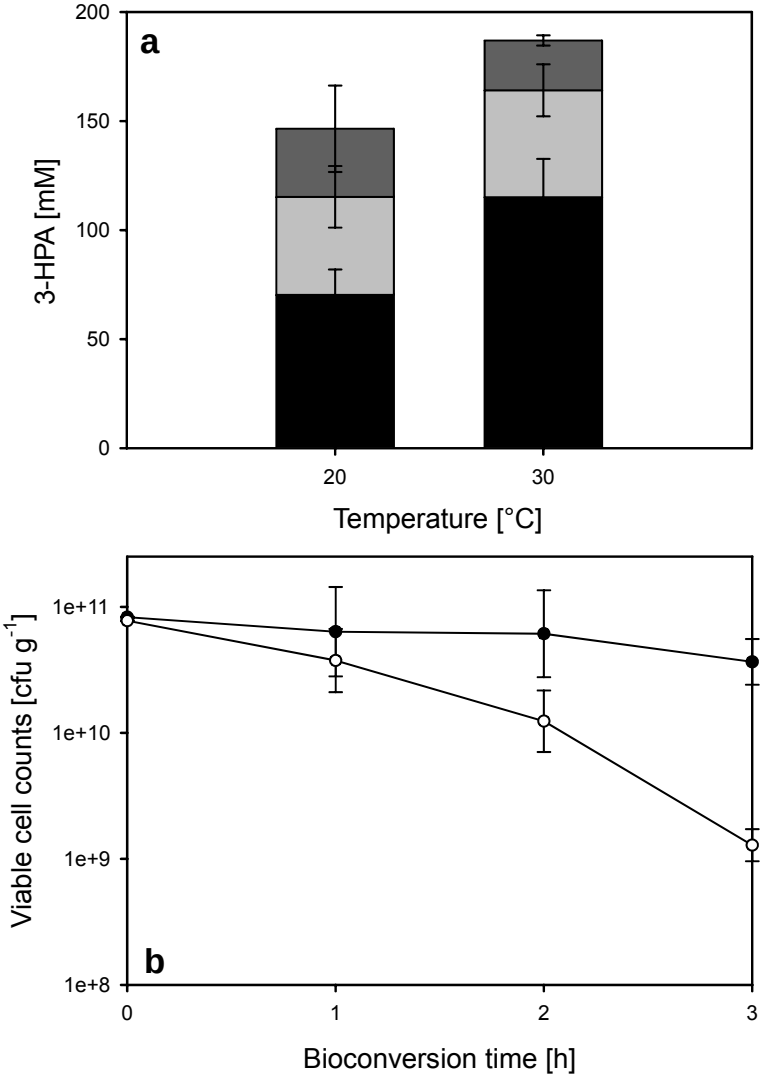

Figure 3.4 Effect of temperature on 3-HPA production (a) and viable cell counts (b) of three successive 1 h batches in 360 mM glycerol solution using 10% (v/v) beads colonised with $5.6\pm3.2\text{x}10^{10}$ cfu g^{-1}. End of first (*black*), second (*pale grey*), and third (*dark grey*) batch (a). *Filled symbols* 20°C, *open symbols* 30°C (b). *Error bars* are standard errors of duplicate experiments.

3.4.4 Effect of biomass concentration on the bioconversion of glycerol

Beads containing different biomass concentrations between $6.2x10^8$ cfu g^{-1} and $1.8x10^{11}$ cfu g^{-1} were tested for 3-HPA production in 360 mM glycerol solution at 20 and 30°C for 1 h in the experimental design (Table 3.1). Additionally low colonised beads were tested at 30°C. Positive linear correlations between initial biomass concentration and 3-HPA production for both 20°C and 30°C after $\log_{10}$ transformation are shown in Figure 3.5. Both correlations show an excellent fit as indicated by their very high coefficient of determination. The effect on 3-HPA production was more pronounced at 20°C than 30°C.

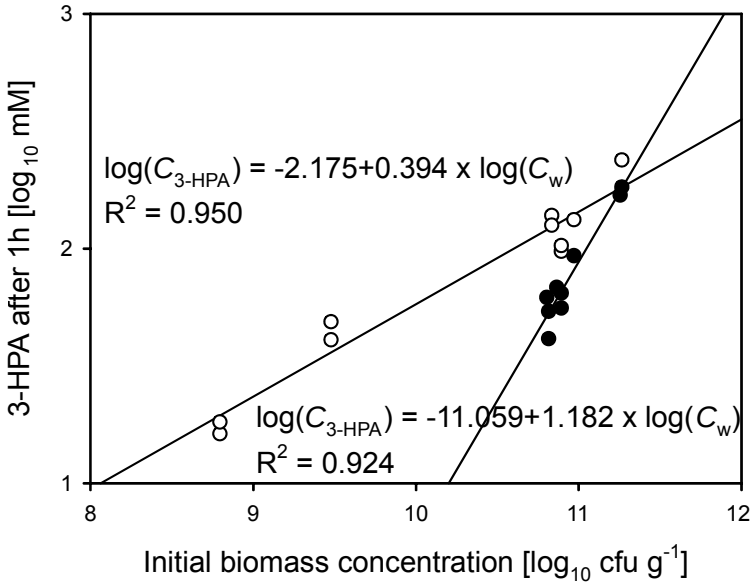

Figure 3.5 3-HPA production in correlation with initial biomass concentration after 1 h bioconversion with immobilised cells at 20°C (*filled circles*) and 30°C (*open circles*) in 360 mM glycerol solution using 10% (v/v) continuously colonised (D = 0.5 or D = 1.5 h^{-1}) beads.

3.4.5 Effect of temperature, type of bioconversion, and initial biomass concentration on 3-HPA accumulation and *L. reuteri* survival

Glycerol bioconversion to 3-HPA using immobilised *L. reuteri* cells at different conditions was fitted to a linear model, and the resulting parameter estimates are listed in Table 3.1.

Table 3.1 Estimated values of linear regression[a] coefficients for maximum cumulative 3-HPA accumulation $C_{3\text{-HPA}}$.

Parameter[b]	Range	Estimate	P-value
Intercept		-3175.197	0.01
T	20, 30°C	4.370	0.042
Type	-1, 1	*ns*[c]	*ns*
log(C_w)	10.7 – 11.3 $\log_{10}$ cfu g^{-1} [d]	296.576	< 0.001

a) The R^2 for the model was 0.731 and df of regression and residuals were 2 and 11, respectively.
b) *T*: temperature [°C]; *Type*: coded incubation type [-1: 1x3 h; 1: 3x1 h]; log(C_w): $\log_{10}$ initial biomass concentration [$\log_{10}$ cfu g^{-1}]
c) *ns*: non-significant (P > 0.05)
d) corresponding to biomass concentrations from 4.8×10^{10} to 1.8×10^{11} cfu g^{-1}.

The linear model was fitted with data from all 14 performed bioconversions having initial biomass concentrations in the range from 4.8×10^{10} to 1.8×10^{11} cfu g^{-1}. The linear regression model was used to estimate interactions between the parameters of an unbalanced design with the non-categorical variable biomass concentration C_w. Analysis of variance (ANOVA) could not be used to test the null hypothesis of non-influence of the factors because ANOVA requires categorical variables. 3-HPA accumulation was influenced by temperature and initial biomass concentration. The non-significant (P > 0.05) factor *Type* was excluded from regression (Table 3.1). Survival of *L. reuteri* cells after bioconversion (Table 3.2) was dependent on accumulated 3-HPA concentration (Pearson's R = -0.534, P < 0.001) and bioconversion temperature (Pearson's R = -0.492, P < 0.001), which was underlayed

by the multicollinearity analysis over all bioconversion data. The factor *Type* showed no significant ($P > 0.05$) influence on the survival.

3.4.6 Reactivation of cells and recolonisation of beads after 3-HPA production

To test recolonisation, beads with different cell loads between $1.3x10^7$ and $7.4x10^{10}$ cfu g^{-1} were incubated immediately after 3-HPA production (3x1 h) in MRS^+ medium, inoculated with 5% (v/v) beads, for 16 h at 37°C and pH 5.5 (Table 3.2). Viable cell counts in beads decreased in all tested cases during MRS^+ incubation. Degree of reduction of viable cell counts after incubation in MRS^+ medium was correlated with viability loss during bioconversion (Pearson's $R = 0.725$, $P = 0.033$), which was influenced by highest 3-HPA concentration and temperature. Glucose was never consumed completely in the recolonisation batches (Table 3.2).

Table 3.2 Batch recolonisation of beads with different cell loads after three successive 1 h 3-HPA productions in 360 mM glycerol at 20°C or 30°C using 10% (v/v) beads. The beads (5% (v/v)) were incubated anaerobically in MRS medium containing 20 mM glycerol at pH 5.5 and 37°C for 16 h. Every condition was tested once.

Initial viable cell concentration [cfu g^{-1} beads]	Bioconversion temperature [°C]	Cumulated 3-HPA [mM] / Highest concentration [mM]	Viable cell concentration after bioconversion [cfu g^{-1} beads]	Survival after bioconversion [%]	Viable cell concentration after MRS incubation [cfu g^{-1} beads]	Viability loss after MRS incubation [%]	Used glucose during MRS incubation [mol %] / [g l^{-1}]	3-HPA production (1 h) after MRS incubation [mM]
$6.7x10^{10}$	20	< 1[a,b]	$7.4x10^{10}$	116	$2.7x10^{10}$	63	62 / 31.8[f]	29
$6.7x10^{10}$	20	133 / 62	$6.4x10^{10}$	101	$1.2x10^{10}$	81	59 / 30.4[f]	21
$9.3x10^{10}$	20	186 / 93	$6.9x10^{10}$	74	$2.1x10^{10}$	70	89 / 14.5[e]	25
$1.8x10^{11}$[c]	20[c]	118[c] / 44[c]	$7.0x10^{10}$[c]	39[c]	$2.5x10^{10}$[c]	64[c]	54 / 28.2[cg]	-[ch]
$1.8x10^{11}$	20	316 / 183	$3.1x10^{10}$	17	$4.8x10^{9}$	84	79 / 11.7[d]	2
$9.3x10^{10}$	30	189 / 133	$1.7x10^{9}$	2	$1.9x10^{4}$	> 99.9	82 / 13.4[e]	< 1[b]
$1.8x10^{11}$	30	275 / 239	$1.3x10^{7}$	< 0.1	$< 10^{3}$	> 99.9	53 / 7.9[d]	< 1[b]

a) Control: incubated in 360 mM sorbitol; b) Below quantification method detection limit; c) 3 successive 15 min incubations; d) Initial glucose concentration: 14.9 g l^{-1}; e) Initial glucose concentration: 16.3 g l^{-1}; f) Initial glucose concentration: 51.3 g l^{-1}; g) Initial glucose concentration: 52.1 g l^{-1}; h) not tested

Reduction of bioconversion time to three 15 min incubations led to a drop in highest and cumulative 3-HPA accumulations, but did not increase *L. reuteri* survival compared to three 1-h bioconversions (Table 3.2). 3-HPA accumulation after recolonisation was always low (< 30 mM) and directly related to the viability after recolonisation. Reduction in cell viability after recolonisation was also observed with control beads without 3-HPA accumulation incubated three times in 360 mM sorbitol solution for 1 h at 20°C.

3.5 Discussion

The aim of this study was to produce 3-HPA biotechnologically using immobilised (IC) *Lactobacillus reuteri* cells. IC technology was chosen because IC grow at high cell densities, which is a crucial factor for the cellular enzymatic process of glycerol bioconversion to 3-HPA (Doleyres *et al.*, 2005) leading to increased volumetric productivity. IC can be easily recovered with no need of centrifugation and reused in repeated batch fermentations (Bertrand *et al.*, 2001), or bioconversion with medium exchange. In this study, the effects of biomass production step, temperature, type of bioconversion, bioconversion time, and initial biomass concentration on 3-HPA accumulation was investigated in 360 mM glycerol solution and compared with free cell (FC) 3-HPA production.

Production of 3-HPA from free *L. reuteri* cells in glycerol has been investigated by multiple researchers (El-Ziney *et al.*, 1998; Lüthi-Peng *et al.*, 2002a; Lüthi-Peng *et al.*, 2002b; Doleyres *et al.*, 2005). 3-HPA accumulation using FC after 2 h in 300 and 400 mM glycerol agreed with published data (Doleyres *et al.*, 2005) and served as control for 3-HPA production with IC.

The feasibility of glycerol bioconversion to 3-HPA using IC was recently demonstrated (Zamudio-Jaramillo *et al.*, 2009). In this study, however, a low maximum 3-HPA yield of approximately 80 mM was reported, which can be partly explained by the selected strain, *L. reuteri* NRRL B-14171/DSM 20016. We showed that this strain accumulates significantly less 3-HPA than the ATCC 55730 strain (data not published). Furthermore, survival after bioconversion was not assessed and

biomass was produced as FC, then immobilised and directly used for bioconversion without prior bead colonisation.

3-HPA accumulation of the present study using IC showed 3.2-fold increased specific productivity compared to FC cultures after 1.5 h at 30°C, with a 4.3-fold lower initial biomass concentration in the reactor but only 1.4-fold lower maximal accumulated 3-HPA. A drawback for industrial applications of IC bioconversions is the 4.8-fold lower mean conversion rate leading to prolonged conversion times. Glycerol bioconversion to 3-HPA using IC shows an initial high rate phase with a rate comparable to FC conversion during 5 to 10 min before the main conversion phase is started. High initial rate could be explained by the dense packed beads leading to local high cell concentrations and therefore high conversion rates. The lower mean rate is explained by diffusion limitations (Arnaud *et al.*, 1992a) for both substrate and inhibitory product, in the present case glycerol and 3-HPA.

Factors influencing 3-HPA accumulation using IC and survival of the producing strain after bioconversion were biomass concentration, temperature (20 or 30°C), and bioconversion time as determined by a linear regression model, which are in agreement with bioconversion using FC (Lüthi-Peng *et al.*, 2002b; Doleyres *et al.*, 2005). Bioconversion type (1x3 h or 3x1 h) showed no influence on cumulative 3-HPA accumulation and cell viability at the end of the process.

3-HPA was accumulated using FC or IC to higher concentrations and at increased velocity with increasing initial biomass concentrations, which is in accordance to reported data with FC (Lüthi-Peng *et al.*, 2002b; Doleyres *et al.*, 2005). Mean biomass concentration of IC was determined to $7.5\pm2.1\text{x}10^{10}$ cfu g^{-1} leading to a mean cumulative 3-HPA production of 194±17 mM after 3 h at 30°C using 10% (v/v) beads. After optimisation of initial biomass concentration to $1.8\text{x}10^{11}$ cfu g^{-1}, superior cumulative 3-HPA accumulations of 316 mM and 275 mM at 20 and 30°C, respectively, were achieved from 360 mM glycerol solution in three successive 1 h incubations.

Bioconversion with IC at 20°C showed a significant reduction of maximal 3-HPA accumulation and significant lower glycerol bioconversion rate than at 30°C agreeing with published data of FC by Lüthi-Peng *et al.* (2002b). In contrast Doleyres *et al.* (2005) observed no difference in maximal 3-HPA accumulation (167±10 mM) between 15°C and 37°C after incubation for 45 min using $1.7\pm0.3\text{x}10^{10}$ cfu ml^{-1} in

200 mM glycerol, thus, a higher initial biomass and lower glycerol concentration was used. After 45 min, glycerol was utilised completely and the bioconversion stopped explaining that no effect of the temperature was observed (Doleyres *et al.*, 2005).

Reactivity of the aldehyde 3-HPA is temperature dependent and increases with increasing temperature (Schauenstein *et al.*, 1977) leading to increased toxicity towards FC (Rasch, 2002; Doleyres *et al.*, 2005). Survival of immobilised *L. reuteri* cells was influenced by the temperature and accumulated 3-HPA. Effect of temperature on survival of immobilised *L. reuteri* is in accordance to data from FC (Doleyres *et al.*, 2005).

Successive incubations for 1 h at 20 and 30°C were performed in an attempt to prevent the producing strain from prolonged contact with high 3-HPA concentration. An increase in 3-HPA production and viability of *L. reuteri* was expected. This hypothesis could not be confirmed. Linear regression analysis showed no significant effect ($P > 0.05$) of the variable *Type* on the total variance of 3-HPA production. In successive incubations, three times higher glycerol input is needed compared to 1x3 h, leading to a low 3-HPA-to-glycerol yield.

MRS consumption during bead colonisation is also an economical factor of the industrial 3-HPA synthesis at large scale. MRS medium is required to grow *L. reuteri*, which is then directly used for bioconversion in the next step. Medium consumption and 3-HPA accumulation are therefore related. IC showed a 2.7-fold increased specific 3-HPA production per consumed MRS medium (63.7 mmol l^{-1}), based on continuous colonisation ($D = 0.5\ h^{-1}$), compared to FC at 30°C with 23.5 mmol l^{-1} (Doleyres *et al.*, 2005).

To test recolonisation immediately after three successive 1 h 3-HPA productions, beads with different cell loads were incubated for 16 h in MRS medium. The greater the impact on viability during bioconversion by high 3-HPA production, the sharper the drop of viability during recolonisation in MRS medium. No growth was also reflected in the low amount of consumed glucose. Cell mortality was also observed in beads stored in 360 mM sorbitol solution suggesting that other factors than toxic 3-HPA accumulation, such as lacking factors or starvation, may contribute to lethal effects during bioconversion. Lethal osmotic stress from 360 mM glycerol or sorbitol could be excluded because osmolarity corresponds to 1.1% NaCl which is not lethal for lactobacilli (Gänzle *et al.*, 1998; Neysens *et al.*, 2003; Prasad *et al.*, 2003; Koch *et*

al., 2007). Lactobacilli are known to be susceptible to sugar starvation as applied here (Valu, 1965; Schepers *et al.*, 2002a; van de Guchte *et al.*, 2002). Valu (1965) reported a decrease in viability when the test strain *Lactobacillus leichmannii* ATCC 7830 was incubated in distilled water exceeding 45 min at 37°C. Incubations of 45 min and less showed no negative impact on the viability and successive growth in MRS medium compared to the control. Starvation stress, however, did not inhibit *L. reuteri* cells growth in the viable cell determination assay on solid MRS agar (37°C, anaerobic, AnaeroGen) after treatment in EDTA solution (incubation 5 min) and dilution in maximum recovery dilute (incubation maximum 60 min at 4°C). This discrepancy between plate counts and bead reactivation on MRS agar and medium, respectively, could be due to several factors. One factor could be the lack of CO_2 during bead reactivation (headspace was flushed with N_2) whereas agar plates are cultivated in CO_2 rich atmosphere because CO_2 is released from AnaeroGen sackets. Lactobacilli are known to require CO_2 for growth especially during early stage (Stevens *et al.*, 2008). This could explain why damaged cells did not show growth during reactivation. Furthermore, for the ability of recycling the beads, precautions should be applied to protect *L. reuteri* viable cells using minimum starvation and contact time.

3.6 Conclusions and Outlook

We demonstrated that 3-HPA production using immobilised *L. reuteri* cells has advantages over free cell production. Specific productivity increased 3.2-fold at 30°C after 1.5 h. Furthermore, centrifugation steps could be avoided. Drawbacks were recognised in the 4.8-fold lower mean conversion rate and 1.4-fold lower maximal accumulated 3-HPA concentration compared to FC cultures. Bioconversion of glycerol was dependent on temperature, biomass concentration in beads, and the colonisation method. Mean cell concentration after continuous colonisation (D = 0.5 h^{-1} or D = 1.5 h^{-1}) was determined to $7.5\pm2.1x10^{10}$ cfu g^{-1} leading to a mean cumulative 3-HPA production of 194±17 mM with 10% beads added for the three 1-h conversion steps at 30°C in 360 mM glycerol. Optimisation of initial biomass concentration to $1.8x10^{11}$ cfu g^{-1} lead to superior cumulative 3-HPA accumulations of

316 mM and 275 mM at 20 and 30°C, respectively. After glycerol bioconversion, re-growth and re-utilisation of beads, however, was not successful under tested conditions. Shorten bioconversion time to one forth did not overcome this limitation, in fact 3-HPA accumulation was decreased by three-fold. With IC, *L. reuteri* viability during 3-HPA accumulation could not be completely maintained and recycling could not be achieved under tested conditions, 3-HPA toxicity needs to be decreased and possible other factors involved in the lack of regrowth of cells after bioconversion needs to be identified. A solution might be *in situ* product removal (ISPR) with FC or IC.

