Influence of Operating Conditions on Reuterin Production by Resting Cells of *Lactobacillus Reuteri* DPC16

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Influence of Operating Conditions on Reuterin Production by Resting Cells of Lactobacillus Reuteri Dpc16

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Attestation of Authorship

I hereby declare that this submission is my work and that, to the best of my Knowledge and belief. It contains no material previously published or written by another person (except where explicitly defined in the acknowledgments), or material which to a substantial extent has been accepted for the award of any other degree or diploma of a university or institution of higher learning.

Signed:

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Date: 10th August 2020

Abstract

Reuterin (3-hydroxypropionaldehyde), secreted from *L. reuteri* strains, is a potentially valuable chemical and broad-spectrum antimicrobial substance. The patented bacteria strain, *L. reuteri* DPC16, is supported by Drapac Ltd, and is successfully used in commerce. However, the low yield of reuterin greatly restricts its commercial use. To improve the conversion of glycerol to reuterin, this project studied the two-step process of reuterin production from glycerol to reuterin by *L. reuteri* DPC16. The influence of initial glycerol concentration, biomass concentration, pH value, culture age, conversion time and temperature on the production of reuterin were investigated. The results showed that maximum reuterin production was achieved by fermenting 350mmol/L (initial concentration) of glycerol for 2h at 25℃ and pH 6.8 using 25g/L of 20h old resting DPC16 cells.

Keywords: *Lactobacillus reuteri* DPC16, reuterin, 3-hydroxypropionaldehyde, bioconversion

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Abbreviations

1, 3-PD	1, 3-propionaldehyde
3-HPA	3-hydroxypropionaldehyde
ATP	Adenosine triphosphate
CAGR	Compound Annual Growth Rate
CFU	Colony-forming unit
DBI	5,6-Dimethylbenzimidazole
DCs	Epithelial and dendritic cells
DCW	Dry cell weight
DDH	Diol dehydratase
DHA	Dihydroxyacetone
DHAP	Dihydroxyacetone phosphate
DNA	Deoxyribonucleic acid
DPC16	*Lactobacillus reuteri* DPC16
EMP	Embden-Meyerhof-Parnas pathway
FAO	Food and Agriculture Organization
GDHt	Glycerol dehydratase
GI	Gastrointestinal
GMO	Genetically modified organism
GSH	Glutathione
HIV	Human immunodeficiency virus
IECs	Intestinal epithelial cells
IgA	Immunoglobulin-A

Kb	Kilobases
LAB	Lactic acid bacteria
Mb	Megabases
MIC	Minimal inhibitory
moDCs	Monocyte-derived dendritic cells
MRS	De Man, Rogosa and Sharpe agar
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
ORFs	Open reading frames
PBS buffer	Phosphate buffer solution
PKP	Phosphoketolase pathway
PPRs	Pattern recognition receptors
SAM	*S*-adenosylmethionine
SCFA	Short-chain fatty acid
TNF-α	Tumor necrosis factor-alpha
WHO	World Health Organization

Chapter 1 General Introduction

In 1928, the first modern antibiotic, penicillin, was discovered by Alexander Fleming (1881-1955), revolutionizing medicine in the 20th century. Since the Second World War, penicillin has been used successfully in medicine. Antibiotics are widely used in various fields such as medicine, human health, aquaculture, and animal husbandry (Gould, 2016). Unfortunately, since antibiotics were effective and easily produced, they were often overused, causing some bacteria to develop drug resistance. The World Health Organization identified antimicrobial resistance as a “serious threat that is no longer a prediction for the future, it is happening right now in every region of the world and has the potential to affect anyone, of any age, in any country" (WHO, 2014).

Since then, biotherapeutics, including probiotics, have been investigated as a new method for protection of human health. Since reuterin, produced by some probiotic bacteria such as *L. reuteri* strain (Vollenweider, 2004), has been reported to have inhibitory activity against some pathogens, it has been the subject of research in recent years. However, its production has not yet been commercialized. This study attempts to determine the individual factors that affect reuterin production ability and the interactions among them.

Chapter 2 Literature Review

2.1 Probiotics

2.1.1 Definition of probiotics

Probiotic has a long history in terms of etymology. The word “probiotics” comes from the Greek adjective that means “fit for life lively” (Fuller, 1992; Alvarez-Olmos, 2001).

In more modern times, Élie Metchnikoff (1908) defined probiotics as: “the dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes” (Metchnikoff, 1908). Since then, the study of probiotics has developed, and newer definitions have emerged. Through a comparison of antibiotics, probiotics were defined as a kind of microorganisms that could stimulate the growth of other kinds of microorganisms (Lilley & Stillwell, 1965). Parker (1974) presented the rudiments of modern concepts of probiotics. He defined probiotics as contributing microorganisms in the intestinal tract to improve the microbial balance. Through more research, the concept of probiotics was broadened. Afrc (1989) provided a new recognition for probiotics which was a living microbial feed supplement that could improve the intestinal microbial balance and which beneficially affects the host, including humans and animals. Havenaar and Huis (1992) further developed the definition through the recognition that probiotics could beneficially affect and improve the properties of the indigenous microflora of a host, including humans or animals, by use of viable mono- or mixed- cultures of microorganisms. In 2001, the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) made an official definition for probiotics as “live microorganisms which when

administered in adequate amounts, confer a health benefit on the host" (Araya et al, 2001, 2002). In the new authority definition, dead microorganisms are not probiotics, regardless of functionality. Certain mechanisms of action do not need living cells due to the delivery of certain enzymes to the intestine. All in all, probiotics are receiving attention worldwide. Through research, the knowledge of probiotics is continuously accumulating. Thus, the probiotics must enter a new era.

2.1.2 Taxonomy of probiotics

In 2002, the FAO and WHO published guidelines for the evaluation of probiotics in food. The report provides guidelines for probiotics as it is necessary to know the genus and species of the probiotic strains. In the laboratory, the taxonomy of probiotics is still changing, not only in terms of morphological, physiological, and biochemical criteria but also on molecular-based phenotypic and genomic characteristics (Alvarez-Olmos et al, 2001).

Generally, a peculiarity of probiotics is that they should possess some general characteristics such as being nonpathogenic and non-antibiotic-resistant. Lactic acid bacteria are often chosen as probiotics. The most common probiotics belong to three genera including *Lactobacillus, Enterococcus,* and *Bifidobacterium,* although there are others (Alvarez-Olmos et al, 2001) (Table 1).

Lactobacillus is a significant component of the lactic acid bacteria group. The *Lactobacillus* genus contains rod-shaped, non-spore-forming bacteria that can convert sugars to lactic acid, are Gram-positive and facultative anaerobic or microaerophilic microorganisms. They are often present in the human gastrointestinal and

genito-urinary tracts (Makarova et al, 2006).

Mendes-Soares et al (2004) determined genomes of *Lactobacillus* which are highly variable in size ranging from 1.2 to 3.3 Mb (megabases). Accordingly, the range of genes used to synthesize protein is from 1,100 to about 3,200 genes. Basharat and Yasmin (2015) observed that there are large numbers of compound microsatellites in the coding region of the genome, which causes lactobacilli easily to mutate and present imperfect and variant motifs. Euzeby (1998) classified the genus into 180 species and divided them into three groups based on their metabolism. The Group one is obligately homofermentative, including *L. acidophilus, L. delbrueckii, L. helveticus,* and *L. salivarius.* Facultatively heterofermentative metabolism methods define the Group two lactobacilli, which contains *L. casei, L. curvatus, L. plantarum,* and *L. sakei.* The last Group contains obligately heterofermentative metabolism pathways, and include *L. brevis, L. buchneri, L. fermentum,* and *L. reuteri. Lactobacillus* species are claimed to play a variety of roles as probiotics. *Lactobacilli* are reported to reduce tumors by binding dietary carcinogens in the colon (El-Nezami, 1998; Goldin, 1996; McIntosh, 1999). Mao (1996) claimed that lactobacilli can have a beneficial effect on chemotherapy-induced enterocolitis, especially for *L. plantarum. Lactobacillus* may also reduce the concentration of fatty acids in the blood by either inhibiting hepatic cholesterol synthesis or redistributing cholesterol from the plasma to the liver (Khalighi et al, 2016). Generally, the effectiveness of lactobacilli depends on their ability to colonize an area of tissue.

Table 1. Common probiotic microorganisms

Lactobacillus species	*acidophilus* *plantarum* *rhamnosus* *paracasei* *fermentum* *reuteri* *johnsonii* *brevis* *casei* *lactis* *delbrueckii gasseri*
Bifidobacterium species	*breve* *infants* *longum* *bifidum* *thermophilum* *adolescentis* *animalis* *lactis*
Bacillus species	*coagulans*
Streptococcus species	*thermophilus*
Enterococcus species	*faecium*
Saccharomyces species	*cerevisiae*

(Khalighi *et al*, 2016)

In mammalian flora, *Bifidobacteria* is a major genus of bacteria within the colon. It is also largely found in the gastrointestinal tract, vagina, and mouth of mammals including humans (Schell et al, 2002; Mayo, 2010). *Bifidobacterium* is a Gram-positive branched anaerobic bacterium, which is normally a nonmotile and non-spore-forming, pleomorphic rod bacteria.

The metabolism of the genus *Bifidobacterium* has a unique glucose utilization pathway in that it can use fructose-6-phosphate phosphoketolase to produce lactic and acetic acids.

Bifidobacterium species such as *B. longum* BB536 act as probiotics and are often

isolated from healthy infants' intestinal tract. It is normally used as a conventional treatment for ulcerative colitis. Furthermore, bifidobacteria can work together with lactobacilli and probiotic yeasts such as *Saccharomyces boulardii*, a combination which seems to reduce the effects of anti-*Helicobacter* therapy (Dupont, 2014; Kondo, 2013; Cremonini et al, 2002).

In nature *Bacillus* species are widely present and often occur in some extreme environments such as high pH, high temperature, and high bile salt concentrations (Slonczewski, 2020). The characteristics of *Bacillus* are Gram-positive bacteria, rod-shaped and they can be obligate aerobes, or facultative anaerobes. The main characteristic of *Bacillus* is that it can form oval endospores to separate the genus from *Lactobacillus.*

Bacillus coagulans is the only species that is recognized as probiotic. It has a similar effect when used therapeutically as *Lactobacillus* and *Bifidobacterium. B. coagulans* can show antibacterial activity due to production of such compounds as coagulin and lactic acid. In an animal model, *Bacillus* spores have been reported to increase the immune response (Duc, 2004; McGroarty, 1993; Hyronimus, 1998).

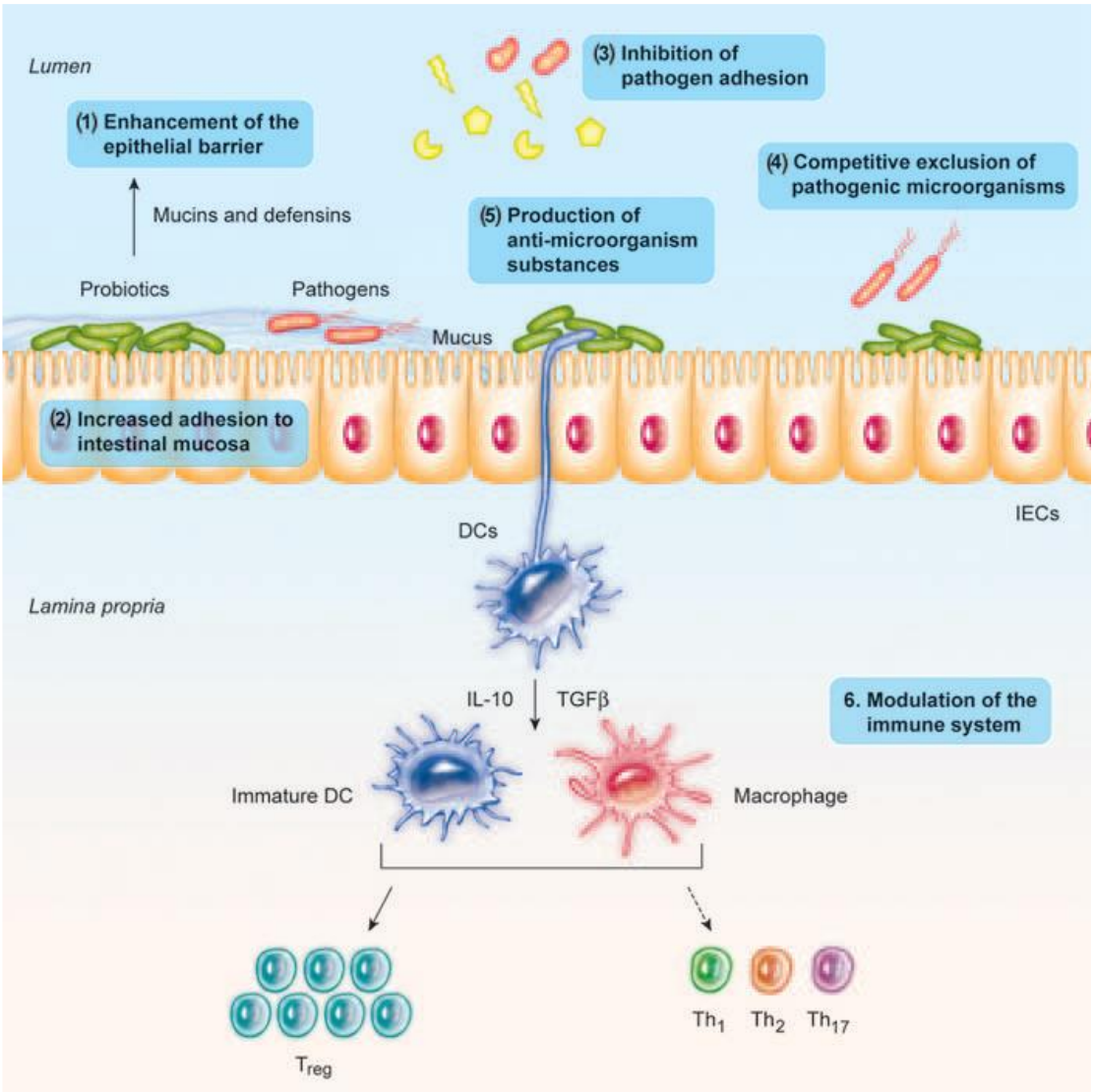
Saccharomyces genus, a yeast, has an important status in food production. *S. cerevisiae* is the only species reported to be probiotic as it can be used for the treatment and prevention of diarrhoea resulting from multiple etiologies (Buts, 2005).

2.1.3 Mechanisms of action of probiotics

Probiotics have been considered as potential treatment options for a variety of diseases when administered in adequate amounts. However, their mechanisms of action have not been completely elucidated. Figure 1 shows the process by which probiotics protect

mucins (glycoproteins) which are the major macromolecular constituents of the epithelial mucus.

Figure 1. Major process of action of probiotics



(Bermudez-Brito, 2012).

Referring to figure 1, the mechanism can be summarized into three main actions which are 1) avoid pathogen adhesion (step 1, 2, 3 and 4); 2) secret antimicrobial compounds (sept 5) and 3) modulate immune system (step 6) (Bermudez-Brito, 2012).

The first mechanism is that probiotic bacteria establish themselves as beneficial

microbial flora in the digestive tract where they enhance epithelial barriers. The mucous layer, antimicrobial peptides, secretory IgA and the epithelial junction adhesion complex constitute the intestinal barrier. Probiotics act as colonization barriers to the competitive exclusion of pathogenic microorganisms (Jacobsen, 1999, Hawrelak, 2013, Fuller, 1997). The maintenance of epithelial integrity is used to protect the host from contact with luminal contents and the intestinal flora. Inflammatory responses such as inflammatory bowel diseases may be induced due to food pathogens reaching submucosa when this barrier function is disrupted (Bermudez-Brito, 2012). Fuller (1991) reported that the association of the gastrointestinal (GI) tract epithelium with pathogenic organisms was a prerequisite for pathogens to colonize the tract. Comparing probiotics and pathogens, probiotics are more able than pathogens to make cellular attachments through competition for adhesion sites (Fuller, 1991). The main mechanism for *bifidobacteria* and *lactobacilli* was mucins and defensins. *Lactobacillus rhamnosus* strain GG and *Lactobacillus plantarum* 229v have been reported to possess the ability to inhibit *Escherichia coli* attachment to human colon cells (Mack, 1999). *L. reuteri* DPC16 can convert glycerol to form reuterin which is used against pathogens. The immobilization of *L. reuteri* DPC16 using an alginate-skim milk encapsulation system can increase the reuterin production through increasing diol dehydratase activity. The immobilization of *L. reuteri* DPC16 cells provides protection to deliver viable cells through the simulated GI tract (Zhao, 2012).

Adhered probiotics are able to inhibit pathogens through the synthesis of antimicrobial compounds, and this is the second possible mechanism of action for probiotics (Rolfe, 2000).

Common probiotics such as *lactobacilli* and *bifidobacteria* produce bacteriocins, which are defined as “compounds produced by bacteria that have a biologically active protein moiety and a bactericidal action” and other antimicrobial compounds (Hawrelak, 2013; Parker, 1974). Recently, biologically active compounds such as hydrogen peroxide, diacetyl, short-chain fatty acids, and reuterin produced by lactic acid bacteria have been identified in a beneficial modification of the microflora (Mishra, 1996).

Thirdly, a hypothesis of probiotic action is that they can protect the host by stimulation of the immune response (Perdigon, 1995). Probiotics can exert an immunomodulatory effect through increased secretion of immunoglobulin-A (IgA), and the number of natural killer cells and macrophages to enhanced phagocytic activity. Furthermore, epithelial and dendritic cells (DCs), acting with monocytes/macrophages and lymphocytes, can also interact with probiotics cells. Host intestinal epithelial cells (IECs) can extensively interact with probiotics. In addition, epithelial and dendritic cells (DCs) play an important role in innate and adaptive immunity when they encounter probiotics. Both IECs and DCs can interact with, and respond to, gut microorganisms through their pattern recognition receptors (PPRs) (Lebeer, 2010).

In addition to this, probiotics may act against pathogens through competitive utilization of nutrients. Probiotics can improve digestive absorption ability through their secretion of various enzymes. *Bacillus* species can secrete protease, lipase and amylase enzymes. Lactic acid bacteria are also a source of vitamins and organic acids, the latter of which can increase peristalsis (Lebeer, 2010). Wilson (1988) reported that most probiotics can utilized monosaccharides, thus causing inhibition of *C. difficile* through a lack of growth substrates.

2.1.4 Applications of probiotics

Probiotics have been used for centuries in fermented foods. In recent times, probiotics have become of interest to food, medicine, and agricultural concerns (Song, 2012; Nagpal, 2012), as they are able to confer a beneficial health benefit effect on the host when administered in adequate amounts. The basic application of probiotics is in a fermented food, where the property of the food can be enhanced by *Lactobacillus* and *Bifidobacterium species*. Such fermented foods include natto, vinegar, pickles and yoghurt. There are several reports that probiotics affect a variety of gastrointestinal and extraintestinal disorders such as diarrhea, inflammatory bowel disease, lactose intolerance and protection against intestinal infections (Wolf, 1950). It has also been suggested that probiotics can benefit oral health in childhood, and confer protection against carcinogens and pro-carcinogens to decrease the risk of cancer (Twetman, 2007; Vasiljevic, 2008). Livestock and pet foods are another application of probiotics. It has been reported that the use of probiotics can improve animal performance, especially dairy production and henneries. The reason is that probiotics provide animals with an additional source of nutrients and digestive enzymes (Krehbiel, 2003; Kalavathy, 2003; Wang, 2007).

Probiotics are also beneficial for agriculture due to the fact that microorganisms break down organic matter into smaller compounds. Plants can then uptake usable compounds through their roots. Commercial plant probiotics are commercially used for the biological control of plant diseases or for biofertilization (Berg, 2009).

2.1.5 Safety and risk of probiotics

In foods and dairy production areas, probiotics have been used safely for a long time. Recently, probiotics have been increasingly used to prevent, mitigate or treat specific diseases. The definition of probiotics demonstrates that in order to exert a beneficial influence, sufficient amounts must be provided (Hojsak, 2013).

The foremost necessity for the quality of probiotics is the strain selection. According to microecology, probiotics are isolated from a natural source and/or natural host environment. However, current commercially used probiotics have been selected in a compromise between health-promoting properties and the technological properties of the strains. The universally used dairy strains appear to have been selected from their natural habitat, the reason being that they are more adaptable to the ecological niche than those selected from elsewhere. Probiotics must not have potential for infectivity or *in situ* toxin production, especially when used in young children, infants, and the elderly or those with weakened immune systems.

There are four types of potential side effects, which include infections, deleterious metabolic activities, excessive immune stimulation in susceptible individuals and gene transfer may also be caused. Some lactic acid bacteria are reported to cause diseases such as bacteremia and infectious endocarditis, and they may harbour drug resistance (Marteau, 2001).

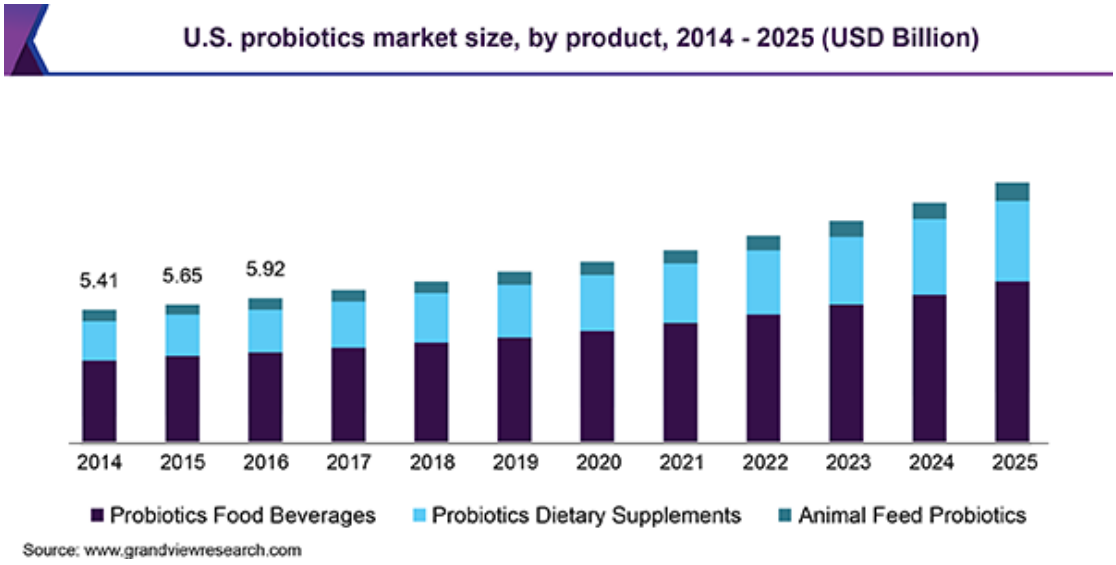
Generally, commercial probiotics are largely and widely used and few adverse effects have been reported. In most studies, comparing experimental groups and control groups, there is no significant statistical evidence of adverse events. However, some

scientists have questioned the safety of probiotics used in immunosuppressed individuals. Two systematic reviews and an Agency for Healthcare Research and Quality have reported that probiotics are safe and adverse effects are uncommon. However, in immunosuppressed individuals, it would be prudent to avoid probiotics (Surawicz, 2016; Elahi, 2007).

2.1.6 Probiotics market size

Globally, the market size in 2008 for probiotics was US $48.38 billion and this number is anticipated to expand to US $77.09 billion in 2025. The compound average growth rate (CAGR) is up to 6.9% (Verma, 2016; Grand View Research, 2019).

