BIOCONVERSION OF BIODIESEL BY-PRODUCTS TO VALUE-ADDED CHEMICALS

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<td>µm</td>
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<tr>
<td>AAC</td>
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<td>alcohol oxidase</td>
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<td>ATP</td>
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<tr>
<td>DCW</td>
<td>dry cell weight</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNS</td>
<td>3,5-dinitrosalicylic acid</td>
</tr>
<tr>
<td>DO</td>
<td>dissolved oxygen</td>
</tr>
<tr>
<td>DSMZ</td>
<td>German Collection of Microorganisms and Cell Cultures</td>
</tr>
<tr>
<td>EB</td>
<td>hydrolysis of rapeseed meal using enzymatic broth</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>FAN</td>
<td>free amino nitrogen</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acids</td>
</tr>
<tr>
<td>g</td>
<td>gram(s)</td>
</tr>
<tr>
<td>G3P</td>
<td>glycerol-3-phosphate</td>
</tr>
<tr>
<td>GK</td>
<td>glycerol kinase</td>
</tr>
<tr>
<td>GM</td>
<td>genetically modified</td>
</tr>
<tr>
<td>GPO</td>
<td>glycerol-3-phosphate oxidase</td>
</tr>
<tr>
<td>GTBE</td>
<td>glycerol tertiary butyl ether</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
</tr>
<tr>
<td>HL</td>
<td>solid-state fermentation followed by fungal autolysis or hydrolysate</td>
</tr>
<tr>
<td>HL+CG</td>
<td>hydrolysate supplemented with crude glycerol</td>
</tr>
<tr>
<td>HL+PG</td>
<td>hydrolysate supplemented with pure glycerol</td>
</tr>
<tr>
<td>HL+SCG</td>
<td>hydrolysate supplemented with synthetic crude glycerol</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram(s)</td>
</tr>
<tr>
<td>L</td>
<td>litre(s)</td>
</tr>
<tr>
<td>LTP</td>
<td>lipid transfer proteins</td>
</tr>
<tr>
<td>mcl-PHAs</td>
<td>medium-chain-length PHAs</td>
</tr>
<tr>
<td>mg</td>
<td>milligram(s)</td>
</tr>
</tbody>
</table>
List of abbreviations and acronyms

min minute(s)
mL millilitre(s)
mm millimetre(s)
MM mineral medium
MM+SCG mineral medium supplemented with synthetic crude glycerol
MW molecular weight
nm nanometre(s)
OD optical density
OD$_{600}$ optical density at 600 nanometres
PBS polybutylene succinate
PCL polycaprolactone
PET polyethylene terephthalate
PG pure glycerol
PHAs polyhydroxyalkanoates
PHB polyhydroxybutyrate
PHH polyhydroxyhexanoate
PHO polyhydroxyoctanoate
PHV polyhydroxyvalerate
PLA polylactic acid
rpm round per minute
SCG synthetic crude glycerol
SCGPE Satake Centre for Grain Process Engineering
scl-PHAs short-chain-length PHAs
sec second(s)
SF submerged fungal fermentation
SFA submerged fungal fermentation followed by fungal autolysis
SSC solid substrate cultivation
SSF solid-state fermentation
TEM transmission electron microscope
T$_g$ glass transition temperature
TKN total Kjeldahl nitrogen
TN total nitrogen
T$_m$ melting temperature
U unit of enzyme activity
USD United States dollar(s)
v/v volume by volume
w/v weight by volume
YE yeast extract
YE+SCG yeast extract solution supplemented with synthetic crude glycerol
Bioconversion of biodiesel by-products to value-added chemicals

Abstract

To mitigate the problems of depleting and soaring price of fossil fuels, the production and use of renewable energy have been vigorously promoted. In Europe, the role of biologically-derived fuels and in particular biodiesel is gradually increasing in prominent. Rapeseed biodiesel is the most widely produced in Europe. As a consequence, enormous amount of by-products from production processes are being generated. Current strategies for managing these by-products (mainly rapeseed meal and crude glycerol) seem not to be economically sustainable. More efficient utilisation could add more value to the production chain which in turn would raise the competitiveness of biodiesel compared to petro-diesel. The aim of the project reported in this thesis was to study the feasibility of producing a value added product, polyhydroxybutyrate (PHB), from by-products generated from rapeseed biodiesel production processes as well as to investigate the effects of methanol, a major impurity in crude glycerol, on growth of Cupriavidus necator, a PHB-producing micro-organism.

The preliminary study of C. necator growth in crude glycerol based media revealed that optimum concentration of crude glycerol was in a range 15-25 g/L. It was also found that slight changes in the carbon to nitrogen ratio of the feedstock did not significantly affect the growth while methanol at concentrations beyond 10 g/L did. A model based on a saturation equation was developed and used to successfully predict the inhibition of growth by methanol. From the developed model, mechanisms of the inhibition were proposed. The model could also be used to predict satisfactorily growth or productivity rates in other systems containing short-chain alcohols. The growth in solutions derived from rapeseed meal (designated as hydrolysate) via solid-state fermentation by Aspergillus oryzae followed by hydrolysis of the fermented solids was also studied. The biomass production was found to increase as a function of initial free amino nitrogen (FAN) concentration presented in the hydrolysate. However, at higher initial FAN concentrations, a lower conversion of nitrogen to biomass was observed.

PHB production was studied using a feedstock which was a mixture of the hydrolysate and crude glycerol. Total biomass concentration reached 28.8 g/L at 120 h with 86% PHB content. PHB productivity and PHB yield on glycerol were 0.21 g/L-h and 0.32 g/g respectively. These results were comparable with those obtained when pure glycerol and synthetic crude glycerol were used, suggesting that, technically, the use of the generic rapeseed- and crude glycerol-based feedstock to produce PHB is feasible.

Overall, the feasibility of producing PHB from rapeseed biodiesel by-products has been demonstrated. The satisfactory result leads to the more important outlook that the generic feedstock derived from rapeseed biodiesel by-products has the potential to be used to produce a wide range of products depending on the micro-organism used. Further development of this process to improve nutrient production efficiency as well as product yields and subsequent integration of the process into the biodiesel production process could well be an important contribution in the development of a sustainable biodiesel industry.
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I would like to show my gratitude to these following people who helped me through my time in Manchester. I would like to profoundly thank Professor Colin Webb for accepting me as his student and being an excellent supervisor since then. Under his supervision, not only have I received excellent guidance, but also have I learned a valuable thing, the way of thinking. These should help me a lot in my careers. I also very much appreciate the time he spent with me during my thesis proofreading. I am very grateful to Dr. Ruohang Wang who, although was very ill at that time, gave me excellent guidance and was like my second supervisor and a mentor during my first year of study.

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Lastly, I would like to thank the Royal Thai Government for the financial support.

Apilak Salakkam
CHAPTER 1

Introduction and scope of the thesis

1.1 Introduction

The compression-ignition engine was invented by Rudolph Diesel in 1893 and subsequently took his name. Interestingly, it was powered by vegetable oil (peanut) and later (around 1937) by what is now known as biodiesel (Meher et al. 2006). Of course, this material was quickly replaced by petro-diesel and it is only recently that the world has become once again interested in biodiesel. The term “biodiesel” was in fact only used for the first time in 1988 in a Chinese paper abstracted in Chemical Abstracts. It next appeared in a publication in 1991 (Knothe 2005) and has since entered into common use.

Biodiesel is the term used to refer to the mono alkyl esters (methyl or ethyl esters) of long chain fatty acids derived from renewable (biological) oils (Al-Zuhair 2007). Biodiesel possesses several advantages over conventional petroleum-derived diesel. For instance, it is biodegradable and has a more favourable combustion emission profile in terms of low carbon emission. It is also less volatile, due to its relatively high flash point (around 160°C), which makes it safer to transport and handle than petro-diesel (Al-Zuhair 2007). In the past, the use of biodiesel was overlooked due to the availability and cheapness of crude oil (Sharma and Singh 2009). However, in recent times, the high energy demand in the industrialised world as well as in the domestic sector, coupled with the rapid depletion of petroleum reserves and environmental concerns make it increasingly necessary to develop renewable and environmentally friendly energy (Fukuda et al. 2001; Meher et al. 2006). Since biodiesel is derived from renewable resources, it is more environmentally friendly than petroleum-based diesel fuel (Ramadhas 2009) and as it can completely displace its petroleum counterpart, it is one of the best choices to reduce the dependence on petro-diesel (Johnson and Taconi 2007).
In the past decade, the biodiesel industry has grown rapidly and is likely to grow much further in the near future. Figure 1.1 shows the growth of biodiesel production from 2002 to 2011. From the figure, it can be seen that the production increases continually, with Germany as the biggest producer. In 2010, The European Union’s total biodiesel production reached 9.6 million metric tons and, based on production capacity, the figure was forecast to rise to 22 million metric tons by 2011 (Emerging Markets Online 2008; European Biodiesel Board 2012). From Figure 1.1, it is clear that the EU plays an important role in this industry as it is the major biodiesel producer in the world. Despite high production capacity, a reduction in EU biodiesel production in 2011 was reported. This was due to increased imports from third countries such as Argentina, Indonesia as well as circumvention measures from North America (European Biodiesel Board 2011). This affected overall world biodiesel production as seen in the figure.

![Figure 1.1: European and world biodiesel production statistics (based on European Biodiesel Board data and BP statistical review of world energy (2011; 2012) - Data for 2011 might be incomplete).](image)

Commercial biodiesel is mostly produced by transesterification of triglycerides to their corresponding esters, with crude glycerol being produced as a co-product (Figure 1.2) (Fukuda et al. 2001; Hanna and Isom 2009). The triglycerides used can be obtained from a variety of feedstocks including animal fats, vegetable, algae and used cooking oils (Demirbas and Fatih Demirbas; Wang et al. 2007; Hanna and Isom 2009). According to the Austrian Biofuel Institute, world biodiesel production is based 84% on
Chapter 1  Introduction and scope of the thesis

rapeseed oil (Bondioli 2005). In Europe, about 80% of biodiesel is processed from rapeseed, and biodiesel consumes about half of the EU’s rapeseed production. According to a report by the United State Department of Agriculture released in 2012, during the marketing year 2011-2012, 19 million metric tons of rapeseed was produced by EU-27 (Foreign Agricultural Service 2006; Foreign Agricultural Service 2012). Given that rapeseed contains about 40% oil (Appelqvist and Ohlson 1972), a rough calculation revealed that 7.6 million tons of rapeseed oil was produced in 2011, of which 3.8 million tons was used for biodiesel production, leaving 9.5 million tons of rapeseed meal as by-product.

As biodiesel production increases, so does production of the by-product or waste. As seen in Figure 1.3, the by-products generated from rapeseed biodiesel production are rapeseed meal and crude glycerol. Rapeseed meal is a residue obtained after oil extraction, and accounts for about 60% of total rapeseed used. Crude glycerol is a co-product from the transesterification reaction, and is often regarded as a waste stream (Johnson and Taconi 2007). The equivalent of about 10% of raw oil ends up as glycerol (Thompson and He 2006; Pagliaro et al. 2007; Willke and Vorlop 2008).
One of the obstacles of using biodiesel is its high price compared to petro-diesel. In order for biodiesel to be able to compete with petro-diesel, apart from fuel tax assistance or government regulations, an effective usage or conversion of by-products generated from the production process could help cut down the production cost (Agarwal and M. Das 2001; Pachauri and He 2006). To date, the poor utilisation of these by-products makes the biodiesel industry uneconomic. Although rich in protein, rapeseed meal is generally used as low-cost animal feed or organic fertiliser (Iriarte et al. 2012; Uçkun Kiran et al. 2012). The presence of anti-nutritional constituents such as phytic acid (Koutinas et al. 2007a), precursor of toxic compounds such as glucosinolates (Tranchino et al. 1983) and high fibre content (Pal Vig and Walia 2001) limit its use in the food industry (Mahajan and Dua 1994). Likewise, the impurities, e.g. methanol and salts (Pachauri and He 2006), limit the use of crude glycerol in many food and drug industries. It is therefore used as an energy source in, for example, swine feed or as a feed supplement (Thompson and He 2006; Hansen et al. 2009).

The aim of the project reported in this thesis was to investigate the use of both rapeseed meal and crude glycerol as raw materials for the fermentative production of value-added chemicals. Polyhydroxybutyrate (PHB) was chosen as a suitable representative for two main reasons. The production cost of PHB is currently much higher than conventional plastic, making it less popular than its counterpart, and a cheaper alternative feedstock would therefore be beneficial. Environmental concerns for the non-degradability of conventional plastic also favour the production of biodegradable plastics such as PHB.
Chapter 1  Introduction and scope of the thesis

The successful development of PHB production from biodiesel by-products could therefore help reduce its price as well as mitigate environmental problems and, at the same time, sustain the biodiesel industry. As significant side aim of this study, the effect of the methanol impurity in biodiesel-derived glycerol on the growth of PHB-producing bacterium, *Cupriavidus necator*, was also studied.

1.2 Scope and structure of the thesis

The fermentative production of PHB from biodiesel by-products was the main area of study in this project, along with a study of the kinetics of methanol inhibition on *C. necator*. In order to conduct experimental work effectively, several topics related to the area of interest were reviewed and are presented in Chapter 2. These cover information about production of biodiesel from rapeseed, solid-state fermentation, PHB, *C. necator* and the kinetics of microbial growth. The objectives and the experimental programme for the project are given in Chapter 3.

The experimental research conducted in the project is detailed in Chapters 4 through 8. The growth of *C. necator* on crude glycerol and improvement of the growth was studied and the results are presented in Chapter 4. Since crude glycerol contains methanol, a substance known for its toxicity to micro-organisms, the inhibitory effects of methanol on growth of *C. necator* was studied and is discussed in Chapter 5. In Chapter 6, the production of nutrient-rich solutions by means of solid-state fermentation followed by hydrolysis is described. These were used to cultivate *C. necator* with the supplement of a carbon source. The growth of the bacterium on the nutrient-rich solution in comparison with a typical nitrogen source is presented in Chapter 7. The productions of PHB in various media in fed-batch fermentation were also conducted and the results are presented in Chapter 8.

Finally, Chapter 9 provides an overall discussion and conclusions, as well as highlighting the findings. Suggestions for further work are also included in this chapter.
2.1 Introduction

In this chapter, several aspects related to the project reported in this thesis are reviewed. These are to give basic understandings of the work to be carried out during the research and begin with the production of biodiesel from rapeseed which is extensively carried out in Europe. Considering the increase in demand and production of biodiesel (see Figure 1.1), by-products from the production processes will be enormous. This gives rise to the project as efficient strategies for waste management are needed in order to add value to the production chain which would in turn contribute to the competitiveness of biodiesel compared to petro-diesel (da Silva et al. 2009). In Section 2.2, the rapeseed biodiesel production process including the current use of by-products generated from the processes (rapeseed meal and crude glycerol) are reviewed. Section 2.3 gives a review of solid-state fermentation (SSF), a technology that has long been applied to heterogeneous substrates like rapeseed meal to produce many products. Basic knowledge and general aspects about SSF as well as applications of SSF are given in this section.

Since polyhydroxybutyrate (PHB) was a product of interest in this project, knowing information about PHB as well as its producers would be beneficial. Section 2.4 presents information about Cupriavidus necator, a PHB-producing bacterium to be used in this project. Included in this section are its morphology, taxonomy and growth conditions as well as a pathway the bacterium uses to synthesise PHB. The general information about PHB including its properties and the applications of PHB are presented in Section 2.5. Brief reviews about commercialisation of PHB and PHB synthesis in various systems are also included in this section.

Although in C. necator PHB accumulation is generally considered to be non-growth associated, the concentration of PHB produced is strongly dependent upon biomass
concentration. Study of the bacterial growth is therefore important in order to produce as much biomass as possible. For this reason, kinetic equations for microbial growth are reviewed in Section 2.6. In this section, the review focuses on inhibition of the growth since this information would be mainly used in the study of the inhibitory effects of methanol on the growth of *C. necator*. Information presented in this chapter was subsequently used as a fundamental knowledge base for the experiments reported in this thesis.

### 2.2 Biodiesel production from rapeseed

In general, commercial rapeseed biodiesel is produced via alkaline catalytic transesterification. Besides rapeseed oil, the reaction requires alcohol and the catalyst. The alcohol that is mostly used is methanol. This is largely because methanol reacts quickly with triglycerides and alkali catalyst is easily dissolved in it as well as it is available at cheap price. However, in some areas where ethanol is less expensive, for example Brazil, fatty acid ethyl esters are produced (Ma and Hanna 1999; Gerpen and Knothe 2005). Several alternative alkalis can be used, such as sodium or potassium methoxide (NaOCH$_3$, KOCH$_3$) and sodium or potassium hydroxide (NaOH, KOH). Of these, sodium hydroxide is the most extensively used. This is also because NaOH is cheaper than the other catalysts (Ma and Hanna 1999).

#### 2.2.1 Production process

The viability of commercial alkaline-catalysed systems depends upon the quality of raw materials and the key parameters are water and free fatty acid (FFA) contents. Water and FFA impurity can cause saponification under alkaline conditions, thereby consuming the catalyst. In addition, the resulting soaps can cause the formation of emulsions, which creates difficulties in recovery and purification of the biodiesel. For this reason, it is necessary to refine and dehydrate the oil before use. Both methanol and NaOH should also be anhydrous (Al-Zuhair 2007).

Figure 2.1 presents the production scheme for rapeseed biodiesel production. As seen in the figure, the process can be divided into two major parts. The first part is oil extraction and oil refining, and the second one is transesterification of oil to produce
biodiesel. For oil extraction, rapeseed is normally processed in two steps: seed crushing and solvent extraction. Rapeseed is usually mechanically pressed in expellers after a preheating step under indirectly heated conditions, giving crude oil. The expeller cake (or pressed cake) is then treated with solvent (commonly hexane) in the extractor, leaving rapeseed meal as waste stream and another portion of crude oil to be refined. Oil refining can be carried out by sequential steps of degumming, bleaching and deodorisation. Oil refining can be carried out by either chemical or physical approach (FEDIOL 2012c; FEDIOL 2012a).

![Diagram](image)

**Figure 2.1** Production scheme for rapeseed biodiesel production by alkaline-catalysed transesterification reaction (adapted from Gerpen (2005a)).

Refined oil from the oil extraction process is transferred into a continuous stirred-tank reactor (CSTR) to mix with methanol and NaOH. The recommended conditions for alkaline-catalysed transesterification are 60°C for 1 h with 6:1 alcohol to oil molar ratio and continuous agitation (Gerpen and Knothe 2005). Note that although the stoichiometry of transesterification (Figure 2.2) requires three moles of alcohol per mole of triglycerides (Fukuda *et al.* 2001), the ratio of 6:1 is used to ensure the completion of the reaction (Meher *et al.* 2006). Transesterification of oil into biodiesel occurs in a stepwise manner with glycerol being produced in the last step as seen in Figure 2.3.
To enhance the completion of the reaction, the transesterification can be carried out in two steps. In the first step, about 80% of the alcohol and catalyst are added into the first reactor. The reacted mixture is then transferred to a separator to remove glycerol before entering the second reactor in which it is mixed with the remaining 20% of the alcohol and catalyst (Gerpen 2005a).

After the reaction, glycerol is removed from the methyl esters (biodiesel) in a separator, by settling or centrifugation. Due to the low solubility of glycerol in the esters, this separation occurs quickly. Water can be added to improve the separation (Gerpen and Knothe 2005). The removal of glycerol is necessary since fuel with excessive glycerol can cause problems with fuel filters and combustion (Gerpen 2005b).

After the glycerol separation step, the biodiesel stream is transferred to a methanol recovery unit then neutralised with acid and washed with water. During neutralisation, soaps produced alongside the transesterification reaction will react with the acid to form fatty acids and water-soluble salts (see Figure 2.4), which are removed with the wash water. The biodiesel stream is then dried in a vacuum flash process. The physical and chemical properties of rapeseed biodiesel are shown in Table 2.1 (Fukuda et al. 2001; Atadashi et al. 2011; Shahid and Jamal 2011).
The glycerol stream discharging from the separator contains about 50% glycerol with residual methanol, catalyst, salt and soaps. In the acidulation and separation unit, this ‘crude’ glycerol is mixed with acid (see Figure 2.1), yielding fatty acids and water-soluble salts. The fatty acids are removed while the salts are left in the crude glycerol. After methanol recovery, the crude glycerol will contain about 80-85% glycerol with traces of methanol, fatty acids and salts (Gerpen and Knothe 2005).

The definitions of some parameters in Table 2.1 are as follows: cetane number is a measure of the autoignition quality characteristics of a fuel. The higher the cetane number, the more easily will the fuel combust in a diesel engine. Cloud point is the temperature at which biowax in biodiesels solidifies, forming a cloudy appearance. Flash point is the lowest temperature at which biodiesels can vaporise to form an ignitable mixture in air. Kinematic viscosity is a measure of resistance to flow of a liquid due to internal friction of one part of the fluid moving over another. Lower heating value is the net heat of combustion for a fuel assuming that all products of combustion are in a gaseous state.
2.2.2 Applications of by-products generated from the production processes

By-products generated from rapeseed biodiesel production processes are mainly rapeseed meal and crude glycerol (see Figure 2.1). In this section, short reviews of the current use of these by-products are given.

**Crude glycerol**

With the increasing amount of crude glycerol discharged from the biodiesel industry, the use of this low-cost, impure glycerol has attracted much attention in the past decade. The compositions of crude glycerol vary depending upon the process of biodiesel production such as separation, acidulation and methanol recovery (see Figure 2.1). Table 2.2 presents ranges of proximate compositions of crude glycerol.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Range reported in the literature</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ash, %</td>
<td>0-7</td>
<td>Kerr et al. (2009)</td>
</tr>
<tr>
<td>Glycerol, %</td>
<td>47-90</td>
<td>Mothes et al. (2007), Ooi et al. (2001)</td>
</tr>
<tr>
<td>Methanol, %</td>
<td>12-28</td>
<td>Hansen et al. (2009), Pyle et al. (2008)</td>
</tr>
<tr>
<td>Moisture, %</td>
<td>0.3-24</td>
<td>Kerr et al. (2009)</td>
</tr>
<tr>
<td>pH</td>
<td>4-9</td>
<td>Kerr et al. (2009)</td>
</tr>
<tr>
<td>Salt, %</td>
<td>0-7</td>
<td>Cavalheiro et al. (2009), Kerr et al. (2009), Mothes et al. (2007)</td>
</tr>
<tr>
<td>Soap, %</td>
<td>15-25</td>
<td>Pyle et al. (2008)</td>
</tr>
</tbody>
</table>

Crude glycerol can be used as an energy source in cattle (Mach et al. 2009; Parsons et al. 2009), poultry (Lammers et al. 2008a) and swine feeds (Lammers et al. 2008b; Lammers et al. 2008c). To add more value to this waste, conversion of crude glycerol to value-added products by means of chemical and biological routes has been extensively studied. For chemical conversion, selective oxidation of the hydroxyl groups in crude glycerol yields several chemicals including dihydroxyacetone (an active ingredient in sunless tanning products) and ketomalonic acid (a building block for household detergents). Etherification can yield glycerol tertiary butyl ether (GTBE) which can be used as an additive in transport fuels (including biodiesel). Hydrogenolysis (dehydration
followed by hydrogenation) of glycerol results in propylene glycerol (1,2-propanediol) which is used as an antifreeze product (Pagliaro et al. 2007). Examples of commercially potential products derived from glycerol via chemical conversion are shown in Figure 2.5.

Figure 2.5  Commercially potential products derived from chemical conversion of glycerol (adapted from Chiu et al. (2005) Pachauri and He (2006) Johnson and Tasconi (2007) and Pagliaro et al. (2006)).

Through the use of micro-organisms, crude glycerol can be converted into many valuable chemicals. For instance, 1,3-propanediol by *Lactobacillus brevis*, dihydroxyacetone by *Gluconobacter oxydans*, succinic acid by *Anaerobiospirillum succiniciproducens*, propionic acid by *Propionibacterium acidipropionici*, citric acid by *Yarrowia lipolytica*, polyhydroxybutyrate by *Cupriavidus necator* and pigments by *Serratia marcescens* (da Silva et al. 2009). Figure 2.6 presents an overview of some possible products obtained from biotransformation of glycerol. Useful information about conversion of crude glycerol into value-added chemicals can be found in the literature by Amaral et al. (2009), da Silva et al. (2009), Johnson and Taconi (2007), Pachauri and He (2006) and Pagliaro et al. (2007).
Rapeseed meal

Rapeseed meal, which is the residue remaining after oil extraction, accounts for approximately 60% by weight of the whole rape seed. Its empirical formula (on an ash-free basis) is $C_{15.18}H_{27.96}O_{8.29}N$ (Çulcuoğlu et al. 2002). The proximate compositions of rapeseed meal are presented in Table 2.3.
Although rich in protein, rapeseed meal cannot be used directly in the food industry (Çalışır et al. 2005). This is because rapeseed meal contains anti-nutritional constituents which, if taken in large quantities, have the potential to adversely affect health and growth by interfering with the absorption of nutrients or minerals (Mikić et al. 2009). These compounds include phytic acid (Koutinas et al. 2007a), precursors of toxic compounds such as glucosinolates (breakdown of glucosinolates yields goitrogenic products) (Fenwick 1982; Tranchino et al. 1983) and fibre content (Pal Vig and Walia 2001). It is therefore used in combination with other materials as a feed or feed supplement for cattle, swine and poultry (Rutkowski 1971).

According to a report by FEDIOL (2012b) oilseeds are mainly used as a source of protein and are the second most important feed ingredient, after cereal, in Europe. Amongst the oilseeds, rapeseed meal is the second most important, accounting for 15% of total protein material, after soybean meal as seen in Figure 2.7.

![Use of protein materials by the EU animal feed sector.](image-url)

**Table 2.3** Proximate compositions of rapeseed meal (oil free, dry basis) (Appelqvist and Ohlson 1972; Niewiadomski 1990).

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein, %</td>
<td>41.67</td>
</tr>
<tr>
<td>Crude fibre, %</td>
<td>14.97</td>
</tr>
<tr>
<td>Ash, %</td>
<td>7.83</td>
</tr>
<tr>
<td>Glucosinolates, %</td>
<td>0.3-0.8</td>
</tr>
</tbody>
</table>
With a well-balanced amino acid profile and its high protein quality, rapeseed meal should have the potential to be used as food or food additive. However, the anti-nutritional constituents in the meal must be removed before it can be used in the food industry (Ghodsvali et al. 2005). In France, a pilot-scale process for protein extraction (albumins and globulins) has been developed to remove major anti-nutritional compounds from rapeseed meal (Chabanon et al. 2007). Another team of workers have purified cruciferin (globulin), napin (albumin) and lipid transfer proteins (LTP) by a series of purification processes consisting of membrane filtration and chromatography. It was reported that starting with 3.5 kg of rapeseed meal, 200 g of cruciferin, 42 g of napin and 5 g of LTP could be extracted (Bérot et al. 2005).

Solid-state fermentation (SSF) by fungi has also been exploited to reduce the anti-nutritional components in rapeseed meal. The use of several fungi has been reported to effectively reduce phenolic (Lacki and Duvnjak 1999), phytic acid (El-Batal and Abdel Kareem 2001) as well as glucosinolates and fibre content (Pal Vig and Walia 2001). Moreover, rapeseed meal has been used as a source of nutrients for various enzyme productions for instance laccase (Hu and Duvnjak 2004) and phytase (El-Batal and Abdel Kareem 2001) via SSF processes. Other applications of rapeseed meal and other oil cakes can be found in a publication by Ramachandran et al. (2007).

In the Satake Centre for Grain Process Engineering (SCGPE), rapeseed meal has been used as a substrate for the production of a generic feedstock by SSF followed by hydrolysis of fermented solids. With a glucose supplement, it was proved feasible to use that feedstock to grow Saccharomyces cerevisiae (Wang et al. 2010). This success indicates that exploiting suitable micro-organisms to produce a generic feedstock and supplementing it with required nutrients to grow a microbe is feasible. It is worthwhile therefore to consider SSF in more detail.

### 2.3 Solid-state fermentation

#### 2.3.1 Overview of solid-state fermentation

Solid-state fermentation (SSF) (also known by the term solid substrate cultivation (SSC) and several other terms) is generally defined as the fermentation of non-soluble
materials in the absence or near absence of free water. Despite no free water, the substrate must possess enough moisture to support growth and metabolism of microorganisms (water activity, $a_w$, of around 0.4 to 0.9) (Cannel and Moo-Young 1980; Pandey 2003; Robinson and Nigam 2003). The moisture in the substrate is in an absorbed or complex form within the solid-matrix, which acts both as supporting material and as a source of nutrients for cell growth (Pandey 1992; Prabhakar et al. 2005; Couto and Sanromán 2006). Due to the lack of free water in SSF, the space between solid particles is normally occupied by a continuous gas phase (Cannel and Moo-Young 1980).

SSF has a long history and has been exploited since antiquity. In ancient times, it was used for bread making by ancient Egyptians in 2,600 B.C. (Pandey 1992) and for production of fermented food in Asia. SSF has been applied for commercial purposes in more recent times and was developed for the pharmaceutical industry in around 1940. SSF is successfully exploited for food, enzymes, fuels, antibiotics, animal feed and also for dye degradation (Prabhakar et al. 2005). A brief history and development of SSF is given in Table 2.4 (Pandey 1992; Pandey 2003).

<table>
<thead>
<tr>
<th>Period</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,600 B.C.</td>
<td>Bread making by Egyptians</td>
</tr>
<tr>
<td>Before birth of Christ</td>
<td>Cheese making by <em>Penicillium roqueforti</em></td>
</tr>
<tr>
<td>in Asia</td>
<td></td>
</tr>
<tr>
<td>2,500 B.P.</td>
<td>Fish fermentation/preservation with sugar, starch, salts etc.</td>
</tr>
<tr>
<td>7th century</td>
<td>Koji process from China to Japan by Buddhist priests</td>
</tr>
<tr>
<td>18th century</td>
<td>Vinegar from pomace, Gallic acid used in tanning, printing etc.</td>
</tr>
<tr>
<td>1860-1900</td>
<td>Sewage treatment</td>
</tr>
<tr>
<td>1900-1920</td>
<td>Fungal enzymes, kojic acid productions</td>
</tr>
<tr>
<td>1920-1940</td>
<td>Fungal enzymes, gluconic acid, citric acid productions</td>
</tr>
<tr>
<td>1940-1950</td>
<td>Development in penicillin production by SSF and submerged</td>
</tr>
<tr>
<td></td>
<td>fermentation</td>
</tr>
<tr>
<td>1950-1960</td>
<td>Steroid transformation by fungal culture</td>
</tr>
<tr>
<td>1960-1980</td>
<td>Production of mycotoxins, protein enriched feed</td>
</tr>
<tr>
<td>1980-present</td>
<td>Various other products such as alcohol and gibberellic acid</td>
</tr>
</tbody>
</table>

In comparison with submerged fermentation (SmF), SSF is simpler and requires less processing energy. SSF also offers many advantages over SmF including higher product concentration, utilisation of low cost substrate (agro-industrial residues) and having low risk of bacterial contamination due to its low moisture level (Prabhakar et al. 2005;
Couto and Sanromán 2006). The basic differences between SSF and SmF are listed in Table 2.5 (Manpreet et al. 2005; Prabhakar et al. 2005; Botella et al. 2009). The advantages and disadvantages of SSF over SmF are presented in Table 2.6 (Manpreet et al. 2005; Couto and Sanromán 2006; Botella et al. 2009).

**Table 2.5** Basic differences between SSF and SmF.

<table>
<thead>
<tr>
<th>SSF</th>
<th>SmF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium is not free-flowing</td>
<td>Medium is free-flowing</td>
</tr>
<tr>
<td>Shallow depth</td>
<td>Greater depth</td>
</tr>
<tr>
<td>Single solid substrate provides C, N, mineral and energy</td>
<td>Requires several ingredients</td>
</tr>
<tr>
<td>Gradients of temperature, pH, C and N concentrations</td>
<td>Uniform</td>
</tr>
<tr>
<td>Less volume</td>
<td>More water, more volume</td>
</tr>
<tr>
<td>Inoculum ratio is large</td>
<td>Inoculum ratio is low</td>
</tr>
<tr>
<td>Intra-particle resistance</td>
<td>No intra-particle resistance (in case of substrate)</td>
</tr>
<tr>
<td>Micro-organisms adhere to solid and grow</td>
<td>Uniformly distribute</td>
</tr>
<tr>
<td>High product concentration</td>
<td>Lower product concentration</td>
</tr>
</tbody>
</table>

**Table 2.6** Advantages and disadvantages of SSF over SmF.

<table>
<thead>
<tr>
<th>Advantage</th>
<th>Disadvantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Higher productivity</td>
<td>Difficulties on scale up</td>
</tr>
<tr>
<td>Better oxygen circulation</td>
<td>Low mixing efficiency</td>
</tr>
<tr>
<td>Low-cost media can be used</td>
<td>Difficult to control process parameters (pH, heat, moisture, nutrient conditions etc.)</td>
</tr>
<tr>
<td>Less effort in downstream processing</td>
<td>Higher impurity products</td>
</tr>
<tr>
<td>Low cost and energy requirement</td>
<td></td>
</tr>
<tr>
<td>It resembles the natural habitat for several micro-organisms</td>
<td></td>
</tr>
<tr>
<td>Liquid waste is not produced</td>
<td></td>
</tr>
</tbody>
</table>

Growth and performance of micro-organisms in SSF are affected by physical, chemical and biochemical factors (Pandey 1992; Pandey et al. 2000). The growth relies on inter- and intra-particle diffusion of gases, such as O$_2$ and CO$_2$, enzymes, nutrients and products of metabolism in the substrate (Mitchell et al. 2003). The general aspects influencing SSF are reviewed below (Manpreet et al. 2005; Prabhakar et al. 2005).
2.3.2 General aspects in SSF

Selection of micro-organisms

The selection of a suitable microbe is essential in SSF (Pandey 1992; Manpreet et al. 2005). Due to the low water content and \( a_w \) value (described later in this section) of solid substrate, SSF provides a suitable environment for many moulds and yeasts that grow in mycelial forms (Jermini and Demain 1989) and some bacterial strains (Robinson et al. 2001; Pandey 2003). For example, *Streptomyces clavuligerus* has been used to produce cephamycin C on wheat rawa (an Indian equivalent of semolina) (Kota and Sridhar 1999) and cephalosporin on barley (Jermini and Demain 1989). *Bacillus subtilis* has also been used to produce iturin A on okara, a by-product from soymilk and tofu production (Ohno et al. 1996; Katayama and Wilson 2008).