4 *In situ* removal and purification of 3-hydroxy-propionaldehyde produced by *Lactobacillus reuteri* from glycerol

Data presented in this chapter were submitted for publication with the following authors: D. P. Rütti, C. Lacroix, T. Jeremic, M. Mathis, A. Díe, S. Vollenweider.

4.1 Abstract

3-hydroxypropionaldehyde (3-HPA or reuterin), a potent antimicrobial compound, is a substance with great potential in the pharmaceutical-, food-, and chemical industry. Until now, it occurs only as intermediate in production of 1,3-propanediol from petro-derived chemicals and its isolation and purification is difficult, expensive, and not performed. In this study, we showed a new approach for the isolation of 3-HPA, biotechnologically produced by *Lactobacillus reuteri*. A complex of an Amberlite anion exchange resin and hydrogensulfite (IRA-SO_3H) was used to extract 3-HPA from the production medium. Elution and concentration of 3-HPA was performed with NaCl, pH adjustment, lyophilisation, and ethanol extraction. Furthermore, the potential of an IRA-SO_3H to bind 3-HPA throughout the production using *L. reuteri* was evaluated. The IRA-SO_3H successfully bound 3-HPA, but inhibited fast and completely the 3-HPA production by *L. reuteri*. To conclude, our data showed the selective extraction of 3-HPA from for the bioconversion medium and for the first time the subsequent recovery of 3-HPA from the ligand. However, future work is needed to adapt this system to an *in situ* product removal process not interfering with the bioconversion.

4.2 Introduction

3-hydroxypropionaldehyde (3-HPA) can be produced biotechnologically from glycerol using *Lactobacillus reuteri* (Doleyres *et al.*, 2005). In water and also after the secretion into the aqueous medium it forms an equilibrium (HPA system or HPA) with its hydrate and its dimer reuterin (Hall and Stern, 1950; Dobrogosz *et al.*, 1989). 3-HPA inhibits the growth of bacteria, moulds, yeasts, and protozoa including human pathogenic and food spoilage organisms (Dobrogosz *et al.*, 1989; El-Ziney and Debevere, 1998) making it interesting for use as an antimicrobial in the health-care- and food industry. As a precursor for the synthesis of 1,3-propanediol (1,3-PDO), acrolein, hydroxypropionic acid, or acrylic acid, 3-HPA has also great potential for the chemical industry (Vollenweider and Lacroix, 2004).

A promising approach for large-scale production of 3-HPA to make it commercially available is the biotechnological conversion of glycerol, using bacteria as biocatalyst. It was previously reported that bacteria of six genera were able to convert glycerol into 3-HPA (Vollenweider and Lacroix, 2004). Out of these, the probiotic *Lactobacillus reuteri* (*L. reuteri*) yielded highest amounts of free 3-HPA (235±3 mM: Doleyres *et al.*, 2005) using a two-step process where viable cells were incubated after growth in 400 mM glycerol solution (El-Ziney *et al.*, 1998; Lüthi-Peng *et al.*, 2002b; Doleyres *et al.*, 2005). However, the accumulation of aldehydes such as 3-HPA is limited by the cell toxicity of these products. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of 3-HPA for *L. reuteri* were determined to be in the range of 30-50 mM and 60-120 mM 3-HPA, respectively (Cleusix *et al.*, 2007), corresponding to 3-HPA concentrations inducing cell-death during its production with the same organism (Vollenweider and Lacroix, 2004; Doleyres *et al.*, 2005). Consequently, to cell inactivation and cell death, 3-HPA production stops and cells cannot be reused for additional production cycles. An economical and large scale process, however, needs high concentrations of viable cells, which could be reused.

One possible solution is the *in situ* removal of the aldehyde during production. The *in situ* product removal (ISPR) of 3-HPA was successfully achieved using semicarbazide, resulting in very high production of 621 mM 3-HPA-semicarbazone

from 761 mM glycerol in small scale experiments with *Klebsiella pneumoniae* (Vancauwenberge *et al.*, 1990). Although these results are promising, the recovery of 3-HPA from the semicarbazone was neither reported (Slininger and Bothast, 1985; Slininger *et al.*, 1990; Vancauwenberge *et al.*, 1990; Vollenweider and Lacroix, 2004), nor successfully performed in our laboratory (data not published).

Other methods can be used to remove aldehydes from watery reaction mixtures and fermentation media: distillation (Raymond, 1984; Stradal and Underwood, 1995), gas stripping (Wecker and Zall, 1987; Zall and Wecker, 1990; Molinari *et al.*, 1995; Simmonds and Robinson, 1998), pervaporation (Lamer *et al.*, 1996), solvent extraction (Duff and Murray, 1989), solid phase extraction (Berger, 1995), and derivatisation (Samuelson, 1949; Huff, 1959; Harris and Hajny, 1960; Raymond, 1984; Igawa *et al.*, 1990; Zall and Wecker, 1990; Shachar-Nishri and Freeman, 1993; Simmonds and Robinson, 1998; Kuzmanovic *et al.*, 2003), and precipitation and filtration from an organic solvent (Stradal and Underwood, 1995). However for 3-HPA, these methods cannot be applied because they are either difficult to scale up (chromatography, membrane separation) or are high energy-consuming (reduced pressure distillation). Furthermore, 3-HPA can not be stripped of an aqueous solution (gas stripping, pervaporation) because of its low volatility, or extracted from aqueous phase as it is too hydrophilic (solvent or solid phase extraction), or re-crystallised because it is heat sensitive (Lüthi-Peng *et al.*, 2002b).

The aim of this study was therefore to develop a method using a ligand, which can bind selectively to 3-HPA in the aqueous bioconversion medium in order to avoid the toxic action of 3-HPA against the producing cells, remove the 3-HPA-ligand adduct from the bioconversion-medium and allow recovery of 3-HPA from the ligand in another solvent. First a screening of ligands was done. One ligand showed reversible binding of 3-HPA and was tested further for its scale-up possibility and biological compatibility adding it to the 3-HPA production process using *L. reuteri* and glycerol, with the aim to develop an ISPR process.

4.3 Material and Methods

4.3.1 Reagents and materials

Unless otherwise specified, chemicals and adsorbents were purchased from Sigma-Aldrich (Buchs, Switzerland). The concentration of the sodium hydrogensulfite solution was 40.0% (w/w), as indicated by the supplier. Ready to use amino-functionalised thin-layer chromatography plates (NH_2F_{254}) were purchased from Merck (Darmstadt, Germany). Sample dilutions for HPLC analysis were done with HPLC grade water (Milli-Q Biocell System, Quantum EX cartridge, Millipak Express 20 final filter, Millipore Corporation). All solutions and media in contact with *Lactobacillus reuteri* were autoclaved at 121°C for 20 min except sodium hydrogensulfite solution that was sterile filtered.

4.3.2 Bacterial strain and inoculum preparation

The stock culture of *Lactobacillus reuteri* (*L. reuteri*) ATCC 55730 (SD2112), obtained from Biogaia (Stockholm, Sweden), was kept at -80°C in a solution containing 10% (w/v) glycerol and 6% (w/v) skim milk powder (Emmi, Lucerne, Switzerland). For inoculum preparation, 1% of frozen *L. reuteri* stock was incubated in MRS medium (Biolife, Milan, Italy) at 37°C for 16 h.

4.3.3 Biomass production

L. reuteri biomass production was done according to Doleyres *et al.* (2005) with slight modifications. *L. reuteri* was grown in Sixfors bioreactors (500 ml useful volume; Infors, Bottmingen, Switzerland) in a 15-h batch culture in 500 ml MRS medium supplemented with 20 mM glycerol, inoculated with a 16 h old culture of *L. reuteri* (1% v/v). Medium was stirred by a three-blade pitched blade impeller at 150 rpm, temperature set at 37°C, and pH was held at 6.0 using 5 M NaOH for optimal cell growth. Nitrogen (N_2) was injected into the bioreactor headspace to assure

anaerobic conditions. Cells were then harvested by centrifugation (3000 *g*, 10 min, 20°C), washed with 500 ml potassium phosphate buffer (Kpi 0.1 M, 20°C, pH 7.0), and used immediately for 3-HPA production.

4.3.4 3-HPA production

3-HPA containing supernatant (3-HPA solution) was produced according to Doleyres *et al.* (2005). Briefly, *L. reuteri* cells from the biomass production step were suspended in 400 mM glycerol solution to obtain an initial viable cell concentration of $1.6x10^{10}$ colony-forming units per ml (cfu ml^{-1}), incubated for 2 h at 20°C, and removed from the supernatant by centrifugation (14000 *g*, 10 min, 20°C).

4.3.5 3-HPA production using *in situ* product removal

The bioconversion step was combined with 3-HPA extraction to test *in situ* product removal (ISPR). Therefore, 18.2% (w/v) IRA-Cl (AMBERLITE® IRA-400, quaternary ammonium in Cl-form) or IRA-SO_3H (IRA-400, hydrogensulfite complex after transformation (as described below in the ligand transformation protocol) were gently stirred (magnetic bar, 20°C) in 10 ml of 400 mM glycerol inoculated with $1.6x10^{10}$ cfu ml^{-1} *L. reuteri*. The amount of resin was chosen according to the theoretical capacity of the resin and expected 3-HPA production. Experiments were performed in 25 ml Erlenmeyer flasks that were continuously flushed with N_2 to avoid oxygen entry. Reported data are means for duplicate experiments.

4.3.5.1 Influence of sodium hydrogensulfite on 3-HPA production

Because hydrogensulfite might be released from IRA-SO_3H during ISPR, various amounts of hydrogensulfite solution (final concentration 0, 1, 2, 5, 12.5, 25, 50, 125 mM) were added at the beginning of the 3-HPA production step from 2 ml 400 mM glycerol solution carried out in 2 ml Eppendorf tubes at 20°C. Concentrations were set according to maximum hydrogensulfite that could be released from the resin.

After 2 h bioconversion, samples for 3-HPA und hydrogensulfite quantification were taken. Reported data are means for duplicate experiments.

4.3.6 Purification of 3-HPA

3-HPA was purified from 3-HPA solution according to Vollenweider *et al.* (2003). The solution from the production was lyophilised and loaded on a silica gel 60 column. 3-HPA was eluted with acetone:ethyl acetate (2:1). After evaporation of the solvent under reduced pressure, 3-HPA was obtained in pure form.

4.3.7 Selection of ligands binding reversibly to 3-HPA

Ligands and conditions tested for 3-HPA binding are displayed in Table 4.1. The potential complexing or adsorbing ligands (approx. 5 g) were added to stirred 3-HPA solution (3 to 30 ml) and incubated for 30 to 180 min. Samples were taken to test 3-HPA remaining in the solution. Ligands with potential retentive properties were used as stationary phase on thin layer or column chromatography (14.7 mm in diameter, 46 mm in height). The tested 3-HPA solution contained 188 mM 3-HPA, 163 mM glycerol, and 19 mM 1,3-PDO (Table 4.1). The 3-HPA binding to the selected ligand, AMBERLITE® IRA-400-hydrogensulfite complex, was further characterised (preparation of ligand, capacity, velocity, influence of temperature and pH). Furthermore, a down-stream process for the recovery of bound 3-HPA was developed.

Table 4.1 List of tested ligands and conditions to bind selectively 3-HPA in fermentation supernatant for *in situ* product removal.

Substance	Principle	Test conditions for 3-HPA binding	Preconditioning	Result [a]	Remarks	Originated from[b]
Polyvinylalcohol (PVA) Mw 89000-98000, 99% hydrolised	Acetalisation of hydroxyl and carbonyl groups catalysed by acids	5 g PVA stirred for 30 min with 30 ml 3-HPA supernatant and 6 drops of 0.1 M HCl		-/-		(Blouin, 1984; Kusudou *et al.*, 2006)
		Variation in time (30-210 min), temperature (20-75°C), 3-HPA amount, and catalyst (HCl and NaOH)				
		4 g PVA in gel form (8.0 g Na_2SO_4) stirred for 90 min with 50 ml water (70°C) and 3 ml 3-HPA $_2SO_4$	A 16 % Na_2SO_4 solution was used to swell PVA for reaction	-/-		
		Variation in time (60-90 min), temperature (60-70°C), and catalyst (H_2SO_4 and citric acid)				
Amberlite IRP-69	Ion exchanger with SO_3^- functional groups	5 g IRP-69 in a column[c], loading with 5 ml 3-HPA supernatant, washing with 30 ml water	Variation of cation: Na^+: used as supplied H^+: 3 g transformed with 20 ml 1 M HCl (2 h), rinse with water	-/-		(Rendina and Cleland, 1981; Bicak and Senkal, 1997)
		3 g IRP-69, stirring for 180 min with 4 ml 3-HPA supernatant and 4 ml water, washing 30 ml water		-/-		
Amberlite CG-400	Reported selective binding of glycol aldehyde to strong basic resin in OH^- form	3 g CG-400 in a column[c], loading with 5 ml 3-HPA supernatant, washing with 30 ml water	Variation of anion: Cl^-: used as supplied OH^-: 3 g transformed with 30 ml 1 M NaOH (2 h), rinse with water until neutral	+/- OH^- -/- Cl^-		(Annan *et al.*, 1965 ; Ouchi *et al.*, 1982)
Amberlite XAD-16	Polymeric, nonionic, hydrophobic adsorbent	2 g XAD-16 resin in water, 0.5 ml 3-HPA supernatant, stirred for 1 h up to 3 days	Washing with water (50 bed volumes)	-/-		(Bagnell *et al.*, 1997 ; Seeram *et al.*, 2005)
Amino-functionalised thin-layer chromatography	Separation by different retention times	Thin-layer chromatography plate ($NH_2F_{254\,s}$), eluant: water and water/ethylene-diamine (1 L/1 ml)		+/	Good separation with water as eluant: Rf(3-HPA) = 0.1[d] Rf(glycerol; 1,3-PDO) = 0.9	(Jost and Hauck, 1983; Zhu *et al.*, 2004)
Amino-functionalised column chromatography[c]	Separation by different retention times	1/3 to 1/2 of the coating of a thin-layer chromatography plate ($NH_2F_{254\,s}$) packed to a column, 0.5-2 ml 3-HPA supernatant		-/	No binding, 3-HPA was eluted with water front from the column. Slow passing when using > 1/2 of the coating	(Jost and Hauck, 1983; Zhu *et al.*, 2004)
		2.5 g aminopropyl functionalised silica gel in a column, loading with 2 ml supernatant, and washing with 5 ml water		(+)/	Only temporarily adsorption of 3-HPA	
		1 g tris(2-aminoethyl)amine polymer-bound resin in a column, loading with 6 drops 3-HPA supernatant, washing with water		-/	No binding, 3-HPA was eluted with water front from the column	
Sodium hydrogen sulfite solution (38-40%), organic solvents	Aldehydes form with hydrogen sulfite a-oxysulphonic acids	Used organic solvents for separation: ethyl acetate, 1,2-dichloroethane, dichloromethane, butanone, hexane	3-HPA adduct formation by equimolar amounts of 3-HPA and hydrogen sulfite (50 mM) during 15 min	+/-[e]	3-HPA and its adducts are too water soluble to be precipitated or extracted by a solvent	(Gabrielson and Samuelson, 1952 ; Williams *et al.*, 1981)
Amberlite IRA-400 in hydrogen sulfite form	Aldehydes and ketones form with hydrogen sulfite a-oxysulphonic acids	5 g IRA-400 in a column, loading with 5 ml 3-HPA supernatant, washing with 30 ml water	Transformation to sulfite form as described with sodium hydrogen sulfite solution, rinse with water	+/+		(Gabrielson and Samuelson, 1952 ; Williams *et al.*, 1981)

a) Ability of binding / Ability of release b) Performed with various aldehydes except 3-HPA c) Effective size of column (14.7 x 46 mm)
d) Retardation factor: ratio of distance travelled by the substance to the mobile phase e) No separation of adduct from supernatant

4.3.7.1 Ligand transformation protocol

Five grams of strongly basic gel-type anion-exchange resin (AMBERLITE® IRA-400, quaternary ammonium in Cl-form) was packed into the column, covered with a porous disk (frit), and converted into the hydrogensulfite form (IRA-SO_3H) by passing 40 ml of a sodium hydrogensulfite solution (40.0%, flow rate: 2-3 ml min^{-1} controlled by a valve) (Samuelson, 1963). The influent was fed from the top of the column. The column was washed with 200 ml bidistilled water until no hydrogensulfite was detected in the effluent (determined with the Ellman test). Per 5 g dry resin, the theoretical capacity was calculated to 10.9 mmol 3-HPA from the known volumetric capacity (1.4 meq ml^{-1}: Sigma-Aldrich) and measured specific volume per dry weight of swelled IRA-400 (1.56 ml g^{-1}).

4.3.7.2 3-HPA reactive extraction protocol

The column containing 5 g IRA-SO_3H was loaded ('loading') with 3-HPA and washed thrice with 10 ml distilled water ('wash') at 25°C. In all steps, a flow rate of 2 ml min^{-1} controlled by a peristaltic pump was maintained (Gabrielson and Samuelson, 1950). For capacity determinations, 5 ml of 3-HPA solution containing different amounts of 3-HPA (14.3, 9.5, 4.8, 0.95, 0.62 mmol) was passed through the column in duplicate. For kinetic characterisation of the binding process, 100 ml 3-HPA solution consisting of 143.4 mM 3-HPA, 249.4 mM glycerol, and 12.8 mM 1,3-PDO was passed through the column. One ml fractions of the effluent were collected and analysed for 3-HPA (purpald assay), glycerol and 1,3-PDO (HPLC) content. The break-through point was reached when 3-HPA was detected in the effluent.

3-HPA-binding was tested at two temperatures (4°C and 20°C) and five pH values (4, 5, 6, 7, and uncontrolled: 3.6) in a full fractional design with 5 ml buffered supernatant containing 0.72 mmol 3-HPA. Buffered 3-HPA supernatant was prepared by adding 9 volumes of 3-HPA supernatant with one volume of ten fold concentrated citrate-phosphate buffer at the defined pH, prepared according to McIlvaine (1921).

Furfural, known to bind to the IRA-SO_3H column system, was chosen as control aldehyde (Gabrielson and Samuelson, 1950). With a flow rate of 2 ml min^{-1}, 600 ml 0.2% (w/v) furfural solution (20.8 mM) was passed through the column to determine

the break-through point. Fractions of 10 ml from the effluent were collected and analysed by HPLC for furfural.

4.3.7.3 Elution and recovery of 3-HPA

3-HPA was eluted from the IRA-SO_3H resin with 200 ml (8x25 ml) of either saturated NaCl solution (brine, 36% (w/v)), 1 M HCl, 1 M NaOH, sodium carbonate solution (0.075 M Na_2CO_3+0.15 M $NaHCO_3$, under CO_2 pressure of 2 bar), hot water (75°C), or ethyl acetate ('eluate'). Then each resin was rinsed with 10 ml distilled water ('rinse'). The eluate was neutralised to pH 7 with 5 M NaOH, freeze-dried, and 3-HPA was extracted with 100 ml absolute ethanol (5 fractions each 20 ml, extraction time 5 min each) at 25°C. The elution test was performed in duplicate.

4.3.8 Viable cell determination

Viable cell counts of *L. reuteri* were determined by plating serially diluted samples (peptonised water with 0.85% (w/v) NaCl) on MRS agar (MRS with 15 g l^{-1} agar; Becton Dickinson AG, Allschwil, Switzerland) plates followed by an anaerobic incubation of the plates for 48 h at 37°C. Reported data are means for duplicate analyses.

4.3.9 Metabolite analyses

Samples for metabolic analyses were immediately centrifuged (14000 *g*, 5 min, 4°C), the supernatant filtered through a 0.45 μm filter and stored at -20°C. All quantifications were performed in duplicate.