Figure 2. Probiotics market size



According to incomplete statistics, probiotic products are concentrated into three main areas which are yoghurt products, other fermented milk products such as cheese, and probiotic supplements including tablets products and capsules products (Feldman, 2018). Feldman et al (2015) reported that probiotics supplements ($3.8 billion)

represented 9.23% of global sales for probiotics products ($41 billion). However, this consumption gap was expected to significantly decrease in many parts of the world (Feldman, 2018). In contrast, Ewa Hudson, Head of Health and Wellness at Euromonitor, predicted that this percentage difference between probiotics supplements and probiotics foods will be raised to 38% in 2021 (Starling, 2016).

Probiotic products, as one of the most high-tech products in the food industry, possess the dual "genes" of nutrition and health and play an important role in leading the healthy transformation of the food industry. Research on probiotics has also become a “hotspot” in many fields such as food science, microbiology, medicine, and nutrition.

2.2 *Lactobacillus reuteri*

2.2.1 History of *L. reuteri*

In the early 20th century, *Lactobacillus reuteri* was classified as *Lactobacillus fermentum* and then it was recorded into lactic acid bacteria (Orla-Jensen, 1919). In the 1960s, Gerhard Reuter a German microbiologist, classified it into a new species which was *Lactobacillus fermentum biotype* II (Reuter, 1965). In 1980, a bacterial strain was isolated from the breast milk of a Peruvian mother and was classified as a new distinct species in the Lactobacillus genus (Kandler et al, 1980). This strain was stored in the American Type Culture Collection as *Lactobacillus reuteri* SD2112 and later as ATCC55730 (Biogaia, 2019).

2.2.2 Morphology and genome structure of *L. reuteri*

L. reuteri has been isolated from the gut of many vertebrates, including mammals. It

also inhabits human breast milk and the vagina and is often a major component of the vaginal microbiota (Morita, 2008; Sinkiewicz, 2010). A biofilm has been reported to be formed by *L. reuteri* to maintain ample populations when it is exposed to harsh environmental conditions (Salas-Jara, 2016; Jones, 2009). The genus *Lactobacillus* belongs to the phylum *Firmiuctes*, class *Bacilli*, order *Lactobacillales,* and family *Lactobacillaceae* (Sinkiewicz, 2010).

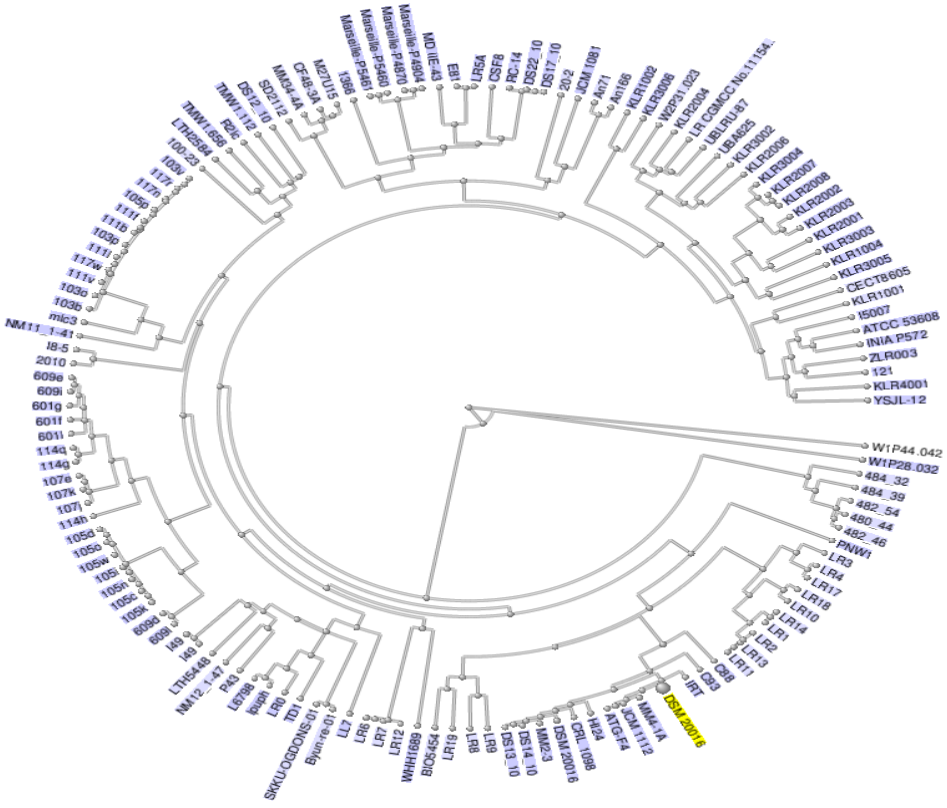
There are over 180 species in the genus, which is divided into three groups on the basis of their metabolism (Refer to Section 2.1.2).

Kandler and Weiss (1986) observed the morphology of *L. reuteri* strains as Gram-positive, anaerobic cells with slightly irregular, bent rods with rounded ends, their size is generally 0.7-1.0 x 2.0-3.0 μm. It was also observed as a non-spore forming, non-motile bacteria. The optimum growth temperature is between 37-42℃, and the optimum pH is about 6.5 (Sinkiewicz, 2010). *L. reuteri* is a heterofermentative lactic acid bacteria which produces lactic acid and other products.

The genomes of *Lactobacillus* spp are reported to be highly variable from 1.2 to 4.9 Mb (megabases) size range. The number of protein-coding genes number ranges from 1,267 to about 4,758 genes (Mendes-Soares, 2014; Sun, 2015). A wealth of compound microsatellites and variant motifs are present in the genus in the coding region of the genome (Basharat, 2015). In 2008, the Kitasato Institute for Life Sciences sequenced the full genome structure for *L. reuteri* JCM1112 (Morita, 2008). The genome contained one circular chromosome and there were no plasmids. There were 2,039,414 nucleotides observed in the genome with a GC content of 38%. The genome of *L. reuteri* JCM112 contains 1901 genes. Protein coding occupied 83% of genes. There are

1,820 open reading frames (ORFs) contained in this chromosome and phage related contained 53%. Comparing the rRNA sequence from all lactobacilli, *L. reuteri* JCM1112 contains two unique areas which result in a 50-kb increase in its genome size. The genes encoding for glycolysis enzymes are present in the unique region I while region II contains nitrate reductase and molybdopterin genes (Morita, 2008). The genome map for *L. reuteri* DSM20016 was sequenced and was 2 Mb gene size and contained 38.9% GC. Through a summary of 140 genome assemblies, the average genome size was reported to be 2.14982Mb, while the median protein count was 1951 and the median GC% was 38.6% (Rosander, 2008). Comparing *L. reuteri* JCM1112 and DSM20016, they have the almost same percentage of GC in the gene map.

Figure 3. The genotype of *L. reuteri*



(Lactobacillus reuteri. https://www.ncbi.nlm.nih.gov/genome/?term=Lactobacillus%20reuteri [Organism]&cmd=DetailsSearch).

Casas (2000) summarized two methods for the identification of *L. reuteri* based on a) the ability to produce a designated substance, reuterin, with antimicrobial activity, and b) PCR amplification. Roos (2002) measured two DNA fragment primers which are used produce reuterin. The first specific 1.5kb DNA fragment for *L. reuteri* strain corresponds to a DEAD-box helicase. Its primer parts are S4 (5' ATTCC AATGG TTCTT GAGGG 3') and R4 (5'CCTTC CACGG CGAA TAAGC 3'). The second specific DNA fragment for the strain is 0.9kb in length, and which is used for reuterin synthesis using glycerol dehydratase. The primer pairs are DHAB1 (5'AACTA CGATA ACATG TTTGC 3') and DHAB7 (5'CCTTC TTCTT CAATT CCGGC A 3'). A wide variety of lactobacilli strains have been used to test the two pairs of primers and it is found that only *L. reuteri* DNA could amplify these genes (Klaenhammer, 1999).

2.2.3 Safety and application of *L. reuteri*

It is accepted that, in order to play a role for probiotics in the intestinal tract, the minimum of viable probiotic cells to be delivered is about 10^6 c.f.u/day (Dunne, 2001). In all studies, *L. reuteri* has been shown to be free from adverse side effects, even when delivered at 1 x 10^{11} c.f.u/day (Wolf, 1994; Casas, 1997). The number of yeasts, *E. coli,* and *Clostridium* were significantly decreased in the testing animals while the colonization rate of *L. reuteri* in the GI tract was reported to be up to 80%.

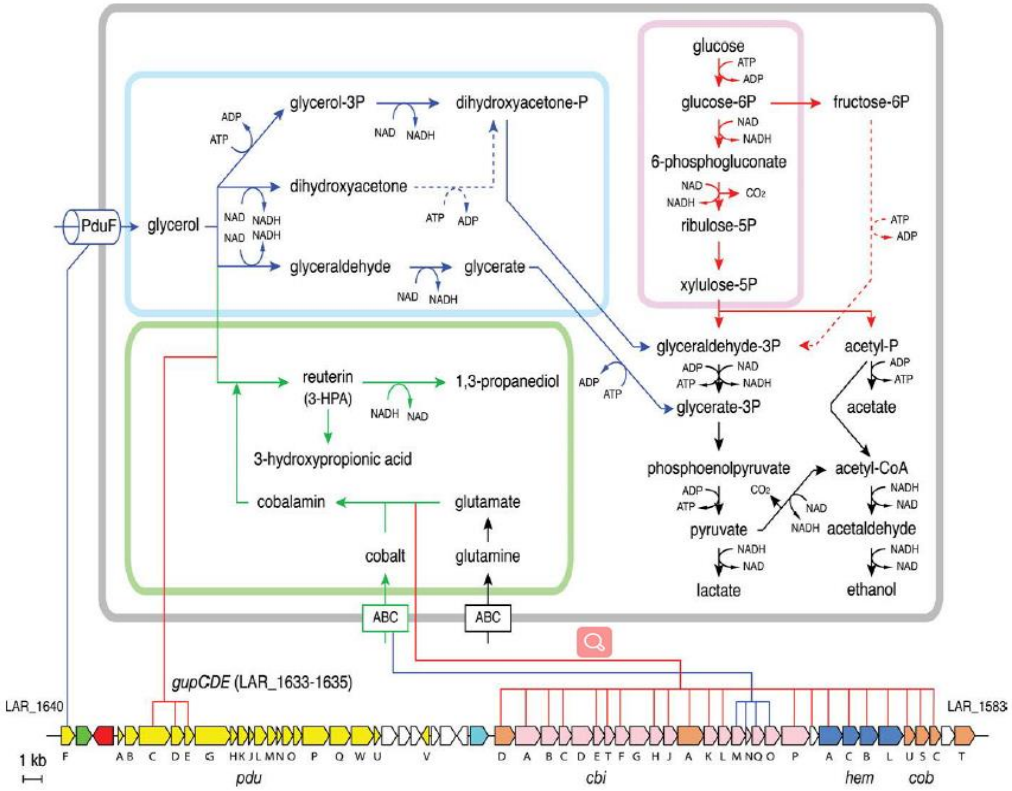
L. reuteri has been reported to have cholesterol-lowering effects and to strengthen immune defenses. It also provided better feed utilization and growth rate, especially for newly hatched and newborn animals, and lowered sickness and mortality rates (Pang, 2011). Piva (1997) fed six adult pigs 1.2 x 10^9 c.f.u/day of *L. reuteri* for 3 weeks and documented that there were no side effects. In human health, the risk of childhood

diarrhoea and the incidence of watery diarrhoea from rotavirus diarrhoea was reduced by feeding *L. reuteri* to infants and children (Isolauri, 1990; Georgieva, 2014). *L. reuteri* has also been reported to reduce infection by *Helicobacter pylori* (Mukai, 2002; Shornikova, 1997). Human immunodeficiency virus (HIV) patients have expressed the same results on intake of *L. reuteri* (10^{10} c.f.u/day) as healthy people (Wolf, 1998). *L. reuteri* has been reported to enhance the absorption of nutrients due to a positive influence on the development of ileal tissue. *In vitro, L. reuteri* strains LT018, LT037, and LT046 isolated from an elderly woman were testified to have good adhesive reproductive capacity and good inhibitory activity against *Escherichia coli, Staphylococcus aureus, Bacillus cereus, Salmonella typhi,* and *Shigella flexneri.* Comparing with other pathogens, *L. reuteri* was also sensitive to four types of common antibiotics including erythromycin, tetracycline, gentamicin, and vancomycin. Based on the above results, *L. reuteri* can be easily suppressed in the human body if necessary (Zhu, 2016).

2.2.4 The metabolic pathways of *L. reuteri*

L. reuteri belongs to the obligate heterofermentative group of lactobacilli and has two relevant metabolic pathways.

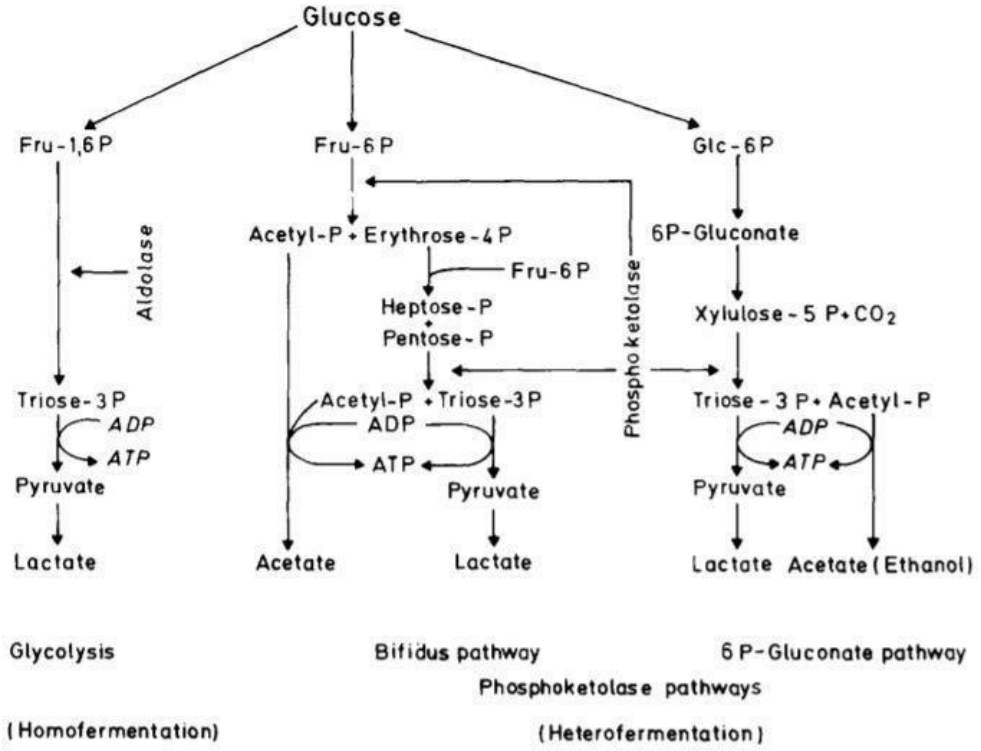
Figure 4. *L. reuteri* JCM112T metabolic pathway



(glucose metabolic pathway: pink outline. glycerol metabolic pathway: blue outline) (Morita, 2008)

The first metabolic pathway is the phosphoketolase-based metabolic pathway that ferments glucose alone and produces lactate, ethanol, and CO_2 as end-products. It is a type of glycolytic pathway.

Figure 5. Glucose fermentation pathways in lactic acid bacteria



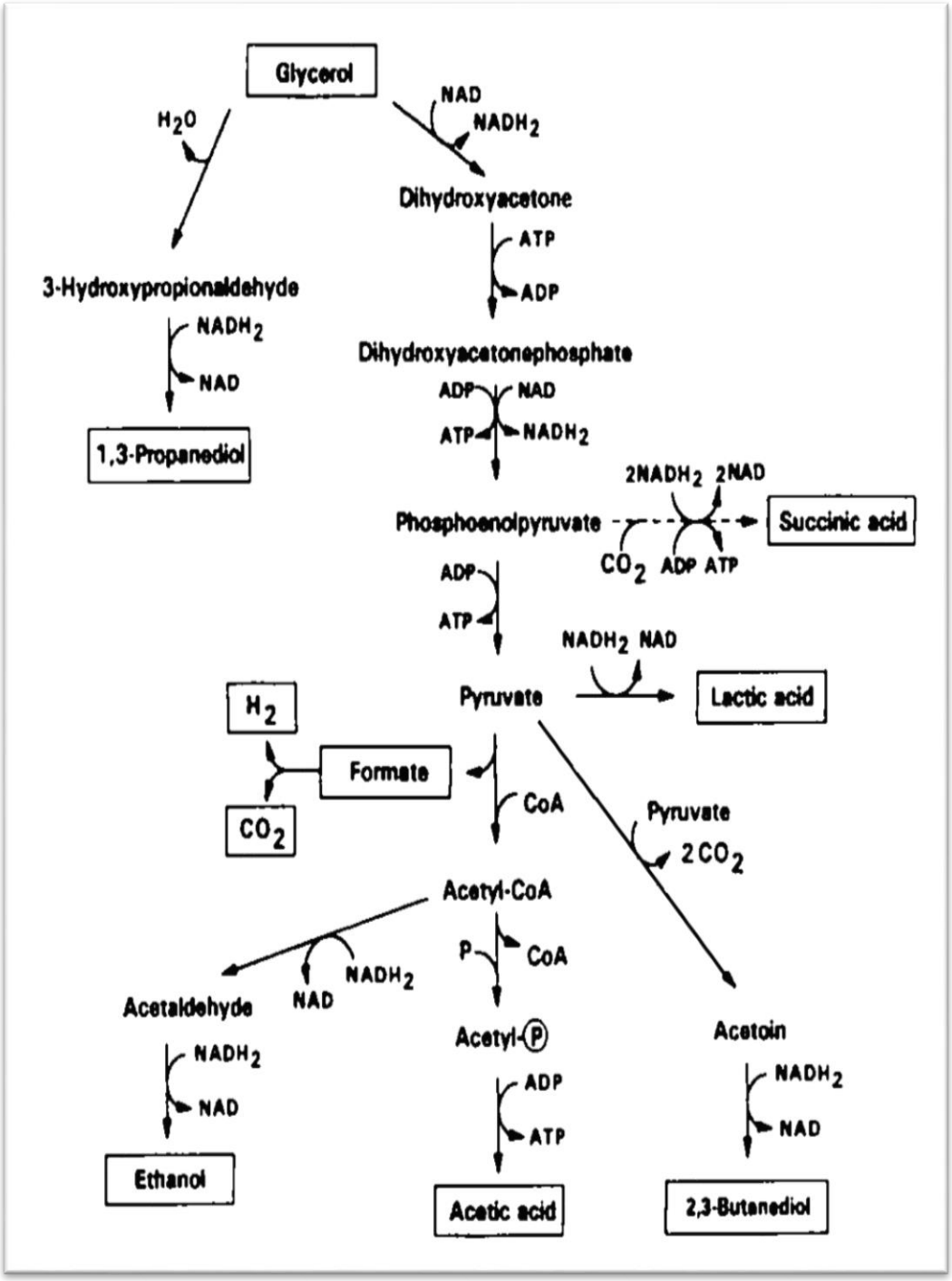
(Kandler, 1983)

The main metabolic flux of this pathway is the PKP model (phosphoketolase pathway). Here, glucose is converted into glucose-6-phosphate and the subsequent metabolites are lactate and acetate. From this pathway, ATP is produced for bacteria to use. At the same time, the Embden-Meyerhof-Parnas pathway (EMP) is also used to produce ATP, NADH, and pyruvate. Acetyl-CoA, which enters the tricarboxylic acid cycle, is produced from pyruvate. Comparing the EMP pathway with the PKP pathway, the latter is used to ferment pentose sugars in heterofermentative LAB (Kandler, 1983). Årsköld (2007) reported that the main flux for *L. reuteri* DSM17938 was the PKP pathway and used the EMP pathway was used as a shunt (Napolean, 2019).

There are two glycerol metabolic pathways in *L. reuteri,* an oxidative pathway and a reductive pathway. When glycerol diffuses into cells, it follows the oxidative pathway by

a dismutation process. The final products of oxidative metabolism are succinic acid, ethanol, acetic acid, 2,3-buntanediol, carbon dioxide and hydrogen.

Figure 6. Glycerol metabolic pathway in *L. reuteri*



(Zeng, 1993)

In *L. reuteri* strains, especially in an anaerobic environment, the glycerol reductive pathway is used (Knietsch, 2003; Zeng, 1993). First, water is removed using glycerol dehydratase in the presence of co-enzyme vitamin B_{12} to produce 3-hydroxypropionaldehyde (3-HPA, reuterin) (Zheng, 2008; Knietsch, 2003). Then, 3-HPA is reduced to 1,3-propanediol (1,3-PD) using 1,3- propanediol-oxidoreductase in the presence of NADH (Wan, 2017; Forage, 1982; Skraly, 1998; Knietsch, 2003). In theory, glycerol can be completely converted to reuterin in an anaerobic environment. In practice, a lower reuterin production yield is seen, caused by the multiple glycerol metabolic pathways and subsequent conversion of reuterin into 1, 3-PD (Krauter, 2012).

2.2.5 The mechanisms of action of *L. reuteri*

L. reuteri has similar mechanisms of action as other probiotics, including strong adhesion ability, and improvement of host immunity. As with other probiotics, *L. reuteri* exhibits strain-specific adhesion as a symbiont in the vertebrate gut using adhesins which are produced as extracellular glycoproteins on the cell surface. Human HT-29 cells have been reported to manifest adhesion of *L. reuteri* cells due to their secreted mucus binding proteins CmbA and MUB (Pang, 2011; Walsham, 2016). Miyoshi (2006) observed that the adhesion surface protein MapA, produced from *L. reuteri* 104R, participated in binding to mucosal mucus and enterocytes. Roos (2002) reported a similar result for *L. reuteri* 1063 to produce a 358kDa protein which increased its binding to mucosal mucus.

Bene (2017) investigated the interaction of *L. reuteri* ATCCPTA6475 and ATCC53608 strains with monocyte-derived dendritic cells (moDCs) through their interactions with

mucus adhesins, CmbA and MUB. The mutual effects were promoted through the mucus adhesins. The anti-and pro-inflammatory effects of the probiotics were mediated by mucus adhesins through their induction of interieukin-10 (IL-10), tumor necrosis factor-alpha (TNF-α), IL-1β, IL-6, and IL-12 cytokines. The moDC-mediated Th1 and Th17 immune responses could be induced by *L. reuteri* with increased IFNg production. The mucus adhesins expressed at the cell surface of *L. reuteri* strains may exert immunoregulatory effects in the gut through modulating the Th1-promoting capacity of DCs upon interaction with C-type lectins (Bene, 2017).

Another mechanism of action of *L. reuteri* is its production of antimicrobial short chain fatty acids (SCFA) such as lactic and acetic acids during the heterofermentative metabolism pathway. The initial hypothesis for SCFA activity against pathogens was based on the general acidic effect, i.e. the lower pH produced by the acids inhibits the pathogens. This mechanism involves disruption of the transmembrane pH-gradient associated with the dissociation of SCFA. At low pH values, SCFA remains undissociated and exerted lipophilic effects. The large amounts of free hydrogen ions within the cells, and the large transmembrane proton gradients which are created, affect bacteria due to interfering with their essential metabolic functions (Baird-Parker, 1980; Booth, 1985). A unique property of *L. reuteri* is the production of reuterin (produced by all strains), reutericin (produced by *L. reuteri* LA6) and reutericyclin (produced by *L. reuteri* LTH2584). The targets of reutericin are Gram-positive bacteria. The mechanism of action of reutericin is the same as that of bacteriocins, which is to produce pores in the target cell membrane. This causes membrane depolarization and small cellular components leak out of the cell (Kabuki, 1997; Kawai, 2001; Kawai 2004). Similarly,

reutericyclin acts against many Gram-positive species and does not affect Gram-negative bacteria. Ganzle (2003) affirmed that reutericyclin act as a proton ionophore to dissipate the transmembrane pH potential through its translocated proton across the cell membrane. Reuterin will be paid most attention because of its potential industrial applications. Its mechanism of action will be discussed below.