Amongst the several groups of micro-organisms that can grow on solid substrates, filamentous fungi have the best capability of growing in the absence of free water. The ability of fungi to spread over and to penetrate inside the solid particles and their ability to produce and excrete large amounts of extra-cellular hydrolytic enzymes make them well suited for SSF. Species of fungi in the genera *Aspergillus*, *Penicillium*, *Rhizopus*, *Mucor*, *Fusarium*, *Alternaria* and *Trichoderma* are commonly used in SSF processes (Pandey 1992; Manpreet et al. 2005).

Selection of substrate

Generally, substrates used in SSF are water insoluble. Three main groups of substrate *i.e.* starchy materials, cellulosic or lignocellulosic materials and those containing soluble sugars are commonly used. Starchy materials include rice, cassava, rice bran, wheat bran and corn meal. Lignocellulosic materials include wheat straw, corn stover, rice stover, pulp and wood whilst substrates containing soluble sugars include grape pomace, sweet sorghum, sugar beet, banana waste and pineapple waste (Manpreet et al. 2005). Amongst these, wheat bran is the most extensively used (Pandey et al. 1999).

The substrate is an important factor affecting SSF processes and its selection depends upon several factors mainly related with cost and availability (Couto and Sanromán 2006). Although both natural and synthetic substances can be used, natural materials, particularly agro-industrial residues, which are cheap and easily available, have received
attention in recent research on SSF (Pandey 1992). However, physical and chemical pre-treatment is generally required to make physical characteristics more suitable for hyphal penetration and the chemical constituents more accessible (Pandey et al. 2000; Manpreet et al. 2005; Couto and Sanromán 2006). High temperature, acid or alkaline treatment and addition of some nutrients are generally used, while chopping and grinding of the substrate are commonly carried out to reduce particle size and to make the interior of the particle more accessible (Manpreet et al. 2005). However, it should be noted that small particle size, although providing a large surface area for cell growth, may result in substrate agglomeration resulting in poor growth. On the other hand, larger particle sizes provide better aeration but also limited surface for cell growth (Pandey et al. 1999). Even with optimally sized particles, the environment presented in SSF is always heterogeneous and therefore potentially subject to unevenness in nutrient concentrations, water activity, pH, and temperature. It is useful therefore to look at parameters affecting SSF in more detail.

**Effects of temperature**

Poor heat transfer is the main cause of temperature gradients occurring during SSF (Manpreet et al. 2005). The heat generated during SSF is directly proportional to the metabolic activity in the system, particularly respiration of micro-organisms during growth (Saucedo-Castañeda et al. 1990; Robinson and Nigam 2003). The temperature at hot spots can be as high as 70°C whereas the optimal growth temperature of micro-organisms used in SSF is normally between 20°C and 40°C, with maximum tolerable temperature usually below 50°C. The temperature gradient can affect bed porosity, slow down microbial activity and cause dehydration of the substrate (Saucedo-Castañeda et al. 1990; Manpreet et al. 2005; Prabhakar et al. 2005). Due to the low thermal conductivity and the heterogeneous nature of the substrate coupled with only limited water present in the system, heat removal can be difficult. Maintaining a desirable temperature in SSF systems is, therefore, very difficult (Saucedo-Castañeda et al. 1990; Prabhakar et al. 2005). A number of cooling methods have been applied for dissipation of heat. For instance, passing coolant across or through the metal trays supporting the substrates, intermittent agitation, circulation of water through a jacket or covering the external fermenter surface with wetted materials. Alternatively, spraying water directly onto the fermenting solids coupled with mixing and forced aeration can also be
effective. Amongst these, forced aeration seems to be the most effective method and can remove up to 80% of heat generated (Manpreet et al. 2005; Prabhakar et al. 2005).

**Effects of pH**

Changes in pH during SSF are mainly caused by production of acids or utilisation of ammonium ions by the micro-organisms resulting in pH reduction, while deamination of urea or other amines will result in release of ammonia, thereby increasing the pH. Although the mechanism of pH change in SSF is well understood, pH control during the process is extremely difficult since free water is absent and change of pH during fermentation cannot easily be monitored (Saucedo-Castañeda et al. 1992; Manpreet et al. 2005; Chutmanop et al. 2008). For this reason, usually the initial pH of the substrate is adjusted before inoculation (Chutmanop et al. 2008). Other reasons for not adding acid or alkali to the medium to correct the pH during fermentation are the lack of mixing, the change in moisture content and the possibility that it will increase the chance of contamination. An initial acidic pH (around 4) is conventionally used to reduce the chance of bacterial contamination and to support the growth of filamentous fungi (Saucedo-Castañeda et al. 1992). Although most substrates used in SSF are known to possess excellent buffering capacity (Chutmanop et al. 2008) and this is particularly true for protein-rich substrates, especially if deamination of the substrate is minimal, the use of micro-organisms which can grow over a wide range of pH is desirable (Manpreet et al. 2005). To minimise pH variation, buffers can be used or, for example, urea can be used as a nitrogen source, relieving the difficulties in controlling pH during fermentation (Saucedo-Castañeda et al. 1992).

**Effects of water activity**

The water activity ($a_w$) concept is used to describe the equilibrium between a heterogeneous system and the surrounding water vapour phase (Gervais and Bensoussan 1994). The $a_w$ of the moist substrate is the ratio of vapour pressure of water above the substrate to vapour pressure of pure water at the same condition. It is measured as relative humidity divided by 100 (Manpreet et al. 2005; Gervais 2008). The $a_w$ value of pure water is 1.00 and it will decrease with the addition of solutes such as NaCl, glucose, sorbitol and glycerol (Jakobsen and Murrell 1977; Manpreet et al. 2005).
Due to the absence of free water in SSF system, micro-organisms that can grow and carry out their metabolic activity at lower $a_w$ values are preferable (Pandey 1992; Manpreet et al. 2005). Generally, bacteria tend to grow at higher $a_w$ values while the optimum range of $a_w$ for the growth of fungi and some strains of yeast is between 0.6 and 0.7 (Pandey 1992). The $a_w$ is an important parameter in the determination of microbial activity in SSF system. It plays an essential role on sporulation and spore germination of both fungi and bacteria. In the course of fungal growth, higher $a_w$ promotes sporulation while lower $a_w$ favours spore germination and mycelia growth (Pandey 1992; Manpreet et al. 2005). For bacteria, sporulation of Bacillus cereus (Jakobsen and Murrell 1977) and Clostridium perfringens (Kang et al. 1969) was found to require a higher $a_w$ than growth.

Alteration of $a_w$ can be used as a tool to modify the metabolic production and excretion of micro-organisms (Pandey 1992). This could be done by, for instance, adjusting relative humidity of the surrounding air (Pandey 1992) or by addition of solutes (Manpreet et al. 2005).

### 2.3.3 Applications of SSF

SSF can be conducted using many different micro-organisms to produce a broad range of products including metabolites, enzymes, foods, flavours and aroma compounds, polymers, organic acids etc. (Pandey et al. 2000; Manpreet et al. 2005; Prabhakar et al. 2005; Couto and Sanromán 2006). A preliminary survey of the SSF literature indicates that yeast and bacteria predominantly involve the production of secondary metabolites while filamentous fungi, especially Aspergillus spp., play an important role in enzyme and organic acid production (Robinson et al. 2001; Manpreet et al. 2005).

Since in this research, SSF would be performed using Aspergillus oryzae, only the SSF applications involving Aspergillus spp. are presented. One of the advantages of SSF is that it offers good oxygen circulation. This is considered suitable for secondary metabolite productions (Robinson et al. 2001; Couto and Sanromán 2006). Examples of secondary metabolites produced under SSF by Aspergillus sp. are mycotoxins, aflatoxins and tetracyclines (Robinson et al. 2001).

Several industrially important enzymes produced under SSF have been extensively studied. The target enzymes include proteases, cellulases, ligninases, xylanases,
pectinases, amylases, glucoamylase, phytases, laccases, polyphenol oxidase, β-galactosidase, and sterol esterase. (Cohen 1973; Lacki and Duvnjak 1999; Pandey et al. 2000; El-Batal and Abdel Kareem 2001; Hu and Duvnjak 2004; Sandhya et al. 2005; Wang et al. 2005; Tőke et al. 2007; Chutmanop et al. 2008; Hui et al. 2008; Nizamuddin et al. 2008; Gotou et al. 2009). A comparison between SSF and SmF for the production of cellulase revealed that SSF could be operated at 10 times lower cost than SmF (Pandey et al. 2000). It was also reported that SSF is more appropriate for the production of other enzymes and thermolabile products than SmF, especially when higher yields can potentially be obtained (dos Santos et al. 2004). Examples of enzymes produced by SSF are given in Table 2.7.

### Table 2.7 Application of SSF for the production of various enzymes by *Aspergillus* spp.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Micro-organism</th>
<th>Substrate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulase</td>
<td><em>Aspergillus niger</em></td>
<td>Wheat bran</td>
<td>Sukumaran et al. (2009)</td>
</tr>
<tr>
<td>Cellulase</td>
<td><em>Aspergillus sp.</em></td>
<td>Soy bean meal</td>
<td>Manpreet et al. (2005)</td>
</tr>
<tr>
<td>Glucoamylase</td>
<td><em>A. awamori</em></td>
<td>Wheat bran</td>
<td>Negi and Banerjee (2009)</td>
</tr>
<tr>
<td>Glucoamylase</td>
<td><em>A. niger</em></td>
<td>Sugarcane bagasse and tapioca</td>
<td>Varzakas et al. (2008)</td>
</tr>
<tr>
<td>Lipase</td>
<td><em>A. niger</em></td>
<td>Coconut cake, gingelly oil cake</td>
<td>Manpreet et al. (2005)</td>
</tr>
<tr>
<td>Pectinase</td>
<td><em>A. niger</em></td>
<td>Citrus wastes, soy bran and wheat bran</td>
<td>Manpreet et al. (2005)</td>
</tr>
<tr>
<td>Phytase</td>
<td><em>A. niger</em></td>
<td>Rapeseed meal</td>
<td>El-Batal and Abdel Kareem (2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wheat bran and rice bran, wheat bran, rice husk, spent</td>
<td>Chutmanop et al. (2008), Sandhya et al. (2005),</td>
</tr>
<tr>
<td></td>
<td></td>
<td>brewing grain, coconut oil cake, palm kernel cake,</td>
<td>Wang et al. (2005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sesame oil cake, jackfruit seed powder, olive oil cake,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>soybean flour, wholemeal wheat flour, agar</td>
<td></td>
</tr>
<tr>
<td>Protease</td>
<td><em>A. oryzae</em></td>
<td>Agar medium</td>
<td>Cohen (1973)</td>
</tr>
<tr>
<td>Sterol esterase</td>
<td><em>Aspergillus sp.</em></td>
<td>Solid wheat bran medium</td>
<td>Tőke et al. (2007)</td>
</tr>
<tr>
<td>Tannase</td>
<td><em>A. oryzae</em></td>
<td>Sugarcane bagasse and rice straw</td>
<td>Paranthaman et al. (2008)</td>
</tr>
<tr>
<td>Xylanase</td>
<td><em>A. niveus, A. niger,</em></td>
<td>Mixture of wheat and yeast extract, mixture of corncock</td>
<td>Betini et al. (2009)</td>
</tr>
<tr>
<td></td>
<td><em>A. ochraceus</em></td>
<td>and wheat bran</td>
<td></td>
</tr>
<tr>
<td>α-amylase</td>
<td><em>A. oryzae</em></td>
<td>Spent brewing grain</td>
<td>Hui et al. (2008)</td>
</tr>
<tr>
<td>β-galactosidase</td>
<td><em>A. oryzae</em></td>
<td>Wheat bran, rice husk</td>
<td>Nizamuddin et al. (2008)</td>
</tr>
</tbody>
</table>

Organic acids are one of the important classes of ingredients used in the food industry. They are mainly used for food acidulation (Krishna 2005). Extensive studies on production of citric acid and lactic acid, the most commonly used organic acids in the food industry have been carried out (Couto and Sanromán 2006). Citric acid is widely used as tart flavour in food and beverage industries. It is also used as an effervescent in powder and tablets in combination with bicarbonate in the pharmaceutical industry, and as buffering agent in the cosmetic and toiletries industry (Pandey et al. 2000; Soccol et
al. 2008). The majority of citric acid available on the market comes from SmF processes by the filamentous fungus, *Aspergillus niger*. However, SSF has recently been reconsidered as a potential alternative to SmF due to its high production efficiency (Couto and Sanromán 2006). The production of citric acid under SSF route also employs *A. niger* as production strain (Pandey *et al.* 2000). The solid substrate used is diverse, including cassava bagasse, sugarcane bagasse, corn husk, coffee husk, pineapple waste, apple pomace, grape pomace, banana, kiwi fruit peel, molasses, rice bran, sweet potato and sawdust with rice hulls (Pandey *et al.* 2000; Krishna 2005; Couto and Sanromán 2006; Soccol *et al.* 2008). The productions of some organic acids are given in Table 2.8 (Pandey *et al.* 2000; Manpreet *et al.* 2005; Couto and Sanromán 2006).

As can be seen above, fungi in the genus *Aspergillus* have an ability to produce a number of enzymes on an extremely wide range of substrates. This attracted the interest in using a fungus to convert agricultural wastes or residues to a feedstock that can support growth and production of products of interest. In the SCGPE, wheat had been used as a substrate for growing *A. awamori* to produce a generic feedstock which was subsequently used to produce polyhydroxybutyrate (PHB) by a bacterium *Cupriavidus necator* (Koutinas *et al.* 2007b). The promising results obtained from that research led to an extension of the study aiming to utilise other agricultural wastes to produce a value-added chemical which is reported in this thesis.

### Table 2.8 Some organic acids produced under SSF by *Aspergillus* spp.

<table>
<thead>
<tr>
<th>Organic acid</th>
<th>Micro-organism</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citric acid</td>
<td><em>A. niger</em>, <em>A. oryzae</em></td>
<td>Cassava bagasse, sugarcane bagasse, corn husk, coffee husk, pineapple waste, apple pomace, grape pomace, banana, kiwi fruit peel, molasses, rice bran, sweet potato, sawdust with rice hulls</td>
</tr>
<tr>
<td>Gluconic acid</td>
<td><em>A. niger</em></td>
<td>Fig</td>
</tr>
<tr>
<td>Kojic acid</td>
<td><em>A. oryzae</em></td>
<td>Steamed rice</td>
</tr>
<tr>
<td>Oxalic acid</td>
<td><em>A. niger</em></td>
<td>Sweet potato</td>
</tr>
</tbody>
</table>

2.4 *Cupriavidus necator*

*C. necator* is a versatile PHB-producing bacterium. It is the most extensively studied and has been commonly used due to its ability to accumulate up to 90% of its dry weight as PHB (Arun *et al.* 2006; Suriyamongkol *et al.* 2007; Chee *et al.* 2010). General
information about morphology, taxonomy and growth conditions for *C. necator* are reviewed below.

### 2.4.1 Morphology

*C. necator* is a gram-negative, coccoid rod shaped bacterium measuring 0.7-0.9×0.9-1.3 µm. It is motile with 2-10 peritrichous flagella (Figure 2.8). It reproduces by binary fission and in older cultures, the rods decrease in size and become rounded. When cultivated on nutrient agar at 27°C for 2 days, colonies are off-white, glistening, mucoid, smooth and convex with entire edge. The colony diameter is 2-4 mm (Makkar and Casida 1987).

![Figure 2.8](image)

**Figure 2.8** A negatively stained *Cupriavidus necator* strain N-1 observed by electron microscopy, bar = 1 µm (Makkar and Casida 1987).

### 2.4.2 Taxonomy

*C. necator* is formerly classified as *Ralstonia eutropha* and *Alcaligenes eutrophus* (German Collection of Microorganisms and Cell Culture (DSMZ) ; Fukui *et al.* 2009). *Wautersia eutropha* is a later synonym of *C. necator*. However, in conformity with rules of the International Code of Nomenclature of Bacteria, the genus name *Cupriavidus* has priority over the genus name *Wautersia*. As a result, all members of the genus *Wautersia* have been reclassified into *Cupriavidus* (Vandamme and Coenye 2004; German Collection of Microorganisms and Cell Culture (DSMZ) 2009). The terms *Cupriavidus necator* derive from Latin words “cuprum” (copper) and “avidus” (eager for, loving) while “necator” means “slayer” (Makkar and Casida 1987; Balkwill 2005).
In 1987, when the genus *Cupriavidus* was proposed by Makkar and Casida, it was most similar to *Alcaligenes*. This was due to its cell morphology, the mol% G+C content of its DNA and its lack of ability to utilise glucose as a sole carbon source. Nevertheless, it can be differentiated from *Alcaligenes* spp. on the basis of its predatory activity and its ability to utilise fructose. Later, *C. necator* was phylogenetically situated within the genus *Ralstonia* based on the analysis of 16S rRNA gene sequences (Yabuuchi et al. 1995). It differs from *R. eutropha* in two other ways: it does not utilise benzoate as a carbon source and it does not cause hemolysis on blood agar. *C. necator* also has lower mol% G+C content than *R. eutropha* (57±1% for the former and 64% to 68.3% for the latter) (Makkar and Casida 1987; Balkwill 2005). Based on these reasons, a new genus, *Cupriavidus*, was placed under the following lineage:\(^1\)

\[\text{Superkingdom: } \text{Bacteria}\]
\[\text{Phylum: } \text{Proteobacteria}\]
\[\text{Class: } \text{Betaproteobacteria}\]
\[\text{Order: } \text{Burkholderiales}\]
\[\text{Family: } \text{Burkholderiaceae}\]
\[\text{Genus: } \text{Cupriavidus}\]

### 2.4.3 Growth conditions

*C. necator* is aerobic and mesophilic with optimal temperature of 27°C but good growth is also obtained at 37°C. It can grow in the pH range 5.5 to 9.2, though optimal pH is between 7.0 and 8.0. Several chemicals including β-hydroxybutyrate, acetate, fructose, lactate, succinate and amino acids except for L-lysine or L-methionine can be used as carbon/nitrogen sources. Makkar and Casida (1987) reported that, for *C. necator* strain N-1, carbon sources that are not utilised include glucose, glycerol, lactose, mannitol mannose, xylose and rhamnose. However, several strains have been reported to be capable of utilising glucose and glycerol as carbon source, for example *C. necator* strain DSM545 and DSM4058 (Mothes et al. 2007; Fiorese et al. 2009).

Media containing 1% NaCl have been found to support the growth, but it is inhibited at 3% NaCl. *C. necator* is a non-obligate predator of various other gram-positive and

---

\(^1\) [http://www.uniprot.org/taxonomy/106590](http://www.uniprot.org/taxonomy/106590)
gram-negative bacteria in soil. It is resistant to copper and growth initiation is stimulated by copper (Makkar and Casida 1987; Balkwill 2005). Under unbalanced growth conditions for instance limitation of essential nutrients such as nitrogen, phosphorus, magnesium, potassium, oxygen or sulphur and excess carbon, C. necator synthesises and accumulates PHB inside its cell as a carbon and energy reserve (Posada et al. 2011).

2.4.4 Polyhydroxybutyrate synthesis in C. necator

As mentioned earlier, C. necator is the most extensively studied micro-organism for PHB production due to its high productivity (Ashby et al. 2004). A glucose-utilising mutant of this bacterium had been reported accumulating up to 80% (w/w) PHB (Holmes 1985). Whereas on glycerol, PHB content of 70% of dry cell mass is achievable (Mothes et al. 2007). The synthesis of PHB is considered the simplest biosynthesis pathway, which involves three enzymes and their encoding genes as shown in Table 2.9 (Suriyamongkol et al. 2007).

Table 2.9 Genes and enzymes for PHB synthesis in C. necator and their functions.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Enzyme</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>phaA</td>
<td>β-ketothiolase</td>
<td>Condense two acetyl-CoA molecules to form acetoacetyl-CoA</td>
</tr>
<tr>
<td>phaB</td>
<td>Acetoacetyl-CoA reductase</td>
<td>Catalyse the reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA, this enzyme is NADPH-dependent</td>
</tr>
<tr>
<td>phaC</td>
<td>PHA synthase</td>
<td>Catalyse the polymerisation of (R)-3-hydroxybutyryl-CoA monomers</td>
</tr>
</tbody>
</table>

The process of PHB synthesis starts with the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA by β-ketothiolase. The second reaction is the reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA by the NADPH-dependent acetoacetyl-CoA reductase. Lastly, (R)-3-hydroxybutyryl-CoA monomers are polymerised by PHA synthase, yielding PHB (Doi et al. 1990; Steinbüchel and Füchtenbusch 1998; Suriyamongkol et al. 2007). PHA synthase, the key enzyme for PHB biosynthesis, in C. necator reacts with substrates containing three to five carbon atoms, with C4-substrate as its preference. As a consequence, PHB polymers obtained from this pathway contain short-chain-length monomers (Suriyamongkol et al. 2007). The pathway is shown in Figure 2.9.
Apart from monomer, *C. necator* can also synthesise PHB copolymers including poly(3-hydroxybutrate-co-3-hydroxyhexanoate), poly(3-hydroxybutyrate-co-4-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (Dennis *et al.* 1998; Amirul *et al.* 2008; Chanprateep *et al.* 2008; Chai *et al.* 2009; Obruca *et al.* 2010). Currently, a glucose-utilising mutant of *C. necator* is used industrially by Metabolix (USA) to produce poly(3-hydroxybutyrate-co-3-hydroxyvalerate). This copolymer is sold under the trade name of Biopol\textsuperscript{TM}\textsuperscript{2}.

\textsuperscript{2} http://www.metabolix.com/privacy.html


2.5 Polyhydroxybutyrate (PHB)

2.5.1 General information about PHB

PHB was discovered as a constituent of *Bacillus megaterium* by French bacteriologist Maurice Lemoigne (1926). PHB is a high molecular weight aliphatic polyester (Poirier *et al.* 1992) belonging to a class of 3-hydroxy acids (Jung *et al.* 2005) (Figure 2.10). It is one of the two major members of the polyhydroxyalkanoates (PHA) family, the other being polyhydroxyvalerate (PHV) (Figure 2.11) (Nolan 2002) and is the most common bacterial PHA in nature. Other PHA polymers include PHP, PHV, PHH and PHO (see Table 2.10 for further detail) (Jogdand 2004; Kahar *et al.* 2004; Green 2010). PHB is a short-chain-length PHA (scl-PHA) with its monomers containing 4 to 5 carbon atoms (Chen 2005).

![Molecular structure of PHB](image)

**Figure 2.10** Molecular structure of PHB, *n* can range from 100 to several thousands.

**Table 2.10** Common PHAs, defined by their respective *R* group.

<table>
<thead>
<tr>
<th><em>R</em>-group</th>
<th>PHA name</th>
<th>Short name</th>
</tr>
</thead>
<tbody>
<tr>
<td>-H</td>
<td>Polyhydroxypropionate</td>
<td>PHP</td>
</tr>
<tr>
<td>-CH₃</td>
<td>Polyhydroxybutyrate</td>
<td>PHB</td>
</tr>
<tr>
<td>-CH₂CH₃</td>
<td>Polyhydroxyvalerate</td>
<td>PHV</td>
</tr>
<tr>
<td>-CH₂CH₂CH₃</td>
<td>Polyhydroxyhexanoate</td>
<td>PHH</td>
</tr>
<tr>
<td>-CH₃CH₂CH₂CH₃</td>
<td>Polyhydroxyoctanoate</td>
<td>PHO</td>
</tr>
</tbody>
</table>
PHB is accumulated as a storage carbon and energy source under unbalanced growth conditions in more than 250 different micro-organisms (Choi and Lee 1997; Huey 2006). Figure 2.12 shows PHB granules inside cells of *C. necator*.

![Figure 2.12](image.png)

**Figure 2.12** Transmission electron microscope image of *C. necator* with PHB granules (grey/white fractions); bar = 0.5 µm.

### 2.5.2 Properties of PHB

**Physical properties**

PHB is a partially crystalline polymer. It is insoluble in water but soluble in chloroform and other chlorinated hydrocarbons (Jogdand 2004; Clarinval and Halleux 2005) such as dichloroethane (Hahn *et al.* 1995). Generally, PHB has similar material properties to polypropylene (PP) and polyethylene (PE), though in terms of the mechanical properties *e.g.* modulus and elongation, it is more similar to high density PE (HDPE) than low
density PE (LDPE). However, it differs from both PP and PE in some characteristics. For instance, it has much less oxygen permeability than those conventional plastics, which makes PHB better for food packaging (Huey 2006). A homopolymer of PHB is stiffer and more brittle than PP. Incorporation of 3-hydroxyvaterate (3HV) can result, in mechanical terms, in a decrease in stiffness and an increase in toughness. Some physical properties of PHB and PP are given in Table 2.11 (Wondu Holding Pty Ltd. 2004). The definitions of some parameters are as follows:

*Crystallinity (%)* is the value used to indicate the amount of crystalline region in polymer with respect to amorphous content. *Extension to break* is presented in percentage. The higher percentage of the extension to break, the less brittleness the material is (Harvie 2000). *Flexural modulus* is used to refer to the stiffness of a material. It is the ratio of stress to strain in flexural deformation. In other words, this is the tendency for a material to bend. *Glass (or glass-to-rubber) transition temperature* (T<sub>g</sub>) is the temperature at which the amorphous phase transitions from a hard and brittle state into rubber-like state (Anderson and Dawes 1990). Below T<sub>g</sub>, the material is brittle while above T<sub>g</sub>, it becomes more elastic. *Melting temperature* (T<sub>m</sub>) is the temperature at which the crystalline phase transforms from solid phase to liquid phase. The material becomes mouldable above this temperature. *Tensile strength* of a material is the maximum force (tension) that it can take before failure, for example breaking.

Table 2.11 Some physical properties of PHB and PP.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PHB</th>
<th>PP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystallinity (%)</td>
<td>65-80</td>
<td>65-70</td>
</tr>
<tr>
<td>Density (g/cm&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>1.23-1.25</td>
<td>0.905-0.94</td>
</tr>
<tr>
<td>Extension to break (%)</td>
<td>6-8</td>
<td>400</td>
</tr>
<tr>
<td>Flexural modulus (GPa)</td>
<td>3.5-4</td>
<td>1.7</td>
</tr>
<tr>
<td>Glass transition temperature, T&lt;sub&gt;g&lt;/sub&gt; (°C)</td>
<td>5-10</td>
<td>-15</td>
</tr>
<tr>
<td>Melting temperature, T&lt;sub&gt;m&lt;/sub&gt; (°C)</td>
<td>171-182</td>
<td>171-186</td>
</tr>
<tr>
<td>Molecular weight, MW (g/mol) (×10&lt;sup&gt;5&lt;/sup&gt;)</td>
<td>1-8</td>
<td>2.2-7</td>
</tr>
<tr>
<td>MW distribution (Polydispersity index)</td>
<td>2.2-3</td>
<td>5-12</td>
</tr>
<tr>
<td>Oxygen permeability (cm&lt;sup&gt;3&lt;/sup&gt;/m&lt;sup&gt;2&lt;/sup&gt;·atm·d)</td>
<td>45</td>
<td>1,700</td>
</tr>
<tr>
<td>Solvent resistance</td>
<td>Poor</td>
<td>Good</td>
</tr>
<tr>
<td>Tensile strength (MPa)</td>
<td>40</td>
<td>39</td>
</tr>
<tr>
<td>UV resistance</td>
<td>Good</td>
<td>Poor</td>
</tr>
<tr>
<td>Other</td>
<td>Due to higher density, PHB sinks in aquatic system</td>
<td>Due to lower density, PP floats in aquatic system</td>
</tr>
</tbody>
</table>

<sup>3</sup> bic-innovation.com/static/bic/knowledge_base/documents/T144535.pdf
**Biological properties**

Biodegradability is an important feature that distinguishes biodegradable plastics from petroleum-based plastics and makes PHB commercially attractive. In aerobic conditions where the polymer is exposed to micro-organisms found in soil, sewage, river bottom and other similar environments, PHB is degraded to carbon dioxide and water. In anaerobic conditions, the products of degradation are carbon dioxide and methane (Jogdand 2004; Wondu Holding Pty Ltd. 2004). The susceptibility of PHB to biodegradation is dependent upon several factors. For instance, the characteristics of the polymer such as thickness, chemical composition, structure, crystallinity and molecular weight (Bonartsev et al. 2007). Environmental conditions, such as the amount of bacteria present, temperature, pH and moisture level also influence the rate of degradation (Anderson and Dawes 1990).

Biodegradation of PHB can occur either intra- or extracellularly as well as *in vivo*. Intracellular PHB degradation proceeds by sequential actions of PHA depolymerase, R-3-hydroxybutyrate dehydrogenase and acetoacetyl-CoA synthetase, to form R-3-hydroxybutyrate and acetyl-CoA. In *Alcaligenes* spp. including *C. necator*, the product of this degradation process is solely R-3-hydroxybutyrate, but the mixture of dimers and monomers of the acid can be obtained in other organisms (Anderson and Dawes 1990; Braunegg et al. 1998).

The biodegradability of PHB *in vivo*, coupled with its biocompatibility, makes it a useful material for many biomedical applications. In animal tissues, degradation of PHB is due to hydrolysis and biodegradation by tissue enzymes (nonspecific esterase) (Bonartsev et al. 2007). Figure 2.13 shows PHB film completely degraded *in vivo* within 3 months (Bonartsev et al. 2007).
In natural environments, PHB is degraded by extracellular PHB depolymerase secreted by numerous organisms (bacteria, fungi and algae) (Vroman and Tighzert 2009) such as *Alcaligenes faecalis*. PHB depolymerase attacks the free hydroxyl terminus of PHB, releasing dimer units (D(−)-3-[D(−)-3-hydroxybutyryloxy]butyric acid) with a trace of monomer (Tanio *et al.* 1982; Anderson and Dawes 1990).

PHB is compostable over a wide range of environmental conditions. Nevertheless, maximum biodegradation rates have been observed at 55% moisture level, 60°C, conditions similar to those used in most large-scale composting plants (Wondu Holding Pty Ltd. 2004). For the commercially available Biopol™, by using the above conditions and C/N ratio of 18, the degradation rate reached close to 100% during a 10-week period (Nolan 2002). The homopolymer of PHB is degraded relatively slowly compared to its copolymers. The presence of 4-hydroxybutyrate (4HB) units results in more rapid degradation under all conditions than PHB. In aerated activated sludge at 30°C, a 3HB-co-9% 4HB film was completely decomposed in 14 days; a 3HB-co-17% 4HB film was decomposed within the same period in soil at 30°C, while PHB took more than 10 weeks (Anderson and Dawes 1990). Figure 2.14 shows the degradation of PHB film incubated for 2 months in soil suspension under microaerobic conditions (Bonartsev *et al.* 2007).
2.5.3 Applications of PHB

The biodegradability and biocompatibility of PHB make it very attractive in various applications. Following are some applications in which PHB has been used or has a promising future as a substitute material to conventional ones.

**Biodegradable packaging materials and disposable items**

PHB is used in small disposable products, such as a degradable shampoo bottle (Wondu Holding Pty Ltd. 2004), and in packaging materials including food packages. It is also used as bags, paper coatings, utensils, diapers, cups etc. Compared to PP, PHB is more rigid and less flexible. This results in lower performance under freezing conditions. Nevertheless, at higher temperature, PHB performs better than PP. Blending PHB with other polymers can improve the polymer physical characteristics, making it suitable for packaging and subsequent storage (Vroman and Tighzert 2009). An example of a commercial copolymer consisting of PHB is Nodax™, developed by Procter & Gamble (P&G, USA) which is now sold by Meredian (USA). It consists of (R)-3-hydroxyalkanoate comonomer units with medium-size-chain side groups and (R)-3-hydroxybutyrate. This copolymer can be manufactured with varying comonomer contents to be used as synthetic paper, foam, resin for coating lamination, elastomeric film (gloves) and so on (Noda et al. 2010).
**Medicine and pharmacy**

Although PHB is biocompatible, it is brittle. For this reason, its application in the biomedical field is limited. Nevertheless, it has the advantageous property of being degraded into 3-hydroxybutyrate, which is naturally found in human blood. A number of medical devices for dental, orthopaedic and skin surgery have therefore been developed using PHB. These include heart valves, surgical suture filaments, screws and plates for cartilage and bone fixation, membranes for periodontal treatment, surgical meshes with PHB coating and wound coverings. For pharmaceutical application, PHB is used in drug delivery system as a matrix material for slow release drugs, in the forms of films, microspheres and microcapsules (Doi 2002; Bonartsev et al. 2007; Suriyamongkol et al. 2007; Vroman and Tighzert 2009). Blending between PHB and other polymers can improve the polymer characteristics. The copolymer of PHB and PHBHx (poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)) showed dramatic improvement in the biocompatibility in the mouse fibroblast cell line L929 compared to PHB (Chen 2005).

### 2.5.4 Commercialisation of PHB

Although PHB can be used in a wide variety of applications, its commercial use remains limited because of its high production cost compared to conventional plastics. The current cost of PHB is around 8-10 times higher than that of petroleum-based plastics. The reasons for this have been due to the utilisation of pure cultures and substrates, the need to maintain sterile conditions and recovery and purification. Among these, recovery and purification represent over half of the overall cost and have been regarded as important factors for greatly reducing the cost. The PHB yield and plant capacity was also found to significantly affect the production cost, while the cost of carbon substrate affected the overall economics of PHB production (Jung et al. 2005; Mudliar et al. 2008). Although the development of fermentation and purification technologies, along with the use of genetic engineering, help cut down the production cost of biodegradable plastics, the prices are still too high. For example, the price for Biopol™ has been reduced from 16 USD/kg to 4 USD/kg, yet it is still too high compared to those for PP and PE (ca. 0.25-0.5 USD/kg). At present, only four PHBs and copolymers have been produced on a large scale for commercial purposes, with annual production of around

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70,000 tons/year. These are PHB, PHBV, P3HB4HB and PHBHHx (Chen and Chen 2010) (see Table 2.12 for detail). Table 2.12 presents PHB and PHB copolymer producing companies worldwide (Clarinval and Halleux 2005; Rudnik 2008; Chen and Chen 2010; Posada et al. 2011).

