Bioconversion of alkylbenzenes by *Yarrowia lipolytica*

by

Aingy Chantel Lind

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Dr K.G. Clarke & Prof M.S. Smit (co-supervisor)

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DECLARATION

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

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Abstract

The abundance of alkane by-products formed in South Africa presents a feedstock opportunity for the production of a wide range of commercially important products, such as long-chain dioic acids and alcohols. These compounds are formed as intermediates through the biological conversion of alkanes, a route which is particularly attractive when compared with chemical conversion due to its operation under milder process conditions. Furthermore, advances in genetic manipulation, which enable the accumulation of a range of metabolic intermediates, make the biological route remarkably flexible. From the literature review *Yarrowia lipolytica* was identified as a promising organism for use in studying alkane bioconversion because of its ability to produce large quantities of fatty acids when grown on *n*-paraffins as a sole carbon source.

The bioconversion of alkanes will not only depend on the genetic modification but also on the process conditions to maximise growth and bioconversion. The overall objective of this project was therefore to investigate the potential of *Y. lipolytica* for alkane bioconversion by defining the conditions that maximise both cell growth and bioconversion. The *Y. lipolytica* strains supplied (TVN348, TVN493 and WT), however, were not yet modified to the extent that accumulation of metabolic intermediates was possible. Use was therefore made of a model system in which the alkane substrate was substituted with an even chain alkylbenzene. Since *Y. lipolytica* is unable to metabolise the benzene ring, the alkylbenzene is converted to the metabolic intermediate, phenyl acetic acid (PAA), and the potential for bioconversion assessed through measuring the accumulation of PAA. The specific objectives of the project were therefore

1) to define and quantify the parameters for the establishment of an effective model system in shake flasks with respect to trace elements, buffering, added nitrogen, oxygen supply, glucose concentration, alkylbenzene substrate and inducer requirements

2) to use the defined model system to identify the most promising strain of *Y. lipolytica* TVN348, TVN493 and WT

3) to use the defined model system and selected strain for evaluation of the influence of time of substrate addition and glucose concentration on cell growth and bioconversion of *Y. lipolytica* under controlled conditions in an instrumented bioreactor

Furthermore, since poor reproducibility in cell growth and bioconversion had been prevalent in previous studies, it was also aimed to identify and statistically quantify the reproducibility between duplicate or triplicate samples in each experiment and between sets of different experiments with respect to PAA formation and cell concentrations.

Studies were conducted in shake flask cultures to define and quantify the parameters for the model system. The parameters assessed included trace elements, buffering, nitrogen concentration, oxygen supply, glucose concentration, alkylbenzene substrate type and possible inducer requirements. Trace elements, phosphate buffering and added nitrogen did not significantly affect the cell growth of *Y. lipolytica* TVN348. The cell concentration of *Y. lipolytica* TVN348 and TVN493 was increased by 65% and 43% respectively for an increase in oxygen supply by decreasing the working volume from 150ml to 50ml, while the cell concentration of *Y. lipolytica* WT was increased by 41% when oxygen supply was increased by switching from non-baffled to baffled flasks in 50ml cultures. Bioconversion was also increased for an increase in oxygen supply: 2.4mM to 29.0mM PAA (*Y. lipolytica* TVN348) and 1.2mM to 21.7mM PAA (*Y. lipolytica* TVN493) for a decrease in working volume; 10.5mM to 46.6mM PAA (*Y. lipolytica* WT) when switching from non-baffled to baffled flasks. These results indicated that adequate oxygen supply is crucial to both growth and bioconversion, and that further study should be conducted in 50ml working volumes. Cell concentrations obtained in 1.6% (wt/v) and 3.2% (wt/v) glucose cultures (3.95×10^8 cells/ml and 4.03×10^8 cells/ml respectively) indicated that cell growth was neither enhanced nor inhibited by 3.2% (wt/v) glucose. Of the range of substrates examined (propylbenzene, butylbenzene, *sec*-butylbenzene, hexylbenzene, ethyltoluene and *tert*-butyltoluene for *Y. lipolytica* TVN348 and TVN493; octylbenzene and decylbenzene for *Y. lipolytica* WT), hexylbenzene was regarded as the best substrate for bioconversion (14.7mM and 14.1mM PAA for TVN348 and TVN493 respectively; 42.6mM PAA for WT). Lastly, the absence of a requirement for an additional inducer such as ethanol or oleic acid was confirmed when PAA was formed from hexylbenzene in the culture containing additional glucose (25.0mM). This suggested that when using hexylbenzene as substrate, bioconversion was induced provided sufficient glucose was available for cell maintenance.

Results from duplicate or triplicate flasks in each individual shake flask experiment were reproducible and conclusions were based solely on results which showed 95% confidence intervals. However, reproducibility problems were experienced with results between different sets of experiments carried out under the same conditions.

The model system was therefore defined by: 1) no addition of trace elements, additional buffering or added nitrogen, 2) cultures grown in 50ml volumes to supply an adequate amount of oxygen crucial for growth and bioconversion, 3) 3.2% (wt/v) glucose and 4) addition of 1% (v/v) hexylbenzene at 24h with no inducer requirements.

Use of the model system in shake flask cultures to identify the most promising of the three strains of *Y. lipolytica* supplied demonstrated that there was no significant difference in cell growth or bioconversion between these strains. *Y. lipolytica* WT (which has no genetic modifications) was therefore used for further investigation until an appropriate strain could be substituted when it became available.

The growth and bioconversion of *Y. lipolytica* WT was further investigated under controlled conditions in a bioreactor. The influence of time of substrate addition (11h, 24h, 48h) and glucose concentration (3.2% and 6.4% (wt/v)) on growth and bioconversion was examined.

When hexylbenzene was added at 48h, cell growth was increased (8.90×10^8cells/ml) when compared to two of the triplicate cultures with hexylbenzene addition at 24h (4.74×10^8cells/ml and 3.92×10^8cells/ml) and the culture with hexylbenzene addition at 11h (2.82×10^8cells/ml). The third of the triplicate cultures with hexylbenzene addition at 24h, on the other hand, exhibited the strongest growth (2.23×10^9cells/ml). The poor reproducibility between the triplicate cultures with hexylbenzene addition as 24h made it difficult to determine whether hexylbenzene addition at 24h or 48h maximised cell growth. Furthermore, the cell growth was not significantly improved when the glucose concentration was increased from 3.2% (wt/v) to 6.4% (wt/v) (7.47×10^8cells/ml for 6.4% glucose culture), however it was also not inhibited.

The highest amount of specific PAA formed by *Y. lipolytica* WT was found when hexylbenzene was added at 11h (7.4×10^{-11}mmol PAA/cell), however the highest accumulated PAA was produced in the culture that exhibited the strongest growth with hexylbenzene addition at 24h (41.4mM). This suggested that the bioconversion of hexylbenzene was maximised when it was added during the active growth phase. It is therefore recommended to conduct fed-batch experiments in future to maintain the active growth phase. Accumulated PAA was increased in 6.4% (wt/v) glucose culture (15.2mM PAA) when compared with two of the 3.2% (wt/v) glucose cultures (5.4mM and 4.3mM PAA). These results indicated that the increased glucose concentration did not inhibit the bioconversion. Furthermore, PAA was formed when 5% (wt/v) residual glucose was observed in the culture, suggesting that the bioconversion of hexylbenzene was not inhibited at glucose

concentrations as high as 5.0% (wt/v). If future work were to be conducted in bioreactor culture where glucose is added in fed-batch operation, glucose concentrations in cultures of up to 5% (wt/v) could be considered for initial studies.

During bioconversion by *Y. lipolytica*, the PAA measured after hexylbenzene exhaustion did not, however, correspond to 100% conversion. Further, poor reproducibility was found in the bioreactor cultures. The disappearance of hexylbenzene without a corresponding accumulation of PAA and poor reproducibility was investigated by determining whether PAA was further degraded or alternatively, whether other metabolic intermediates were being formed and accumulated from the hexylbenzene. However, substitution of the hexylbenzene with PAA as substrate confirmed that PAA could not be metabolised. Further, NMR analyses of both the aqueous and organic phases of the culture did not identify any additional metabolic intermediates. It is recommended that additional analyses be conducted on the aqueous and organic phases to further assess the possible accumulation of intermediates.

The development of the model system in shake flask cultures demonstrated the importance of adequate oxygen supply for both cell growth and bioconversion. It was also shown that no inducer was needed because hexylbenzene acted as its own substrate inducer. Furthermore, comparison of *Y. lipolytica* strains TVN348, TVN493 and WT under the defined conditions of the model system revealed that the genetically modified strains (TVN348, TVN493) did not exhibit enhanced bioconversion. Bioreactor cultures using the model system under controlled conditions further showed that bioconversion was not inhibited at a 5% (wt/v) residual glucose concentration and suggested that bioconversion was maximised when hexylbenzene was added during active growth phase. This informs on future work, suggesting fed-batch operation in order to extend the active growth phase, where glucose concentrations in the bioreactor of up to 5% (wt/v) can be considered.

Opsomming

Die groot hoeveelheid alkaan by-produkte wat in Suid Afrika gevorm is lewer 'n voerstof geleentheid vir die produksie van 'n wye reeks produkte wat van commersiële belang is, soos lang-ketting dioïse sure en alkohole. Dié chemiese verbindinge is geproduseer deur 'n biologiese omsetting van alkane, waar dit particularly attractive is as dit met die chemiese omsetting vergelyk word. Verder maak vorderings in genetiese manipulasie, wat die akkumulasie van 'n reeks metaboliese oorgangs in staat stel, die biologiese roete opmerklik handelbaar. Van die literatuur studie is *Yarrowia lipolytica* as 'n belowende organisme vir die studie van alkaan biologiese omsettings aangedui. Dit is as gevolg van die organisme se vermoë om groot hoeveelhede sure te produseer as dit op alkane gegroei is.

Die biologiese omsetting van alkane sal nie net op die organisme se genetiese manipulasie afhang nie, maar asook op die proses kondisies, wat sel groei en biologiese omsetting maksimeer. Die algemene doel van dié projek was dus om die potensiaal van *Y. lipolytica* vir die biologiese omsetting van alkane te ondersoek deur die kondisies te definieer wat albei sel groei en biologiese omsetting maksimeer. Die *Y. lipolytica* rasse (TVN348, TVN493 en WT) wat verskaf is was nie gemodifiseer tot die mate dat akkumulasie van metaboliese oorgangs moontlik is nie. 'n Model sisteem was dus gebruik waar die alkaan substraat met 'n alkiel benseen vervang was. Siende dat *Y. lipolytica* nie in staat is om die benseen ring te metaboliseer nie is die alkiel benseen omgekeer na die metaboliese oorgangs, feniel asynsuur (FAS). Die potensiaal vir biologiese omsetting is dus beraam deur die akkumulasie van FAS te meet. Die spesifieke doele van die projek was dus

1) om die parameters van die model sisteem in skud vlesse te definieer en om hoeveelhede te bepaal met betrekking tot spoor elemente, addisionele buffers, addisionele stikstof, suurstof verskaffing, glukose konsentrasie, alkielbenseen substraat en induseerder behoeftes

2) om die gedefinieerde model sisteem te gebruik om die mees belowende ras van *Y. lipolytica* TVN348, TVN493 en WT aan te dui

3) om die gedefinieerde model sisteem en geselekteerde ras te gebruik om die invloed van tyd van substraat byvoeging en glukose konsentrasie op sel groei en biologiese omsetting in 'n geïnstrumenteerde bioreaktor onder gekontroleerde kondisies te bereken.

Verder, siende dat daar 'n swak herhaalbaarheid in vorige studies aangedui is vir sel groei en biologiese omsetting, was daar ook die doel om die herhaalhaarbeid tussen duplikaat en triplikaat monsters in elke eksperiment en tussen verskillende eksperimente met betrekking tot FAS produksie en sel konsentrasie te identifiseer en statisties te bepaal.

Studies is in skud vlesse gevoer vir om van die parameters van die model sisteem te definieer en om hoeveelhede te bepaal. Die parameters wat in skud vlesse beraam is sluit spoor elemente, bufferwerking, stikstof konsentrasie, suurstof verskaffing, glukose konsentrasie, alkiel benseen substraat tipe en moontlike induseerder vereistes in. Die sel konsentrasie van *Y. lipolytica* TVN348 en TVN493 was respektiewelik met 65% en 43% vermeerder vir 'n toeneming in suurstof verskaffing (deur 'n afneming in werkende volume van 150ml na 50ml kulture). Verder was die sel konsentrasie van *Y. lipolytica* WT met 41% vermeerder vir 'n toeneming in suurstof verskaffing (met die oorskakeling van 50ml kulture in gewone skud vlesse na skot skud vlesse toe). Die biologiese omsetting was ook vermeerder vir 'n toeneming in suurstof verskaffing: 2.4mM na 29.0mM FAS (*Y. lipolytica* TVN348) en 1.2mM na 21.7mM FAS (*Y. lipolytica* TVN493) vir 'n afneming in werkende volume; 10.5mM na 46.6mM FAS (*Y. lipolytica* WT) as gewone skud vlesse met skot skud vlesse vervang is. Dié resultate het aangedui dat dit baie belangrik is vir albei sel groei en biologiese omsetting dat die organisme 'n voldoende hoveelheid suurstof kry, en dat verdere werk in 50ml kultuur volumes uitgereik moet word. Sel konsentrasies wat in 1.6% en 3.2% glukose kulture verkrygbaar was (respektiewelik 3.95×10^8 selle/ml en 4.03×10^8 selle/ml) het aangedui dat sel groei nie versterk of verhinder is deur 'n 3.2% glukose konsentrasie nie. Van die reeks alkielbenseen substrate wat ondersoek was (propielbenseen, butielbenseen, *sec*-butielbenseen, hexielbenseen, etieltolueen en *tert*-butieltolueen vir *Y. lipolytica* TVN348 en TVN493; oktielbenseen en decylbenseen vir *Y. lipolytica* WT) is hexielbenseen as die beste substraat vir biologiese omsetting aangedui (14.7mM en 14.1mM FAS vir TVN348 en TVN493; 42.6mM FAS vir WT). Ten laaste, is die afwesigheid van die vereiste vir 'n addisionele induseerder soos etanol or oliesuur is ook bevestig toe daar FAS, in die kultuur wat addisonele glukose gehad het, gevorm is (25.0mM). Dié het voorgestel dat die biologiese omsetting sonder 'n induseerder voort kan as hexielbenseen as substraat gebruik word mits dat daar 'n voldoende hoveelheid glukose in die kultuur is.

Resultate van die duplikaat of triplikaat vlesse in elke individuele skud vles eksperiment was herhaalbaar. Verder is gevolgtrekkings van die eksperimente op 95% sekerheids tussentye gebaseer. Swak herhaalbaarheid is egter soms tussen stelle van identiese eksperimente gevind.

Die model sisteem was dus gedefinieer: 1) geen spoor elemente, addisionel buffers of addisionele stikstof is bygevoeg nie, 2) kulture is in 50ml werkende volumes gegroei om die belangrike suurstof vir sel groei en biologiese omsetting te verskaf, 3) 3.2% glukose and 4) 1% (v/v) hexielbenseen is teen 24uur bygevoeg sondere enige induseerders.

Gebruik van die model sisteem in skud vles kulture om die mees belowende ras van die drie rasse van die verskafde *Y. lipolytica* aan te dui het bewys dat daar geen betekenisvolle verskil in sel groei of biologiese omsetting tussen die drie rasse was nie. *Y. lipolytica* WT (wat geen genetiese veranderings het nie) was dus gebruik vir verdere ondersoeking totdat 'n geskikte ras vervang kan word sodra dit beskikbaar is.

Die sel groei en biologiese omsetting van *Y. lipolytica* WT was onder gekontroleerde kondisies in 'n bioreactor ondersoek. Die invloed van die tyd van substraat byvoeging (11 uur, 24 uur en 48 uur) en glukose konsentrasies (3.2% en 6.4%) was ondersoek.

Toe hexiel benseen teen 48 uur bygevoeg is was die sel groei vermeerder (8.90×10^8selle/ml) as dit met twee van die drie kulture met hexiel benseen bygevoeging teen 24 uur (4.74×10^8selle/ml en 3.92×10^8selle/ml), en met die kultuur met hexiel benseen byvoeging teen 11 uur vergelyk word (2.82×10^8selle/ml). Die derde kultuur van die drie kulture met hexiel byvoeging teen 24 uur het egter die sterkste groei vertoon (2.23×10^9selle/ml). Die swak herhaalbaarheid tussen die kulture waar hexiel benseen teen 24 uur bygevoeg is maak dit moeilik om te bepaal of sel groei verbeter is in kulture met 24 uur of 48 uur byvoeging. Verder is die sel groei nie betenisvol vermeerder toe die glukose konsentrasie van 3.2% tot 6.4% (7.47×10^8selle/ml vir 6.4% glukose kultuur) vermeerder is nie. Die sel groei is egter ook nie verhinder teen 6.4% glukose nie.

Die hoogste spesifieke FAS produksie by *Y. lipolytica* WT is in die kultuur waar hexielbenseen teen 11 uur bygevoeg is, gevind (7.4×10^{-11}mmol FAS/sel). Die hoogste geakkumuleerde FAS is egter in die kultuur waar hexielbenseen teen 24 uur bygevoeg is, gevind. Dit stel voor dat die biologiese omsetting van hexielbenseen gemaksimeer was toe dit gedurende die aktiewe groei fase bygevoeg was. Dit is dus verder voorgestel om eksperimente onder "fed-batch" kondisies te ondersoek wat die aktiewe groei fase onderhou. Geakkumuleerde FAS was in die 6.4% glukose kultuur vermeerder (15.2mM FAS) as dit met twee van die 3.2% glukose kulture vergelyk word (5.4mM en 4.3mM FAS). Dié resultate het aangedui dat 'n hoer glukose konsentrasie van 6.4% nie die biologiese omsetting verhinder het nie. Verder is FAS in die kultuur gevorm toe daar nogsteed 5% oorblywende

glukose in die kultuur gemeet is, wat aandui dat die biologiese omsetting van hexielbenseen nie teen 5% glukose verhinder is nie. As verdere werk in bioreaktor eksperimente uitgevoer is waar glukose onder "fed-batch" kondisies bygevoeg is, kan konsentrasies tot en met 5% aanvanklik beskou word.

Die afgemete FAS na hexiel benseen uitputting het nie met 100% omsetting in enigeen van die kulture ooreenstem nie. Verder is swak herhaalbaarheid in bioreaktor kulture gevind. Die verdwyning van hexielbenseen sonder die ooreenstemmende FAS, en die swak herhaalbaarheid is ondersoek deur om te bepall of FAS verder gebruik was en of daar alternatiewe meta wat voorstel dat hexiel benseen omgesit is in addisionele metaboliese oorgangs gevorm is. Die plaasvervanging met FAS as substraat het bevestig dat dit nie moontlik was vir die organisme om FAS te metaboliseer nie. Verder kon KMR analises van albei die waterige en organiese fases nie bewys of daar ander metaboliese oorgangs gevorm word nie. Dit is voorgestel dat addisionele analises op die waterige en organiese fases ondersoek is om verder die akkumulasie van metaboliese oorgangs te skat.

Die ontwikkeling van die model sisteem in skud vlesse het die belangrikheid van 'n voldoende verskaffing van suurstof vir albei sel groei en biologiese omsetting bewys. Dit is ook bewys dat geen induseerder benodig vir die biologiese omsetting van hexielbenseen nie, en veral dat hexielbenseen as sy eie induseerder optree. Verder is daar, onder die kondisies van die model sisteem, geen verskil in biologiese omsetting tussen *Y. lipolytica* TVN348, TVN493 en WT gevind nie, wat aandui dat die biologiese omsetting in die gemodifeerde rasses (TVN348, TVN493) nie suksesvol was nie. Bioreaktor kulture wat die model sisteem gebruik het het bewys dat die biologiese omsetting nier verhinder is as daar 5% oorblywende glukose in die kultuur is nie en het aangedui dat die biologiese omsetting gemaksimeer is as hexielbenseen gedurend die aktiewe groei fase bygevoeg is. Hierdie ontdekkings kan by toekomstige ingesluit word omdat dit aandui dat as eksperimente onder "fed-batch" kondisies bygevoeg word sal die aktiewe groei fase verleng word, en verder kan glukose konsentrasies tot en met 5% beskou word.

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1. Introduction

The gas to liquid technology in South Africa produces an abundance of alkane by-products (Sasol Annual Facts, 2007). These by-products present a feedstock opportunity for the production of a wide range of commercially important products and intermediates. Products of interest include long-chain alcohols and dioic acids. Long-chain alcohols are important raw materials in the detergent and perfume industries and dioic acids are chemical intermediates in the manufacture of polyamides and polyesters.

Long-chain dioic acids and alcohols are generally derived chemically from hydrocarbons (Ullmann 2002). Dioic acids are produced by oxidative cleavage or oxidation while alcohols are produced by a catalysed liquid phase oxidation. Chemical processes, however, are operated at high temperatures and pressures and are further subjected to an increase in cost with an increase in carbon chain length (Ullmann 2002). The biological conversion from alkane feed stocks, on the other hand, takes place at ambient conditions and is therefore an attractive route to pursue. The possibility of genetic modification to enable organisms to accumulate metabolic intermediates makes the biological route exceptionally versatile.

The optimisation of the biological conversion to alcohol or dioic acid intermediates is made possible by both the availability of a suitably modified organism (genetically enhanced for accumulate of desired product intermediates) and process modifications to provide conditions for maximal growth. The yeast, *Yarrowia lipolytica*, has been reported as a promising organism for use in studies on alkane bioconversion. This is due to the large amount of proteins that are easily secreted by the organism, making it a suitable candidate for genetic modification (Beckerich *et al.* 1998). Studies already in progress aim to genetically modify strains of *Y. lipolytica* such that its ability to accumulate metabolic intermediates may be enabled and its capacity for bioconversion is enhanced (Smit, personal communication). It is therefore the focus of this study to modify the process. This will be achieved by quantifying the organism's potential for the accumulation of intermediates, and investigating parameters that affect conditions for optimal bioconversion of *Y. lipolytica*.

The *Y. lipolytica* strains supplied for this study have not yet been modified to the extent that accumulation of metabolic intermediates is possible. Instead, multiple copies of the gene encoding for the enzyme, Cytochrome P450, have been inserted. Cytochrome P450 is responsible for the initial oxidation of hydrocarbons and the over-expression of this enzyme therefore enhances the

organism's potential for bioconversion. Since accumulation of metabolic intermediates is not possible with the strains supplied, use is made of a model system. The model system uses straight chain alkylbenzenes in the place of alkane substrates. *Y. lipolytica* are unable to metabolise the benzene ring in alkylbenzenes and so will accumulate phenyl acetic acid (PAA) for even chained alkylbenzenes. The acids are readily measurable intermediates and their quantification is a direct measurement of the organisms' potential to biologically convert hydrocarbons.

The scope of this project incorporates preliminary studies conducted in shake flasks to develop the model system. Cultures in shake flasks are limited by the control of certain process parameters, which include pH regulation and oxygen supply. Subsequent cultures in an instrumented bioreactor overcome these limitations under controlled conditions. Further evaluation of the organism's potential for bioconversion with the use of the developed model system will be undertaken. Additionally, since more than one strain is supplied, strain selection will also be required.

The aim of this project is to investigate conditions, which maximise both cell growth and bioconversion by *Y. lipolytica* to increase accumulated and specific product formation, respectively. A range of parameters, which influence both cell growth and bioconversion, will therefore be investigated. In addition to this, reproducibility in bioconversion and cell growth between sets of cultivations are known to be a problem for these strains of *Y. lipolytica*, therefore it will also be addressed in the project.

The main objectives of this project are therefore to:

- Develop the model system in shake flasks in terms of process conditions for the bioconversion of alkylbenzenes by *Y. lipolytica*

- Identify and statistically quantify reproducibility between duplicate or triplicate samples in each experiment and between sets of different experiments, both with respect to PAA formation and cell concentrations

- Use the model system to identify the most promising strain of supplied *Y. lipolytica* strains, namely TVN348, TVN493 or WT

- Use the model system and selected strain in an instrumented bioreactor to further evaluate the influence of process conditions on the growth and bioconversion of *Y. lipolytica* under controlled conditions

The thesis is organised to provide a literature review that investigates the application of biological systems, the micro-organisms of interest and their existing modifications, alkane metabolism and process considerations for the study (Chapter 2). Chapter 3 describes the materials and methods used in the study while the results are presented and discussed in Chapters 4 and 5. Chapter 4 details the development of the model system in shake flask culture, while Chapter 5 describes the use of the model system to identify the most promising strain and for further investigation of time of hexylbenzene addition and glucose concentration in bioreactor culture. Both discussion chapters address the reproducibility between experiments. The conclusions and recommendations drawn from these discussions are then summarised in Chapter 6.

2. Literature survey

2.1 Introduction

The aim of this review is to investigate the existing knowledge on the capacity of micro-organisms to produce commercial products from *n*-alkane sources. The review is commenced by investigation of the commercial applications of products obtained through chemical or biological conversion of hydrocarbons. After identifying the biological conversion as an attractive route to pursue, an investigation of the micro-organisms capable of hydrocarbon degradation is undertaken. The metabolism of *n*-alkanes is also discussed in order to gain a better understanding of the reactions taking place and the enzymes involved. Finally, an investigation is made of the process challenges, which influence the bioconversion of hydrocarbons. Process challenges include the influence of operational parameters, hydrocarbon- type, concentration and time of addition, carbohydrate energy co-substrate- type and concentration, and oxygen supply.

The identification of products of commercial interest, a suitable organism and process challenges forms the scope of this review. The primary focus lies in the production of long-chain dioic acids and alcohols from *n*-alkanes. Particular attention is therefore given to such processes, from reported literature, in all aspects of the review. Furthermore, specific emphasis is placed on genetically modified organisms and in particular the yeast, *Yarrowia lipolytica*. Upon investigation of the process challenges, a route forward may be proposed to determine conditions that optimise the bioconversion of alkanes.

2.2 Commercial applications of products obtained through chemical or biological conversion of hydrocarbons

The range of products available from the chemical conversion of hydrocarbons is extensive. Some of these products and the methods for their production (Ullmann 2002) include:

- Acetylene - produced from pure or a mixture of saturated hydrocarbons by pyrolysis at temperatures above $1000^{o}C$

- Synthesis gas (carbon monoxide and hydrogen) - produced by the steam-catalysed or non-catalysed partial oxidation of saturated hydrocarbons

- Dicarboxylic (dioic) acids - produced by the oxidative cleavage of cyclic hydrocarbons

- Long-chain alchohols - produced by a catalysed liquid phase oxidation of long-chain *n*-alkanes

The primary use of acetylene is as raw material for detergents. More specifically long-chain dioic acids are chemical intermediates in the manufacture of polyamides and polyesters, whereas long-chain alcohols are used in detergents, perfumes and insecticides (Ullmann 2002).

An advantage of chemical processes is the wide range of commodities that can be produced from a wide range of hydrocarbons. Disadvantages that arise from employing chemical processes, however, are that they are increasingly costly with an increase in carbon chain length and/or that they sustain hazardous operational parameters. In the biological conversion of hydrocarbons, micro-organisms have the potential to oxidise some hydrocarbons and, in particular, long-chain alkanes as their sole carbon source at ambient conditions (Bühler & Schindler 1984). Furthermore, advances in genetic manipulations, which enable the accumulation of metabolic intermediates and expand the range of available commodities, provide a further advantage of biological over chemical processing of alkanes (Groguenin *et al.* 2004, Picataggio *et al.* 1992). The reduction in cost- and safety issues, together with the added advantage of genetic manipulation, makes the biological route an attractive route to pursue.

Literature that deals with the biological production of long-chain dioic acids from *n*-alkanes is abundant (Table 2.1). The biological production of citric acid from hydrocarbons is not as common; however, citric acid is also produced from biological growth on glucose (Antonucci *et al.* 2001, Rane & Sims 1993). Furthermore, there is limited information regarding the biological production of long-chain alcohols. This may partially be due to the difficulty in genetically modifying organisms for the accumulation of alcohols (Smit, personal communication).