2.2.6 Previous studies of *L. reuteri* DPC16

L. reuteri DPC16 was patented by Bioactives Research New Zealand Limited (used to be named as Dragon Pacific Limited) under the New Zealand Patents Acts 1953. The original *L. reuteri* DPC16 strain was isolated from the feces of a healthy Caucasian male. The 16S rRNA gene of *L. reuteri* DPC16 was isolated and affiliated to the *Lactobacillus* genus with 99.3-99.6% similarity. It was confirmed to be a novel strain of *L. reuteri* (Lu, 2007; Shu & Liu, 2008). The immune cell activities were also enhanced, as was inhibition of viral pathogens. *L. reuteri* DPC16 was reported to provide good gastrointestinal stability; during a 10 days posting-dosing period, wet human feces contained more than 10^4 cells per gram after it had been dosed as a single oral food sample containing 10^9 organisms (Shu & Liu, 2008). *L. reuteri* DPC16 exerted the greatest antimicrobial effect against foodborne pathogens during a study involving 18 lactic acid bacteria strains grown under controlled conditions in 40% CO_2 and 60% N_2 (Lu, 2007). This study also showed that both fresh *L. reuteri* DPC16 cells and its culture supernatant had antimicrobial effects against both Gram-positive and Gram-negative bacteria in a wide range of pH and temperatures (as low as 10℃). Reuterin was the main antagonistic compound produced by *L. reuteri* DPC16 against

pathogens, although lactic acid may have also played a role (Lu, 2007). Furthermore, Lu (2007) reported that gene expression in *L. monocytogenes* was affected in the presence of *L. reuteri* DPC16 supernatant.

It was suggested that a novel strategy incorporating both *L. reuteri* DPC16 or its fermentative products and a modified atmosphere rich in CO_2 in food products could potentially control foodborne pathogens (Lu, 2007).

Bian et al (2010) investigated the antimicrobial activity of the supernatant of *L. reuteri* DPC16 against normal gastrointestinal microflora and gastric mucus *in vitro*. The study found that both fresh *L. reuteri* DPC16 cells and its cell-free supernatant had a very strong antimicrobial effect against foodborne pathogens. This activity developed in a sigmoidal manner during growth and the maximum activity was present after 6-8h, and was maintained at the same level thereafter. They also reported that the activity of the supernatant was pH-independent over the range of pH 4.6 to 6.5. At high concentrations, the supernatant showed a bactericidal effect against the pathogens while at low concentrations it showed a bacteriostatic effect.

However, the supernatant of *L. reuteri* DPC16 was also observed to reduce the viability of *L. reuteri* DPC16 itself, which suggests that this glycerol-derived supernatant had a lethal effect on its own cells. Nevertheless, compared to pathogens, this lethal effect was exerted to a much lesser extent. In a study on how to deliver cells of DPC16 to the target site in the colon, Zhao et al (2012) found that an alginate-skim milk-$CaCl_2$ immobilisation system was an effective and efficient method to protect the viability and physiological properties of the cells during passage through simulated gastric and small intestinal fluids. They reported that the optimal concentration of

alginate was 3% (w/v), optimal skim milk concentration was 8% (w/v) and the concentration of calcium chloride was 0.3M. Based on this formula, immobilized *L. reuteri* DPC16 cells could survive passage through simulated gastrointestinal fluid, followed by the release of free cells in the simulated colonic fluid. The study also found that the functional properties and growth kinetics of *L. reuteri* DPC16 cells recovered after release in the simulated colonic fluid were not diminished. In addition, the ability of recovered cells to adhere to epithelial cells and their ability to inhibit the adhesion of *E. coli* to epithelial cells after passage through the gastrointestinal tract was also unimpaired. Interestingly, immobilization of *L. reuteri* DPC16 caused an enhancement of antimicrobial activity, probably due to increased activity of the enzyme (diol dehydratase) that is responsible for reuterin production from glycerol (Zhao, 2012). Tian (2013) performed a confirmatory identification of *L. reuteri* DPC16 strain and its antibacterial compound, reuterin, by using 16s rRNA sequencing. The study showed that *L. reuteri* was able to tolerate pH 2 and physiological concentrations of bile salts and that it was also able to adhere to the Caco-2 human epithelial monolayer. When used in combination with bovine lactoferrin, a synergistic inhibitory effect was shown against pathogenic bacteria. Reuterin is a unique antimicrobial substance synthesized by *L. reuteri* when it is incubated with glycerol (Bian et al, 2010). Resting cells of *L. reuteri* DPC16, harvested from MRS broth and incubated with glycerol, can utilize the glycerol to produce reuterin. Hence, although reuterin can be produced during growth, it can also be produced by harvested cells and the activity can be maintained for a considerable period. This provides evidence that a secondary fermentation process using harvested cells may be feasible and desirable for maximum reuterin production

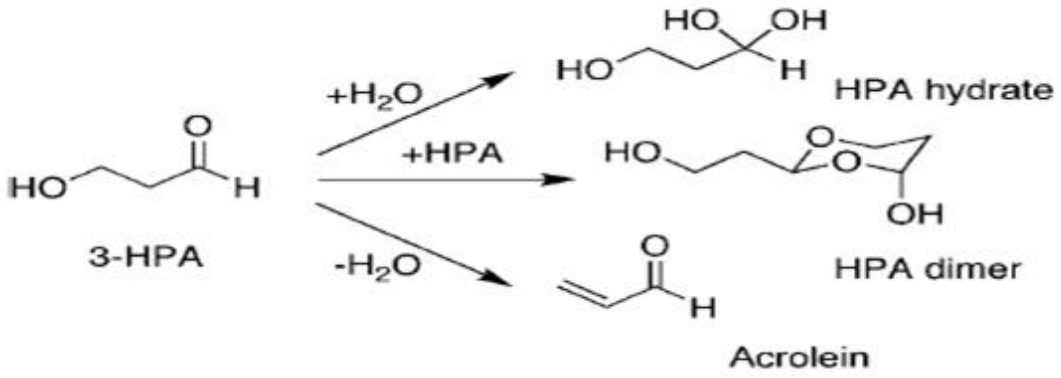
(Bian, 2008).

2.3 Reuterin

2.3.1 General information about reuterin

Voisenet (1910) first observed 3-hydroxypropionaldehyde (3-HPA, reuterin) which was produced from glycerol during *Bacillus amaracrylus* spoilage of wine. In 1950, Hall and Stern (1950) reported 3-HPA and its monomeric form to be a dimeric equilibrium in solution. Then, Nielsen (1981) found a third component, HPA-hydrate, in the HPA system by using NMR. The molecular formula of 3-hydroxypropionaldehyde is $C_3H_6O_2$ and the molecular weight is 74.09g/mol. Since 3-hydroxypropionaldehyde possesses hydroxyl and aldehydes groups, the molecule is very soluble in water and is also soluble in ethanol, ether, acetone and other polar solvents. The active aldehyde group can form a hydrogen bond with water (Talarico, 1989). In aqueous solution, since the chemically active aldehyde group present in 3-hydroxypropionaldehyde is significant, 3-HPA undergoes a reversible dimerization and hydration. Talarico et al (1989) reported that 3-HPA can exist in solution and form a dynamic equilibrium mixture with three chemical forms: monomeric, hydrated monomeric and cyclic dimeric. In solution, 3-HPA can combine with water to form HPA hydrate, and it can also be dehydrated to form acrolein. Two 3-HPA molecules can combine to form the HPA dimer. The three systems can mutually transform simultaneously and become an equilibrium mixture (Fig 7).

Figure 7. The structure of reuterin



(Laura et al, 2010).

Vollenweider et al (2003) reported that the concentration of reuterin in aqueous solution affected the distribution of the three compound forms in the HPA equilibrium. The HPA cyclic dimer is the main component of the HPA system at high concentrations. On the other hand, as the concentration of HPA decreased, the mole-fraction of HPA hydrate increased and it decreased the concentration of cyclic dimer. The final equilibrium of the mole-fraction of HPA is 1.2M. Significant amounts of the monomeric form are never detected, and it is always analysed with HPA hydrate. The antimicrobial activity of HPA is dependent on the three forms working together (Vollenweider et al, 2003).

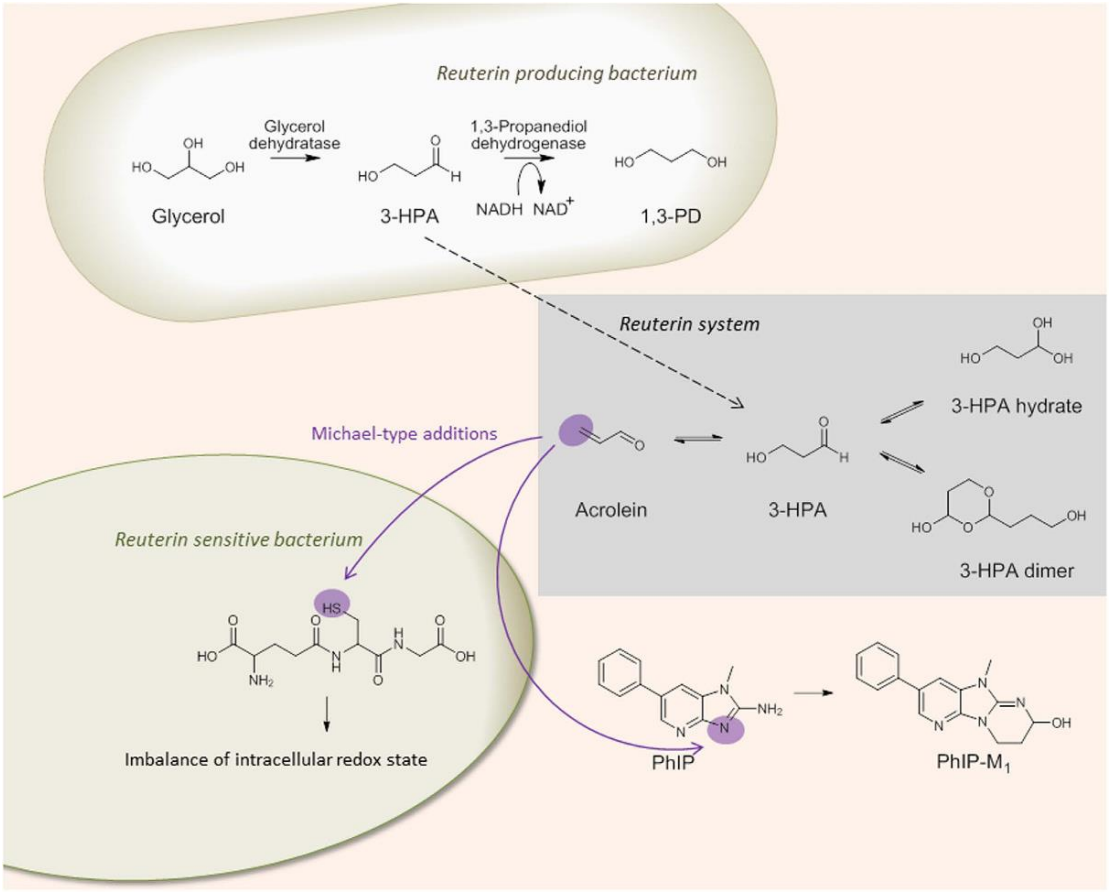
The 3-HPA dimer was patented and named as reuterin. Hence, this was reported to be the antibiotic produced by *L. reuteri* strains and to be responsible for its probiotic effects (Dobrogosz, 1988). Reuterin can inhibit a wide spectrum of microorganisms and it can resist many extreme conditions such as a nuclease, protease, and lipolytic enzymes. Reuterin is also active at a wide range of pH values and a wide range of temperatures (Talarico et al, 1988; Salminen, 1998; Dobrogosz and Lindgren,

1995; El-Ziney et al, 1999)

2.3.2 Mechanism of antimicrobial action of reuterin

Because of the different forms of reuterin that exist in solution, its mechanism has been difficult to determine. Two hypotheses to explain its mechanism have been proposed. (Fig 8).

Figure 8. Components of the reuterin system and proposed mechanism of action



(Engels, 2016)

The first hypothesis is based on the aldehyde group in acrolein, which is formed from reuterin, and which can react with free thiol groups. Thus, reactions with glutathione (GSH) and modification of proteins such as functional enzymes cause toxicity in

microbial cells

Acrolein is more reactive to nucleophilic addition than is 3-HPA, due to the presence of the highly reactive and electrophilic α, β-unsaturated aldehyde group (Engels, 2016). The second hypothesis for reuterin's antimicrobial activity is related to the its dimer form. The structure of the HPA dimer is similar to that of a ribose sugar, and which works as a competitive inhibitor of the enzyme ribonucleotide reductase, thus blocking DNA synthesis. However, this mechanism is difficult to be determined due to the enzyme active site containing a thiol group (Schaefer, 2010).

2.3.3 Applications of reuterin

Recently, reuterin has become of interest because of its antimicrobial activity, and it has been reported to be potent against bacteria, yeast, fungi, and protozoa (Talarico, 1988; Axelsson, 1989; Chung, 1989; Talarico, 1989). Furthermore, reuterin can be used as a preservative to increase the shelf life of certain foods, fodder, and beverages. Reuterin has been suggested to be used as a biological bacteriostatic agent in non-high temperature sterilization production of materials such as milk. Reuterin has been evaluated to present positive effects to prevent the growth of spoilage and pathogenic microorganisms in dairy products (EL-Ziney, 2008). Arqués (2004) reported work on reuterin in milk, acting against *Listeria monocytogenes, Staphylococcus aureus, E. coli O157: H7, Salmonella choleraesuis ssp. Choleraesuis, Yersinia enterocolitica, Aeromonas hydrophila ssp. Hydrophila,* and *Campylobacter jejuni.* Reuterin can be used in infant food to modulate the infant intestinal flora; it can also be used as a gum additive for the prevention of dental caries. Since reuterin has limited toxicity for the

human body, it can be used as a sanitizer and tissue fixing agent. Finally, reuterin has been suggested to be used as an anti-infectious agent instead of antibiotics to treat animal pathogens (Pang, 2011). In New Zealand, Drapac Ltd has developed the addition of reuterin into honey to improve its antibiotic activity (Zhao, 2012; Mohan, 2020).

2.3.4 Safety of reuterin

Safety issues are of utmost importance and must be seriously considered for human application. Vimont (2019) reported that, using purified reuterin, microbial growth inhibition occurred at 11mM or less. For fungicidal activity, the reuterin concentration was 15.6mM. In yoghurt, when the concentration of reuterin was 1.38mM, it showed a fungistatic effect; when the concentration of reuterin increased to 6.9mM, it showed a fungicidal effect. Yunmbam (1993) reported that reuterin killed 61% of treated *Trypanosoma bocagei* in 7 days. The MIC values for reuterin against *Lactobacillus* strains were at least 2 times higher than for *E. coli.* Reuterin indicated a moderate toxicity, the 50% of death after intraperitoneal injection to mice was approximately 250 mg/kg by weight (Yunmbam *et al,* 1993). Reuterin converted to acrolein (prop-2-enal) by thermal dehydration (Vollenweider *et al*, 2004). The toxic of acrolein is not hazardous to health at concentrations and acrolein also can be naturally present in certain foods (Abraham *et al,* 2011). Fernández-Cruz *et a*l (2016) reported that the toxicity of reuterin was only four times more than that of diacetyl and its much less toxic than acrolein in the human hepatoma cell line HepG2.

Therefore, reuterin has expressed high potential as a food preservative, especially in its biochemical properties and antibacterial and antifungal activities.

2.4 Production of reuterin

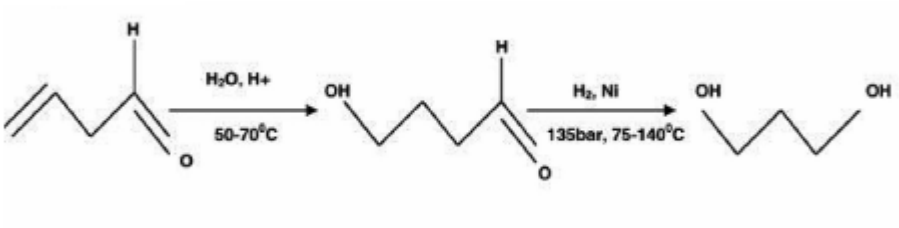
2.4.1 Chemical method

Reuterin is an intermediate during factory production of 1, 3-propanediol (1, 3-PDO) since the latter is currently in demand for over 100 million kg annually worldwide in the 21st century (Németh, 2003).

At present, there are three chemical methods that are used for producing 1, 3-PDO and two of them are successfully used commercially *via* hydrogenation of 3-HPA.

The German company, DuPont, synthesized 1, 3-PDO from acrolein, through 3-HPA as an intermediate (Refer to Fig 9).

Figure 9. Chemical synthesis of 1, 3-PD from acrolein

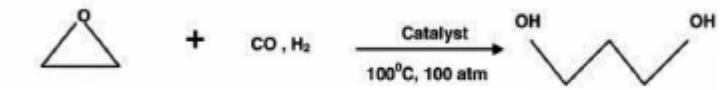


(Przystalowska, 2015)

In this method, 3-HPA is an intermediate in 1, 3-PDO production by a hydrogenation reaction at high pressure in the presence of a catalyst. The acrolein conversion is about 40% - 60% while the 3-HPA represents about 75% - 85% of that converted. However, the product is difficult to separate. Generally, the benefit of this method is its gentle conditions, and the hydrogenation is a simple reaction. However, the disadvantages are its high cost and the toxicity of acrolein (Xiao, 2009)

The Anglo-Dutch company, Shell, applied for a patent for a 1,3-PDO synthesis method using ethylene oxide (Xiao, 2009) (Refer to Fig 10).

Figure 10. Chemical synthesis of 1,3-PDO from ethylene oxide



(Przystalowska, 2015)

The main processing for this method is hydroformylation. 3-HPA is produced through a carbonylation reaction between ethylene oxide and carbon monoxide and hydrogen using a catalyst at 100℃ and 100atm pressure. Then 3-HPA is hydrogenated to produce 1, 3-PDO. The reuterin yield is up to 80%. The benefit of this method to produce reuterin is low production cost, safety, and high product quality. The disadvantage of this method is the high cost for equipment and high technical difficulty (Xiao, 2009).

Generally, reuterin production using chemical methods is significant. Raw materials have good reconversion yields of up to 80% while the reuterin yield can be 80% to 85% of that converted. However, the use of high pressure and temperature, expensive catalysts, moderate process efficiency and environmental noxiousness restrict the practicability of these two chemical synthesis methods (Xiao, 2009; Przystalowska, 2015).

The biosynthetic method to produce reuterin is a novel and significant method. The

benefit of this method is “greenness”, but no cost data are yet available. However, since reuterin is toxic to bacteria, a problem for the biosynthesis method to produce reuterin is to overcome this toxicity to allow maximum production. This is a challenge for the future.

2.4.2 Bacterial species used to produce reuterin

In vivo, reuterin can be produced from glycerol in an enzymatic step. The benefit of reuterin produced using this biotechnological method is that renewable substrates can be used under mild reaction conditions. On the other hand, the main challenge for biotechnological production is to ensure maximum yields and production rates followed by recovery of pure product, to minimize the cost.

There are six different genera of bacteria including *Bacillus* (Voisenet, 1914a); *Klebsiella* (Aerobacter) (Abeles et al,1960; Reymolds et al, 1939; Slininger et al,1983); *Citrobacter* (Mickelson and Werkman, 1940); *Enterobacter* (Barbirato et al, 1996); *Clostridium* (Humphreys, 1924); and *Lactobacillus* (Mills et al, 1954; Serjak et al, 1954) that have been reported to convert glycerol into reuterin (Vollenweider, 2004) (Refer to Table 2). From Table 2, it is noticeable that different genera have different abilities to produce reuterin.

Since reuterin can be further converted to PDO, it is the important to restrict this in any reuterin production method. *K. pneumoniae* and *L. reuteri* appear to have superior reuterin production ability (Table 3).

Table 2. Production reuterin and 1, 3-PDO from glycerol under different conditions

Species	Strain	Glycerol (mM)	3-HPA (mM)	PDO (mM)	Reference
Lactobacillus sp.	NRRL B-1720	2.34	+[a]	2	Sobolov and Smiley, 1960
Lactobacillus sp.	NRRL B-1720	326	95	nd[b]	Slininger et al, 1983
Enterobacter agglomerans	CNCM 1210	725	30		Barbirato et al, 1996
		830		237	
Citrobacter freudii	ATCC 8090	760	17	400	
		740			
Klebsiella pneumonia	ATCC 25955	760	24		
		780		429	
K. pneumonia	ATCC 8724	333	177[c]	nd	Slininger et al, 1983
K. pneumonia	ATCC 8724	326	220[c]	bd	Slininger and Bothast, 1985
K. pneumonia	ATCC 8724	760	621[c]	nd	Vancauwenberge et al, 1990
Lactobacillus reuteri	1063	250	140	50	Talarico et al, 1988
L. reuteri	ATCC 53608	200	170[d]	nd	Lüthi-Peng et al, 2002b; Vollenweider et al, 2003

([a] Present, [b] Not determined, [c] Production in buffered semicarbazide, [d] Production in water) (Vollenweider, 2004)

Table 3. Optimal factors for reuterin production using different *L. reuteri* strains

Strain	Glycerol (mM/L)	Biomass (gdry/L[a] gwet/L[b])	Temp	pH	Cell age	Conversion time	Reuterin (Mm/L)	Yield (%)	Reference
ATCC 53608	200	30[a]	37℃	6	8h	2h	170	85	Lüthi-Peng, 2002
CG001	200	25.3[a]	30℃	6.2	24h	4h	195.8	97.9	Chen, 2011
ATCC 53608	400	111[b]	30℃	6.2	26h	2h	241.2	60.3	Wan, 2017

Table 3 shows that different *L. reuteri* strains have been reported for optimal reuterin production. Different reuterin yield were observed in the same bacterial species.

Figure 11. Reuterin production and initial glycerol for different bacterial strains

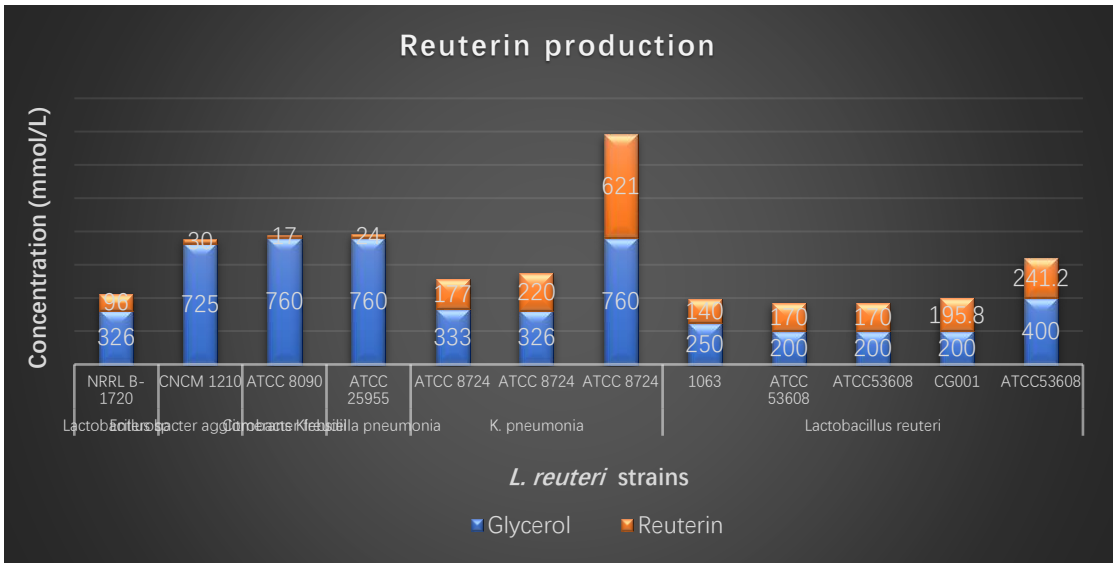


Figure 11 shows that both *K. pneumonia* and *L. reuteri* have possibilities to be used for producing reuterin using the biosynthesis method. *K. pneumonia* has shown a reuterin yield of about 50%, while *L. reuteri* yields of up to 80% have been observed.