Table 2.12 Worldwide PHB and PHB copolymer producers.

<table>
<thead>
<tr>
<th>Type of PHAs</th>
<th>Company and origin</th>
<th>Trade name</th>
<th>Price (USD/kg)</th>
<th>Micro-organism used</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHB</td>
<td>Biomer, Germany</td>
<td>Biomer®</td>
<td>3.75-6.25 (2010)</td>
<td>Al. latus</td>
</tr>
<tr>
<td>PHB</td>
<td>BTF, Austria</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>PHB</td>
<td>Chemie Linz, China</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>PHB</td>
<td>Jiangsu Nan Tian, China</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>PHB</td>
<td>Mitsubishi Gas Chemical, Japan</td>
<td>Biogreen™</td>
<td>2.75 (2010)</td>
<td>Unknown</td>
</tr>
<tr>
<td>PHB</td>
<td>Tianjin Northern, China</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>PHB, PHBV*</td>
<td>BASF, Germany</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>PHB, PHBV</td>
<td>Monsanto, USA</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>PHBV</td>
<td>ICI, UK</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>PHBV</td>
<td>Zhejiang Tian An, China</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>PHB/PBV</td>
<td>Metabolix, USA</td>
<td>Biopol™</td>
<td>4 (2005)</td>
<td>Glucose-utilising mutant of C. necator</td>
</tr>
<tr>
<td>P3HB4HB**</td>
<td>Tianjin Green Bioscience, China</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>PHBHHx***</td>
<td>Jiangmen Biotech Center, China</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>PHA/PHB/PHO****</td>
<td>Metabolix, USA</td>
<td>Metabolix PHA</td>
<td>Unknown</td>
<td>Recombinant E. coli K12</td>
</tr>
<tr>
<td>PHA copolymer</td>
<td>Meredian, USA</td>
<td>Nodax™</td>
<td>Unknown</td>
<td>Aeromonas caviae and C. necator</td>
</tr>
<tr>
<td>Several PHAs</td>
<td>ADM, USA (with Metabolix)</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>Several PHAs</td>
<td>Kaneka, Japan (with P&amp;G)</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>Several PHAs</td>
<td>Procter &amp; Gamble (P&amp;G), USA</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

* PHBV is poly(\(R\))-3-polyhydroxybutyrate-co-3-hydroxyvalerate  
** P3HB4HB is poly(\(R\))-3-polyhydroxybutyrate-co-4-hydroxybutyrate  
*** PHBHHx is poly(\(R\))-3-polyhydroxybutyrate-co-(\(R\))-3-hydroxyhexanoate  
**** PHO is polyhydroxyoctanoate

2.5.5 PHB synthesis

The PHB polymer is synthesised by hundreds of micro-organisms and stored in their cytoplasm as insoluble inclusions (Steinbüchel and Füchtenbusch 1998). Bacteria that are capable of producing PHAs can be divided into two groups based on the culture condition required for PHA synthesis. The first group requires limitation of an essential nutrient such as nitrogen, phosphorus, magnesium or sulphur for the synthesis of PHA from excess carbon source. Included in this group are, for example, C. necator and Pseudomonas oleovorans. Since in this research, C. necator was to be used to produce
PHB and due to the PHB accumulation occurs in a non-growth associated manner, fed-batch process is likely to be a suitable approach. To achieve high cell density, the bacterium is maintained in complete medium then an essential nutrient is limited to allow efficient PHB accumulation (Posada et al. 2011). The bacteria in the second group do not require nutrient limitation for PHA synthesis and can accumulate the polymer during their growth. The bacteria in this group are Alcaligenes latus, Azotobacter vinelandii and recombinant Escherichia coli (Steinbüchel and Füchtenbusch 1998; Rudnik 2008). Substrates used in PHB research and for PHB production are also as diverse as the PHB producers. Carbohydrates (glucose, fructose, sucrose, lactose), alcohol (methanol, glycerol), alkanes (hexane to dodecane), organic acids (butyrate upwards), fossil resources (methane, mineral oil, lignite, hard coal), chemicals (propionic acid), renewable resources (starch, cellulose, plant oils) and by-products (molasses, whey, crude glycerol) are amongst the substrates used (Fukui and Doi 1998; Steinbüchel and Füchtenbusch 1998; Ghatnekar et al. 2002; Fonseca et al. 2008; Lee et al. 2008; Rudnik 2008; Cavalheiro et al. 2009; Lee et al. 2009).

Apart from PHB synthesis in C. necator presented earlier, other prevalent micro-organisms and biological systems are also of interest from an industrial point of view. Brief reviews about PHB synthesis in those systems are given below.

**PHB synthesis in bacteria**

Interesting bacterial strains that are capable of producing PHB include, for example, Alcaligenes latus, Pseudomonas oleovorans, Azotobacter vinelandii and Paracoccus denitrificans. Amongst these, Al. latus and Az. vinelandii are known to produce PHB in a growth-associated manner and do not require nutrient limitation for PHB synthesis (Wang and Lee 1997). Also, they can accumulate PHB at high quantities (more than 70% of cell dry weight) (Page 1989; Yamane et al. 1996; Braunegg et al. 1998), close to that observed in C. necator.

*Ps. oleovorans* produces short-chain-length (scl) PHAs rather than medium-chain-length (mcl) PHAs (C6 to C14) that most Pseudomonads do. This is because *P. oleovorans* contains the genes for scl-PHA synthesis. Ashby et al. (2004) reported that it produced solely PHB from crude glycerol obtained from soybean oil biodiesel production process. The PHB content was more than 30% of dry weight.
*Pa. denitrificans* is another bacterium that can accumulate PHB at high percentages (70% of dry mass). This bacterium can use a variety of carbon substrates for PHB synthesis including glucose, fructose, glycerol and crude glycerol (Mothes *et al.* 2007). The ability to utilise cheap carbon substrate makes this bacterium an interesting candidate for PHB production.

In recent years, knowledge in the field of genetic engineering and molecular microbiology have been exploited to enhance PHB production in micro-organisms (Suriyamongkol *et al.* 2007). The genes associated with PHB synthesis (*pha* genes) isolated from various PHB producers, such as *C. necator* and *Al. latus*, have been introduced into *Escherichia coli* and expressed. It has been reported that PHB accumulation level in recombinant *E. coli* can be as high as that in the natural producers. *E. coli* also has several advantages over wild-type PHA producers including faster growth, utilisation of a wide range of carbon sources and the fragility of cells which allows easy recovery of the polymer (Choi and Lee 1999; Ahn *et al.* 2001). Moreover, since *E. coli* does not possess PHA depolymerase, the synthesised PHB is not degraded during the production processes. Molecular weight of PHB produced by recombinant *E. coli* can also be controlled (Choi and Lee 1999; Wondu Holding Pty Ltd. 2004; Suriyamongkol *et al.* 2007).

**PHB synthesis in eukaryotic cells**

*Saccharomyces cerevisiae* harbouring the PHA synthase gene from *C. necator* was used to demonstrate the production of PHB in eukaryotes. The PHB accumulation, however, is very low (0.5% of dry weight) (Leaf *et al.* 1996). There has also been an attempt to use insect cells as a model for PHB production. Fall armyworm cells (*Spodoptera frugiperda*) were transfected with modified eukaryotic fatty acid synthase, which did not extend fatty acid beyond hydroxybutyric acid, and *phaC* gene from *C. necator*. The PHB accumulation was observed, but at very low level (less than 1% of cell dry weight) (Williams *et al.* 1996). Despite low level of PHB production, studies of PHA formation in yeast and insect cells can provide useful information about how to incorporate PHA synthesis pathway into plants (Suriyamongkol *et al.* 2007).
**PHB synthesis in transgenic plants**

PHA production in plants is considerably less expensive than in micro-organisms because plants only rely on water, soil nutrients, CO$_2$ and sunlight, while micro-organisms grow under sterile conditions in costly fermentation processes (Suriyamongkol *et al.* 2007). The first attempt on the PHB production in plants was conducted on *Arabidopsis thaliana* harbouring the PHA genes (*phaB* and *phaC*) of *C. necator*. However, PHB accumulation was low. When all the genes (*phaA*, *phaB* and *phaC*) were expressed in the chloroplast of *Ar. thaliana*, the PHB accumulation was improved to 14% of dry weight (Steinbüchel and Füchtenbusch 1998). Genetically modified (GM) oilseed, such as GM soybean and GM rapeseed, were also used to produce up to 85% yield of PHB, with oil and meal as co-products (Bohlmann 2004). Apart from oilseed, Somleva *et al.* (2008) reported the use of switchgrass (*Panicum virgatum* L.) harbouring multiple-gene pathway for the synthesis of PHB. The PHB content was found to be 3.72% of dry weight in leaf tissues. A number of crops have been tested for PHAs productions. These include maize, sugarcane, flax, cotton, alfalfa, tobacco, potato and oilseed crops (rapeseed, sunflower and soybean) (Braunegg *et al.* 1998; Somleva *et al.* 2008).

It has been projected that bioplastics derived from plants would have the potential to compete with petroleum-based plastics in terms of price if the yields of PHAs were 20% to 50% of the crop (Wondu Holding Pty Ltd. 2004). The cost of plant-derived PHAs production will depend upon several factors for instance crop used, content of PHAs and ease of extraction. On the basis of market prices for corn starch, soybean oil, sucrose and glucose of between 0.25-0.5 USD/kg, Steinbüchel and Füchtenbusch (1998) anticipated that PHB may be produced at less than 0.5 USD/kg.

**2.6 Kinetics of bacterial growth**

PHB synthesis in *C. necator* is generally considered to be non-growth associated which implies that the production of PHB is not directly influenced by the rate of growth but rather by cell concentration. However, experimental results indicate that the commencement of PHB accumulation occurs during the growth phase (Mulchandani *et al.* 1989). It is therefore possible to use the product formation model originally proposed
by Luedeking and Piret (1959) to describe the accumulation of PHB since the model incorporates both growth associated and non-growth associated terms (Mulchandani et al. 1989; Qaderi et al. 2012). The Luedeking-Piret model is shown in Equation 2.1.

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X$$  \hspace{1cm} (2.1)

Where $P$ is the product concentration (g/L), $t$ is fermentation time (h), $X$ is the cell concentration (g/L), $\alpha$ is the growth associated constant (g_product/g_cell) and $\beta$ is the non-growth associated constant (g_product/g_cell·h).

The term $\alpha \frac{dX}{dt}$ represents the growth associated product formation and the term $dX/dt$ represents the rate of cell growth. During the PHB accumulation phase, the bacterial cell components consist of two main parts i.e. PHB ($P$) and the residual biomass ($X_R$). This residual biomass is still catalytically active and therefore responsible for the metabolic activity of the cells. For this reason, Equation 2.1 should strictly be rewritten to represent the accumulation of PHB in the cells as shown in Equation 2.2.

$$\frac{dP}{dt} = \alpha \frac{dX_R}{dt} + \beta X_R = \alpha \frac{d(X-P)}{dt} + \beta(X-P)$$  \hspace{1cm} (2.2)

However, the basic of the Luedeking-Piret model (Equation 2.1) is normally used to represent the system (Qaderi et al. 2012). The rate of cell growth, $dX/dt$, is equal to $\mu X$, where $\mu$ is the specific growth rate and can be related to the substrate concentration by Monod’s equation (Equation 2.3).

$$\mu = \frac{\mu_{\text{max}} S}{K_s + S}$$  \hspace{1cm} (2.3)

Where $\mu_{\text{max}}$ is the maximum specific growth rate (h$^{-1}$), $S$ is the concentration of limiting substrate (g/L) and $K_s$ is the concentration of substrate for which $\mu$ is equal to $\mu_{\text{max}}/2$ (g/L). $K_s$ is also known as the Monod constant. The relationship between $\mu$ and $S$ is represented graphically in Figure 2.15.
The Monod equation implies that the growth of a micro-organism is not inhibited by the substrate however high the substrate concentration is. However, in reality, high concentrations of substrate can suppress microbial growth. This is known as substrate inhibition and can be caused by changes in medium physicochemical properties, such as osmotic pressure, ionic strength, dielectric constant, etc. These changes can alter the cell membrane activity as well as changes in adsorption or complexation between enzymes, coenzymes and substrates which in turn affect the enzyme activity (Mulchandani et al. 1989). Such effects can be incorporated into the Monod equation, for example, as shown in Equation 2.4, where an inhibition constant ($K_i$) is used to account for the effect. Plots of Equation 2.4 with varying $K_i$ values are shown in Figure 2.16. Note that the equation can vary in form depending on several factors such as microbial strain and cultivation conditions.

$$
\mu = \frac{\mu_{\text{max}} S}{K_s + S + K_i S^2}
$$  \hspace{1cm} (2.4)

**Figure 2.15** Graphical representation of Equation 2.3.
Specific growth rate ($h^{-1}$)

Equation 2.4

<table>
<thead>
<tr>
<th>Ki</th>
<th>0.001</th>
<th>0.01</th>
<th>0.05</th>
<th>0.1</th>
</tr>
</thead>
</table>

Figure 2.16 Specific growth rate influenced by substrate concentrations according to Equation 2.4.

Inhibition can occur at a cellular level. Overproduction of one type of molecule by one pathway can result in an inhibition called feedback inhibition where the product produced by one pathway inhibits the enzyme that catalyses an early step of the pathway, resulting in that metabolic pathway switching off. Micro-organisms use this mechanism to control their biosynthesis pathway. The benefit of this type of inhibition is that the micro-organism can avoid wasting nutrient resources (Gerhart and Pardee 1962). An example of this phenomenon is the Pasteur effect, which occurs in yeasts (Mulchandani and Luong 1989). Since yeasts are generally facultative anaerobes, they can produce energy by two metabolic pathways, glycolysis and the citric acid cycle. When the yeast is cultivated under an anaerobic conditions, the product of glycolysis pathway, pyruvate, is turned into ethanol and carbon dioxide. The energy produced from glycolysis is low, 2 adenosine triphosphate (ATP) per molecule of glucose. Whereas when it is grown under aerobic conditions, pyruvate is converted into acetyl-CoA and used in the citric acid cycle. Complete oxidation of glucose to CO$_2$ under aerobic conditions yields 30-32 ATP per molecule of glucose, about 15 times as much energy as is produced by glycolysis (Nelson and Cox 2008). The ATP produced from the citric acid cycle acts as an inhibitor for phosphofructokinase-1 in glycolysis. As a consequence, the yeast switches from glycolysis to respiration (Mulchandani and Luong 1989).

Another type of microbial inhibition is product inhibition. The accumulation of product formed during the cultivation of a micro-organism can affect cell growth as well as
product formation. At sufficiently high concentrations, many metabolic products, such as ethanol, butanol, acetone, lactic acid, etc. can completely stop cell growth (Mulchandani and Luong 1989; Sinclair and Cantero 1990). An example of product inhibition is ethanol inhibition in yeast fermentation. In this case, ethanol acts as a non-competitive inhibitor on microbial growth. This can be demonstrated by addition of ethanol to log phase yeast cultures, which results in reduction in maximum specific growth rate but not $K_s$ (Mulchandani and Luong 1989). The mechanism for ethanol toxicity is possibly due to its effects on protein synthesis. Cell viability is also affected by increasing concentrations of ethanol, as enzymes in the cells are irreversibly denatured. Figure 2.17 shows an example of the reduction in specific growth rate of *Saccharomyces cerevisiae* influenced by increasing concentrations of ethanol.

![Figure 2.17](image)

**Figure 2.17** Specific growth rate of *Saccharomyces cerevisiae* inhibited by increasing concentrations of ethanol as a product of fermentation (data taken from Han and Levenspiel (1988)).

In an attempt to describe such inhibition in microbial growth, many researchers have proposed kinetic models as shown in Table 2.13 (Han and Levenspiel 1988; Mulchandani and Luong 1989).
Table 2.13 Examples of substrate and product inhibition models reported in literature.

<table>
<thead>
<tr>
<th>Equation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Substrate inhibition models</strong></td>
<td></td>
</tr>
<tr>
<td>[ \mu = \mu_{\text{max}} \frac{S(1 + S/K)}{K_s + S + S^2/K_{si}} ]</td>
<td>a</td>
</tr>
<tr>
<td>[ \mu = \mu_{\text{max}} \frac{1}{1 + (K_s/S) + (S/K_1)} ]</td>
<td>b</td>
</tr>
<tr>
<td>[ \mu = \mu_{\text{max}} \frac{S}{K_s + S + (S^2/K_2)} ]</td>
<td>b</td>
</tr>
<tr>
<td>[ \mu = \mu_{\text{max}} \frac{S}{K_s + S} \exp(-S/K_3) ]</td>
<td>b</td>
</tr>
<tr>
<td>[ \mu = \mu_{\text{max}} \frac{S}{K_s + S}; \text{when } S &lt; S' ]</td>
<td>b</td>
</tr>
<tr>
<td>[ \mu = \mu_{\text{max}} \frac{S}{K_s + S} - K_4(S - S'); \text{when } S &gt; S' ]</td>
<td>b</td>
</tr>
<tr>
<td>[ \mu = \mu_{\text{max}} \left(1 - \frac{S}{S'}\right)^n \frac{S}{S + K_s(1 - S/S')^m} ]</td>
<td>b</td>
</tr>
<tr>
<td><strong>Product inhibition models</strong></td>
<td></td>
</tr>
<tr>
<td>[ \mu = \mu_{\text{max}} \frac{S}{K_s + S} \left(1 - (K \times P)\right) ]</td>
<td>b</td>
</tr>
<tr>
<td>[ \mu = \mu_{\text{max}} - K(P - K_1) ]</td>
<td>b</td>
</tr>
<tr>
<td>[ \mu = \mu_{\text{max}} \frac{1 - P}{P'} \frac{S}{K_s + S} ]</td>
<td>b</td>
</tr>
<tr>
<td>[ \mu = \mu_{\text{max}} \frac{S}{K_s + S} \exp(-K \times P) ]</td>
<td>b</td>
</tr>
<tr>
<td>[ \mu = \mu_{\text{max}} \left(K_s \left(1 + \frac{K_1}{K_1 + P}\right) \right) ]</td>
<td>b</td>
</tr>
<tr>
<td>[ \mu = \mu_{\text{max}} \left(1 - \frac{P}{P'}\right)^{0.5} \frac{S}{K_s + S} ]</td>
<td>b</td>
</tr>
<tr>
<td>[ \mu = \mu_{\text{max}} \left(1 - \frac{P}{P'}\right)^n \frac{S}{K_s + S} ]</td>
<td>b</td>
</tr>
</tbody>
</table>

**Nomenclature**

- \( \mu \) The specific growth rate (time\(^{-1}\))
- \( \mu_{\text{max}} \) The maximum specific growth rate (time\(^{-1}\))
- \( S \) The substrate concentration (substrate/volume)
- \( S' \) The critical substrate concentration, above which reaction cannot proceed (substrate/volume)
- \( K_s \) The Monod constant (substrate/volume)
- \( K_{si} \) The substrate inhibition constant (substrate/volume)
- \( P \) The Product concentration (product/volume)
- \( P' \) The critical product concentration, above which reaction cannot proceed (product/volume)
- \( K, K_1, K_2, K_3, m, n \) Constants

a: data taken from Mulchandani and Luong (1989)
b: data taken from Han and Levenspiel (1988)
2.7 Conclusion

Although the growing biodiesel industry gives an alternative for customers to reduce the use of petroleum-based diesel, the vast amount of residues generated from the production processes also cause problems in waste management. In Europe, the majority of biodiesel is processed from rapeseed from which rapeseed meal and crude glycerol are generated as by-product streams. Although these can be used in several applications, such as in animal feed industry, utilising them in this way may not be economically sustainable and they are often therefore regarded as wastes.

Production of value-added products from these wastes can help mitigate the problem of waste management and at the same time help sustain the biodiesel production process. The concept of converting agricultural residues including rapeseed meal to a generic feedstock by solid-state fermentation (SSF) followed by hydrolysis of fermented solids to be used in fermentations has long been developed in the Satake Centre for Grain Process Engineering (SCGPE). For example, a generic feedstock produced from wheat by the use of Aspergillus awamori has been successfully developed (Webb and Wang 1997). This feedstock was subsequently used to produce polyhydroxybutyrate (PHB) by Cupriavidus necator (Xu 2007). The present research was an extension of this previous study and exploited the information presented in this chapter to develop a process for producing a value-added product from rapeseed biodiesel by-products. Aiming at utilising rapeseed meal and crude glycerol to produce PHB, several objectives were set and are presented in Chapter 3.
CHAPTER 3

Objectives and experimental programme

The overall goal of the project reported in this thesis was to demonstrate the feasibility of utilising rapeseed biodiesel by-products to produce value-added chemicals, taking polyhydroxybutyrate (PHB) as a target product as mentioned in Chapter 1. The experimental work was divided into two major parts, investigating the bioconversion of rapeseed biodiesel by-products into PHB and the inhibitory effects of methanol on the bacterial growth. Within the major aim, several objectives were set as follows:

- To adapt the bacterium \textit{C. necator} to be able to grow on glycerol
- To fully investigate the effect of methanol on growth of \textit{C. necator}
- To produce nutrient-rich media from rapeseed meal for use as nutrient source
- To investigate the effect of rapeseed meal-derived media on growth of \textit{C. necator}
- To produce PHB using feedstock derived from rapeseed biodiesel by-products

For each of the above objectives an experimental programme was designed and is detailed as follows:

\textit{To adapt the bacterium \textit{C. necator} to be able to grow on glycerol}

This study was carried out to acquire knowledge about the bacterial growth on crude glycerol as a carbon source. \textit{Cupriavidus necator} was cultivated in mineral medium supplemented with varied concentrations of crude glycerol to observe its ability to grow on this carbon source. Adaptation of the bacterium to high crude glycerol concentrations might be required to improve its growth rate. Other aspects on carbon source \textit{i.e.} the effect of carbon to nitrogen ratios and the effect of methanol on the bacterial growth were also investigated.
To fully investigate the effect of methanol on growth of C. necator

It is interesting to study the effect of methanol on the growth of the micro-organism since, at high levels, methanol impurities in the crude glycerol stream could significantly affect the growth. The work conducted in this part of the project was a preliminary study to confirm if methanol does have a negative effect on C. necator growth. A full investigation was subsequently carried out in an attempt to develop a model to describe the effect. In order to observe this effect, C. necator was grown in media containing increasing concentrations of methanol (0-125 g/L). Due to the large number of concentrations tested, these experiments were conducted at flask scale with three samples being taken per concentration. To study the growth of the bacterium in media containing methanol, specific growth rate at each methanol concentration was determined. The results obtained provided the information for the development of a model to describe the effect. To test the applicability of the developed model, sets of data for microbial growth in media containing alcohols were extracted from publications and used to fit with the model.

To produce nutrient-rich media from rapeseed meal for use as nutrient source

In this research, crude glycerol was used as both a carbon source for growing the bacterium and as a carbon substrate for PHB production, whereas rapeseed meal was used as a nutrients source. However, due to the solid, heterogeneous nature of rapeseed meal, a transformation of this substrate into a liquid form was necessary to be able for this to be used in submerged fermentations. A two-step process, which had previously been developed in the SCGPE, consisting of solid-state fermentation (SSF) by a fungus followed by hydrolysis of the fermented solids was employed for this purpose. A vigorous proteolytic enzyme producer, Aspergillus oryzae, was used since rapeseed meal contains high levels of protein. Observations and determination of some parameters were made to follow the growth of the fungus and the production of key components. The nutrient-rich solution obtained from this two-step process was used as source of nutrients for growing C. necator in a combination with crude glycerol as a main carbon source. However, since this solution was not formulated, it might have had some effect on the bacterial growth. It was therefore worth investigating the effect of this solution on growth of the bacterium to find suitable conditions.
To investigate the effect of rapeseed meal-derived media on growth of C. necator

The principal component in the nutrient-rich media obtained from the two-step process is free amino nitrogen (FAN). This FAN was to be used as an indicator to follow the utilisation of nitrogen source in this research. For this reason, the work carried out in this part of the project focused on the effect of initial concentration of FAN on growth of C. necator. The bacterium was grown in the nutrient-rich media at different initial concentrations supplemented with synthetic crude glycerol, which was formulated to mimic real crude glycerol. Growth of the bacterium in terms of dry cell weight as well as FAN utilisation was determined and compared to those obtained from systems containing yeast extract and synthetic crude glycerol. The result obtained from this study was used together with those obtained from the previous studies to grow C. necator and produce PHB in the most suitable conditions.

To produce PHB using feedstock derived from rapeseed biodiesel by-products

To produce PHB, C. necator was grown in the nutrient-rich media supplemented with crude glycerol. Fermentations were carried out in fed-batch mode by periodic addition of carbon source into the system. Bacterial growth and nutrient utilisation as well as PHB production were observed then compared with those obtained from various systems (mineral medium and yeast extract solution supplemented with synthetic crude glycerol and the nutrient-rich solution supplemented with either pure glycerol or synthetic crude glycerol). A preliminary estimate of economic feasibility, based on the results obtained from the study, was evaluated. An overall production scheme for PHB using feedstock derived from rapeseed biodiesel production process was then developed.

Based on the objectives and descriptions given above, an overview of the entire experimental programme can be visualised and is shown in Figure 3.1. Each of the studies is reported in a separate chapter of the thesis and this is also indicated in Figure 3.1.
Figure 3.1 Research scheme for the project with indication of where in the thesis results are reported.
CHAPTER 4

Growth of *Cupriavidus necator* DSM4058

4.1 Introduction

Growth characteristics of a micro-organism are amongst the factors for which information is needed if the micro-organism is to be fully exploited. Knowing what each micro-organism does or does not require can help in manipulating conditions in which the micro-organism is to be grown. Growing the micro-organism in its desired, or optimum, condition can boost its performance, resulting in better growth or productivity which in turn reduces the dissipation of energy and cost of production. In this study the bacterial strain of *Cupriavidus necator* DSM4058 was used. As it was to be cultivated in media containing crude glycerol as the main carbon source and hydrolysate derived from rapeseed meal as the nitrogen source (discussed in Chapter 7) with no established data about this available, it was necessary to study its growth behaviour in order to be able to grow it in the most suitable condition.

In this chapter, only aspects involving the growth of *C. necator* on crude glycerol are discussed. Although not all, some of the important ones are included in this chapter. These begin with the reactivation of the lyophilised bacterium (Section 4.4.1) followed by study of the bacterial growth in a range of crude glycerol concentrations (Section 4.4.2). The kinetic parameters of growth were also determined in order to predict/fit the experimental data. It was also interesting to know whether serial sub-cultivation in unfavourable conditions could improve the ability of the bacterium to grow under such conditions. Therefore an experiment, designed to investigate that, was performed and is discussed in Section 4.4.3. In addition, since it can be present in crude glycerol as an impurity, the effect of methanol on growth of the bacterium is also taken into account. Methanol is well known for its toxicity to many micro-organisms (Whittenbury *et al.* 1970). Growth of *C. necator* in the media containing a range of methanol is described in Section 4.4.4. In addition, the effect of carbon to nitrogen ratio on growth of the bacterium was investigated and is discussed in Section 4.4.5. All
results obtained were used as fundamental knowledge for growing *C. necator* in subsequent experiments.

### 4.2 Materials

#### 4.2.1 Micro-organism

The bacterium strain *Cupriavidus necator* DSM4058 was used in this study. It was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), Germany as lyophilised cells. General information about this bacterium is presented in Section 2.4.

After the reactivation and adaptation, the culture of *C. necator* that was capable of growing at a concentration of at least 48 g/L crude glycerol was stored at −30°C using 15% dimethyl sulfoxide (DMSO) as cryopreservative for further experiments.

#### 4.2.2 Crude glycerol

The crude glycerol used in this study was provided by Double green Ltd., Hull, UK. It was stored in sealable glass bottle at room temperature. Its partial compositions are presented in Section 8.4.1.

#### 4.2.3 Culture media

A complex medium, nutrient broth, was used to reactivate the lyophilised cells as recommended by the supplier. After that the bacterium was sub-cultivated in mineral medium which was comprised of (per L) Na$_2$HPO$_4$ 2.44 g, KH$_2$PO$_4$ 1.52 g, (NH$_4$)$_2$SO$_4$ 0.5 g, MgSO$_4$.7H$_2$O 0.2 g, CaCl$_2$.2H$_2$O 0.05 g. Either crude or pure glycerol (≥99% pure with ≤0.1% sulfated ash and ≤1.0% water, Sigma-Aldrich) was used as sole carbon source at various concentrations. Tap water was used instead of distilled water in order to compensate the lack of some trace elements in the media. All media were sterilised at 121°C for 20 min. After the sterilisation cycle completed, flasks containing sterile medium were vigorously shaken to dissolve any precipitate before leaving to cool to room temperature prior to use.
4.3 Methods

4.3.1 Reactivation and storage of C. necator

The lyophilised C. necator was reactivated by serial sub-cultivation in nutrient broth. Firstly, a small portion of the lyophilised bacterium was aseptically transferred from an ampoule into universal bottles containing 10 mL of nutrient broth. The culture was incubated at 30°C until the media became turbid. After that, the culture was sub-cultivated twice further in nutrient broth using 10% inoculum size. The active bacterium was used as inoculum for preparation of the bacterial stock culture in nutrient broth. After 2 days of incubation in nutrient broth, the cells were harvested by centrifugation at 10,000 rpm (Eppendorf, Centrifuge 5804) for 10 min then re-suspended in nutrient broth containing 15% DMSO as cryopreservative. The culture was stored at −30°C as the stock cell culture.

The bacterium obtained after an experiment with 48.08 g/L crude glycerol was also preserved for using as the stock culture for further experiments. For a short term storage, the culture was streaked onto mineral agar (3.5% (w/v) agar) containing 48.08 g/L crude glycerol and incubated for 5-6 days until single colonies were clearly observed then stored at 4°C. The bacterium was sub-cultivated every month to maintain its viability. For long term storage, the bacterium was grown in mineral medium containing 48.08 g/L crude glycerol for 2 days. Then it was harvested by centrifugation and re-suspended in cryopreservation medium consisting of mineral medium supplemented with 50 g/L pure glycerol and 15% DMSO then stored at −30°C.

4.3.2 Growth of C. necator and improvement of bacterial growth in mineral media containing glycerol as sole carbon source

Inoculum cultures were prepared by growing the reactivated bacterium in sterile mineral media at 30°C for 18 h in an orbital shaker (Gallenkamp) set at 200 rpm. In order to study the bacterial growth at various crude glycerol concentrations, 500 mL Erlenmeyer flaks containing 150 mL mineral media supplemented with 1.92 g/L crude glycerol were inoculated with 3 mL of 18-hour-old inoculum cultures then incubated at 30°C, 200 rpm for 2-3 days. The cultures obtained after these fermentations were then used as further inocula for serial sub-cultivation in media containing higher crude glycerol.
concentrations. The concentration of crude glycerol was gradually increased from 1.92 to 48.08 g/L through 13 sequential batch fermentations. Samples were taken periodically to measure optical density at 600 nm (OD$_{600}$) (Shimadzu, UV mini 1240). A growth curve of the bacterium at each crude glycerol concentration was generated and used to determine its specific growth rate.

In order to adapt the bacterium to high glycerol concentrations, cultures obtained at 48.08 g/L glycerol were serially sub-cultivated in mineral media containing 48.08 g/L crude glycerol for 6 cycles to improve its specific growth rate.

4.3.3 Inhibitory effects of methanol on growth of *C. necator*

To investigate the inhibitory effects of methanol, 500 mL Erlenmeyer flasks containing 100 mL mineral media supplemented with methanol in the range 0 to 12.08 g/L, along with 50 g/L pure glycerol and 50 g/L NaCl, were inoculated with 3 mL of 18-hour-old inoculum culture and incubated at 30°C in an orbital shaker (200 rpm) for 48 h. Samples were taken hourly during the first 8 h and then periodically afterward for optical density measurement at 600 nm. All inocula were freshly prepared using medium with no methanol, to avoid acclimatisation of the bacterium to the methanol. All experiments were done in triplicate and the results are reported as average values with standard deviation.

4.4 Results and discussions

4.4.1 Reactivation and storage of *C. necator*

The bacterium was successfully reactivated after being serially sub-cultivated in nutrient broth for 3 cycles. Figure 4.1 shows the first batch culture of lyophilised *C. necator* in nutrient broth after being incubated for 5 days. After being serially sub-cultivated in nutrient broth and adapted in glycerol-based media as described earlier, the bacterium was preserved for both short term and long term storage. For short term storage, after clear single colonies with diameters of at least 1 mm were observed, universal bottles containing agar slants and Petri dishes with those single colonies were sealed with Parafilm and stored at 4°C. Figure 4.2 shows the appearance of the bacterial single
colonies. Each colony is in circular form with entire edge, white opaque in colour and smooth appearance. However, during fermentation on agar media, it was noticed that the bacterium grew very slowly and only a few single colonies were observed. It was possible that the high agar concentration (3.5% w/v) used in this experiment affected the growth of the bacterium. This might be because the agar was too rigid for the bacterium to grow on. However, when lower agar concentration (2% w/v) was used, the agar failed to retain its shape when Petri dishes were tilted. Some researchers have investigated the effect of agar concentration on growth of some bacteria and reported that agar concentration has little effect in the early stages of cell growth but tended to yield higher cell numbers at lower concentration, especially for motile bacteria. In addition, it was found that increased agar concentration resulted in smaller colony diameter (Mitchell and Wimpenny 1997).

For long term storage, after centrifuging and resuspending in cryopreservation medium, 1 mL of cell suspension was transferred into each sterile 1.5 mL microcentrifuge tube, sealed with Parafilm and stored at −30°C.