4.3.9.1 Quantification of 3-HPA, glycerol, 1,3-propanediol, and furfural

3-HPA, glycerol, 1,3-PDO, and furfural were determined by HPLC (LaChrom HPLC system, Merck Hitachi, Dietikon, Switzerland). Separation was performed on a Biorad Aminex HPX-87H column (300 x 7.8 mm; Bio-Rad Laboratories AG, Reinach BL, Switzerland) with 10 mM sulphuric acid (HPLC grade) at a flow rate of 0.6 ml min^{-1} and temperature of 40°C. Quantification was performed by refractive

index detector with external standards of known amounts of commercially pure substances (glycerol, 1,3-PDO, furfural), except for 3-HPA, which was produced in our laboratory (Vollenweider *et al.*, 2003).

4.3.9.2 Quantification of 3-HPA in presence of hydrogensulfite

Hydrogensulfite, having the same retention time as 3-HPA, interfered with the HPLC quantification. 3-HPA was in this case quantified using a colorimetric method (purpald assay: Dickinson and Jacobsen, 1970) which was further developed to allow quantitative determination. A 1 ml sample was added to 1 ml purpald solution (2% (w/v) purpald in 2 M NaOH) and incubated under aeration by inverting the tubes for 20 min at 25°C. The absorption was measured at 550 nm. Calibration was performed with known amounts of pure 3-HPA.

4.3.9.3 Quantification of total hydrogensulfite

Total sulfite was quantified by a colorimetric method (Ellman test) adapted from Ellman (1959). In this method, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) reacts with sulfite under neutral or weak basic conditions to a yellow coloured anion which is quantified at 412 nm. For this, 990 µl sample diluted with 0.2 M potassium phosphate buffer (Kpi, pH 7.0) was mixed with 10 µl DNTB (4% (w/v) in dimethyl sulfoxide) and incubated for 10 min at 25°C. Calibration was performed with known amounts of hydrogensulfite. All solutions were prepared just prior use and handled to avoid oxygen entry.

4.3.9.4 Quantification of free hydrogensulfite

Unbound hydrogensulfite was quantified by triiodide tritration using starch solution as indicator, using a method adapted from Frigerio (1969). Therefore, a titrated amount of triiodine solution (iodine, 0.33%, and potassium iodide, 0.66%) was added to 10 µl sample, 3000 µl water, and 500 µl starch solution (1% (w/v); BD, Allschwil, Switzerland) until a stable violet colour was developed. Calibration was performed with known amounts of hydrogensulfite. All solutions were prepared just prior use and handled to avoid oxygen entry.

4.3.10 Statistical analyses

ANOVA was performed using the general linear model of SPSS 16.0 (SPSS, Chicago, IL). Significant differences among treatment means were tested using the LSD multiple comparison test, with a level of significance of 0.05.

4.4 Results

4.4.1 Selection of a 3-HPA binding ligand for purification

Various ligands were screened for selective and reversible binding to 3-HPA (Table 4.1). 3-HPA did not bind to polyvinyl-alcohol, Amberlite IRP-69 (SO_3^- functional groups), or Amberlite XAD-16 (nonionic hydrophobic adsorbent) under tested conditions. An irreversible binding to 3-HPA was found for a strong basic ion exchange resin in the hydroxyl form (Amberlite CG-400). Amino-functionalised thin-layer chromatography showed good separation of 3-HPA from glycerol and 1,3-PDO but this property was lost during implementation to a column chromatography.

Sodium hydrogensulfite in contrast was found to reversibly bind to 3-HPA. The degree of adduct formation of 3-HPA with hydrogensulfite was determined as a difference of the total and the free, unreacted, hydrogensulfite after incubation of equimolar amounts of 3-HPA and sodium hydrogensulfite (50 mM, pH 3.5, 25°C) for 15 min. Under test conditions, 73.9±0.2% of 3-HPA was present as adduct.

The 3-HPA-sulfite-adduct was too hydrophilic to be extracted into a solvent phase or to be precipitated by known methods from the aqueous phase. The separation was then performed by an ionic binding of the adduct to a carrier (strongly basic anion exchange resin with quaternary ammonium, Amberlite IRA-400). This method was optimised, by binding first the hydrogensulfite to the above anion exchange resin (IRA-SO_3H) which was then used in a column to withdraw 3-HPA from the medium.

4.4.2 Control with furfural

As a control with known binding ability to the IRA-SO_3H system furfural was used. Therefore, 600 ml furfural solution (20.8 mM) containing 12.48 mmol furfural was passed through the column (Figure 4.1, *open circles*, second x-axis). After a volume of 250 ml, furfural appeared in the effluent (break-through point). An amount of 5.2 mmol furfural or 48 mol % of the column capacity was absorbed. With increasing flow through volume, furfural concentration increased in the effluent. After 600 ml flow through, a total of 8.16 mmol furfural was found to be loaded onto the column.

4.4.3 Determination of capacity

The capacity of IRA-SO_3H (5 g) for 3-HPA was determined using 0.62, 0.95, 4.8, 9.5, and 14.3 mmol 3-HPA (in a total volume of 5 ml with distilled water, Table 4.2). The maximum uptake was 9.3±0.6 mmol after loading with 14.3 mmol 3-HPA corresponding to 85.4±5.2 mol % of the column capacity. Loading with 0.62, 0.95, and 4.8 mmol resulted in a high uptake of 84.9±1.5 mol % and low leakage of the applied 3-HPA.

4.4.4 Determination of dynamics by a break-through curve

The sorption kinetics of 3-HPA on IRA-SO_3H (5 g) was determined by passing 14.3 mmol 3-HPA in a volume of 100 ml (undiluted supernatant) through the column. 3-HPA was quantified in the effluent and visualised in a break-through curve (Figure 4.1). In total, 9.43 mmol 3-HPA remained on the resin, corresponding to 86.5 mol % of the theoretical capacity. However, already after 6 ml (column volume 7.8 ml), 3-HPA could be detected in the effluent and its concentration increased steadily forming a ‘s’-shaped curve (Figure 4.1, *filled circles*).

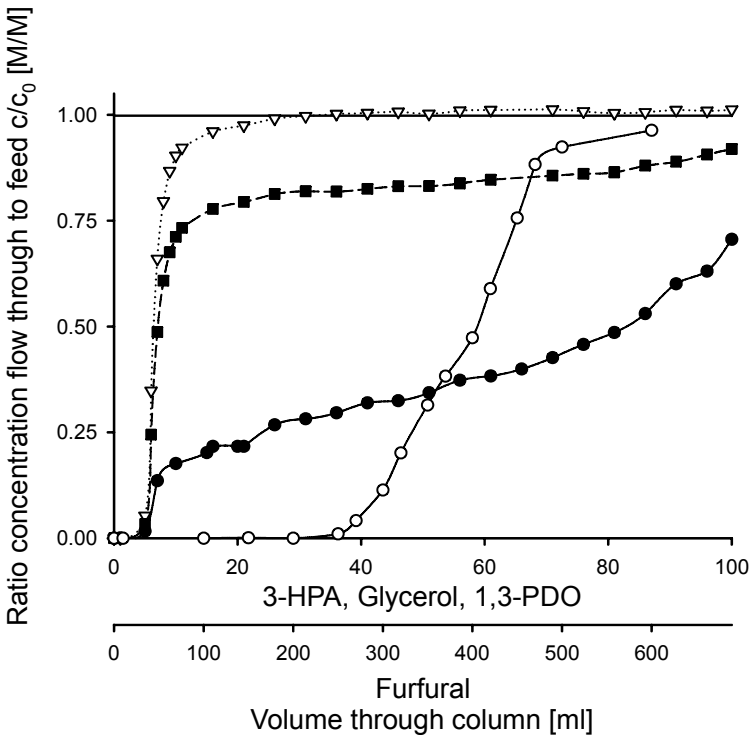

Figure 4.1 Breakthrough curve of 3-HPA and furfural from the IRA-SO_3H resin (5 g). Feed were 100 ml supernatant, containing 3-HPA (143 mM, purpald assay, *filled circles*), glycerol (249 mM, HPLC, *open triangles*), 1,3-propanediol (13 mM, HPLC, *filled squares*), and 600 ml furfural solution (control, 20.8 mM, HPLC, Gabrielson and Samuelson 1950, *open circles*). Flow rate was 2 ml min^{-1}. Different volume scales are adjusted according to the amount of 3-HPA and furfural in the feed.

Table 4.2 Binding of different amounts of 3-HPA on 5 g IRA-SO_3H after loading (5 ml) and three washing steps (10 ml water each). Total 3-HPA leakage is calculated from the difference between applied and bound 3-HPA. Values are means and standard errors of duplicate experiments.

Amount of applied 3-HPA [mmol]	Amount of 3-HPA bound to IRA-SO_3H [mmol]	Used capacity of IRA-SO_3H [mol %][a]	Total 3-HPA leakage [mol %][b]	Total 3-HPA leakage [mmol]
0.62	0.52±0.01	4.8±0.1%	15.0±1.7%	0.09±0.01
0.95	0.82±0.02	7.5±0.2%	14.0±2.1%	0.13±0.02
4.8	4.01±0.21	36.7±2.0%	16.1±4.9%	0.77±0.24
9.5	7.16±0.07	65.7±0.6%	24.6±0.7%	2.34±0.07
14.3	9.31±0.56	85.4±5.2%	34.8±4.0%	4.96±0.59

a) Ratio of bound 3-HPA [mmol] to theoretical capacity (10.9 mmol)

b) Ratio of 3-HPA loss [mmol] to applied 3-HPA [mmol]

4.4.5 Binding of by-products of the glycerol bioconversion to IRA-SO_3H

Glycerol and 1,3-PDO showed very poor binding to the IRA-SO_3H (Figure 4.1). From 24.9 mmol glycerol fed in a volume of 100 ml (undiluted supernatant), only 1.5 mmol was retained in the column, which was accumulated during the first 26 ml effluent (Figure 4.1, *open triangles*). In general, the amount of glycerol retained by the column was in-between 0.07 and 1.69 mmol, and the amount washed out by brine (saturated NaCl solution) was 0.26±0.23 mmol (results not shown).

From 1.3 mmol 1,3-PDO fed in a volume of 100 ml (undiluted supernatant), 0.27 mmol was retained in the column, and could not be eluted by brine (Figure 4.1, *filled squares*).

4.4.6 Influence of temperature and pH on binding properties of the column

The loading potential of the IRA-SO_3H resin (5 g) for 3-HPA was tested at 4°C and 20°C and at pH 4, 5, 6, 7, and 3.6 (uncontrolled, pH of the supernatant from the bioconversion). The average uptake of 3-HPA (5 ml of a 143 mM undiluted supernatant, corresponding to 0.72 mmol) at 20°C was significantly ($P < 0.05$) higher than at 4°C with 0.53±0.06 and 0.42±0.09 mmol bound 3-HPA, respectively. The pH in the range from 3.6 to 7 showed no significant influence ($P > 0.05$) on the binding. The mean 3-HPA uptake at the pH from 3.6 to 7 was determined to 0.48±0.01, 0.44±0.14, 0.39±0.06, 0.53±0.05, and 0.52±0.04 mmol 3-HPA, respectively.

4.4.7 Elution of 3-HPA from the IRA-SO_3H resin

Six IRA-SO_3H resin containing columns were each loaded with 0.62 mmol 3-HPA and washed. Under these conditions 0.53±0.02 mmol 3-HPA was bound to the column. 3-HPA was eluted with 6 different conditions and concentration of 3-HPA in 25 ml eluent fractions was measured (Figure 4.2). Brine (saturated NaCl solution,

36%) showed best and fastest elution ability with 0.40±0.02, 0.47±0.02, 0.50±0.01 and 0.51±0.00 mmol, corresponding to 75.6%, 90.0%, 95.9, and 98.2% recovered 3-HPA after 25, 50, 100, and 200 ml elution volume, respectively (Figure 4.2, *filled circles*). Sodium carbonate solution (0.075 M Na_2CO_3 and 0.15 M $NaHCO_3$,) showed good elution with 0.49±0.03 mmol recovered 3-HPA after 200 ml, but a pressure of 2 bar had to be applied to prevent CO_2 gas formation (Figure 4.2, *filled triangles*). With HCl (1 M) and NaOH (1 M) the amount of 3-HPA recovered with 200 ml was 0.47±0.04 mmol and 0.19 mmol, respectively (Figure 4.2, *open circles* and *open triangles*, respectively). Hot water (75°C) and ethyl acetate showed no elution capacity for 3-HPA (data not shown).

Brine was then used to test the recovery of 3-HPA from resins with higher 3-HPA loading. For all tested loadings (0.82, 4.0, 7.2, and 9.3 mmol) 74.3±3.3 mol % 3-HPA could be recovered by elution with 200 ml brine solution. Total hydrogensulfite analysis in brine eluate after loading with 9.3 mmol 3-HPA showed that 9.1 mmol sulfite was released from the column simultaneously to the recovery of 3-HPA. Therefore, 3-HPA was recovered as adduct.

4.4.8 Recovery of 3-HPA from eluant

After 3-HPA was selectively extracted from the crude production supernatant (143 mM 3-HPA, 249 mM glycerol, 13 mM 1,3-PDO, Figure 4.3a) to IRA-SO_3H and liberated with brine, the 3-HPA adduct was recovered from the latter. 3-HPA cleavage from the adduct and recovery were performed by neutralisation, water removal by lyophilisation, and solvent extraction of the 3-HPA with ethanol. After ethanol removal under reduced pressure, the remaining 3-HPA was analysed by HPLC (Figure 4.3b). The purity of 3-HPA was 34.1 mol %. NaCl the major compound (59.3 mol %), and traces of glycerol (3.3 mol %), ethanol (3.2 mol %), and 1,3-PDO (0.1 mol %) were detected.

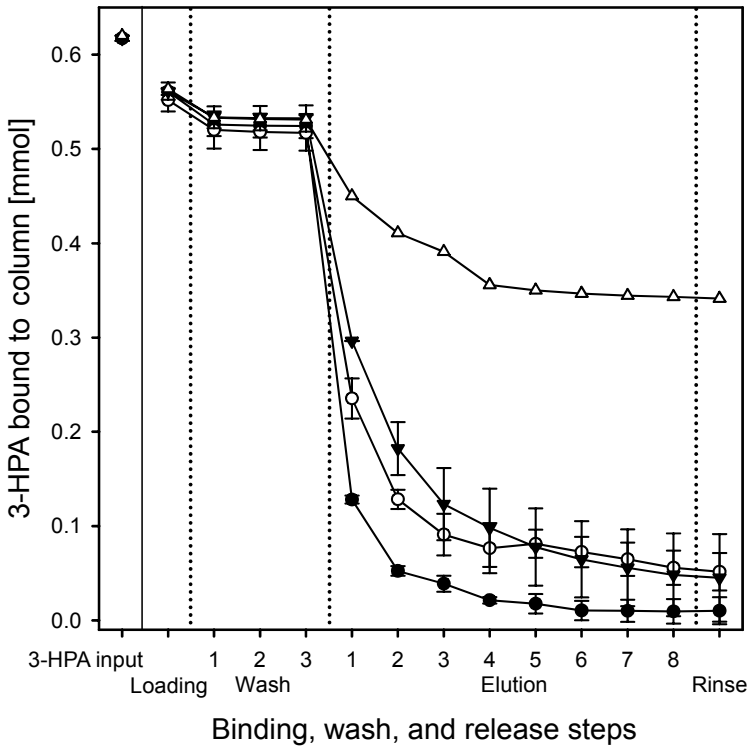

Figure 4.2 Binding and release of 3-HPA. Amount of 3-HPA (mmol) remaining in the column filled with IRA-SO_3H resin (5 g). After identical loading (5 ml) and wash (10 ml) steps, 3-HPA, determined by purpald assay, was eluted (8 x 25 ml) by either saturated NaCl solution (*filled circles*), 1 M HCl (*open circles*), 0.075 M Na_2CO_3+0.15 M $NaHCO_3$ solution (*filled triangles*), or 1 M NaOH (*open triangles*). *Error bars* show standard errors of duplicate experiments.

Recovery calculations of two independent 3-HPA purifications split into binding, elution, and ethanol extraction are displayed in Table 4.3. One purification was performed using concentrated 3-HPA supernatant (14.3 mmol 3-HPA) leading to high loaded resin (85.4 mol % of IRA-SO_3H capacity, 65.1 mol % 3-HPA recovery) and the other using unconcentrated 3-HPA supernatant (0.95 mmol 3-HPA, 86.0 mol % 3-HPA recovery) leading to low loaded IRA-SO_3H (7.5 mol %). The final recovery of pure 3-HPA was dependent on the initial 3-HPA amount and was determined as 14.5 mol % for highly and 45.3 mol % for low loaded resin. The relative recoveries of every purification step was with low 3-HPA amount superior to the recoveries obtained with high 3-HPA amount.

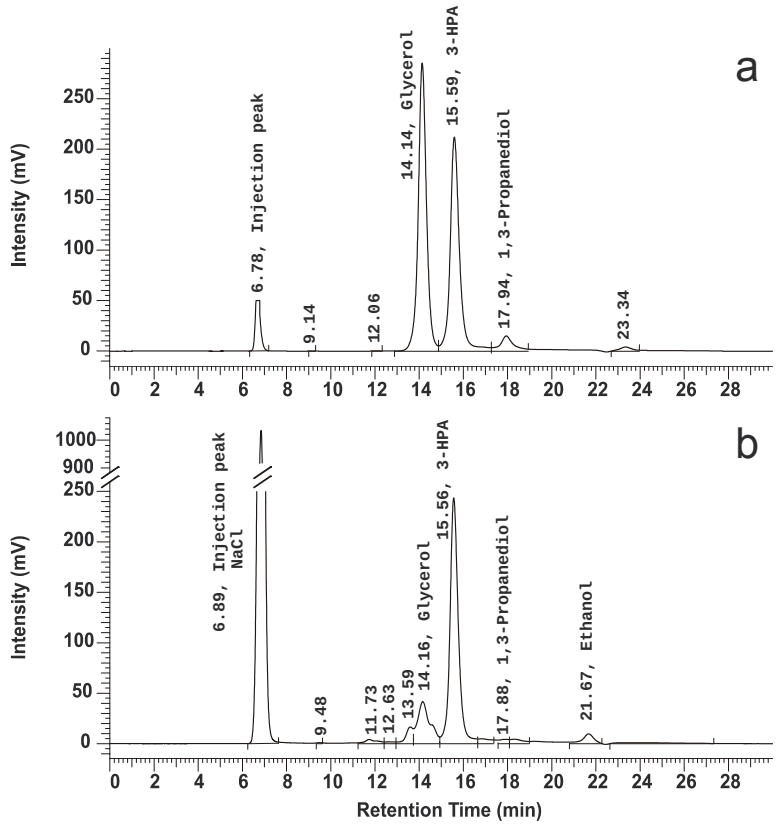

Figure 4.3 HPLC chromatograms after production of 3-HPA with *Lactobacillus reuteri* in 400 mM glycerol solution: (a) crude supernatant, after (b) elution with NaCl and extraction with ethanol.

Table 4.3 Recovery from the IRA-SO_3H resin of 3-HPA by elution with NaCl and by ethanol extraction of the lyophilised eluant.

Purification step	Amount of 3-HPA [mmol]	Used capacity of IRA-SO_3H [mol %][a]	3-HPA recovery of the purification step [mol %]	3-HPA recovery calculated from initial 3-HPA amount [mol %]
Purification with highly loaded resin				
3-HPA supernatant	14.3			100%
Bound to IRA-SO_3H	9.31	85.4%	65.1%	65.1%
Elution	7.08		76.0%	49.5%
Ethanol extraction	2.08		29.4%	**14.5%**
Purification with low loaded resin				
3-HPA supernatant	0.95			100%
Bound to IRA-SO_3H	0.82	7.5%	86.0%	86.0%
Elution	0.82		100.4%	86.3%
Ethanol extraction	0.43		52.5%	**45.3%**

a) Ratio of bound 3-HPA [mmol] to theoretical capacity (10.9 mmol)

4.4.9 3-HPA production using *in situ* product removal

The developed purification method was combined with bacterial 3-HPA production *in situ*. Pre-tests showed that a continuous flushing of the head space of the bioconversion with sterile nitrogen was necessary to ensure a high 3-HPA production of the control (248±17 mM 3-HPA, Figure 4.4, *dashed lines*). Without protection from air oxygen, a 3.5-fold reduction of produced 3-HPA (71±16 mM) was observed.