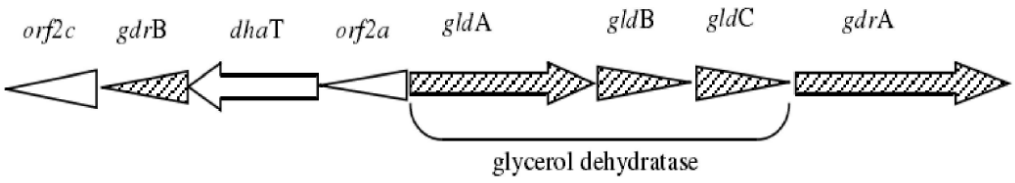
The enzyme, glycerol dehydratase, isolated from *K. pneumonia,* has been studied more than its equivalent from *L. reuteri* (Zeng, 1993). However, *K. pneumonia* is a pathogen, and has potential risk if used to produce reuterin commercially. Thus, *L. reuteri* strains have received more attention for producing reuterin.

2.4.3 Glycerol dehydratase enzyme

Glycerol dehydratase (GDHt) is the enzyme that converts glycerol to reuterin. The various strains which used glycerol as fermentation substrate can be used to isolate glycerol dehydratase. The first isolated glycerol dehydratase came from *Aerobacter aerogenes* and its molecular weight was 188kDa (Schneider, 1966). Glycerol dehydratase has also been purified from *L. reuteri* strains. The enzyme is classified into

two types depending on the presence or absence of AdoCBI-dependence (co-enzyme Vb_{12}). The first type of glycerol dehydratase is the AdoCBI-dependent enzyme. The bacteria that contain this type include *Klebsiella pneumoniae*, *Citrobacter and Clostridium pasterianum*. The coenzyme-Vb_{12} dependent glycerol dehydratase has facultative anaerobic activity, and thus is active in both a microaerobic and anaerobic environment. The second glycerol dehydratase is the AdoCBI-independent enzyme which has been isolated from *Clostridium butyricum*. AdoCBI-independent glycerol dehydratase is very sensitive to oxygen, and is an anaerobic enzyme. Glycerol is a suicide substrate for all types of glycerol dehydratase. The *in situ* reactivation system for AdoCBI-dependent glycerol dehydratase involves the addition of external co-enzyme Vb_{12} and ATP. The AdoCBI-independent glycerol dehydratase can be reactivated through *S*-adenosylmethionine (SAM) systems (Toraya, 2000). The coding gene for glycerol dehydratase located is located on the *dha* operon (Fig 12).

Figure 12. The *dha* operon



(Wan, 2017)

The genes for active glycerol dehydratase are *gdrA* and *gdrB* are located on the side of the *dha* operon (Wan, 2017).

The molecular weights of glycerol dehydratase isolated from different sources are

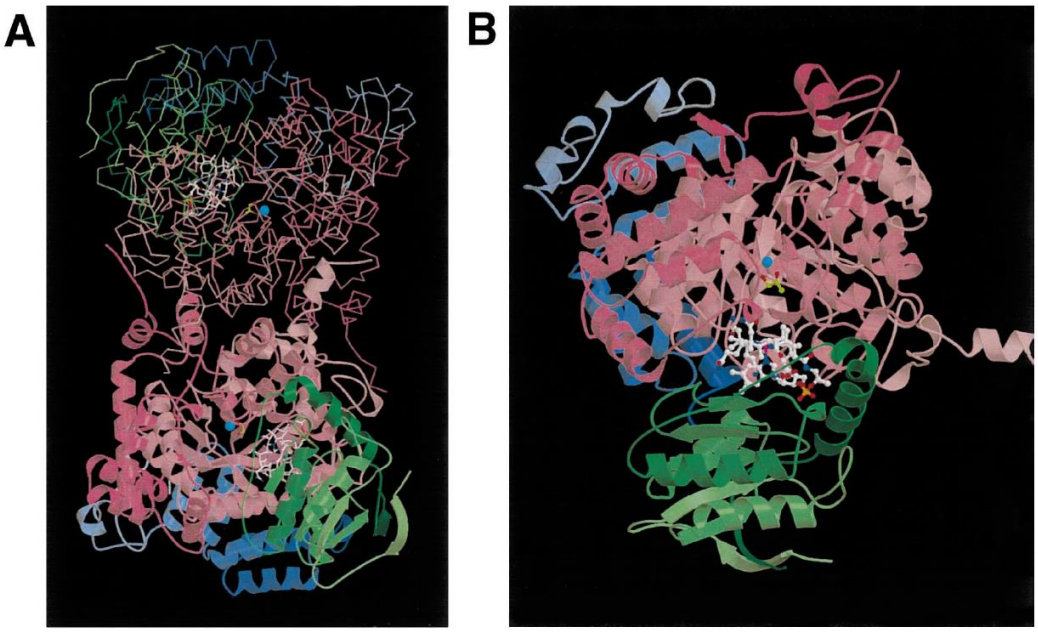
similar (188-196kDa), but the optimum pH (6.0-9.0) and temperature (28-37℃) values are different. It may be caused by heterology of glycerol dehydratase. The different sources of glycerol dehydratase have similar protein structure, and similar molecular weights. In contrast, different sources of glycerol dehydratase have different cation selectivity and different affinities for co-enzyme Vb_{12} and its substrate. Although it is only 60% similar for β units and γ units in glycerol dehydratase which isolated from *L. reuteri* and *K. pneumoniae,* the glycerol dehydratase isolated from *L. reuteri* was co-enzyme Vb_{12}-dependent. The same *in situ* reactivation system has been reported for glycerol dehydratase found in *L. reuteri*. The glycerol dehydratase isolated from *L. reuteri* has been shown to be co-enzyme Vb_{12}-dependent. Thus, *in situ* reactivation can be achieved using external co-enzyme Vb_{12} and ATP (Zeng, 1993).

2.4.4 The structure of coenzyme Vb_{12} glycerol dehydratase

The crystal structure of glycerol dehydratase exists as a dimer of a heterotrimer structure, $(\alpha\beta\gamma)_2$, which is produced through single α, β and γ units. The overall structure of glycerol dehydratase exists as a non-crystallographic two-fold axis. The dimerization of the heterotrimer is caused by the interaction between two α subunits and separately bound with two β subunits and two γ subunits. A barrel structure, named as TIM (triose-phosphate isomerase) barrel, is built by 8 paralleled β subunits and includes an α subunit such as $(\beta/\alpha)_8$ barrel structure. One molecule of CN-Cbl is bound by one heterotrimer (α β γ). In between the α and β subunits, the cobalamin molecule is located and orients the upper (Coβ) ligand to the direction of the α subunit. The α subunits work as the main activity center, due to being buried deep near the essential cofactor, the

substrate 1, 2-propanediol and K^+, in the barrel. The outer parts of the barrel are surrounded by the N-terminal and C-terminal regions of α subunits consisting of many α helices and a few β strands, while the γ subunit consists mainly of α helices. In the central part, the Rossmann-fold like the structure of β subunits contact with the lower ligand of cobalamin, which plays another important role. Surrounding the Rossmann-fold structure, many α helices, and a few anti-parallel β strands contribute to the remaining parts (Fig 13) (Wang, 2011; Toraya, 2003).

Figure 13. The structure of glycerol dehydratase



(A, $(\alpha\ \beta\ \gamma)_2$, overall structure. B, (α β γ) heterotrimer unit. Pink, green, and blue colours are used for the α, β, and γ subunits, respectively, darkening continuously from the N-terminal to the C-terminal sides.) (Toraya, 2003).

There is no enzyme activity present in any single or two combined α, β, or γ subunits. There is weak enzyme activity present when α, β, or γ subunits mix in equal mole proportions as in natural glycerol dehydratase. It is speculated that a simple mix of α, β,

or γ subunits cannot form the reasonable spatial conformation of natural glycerol dehydratase structure (Wang, 2011).

2.4.5 Factors affecting glycerol dehydratase

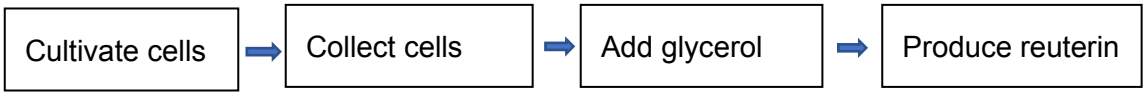
Glycerol dehydratase is a class Ⅱ biocatalyst which uses adenosylcobalamin as coenzyme. The 3-HPA is produced from glycerol using an electron donor which is produced through the combined actions of glycerol dehydratase and co-enzyme Vb_{12}. The different sources of glycerol dehydratases have different cation selectivities, coenzyme affinities and substrate specificities, thus leading to different optimum pH values and temperatures (Zhang, 2009). The enzymes from *L. reuteri* ATCC53608 and *L. reuteri* CG001 have been reported to be differently affected by the cells age, dissolved oxygen, metal ions, pH values and concentration of coenzyme Vb_{12}. The optimum temperature of glycerol dehydratase from *L. reuteri* is 37℃, which is as same as that isolated from *K. pneumoniae* and *C. freundii*. Cells in the lag phase have the highest glycerol dehydratase activity. However, higher dissolved oxygen levels inhibit glycerol dehydratase activity. For the common metal ions, Mg^{2+} and Mn^{2+} enhanced the enzyme activity, while it was inhibited by Ca^{2+}, Co^{2+}, Fe^{3+}, $Zn^{2+,}$ and Cu^{2+} ions. Interestingly, neither Na^{+} nor Fe^{2+} had any significant effect. Previously, Talarico *et al* (1990), using the enzyme isolated from *L. reuteri* 1063, reported that the optimum temperature was 30℃. Ma (2013) further characterized the optimum temperature and pH value as 37℃ and 6.2, respectively. Furthermore, reactive glycerol dehydratase is another main factor that affects reuterin production, due to glycerol being a suicide substrate for it. Any extra added coenzyme Vb_{12} and ATP allows inactive coenzyme

Vb_{12} to recombine with glycerol dehydratase to reactivate it. The glycerol dehydratase isolated from *L. reuteri* has such a reactivation mechanism, and can be reactivated when coenzyme Vb_{12} and ATP are present (Ma, 2013).

2.4.6 Current status of reuterin production using biosynthesis

Currently, biotransformation using resting cells is the main method being studied to produce reuterin using microorganisms (Fig 14).

Figure 14. Process flow of reuterin production using free-whole-cell biotransformation



Although both *K. pneumoniae* and *L. reuteri* have been reported to have good glycerol conversion ability, researchers have attempted to use genetically modified organisms through insertion of the glycerol dehydratase genes into *E. coli* which is then used as a host organism to produce reuterin. Genetically modified *E. coli* K-12 which was provided with glycerol dehydratase dhaB from *K. pneumoniae* DSM2026 has been produced (Raj, 2008). However, the main problem for using the GM-organism to produce reuterin was the low yield caused by the unstable glycerol dehydratase activity in the host *E. coli* (Niu, 2015). Furthermore, reuterin was toxic to the bacteria and a high concentration of reuterin can even inhibit *L. reuteri* itself (Doleyres, 2005). It has been reported that higher temperatures could promote this conversion while a lower temperature could help reuterin stability. In addition, *L. reuteri* cells further converted reuterin to 1, 3-PD as a means of removing the toxic aldehyde. Scavenger compounds such as bisulphite and semicarbazide have been used to combined with the reuterin to reduce its toxicity and

increase its yield. The combined reuterin-scavenger-adduct compound could be separated through ion-exchange resin under acidic conditions. In this way, reuterin production was 35 times higher than that without using scavenger (Krauter, 2010). However, some scavenger was also toxic for the cells and reuterin-scavenger-adduct is difficult to be purified and separated into reuterin and scavenger.

Since *L. reuteri* has been reported to have the strongest glycerol conversion ability, it has been used by most groups for reuterin production from glycerol. However, the yield of reuterin produced by *L. reuteri* was generally low. Environmental factors such as temperature, pH, incubation time, cell age, glycerol concentration and cell biomass mass concentration, which will be discussed in chapter 4, are important for the conversion. In addition, the oxygen concentration and glucose concentration are two other factors that have been used to affect reuterin production. It is evident that *L. reuteri* produces glycerol dehydratase when grown anaerobically on glycerol (Toraya, 1980). The presence of glucose in the glycerol solution mitigates against reuterin production, the reason being that NAD^+ is continually produced when *L. reuteri* converts glycerol to reuterin. The reuterin can be converted into 1, 3-PD using propanediol dehydrogenase depending on the NAD^+-concentration. Reuterin production by *L. reuteri* has been reported to depend on the glucose: glycerol molar ratio. For the maximum of reuterin production the ratio has been stated as 0.33 (Lüthi-Peng, 2002).

2.4.7 The significance of this project

Reuterin is an antimicrobial compound that has potential to be used in such areas as the food industry and medicine. In the chemical field, reuterin could also be used in the

synthesis of acrolein, acrylic acid and 1, 3-propanediol. The biotechnology method to produce reuterin may be a cheaper, gentler method with only one chemical conversion step from glycerol to reuterin using glycerol dehydratase. In theory, in the bioconversion method to produce reuterin, glycerol can be completely converted to reuterin (Refer to Section 2.2.4). In practice, however the supplied glycerol cannot all be used to participate in the reaction. The first reason for this is that glycerol can poison glycerol dehydratase during its conversion, and the second reason is that part of the glycerol may be converted into other products such as ethanol, 2, 3-butanediol, acetic acid, lactic acid and succinic acid (Zeng, 1993).

Although, *K. pneumoniae* is harmful to humans and the environment, it has been the major organism studied to produce reuterin. Compared to *K. pneumoniae, L. reuteri* is more effective in reuterin production from a pure glycerol solution and is much safer. *L. reuteri* DPC16 is the strain patented by Drapac Ltd, and is currently being grown commercially for reuterin production. Currently, projects for reuterin production using *L. reuteri* are focused on its accumulation and application in a food product. However, there are few reports on the factors that will maximise reuterin production. The relatively low reuterin production yield and discontinuity of the process restrict its production. Thus, optimization of the conversion conditions will be a significant step forward.

2.4.8 Main research content

In this project, the main purpose was to improve the reuterin yield from glycerol by using whole *L. reuteri* DPC16 cells in a resting biotransformation process. Individual factors such as temperature, glycerol concentration, pH, biomass concentration, cell age and incubation time were analysed to determine their effects on reuterin production. Then,

the rank of effects on reuterin production for the six single factors was determined using statistical methods.

Chapter 3 Materials and Methods

3.1 Experimental materials

3.1.1 Bacterial strain and media

The bacterial strain used was *Lactobacillus reuteri* DPC16, provided by Drapac Ltd New Zealand as a frozen culture. The growth media employed were MRS and MRS agar, obtained from Fort Richard Laboratories Ltd, 12 Huia Road, Otahuhu Auckland 1640 New Zealand. All media were sterilized at 120℃ for 15 min. Details of other media that were used in the experimental trial are given below.

3.1.2 Experimental reagents and equipment

Experimental reagents included:

Acrolein (purity 97%) solution, purchased from O2Si smart solutions Ltd, 7290-B Investment Drive, North Charleston, SC 29418, USA. Sigma-Aldrich New Zealand Co (A subsidiary of Merck Ltd), Private Bag 92162 Auckland 1142 New Zealand, supplied L-tryptophan powder (purity 99.5%), glycerol analysis standard solution (purity 98.5%), 1,3-propanediol standard solution (purity 98%) and D-(+)-glucose standard powder (purity 99.5%). Toluene and concentrated hydrochloric acid were obtained from the AUT University laboratory.

Experimental equipment included:

Holten LaminAir clean bench HB 2460; Contherm mitre culture incubator (4000 series) with carbon dioxide (gas code 169); Eppendorf Centrifuge (5810R) and Gyrozen Centrifuge (1580R) with 50ml and 15ml sterilized centrifuge tubes; Olympus microscope (CX31); Agilent Technologies HPLC (1200 series); Hitachi spectrophotometer (U-3900)

with fused quartz microcalorimetric cuvette (10-2mm width) and Pharmacia biotech spectrophotometer (Ultrospec 2000) with cuvette (4.5ml-10mm width); Sterilized empty Petri dishes.

3.2 Experimental methods

3.2.1 Activation of *L. reuteri* DPC16 cells to prepare seed culture

The frozen *L. reuteri* DPC16 was defrosted at room temperature, then 1ml was transferred into 10ml of sterilized MRS broth and incubated for 24h at 37℃ with 5% of CO_2. This was the first-generation culture. After the first-generation incubation, 1ml of this culture was transferred into another 10ml sterilized MRS broth and incubated for another 24h under the same conditions. The second-generation culture was then used as the seed culture.

3.2.2 Preparation of *L. reuteri* DPC16 glycerol seed cultures

According to the method described by Wan (2017), glycerol and distilled water were mixed to form 50% (v/v) glycerol-water solution and sterilized at 120℃ for 15 min. Seed culture (1.6ml) and 0.8ml of glycerol solution (50% v/v) were transferred into a centrifuge tube. The final glycerol solution was approximate 15% (v/v) in this tube. Glycerol DPC16 seed culture tubes were stored at -80℃.

3.2.3 Time course of *L. reuteri* DPC16 batch fermentation

The seed culture tube was incubated for 24h at 37℃ in 5% CO_2 and then transferred into 50ml sterilized MRS broth. The cultures were incubated for 36h at 37℃ with 5% CO_2. Samples (3ml) were harvested after 0h, 4h, 8h, 12h, 16h, 20h, 24h, 26h, and 36h

of incubation. The absorbance at 620nm of each sample was measured using a blank of 1% peptone water. This experiment was performed in triplicate and the average values were used to draw the growth curve.

3.2.4 Dry cell weight (DCW) measurement

L. reuteri DPC16 was incubated as described above and 13ml samples were taken every 4h. Three ml of each sample were used for measuring absorbance values at 620nm while 10ml were placed into pre-weighed 15ml centrifuge tubes for dry weight measurement. Samples were centrifuged at 4000 rpm for 10min at 4℃. The supernatant was removed, and the sediment was washed twice with 1% peptone water. Finally, the centrifuge tubes were dried at 80℃ until constant weight.

3.2.5 Standard curve between absorbance values and viable cell count

One ml of active *L. reuteri* DPC16 culture was transferred to 9ml of 1% of sterilized peptone water to produce a 10^{-1} diluted culture suspension. Further dilutions were made to 10^{-8} and 0.1ml of each dilution was spread-plated onto MRS agar and incubated for 48h at 37℃ in 5% CO_2 to determine the viable cell count.

3.2.6 Reuterin production from glycerol

Cells were grown in MRS medium, harvested and washed twice in sterile distilled water before re-suspension in glycerol solution for bioconversion to reuterin. In each conversion, 1.3ml of glycerol was used to convert into reuterin. To determine the effects of individual factors on the conversion, a range of each was used, as follows:

Biomass concentration: 9, 11, 17, 21, 25, 30 g dry cell weight/L.

Glycerol concentration: 150, 200, 300, 350, 400, 450 mmol/L.

Culture age at harvesting from MRS growth medium: 12, 16, 20, 24, 28, 32 h.

Incubation time: 0.5, 1, 1.5, 2, 3, 4 h.

Temperature: 20, 25, 30, 37, 42 ℃.

pH (adjusted using 0.2M phosphate buffer): 6, 6.2, 6.8, 7.2, 7.5, 8.

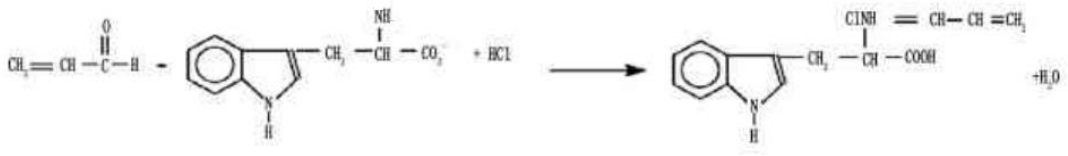
After the conversion, cultures were centrifuged at 4000 rpm for 10min at 4℃. The supernatant solutions were stored at -80℃ before analysis.

3.3 Analytical methods

3.3.1 Preparation of standard acrolein solution and tryptophan solutions

Acrolein undergoes a condensation reaction with L-tryptophan to produce a colour compound (Fig 15).

Figure 15. The formula of acrolein and L-tryptophan



The method based on Circle *et al*. (1945), was used with some modifications. A standard curve was prepared using various concentrations of acrolein with L-tryptophan. The mixtures were heated for 20 min at 40℃, to give a purple colour. The absorbance of the solutions was measured at 570 nm. The standard curve of acrolein concentration was established as shown in Fig 16.

A standard acrolein solution of 7.14 mmol/L was prepared and stored at 4℃. A solution

of L-tryptophan was prepared at a concentration of 19.7424mmol/L.

Figure 16. Standard curve for acrolein concentration

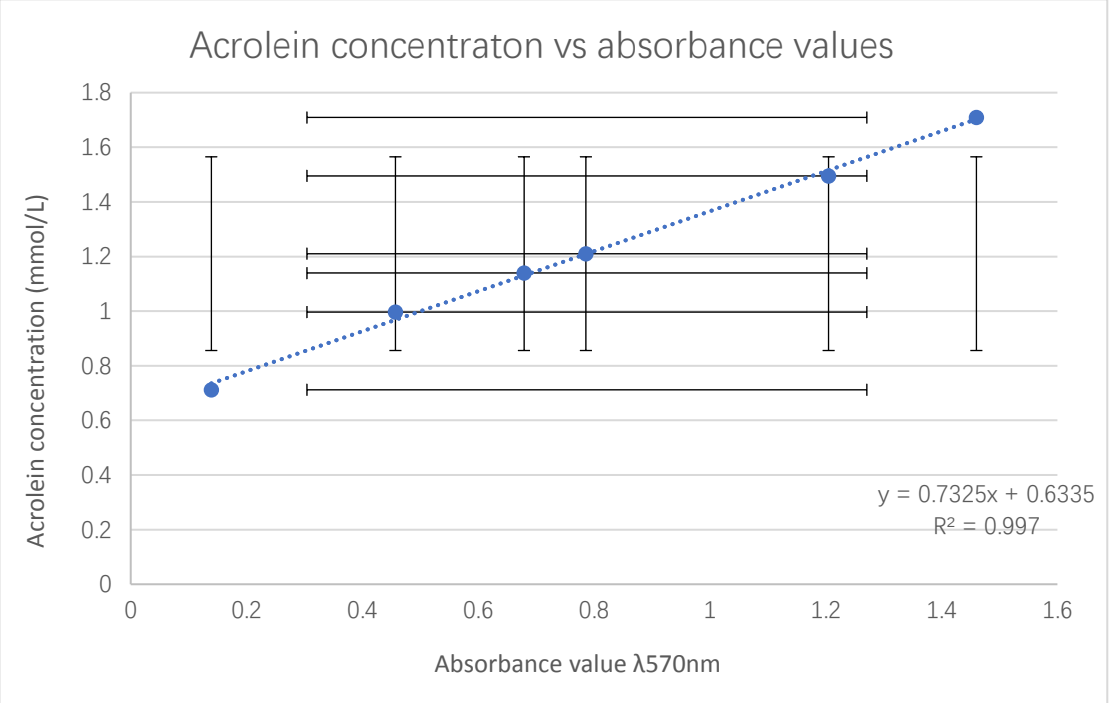


Figure 16 shows a linear relationship between acrolein concentration and the absorbance values. The formula is Y=0.7325X + 0.6335 (Y: Acrolein concentration, X: Absorbance values, R^2=0.997).