Table 2.1 Biological production of commodities from hydrocarbons

Product	Commercial application	Hydrocarbon source	Micro-organism used	Reference
Citric acid	Raw material in pharmaceuticals and food	n-paraffins	*Candida lipolytica*	Akiyama *et al.* 1973
		ethanol	*Yarrowia lipolytica*	Arzumanov *et al.* 2000
Long-chain dioic acids	Raw material in emulsified agents, lubricants, plastics, perfume, pharmaceuticals	n-alkanes	*Candida tropicalis*	Hara *et al.* 2001; Jiao *et al.* 2001; Lin *et al.* 2000
		chloro alkanes	*Cunninghamella elegans*	Murphy & Perry 1984
		chloro alkanes	*Penicillium zonatum*	Murphy & Perry 1984
		chloro alkanes	*Candida lipolytica*	Murphy & Perry 1984
		C_9 to C_{18} alkanes	*Candida cloacae*	Uchio & Shiio 1972
		C_{10} to C_{14} alkanes	*Corynebacterium*	Kester & Foster 1962
		C_{11} to C_{16} alkanes	*Candida tropicalis*	Hill *et al.* 1986
		C_{14} to C_{18} alkanes	*Candida lipolytica*	Klug & Markovetz 1967
		n-pentadecane	*Cryptococcus neoformans*	Chan & Kuo 1997
		n-pentadecane	*Pseudomonas aeruginosa*	Chan *et al*. 1997
		n-pentadecane	*Rhodococcus opacu*	Alvarez 2003
		C_{15} to C_{16} alkanes	*Nocardia asteroides*	Alvarez 2003
		C_{15} to C_{16} alkanes	*Rhodococcus erythropolis*	Alvarez 2003
		C_{15} to C_{16} alkanes	*Rhodococcus fascians*	Alvarez 2003
		n-hexadecane	*Gordonia amarae*	Alvarez 2003
		n-hexadecane	*Nocardia globerula*	Alvarez 2003
		n-hexadecane	*Nocardia restricta*	Alvarez 2003
Long-chain alcohols	Raw material in perfume and detergents	C_{14} to C_{18} alkanes	*Candida lipolytica*	Klug & Markovetz 1967
		C_{16}, C_{18} alkanes	*Candida tropicalis*	Cheng *et al.* 2005
		n-alkanes	*Pseudomonas putida*	Mathys *et al*. 1998

To expand the range of commodities formed through biological production it will be important to develop bioprocesses through process operation and modification of the organism's potential for bioconversion. It is therefore necessary to investigate micro-organisms previously studied in light of hydrocarbon utilisation. These organisms are discussed in detail in section 2.3 and the routes for genetic modification are investigated.

2.3 Hydrocarbon-degrading micro-organisms

The degradation or assimilation of certain hydrocarbons by micro-organisms takes place preferentially over other hydrocarbons (Bühler & Schindler 1984), i.e.:

a)   Aliphatic paraffins are more readily degraded than aromatic hydrocarbons

b)   Long-chain paraffins are preferred over short-chain paraffins

c)   Saturated- is preferred over unsaturated compounds and

d)   Straight chain hydrocarbons are more readily degraded than branched chains

The use of aliphatic hydrocarbons, as substrate in biological processes, has been studied extensively. Most of these studies involved the utilisation of n-alkanes with a carbon chain length of C_9 to C_{18}, because they are degraded better than other n-alkanes. Furthermore, n-alkanes with lower carbon content are toxic due to a higher solubility in aqueous solutions (Klug and Markovetz, 1967). It is important to note that, in addition to hydrocarbon bioconversions, micro-organisms metabolise sugar sources as well. Often the micro-organism is supplemented by both hydrocarbon and sugar source, which forms an important focus of this study. Cultures grown on sugar are well established and investigation of these processes are well documented (Akiyama *et al.* 1973, Amaral *et al.* 2006, Antonucci *et al.* 2001, Blasig *et al.* 1984, Blázquez *et al.* 1993, Dynesen *et al.* 1998, Fickers *et al.* 2006, Ghribi *et al.* 2006, Green *et al.* 2000, Hara *et al.* 2001, Papanikoaou & Aggelis 2002, Rane & Sims 1993, Yun & Ryu 2001).

Examples of the accumulation of metabolic intermediates in hydrocarbon- and/or sugar metabolism with non-genetically modified organisms include the production of citric acid from n-paraffins, glucose or ethanol by *Yarrowia lipolytica* (Akiyama *et al.* 1973, Arzumanov *et al.* 2000, Antonucci *et al.* 2001).

Other examples include the formation of fatty acids from *n*-alkanes by various micro-organisms (Blasig *et al.* 1984, Chan *et al.* 1997, Jiao *et al.* 2001, Cheng *et al.* 2005). That said, the degradation by micro-organisms usually prevents the efficient accumulation of compounds that are of the same carbon chain length as carbon substrate (Picataggio *et al.* 1992). Consequently, genetic modification of the organism aims to enable such accumulations. In a hypothetical situation, the genes, encoding for enzymes responsible for the conversion of the hydrocarbon, would firstly be cloned so that multiple copies could be inserted for over-expression of the enzyme. Maximum conversion of the hydrocarbon could then take place (Madzak *et al.* 2004). Secondly, efficient accumulation of the product intermediate could be achieved by blocking further metabolism, as performed by Picataggio *et al.* (1992). Using *Candida tropicalis*, Picataggio *et al.* (1992) disrupted the genes encoding for the β-oxidation pathway, which allows the organism to obtain energy from hydrocarbons for growth. This was for the production of long-chain dicarboxylic acids from alkanes. Blocking further metabolism necessitates an alternative carbon and energy source (not a hydrocarbon) for cell growth and maintenance. For that reason the influence of sugar (the alternative carbohydrate energy source and co-substrate) metabolism on the bioconversion of hydrocarbons is an important factor to consider when using genetically modified organisms that cannot obtain sufficient energy from the hydrocarbon source (substrate) alone (Picataggio *et al.* 1992).

In subsections 2.3.1 and 2.3.2 that follow, bacteria and yeasts are investigated to identify a suitable organism for hydrocarbon utilisation and/or genetic modification.

2.3.1 Bacteria

Studies conducted by Kester & Foster (1962) showed that the genus *Corynebacterium* degrades C_3 to C_{15} alkanes and C_{17} to C_{18} alkanes. More specifically *Corynebacterium hydrocarboclastus* grows maximally on *n*-C_{14} to *n*-C_{19} alkanes (Shiio & Uchio 1968).

Al-Hadhrami *et al.* (1995) investigated the application toward the biodegradation of oil spills in the Gulf of Oman. They found that *Pseudomonas aeruginosa* was the only organism out of a mixture of eight bacteria (namely *Micrococcus luteus*, *Klebsiella cepacia*, *Moraxella phenylpyruvica*, *Xanthomonas maltophilia*, *Ochrobactrum anthoropi*, *Vibrio fischeri* and *Enterococcus casseliflavens*) to survive in an oil-water-air-mixture. The crude oil contained C_{14} to C_{30} alkanes, all of which were degraded by *P. aeruginosa*. Another study compared the degradation of crude oil by *Pseudomonas* sp., *Acinetobacter* sp. and *Bacillus* sp. (Verma *et al.*

2006); however, it was the *Bacillus* sp. that degraded the most crude oil (C_{12} to C_{30} aliphatic and aromatic hydrocarbons).

Chan *et al.* (1997) used *P. aeruginosa* to degrade *n*-pentadecane to tridecane 1,13-dicarboxylic acid for industrial use. The same bioconversion of *n*-pentadecane to tridecane 1,13-dicarboxylic acid by *Cryptococcus neoformans* was investigated (Chan & Kuo 1997).

Numerous species of anaerobic bacteria degrade aromatic or aliphatic hydrocarbons. Examples include the degradation of $C_6 - C_{20}$ alkanes by the *Azoarcus* species and *Desulfobacterium cetonicum*. *Thauera aromatica*, *Desulfobacula toluolica* and *Desulfobacterium cetonicum* degrade toluene (Spormann & Widdel 2000). Furthermore, actinomycetes bacteria belonging to the genera *Rhodococcus (R. fascians, R. erythropolis* and *R. opacus), Nocardia (N. asteroides, N. globerula* and *N. restricta), Gordonia (G. amarae) and Dietzia* are able to degrade gas-oil, pristane, naphthalene, hexadecane and phenyldecane for cell growth (Alvarez 2003).

Mohanty & Mukherji (2008) reported that Gram-positive *Exiguobacterium aurantiacum* and Gram-negative *Burkholderia cepacia* degraded diesel which contained C_9 to C_{26} alkanes. Both cultures degraded C_{17} to C_{19} alkanes faster than C_{10} to C_{16} alkanes, which is contrary to the findings by Setti *et al.* (1995). Setti *et al.* reported that the rate of C_{12} to C_{16} alkane degradation was linearly correlated with the inverse of carbon chain length. This suggests that each organism has its own preferential use of hydrocarbons, which substantiates the need always to investigate an organism with respect to specific hydrocarbon degradation.

Finally, in a review by Bühler and Schindler (1984) additional bacteria have been reported as hydrocarbon degraders, namely *Achromobacter, Acinetobacter, Alcaligenes, Arthrobacter, Aermononas, Bacillus, Brevibacterium, Flavobacterium, Micrococcus, Mycobacterium, Streptomyces* and *Vibrio*.

2.3.2 Yeasts

A large range of yeasts is known to utilise the hydrocarbons efficiently to produce valuable products. These include both the wild type and genetically modified (and recombinant) yeasts.

2.3.2.1 <u>Wild yeasts</u>

Lodderomyces elongisporus degraded *n*-C_{14}, *n*-C_{16} and *n*-C_{17} alkanes to yield the corresponding fatty acid (Blasig *et al.* 1984). *Candida tropicalis* was also

reported as able to convert *n*-alkanes such as tridecane (Jiao *et al.* 2001), hexadecane and octadecane (Cheng *et al.* 2005) to fatty acids effectively.

Studies on the degradation of hexadecane by *Candida maltosa* are well-documented (Riege *et al.* 1989, Wiedmann *et al.* 1988, Scheller *et al.* 1998). *C. maltosa* degraded C_{11}-C_{19} alkanes and solid alkanes up to a chain length of 28 carbons, although the fatty acid produced was predominantly C_{16} to C_{18} fatty acids, regardless of the substrate's carbon chain length (Blasig *et al.* 1989).

In an investigation the yeasts *Candida tropicalis, Candida maltosa, Pichia stipitis* and *Yarrowia lipolytica*, and bacteria *Acinetobacter calcoaceticus, Mycobacterium fortuitum, Nocardia coynebacteroides, Pseudomonas aeruginosa, Pseudomonas aureofaciens, Pseudomonas oleovorans, Rhodococcus erythropolis* and *Rhodococcus rhodochrous*, were compared to one another with regard to alkane assimilation of decane and hexadecane (Schmitz *et al.* 2000). It was found that yeasts overgrew bacteria. Furthermore, strains of *Candida maltosa* and *Yarrowia lipolytica* showed enhanced growth over strains of *Candida tropicalis*, *Candida shehatae* and *Pichia stipitis* (Schmitz *et al.* 2000).

Of the two most efficient hydrocarbon utilising yeasts (i.e. *Candida maltosa*, *Yarrowia lipolytica*), focus has been placed on *Y. lipolytica* because they are able to produce particularly high amounts of organic acids when grown on *n*-paraffins as a sole carbon source (Barth and Gaillardin 1997, Finogenova *et al.* 2005). *Y. lipolytica* is a dimorphic, non-pathogenic yeast (Juretzek *et al.* 2001) and forms part of the group of "non-conventional" yeasts. Yeasts were placed into this group of species if they were not studied for areas of fundamental research (Van Dijken 2001), such as with *Saccharomyces cerevisiae*. The genera were originally classified as *Candida* because no sexual state had been described but was subsequently changed to *Yarrowia* in 1980. Furthermore, *Yarrowia* showed prospects for industrial application because of its ability to secrete large amounts of proteins (Beckerich *et al.* 1998).

Examples of hydrocarbon utilisation by *Y. lipolytica* include a study using *C. lipolytica* for the production of biomass by degradation of a C_{12} alkane (Moo-Young *et al.* 1971, Whitworth *et al.* 1973). *Y. lipolytica* can also be applied in the treatment of olive mill wastewater (Lanciotti *et al.* 2005, Scioli & Vollaro 1997). The COD level was reduced by *Y. lipolytica,* which degraded the organic fraction. The organic fraction contained sugar, tannins, polyphenols, polyalcohols, pectins and lipids.

Y. lipolytica are able to play a role the transformation of compounds. Murphy & Perry (1984) reported a 50% degradation of chlorinated alkanes (1-chlorohexadecane and 1-chlorooctadecane) by *Y. lipolytica* to chlorinated fatty acids in the range of C_{14} to C_{18}. *Y. lipolytica* was also applied in groundwater treatment because it was found capable of transforming 2,4,6-trinitrotoluene, a man-made explosive, to a safer aminodinitrotoluene, in the presence of another carbon source, glucose (Jain *et al.* 2004).

Y. lipolytica may also be used for the production of lipase, which is an industrial enzyme used in the production of detergent. This was achieved by degrading olive oil or corn oil (Corzo & Revah 1999) as well as oleic acid (Fickers *et al.* 2003).

The production of cellular- fluid and lipids by degradation of C_{14} to C_{18} *n*-alkanes and 1-alkenes was assessed with *C. lipolytica* (Klug & Markovetz 1967). It was found that fatty acids and primary and secondary alcohols of the same chain length of the substrate were present in the culture. These results indicate the potential for accumulation of metabolic intermediates having the same carbon chain length as the substrate (by genetic manipulation) when alkanes are degraded by *Y. lipolytica*.

The wide application of *Y. lipolytica* in the industry and its ability to degrade a range of hydrocarbons, produce large amounts of fatty acids and secrete large amounts of protein, makes it a model organism for use in further study of the production of long-chain fatty acids or alcohols by alkane degradation. Routes for genetic manipulation, which will aid in the accumulation of these products, are investigated in the next section.

2.3.2.2 <u>Genetically modified yeasts</u>

The accumulation of metabolic intermediates produced during hydrocarbon metabolism can be increased when mutant strains of micro-organisms are used. Examples include a mutant strain of *Candida cloacae* unable to metabolise dicarboxylic acids, which produced over 5-fold more C_{12} dicarboxylic acid from C_{12} alkane than the wild type (Uchio & Shiio 1972). Hill *et al.* (1986) reported the increased production of long-chain dicarboxylic acids by a mutant strain of *Candida tropicalis* through use of C_{11} to C_{16} *n*-alkanes. The mutated strain was unable to degrade dioic acids or *n*-alkanes, such that the corresponding fatty acid was accumulated. It was found that although a higher yield on glycerol was obtained when hexadecane was used as substrate; the highest conversion of substrate to dioic acid was achieved with dodecane (91.13%). In another study a mutant strain of *C. tropicalis*,

which was unable to metabolise dicarboxylic acids, converted alkanes to undecane 1,11-dicarboxylic acids (Lin *et al.* 2000).

Where efficient accumulation of metabolic intermediates during hydrocarbon degradation is necessary, genetically engineered micro-organisms may enhance the potential to prevent further degradation of the intermediate. Picataggio *et al.* (1992) investigated the production of dodecanedioic acid by a genetically modified *C. tropicalis*. The modification involved the disruption of the β-oxidation pathway (which breaks down the fatty acid) and the amplification of genes encoding for cytochrome P450 and the NADPH-cytrochrome reductase (responsible for oxidation of the alkane to alcohol). In so doing, 100% conversion was achieved with a 30% increase in productivity (Picataggio *et al.* 1992).

Yarrowia lipolytica has already been identified as particularly attractive for foreign gene expression because of its capability to secrete large proteins in high amounts (Gaillardin & Heslot 1988, Casaregola *et al.* 2000). In addition to this, *Y. lipolytica* was reported to secrete higher amounts of important proteins than other yeast species (Kerscher *et al.* 2002).

For the biological production of citric acid from C_{12} to C_{15} *n*-paraffins, mutant strains of *C. lipolytica* were used to economise the process by producing a higher amount of citric acid than isocitric acid (Akiyama *et al.* 1973). The citric acid to isocitric acid ratio improved from 60:40 to 97:3. Waché *et al.* (2002) and Groguenin *et al.* (2004) found that by modifying the β-oxidation pathway of *Yarrowia lipolytica*, they were able to increase the production of the aroma compound γ-decalactone. Groguenin *et al.* (2004) reported the increase in γ-decalactone production from a hydroxylated C_{18} fatty acid, ricinoleic acid as 10-fold. Cytochrome P450 genes from *Rhodotorula minuta* were successfully cloned and over-expressed in *Y. lipolytica* (Shiningavamwe *et al.* 2005). This heterologous over-expression aided in the increased hydroxylation activity (which converts the alkane to alcohol) obtained with *Y. lipolytica*. The work done by Shiningavamwe *et al.* (2005) was paralleled and continued by Van Rooyen (2005) who investigated the bioconversion of undecane, dodecane, undecene, 5-methyl-undecane and hexylbenzene by modified *Y. lipolytica* strains. The range of strains used had β-oxidation pathways disrupted by the gene deletion and/or had multiple copies cytochrome P450 genes inserted.

Although not related to alkane degradation, further examples of genetic modification of *Y. lipolytica* promote the use of this organism. The over-expression of β-Galactosidase (in the β-oxidation cycle) in *Y. lipolytica* was investigated with respect to the promoter genes inserted, which regulate gene expression (Juretzek *et al.* 2000). It was found that *pICL1*, *pPOX2* and *pPOT1*

were the strongest promoters available. These promoters have an advantage over other promoters because they are induced by cheap carbon sources (Juretzek *et al.* 2000). In another study, genes encoding for cytokinin oxidase from *Zea mays* were cloned into *Y. lipolytica* to achieve high-level heterologous protein expression (Kopečny *et al.* 2005).

The large range of degradable hydrocarbons and opportunities for high-level expression of proteins makes *Yarrowia lipolytica* a suitable organism for further study in the production of long-chain dioic acids and alcohols. The next step is to investigate the metabolism of alkanes by yeast to gain insight on the conversion of alkane to its metabolic intermediates.

2.4 Alkane metabolism

Yeasts have been identified as promising organisms for the study of the conversion of long-chain *n*-alkanes to long-chain dioic acids or alcohols. It is important to understand how yeasts assimilate the alkanes to produce energy. The focus of this part of the review is to investigate the processes involved in producing metabolic intermediates. An exploration of the potential for genetic modification in these steps, which would enable and enhance accumulation of long-chain dioic acids or alcohols, is also undertaken. The alkane metabolism described by Tanaka & Fukui (1980) are summarised in section 2.4, unless otherwise stated.

2.4.1 General metabolism in overview

The metabolism of *n*-alkanes differs from carbohydrate metabolism in that the assimilation is associated with the flow of carbon from alkane substrates to the syntheses of cellular carbohydrates, through the formation of fatty acids. The β-oxidation pathway that follows converts the fatty acid to acetyl-CoA, thereby generating energy from the substrate (Figure 2.1). The assimilation of alkanes by yeasts can be divided into several steps and are discussed in the following sub-sections, namely

(2.4.1.1) Alkane uptake and initial oxidation of alkanes

(2.4.1.2) Oxidation of alcohols to fatty acids

(2.4.1.3) The activation of fatty acids to their Coenzyme A esters

(2.4.1.4) Metabolism of fatty acyl-CoA through β-oxidation

(2.4.1.5) Synthesis of cellular fatty acids or tricarboxylic acid cycle intermediates
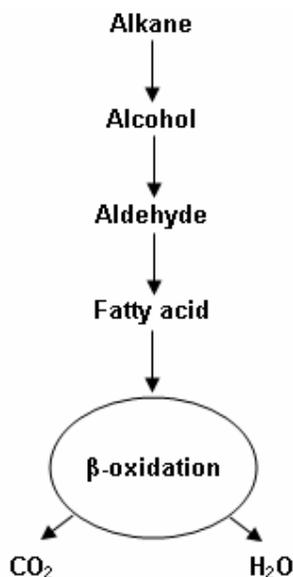
Figure 2.1 Alkane metabolism in overview

2.4.1.1 <u>Alkane uptake and initial oxidation of alkanes</u>

The first step in alkane assimilation is the uptake of the alkane by cells and the transport thereof to the site where it undergoes the initial oxidation to form an alcohol (Figure 2.2). The alkane can be taken up either by (i) direct contact between the alkane droplets and microbial cells or (ii) as an alkane made soluble by emulsification. Once the alkane has been taken up by the cells, it undergoes initial oxidation by cytochrome P450 mono-oxygenase enzymes. The P-450 cytochrome uses the co-enzyme NADPH reductase, and oxygen as substrate. The site of oxidation of the oxygen atom can be monoterminal, diterminal or subterminal (Figure 2.3). Studies for yeasts, however, have shown that monoterminal oxidation is predominant, leading to the formation of carboxylic acids, while diterminal oxidation has been found to lead to the formation of dicarboxylic acids (Shiio & Uchio 1971). The synthesis of cytochrome P450 was found to be enhanced by alkanes and their derivatives (Mauersberger *et al.* 1981), i.e. the alkane and/or derivative present induces the synthesis of P450 enzyme.
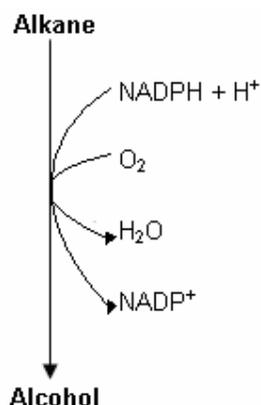
Figure 2.2 Formation of alcohol in alkane metabolism (Fukui & Tanaka 1981)
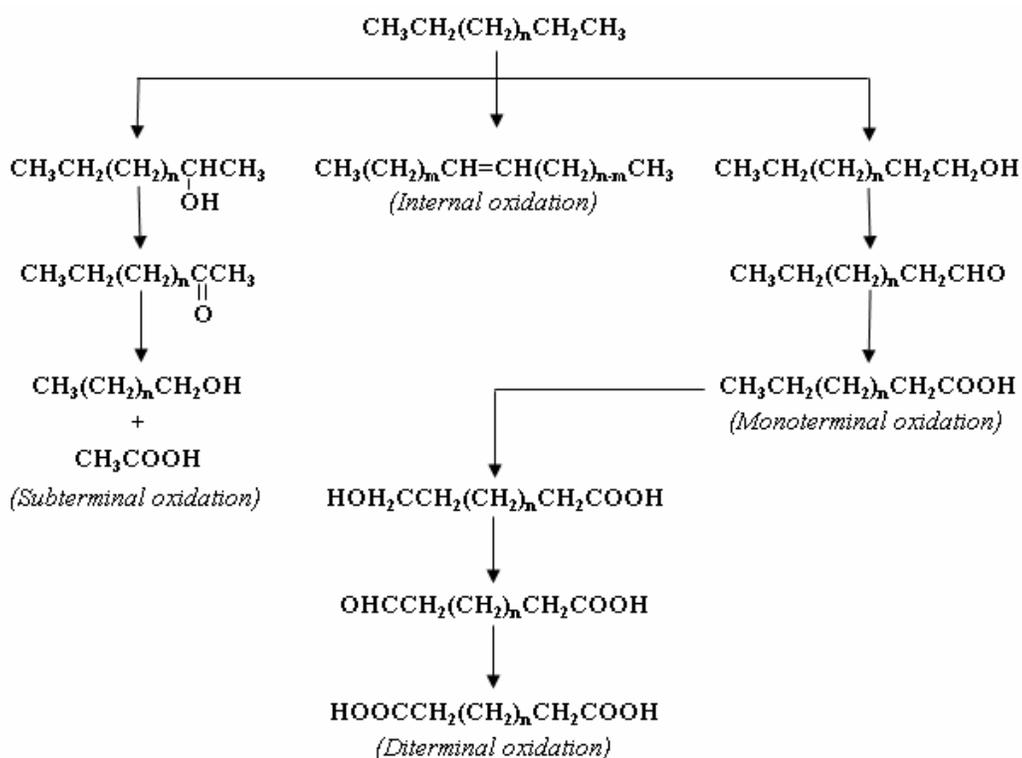
Figure 2.3 Microbial oxidation pathways of alkanes (Fukui & Tanaka 1981)

2.4.1.2 Oxidation of alcohols to fatty acids

During this stage, the long-chain alcohol formed is oxidised to form the fatty acid via aldehyde formation (Figure 2.4). The NAD+-linked alcohol dehydrogenase and aldehyde dehydrogenase, which facilitates the alcohol-to-aldehyde-to-fatty acid reactions, is specific to long-chain substrates or alkanes

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and is induced by the presence of alkanes, long-chain alcohols or aldehydes respectively. The alcohol dehyrdogenase of *Candida tropicalis* oxidises alcohols with a carbon content of $C_6 - C_{14}$ however, yeasts in general may process alkanes of carbon chains up to C_{19}.

Alcohol

NAD$^+$

NADH + H$^+$

Aldehyde

NAD$^+$

NADH + H$^+$
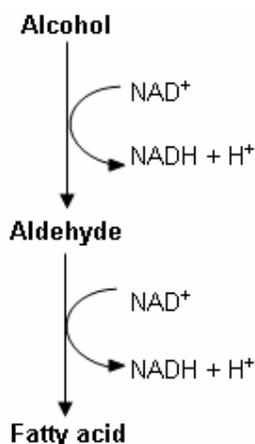
Fatty acid

Figure 2.4 Formation of fatty acid through aldehyde formation (Fukui & Tanaka 1981)

2.4.1.3 <u>The activation of fatty acids to their Coenzyme A esters</u>

Fatty acids that have been formed must be activated by acyl-Coenzyme A (CoA) synthetase, for conversion to their corresponding CoA esters (or fatty acyl-CoA), before any further metabolism of the fatty acid can take place. Acyl-CoA synthetase is classified as type I or type II. Synthetase I is observed in cells grown on glucose and oleic acid and it can be said that synthetase I is responsible for the producing acyl-CoA so that cellular lipids may be formed. Synthetase II, on the other hand, has been found only in cells grown on oleic acid and is responsible for the degradation of acyl-CoA by the β–oxidation system to form acetyl-CoA.

2.4.1.4 <u>Metabolism of fatty acyl-CoA through β-oxidation</u>

The β-oxidation system could be described as follows: In the first step, acyl-CoA is oxidised by FAD (acyl-CoA oxidase) to form enoyl-CoA. The second step involves the hydration of enoyl-CoA by enoyl CoA hydratase to form hydroxyacyl CoA. In the third step hydroxyacyl CoA is oxidised by NAD$^+$ (hydroxyacyl CoA dehydrogenase) to form ketoacyl CoA. The final step involves the cleavage of the ketoacyl CoA by the thiol group of another

molecule of CoA so that an acetyl CoA molecule is formed together with an acyl CoA molecule, which is now two carbons shorter (Figure 2.5).
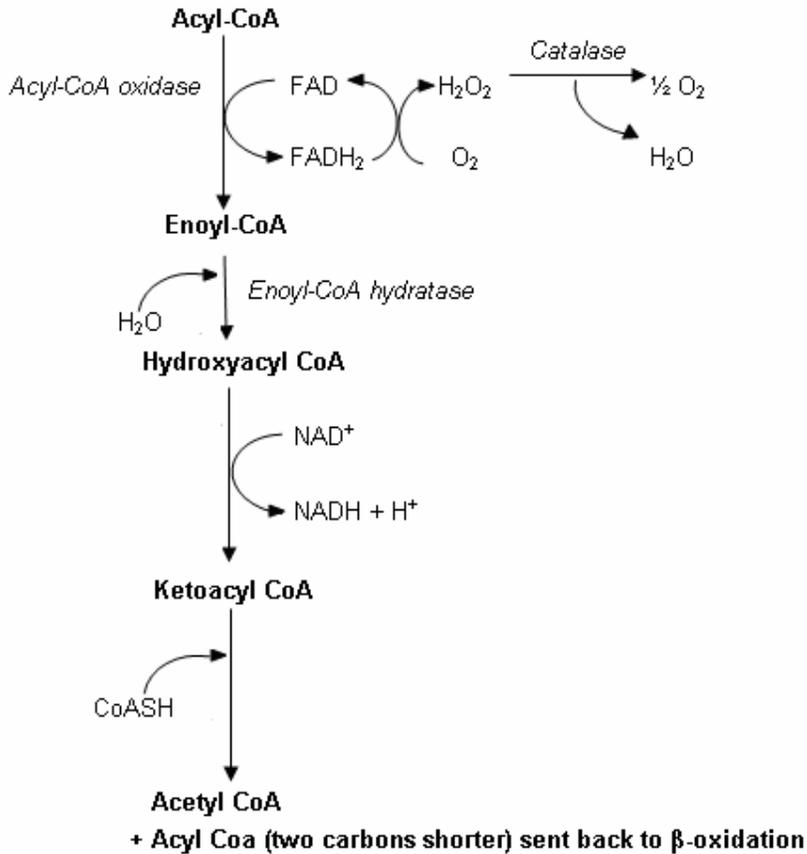
Figure 2.5 Fatty acid β-oxidation system (Fukui & Tanaka 1981)

2.4.1.5 <u>Synthesis of cellular fatty acids or tricarboxylic acid cycle intermediates</u>

A fraction of fatty acids derived from alkanes is incorporated into cellular lipids without degradation. These are derived via the *de novo* synthesis from acetyl-CoA, chain elongation of the substrate carbon skeleton with one or more acetyl units, and/or intact incorporation of the substrate carbon chain.