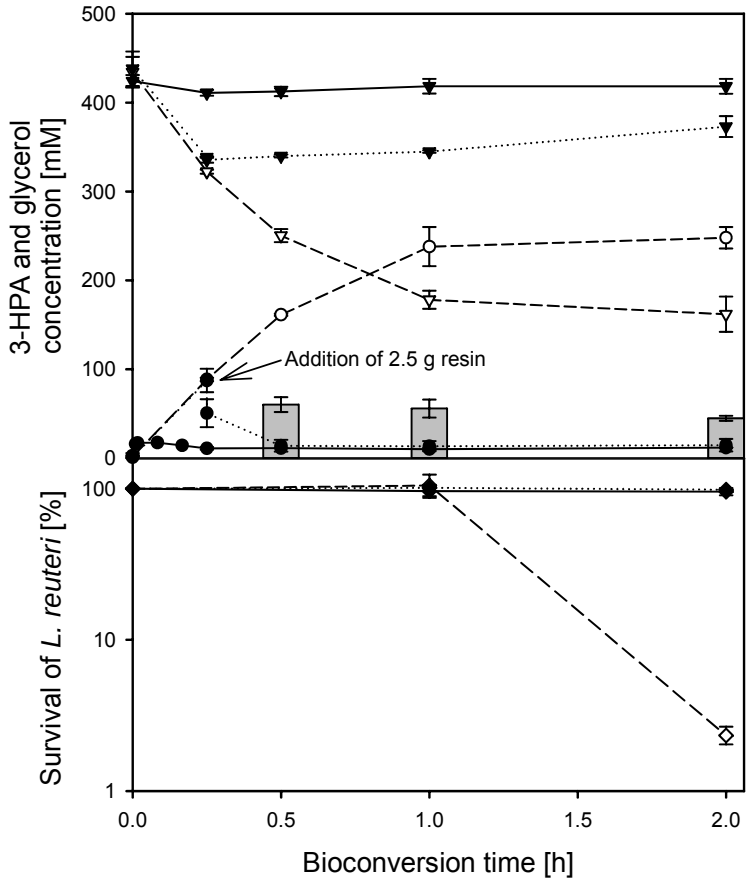

Figure 4.4 *In situ* product removal of 3-HPA during its production with *Lactobacillus reuteri* ($1.6x10^{10}$ cfu ml^{-1}) cells in the presence of 18.2% (w/v) IRA-SO_3H in 400 mM glycerol solution at 20°C (*solid lines*). *Dotted lines*: *in situ* product removal with resin addition after 15 min conversion. *Dashed lines*: control without resin. The amounts of glycerol (*triangles*) was monitored by HPLC analysis and 3-HPA (*circles*) by purpald assay (a); survival (*diamonds*) was monitored by plating out on MRS agar (b). *Grey bars* show difference between measured and initial glycerol amount (delayed resin addition). *Error bars* show standard errors of duplicate experiments.

The effect of IRA-SO_3H addition (18.2% (w/v)) on 3-HPA production in 400 mM glycerol containing $1.6x10^{10}$ cfu ml^{-1} *L. reuteri* cells at 20°C was tested. Resin addition at start sharply inhibited glycerol bioconversion and very low amount of 3-HPA (14±1 mM) was accumulated (Figure 4.4, *solid line*) after an incubation time of 2 h. Subsequent elution of the resin followed by 3-HPA determination (purpald assay) confirmed that almost no 3-HPA was loaded. When IRA-SO_3H was added to the bioconversion after 15 min, glycerol conversion stopped immediately at this point and 3-HPA concentration rapidly decreased (Figure 4.4, *dotted line*). Elution of the resin (ISPR 15 min delayed) with 100 ml saturated NaCl solution recovered 85 mol % of the converted 3-HPA within the first 15 min (0.89 mmol), showing the feasibility of the system.

Cell viability dropped drastically to 2±1% after 3-HPA production (248±17 mM) without IRA-SO_3H addition (Figure 4.4), whereas a 97±3% survival of *L. reuteri* was observed in presence of IRA-SO_3H showing that only bioconversion was influenced by IRA-SO_3H but not cell viability. Total sodium hydrogensulfite in the glycerol solution of the bioconversion with IRA-SO_3H was quantified to 25±5 mM after 2 h.

4.4.9.1 Effect of sodium hydrogensulfite and IRA-400 quartenary ammonium resin on 3-HPA production

Because the addition of IRA-SO_3H inhibited instantly 3-HPA production, the influence of the two main factors, sodium hydrogensulfite and resin (IRA-Cl), were tested separately on bioconversion. The effects of sodium hydrogensulfite concentration ranging from 0 mM to 125 mM on 3-HPA bioconversion was studied using an initial biomass concentration of $1.6x10^{10}$ cfu ml^{-1} at 20°C, and 2 h (Figure 4.5). In comparison with the control (no hydrogensulfite), no difference in 3-HPA production for up to 25 mM sodium hydrogensulfite could be detected. At concentrations exceeding 25 mM, 3-HPA accumulation decreased with increasing hydrogensulfite concentrations. Hydrogensulfite was determined quantitatively at the end of the production using the Ellman test.

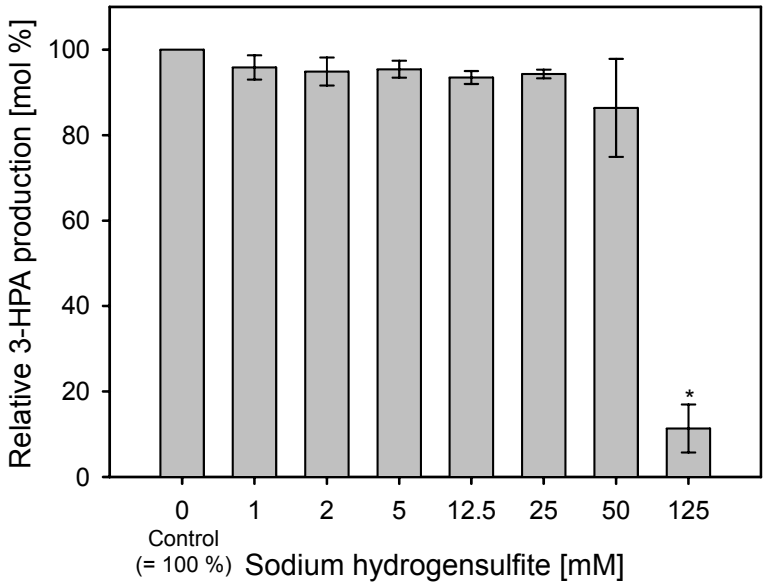

Figure 4.5 Bioconversion of glycerol to 3-HPA in presence of sodium hydrogensulfite using $1.6x10^{10}$ cfu ml^{-1} *Lactobacillus reuteri* cells in 400 mM glycerol solution at 20°C for 2 h. Concentration of 3-HPA is expressed in percentage of the control (0 mM hydrogensulfite) and was quantified by purpald assay. *Error bars* show standard errors of duplicate experiments. *Asterisk* shows significance ($P < 0.05$) between treatments.

The influence of IRA-Cl (18.2% (w/v) resin in 400 mM glycerol) on 3-HPA production was investigated for an initial biomass concentration of $1.6x10^{10}$ cfu ml^{-1} at 20°C and with continuous flushing the headspace with N_2. The highest production in the presence of the resin was significantly ($P < 0.05$) lower (144±25 mM 3-HPA) than for the control with no resin added (248±17 mM 3-HPA). After 1 h incubation, a similar survival of *L. reuteri* for both control and resin treatment were observed, averaging 101±11%. After 2 h, survival dropped to 10±9% (control) and 8±8% (with resin) in parallel to 3-HPA production.

4.5 Discussion

The aim of this study was to develop an efficient and easy purification method for 3-HPA and afterwards to combine this method with the 3-HPA production *in situ*. Simultaneous entrapment of 3-HPA during production would avoid the production of degradation products such as 1,3-propanediol, thus increasing the conversion yield of

glycerol to 3-HPA, and enable high viability of the production strain. The screening of various ligands showed that only one compound, sodium hydrogensulfite could selectively bind 3-HPA in an aqueous medium and subsequent release in suitable conditions. Sulfites are known to bind to carbonyl groups (Kerp, 1904a; Kerp, 1904b) forming α-oxysulfonic acids (Samuelson and Westlin, 1947) and are used to recover acetaldehyde (Harris and Hajny, 1960; Igawa *et al.*, 1990; Zall and Wecker, 1990), formaldehyde (Igawa *et al.*, 1990), lactaldehyde and pyruvaldehyde (Huff, 1959), benzaldehyde (Simmonds and Robinson, 1998; Kuzmanovic *et al.*, 2003), and the ketone acetone (Igawa *et al.*, 1990) in laboratory or industrial scale (Williams *et al.*, 1981).

According to our data, the adduct is too hydrophilic to be extracted into a solvent phase or to be precipitated by known methods from the aqueous phase. The solution was to adhere the 3-HPA-sulfonate to a water-insoluble strong basic ion exchange resin. This adsorption was simplified by transforming the resin with hydrogensulfite to hydrogensulfite form (IRA-SO_3H) first before using IRA-SO_3H to selectively withdraw 3-HPA from the 3-HPA containing supernatant. The good selectivity of the resin for the aldehyde was demonstrated by the fact that glycerol and 1,3-propanediol bound only weakly to the resin.

The measured capacity of IRA-SO_3H was determined by loading 14.3 mmol 3-HPA in either 5 ml or 100 ml supernatant to 85 mol % of theoretical capacity. Therefore, IRA-SO_3H can be loaded with 3-HPA to the region of the theoretical maximum capacity independently of the influent volume. The large leakage of up to 35 mol % of the applied amount of 3-HPA, however, will impact negatively the production costs. To reduce 3-HPA leakage, the amount of applied 3-HPA can be reduced. However, we showed 3-HPA leakage remained high even for low amount of applied 3-HPA (Table 4.2). Another solution could be to circulate the medium through the column. A limitation of the latter could be an instable 3-HPA-adduct leading to unwanted release from IRA-SO_3H. According to our data, the 3-HPA-adduct is stable enough to circulate a volume of the supernatant at least 13 times the column volume.

In comparison with other aldehydes, 3-HPA shows a complete different binding. According to Gabrielson and Samuelson (1950), 4.1 mmol acetaldehyde, benzaldehyde, and salicylaldehyde (in 50 ml volume each) were adsorbed quantitatively (< 0.01% loss) on IRA-SO_3H with a capacity of 14.8 mmol

corresponding to an uptake of 3.0 mmol aldehyde calculated for the investigated system. Acetone, a ketone, was adsorbed well (approx 2.5% loss). Furthermore, 8.5 mmol furfural corresponding to 57 mol % of the theoretical capacity was reported to adsorb without leakage (Gabrielson and Samuelson, 1950). In our study, a constant 3-HPA leakage of about 15% was measured for applied 3-HPA amounts equal or lower to 4.8 mmol. The amount of bound 3-HPA is correlated with the stability of the adduct. The stability was assessed by the quantification of free hydrogensulfite of 3-HPA after incubation of equimolar amounts of aldehyde and sulfite at pH 3.5. The amount of free sulfite is lower than a mixture of equimolar amounts of acetone and sulfite (37 mol % free sulfite) (Rehm *et al.*, 1965). Therefore, 3-HPA-sulfonate is at least as stable as acetone-sulfonate and a quantitative uptake of up to 3.0 mmol 3-HPA is expected.

A different explanation might be found in the preference of 3-HPA to form dimers and hydrates (Vollenweider *et al.*, 2003) like formaldehyde (Walker, 1964). Gabrielson and Samuelson (1950) reported some difficulties to absorb quantitatively formaldehyde on IRA-SO_3H. The authors speculated that the dimer or polymer must be transformed to the aldehyde prior adsorption. In case of formaldehyde, doubling of the resin and decrease of the flow rate enabled a quantitative uptake.

For the recovery of 3-HPA, saturated NaCl solution was selected for best recovery performance (elution speed, final recovery, and handling). Because hydrogensulfite was eluted by NaCl along with 3-HPA from the resin, 3-HPA is recovered as adduct. This data is in agreement with Walton (1957) who reported that aldehydes were recovered as adduct from IRA-SO_3H by elution with NaCl. Thereby, liberated 3-HPA must be first cleaved from the adduct before separation from eluant. This could be performed by pH shift to alkaline as shown before for other aldehydes than 3-HPA (Rehm *et al.*, 1965). For the cleavage of 3-HPA-sulfonate, a neutral pH was chosen because the stability of aldehydes is reduced significantly at alkaline pH as shown with glutaraldehyde (Trelstad, 1969).

Ethanol was selected due to low solubility of NaCl, cost and recycling possibility. Carry over of salt in the water/ethanol mixture was prevented by water removal prior ethanol extraction. NaCl is soluble in pure ethanol to a degree of only 0.055% (w/w) that is increased to 8.9 and 26.5% (w/w) in 50% (w/w) ethanol and water, respectively (Pinho and Macedo, 1996). Since 3-HPA is converted to acrolein at

temperatures above 37°C (Lüthi-Peng *et al.*, 2002b; Vollenweider and Lacroix, 2004), lyophilisation was chosen for water removal at laboratory scale. However, because lyophilisation is a low output and expensive process, further techniques need to be developed for water removal at larger scales.

The conditions for optimal bioconversion of glycerol to 3-HPA were reported to a temperature of 20°C and low pH of 4 to 5 (Doleyres *et al.*, 2005). Our studies showed that 3-HPA uptake at 20°C was higher than at 4°C and that pH (3.6 to 7) had no significant influence on the absorption of 3-HPA. Therefore, 3-HPA production with *in situ* product removal with the IRA-SO_3H system could be performed at optimal conditions.

The newly developed purification method was combined with 3-HPA production from glycerol with free *L. reuteri* cells to develop an *in situ* product removal process. However, the production of 3-HPA was blocked immediately after addition of IRA-SO_3H. Trials to evaluate the key factors for inhibition, IRA-400 exchange resin or hydrogensulfite, only revealed a partial inhibition of 3-HPA production. A possibility could be a decomposition product from the resin or other effects on cofactors for the enzyme glycerol dehydratase. Both vitamin B_{12} and enzyme being intracellular, the capture of the important cofactor vitamin B_{12} for this enzyme by IRA-SO_3H is not likely.

To conclude our data showed a new method to selectively extract 3-HPA from bioconversion medium by the anion exchange resin IRA-400 in hydrogensulfite form, that method can be upscaled, and that the 3-HPA can be recovered. However, additional work is needed to screen for an appropriate anion exchange resin for an *in situ* product removal process not interfering with the bioconversion and to avoid the expensive lyophilisation step before ethanol removal of NaCl.

5 General conclusions and outlook

This thesis focused on the optimisation of biotechnological production of 3-hydroxypropionaldehyde, a broad spectrum antibiotic and a precursor for biopolymers, using live intact *Lactobacillus reuteri* ATCC 55730 cells in a two-step process. Highest reported amount of free 3-HPA occurred under these conditions (Doleyres *et al.*, 2005).

The first hypothesis of this study claimed that 3-HPA accumulation is increased by adding cofactors of the glycerol dehydratase, oxidative cell protector glutathione, or glucose to the bioconversion step. This hypothesis could not be confirmed. None of the tested supplements showed stimulation on 3-HPA production. Addition of the tripeptide GSH, which is involved in detoxification processes and maintaining a reduced intracellular environment, showed no influence on *L. reuteri* survival and 3-HPA accumulation during bioconversion. These data suggested that *L. reuteri* was not able to uptake the delivered substances during starvation or these were already provided in sufficient amounts from biomass production step.

Glycerol bioconversion by *L. reuteri* ATCC 55730 was favoured under anaerobic conditions, but 3-HPA accumulation was not influenced by oxygen as long as glycerol solution and buffer were sterilised by autoclaving. In the case of stirred ISPR processes or glycerol conversion in 2 ml microtiter plates, oxygen removal techniques needed to be applied.

Glucose was reported to stimulate 3-HPA accumulation and glycerol bioconversion using low biomass concentration (Lüthi-Peng *et al.*, 2002a). With optimised initial biomass concentration according to Doleyres *et al.* (2005), we showed that glucose decreased 3-HPA accumulation and glycerol conversion, together with increased lactic acid and 1,3-PDO excretion. Similarly, addition of phosphate-citrate buffer for pH control decreased significantly 3-HPA accumulation. For our conditions, unbuffered, unadjusted, and unsupplemented 400 mM glycerol solution is therefore optimal for highest 3-HPA production.

The next approach to enhance 3-HPA accumulation of providing glucose and GSH during washing was successful. Washing with Kpi^{+} (Kpi with 100 mM glucose, 1

mM glutathione, 1 mM $MgCl_2$) enhanced significantly both successive 3-HPA production steps compared with the control (Kpi and Kpi/Kpi, respectively) while the by product 1,3-propanediol increased only marginally. Survival of *L. reuteri*, however, dropped during washing with Kpi^+ compared with the control (Kpi and Kpi/Kpi, respectively). During washing in buffer, the producing strain is therefore able to uptake the provided supplements leading to increased 3-HPA accumulation. The observed drop in survival indicated on the other hand that resting *L. reuteri* cells were more tolerant to 3-HPA toxicity than metabolic active cells.

In order to increase productivity of cells, survival after bioconversion and eliminate centrifugation steps, immobilised cell technology was applied. A 3.2-fold increase in specific productivity of 3-HPA was observed with 4.3-fold fewer cells in the bioreactor compared to free cells. Increased specific productivity and survival, however, were at the expense of three-fold lower mean conversion rate and 1.4-fold lower maximal accumulated 3-HPA concentration. Bioconversion of glycerol using IC was dependent on temperature, biomass concentration in beads, and the colonisation method. Attempts to increase specific 3-HPA yield by bead recolonisation in MRS medium and successive glycerol bioconversion failed, since *L. reuteri* cells were irreversibly damaged during starvation and because of toxic 3-HPA. Shorten bioconversion time to one forth was not able to decrease starvation effects, but 3-HPA accumulation was decreased three-fold. 3-HPA accumulation is directly linked to viability of *L. reuteri*, therefore growth promoting factors and physiological solution (supplement for maximal viability recovery) were tested on viability and 3-HPA accumulation. These supplements showed repressing effects on 3-HPA accumulation that glycerol bioconversion is optimally carried out in mere glycerol solution according to our data. Although physiological solution showed no significant decrease in 3-HPA accumulation and might help coping *L. reuteri* with starvation, this approach was not further investigated due to development of a novel purification method applicable in an *in situ* product removal process (ISPR).

The third and fourth objectives were therefore to develop an efficient affinity like purification for 3-HPA and to combine the optimised fermentation process parameters with the novel purification process to an ISPR process. The new purification method based on selective extraction of 3-HPA from bioconversion medium was successfully performed using an anion exchange resin IRA-400 in hydrogensulfite form.

Subsequent down stream process consisted in release of 3-HPA adduct by elution with brine, water removal of the eluate (lyophilisation), and extraction of pure 3-HPA from the dry salt by EtOH. This method can be upscaled to large scale processes. The overall yield of the reactive extraction and down stream process ranged between 14.5 and 45.3 mol % whereas the EtOH extraction step was identified as the most inefficient step with low recoveries of between 29.4 and 52.5 mol %. Additional work is therefore needed to optimise 3-HPA extraction from elute. Due to leakage during loading of 3-HPA to IRA-SO_3H, losses up to 24.6±0.7% were observed. These losses might be reduced by circulation of bioconversion medium since very similar 3-HPA amounts were loaded on IRA-SO_3H by either 100 ml (13 resin volumes) or 5 ml liquid. Increase in uptake of 3-HPA needs to be experimentally proven.

After successful development of novel purification, IRA-SO_3H was added to glycerol bioconversion forming an ISPR. Bioconversion stopped instantly when IRA-SO_3H was added. Interestingly, neither IRA-400 nor hydrogensulfite showed same drastic impact on glycerol conversion. Additional work is needed to screen for an appropriate anion exchange resin for an ISPR process not interfering with the bioconversion.