![Figure 4.1](image.jpg) Reactivated *C. necator* in nutrient broth.
4.4.2 Growth of *C. necator* in mineral medium containing glycerol as sole carbon source

The active bacterium obtained after reactivation in nutrient broth was grown in mineral media containing crude glycerol ranging from 1.92 to 48.08 g/L to observe its ability to grow on crude glycerol. Growth curves at the various concentrations of crude glycerol are illustrated in Figure 4.3.

It should be noted that the media obtained after sterilisation were not completely clear. Slightly high optical density values of the medium at the beginning were caused by...
precipitation during sterilisation. This made the medium slightly opaque. The decrease in optical density after shaking the media might, therefore, be achieved by dissolution of the salts. Apart from that, addition of crude glycerol to the media affected its colour. Since the colour of crude glycerol is dark brown, it changed media colour from colourless to slightly yellowish. In order to measure the optical density accurately, a blank was prepared by adding crude glycerol to tap water at the same concentration as that in each experiment. To avoid the change of turbidity, it was centrifuged at 10,000 rpm for 10 min prior to the measurement.

From Figure 4.3, it is obvious that crude glycerol at a concentration of 1.92 g/L was not favourable for cell growth. A short exponential phase coupled with significantly lower cell concentration in terms of optical density suggested that this concentration was too low to favour cell growth. Increases in concentration of crude glycerol to 5.77 g/L and above resulted in higher cell concentration and longer exponential phase. However, it was noticed that increased crude glycerol concentration also resulted in longer lag phase. This effect was clearly seen when the concentration of crude glycerol was increased to more than 32 g/L. The lag phase of the growth beyond this concentration was found to be very much longer than at the lower concentrations. This might be because of substrate inhibition (discussed later in this section), or possibly the inhibitory effect of methanol, which was present in crude glycerol as an impurity (the effect of methanol on growth of the bacterium is discussed in detail in Chapter 5). Despite longer lag phase, cell concentrations at the end of experiments were similar to those obtained at lower concentrations.

In order to compare the growth of the bacterium at each concentration of crude glycerol, growth curves were analysed to determine specific growth rate ($\mu$) from a log-linear plot of optical density versus concentration of crude glycerol (Figure 4.4).
Chapter 4  Growth of *C. necator*
Figure 4.4  Log-linear plots of *C. necator* growth in mineral media containing 1.92 to 48.08 g/L crude glycerol, based on optical density measurements.

The specific growth rate of the bacterium at each concentration was determined by fitting the steepest region (log phase of growth) of each curve with an exponential equation, which in turn generated the value of \( \mu \) according to the following equation.

\[
X = X_0 e^{\mu t}
\]  \hspace{1cm} (4.1)

Where  
- \( X \) is the cell concentration (OD unit)  
- \( X_0 \) is the initial cell concentration (OD unit)  
- \( \mu \) is the specific growth rate (h\(^{-1}\))  
- \( t \) is time after inoculation (fermentation time) (h)
In the log-linear plot, Equation 4.1 is obviously a straight line, for which the gradient is equal to $\mu$. However, since the determination of the gradient is dependent on which data points are used, and this is a subjective decision, average values of $\mu$ (along with standard deviations) based on 2 or 3 overlapping ranges of data per growth curve were determined. At least 3 data points were included in each determination. Figure 4.5 shows an example set of data with the several lines used to determine average $\mu$.

\[ \text{Crude glycerol concentration (g/L)} \]

**Figure 4.5** Example set of data with lines used to determine values of $\mu$ from which an average $\mu$ was calculated.

\[ \text{Specific growth rate (h}^{-1}) \]

**Figure 4.6** Specific growth rates of *C. necator* grown in mineral media containing 1.92 to 48.08 g/L crude glycerol.
A plot of $\mu$ against crude glycerol concentration demonstrates the effect on the growth as shown in Figure 4.6. It is likely that the optimum concentration of crude glycerol for cell growth was in the range 15-25 g/L. Specific growth rate of the bacterium tended to increase when concentration of crude glycerol was increased up to 21.15 g/L. The highest $\mu$ of all 13 fermentations was 0.126 h$^{-1}$ obtained when 17.31 g/L crude glycerol was used. Increasing the concentration beyond 21.15 g/L was found to negatively influence the growth as $\mu$ declined and finally dropped to 0.084 h$^{-1}$ when 48.08 g/L crude glycerol was used. Extrapolation of the graph, starting at 21.15 g/L, would suggest that the bacterium would stop growing completely at about 116 g/L crude glycerol. The result suggests that growth of the bacterium is dependent upon crude glycerol concentration since it was the only independent variable in this study. This result agrees well with a report by Zeng et al. (1994) which states that many chemicals including glycerol have inhibitory effect on the growth of Clostridium butyricum and Klebsiella pneumonia. Although C. necator was not used in Zeng’s study, it was stated that the inhibitory effect of the substrate in the glycerol fermentation was irrespective of strain tested.

The data of average $\mu$ and crude glycerol concentration were used to determine maximum specific growth rate ($\mu_{\text{max}}$) and Monod constant ($K_s$). Three different methods, namely Lineweaver-Burk plot, Hanes-Woolf plot and Eadie-Hofstee plot were used. The $\mu_{\text{max}}$ and $K_s$ were then subsequently used in the Monod equation (Equation 4.5) to fit the experimental data. All three linearised versions of the Monod equation have advantages and disadvantages. For example, the Eadie-Hofstee plot is, although not very good at predicting $\mu_{\text{max}}$, efficient for predicting $K_s$ (Berges et al. 1994). The Lineweaver-Burk and Hanes-Woolf plots, on the other hand, are good at predicting $\mu_{\text{max}}$ (Bailey and Ollis 1986). Fitting data with different methods can result in different or even contradictory outcomes (Berges et al. 1994), therefore a comparison of the results from the various methods will benefit the determination of the kinetic parameters.

The first method, the Lineweaver-Burk or double reciprocal plot (Equation 4.2), is a plot of 1/reaction velocity (in this case, specific growth rate) against 1/substrate concentration. This classical method is prone to error as the Y-axis takes the reciprocal of the rate of reaction which in turn increases any small error in measurement (Doran 1995). Moreover, since it unevenly weights data points, it exaggerates the influence of
low substrate concentrations. These are furthest from the origin and will tend to determine the slope of the plot (Bailey and Ollis 1986). Typically, this plot gives a straight line with Y-axis intercept and slope equal to \(1/\mu_{\text{max}}\) and \(K_s/\mu_{\text{max}}\) respectively. But in this study the graph did not yield a straight line. Instead, it gave a V-shaped plot which bends upwards sharply as it approaches the Y-axis. This hinted that there was substrate inhibition (Schulz 1994). Hence, to determine \(1/\mu_{\text{max}}\) and \(K_s/\mu_{\text{max}}\), the linear portion of the curve (at crude glycerol concentration between 1.92 and 21.15 g/L) was extrapolated to the Y-axis and the intercept and slope of the line were interpreted as \(1/\mu_{\text{max}}\) and \(K_s/\mu_{\text{max}}\) respectively (Figure 4.7). Maximum specific growth rate and \(K_s\) obtained from this approach were 0.1259 h\(^{-1}\) and 0.8127 g/L respectively.

\[
\frac{1}{\mu} = \frac{K_s}{\mu_{\text{max}} S} + \frac{1}{\mu_{\text{max}}} \tag{4.2}
\]

Where  
\(\mu\) is the specific growth rate (h\(^{-1}\))  
\(\mu_{\text{max}}\) is the maximum specific growth rate (h\(^{-1}\))  
\(K_s\) is the Monod constant (g/L)  
\(S\) is the substrate concentration (g/L)

![Figure 4.7 Lineweaver-Burk plot of C. necator specific growth rate in mineral media containing 1.92 to 48.08 g/L crude glycerol.](image)

The Second method, the Hanes-Woolf method, is a plot of the ratio of substrate concentration to the reaction velocity against the substrate concentration (Equation 4.3). An advantage of this method is that it spreads out the data for higher values of \(\mu\)
resulting in quite accurate determination of slope (Bailey and Ollis 1986). The Hanes-Woolf plot gives Y-axis intercept and slope equal to $K_s/\mu_{\text{max}}$ and $1/\mu_{\text{max}}$ respectively.

The range of crude glycerol concentration used to determine the kinetic parameters was the same as that used for the Lineweaver-Burk plot. Maximum specific growth rate obtained from this method (Figure 4.8) was 0.1297 $h^{-1}$ while $K_s$ was 1.1408 g/L.

\[
\frac{S}{\mu} = \frac{S}{\mu_{\text{max}}} + \frac{K_s}{\mu_{\text{max}}} \tag{4.3}
\]

![Figure 4.8](image.png) Hanes-Woolf plot of \textit{C. necator} specific growth rate in mineral media containing 1.92 to 48.08 g/L crude glycerol.

The last method used to determine $\mu_{\text{max}}$ and $K_s$ was the Eadie-Hofstee plot (Equation 4.4). This method can be used to determine $K_s$ effectively. However, as both ordinate and abscissa of the plot are dependent on the reaction velocity, any experimental error will be present in both axes and could lead to large errors (Bailey and Ollis 1986). The Eadie-Hofstee plot interprets Y-axis intercept and slope as $\mu_{\text{max}}$ and $-K_s$ respectively (Figure 4.9). Similar to the previous two methods, the range of crude glycerol concentration used was between 1.92 and 21.15 g/L. The $\mu_{\text{max}}$ and $K_s$ obtained was 0.1262 $h^{-1}$ and 0.8109 g/L respectively.
\[ \mu = -K_s \frac{\mu}{S} + \mu_{\text{max}} \] (4.4)

![Graph showing specific growth rate vs. crude glycerol concentration](image)

**Figure 4.9**  Eadie-Hofstee plot of *C. necator* specific growth rate in mineral media containing 1.92 to 48.08 g/L crude glycerol.

It is interesting that although all these three methods are weighted on different variables or parameters, they gave close values of \( \mu_{\text{max}} \) and \( K_s \) with standard deviation (SD) of 0.0021 and 0.19 for \( \mu_{\text{max}} \) and \( K_s \) respectively as shown in Table 4.1.

**Table 4.1**  Maximum specific growth rates and Monod constants obtained from Lineweaver-Burk, Hanes-Woolf and Eadie-Hofstee plots.

<table>
<thead>
<tr>
<th>Method</th>
<th>Maximum specific growth rate (h(^{-1}))</th>
<th>Monod constant (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lineweaver-Burk plot</td>
<td>0.1259</td>
<td>0.8127</td>
</tr>
<tr>
<td>Hanes-Woolf plot</td>
<td>0.1297</td>
<td>1.1408</td>
</tr>
<tr>
<td>Eadie-Hofstee plot</td>
<td>0.1262</td>
<td>0.8109</td>
</tr>
<tr>
<td>Average</td>
<td>0.1273</td>
<td>0.9215</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.0021</td>
<td>0.19</td>
</tr>
</tbody>
</table>

The \( \mu_{\text{max}} \) and \( K_s \) values obtained from all three methods were used in the Monod equation (Equation 4.5) to fit the experimental data. The result shown in Figure 4.10 reveals that specific growth rate of *C. necator* in the media containing crude glycerol is reasonably predictable up to around 20 g/L. However, the data cannot be predicted by the Monod equation at higher concentrations.
Specific growth rate \( (\mu) \) of \( C. \) necator fitted with curves obtained from the Monod equation using parameters from the Lineweaver-Burk, Hanes-Woolf and Eadie-Hofstee plots.

Figure 4.10

It is worth noting that the Lineweaver-Burk and Eadie-Hofstee plots gave similar \( \mu_{\text{max}} \) and \( K_s \) values, so the lines of the two equations are similar. From Figure 4.10 it is obvious that the growth of \( C. \) necator was inhibited at concentrations of crude glycerol higher than about 20 g/L. As hinted by the V-shape plot in the Lineweaver-Burk plot, a substrate inhibition equation of the form proposed by Han and Levenspiel (1988) could be useful in describing the observed data. Equation 4.6 was, therefore, tested to fit the experimental data.

\[
\mu = \mu_{\text{max}} \left( 1 - \frac{S}{S^*} \right)^n \frac{S}{S + K_s \left( 1 - \frac{S}{S^*} \right)^m}
\]  

(4.6)

Where

- \( \mu \) is the specific growth rate (h\(^{-1}\))
- \( \mu_{\text{max}} \) is the maximum specific growth rate (h\(^{-1}\))
- \( S \) is the substrate concentration (g/L)
- \( S^* \) is the substrate concentration that completely inhibits cell growth (g/L)
- \( K_s \) is the Monod constant (g/L)
- \( n \) and \( m \) are constants
The graph fitted with the Han and Levenspiel equation is shown in Figure 4.11. Although the equation did not fit the entire range of experimental data, it usefully predicted the specific growth rate of the bacterium at concentrations above where inhibition begins (21 g/L).

![Graph showing specific growth rates of C. necator](image)

**Figure 4.11** Specific growth rates of *C. necator* showing the model by Han and Levenspiel (1988) (Equation 4.6) for concentrations above 21 g/L

According to the Han and Levenspiel method of determining $\mu_{\text{max}}$ and $K_s$, a slightly higher $\mu_{\text{max}}$ of 0.1525 h$^{-1}$ and a lower $K_s$ of 0.7987 g/L compared to those obtained from the previous three equations were obtained. Nevertheless, the important thing that this plot confirms is that there was significant inhibition during the fermentations. For this reason if this bacterium were to be used at 50 g/L glycerol, it would be necessary to improve its tolerance to such a high concentration.

In order to improve the tolerance of the bacterium in terms of specific growth rate, it was serially sub-cultivated in mineral media containing 48 g/L crude glycerol as described below.

### 4.4.3 Improvement of the specific growth rate of *C. necator* by serial sub-cultivation

Since the substrate inhibition reduced the growth rate of *C. necator* by more than 30%, good growth in medium containing more than 48 g/L crude glycerol will be difficult to achieve. Therefore, an attempt to adapt the bacterium to grow at high concentrations of
crude glycerol was conducted, based on an assumption that serial sub-cultivation at the high crude glycerol concentration would help it to adapt. The results showed that the bacterium did indeed gradually adapt itself to tolerate high crude glycerol concentration through only 6 cycles of sub-cultivation (Figure 4.12). However, it was noticed that in the 6\textsuperscript{th} cycle, for which the inoculum was taken from the 5\textsuperscript{th} cycle at 24 h, the optical density values took longer to increase. This was mainly due to low cell concentration in the inoculum. This effect was studied earlier by Xu (2007). It was reported that both age and size of seed culture have an effect on growth of the culture. However, when plotted on a log-linear scale, no increase in lag phase was observed (Figure 4.13D).

![Figure 4.12](image)

**Figure 4.12**  Growth of *C. necator* serially sub-cultivated in mineral media containing 48.08 g/L crude glycerol.

Log-linear plots of the bacterial growth in media containing 48.08 g/L crude glycerol are shown in Figure 4.13. It was found that the lag phase was shortened from over 30 h to only 6 h after 3 successive sub-cultivations.
Figure 4.13 Improvement of *C. necator* growth after sub-cultivation for 1 cycle (A), 3 cycles (B), 5 cycles (C) and 6 cycles (D).

Using the same approach as explained for Figure 4.6, values of $\mu$ were determined and reported as averages (along with standard deviations) based on 2-3 overlapping ranges of data per growth curve. The results are shown in Figure 4.14.

Figure 4.14 Improvement of specific growth rate of *C. necator* after being serially sub-cultivated in mineral media containing 48 g/L crude glycerol.

Figure 4.14 shows the dramatic improvement in $\mu$ after serial sub-cultivation for 6 cycles. The $\mu$ obtained after the 6th cycle was more than 2.4 times that of the first cycle. The cells obtained from cycle 6 were preserved in cryopreservation medium at $-30^\circ$C as a new stock culture for subsequent experiments.
Although the growth of *C. necator* was inhibited at higher than 21 g/L crude glycerol, it was observed that methanol, which was present as an impurity, also has an inhibitory effect on the micro-organism. It was suspected that the inhibition occurring in this study might be the result of combined substrate and methanol inhibition. Therefore the effect of methanol on the growth of the bacterium was investigated.

### 4.4.4 Inhibitory effects of methanol on growth of *C. necator*

As an impurity left over from biodiesel production, methanol may have a significant effect on growth or performance of micro-organisms, if crude glycerol were to be used as the fermentation substrate. The objective of this study was to investigate the effect of increasing methanol concentration (0 to 12 g/L, equivalent to 0 to 25% (v/v) methanol in crude glycerol) on the specific growth rate of *C. necator*.

Growth of *C. necator* in mineral media containing 0 to 12.08 g/L methanol is shown in Figure 4.15. It can be seen that increasing the amount of methanol affected the rate at which the bacterium could grow (in terms of optical density). Note that since there were discontinuities between 8 h and 24 h of fermentations, dotted lines were used to connect the data points during this period. Methanol also affected the final cell concentration (at 48 h when the fermentation was stopped) as shown below.

![Figure 4.15](image)

*Figure 4.15* Growth of *C. necator* in mineral media containing 50 g/L glycerol with various amounts of methanol.
The specific growth rate of the bacterium at each methanol concentration was determined from log-linear plots of optical density against fermentation time using the same method as described in Section 4.4.2. All the data points shown in Figure 4.16 are the average values of optical density calculated from 3 replicate samples.

![Log-linear plots of optical density at 600 nm against fermentation time of C. necator grown in mineral media containing 50 g/L glycerol and 0 to 12.08 g/L methanol.](image)

Figure 4.16 Log-linear plots of optical density at 600 nm against fermentation time of C. necator grown in mineral media containing 50 g/L glycerol and 0 to 12.08 g/L methanol.

The plot of specific growth rate against methanol concentration clearly shows a reduction in specific growth rate as the amount of methanol present in the medium increased (Figure 4.17). The highest $\mu$ of 0.42 h$^{-1}$ was obtained from an experiment with 0 g/L methanol and it tended to decline continually as methanol concentration was increased. This suggested that methanol has an inhibitory effect on the growth of the bacterium. The result shown in Figure 4.17 led to a further investigation of this effect which is discussed, more fully, in Chapter 5.
Chapter 4  Growth of C. necator

4.4.5 Effect of carbon to nitrogen ratio on growth of C. necator

Based on the formula of mineral medium with 50 g/L glycerol as the sole carbon source (Section 4.2.2), the C/N ratio used to grow the bacterium was 197. The ratio of C to N found generally in bacterial cells is only 3.3-5 (Lawford and Rousseau 1995), so the ratio of 197 seems to be excessive. However, the objective of this research was to produce PHB from crude glycerol, rather than cells, so the high ratio should encourage PHB production. It was interesting, therefore, to check whether small changes in C/N ratio would significantly affect the growth of the bacterium. Thus, a study of ratios between 197 and 236 was conducted.

Apart from fixing the concentration of glycerol, a constant methanol concentration (48 g/L) was also used. Methanol at this concentration was chosen because, from Figure 5.4, it gave a middle value of C. necator specific growth rate, having a moderate inhibitory effect. Three C/N ratios of 197, 210 and 236 were used to explore the effect on growth. These ratios covered the range resulting from the addition of methanol used in the study of its inhibitory effects on C. necator growth (Chapter 5) and were used to check that the effects reported in Chapter 5 were not due to changes in C/N ratio. To achieve the desired C/N ratios, varied amounts of (NH₄)₂SO₄ of 0.469, 0.439 and 0.391 g/L were used. The results are shown in Figure 4.18.
The growth profiles of the bacterium during the first 7 h were almost identical. The lag
phase was only about 1 h. After this, the cell concentration in terms of optical density
increased exponentially from 2 to 7 h. Since no samples were taken between 7 h and
24 h of fermentation, dotted lines are used to link the data points in this period. The
bacterium entered the stationary phase at 30 h and all the optical density values at 48 h
were similar.

The specific growth rate of the bacterium during the first 7 h was determined using the
same approach as described earlier and it can be seen in Figure 4.19 that the values were
very similar.

Figure 4.18  Growth of *C. necator* grown in mineral media with C/N ratio of 197, 210 and 236.

Figure 4.19  Specific growth rate of *C. necator* in media containing various C/N ratios.
A one-way analysis of variance (one-way ANOVA) with a significance level of 0.05 was performed to analyse the difference between these values of $\mu$. The analysed result revealed that there was no significant difference between the data points.

### 4.5 Conclusion

The bacterium, *Cupriavidus necator* DSM4058, with an ability to synthesise and accumulate polyhydroxybutyrate (PHB) was selected, based on the literature survey, for research into growth and PHB production using glycerol as substrate. After being re-activated, growth behaviour of the bacterium was investigated over a range of substrate and inhibitor concentrations. The plot of *C. necator* specific growth rate against crude glycerol concentration fitted with the Monod equation revealed that crude glycerol had an inhibitory effect on growth, which could be categorised as substrate inhibition (Figure 4.10). The inhibition started at 21 g/L crude glycerol and the effect was greater when higher levels of crude glycerol were present in the medium. From the range studied, it appeared that the optimum concentration of crude glycerol for growing *C. necator* was between 15 and 25 g/L. In order to predict the specific growth rate of the bacterium at concentrations beyond 21 g/L, a substrate inhibition equation of the form proposed by Han and Levenspiel (1988) was used and gave a regression coefficient of 0.83. However, most of the predicted values were lower than the empirical values (Figure 4.11).

*C. necator* was shown to be able to acclimatise to unfavourable growth conditions due to high substrate concentration (50 g/L crude glycerol). Serial sub-cultivation proved effective for gradually adapting the bacteria to grow at such high concentration. The specific growth rate improved 2.4 times from 0.09 to 0.21 h$^{-1}$ (Figure 4.14) and the bacterial culture was stored for use as a new stock culture for subsequent experiments. In addition, the effect of methanol on growth was investigated. It was found that methanol had an inhibitory effect on cell growth and also caused a reduction in final cell concentration (Figure 4.17). This led to a full investigation of this effect which is discussed in the next chapter.

Finally, over the range studied, increasing C/N ratio was found to have no effect on either cell concentration (Figure 4.18) or specific growth rate (Figure 4.19). Reducing
the concentration of nitrogen source by 16.6% resulted in the same final cell concentration and the same rate of growth. This information could be useful in a large scale production as the concentration of crude glycerol can vary.
CHAPTER 5

Inhibitory effects of methanol on the growth of

*Cupriavidus necator*

5.1 Introduction

As discussed in Chapter 4, since methanol can negatively influence the growth of *C. necator*, further investigation was of interest. Despite many kinetic models of both substrate and product inhibition having been proposed, there is no generic form of model available to describe these phenomena. They are likely to differ depending on microbial strain and cultivation conditions (Mulchandani and Luong 1989). In addition, although methanol is widely known for its toxicity to a number of micro-organisms (Çelik et al. 2008), only a handful of reports on the kinetics of its toxicity are available. To maintain high cell activity in inhibitor-containing systems, understanding microbial performance is important. It is therefore desirable to describe or, better still, predict the behaviour of a micro-organism in such a system.

In this chapter, experiments designed to investigate the effect of methanol on the growth of *C. necator* are reported (Section 5.4.1). An attempt was also made to develop a model to predict the specific growth rate of the organism in media containing methanol (Section 5.4.2). It was hoped that this could be used for prediction of methanol toxicity on micro-organisms in general. A set of growth data was fitted with various forms of product inhibition equation. A saturation equation, which was developed in this study, was also used. Finally, the applicability of the proposed model was tested with several sets of extracted data from various publications (Section 5.4.3).
5.2 Materials

5.2.1 Micro-organism

The bacterium *Cupriavidus necator* DSM4058, which was capable of growing at 50 g/L glycerol, was used. Inocula were prepared by transferring thawed stock culture into a mineral medium containing 50 g/L glycerol with no methanol and incubated at 30°C for 48 h. The culture was then sub-cultivated and incubated at 30°C for 18 h.

5.2.2 Culture medium

The bacterium was grown in mineral medium which comprised (per L) Na$_2$HPO$_4$ 2.44 g, KH$_2$PO$_4$ 1.52 g, (NH$_4$)$_2$SO$_4$ 0.5 g, Mg$_2$SO$_4$.7H$_2$O 0.2 g, CaCl$_2$.2H$_2$O 0.05 g. Pure glycerol was used as sole carbon source at a concentration of 50 g/L. Tap water was used instead of distilled water in order to compensate the lack of some trace elements in the medium. Methanol at concentrations between 0 and 125 g/L were used. The medium was dispensed into 500 mL Erlenmeyer flasks, which were then plugged with cotton wool and covered with aluminum foil (held in place by masking tape) then sterilised at 121°C for 20 min. After the sterilisation cycle, flasks containing sterile medium were vigorously shaken to dissolve any precipitate before leaving to cool to room temperature prior to use. To avoid the risk of contamination, samples were not taken at this stage and so the initial methanol concentrations were not confirmed as those calculated and used for the presentation of the results.

5.3 Method

To investigate the inhibitory effects of methanol in more detail than in the experiment discussed in the previous chapter, the concentration of methanol was varied over the range 0 to 125 g/L. Inocula (3 mL) from an 18 h culture were transferred into 500 mL Erlenmeyer flasks containing 100 mL mineral media supplemented with methanol, along with 50 g/L pure glycerol and 50 g/L NaCl. Incubation was carried out at 30°C in an orbital shaker (200 rpm) for 48 h. Samples were taken hourly during the first 8 h and then periodically afterward for optical density measurement. All inocula were freshly prepared using medium with no methanol, to avoid any adaptation of the bacterium to
Inhibitory effects of methanol on *C. necator* growth

All fermentations were carried out in triplicate and the results are reported as average values with standard deviation.

### 5.4 Results and discussions

#### 5.4.1 Growth of *C. necator* in the presence of methanol

As discussed earlier in Section 4.4.4, increasing the methanol concentration caused a reduction in *C. necator* growth. This effect was more obvious when the concentration was increased further up to 48 g/L (Figure 5.1). As a result, the gradient of the curves decreased with increasing methanol concentration. It was also observed that the optical density values at 48 h for any concentration above 25 g/L methanol were less than half the value at 0 g/L. Note that due to the wide range of concentrations studied, this experiment was carried out in several sets. These resulted in the discontinuities seen in Figure 5.1 but did not alter the overall trends observed.

![Figure 5.1](image-url)

**Figure 5.1** Growth of *C. necator* in mineral media containing 50 g/L glycerol and 0 to 48.34 g/L methanol.

Log-linear plots at each methanol concentration (see Appendix A) were used to determine $\mu$ using the same method as described in Section 4.4.2. The values of $\mu$ were
then plotted against methanol concentration to demonstrate the inhibitory effects as shown in Figure 5.2. The highest $\mu$ of 0.42 h$^{-1}$ was obtained from the experiment with 0 g/L methanol. It was noticed that methanol at all the concentrations tested had a negative influence on growth rate, indicating that the bacterium does not utilise methanol as a carbon source. Over the range 0 to 48 g/L, it appeared that the reduction in specific growth rate was more or less linearly related to concentration. A similar observation had previously been reported by Swartz and Cooney (1981) for the growth of a yeast.

![Graph showing specific growth rate vs methanol concentration]

**Figure 5.2** Specific growth rate of *C. necator* in mineral medium containing 50 g/L glycerol and 0 to 48.34 g/L methanol. Data have been fitted by the Swartz and Cooney linear model.

If the graph in Figure 5.2 is extrapolated, no growth should be observed when the methanol concentration is increased to around 90 g/L. To investigate this, the concentration of methanol was increased further up to 125 g/L. Surprisingly, the bacterium was still able to grow, even at this high level, although the optical density values were very low as shown in Figure 5.3.
Figure 5.3  Growth of *C. necator* in mineral media containing 50 g/L glycerol and 0 to 125 g/L methanol.
As can be seen from the figure, the growth at 125 g/L methanol was very low and $\mu$ was only 0.0279 h\(^{-1}\). The extended plot of specific growth rate against methanol concentration (Figure 5.4) reveals the continued reduction in the growth as the concentration of methanol was increased. At lower than 10 g/L, methanol caused only a slight reduction in $\mu$ though the effect was greater when the concentration was increased up to 90 g/L. Beyond 90 g/L, increasing the methanol concentration had very little further effect on cell growth. This might suggest a limit to the toxicity of methanol on \textit{C. necator}. Note that where no variation bar is shown is it is because the standard deviation is smaller than the data symbol.

![Graph showing specific growth rate of C. necator grown in mineral medium containing 50 g/L glycerol and 0 to 125 g/L of methanol.](image)

**Figure 5.4** Specific growth rate of \textit{C. necator} grown in mineral medium containing 50 g/L glycerol and 0 to 125 g/L of methanol.

The log-linear plots of the bacterial growth (Appendix A) also revealed relatively small increases in the lag phase, as methanol concentration was increased. However, extension to the lag phase was never more than about 2 h over the whole range of methanol concentrations tested. From these results, it can be inferred that the long lag phase observed in earlier experiments (Section 4.4.2) was caused by the increasing crude glycerol concentration (substrate inhibition) rather than by the presence of methanol.
5.4.2 Fitting the data

In order to predict $\mu$ for the bacterium, the simple linear model is clearly no longer sufficient. Different forms of product inhibition equation including a saturation equation were tested to fit the extended set of experimental data. The results are shown in the following section.

Tests of product inhibition equations

The product inhibition equations were considered potentially applicable because the concentration of methanol, in this study, was gradually increased. This is similar to product formation during fermentations e.g. ethanol production (Sangkharak 2011). Figure 5.5 shows that the predicted $\mu$ calculated using an equation proposed by Ghose and Tyagi (Equation 5.1) (Han and Levenspiel 1988) did not fit with the data. Using the value of $\mu_{\text{max}}$ obtained from the experiment (0.4204 h$^{-1}$) and $K_s$ which was obtained from Han and Levenspiel’s method (0.0855 g/L), the model predicted that $\mu$ would decrease sharply from 0.42 to 0 if the concentration of methanol were to be increased from 0 to 90 g/L as shown in Figure 5.5.

$$
\mu = \mu_{\text{max}} \left(1 - \frac{C_R}{C_R^*} \right) \frac{C_A}{K_s + C_A}
$$

Where $\mu$ is the specific growth rate (h$^{-1}$)  
$\mu_{\text{max}}$ is the maximum specific growth rate (h$^{-1}$)  
$C_R$ is the inhibitor concentration (g/L)  
$C_R^*$ is the critical inhibitor concentration above which growth is zero (g/L)  
$C_A$ is the substrate concentration (g/L)  
$K_s$ is the Monod constant (g/L)  
n and m are constants

Bazua and Wilke (1977) proposed an equation to describe the effect of ethanol, as a product, on a continuous fermentation with Saccharomyces cerevisiae (Equation 5.2). This model fitted the data for inhibition of C. necator reasonably well up to 22 g/L methanol (Figure 5.5). However, beyond 22 g/L, the model predicted that the growth would decrease more slowly and stop at about 90 g/L.
Chapter 5  Inhibitory effects of methanol on C. necator growth

\[
\mu = \mu_{\text{max}} \left(1 - \frac{C_R}{C_R^*}\right)^{0.5} \frac{C_A}{K_s + C_A}
\]  \hspace{1cm} (5.2)

The next equation tested was proposed by Levenspiel (Equation 5.3) \cite{Han1988}. The model fitted the data well up to 70 g/L methanol. However, it could not be used to describe the growth at concentrations lower than 4 g/L as it exaggerated the values of \(\mu\). Beyond 70 g/L, the model predicted that the growth would decrease sharply and reach zero at 90 g/L as shown in Figure 5.5.

\[
\mu = \mu_{\text{max}} \left(1 - \frac{C_R}{C_R^*}\right)^n \frac{C_A}{K_s + C_A}
\]  \hspace{1cm} (5.3)

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.5.png}
\caption{Specific growth rate of C. necator in mineral medium containing 50 g/L glycerol and 0 to 125 g/L methanol fitted using Equation 5.1, 5.2 and 5.3.}
\end{figure}

Note that the above three models are of the same form but with slight modification (different exponent). In 1988, Han and Levenspiel proposed a generalised Monod equation, based on the same form, which takes into account inhibition effect caused by either high concentration of substrate or cell, or product or other inhibitory substance. Equation 5.4 fits the data reasonably well up to about 48 g/L methanol. However, the
model predicted that the growth would continue to decrease sharply beyond 48 g/L methanol and it would stop completely at about 80 g/L as seen in Figure 5.6. Unfortunately, although this equation seems to be more developed compared to the previous three equations, it did not fit the whole range of data.

\[
\mu = \mu_{\text{max}} \left(1 - \frac{C_R}{C_R^*}\right)^n \frac{C_A}{C_A + K_s \left(1 - \frac{C_R}{C_R^*}\right)^m}
\]

(5.4)

Figure 5.6  Specific growth rate of *C. necator* in mineral medium containing 50 g/L glycerol and 0 to 125 g/L methanol fitted using Equation 5.4.

A proposed alternative model

Since none of the equations tested above could accurately predict the entire range of methanol inhibition, it was decided to consider more fundamentally what mechanism might be involved in the inhibition effect.

As a gram negative bacterium, *C. necator* will take up glycerol by energy-independent diffusion via an integral membrane protein, *i.e.* a glycerol facilitator (Darbon *et al.* 1999). Figure 5.4 shows clearly that the presence of methanol affects the growth of *C. necator* in a negative way. If the methanol, as a ligand molecule, were to bind to the glycerol facilitator, it might reduce its affinity for glycerol. In this way it could hamper
the ability to transport glycerol into the bacterial cell, thereby slowing growth. The more methanol that is present, the greater the effect would be. However, in Figure 5.4, it is clear that at concentrations higher than 90 g/L, the methanol had little further effect on growth rate. This could be because most of the glycerol facilitators had become saturated by methanol molecules. It is possible therefore that a saturation type model might describe the inhibitory effect of methanol on *C. necator* better than those presented above.

A saturation equation (Equation 5.5), originally used by Hill (1910) to describe the binding of oxygen to haemoglobin, was adapted to model the specific growth rate.

\[
Y = \frac{Y_{\text{max}}X^\alpha}{C^\alpha + X^\alpha}
\]  

(5.5)

Where  \( Y \) is the dependent variable,  \( x \) is the independent variable,  \( y_{\text{max}} \),  \( C \) and  \( \alpha \) are parameters.