A large portion, however, is degraded by β-oxidation to acetyl-CoA, in the case of even and odd chain alkanes, and propionyl-CoA, in the case of odd chain alkanes (Figure 2.6). In the case of yeasts growing under gluconeogenic conditions, the glyoxylate cycle plays a pivotal role in the provision of precursors for biosynthesis of cellular components, such as carbohydrates and proteins. The tricarboxylic-acid (TCA) cycle produces carbon dioxide and the reducing power linked to the respiratory system to

produce energy. In addition to the TCA cycle the glyoxylate cycle produces one molecule of C_4 compounds, such as malate and succinate, from two molecules of acetyl-CoA by condensation with oxalacetate and glyoxylate (Figure 2.7). The key enzymes of the glyoxlyate cycle are isocitrate lyase and malate synthase.

$$CH_3(CH_2)_{2n}CH_3 \rightarrow CH_3(CH_2)_{2n}COOH \xrightarrow{(n+1)CoASH} (n+1)CH_3CO-SCoA$$

Even chain alkane   **Acetyl-CoA**

$$CH_3(CH_2)_{2n+1}CH_3 \rightarrow CH_3(CH_2)_{2n+1}COOH \xrightarrow{(n+1)CoASH} nCH_3CO-SCoA$$
$$+ CH_3CH_2CO-SCoA$$

Odd chain alkane   **Acetyl-CoA**
                                                        + Propionyl-CoA
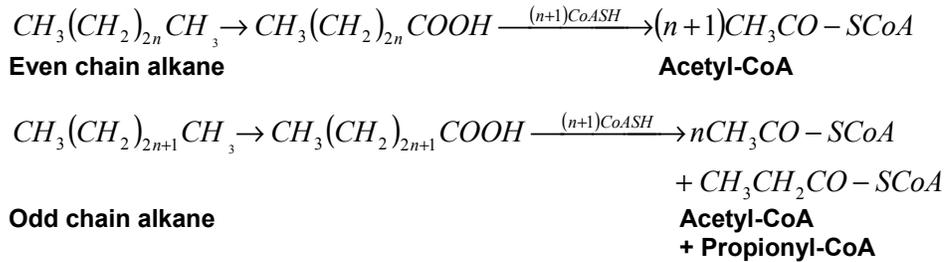
Figure 2.6 Formation of acetyl-CoA and propionyl-CoA from alkanes (Fukui & Tanaka 1981)
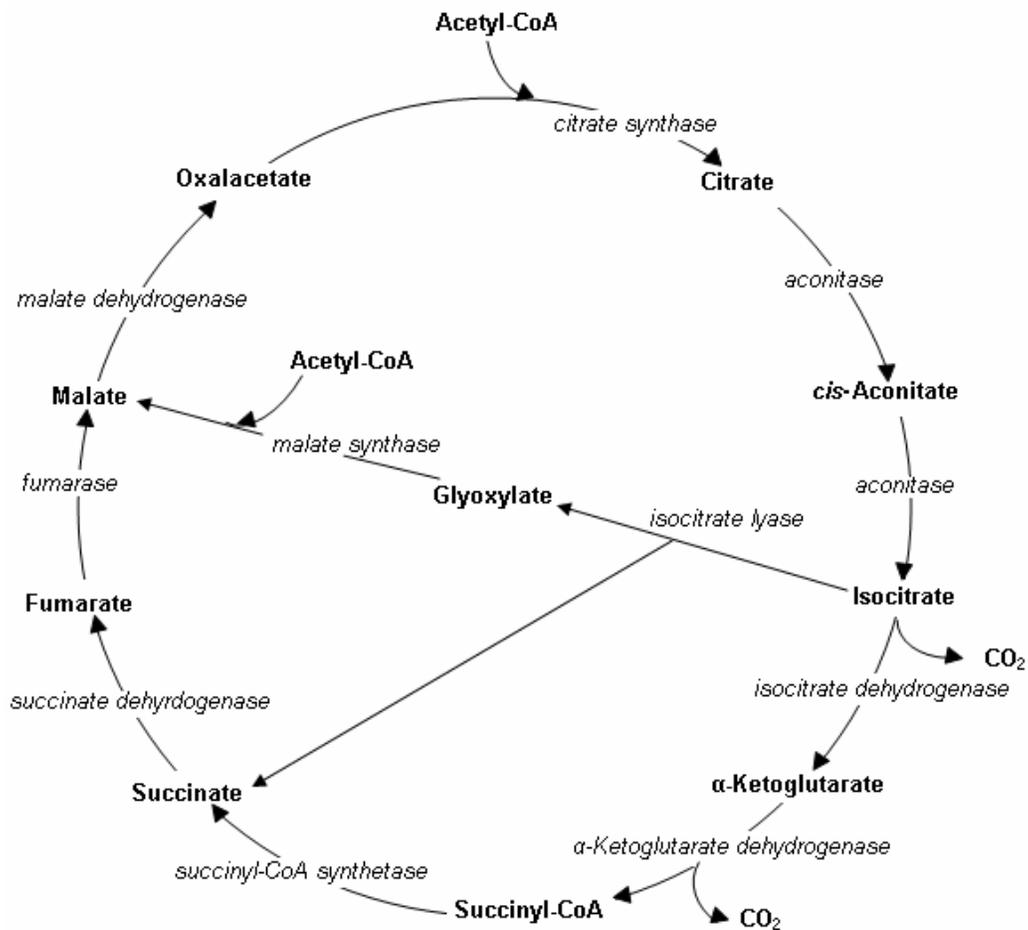
Figure 2.7 The TCA and glyoxylate cycles (Fukui & Tanaka 1981)

2.4.2 Alkane hydroxylation by cytochrome P450 mono-oxygenase

Cytochrome P450 enzymes are responsible for alkane hydroxylation, as previously described in subsection 2.4.1.1. For an enhanced bioconversion of alkanes, a key proposal would be to over-express the synthesis of cytochrome P450's or to provide process conditions optimal for P450 synthesis. A closer look is taken at the cytochrome P450 system to gain a better understanding of what processes take place.

Cytochrome P450's are a class of enzyme involved in both anabolic and catabolic metabolic processes and interact with a large variety of substrates, making them functionally diverse (De Mot & Parret 2002). Cytochrome P450 mono-oxygenases are part of a super family of heme-containing enzymes (containing red pigments) (Kelly *et al.* 2003). They are named mono-oxygenases because they incorporate one oxygen atom into the substrate during hydroxylation (Hannemann *et al.* 2007). The 'P' in P450 stands for pigment and '450' describes the absorption peak of the CO-bound complex at 450nm (Kelly *et al.* 2003). Cytochrome P450 genes are identified by the abbreviation CYP followed by a number denoting the family of proteins and a letter designating a sub-family and a number representing the individual gene within the sub-family (Hanneman *et al.* 2007). In 2007, 267 families with more than 5000 genes were established. Examples include the CYP101 family contained by *Pseudomonas putida* (Hannemann *et al.* 2007), CYP19 family contained by *Halichoeres tenuispinis* (Choi *et al.* 2005), the CYP51 family (Schunck *et al.* 1989, De Mot & Parret 2002) and the CYP52 family contained by *Candida maltosa* (Cheng *et al.* 2005, Zimmer *et al.* 1996).

The CYP systems catalyse the following reaction:

$$RH + O_2 + NAD(P)H + H^+ \rightarrow ROH + H_2O + NAD(P)^+$$
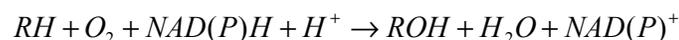
Cytochrome P450 enzymes are directly bonded to a ferric ion. The activation of P450 starts with the binding of the alkane (RH) with the first electron. This electron is transferred from NADPH via an electron transfer chain with NAD(P)H-cytochrome P450 reductase (CPR) (De Mot & Parret 2002). Next, the oxygen binds to the P450 enzyme and accepts the second electron from NADPH. A ferric peroxy anion is produced (now $O_2RH(P\text{-}450)Fe^{2+}$). The anion is then protonated to form the ferric hydroperoxy complex ($O_2RH(P\text{-}450)Fe^{3+}$) which undergoes heterolytic cleavage to form a ferryl species. This species attacks the alkane substrate to yield the hydroxylated product (water and alcohol) with the addition of an electron. The P450 then dissociates to let the cycle start again (Bernhardt 2006) (Figure 2.8).

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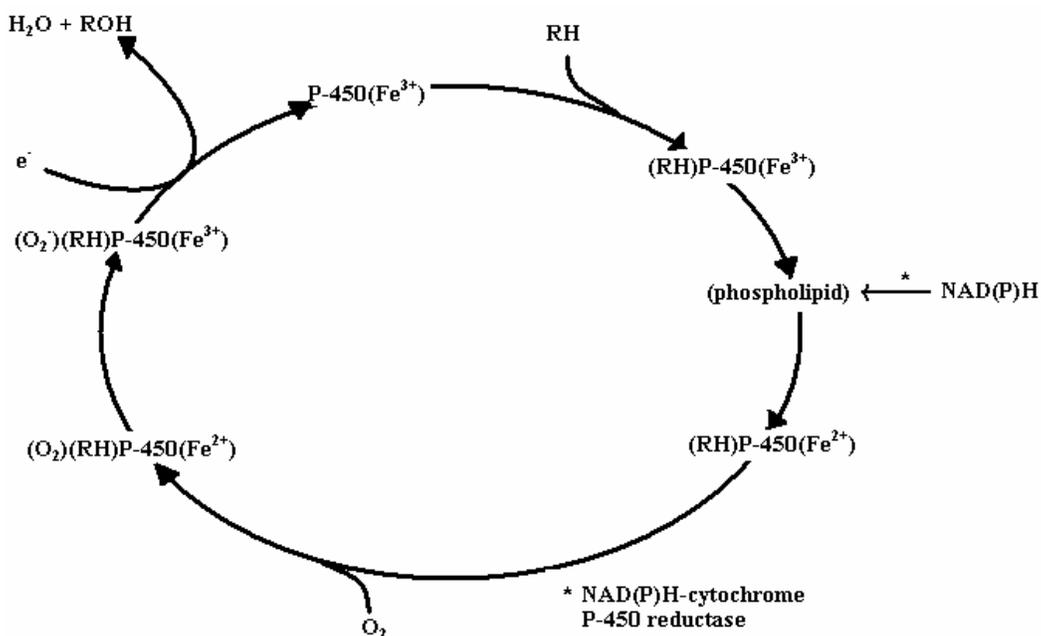
Figure 2.8 Proposed mechanism of the hydroxylation in liver microsomes (Fukui & Tanaka 1981)

The mechanism of the P450 cycle suggests that the availability of oxygen will play a major role in the effective oxidation by Cytochrome P450. Furthermore, in respect of genetic modification it has been reported that the maintenance of a balance in the expression of Cytochrome P450- mono-oxygenase and reductase is critical in achieving optimised P450 activity (Yadav & Loper 2000). Recall the cloning and amplification of CYP *and* CPR genes in *Candida tropicalis* to improve the bioconversion of hydrocarbons (Picataggio *et al.* 1992).

Studies conducted by Iida *et al.* (1998) classified *Y. lipolytica* as part of the CYP52 family. The CYP52 family participates in the terminal oxygenation of various *n*-alkanes species. Cloning of the CYP gene was successful in *Y. lipolytica* and the isolated gene was named *YlALK1*. This gene was induced by *n*-tetradecane and repressed by glycerol, whereas glucose did not repress the synthesis of cytochrome P450. *YlALK1* was also induced by *n*-decane (Sumita *et al.* 2002). In a parallel study by Iida *et al.* 2000, several new genes (*YlALK1* to *YlALK6*) were isolated for cloning of the CYP gene. It was found that *YlALK1* functioned to assimilate *n*-decane and longer molecules, whereas *YlALK2* was involved in the assimilation molecules longer than *n*-dodecane. *YlALK3* to *YlALK6* were not significantly involved in the assimilation of C_{10} to C_{16} *n*-alkanes (Iida *et al.* 2000).

The induction of the *YlALK1* to *YlALK6* genes is related to promoters, which regulate heterologous gene expression in *Yarrowia lipolytica* and these were investigated by Juretzek *et al.* 2000. Some of the genes expressed include alkaline extracellular protease (*pXPR2*), isocitrate lyase (*pICL*), acyl-CoA oxidases 1, 2, 5 (*pPOX1*, *pPOX2* and *pPOX5*, respectively) and 3-oxo-acyl-CoA thiolase (*pPOT1*) (Madzak *et al.* 2004). Of all the available promoters the *XPR2* promoter is mostly studied and used, however it is only active at a pH above 6 and requires high levels of peptones (Madzak *et al.* 2004). It cannot be induced in minimal media. The *pICL, pPOX2* and *pPOT1* promoters were stronger than *pXPR2* in conditions where inducers such as oleic acid and ricinoleic acid methyl ester were added (Juretzek *et al.* 2000). It was found that these three promoters were inducible by fatty acid derivatives or alkanes. The *ICL* promoter was additionally induced by acetate or ethanol and not fully repressed by glucose or glycerol, unlike *pPOX2* and *pPOT1* (Juretzek *et al.* 2000).

For the accumulation of metabolic intermediates from hydrocarbon utilisation, an alternative carbon co-substrate is required for cell growth and maintenance. This is usually supplied in the form of glucose or glycerol. The partial or full repression of promoters by either glucose or glycerol (Juretzek *et al.* 2000) demonstrates the importance in developing a system that is beneficial for both cell growth and gene expression in the P450 system. This leads to the suggestion that co-substrate concentration will play a vital role in optimising process conditions.

2.5 Process operation

The motivation for selecting *Yarrowia lipolytica* as a model organism for alkane utilisation and foreign gene expression has been described. Further investigation of the involved reactions and enzymes in alkane metabolism has aided in a better understanding of what factors will play a major role in maximising the potential of *Y. lipolytica* to degrade alkanes. This potential is defined by cell growth and bioconversion of alkanes, both of which are dependent on process conditions. In this section the conditions, under which yeasts grow and convert alkanes optimally, was investigated.

Operational parameters investigated include temperature, pH and culture media. The influence of carbon source and concentration was also investigated. The carbon sources comprise hydrocarbon substrates for bioconversion and carbohydrate co-substrate for cell growth. Finally, an

assessment was made of the influence of oxygen supply on growth and bioconversion.

2.5.1 Operational parameters

2.5.1.1 Temperature

A range of temperatures from 20°C to 37°C has been used for cell growth and bioconversion by *Yarrowia lipolytica*. The majority of processes are operated at 28°C and 30°C (Akiyama *et al.* 1973, Amaral *et al.* 2006, Antonucci *et al.* 2001, Corzo & Revah 1999, Gunkel *et al.* 1999, Jain *et al.* 2004, Juretzek *et al.* 2000 & 2001, Klug & Markovetz 1967, Kopecny *et al.* 2005, Luo *et al.* 2000, Moo-Young *et al.* 1971, Morgunov *et al.* 2004, Papanikolaou *et al.* 2002, Park *et al.* 1998, Scioli & Vollaro 1997, Song *et al.* 2006, Sumita *et al.* 2002, Thevenieau *et al.* 2006, Whitworth *et al.* 1973, Yamagami *et al.* 2001).

Rane & Sims (1993) assessed the effect of temperature on cell growth of *Y. lipolytica* at 27°C, 32°C and 37°C and it was found that the highest biomass was achieved in cultures operated at 27°C. This supported Corzo & Revah (1999) who investigated the influence of temperatures of 26°C, 30°C and 34°C on growth and production of lipase by *Y. lipolytica*. After construction of a model, they concluded that 29°C was the temperature necessary for optimum growth conditions. Kawasse *et al.* (2003) found that by increasing the temperature from 29°C to 37°C in *Y. lipolytica* cultures the organism was placed under thermal stress such that mycelial cells formed, which negatively affected growth performance. This is referred to as dimorphism, which is the ability of fungi to grow in two distinct forms, namely single oval cells (yeast-like form) or as a filament (mycelial form) (Kawasse *et al.* 2003). This happens in response to environmental and nutritional conditions and yeasts have been reported to convert alkanes optimally when they are in a yeast-like form (Zinjarde *et al.* 1998). All operating temperatures found in literature for *Y. lipolytica* are listed in Table 2.2. Unless otherwise stated, cultures were grown in shake flasks.

Table 2.2 Operating temperatures of *Yarrowia lipolytica* cultures

Temperature	Growth	Reference
$20^{o}C$	$OD_{(660nm)} = \pm0.6$	Alkasrawi *et al.* 1999
$25^{o}C$	$OD_{(620nm)} = 9$	Shiningavamwe *et al.*
	n.d.g *	Guerzoni *et al.* 2001
	n.d.g	Lanciotti *et al.* 2005
$26^{o}C$	$\pm14.5g/l$	Corzo & Revah 1999
	n.d.g	Ramon *et al.* 1999
$27^{o}C$	3.5×10^{7}cells/ml	Groguenin *et al.* 2004
	n.d.g	Gomes *et al.* 2007
	n.d.g	Rane & Sims 1993
	n.d.g	Waché *et al.* 1998, 2002
$28^{o}C$	4.5g/l	Park *et al.* 1998
	7g/l	Papanikolaou *et al.* 2002
	8g/l	Amaral *et al.* 2006
	6g/l and 10.5g/l (growth in shake flask and bioreactor)	Morgunov *et al.* 2004
	$OD_{(600nm)} = 0.1$ to 20 (varied due to range of substrates used)	Thevenieau *et al.* 2006
	$OD_{(600nm)} = 14$	Kopecny *et al.* 2005
	$OD_{(660nm)} = 2.9$	Gunkel *et al.* 1999
	DNA = 27g/l	Akiyama *et al.* 1973
	n.d.g	Juretzek *et al.* 2000, 2001
$29^{o}C$	1.3×10^{9}cells/ml	Fickers *et al.* 2003, 2005, 2006
	n.d.g	Kawasse *et al.* 2003
$30^{o}C$	8g/l	Moo-Young *et al.* 1971
	±12.2 g/l	Corzo & Revah 1999
	22g/l (growth in bioreactor)	Scioli & Vollaro 1997
	30g/l (growth in bioreactor)	Antonucci *et al.* 2001
	$OD_{(660nm)} = 1.2$	Jain *et al.* 2004
	n.d.g	Klug & Markovetz 1967
	n.d.g	Luo *et al.* 2000
	n.d.g	Song *et al.* 2006
	n.d.g	Sumita *et al.* 2002
	n.d.g	Whitworth *et al.* 1973
	n.d.g	Yamagami *et al.* 2001
$32^{o}C$	*n.d.g*	Rane & Sims 1993
$34^{o}C$	$\pm7.3g/l$	Corzo & Revah 1999
$37^{o}C$	*n.d.g*	Kawasse *et al.* 2003
	n.d.g	Rane & Sims 1993

* *n.d.g.* = no data given

No significant difference in cell growth of *Y. lipolytica*, between operating temperatures of $28^{o}C$ and $30^{o}C$, was found in literature. Selecting either of the two operating temperatures is considered feasible. To keep in accordance

with operating temperatures used in collaborative projects using the same strains (Van Rooyen 2005, Smit personal communication), 28°C was selected for cultivation in this project.

2.5.1.2 pH

The operating pH for yeasts in the literature were varied and appeared to be dependent on the product of interest. A literature investigation has been made of the pH range optimal for growth of yeasts in general. This is followed by the pH range optimal for growth of *Yarrowia lipolytica* in alkane and sugar degradation.

A pH of 4.6 was used for fatty acid production from *n*-alkanes by *Candida maltosa* (Blasig *et al.* 1989). Bednarski *et al.* (2004) used an initial pH of 5.5 for cultures of *Candida antarctica* and *Candida apicola*. They compared the effect of glycolipid content when the pH was maintained at 5.5 and when not maintained. The pH was maintained at 5.5 by adding 1M sodium hydroxide or 1M hydrochloric acid, otherwise the pH decreased to as low as 2.0. It was found that, in the case of cultures where the pH was maintained, an increase of 3g/l in glycolipid content was found (Bednarski *et al.* 2004). In another study, a pH of 6.5 was used for the first 24h of cultivation of *Candida tropicalis* during the formation of long-chain dicarboxylic acids from pure *n*-alkanes (Hill *et al.* 1986). The pH was subsequently raised to pH 8 when *n*-dodecane was added fed-batchwise and maintained at 8 by adding 9N NaOH. The fact that pH was not maintained at 8 from the start of culture growth suggests that a 6.5pH was optimal for initial cell growth of *C. tropicalis*. Jiao et al. (2001) repeated this work with an initial pH of 6.5 after which the pH was adjusted to 8. This was done 20h after the start of culture growth when tridecane was added as bioconversion substrate. The purpose of the study was for the production of dicarboxylic acids. Shiio & Uchio (1972) and Picataggio et al. (1992) carried out similar pH control. Hill *et al.* (1986) explained how dioic acids formed are highly soluble in water as an alkaline salt, whereas the solubility is low in water as a free acid. This could be the motivation for maintaining a high pH in cultures for the production of long-chain dicarboxylic acids. For various organisms, including *Candida cloacae*, a pH of 7 was maintained by adding 1N KOH twice daily for the production of long-chain dicarboxylic acids (Shiio & Uchio, 1971). Finally, in another study the pH was adjusted to pH 7 when comparing the utilisation of a C_{14} alkane by a range of micro-organisms (Schmitz *et al.* 2000). These organisms included *C. maltosa*, *C. tropicalis* and *Y. lipolytica* (Schmitz *et al.* 2000).

A wide pH range of 4.6 to 8 can therefore be recommended for growth of yeasts in general. For the production of long-chain dicarboxylic acids,

however, it is necessary to maintain the pH above 7, due to the low solubility of dicarboxylic acids (from hydrocarbons) at lower pH.

The pH conditions optimal for growth of *Y. lipolytica* were investigated. In an experiment conducted by Praphailong & Fleet (1997) it was found that *Y. lipolytica*, among a few other yeast species, was able to grow optimally at pH 2.5 to 8. Some (*Pichia membranaefaciens*, *Kloeckera apiculata*, *Zygosaccharomyces rouxii*) were only able to grow strongly up to a pH of 7. Corzo & Revah (1999) investigated the effect of pH on the growth and production of lipase by *Y. lipolytica*. Cultivations were carried out in a pH of 3, 4.5 and 6. Construction of a mathematical model predicted that a pH of 4.5 was required for optimum growth in that pH range (Corzo & Revah 1999). Further pH ranges of 4.5 to 6.8 were used in cultures containing *Y. lipolytica* (Moo-Young *et al.* 1971, Akiyama *et al.* 1973, Whitworth *et al.* 1973, Rane & Sims 1993, Scioli & Vollaro 1997, Park *et al.* 1998, Juretzek *et al.* 2000, Antonucci *et al.* 2001, Papanikolaou *et al.* 2002, Fickers *et al.* 2003, Morgunov *et al.* 2004, Gomes *et al.* 2007). Cell growth on hydrocarbons and carbohydrates was investigated by Moo-Young *et al.* (1971), Whitworth *et al.* (1973), Scioli and Vollaro (1997), Park *et al.* (1998) and Juretzek *et al.* (2000). The production of lipids, lipase and citric acid was investigated by Akiyama *et al.* (1973), Rane and Sims (1993), Antonucci *et al.* (2001), Papanikolaou *et al.* (2002) and Fickers *et al.* (2003). Finally Morgunov *et al.* (2004) investigated the production of pyruvic acid and Gomes *et al.* (2007) the production of γ-decalactone.

Y. lipolytica appears to be more tolerant of varied pH conditions since cell growth is possible in a pH range of 2.5 to 8. For the accumulation of metabolic intermediates during alkane degradation, however, the same principles of a higher pH should apply (Shiio & Uchio 1972, Hill *et al.* 1986, Picataggio *et al.* 1992, Jiao *et al.* 2001). In a study by Shiningavamwe *et al.* (2005), a pH8 buffer was added for the production of *para*-hydroxybenzoic acid from benzoic using a genetically modified *Y. lipolytica*. The purpose of the study was to investigate the hydroxylase activity of Cytochrome P450, which is the enzyme of interest for this study.

The information supplied in the literature reveals that the evaluation of pH differed. Many authors did not even report pH used in cultures, suggesting that it was not of importance to them (Klug & Markovetz 1967, Blasig *et al.* 1984, Groguenin *et al.* 2004, Jain *et al.* 2004, Amaral *et al.* 2006). For shake flask cultures, the pH could not be rigorously controlled throughout the monitoring period of culture growth. Consequently, one would not know if a significant drop or rise in pH contributed towards any changes in cell growth, product formation or enzyme activity. This limitation in shake flasks could

serve as a motivation for investigating cultivations in a controlled bioreactor where the pH can be monitored and maintained.

2.5.1.3 <u>Carbon to nitrogen ratio in culture media</u>

The C:N ratios from literature were calculated by determining the amount of carbon and nitrogen supplied in the media. Of those that reported cell growth the C:N ratio ranged from 2.71 to 31.73 (Scioli and Vollaro 1997, Corzo and Revah 1999, Antonucci *et al.* 2001, Papanikolaou and Aggelis 2002, Bednarski *et al.* 2004, Bourel *et al.* 2004, Amaral *et al.* 2006). Upon investigation of the corresponding cell growth, there does not seem to be a clear correlation between the C:N and cell growth (Table 2.3). However, a C:N ratio of 4 to 8 does appear to provide favourable conditions for maximising cell growth.

Table 2.3 Carbon to Nitrogen ratios of micro-organisms

C:N	Cell growth (g/l)	Reference
2.71	22	Scioli and Vollaro 1997
4.39	10	Amaral *et al.* 2006
6.05	16	Bourel *et al.* 2004
8.57	14	Corzo & Revah 1999
8.89	25	Antonucci *et al.* 2001
20.51	7	Papanikolaou and Aggelis 2002
31.28	10	Bednarski *et al.* 2004

2.5.2 Hydrocarbon type, concentration and time of addition

The main hydrocarbon source of interest is *n*-alkanes. Literature established that *Yarrowia lipolytica* are capable of using alkanes as the sole carbon source for growth (Moo-Young *et al.* 1971, Blasig *et al.* 1988, Riege *et al.* 1989). However, the toxicity of alkanes increases with decreasing carbon chain length due to their increased solubility in aqueous solutions (Klug & Markovetz, 1967). A probable result is that compounds become more volatile,

creating an error in analytical methods since the substrate may have been partly evaporated and degraded, which is not desirable.

The average chain length of alkane substrates used in literature ranged from C_8 to C_{19} with concentrations varying from 0.3% (v/v) to 20% (v/v). All substrates were added in batch to the culture except for studies conducted by Hill *et al.* (1986) and Picataggio *et al.* (1992) where the substrate was added in fed-batch (Table 2.4). When this occurred, the co-substrate was supplied at the start in the form of glucose or glycerol.

Table 2.4 Alkane substrates, concentrations and methods of addition by yeasts

Alkane type	Concentration	Type/time of alkane addition (h)	Organism	Reference
C_8 to C_{18}	1% (wt/v)	Batch at 0 h	*C. cloacae*	Green *et al.* 2000
n-C_{11} to n-C_{16}	20ml/h	Batch at 0 h; Fed-batch at 24 h	*C. tropicalis*	Hill *et al.* 1986
C_{10} to C_{16}	1% (wt/v)	Batch at 0 h	*Y. lipolytica*	Thevenieau *et al.* 2006
n-C_{11} to n-C_{19}	3% (v/v)	Batch at 0 h	*C. maltosa*	Blasig *et al.* 1988
n-C_{12}	0.5 to 1% (v/v)	Batch at 0 h	*C. lipolytica*	Moo-Young *et al.* 1971
n-C_{12}	1% (v/v)	Batch at 0 h	*C. lipolytica*	Whitworth *et al.* 1973
n-C_{12}	2% (v/v)	Batch at 0 h	*Y. lipolytica*	Yamagami *et al.* 2001
C_{12}	1% (v/v)	Batch at 0 h	*Y. lipolytica*	Alkasrawi *et al.* 1999
n-C_{12} to n-C_{15}	8% (wt/v)	Batch at 0 h	*C. lipolytica*	Akiyama *et al.* 1973
C_{12}, C_{16}	1% (v/v)	Batch at 0 h	*Y. lipolytica*	Juretzek *et al.* 2000
n-C_{12}, n-C_{16}	10% (v/v)	Batch at 0 h	*C. cloacae*	Uchio & Shiio 1972
n-C_{13}	10% (v/v)	Batch at 0 h	*C. tropicalis*	Jiao *et al.* 2001
C_{13} to C_{18}	0.5 to 3% (wt/v)	Fed-batch at 18 h	*C. tropicalis*	Picataggio *et al.* 1992
C_{14}	2% (v/v)	Batch at 0 h	*Y. lipolytica*	Iida *et al.* 1998
n-C_{14}, n-C_{16}, n-C_{17}	0.3% (v/v)	Batch at 40 h	*Lodderomyces elongisporus*	Blasig *et al.* 1984
n-C_{14} to n-C_{18}	1% (v/v)	Batch at 0 h	*C. lipolytica*	Klug & Markovetz 1967
n-C_{16}	0.7% (wt/v)	Batch at 0 h	*C. lipolytica*	Akiyama *et al.* 1973
C_{16}	0.8 to 1.6% (wt/v)	Batch at 0 h	*Y. lipolytica*	Finogenova *et al.* 2005
n-C_{16}	5 to 20% (v/v)	Batch at 0 h	*C. cloacae*	Uchio & Shiio

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				1972
n-C$_{16}$, n-C$_{18}$	1% (v/v)	Batch at 0 h	*C. tropicalis* and *C. cloacae*	Cheng *et al.* 2005

In the study by Uchio & Shiio (1972) a range of hexadecane concentrations were used for the production of n-C$_{16}$ dicarboxylic acid. These concentrations were 5%, 7.5%, 10%, 15% and 20% (v/v). It was found that all hexadecane was consumed only when substrate concentrations were between 5% and 10% (v/v).