3.3.2 Determination of reuterin, 1,3-propanediol (1,3-PD), and glucose using HPLC

An Agilent Technologies HPLC (1200 series) was used to determine reuterin, 1,3-propanediol and glucose concentrations. Separation proceeded at 55℃ using an Aminex HPX-87H ion exclusion column (300nm*7.8mm). The eluent solution was 5 mmol/L H_2SO_4 at a flow rate of 0.5ml/min. The concentrations were measured using an external standard method and were recorded in the database. The data base of Qingdao Institute of Bioenergy and Bioprocess Technology had the standard glycerol

concentration curve, which was used to determine the glycerol concentration.

3.3.3 Statistics method to analyze data

Statistics software, Mintab 19 and IBM SPSS Station 21, were used to analyze data. The one-way ANOVA method and the PCA method were used to separately describe the relationship between single factors and produced reuterin concentration.

Chapter 4 Experimental results

4.1 The growth of *Lactobacillus reuteri* DPC16 in batch culture

4.1.1 The growth curve of *Lactobacillus reuteri* DPC16

Figure 17. Growth curve of *L. reuteri* DPC16 (h)

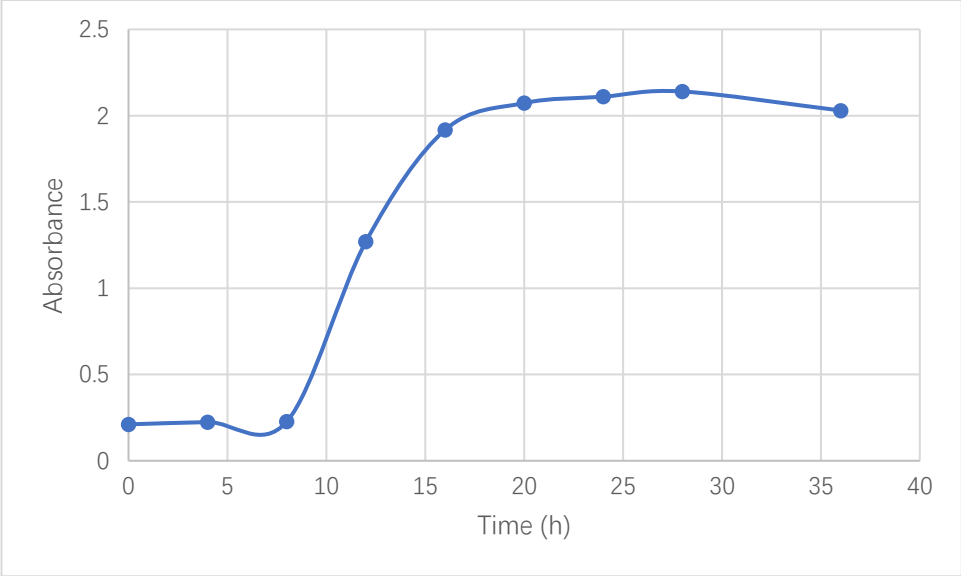


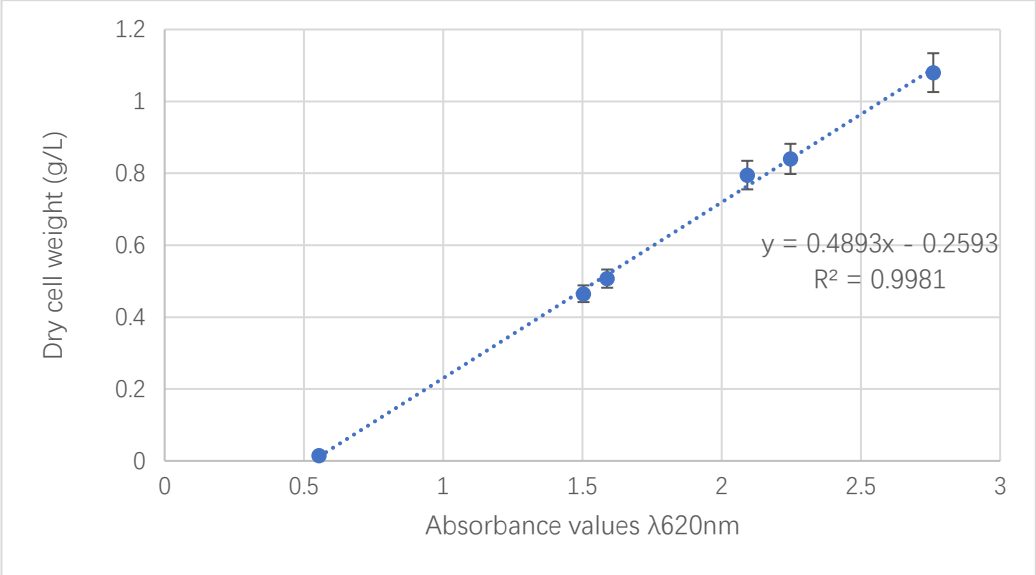
Figure 17 shows the growth kinetic curve of *Lactobacillus reuteri* DPC16 with four phases. The lag phase of *L. reuteri* DPC16 was from 0 to 8h, the log phase was from 8 to 16h and the stationary phase was after 16h. The death phase of *L. reuteri* commenced at 30h. In order to obtain enough fresh *L. reuteri* DPC16 cells, cells should be harvested during incubation from 12h to 32h.

4.1.2 The standard curve of dry cell weight *versus* absorbance

Figure 18 shows a linear relationship between dry cell weight and absorbance values. The formula for dry cell weight was Y = 0.4893X - 0.2593 (Y: dry DPC16 cell weight, X: absorbance values, R^2 = 0.9981). The absorbance value of one dry gram of DPC16

cells was 2.577.

Figure 18. The relationship between dry cells weight and absorbance values



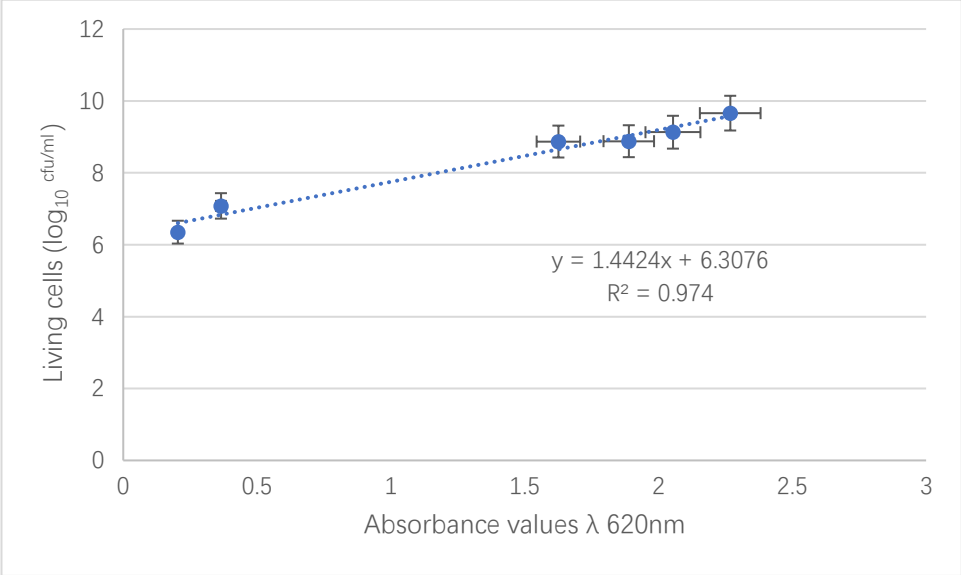
Dry cell weight instead of absorbance value was used as the unit of cells to reduce inherent errors. DPC16 is a cell-adhered culture which may affect its biomass concentration.

Talarico (1988), Lüthi-Peng (2002) and Chen (2011) all used dry cell weight to describe biomass concentrations.

4.1.3 The standard curve of viable cell counts of DPC16 *versus* absorbance

Figure 19 exhibits the relationship between viable cell counts (c.f.u) and absorbance values (λ=620nm) of DPC16.

Figure 19. Standard curve of viable cell counts against absorbance values.



The viable cell counts and their absorbance values showed a linear relationship. The formula of this relationship was

Y = 1.4424X + 6.3076 (Y: Living cells (log_{10} c.f.u/ml), X: absorbance values, R^2 = 0.974).

One gram of dry DPC16 cells contained 10.025 (log_{10} c.f.u/ml) of living cells.

4.2 Reuterin production using resting-cells

In theory, glycerol is completely converted to reuterin (1 mole of glycerol is converted to 1 mole of reuterin). However, glycerol is also converted to other compounds, and a part of the produced reuterin is further converted to 1, 3-PD and another unknown compound (Refer to Section 2.2.4).

The percentage of glycerol consumption, the consumed glycerol and the reuterin yield were calculated as below:

(%) glycerol consumption= consumed glycerol (mmol/L) / initial glycerol concentration (mmol/L)

Consumed glycerol (mmol/L) = initial glycerol concentration (mmol/L) – residual glycerol

concentration (mmol/L)

Reuterin yield (%) = produced reuterin concentration (mmol/L) / initial glycerol concentration (mmol/L)

4.2.1 Influence of biomass concentration on reuterin production

The effect of biomass concentrations on the glycerol bioconversion was studied using cells harvested after 24h of growth. The DPC16 cells were suspended at pH 6.2 and 37℃ in 300mmol/L glycerol solution and the conversion was measured after 1h of incubation.

Table 4. One-way ANOVA: biomass *versus* consumed glycerol, produced reuterin and 1, 3-PD

Substance (mmol/L)	Analysis scope	Sum of Squares	df	Mean Square	P-Value	R-sq (pred)
Consumed Glycerol	Between Groups	10265.892	5	2053.178	.001	52.2%
	Within Groups	2769.174	12	230.764		
	Total	13035.066	17			
Produced Reuterin	Between Groups	7265.953	5	1453.191	.004	40.14%
	Within Groups	2633.682	12	219.473		
	Total	9899.635	17			
Produced 1,3-PD	Between Groups	87.178	5	17.436	.000	78.15%
	Within Groups	9.376	12	.781		
	Total	96.554	17			

(Significance level α = 0.05)

Table 4 shows the result of one-way ANOVA of the effect of biomass concentration on glycerol consumption, reuterin production and 1, 3-PD production. Biomass concentration had a significant effect on consumed glycerol (P-values <0.05), reuterin production (P values <0.05) and 1,3-PD production (P-values <0.05). The R-square value for biomass concentration and consumed glycerol was 52.2%, which showed a

weak linear relationship. Biomass concentration also presented a weak linear relationship with produced reuterin (R-square= 40.14%). R-square for biomass concentration and produced 1, 3-PD was 78.15%, which was a relatively strong linear relationship.

Figure 20. Effect of biomass on reuterin production

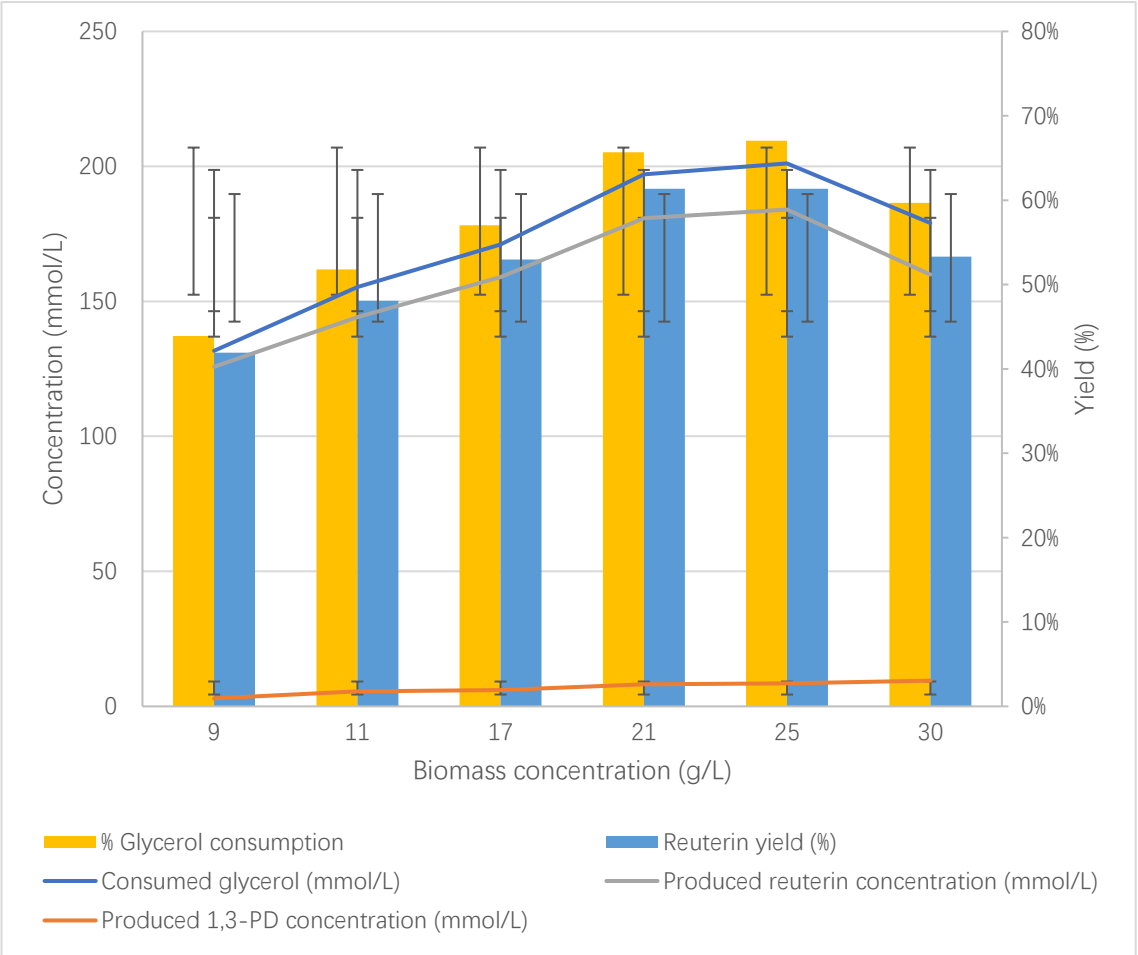


Figure 20 shows that as the biomass concentration increased from 9g/L to 21g/L, the amounts of glycerol consumed and reuterin produced significantly increased. The peak point occurred at 21g/L of biomass concentration and this peak was maintained high until the biomass concentration rose to 25g/L. After the peak point, the consumed

glycerol and reuterin production both decreased. Furthermore, there were very small amounts of 1, 3-PD (average produced 1, 3-PD values were 6.80mmol/L) converted from reuterin and its concentration was proportional to the biomass concentrations. The percentage of glycerol consumption and reuterin yield followed similar trends. The peak point for percentage of glycerol consumption (67.03%) occurred at 25g/L of biomass concentration, while the peak for the reuterin yield (61.33%) occurred at biomass concentrations of 21g/L and 25g/L.

Table 5. The reuterin production and unit yield per DPC16 at different biomass concentrations

Biomass (g/L)	Produced reuterin (mmol)	Reuterin unit yield (g/ (log_{10} c.f.u))
9	0.1258	1.395
11	0.1442	1.308
17	0.1589	0.933
21	0.1808	0.859
25	0.1840	0.735
30	0.1600	0.532

(The number of viable DPC16 cells (log_{10} c.f.u/mL) was calculated in 1.0ml of samples (Refer to Section 4.1.3). One gram of dry cell DPC16 contained 10.020 (log_{10} c.f.u/ml) of viable cells)

Table 5 shows the relationship between reuterin unit yield and biomass concentration, which was an inverse relationship. As biomass concentration increased, reuterin production yield decreased. The maximum reuterin yield (1.395) was found when the initial biomass was 9g/L. The reuterin yield (1.395) was nearly three times greater than that found at 30g/L of biomass (0.532). The 11g/L of biomass showed nearly the same reuterin unit yield (1.308 g/ log_{10} c.f.u). When reuterin presented maximum production (at biomass concentrations of 21g/L and 25g/L), the reuterin unit yields were only 0.859 g/log_{10} c.f.u and 0.735 g/ log_{10} c.f.u, respectively, which were approximately half that of

maximum unit yield.

Under the experimental conditions used, the biomass concentration that maximized reuterin production was 21g/L. This result is similar to that reported by Lüthi-Peng (2002) using *Lactobacillus reuteri* ATCC53608 and Chen (2017) using *Lactobacillus reuteri* CG001. However, Chen (2011) reported that the reuterin production continued to increase as the biomass concentration increased. In his study, the yield of reuterin reached 97.9% at 25.3g/L of CG001 cells.

4.2.2 Influence of pH on reuterin production

The effect of pH on the glycerol bioconversion was studied using cells harvested after 24h of growth. The DPC16 cells (25g/L dry cell weight) were suspended at 37℃ in 300mmol/L glycerol solution and the conversion was measured after 1h of incubation. The pH values of the glycerol solutions were adjusted to 6.0, 6.2, 6.8, 7.2, 7.5, and 8.0 by pretreating the PBS buffer, separately.

Table 6. One-way ANOVA: pH *versus* consumed glycerol, produced reuterin and 1, 3-PD

Substance (mmol/L)	Analysis scope	Sum of Squares	df	Mean Square	P-Values	R-sq (pred)
Consumed Glycerol	Between Groups	2100.536	5	420.107	.045	3.91%
	Within Groups	1565.864	12	130.489		
	Total	3666.400	17			
Produced Reuterin	Between Groups	1763.618	5	352.724	.098	0.00%
	Within Groups	1753.399	12	146.117		
	Total	3517.018	17			
Produced 1,3-PD	Between Groups	179.552	5	35.910	.008	31.29%
	Within Groups	78.938	12	6.578		
	Total	258.490	17			

(Significance level α = 0.05)

Table 6 shows the effect of pH on consumed glycerol, produced reuterin, and produced 1, 3-PD. The results showed that pH values had a significant effect on consumed glycerol and produced 1, 3-PD (P-values < 0.05). However, pH values had no significant effect on produced reuterin (P-values >0.05). Meanwhile, the R-square value indicated that pH values did not show good linear regression due to the small numerical values.

Figure 21. Effect of pH on reuterin production

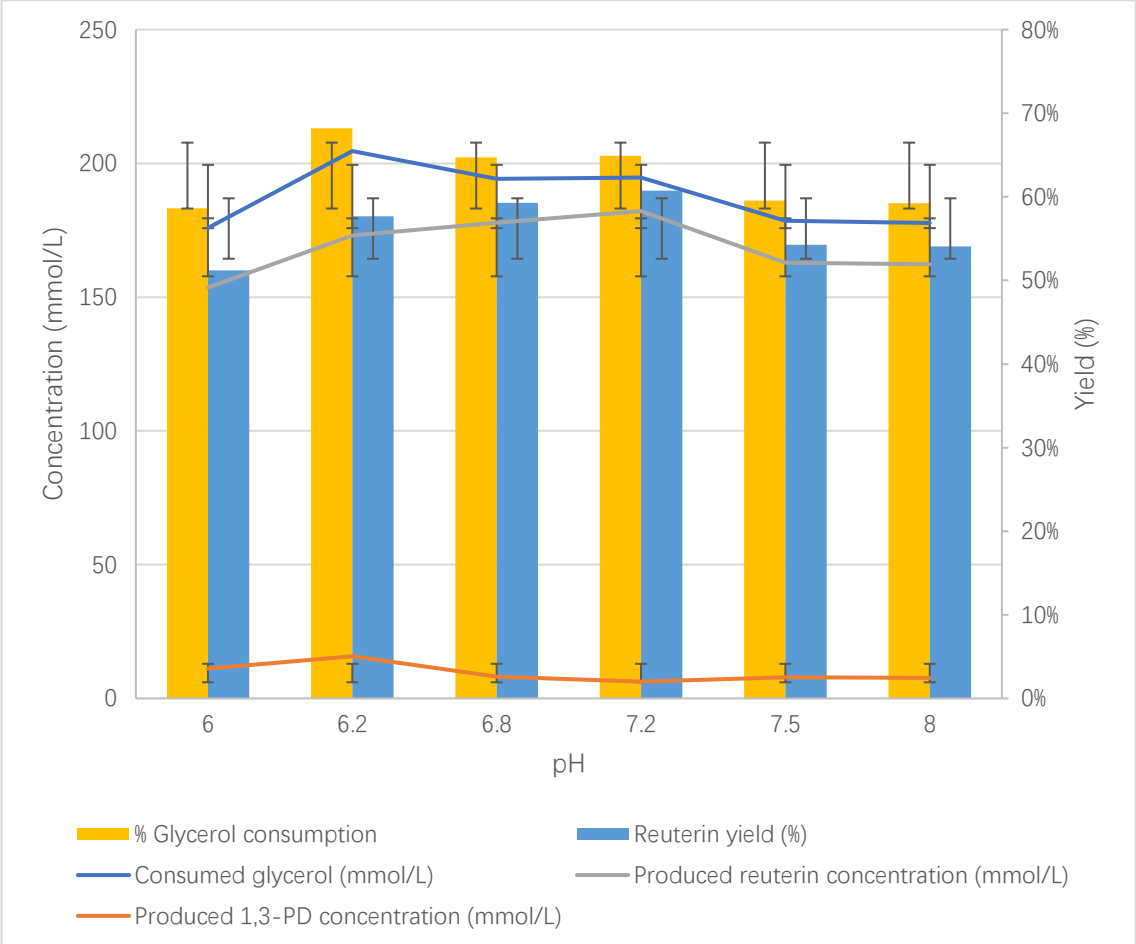


Figure 21 shows the relationship between different pH values and concentrations of glycerol, reuterin, and 1, 3-PD. The peak point for consumed glycerol occurred at pH 6.2. This consumption was significantly increased from that at pH 6.0. After pH 6.2, the

consumed glycerol decreased until the pH reached 7.5 and it maintained low until pH 8.0. In contrast, production of reuterin increased from pH 6.0 to pH 7.2, after which it decreased by a small amount. When the pH value was above 7.5, which is the extreme pH value for isolated glycerol dehydratase, the activity of intracellular glycerol dehydratase maintained stable. Production of 1, 3-PD peaked at pH 6.2, but was at a relatively low concentration. The percentage of glycerol consumption showed a peak of 68.20% of that supplied at pH 6.2. As the pH value increased, the percentage of glycerol consumption decreased. In contrast, as the pH increased, the yield of reuterin increased from 51.2% (at pH 6.0) to 60.72% (at pH 7.2), before decreasing to 54.09% (at pH 8.0).

Table 7. The reuterin production and unit yield per DPC16 at different pH values

pH	Produced reuterin (mmol)	Reuterin unit yield (g/ (log_{10} c.f.u))
6.0	0.1997	0.797
6.2	0.2250	0.898
6.8	0.2312	0.923
7.2	0.2368	0.945
7.5	0.2117	0.845
8.0	0.2110	0.842

(The number of viable DPC16 cells (log_{10} c.f.u/mL) was calculated in 1.0ml of samples (Refer to Section 4.1.2). 25g/L of biomass was used to produce reuterin. One gram of dry cell DPC16 contained 10.020(log_{10} c.f.u/ml) of viable cells)

Table 7 shows that the reuterin unit yield increased from pH 6.0 to pH 7.2, then decreased after pH 7.2. After pH 7.2, the reuterin unit yield was maintained as the pH values increased. The peak point of reuterin unit yield (0.923mmol/log_{10} c.f.u) presented at pH 7.2 which was slightly higher than that presented at pH 6.8 (reuterin unit yield was 0.923 mmol/log_{10} c.f.u). At the optimum pH for growth of *L. reuteri* DPC16 (6.2), the reuterin unit yield was 0.898 mmol/log_{10} c.f.u. The optimum pH value for producing reuterin using DPC16 was 7.2. This result was similar to that observed by Chen (2011)

and Lüthi-Peng (2002), with an optimized pH of 7.2. However, Wan (2017) reported that the optimum pH value for reuterin production by *L. reuteri* ATCC53608 was 6.2. The optimum initial pH value for *L. reuteri* IMAU10240 to convert glycerol to reuterin was reported to be pH 6.5 (Yao, 2016).