Subsequent research is focused on the combination of IC with ISPR. Two approaches could be pursued. Firstly, immobilised *L. reuteri* cells are incubated in glycerol solution with IRA-SO_3H added to the bioconversion medium or medium is circulated through the IRA-SO_3H column. Secondly, during continuous colonisation of beads, free *L. reuteri* cells are released at high concentration with the medium. These cells might be used directly for the bioconversion. To reduce by-product 1,3-PDO formation, a knock-out of the encoding gene of 1,3-propanediol dehydrogenase might be engineered. Word for a genetically modified *L. reuteri* without active 1,3-propanediol dehydrogenase is currently in progress at our laboratory.

6 References

3-Hydroxypropionaldehyde, MSDS [Online]. Oct 6, 2003. The Physical and Theoretical Chemistry Laboratory, Oxford University, Oxford, UK. http://msds.chem.ox.ac.uk/HY/3-hydroxypropionaldehyde.html (accessed Nov 2, 2009).

Abeles, R. H., A. M. Brownstein, and C. H. Randles. 1960. β-hydroxypropionaldehyde, an intermediate in the formation of 1,3-propanediol by *Aerobacter aerogenes*. Biochim Biophys Acta 41:530-531.

Acrolein, MSDS [Online]. Aug 21, 2005. The Physical and Theoretical Chemistry Laboratory, Oxford University, Oxford, UK. http://msds.chem.ox.ac.uk/AC/acrolein.html (accessed Nov 11, 2009).

Ahrné, S., S. Nobaek, B. Jeppsson, I. Adlerberth, A. E. Wold, and G. Molin. 1998. The normal *Lactobacillus* flora of healthy human rectal and oral mucosa. J Appl Microbiol 85:88-94.

Annan, W. D., E. Hirst, and D. J. Manners. 1965. Constitution of laminarin. Part IV. Minor component sugars. J Chem Soc:220-226.

Arnaud, J. P., C. Lacroix, and F. Castaigne. 1992a. Counterdiffusion of lactose and lactic-acid in kappa-carrageenan locust bean gum gel beads with or without entrapped lactic-acid bacteria. Enzyme Microb Technol 14:715-724.

Arnaud, J. P., C. Lacroix, and L. Choplin. 1992b. Effect of agitation rate on cell release rate and metabolism during continuous fermentation with entrapped growing. Biotechnol Tech 6:265-270.

Arqués, J. L., J. Fernández, P. Gaya, M. Nuñez, E. Rodríguez, and M. Medina. 2004. Antimicrobial activity of reuterin in combination with nisin against food-borne pathogens. Int J Food Microbiol 95:225-229.

Arqués, J. L., E. Rodríguez, M. Nuñez, and M. Medina. 2008. Inactivation of Gram-negative pathogens in refrigerated milk by reuterin in combination with nisin or the lactoperoxidase system. Eur Food Res Technol 227:77-82.

Arsköld, E., E. Lohmeler-Vogel, R. Cao, S. Roos, P. Rådstöm, and E. W. J. van Niel. 2008. Phosphoketolase pathway dominates in *Lactobacillus reuteri* ATCC 55730 containing dual pathways for glycolysis. J Bacteriol 190:206-212.

Atlas, R. M. 1999. Probiotics - snake oil for the new millennium? Environ Microbiol 1:377-382.

Axelsson, L., T. C. Chung, W. J. Dobrogosz, and S. E. Lindgren. 1989. Production of a broad-spectrum antimicrobial substance produced by *Lactobacillus reuteri*. Microb Ecol Health Dis 2:131-136.

Bagnell, L., M. Bliese, T. Cablewski, C. R. Strauss, and J. Tsanaktsidis. 1997. Environmentally benign procedures for the preparation and isolation of 3-methylcyclopent-2-en-1-one. Aust J Chem 50:921-925.

Barbirato, F., J. P. Grivet, P. Soucaille, and A. Bories. 1996. 3-hydroxypropionaldehyde, an inhibitory metabolite of glycerol fermentation to 1,3-propanediol by enterobacterial species. Appl Environ Microbiol 62:1448-1451.

Baumgart, J., and B. Becker, eds. 2004. Mikrobiologische Untersuchung von Lebensmitteln, 5. akt. und erw. Auflage. Behr's Verlag, Hamburg.

Berger, R. G. 1995. Aroma biotechnology. Springer, Berlin [etc], pp. 139-162.

Bertrand, N., I. Fliss, and C. Lacroix. 2001. High nisin-Z production during repeated-cycle batch cultures in supplemented whey permeate using immobilized *Lactococcus lactis* UL719. Int Dairy J 11:953-960.

Bicak, N., and B. F. Senkal. 1997. Aldehyde separation by polymer-supported oligo(ethyleneimines). J Polym Sci [A1] 35:2857-2864.

Blouin, J. J. 1984. Continuous process for making solid, free-flowing water dispersible PVA-aldehyde reaction product. US Patent No. 4,472,552.

Brenda. The Comprehensive Enzyme Information System. http://www.brenda-enzymes.info.

Burgess, C., M. O'Connell-Motherway, W. Sybesma, J. Hugenholtz, and D. van Sinderen. 2004. Riboflavin production in *Lactococcus lactis*: Potential for in situ production of vitamin-enriched foods. Appl Environ Microbiol 70:5769-5777.

Cachon, R., P. Anterieux, and C. Divies. 1998. The comparative behavior of *Lactococcus lactis* in free and immobilized culture processes. J Biotechnol 63:211-218.

Caglar, E., S. K. Cildir, S. Ergeneli, N. Sandalli, and S. Twetman. 2006. Salivary mutans streptococci and lactobacilli levels after ingestion of the probiotic bacterium *Lactobacillus reuteri* ATCC 55730 by straws or tablets. Acta Odontol Scand 64:314-8.

Casas, I. A., and W. J. Dobrogosz. 2000. Validation of the probiotic concept: *Lactobacillus reuteri* confers broad-spectrum protection against disease in humans and animals. Microb Ecol Health Dis 12:247-285.

Casas, I. A., and B. Mollstam. 1998. Treatment of diarrhea. US Patent No. 5,837,238.

Champagne, C. P., C. Lacroix, and I. Sodini-Gallot. 1994. Immobilized cell technologies for the dairy industry. Crit Rev Biotechnol 14:109-134.

Chen, C. N., H. W. Sung, H. F. Liang, and W. H. Chang. 2002. Feasibility study using a natural compound (reuterin) produced by *Lactobacillus reuteri* in sterilizing and crosslinking biological tissues. J Biomed Mater Res 61:360-369.

Chen, Y. C., W. H. Chang, Y. Chang, C. M. Huang, and H. W. Sung. 2004. A natural compound (reuterin) produced by *Lactobacillus reuteri* for hemoglobin polymerization as a blood substitute. Biotechnol Bioeng 87:34-42.

Chung, T. C., L. Axelsson, S. E. Lindgren, and W. J. Dobrogosz. 1989. *In vitro* studies on reuterin synthesis by *Lactobacillus reuteri*. Microb Ecol Health Dis 2:137-144.

Cinquin, C., G. Le Blay, I. Fliss, and C. Lacroix. 2004. Immobilization of infant fecal microbiota and utilization in an *in vitro* colonic fermentation model. Microb Ecol 48:128-138.

Circle, S. J., L. Stone, and C. S. Boruff. 1945. Acrolein determination by means of tryptophane. Ind Eng Chem Res 17:259-262.

Cleusix, V., C. Lacroix, S. Vollenweider, M. Duboux, and G. Le Blay. 2007. Inhibitory activity spectrum of reuterin produced by *Lactobacillus reuteri* against intestinal bacteria. BMC Microbiol 7:101.

Cleusix, V., C. Lacroix, S. Vollenweider, and G. Le Blay. 2008. Glycerol induces reuterin production and decreases *Escherichia coli* population in an *in vitro* model of colonic fermentation with immobilized human feces. FEMS Microbiol Ecol 63:56-64.

Condon, S. 1987. Responses of lactic acid bacteria to oxygen. FEMS Microbiol Lett 46:269-280.

Cotter, P. D., C. Hill, and R. P. Ross. 2005. Bacteriocins: Developing innate immunity for food. Nat Rev Microbiol 3:777-788.

Culligan, E. P., C. Hill, and R. D. Sleator. 2009. Probiotics and gastrointestinal disease: successes, problems and future prospects. Gut Pathog 1:19.

Daeschel, M. A. 1989. Antimicrobial substances from lactic acid bacteria for use as food preservatives. Food Technol 43:164-167.

Daniel, R., T. A. Bobik, and G. Gottschalk. 1999. Biochemistry of coenzyme B_{12}-dependent glycerol and diol dehydratases and organization of the encoding genes. FEMS Microbiol Rev 22:553-566.

Daniel, R., and G. Gottschalk. 1992. Growth temperature-dependent activity of glycerol dehydratase in *Escherichia coli* expressing the *Citrobacter freundii* dha regulon. FEMS Microbiol Lett 100:281-286.

de Man, J. C., M. Rogosa, and M. E. Sharpe. 1960. A medium for the cultivation of Lactobacilli. J Appl Bacteriol 23:130-135.

Dickinson, R. G., and N. W. Jacobsen. 1970. A new sensitive and specific test for the detection of aldehydes: Formation of 6-mercapto-3-substituted-s-triazolo[4,3-b]-s-tetrazines. J Chem Soc D:1719-1720.

Dickinson, R. G., and N. W. Jacobsen. 1975. 6-Substituted s-Triazolo[4,3-b]-s-tetrazine-3-thiols - Sensitive and specific test for aldehydes. J Chem Soc Perkin I:975-979.

Dobrogosz, W. J., I. A. Casas, G. A. Pagano, B.-M. Sjöberg, T. L. Talarico, and M. Karlsson. 1989. *Lactobacillus reuteri* and the enteric microbiota *in* The regulatory and protective role of the normal microflora (R. Grubb, T. Midtvedt, and E. Norin, eds.). Macmillan Press Ltd., London.

Dobrogosz, W. J., and S. E. Lindgren. 1988. Antibiotic reuterin. US Patent PCT/US88/01423.

Dobrogosz, W. J., and S. E. Lindgren. 1994. Method of determining the presence of an antibiotic produced by *Lactobacillus reuteri*. US Patent No. 5,352,586.

Dobrogosz, W. J., and S. E. Lindgren. 1995. Method for inhibiting microorganism growth. US Patent No. 5,439,678.

Doleyres, Y., P. Beck, S. Vollenweider, and C. Lacroix. 2005. Production of 3-hydroxypropionaldehyde using a two-step process with *Lactobacillus reuteri*. Appl Microbiol Biotechnol 68:467-474.

Doleyres, Y., I. Fliss, and C. Lacroix. 2004a. Continuous production of mixed lactic starters containing probiotics using immobilized cell technology. Biotechnol Progr 20:145-150.

Doleyres, Y., I. Fliss, and C. Lacroix. 2004b. Increased stress tolerance of *Bifidobacterium longum* and *Lactococcus lactis* produced during continuous mixed-strain immobilized-cell fermentation. J Appl Microbiol 97:527-539.

Doleyres, Y., and C. Lacroix. 2005. Technologies with free and immobilised cells for probiotic bifidobacteria production and protection. Int Dairy J 15:973-988.

Duff, S. J. B., and W. D. Murray. 1989. Oxidation of benzyl alcohol by whole cells of *Pichia pastoris* and by alcohol oxidase in aqueous and nonaqueous reaction media. Biotechnol Bioeng 34:153-159.

Duwat, P., S. Sourice, B. Cesselin, G. Lamberet, K. Vido, P. Gaudu, Y. Le Loir, F. Violet, P. Loubiere, and A. Gruss. 2001. Respiration capacity of the fermenting bacterium *Lactococcus lactis* and its positive effects on growth and survival. J Bacteriol 183:4509-4516.

El-Ziney, M. G., N. Arneborg, M. Uyttendaele, J. Debevere, and M. Jakobsen. 1998. Characterization of growth and metabolite production of *Lactobacillus reuteri*

during glucose/glycerol cofermentation in batch and continuous cultures. Biotechnol Lett 20:913-916.

El-Ziney, M. G., and J. Debevere. 1998. The effect of reuterin on *Listeria monocytogenes* and *Escherichia coli* O157:H7 in milk and cottage cheese. J Food Prot 61:1275-1280.

El-Ziney, M. G., T. van den Tempel, J. Debevere, and M. Jakobsen. 1999. Application of reuterin produced by *Lactobacillus reuteri* 12002 for meat decontamination and preservation. J Food Prot 62:257-261.

Ellman, G. E. 1959. Tissue sulfhydryl groups. Arch Biochem Biophys 82:70-77.

FAO/WHO. 2002. Joint FAO/WHO Working Group Report on Drafting Guidelines on the Evaluation of Probiotics in Food. London, Ont. Canada.

Ferguson, G. P., and I. R. Booth. 1998. Importance of glutathione for growth and survival of *Escherichia coli* cells: Detoxification of methylglyoxal and maintenance of intracellular K^+. J Bacteriol 180:4314-4318.

Freeman, A., J. M. Woodley, and M. D. Lilly. 1993. *In-situ* product removal as a tool for bioprocessing. Biotechnology 11:1007-1012.

Frigerio, N. A., and M. J. Shaw. 1969. A simple method for determination of glutaraldehyde. J Histochem Cytochem 17:176-181.

Fuller, R. 1991. Probiotics in human medicine. Gut 32:439-442.

Gabrielson, G., and O. Samuelson. 1950. Utilization of ion exchangers in analytical chemistry. XVI: Adsorption of aldehydes and ketones. Sven Kem Tidskr 62:214-223.

Gabrielson, G., and O. Samuelson. 1952. Utilization of ion exchangers in analytical chemistry. XIX: Separation of aldehydes and ketones from organic acids. Acta Chem Scand 6:729-737.

Gänzle, M. G. 2004. Reutericyclin: biological activity, mode of action, and potential applications. Appl Microbiol Biotechnol 64:326-332.

Gänzle, M. G., M. Ehmann, and W. P. Hammes. 1998. Modeling of growth of *Lactobacillus sanfranciscensis* and *Candida milleri* in response to process parameters of sourdough fermentation. Appl Environ Microbiol 64:2616-2623.

Gänzle, M. G., A. Holtzel, J. Walter, G. Jung, and W. P. Hammes. 2000. Characterization of reutericyclin produced by *Lactobacillus reuteri* LTH2584. Appl Environ Microbiol 66:4325-4333.

Gänzle, M. G., N. Vermeulen, and R. F. Vogel. 2007. Carbohydrate, peptide and lipid metabolism of lactic acid bacteria in sourdough. Food Microbiol 24:128-138.

Garai-Ibabe, G., I. Ibarburu, I. Berregi, O. Claisse, A. Lonvaud-Funel, A. Irastorza, and M. T. Dueñas. 2008. Glycerol metabolism and bitterness producing lactic acid bacteria in cidermaking. Int J Food Microbiol 121:253-261.

Gibson, G. R., and R. Fuller. 2000. Aspects of in vitro and in vivo research approaches directed toward identifying probiotics and prebiotics for human use. J Nutr 130:391S-395S.

Glutaraldehyde, MSDS [Online]. Aug 7, 2009. The Physical and Theoretical Chemistry Laboratory, Oxford University, Oxford, UK. http://msds.chem.ox.ac.uk/GL/glutaric_dialdehyde.html (accessed Nov 12, 2009).

Guarner, F., G. Perdigon, G. Corthier, S. Salminen, B. Koletzko, and L. Morelli. 2005. Should yoghurt cultures be considered probiotic? Br J Nutr 93:783-786.

Hall, R. H., and E. S. Stern. 1950. Acid-catalysed hydration of acrylaldehyde: Kinetics of the reaction and isolation of β-hydroxypropionaldehyde. J Chem Soc 1950:490-498.

Hammes, W. P. 1998. Bewertung der gesundheitlichen Unbedenklichkeit von Milchsäurebakterien und Probiotika. Monatsschr Kinderheilkd 146:31-38.

Hansen, E. B. 2002. Commercial bacterial starter cultures for fermented foods of the future. Int J Food Microbiol 78:119-131.

Harris, J. F., and G. J. Hajny. 1960. Glycerol production: a pilot-plant investigation for continuous fermentation and recovery. J Biochem Microbiol Tech Eng 2:9-24.

Hartmanis, M. G., and T. C. Stadtman. 1986. Diol metabolism and diol dehydratase in *Clostridium glycolicum*. Arch Biochem Biophys 245:144-152.

Holzapfel, W. H., P. Haberer, R. Geisen, J. Bjorkroth, and U. Schillinger. 2001. Taxonomy and important features of probiotic microorganisms in food and nutrition. Am J Clin Nutr 73:365S-373S.

Honda, S., T. Toraya, and S. Fukui. 1980. In situ reactivation of glycerol-inactivated coenzyme B_{12}-dependent enzymes, glycerol dehydratase and diol dehydratase. J Bacteriol 143:1458-1465.

Huber, K., H. Esterbauer, H. J. Jäger, and D. Grill. 1987. Detection of sulphite in plants. Environ Pollut 46:127-136.

Huff, E. 1959. Chromatography of mixtures of lactaldehyde, acetol, and pyruvaldehyde on bisulfite ion exchange columns. Anal Chem 31:1626-1629.

Humphreys, F. B. 1924. Formation of acrolein from glycerol by *B. welchii*. J Infect Dis 35:282-290.

Igawa, M., Y. Fukushi, T. Hayashita, and M. R. Hoffmann. 1990. Selective transport of aldehydes across an anion-exchange membrane via the formation of bisulfite adducts. Ind Eng Chem Res 29:857-861.

Imase, K., A. Tanaka, K. Tokunaga, H. Sugano, H. Ishida, and S. Takahashi. 2007. *Lactobacillus reuteri* tablets suppress *Helicobacter pylori* infection - a double-blind randomised placebo-controlled cross-over clinical study. Kansenshogaku zasshi 81:387-93.

Ito, Y., Y. Kawai, K. Arakawa, Y. Honme, T. Sasaki, and T. Saito. 2009. Conjugative plasmid from *Lactobacillus gasseri* LA39 that carries genes for production of and immunity to the circular bacteriocin gassericin A. Appl Environ Microbiol 75:6340-6351.

Jin, L., L. Tao, S. I. Pavlova, J. S. So, N. Kiwanuka, Z. Namukwaya, B. A. Saberbein, and M. Wawer. 2007. Species diversity and relative abundance of vaginal lactic acid bacteria from women in Uganda and Korea. J Appl Microbiol 102:1107-1115.

Johnson, B. C., A. Stroinski, and Z. Schneider. 1975. Glycerol dehydratase from *Aerobacter aerogenes*. Methods Enzymol 42:315-323.

Jost, W., and H. E. Hauck. 1983. High-performance thin-layer chromatographic precoated plates with amino modification and some applications. J Chromatogr 261:235-244.

Kandler, O., and K.-O. Stetter. 1973. Der Beitrag neuerer biochemischer Merkmale für die Systematik der Laktobazillen *in* 3. Symposium Technische Mikrobiologie, Berlin.

Kandler, O., K.-O. Stetter, and R. Köhl. 1980. *Lactobacillus reuteri* sp. nov., a new species of heterofermentative Lactobacilli. Zentralbl Bakteriol Mikrobiol Hyg, I. Abt Orig C 1:264-269.

Kastner, S., V. Perreten, H. Bleuler, G. Hugenschmidt, C. Lacroix, and L. Meile. 2006. Antibiotic susceptibility patterns and resistance genes of starter cultures and probiotic bacteria used in food. Syst Appl Microbiol 29:145-155.

Kawai, Y., Y. Ishii, K. Arakawa, K. Uemura, B. Saitoh, J. Nishimura, H. Kitazawa, Y. Yamazaki, Y. Tateno, T. Itoh, and T. Saito. 2004. Structural and functional differences in two cyclic bacteriocins with the same sequences produced by lactobacilli. Appl Environ Microbiol 70:2906-2911.

Kerp, W. 1904a. Über die schweflige Säure im Wein. 1. Abhandlung. Allgemeines. Arbeiten aus dem Kaiserlichen Gesundheitsamte 21:141-155.

Kerp, W. 1904b. Über die schweflige Säure im Wein. 2. Abhandlung. Über die aldehydschweflige Säure im Wein. Arbeiten aus dem Kaiserlichen Gesundheitsamte 21:156-179.