In studies conducted by Hill *et al.* (1986) and Picataggio *et al.* (1992) the alkane substrate used for dicarboxylic acid production was only added after growth on a carbohydrate. The substrate was added either 18h or 24h after the start of cultivation. In a different study where another hydrocarbon substrate was used, methyl ricinoleate was added to *Y. lipolytica* culture 19h after inoculation (Groguenin *et al.* 2004). From these three examples by Hill *et al.*, Picataggio *et al.* and Groguenin *et al.*, it appears desirable to add the alkane when cells are growing actively, i.e. after 18h. Initial studies can therefore be undertaken where the hydrocarbon substrate is added within 18 to 24 h of cultivation. It is suspected that the time of substrate addition will play a major role in the potential of bioconversion of alkanes by *Y. lipolytica*.

2.5.3 Carbohydrate type and concentration

As previously mentioned in section 2.3, a carbon energy source is needed as a co-substrate where genetic modification or mutant strains prevent the organism from deriving any or enough energy from alkanes (Jain *et al.* 2004). The carbohydrate source most commonly used is glucose (Picataggio *et al.* 1992, Green *et al.* 2000, Waché *et al.* 2002, Groguenin *et al.* 2004, Jain *et al.* 2004, Shiningavamwe *et al.* 2005, Gomes *et al.* 2007) and is sometimes referred to as the "preferred substrate" for yeast growth because yeasts grow abundantly on glucose (Wills, 1996). Other sources include glycerol (Hill *et al.* 1986, Blasig *et al.* 1984) and sucrose (Lin *et al.* 2000, Uchio & Shiio 1972).

A glucose concentration range of 0.12% to 7.5 % (wt/v) has been used as co-substrate during conversion of a hydrocarbon substrate by micro-organisms. These include:

(a) 0.12% to 1.4% (wt/v) glucose for growth of *Y. lipolytica* with 2,4,6-dinitrotoluene (TNT) addition at 0h (Jain *et al.* 2004). It was found that 1.4% (wt/v) glucose achieved the highest cell concentration and bioconversion of TNT in this range of glucose concentrations.

(b) 0.5% to 2.5% (wt/v) glucose for growth of *Candida cloacae* with dodecanoic acid addition at 0h (Green *et al.* 2000). This study was conducted for the production of dodecanedioic acid. It was found that the highest amount of dodecanedioic acid was produced when 0.5% (wt/v) glucose was used.

(c) 1.5% to 2% (wt/v) glucose for growth of *Y. lipolytica* (Gomes *et al.* 2007, Groguenin *et al.* 2004). Before the bioconversion substrate (methyl ricinoleate) was added, however, cells were harvested and re-suspended in a buffered medium that contained zero glucose.

(d) 2% (wt/v) glucose for growth of *Y. lipolytica* before inducing substrate and benzoic acid addition at 36h and 54h, respectively (Shiningavamwe *et al.* 2005)

(e) 7.5% (wt/v) glucose for the growth of *C. tropicalis* before dodecane addition at 18h in a bioreactor (Picataggio *et al.* 1992)

In the study reported in (b) above, Green *et al.* (2000) proposed that higher glucose concentrations (≥1% (wt/v)) repressed Cytochrome P450 reductase activity. It is interesting to note, however, that in the control where no glucose was added, the dodecanedioic acid concentration was lower than in the 0.5% (wt/v) glucose culture (zero glucose added: 0.55g/l; 0.5% glucose added: 1.1g/l). This finding suggests that some carbon was still needed for the bioconversion to take place and that a threshold glucose level may exist below bioconversion may take place. Based on the information supplied by Green *et al.* (2000) it can be assumed that this threshold level lies somewhere between 0% and 1% (wt/v) glucose.

It is expected that the glucose concentration should play a vital role in determining conditions that optimise the bioconversion of alkanes. The concept of glucose repression may also be applicable to studies where an alkane is used as the secondary carbon source.

2.5.4 Oxygen supply

It has been reported that sufficient oxygen supply has important effects on the growth rate, biomass yield and composition, and enzyme composition during alkane degradation by micro-organisms (Riege *et al.* 1989). It was found by Riege *et al.* (1989) that protein formation and alkane utilisation by *C. maltosa* was decreased in studies where the oxygen was limited at sufficient concentrations of the alkane source.

Alternatively, in another study conducted by Kawasse *et al.* (2003), *Y. lipolytica* was subjected to oxidative stress (high oxygen supply) by the addition of hydrogen peroxide (H_2O_2) concentrations up to 20mM. Although biomass was enhanced with increase in oxygen supply, dimorphism occurred at high oxygen levels, and mycelial cells formed. This suggests that too much oxygen is also not desirable, especially not for the bioconversion of alkanes. Jiao *et al.* (2001) concluded further that concentrations of H_2O_2 above 2mM inhibited the induction of Cytochrome P450 enzymes.

It will be important to supply *Y. lipolytica* with just the right amount of oxygen. This will prevent the decreased alkane degradation in low oxygen conditions, and the formation of mycelial cells in high oxygen conditions.

2.6 Introduction to current study and model system

The preceding sections in Chapter 2 have developed a basis for the bioconversion of alkanes to commercially important long-chain dioic acids and alcohols by a genetically modified *Yarrowia lipolytica*. The aim of this project is to quantify the potential capacity of *Y. lipolytica* for the said bioconversion. This will be performed by investigation of process conditions that optimise cell growth of *Y. lipolytica* and bioconversion of hydrocarbons.

Biological processes presented in this literature survey have made use of cultures in shake flasks and bioreactors. Although initial studies in shake flasks have its advantages when making preliminary findings, studies in bioreactor cultures are necessary for examination under controlled conditions. The limitations in using shake flasks include oxygen supply, pH control and limited sampling. In this project, optimisation of process conditions will be investigated in shake flasks initially. Subsequent cultivations will be carried out in bioreactor cultures. This will allow the temperature to be controlled at $28^{\circ}C$, the pH to be maintained above 7 or 8 and the oxygen supply to be controlled at an optimum level.

In the current study, strains of *Y. lipolytica* (TVN348, TVN493, WT) have been supplied by the University of the Free State. TVN348 and TVN493 have multiple copies of Cytochrome P450 genes inserted to over-express the synthesis of the enzyme responsible for alkane to alcohol conversion. All strains, however, have functional β-oxidation pathways and therefore no accumulation of metabolic intermediates is possible. A model system is therefore proposed in which *Y. lipolytica*'s potential for bioconversion may still be assessed (Van Rooyen 2005). In the model system, the alkane substrate is substituted with an alkylbenzene. The benzene ring of the alkylbenzene cannot be metabolised by *Y. lipolytica* (Mauersberger *et al.*, 1996), thus either

phenyl acetic acid (PAA) or benzoic acid (BA) is formed and accumulated (Figure 2.9). PAA and BA may be quantified by analytical methods and therefore the bioconversion of the alkylbenzene may be directly measured. Furthermore, since *Y. lipolytica* cannot derive energy from the alkylbenzene, a co-substrate is needed for cell growth and maintenance. Glucose is supplied as co-substrate and in so doing, the system using a substrate and co-substrate is modelled.
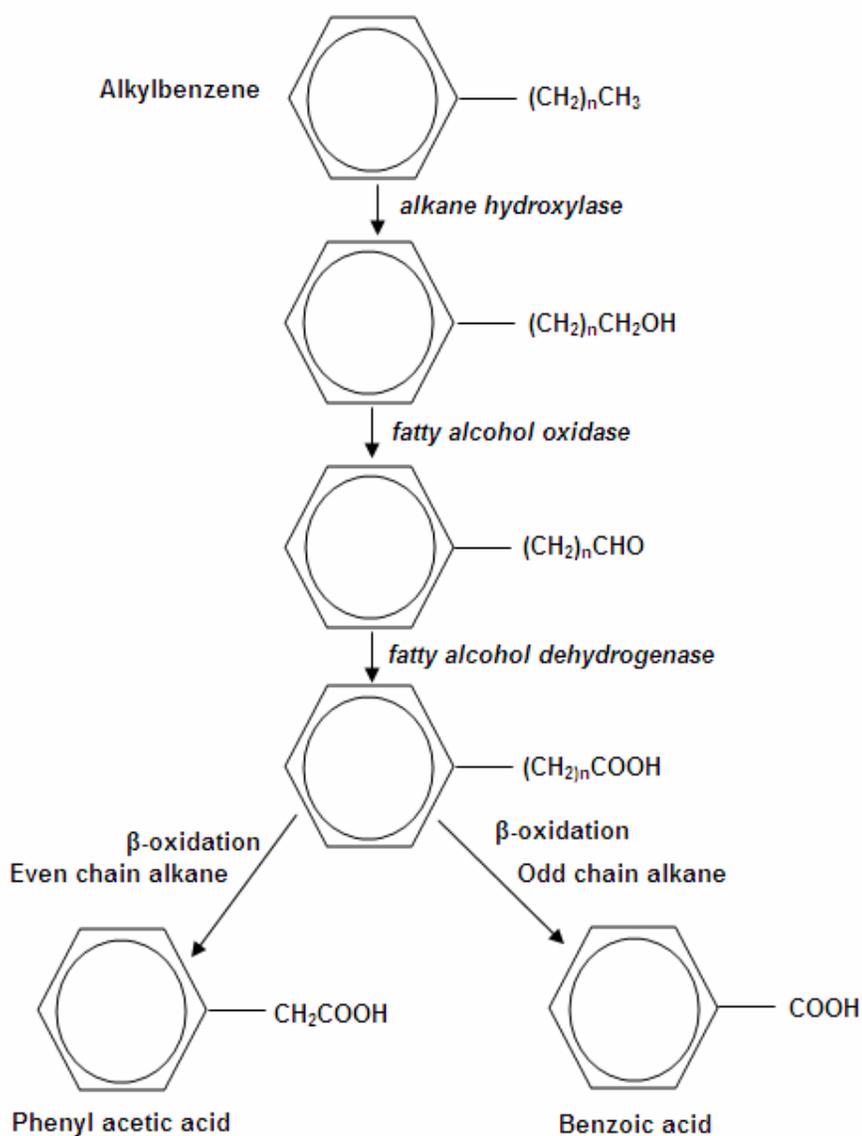
Figure 2.9 The conversion of phenylalkanes by yeasts (Mauersberger *et al.* 1996)

It has been hypothesized in a previous study using this model system (Van Rooyen 2005) that *Y. lipolytica* does not recognise the alkylbenzene as alkane or intermediate and therefore requires another chemical, which will serve to induce the synthesis of the Cytochrome P450 enzyme. The difficulty that arises when using the model system is that if *n*-alkanes are used as an inducer then the product formed from the bioconversion of alkylbenzenes, phenyl acetic acid, is not a complete measure of the conversion of alkanes (conversion of *n*-alkane inducers cannot be quantified). Van Rooyen (2005) investigated the use of a variety of inducers (dodecane, oleic acid, ethanol, glucose and stearic acid) and proposed that ethanol was the best inducer for synthesis of Cytochrome P450 enzymes. Further, the possible toxic effect of alkylbenzenes may inhibit the growth of micro-organisms (Smit, personal communication). It may be suggested that the cells reach a sustainable level before either the inducer or substrate can be added (Van Rooyen 2005). This level still needs to be investigated. Van Rooyen (2005) added the inducer and alkylbenzene at 24h and 48h respectively. This is an area in research that has not been investigated extensively and will form part of the focus of this study.

Y. lipolytica cultured in the model system has been demonstrated to exhibit poor reproducibility, especially with respect to PAA formation (Van Rooyen 2005, Ramorobi 2008). For instance, identical studies conducted by Van Rooyen (2005) in shake flasks have shown differences in PAA formation from hexylbenzene by *Y. lipolytica* TVN493 from 50.5mM to 65.0mM. Additionally, identical studies conducted by Ramorobi (2008) with *Y. lipolytica* TVN348 have shown differences in PAA formation from hexylbenzene of 45mM to 58mM. Moreover, differences in *Y. lipolytica* TVN348 cell growth in replicate samples also showed poor reproducibility ranging from 6.50g/l to 8.45g/l at 48h (Ramorobi 2008).

2.7 Hypotheses

From the foregoing sections, it is clear that many parameters influence the bioconversion of alkanes. Apart from the genetic modification, choice of substrate and co-substrate and their concentrations are expected to play a major role in defining the optimum conditions for bioconversion. Parameters that influence both cell growth and bioconversion will also be assessed in conjunction with the model system described for the current study in Section 2.6. The following hypotheses are proposed:

- *Yarrowia lipolytica* strains TVN 348, TVN 493 and WT can utilise glucose for cell growth and maintenance and can convert even chain

alkylbenzenes to phenyl acetic acid while using glucose as a co-substrate for cell growth and maintenance

- Cell growth and bioconversion are dependent on media constituents and process conditions. These include oxygen supply, glucose concentrations, substrate- type and addition and inducer requirements.

- The bioconversion of alkylbenzenes by genetically modified strains of *Yarrowia lipolytica* is increased when compared with the unmodified strain

3. Materials and Methods

Studies were conducted to determine the growth of *Yarrowia lipolytica* strains in liquid medium. Investigations were also conducted to determine yeast bioconversions of different alkylbenzene substrates into their respective products in liquid cultures. In this chapter, the micro-organisms supplied are defined. The media and culture maintenance and the culture methodology are also detailed. Finally, the analytical methods for measuring cell-, substrate- and product concentration are described.

Appendix A details the suppliers of all chemicals described in this chapter.

3.1 Micro-organisms

Three strains of *Y. lipolytica* were received from the Department of Microbial, Biochemical and Food Technology at the University of the Free State, South Africa. *Y. lipolytica* TVN493 and TVN348 differ in the Cytochrome P450 (CYP) cloned genes inserted. Strain TVN493 contains multiple copies of an endogenous CYP gene while strain TVN348 contains multiple copies of a foreign CYP gene from *Rhodotorula retinophila*. These genes are responsible for the expression of Cytochrome P450 enzymes, which oxidise the alkane substrate. Both TVN493 and TVN348 have the P450 reductase (CPR) gene cloned which is also responsible for the expression of enzymes responsible for alkane oxidation. Strain WT has neither CYP nor CPR genes cloned. All strains have intact β–oxidation pathways. The properties of the yeast strains used in this study are summarised in Table 3.1.

Table 3.1 Strains used in this study

Y. lipolytica Strain	CYP Gene cloned	Hydroxylase activity	CYP Gene source	CPR gene cloning	Constructed by
TVN493	CYP52F1	Alkane	*Yarrowia lipolytica*	Yes	Dr M.E. Setatie (Van Rooyen 2005)
TVN348	CYP557A1	Fatty acid hydroxylase	*Rhodotorula retinophila*	Yes	Dr A.N. Shiningavamwe (Van Rooyen 2005)
WT	None	-	-	No	-

3.2 Media and culture maintenance

3.2.1 Media

A medium containing yeast extract, peptone and glucose (YPD) was used in this study. The liquid media used for inoculum cultures contained 10g/l yeast extract, 10g/l peptone and 20g/l glucose (YPD_2). The liquid media used for test cultures contained 10g/l yeast extract, 20g/l peptone and either 20g/l, 40g/l or 80g/l glucose (YP_2D_2, YP_2D_4 or YP_2D_8). Where indicated in the text, added phosphate buffering, trace element solution and added nitrogen was added to liquid medium. The composition of the phosphate buffer, trace element solution and added nitrogen are summarised in Table 3.2.

Table 3.2 Nutrients additional to standard YPD medium

Components	Concentration (g/l)
Phosphate buffer: *di*-Potassium hydrogen orthophosphate anhydrous Potassium dihydrogen orthophosphate	 0.500 0.500
Trace element solution: Magnesium sulphate Ferrous sulphate Manganese sulphate Zinc sulphate	 2.000 1.000 0.300 0.200
Added nitrogen: Ammonium sulphate	 4.714

Solid media for both plates and slants contained 10g/l yeast extract, 20g/l peptone, 20g/l glucose and 20g/l bacteriological agar.

For preparation of the liquid media, Solution A and B were prepared. Solution A contained yeast extract and peptone in half the volume of required distilled water, while Solution B contained glucose in the remaining volume. The two solutions were sterilised separately to prevent the Malliard reaction from taking place in which the glucose is consumed. Both Solutions (A and B) were sterilised in a vertical steam steriliser (Gemmy) at 121°C for 20min. After sterilisation, the two solutions were allowed to cool before combining and aseptic transfer to shake flasks for pre- or test cultures in a laminar flow cabinet (Clearflow).

The trace element solution was prepared by aseptically adding the trace elements to sterilised water to make a concentration ten times stronger (10X) than displayed in Table 3.2 so that 10% of the final working volumes in shake flasks was made up of the concentrated trace element solution.

For preparation of the solid media, Solution A was prepared and the agar was added to Solution B before sterilisation. The cooled solutions were combined before setting and poured into agar plates using aseptic techniques.

3.2.2 Culture maintenance

Cultures were prepared for long-term storage in aqueous solution of 30% (v/v) glycerol by transferring the strains from solid agar plates to YPD_2 liquid medium. The liquid culture was incubated at 28^oC and 180rpm in an incubator shaker (Labcon) over a period of 36h.

After cultivation in liquid medium, volumes of culture were transferred aseptically to the sterile 80% glycerol solutions such that the final concentration of glycerol present was 30%. These stocks were stored at -80^oC.

The cultures were revived for use in experiments by streaking directly onto solid media on agar plates. These cultures were incubated at 28^oC for at least 24h and used immediately.

3.3 Culture methodology

Culture methodologies included both shake flask- and bioreactor studies. Shake flask studies were conducted to develop the model system, whereas bioreactor studies were conducted to use the model system under controlled conditions.

3.3.1 Shake flask culture

Shake flask studies were carried out in triplicate, unless otherwise stated. Cultures were incubated at 28^oC and 180rpm in 500ml Erlenmeyer flasks on an incubator shaker. The 50ml inoculum was developed by incubation of culture in YPD_2 liquid media for 24h to 36h, depending on cells reaching an OD_{620} of approximately 2. In test cultures, for a working volume of 50ml, shake flasks contained 40ml YP_2D_2 or YP_2D_4 liquid media, 5ml phosphate buffer (0.5M, pH8) and 5ml inoculum from inoculum cultures. It was assumed that all glucose was consumed in inoculum cultures, and therefore only 40ml of the

50ml test culture volume (i.e. 80%) contained glucose. Nominal glucose concentrations of 20g/l or 40g/l were therefore reported as the actual glucose concentration in the test culture, i.e. 16g/l (1.6%) or 32g/l (3.2%). During sampling no more than 10% (v/v) of the liquid medium was removed throughout the cultivation.

Where indicated in the text, alkylbenzene substrates were added to test cultures. A range of alkylbenzenes were used, namely ethyltoluene, propylbenzene, butylbenzene, *sec*-butylbenzene, *tert*-butyltoluene, hexylbenzene, octylbenzene and decylbenzene. Unless otherwise stated, 1% (v/v) alkylbenzene was added at 24h. In an investigation where the influence of ethyltoluene, propylbenzene, butylbenzene, *sec*-butylbenzene, *tert*-butyltoluene and hexylbenzene on the bioconversion of substrate was determined, alkylbenzenes were added in 0.5% (v/v) aliquots at 48h, 72h and 96h. This was to overcome the toxic effect of alkylbenzenes containing alkyl chain lengths with less than 6 carbons.

The compounds ethanol and oleic acid were also added to test cultures because these compounds had been reported as inducers for alkane bioconversion (Van Rooyen 2005). Where indicated in the text, 1% (v/v) ethanol or 1% (v/v) oleic acid was added at 24h after which the alkylbenzene substrate was added at 48h.

3.3.2 Bioreactor culture methodology

A 7L NBS Bioflo 110 stirred tank bioreactor (working volume 2L) was inoculated with a 10% (v/v) inoculum culture. The inoculum was prepared by aseptically transferring 5 loops of culture from agar plates to 200 ml YPD_2 medium in a 3L Erlenmeyer flask and incubated at 28^oC and 200rpm for up to 36h, depending on cells reaching an OD_{620} of approximately 2.

The bioreactor medium comprised 1.6L YP_2D_4 or YP_2D_8 medium and 200ml phosphate buffer (0.5M, pH8). As in shake flask culture, the actual glucose concentration was reported as 80% of the nominal glucose concentrations of 40g/l or 80g/l, i.e. 32g/l (3.2%) or 64g/l (6.4%). The medium also contained 0.025% (v/v) silica Antifoam A supplies to prevent foaming in the culture. During cultivation, air was supplied at 0.8vvm. The temperature was maintained at 28^oC while the pH was controlled to above 7.6 with the addition of 2M sodium hydroxide. An aqueous solution containing 2% (v/v) Antifoam A was manually added drop-wise to the bioreactor when required. When severe foaming occurred, as much as 5ml antifoam solution was added. To prevent the volatilisation of compounds in the culture, the outlet condenser was cooled with icy water. A loop placed in the outlet tube from the condenser was clear

of condensate, indicating that all condensate was returned to the bioreactor. Furthermore, for safety measures the outlet from the condenser was released into an extractor.

Initially the dissolved oxygen was maintained above 20% by manually adjusting the agitation between 400rpm and 600rpm. The dissolved oxygen was subsequently controlled to a set point of 22.5% through automatic adjustment of the agitation between the limits of 200rpm and 600rpm, i.e. a drop or rise in dissolved oxygen at 22.5% would respectively increase or decrease the agitation speed automatically.

3.4 Analytical methods

Cell concentration was determined by estimating the optical density and cell count. The alkylbenzene substrate- and the product concentrations were measured with a gas chromatograph. The concentration of the co-substrate, glucose, was determined by means of a colorimetric method using a dinitrosalicylic acid reagent (DNS) for determining reducing sugars.

3.4.1 Cell concentration

3.4.1.1 <u>Optical density (OD)</u>

Cell concentration was measured by density using a spectrometer (Varian) at 620nm. The samples for absorbance readings were prepared by pipetting a 500µl culture sample into a microcentrifuge tube and centrifuging it for 5min at 10,000rpm (Eppendorf Minispin Plus). The supernatant was used for glucose analyses while the pellet was re-suspended in a 500µl physiological salt solution (0.9% (wt/v) aqueous sodium chloride) and vortexed (VM-300). The re-suspended sample was then diluted with physiological salt solution so that the absorbance reading was obtained within the linear range below 0.8. The dilution factor was recorded. The OD could therefore be calculated by

$$OD = Absorbance \times Dilution\ factor \qquad\qquad 3.1$$

The spectrophotometer tare was set with a sample of physiological salt solution and sample readings were taken by using 1.5ml glass cuvettes.

3.4.1.2 <u>Cell counts</u>

The cells were counted using a haemocytometer (Nieubauer). The haemocytometer has a depth of 0.1mm; the smallest squares have an area of 0.0025m^2. 100µl samples were pipetted into microcentrifuge tubes. These

samples were diluted with distilled water to facilitate counting and the dilution factor recorded. The microcentrifuge tubes were vortexed to ensure proper mixing. 10µl of the well-agitated samples were then analysed through a microscope (Zeiss) at 400x magnification. Ten (10x) $0.0025m^2$ squares were selected for counting cells to determine the average cell concentration. The cell counts could be calculated by

$$Cell\ count\ concentration = \frac{Cells / square}{Area \times Depth} \times Dilution\ factor \quad \text{(cells/ml)} \qquad 3.2$$

The morphology of *Yarrowia lipolytica* includes cells that are round (yeast-like form) and elongated (mycelial form). Both forms were counted as cells. Where budded, cells were counted as individuals when they were at least two-thirds the size of adjoined cells. Additionally, cells straddling the line in the smallest squares were included in the count if two thirds or more lay within the square.

3.4.2 Substrate concentration

3.4.2.1 Glucose concentration

The analytical method described by Miller (1959) for the determination of reducing sugars was used for the analysis of residual glucose concentrations, and modified by using different component concentrations and cooling method (M. Johnstone-Robertson, personal communication).

Table 3.3 lists the composition of the dinitrosalicylic acid (DNS) reagent.

Table 3.3 Dinitrosalicylic acid reagent

Compound	Amount
3,5-dinitrosalicylic acid	5.3g
Rochelle salts	153g
Sodium hydroxide	9.9g
Sodium-metabisulphite	4.15g
Phenol	3.8g
Distilled water	708ml

All the above constituents excluding phenol were dissolved in the water. Phenol was melted at medium heat in a microwave prior to mixing with the rest of the components. The solution was then stored in a container covered with foil to protect the reagent from light.

During analysis, 500μl samples were taken from the test cultures and placed into microcentrifuge tubes. These were centrifuged for 5min at 10,000rpm. The supernatant was diluted 20 or 40 times for samples containing 16g/l and 32g/l initial glucose respectively such that the dilution would contain between 0 and 0.8g/l glucose. 200μl of this diluted solution was then added to 600μl DNS reagent in test tubes. The test tubes were heated for 5min in boiling water, after which they were removed and cooled on ice for 5min, after which a dark orange colour was obtained. The orange colour was developed as a result of the reduction of 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid while the aldehyde groups in the reagents were oxidised to carboxyl groups (Miller 1959). The samples were then diluted with 8.2ml distilled water to provide a linear range for absorbance readings on the spectrophotometer. Absorbance was determined at 523nm in 1.5ml glass cuvettes in a spectrophotometer (Varian).

The tare was set with a sample prepared with zero glucose. With known concentrations of glucose up to 1g/l, a calibration curve could be set up to relate the absorbance readings to glucose concentration. Four standard curves were constructed over the period of the study with coefficients of determination (R^2) above 0.93. Appendix B.1 details the standard calibration curves.

3.4.2.2 <u>Alkylbenzene concentration</u>

The alkylbenzene concentration was measured with a gas chromatograph (Varian). As sampling of a hydrophobic compound was a problem in shake flasks, the alkylbenzene substrate was only measured in bioreactor culture. The extraction procedure was as follows: 500μl samples of the culture were placed into microcentrifuge tubes and acidified with 50μl 1M hydrochloric acid. The samples were extracted with 300μl *tert*-butyl-methyl-ether (TBME) containing 0.5% (w/v) undecanol as the internal standard, by vortexing at high speed for 10min. The aqueous and non-aqueous phases were separated by centrifugation (10,000rpm for 10min). The organic fraction was removed for sampling and the procedure repeated on the aqueous fraction for a second extraction. The extracts were then combined. The efficiency of the extraction procedure was assessed by analysing duplicate or triplicate sample extracts of the same sample. The standard deviation between samples indicated that the extraction efficiency was adequate (Appendix C.1).

Initially samples were methylated which shortened the overall retention times. The samples were methylated by adding 50µl of the extract to 50µl trimethylsulfonium hydroxide in column 1 (Table 3.4), however, due to the deterioration of the column the results from this column were not satisfactorily reproducible. Column 2 (Table 3.4) was therefore used where methylation of samples was not required.

Table 3.4 Gas chromatograph conditions

GC Conditions	Column 1	Column 2
Gas chromatograph:	Varian CP 3880	Varian 3400
Column type:	Varian WCOT Fused silica column with specifications: Length - 30m Film thickness - 1µm Inside diameter - 0.53mm Outside diameter - 0.7mm	Phenomenex Zebron ZB-5 column with specifications: Length – 60m Film thickness – 0.25µm Inside diameter - 0.32mm
Column flow:	5-6ml/min	5-6ml/min
Head pressure:	5psi	10psi
Split ratio:	50:1	50:1
Flame gas:	Hydrogen – 30ml/min Air – 300ml/min	Hydrogen – 30ml/min Air – 300ml/min
Make-up gas:	Helium – 30ml/min	Helium – 30ml/min
Detector:	Flame ionisation detector (FID) $300^{o}C$	Flame ionisation detector (FID) $300^{o}C$
Injector:	$220^{o}C$	$220^{o}C$
Oven:	- $120^{o}C$ held for 5min - Increase to $230^{o}C$ at $10^{o}C$/min - Hold for 5min (Total time 21min)	- $120^{o}C$ held for 1min - Increase to $220^{o}C$ at $10^{o}C$/min

A sample chromatogram is displayed in Appendix C.2. The retention times for undecanol and hexylbenzene, using Column 1 conditions, were 10.80min and 4.90min respectively. Similarly, the retention times for undecanol and hexylbenzene, using Column 2 conditions, were 14.13min and 12.96min respectively.

Alkylbenzene substrate concentration was only measured in later bioreactor studies where the accuracy of sampling hexylbenzene could be increased. A standard calibration curve of hexylbenzene concentration was therefore set up using only the conditions of Column 2. The calibration curve of hexylbenzene is displayed in Appendix B.2.

3.4.3 Product concentration

3.4.3.1 Phenyl acetic acid

The product, phenyl acetic acid (PAA), and alkylbenzene substrate were measured in the same sample with the method described section 3.4.2.2. The retention time for PAA, using Column 1 conditions, was 9.25min. Similarly the retention time for PAA, using Column 2 conditions, was 12.26min. Standard calibration curves for PAA are displayed in Appendix B.3.

3.4.3.2 Metabolic intermediates

The accumulation of possible metabolic intermediates other than PAA was analysed by using nuclear magnetic resonance (NMR). The process was carried out on the hydrophobic extract and on the aqueous extract.