4.2.3 Influence of glycerol concentration on reuterin production

The effect of the glycerol concentration on its conversion was studied using DPC16 cells harvested at 24h of growth. The cells (25g/L) were suspended at pH 6.2 and 37℃ in a series of glycerol solutions and the conversion was measured after 1h of incubation. Concentrations of glycerol solutions were 150mmol/L, 200 mmol/L, 300 mmol/L, 350 mmol/L, 400 mmol/L, and 450 mmol/L.

Table 8. One-way ANOVA: initial glycerol concentration *versus* consumed glycerol, produced reuterin and 1, 3-PD

Substance (mmol/L)	Analysis scope	Sum of Squares	df	Mean Square	P-Values	R-sq (pred)
Consumed Glycerol	Between Groups	47289.055	5	9457.811	.000	84.04%
	Within Groups	3610.175	12	300.848		
	Total	50899.230	17			
Produced Reuterin	Between Groups	48897.793	5	9779.559	.000	83.57%
	Within Groups	3851.368	12	320.947		
	Total	52749.161	17			
Produced 1,3-PD	Between Groups	17.485	5	3.497	.269	0.00%
	Within Groups	28.449	12	2.371		
	Total	45.934	17			

(Significance level α = 0.05)

Table 8 shows that the initial glycerol concentration had significant effect on consumed glycerol (P values <0.05) and produced reuterin (P values<0.05). Initial glycerol

concentration had no significant effect on produced 1, 3-PD (P values >0.05). It was worth noting that the initial glycerol concentration demonstrated a linear relationship with consumed glycerol (R-sq=84.04%) and produced reuterin (R-sq=83.57%). However, initial glycerol concentration had no linear relationship with produced 1, 3-PD (R-sq=0.00%).

Figure 22. Effect of glycerol concentration on reuterin production

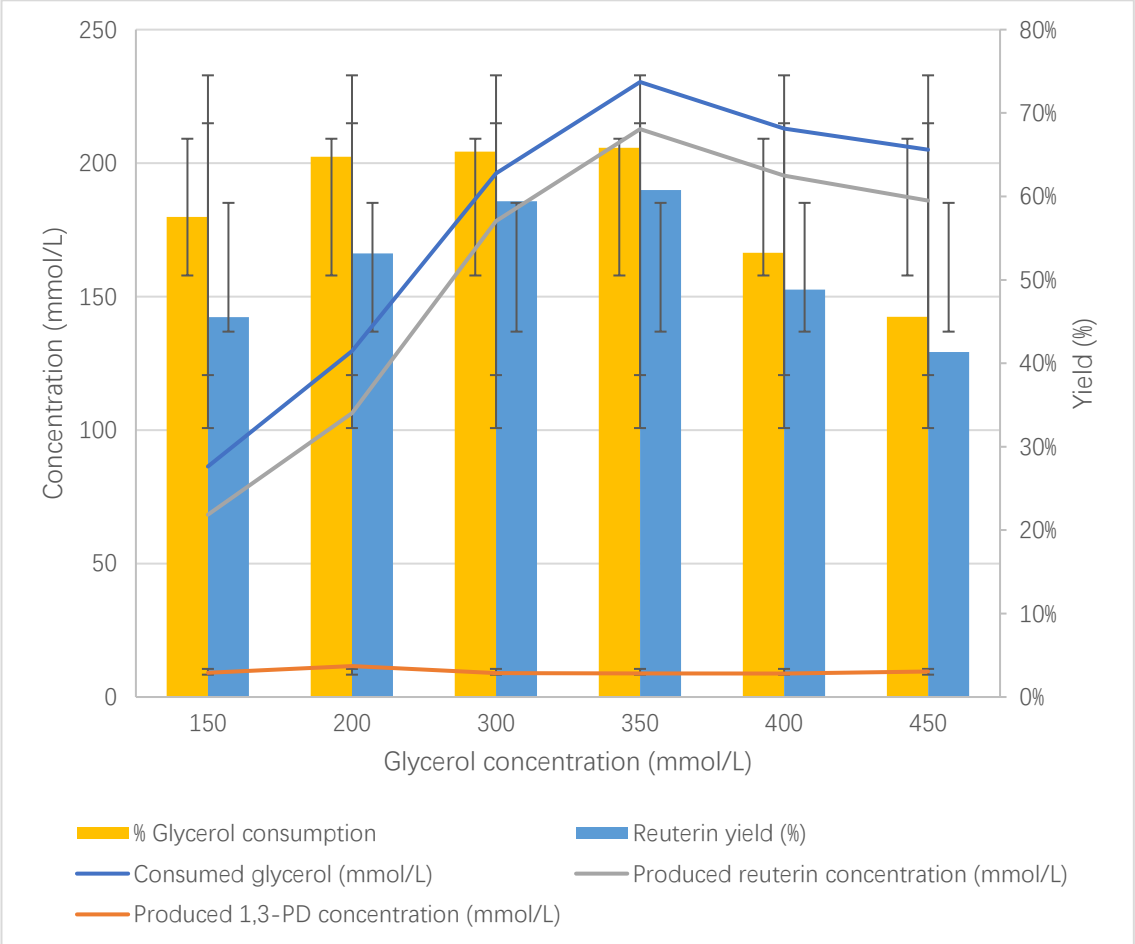


Figure 22 shows that the concentrations of consumed glycerol increased as the initial glycerol concentration increased from 150mmol/L to a concentration of 350mmol/L. When the initial glycerol concentration exceeded 350mmo/L, the consumed glycerol decreased. A similar trend was observed for reuterin production. The peak of reuterin

production occurred at the 350mmol/L of initial glycerol. Concentrations of 1,3-PD produced remained low throughout. The percentage of glycerol consumption was 57.56% of that supplied at 150mmol/L of initial glycerol concentration. Interestingly, although initial glycerol concentrations increased from 200mmol/L to 350mmol/L, the percentage of glycerol consumption remained around 65% of that supplied. The peak point of glycerol consumption was seen at 350mmol/L, after which it decreased sharply. The relationship between reuterin yield and initial glycerol concentration was similar to the relationship between the percentage of glycerol consumption and initial glycerol concentration. The reuterin yield was 45.53% at 150mmol/L of initial glycerol concentration. As the initial glycerol concentration increased, the reuterin yield increased to the peak point (60.78%) at 350mmol/L of initial glycerol concentration. After this point, reuterin yield decreased as initial concentration decreased.

Table 9. The reuterin production and unit yield per DPC16 at different initial glycerol concentrations

Glycerol concentration (mmol /L)	Produced reuterin (mmol)	Reuterin unit yield (g/$\log_{10}$ c.f.u)
150	0.0888	0.354
200	0.1383	0.552
300	0.2317	0.925
350	0.2766	1.104
400	0.2540	1.014
450	0.2418	0.965

(The number of viable DPC16 cells ($\log_{10}$ c.f.u/mL) was calculated in 1.0ml of samples (Refer to Section 4.1.2). 25g/L of biomass was used to produce reuterin. One gram of dry cell DPC16 contained 10.020($\log_{10}$ c.f.u/ml) of viable cells)

Table 9 shows the reuterin yield at different initial glycerol concentrations based on biomass. At the lowest initial glycerol concentration (150mmol/L), *L. reuteri* DPC16

showed the weakest reuterin-producing ability (0.354 mmol/log_{10} c.f. u). This yield of reuterin increased to a peak point (1.104mmol/log_{10} c.f. u) when the initial glycerol concentration was 350mmol/L. Thereafter, as the concentration of glycerol was over 350mmol/L, the reuterin unit yield decreased. Overall, the optimum initial glycerol concentration was 350mmol/L for the conversion of glycerol to reuterin. Lüthi-Peng (2002), Chen (2011), and Wan (2017) obtained nearly similar results for initial glycerol concentrations which optimized the reuterin yield (less than 300mmol/L using *L. reuteri* CG001 and *L. reuteri* ATCC53608). It is clear that initial glycerol concentration should be increased to no more than 350mmol/L for effective reuterin production.

4.2.4 Influence of temperature on reuterin production

The effect of temperature on glycerol bioconversion was studied using cells harvested at 24h of growth. The cells (25g/L) were suspended at pH 6.2 and incubated at a series of temperatures in 300mmol/L of glycerol solution, and the conversion rate was measured after 1h of incubation. Tested temperatures were 20℃, 25℃, 30℃, 37℃, and 42℃.

Data analysis (Table 10) shows that temperature had significant effects on consumed glycerol (P-values<0.05), produced reuterin (P-values<0.05) and produced 1, 3-PD (P-values<0.05). However, temperature had no strong linear relationship with consumed glycerol (R-sq=54.71%), produced reuterin (R-sq=37.67%) or produced 1, 3-PD (R-sq=52.83%).

Table 10. One-way ANOVA: temperature *versus* consumed glycerol, produced reuterin and 1, 3-PD

Substance (mmol/L)	Analysis scope	Sum of Squares	df	Mean Square	P-Values	R-sq (pred)
Consumed Glycerol	Between Groups	2454.189	4	613.547	.002	54.71%
	Within Groups	618.535	10	61.853		
	Total	3072.724	14			
Produced Reuterin	Between Groups	3418.057	4	854.514	.008	37.67%
	Within Groups	1309.548	10	130.955		
	Total	4727.605	14			
Produced 1,3-PD	Between Groups	268.472	4	67.118	.002	52.83%
	Within Groups	71.210	10	7.121		
	Total	339.682	14			

(Significance level α = 0.05)

Figure 23. Effect of temperature on reuterin production

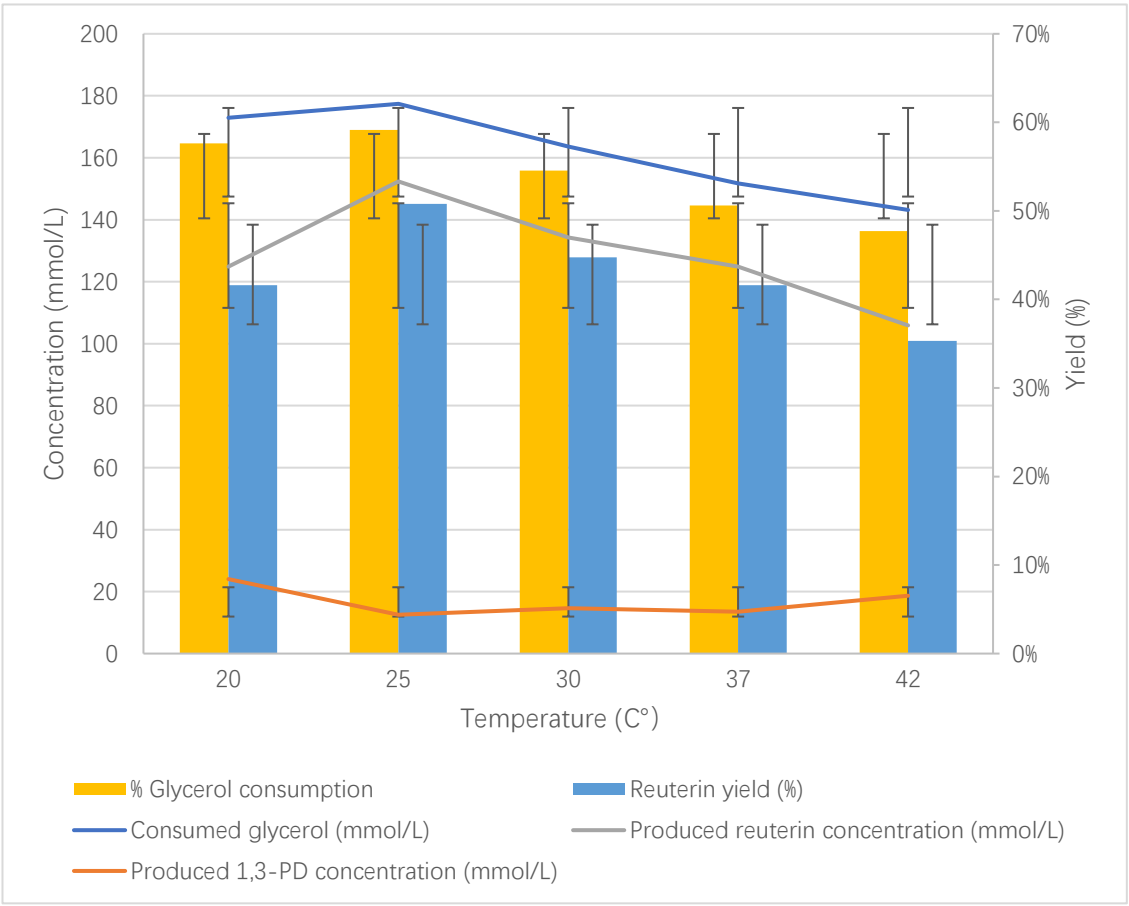


Figure 23 shows that at the lower temperatures (25℃ and below), the concentrations of consumed glycerol were similar, and the peak value of glycerol consumed (177.40 mmol/L) occurred at 25℃. As the temperature increased, the consumption of glycerol decreased sharply. The trend of reuterin production was similar to that of glycerol consumption, and the peak value of reuterin production was at 25℃. As the temperature increased, reuterin production decreased sharply. Interestingly, the production of 1,3-PD followed an opposite trend. The highest production of 1, 3-PD was achieved at 20℃. Furthermore, the glycerol consumption percentage values were nearly same at 20℃ (57.64%) and 25℃ (59.3%). After 25℃, the glycerol consumption percentage continually decreased as temperature increased. The percentage of glycerol consumption values were 54.54%, 50.50% and 57.72% when temperatures were 30℃, 37℃ and 42℃, respectively. However, reuterin yield was changed sharply as temperature changed. The reuterin yield was 41.62% when the temperature was 20℃. This yield was sharply increased to 50.78% to be the peak point at 25℃. After 25℃, as the temperature increased, the reuterin yield sharply decreased to 35.30% when the temperature was 42℃.

Table 11. The reuterin production and unit yield per DPC16 at different temperatures

Temperature (℃)	Produced reuterin (mmol)	Reuterin unit yield (g/ (log_{10} c.f.u))
20	0.1623	0.648
25	0.1981	0.791
30	0.1745	0.697
37	0.1623	0.648
42	0.1377	0.550

(The number of viable DPC16 cells (log_{10} c.f.u/mL) was calculated in 1.0ml of samples (Refer to Section 4.1.2). 25g/L of biomass was used to produce reuterin. One gram of dry cell DPC16 contained 10.020(log_{10} c.f.u/ml) of viable cells)

Table 11 shows that reuterin unit yield changed slightly from 0.648 mmol/log_{10} c.f.u (at 20℃) to 0.648 mmol/log_{10} c.f.u (at 37℃). The highest value of reuterin unit yield was 0.791 mmol/log_{10} c.f.u, which was at 25℃. After 37℃, the reuterin unit yield significantly decreased to 0.550 mmol/log_{10} c.f.u at 42℃.

Overall, the optimum temperature for bioconversion of glycerol to reuterin by *L. reuteri* DPC16 was 25℃. This result was similar to that of Doleyres (2005) who reported that a lower temperature was beneficial to reuterin production. However, Lüthi-Peng (2002) and Chen (2011) drew a different conclusion, reporting that a higher temperatures (30℃ and 37℃) promoted the glycerol-reuterin conversion.

4.2.5 Influence of incubation time on reuterin production

The effect of incubation time on the glycerol bioconversion was studied using cells harvested at 24h of growth. The cells (25g/L) were suspended at pH 6.2 and 37℃ in 300mmol/L of glycerol solution and the glycerol-reuterin conversion was measured at a series of incubation times. Tested incubation times were 0.5h, 1h, 1.5h, 2h, 3h, and 4h.

Table 12 shows that incubation time had a significant effect on consumed glycerol, produced reuterin and produced 1,3-PD (P-values<0.05). Furthermore, incubation time showed a strong linear relationship with consumed glycerol, produced reuterin and produced 1, 3-PD (R-square values over 70%).

Table 12. One-way ANOVA: incubation time *versus* consumed glycerol, produced reuterin and 1, 3-PD

Substance (mmol/L)	Analysis scope	Sum of Squares	df	Mean Square	P-Values	R-sq (pred)
Consumed Glycerol	Between Groups	3941.520	4	985.380	.000	76.06%
	Within Groups	729.505	10	72.951		
	Total	4671.026	14			
Produced Reuterin	Between Groups	5561.077	4	1390.269	.000	72.77%
	Within Groups	780.445	10	78.045		
	Total	6341.522	14			
Produced 1,3-PD	Between Groups	397.337	4	99.334	.000	91.82%
	Within Groups	23.641	10	2.364		
	Total	420.978	14			

(Significance level α = 0.05)

Figure 24. Effect of incubation time on reuterin production of DPC16

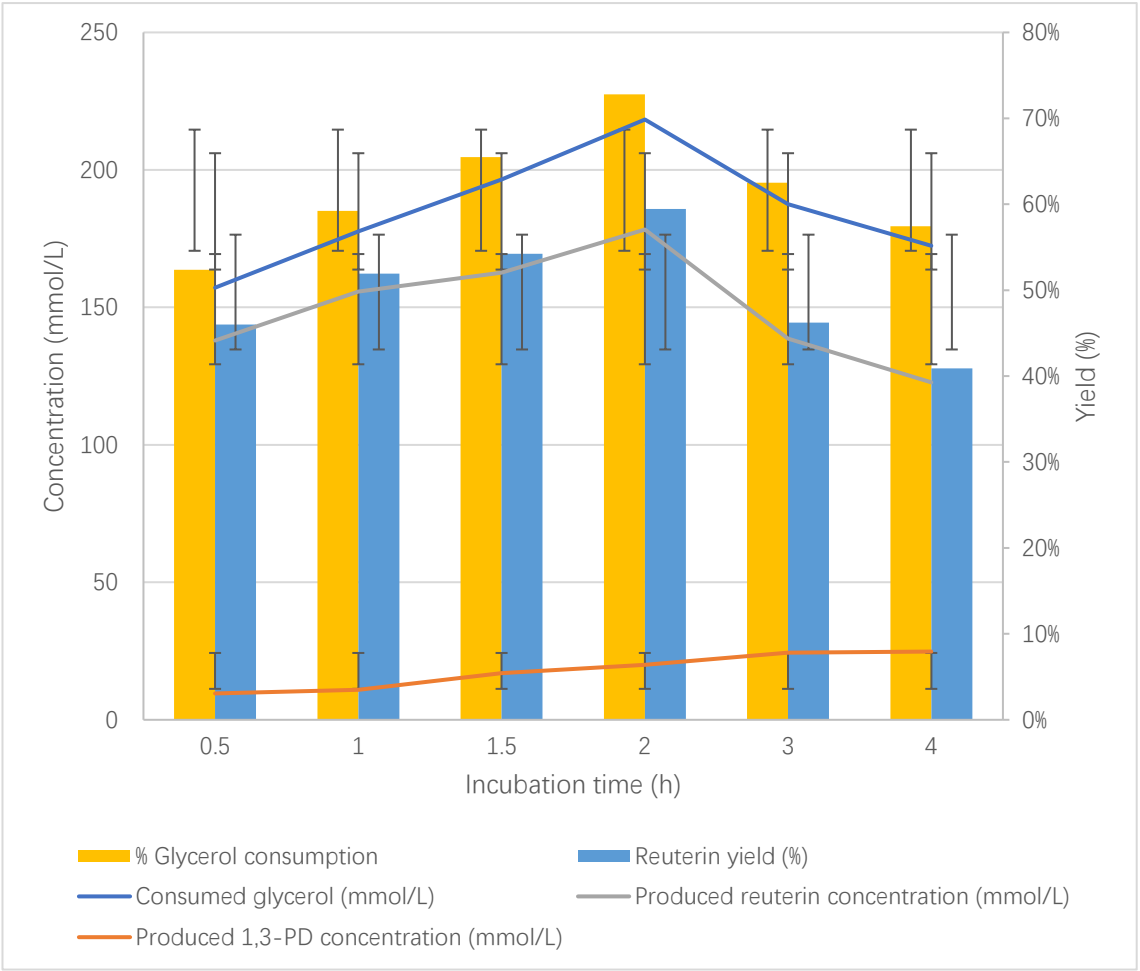


Figure 24 shows that the concentration of glycerol consumed increased as the incubation time increased to 2h, after which it decreased. The effect of the conversion time on reuterin production followed a similar trend as that on glycerol consumption. The maximum percentage of glycerol consumption (72.77%) was observed at 2h. Interestingly, as the incubation time increased, the production of 1,3-PD increased throughout the whole period of incubation. The maximum values of glycerol consumption percentage and the reuterin yield during the entire incubation time (4h) were 72.77% and 59.44%, respectively. They were both at 2h incubation time.

Table 13. The reuterin production and yield unit per DPC16 after different incubation time

Conversion time (h)	Produced reuterin (mmol)	Reuterin unit yield (g/ (log_{10} c.f.u))
0.5	0.1794	0.716
1	0.2024	0.808
1.5	0.2114	0.844
2	0.2318	0.925
3	0.1803	0.720
4	0.1595	0.637

(The number of viable DPC16 cells (log_{10} c.f.u/mL) was calculated in 1.0ml of samples (Refer to Section 4.1.2). 25g/L of biomass was used to produce reuterin. One gram of dry cell DPC16 contained 10.020(log_{10} c.f.u/ml) of viable cells)

Table 13 shows that reuterin unit yield was increased from 0.716 mmol/log_{10} c.f.u (0.5h incubation time) to 0.925 mmol/log_{10} c.f.u (2h incubation time). The 2h of incubation time presented the peak point. Thereafter, the reuterin unit yield started to decrease and reached its lowest value (0.637mmol/log_{10} c.f.u) at 4h incubation time. Lüthi-Peng (2002) reported that the optimum incubation time for *L. reuteri* ATCC53608 was 3h, while Wan (2017) reported an optimum incubation time of only 1h.

4.2.6 Influence of culture age on reuterin production of DPC16

The effect of culture age on glycerol bioconversion was studied using cells harvested after a series of different growth periods. The cells (25 g/L) were suspended at pH 6.2 and 37℃ in 300mmol/L of glycerol solution and the conversion was measured after 1h incubation time. Tested culture ages were 12h, 16h, 20h, 24h, 28h, and 32h.

Table 14. One-way ANOVA: culture age versus consumed glycerol, produced reuterin and 1,3-PD

Substance (mmol/L)	Analysis scope	Sum of Squares	df	Mean Square	P-Values	R-sq (pred)
Consumed Glycerol	Between Groups	2300.139	5	460.028	.674	0.00%
	Within Groups	8619.506	12	718.292		
	Total	10919.645	17			
Produced Reuterin	Between Groups	1750.383	5	350.077	.791	0.00%
	Within Groups	8917.485	12	743.124		
	Total	10667.868	17			
Produced 1,3-PD	Between Groups	145.642	5	29.128	.000	71.15%
	Within Groups	21.421	12	1.785		
	Total	167.063	17			

(Significance level α = 0.05)

Table 14 shows that culture age had a significant effect on produced 1, 3-PD (P-values<0.05) and there was a strong linear relationship (R-sq=71.15%). However, there was no significant effect between culture age (P-values>0.05) and produced reuterin (P-values>0.05). There was not a strong linear relation between culture age and consumed glycerol (R-se=0.00%) or produced reuterin (R-sq=0.00%), separately.

Figure 25 shows the relationship between culture age at harvest on reuterin production. The concentrations of consumed glycerol and reuterin production both showed peaks at

a harvest age of 20 h. Interestingly, glycerol conversion and reuterin production values, and reuterin yield did not vary considerably for DPC16 cells harvested at different times.