This equation, related in form to the logistic function, is widely used to analyse receptor occupancy, especially in biochemistry, physiology and pharmacology (Weiss 1997; Goutelle *et al.* 2008). It was rewritten to be used in this study as follows (Equation 5.6).

\[
\mu = \frac{\mu_{\text{max}}S^b}{C^b + S^b}
\]  

(5.6)

Where  \( \mu \) is the specific growth rate (h\(^{-1}\))  
\( \mu_{\text{max}} \) is the maximum specific growth rate (h\(^{-1}\))  
\( S \) is the methanol concentration (g/L)  
\( C \) is the concentration of methanol that results in half \( \mu_{\text{max}} \) (g/L)  
\( b \) is the Hill coefficient, which should in principle be an integer.

The implication of the adapted form of the equation is that the ratio \( \mu/\mu_{\text{max}} \) is directly proportional to the fractional availability of facilitator binding sites. In other words, if the availability of the facilitator is reduced to half its original value, because of blockage by methanol molecules, then the specific growth rate will be similarly reduced to half its original value.

By using the value of \( \mu_{\text{max}} \) obtained from the fermentations (0.4204 h\(^{-1}\)) and the appropriate value for  \( C \) from Figure 5.4 (46 g/L), the Hill coefficient,  \( b \), for this system could be determined using the Solver function in Microsoft Excel. The best-fit value of
−2.06 is very close to −2 and so a value of −2 was used in modelling the system (Figure 5.7). Despite this approximation, the resultant model still gave an excellent fit with a regression coefficient of 0.985 as shown in Figure 5.7.

**Figure 5.7** Specific growth rate of *C. necator* grown in mineral medium containing 50 g/L glycerol and 0 to 125 g/L methanol predicted using the proposed model (Equation 5.6).

Compared to the other equations tested, this proposed model gives by far the best fit, particularly up to 80 g/L methanol. It was noticed that at higher than 90 g/L, the model slightly overestimated the values of *µ*. However, as mentioned earlier, obtaining the curve gradient involves a subjective judgement, which may result in inexact values of *µ*. The negative value of the Hill coefficient (−2) indicated negative cooperativity between ligands (methanol) and receptors (glycerol facilitator) (Yifrach 2004). This occurs when a ligand molecule binds to a receptor and reduces the affinity for other ligands. In this case it might reduce the ability to transport glycerol into the bacterial cell. The value of the coefficient (−2) indicates that in order to efficiently bind to a receptor, two molecules of methanol are required (Figure 5.8). However, beyond 90 g/L, increasing methanol concentration did not affect the growth rate further. This was probably because, according to the equation, most of the glycerol facilitators were by now saturated with methanol molecules, so only a small further amount of glycerol could be taken up.
Further examination of the literature has shown that a methane metabolism is present in *Ralstonia eutropha* (now *C. necator*). In that pathway, formaldehyde (CH$_2$O) is an intermediate, produced from methanol and transformed into formic acid (CH$_2$O$_2$) (Kanehisa Laboratories 2010). The accumulation of formaldehyde or formic acid could alter the structure of membrane proteins (Rigomier *et al.* 1980; Swartz and Cooney 1981). This affects the oxidative pathway that bacteria use to assimilate glycerol (Babel and Hofmann 1982) and membrane-associated enzyme activity such as electron transport and oxidative phosphorylation (Ingram 1976; Fletcher 1983). This alteration of cell membrane protein could result in malfunction of the glycerol facilitator. Increasing the methanol concentration would result in a higher degree of membrane protein deterioration as shown in Figure 5.9. Note that, from the value of the coefficient, it might also need two formaldehyde molecules to inactivate a glycerol facilitator.
5.4.3 Applicability of the proposed model to other systems

The applicability of Equation 5.6 was tested for other systems. Sets of data extracted from publications by Swartz and Cooney (1981), Converti et al. (2000), Han and Levenspiel (1988), Caldwell and Murray (1986) and Wayman and Tseng (1976) were used.

Example 1

Swartz and Cooney reported the growth of yeast, *Hansenula polymorpha*, in synthetic media containing 4 to about 40 g/L methanol (Figure 5.10).
Chapter 5  Inhibitory effects of methanol on C. necator growth

The authors reported that at less than 1 g/L methanol, the growth of the yeast obeyed the Monod equation and the inhibition began at 6.5 g/L methanol. Yeast growth decreased linearly and, according to the extrapolation of the graph starting at 6.5 g/L, the growth would stop completely at a concentration of around 58 g/L. A linear equation proposed by the authors is shown below.

\[
\mu = 0.225 - 2.87 \times 10^{-3} S
\]

Where  
\( \mu \) is the specific growth rate (h\(^{-1}\))
\( S \) is the methanol concentration (g/L)

The above equation satisfactorily fitted the plot in the range 6.5 to 40 g/L methanol as shown in Figure 5.10. The authors also stated that the growth stopped completely at 80 g/L methanol. However, as experienced from this study, the growth of microorganisms might not completely stop. So Equation 5.6 was used to fit the extracted data, using the Hill coefficient of −2, to demonstrate the other possibility of the yeast growth. The fitted curve with regression coefficient of 0.9733 is shown in Figure 5.10.

As seen from the figure, Equation 5.6 can be used to successfully predict the growth of H. polymorpha and extrapolation suggests that growth would decrease exponentially

---

**Figure 5.10**  Specific growth rate of H. polymorpha influenced by methanol, fitted with linear equation and Equation 5.6.
and would not completely stop at 80 g/L methanol as the authors suggested. However, the growth rate at this stage would be very low.

**Example 2**

A set of data extracted from a publication by Converti et al. (2000) was also fitted reasonably well by Equation 5.6 using the Hill coefficient of −1 (Figure 5.11). The extracted data were for the maximum specific productivity ($V_{\text{max}}''$ as appeared in the publication) of xylitol by yeast, *Candida guilliermondii*, inhibited by methanol. The equation fitted the values of $V_{\text{max}}''$ well over the range 0-100 g/L methanol with the regression coefficient of 0.985.

![Graph](image)

**Figure 5.11** Maximum specific productivity of xylitol in the presence of methanol by *C. guilliermondii* fitted with Equation 5.6.

**Example 3**

Data for ethanol production in continuous fermentation with *Saccharomyces cerevisiae* was extracted from a publication by Bazua and Wilke (1977). The regression coefficient obtained when Equation 5.6 was used was 0.9753 (with the Hill coefficient of −4). The model predicts that specific growth rate would decrease exponentially and reach 0.01 h$^{-1}$ at 150 g/L methanol as shown in Figure 5.12.
Chapter 5  Inhibitory effects of methanol on *C. necator* growth

![Graph showing specific growth rate vs. ethanol concentration](image)

**Figure 5.12** Specific growth rate of *S. cerevisiae* inhibited by ethanol as a product of fermentation fitted with Equation 5.6.

**Example 4**

The effect of ethanol on growth yield of *Bacteroides ruminicola* subsp. *brevis* strains GA33 and B_{14} (Caldwell and Murray 1986) was also estimated. Equation 5.6 fitted well with the extracted data. The regression coefficient for strain GA33 was 0.9885 (with the Hill coefficient of −4) and for B_{14} was 0.9992 (Hill coefficient of −12). However, it was noticed that despite the high regression coefficient the model exaggerated the value of yield at low inhibitor concentrations (for strain B_{14}). This could be a problem when dealing with inhibitors that can be consumed by micro-organisms. For this reason, employing a substrate inhibition equation might be preferred at low inhibitor concentrations.
**Example 5**

A set of data for *Pseudomonas methanica* growing on methanol in the range 1.5 to 50 mL/L was extracted from a publication by Wayman and Tseng (1976). Equation 5.6 with the Hill coefficient of −2 gave a very good fit, with regression coefficient of 0.9832 (Figure 5.14). The model predicted that \( \mu \) would decrease from the highest value and the growth would not stop at less than 120 mL/L methanol. This differed from the result reported by the authors as their model predicted that the growth would rise from about 0.01 to 0.2 h\(^{-1}\) (\( \mu_{\text{max}} \)) then decrease as a function of methanol concentration to zero at around 55 mL/L. This was because the model proposed by the authors considered methanol as the substrate for the bacterium while the saturation model treats it as an inhibitor.
Chapter 5  Inhibitory effects of methanol on C. necator growth

Figure 5.14  The growth of P. methanica inhibited by methanol, fitted by Equation 5.6.

5.5 Conclusion

Growth of micro-organisms depends on many factors (Mulchandani and Luong 1989) and can be inhibited in three ways i.e. the presence of an inhibitor, increasing the substrate concentration (substrate inhibition) and increase in the product concentration (product inhibition) (Wayman and Tseng 1976). As an inhibitor, increasing the concentration of methanol in the media caused the reduction in specific growth rate (μ) of C. necator. The overall effect was clearly seen when the methanol concentration was increased up to 125 g/L. It is important to note that the concentrations of methanol were not measured and so the values reported are the concentrations that were made up before autoclaving. Since it is possible that some methanol could have evaporated following sterilisation, the concentrations reported represent maximum values. The final cell concentration (at 72 h of fermentation) was also affected. This was considered due to the low rate of bacterial propagation caused by methanol inhibition.

The specific growth rate of the bacterium decreased as a function of methanol concentration and could not be predicted with a simple linear equation. Attempts were made to fit the experimental data with various product inhibition equations from the literature, but all failed. This was probably because methanol was not the product of the
fermentation. However, a proposed model based on the saturation (or logistic) equation was successfully used to fit the data with relatively high regression coefficient compared to other inhibition equations.

The inhibitory effect of methanol was also confirmed by the study of the effect of C/N ratio on the growth (Section 4.4.5). The reduction in nitrogen source concentration caused by increasing the concentration of methanol did not have any effect on specific growth rate at a significance level of 0.05 (Section 4.4.5). This confirmed that the reduction in $\mu$ was solely caused by increasing concentration of methanol.

The applicability of the proposed equation (Equation 5.6) was tested with several sets of data extracted from publications. All data sets were fitted well with the proposed saturation model regardless of micro-organism or type of alcohol inhibitor (regression coefficient of over 97%). From the results, it could be concluded that this equation can be used to describe or predict the specific growth rate in systems containing methanol or ethanol. However, it was found that the model did not fit perfectly at low concentrations of inhibitor if the inhibitor could be utilised as a substrate. In this case, the classic substrate limitation model (the Monod equation) may be more applicable. The prediction of specific growth rate (or even specific productivity) in some cases can be helpful in dealing with substrates containing methanol. As pre-treatment of the substrate can add to overall production costs, knowing the growth rate at actual concentrations of methanol in the substrate would help a producer to make a better decision regarding substrate preparation.
CHAPTER 6

Production of nutrients from rapeseed meal

6.1 Introduction

Amongst several elements in microbial cells, nitrogen is often the key for cellular replication. It is a constituent of all proteins, which account for about 40-60% of cell dry weight (Christias et al. 1975; Moran et al. 2010). Although micro-organisms are able to utilise nitrogen from a wide range of organic and mineral compounds, not all forms can be assimilated by all microbes. In the natural environment, complex nitrogenous substances have to be broken down into smaller units by extracellular enzymes secreted by the micro-organisms. These small units, in the form of free amino nitrogen (FAN) or oligopeptides may be either mineralised to ammonium ions (NH₄⁺) or taken up into cells directly (Geisseler et al. 2010). On the other hand, in laboratory or industrial culture, readily assimilable nutrients, either organic or inorganic, are provided. As for nitrogen source, both organic and inorganic substances can be used. Ammonium chloride (NH₄Cl), ammonium sulphate ((NH₄)₂SO₄) and ammonium nitrate (NH₄NO₃) are amongst the most common inorganic nitrogen sources used, while yeast extract, tryptone, peptone and meat extract are the more commonly used organic sources (Abou-Zeid et al. 1981; Kalil et al. 2008).

Several authors state that yeast extract is a good nitrogen source because it contains wide variety of amino acids, peptides, water-soluble vitamins and carbohydrates (Smith et al. 1975; Jackson et al. 1998). On the other hand, inorganic nitrogen sources probably provide only nutrients that are adequate to meet the minimum requirements for growth (Costa et al. 2002). Although yeast extract is effective and extensively used, it is expensive and therefore responsible for high production costs. Reducing the amount of yeast extract is not a solution, as lower concentrations would result in lower cell proliferation (Huang et al. 2011). Therefore, finding cheaper alternative sources of nitrogen may give rise to a cheaper production process.
The idea of using rapeseed meal as a substrate for the production of nutrients is not new. It has been proved feasible to produce a nitrogen-rich solution for use as a yeast extract substitute (Wang et al. 2010). In the present study, production of nutrients from rapeseed meal was carried out. First of all, some characteristics of the rapeseed meal, both chemical and physical, were determined (Section 6.4.1). Next, solid-state fermentation (SSF) of rapeseed meal was performed in order to produce nutrients. The production of key components was followed and results are discussed in Section 6.4.2. In order to make the most of the rapeseed meal, fermented solids obtained after SSF were subsequently hydrolysed to increase the FAN content. The results are discussed in Section 6.4.3. Finally, based on FAN content, the quality of the hydrolysate was compared with other nitrogen sources. The results are discussed in Section 6.4.4.

6.2 Materials

6.2.1 Rapeseed meal

Rapeseed meal was obtained from Brocklebank Oilseed Processing Division, Cargill PLC, Liverpool, UK. It was ground using a kitchen blender then passed through a 1.7 mm sieve to remove other debris and stored at room temperature in an air-tight plastic container. Prior to carrying out chemical analysis, the meal was ground with a small hammer mill (Glen Creston, type DFH48) fitted with a 0.8 mm sieve for 2-3 times for homogenisation and accurate sampling.

6.2.2 Micro-organism

The micro-organism used was a filamentous fungus, *Aspergillus oryzae*. It was isolated from a soy sauce starter provided by Amoy Food Ltd., Hong Kong. The fungal spores were stored on silica sand at −30°C. Working spore suspension was prepared at once and stored in 1.5 mL microcentrifuge tubes at −30 °C.

In order to prepare a spore suspension, a small portion of silica sand containing dormant spores of *A. oryzae* was suspended in sterile distilled water using a vortex mixer. The sand was left to settle for a few minutes, then less than 0.5 mL of the liquid phase was
transferred to 500 mL flasks containing 100 mL sporulation agar\(^5\). The flasks were then gently shaken to let the liquid spread over the entire agar surface. The incubation was carried out at 35°C. After the fungus had grown over the agar surface and green spores were produced, 15 mL sterile distilled water and a few sterile glass beads were transferred into the first of several flasks. The flask was then shaken vigorously to remove fungal spores. The resultant spore suspension was then aseptically transferred into a second flask to extract more spores. The washing process was carried out successively until all the flasks had been extracted. Another 15 mL sterile distilled water was added into the first flask again to wash residual spores. The process was done successively as described earlier. The spore suspension thus obtained was temporarily stored in a 50 mL bottle before one mL of the suspension was transferred into each of 20-30 sterile 1.5 mL micro-centrifuge tubes. The tubes were then sealed with Parafilm and stored at −30°C as the stock culture.

### 6.3 Methods

#### 6.3.1 Partial characterisation of rapeseed meal

Some characteristics of the meal were previously determined by Dr. Ruohang Wang, a former member of Satake Centre for Grain Process Engineering, School of Chemical Engineering and Analytical Science, Faculty of Engineering and Physical Sciences, The University of Manchester, UK. In this study, chemical characteristics including pH, starch, reducing sugars and glucose content were determined. As for physical characteristic, bulk and particle density of the meal were measured.

**Determination of starch content**

Starch content was determined using a Megazyme analysing kit (Megazyme International Ireland Ltd.). Approximately 0.1 g of rapeseed meal was suspended in 3 mL \(\alpha\)-amylase (300 U) in a centrifuge tube then installed in a boiling water bath for 6 min. After adding 4 mL sodium acetate buffer and 0.1 mL amyloglucosidase (200 U), the tube was placed in a 50°C water bath for 30 min. The tube was then centrifuged at 3,000 rpm for 10 min (Heraeus model Labofuge 300). The supernatant (0.1 mL) was

\(^5\) Sporulation agar consisted of 30-50 g/L rapeseed meal and 2.5% (w/v) agar
mixed with 3 mL GOPOD reagent then incubated at 50°C for 20 min. Glucose control and reagent blank were prepared by mixing 0.1 mL D-glucose standard solution (1 mg/mL) and 0.1 mL distilled water with 3 mL GOPOD reagent respectively. The absorbance values of the samples and glucose control were measured at 510 nm against the reagent blank. The starch content was calculated according to Equation 6.1 and expressed as percentage on a dry basis (db).

\[
\text{Starch} = A_{510} \times F \times 71 \times \frac{1}{1,000} \times \frac{100}{W} \times \frac{162}{180}
\]

(6.1)

Where \( \text{Starch} \) is the content of starch in sample (%)

\( A_{510} \) is the absorbance at 510 nm

\( F \) is \( \frac{100}{\text{Absorbance of 100 µg glucose}} \)

71 is the volume correction (0.1 mL taken from 7.1 mL)

\( \frac{1}{1,000} \) is the conversion factor for conversion of µg to mg

\( \frac{100}{W} \) is the factor to express starch as a percentage of meal weight

\( W \) is weight of sample (mg)

\( \frac{162}{180} \) is an adjustment from free glucose to anhydro-glucose

**Determination of reducing sugars content**

Free reducing sugars were extracted by suspending 3 g of rapeseed meal in 5 mL distilled water using a vortex mixer. The content was left at room temperature for 5 min then centrifuged at 3,000 rpm for 5 min. The supernatant was collected and used for the determination of reducing sugars.

The supernatant (1 mL) was mixed with 1 mL 3,5-dinitrosalicylic acid (DNS) reagent in test tubes then installed in a boiling water bath for 15 min. After cooling under running water, 9 mL distilled water was added into the tubes. The absorbance was measured at 540 nm against a blank. The reagent blank was prepared by using distilled water in place of the sample. Reducing sugars content was calculated according to Equation 6.2 and expressed as mg reducing sugar/g meal (db).
\[
Reducing \ sugars = A_{540} \times Slope \times DF \times \frac{V}{W}
\]

Where \(Reducing \ sugars\) is the concentration of reducing sugars (mg/g)  
\(A_{540}\) is the absorbance at 540 nm  
\(Slope\) is the slope obtained from a maltose standard curve  
\(DF\) is the dilution factor  
\(V\) is the volume of distilled water used to suspend rapeseed meal (mL)  
\(W\) is the weight of rapeseed meal (g)

**Determination of glucose content**

Glucose content was determined using a Megazyme analysing kit. Firstly, glucose was extracted by suspending 3 g of the meal in 10 mL \(\alpha\)-amylase (3,000 U) in centrifuge tubes then placed in a boiling water bath for 30 min. Secondly, 4 mL sodium acetate buffer and 1 mL amylloglucosidase (200 U) were added into the tubes and incubated at 50°C for 30 min. The content was then centrifuged at 3,000 rpm for 10 min. The supernatant (0.1 mL) was taken and mixed with 3 mL GOPOD reagent. The mixture was incubated at 50°C for 20 min. Absorbance values were measured at 510 nm against a reagent blank. Glucose control and reagent blank were prepared by mixing 0.1 mL D-glucose standard solution (1 mg/mL) and 0.1 mL distilled water with 3 mL GOPOD reagent respectively. Glucose content was calculated using Equation 6.3 and expressed as percentage (db).

\[
Glucose = A_{510} \times \frac{100}{Abs \ of \ glucose} \times 150 \times \frac{1}{1,000} \times \frac{100}{3,000}
\]

Where \(Glucose\) is the content of glucose in sample (%)  
\(A_{510}\) is the absorbance at 510 nm  
\(Abs \ of \ glucose\) is the absorbance value of standard glucose solution  
150 is the volume correction (0.1 mL taken from 15 mL)  
\(\frac{1}{1,000}\) is the conversion factor for converting µg to mg  
\(\frac{100}{3,000}\) is the factor to express starch as a percentage of meal weight  
3,000 is the weight of rapeseed meal in mg
**Measurement of pH**

The pH was measured by suspending 1 g of rapeseed meal in 10 mL distilled water in a universal bottle. The mixture was stirred for 1 hour at room temperature. The meal was allowed to settle before pH was measured.

**Determination of rapeseed meal density and voidage**

Bulk density is the density of a solid sample referred to the bulk volume occupied collectively by the solids. It was determined by transferring a certain amount of rapeseed meal into a pre-weighed measuring cylinder. Weight and volume of the meal were recorded then the bulk density was calculated using Equation 6.4.

\[
\rho_b = \frac{W_{\text{meal}}}{V_{\text{meal}}}
\]  
(6.4)

Where \( \rho_b \) is the bulk density of rapeseed meal (g/mL)  
\( W_{\text{meal}} \) is the weight of rapeseed meal (g)  
\( V_{\text{meal}} \) is the bulk volume of rapeseed meal (mL)

Particle density of the meal was determined using the conventional water displacement method. A certain amount of rapeseed meal was transferred into a pre-weighed measuring cylinder, the weight was recorded. Distilled water was added into the cylinder to flood the sample then the total weight and total volume were recorded. In order to convert weight of water into volume, density of water at the temperature when the experiment was carried out was used. The calculated volume of water was then used to calculate the particle density using Equation 6.5.

\[
\rho_p = \frac{W_m}{V_{\text{total}} - \frac{W_w}{\rho_w}}
\]  
(6.5)

Where \( \rho_p \) is the particle density of rapeseed meal (g/mL)  
\( W_m \) is the weight of rapeseed meal (g)  
\( V_{\text{total}} \) is the total volume of rapeseed meal-water mixture (mL)  
\( W_w \) is the weight of distilled water (g)  
\( \rho_w \) is the density of water (g/mL)
The voidage of rapeseed meal was estimated using bulk and particle density and reported in percentage (Nimmo 2004) (Equation 6.6).

\[
\varepsilon = \left(1 - \frac{\rho_b}{\rho_p}\right) \times 100
\]

(6.6)

Where \( \varepsilon \) is the voidage (%)
\( \rho_b \) is the bulk density (g/mL)
\( \rho_p \) is the particle density (g/mL)

### 6.3.2 Production of nutrient-rich solution

The production of a nutrient-rich solution was carried out in two steps. Firstly, solid-state fermentation (SSF) of rapeseed meal by \( A. \) oryzae was conducted. The fermented matter obtained was then hydrolysed as described below.

**Solid-state fermentation of rapeseed meal**

A certain amount of rapeseed meal was moistened with the required amount of tap water to attain 65% moisture in a 1 L bottle. The content was sterilised at 121°C for 20 min and left overnight prior to inoculation. \( A. \) oryzae spore suspension (ca. \( 10^6 \) spores/g meal) was mixed with the meal using a sterile aluminium rod. After mixing well, 10-13 g of the content was transferred into disposable 9-cm Petri dishes and incubated at 35°C for 3 days. Samples were taken periodically by taking the whole content of Petri dishes. The samples were stirred in a small beaker and used for determination of moisture content, reducing sugars, free amino nitrogen (FAN) and protease activity. The experiment was conducted in triplicate.

Simultaneously, another set of experiments was carried out in order to measure the reduction in weight of fermenting solids over the course of the SSF. This was conducted in triplicate plus a blank dish (rapeseed meal with no inoculation) as a control. In order to measure the reduction in weight, all dishes including the blank were periodically weighed using a 4-decimal balance.
Hydrolysis of fermented solids

Fermented solids obtained from SSF were blended with the required amount of distilled water to attain a 10-15 g/L suspension (db) using a kitchen blender. The experiment was conducted by transferring the mixture into a screw cap bottle then incubating at 55°C for 3 days. The mixture was stirred intermittently to help mixing using a magnetic stirrer. About 10 mL mixture was taken periodically and centrifuged at 4,000 rpm for 10 min. The supernatants were used to determine reducing sugars and FAN concentration. The residues were used to measure dry matter weight.

Determination of moisture content

Moisture content of fermenting solid was measured by placing 3-4 g of sample into pre-dried and pre-weighed containers then drying at 95°C for 24 h. The containers with dry mass were weighed and moisture content was calculated according to Equation 6.7.

\[
M_C = \left(1 - \frac{W_2}{W_1}\right) \times 100
\]  

(6.7)

Where

- \(M_C\) is the moisture content (%)
- \(W_1\) is the weight of sample before drying (g)
- \(W_2\) is the weight of dry sample (g)

Determination of reducing sugars concentration

For determination of reducing sugars concentration in samples taken from the SSF, the liquid phase obtained after suspending one gram of sample in 10 mL distilled water was used. As for samples taken from the hydrolysis reaction, the reaction mixture was centrifuged and the supernatant was used.

The liquid sample (0.5 mL) was mixed with 0.5 mL DNS reagent in 10 mL centrifuge tube (a blank was prepared using distilled water). The tube was installed in a boiling water bath for 15 min. After addition of 4.5 mL distilled water into the tube, the absorbance at 540 nm was measured against the blank. Reducing sugars concentration was calculated according to Equation 6.8 and expressed as mg reducing sugars/g meal (db).
Reducing sugars = \( A_{540} \times \text{Slope} \times \text{DF} \times \frac{V}{W} \) \hspace{1cm} (6.8)

Where
- **Reducing sugars** is the concentration of reducing sugars (mg/g)
- \( A_{540} \) is the absorbance value at 540 nm
- **Slope** is the slope obtained from maltose standard curve
- **DF** is the dilution factor
- \( V \) is the volume of distilled water used to suspend fermenting solid (mL) or the volume of liquid sample for hydrolysis experiment (mL)
- \( W \) is the weight of dry matter in the sample (g)

**Determination of free amino nitrogen (FAN) concentration**

FAN content was determined using the ninhydrin method (Abernathy *et al.* 2009). Appropriately diluted samples (1 mL) (1 mL distilled water for blank) were mixed with 0.5 mL colour reagent in test tubes. The tubes were capped and installed in a boiling water bath for 16 min. After cooling under running water, 2.5 mL dilution reagent was added. Absorbance at 570 nm was measured against the blank. FAN concentration was calculated according to Equation 6.9 and expressed as mg FAN/g meal (db).

\[ \text{FAN} = A_{570} \times \text{Slope} \times \text{DF} \times \frac{V}{W} \] \hspace{1cm} (6.9)

Where
- **FAN** is the concentration of FAN (mg/g)
- \( A_{570} \) is the absorbance at 570 nm
- **Slope** is the slope obtained from glycine standard curve
- **DF** is the dilution factor
- \( V \) is the volume of distilled water used to suspend fermenting solid (mL) or the volume of liquid sample for hydrolysis experiment (mL)
- \( W \) is the weight of dry matter in the sample (g)

**Assay of protease activity**

Crude protease was obtained by suspending 1 g of fermenting solid in 10 mL distilled water then centrifuged at 4,000 rpm for 10 min. The supernatant was stored at −30°C for the assay.

Activity of the crude enzyme was determined using 2.5% (w/v) casein solution as a substrate. Two 1.5 mL micro-centrifuge tubes containing 0.5 mL trichloroacetic acid
(10% (w/v)) were prepared for each sample. Substrate solution (1 mL) was mixed with 1 mL crude enzyme in a 10 mL centrifuge tube then 0.5 mL of the mixture was immediately transferred into the first of the two micro-centrifuge tubes to stop the enzyme reaction. The centrifuge tubes containing the rest of the mixture were then incubated in a 55°C water bath to react for 30 min. After that, 0.5 mL of the reaction mixture was transferred into the second micro-centrifuge tube to terminate the reaction. Both micro-centrifuge tubes were centrifuged at 13,000 rpm for 5 min. The supernatants were used to determine the concentration of FAN before and after enzymatic degradation. The FAN values were subsequently used to calculate the activity of crude protease using Equation 6.10. One unit of protease was defined as the amount of protease that releases 1 µg FAN from casein within one minute under the reaction conditions.

\[
\text{Activity} = \frac{(FAN_{30} \times D_{30}) - (FAN_0 \times D_0)}{30 \times M} \times 1,000
\]  

(6.10)

Where

- \( Activity \) is the activity of protease (unit/g)
- \( FAN_0 \) is the FAN concentration at 0 min (mg/L)
- \( FAN_{30} \) is the FAN concentration at 30 min (mg/L)
- \( D_0 \) is the dilution factor of enzyme solution at 0 min
- \( D_{30} \) is the dilution factor of enzyme solution at 30 min
- 30 is the period of reaction (min)
- \( M \) is the fermenting solid concentration (g/L)
- 1,000 is the conversion factor for mg to µg

### 6.4 Results and discussions

#### 6.4.1 Partial characterisation of rapeseed meal

In this study, starch content, total reducing sugar, glucose content, pH and density of the rapeseed meal were determined. The results are shown in Table 6.1.
### Table 6.1  Some characteristics of rapeseed meal used in this study.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ash content* (%, db)</td>
<td>7.48</td>
</tr>
<tr>
<td>Bulk density (g/mL)</td>
<td>0.54</td>
</tr>
<tr>
<td>Free amino nitrogen* (mg/g meal, db)</td>
<td>0.65</td>
</tr>
<tr>
<td>Free phosphorus* (mg/g meal, db)</td>
<td>0.81</td>
</tr>
<tr>
<td>Glucose content (%, db)</td>
<td>0.17</td>
</tr>
<tr>
<td>Moisture content* (%)</td>
<td>10.3</td>
</tr>
<tr>
<td>Oil content* (%, db)</td>
<td>2.45</td>
</tr>
<tr>
<td>Particle density (g/mL)</td>
<td>1.18</td>
</tr>
<tr>
<td>pH</td>
<td>5.71</td>
</tr>
<tr>
<td>Reducing sugars (mg/g meal)</td>
<td>0.37</td>
</tr>
<tr>
<td>Starch content (%, db)</td>
<td>0.49</td>
</tr>
<tr>
<td>Total Kjeldahl nitrogen* (mg/g meal, db)</td>
<td>62.19</td>
</tr>
<tr>
<td>Total phosphorus* (mg/g meal, db)</td>
<td>16.89</td>
</tr>
<tr>
<td>Voidage (%)</td>
<td>53.76</td>
</tr>
</tbody>
</table>

*Determined by Dr. Ruohang Wang

As seen from the table, the meal contained a low level of oil content. This was because the majority of the oil had been pressed out and extracted by solvent extraction. The moisture content of the meal was also low and as it was stored in a sealed container, the value of 10.3% was used as actual moisture content in all calculations. The phosphorus content of the meal was 16.89 g/kg meal (db), complying with the range 9-19 g/kg reported in the literature (Rutkowski 1971). Phosphorus is present generally in the forms of phospholipids, phytic compounds, nucleic acids and inorganic compounds (Appelqvist and Ohlson 1972). It was assumed that there was no loss of phosphorus during the oil extraction process.

Analysis of ash content is a method to determine non-volatile inorganic matter. Ash consists of oxides and salts containing anions such as phosphates, chlorides, sulphates, and other halides and cations such as sodium, potassium, calcium, magnesium, iron, and manganese (Food-science 2008). A normal value of ash content in rapeseed is between 7% and 8% and the variation among samples appears to be rather small (Appelqvist and Ohlson 1972). In this study, ash content of the rapeseed meal was found to be 7.48%.

Protein is one of the major component categories in rapeseed, being normally present in the range 30-45% (Rutkowski 1971; Chabanon et al. 2007). Protein content can be estimated by multiplying the total Kjeldahl nitrogen (TKN) value by a factor of 6.25. However, Bell (1984) suggested that it was inappropriate to use this number for
estimation of protein content in cereal grains and oilseeds. This is because the value is uncertain and the factor should only be used when the exact percentage of nitrogen in protein is known (Gutheil and Bailey 1993). Many researchers suggested that the correct factor for rapeseed should be 5.53 (Appelqvist and Ohlson 1972; Bell 1984). By using the factors of 5.53 and 6.25, the calculated protein content of rapeseed meal was 34.39% and 38.87% respectively.

Although rapeseed meal is rich in protein, fungi could not use this complex nitrogenous material directly. They must firstly produce protein-degrading enzymes to hydrolyse the protein into smaller molecules. In this study, free amino nitrogen (FAN), a collective term referring to amino acids, dipeptides and tripeptides (Thomas and Ingledew 1990), was used as an indicator to follow the production of small nitrogenous molecules. Although, some FAN was present in the raw rapeseed meal, this is at low levels (0.65 mg/g meal). However, it is sufficient for the initiation of fungal growth (results are shown in Section 6.4.2).

The bulk and particle density of rapeseed meal were determined and used to calculate bulk voidage. The bulk density was found to be 0.54 g/mL while the particle density was 1.18 g/mL. The estimated voidage was 53.76%. Çalışır (2005) proposed equations for estimating bulk density and voidage of rapeseed as follows.

**Bulk density:**

\[ \rho_b = 616.74 - 1.4518 M_c \]

**Voidage:**

\[ \varepsilon = 44.659 + 0.6656 M_c \]

Using the moisture content of 10.3% in the above equations, close values of the bulk density (0.6 g/mL) and the voidage (51.5%) were obtained. From the above equations, it is obvious that moisture content has an influence on both properties. Although the stocked rapeseed meal in the laboratory followed the above relationship, working rapeseed meal with 65% moisture content did not. The bulk density of 65% moisture rapeseed meal was 1.09 g/mL and its voidage was 12.63%. These results contradicted the equations above as bulk density increased and the voidage decreased when
increasing the moisture content. This was possibly because the water added into the meal caused it to compact more densely than the drier meal. This resulted in more particles packing into a small volume, which in turn reduced the voidage. The pictures of intact rapeseed and rapeseed meal are shown in Figure 6.1.

![Figure 6.1](image)

**Figure 6.1** Intact rapeseed (A) and rapeseed meal (B).

Although the constituents of rapeseed vary depending on many factors such as cultivar, season of cultivation and growing location (Hu and Duvnjak 2004), the analysis of chemical and physical characteristics of the meal revealed that they were all within the ranges found in the literature (Niewiadomski 1990; Çalışır et al. 2005).