For the hydrophobic extract, 200ml sample was prepared by extraction of the organic phase as per the method described in the sub-section 2.4.2.2. The TBME was then removed in a rotary evaporator and the remaining precipitate dissolved in chloroform. The dissolved precipitate was then subjected to 300MHz ^{1}H-NMR spectroscopy (Varian VNMRS 300). The aqueous extract was prepared by filtering 500ml culture (sampled at the end of the analysis period) through 0.2μm filter paper and freeze drying the filtered liquid. The dried sample was then dissolved in distilled water and subjected to 600MHz ^{1}H-NMR spectroscopy (Varian Unity*Inova* 600).

4. Results and Discussion I – Development of the model system

To evaluate the potential of *Yarrowia lipolytica* for growth and bioconversion using the model system, parameters that influence cell growth and bioconversion were assessed in shake flasks. The first priority was to develop the parameters of the model system so that cell growth and bioconversion could be maximised. To develop the model system the influence of trace elements, added nitrogen, buffering, oxygen supply, glucose concentration, substrate type and ethanol or oleic acid addition on bioconversion and/or cell growth was investigated. These parameters were assessed in shake flasks and the results presented in Chapter 4.

Unless otherwise stated, shake flask cultivations were conducted in triplicate. Where applicable, the maximum to minimum range of the parameter for individual data points are indicated graphically. Sample calculations are detailed in Appendix D and raw experimental data have been tabulated in Appendix E.1.

4.1 Influence of trace elements, buffering and added nitrogen on cell growth

The influence of trace elements, buffering and added nitrogen on cell growth of *Y. lipolytica* was assessed. This was done by comparing the growth in three different media. In the first medium (Medium 1) a standard YP_2D_4 medium was used. In the second medium (Medium 2) the phosphate buffer and trace element solution (described in subsection 3.2.1) was added to the YP_2D_4 medium. In the third medium (Medium 3) a phosphate buffer, trace element solution and added nitrogen was added to the YP_2D_4 medium (Table 4.1).

Table 4.1 Trace elements, buffering and additional nitrogen composition in YP_2D_4 medium

Components (additional to YP_2D_4 medium)	Amount		
	Medium 1	Medium 2	Medium 3
Phosphate buffer	0	1g/l (0.135g)	1g/l (0.135g)
Trace element solution	0	10 vol% (15ml)	10 vol% (15ml)
Ammonium sulphate	0	0	4.714g/l (0.636 g)

A comparison made between Medium 1 and Medium 2 would assess the combined influence of buffering and trace elements on cell growth. A comparison made between Medium 2 and Medium 3 would assess the influence of added nitrogen on cell growth.

Y. lipolytica TVN348 was cultivated in Medium 1, 2 and 3 (Table 4.1) in volumes of 150ml in 500ml Erlenmeyer flasks. Cultures in Medium 1 and 2 were carried out in quadruplicate while cultures in Medium 3 were carried out in triplicate. The growth on 3.2% (wt/v) glucose was analysed without addition of alkylbenzene over 43h. *Y. lipolytica* TVN348 was the only strain used for this study. The maximum cell concentrations obtained were 1.76×10^8cells/ml, 2.00×10^8cells/ml and 2.06×10^8cells/ml for cultures in Medium 1, 2 and 3, respectively (Figure 4.1). The standard deviation between triplicate samples ranged from 7.6×10^5cells/ml at 3h to 2.95×10^7cells/ml at 43.5h, and it can be said with a 95% confidence interval that there was no significant difference in cell growth between the three media used. Confidence intervals use the standard deviation between duplicate or triplicate samples to determine whether there is a statistically significant difference between two parameters with a 95% confidence. The steps for calculating these intervals are demonstrated in Appendix D.1.

The pH of inoculum cultures varied between 6.10 and 6.23. After 31h of cultivation when all growth levelled, the pH of the culture using Medium 1 and 2 was 7.6 and the average pH of the culture using Medium 3 was 7.3. The <5% difference is considered insignificant. The results of this experiment suggested that the growth of the organism was limited by something other than the buffering capacity, trace elements or nitrogen concentration.
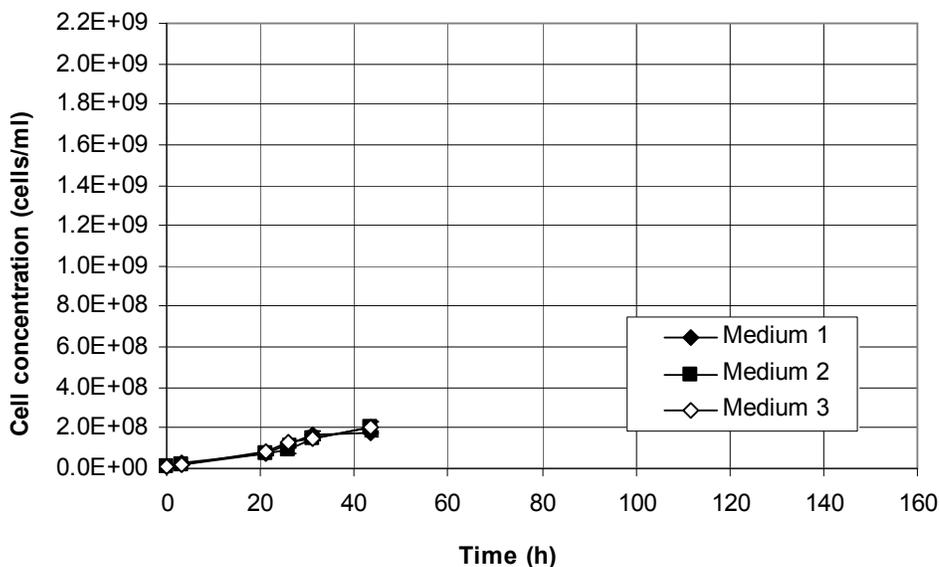
Figure 4.1 Determination of the effect of differing media compositions and buffering capacity (according to Table 4.1) on the growth of *Y. lipolytica* TVN348 grown on 3.2% (wt/v) glucose in 150ml shake flask culture

4.2 Influence of oxygen supply on cell growth and bioconversion

The influence of oxygen supply was assessed in two ways: 1) by comparing different culture volumes in shake flasks, and 2) by comparing cultures in baffled- and non-baffled flasks. Oxygen supply is increased when the working volume is decreased because it provides a larger surface area for oxygen transfer to take place. Similarly, baffled flasks also increase the oxygen supply because the indents along the wall of the flask increase the turbulence in the culture medium, thereby increasing the surface area.

4.2.1 Influence of oxygen supply on cell growth

For the assessment of the influence of oxygen supply on growth of *Y. lipolytica* TVN348 and TVN493, the cell growth of these strains were compared in culture volumes of 50ml, 70ml and 150ml. All the flasks were inoculated with the same inoculum (2.00×10^7cells/ml and 3.12×10^7cells/ml for TVN348 and TVN493 cultures respectively) and cultivated in the YP_2D_2 culture medium (from section 3.3.1). 50ml Cultures were carried out in sextuplicate, 70ml cultures in triplicate and 150ml cultures in a single flask. *Y. lipolytica* WT was not included in this study, as it had not been supplied at this time.

The cell concentrations obtained in *Y. lipolytica* TVN348 cultures (at 48h) were 3.24×10^8 cells/ml, 4.48×10^8 cells/ml and 5.32×10^8 cells/ml in 150ml, 70ml and 50ml cultures, respectively (Figure 4.2). The cell concentrations obtained in *Y. lipolytica* TVN493 cultures (at 48h) were 3.36×10^8 cells/ml, 3.99×10^8 cells/ml and 4.81×10^8 cells/ml in 150ml, 70ml and 50ml cultures, respectively (Figure 4.3). The 65% increase in cell concentration of *Y. lipolytica* TVN348 from 150ml to 50ml cultures was significant (95% confidence interval: $5.32 \times 10^8 \pm 5.06 \times 10^7$ cells/ml). Similarly, the 43% increase in cell concentration of *Y. lipolytica* TVN493 from 150ml to 50ml cultures was also significant (95% confidence interval: $4.81 \times 10^8 \pm 1.18 \times 10^8$ cells/ml). These results show that that the cell growth in 150ml cultures was limited by oxygen supply when compared with growth in 50ml cultures, and the increase in oxygen supply had a significant influence on the cell growth of *Y. lipolytica*.
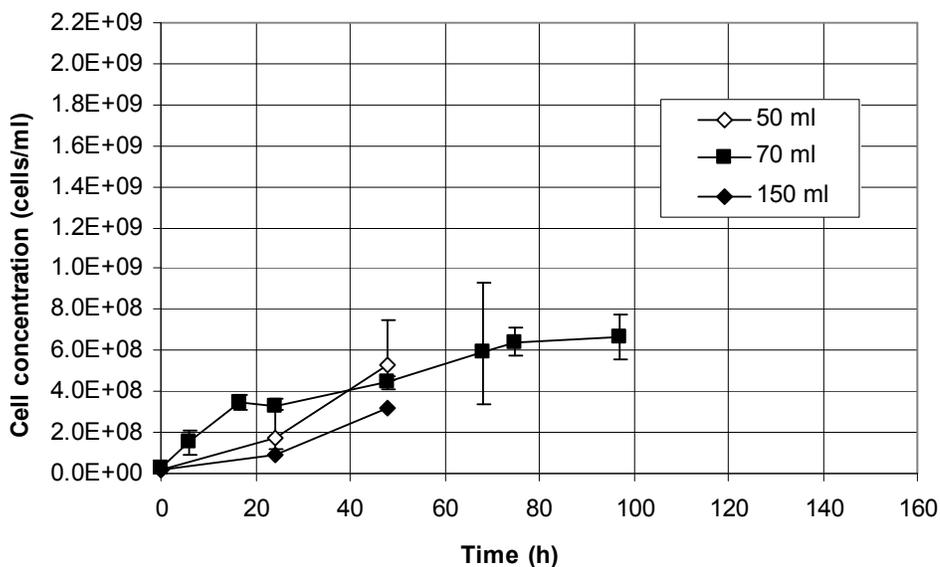
Figure 4.2 Determination of the effect of various working volumes on the growth of *Y. lipolytica* TVN348 grown on 1.6% (wt/v) glucose. 1% (v/v) Ethanol added at 24h, 1% (v/v) hexylbenzene added at 48h.
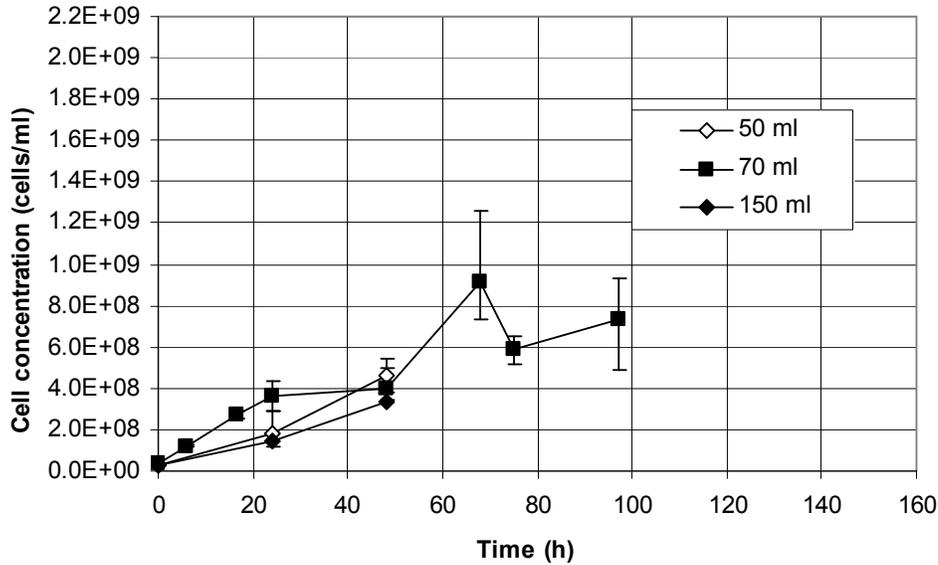
Figure 4.3 Determination of the effect of various working volume on the growth of *Y. lipolytica* TVN493 grown on 1.6% (wt/v) glucose. 1% (v/v) Ethanol added at 24h, 1% (v/v) hexylbenzene added at 48h.

Comparison of the maximum specific growth rates calculated over the first 24h yields for *Y. lipolytica* TVN348 yields $0.06h^{-1}$, $0.08h^{-1}$ and $0.09h^{-1}$ in 150ml, 70ml and 50ml cultures, respectively (selected over first 24h from Figure 4.4). Similarly, the maximum specific growth rates for *Y. lipolytica* TVN493 were $0.06h^{-1}$, $0.08h^{-1}$ and $0.07h^{-1}$ in 150ml, 70ml and 50ml cultures, respectively (selected over first 24h from Figure 4.5). The first data points for 70 ml cultures in figures 4.4 and 4.5 were not considered as the maximum specific growth rates because it is only 6h after inoculation. It was suggested that an error in measuring the initial cell concentration may have occurred where the initial concentration was measured lower than the actual value. The 47% increase in maximum specific growth rate by TVN348 from 150ml to 50ml cultures was significant (95% confidence interval: 0.091 ± 0.013 h^{-1}). The 14% increase in maximum specific growth rate by TVN493 from 150ml to 50ml cultures was, however, not significant (95% confidence interval: 0.073 ± 0.016 h^{-1}). The marginal or insignificant increase in maximum specific growth rate for increase in oxygen supply suggests that, at low cell concentrations, there was enough oxygen to support cell growth.
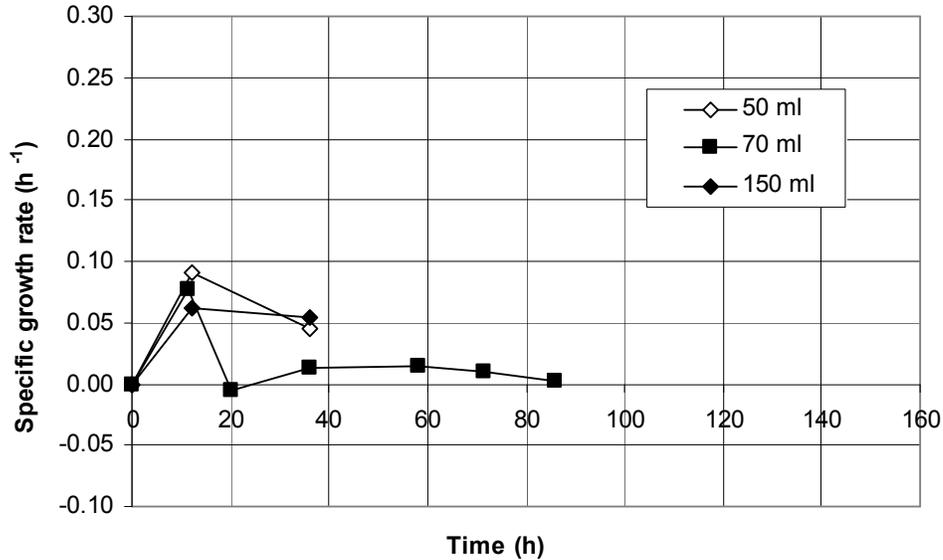
Figure 4.4 Evaluation of specific growth rates of *Y. lipolytica* TVN348 during cultivations in various working volumes grown on 1.6% (wt/v) glucose. 1% (v/v) Ethanol added at 24h, 1% (v/v) hexylbenzene added at 48h.
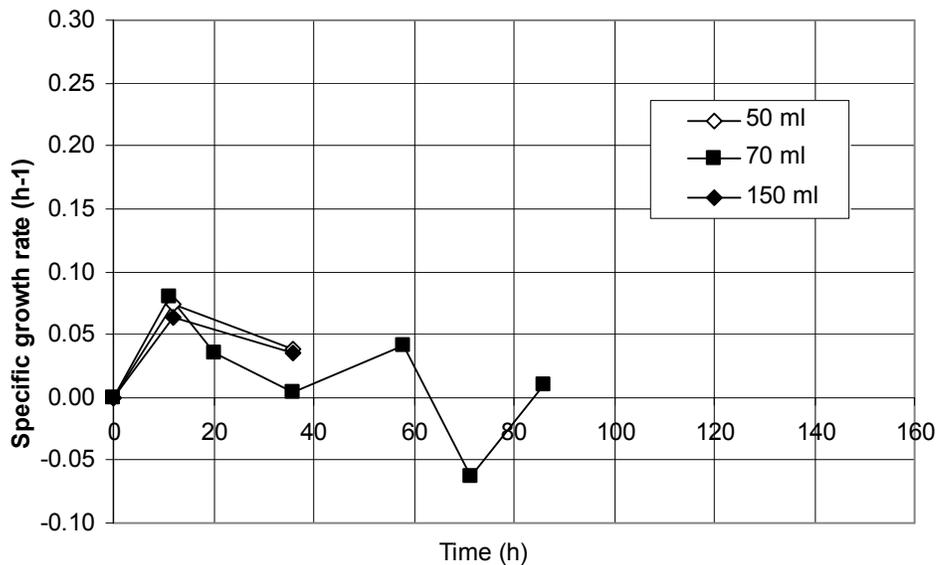
Figure 4.5 Evaluation of specific growth rates of *Y. lipolytica* TVN493 during cultivation in various working volumes grown on 1.6% (wt/v) glucose. 1% (v/v) Ethanol added at 24h, 1% (v/v) hexylbenzene added at 48h.

The influence of oxygen on cell growth of *Y. lipolytica* was examined by varying the oxygen supply in a second case using baffled and non-baffled flasks. Cultivations of *Y. lipolytica* WT in 50ml YP$_2$D$_4$ medium (section 3.2.1)

were carried out in duplicate in both baffled and non-baffled flasks. *Y. lipolytica* WT was used in this case because these studies were conducted at a later stage in research where this strain had already been selected for future study. The final cell concentrations of *Y. lipolytica* WT were 1.54×10^9 cells/ml and 2.17×10^9 cells/ml in non-baffled- and baffled flasks, respectively (Figure 4.6). The 41% increase in cell concentration from non-baffled- to baffled flasks was significant (95% confidence interval: $1.54 \times 10^9 \pm 8.27 \times 10^7$ cells/ml). However, the optical density (OD) observed in non-baffled- and baffled flask cultures were similar at 72h, i.e. 37.6, even though the cell concentration increased by 51% from baffled flasks (1.20×10^9 cells/ml) to baffled flasks (1.81×10^9 cells/ml) (Figure 4.6). This suggests that cells growing in the non-baffled culture were larger in biomass than cells growing in the baffled culture. It is proposed that larger cells were observed in non-baffled flask culture because the oxygen supplied was sufficient for cell maintenance, but not cell multiplication. Cell growth of *Y. lipolytica* is therefore limited by oxygen supply even in 50ml culture volumes in shake flasks.

Comparison of the maximum specific growth rates calculated over the first 24h yields approximately 0.5 for both non-baffled flasks and baffled flasks, respectively (Figure 4.7). The difference in growth rate between non-baffled and baffled flask cultures is not significant.
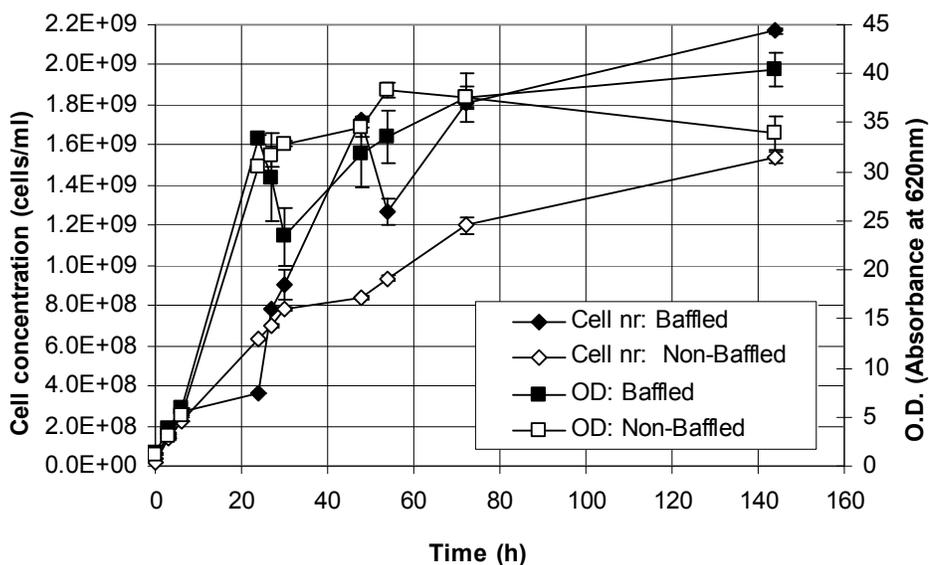
Figure 4.6 Determination of the effect of baffled and non-baffled flasks on the growth of *Y. lipolytica* WT grown on 3.2% (wt/v) glucose in 50ml culture volumes. 1% (v/v) Hexylbenzene added at 24h.
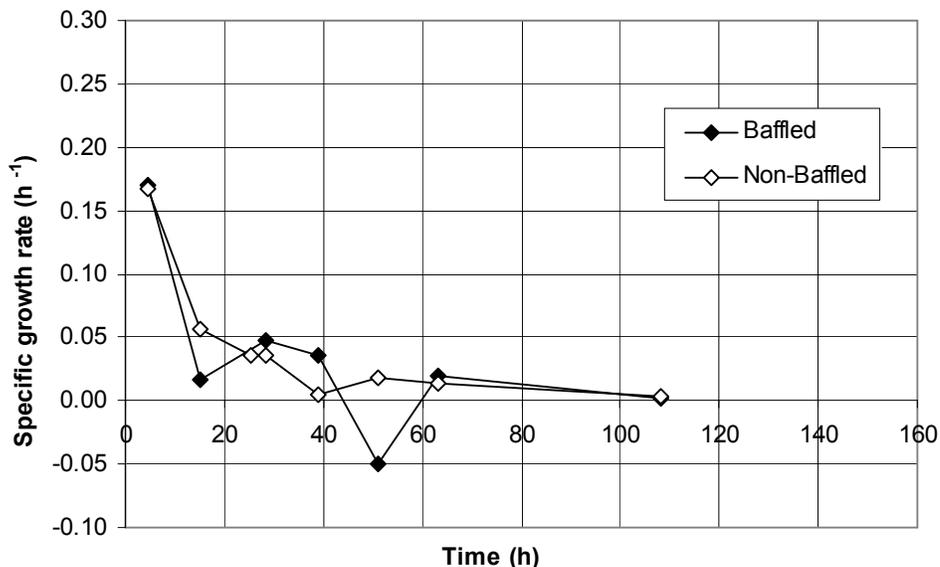
Figure 4.7 Evaluation of specific growth rates of *Y. lipolytica* WT during cultivations in baffled and non-baffled flasks grown on 3.2% (wt/v) glucose in 50ml culture volumes. 1% (v/v) Hexylbenzene added at 24h.

The increase in cell growth with an increase in oxygen supply by 1) decrease in working volume and 2) use of baffled flasks over non-baffled flasks demonstrates the importance of oxygen supply for cell growth in all strains (TVN348, TVN493 and WT). It is therefore imperative that an adequate amount of oxygen is supplied during *Y. lipolytica* cultivations to obtain maximum cell concentration.

4.2.2 Influence of oxygen supply on bioconversion

The influence of oxygen supply on bioconversion was assessed in the same cultivations described in subsection 4.2.1, i.e. by comparing product formations by 1) *Y. lipolytica* TVN348 and TVN493 in 50ml, 70ml and 150ml culture volumes and 2) *Y. lipolytica* WT in baffled and non-baffled flasks.

In the assessment using different culture volumes of *Y. lipolytica* TVN348 and TVN493, 1% (v/v) ethanol and 1% (v/v) hexylbenzene was added at 24h and 48h, respectively. Here 50ml and 150ml cultures were carried out in a single flask while 70ml cultures were carried out in triplicate. At 120h *Y. lipolytica* TVN348 produced phenyl acetic acid (PAA) with concentrations of 2.38mM, 5.0mM and 29.0mM in 150ml, 70ml and 50ml cultures, respectively (Figure 4.8). Although hexylbenzene was not measured in shake flask studies, this would correspond to a potential hexylbenzene conversion increase from 4.5% to 18.8% to 53.2%. At 120h, *Y. lipolytica* TVN493 produced PAA with

concentrations of 1.2mM, 7.5mM and 21.7mM PAA in 150ml, 70ml and 50ml cultures, respectively (Figure 4.9). Similarly, this would correspond to a potential hexylbenzene conversion increase from 2.3% to 30.3% to 40.9%. These results indicate that an increased oxygen supply also increased the bioconversion.

The accumulated PAA produced by *Y. lipolytica* TVN493 in 50ml culture may be compared with an identical study conducted by Van Rooyen (2005). In the current study, 21.7mM PAA had accumulated after 120h whereas in the study by Van Rooyen approximately 35mM PAA (read off a graph) had been accumulated by this time. It is not known whether the higher PAA obtained in Van Rooyen's work was due to an increased cell growth since cell growth data was not provided. No conclusion could be made about the difference. Further, due to the significant difference in PAA formation for identical cultivations of *Y. lipolytica* TVN493, the difference between PAA formations of *Y. lipolytica* TVN348 and *Y. lipolytica* TVN493 was considered insignificant.

The 480% and 190% increase in product formation (by *Y. lipolytica* TVN348 and TVN493 respectively) from 70ml to 50ml cultures (at 120h) indicates the organism's sensitivity to the oxygen available. This strongly suggests that *Yarrowia* may be dependent on a specific supply of oxygen for optimal bioconversion to take place. That said, upon further analysis of the PAA formed by *Y. lipolytica* TVN493, it was found that the maximum PAA accumulated in the 70ml cultures at 148h (20.2mM PAA for maximum range in 70ml culture) was similar to the PAA formed in the 50ml culture at 120h (21.7mM) (Figure 4.9). An equivalent amount of PAA was therefore accumulated in 70ml cultures, but only at a later stage. It could be argued that, if the cultivation period had been extended to 148h, the amount of accumulated PAA in the 50ml culture might have increased. This was certainly the case in Van Rooyen's work where 100% conversion of hexylbenzene was achieved after 148h. Glucose was exhausted within 48h of cultivation in 50ml culture volumes, however, and so at 120h cells were already dying. This suggests that, in addition to the supply of adequate oxygen, the addition of glucose during cultivation will maximise the potential for both cell growth and bioconversion.
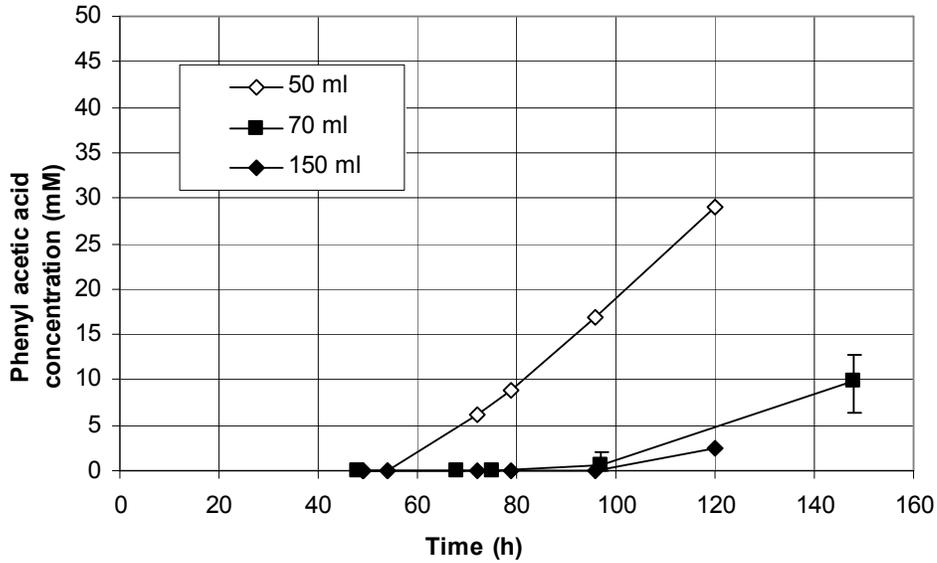
Figure 4.8 Determination of the effect of various working volumes on phenyl acetic acid production by *Y. lipolytica* TVN348 grown on 1.6% (wt/v) glucose. 1% (v/v) Ethanol added at 24h, 1% (v/v) hexylbenzene added at 48h.
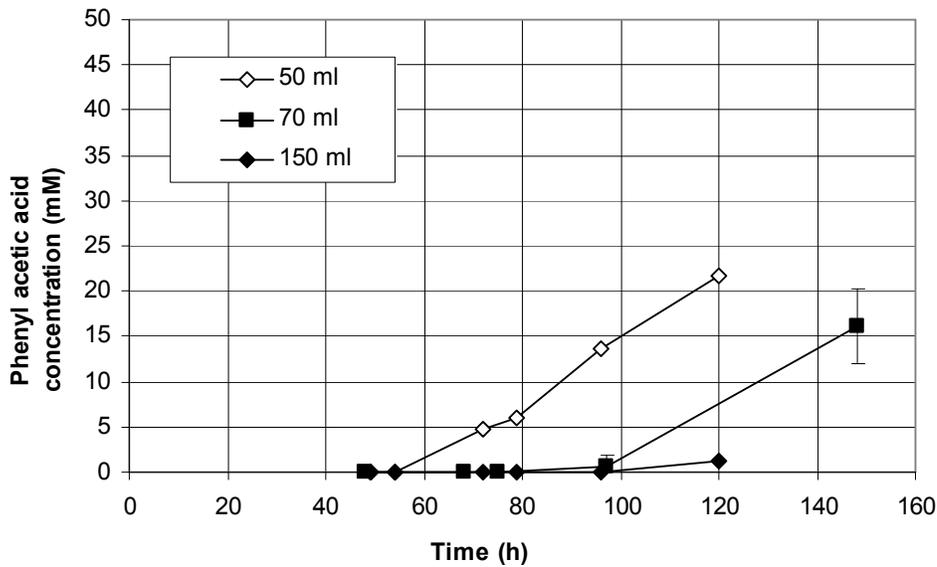
Figure 4.9 Determination of the effect of various working volumes on phenyl acetic acid production by *Y. lipolytica* TVN493 grown on 1.6% (wt/v) glucose. 1% (v/v) Ethanol added at 24h, 1% (v/v) hexylbenzene added at 48h.