Figure 25. Effect of culture age on reuterin production of DPC16

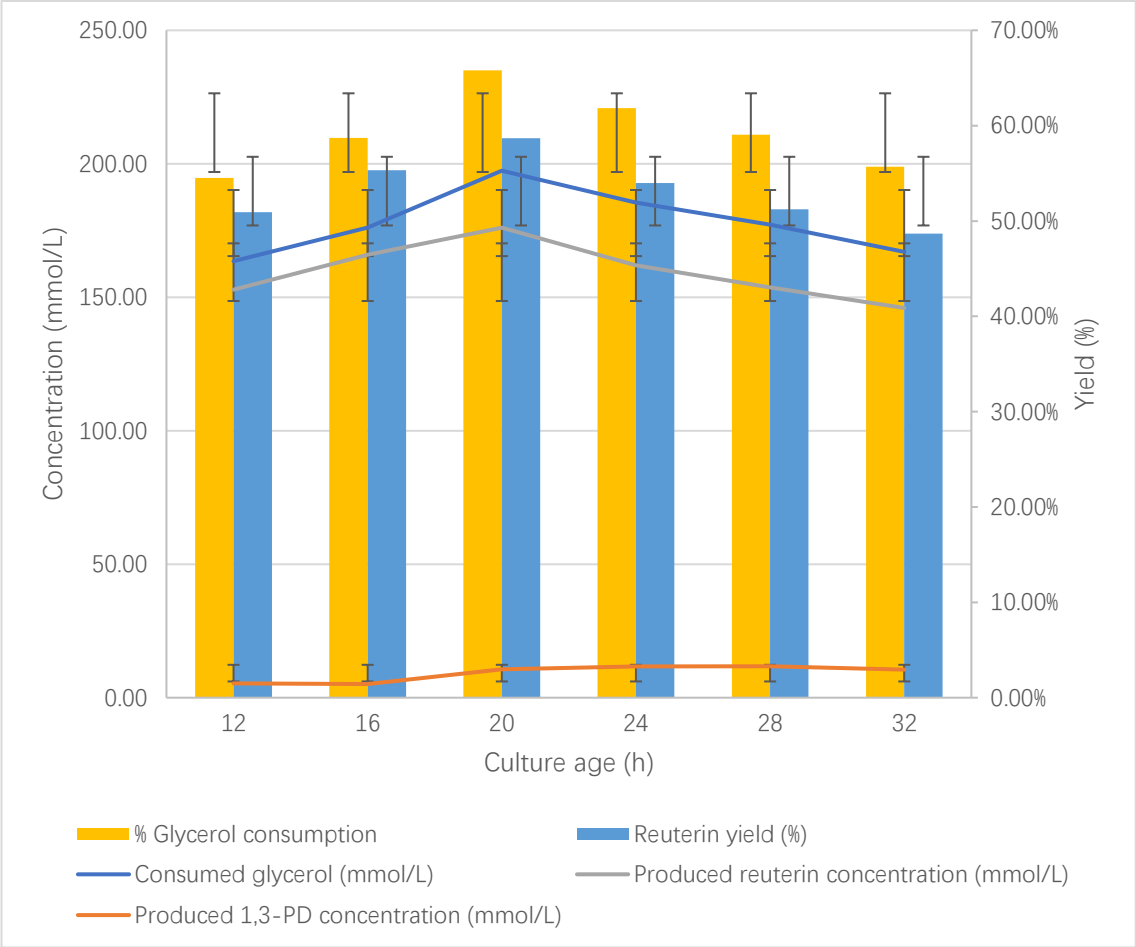


Table 15. The reuterin production and unit yield of DPC16 at different harvest times

Culture age (h)	Produced reuterin (mmol)	Reuterin unit yield (g/ (log_{10} c.f.u))
12	0.1986	0.793
16	0.2157	0.861
20	0.2289	0.914
24	0.2105	0.840
28	0.1997	0.797
32	0.1898	0.758

(The number of viable DPC16 cells (log_{10} c.f.u/mL) was calculated in 1.0ml of samples (Refer to Section 4.1.2). 25g/L of biomass was used to produce reuterin. One gram of dry cell DPC16 cell contained 10.020(log_{10} c.f.u/ml) of viable cells)

Table 15 shows that reuterin unit yield was increased from 12h (0.793 mmol/log_{10} c.f.u) to 20h culture age (0.914mmol/log_{10} c.f.u) which was the peak point. Thereafter, the reuterin unit yield kept decreasing as culture age increased. Referring to Section 4.1.1, reuterin unit yield did not fluctuate greatly from 16h to 28h. The reuterin unit yield decreased sharply when culture age entered the death phase (32h).

In summary, the optimum culture age of DPC16 cells for harvesting cells for reuterin production was 20h. A previous report showed a similar result, the optimum culture age for *L. reuteri* CG001 being at 16h to 24h (Chen, 2011). However, an optimum culture age for *L. reuteri* ATCC53608 was reported to be 8h (Lüthi-Peng, 2002). Wan (2017) reported an optimum in the early stationary phase (26h).

4.2.7 Statistical analysis for reuterin production

Referring to Sections 4.2.1 to 4.2.6, i.e. biomass concentration, pH, glycerol concentration, temperature, incubation time, and culture age, were separately named X1 to X6 and used to analyse their effects on reuterin production. In order to determine the relationships between these six factors, the PCA method was used.

Figure 26 shows that there are seven variables affecting reuterin production. The first factor explains 24.173% of the variation among samples while the following three factors explain similar variations of 15.513%, 15.404% and 15.199%, successively. The last factor explains only 4.665% of the variation among samples.

Figure 26. Eigenvalues analysis for 6 single factors affecting reuterin production

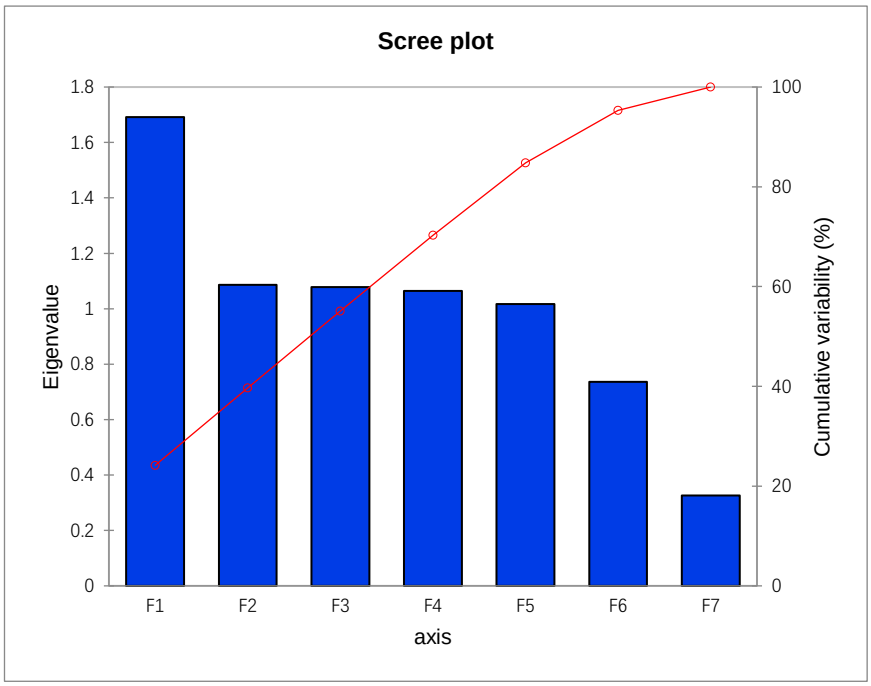


Figure 27. Sample configuration in the first and second dimensions of the PCA

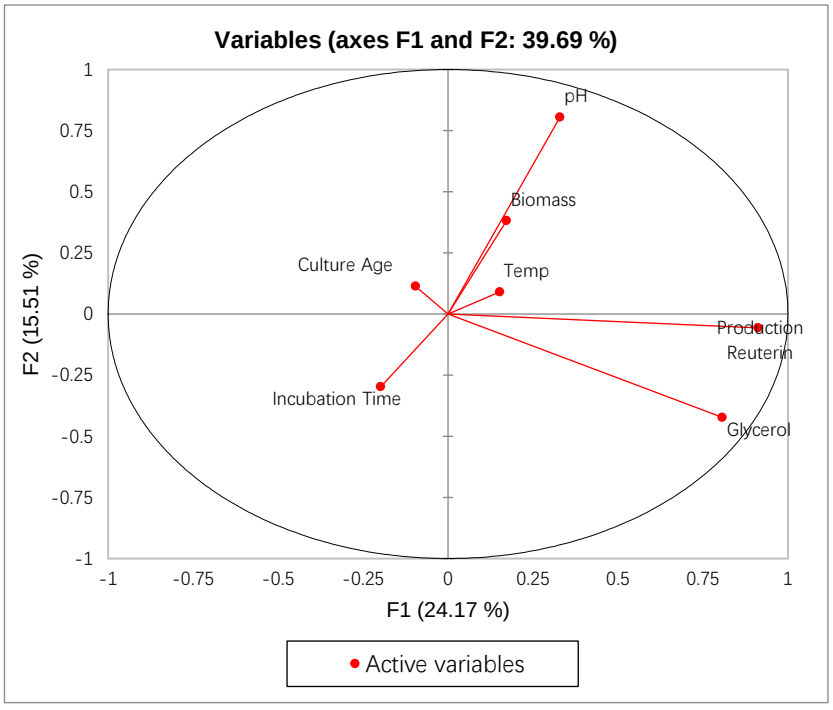


Figure 27 shows the analysis map data of the factors affecting reuterin production. A total of 39.69% of variation between samples was explained. The first axis explained

24.17% of the total variation, and the second axis up to 27.29% variance. The first component (F1) indicated that the culture age and glycerol concentration had opposite effects on reuterin production. At the second axis, the incubation time worked in opposition to the pH, biomass concentration and temperature. Reuterin production and glycerol concentration were highly correlated.

Table 16. Correlation Matrix for factors affecting reuterin production

		Biomass	pH	Glycerol	Temp	Incubation Time	Culture Age	Produced Reuterin
Correlation	Biomass	1.000	.096	.009	-.069	.079	-.034	.113
	pH	.096	1.000	-.011	.083	-.096	.041	.212
	Glycerol	.009	-.011	1.000	.008	-.009	.004	.612
	Temp	-.069	.083	.008	1.000	.069	-.029	.103
	Incubation Time	.079	-.096	-.009	.069	1.000	.034	-.131
	Culture Age	-.034	.041	.004	-.029	.034	1.000	-.072
	Produced Reuterin	.113	.212	.612	.103	-.131	-.072	1.000

Table 16 shows the correlation matrix between the six factors and reuterin production. Through comparison of the correlation values between the six factors and produced reuterin, it can be seen that biomass concentration, pH, glycerol concentration and temperature were positively correlated with reuterin production. On the other hand, incubation time and culture age showed negative correlation with reuterin production. The correlation value between reuterin production and glycerol concentration was 0.612, and this was the main influencing factor. The second influencing factor for reuterin production was pH (0.212). The following influencing factors for reuterin production were incubation time (the third influence factor, -0.131), biomass concentration (the fourth influence factor, 0.113) and temperature (the fifth influence factor, 0.103). The

correlation value of -0.072 for culture age was the least influencing factor for reuterin production.

All in all, the rank of effect on reuterin production for six single factors are glycerol concentration> pH > incubation time > biomass concentration > temperature > culture age. Wan (2017) showed a slightly different result for the rank of the effect of reuterin production, which was glycerol> biomass> incubation time> temperature> pH. The difference in rank could be caused by the strain used.

Chapter 5 Discussion

Theoretically, the main factor affecting reuterin production of *Lactobacillus reuteri* DPC16 cells is the enzyme glycerol dehydratase. In previous Section 2.4.3, factors affecting glycerol dehydratase were stated. However, other factors need to be considered when using a whole-cell method.

5.1 Reuterin production in single factor experiments

5.1.1 Effect on reuterin production of biomass concentration

It was expected that reuterin production would be in direct production to the biomass concentration. However, in actuality, there was a critical value of biomass which affected reuterin production using resting *L. reuteri* DPC16 cells (Refer to 4.2.1, optimum biomass was 21g/L). Biomass values below or above this value both led to a decrease in reuterin production. The mechanism of biomass concentration is probably caused by the extent of surface area between *L. reuteri* DPC16 cells and the glycerol solution (Wan, 2017). At lower concentrations of cells, the bacteria were sufficiently immersed in the glycerol solution, which contributed to a better reuterin production ability. As the biomass increased, the contact area between the cells and glycerol solution decreased, probably due to cell aggregation, mass transfer resistance increased, and the unit reuterin production ability of DPC16 decreased (Refer to Table 6). During the experiment, it was observed that *L. reuteri* DPC16 cells tended to sediment and adhere to the bottom and walls of the bottles. Thus, the adhesion tendency of DPC16 might contribute to the lower reuterin production at higher concentrations of DPC16 biomass. Hence, reuterin production could possibly be increased by stirring as it would increase

the contact area of the DPC16 cells (Sardari, 2013). This could be verified in further experiments. In industrial production, to determine the optimized production conditions, it will be important to balance the biomass concentration and glycerol concentration.

5.1.2 Effect on reuterin production of pH values

Different *L. reuteri* strains are reported to have different optimum pH values to convert glycerol to reuterin due to differences in the enzyme glycerol dehydratase. Generally speaking, the conversion of glycerol to reuterin prefers a neutral to acidic environment (pH 6.2 to pH 7.2). Wan (2017) reported that in glycerol solution without PB buffer (providing different pH environments), the reuterin production of *L. reuteri* ATCC53608 cells was 5% lower than in glycerol solution adjusted using PB buffers. The explanation was that *L. reuteri* produced short-chain fatty acids during its metabolism (Morita, 2008), and that these could lower the glycerol solution's pH values. The more acidic environment (pH< 6.2) could reduce the reuterin production ability of DPC16. Referring to Section 4.2.3, *L. reuteri* DPC16 preferred a neutral environment for growth (pH 6.8 to pH 7.2), which also matches the pH value in the intestinal tract (pH6.8 to pH 7.2). The reuterin unit yield for *L. reuteri* DPC16 was not significantly different from pH 6 to pH 8. Interestingly, the pH value significantly affected isolated glycerol dehydratase from various microorganism strains. The optimum pH value of glycerol dehydratase isolated from *K. pneumoniae* and *C. freundii* was pH 8.5, which is considerably higher than the optimum pH value using intact resting cells of strain DPC16 in the present work. Overall, the optimum pH value for reuterin production using resting cells of *L. reuteri* DPC16 is nearly neutrality.

5.1.3 Effect on reuterin production of glycerol concentration

Glycerol is the substrate for reuterin production; its concentration significantly affected reuterin production and yield. The present results showed that there was an optimum concentration of glycerol, above which its conversion and reuterin production declined. The most likely reason for this is that reuterin is toxic to the cells, and once a certain concentration is reached, production ceases.

5.1.4 Effect on reuterin production of temperature

The temperature affected reuterin production through two mechanisms. First, it is expected that as the temperature rises, the reaction rate will increase. However, conversely, as the temperature rises, enzymes become denatured and inactivated. The present results show that, using resting cells, the optimum temperature for conversion of glycerol to reuterin was 25^{o}C, which is considerably lower than the optimum growth temperature for this organism (37^{o}C). This illustrates the value of using a 2-step process, whereby organism growth can be optimized in the first step, and reuterin production in the second step.

5.1.5 Effect on reuterin production of incubation time

The present results showed that, under the experimental conditions used, the maximum production of reuterin occurred after 2 hours of incubation, after which its concentration decreased. This can be partly explained by further conversion of reuterin to 1,3-propanediol, but this does not explain all the losses. Possibly, glycerol was also converted into dihydroxyacetone, producing $NADH_2$. This extra $NADH_2$ could also be used in the conversion of reuterin to 1,3-PD. In addition, the toxic effect of reuterin on

the cells must also be considered.

An additional factor to consider is the lack of an additional energy source for the cells. Possibly, the glycerol conversion to reuterin may be maintained by providing a source of energy such as glucose. This could be tested in future experiments.

5.1.6 Effect on reuterin production of culture age

The present results showed that culture age had only a minor effect on reuterin production and yield. However, it is apparent that the stationary phase of growth is the most suitable for cell harvesting when using resting cells in a 2-step process.

5.1.7 Effect on reuterin production of other conditions

First, the presence of oxygen was not controlled or measured during the present work. Having now optimized some of the main operating conditions, it would be appropriate to test if anaerobic conditions would affect the bioconversion.

Secondly, as mentioned earlier, the addition of glucose as an energy source during the conversion should be studied. Glucose could affect reuterin production through two methods. First, its presence could change the NAD/NADH ratios during the reaction. A ratio of glucose to glycerol of no more than 0.33 has been proposed for optimum conversion (Doleyres, 2005), thus assisting maintenance of appropriate NAD/NADH ratios within the cell.

Chapter 6 Conclusion and Research Prospects

6.1 Conclusion

In this work, the conversion of glycerol to produce reuterin using *L. reuteri* DPC16 is affected by culture age, glycerol concentration, pH value, temperature, and biomass concentration. The glycerol concentration is the main influencing factor. The highest reuterin yield observed was 61.33% using 24h pre-cultured cells, at a concentration of 21 g/L to convert 300mmol/L of initial glycerol solution in 1h at 37℃ and pH 6.2. Cells harvested after approximately 20h of growth were the most useful for the glycerol to reuterin transformation at 25℃ and pH 6.8. The optimum biomass concentration was 25g/L and using an incubation time of 2h.

6.1 Future research

In this project only the influence of single factors (glycerol concentration, biomass concentration, temperature, pH, culture age and incubation time) on reuterin production have been examined. The interrelationships among the six factors and optimum reuterin production conditions were not analyzed. In further research, these interrelationships should be studied. Furthermore, because high concentrations of reuterin are toxic to *L. reuteri* DPC16 techniques must be found to mitigate against this.

As stated earlier, the effects of oxygen and glucose on the conversion require examination.

Furthermore, in this project, all the data are from laboratory experiments. For commercial use, the optimum conversion conditions for producing reuterin remain to be researched. It requires design of a pilot plant to find industry reuterin production

conditions.

Finally, reuterin can also be researched for further uses. The company Drapac Ltd subsidizes research for reuterin used as a food additive.

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Appendix

Appendix 1 Ingredients of cultural growth media

Table A. 1 Composition of MRS broth (Difo, Michigan; USA)

Ingredients	Amount (g per liter of distilled water)
Protease peptone	10.0
Beef extract	10.0
Yeast extract	5.0
Polysorbate 80	1.0
Ammonium citrate	2.0
Sodium acetate	5.0
Dipotassium phosphate	2.0
Magnesium sulphate	0.1
Manganese sulphate	0.05
Glucose	20.0 (equivalent to 110mM)
Medium pH value: 6.5± 0.2	

Table A. 2 Composition of MRS Agar (Difo, Michigan; USA)

Ingredients	Amount (g per liter of distilled water)
Protease peptone No.3	10.0
Beef extract	10.0
Yeast extract	5.0
Dextrose	20.0
Polysorbate 80	1.0
Ammonium citrate	2.0
Sodium acetate	5.0
Magnesium sulphate	0.1
Manganese sulphate	0.05
Dipotassium Phosphate	2
Agar	15.0
Medium pH value: 6.5± 0.2	

Table A. 3 Standard acrolein curve concentration

No.	Pure Acrolein solution mL	L-tryptophan solution mL	Total volume mL	Acrolein concentration mmol/L
1	0.1	0.9	1	0.7120
2	0.14	0.86	1	0.9968
3	0.16	0.84	1	1.1392
4	0.17	0.83	1	1.2104
5	0.21	0.79	1	1.4952
6	0.24	0.76	1	1.7088
Acrolein solution:7.14 mmol/L				
L-tryptophan: 19.7424mmol/L				

Appendix 2 Raw data

Table B. 1 Optical Density (OD) values

Factors: Glycerol concentration, Incubation time: 24h Temperature: 37℃	
No.	OD: (λ=620mm)
1	2.613
2	2.614
3	2.653
Factors: pH, Incubation time: 24h Temperature: 37℃	
No.	OD: (λ=620mm)
1	1.894
2	1.962
3	1.876
4	2.018
5	1.942
Factors: Biomass, Incubation time: 24h Temperature: 37℃	
No.	OD: (λ=620mm)
1	2.583
2	2.480
3	2.553
Factors: Temperature, Incubation time: 24h Temperature: 37℃	
No.	OD: (λ=620mm)
1	2.562
2	2.514
3	2.538
Factors: Incubation time, Incubation time: 24h Temperature: 37℃	
No.	OD: (λ=620mm)
1	2.613
2	2.614
3	2.594
4	2.6813

Factors: Culture age, Incubation time: 24h Temperature: 37℃		
Incubation time	No.	OD: (λ=620mm)
12	1	1.698
	2	1.640
	3	1.644
	4	1.661
16	1	1.859
	2	2.108
	3	2.204
20	1	2.014
	2	2.042
	3	1.982
24	1	2.165

	2	2.024
	3	2.274
28	1	2.462
	2	2.652
	3	2.114
32	1	2.429
	2	2.440
	3	2.401
Factors: DOE experiment, Incubation time: 24h Temperature: 37℃		
Incubation time	No.	OD: (λ=620mm)
16	1	2.831
	2	2.556
	3	2.422
20	1	2.985
	2	2.632
	3	2.801
24	1	2.994
	2	2.850
	3	2.829

Table B. 2 HPLC values

Factor: Biomass Standard sample size 10 μl					
No.	Tubes Name	Dilute times	Sample size ((μl)	Glycerol conc. (ppm)	1,3-PD conc. (ppm)
1	B9-1	10	50	7787.5498	114.20056
2	B9-2	10	50	7687.6275	113.78001
3	B9-3	20	50	3886.8562	54.78991
4	B11-1	10	50	7282.8117	175.86517
5	B11-2	10	50	6312.3487	222.39385
6	B11-3	20	50	3194.0182	118.57587
7	B17-1	10	50	5961.2688	244.98744
8	B17-2	10	50	5954.9613	224.98744
9	B17-3	20	50	2948.1580	110.57411
10	B21-1	10	50	6224.9693	282.45577
11	B21-2	10	50	4309.6576	320.09406
12	B21-3	20	50	1847.1245	162.0496
13	B25-1	10	50	5646.6435	333.13066
14	B25-2	10	50	3989.7330	316.15214
15	B25-3	20	50	2012.6826	163.14044
16	B30-1	10	50	5517.3824	324.32536
17	B30-2	10	50	5600.7009	443.91366
18	B30-3	20	50	2797.5542	159.47111
Factor: pH Standard sample size 10 μl					
No.	Tubes Name	Dilute	Sample size	Glycerol conc.	1,3-PD

		times	((µl)	(ppm)	conc. (ppm)
1	pH6-1	10	50	5254.15858	510.39655
2	pH6-2	10	50	5957.78022	340.48703
3	pH6-3	20	50	2965.78114	210.58777
4	pH6.2-1	10	50	4861.22359	641.38697
5	pH6.2-2	10	50	4155.13929	568.39065
6	pH6.2-3	20	50	2080.17144	295.12566
7	pH6.8-1	10	50	5186.57123	255.14114
8	pH6.8-2	10	50	4447.792	343.48953
9	pH6.8-3	20	50	2490.47144	167.25571
10	pH7.2-1	10	50	5993.08971	312.4115
11	pH7.2-2	10	50	3992.14197	405.99677
12	pH7.2-3	20	50	2276.25014	200.54415
13	pH7.5-1	10	50	5913.08971	305.90555
14	pH7.5-2	10	50	5303.14742	298.00111
15	pH7.5-3	20	50	2776.22227	148.45221
16	pH8-1	10	50	5472.29833	325.79021
17	pH8-2	10	50	5820.26322	265.97012
18	pH8-3	20	50	2801.29333	144.1147