### 6.4.2 Solid-state fermentation (SSF) of rapeseed meal by *Aspergillus oryzae*

SSF was carried out in order to extract assimilable nutrients from rapeseed meal. Due to the high protein content, the use of vigorous proteolytic enzyme producers such as *Aspergillus niger* and *A. oryzae* (Pandey et al. 1999; Van der Aa et al. 2002) is preferred. Figure 6.2 shows the growth of *A. oryzae* on rapeseed meal from the beginning until 72 h of incubation.
Figure 6.2  Growth of *A. oryzae* on rapeseed meal at the beginning of SSF (A), 24 h (B) and 72 h (C), tightly compacted substrate due to the growth of the fungus at 72 h (D).

From the above figure, it is clearly seen that, despite the low level of FAN in raw rapeseed meal, growth of the fungus successfully commenced. At 24 h, some white mycelia were observed and the meal was compacted loosely due to the penetration and covering of the hyphae. At the end of experiment, the meal was compacted more tightly and the mycelia covered the entire surface of the substrate. When the meal was cut to examine the growth of the fungus inside the substrate bed, it was found that there were hyphae penetrating through the bed resulting in firm compaction of the substrate (Figure 6.2D).

The reduction in weight of both fermented solids and uninoculated control was periodically measured throughout the course of the SSF. Assuming that no chemical or biochemical reaction occurred in the uninoculated control, the loss of solid weight was caused only by the evaporation of water. The differences between weight of fermenting solids and the uninoculated control indicated that besides the loss of water, there must be other factors affecting the overall weight. This net weight loss was therefore assumed to be due to fungal growth. Since in the experiment, the initial weight of the meal in the SSF and the control were different, normalised ratios of solid weight at time $t$ (*i.e.* $W_t$) to the initial solid weight ($W_0$) were used to demonstrate this phenomenon as shown in Figure 6.3.
The heterogeneous nature of solid substrates, such as rapeseed meal, causes difficulties in the regulation of parameters (Varzakas et al. 2008) and the observation of variables. As seen from Figure 6.2, it is virtually impossible to separate the fungal mycelia to measure growth. For this reason, several workers have used indirect methods to follow the growth. These include measurement of glucosamine concentration (Chutmanop et al. 2008), calculation of weight reduction ratio (Wang et al. 2010) and CO₂ evolution rate measurement (Koutinas et al. 2003a). In this experiment, the reduction in fermenting solid weight was used to calculate weight reduction ratio during SSF to represent the growth profile of the fungus (Figure 6.4). By assuming that the reduction of fermenting solid weight is linearly related to the fungal growth, a plot of weight reduction ratio (calculated using Equation 6.11) against fermentation time could be presented as a growth curve. The results shown in Figure 6.4 are the average values of three replicates with standard deviations.

$$R = \left( \frac{W_i - W_{bi}}{W_i/W_{bi}} \right) \times 100$$  \hspace{1cm} (6.11)

Where  
R is the weight reduction ratio (%)  
Wᵢ is the weight loss of sample at time t  
Wᵢ is the initial weight of sample  
Wₑ is the weight loss of uninoculated control at time t  
Wₑ is the initial weight of uninoculated control
Figure 6.4  Changes in weight reduction ratios during A. oryzae fermentation of rapeseed meal.

An implicit assumption in this approach is that the same level of water evaporation will occur in the fermenting sample as in the uninoculated sample. This might not however be the case. Growth and respiration of the fungus will result in metabolic heat and water being generated. This might cause more water to evaporate than in the uninoculated control. On the other hand, when the hyphae penetrate and the mycelium covers the substrate bed, this helps to retain moisture in the system and so the rate of evaporation may be lower than in the uninoculated control. Given that the difference in weight loss between the fermenting and the uninoculated samples is small relative to the total weight loss, it is difficult to estimate how important the above factors might be. Nonetheless, differences between weight losses must still give an indication of the extent of growth.

The result confirms that growth of A. oryzae was successfully initiated despite only small amounts of reducing sugars and FAN being present in the meal. After inoculation, the fungus spent about 6 h in the lag period. This was a period of spore swelling and germination (Gottlieb 1950; Wang et al. 2010), associated with an uptake of moisture, implied by the negative difference in moisture content during the first 6 h (Figure 6.5). Also, during this period, the production and secretion of extracellular hydrolytic enzymes will have taken place (Pereira et al. 2007). A rapid increase in weight reduction ratio, from 0.16% to 2.4% between 6 and 30 h, indicated that the fungus was
in the exponential growth phase. By using the maximum weight reduction ratio of 2.53% and initial weight reduction ratio of 0.16% (at the commencement of the exponential phase), a model proposed by Okazaki et al. (1980) (Equation 6.12) was used to determine the specific growth rate ($\mu$) as 0.17 h$^{-1}$. However, the model only satisfactorily fitted the graph in the range 6 to 48 h (Figure 6.4). The fungus entered stationary phase after 30 h and stayed in this stage for 18 h, after which the ratio decreased, eventually reaching 1.72%.

$$R = \frac{R_m}{1 + \left(\frac{R_m}{R_i} - 1\right)e^{-\mu t}}$$

(6.12)

Where

- $R$ is the weight reduction ratio (%)
- $R_m$ is the maximum weight reduction ratio (%)
- $R_i$ is the initial weight reduction ratio (%)
- $\mu$ is the specific growth rate (h$^{-1}$)
- $t$ is the fermentation time (h)

The differences between moisture content of fermenting solids and uninoculated control, calculated on a dry basis, can be used to demonstrate the water consumption and metabolic water production during the SSF as shown in Figure 6.5. During the first 6 h, as discussed earlier, water was absorbed by fungal spores for spore germination. This resulted in the reduction of moisture content by nearly 2%. The result shows that the water production started after 6 h, when the spores germinated and started growing. The percentage of difference increased rapidly between 6 and 30 h corresponding to the exponential growth phase. When the fungus entered stationary phase, the production of water reduced significantly due to the reduction in fungal growth. Note that the shift in moisture content at 54 h was probably due to droplets of condensate falling back into the substrate, which can sometimes be observed during incubation.
The water content of fermenting solids can change easily due to the change of environment. In order to prevent the solids from drying out, SSF can be conducted in a humidified atmosphere e.g. at 80% relative humidity (Hui et al. 2008). However, in this study all SSF experiments were performed in an incubator circulating with dry air. Therefore, the lids of the Petri dishes were left on at all times to prevent the rapid evaporation of water. Keeping the lids on has a significant influence on the moisture content of the fermenting solid as shown in Figure 6.6.
It can be seen that in the experiment without a lid, the moisture content decreased very quickly from 66.8% to 7.3% within 30 h. As a result, no growth was observed. From Figure 6.6, it was obvious that the presence of a lid helps in retention of the moisture. The reduction in moisture content for the plates with lids was very slow compared to those without. Interestingly, it was further noticed that in the experiment that was inoculated with A. oryzae, the moisture content reduced at an even lower rate. This could be because, as mentioned earlier, the fungal mycelium and hyphae helped prevent the water vapour from escaping the system. However, keeping the lids on also has a drawback. Droplets formed beneath the lids fell back to the fermenting solids. This resulted in uneven distribution of the moisture in the system and increased the overall moisture content of the substrate. For this reason, during sampling, the whole content was removed from the dish and stirred using a glass rod to ensure good distribution.

Enzyme production in A. oryzae is associated with growth (Cohen 1973). In order to take up surrounding nutrients, the fungus secretes extracellular enzymes such as amylases and proteases to hydrolyse large nutrient molecules (e.g. starch and protein) into smaller units (Thomas and Ingledew 1990). Although in this study amylase production was not investigated, the product of its reaction, reducing sugars, was
determined. Figure 6.7 illustrates the production of reducing sugars during the course of SSF. The results are the average values of three replicates and show standard deviations.

![Graph showing the production of reducing sugars during SSF](image.png)

**Figure 6.7** Net production of reducing sugars during SSF by *A. oryzae*.

The concentration of reducing sugars decreased from 6.94 to 3.31 mg/g meal during the first 6 h. This was because the fungus utilised it for the initiation of growth (Chutmanop *et al.* 2008). When the fungus grew and started producing amylolytic enzymes, the concentration of reducing sugars increased steadily and reached 9.98 mg/g meal at 48 h. The increase in reducing sugars content was because the rate of enzyme activity was high enough to exceed the rate of sugar utilisation by fungal cells (Rob *et al.* 2002). The rate of reducing sugars production decreased after the fungus entered the stationary phase. This might be because there was surplus sugar in the environment as suggested by Chutmanop and colleagues (2008). However, due to the low starch content in rapeseed meal (see Table 6.1), the concentration of reducing sugars was relatively low.

Protease activity was determined over the course of the SSF. It was found that the profile of protease activity was very similar to that of fungal growth. It was reported that the ability of *A. oryzae* to produce extracellular protease is inducible (Battaglino *et al.* 1991) and it was confirmed for this strain by Wang *et al.* (2005). From Figure 6.8, it is clearly seen that protease activity increased sharply during the exponential growth phase (between 6 and 30 h). After the fungus entered the stationary phase, no more protease activity was detected and it declined to 118 U/g at the end of experiment. This was because at this phase of growth, the fungus had low cell activity.
The production of FAN was also investigated. In this study, FAN was used as an indicator to follow the production of assimilable nitrogenous substances. It was found that as soon as protease activity appeared to increase, FAN content rose, along with the enzyme activity. Although the fungus utilised the FAN as its nitrogen source, high levels of enzyme activity sustained the production of FAN over the period of study (Wang et al. 2010). The rate of FAN production decreased at 48 h due to the reduction in protease activity. However, as seen in the figure, it still increased until the end of the experiment. This suggested that most of the protease released earlier was still active and continued to digest the protein in the meal.

Due to the high protein content in rapeseed meal (38.87%), conducting only SSF might not be able to digest all of it. Therefore, hydrolysis of fermented solids was subsequently carried out in order to digest the remaining protein. The results of the hydrolysis are discussed in the next section.

### 6.4.3 Hydrolysis of fermented solids

Subsequent hydrolysis of fermented solids proved to be effective since it could improve nutrient production after the SSF. The increase in reducing sugars and particularly FAN concentration throughout the course of reaction implied that plenty of substrate still
remained in the meal after the SSF. Since the fermented mass was blended to increase the suspension ability of the solid matter and incubated in an oxygen limited condition, no growth of the fungus was observed. During the incubation, not only the hydrolysis reaction took place, but also in this condition, the autolysis of the fungal cells occurred. During autolysis, many enzymes such as protease (Santamaria and Reyes 1988), phosphatase, nuclease (Reyes et al. 1990), glucanase and chitinase (White et al. 2002) are working synergistically to digest building blocks in fungal cells and cell walls. This results in disruption of the cells (Koutinas et al. 2005) and release of intracellular nutrients such as amino acids, peptides, nucleotides, phosphorus, vitamins and some trace elements, into the hydrolysate. Figure 6.9 shows the reaction mixture at 48 h.

As a result of hydrolysis and fungal autolysis, solids concentration decreased exponentially from 11.7 to 8.15 g/L (db) within 72 h as shown in Figure 6.10. The slow rate of hydrolysis observed towards the end of the experiment might be because of product inhibition caused by very high FAN concentration (Figure 6.11) as suggested by Wang et al. (2010).

In order to predict the reduction in solids concentration, an equation proposed by Moresi et al. (1995) was used (Equation 6.13). It fitted the experimental data well as seen in Figure 6.10 with the reaction constant of 0.045 h$^{-1}$.

$$W = W_0 \left[ \left( D \times e^{-kt} \right) + \left( 1 - D \right) \right]$$

(6.13)

Where

- $W$ is the dry solids concentration (g/L)
- $W_0$ is the initial dry solids concentration (g/L)
- $D$ is the ratio of dry matter loss to initial dry solids concentration
- $k$ is first-order reaction constant (h$^{-1}$)
- $t$ is reaction time (h)
The production of reducing sugars and FAN was also investigated. At the beginning of the reaction, the mixture contained 0.56 mg reducing sugars/g solid and 56.37 mg FAN/g solid. At the end of the experiment, reducing sugars and FAN at a concentration of 10.76 mg/g and 84.48 mg/g respectively were obtained. Figure 6.11 shows the production of these components during the hydrolysis and fungal autolysis. FAN production was considered to be linked to the activity of both extracellular protease released during the SSF and intracellular protease from autolysis. The concentration of FAN rose sharply during the first 24 h then reached the plateau with not much more being produced. This probably due to the inhibition of enzyme by product inhibition as described earlier. As for reducing sugars production, it was a result of amylolytic enzymes and gluca nase activity that digested fungal cell walls and probably starch remaining after SSF (Fleet and Phaff 1974). Furthermore, chitinase, which is an inducible enzyme triggered by chitin oligomers (White et al. 2002), might digest chitin, releasing N-acetyl-D-glucosamine which is a glucose derivative into the liquid. However, reducing sugars were detected at a low concentration. This was mainly due to low starch content in rapeseed meal.

An equation proposed by Koutinas et al. (2003b) (Equation 6.14) was used to fit the data for components production during hydrolysis and fungal autolysis. The first-order reaction constants for reducing sugars and FAN production were 0.045 h\(^{-1}\) and 0.071 h\(^{-1}\) respectively.
\[ X_i = X_{if} \left(1 - e^{-kt} \right) \]  

(6.14)

Where  
- \( X_i \) is the concentration of component \( i \) (mg/g solid)  
- \( X_{if} \) is the final concentration of component \( i \) (mg/g solid)  
- \( k_i \) is the first-order reaction constant for component \( i \) (h\(^{-1}\))  
- \( t \) is the reaction time (h)

**Figure 6.11** Production of FAN and reducing sugars during the hydrolysis of fermented solids and fungal autolysis.

The hydrolysate obtained after each experiment was centrifuged and filtered through filter paper (Whatman No.1), then pooled to store together. It was analysed for its FAN content and stored at \(-30^\circ\)C for subsequent experiments.

### 6.4.4 Comparison of FAN content in various nitrogen sources

The hydrolysate was analysed for its FAN content and compared with other commercial nitrogen sources *i.e.* yeast extract, beef extract and a complex medium, nutrient broth. Since there are several yeast extracts available in the market, two brands which were available in our laboratory, were used. The concentration of each nitrogen source used was the normal concentration used in media preparations, except for beef extract at 10 g/L which was prepared only for comparison. The hydrolysate was used undiluted.
Chapter 6  Production of nutrients from rapeseed meal

Figure 6.12 shows the concentration of FAN in each sample. It was found that beef extract at a concentration of 10 g/L had the highest FAN content (1,100 mg/L) followed by the hydrolysate (ca. 700 mg/L FAN). From this experiment it was found that one of the yeast extracts had about 680 mg/L FAN while the other had only 480 mg/L. It was interesting that there was a big difference in FAN content between the two brands of yeast extract. Du et al. (2008) also reported a different concentration of FAN (500 mg/L) for 10 g/L yeast extract. The difference in compositions in yeast extract may be attributed to many factors, for instance type of yeast, the treatment used to extract nutrients from yeast cells as well as downstream processes (EURASYP 2010).

Nutrient broth comprised several nitrogen sources at various concentrations such as peptone, yeast extract and meat extract (Merck 2002; German Collection of Microorganisms and Cell Culture (DSMZ) 2007). For this reason, its compositions vary depending on the formulation. In this study, 13 g/L commercial nutrient broth containing peptone (5 g/L) and meat extract (3 g/L) was used. The FAN content was found to be about 600 mg/L. Beef extract at a normal concentration (3 g/L) (Neogen Corporation 2009) had FAN content of 480 mg/L. Note that although both beef extract and yeast extract have similar total nitrogen (TN) content; 11-14% for beef extract (Neogen Corporation 2009) and 8-12% for yeast extract (EURASYP 2010), the amount of FAN can vary.

![Figure 6.12](image.png)

**Figure 6.12**  FAN content of the fungal hydrolysate and commercial nitrogen sources; BE is beef extract, YE is yeast extract, NB is nutrient broth and HL is the hydrolysate.
6.5 Conclusion

The high protein content (38.87%, TKN×6.25) in rapeseed meal makes it a promising raw material for production of nitrogen source for microbial cultivation. By using biological treatment, which is carried out under mild conditions, the small molecules of nitrogenous substances can be extracted. Due to the physical characteristics of rapeseed meal, the appropriate method of using it as fermentation substrate is solid-state fermentation (SSF) by filamentous fungi. In this study, SSF of rapeseed meal by *Aspergillus oryzae* was conducted. Protease, the key enzyme in this experiment, was found to have high activity (148 U/g, db). This resulted in FAN production of 15.3 mg/g (db) at 72 h of fermentation (Figure 6.8). Hydrolysis of fermented matter along with fungal autolysis was subsequently carried out. At the end of reactions, 28.11 mg/g FAN (db) was obtained (Figure 6.11). This two-step process yielded overall FAN production of 43.4 mg/g meal (718 mg/L).

A comparison, based on FAN content, between the hydrolysate and commercial nitrogen sources was made in order to demonstrate the potential of the hydrolysate. It was found that, as a nitrogen source, the hydrolysate could probably compete with other sources of nitrogen as it had the highest FAN concentration at normal concentrations. For a volume of 1 litre, the FAN concentration of 700 mg/L obtained from rapeseed meal is equivalent to about 10 g/L of one brand of yeast extract, 20 g/L of another brand of yeast extract, 15 g/L nutrient broth and 5 g/L beef extract.

Despite the long processing time for hydrolysate production (3 days for SSF and 3 days for the hydrolysis), the ready availability of rapeseed meal, the simple operation procedures and the resultant FAN-rich hydrolysate make this approach attractive for production of alternative nutrients to commercial nitrogen sources. Since the hydrolysate contains high FAN concentration and micro-organisms can directly use it as a nitrogen source, only a carbon source is required. The possibility of using the hydrolysate as the sole non-carbon nutrient source for bacterial fermentation was therefore investigated and the results are presented and discussed in Chapter 7.
CHAPTER 7

Growth of *Cupriavidus necator* in solutions derived from rapeseed meal

7.1 Introduction

Sources of nitrogen for microbial cultivation can be diverse. Inorganic nitrogenous chemicals *e.g.* ammonium chloride (NH$_4$Cl), ammonium sulphate ((NH$_4$)$_2$SO$_4$), ammonium nitrate (NH$_4$NO$_3$), potassium nitrate (KNO$_3$) and calcium nitrate (Ca(NO$_3$)$_2$) can be used (Abou-Zeid *et al.* 1981; Kalil *et al.* 2008). These chemicals are often used in defined media for which chemical compositions are known. The advantage of this kind of media is that it gives more reproducible results compared to complex media (Hodges *et al.* 1980) as well as a controllable medium compositions. However, it is difficult to define fully the nutrient requirements of a particular micro-organism. Complex media, on the other hand, contain undefined components. This type of media is usually rich in nutrients and minerals and is generally more complete than defined media. They contain water soluble extracts, *e.g.* peptone, tryptone and yeast extract, which are derived from natural sources (Sigma-Aldrich 2011b). Because of this, their exact compositions are unknown and can vary from batch to batch. Despite this disadvantage, undefined media are frequently used to cultivate micro-organisms as not only essential elements are present but also other vitamins and trace elements. The aim of this study was to investigate the potential use of solutions derived from rapeseed meal as an alternative to a selected undefined organic nitrogen source to grow a micro-organism.

Several approaches were carried out to prepare solutions from rapeseed meal. In this chapter, the key components, *i.e.* total nitrogen (TN) and free amino nitrogen (FAN) content, of rapeseed meal-derived solutions are compared with those of yeast extract, a typical nitrogen source. The results are shown in Section 7.4.1. A medium obtained using a strategy that gave highest FAN and TN values was used to grow *C. necator* for
comparison with yeast extract. The results are shown in Section 7.4.2. Next, the rapeseed medium was used to grow the bacterium with various initial FAN concentrations to investigate its effect. The results are presented and discussed in Section 7.4.3.

### 7.2 Materials

#### 7.2.1 Micro-organism

The bacterium used in this study was *Cupriavidus necator* DSM4058. The material and methods for inoculum preparation were as described in Section 5.2.1 and Section 5.2.2.

#### 7.2.2 Culture media

For the study of *C. necator* growth in a rapeseed meal-derived solution, the medium was filtered through 0.45 µm membranes (Millipore, Durapore PVDF), then diluted to attain a final FAN concentration of 200 mg/L. Yeast extract solution was prepared using a calculated amount to attain 200 mg/L FAN. The solutions were supplemented with 50 g/L pure glycerol (≥ 99%) and sterilised at 121°C for 20 min. All the media were left to cool to room temperature prior to inoculation.

For the study of effect of initial FAN concentration on bacterial growth, yeast extract and hydrolysate were used. Calculated amounts of yeast extract were dissolved in 1 litre distilled water to obtain solutions containing FAN in the range 90 to 680 mg/L. The fungal hydrolysate was centrifuged at 10,000 rpm for 10 min then filtered through 0.45 µm membrane. It was then diluted with distilled water to attain FAN concentrations ranging from 50 to 660 mg/L. All the media were supplemented with 50 g/L synthetic crude glycerol and sterilised at 121°C for 20 min. Synthetic crude glycerol was formulated based on the literature. It consisted of 65% (v/v) pure glycerol, 10% (v/v) methanol (≥99.8%) and 5% (w/v) NaCl (Cargill 2007; Pyle *et al.* 2008; Kerr *et al.* 2009).
7.3 Methods

7.3.1 Preparation of rapeseed meal-derived solutions

All the rapeseed meal-derived solutions, except for the fungal hydrolysate (HL), were prepared by Esra Uçkun Kiran, Middle East Technical University, Turkey, during a research visit to the SCGPE, according to the following procedures (Uçkun Kiran et al. 2012):

Commercial enzymatic treatment (CE): Commercial protease (P4860, Protease from *Bacillus licheniformis*) was purchased from Sigma-Aldrich. The activity of the enzyme was 49 U/mL at 55°C, pH 7. In the experiment, an enzyme loading of $5 \times 10^{-3}$ U/g meal was used to hydrolyse 10% (w/v, db) rapeseed meal suspension.

Submerged fungal fermentation (SF): A suspension of rapeseed meal (100 g/L) was sterilised at 121°C for 20 min. After cooling to room temperature, it was inoculated with *A. oryzae* spores suspension ($10^6$ spores/g meal) then incubated at 30°C for 72 h.

Submerged fungal fermentation followed by fungal autolysis (SFA): The fungal culture was prepared using the method of SF described above. After 72 h of incubation, the culture flask was placed in a 55°C water bath for 72 h to allow further hydrolysis of the substrate and fungal autolysis.

Hydrolysis of rapeseed meal using enzymatic broth (EB): Enzymatic broth was produced according to the SF method described above. At 72 h of incubation, the culture was filtered through filter paper (Whatman No. 1) to obtain a crude enzyme solution, which was used at 10% (v/v) to hydrolyse 10% (w/v) rapeseed meal at 55°C.

Solid-state fermentation followed by fungal autolysis (HL): The required amount of rapeseed meal with a moisture content of 65% was sterilised at 121°C for 20 min. After cooling to room temperature, the meal was inoculated with $10^6$ *A. oryzae* spores/g meal then incubated at 35°C for 3 days. The fermented solids was then suspended in distilled water and homogenised using a kitchen blender. The mixture was incubated at 55°C for 3 days.
7.3.2 Determination of total nitrogen (TN) concentration

Total nitrogen (TN) analysis was carried out using a Total Nitrogen, LR, Test ‘N Tube™ kit obtained from Hach (2007). For this method, an alkaline persulphate digestion converts all forms of nitrogen to nitrate. Sodium metabisulphite is added after the digestion to eliminate halogen oxide interferences. Nitrate then reacts with chromotropic acid under strongly acidic conditions to form a yellow complex of which the colour intensity is proportional to total nitrogen concentration.

Briefly, 2 mL of appropriately diluted sample was mixed with Total Nitrogen Persulphate Reagent in a Total Nitrogen Hydroxide Digestion Reagent vial (a blank was prepared using organic-free water supplied with the kit). The mixture was vigorously shaken for 30 sec, then heated at 105°C for exactly 30 min and left to cool to room temperature. Next, the Total Nitrogen Reagent A powder was added into the vial, and then shaken for 15 sec. The mixture was left to react for 3 min. Subsequently, the Total Nitrogen Reagent B was added and the vial was shaken for 15 sec and left for 2 min to react. After that, 2 mL reacted mixture was transferred to a Total Nitrogen Reagent C vial. The vial was capped and inverted slowly 10 times to mix and left to react for 5 min. The absorbance value of the sample was then measured at 410 nm against the blank.

7.3.3 Growth of C. necator in rapeseed meal-derived solutions

Sterile media (100 mL) with initial FAN concentration of 200 mg/L were inoculated with three mL of inocula then incubated at 30°C, 200 rpm for 54 h. Samples were taken every 2 h during the first 8 h then periodically after that for optical density measurement.

7.3.4 Effect of initial FAN concentration on growth of C. necator

Initial FAN concentration in the media was varied between 0 and 680 mg/L. All fermentations were conducted in a 1.5L bioreactor (Electrolab, model 351 equipped with 300 stirrer control) with 1 litre working volume. Dissolved oxygen (DO) (Broadley James Corporation, Oxyprobe D140) and pH probe (Sentek, Sterprobes) were connected to the bioreactor and calibrated prior to sterilisation. The assembled bioreactor containing culture medium was sterilised at 121°C for 20 min. All probes
including temperature probe were connected to the controller of the bioreactor immediately after sterilisation. The bioreactor was left overnight prior to inoculation.

The sterile medium was aseptically inoculated with 100 mL inoculum. Aeration rate was set at 1.5 volume per volume per minute (vvm). DO set point was 25% while agitation speed (300-1,200 rpm) was controlled by DO level. The pH was left uncontrolled. Inlet and outlet air was filter sterilised using 0.2 µm membrane (Sartorius, Midisart 2000). Antifoam (Sigma-Aldrich, Antifoam Y-30 emulsion) was added into the bioreactor during the exponential growth phase of the bacterium with an amount of 3 drops in every 2 h. About 10 mL samples were taken every 2 h during the first 8 h and periodically afterward until the fermentation was stopped at 72 h. Samples were used to measure optical density at 600 nm as well as to determine FAN concentration and dry cell weight.

### 7.3.5 Determination of dry cell weight

In order to measure dry weight, one mL of sample was transferred into a pre-dried and pre-weighed 1.5 mL micro-centrifuge tube then centrifuged at 13,000 rpm for 5 min. The supernatant was used to determine FAN concentration while the residue was washed twice with distilled water then dried at 60°C overnight. Dry cell weight (DCW) was calculated according to Equation 7.1 as shown below.

\[
DCW = \left( W_2 - W_1 \right) \times 1,000
\]  

(7.1)

Where

- \( DCW \) is dry cell weight (g/L)
- \( W_1 \) is weight of pre-dried and pre-weighed micro-centrifuge tube (g)
- \( W_2 \) is weight of micro-centrifuge tube containing dry cell (g)
- 1,000 is conversion factor for g/mL to g/L

### 7.3.6 Determination of free amino nitrogen concentration

The method used to determine FAN concentrations is described in Section 6.3.2.
7.4 Results and discussions

7.4.1 Comparison of free amino nitrogen (FAN) and total nitrogen (TN) concentrations in nitrogen-rich solutions

The concentrations of FAN and TN in solutions derived from rapeseed meal are compared in order to demonstrate the different outcomes of each method. Amongst the submerged systems tested (SF, EB, CE and SFA), a solution obtained from submerged fungal fermentation followed by fungal autolysis (SFA) had the highest FAN (300 mg/L) and TN (1,845 mg/L). However, SSF followed by hydrolysis (HL) gave higher FAN and TN content of up to 703 mg/L and 1,962 mg/L respectively. Note that the fungal hydrolysate used in this experiment was obtained from two production batches, designated as HL #1 and HL #2. Two brands of yeast extract, YE #1 and YE #2 were also used. However, TN for one of the hydrolysates and yeast extract samples was not determined.

The result shows that FAN concentrations of solutions from submerged fermentation and enzymatic treatments (SF, EB and CE) were relatively low (Figure 7.1). This was because the protein in the meal was only digested either by enzymes secreted by A. oryzae or by commercial protease, unlike the fermentations that were followed by hydrolysis and fungal autolysis (HL and SFA), during which the majority of the protein was digested. It was discussed earlier in Chapter 6 that hydrolysis and fungal autolysis can be carried out after fungal fermentation to increase overall nutrients production (Koutinas et al. 2005).

Amongst SF, EB and CE, the highest TN values for CE indicated that commercial protease was more efficient than the crude ones in terms of protein digestion. HL was found to be the most effective approach for protein digestion followed by SFA. Interestingly, YE #2 contained only 303 mg/L FAN and 532 mg/L TN. These were relatively low compared to HL #1, HL #2 and YE #1. It was mentioned earlier that type of yeast, extraction method and downstream processes can affect the quality of yeast extract. Significant variation in FAN and TN content is, therefore, possible.
As discussed above, the HL media gave the highest TN and FAN values. This approach was therefore used to produce a medium from rapeseed meal for subsequent experiments. The fungal hydrolysate, also designated as HL, along with yeast extract (YE) were used as the sole (non-carbon) nutrient source for growing *C. necator* along with pure glycerol (50 g/L) as the carbon source. A comparison shown in Figure 7.2 indicates that YE supported cell growth better than HL in the early stage of fermentation. This might be because the nutrients in YE are more readily accessible and there were some inhibitory substances in HL. However, the exponential growth phase of the bacterium in HL lasted longer than in YE. This resulted in higher cell concentrations (in terms of optical density) later in the fermentation. It should be taken into consideration that after sterilisation, some precipitation occurred in HL. This resulted in cloudiness of the medium before inoculation and might be the reason of higher OD values in this experiment. Nevertheless, this precipitation did not affect the concentration of FAN in the medium.

Media obtained from other strategies, *i.e.* SF, EB, CE and SFA, were also used to cultivate the bacterium. All the media were found to support cell growth but the OD values were heavily influenced by the precipitate formed after sterilisation, resulting in

---

**Figure 7.1** FAN and TN concentrations in rapeseed meal-derived solutions compared with those of yeast extract.

### 7.4.2 Growth of *C. necator* in a medium derived from rapeseed meal

As discussed above, the HL media gave the highest TN and FAN values. This approach was therefore used to produce a medium from rapeseed meal for subsequent experiments. The fungal hydrolysate, also designated as HL, along with yeast extract (YE) were used as the sole (non-carbon) nutrient source for growing *C. necator* along with pure glycerol (50 g/L) as the carbon source. A comparison shown in Figure 7.2 indicates that YE supported cell growth better than HL in the early stage of fermentation. This might be because the nutrients in YE are more readily accessible and there were some inhibitory substances in HL. However, the exponential growth phase of the bacterium in HL lasted longer than in YE. This resulted in higher cell concentrations (in terms of optical density) later in the fermentation. It should be taken into consideration that after sterilisation, some precipitation occurred in HL. This resulted in cloudiness of the medium before inoculation and might be the reason of higher OD values in this experiment. Nevertheless, this precipitation did not affect the concentration of FAN in the medium.

Media obtained from other strategies, *i.e.* SF, EB, CE and SFA, were also used to cultivate the bacterium. All the media were found to support cell growth but the OD values were heavily influenced by the precipitate formed after sterilisation, resulting in
difficulty in comparing the results. For this reason, only the comparisons between HL and YE are presented.

Figure 7.2  Growth of *C. necator* in terms of optical density at 600 nm in HL and YE supplemented with 50 g/L synthetic crude glycerol.

Figure 7.3 shows specific growth rate of *C. necator* during the first 8 h of incubation determined using the method described in Section 4.4.2. It was found that YE gave higher \( \mu \) (0.4 h\(^{-1}\)) which was more than 1.6 times as much as that obtained from HL. However, due to the fact that HL gave a high cell concentration under the length of time studied, it might have a potential to be used as a non-carbon nutrient source for PHB production. Note that since no samples were taken between 8 h and 24 h, dotted lines are used to link the data points as seen in Figure 7.2. This did not affect the calculation of the specific growth rate, which was based only on data up to 8 h and therefore represents the maximum attainable value.
With the same initial concentration of carbon source and FAN in all fermentations, the differences in growth might be caused by unequal amounts of other nutrients or even nitrogen in terms of TN (Uçkun Kiran et al. 2012) as seen in Figure 7.1. In this experiment, only FAN was used to observe the changes in concentration of nitrogen sources. But in reality, micro-organisms can also utilise other forms of nitrogen such as inorganic nitrogen, long-chain amino acids or protein for their growth (Uçkun Kiran et al. 2012).

The results of FAN and TN concentrations (Figure 7.1) indicated that more nitrogenous substrate was transformed into accessible source of nitrogen via the HL strategy compared with other methods. Coupling with high cell density, this method was considered the most appropriate method for production of nutrients from rapeseed meal.

### 7.4.3 Effect of initial FAN concentration on growth of *C. necator*

In this study, *C. necator* was grown in media consisting of various concentrations of HL and YE supplemented with synthetic crude glycerol (50 g/L). Growth of the bacterium in terms of dry cell weight (DCW) was followed along with FAN consumption. The results are shown in Figure 7.4.
From the results, it was found that even at 0 mg/L FAN, the bacterium was still able to grow. This is probably because there was some \((\text{NH}_4)_2\text{SO}_4\) left in the inoculum when it was transferred to the bioreactor, so that the bacterium utilised it for growth. Apart from this, it was obvious that growth of the bacterium directly related to the amount of FAN consumed. Since the bacterium utilised FAN for its growth, FAN concentration decreased dramatically during the exponential phase of growth. The consumption of FAN reduced significantly when cells entered the stationary phase. It is interesting that cells did not consume the entire FAN in the medium. This might be because of the
depletion of carbon source or other nutrients or perhaps because not all of the amino acids present can be utilised by the bacterium.

Growth of the bacterium in YE solutions showed a similar profile as the HL, except that there was no observable lag phase. FAN utilisation in yeast extract solutions increased rapidly during the exponential growth phase before remaining constant after the cells entered stationary phase. The same explanation for the HL experiments can be applied to these phenomena. Growth of \textit{C. necator} in yeast extract solutions are shown in Figure 7.5.