In the assessment where oxygen supply was varied by 50ml, 70ml and 150ml culture volumes, varied cell concentrations of *Y. lipolytica* were obtained at

48h when the substrate was added (3.24×10^8cells/ml, 4.48×10^8cells/ml and 5.32×10^8cells/ml for TVN348; 3.36×10^8cells/ml, 3.99×10^8cells/ml and 4.81×10^8cells/ml for TVN493). As a result, the accumulated PAA formation would inevitably be dependent on the cell concentration. In order to eliminate the effect of different cell concentrations, a study was conducted where the influence of oxygen supply on bioconversion was assessed in cultures that had the same cell concentration at the time of substrate addition. This was done by cultivating *Y. lipolytica* TVN348 and TVN493 in a series of 50ml culture volumes for 48h during which time 1% (v/v) ethanol was added at 24h. Just before 1% (v/v) hexylbenzene addition at 48h, the 50ml cultures were combined such that new culture volumes were obtained, namely 50ml, 100ml and 150ml, all containing the same cell concentration (5.32×10^8cells/ml for TVN348 and 4.81×10^8cells/ml for TVN493). At 120h, *Y. lipolytica* TVN348 produced PAA with concentrations of 3.8mM, 15.9mM and 28.2mM in 150ml, 100ml and 50ml cultures, respectively (Figure 4.10). This would correspond to a potential hexylbenzene conversion increase from 7.1% to 30.0% to 53.2%. At 120h, *Y. lipolytica* TVN493 produced PAA with concentrations of 7.4mM, 16.5mM and 21.7mM in 150ml, 100ml and 50 ml cultures, respectively (Figure 4.11). Similarly, this would correspond to a potential hexylbenzene conversion increase from 14.0% to 31.2% to 40.9%. These results further demonstrate and confirm the importance of oxygen supply for bioconversion in *Y. lipolytica* TVN348 and TVN493.
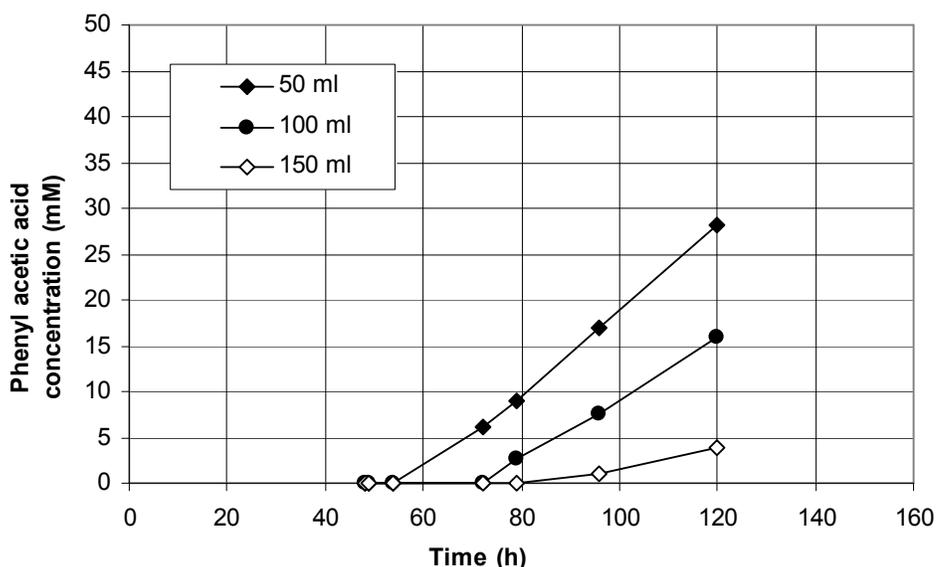
Figure 4.10 Determination of the effect of various working volumes on phenyl acetic acid production by *Y. lipolytica* TVN348 cultures containing equivalent cell concentrations at the time of substrate addition. Grown on 1.6% (wt/v) glucose, 1% (v/v) ethanol added at 24h, 1% (v/v) hexylbenzene added at 48h.
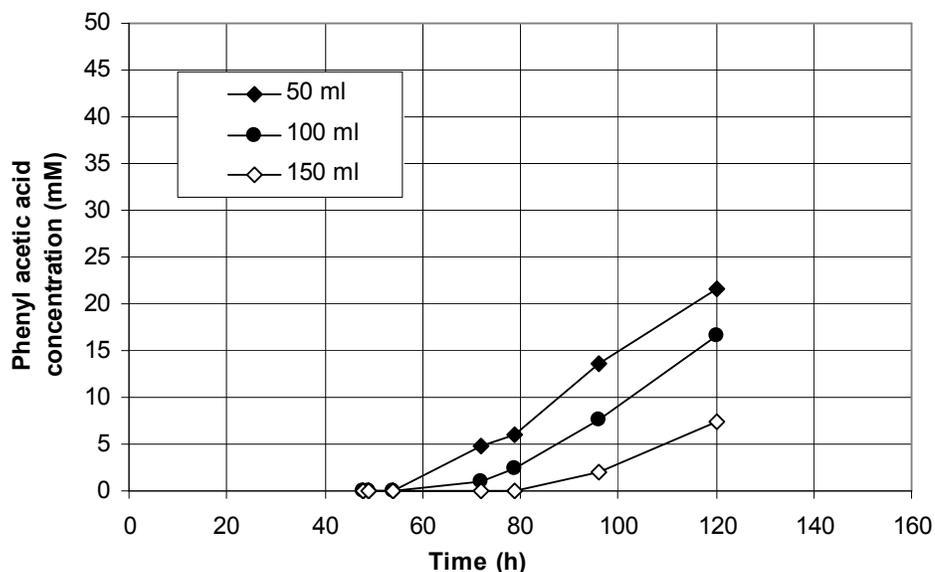
Figure 4.11 Determination of the effect of various working volumes on phenyl acetic acid production by *Y. lipolytica* TVN493 cultures containing equivalent cell concentrations at the time of substrate addition. Grown on 1.6% (wt/v) glucose, 1% (v/v) ethanol added at 24h, 1% (v/v) hexylbenzene added at 48h.

Higher PAA was also produced by *Y. lipolytica* WT with an increase in oxygen supply. Using baffled- and non-baffled flasks of *Y. lipolytica* WT in 50ml culture, 1% (v/v) hexylbenzene was added at 24h. The experiment was conducted in duplicate. Ethanol was not added in this case because it was conducted after it was established that ethanol was not necessary for induction (section 4.5). At 144h *Y. lipolytica* WT produced PAA with concentrations of 10.5mM and 46.6mM PAA for non-baffled and baffled flasks, respectively (Figure 4.12). This would correspond to a potential hexylbenzene conversion increase of over 4.4-fold from 19.8% to 88.0%. These results indicate that a significant increase in PAA formation was obtained with increase in oxygen supply in baffled flasks. However, analysis of the specific product formations by *Y. lipolytica* WT in non-baffled- and baffled flasks revealed only a 2.9-fold increase from 6.8×10^{-12}mmol PAA/cell (non-baffled) to 2.0×10^{-8}mmol PAA/cell (baffled) (Figure 4.13). The higher PAA formed in baffled flask cultures was therefore partly due to the increased cell concentration. Since different strains and substrate addition times were used for the two ways in which the influence of oxygen supply was assessed, a comparison of PAA formations between *Y. lipolytica* TVN348, TVN493 and WT cultures cannot be made.
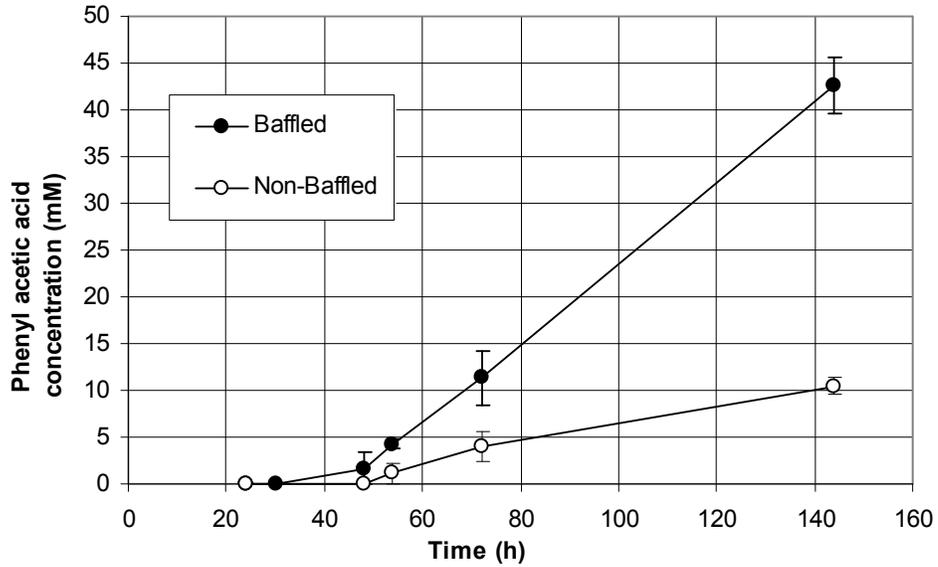
Figure 4.12 Determination of the effect of baffled and non-baffled flasks on phenyl acetic acid production by Y. lipolytica WT grown on 3.2% (wt/v) glucose in 50ml culture volumes. 1% (v/v) Hexylbenzene added at 24h.
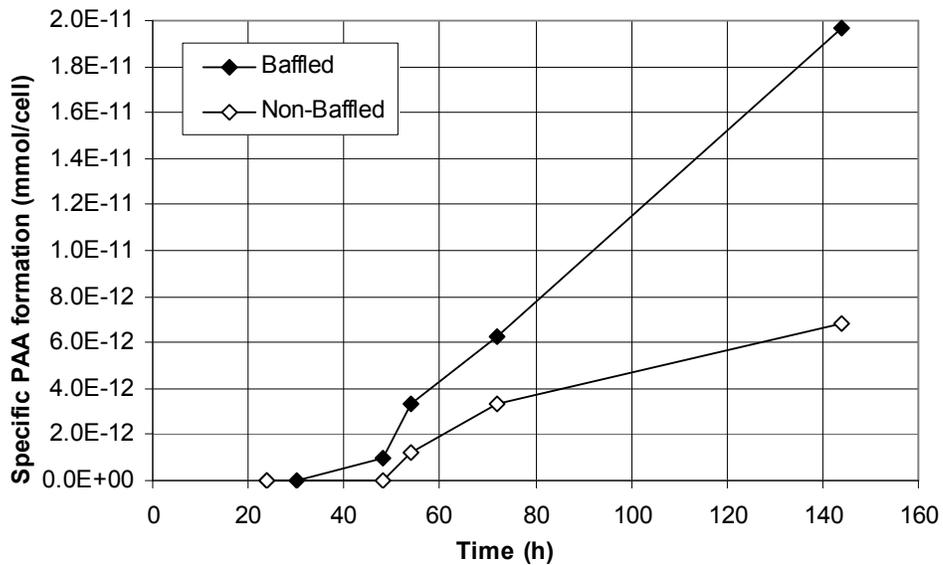
Figure 4.13 Evaluation of specific phenyl acetic acid production by *Y. lipolytica* WT in baffled in non-baffled flasks grown on 3.2% (wt/v) glucose in 50ml culture volumes. 1% (v/v) Hexylbenzene added at 24h.

The findings reported in this section suggest oxygen supply to be a major contributing factor to the limitation of growth and bioconversion in shake

flasks. These results suggest a sound rationale for conducting cultivations in a controlled bioreactor where adequate oxygen supply can be ensured.

4.3 Influence of glucose concentration on cell growth

The influence of glucose concentration on cell growth was evaluated by comparing *Y. lipolytica* TVN348 cultures grown on 1.6% (wt/v) and 3.2% (wt/v) glucose in YP_2D_2 and YP_2D_4 medium (subsection 3.2.1), respectively. These experiments were carried out in 50ml culture volumes and non-baffled flasks.

At 37.5h, the cell concentration of *Y. lipolytica* TVN348 increased from 3.95×10^8 cells/ml to 4.03×10^8 cells/ml for an increase in glucose concentration from 1.6% to 3.2% (wt/v) (Figure 4.14). The difference in cell concentration for 1.6% and 3.2% (wt/v) glucose cultures is insignificant (95% confidence interval: $3.95 \times 10^8 \pm 1.93 \times 10^8$ cells/ml). The optical density (OD), however, increased significantly from 24.20 to 31.72 at 37.5h (95% confidence interval: 24.20 ± 7.68). The 31% higher OD in 3.2% (wt/v) glucose cultures can be accounted for by the change in morphology in the different glucose cultures. In the 1.6% glucose cultures there were many yeast-like cells present. In the 3.2% glucose cultures, many mycelial cells had formed which were larger than the yeast-like cells. The occurrence of dimorphism (described in subsection 2.5.1.1) in 3.2% glucose cultures indicates that cells were placed under stress under these conditions. Kawasse *et al.* (2003) reported dimorphism occurrences when the supplied nitrogen concentration was low. Since the carbon to nitrogen ratio increased from 1.6% glucose cultures to 3.2% glucose cultures, it could be suggested that the cell growth of *Y. lipolytica* TVN348 was limited by nitrogen concentration in 3.2% (wt/v) glucose cultures, which is why dimorphism occurred. Alternatively, it could be argued that a higher glucose concentration demands a higher oxygen supply. Further oxygen limitation in shake flasks could then cause dimorphism to occur. If this were so, increased oxygen could be supplied by conducting experiments in baffled flasks. Cultivations in an aerated bioreactor would further overcome oxygen limitation and it is expected that bioreactor cultures of *Y. lipolytica* would be able to grow in glucose concentrations higher than 3.2% (wt/v).
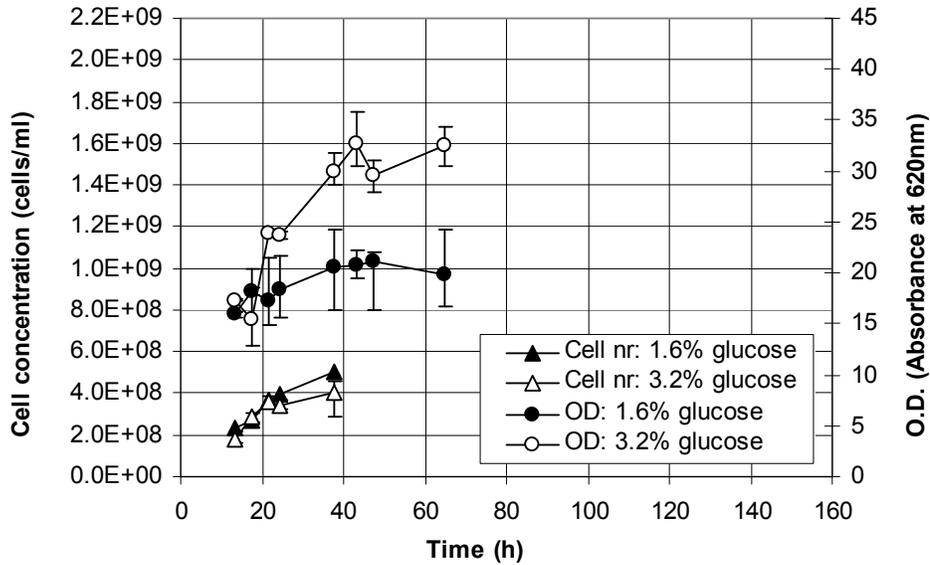
Figure 4.14 Determination of the effect of glucose concentrations on the growth of *Y. lipolytica* TVN348 in 50ml culture volumes.

Comparison of the maximum specific growth rates of *Y. lipolytica* TVN348 calculated as an average between 15.5h and 19.5h yields $0.06h^{-1}$ and $0.09h^{-1}$ in 1.6% and 3.2% glucose cultures, respectively (Figure 4.15). The increase in specific growth rate with increase in glucose concentration was not significant due to the large variation within a sample (95% confidence interval: 0.06 ± 0.04 h^{-1}). Although the cell concentration and maximum specific growth rate were not significantly increased by increase in glucose concentration, cell growth was also not inhibited by 3.2% (wt/v) glucose concentration.
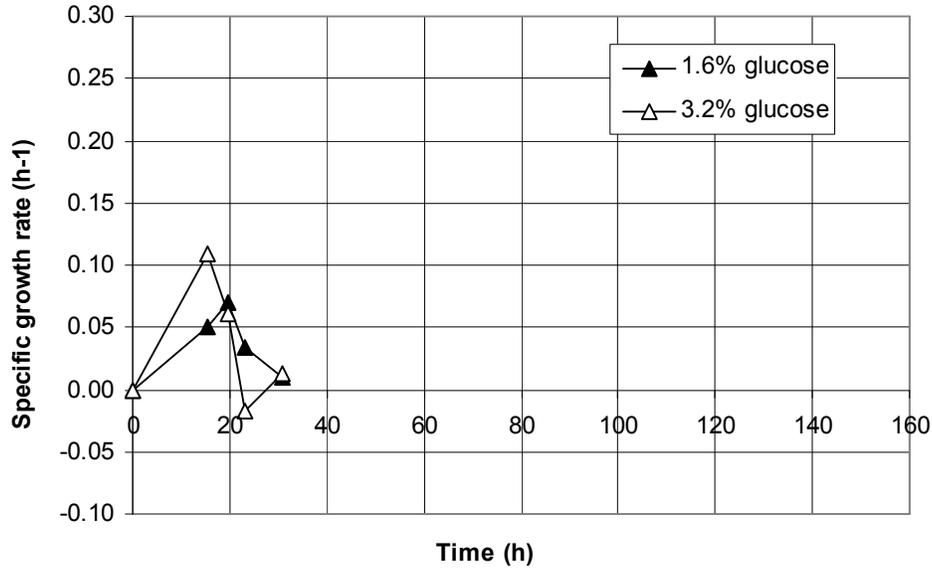
Figure 4.15 Evaluation of specific growth rates of *Y. lipolytica* TVN348 in cultivations using differing glucose concentrations in 50ml culture volumes.

The residual glucose concentrations and yields on glucose, in 1.6% (wt/v) and 3.2% (wt/v) glucose cultures, were compared. Glucose was exhausted by *Y. lipolytica* TVN348 within 48h in 1.6% glucose cultures whereas in 3.2% glucose cultures glucose was only exhausted after 65h (Figure 4.16). Further, the marginal increase in yield from 2.55×10^{10} cells/g glucose to 2.71×10^{10} cells/g glucose from 1.6% to 3.2% can be accounted for by the presence of the larger mycelial cells present in the 3.2% (wt/v) glucose cultures, resulting in a higher OD for the same cell concentration.
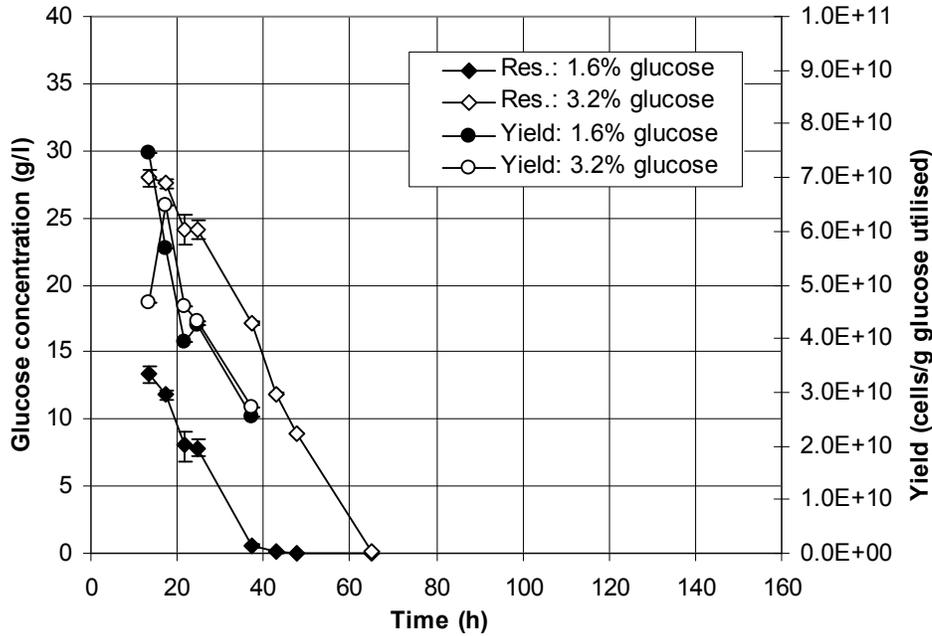
Figure 4.16 Determination of the effect of glucose concentrations on glucose utilisation and yields by *Y. lipolytica* TVN348 in 50ml culture volumes (Abbreviation: Res. – residual glucose).

In studies conducted by Dynesen *et al.* (1998) and Green *et al.* (2000) (section 2.5.3), both reported an inhibition of bioconversion of other substrates (dodecanoic acid, sucrose) when glucose concentration was above 0.5% (wt/v) in *S. cerevisiae* and *C. cloacae* cultures respectively. This 0.5% glucose level was achieved in the current 1.6% and 3.2% glucose cultures at 30h and 55h, respectively (Figure 4.16). From these other studies, it could be proposed that if more glucose were added to cultures to maintain this level after 30h or 55h (depending on the glucose concentrations in cultures) the cell growth would improve while optimal bioconversion would be achieved.

A 3.2% (wt/v) glucose concentration neither enhanced nor inhibited cell growth of *Y. lipolytica* TVN348. If the formation of mycelial cells was caused by lack of oxygen supply, then further study in baffled flasks or bioreactor culture would overcome the oxygen limitation and 3.2% or higher glucose concentrations may be expected to yield improved results.

4.4 Influence of alkylbenzene substrate on bioconversion

A range of alkylbenzene substrates (propylbenzene, butylbenzene, hexylbenzene, ethyltoluene, *sec*-butylbenzene, *tert*-butyltoluene, octylbenzene and decylbenzene) were assessed for their influence on bioconversion. Two investigations were carried out. The first investigation compared the influence of propylbenzene, butylbenzene, hexylbenzene, ethyltoluene, *sec*-butylbenzene and *tert*-butyltoluene on bioconversion by *Y. lipolytica* TVN348 and TVN493. *Y. lipolytica* WT was not included, as it had not yet been supplied. The second investigation compared the influence of hexylbenzene, octylbenzene and decylbenzene on bioconversion by *Y. lipolytica* WT. *Y. lipolytica* TVN348 and TVN493 were not included in the second investigation as WT had already been selected for use in future study at this stage (section 5.1).

4.4.1 Influence of propylbenzene, butylbenzene, hexylbenzene, ethyltoluene, *sec*-butylbenzene and *tert*-butyltoluene on bioconversion

The bioconversion of propylbenzene, butylbenzene, hexylbenzene, ethyltoluene, *sec*-butylbenzene and *tert*-butyltoluene by *Y. lipolytica* TVN348 and TVN493 cultures was assessed. Experiments were conducted in YP_2D_2 medium and single flask for each substrate. 1% Ethanol (v/v) was added at 24h and 0.5% (v/v) alkylbenzene substrate was added in three aliquots at 48h, 72h and 96h. This is contrary to the previous studies where 1% (v/v) hexylbenzene was added at 48h (subsection 4.2.2). The substrates in this study were added in three 0.5% aliquots to reduce the possible effect of toxicity of the shorter chain alkylbenzenes (propylbenzene, butylbenzene, *sec*-butylbenzene).

The only substrate to form a product was hexylbenzene. At 120h 14.7mM and 14.1mM PAA was formed by *Y. lipolytica* TVN348 and TVN493, respectively. Although hexylbenzene was not measured, this would correspond to a potential hexylbenzene conversion of 27.8% and 26.6%. The marginal difference in PAA formation between *Y. lipolytica*'s TVN348 and TVN493 indicates that there was no significant difference between the bioconversion in the two strains. The product formations from bioconversion of propylbenzene, butylbenzene, hexylbenzene, ethyltoluene, *sec*-butylbenzene and *tert*-butyltoluene by *Y. lipolytica* TVN348 and TVN493 using are illustrated in Figure 4.17 and Figure 4.18, respectively.

The PAA obtained by *Y. lipolytica* TVN348 previously (at 120h), however, was 97% higher at 29.0mM PAA (Figure 4.8). Similarly, for *Y. lipolytica* TVN493 the previous PAA obtained was 54% higher at 21.7mM (Figure 4.9) when

compared with this later PAA (14.1mM at 120h). It should be noted, however, that the substrates in this section were added later (48h, 72h and 96h) than in the previous study (48h) therefore the studies cannot be directly compared. It is expected that the amount of accumulated PAA in this section would increase beyond 120h of cultivation and eventually equate with the PAA formation in the previous study. Due to the exhaustion of glucose within 48h, however, the cultivation was not extended.

The times of hexylbenzene addition also prevented a direct comparison of PAA formation by *Y. lipolytica* TVN493 in this section to Van Rooyen's work (2005) (35mM PAA) where 1% (v/v) hexylbenzene was added at 48h.
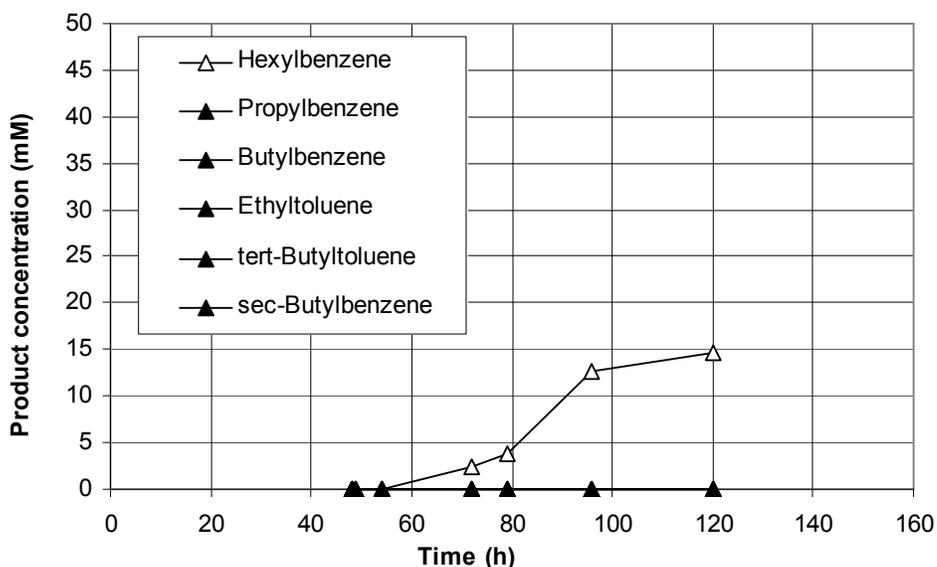
Figure 4.17 Determination of the effect of alkylbenzene substrates on product formation by *Y. lipolytica* TVN348 grown on 1.6% (wt/v) glucose in 50ml culture volumes. 1% (v/v) Ethanol added at 24h, 0.5% (v/v) substrate added at 48h, 72h and 96h.
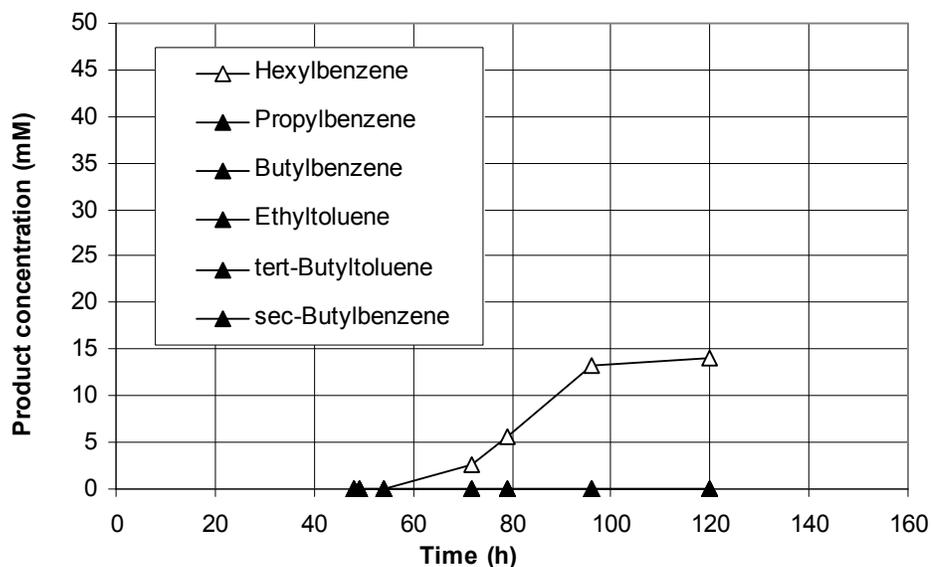
Figure 4.18 Determination of the effect of alkylbenzene substrates on product formation by *Y. lipolytica* TVN493 grown on 1.6% (wt/v) glucose in 50ml culture volumes. 1% (v/v) Ethanol added at 24h, 0.5% (v/v) substrate added at 48h, 72h and 96h.