Kleerebezem, M., and J. Hugenholtz. 2003. Metabolic pathway engineering in lactic acid bacteria. Curr Opin Biotechnol 14:232-237.

Klein, G., C. Hallmann, I. A. Casas, J. Abad, J. Louwers, and G. Reuter. 2000. Exclusion of *vanA*, *vanB* and *vanC* type glycopeptide resistance in strains of *Lactobacillus reuteri* and *Lactobacillus rhamnosus* used as probiotics by polymerase chain reaction and hybridization methods. J Appl Microbiol 89:815-824.

Koch, S., G. Oberson, E. Eugster-Meier, L. Meile, and C. Lacroix. 2007. Osmotic stress induced by salt increases cell yield, autolytic activity, and survival of lyophilization of *Lactobacillus delbrueckii* subsp. *lactis*. Int J Food Microbiol 117:36-42.

Krämer, J. 1997. Lebensmittel-Mikrobiologie, 3. Auflage. Verlag Eugen Ulmer, Stuttgart.

Kusudou, T., Y. Kumaki, and N. Fujiwara. 2006. Polyvinyl acetal and its use. US Patent No. 6,992,130.

Kuzmanovic, B., N. J. M. Kuipers, A. B. de Haan, and K. Gerard. 2003. Reactive extraction of carbonyl compounds from apolar hydrocarbons using aqueous salt solutions. Ind Eng Chem Res 42:2885-2896.

Lacroix, C., F. Grattepanche, Y. Doleyres, and D. Bergmaier. 2005. Immobilized cell technology for the dairy industry *in* Application of cell immobilization biotechnology (V. Nevidovic, and R. Willaert, eds.). Springer, Heidelberg and Berlin, Germany.

Lamer, T., H. E. Spinnler, I. Souchon, and A. Voilley. 1996. Extraction of benzaldehyde from fermentation broth by pervaporation. Process Biochem 31:533-542.

Leistner, L. 1978. Hurdle effect and energy saving *in* Food Quality and Nutrition (W. K. Downey, ed.). Applied Science Publishers, London, UK. pp. 553-557.

Leistner, L. 2000. Basic aspects of food preservation by hurdle technology. Int J Food Microbiol 55:181-186.

Lerche, M., and G. Reuter. 1962. Das Vorkommen aerob wachsender gram-positiver Stäbchen des Genus *Lactobacillus beijerinck* im Darminhalt erwachsener Menschen. Zentralbl Bakteriol, I. Abt Orig 185:446-481.

Lindgren, S. E., and W. J. Dobrogosz. 1990. Antagonistic activities of lactic acid bacteria in food and feed fermentations. FEMS Microbiol Rev 87:149-163.

Lionetti, E., V. L. Miniello, S. P. Castellaneta, A. M. Magista, A. de Canio, G. Maurogiovanni, E. Ierardi, L. Cavallo, and R. Francavilla. 2006. *Lactobacillus reuteri* therapy to reduce side-effects during anti-*Helicobacter pylori* treatment in children: a randomized placebo controlled trial. Aliment Pharmacol Ther 24:1461-1468.

Lüthi-Peng, Q., F. B. Dileme, and Z. Puhan. 2002a. Effect of glucose on glycerol bioconversion by *Lactobacillus reuteri*. Appl Microbiol Biotechnol 59:289-296.

Lüthi-Peng, Q., S. Schärer, and Z. Puhan. 2002b. Production and stability of 3-hydroxypropionaldehyde in *Lactobacillus reuteri*. Appl Microbiol Biotechnol 60:73-80.

Mahapatra, A. K., K. Muthukumarappan, and J. L. Julson. 2005. Applications of ozone, bacteriocins and irradiation in food processing: A review. Crit Rev Food Sci Nutr 45:447-461.

Makarova, K., A. Slesarev, Y. Wolf, A. Sorokin, B. Mirkin, E. Koonin, A. Pavlov, N. Pavlova, V. Karamychev, N. Polouchine, V. Shakhova, I. Grigoriev, Y. Lou, D. Rohksar, S. Lucas, K. Huang, D. M. Goodstein, T. Hawkins, V. Plengvidhya, D. Welker, J. Hughes, Y. Goh, A. Benson, K. Baldwin, J. H. Lee, I. Diaz-Muniz, B. Dosti, V. Smeianov, W. Wechter, R. Barabote, G. Lorca, E. Altermann, R. Barrangou, B. Ganesan, Y. Xie, H. Rawsthorne, D. Tamir, C. Parker, F. Breidt, J. Broadbent, R. Hutkins, D. O'Sullivan, J. Steele, G. Unlu, M. Saier, T. Klaenhammer, P. Richardson, S. Kozyavkin, B. Weimer, and D. Mills. 2006. Comparative genomics of the lactic acid bacteria. Proc Natl Acad Sci USA 103:15611-15616.

Marteau, P. R., M. de Vrese, C. J. Cellier, and J. Schrezenmeir. 2001. Protection from gastrointestinal diseases with the use of probiotics. Am J Clin Nutr 73:430S-436S.

Masson, F., C. Lacroix, and C. Paquin. 1994. Direct measurement of pH profiles in gel beads immobilizing *Lactobacillus helveticus* using a pH sensitive microelectrode. Biotechnol Tech 8:551-556.

McIlvaine, T. C. 1921. A buffer solution for colorimetric comparison. J Biol Chem 49:183-186.

Mills, D. E., W. D. Baugh, and H. A. Conner. 1954. Studies on the formation of acrolein in distillery mashes. Appl Microbiol 2:9-13.

Molin, G., M. L. Johansson, M. Stahl, S. Ahrné, R. Andersson, B. Jeppsson, and S. Bengmark. 1992. Systematics of the *Lactobacillus* population on rat intestinal mucosa with special reference to *Lactobacillus reuteri*. Antonie van Leeuwenhoek 61:175-183.

Molinari, F., R. Villa, M. Manzoni, and F. Aragozzini. 1995. Aldehyde production by alcohol oxidation with *Gluconobacter oxydans*. Appl Microbiol Biotechnol 43:989-994.

Morita, H., H. Toh, S. Fukuda, H. Horikawa, K. Oshima, T. Suzuki, M. Murakami, S. Hisamatsu, Y. Kato, T. Takizawa, H. Fukuoka, T. Yoshimura, K. Itoh, D. J. O'Sullivan, L. L. Mckay, H. Ohno, J. Kikuchi, T. Masaoka, and M. Hattori. 2008. Comparative genome analysis of *Lactobacillus reuteri* and

Lactobacillus fermentum reveal a genomic island for reuterin and cobalamin production. DNA Res 15:151-161.

Naito, S., H. Hayashidani, K. Kaneko, M. Ogawa, and Y. Benno. 1995. Development of intestinal lactobacilli in normal piglets. J Appl Bacteriol 79:230-236.

Nef, J. U. 1904. Dissociationsvorgänge in der Glycol-Glycerinreihe. Justus Liebigs Ann Chem 335:247-333.

Neysens, P., W. Messens, and L. De Vuyst. 2003. Effect of sodium chloride on growth and bacteriocin production by *Lactobacillus amylovorus* DCE 471. Int J Food Microbiol 88:29-39.

Nunoshiba, T., and K. Yamamoto. 1999. Role of glutathione on acrolein-induced cytotoxicity and mutagenicity in *Escherichia coli*. Mutat Res 442:1-8.

O'Brien, J. R., C. Raynaud, C. Croux, L. Girbal, P. Soucaille, and W. N. Lanzilotta. 2004. Insight into the mechanism of the B_{12}-independent glycerol dehydratase from *Clostridium butyricum*: Preliminary biochemical and structural characterization. Biochemistry 43:4635-4645.

O'Brien, P. J., A. G. Siraki, and N. Shangari. 2005. Aldehyde sources, metabolism, molecular toxicity mechanisms, and possible effects on human health. Crit Rev Toxicol 35:609-662.

Ouchi, T., H. Moriwaki, N. Murayama, and M. Imoto. 1982. Vinyl polymerization. 402. Radical polymerization of vinyl monomers initiated by anion-exchange resin [Crosslinked poly(p-methylstyrene)-trimethyl ammonium-chloride]. J Polym Sci [A1] 20:1133-1138.

Parvez, S., K. A. Malik, S. Ah Kang, and H. Y. Kim. 2006. Probiotics and their fermented food products are beneficial for health. J Appl Microbiol 100:1171-1185.

Pasteur, L. 1858a. Nouvelles recherches sur la fermentation alcoolique. CR Acad Sci XLVII:224.

Pasteur, L. 1858b. Production constante de glycérine dans la fermentation alcoolique. CR Acad Sci XLVI:857.

Pasteur, L. 1866. Études sur le vin. Ses maladies et les causes qui les provoquent, procédés nouveaux pour le conserver et pour le vieillir. Imprimerie Impériale, Paris.

Pérez, A. A. 2001. Enhanced microbial production of natural flavors via *in-situ* product adsorption. Dissertation 14074. Institute of Food Science and Nutrition. ETH Zurich, Zurich.

Pinho, S. P., and E. A. Macedo. 1996. Representation of salt solubility in mixed solvents: A comparison of thermodynamic models. Fluid Phase Equilib 116:209-216.

Poppe, L., W. E. Hull, R. Nitsche, T. Graf, E. Stupperich, and J. Retey. 1999. (Hydroxyalkyl)cob(III)alamins as competitive inhibitors in coenzyme B-12-dependent enzymic reactions: H-1-NMR structure analysis and kinetic studies with glycerol dehydratase and diol dehydratase. Helv Chim Acta 82:1250-1265.

Prasad, J., P. McJarrow, and P. Gopal. 2003. Heat and osmotic stress responses of probiotic *Lactobacillus rhamnosus* HN001 (DR20) in relation to viability after drying. Appl Environ Microbiol 69:917-925.

Propanal, MSDS [Online]. Aug 24, 2005. The Physical and Theoretical Chemistry Laboratory, Oxford University, Oxford, UK. http://msds.chem.ox.ac.uk/PR/propionaldehyde.html (accessed Nov 11, 2009).

Rasch, M. 2002. The influence of temperature, salt and pH on the inhibitory effect of reuterin on *Escherichia coli*. Int J Food Microbiol 72:225-231.

Raymond, W. R. 1984. Process for the generation of acetaldehyde from ethanol. US Patent No. 4,481,292.

Rehm, J.-H., P. Wallnöfer, and H. Keskin. 1965. Beitrag zur Kenntnis der antimikrobiellen Wirkung der schwefligen Säure. IV. Mitteilung: Dissoziation und antimikrobielle Wirkung einiger Sulfonate. Z Lebensm Unters Forsch A 127:72-85.

Rehm, J.-H., and H. Wittmann. 1962. Beitrag zur Kenntnis der antimikrobiellen Wirkung der schwefligen Säure. II. Mitteilung: Die Wirkung der dissoziierten und dissoziierten Säure auf verschiedene Mikroorganismen. Z Lebensm Unters Forsch A 118:413–429.

Reid, G. 1999. The scientific basis for probiotic strains of *Lactobacillus*. Appl Environ Microbiol 65:3763-3766.

Rendina, A. R., and W. W. Cleland. 1981. Separation of aldehydes and ketones by chromatography on Dowex-50 in the ethylenediamine form. Anal Biochem 117:213-218.

Rentschler, H., and H. Tanner. 1951. Das Bitterwerden der Rotweine. Mitt Gebiete Lebensm Hyg 42:463-475.

Reuter, G. 1965. Das Vorkommen von Laktobazillen in Lebensmitteln und ihr Verhalten im menschlichen Intestinaltrakt. Zentralbl Bakteriol, I. Abt Orig 197:468-487.

Rodriguez, E., J. L. Arques, R. Rodriguez, M. Nunez, and M. Medina. 2003. Reuterin production by lactobacilli isolated from pig faeces and evaluation of probiotic traits. Lett Appl Microbiol 37:259-263.

Römpp Online Enzyklopädie. Keyword: Aldehyde. Thieme Chemistry, Stuttgart, Germany. http://www.roempp.com.

Rosander, A., E. Connolly, and S. Roos. 2008. Removal of antibiotic resistance gene-carrying plasmids from *Lactobacillus reuteri* ATCC 55730 and characterization of the resulting daughter strain, *L. reuteri* DSM 17938. Appl Environ Microbiol 74:6032-6040.

Ruiz-Palacios, G., M. L. Guerrero, M. Hilty, M. Dohnalek, P. Newton, J. J. Calva, T. Costigan, F. Tuz, and F. Arteaga. 1996. Feeding of a probiotic for the prevention of community-acquired diarrhea in young Mexican children. Pediatr Res 39 Part 2:184A, Abstract 1089.

Saggioroa, A., M. Carolia, M. Pasinia, F. Bortoluzzia, L. Girardia, and G. Piloneb. 2005. *Helicobacter pylori* eradication with *Lactobacillus reuteri*. A double-blinded placebo-controlled study. Dig Liver Dis 37:S88.

Samuelson, O. 1949. Sätt att rena alkoholhaltiga vätskor. SW Patent No. 125443.

Samuelson, O. 1963. Ion exchange separations in analytical chemistry. Almqvist & Wiksell, Stockholm.

Samuelson, O., and A. Westlin. 1947. Über das lose gebundene SO_2 in Sulfitlauge. Sven Kem Tidskr 59:244-253.

Santos, F., A. Wegkamp, W. M. de Vos, E. J. Smid, and J. Hugenholtz. 2008. High-level folate production in fermented foods by the B_{12} producer *Lactobacillus reuteri* JCM1112. Appl Environ Microbiol 74:3291-3294.

Sauvageot, N., K. Gouffi, J. M. Laplace, and Y. Auffray. 2000. Glycerol metabolism in *Lactobacillus collinoides*: production of 3-hydroxypropionaldehyde, a precursor of acrolein. Int J Food Microbiol 55:167-170.

Savino, F., E. Pelle, E. Palumeri, R. Oggero, and R. Miniero. 2007. *Lactobacillus reuteri* (American type culture collection strain 55730) versus simethicone in the treatment of infantile colic: A prospective randomized study. Pediatrics 119:E124-E130.

Saxelin, M., H. Rautelin, S. Salminen, and P. H. Makela. 1996. Safety of commercial products with viable *Lactobacillus* strains. Infect Dis Clin Pract 5:331-335.

Schauenstein, E., H. Esterbauer, and H. Zollner. 1977. Aldehydes in biological systems: Their natural occurance and biological activities. Pion Limited, London.

Schepers, A. W., J. Thibault, and C. Lacroix. 2002a. *Lactobacillus helveticus* growth and lactic acid production during pH-controlled batch cultures in whey permeate/yeast extract medium. Part I. multiple factor kinetic analysis. Enzyme Microb Technol 30:176-186.

Schepers, A. W., J. Thibault, and C. Lacroix. 2002b. *Lactobacillus helveticus* growth and lactic acid production during pH-controlled batch cultures in whey permeate/yeast extract medium. Part II. kinetic modeling and model validation. Enzyme Microb Technol 30:187-194.

Schlegel, H. G. 1972. Allgemeine Mikrobiologie, 2. Auflage. Georg Thieme Verlag, Stuttgart, pp. 165-166.

Schleifer, K. H., and W. Ludwig. 1995. Phylogeny of the genus Lactobacillus and related genera. Syst Appl Microbiol 18:461-467.

Schneider, Z., and B. C. Johnson. 1971. Studies on the thermal dissociation of glycerol dehydrase. Arch Biochem Biophys 144:491-495.

Schneider, Z., E. G. Larsen, G. Jacobson, B. C. Johnson, and J. Pawelkiewicz. 1970. Purification and properties of glycerol dehydrase. J Biol Chem 245:3388-3396.

Schütz, H., and F. Radler. 1984. Anaerobic reduction of glycerol to 1,3-propanediol by *Lactobacillus brevis* and *Lactobacillus buchneri*. Syst Appl Microbiol 5:169-178.

Seeram, N., R. Lee, M. Hardy, and D. Heber. 2005. Rapid large scale purification of ellagitannins from pomegranate husk, a by-product of the commercial juice industry. Separ Purif Technol 41:49-55.

Seifert, C., S. Bowien, G. Gottschalk, and R. Daniel. 2001. Identification and expression of the genes and purification and characterization of the gene products involved in reactivation of coenzyme B_{12}-dependent glycerol dehydratase of *Citrobacter freundii*. Eur J Biochem 268:2369-2378.

Serjak, W. C., W. H. Day, J. M. Van Lanen, and C. S. Boruff. 1954. Acrolein production by bacteria found in distillery grain mashes. Appl Microbiol 2:14-20.

Shachar-Nishri, Y., and A. Freeman. 1993. Continuous production of acetaldehyde by immobilized yeast with *in situ* product trapping. Appl Biochem Biotechnol 39:387-399.

Shornikova, A. V., I. A. Casas, E. Isolauri, H. Mykkanen, and T. Vesikari. 1997a. *Lactobacillus reuteri* as a therapeutic agent in acute diarrhea in young children. J Pediatr Gastroenterol Nutr 24:399-404.

Shornikova, A. V., I. A. Casas, H. Mykkanen, E. Salo, and T. Vesikari. 1997b. Bacteriotherapy with *Lactobacillus reuteri* in rotavirus gastroenteritis. Pediatr Infect Dis J 16:1103-1107.

Simmonds, J., and G. K. Robinson. 1998. Formation of benzaldehyde by *Pseudomonas putida* ATCC 12633. Appl Microbiol Biotechnol 50:353-358.

Slininger, P. J., and R. J. Bothast. 1985. Optimizing aerobic conversion of glycerol to 3-hydroxypropionaldehyde. Appl Environ Microbiol 50:1444-1450.

Slininger, P. J., R. J. Bothast, and K. L. Smiley. 1983. Production of 3-hydroxypropionaldehyde from glycerol. Appl Environ Microbiol 46:62-67.

Slininger, P. J., J. E. Vancauwenberge, and R. J. Bothast. 1990. Production of 3-hydroxypropionaldehyde from glycerol by *Klebsiella pneumoniae*. US Patent No. 4,962,027.

Smiley, K. L., and M. Sobolov. 1962. A cobamide-requiring glycerol dehydrase from an acrolein-forming *Lactobacillus*. Arch Biochem Biophys 97:538-543.

Sobolov, M., and K. L. Smiley. 1960. Metabolism of glycerol by an acrolein-forming *Lactobacillus*. J Bacteriol 79:261-266.

Sönke, A., A. Wiezer, H. Bendfeldt, T. Waschkowitz, C. Toeche-Mittler, and R. Daniel. 2004. Insights into the Genome of the Enteric Bacterium *Escherichia blattae*: Cobalamin (B_{12}) Biosynthesis, B_{12}-Dependent Reactions, and Inactivation of the Gene Region Encoding B_{12}-Dependent Glycerol Dehydratase by a New Mu-Like Prophage. J Mol Microbiol Biotechnol 8:150-168.

Sponholz, W.-R. 1993. Wine spoilage by microorganisms *in* Wine Microbiology and Biotechnology (G. H. Fleet, ed.). Harwood Academic Publishers, Chur.

Sriramulu, D. D., M. Liang, D. Hernandez-Romero, E. Raux-Deery, H. Lunsdorf, J. B. Parsons, M. J. Warren, and M. B. Prentice. 2008. *Lactobacillus reuteri* DSM 20016 produces cobalamin-dependent diol dehydratase in metabolosomes and metabolizes 1,2-propanediol by disproportionation. J Bacteriol 190:4559-4567.

Stark, D., and U. von Stockar. 2003. In situ product removal (ISPR) in whole cell biotechnology during the last twenty years. Adv Biochem Eng Biotechnol 80:149-175.

Stevens, M. J. A., S. Vollenweider, and C. Lacroix. 2010. Potential of reuterin produced by *Lactobacillus reuteri* as broad spectrum preservative in food *in* Protective cultures, antimicrobial metabolites and bacteriophages for food and beverage biopreservation (C. Lacroix, ed.). Woodhead Publishing, Cambridge, UK.

Stevens, M. J. A., A. Wiersma, W. M. de Vos, O. P. Kuipers, E. J. Smid, D. Molenaar, and M. Kleerebezem. 2008. Improvement of *Lactobacillus plantarum* aerobic growth as directed by comprehensive transcriptome analysis. Appl Environ Microbiol 74:4776-4778.