\textbf{Figure 7.5} Growth of \textit{C. necator} in yeast extract solutions containing 50 g/L synthetic crude glycerol and 88 to 678 mg/L FAN.

The specific growth rate of the bacterium at each initial FAN concentration was determined using the method described in Section 4.4.2. The results are shown in Figure 7.6. Note that no variation bar is shown because the standard deviation is smaller than the data symbol.
Chapter 7  Growth of *C. necator* in solutions derived from rapeseed meal

The value of $\mu$ at 0 mg/L FAN (0.07 h$^{-1}$) is shown in Figure 7.6 to demonstrate the growth of the bacterium when there was no organic nitrogen in the medium. When the hydrolysate was used to grow *C. necator*, it was found that $\mu$ increased as the initial FAN concentration increased (0.13 h$^{-1}$ to 0.19 h$^{-1}$). Likewise, although it started at higher value, $\mu$ in YE increased at more or less the same rate as in HL (0.23 h$^{-1}$ to 0.32 h$^{-1}$). The explanation for the higher $\mu$ in YE is that the calculation of $\mu$ was done using data points during the first 8 h of incubation. During this period, as shown in Figure 7.2, YE supported cell growth better than HL. Hence, the higher $\mu$.

The data for $\mu$ in both HL and YE, and initial FAN concentrations were used to estimate maximum specific growth rate ($\mu_{max}$) and Monod constant ($K_s$) using a Lineweaver-Burk plot (see Section 4.2 for detail). The Lineweaver-Burk plots for HL and YE are shown in Figure 7.7. The estimated $\mu_{max}$ and $K_s$ for HL system were 0.19 h$^{-1}$ and 25.9 mg/L respectively, whereas they were 0.36 h$^{-1}$ and 50 mg/L respectively in the YE system. The plots of the Monod equation using the estimated parameters above suggested that FAN is the limiting substrate for *C. necator* growth (Figure 7.8).

**Figure 7.6**  Specific growth rates of *C. necator* in fungal hydrolysate and yeast extract solutions influenced by increasing initial FAN concentrations.
Biomass production (calculated by subtracting the DCW at 72 h by the initial DCW) in HL and YE at the end of fermentations were used to demonstrate the increase in the production of biomass when the initial FAN concentration increased. As seen in Figure 7.9, although they increased steadily at low initial FAN concentrations, biomass production did not follow a linear relationship (orange broken line). Instead, apparent yield reduced as FAN concentration increased. This could be because at higher concentrations of FAN, a lower proportion is utilised. However, closer inspection of FAN consumption shows clearly that for both HL and YE, the proportion of FAN consumed is constant (Figure 7.10). So, since a constant proportion of the FAN was...
consumed, the curve in Figure 7.9 must indicate a lower conversion of nitrogen to biomass at higher initial FAN concentrations. It is interesting to note that the percentage consumption, while constant, was not 100% and was different for the two substrates (65% for HL and 90% for YE).

**Figure 7.9** Biomass productions at 72 h of fermentation in HL and YE systems containing various FAN concentrations and 50 g/L synthetic crude glycerol.
From Figure 7.9, it can be seen that despite lower $\mu$ values, biomass production in HL increased as the volume of the hydrolysate in the media increased and the trends in biomass production in both substrates were similar. This might be because, in the HL experiments, *C. necator* consumed other sources of nitrogen together with FAN, resulting in high biomass production. On the other hand, in the case of YE, since the lengths of the log phase in all concentrations tested were similar, the increases in DCW were considered due to the increases in $\mu$. The data point at 0 mg/L FAN is included in the figure to demonstrate the production of biomass from 0 to 660 mg/L FAN (for HL) and from 0 to about 680 mg/L FAN (for YE).

The results shown in Figure 7.8 indicate that $\mu_{\text{max}}$ was reached when the initial FAN concentration was higher than about 400 mg/L. It also suggests that $\mu_{\text{max}}$ is different for the two nutrient sources. According to Figure 7.9, biomass production tends to increase as the initial FAN concentration increases. However, the yield of biomass per unit mass of FAN reduces with increasing FAN concentration. Provided that the nutrient-rich solution produced by SSF followed by hydrolysis of fermented solids contains only 600-700 mg/L at the highest, the initial FAN concentration used to grow *C. necator* should be at least 600 mg/L. The fermentation data for HL and YE are summarised in Table 7.1.
Chapter 7  Growth of *C. necator* in solutions derived from rapeseed meal

### Table 7.1 Comparison of fermentation data between HL and YE systems.

<table>
<thead>
<tr>
<th>Fermentation data</th>
<th>No organic nitrogen</th>
<th>Initial FAN concentration in HL (mg/L)</th>
<th>Initial FAN concentrations in YE (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50  275  345  660  90  300  450  680</td>
<td></td>
</tr>
<tr>
<td>Fermentation time (h)</td>
<td>72  72  72  72  72  72  72  72</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial DCW (g/L)</td>
<td>0.1  0.2  0.2  0.1  0.3  0.6  0.1  0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final DCW (g/L)</td>
<td>0.3  1.5  5.4  6.5  8.5  2.4  6  7.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell productivity (g/L·h)</td>
<td>0.003  0.02  0.07  0.09  0.12  0.03  0.08  0.1  0.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specific growth rate (h⁻¹)</td>
<td>0.07  0.13  0.15  0.19  0.19  0.23  0.26  0.40  0.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final FAN concentration (mg/L)</td>
<td>- 12.39  108.51  161.72  211.53  8.18  27.17  45.89  62.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAN consumption rate (mg/L·h)</td>
<td>- 0.55  2.32  2.55  6.26  1.10  3.84  5.576  8.55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yield (g cell/mg FAN)</td>
<td>- 0.03  0.03  0.03  0.02  0.03  0.02  0.02  0.01</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 7.5 Conclusion

The potential use of media derived from rapeseed meal as an alternative to commercial nitrogen sources was investigated and is reported in this chapter. This began with the comparison of FAN and total nitrogen (TN) concentrations. The use of enzyme to digest protein in the meal was found to be ineffective. Media obtained from submerged fungal fermentation (SF), enzymatic broth (EB) and commercial enzyme (CE) treatment had relatively low levels of FAN. However, CE appeared to be a good choice for TN extraction. Amongst the strategies tested, solid-state fermentation followed by hydrolysis of fermented solids (HL) gave the highest values of FAN and TN followed by SF with autolysis (SFA). Comparing with yeast extract, HL gave almost the same amount of FAN (YE #1) with much more TN content (YE #2) (Figure 7.1). However, it should be taken into consideration that the compositions of yeast extract can vary as discussed in Section 7.4.1. Growth of *C. necator* in rapeseed meal-derived media (HL) was compared to that in YE. It was found that YE supported cell growth better than HL during the early stages of fermentation. However, the final dry cell weight (DCW) obtained from the two fermentations were similar.

The importance of the initial FAN concentration on growth of *C. necator* was demonstrated. It was found that initial FAN concentration has an influence on both specific growth rate ($\mu$) and biomass production. The results shown in Figure 7.8 indicate that FAN is the limiting substrate for *C. necator* growth. Increases in the initial FAN concentrations resulted in increases in $\mu$. Maximum specific growth rate ($\mu_{\text{max}}$) in both HL and YE experiments was obtained when the initial FAN concentrations were
higher than 400 mg/L. Biomass production in both HL and YE was heavily influenced by the initial FAN concentration. At low FAN concentrations, biomass production increased rapidly then decreased continually as initial FAN concentration was increased (Figure 7.9). The results presented in this chapter lead to a conclusion that HL has the potential to be used as an alternative to YE for growing *C. necator*. Based on the results of specific growth rate and biomass production, the initial FAN concentration used to grow *C. necator* should be at least 600 mg/L. The production of polyhydroxybutyrate by *C. necator* growing in HL supplemented with various carbon sources are discussed in Chapter 8.
CHAPTER 8

Production of polyhydroxybutyrate in feedstock derived from biodiesel by-products

8.1 Introduction

To date, there are several reports claiming the use of biodiesel by-products to produce chemicals that have a commercial potential. However, none have focused on the solid residues but simply considered the crude glycerol by-product (Papanikolaou et al. 2000; Chi et al. 2007; Mothes et al. 2007; Çelik et al. 2008; Shams Yazdani and Gonzalez 2008; Cavalheiro et al. 2009; Tang et al. 2009; Liu et al. 2011). To make the biodiesel industry more sustainable, the development of processes using the integrated biorefinery concept would be beneficial. In other words, all biomass and chemicals involved in the process should be of use to some extent in the production of value-added by-products. Since in Europe, the majority of biodiesel is processed from rapeseed oil (Foreign Agricultural Service 2006; da Silva et al. 2009), the utilisation of rapeseed residues (the cake or meal remaining after oil extraction) is of interest. By using all by-products the rapeseed biodiesel industry would be enhanced and the economics improved (Choi 2008).

With this in mind, rapeseed meal was investigated as a potential source of nutrients (see Chapter 6) and its use along with crude glycerol is reported here for the fermentative production of polyhydroxybutyrate (PHB). PHB was chosen as a representative because its biodegradable thermoplastic property (Doi 2002) can help reduce the dependence on conventional plastic. By using the fungal hydrolysate, which was derived from rapeseed meal, as a non-carbon nutrient and crude glycerol as a carbon source, it was hoped that a PHB-producing bacterium could be grown and successfully used to produce PHB.

In this chapter, a partial characterisation of crude glycerol is presented (Section 8.4.1). Cupriavidus necator was cultivated, in fed-batch mode, as the micro-organism for PHB
production. The results are discussed in Section 8.4.2. Based on the results reported in Chapter 6 and in this chapter, a preliminary economic assessment and a flow diagram for PHB production from rapeseed biodiesel by-products is presented in Section 8.4.3.

8.2 Materials

8.2.1 Micro-organism

The bacterium strain used in this study was *Cupriavidus necator* DSM4058. The method of inoculum preparation was described in Section 5.2.1.

8.2.2 Crude glycerol

The crude glycerol used in this study was obtained from a working biodiesel plant and was provided by Double green Ltd., Hull, UK. It was stored in sealable glass bottles at room temperature.

8.2.3 Culture media

In this study, three different media, *i.e.* mineral medium, yeast extract solution and fungal hydrolysate, were used to examine the effect of different nitrogen sources on bacterial growth and PHB production.

Since, in the previous fermentations, the mineral medium used gave low concentrations of cells, the formulation of the medium was changed to an alternative reported by Kim *et al.* (1994). The new medium comprised (per L) pure glycerol 50 g, (NH₄)₂SO₄ 1 g, KH₂PO₄ 1.5 g, Na₂HPO₄.2H₂O 4.5 g, MgSO₄.7H₂O 0.2 g, and trace element solution⁶ 1 mL. For fed-batch fermentation, the mineral medium comprised (per L) synthetic crude glycerol⁷ 50 g, (NH₄)₂SO₄ 4 g, KH₂PO₄ 13.3 g, MgSO₄.7H₂O 1.2 g, citric acid 1.7 g and trace element solution 10 mL. The pH was adjusted to 6.8 with 5M NaOH. The medium was sterilised at 121°C for 20 min. Glycerol and MgSO₄.7H₂O were sterilised separately and aseptically added to the medium after cooling to room temperature.

⁶ Trace elements solution comprised (per L) FeSO₄.7H₂O 10 g, ZnSO₄.7H₂O 2.25 g, CuSO₄.5H₂O 1 g, MnSO₄.4-5H₂O 0.5 g, CaCl₂.2H₂O 2 g, (NH₄)₆Mo₇O₂₄ 0.1 g and 37% HCl 10 mL.

⁷ The formula of synthetic crude glycerol is presented in Section 7.2.2.
Yeast extract solution was prepared by dissolving an appropriate amount of yeast extract, in distilled water, calculated to provide a desired FAN concentration. The pH was adjusted to 6.8 with 5M NaOH. The medium was sterilised at 121°C for 20 min. synthetic crude glycerol at a concentration of 50 g/L was sterilised separately and aseptically added to the medium after cooling to room temperature.

The fungal hydrolysate containing about 600 mg/L FAN was filtered through a 0.45 µm membrane (Millipore, Durapore PVDF) then sterilised at 121°C for 20 min. Carbon source (pure glycerol, synthetic crude glycerol and crude glycerol) at a concentration of 50 g/L was sterilised separately and added to the medium after cooling to room temperature.

8.3 Methods

8.3.1 Partial characterisation of crude glycerol

Some characteristics of crude glycerol were determined and the results obtained were used as information for subsequent experiments. In this study, glycerol, methanol, free fatty acids contents and pH as well as density were determined according to the following procedures:

**Determination of glycerol concentration**

Concentration of glycerol was determined using a GL6 analyser and a reagent kit (GMRD-185) obtained from Analox instruments Ltd., London, UK. Glycerol is phospholyrated by adenosine triphosphate (ATP) in the presence of glycerol kinase (GK), resulting in the formation of glycerol-3-phosphate (G3P). Concurrently, G3P is oxidised by glycerol-3-phosphate oxidase (GPO) to dihydroxyacetone phosphate (DAP) and hydrogen peroxide. According to Equation 2) shown below, the rate of oxygen consumption is directly proportional to glycerol concentration.

\[
\begin{align*}
  1) & \quad \text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{G3P} \\
  2) & \quad \text{G3P} + \text{O}_2 \xrightarrow{\text{GPO}} \text{DAP} + \text{H}_2\text{O}_2
\end{align*}
\]
In order to measure glycerol concentration, an appropriately diluted sample (7 µL) was mixed with 250 µL distilled water using a vortex mixer, then 7 µL of the mixture was injected into the reaction chamber of the analyser. The machine was calibrated using 2.0% (w/v) standard glycerol solution provided with the kit.

**Determination of methanol concentration**

Concentration of methanol was determined using a GL6 analyser and a reagent kit (GMRD-125) obtained from Analox instruments Ltd. Methanol is oxidised by the enzyme alcohol oxidase (AOD) in the presence of molecular oxygen as shown in the equation below

\[
\text{Methanol} + \text{O}_2 \xrightarrow{\text{AOD}} \text{Formaldehyde} + \text{H}_2\text{O}_2
\]

Under the conditions of the assay, the rate of oxygen consumption is directly proportional to the methanol concentration.

In order to measure the concentration of methanol, an appropriately diluted sample (5 µL) was mixed with distilled water (500 µL) using a vortex mixer, then 5 µL of the mixture was injected into the reaction chamber of the analyser. The machine was calibrated using 2.0% (w/v) standard methanol solution provided with the kit.

**Determination of free fatty acids (FFA) content**

The amount of FFA was determined by titrating the sample with 1 M KOH using phenolphthalein (1% (w/v) in a 50% ethanol solution) as an indicator. Since the crude glycerol was viscous and its colour was dark brown, it was diluted 2-4 times for the ease of titration. Diluted crude glycerol was mixed with 2-3 drops of phenolphthalein and placed on a magnetic stirrer then titrated until its colour turned to pink. According to the equation below, mole ratio of FFA to KOH is 1:1. This implies that the amount of 1 M KOH used is equivalent to the amount of FFA in a sample. Note that the general formula for a fatty acid chain is represented as \( R\text{-COOH} \), where \( R \) is an alkyl group.

\[
R\text{-COOH} + \text{KOH} \rightarrow R\text{-COOK} + \text{H}_2\text{O}
\]


**Measurement of pH**

Crude glycerol was diluted with distilled water in a 100 mL bottle to obtain a 10% (v/v) solution. This was stirred using magnetic stirrer while pH was being measured.

**Measurement of density**

The density of the crude glycerol was measured using 10 mL pycnometer.

### 8.3.2 Production and purification of PHB for use as a standard

The bioreactor setup and operation are as described in Section 7.3.4 except that the pH was controlled at 6.8. The bioreactor containing 900 mL mineral medium was sterilised at 121°C for 20 min. Pure glycerol and MgSO₄·7H₂O were autoclaved separately and aseptically added into the bioreactor after cooling to room temperature.

Fed-batch fermentation was carried out by feeding about 100 mL pure glycerol into the system every 24 h until the fermentation was stopped at 120 h. After that, the culture was centrifuged at 10,000 rpm for 15 min to separate the cells from the medium. The cells were washed twice with distilled water and then with acetone and dried at 55°C until constant weight.

In order to extract PHB from the dry cell, a method described by Hahn *et al.* (1995) was used. A certain volume of dry biomass was suspended in 50 volumes of chloroform for 48 h at 30°C and 200 rpm. The mixture was then filtered through 0.45 µm membranes (Gelman Laboratory, FP-450). The clear solution was mixed with 5 volumes of non-solvent solution to precipitate PHB. The non-solvent solution was a mixture of methanol and distilled water at a ratio of 7:3 (v/v). Purified PHB was obtained by filtering the mixture using a 0.45 µm membrane then drying at 55°C. The purified PHB was stored in a universal bottle at room temperature. Figure 8.1 shows the purified PHB used as a standard in this study.
8.3.3 Production of PHB in fed-batch fermentation

The bioreactor setup and operation are described in Section 7.3.4. Figure 8.2 shows the setup used in this study.

Fed-batch fermentations were carried out by feeding a required amount of stock carbon source solution to the bioreactor every 24 h to achieve a concentration of around 50 g/L using a peristaltic pump. About 10 mL of the culture were taken every 3 h during the first 6 h and periodically afterwards until the fermentation was stopped at 120 h. Samples were used to measure optical density and dry cell weight as well as to determine glycerol, methanol, PHB and FAN concentrations (where applicable). Note that for the experiments with the fungal hydrolysate, the pH of the medium was adjusted to 5.5-6.0 then left uncontrolled during the first 24 h due to precipitation at pH above 6.5. After that the pH was controlled at 6.8.
8.3.4 Determination of dry cell weight

Culture broth (5 mL) was centrifuged at 10,000 rpm for 10 min then the residue was washed twice with distilled water. It was then suspended in acetone and transferred into a pre-dried and pre-weighed universal bottle. The suspension was dried at 55°C overnight then moved to a desiccator to cool to room temperature. Dry cell weight (DCW) was calculated according to Equation 8.1.

\[
DCW = \frac{W_2 - W_1}{V} \times 1,000
\]  

(8.1)

Where \( DCW \) is the dry cell weight (g/L)
- \( W_1 \) is the weight of a pre-dried and pre-weighed universal bottle (g)
- \( W_2 \) is the weight of a universal bottle containing dry cells (g)
- \( V \) is the volume of culture broth (mL)
- 1,000 is the factor for conversion of mL to L

8.3.5 Determination of PHB concentration

Dry cells obtained after DCW measurement were used for quantification of PHB using gas chromatography (GC). Following the method of Riis and Mai (1988), 1-propanol and 37% hydrochloric acid was used to hydrolyse and transesterify PHB using 1,2-dichloroethane (DCE) as a solvent.

To a universal bottle containing dry cells 2 mL DCE and 2 mL acidified propanol (4 volumes of propanol: 1 volume of hydrochloric acid) were added. The bottles were tightly capped and installed in a boiling water bath for 2 h. After cooling to room temperature, 4 mL distilled water were added and the mixture was mixed using a vortex mixer for 20-30 sec. The solvent (denser) phase was then used to determine the concentration of PHB.

A GC (Varian, model CP-3800) assembled with autosampler (Combi/Pal) was used. Software used was Varian Star Workstation version 6.20. Column, detector and carrier gas were Poraplot Q-HT 10×32 mm column, flame ionisation detector and helium respectively. Injection volume was 1 µL. The injection temperature was 230°C. The initial temperature was 120°C and it was gradually increased to 230°C after 3 min and remained at 230°C until the end. Detection temperature was 200°C. The area of the
peak obtained from the GC was used to determine the concentration of PHB using Equation 8.2. Figure 8.3 shows an example of a PHB calibration curve.

\[
PHB = \frac{Area}{Slope} \times \frac{1,000}{V}
\]  

(8.2)

Where  

\(PHB\) is the concentration of PHB (g/L)  
\(Area\) is the area of a PHB peak obtained from GC (counts)  
\(Slope\) is the slope of a PHB calibration curve  
1,000 is the conversion factor for mL to L  
\(V\) is the volume of culture broth used to determine DCW (mL)

![PHB Calibration Curve](image)

**Figure 8.3** An example of PHB calibration curve used to calculate the concentration of PHB in dry cell samples.

---

### 8.4 Results and discussions

#### 8.4.1 Partial characterisation of crude glycerol

Generally, crude glycerol contains glycerol as a major component with traces of methanol, water, soaps and free fatty acids (FFA), unconverted triglycerides, residual fatty acid alkyl esters etc. (Ashby et al. 2004; Meher et al. 2006). According to the literature, the compositions of crude glycerol can vary over a wide range. The glycerol content is usually relatively high, yet the stream cannot be used directly by other industries due to the impurities (Amaral et al. 2009). For this reason, further purification (to 99% or higher) is required if the crude glycerol is to be used in food, cosmetics or...
other products (Choi 2008; U.S. Department of Energy 2011). For fermentation on the other hand, purity is not necessarily an issue (Amaral et al. 2009). The main factors affecting the glycerol concentration in the crude glycerol stream of a biodiesel process are the efficiency of the separation process and acidulation and methanol recovery (See Figure 2.1).

Despite passing through several purification units, traces of methanol, free fatty acids and other impurities always remain and were, therefore, still present in the crude glycerol used in this study. The partial composition of crude glycerol used in this study is shown in Table 8.1. Glycerol content of crude glycerol was 73.3% (v/v) equivalent to 916 g/L and methanol was the biggest impurity at 12.4%. As discussed earlier in Chapter 5 methanol can inhibit the growth of *C. necator*, so it is important to know how much is present. This value is towards the low end of the range reported in the literature (0.01% to 37.5%) (Papanikolaou et al. 2000; Thompson and He 2006; Mothes et al. 2007; Pyle et al. 2008; Cavalheiro et al. 2009; Hansen et al. 2009; Tang et al. 2009). However, in an efficient biodiesel process, the majority of methanol should be recovered for reuse in the transesterification process. Apart from cutting overall biodiesel production costs, the recovery of methanol will result in higher quality crude glycerol, making its subsequent use easier since small amounts of methanol can be removed by boiling or autoclaving (Choi 2008; Pyle 2008).

### Table 8.1 Characteristics of crude glycerol used in this study.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value/appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol (g/L)</td>
<td>916.67±5.77</td>
</tr>
<tr>
<td>Methanol (g/L)</td>
<td>98±1.41</td>
</tr>
<tr>
<td>Others (% v/v)</td>
<td>10.93</td>
</tr>
<tr>
<td>Free fatty acids (% v/v)</td>
<td>3.33±1.15</td>
</tr>
<tr>
<td>Salt (NaCl) (g/L)</td>
<td>0 – 70*</td>
</tr>
<tr>
<td>pH of a 10% (v/v) aqueous solution</td>
<td>6.43</td>
</tr>
<tr>
<td>Density (g/mL)</td>
<td>1.19±0.02</td>
</tr>
<tr>
<td>Colour</td>
<td>Dark brown</td>
</tr>
</tbody>
</table>

*Not determined in this study

Some of the FFA present in raw rapeseed oil can remain in the crude glycerol stream. Also, FFA is formed during the acidulation process. Although, according to Figure 2.1, FFA is removed to refine the crude glycerol, traces of FFA can be found. The amount of FFA found in the crude glycerol used in this study was 3.3% (v/v). The ‘others’ component of the crude glycerol was defined as 100 – [glycerol content + methanol...
content + FFA content]. This 10.9% portion of the crude glycerol will include mono- and diacylglycerides, ash, water, soaps and organic non-glycerol substances (Pyle et al. 2008; Cavalheiro et al. 2009; Hansen et al. 2009; Tang et al. 2009). However, since water generally accounts for around 10% of total crude glycerol compositions (ABG Inc. 2007), this portion was considered to consist primarily of water. Sodium chloride (NaCl) can also be present in the crude glycerol stream. The range of NaCl reported in the literature is between 0.01% and 6.6% (Mothes et al. 2007; Kerr et al. 2009). Use of crude glycerol containing high NaCl content for fermentation can result in reduction of the microbial performance. PHB accumulation by *C. necator* in media containing NaCl-contaminated crude glycerol has been reported to be significantly reduced due to osmoregulation caused by accumulation of NaCl (Mothes et al. 2007).

Due to the low level of water in the crude glycerol, pH measurements were made in aqueous solutions. A solution of 10% (v/v) crude glycerol in distilled water was found to have a pH of 6.43, which is within the reported range of 4 to 9 crude glycerol (SRS Engineer Corp. 2008; Kerr et al. 2009). Density of crude glycerol can vary over a wide range, depending on its composition. Published data on material safety data sheets range from 0.94 to 1.38 g/mL (Beaver Biodiesel LLC; Cargill 2007). The density of the crude glycerol used in this study (1.19 g/mL) was the same as that of synthetic crude glycerol and slightly lower than pure glycerol (1.25-1.26 g/mL) (www.sciencelab.com 2010; Sigma-Aldrich 2011a).

### 8.4.2 Growth of *C. necator* and PHB production in various media

In this section the growth of *C. necator* in fed-batch fermentation and PHB production in mineral medium (MM), yeast extract solution (YE) and the hydrolysate (HL) are discussed. The mineral medium was used to demonstrate the bacterial performance in defined medium whereas YE was used as a (non-carbon) nutrient source to show the performance of the bacterium in a typical complex nutrient source. The hydrolysate used in this study was supplemented variously with pure glycerol (PG), synthetic crude glycerol (SCG) and crude glycerol (CG) in order to compare the effect of carbon source quality on growth and PHB production.
Growth of *C. necator* and PHB production in mineral medium

The bacterial growth and PHB production in mineral medium are shown in Figure 8.4. It was observed that the cells grew quickly after inoculation. A log-linear plot of DCW against fermentation time revealed that growth entered the exponential phase after 3 h and total biomass steadily increased for about 45 h. During this period, glycerol consumption rate was found to be highest (0.47 g/L·h). Although total biomass continued to increase until 72 h, cell concentrations referred to as non-PHB biomass stopped increasing after 30 h and tended to decrease thereafter. This suggested that some nutrient elements, possibly nitrogen, might be limited, affecting cell proliferation and probably leading to cell autolysis (Xu 2007). Since during this fermentation, total nitrogen concentration was not determined, the time of nitrogen limitation was therefore not known. However, as can be seen in the figure, PHB accumulation was observed at 24 h. This implies that the concentration of nitrogen decreased to the level that stimulates PHB synthesis in the cells. This coupled with periodic addition of synthetic crude glycerol into the system resulted in excess carbon with a limitation of nitrogen, promoting PHB synthesis (Mazur *et al.* 2009). So, the increase in total biomass after 30 h was due to intracellular PHB accumulation. The highest total biomass obtained from this defined medium was 9.36 g/L at 72 h but this decreased to 8 g/L by the end of the experiment.
From the results shown in Figure 8.4, it is clear that the increase in total biomass was due to PHB accumulation. The concentration of PHB rose sharply between 30 h and 54 h reaching about 7 g_{PHB}/L at an accumulation rate of 0.26 g_{PHB}/L·h. A transient PHB concentration of 8.26 g/L was achieved at 72 h when the biomass reached the highest concentration (9.36 g/L), resulting in more than 88% (w/w) PHB content. The decrease in PHB content to around 77% at the end of the experiment might be because of the release of PHB into the medium due to cell autolysis. The plot of volumetric PHB productivity calculated during the course of the fermentation along with glycerol consumption rate against fermentation time revealed similar trends as shown in Figure 8.5. This implies that there is a strong link between glycerol consumption and PHB accumulation.
Growth and PHB production in a yeast extract solution

The growth in YE was similar in trend to that in mineral medium. A log-linear plot of DCW against fermentation time showed that the exponential phase was between 3 h and around 48 h. During this period, cell concentration rose sharply from 0.18 g/L to 22.14 g/L while FAN concentration decreased dramatically from 945 mg/L to 173 mg/L with a glycerol consumption rate of 0.77 g/L·h. The non-PHB biomass stopped increasing after 48 h and began to slightly decrease towards the end of the fermentation, similar to what happened in the MM+SCG medium. Again, the increase in total biomass was considered due to PHB accumulation since no increase in non-PHB biomass was observed after 48 h. Total biomass obtained at 120 h was 24.8 g/L. The final non-PHB biomass of 5.2 g/L obtained from this experiment was nearly 3 times higher than that obtained from mineral medium. The improved growth in this medium was probably due to the completeness of yeast extract compared to mineral medium. The results are shown in Figure 8.6.
Figure 8.6 Growth and PHB accumulation of *C. necator* in yeast extract solution containing, initially, 50 g/L synthetic crude glycerol, with fed-batch operation.

Concerning FAN utilisation, it was found that a total of 835 mg/L FAN was consumed during the course of the fermentation, equivalent to about 88% utilisation. From the result, it was found that *C. necator* accumulated PHB at 79% of its dry weight and since the bacterial elemental formula (without PHB) is C₄H₈O₂N (Bormann and Roth 1999), the formula for cells containing 79% PHB can be calculated as C₄.6H₇.32O₂.3N₀.21. On this basis, the cellular nitrogen will account for 2.88% and, in order to achieve 24.8 g/L biomass, 0.71 g nitrogen would therefore be required. With an initial concentration of about 0.95 g/L FAN in the medium, this means that approximately 75% of the FAN would have been required by the biomass. The difference between the directly calculated figure (88%) and the theoretical figure (75%) might be accounted for by the secretion of extracellular enzymes or the loss of nitrogenous substances via metabolism.

Similar to that observed in mineral medium, the accumulation of PHB could be detected at 24 h and was in parallel with the total biomass. The concentration of PHB rose sharply from 0.98 g/L to 15.1 g/L between 24 h and 48 h (the productivity increased 14 fold from 0.04 to 0.59 g/L·h). The rate of accumulation decreased after 48 h and no
more PHB production was observed after 72 h. The constant concentration of PHB indicated that it was neither utilised nor released into the medium during the fermentation, unlike in the mineral medium. This implies that there were adequate nutrients to sustain growth. The relationship between glycerol consumption rate and PHB productivity found in the MM+SCG experiment was found to be similar in this experiment (Figure 8.7).

**Figure 8.7** PHB productivity and glycerol consumption rate during the course of *C. necator* fed-batch fermentation in a yeast extract solution supplemented with synthetic crude glycerol.

**Growth and PHB production in rapeseed hydrolysate**

In this section, bacterial growth and PHB production in the hydrolysate supplemented with glycerol at different purity levels are discussed. Figure 8.8A through 8.8C depicts the performance of *C. necator* in the hydrolysate with pure glycerol (HL+PG), synthetic crude glycerol (HL+SCG) and also with real crude glycerol (HL+CG) as main carbon source.

The biomass production in all three fermentations was similar. Log-linear plots of DCW against fermentation time shown in Figure 8.9 reveal that the cells entered both exponential growth phase and stationary phase at nearly the same time. However, a further decrease in DCW after inoculation in HL+SCG and HL+CG was noticed. This could have been because of cell disruption (cell debris could be observed under transmission electron microscope (TEM) at 6 h, Figure 8.11) though the cause of this is unclear. A possibility is that the precipitate formed after sterilisation started to dissolve, resulting in apparent DCW reduction.
Figure 8.8 Growth and PHB production of *C. necator* in rapeseed hydrolysate supplemented with pure glycerol (A), synthetic crude glycerol (B) and crude glycerol (C) in fed-batch operation.
From the results, it was seen that the total biomass increased steadily for about 45 h (between 3 h and 48 h). Biomass productivities during this period in all fermentations were very close (0.45 g/L·h for HL+PG and HL+SCG and 0.4 g/L·h for HL+CG). This indicates that the impurities that were present in the media did not have any significant effect on biomass production. Even methanol that was found to be as high as 5 g/L in the HL+CG experiment did not significantly affect the growth. These results agree well with those reported in Chapter 5 where a significant effect of methanol was observed only beyond 10 g/L methanol. From Figure 8.8, it can be seen that PHB accumulation increased rapidly after the depletion of FAN while non-PHB biomass tended to decrease due to cell lysis. For this reason, the increase in DCW was again considered due to PHB accumulation. Final biomass concentrations measured at 120 h for HL+PG, HL+SCG and HL+CG were 28.86, 25.1 and 28.86 g/L respectively.

![Log-linear plots of C. necator biomass against fermentation time in rapeseed hydrolysate supplemented variously with pure glycerol, synthetic crude glycerol and crude glycerol.](image)

FAN utilisation in all three fermentations was also very similar. Total FAN utilisation of 91.6%, 92% and 93.6% was observed in HL+PG, HL+SCG and HL+CG respectively. These resulted in residual FAN concentrations of 50, 51 and 38.4 mg/L. It is interesting that FAN consumption in these experiments were more than those reported in Chapter 7 (about 65%). This suggests that different amounts of accessible nitrogen are present in the hydrolysates obtained from different batches. By using the method described earlier to calculate the theoretical amount of nitrogen in the biomass, it was found that there was 0.51, 0.65 and 0.71 g nitrogen in the biomass obtained from
HL+PG, HL+SCG and HL+CG respectively. Surprisingly, the calculated figure for HL+SCG and HL+CG appeared to be higher than the total FAN available (ca. 0.6 g/L). However, since the hydrolysate also contains larger nitrogenous molecules such as di-, oligo- or polypeptides, as discussed in Section 7.4.2, it is likely that the bacterium utilised these. *C. necator* has been reported to grow successfully on soy cake, which is rich in protein (RusSoya 2010), via SSF to produce PHB (Oliveira *et al.* 2007). This helps confirm that this bacterium is able to produce extracellular proteolytic enzymes necessary to hydrolyse protein substrates for its growth.

By plotting the production of PHB in the hydrolysate media together with those observed in other media, it was found that the trend in PHB accumulation in all media were quite similar (Figure 8.10). The curve was fitted with a logistic equation trend line with regression coefficient of 0.97. The logistic parameters were: maximum PHB accumulation, 84%; time at which PHB accumulation reached half the maximum, 32 h; and an exponent of 5. The equation is as shown below.

\[
\text{PHB} = \frac{84t^5}{(32^5 + t^5)}
\]

Where PHB is the PHB content (%) and *t* is the fermentation time (h).