4.4.2 Influence of hexylbenzene, octylbenzene and decylbenzene on bioconversion

In a subsequent study, the bioconversion of hexylbenzene, octylbenzene and decylbenzene by *Y. lipolytica* WT was compared. Experiments were conducted in YP_2D_4 medium and in duplicate baffled flasks. 1% (v/v) substrate was added at 24h. Ethanol was not added in this study because ethanol had been found unnecessary for induction by the time this experiment was conducted (section 4.5). Hexylbenzene was therefore added at 24h so that PAA could be produced sooner (note that the culture using hexylbenzene in this study is the same culture described in Figure 4.12).

PAA was formed with all three substrates. 42.6mM PAA, 9.1mM PAA and 7.0mM PAA was achieved using hexylbenzene, octylbenzene and decylbenzene, respectively (Figure 4.19). Although substrates were not measured, this would correspond to a potential substrate conversion of 80.4%, 17.1% and 13.3% for hexylbenzene, octylbenzene and decylbenzene, respectively. The amount of PAA produced by bioconversion of hexylbenzene was significantly higher than cultures containing octylbenzene or decylbenzene.
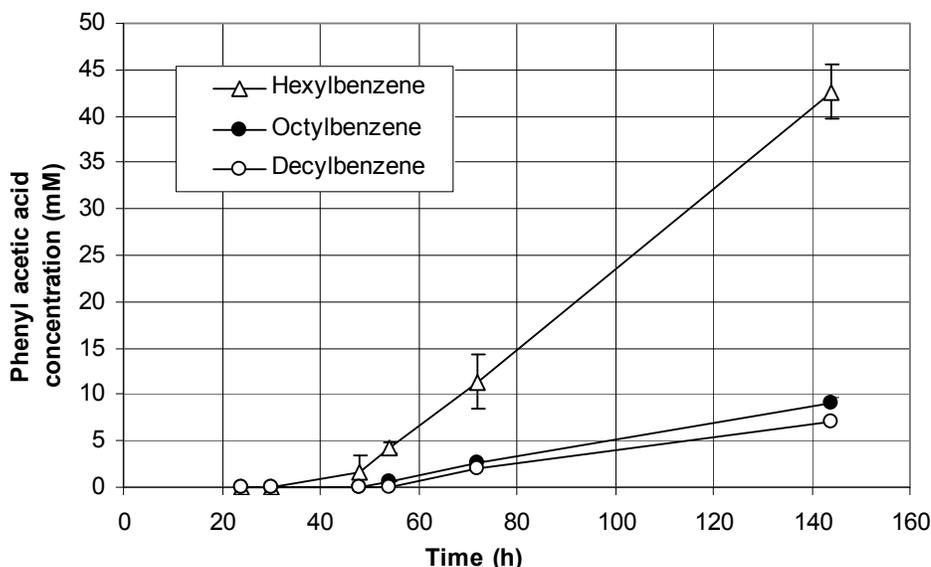
Figure 4.19 Determination of the effect of alkylbenzene substrates on product formation by *Y. lipolytica* WT grown on 3.2% (wt/v) glucose in 50ml culture volumes. 1% (v/v) Substrate added at 24h.

These results confirm that hexylbenzene was the only substrate to form a product from the bioconversion of propylbenzene, butylbenzene, hexylbenzene, ethyltoluene, *sec*-butylbenzene and *tert*-butyltoluene by *Y. lipolytica* TVN348 and TVN493. The highest amount of PAA was also formed by hexylbenzene when the bioconversion of hexylbenzene, octylbenzene and decylbenzene by *Y. lipolytica* WT were compared. These results suggest that hexylbenzene is the most suitable substrate for use in the model system. It was consequently used in all further study.

4.5 Inducer requirement

The requirement for an inducer was the final parameter investigated for the development of the model system. In the report by Van Rooyen (2005), it was suggested that ethanol or oleic acid was necessary for the induction of the bioconversion of alkylbenzenes because it was thought that alkylbenzenes could not induce the enzymes needed for alkane bioconversion. For that reason, the influence of ethanol and oleic acid, on the bioconversion of hexylbenzene by *Y. lipolytica* TVN348, was assessed. In this study 1% (v/v) ethanol or oleic acid was added at 24h and 1% (v/v) hexylbenzene was added at 48h. These were compared to two control systems where 1) 1% (wt/v) glucose was added at 24h as a carbon substitute for ethanol or oleic acid and

2) no compound was added at 24h. Both control systems included the addition of 1% (v/v) hexylbenzene at 48h. Experiments were conducted in YP_2D_2 media and 50ml non-baffled flasks, using single flasks for each compound (ethanol and oleic acid) and control system.

The PAA produced by *Y. lipolytica* TVN348 (at 120h) was 25.0mM PAA, 27.5mM PAA and 31.5mM PAA in cultures containing (added) glucose, ethanol and oleic acid, respectively (Figure 4.20). Although hexylbenzene was not measured, this would correspond to a potential hexylbenzene conversion of 47.2%, 51.9% and 59.4%. No product was formed in the control where neither ethanol, oleic acid nor glucose was added. These results indicate that the highest PAA was obtained when oleic acid was added to culture. The production of PAA by *Y. lipolytica* TVN348 culture using ethanol (27.5mM) compares with previous TVN348 culture using identical conditions (29.0mM) (Figure 4.8). When using *Y. lipolytica* TVN493, Van Rooyen (2005) reported that (at 120h) approximately 27mM PAA, 33mM PAA and 24mM PAA was produced in cultures containing (added) glucose, ethanol and oleic acid, respectively. The highest PAA obtained in Van Rooyen's work was therefore with ethanol and not oleic acid, which contradicts the finding in this study. The differences in PAA formation between the culture conditions in this section are, however, not considered significant (confidence intervals are not given here because the experiments in this study were conducted in single flasks). What is more important is the finding that PAA was still formed in the control where glucose was added. This suggests that glucose did not repress Cytochrome P450 enzymes. It also indicates that oleic acid and ethanol were not acting as inducers; rather they were supplying the organism with the necessary carbon for cell growth and maintenance. Since glucose is exhausted within 24h of cultivation when 1.6% (wt/v) glucose cultures are used (Figure 4.16), no carbon is left for cell growth and maintenance. It is suggested that the organism was starved in the control where neither ethanol, oleic acid nor glucose was added at 24h and could therefore not convert the hexylbenzene to PAA.
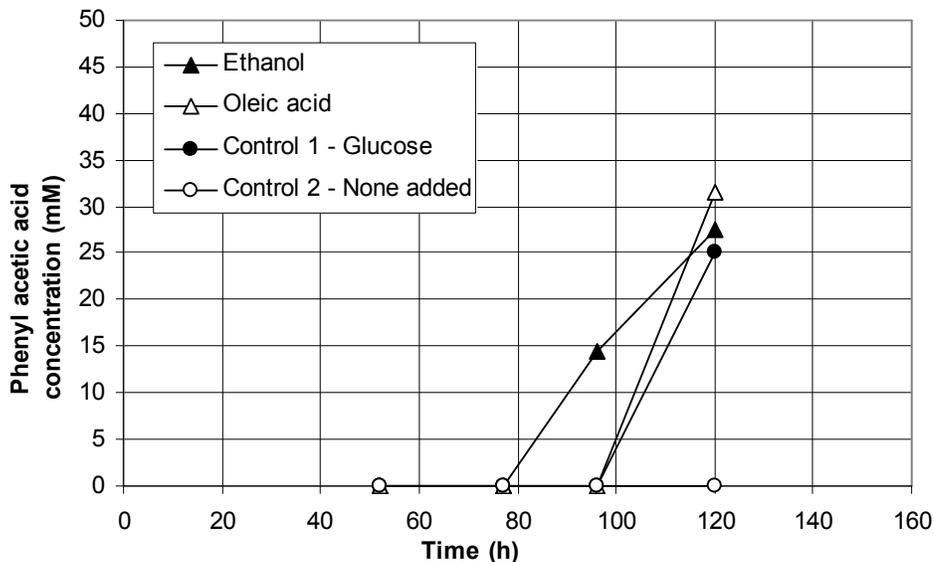
Figure 4.20 Determination of the effect of addition of ethanol or oleic acid on phenyl acetic acid production by *Y. lipolytica* TVN348 grown on 1.6% (wt/v) glucose in 50ml culture volumes. 1% (v/v) Ethanol/oleic acid/glucose added at 24h, 1% (v/v) hexylbenzene added at 48h.

It was proposed that inducers are not necessary in the case where hexylbenzene is used as substrate and that hexylbenzene acts as its own inducer for Cytochrome P450 synthesis. Therefore, in development of the model system, inducer requirements were eradicated and it was decided to supply the organism with adequate carbon in the form of glucose. This decision simplifies further analysis and is convenient because it closely models the conversion of alkanes where no inducer will be necessary.

In conclusion, results from duplicate or triplicate samples in each of the experiments were reproducible as indicated by the minimum and maximum error bars. Furthermore, conclusions were based solely on results which showed 95% confidence intervals. However, reproducibility problems were sometimes experienced with results between different sets of identical experiments. The poor reproducibility between different sets of identical experiments was similar to that experienced by Van Rooyen (2005) and Ramorobi (2008).

4.6 Model system defined

By assessing the influence of various parameters on the cell growth and bioconversion of *Y. lipolytica*, the parameters of the model system could be developed. The findings made in chapter 4, which led to the development of the model, are summarised in Table 4.2.

Table 4.2 Model system defined in shake flasks

Parameter	Effect	Definition in model system
Trace elements, buffering, added nitrogen	Negligible	None added
Oxygen supply	Adequate supply crucial	Maintain adequate oxygen (dissolved oxygen >20%)
Glucose concentration	Not inhibitory at 3.2%	3.2% (i.e. YP_2D_4 medium)
Substrate type	Hexylbenzene (substrate inducer)	1% (v/v) Hexylbenzene
Additional inducer requirements	None needed	None added

5. Results and Discussion II - Use of the model system

The model system was defined in chapter 4 and its properties summarised in Table 4.2. With a defined model system now in place, investigation could be undertaken to identify the most promising strain out of *Y. lipolytica* TVN348, TVN493 and WT in shake flasks for further examination under defined conditions. Once a strain had been selected, bioreactor studies investigated the cultivation of *Y. lipolytica* under controlled conditions. Initial bioreactor studies, however, suggested that the bioconversion did not compare well with shake flask studies.

Sample calculations are detailed in Appendix D and raw experimental data have been tabulated in Appendix E.2.

5.1 Identification of the most suitable strain for cell growth and bioconversion of hexylbenzene

In this study the cell growth and bioconversion of *Y. lipolytica* TVN348, TVN493 and WT were compared in shake flasks using the defined model system. Experiments were carried out in triplicate and since an inducer was not required, it was decided to add 1% (v/v) hexylbenzene sooner at 24h.

5.1.1 Comparison of growth of *Y. lipolytica* TVN348, TVN493 and WT

The cell concentrations obtained at 161h were 4.00×10^8cells/ml, 4.11×10^8cells/ml and 4.31×10^8cells/ml for *Y. lipolytica* TVN348, TVN493 and WT, respectively (Figure 5.1). Similarly, the OD obtained at 161h was 22.33, 23.17 and 25.19 (Figure 5.1). The maximum specific growth rates obtained (over the first 24h) were $0.06h^{-1}$, $0.07h^{-1}$ and $0.06h^{-1}$ (Figure 5.2). The growth of *Y. lipolytica* TVN348 and TVN493 were not significantly different from *Y. lipolytica* WT (cell number: 95% confidence interval $4.31 \times 10^8 \pm 4.8 \times 10^7$ cells/ml; OD: 95% confidence interval 25.19 ± 2.94). Similarly, there was also no significant difference in maximum specific growth rate (0.06 ± 0.02 h^{-1}) between the three strains.

The standard deviation between triplicate samples of the same organism (at 161h) was ≤10% of the average value (3.96×10^7cells/ml, 3.84×10^7cells/ml and 2.45×10^7cells/ml for *Y. lipolytica* TVN348, TVN492 and WT, respectively). This indicates that good reproducibility between cultures of the same set of

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experiments was obtained. That said, if the growth of *Y. lipolytica* WT referred to in Figure 4.6 is compared to *Y. lipolytica* WT in this section, it is found that the maximum cell concentration of 1.54×10^9 cells/ml obtained in Figure 4.6 was over 3.5-fold higher than WT in this study (4.31×10^8 cells/ml). This indicates that in some cases poor reproducibility between sets of experiments was obtained.
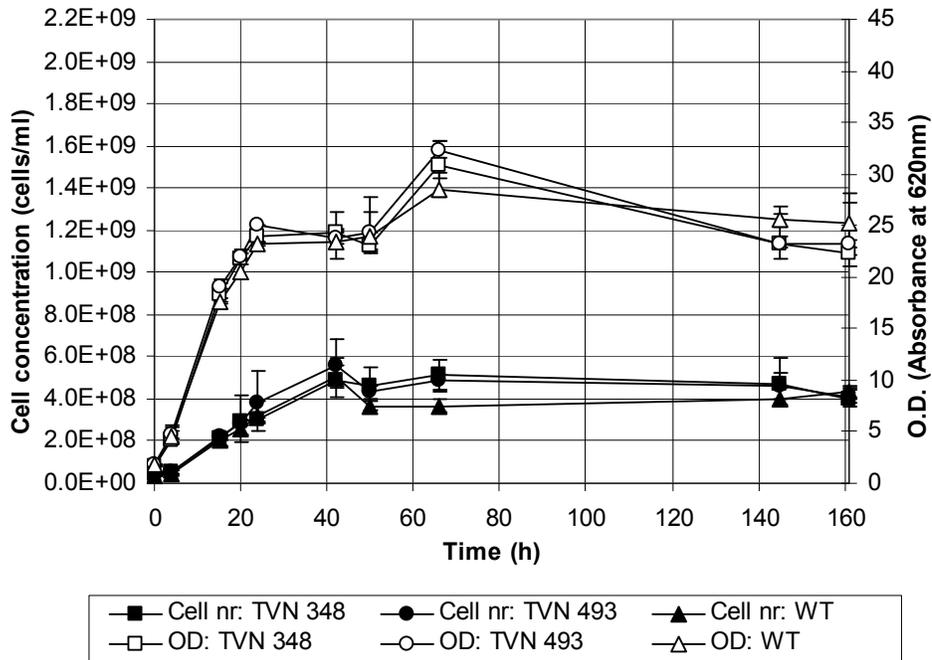
Figure 5.1 Determination of growth of *Y. lipolytica* TVN348, TVN493 and WT grown on 3.2% (wt/v) glucose in 50ml culture volumes. 1% (v/v) Hexylbenzene added at 24h (Abbreviation: OD – optical density).
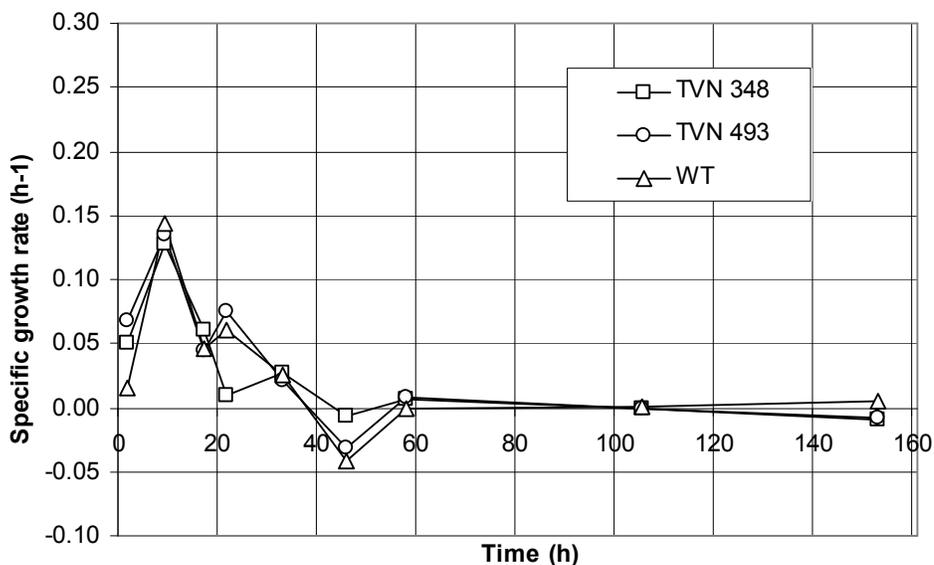
Figure 5.2 Evaluation of specific growth rates of *Y. lipolytica* TVN348, TVN493 and WT grown on 3.2% (wt/v) glucose in 50ml culture volumes. 1% (v/v) Hexylbenzene added at 24h.

5.1.2 Comparison of bioconversion of hexylbenzene by *Y. lipolytica* TVN348, TVN493 and WT

The PAA formation and conversion by *Y. lipolytica* TVN348, TVN493 and WT at 145h and 161h are detailed in Table 5.1. The PAA formed by *Y. lipolytica* TVN348 and TVN493 during cultivation was not significantly different from *Y. lipolytica* WT at either 145h or 161h (95% confidence interval for *Y. lipolytica* WT at 145h: 47.9±34.0 mM PAA; and at 161h: 54.8±31.8 mM PAA).

Table 5.1 PAA formation by *Yarrowia lipolytica* strains using the model system

Y. lipolytica strain	Average PAA formation ± standard deviation (mM)		Conversion ± standard deviation (%)*	
	At 145 h	**At 161 h**	**At 145 h**	**At 161 h**
TVN348	44.6 ± 12.7	34.6 ± 11.0	84.0 ± 23.9	65.1 ± 20.8
TVN493	46.6 ± 20.9	40.2 ± 7.8	87.8 ± 39.4	75.8 ± 14.7
WT	47.9 ± 17.4	54.8 ± 16.2	90.3 ± 32.7	103.3 ± 30.6

*Hexylbenzene was not measured; the conversion is reported as a potential hexylbenzene conversion

With sample standard deviations as large as 45% (TVN493 at 145h), the reproducibility in product formation was poor between samples of the same set of experiments despite the satisfactory reproducibility reported in cell growth (Figure 5.1). If the PAA formation by *Y. lipolytica* WT referred to in Figure 4.12 is compared to *Y. lipolytica* WT in this section, it is found that the maximum PAA of 10.5mM obtained (at 144h) was over 4.5-fold *lower* than WT in this study (47.9mM at 145h). This indicates that reproducibility in PAA production between sets of cultivations was also poor, correlating with previous studies by Van Rooyen (2005) and Ramorobi (2008).

There is a clear error in the 103.3% conversion reported for *Y. lipolytica* WT at 161h (Table 5.1). This could be due to an error in analysis. Furthermore, a decrease in accumulated PAA from 145h to 161h was observed for *Y. lipolytica* TNV348 and TVN493. Due to the standard deviation between samples, the decrease in PAA is not significant (95% confidence interval for TVN348: 44.6±24.9 mM PAA; 95% confidence interval for TVN493: 46.6±41.0 mM PAA).

Under the conditions of the experiment, no significant difference in cell growth or bioconversion could therefore be found between *Y. lipolytica* TVN348, TVN493 and WT. From the data it would appear that the over-expression of cytochrome P450 enzymes has not been successful in enhancing the bioconversion of hydrocarbons by *Y. lipolytica* TVN348 and TVN493. Subsequently it was found that although the strains contained the inserted genes, they were not able to show any enhanced activity (Smit, personal communication). For that reason, *Y. lipolytica* WT was selected for use in further study until appropriate genetically modified strains become available.

5.2 Bioreactor studies

With the developed model system and the selected strain *Y. lipolytica* WT, further investigation could be continued using the model system and organism under controlled conditions. Two parameters were assessed for their influence on cell growth and bioconversion, namely the time of substrate addition and glucose concentration. The influence of time of substrate addition was evaluated at 11h, 24h and 48h to determine whether either cell growth or bioconversion could be improved. The influence of glucose concentration was evaluated at 3.2% (wt/v) glucose and doubled to 6.4% (wt/v) to determine the effect on cell growth and bioconversion.

Single cultivations were carried out for the assessment of each parameter, except when hexylbenzene was added at 24h to 3.2% (wt/v) glucose cultures, in which case triplicate batches were carried out. Where indicated in the text, "24h (3x)" refers to the triplicate cultivations with hexylbenzene addition at 24h. Where indicated in the text and figures "24h (1)", "24h (2)" and "24h (3)" refer to the first, second and third cultivation with hexylbenzene addition at 24h.

The three cultivations differed only in the method of dissolved oxygen control:

a) In the 1st batch cultivation dissolved oxygen (DO) was automatically controlled to 22.5% by varying the agitation rate between the limits of 200rpm to 750rpm.

b) In the 2nd batch cultivation, dissolved oxygen was manually controlled above 20% by maintaining the agitation rate at 400rpm. In this cultivation it was not necessary to increase the agitation rate above 400rpm because DO was always above 20%.

c) In the 3rd batch cultivation, dissolved oxygen was manually controlled above 20% within the first 24h by adjusting the agitation rate. Thereafter it was automatically controlled to 20% by varying the agitation rate between the limits of 200rpm to 600rpm.

Unless otherwise stated, bioreactor methodology described in section 3.3.2 was used.

5.2.1 Influence of parameters on cell growth

The parameters assessed for the influence on cell growth were time of hexylbenzene addition and glucose concentration.

5.2.1.1 Influence of time of substrate addition on cell growth

The influence of the addition of 1% (v/v) hexylbenzene at 11h, 24h and 48h, on the growth of *Y. lipolytica* WT was assessed. These cultures (grown on 3.2% (wt/v) glucose) were compared to *Y. lipolytica* WT cultivated under the same conditions with no addition of hexylbenzene.

The cell number concentration obtained in *Y. lipolytica* WT cultures (at approximately 120h) was 2.82×10^8cells/ml, 2.23×10^9cells/ml, 4.74×10^8cells/ml, 3.92×10^8cells/ml and 8.90×10^8cells/ml for the addition of hexylbenzene at 11h, 24h (3x) and 48h, respectively (Figure 5.4). The final cell number concentration obtained in the *Y. lipolytica* WT culture, with no hexylbenzene addition, was 1.01×10^9cells/ml.
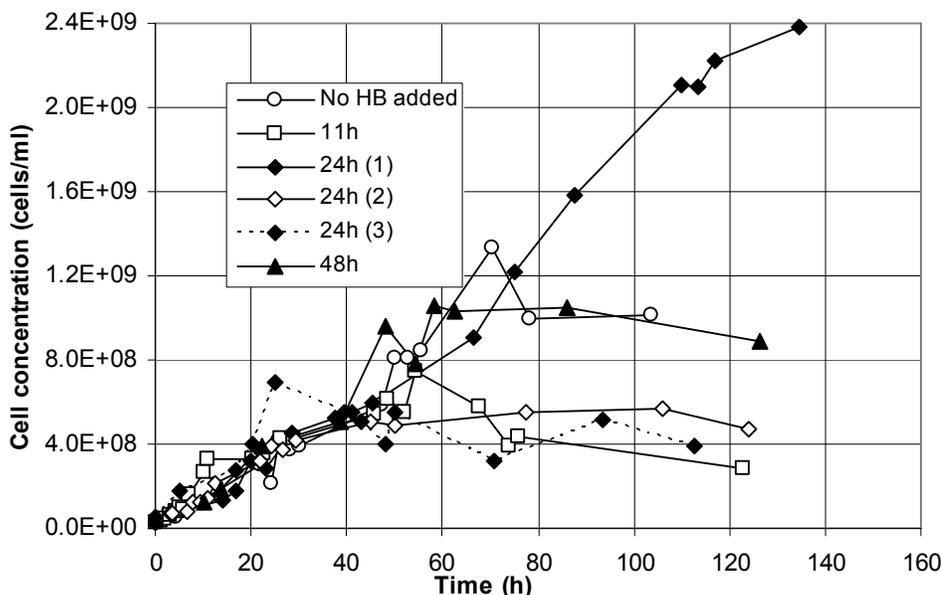
Figure 5.3 Determination of the effect of time of hexylbenzene addition on growth (cell number) of *Y. lipolytica* WT grown on 3.2% (wt/v) glucose in bioreactor culture. 1% (v/v) Hexylbenzene either not added, or added at 11h, 24h or 48h (Abbreviation: HB – hexylbenzene).

The cell number of *Y. lipolytica* WT culture was increased with hexylbenzene addition at 48h when compared to 1) hexylbenzene addition at 11h and 2) hexylbenzene addition at 24h in two of the triplicate cultivations. The marginal difference in maximum cell number concentration between the culture with hexylbenzene addition at 48h (8.90×10^8 cells/ml) and the culture with no hexylbenzene addition (1.01×10^9 cells/ml) indicates that *Y. lipolytica* WT was unaffected by the presence of hexylbenzene when it was added at 48h. Furthermore, the cell number concentration in the culture with hexylbenzene addition at 11h was not significantly different from two of the triplicate cultures with hexylbenzene addition at 24h. The highest cell number concentration obtained, however, was in one of the triplicate cultures with hexylbenzene addition at 24h (2.23×10^9 cells/ml). This was at most 5.7-fold higher than other cultures with hexylbenzene addition at 24h, and 7.9-fold higher than the remaining cultures.

Figure 5.3 suggests that the growth of all cultures over the first 45h is similar and is confirmed by the logarithmic cell concentration of *Y. lipolytica* WT, which indicates that the specific growth rate is also similar (Figure 5.4). Monitored process conditions indicate that the oxygen demand for the best performing 24h addition cultivation was higher than the other 24h addition cultivations such that the agitation was increased up to 750 rpm. The final pH

in the best performing 24h culture was also higher (8.8) than other cultures (8.0 and lower). The poor reproducibility in cell concentration between cultures with hexylbenzene addition as 24h could not be explained. The possibility of a negative effect of agitation was eliminated given that the agitation was highest in batch culture with the highest cell concentration. Nevertheless, a comparison of growth of *Y. lipolytica* WT cultures in shake flasks (Figure 5.1) (4.00×10^8 cells/ml at approximately 130h) indicated that cell growth was improved in the bioreactor.
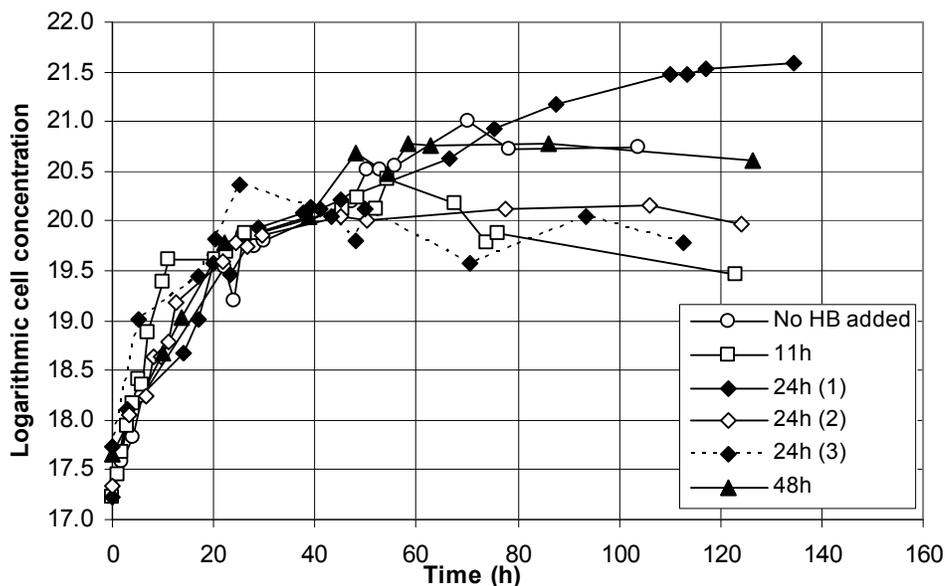
Figure 5.4 Determination of the effect of time of hexylbenzene addition on logarithmic growth (cell number) of *Y. lipolytica* WT grown on 3.2% (wt/v) glucose in bioreactor culture. 1% (v/v) Hexylbenzene either not added, or added at 11h, 24h or 48h (Abbreviation: HB – hexylbenzene).