Factor: Glycerol concentration Standard sample size 10 µl					
No.	Tubes Name	Dilute times	Sample size ((µl)	Glycerol conc. (ppm)	1,3-PD conc. (ppm)
1	G150-1	10	50	2932.47321	321.54797
2	G150-2	10	50	2939.19981	350.97469
3	G150-3	20	50	1460.54714	151.87411
4	G200-1	10	50	3209.07882	357.78736
5	G200-2	10	50	3297.14777	475.63787
6	G200-3	20	50	1613.00028	209.65224
7	G300-1	10	50	6420.13749	383.65224
8	G300-2	10	50	3542.85056	282.66011
9	G300-3	20	50	2191.00011	150.65224
10	G350-1	10	50	6963.43159	260.83265
11	G350-2	10	50	4578.91218	310.58229
12	G350-3	20	50	2491.01487	191.14487
13	G400-1	10	50	8610.57766	363.02644
14	G400-2	10	50	8608.9766	371.05265
15	G400-3	20	50	4310.49552	108.07487
16	G450-1	10	50	11279.30544	359.60141
17	G450-2	10	50	11200.294	331.38771
18	G450-3	20	50	5678.47713	169.15957

Factor: Temperature Standard sample size 10 µl					
No.	Tubes Name	Dilute times	Sample size ((µl)	Glycerol conc. (ppm)	1,3-PD conc. (ppm)
1	Tem20-1	10	10	1154.64785	182.14224
2	Tem20-2	10	10	1195.54123	176.28773
3	Tem20-3	10	10	1160.54328	190.17786
4	Tem25-1	10	10	1123.29264	100.79334
5	Tem25-2	10	10	1130.35871	96.49731
6	Tem25-3	10	10	1133.51739	88.53111
7	Tem30-1	10	10	1358.0614	162.68125
8	Tem30-2	10	10	1306.18477	82.37521
9	Tem30-3	10	10	1103.70051	90.18566
10	Tem37-1	10	10	1390.87211	102.68711
11	Tem37-2	10	10	1339.93713	104.79251
12	Tem37-3	10	10	1364.21205	99.87711
13	Tem42-1	10	10	1456.39414	142.05441
14	Tem42-2	10	10	1521.24116	144.27791
15	Tem42-3	10	10	1355.2609	138.98777

Factor: Incubation time Standard sample size 10 µl					
No.	Tubes Name	Dilute times	Sample size ((µl)	Glycerol conc. (ppm)	1,3-PD conc. (ppm)
1	T0.5-1	10	10	1370.17114	69.30161
2	T0.5-2	10	10	1261.30117	76.54744
3	T0.5-3	10	10	1315.00114	72.97411
4	T1-1	10	10	1125.75863	74.09516
5	T1-2	10	10	1003.69812	93.30882
6	T1-3	10	10	1251.31877	82.4715
7	T1.5-1	10	10	972.16524	129.75027
8	T1.5-2	10	10	994.16014	137.13763
9	T1.5-3	10	10	893.68937	119.16659
10	T2-1	10	10	754.56157	156.58003
11	T2-2	10	10	769.92211	138.80701
12	T2-3	10	10	732.1734	161.26145
13	T3-1	10	10	1059.43485	185.09889
14	T3-2	10	10	1023.65862	205.13866
15	T3-3	100	50	512.50057	83.54853
16	T4-1	10	10	1050.01147	188.7524
17	T4-2	10	10	1253.07821	191.53866
18	T4-3	10	10	1223.64755	186.06145

Factor: Culture age Standard sample size 10 μl					
No.	Tubes Name	Dilute times	Sample size ((μl)	Glycerol conc. (ppm)	1,3-PD conc. (ppm)
1	A12-1	100	50	578.03227	8.90052
2	A12-2	10	10	1308.1700	49.77388
3	A12-3	10	10	1306.20782	48.43221
4	A16-1	10	10	1068.00271	42.60481
5	A16-2	10	10	1185.10752	35.74112
6	A16-3	10	10	1168.17000	32.17551
7	A20-1	10	10	1198.00141	83.08086
8	A20-2	10	10	1345.03744	74.44771
9	A20-3	50	50	291.00052	73.36769
10	A24-1	10	10	1041.23147	83.26437
11	A24-2	10	10	928.10067	89.07922
12	A24-3	10	10	1194.54527	82.15337
13	A28-1	10	10	1188.65241	92.21064
14	A28-2	10	10	1080.27874	80.10019
15	A28-3	10	10	1124.09144	82.20558
16	A32-1	10	10	1228.20137	71.12279
17	A32-2	10	10	1138.23755	88.22084
18	A32-3	10	10	1307.14987	68.1368

Table B. 3 One-way Anova data

One-way ANOVA: Consumed glycerol (mmol/L) versus Biomass (g/L)

Method					
Null hypothesis			All means are equal		
Alternative hypothesis			Not all means are equal		
Significance level			α = 0.05		
Equal variances were assumed for the analysis					
Analysis Variance					
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Biomass (g/L)	5	10266	2053.2	8.90	0.001
Error	12	2769	230.8		
Total	17	13035			
Model Summary					
S	R-sq		R-sq(adj)		R-sq(pred)
15.1910	78.76%		69.90%		52.20%
Means					

Biomass(g/L)	N	Mean	StDev	95% CI
9	3	131.694	1.176	(112.585, 150.804)
11	3	155.34	11.72	(136.23, 174.45)
17	3	171.050	0.778	(151.940, 190.159)
21	3	197.0	28.7	(177.9, 216.1)
25	3	201.1	20.6	(182.0, 220.2)
30	3	179.008	1.011	(159.899, 198.118)
Pooled StDev = 15.1910				

One-way ANOVA: Produced reuterin (mmol/L) versus Biomass (g/L)

Method					
Null hypothesis		All means are equal			
Alternative hypothesis		Not all means are equal			
Significance level		α = 0.05			
Equal variances were assumed for the analysis					
Analysis Variance					
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Biomass (g/L)	5	7266	1453.2	6.62	0.004
Error	12	2634	219.5		
Total	17	9900			
Model Summary					
S	R-sq		R-sq(adj)		R-sq(pred)
14.8146	73.40%		62.31%		40.14%
Means					
Biomass(g/L)	N	Mean	StDev	95% CI	
9	3	125.779	1.142	(107.143, 144.415)	
11	3	144.20	10.13	(125.57, 162.84)	
17	3	158.939	1.335	(140.303, 177.575)	
21	3	180.8	27.5	(162.1, 199.4)	
25	3	184.0	20.9	(165.4, 202.6)	
30	3	159.96	4.33	(141.32, 178.59)	
Pooled StDev = 15.1910					

One-way ANOVA: Produced 1,3-PD (mmol/L) versus Biomass (g/L)

Method					
Null hypothesis			All means are equal		
Alternative hypothesis			Not all means are equal		
Significance level			α = 0.05		
Equal variances were assumed for the analysis					
Analysis Variance					
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Biomass (g/L)	5	87.178	17.4357	22.31	0.000
Error	12	9.376	0.7813		
Total	17	96.555			
Model Summary					
S	R-sq		R-sq(adj)		R-sq(pred)
0.883939	90.29%		86.24%		78.15%
Means					
Biomass(g/L)	N	Mean	StDev	95% CI	
9	3	2.9576	0.0672	(1.8456, 4.0695)	
11	3	5.567	0.841	(4.455, 6.679)	
17	3	6.055	0.336	(4.943, 7.167)	
21	3	8.119	0.604	(7.007, 9.231)	
25	3	8.547	0.225	(7.436, 9.659)	
30	3	9.53	1.86	(8.41, 10.64)	
Pooled StDev = 0.883939					

One-way ANOVA: Consumed glycerol (mmol/L) versus Conversion time

Method					
Null hypothesis			All means are equal		
Alternative hypothesis			Not all means are equal		
Significance level			α = 0.05		
Equal variances were assumed for the analysis					
Analysis Variance					
Source	DF	Adj SS	Adj MS	F-Value	P-Value

Biomass (g/L)	5	6713.9	1342.79	20.16	0.000
Error	12	799.4	66.62		
Total	17	7513.3			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
8.16185	89.36%	84.93%	76.06%

Means

Biomass(g/L)	N	Mean	StDev	95% CI
0.5	3	157.15	5.91	(146.88, 167.42)
1.0	3	177.63	13.44	(167.36, 187.89)
1.5	3	196.48	5.74	(186.21, 206.74)
2.0	3	218.32	2.06	(208.05, 228.58)
3.0	3	187.50	2.20	(177.23, 197.77)
4.0	3	172.34	11.92	(162.08, 182.61)

Pooled StDev = 8.16185

One-way ANOVA: Produced reuterin (mmol/L) versus Conversion time

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis

Analysis Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Biomass (g/L)	5	6026.1	1205.21	17.43	0.000
Error	12	829.6	69.14		
Total	17	6855.7			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
8.31480	87.90%	82.86%	72.77%

Means

Biomass(g/L)	N	Mean	StDev	95% CI
0.5	3	137.98	4.96	(127.52, 148.44)
1.0	3	155.73	12.22	(145.28, 166.19)
1.5	3	162.65	8.07	(152.19, 173.11)

2.0	3	178.307	1.512	(167.848, 188.767)
3.0	3	138.67	5.33	(128.21, 149.13)
4.0	3	122.72	12.04	(112.26, 133.18)
Pooled StDev = 8.31480				

One-way ANOVA: Produced 1,3-PD (mmol/L) versus Conversion time

Method					
Null hypothesis		All means are equal			
Alternative hypothesis		Not all means are equal			
Significance level		α = 0.05			
Equal variances were assumed for the analysis					
Analysis Variance					
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Biomass (g/L)	5	639.00	127.800	63.65	0.000
Error	12	24.09	2.008		
Total	17	663.09			
Model Summary					
S	R-sq		R-sq(adj)		R-sq(pred)
1.41698	96.37%		94.85%		91.82%
Means					
Biomass(g/L)	N	Mean	StDev	95% CI	
0.5	3	9.586	0.476	(7.804, 11.369)	
1.0	3	10.946	1.266	(9.164, 12.729)	
1.5	3	16.912	1.187	(15.130, 18.695)	
2.0	3	20.005	1.557	(18.222, 21.787)	
3.0	3	24.42	2.50	(22.63, 26.20)	
4.0	3	24.811	0.360	(23.028, 26.593)	
Pooled StDev = 1.41698					

One-way ANOVA: Consumed glycerol (mmol/L) versus Culture age

Method	
Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05
Equal variances were assumed for the analysis	

Analysis Variance					
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Biomass (g/L)	5	2300	460.0	0.64	0.674
Error	12	8620	718.3		
Total	17	10920			

Model Summary			
S	R-sq	R-sq(adj)	R-sq(pred)
26.8011	21.06%	0.00%	0.00%

Means				
Biomass(g/L)	N	Mean	StDev	95% CI
12	3	163.52	9.48	(129.81, 197.24)
16	3	176.16	6.87	(142.45, 209.88)
20	3	197.4	62.0	(163.7, 231.1)
24	3	185.48	14.52	(151.76, 219.19)
28	3	177.18	5.92	(143.47, 210.90)
32	3	167.03	9.18	(133.31, 200.74)

Pooled StDev = 26.8011

One-way ANOVA: Produced reuterin (mmol/L) versus Culture age

Method	
Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis

Analysis Variance					
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Biomass (g/L)	5	1750	350.1	0.47	0.791
Error	12	8917	743.1		
Total	17	10668			

Model Summary			
S	R-sq	R-sq(adj)	R-sq(pred)
27.2603	16.41%	0.00%	0.00%

Means				
Biomass(g/L)	N	Mean	StDev	95% CI

12	3	152.80	14.49	(118.50, 187.09)
16	3	165.94	5.61	(131.65, 200.23)
20	3	176.1	62.7	(141.8, 210.4)
24	3	161.94	13.60	(127.65, 196.24)
28	3	153.65	7.67	(119.36, 187.94)
32	3	145.99	6.43	(111.70, 180.28)
Pooled StDev = 27.2603				

One-way ANOVA: Produced 1,3-PD (mmol/L) versus Culture age

Method					
Null hypothesis		All means are equal			
Alternative hypothesis		Not all means are equal			
Significance level		α = 0.05			
Equal variances were assumed for the analysis					
Analysis Variance					
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Biomass (g/L)	5	145.64	29.128	16.32	0.000
Error	12	21.42	1.785		
Total	17	167.06			
Model Summary					
S	R-sq		R-sq(adj)		R-sq(pred)
1.33609	87.18%		81.83%		71.15%
Means					
Biomass(g/L)	N	Mean	StDev	95% CI	
12	3	5.36	2.51	(3.68, 7.04)	
16	3	5.110	0.735	(3.430, 6.791)	
20	3	10.676	0.738	(8.996, 12.357)	
24	3	11.768	0.516	(10.087, 13.448)	
28	3	11.768	0.898	(10.088, 13.449)	
32	3	10.518	1.503	(8.838, 12.199)	
Pooled StDev = 1.33609					

One-way ANOVA: Consumed glycerol (mmol/L) versus Initial glycerol (mmol/L)

Method	
Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal

Significance level			α = 0.05		
Equal variances were assumed for the analysis					
Analysis Variance					
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Biomass (g/L)	5	47289	9457.8	31.44	0.000
Error	12	3610	300.8		
Total	17	50899			
Model Summary					
S	R-sq		R-sq(adj)		R-sq(pred)
17.3449	92.91%		89.95%		84.04%
Means					
Biomass(g/L)	N	Mean	StDev	95% CI	
150	3	86.347	0.199	(64.528, 108.165)	
200	3	129.546	1.015	(107.727, 151.364)	
300	3	196.2	32.1	(174.3, 218.0)	
350	3	230.4	27.7	(208.6, 252.2)	
400	3	212.933	0.142	(191.114, 234.751)	
450	3	205.047	1.701	(183.228, 226.866)	
Pooled StDev = 17.3449					

One-way ANOVA: Produced reuterin (mmol/L) versus Initial glycerol (mmol/L)

Method					
Null hypothesis			All means are equal		
Alternative hypothesis			Not all means are equal		
Significance level			α = 0.05		
Equal variances were assumed for the analysis					
Analysis Variance					
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Biomass (g/L)	5	48898	9779.6	30.47	0.000
Error	12	3851	320.9		
Total	17	52749			
Model Summary					
S	R-sq		R-sq(adj)		R-sq(pred)
17.9150	92.70%		89.66%		83.57%

Means				
Biomass(g/L)	N	Mean	StDev	95% CI
150	3	68.290	1.515	(45.754, 90.826)
200	3	106.38	4.23	(83.84, 128.91)
300	3	178.3	35.1	(155.7, 200.8)
350	3	212.7	25.4	(190.2, 235.3)
400	3	195.36	4.70	(172.82, 217.89)
450	3	186.01	2.06	(163.47, 208.55)
Pooled StDev = 17.9150				

One-way ANOVA: Produced 1,3-PD (mmol/L) versus Initial glycerol (mmol/L)

Method					
Null hypothesis		All means are equal			
Alternative hypothesis		Not all means are equal			
Significance level		α = 0.05			
Equal variances were assumed for the analysis					
Analysis Variance					
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Biomass (g/L)	5	17.49	3.497	1.48	0.269
Error	12	28.45	2.371		
Total	17	45.93			
Model Summary					
S	R-sq		R-sq(adj)		R-sq(pred)
1.53972	38.07%		12.26%		0.00%
Means					
Biomass(g/L)	N	Mean	StDev	95% CI	
150	3	9.028	0.662	(7.091, 10.965)	
200	3	11.585	1.635	(9.648, 13.522)	
300	3	8.948	1.491	(7.011, 10.885)	
350	3	8.820	1.694	(6.883, 10.756)	
400	3	8.79	2.42	(6.85, 10.72)	
450	3	9.519	0.408	(7.582, 11.456)	
Pooled StDev = 1.53972					

One-way ANOVA: Consumed glycerol (mmol/L) versus pH

Method					
Null hypothesis			All means are equal		
Alternative hypothesis			Not all means are equal		
Significance level			α = 0.05		
Equal variances were assumed for the analysis					
Analysis Variance					
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Biomass (g/L)	5	2101	420.1	3.22	0.045
Error	12	1566	130.5		
Total	17	3666			
Model Summary					
S	R-sq		R-sq(adj)		R-sq(pred)
11.4232	57.29%		39.50%		3.91%
Means					
Biomass(g/L)	N	Mean	StDev	95% CI	
6.0	3	175.89	8.66	(161.52, 190.26)	
6.2	3	204.61	8.82	(190.24, 218.98)	
6.8	3	194.20	8.28	(179.83, 208.57)	
7.2	3	194.8	22.4	(180.4, 209.1)	
7.5	3	178.61	6.66	(164.24, 192.98)	
8.0	3	177.69	3.82	(163.32, 192.06)	
Pooled StDev = 11.423					

One-way ANOVA: Produced reuterin (mmol/L) versus pH

Method					
Null hypothesis			All means are equal		
Alternative hypothesis			Not all means are equal		
Significance level			α = 0.05		
Equal variances were assumed for the analysis					
Analysis Variance					
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Biomass (g/L)	5	1764	352.7	2.41	0.098

Error	12	1753	146.1		
Total	17	3517			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
12.0879	50.15%	29.37%	0.00%

Means

Biomass(g/L)	N	Mean	StDev	95% CI
6.0	3	153.60	5.07	(138.40, 168.81)
6.2	3	173.07	10.72	(157.86, 188.27)
6.8	3	177.84	6.50	(162.64, 193.05)
7.2	3	182.2	25.3	(167.0, 197.4)
7.5	3	162.82	6.88	(147.62, 178.03)
8.0	3	162.27	2.34	(147.07, 177.48)

Pooled StDev = 12.0879

One-way ANOVA: Produced 1,3-PD (mmol/L) versus pH

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis

Analysis Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Biomass (g/L)	5	179.55	35.910	5.46	0.008
Error	12	78.94	6.578		
Total	17	258.49			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2.56479	69.46%	56.74%	31.29%

Means

Biomass(g/L)	N	Mean	StDev	95% CI
6.0	3	11.15	2.23	(7.92, 14.37)
6.2	3	15.771	0.985	(12.545, 18.997)
6.8	3	8.176	1.278	(4.949, 11.402)
7.2	3	6.29	5.59	(3.07, 9.52)
7.5	3	7.8925	0.1291	(4.6662, 11.1189)

8.0	3	7.710	0.795	(4.484, 10.936)
Pooled StDev = 2.56479				

One-way ANOVA: Consumed glycerol (mmol/L) versus Temp

Method					
Null hypothesis		All means are equal			
Alternative hypothesis		Not all means are equal			
Significance level		α = 0.05			
Equal variances were assumed for the analysis					
Analysis Variance					
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Biomass (g/L)	4	2454.2	613.55	9.92	0.002
Error	10	618.5	61.85		
Total	14	3072.7			
Model Summary					
S	R-sq		R-sq(adj)		R-sq(pred)
7.86465	79.87%		71.82%		54.71%
Means					
Biomass(g/L)	N	Mean	StDev	95% CI	
20	3	172.92	2.40	(162.81, 183.04)	
25	3	177.396	0.569	(167.279, 187.514)	
30	3	163.61	14.60	(153.50, 173.73)	
37	3	151.77	2.77	(141.66, 161.89)	
42	3	143.16	9.08	(133.05, 153.28)	
20	3	172.92	2.40	(162.81, 183.04)	
Pooled StDev = 7.86465					

One-way ANOVA: Produced reuterin (mmol/L) versus Temp

Method	
Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05
Equal variances were assumed for the analysis	

Analysis Variance					
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Biomass (g/L)	4	3418	854.5	6.53	0.008
Error	10	1310	131.0		
Total	14	4728			

Model Summary			
S	R-sq	R-sq(adj)	R-sq(pred)
11.4436	72.30%	61.22%	37.67%

Means				
Biomass(g/L)	N	Mean	StDev	95% CI
20	3	124.857	1.634	(110.136, 139.578)
25	3	152.354	1.130	(137.633, 167.075)
30	3	134.2	23.4	(119.5, 149.0)
37	3	124.85	2.58	(110.12, 139.57)
42	3	105.90	9.78	(91.18, 120.62)
20	3	124.857	1.634	(110.136, 139.578)

Pooled StDev = 11.4436

One-way ANOVA: Produced 1,3-PD (mmol/L) versus Temp

Method	
Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis

Analysis Variance					
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Biomass (g/L)	4	268.47	67.118	9.43	0.002
Error	10	71.21	7.121		
Total	14	339.68			

Model Summary			
S	R-sq	R-sq(adj)	R-sq(pred)
2.66853	79.04%	70.65%	52.83%

Means				
Biomass(g/L)	N	Mean	StDev	95% CI

20	3	24.033	0.916	(20.600, 27.466)
25	3	12.521	0.818	(9.088, 15.954)
30	3	14.69	5.82	(11.25, 18.12)
37	3	13.465	0.324	(10.032, 16.897)
42	3	18.632	0.349	(15.199, 22.065)
20	3	24.033	0.916	(20.600, 27.466)
Pooled StDev = 2.66853				

Table B. 4 Statistics values

Covariance analysis

Correlation Matrix

		Biomass	pH	Glycerol	Temp	Incubation Time
Correlation	Biomass	1.000	.096	.009	-.069	.079
	pH	.096	1.000	-.011	.083	-.096
	Glycerol	.009	-.011	1.000	.008	-.009
	Temp	-.069	.083	.008	1.000	.069
	Incubation Time	.079	-.096	-.009	.069	1.000
	Culture Age	-.034	.041	.004	-.029	.034
	Produced Reuterin	.113	.212	.612	.103	-.131

Correlation Matrix

		Culture Age	Produced Reuterin
Correlation	Biomass	-.034	.113
	pH	.041	.212
	Glycerol	.004	.612
	Temp	-.029	.103
	Incubation Time	.034	-.131
	Culture Age	1.000	-.072
	Produced Reuterin	-.072	1.000

Communalities

	Raw		Rescaled	
	Initial	Extraction	Initial	Extraction
Biomass	15.490	.303	1.000	.020
pH	.169	.013	1.000	.077
Glycerol	2022.772	2022.770	1.000	1.000
Temp	14.354	.232	1.000	.016

Incubation Time		.403	.010	1.000	.025
Culture Age		8.992	.084	1.000	.009
Produced Reuterin		1033.731	1033.718	1.000	1.000
Extraction Method: Principal Component Analysis.					

Total Variance Explained

Component		Initial Eigenvalues[a]			Extraction Sums of Squared Loadings	
		Total	% of Variance	Cumulative %	Total	% of Variance
Raw	1	2541.702	82.099	82.099	2541.702	82.099
	2	515.428	16.649	98.747	515.428	16.649
	3	16.050	.518	99.266		
	4	13.302	.430	99.695		
	5	8.890	.287	99.983		
	6	.386	.012	99.995		
	7	.153	.005	100.000		
Rescaled	1	2541.702	82.099	82.099	1.574	22.489
	2	515.428	16.649	98.747	.572	8.176
	3	16.050	.518	99.266		
	4	13.302	.430	99.695		
	5	8.890	.287	99.983		
	6	.386	.012	99.995		
	7	.153	.005	100.000		

Component Matrix[a]

	Raw		Rescaled	
	Component		Component	
	1	2	1	2
Biomass	.173	.523	.044	.133
pH	.025	.111	.060	.271
Glycerol	43.484	-11.484	.967	-.255
Temp	.150	.457	.040	.121
Incubation Time	-.031	-.095	-.049	-.150
Culture Age	-.061	-.283	-.020	-.094
Produced Reuterin	25.510	19.569	.793	.609

Extraction Method: Principal Component Analysis.[a]
a. 2 components extracted.