Stradal, J. A., and G. L. Underwood. 1995. Process for producing hydroxyacetaldehyde US Patent No. 5,393,542.

Stroinski, A., J. Pawelkiewicz, and B. C. Johnson. 1974. Allosteric interactions in glycerol dehydratase. Purification of enzyme and effects of positive and negative cooperativity for glycerol. Arch Biochem Biophys 162:321-330.

Sung, H. W., C. N. Chen, Y. Chang, and H. F. Liang. 2002. Biocompatibility study of biological tissues fixed by a natural compound (reuterin) produced by *Lactobacillus reuteri*. Biomaterials 23:3203-3214.

Sung, H. W., C. N. Chen, H. F. Liang, and M. H. Hong. 2003. A natural compound (reuterin) produced by *Lactobacillus reuteri* for biological-tissue fixation. Biomaterials 24:1335-1347.

Sung, H. W., R. N. Huang, L. L. H. Huang, and C. C. Tsai. 1999. *In vitro* evaluation of cytotoxicity of a naturally occurring cross-linking reagent for biological tissue fixation. J Biomater Sci Polym Ed 10:63-78.

Sybesma, W., M. Starrenburg, L. Tijsseling, M. H. N. Hoefnagel, and J. Hugenholtz. 2003. Effects of cultivation conditions on folate production by lactic acid bacteria. Appl Environ Microbiol 69:4542-4548.

Talarico, T. L., I. A. Casas, T. C. Chung, and W. J. Dobrogosz. 1988. Production and isolation of reuterin, a growth inhibitor produced by *Lactobacillus reuteri*. Antimicrob Agents Chemother 32:1854-1858.

Talarico, T. L., and W. J. Dobrogosz. 1989. Chemical characterization of an antimicrobial substance produced by *Lactobacillus reuteri*. Antimicrob Agents Chemother 33:674-679.

Talarico, T. L., and W. J. Dobrogosz. 1990. Purification and characterization of glycerol dehydratase from *Lactobacillus reuteri*. Appl Environ Microbiol 56:1195-1197.

Tannock, G. W. 1999. Probiotics: A critical review. Horizon Scientific Press, Wymondham, Norfolk, UK.

Taranto, M. P., J. L. Vera, J. Hugenholtz, G. F. De Valdez, and F. Sesma. 2003. *Lactobacillus reuteri* CRL1098 produces cobalamin. J Bacteriol 185:5643-5647.

Teuber, M. 1999. Spread of antibiotic resistance with food-borne pathogens. Cell Mol Life Sci 56:755-763.

Teuber, M., L. Meile, and F. Schwarz. 1999. Acquired antibiotic resistance in lactic acid bacteria from food. Antonie van Leeuwenhoek 76:115-137.

Toba, T., S. K. Samant, E. Yoshioka, and T. Itoh. 1991. Reutericin 6, a new bacteriocin produced by *Lactobacillus reuteri* LA-6. Lett Appl Microbiol 13:281-286.

Toraya, T. 1998. Recent structure-function studies of B_{12} coenzymes in diol dehydrase *in* Vitamin B_{12} and B_{12}-Proteins (B. Kräutler, D. Arigoni, and B. T. Golding, eds.). Wiley-VCH, Weinheim, Germany.

Toraya, T., and S. Fukui. 1977. Immunochemical evidence for the difference between coenzyme-B_{12}-dependent diol dehydratase and glycerol dehydratase. Eur J Biochem 76:285-289.

Trauth, E., J. P. Lemaitre, C. Rojas, C. Divies, and R. M. Cachon. 2001. Resistance of immobilized lactic acid bacteria to the inhibitory effect of quaternary ammonium sanitizers. Lebensm Wiss Technol 34:239-243.

Trelstad, R. L. 1969. The effect of pH on the stability of purified glutaraldehyde. J Histochem Cytochem 17:756-757.

Tubelius, P., V. Stan, and A. Zachrisson. 2005. Increasing work-place healthiness with the probiotic *Lactobacillus reuteri*: a randomised, double-blind placebo-controlled study. Environ Health 4:25.

Valeur, N., P. Engel, N. Carbajal, E. Connolly, and K. Ladefoged. 2004. Colonization and immunomodulation by *Lactobacillus reuteri* ATCC 55730 in the human gastrointestinal tract. Appl Environ Microbiol 70:1176-1181.

Valu, J. A. 1965. Survival of *Lactobacillus leichmannii* in relation to vitamin B_{12} assays. Appl Microbiol 13:486-490.

van Coillie, E., J. Goris, I. Cleenwerck, K. Grijspeerdt, N. Botteldoorn, F. Van Immerseel, J. de Buck, M. Vancanneyt, J. Swings, L. Herman, and M. Heyndrickx. 2007. Identification of lactobacilli isolated from the cloaca and vagina of laying hens and characterization for potential use as probiotics to control *Salmonella* Enteritidis. J Appl Microbiol 102:1095-1106.

van de Guchte, M., P. Serror, C. Chervaux, T. Smokvina, S. D. Ehrlich, and E. Maguin. 2002. Stress responses in lactic acid bacteria. Antonie van Leeuwenhoek 82:187-216.

Vancauwenberge, J. E., P. J. Slininger, and R. J. Bothast. 1990. Bacterial conversion of glycerol to beta-hydroxypropionaldehyde. Appl Environ Microbiol 56:329-332.

Veiga-da-Cunha, M., and M. A. Foster. 1992a. 1,3-Propanediol: NAD^+ oxidoreductases of *Lactobacillus brevis* and *Lactobacillus buchneri*. Appl Environ Microbiol 58:2005-2010.

Veiga-da-Cunha, M., and M. A. Foster. 1992b. Sugar-glycerol cofermentations in *lactobacilli*: the fate of lactate. J Bacteriol 174:1013-1019.

Vogel, R. F., G. Bocker, P. Stolz, M. Ehrmann, D. Fanta, W. Ludwig, B. Pot, K. Kersters, K. H. Schleifer, and W. P. Hammes. 1994. Identification of lactobacilli from sourdough and description of *Lactobacillus pontis* sp. nov. Int J Syst Bacteriol 44:223-229.

Voisenet, M. E. 1910. Formation d'acroléine dans la maladie de l'amertume des vins. CR Acad Sci 150:1614 -1616.

Voisenet, M. E. 1914a. Nouvelles recherches sur un ferment, contenu dans les eaux, agent de déshydratation de la glycérine. CR Acad Sci:734 -736.

Voisenet, M. E. 1914b. Sur un ferment, contenu dans les eaux, agent de déshydratation de la glycérine. CR Acad Sci 158:195-197.

Vollenweider, S., S. Evers, K. Zurbriggen, and C. Lacroix. 2010. Unraveling the HPA system; An active antimicrobial agent against human pathogens. J Biol Chem: in press.

Vollenweider, S., G. Grassi, I. König, and Z. Puhan. 2003. Purification and structural characterization of 3-hydroxypropionaldehyde and its derivatives. J Agric Food Chem 51:3287-3293.

Vollenweider, S., and C. Lacroix. 2004. 3-Hydroxypropionaldehyde: applications and perspectives of biotechnological production. Appl Microbiol Biotechnol 64:16-27.

Walker, F. J. 1964. Formaldehyde. Reinhold, New York, pp. 52-78.

Walton, H. F. 1957. Ion exchangers in organic chemistry *in* Ion exchangers in organic and biochemistry (C. Calmon, and T. R. E. Kressman, eds.). Interscience, New York, pp 640-657.

Wecker, M. S., and R. R. Zall. 1987. Fermentation strategies: acetaldehyde or ethanol? Process Biochem 22:135-138.

Weizman, Z., G. Asli, and A. Alsheikh. 2005. Effect of a probiotic infant formula on infections in child care centers: comparison of two probiotic agents. Pediatrics 115:5-9.

Wiederholt, K. M., and J. L. Steele. 1994. Glutathione accumulation in lactococci. J Dairy Sci 77:1183-1188.

Williams, P. J., C. R. Strauss, K. E. Klingner, S. R. Obst, and G. L. G. Anderson. 1981. Development of a process for the deodorization of spirit recovered from grape marc. Food Technol Austr 33:12-13.

Willke, T., and K. D. Vorlop. 2004. Industrial bioconversion of renewable resources as an alternative to conventional chemistry. Appl Microbiol Biotechnol 66:131-142.

Wolf, B. W., K. A. Garleb, D. G. Ataya, and I. A. Casas. 1995. Safety and tolerance of *Lactobacillus reuteri* in healthy adult male subjects. Microb Ecol Health Dis 8:41-50.

Wolf, B. W., K. B. Wheeler, D. G. Ataya, and K. A. Garleb. 1998. Safety and tolerance of *Lactobacillus reuteri* supplementation to a population infected with the human immunodeficiency virus. Food Chem Toxicol 36:1085-1094.

Yunmbam, M. K., and J. F. Roberts. 1992. The *in vitro* efficacy of reuterin on the culture and bloodstream forms of *Trypanosoma brucei brucei*. Comp Biochem Physiol C 101:235-238.

Yunmbam, M. K., and J. F. Roberts. 1993. *In vivo* evaluation of reuterin and its combinations with suramin, melarsoprol, DL-alpha-difluoromethylornithine and bleomycin in mice infected with *Trypanosoma brucei brucei*. Comp Biochem Physiol C 105:521-524.

Zall, R. R., and M. Wecker. 1990. Microbiological production of acetaldeyde. US Patent No. 4,900,670.

Zamudio-Jaramillo, M. A., A. Hernandez-Mendoza, V. J. Robles, P. G. Mendoza-Garcia, J. J. Espinosa-de-los-Monteros, and H. S. Garcia. 2009. Reuterin production by *Lactobacillus reuteri* NRRL B-14171 immobilized in alginate. J Chem Technol Biotechnol 84:100-105.

Zhu, P., X. Chen, and C. G. Roberts. 2004. Device and method of use for aldehyde removal. US Patent No. 6,776,904.

7 Appendix: Quantification of hydrogensulfite and 3-HPA

7.1 Abstract

3-Hydroxypropionaldehyde (3-HPA) is a versatile chemical for the chemical and food industry and can be synthesised biotechnologically by *Lactobacillus reuteri*. Purification might be achieved by binding reversibly to hydrogensulfite on an anion exchange resin. hydrogensulfite, however, interfered with known methods of 3-HPA quantification (tryptophan assay and HPLC). New, simple, rapid, and exact methods for the quantification of total hydrogensulfite, free hydrogensulfite, and 3-HPA, based on Ellman test, iodine titration, and purpald assay, respectively, were adapted and tested for the usability in the 3-HPA production system.

7.2 Quantification of hydrogensulfite using Ellman test

7.2.1 Introduction

hydrogensulfite can be used to complex 3-HPA during purification of 3-HPA and may possibly encounter the producing strain *Lactobacillus reuteri*. The determination of toxic hydrogensulfite is necessary. A simple and rapid method was described by Ellman (1959) for determination of sulphydryl groups and adapted to quantify hydrogensulfite in 1970 (Humphreys). In this method, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) reacts quantitatively with sulfite under neutral or weak basic conditions to a yellow coloured anion, which can be quantified at 412 nm.

Free hydrogensulfite was used as an indicator for the degree of formed adduct and its stability after quantification of the unreacted hydrogensulfite (Rehm and Wittmann,

1962; Rehm *et al.*, 1965). Free hydrogensulfite can be oxidised to sulfate with the oxidising agent iodine without affecting the complex (Frigerio and Shaw, 1969). Quantification of free hydrogensulfite could be performed by titrating against iodine. The aim of this experiment was to test the application of the methods in the 3-HPA production environment. Therefore, the interaction of possible present disturbing substances, 3-HPA, glycerol, glucose, acetic acid, and MRS medium, was investigated on the total hydrogensulfite quantification.

7.2.2 Material and methods

7.2.2.1 Reagents and materials

All reagents were purchased from Sigma-Aldrich (Buchs, Switzerland) at ≥ 98.5% purity unless otherwise specified. 3-HPA was produced biotechnologically from glycerol and purified on site according to Vollenweider *et al.* (2003).

7.2.2.2 Enzymatic determination of sodium hydrogensulfite solution

As reference method, the enzymatic test for hydrogensulfite (Boehringer Mannheim R-Biopharm) was used. hydrogensulfite is oxidised to sulfate and superoxide that reacts in a successive step with NADH to NAD^+. Diminishing NADH can be quantified at 340 nm using Beer–Lambert law. The quantification was performed in quadruplicate according to the user's manual but at one-fourth volume.

7.2.2.3 Quantification of total sulfite concentration

Total sulfite was quantified by a colorimetric method (Ellman test) adapted from Huber (1987). A 990 µl sample, previously diluted with 0.2 M potassium phosphate buffer (Kpi, pH 7.0) to a hydrogensulfite concentration below 0.1 mM final concentration, was pipetted into a photometrical cuvette. The reaction was started by adding 10 µl DTNB (4% (w/v) in dimethyl sulfoxide, ≥ 98.0%) and the assay was mixed with the pipette. After incubation of exactly 10.0 min at 20°C, the extinction

was measured with a photo spectrometer (Varian Cary 1 Bio) at 412 nm. Calculation of the concentration was performed using a standard curve with known amounts of hydrogensulfite obtained under same conditions. All solutions were prepared just prior use and mixed carefully to minimise oxygen entry.

Standard preparation

Calibration was performed with known amounts of hydrogensulfite by dissolving sodium sulfite. The extinctions of five evenly distributed final concentrations of hydrogensulfite between 0 and 80 µM were determined in double.

Sample preparation

Depending on the hydrogensulfite concentration, the watery samples were pre-diluted with bidistilled water and were added to the Ellman test according to Table 7.1. After addition of the corresponding amount of Kpi (Table 7.1), the quantification was started by pipetting 10 µl DTNB solution according to the protocol.

Table 7.1 Dilution scheme for the Ellman test for hydrogensulfite quantification depending of concentration range of original sample. The sample dilution is applied directly in the cuvette, pre-dilution is done externally.

Sample volume [µl]	Sample dilution	Pre-dilution	Total dilution	Concentration range [mM] min	max	Buffer [µl]
40	25x	1x	25x	0.67	2.68	950
20	50x	1x	50x	1.34	5.36	970
10	100x	1x	100x	2.68	10.72	980
40	25x	10x	250x	6.7	26.8	950
40	25x	20x	500x	13.4	53.6	950
20	50x	20x	1000x	26.8	107.2	970
40	25x	50x	1250x	33.5	134	950
10	100x	20x	2000x	53.6	214.4	980
20	50x	50x	2500x	67	268	970
20	50x	100x	5000x	134	536	970
10	100x	100x	10000x	268	1072	980

7.2.2.4 Quantification of free sulfite concentration

Unbond hydrogensulfite was quantified by triiodide tritration using starch solution as indicator adapted from Frigerio (1969). A measured amount of triiodine solution (0.01 M I_2 + 0.04 M KI; № HT90232) was added to 10 µl sample, 3000 µl bidistilled

water, and 500 µl starch solution (1% (w/v); BD, Allschwil, Switzerland) until a stable violet colour developed. Calibration was performed with known amounts of hydrogensulfite. All solutions were prepared just prior use and handled with care to avoid oxygen entry.

Standard preparation

Calibration was performed with 0, 40, 80, 120, 160, and 200 µM hydrogensulfite (sample concentration) in double.

Sample preparation

Formation of the 3-HPA hydrogensulfite adduct was investigated in a 3x3 full factorial design. Three amounts (0, 50, and 100 mM) of 3-HPA and sodium hydrogensulfite were mixed and incubated for 15 min at 20°C (Rehm *et al.*, 1965). Then, 10 µl of the mixture was added to 3000 µl bidistilled water and 500 µl starch solution and titrated with triiodine solution according to the protocol. The amount of formed adduct was calculated by the subtraction of free hydrogensulfite from the introduced amount.

7.2.3 Results and Discussion

7.2.3.1 Standard curve

The calibration curve is displayed in Figure 7.1. A linear regression was found (R^2=0.999) with a linearity factor of 0.0125 μM^{-1}. The molar extinction coefficient ε of the DTNB-anion, calculated from this calibration curve, was 12500 l mol^{-1} cm^{-1} agreed with reported values: 13190±350 l mol^{-1} cm^{-1} for sulphite (Huber *et al.*, 1987) and 13600 l mol^{-1} cm^{-1} for thiol groups (Ellman, 1959). The slightly lower value can be explained by oxidation of the sulfite during preparation and quantification by aerial oxygen.

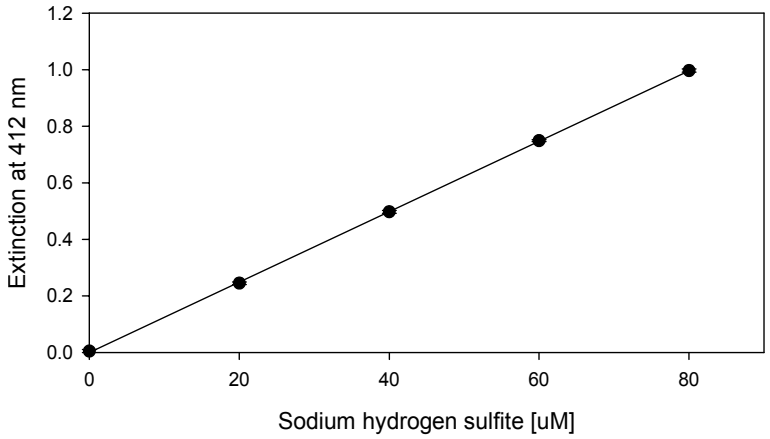

Figure 7.1 Standard curve for the hydrogen sulifte quantification using Ellman test. *Error bars* standard errors of 4 replicas.

7.2.3.2 Interaction with 3-HPA, acetic acid, medium MRS, glucose, and glycerol

After successful adaptation of the method to detect hydrogensulfite in aqueous samples, interacting ingredients possible present from the 3-HPA production step are tested for negative effect (Figure 7.2). No interaction on both the recovery and detection limit was found. However, a fair accuracy of the method under performed conditions (±5%) was found as seen in the standard errors of the control. This value corresponds well to the standard error of the calibration curve (±3.4%) indicating the limit of the method. If a higher accuracy is needed, working under complete anaerobic conditions might decrease the variability due to lowest oxidation of the hydrogensulfite.

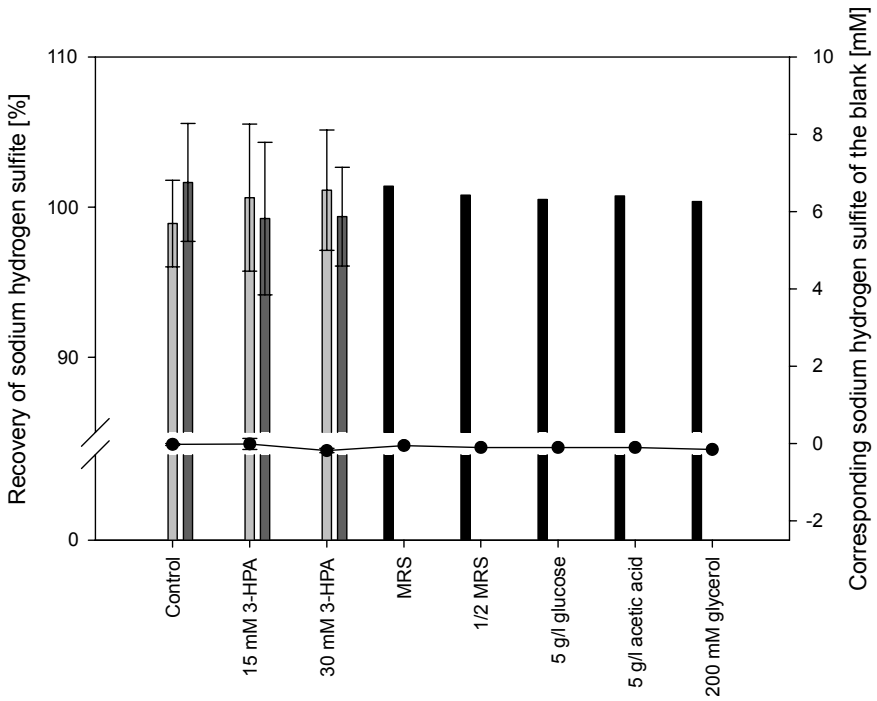

Figure 7.2 Interaction of different compounds with the quantification of hydrogensulfite using Ellman test at different sodium hydrogensulfite concentrations: *black* 2.66 mM, *light grey* 15 mM, *grey* 30 mM. *Straight line* shows measured hydrogensulfite concentration of a blank with no sodium hydrogensulfite but with added compound. *Error bars* show standard errors of triplicate experiments.