![Figure 8.10](image)

**Figure 8.10** Accumulation of PHB (%) in *C. necator* grown in various media supplemented with pure, synthetic crude and crude glycerol and fitted with a logistic equation.
Samples taken from the HL+SCG experiment were used to observe the accumulation of PHB at different times throughout the course of the fermentation. Appropriately diluted samples were first mixed with fixative solution (a mixture of 2.5% glutaraldehyde and 4% formaldehyde in 0.1M cacodylate buffer). These were then post fixed with 1% osmium tetroxide, 1% tannic acid solution and 1% uranyl acetate then dehydrated in alcohol. The samples were embedded in epoxy resin and sectioned to be observed under TEM. The results are shown in Figure 8.11. Figure 8.11A shows cells at 6 h. As can be seen, no PHB granules were observed and there were cell debris scattered in the medium. The bubbles around the cell at the centre of the image might somehow have damaged the cell, leading to cell disruption (personal communication with Dr. Aleksandr Mironov, Faculty of Life Sciences, the University of Manchester). At 24 h, when the culture was in the exponential growth phase and the concentration of FAN had decreased to less than half the initial concentration, small granules of PHB were observed (Figure 8.11B). In Figure 8.7C a dead cell disrupting and releasing PHB into the medium was spotted. Beyond 75 h, the proportion of cell volumes occupied by PHB granules were not much different. By 120 h, about 5% PHB-free cells, 5% dead cells and 95% PHB-containing cell were estimated (Figure 8.11F). Note that although C. necator has flagella, it was difficult to see them using this method because the embedded cells could be sectioned in any direction and angle. In order to observe the flagella arrangement, the cells should be negatively stained and observed under electron microscopy (Makkar and Casida 1987). However, in this study the presence of flagella was not of particular importance compared to the presence of PHB.
Figure 8.11 TEM images of *C. necator* accumulating PHB as intracellular granules at different times through the course of a fermentation. PHB granules are the grey/white fractions inside the cells.

**Yield and productivity**

Since growing *C. necator* to accumulate PHB in hydrolysate media supplemented with crude glycerol was successfully achieved, this confirmed that biodiesel by-products have the potential to be used as raw materials for PHB production. In order to assess this potentiality further, yield and PHB productivity in hydrolysate systems were determined.

Yamane (1993) proposed an equation for determining the theoretical yield of PHB on carbon source based on chemical stoichiometry (Equation 8.3). Assuming that no loss of
carbon occurs during PHB synthesis, theoretical yield of PHB, $Y_{pc}^{(theor)}$, on compounds having the composition of $C_xH_yO_z$ can be derived straightforwardly as follows.

$$\frac{4n}{x}C_xH_yO_z \rightarrow (C_4H_8O_2)_n + n\left(\frac{2y}{x} - 3\right)H_2O + n\left(\frac{1}{2} - \frac{y}{x} - \frac{2z}{x}\right)O_2$$

From the above equation, $Y_{pc}^{(theor)}$ is calculated as:

$$Y_{pc}^{(theor)} = \frac{43x}{2(12x + y + 16z)}$$ (8.3)

In the case of glycerol, which has the elemental formula $C_3H_8O_3$, $Y_{pc}^{(theor)}$ is 0.7 g/g. However, in practice, the carbon source is not only used for PHB synthesis but also for biomass production and respiration (carbon will be lost from the system in the form of CO$_2$). For this reason Equation 8.3 can only be used as a reference point for the highest possible yield that can be achieved theoretically. Yamane (1993) also proposed definitions based on experimental observation that are more applicable as follows.

$$Y_{pc}^{(theor)} = \frac{\Delta p}{-\Delta s_1}$$ (8.4)

$$Y_{pc}^{(observed)} = \frac{\Delta p}{-\Delta s_{total}}$$ (8.5)

$$Y_{s/c} = \frac{\Delta x_{residual}}{-\Delta S_2}$$ (8.6)

Where $Y_{pc}^{(theor)}$ is the theoretical yield of PHB on glycerol ($g_{PHB}/g_{Glycerol}$) $Y_{pc}^{(observed)}$ is the observed yield of PHB on glycerol ($g_{PHB}/g_{Glycerol}$) $Y_{s/c}$ is the yield of non-PHB biomass on glycerol ($g_{Cell}/g_{Glycerol}$) $\Delta p$ is the PHB concentration (g/L) $-\Delta s_1$ is the total amount of glycerol consumed (g/L) $-\Delta s_{total}$ is the amount of glycerol converted to PHB (g/L) $-\Delta S_2$ is the amount of glycerol converted to non-PHB biomass (g/L)
\( \Delta x_{\text{residual}} \) is the concentration of non-PHB biomass (g/L)

Better still, the relationship between PHB content and the ratio of observed yield to theoretical yield \( \left( \frac{Y_{\text{PHB/Gly}}^{\text{(observed)}}}{Y_{\text{PHB/Gly}}^{\text{(theoretical)}}} \right) \) was demonstrated. This relationship can be used to estimate theoretical yield very easily as shown below.

\[
Y_{p/c}^{\text{theoretical}} = \frac{C_p}{Y_{s/c} + \left( 1 - \frac{Y_{p/c}^{\text{theoretical}}}{Y_{s/c}} \right) C_p}
\]  
(8.7)

Since the ratio of \( \frac{Y_{p/c}^{\text{theoretical}}}{Y_{s/c}} \) varies in a narrow range (1.1-1.3), the dependence of the value of \( \frac{Y_{p/c}^{\text{observed}}}{Y_{p/c}^{\text{theoretical}}} \) on the variation of this ratio is not significant and the ratio of \( Y_{p/c}^{\text{observed}} \) to \( Y_{p/c}^{\text{theoretical}} \) is roughly proportional to \( C_p \) (Yamane 1993). Equation 8.7 can therefore be rewritten as follows.

\[
Y_{p/c}^{\text{theoretical}} = \frac{Y_{p/c}^{\text{observed}}}{C_p}
\]  
(8.8)

Where \( C_p \) is the PHB content of the total biomass (g PHB/g biomass).

The theoretical yields calculated using Equation 8.8, observed yield and yield of non-PHB biomass on glycerol obtained from all the fermentations reported in this chapter are presented in Table 8.2. Note that theoretical yields were not the same in each case because this value depends on the fermentation conditions.

<table>
<thead>
<tr>
<th>Fermentation data</th>
<th>MM+SCG</th>
<th>YE+SCG</th>
<th>HL+PG</th>
<th>HL+SCG</th>
<th>HL+CG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed yield of PHB on glycerol (g/g)</td>
<td>0.14</td>
<td>0.24</td>
<td>0.29</td>
<td>0.29</td>
<td>0.32</td>
</tr>
<tr>
<td>Theoretical yield of PHB on glycerol (g/g)</td>
<td>0.18</td>
<td>0.31</td>
<td>0.34</td>
<td>0.36</td>
<td>0.37</td>
</tr>
<tr>
<td>Observed yield of non-PHB biomass on glycerol (g/g)</td>
<td>0.18</td>
<td>0.31</td>
<td>0.34</td>
<td>0.36</td>
<td>0.37</td>
</tr>
</tbody>
</table>

From the results, it is clear that MM+SCG gave the lowest yield of PHB, at only 0.14 gPHB/gGlycerol, which was just half of those obtained from the hydrolysate-based systems. The theoretical yields achieved with the hydrolysate systems were around 50% of the highest possible theoretical yield of 0.7 g/g (i.e. with all carbon being converted
to PHB). Yields obtained from YE were slightly lower than those obtained from the hydrolysate systems.

The key results for PHB production from this study along with those from various publications are shown in Table 8.3. The PHB productivity and yield of PHB on substrate can be as high as 2.42 \( \text{g}_{\text{PHB}}/\text{L} \cdot \text{h} \) and 0.43 \( \text{g}_{\text{PHB}}/\text{g}_{\text{Substrate}} \) respectively. However, while the productivity varies depending on fermentation conditions the yield, in most systems, is found to fluctuate over only a narrow range (0.3-0.36 g/g). This agrees well with the results shown in Figure 8.10 that the media composition did not have significant effect on PHB synthesis in \( C. \) necator. From the table, it can be seen that the system used in this study (rapeseed hydrolysate supplemented with glycerol at different quality) gave relatively low biomass concentration and therefore PHB productivity. This was possibly because the biomass production before the commencement of PHB accumulation was low and the fermentation times were long. The production of PHB has not been optimised in this study and the results presented are very preliminary. It is likely however that biomass and PHB yields can be significantly improved, as can the production of the generic feedstock.

**Table 8.3** Comparison of key results in the production of PHB by \( C. \) necator in various systems

<table>
<thead>
<tr>
<th>Reference</th>
<th>Carbon source</th>
<th>Total biomass concentration (g/L)</th>
<th>PHB concentration (g/L)</th>
<th>Productivity ( (\text{g}_{\text{PHB}}/\text{L} \cdot \text{h}) )</th>
<th>Yield ( (\text{g}<em>{\text{PHB}}/\text{g}</em>{\text{Substrate}}) )</th>
<th>Fermentation mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cavalheiro \textit{et al.} (2009)</td>
<td>Pure glycerol</td>
<td>82.5</td>
<td>51.2</td>
<td>0.6-1.5</td>
<td>0.36</td>
<td>Fed-batch</td>
</tr>
<tr>
<td></td>
<td>Crude glycerol</td>
<td>68.8</td>
<td>26.1</td>
<td>0.84</td>
<td>0.34</td>
<td>Fed-batch</td>
</tr>
<tr>
<td>Du \textit{et al.} (2001)</td>
<td>Glucose</td>
<td>42.4</td>
<td>30.5</td>
<td>1.23</td>
<td>0.36</td>
<td>Continuous</td>
</tr>
<tr>
<td>Khanna and Srivastava (2008)</td>
<td>Fructose</td>
<td>27.7</td>
<td>5.5</td>
<td>0.55</td>
<td>-</td>
<td>Continuous</td>
</tr>
<tr>
<td>Kim \textit{et al.} (1994)</td>
<td>Glucose</td>
<td>164</td>
<td>121</td>
<td>2.42</td>
<td>0.3</td>
<td>Fed-batch</td>
</tr>
<tr>
<td>Koutinas \textit{et al.} (2007b)</td>
<td>Glucose</td>
<td>75.4</td>
<td>45.2</td>
<td>0.29</td>
<td>0.43</td>
<td>Fed-batch</td>
</tr>
<tr>
<td>This work</td>
<td>Pure glycerol</td>
<td>28.86</td>
<td>25.22</td>
<td>0.21</td>
<td>0.29</td>
<td>Fed-batch</td>
</tr>
<tr>
<td></td>
<td>Synthetic crude glycerol</td>
<td>25.1</td>
<td>20.44</td>
<td>0.17</td>
<td>0.29</td>
<td>Fed-batch</td>
</tr>
<tr>
<td></td>
<td>Crude glycerol</td>
<td>28.86</td>
<td>24.75</td>
<td>0.21</td>
<td>0.32</td>
<td>Fed-batch</td>
</tr>
</tbody>
</table>

The PHB productivities in all the media used in this study, calculated by dividing the PHB concentration by fermentation time (120 h), were found to be 0.05, 0.16, 0.21, 0.17, 0.21 \( \text{g}_{\text{PHB}}/\text{L} \cdot \text{h} \) for MM+SCG, YE+SCG, HL+PG, HL+SCG and HL+CG respectively. However, most of the production in most cases had been completed by
72 h and so these values would be significantly higher if based on the time at which PHB concentrations reached their maximum values \textit{(i.e.} 0.11, 0.27, 0.32, 0.27 and 0.32 g_{PHB}/L·h respectively). The fermentation data determined from this study are summarised in Table 8.4.

### Table 8.4  Summary of fermentation data for the production of PHB on various media used in this study.

<table>
<thead>
<tr>
<th>Fermentation data</th>
<th>MM+SCG</th>
<th>YE+SCG</th>
<th>HL+PG</th>
<th>HL+SCG</th>
<th>HL+CG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermentation time (h)</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>Initial cell concentration (g/L)</td>
<td>0.64</td>
<td>0.42</td>
<td>0.6</td>
<td>0.58</td>
<td>0.66</td>
</tr>
<tr>
<td>Total biomass at 120 h (g/L)</td>
<td>8.00</td>
<td>24.76</td>
<td>28.86</td>
<td>25.10</td>
<td>28.86</td>
</tr>
<tr>
<td>PHB (g/L)</td>
<td>6.18</td>
<td>19.53</td>
<td>25.22</td>
<td>20.44</td>
<td>24.75</td>
</tr>
<tr>
<td>Non-PHB biomass (g/L)</td>
<td>1.82</td>
<td>5.23</td>
<td>3.64</td>
<td>4.66</td>
<td>4.11</td>
</tr>
<tr>
<td>PHB content (%)</td>
<td>77.30</td>
<td>78.87</td>
<td>87.38</td>
<td>81.43</td>
<td>85.76</td>
</tr>
<tr>
<td>Non-PHB biomass content (%)</td>
<td>22.70</td>
<td>21.13</td>
<td>12.62</td>
<td>18.57</td>
<td>14.24</td>
</tr>
<tr>
<td>PHB productivity (g/L·h)</td>
<td>0.05</td>
<td>0.16</td>
<td>0.21</td>
<td>0.17</td>
<td>0.21</td>
</tr>
<tr>
<td>Glycerol consumed (g/L)</td>
<td>44.1</td>
<td>81</td>
<td>86.1</td>
<td>70.6</td>
<td>77</td>
</tr>
<tr>
<td>Glycerol converted to PHB (g/L)</td>
<td>34.09</td>
<td>63.88</td>
<td>75.23</td>
<td>57.49</td>
<td>66.03</td>
</tr>
<tr>
<td>Glycerol converted to non-PHB biomass (g/L)</td>
<td>10.01</td>
<td>17.12</td>
<td>13.87</td>
<td>13.11</td>
<td>10.97</td>
</tr>
<tr>
<td>Yield of PHB on glycerol (theoretical) (g/g)</td>
<td>0.18</td>
<td>0.31</td>
<td>0.34</td>
<td>0.36</td>
<td>0.37</td>
</tr>
<tr>
<td>Yield of PHB on glycerol (observed) (g/g)</td>
<td>0.14</td>
<td>0.24</td>
<td>0.29</td>
<td>0.29</td>
<td>0.32</td>
</tr>
<tr>
<td>Yield of non-PHB biomass on glycerol (g/g)</td>
<td>0.18</td>
<td>0.31</td>
<td>0.34</td>
<td>0.36</td>
<td>0.37</td>
</tr>
<tr>
<td>Initial FAN concentration (g/L)</td>
<td>-</td>
<td>0.95</td>
<td>0.59</td>
<td>0.63</td>
<td>0.61</td>
</tr>
<tr>
<td>Residual FAN concentration (g/L)</td>
<td>-</td>
<td>0.11</td>
<td>0.05</td>
<td>0.05</td>
<td>0.04</td>
</tr>
<tr>
<td>FAN consumed (g/L)</td>
<td>-</td>
<td>0.84</td>
<td>0.54</td>
<td>0.58</td>
<td>0.57</td>
</tr>
<tr>
<td>FAN utilisation (%)</td>
<td>-</td>
<td>87.93</td>
<td>91.60</td>
<td>91.99</td>
<td>93.66</td>
</tr>
<tr>
<td>Yield of non-PHB biomass on FAN (g/g)</td>
<td>-</td>
<td>6.27</td>
<td>6.70</td>
<td>7.98</td>
<td>7.25</td>
</tr>
</tbody>
</table>

#### 8.4.3 Economic feasibility and proposed production scheme for PHB from biodiesel by-products

In Section 8.4.2, the possibility of producing PHB from rapeseed biodiesel by-products has been demonstrated. The study of economic feasibility and process development as well as scaling up is therefore the next step towards industrialised PHB production. The economic feasibility of using biodiesel by-products as raw materials for PHB production was evaluated by comparing the value of the PHB produced to the direct value of the raw materials (rapeseed meal and crude glycerol) as shown in Table 8.5 for a basis of 10,000 kg PHB produced via the process shown in Figure 8.13.
It can be seen from the table that the values of both crude glycerol and rapeseed meal are very low. Purification of crude glycerol to obtained 99.5% purity is an obvious option to increase its value. As can be seen, the price of refined glycerol is almost four times that of crude glycerol. However, the purification process, which includes filtration, chemical addition and fractional vacuum distillation, is expensive and economically unfeasible for small and medium scale plants (Posada et al. 2011). From the biodiesel producer point of view, converting the by-products to PHB might be more economic, particularly if the residual biomass after the fermentation and PHB extraction can be sold as a protein-rich feed. Choi and Lee (1997) presented a breakdown of the costs associated with producing PHB from various systems. This showed that the raw materials accounted for 39% of the total production cost (Figure 8.12) so, using low cost raw materials such as rapeseed meal and crude glycerol could help to reduce the production cost considerably.

![Figure 8.12 Breakdown of average PHB production costs based on a variety of carbon sources (adapted from Choi and Lee (1997)).](image-url)
A flow sheet for PHB production with estimated mass balance for a production of 10,000 kg PHB per annum is proposed. The flow sheet includes both upstream processes (nutrient-rich solution preparation) as well as economic downstream processes (PHB extraction and purification) chosen from the literature (Posada et al. 2011) and is shown in Figure 8.13. The mass balance for upstream processing was calculated based on results shown in Chapter 6, whereas the results obtained from the HL+CG experiment were used for calculation of the mass balance for PHB fermentation.
Figure 8.13  Proposed flow sheet for PHB production from rapeseed biodiesel by-products.
Chapter 8  PHB production

8.5 Conclusion

In this chapter, the feasibility of producing PHB from biodiesel by-products is demonstrated. Two waste streams, namely rapeseed meal and crude glycerol, were used in this study. The rapeseed meal was used to produce a nutrient-rich solution (see Chapter 6) and combined with the crude glycerol as the main carbon source for \textit{C. necator} fermentation. The crude glycerol used in this study comprised 73% glycerol, 12% methanol and 11% water.

PHB is synthesised and accumulated as an intracellular energy and carbon storage material under nutrient-limiting conditions (Brandl \textit{et al}. 1988; Fukui and Doi 1998; Ahn \textit{et al}. 2001). For this reason, the amount of PHB produced is highly dependent on the number of microbial cells present. As seen from Figure 8.10 the accumulation of PHB in microbial cells was similar in all media tested, this means that the medium that support the best cell growth was the most suitable for PHB production. Amongst the media tested, the hydrolysate was considered the best for supporting cell growth. Regardless of the purity of glycerol, the hydrolysate gave the highest biomass concentration as well as the highest yield of biomass per unit of glycerol (28.86 g/L and 0.37 g/g respectively for the HL+CG experiment). It should be noted that the concentration of yeast extract used in the YE+SCG experiment was 4 times the normal concentration but even so, cell growth in the hydrolysate was still better than in yeast extract. The above conclusion was confirmed by the figures given in Table 8.4, especially for PHB concentration and productivity. Clearly, the hydrolysate derived from rapeseed meal and crude glycerol can be used very successfully to produce PHB.

The process for converting biodiesel by-products to PHB is also likely to be more economic than direct sale (Table 8.5). On a basis of 10,000 kg PHB, converting the by-products to PHB can yield 37,500 USD while only 13,900 USD would be obtained from direct sale of these by-products. Also, from the PHB producer point of view, using low cost substrates could reduce the production cost, which in turn enables a reduction in the price of PHB in the market.
Chapter 9

Conclusions and recommendations

9.1 Overall discussion and conclusions

In this project, the by-products generated from rapeseed biodiesel production processes were used as raw materials for the production of polyhydroxybutyrate (PHB). The research started with the study of the growth of PHB-producing bacterium, *Cupriavidus necator* DSM4058, in media containing crude glycerol as sole carbon source (Chapter 4). The optimum crude glycerol concentration for cell growth was found to be in the range 15-25 g/L (Figure 4.6). An average maximum specific growth rate ($\mu_{\text{max}}$) and a Monod constant ($K_s$) obtained from three different approaches (Lineweaver-Burk, Hanes-Woolf and Eadie-Hofstee plots) were 0.13 h$^{-1}$ and 0.92 g/L respectively. Increasing the amount of crude glycerol in the media resulted in longer lag phase. Growth of the bacterium seemed to obey the Monod equation up to 21 g/L crude glycerol. Increasing the crude glycerol concentration beyond this was found to influence cell growth negatively due to substrate inhibition (Figure 4.10).

In order to mitigate the effect of substrate inhibition on the bacterial growth, serial sub-cultivation in media containing more than 48 g/L crude glycerol was carried out. This encouraged adaptation of the bacterium to higher levels of crude glycerol for subsequent experiments. A dramatic improvement in $\mu_{\text{max}}$ (from 0.09 h$^{-1}$ to 0.21 h$^{-1}$) was achieved after six cycles of sub-cultivation in mineral medium containing 48 g/L crude glycerol (Figure 4.14). The adapted bacterium was preserved at −80°C and used as a stock culture for further experiments. A preliminary study of methanol inhibitory effect on the bacterial growth was conducted. The range studied was between 0 and 12 g/L, representing 0 to 25% (v/v) methanol in the crude glycerol stream. The results showed that increasing methanol concentrations could suppress the specific growth rate but did not affect the lag phase of growth (Section 4.4.4). This confirmed that the extended lag phase observed previously was caused by increasing concentration of glycerol in the medium. The effect of C/N ratios (between 197 and 236) on the growth was also
studied. In this experiment, fixed concentrations of glycerol and methanol of 50 g/L and 48 g/L respectively were used. The results suggested that this slight increase in C/N ratio did not affect the growth of the bacterium (Figure 4.19). This was confirmed by a one-way analysis of variance.

The preliminary study of the effects of methanol on growth suggested that it had a negative influence on cell growth. A full investigation of this inhibitory effect was therefore conducted since methanol was found to be one of the major impurities in the crude glycerol that was to be used to grow C. necator. Increasing methanol concentrations up to 48 g/L resulted in a reduction of $\mu_{\text{max}}$ which was initially considered to follow a linear relationship (Figure 5.2). However, increasing the concentration further was found to affect growth in a non-linear manner (Figure 5.4). Several product inhibition models based on the Monod equation were tested to fit the experimental data but none were satisfactory. Considering that a saturation mechanism might be involved in this phenomenon, a saturation equation was adapted and tested. A satisfactory fit was obtained with this model, with a regression coefficient of 0.985 (Figure 5.7) and so it was tested further against data from the literature. The model provided an excellent description for a variety of different data sets, where short-chain alcohols were present. Results are presented in Section 5.4.3.

Rapeseed meal was used in this study to produce nutrient-rich solution for use as an alternative to yeast extract. Solid-state fermentations of the meal using Aspergillus oryzae followed by hydrolysis of fermented solids were employed to extract nutrients from rapeseed meal, especially free amino nitrogen (FAN) which was followed in this study as a nitrogen source for C. necator growth. This strategy yielded overall FAN concentration of 43.4 mg FAN/g meal (718 mg/L) (Section 6.4.2 and Section 6.4.3). Comparing with other nitrogen sources, it was found that the FAN obtained from rapeseed meal was equivalent to 10-20 g/L yeast extract (depending on brand), 15 g/L nutrient broth and 5 g/L beef extract (Figure 6.12).

The production of nutrient solution by other strategies, namely commercial enzymatic treatment (CE), submerged fungal fermentation (SF), SF followed by fungal autolysis (SFA) and hydrolysis of rapeseed meal using enzymatic broth (EB) were also tested and compared with solid-state fermentation followed by hydrolysis of fermented solids (HL). It was found that the hydrolysate gave the highest FAN as well as the highest total
nitrogen (TN) concentrations (Figure 7.1). With the same carbon source and initial FAN concentration, growth (in terms of optical density at the end of experiments) in the hydrolysate was comparable with that in yeast extract as shown in Figure 7.2.

Since the hydrolysate could be used to grow *C. necator*, a study of the effect of initial FAN concentration on growth was subsequently conducted to investigate suitable initial FAN concentrations for *C. necator* fermentation. The main carbon source used was synthetic crude glycerol. The maximum specific growth rate of *C. necator* grown in the hydrolysate was found to be significantly lower than that in yeast extract solutions (0.19 h\(^{-1}\) for the hydrolysate against 0.36 h\(^{-1}\) for yeast extract) (Figure 7.8). However, it was considered that the bacterium consumed other sources of nitrogen together with FAN, resulting in high biomass production. This resulted in comparable biomass production in these two substrates as shown in Figure 7.9. FAN utilisation in the two substrates was different with only 65% of the FAN in the hydrolysate being consumed while 90% of the FAN in yeast extract was used to produce the same amount of biomass. This suggests a difference in the quality of the substrates. The hydrolysate might contain some amino acids or oligopeptides that the bacterium cannot assimilate but it might also be able to use other nitrogen compounds available in the media. It is also worth noting that the conversion of nitrogen to biomass decreased at higher initial FAN concentrations in both systems (Figure 7.9).

PHB production using the rapeseed biodiesel by-products was investigated. Rapeseed meal was used in the form of hydrolysate supplemented with crude glycerol. Other sources of carbon *i.e.* pure glycerol and synthetic crude glycerol were also tested. The bacterial growth and PHB production in the generic feedstock (the hydrolysate supplemented with crude glycerol) were compared with those in other media (mineral medium and yeast extract solution supplemented with synthetic crude glycerol and the hydrolysate supplemented with pure glycerol and synthetic crude glycerol). The results revealed that PHB accumulation (%) in all media were similar. This implied the quality or components present in the media did not adversely affect the ability to convert glycerol into PHB in *C. necator*. On the other hand, media compositions did significantly affect growth. It was found that organic nutrients in yeast extract and the hydrolysate supported the growth better than inorganic nutrients in the mineral medium.
Amongst organic nutrients, the hydrolysate was considered better than yeast extract since it gave similar results with lower initial FAN concentrations (Table 8.4). However, it should be noted that FAN utilisation in the hydrolysate observed during PHB production experiments was higher than that observed previously (90% against only 65% in the previous experiments). This suggested that the quality of the hydrolysate varies from batch to batch since it contains variable amounts of FAN. Considering the yield of PHB on glycerol (0.32 g/g) and yield of non-PHB biomass on FAN (7.25 g/g), which were similar to those observed in the yeast extract experiment (YE+SCG) and the hydrolysate supplemented with either pure glycerol (HL+PG) or synthetic crude glycerol (HL+SCG), it can be concluded that the rapeseed biodiesel by-products have the potential to be used as raw materials for PHB production. Based on these findings, a full process was proposed (Figure 8.13) for the production of PHB from rapeseed biodiesel by-products. The operating conditions for unit operations involved in this process are summarised in Table 9.1.

Table 9.1 Operating conditions for unit operations used in the production of PHB from rapeseed biodiesel by-products.

<table>
<thead>
<tr>
<th>Unit operation</th>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solid-state fermentation</td>
<td>Fermentation time</td>
<td>72 h</td>
</tr>
<tr>
<td></td>
<td>Initial moisture content</td>
<td>65%</td>
</tr>
<tr>
<td></td>
<td>Inoculum size</td>
<td>$1 \times 10^6$ <em>A. oryzae</em> spores/g meal</td>
</tr>
<tr>
<td></td>
<td>Temperature</td>
<td>35°C</td>
</tr>
<tr>
<td>Hydrolysis of fermented solids</td>
<td>Initial solid concentration</td>
<td>10-15 g/L</td>
</tr>
<tr>
<td></td>
<td>Reaction temperature</td>
<td>55°C</td>
</tr>
<tr>
<td></td>
<td>Reaction time</td>
<td>72 h</td>
</tr>
<tr>
<td></td>
<td>Stirring</td>
<td>Periodically</td>
</tr>
<tr>
<td>PHB bioconversion</td>
<td>Aeration rate</td>
<td>1.5 vvm</td>
</tr>
<tr>
<td></td>
<td>Agitation speed</td>
<td>300-1,200 rpm</td>
</tr>
<tr>
<td></td>
<td>Antifoam</td>
<td>Used during the exponential growth phase (3 drops in every 2 h)</td>
</tr>
<tr>
<td></td>
<td>Dissolved oxygen</td>
<td>More than 25%</td>
</tr>
<tr>
<td></td>
<td>Fermentation time</td>
<td>120 h</td>
</tr>
<tr>
<td></td>
<td>Initial FAN concentration</td>
<td>600-700 mg/L</td>
</tr>
<tr>
<td></td>
<td>Inoculum size</td>
<td>10% of working volume</td>
</tr>
<tr>
<td></td>
<td>Glycerol concentration</td>
<td>50 g/L</td>
</tr>
<tr>
<td></td>
<td>pH</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td>Stock crude glycerol concentration</td>
<td>Can be used as it is</td>
</tr>
<tr>
<td></td>
<td>Temperature</td>
<td>30°C</td>
</tr>
<tr>
<td></td>
<td>Working volume</td>
<td>1 litre</td>
</tr>
</tbody>
</table>
9.2 Recommendations for further work

Although the study presented in this thesis has established the potential for the bioconversion of biodiesel by-products to value-added chemicals, it only represents preliminary investigations. There are therefore many more experiments that can and should be performed before the process can be fully evaluated. For example, further work on the mechanism of methanol inhibition in \textit{C. necator} culture as well as the improvement of PHB production is recommended.

9.2.1 Study of methanol inhibition mechanism in \textit{C. necator} culture

For a better understanding of the inhibition mechanism of methanol on \textit{C. necator}, methanol uptake as well as formaldehyde production during the bacterial cultivation should be determined. This could resolve the question of whether methanol reacts with the glycerol facilitators or is consumed and converted into formaldehyde inside the cells.

9.2.2 Enhancement of PHB production from rapeseed biodiesel by-products

To increase the production of PHB from rapeseed biodiesel by-products, it will be necessary to develop and optimise the process further. A number of approaches would be worth pursuing as follows.

\textit{Improving free amino nitrogen production from rapeseed meal}

In this project, solid-state fermentation (SSF) by \textit{Aspergillus oryzae} was conducted followed by hydrolysis of fermented solids to produce a nutrient-rich solution. During SSF, the fungus produces several enzymes including intra- and extra-cellular protein digesting enzymes. The hydrolysis of fermented solids obtained from SSF is the process that exploits these enzymes to digest remaining proteins in the solids. To increase overall production of free amino nitrogen (FAN), unhydrolysed rapeseed meal could be periodically added into the reaction vessel to increase the substrate for the proteolytic enzymes. This approach has proved effective in a wheat system where fresh wheat was suspended in hydrolysate for several cycles. By using this approach, the possibility of
producing a nutrient solution of high concentration with reduction in final feedstock volume could be realised (Koutinas et al. 2003b).

**Improvement of C. necator growth and PHB accumulation**

Since PHB accumulation is dependent upon cell concentration, improvement in cell proliferation can result in higher PHB production. Fed-batch fermentation can be carried out using a feeding solution containing not only carbon source, but also some amount of nitrogen to sustain the growth of the bacterium. Excess nitrogen in the system prevents the bacterium from accumulating PHB, by channelling the metabolism towards cell proliferation. Xu (2007) studied the effects of different feeding solutions on cell growth and PHB accumulation and found that, for a similar concentration of glucose, feeding solutions containing 1,480 mg/L FAN could yield 63% higher non-PHB biomass than feeding a solution containing 300 mg/L FAN. As a result, Xu (2007) could produce 25% more PHB. It has also been reported that C/N ratio can influence poly-3-hydroxybutyrate-co-4-hydroxybutyrate accumulation in *Cupriavidus* sp. USMAA1020 (Amirul et al. 2008). By controlling C/N ratio of the feeding solution, it could be possible to improve PHB accumulation in *C. necator*. In this project, only carbon source was used as a feeding solution, so further research should concentrate on the use of different feeding solutions and/or feeding strategies for enhancing cell growth and PHB production.

Another strategy is to increase the inoculum size in order to improve the growth. By shortening the fermentation time, the productivity will be increased. Yamane *et al.* (1996) found that increasing initial cell concentration from 1.1 g/L to 13.7 g/L, reduced the required fermentation time by 12.5 h to just 18 h. This could also help reduce operation time and production cost in a large scale PHB production.

**9.2.3 Scaling-up of the process**

The feasibility of scaling-up the process from laboratory to pilot scale is worthy of investigation. Although the biomass and PHB concentrations obtained from this research were not comparable to those reported in other systems (Xu 2007; Cavalheiro *et al.* 2009), the production of PHB from rapeseed meal and crude glycerol still yield a more valuable product compared to direct sale of these by-products as shown in
Table 8.5. Better still, if the application of feeding strategy for enhancement of PHB production is successful, it would make the process even more viable.

9.2.4 Study of the possibility of using the generic feedstock for production of other chemicals

The applicability of the generic feedstock derived from rapeseed biodiesel by-products for PHB production was shown to be feasible in this project. This can be used as a model for production of other chemicals such as succinic acid and dihydroxyacetone. Succinic acid, a building block for the production of various commodity and specialty chemicals, can be produced from glycerol by the use of Actinobacillus succinogenes (Vlysidis et al. 2011). Likewise, dihydroxyacetone, a precursor for pharmaceuticals and fine chemicals synthesis, has also been produced from glycerol by Gluconobacter oxydans (Gätgens et al. 2007; Wei et al. 2007). To reduce the cost of the production process, the generic feedstock containing the hydrolysate and crude glycerol could be used.

9.3 Publications from this project


References


References


German Collection of Microorganisms and Cell Culture (DSMZ) (2009). Bacterial Nomenclature up-to-date May 2009 (Approved lists, Validation lists). Braunschweig, Germany, DMSZ.


Hach (2007). Total nitrogen Persulfate digestion method. HACH Company USA.


References


References


References


Appendices

Appendices included in this thesis are as follows.

Appendix A

Log-linear plots of optical density at 600 nm against fermentation time of *C. necator* grown in mineral media containing 50 g/L glycerol and 0 to 125 g/L methanol.
Appendix B

Log-linear plots of dry cell weight of *C. necator* grown in the hydrolysate containing 0 to 660 mg/L FAN supplemented with 50 g/L synthetic crude glycerol.
Log-linear plots of dry cell weight of *C. necator* grown in yeast extract solutions containing 90 to 680 mg/L FAN supplemented with 50 g/L synthetic crude glycerol.