7.2.3.3 Degree of formation of the 3-HPA- hydrogensulfite adduct

The degree of adduct formation of 3-HPA with hydrogensulfite was determined by quantification of free, unreacted hydrogensulfite after incubation of 0-100 mM 3-HPA and sodium hydrogensulfite for 15 min (Rehm *et al.*, 1965). Under test conditions, 0.0, 39.6, 68.7, and 71.9 mol % of 3-HPA was present as adduct in solutions with a 3-HPA-to- hydrogensulfite ratio of 0, 0.5, 1, and 2 M M^{-1}, respectively.

7.3 Quantification of 3-HPA in presence of sulfite using purpald assay

7.3.1 Introduction

To date 3-HPA is quantified either photometrically using tryptophan assay (Circle *et al.*, 1945; Slininger *et al.*, 1983) or by HPLC (10 mM H_2SO_4, 0.6 ml min^{-1}; Adapted from Talarico *et al.*, 1988). Both methods, however, interfered with hydrogensulfite, a 3-HPA complexing agent during purification, leading possibly to wrong results (Figure 7.3).

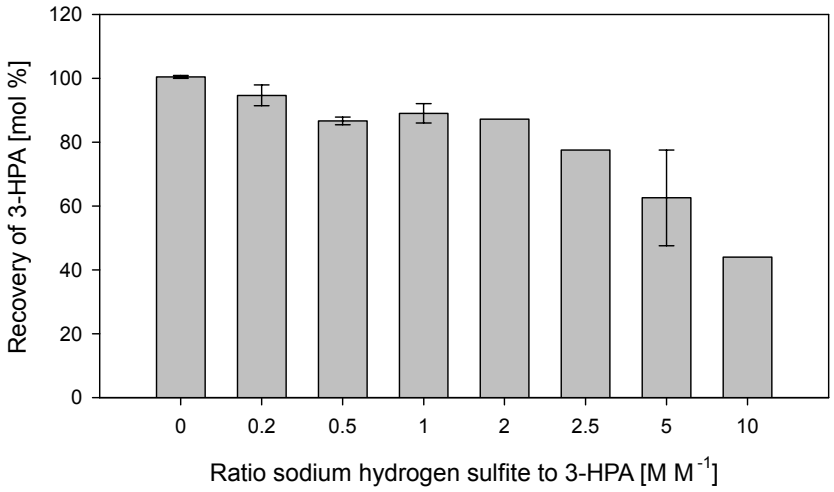

Figure 7.3 Interaction of hydrogensulfite with the quantification of 3-HPA using tryptophan assay (Slininger *et al.*, 1983). *Error bars* show standard errors of duplicate experiments.

An alternative method for 3-HPA quantification was adapted from a qualitative method to detect aldehydes (Dickinson and Jacobsen, 1970). 4-Amino-3-hydrazino-5-mercapto-1,2,4-triazole (purpald) condenses under basic conditions with the acyl group of the aldehyde to form *inter alia* an unstable, oxygen labile intermediate (1,2,3,4-tetrahydro-6-mercapto-3-substituted-s-triazolo[4,3-b]-s-tetrazine). At the liquid-air interface, the intermediate is rapidly oxidised (< 1 min) to a purple 6-mercapto-3-substituted-s-triazolo[4,3-b]-s-tetrazine, which was quantified by photo spectrometry (Dickinson and Jacobsen, 1970).

The new method was tested for interaction with hydrogensulfite and other substances originated from a 3-HPA production step such as glycerol, acetic acid, lactic acid, glucose, and 1,3-propanediol.

7.3.2 Material and methods

7.3.2.1 Reagents and materials

All reagents including purpald (4-Amino-3-hydrazino-5-mercapto-1,2,4-triazole) were purchased from Sigma-Aldrich (Buchs, Switzerland) at ≥ 98.5% purity unless otherwise specified. Sodium hydrogensulfite solution (38-40%) was analysed to 40.0% (w/w) by an enzymatic assay (see 7.2.2.2). 3-HPA was produced biotechnologically and purified on site according to Vollenweider *et al.* (2003).

7.3.2.2 Procedure of 3-hydroxypropionaldehyde quantification

A 1 ml sample, previously diluted with bidistilled water to a 3-HPA concentration below 0.6 mM, was pipetted into a 10 ml test tube. The reaction was started by adding 1 ml purpald solution (2% (w/v) in 2 M NaOH). After incubation of exactly 20.0 min at 20°C under aeration by constantly inverting the tubes, the extinction was measured with a photo spectrometer (Varian Cary 1 Bio) at 550 nm. Calculation of the concentration was performed using a calibration curve obtained under same conditions. The purpald solution was prepared by adding 10 ml 2 M NaOH to 200 mg purpald just prior use as dissolved purpald is sensitive to oxygen.

Standard preparation

Calibration was performed with seven evenly distributed concentrations (final in cuvette) of 3-HPA between 0 and 300 µM in double.

Sample preparation

Depending on the 3-HPA content, watery samples were pre-diluted and were added to the purpald assay according to Table 7.2. After addition of the corresponding amount of water (Table 7.2), the quantification was started by pipetting 1 ml purpald solution according to the protocol.

Table 7.2 Dilution scheme for the purpald assay for 3-HPA quantification depending of concentration range of original sample. The sample dilution is applied directly in the cuvette, pre-dilution is done externally.

Sample volume [µl]	Sample dilution	Pre-dilution	Total dilution	Concentration range [mM] min	max	H_2O [µl]
500	4x	1x	4x	0.2	1.2	500
100	20x	1x	20x	1	6	900
50	40x	1x	40x	2	12	950
20	100x	1x	100x	5	30	980
100	20x	10x	200x	10	60	900
100	20x	20x	400x	20	120	900
50	40x	20x	800x	40	240	950
100	20x	50x	1000x	50	300	900

7.3.2.3 Recording or absorption spectrum of 3-HPA-purpald complex

The absorption spectrum of the 3-HPA-purpald complex was recorded after incubation of 0, 80, and 230 µM 3-HPA according to the protocol. The spectrum was recorded between 200 and 800 nm in 1 nm steps within 1 min.

7.3.3 Results and discussion

7.3.3.1 Adaptation of qualitative purpald test to quantitative determination

The dilution of the purpald solution had to be taken into account leading to doubled insert of purpald. The amount of purpald could then be halved without any detectable loss in sensitivity and accuracy in a successive optimisation step. The incubation time needs to be followed exactly since no maximal extinction is formed. This could be achieved by starting the samples staggered and measuring their corresponding extinctions in same staggered order after incubation. Needed oxygen entry for colour development was achieved by constantly inverting the samples during incubation without bubble formation that could interfere with the photometrical method.

7.3.3.2 Standard curve

The calibration curve is displayed in Figure 7.4. A quadratic regression was found with R^2=0.999 (Equation 5.1).

$$\Delta E = a_2 \cdot C_{\mathrm{HPA}}^2 + a_1 \cdot C_{\mathrm{HPA}} = 5.628 \cdot C_{\mathrm{HPA}}^2 + 2.983 \cdot C_{\mathrm{HPA}} \quad (5.1)$$

where ΔE is the measured extinction, $C_{3\text{-HPA}}$ 3-HPA concentration, and a_1-a_2 are equation coefficients. Solving of the quadratic equation leads to 3-HPA concentration from measured extinction (Equation 5.2).

$$C_{\mathrm{HPA}} = \frac{-a_1 + \sqrt{a_1^2 + 4a_2\Delta E}}{2a_2} = \frac{-2.983 + \sqrt{8.8983 + 22.512(E - E_0)}}{11.256} \quad (5.2)$$

where E is the measured extinction at 550 nm and E_0 extinction of the blank.

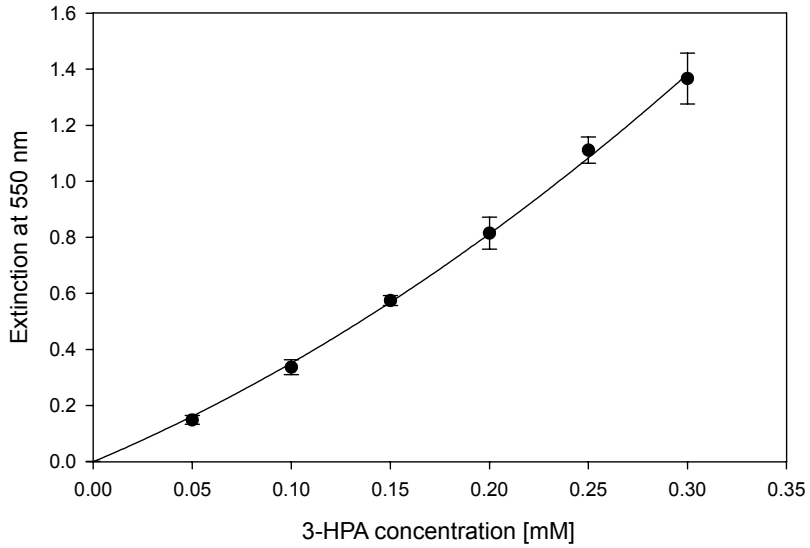

Figure 7.4 Standard curve for 3-HPA quantification using purpald assay. *Error bars* standard errors of 10 replicas.

7.3.3.3 Absorption spectrum of 3-HPA-purpald complex

The blank sample showed a maximum at 528 nm while 3-HPA containing samples showed a relative maximum at 536 and 537 nm for 80 and 230 µM, respectively, with only a slight decrease in the extinction at 550 nm. Both wavelengths 537 and 550 nm could therefore be used for quantification. The absorption maximum of the 3-HPA purpald derivate is in the same range like other aldehydes (525-549 nm), for instance propanal (532 nm), as published by Dickinson (1975).

7.3.3.4 Interaction with sodium hydrogensulfite, acids, 1,3-propanediol, and glucose

Sodium hydrogensulfite, lactic acid, acetic acid, 1,3-propanediol, and glucose are substances that might be carried over from the 3-HPA production step and might have a negative effect on 3-HPA quantification (Figure 7.5). No interaction on both recovery and detection limit was found.

7.3.3.5 Interaction with glycerol

Glycerol showed an interaction with 3-HPA quantification in a preliminary experiment. Further investigations revealed an approximate linear correlation between the introduced glycerol and measured 3-HPA concentration leading to wrong high results (Figure 7.6). This is unexpected because alcohol groups do not react with amine groups under test conditions as seen with 1,3-propanediol. An explanation might be some undeclared impurities such as aldehydes in the range below 1% that react with purpald and distort the quantification due to the high sensitivity of the method.

For the quantification of 3-HPA from bioconversion, however, this issue should not be overestimated because high 3-HPA content decreased the error originated from glycerol by sample dilution.

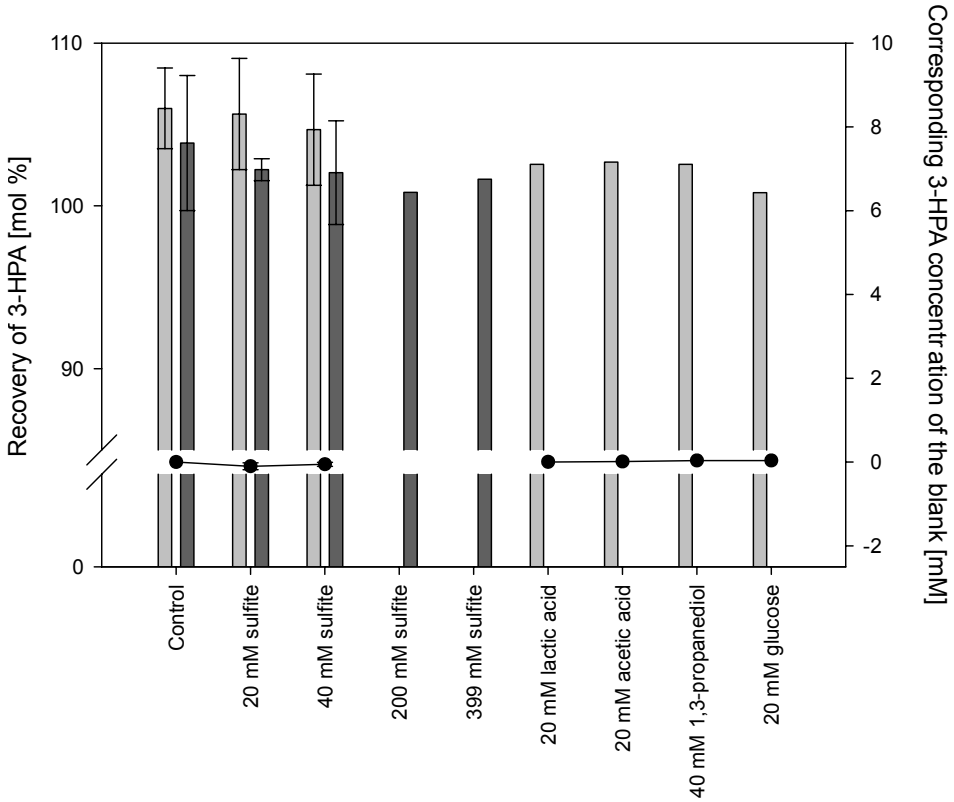

Figure 7.5 Interaction of different compounds with quantification of 3-HPA using purpald assay at different 3-HPA concentrations: *grey* 15 mM, *dark grey* 30 mM. *Straight line* shows measured 3-HPA concentration of a blank without 3-HPA but with added compound. *Error bars* show standard errors of triplicate experiments.

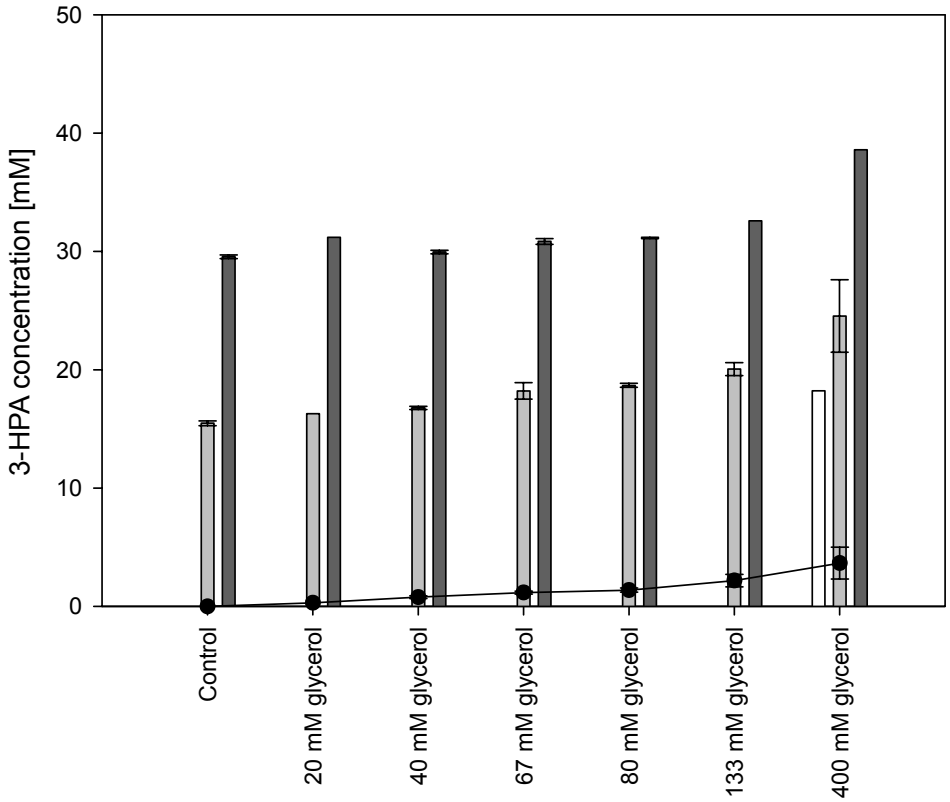

Figure 7.6 Interaction of different concentrations of glycerol with the quantification of 3-HPA using purpald assay at different 3-HPA concentrations: *empty* 7.5 mM, *grey* 15 mM, *dark grey* 30 mM. *Straight line* shows measured 3-HPA concentration of a blank with added glycerol. *Error bars* show standard errors of quadruplicate experiments.

7.4 Conclusions

Three novel methods for total hydrogensulfite, free hydrogensulfite, and 3-HPA quantification were developed and already used for hydrogensulfit and 3-HPA qunatification in chapter 4. Handled correctly, all methods are simple, rapid, exact, selective, and suitable for the quantification of the corresponding substance in a 3-HPA production environment.

The quantification of total hydrogensulfite is based on a method published by Ellman (1959) for sulphydryl groups quantification. 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) reacts quantitatively with sulfite under neutral or weak basic conditions to a yellow coloured anion which can be quantified at 412 nm. Because hydrogensulfite is oxidised with aeral oxygen, carefully freshly prepared solutions were used for the test.

To quantify 3-HPA, a method for qualitative aldehyde detection was adapted (Dickinson and Jacobsen, 1970). This method, however, only quantifies 3-HPA accurately, when 3-HPA concentration exceedes glycerol concentration because an interaction was found. This means for a glycerol bioconversion process that 3-HPA is overestimated by 10 to 15% at the beginning, but when the glycerol has been converted, 3-HPA is accurately quantified.

Acknowledgements

First, let me thank Prof. Christophe Lacroix for giving me the opportunity to work on this interesting project and his support and confidence in me and my work. I would like to thank especially Sabine Vollenweider for her co-supervision and her support during my overall study time. Her inputs are very much appreciated.

I thank Dr. Jenny Weißbrodt for the co-examination and review of the thesis.

Many thanks go to my students Xavier Mauler and Daniel Wiederkehr for their valuable contributions to this project, Marc Stevens and Peter Mertes for fruitful scientific discussions, Alfonso Díe for his valuable support with the quantification methods, and Markus Mathis and Tatjana Jeremic for their valuable contribution and inputs to the purification of 3-HPA.

“Kiki” Venakis’ work in the “kitchen” and coffee room are very much appreciated and I would like to thank her for always having cleaned glassware and lending an open ear for our small problems. Of course, I also thank all my former and present colleagues of the Laboratory of Food Biotechnology for their everlasting help and support as well as for the friendly working atmosphere.

I thank Marc Stevens, Jorge Sierra, and Marcus Yaffee for their endurance and patience in proofreading my publications and the final thesis.

This study was carried out with the appreciated support of the ETH Zurich grant (TH-47/04-03), the Walter Hochstrasser Foundation, and the BNF programme (Bern, Switzerland).

Finally, I thank my family and my friends for their constant love, faith, and motivation during my studies and PhD thesis.

Curriculum Vitae

David Paul Rütti
born July 24, 1980
Citizen of Ersigen BE
Switzerland

Education

2003-2009	Research assistant and Ph.D. student in the Laboratory of Food Biotechnology at the Institute of Food Science and Nutrition, ETH Zürich
2004	Diploma in Food Science (MSc Food Science) with major in Food Biotechnology, Food Chemistry, and Human Nutrition
1999-2004	Studies in Food Science at the Swiss Federal Institute of Technology (ETH), Zürich
1995-1999	Kantonsschule am Burggraben St. Gallen, Matura Typus C
1993-1995	Secondary school in St. Gallen
1987-1993	Primary education in Wittenbach (SG)

Congresses

Barcelona (2007)	13th European Congress on Biotechnology. Poster presentation entitled 'Biotechnological production of 3-hydroxypropionaldehyde combined with an *in-situ* product removal process to purify this compound'
Lausanne (2006)	XIVth International Workshop on Bioencapsulation. Poster presentation entitled 'Production of 3-hydroxypropionaldehyde with immobilized *Lactobacillus reuteri* cells'