The optical density observed in *Y. lipolytica* WT cultures (at approximately 120h) was 29.22, 42.36, 14.70, 29.10 and 45.30 for cultures for the addition of hexylbenzene at 11h, 24h (3x) and 48h, respectively (Figure 5.5). The final optical density observed in the *Y. lipolytica* WT culture, with no hexylbenzene addition, was 48.69.
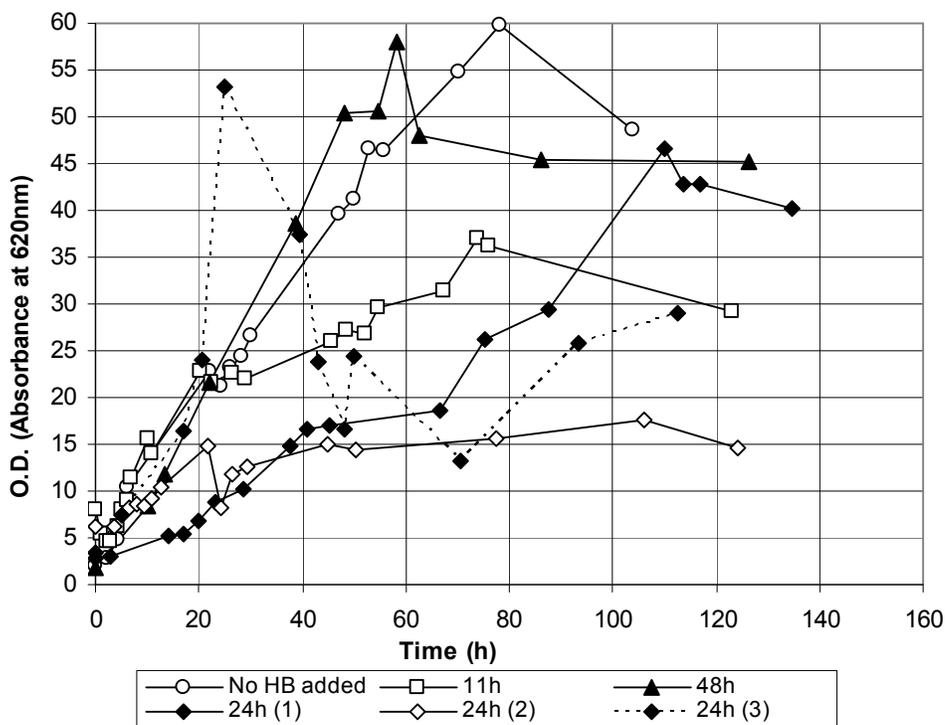
Figure 5.5 Determination of the effect of time of hexylbenzene addition on growth (OD) of *Y. lipolytica* WT grown on 3.2% (wt/v) glucose in bioreactor culture. 1% (v/v) Hexylbenzene either not added, or added at 11h, 24h or 48h (Abbreviation: HB – hexylbenzene).

The optical density of *Y. lipolytica* WT culture was increased with hexylbenzene addition at 48h when compared to 1) hexylbenzene addition at 11h and 2) hexylbenzene addition at 24h in all three triplicate cultivations (although only marginally when compared to 24h (1)). The marginal difference in optical density between the culture with hexylbenzene addition at 48h (45.30) and the culture with no hexylbenzene addition (48.69) correlates with the observed cell concentrations, and confirms that *Y. lipolytica* WT was unaffected by the presence of hexylbenzene when it was added at 48h. The optical density of the triplicate cultures, with hexylbenzene addition at 24h, appear closer in relation to one another because the OD of the culture containing the highest cell concentration was at most 2.9-fold higher than the other 24h cultures. This does not correlate with the observed cell number concentrations and could be because of morphological change between the triplicate 24h addition cultivations.

Glucose was exhausted within 70h of cultivation in all the *Y. lipolytica* WT bioreactor cultures grown on 3.2% (wt/v) glucose (although glucose not measured for 24h (2)), with the exception of two of the triplicate cultures with hexylbenzene addition at 24h (Figure 5.6). In the first case (24h (3)), 1%

(wt/v) glucose still remained in the culture at the end of cultivation (115h). The yield on glucose, however, for this cultivation was equivalent to the other culture where glucose was exhausted within 70h (Figure 5.7). The lower cell concentration in this cultivation therefore accounts for the glucose not being consumed. In the second case (24h (1)), glucose was only exhausted after 110h due to a 40h lag in glucose utilisation. The lag could not be explained, especially since the glucose curve in mention corresponded to the culture where the highest cell concentration was observed (2.23×10^9 cells/ml). As a result, the observed maximum yield on glucose was over 10-fold higher than the rest of the cultivations described in this section (Figure 5.7).

Caution should be exercised when interpreting the results of the yield data because this has been based on glucose as the sole carbon source. However, for the first 40h, glucose utilisation was negligible, suggesting utilisation of another carbon source, possibly the alkyl chain on hexylbenzene or carbon present in the yeast extract in the media.
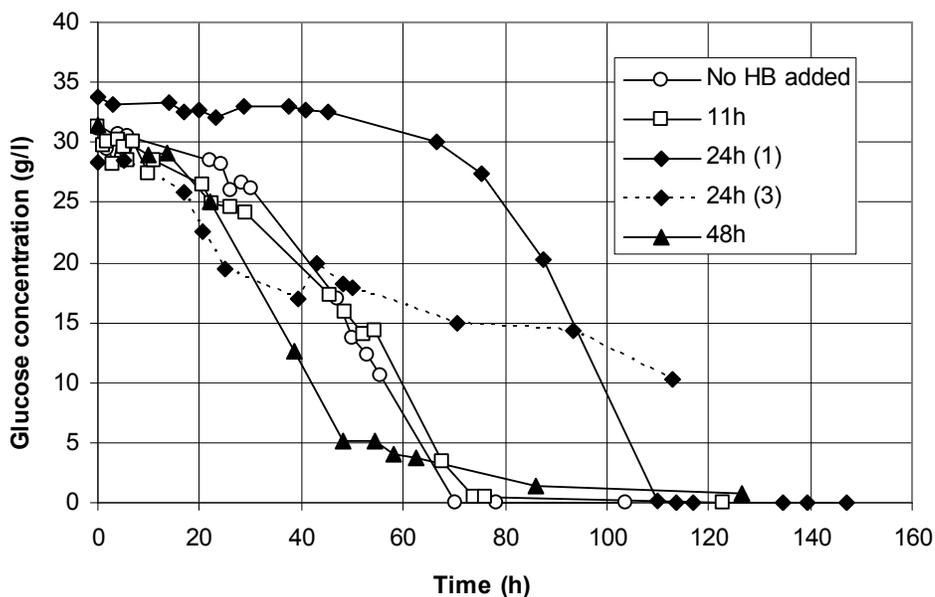
Figure 5.6 Determination of the effect of time of hexylbenzene addition on glucose utilisation by *Y. lipolytica* WT grown on 3.2% (wt/v) glucose in bioreactor culture. 1% (v/v) Hexylbenzene either not added, or added at 11h, 24h or 48h (Abbreviation: HB - hexylbenzene).
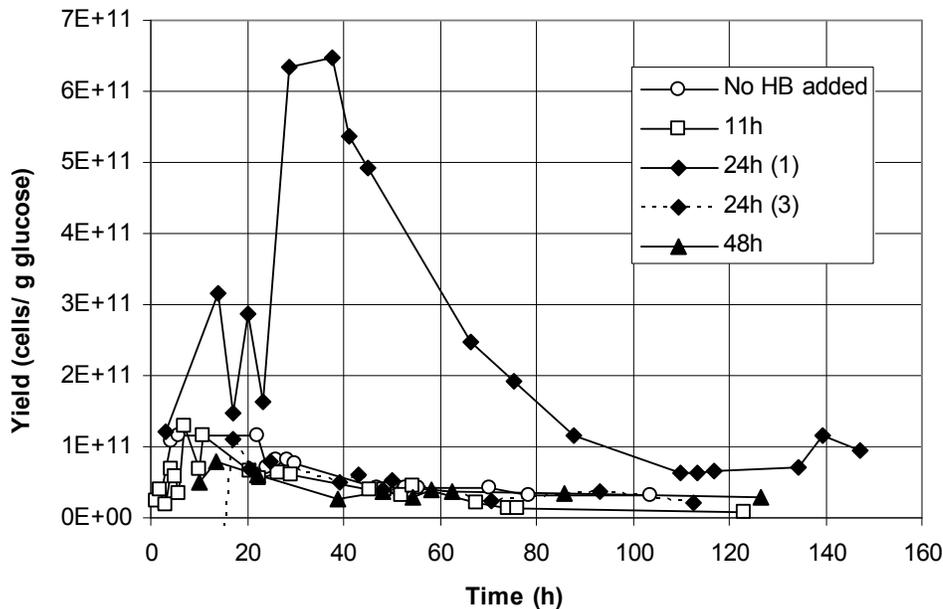
Figure 5.7 Determination of the effect of time of hexylbenzene addition on yield of *Y. lipolytica* WT grown on 3.2% (wt/v) glucose in bioreactor culture. 1% (v/v) Hexylbenzene either not added, or added at 11h, 24h or 48h (Abbreviation: HB - hexylbenzene).

The cell growth of *Y. lipolytica* WT with hexylbenzene addition at 48h was improved when compared to cultures with hexylbenzene addition at 11h and 24h save one of the triplicate cultures with hexylbenzene addition at 24h. However, since the highest cell growth was observed in one of the triplicate cultures (with hexylbenzene addition at 24h), it was decided that for further investigation hexylbenzene would be added at 24h.

5.2.1.2 <u>Influence of glucose concentration on cell growth</u>

The influence of 3.2% (wt/v) and 6.4% (wt/v) glucose on the growth of *Y. lipolytica* WT was assessed. In these cultures 1% (v/v) hexylbenzene was added at 24h. (Note that the 3.2% (wt/v) glucose cultures referred to here are the same triplicate cultivations described in the foregoing section.)

The cell number in *Y. lipolytica* WT cultures (at approximately 120h) was 2.23×10^9 cells/ml, 4.74×10^8 cells/ml, 3.92×10^8 cells/ml and 7.47×10^8 cells/ml for the triplicate 3.2% (wt/v) glucose cultures and 6.4% (wt/v) glucose culture, respectively (Figure 5.8). The cell number of *Y. lipolytica* WT was increased up to 1.9-fold higher in the 6.4% (wt/v) glucose when compared to two of the triplicate 3.2% (wt/v) glucose cultures. However, over the course of the first 100h of cultivation there was no significant difference in growth between the 6.4% (wt/v) and two of the 3.2% (wt/v) glucose cultures.
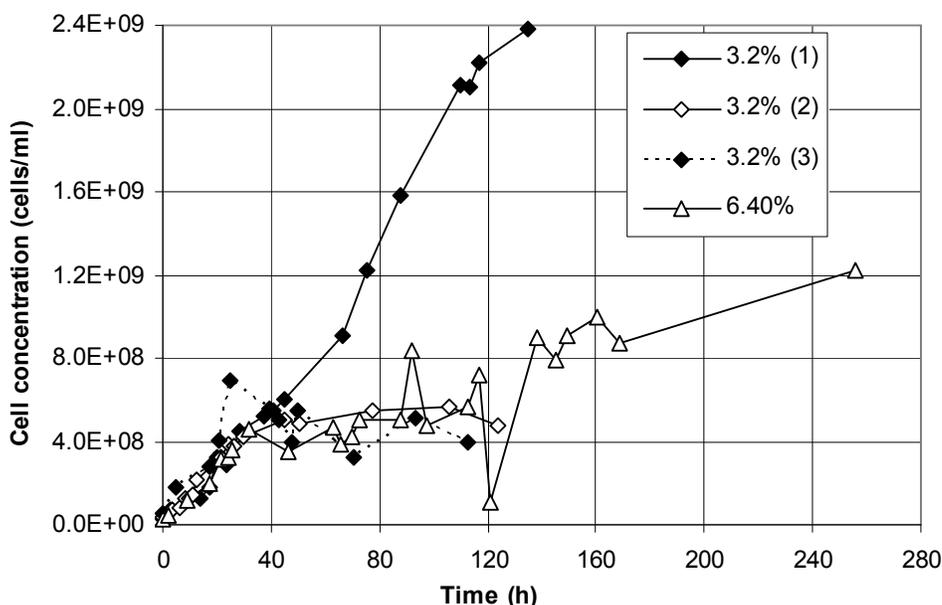
Figure 5.8 Determination of the effect of glucose concentration on growth (cell number) of *Y. lipolytica* WT grown on 3.2% or 6.4% (wt/v) glucose in bioreactor culture. 1% (v/v) Hexylbenzene added at 24h.

The optical density observed in *Y. lipolytica* WT cultures (at approximately 120h) was 42.36, 14.70, 29.10 and 30.57 for the triplicate 3.2% (wt/v) glucose cultures and 6.4% (wt/v) glucose culture, respectively (Figure 5.9). The optical density of *Y. lipolytica* WT in the 6.4% (wt/v) glucose culture was only increased when compared to one of the triplicate 3.2% (wt/v) glucose cultures. In this study, large mycelial cells were not observed. This suggests that cells were not placed under stress in the 6.4% (wt/v) glucose culture. In section 4.3 where the influence of 1.6% (wt/v) and 3.2% (wt/v) glucose concentration on cell growth was assessed in shake flasks, mycelial shaped cells were observed in the higher glucose cultures. It was suggested that either nitrogen or an oxygen limitation caused dimorphism to occur. Since no extra nitrogen was added to the bioreactor culture medium, it could be suggested that the adequate oxygen supply prevented the formation of mycelial cells. Consequently, this suggests that dimorphism occurred in shake flask cultures containing higher glucose concentration (i.e. 3.2%) because of a lack of oxygen supply, and not nitrogen concentration.
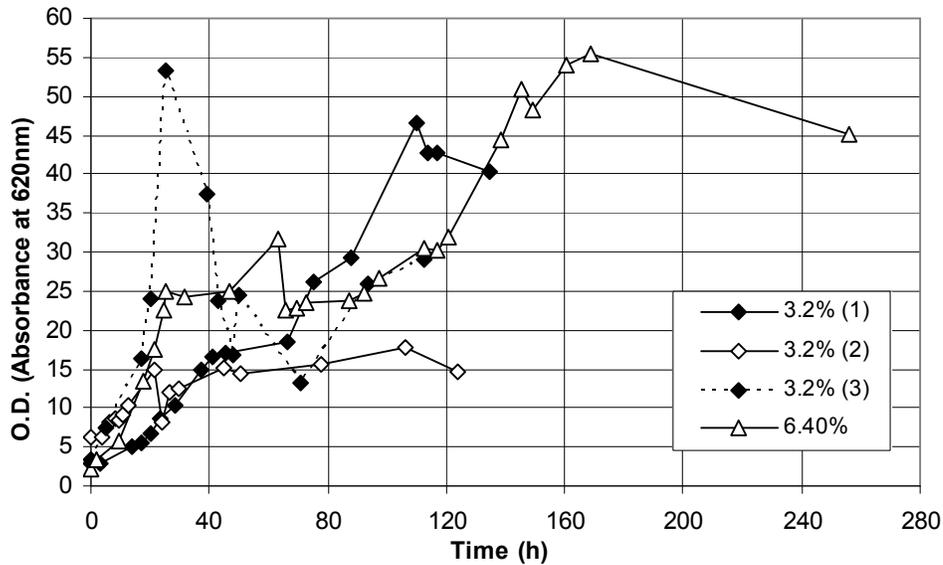
Figure 5.9 Determination of the effect of time of glucose concentration on growth (O.D.) of *Y. lipolytica* WT grown on 3.2% or 6.4% (wt/v) glucose in bioreactor culture. 1% (v/v) Hexylbenzene added at 24h.

Glucose was exhausted within 160h of cultivation in the *Y. lipolytica* WT bioreactor culture containing 6.4% (wt/v) glucose (Figure 5.10). (Note that glucose was not measured for 3.2% (2).) A lag in glucose utilisation is not observed and so the maximum yield on glucose is similar to other 3.2% (wt/v) glucose cultures, with the exception of one the triplicate 3.2% (wt/v) glucose cultures (Figure 5.11).
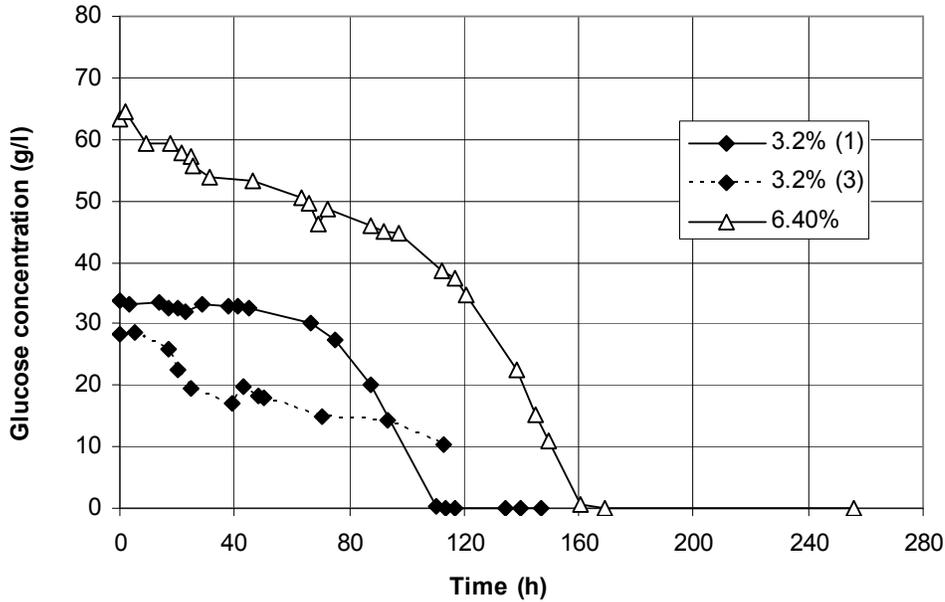
Figure 5.10 Determination of the effect of glucose concentration on glucose utilisation by *Y. lipolytica* WT grown on 3.2% and 6.4% (wt/v) glucose in bioreactor culture. 1% (v/v) Hexylbenzene added at 24h.
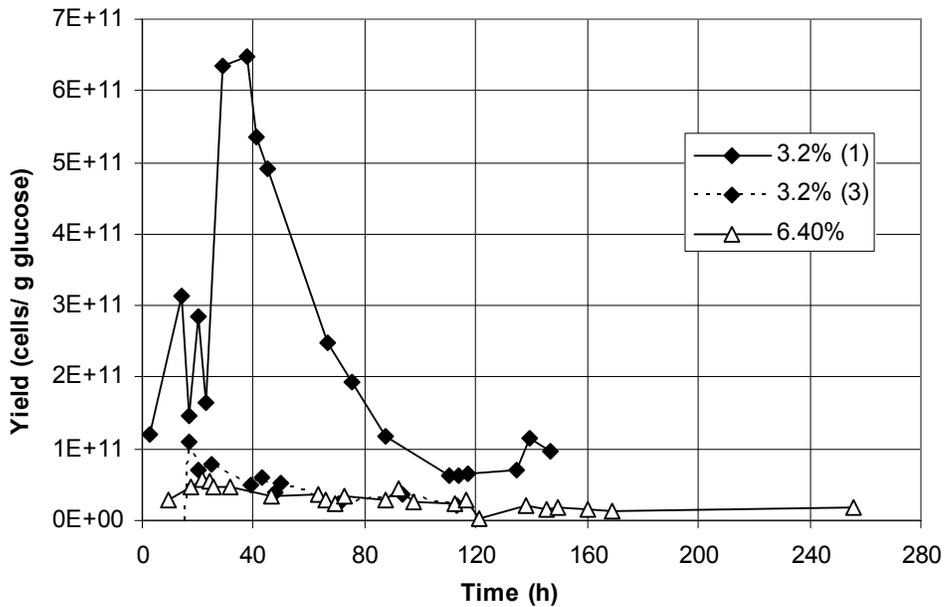
Figure 5.11 Determination of the effect of glucose concentration on yield of *Y. lipolytica* WT grown on 3.2% (wt/v) glucose in bioreactor culture. 1% (v/v) Hexylbenzene added at 24h.

The cell growth of *Y. lipolytica* WT was not inhibited in 6.4% (wt/v) glucose cultures and dimorphism did not occur. Cultivation in 3.2% (wt/v) and 6.4%

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(wt/v) glucose are therefore both considered feasible options for maximising cell growth.

From Figures 5.3, 5.5, 5.8 and 5.9 it was noted that the O.D. and the cell number did not appear to correlate well with one another. In the best performing 24h hexylbenzene addition culture, O.D. and cell count correlated with a coefficient of determination of 0.95 (Figure 5.12). This was noticeably different from all other bioreactor cultures which together correlated with a coefficient of determination of 0.79. At no stage was any morphology other than yeast-like cells noted and the analyses were carried out in an identical fashion. Further investigation into the analytical technique for the absorbance readings should be considered, e.g. by determining whether the absorbance reading of the supernatant changes.
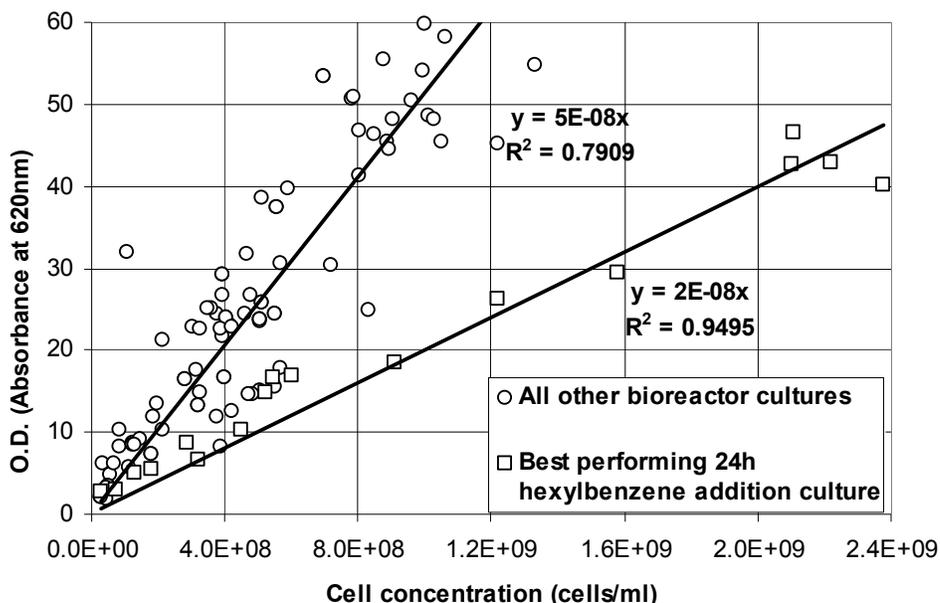
Figure 5.12 Evaluation of the relationship of O.D. versus cell counts for all bioreactor cultures in this study, i.e. 3.2% glucose with no hexylbenzene addition, 3.2% glucose with hexylbenzene at 11h, 3.2% glucose with hexylbenzene addition at 24h, 3.2% glucose with hexylbenzene addition at 48h, 6.4% glucose with hexylbenzene addition at 24h.

5.2.2 Influence of parameters on bioconversion

The parameters assessed for the influence on bioconversion were time of hexylbenzene addition and glucose concentration.

5.2.2.1 <u>Influence of time of substrate addition on bioconversion</u>

The influence of the addition of 1% (v/v) hexylbenzene at 11h, 24h and 48h on the production of PAA by *Y. lipolytica* WT was assessed. The growth curves described in subsection 5.2.1.1 relate to the same cultivations described in this section.

The PAA formed by *Y. lipolytica* WT cultures (at approximately 120h) was 20.8mM PAA, 41.4mM PAA, 5.4mM PAA, 4.3mM PAA and 2.4mM PAA for the addition of hexylbenzene at 11h, 24h (3x) and 48h, respectively (Figure 5.13). Similarly, the specific PAA formations were 7.4×10^{-11}mmol PAA/cell, 1.9×10^{-11}mmol PAA/cell, 1.1×10^{-11}mmol PAA/cell, 1.1×10^{-11}mmol PAA/cell and 2.7×10^{-12}mmol PAA/cell (Figure 5.14).
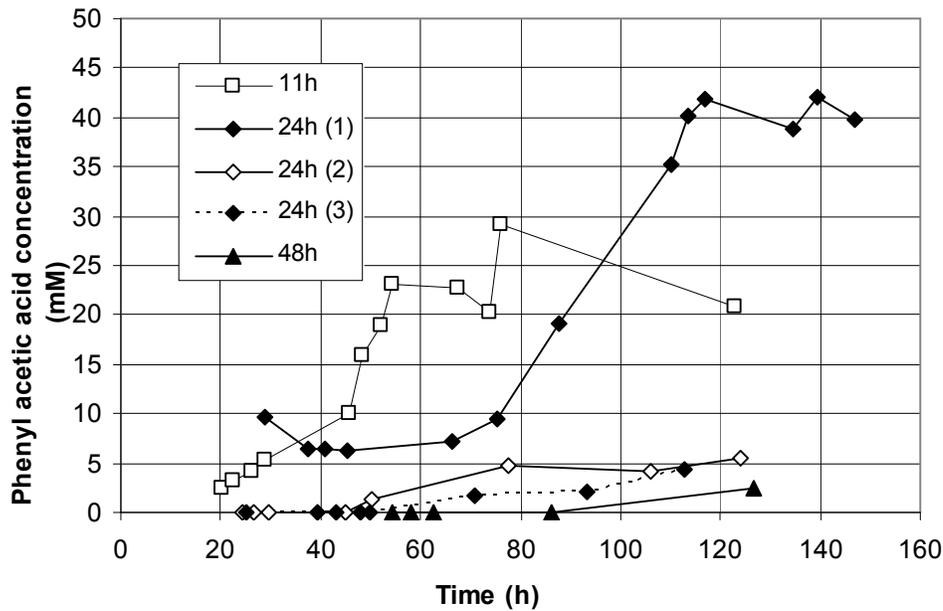
Figure 5.13 Determination of the effect of time of hexylbenzene addition on PAA formation by *Y. lipolytica* WT grown on 3.2% (wt/v) glucose in bioreactor culture. 1% (v/v) Hexylbenzene added at 11h, 24h or 48h.
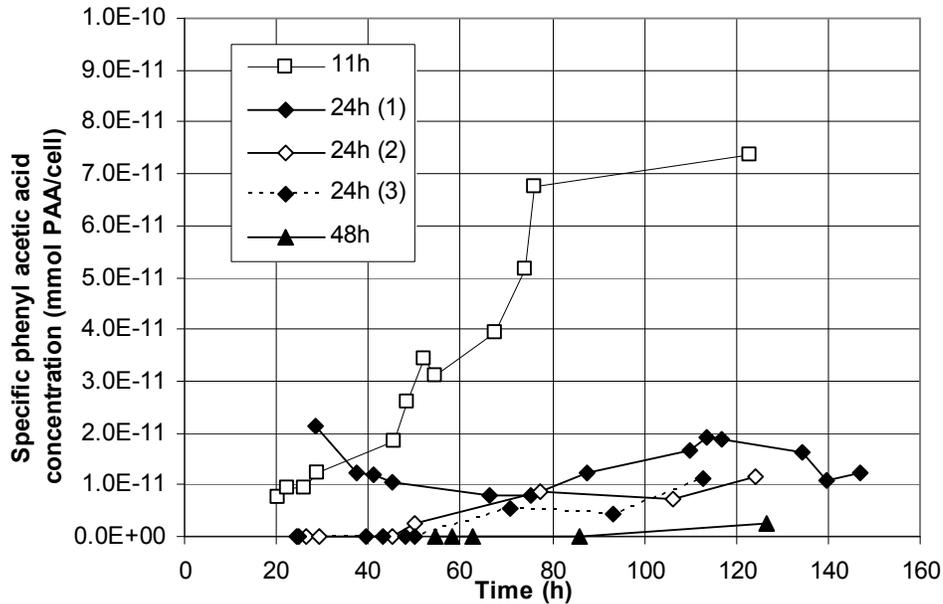
Figure 5.14 Determination of the effect of time of hexylbenzene addition on specific PAA formation by *Y. lipolytica* WT grown on 3.2% (wt/v) glucose in bioreactor culture. 1% (v/v) Hexylbenzene added at 11h, 24h or 48h.

The PAA accumulated by *Y. lipolytica* WT culture was increased with hexylbenzene addition at 11h when compared to 1) hexylbenzene addition at 24h in two of the triplicate cultivations and 2) hexylbenzene addition at 48h. Furthermore, even though the highest amount of accumulated PAA was observed in one of the triplicate cultures with hexylbenzene addition at 24h (41.4mM), the specific PAA observed in the culture with hexylbenzene addition at 11h, was 3.9-fold higher than the best 24h addition culture (1.9×10^{-11}mmol PAA/cell$\rightarrow 7.4 \times 10^{-11}$mmol PAA/cell). Since *Y. lipolytica* cells were growing actively at 11h, these results suggest that substrate bioconversion is maximised when the substrate is added during the active growth phase. It may further be suggested to conduct fed-batch experiments to maintain the active growth phase.

The reproducibility between identical cultivations remains problematic since the standard deviation between cultures, with hexylbenzene addition at 24h, was 21.4mM PAA (accounting for approximately 40% conversion). Analysis of the monitored pH values (Figure 5.15) shows a steady rise in pH from 80h onwards for the best performing 24h addition culture (up to 8.8) (Note that the pH for the culture with hexylbenzene addition at 11h was not monitored). This corroborates with the rise in PAA formation at 80h (Figure 5.13). It may be suggested that the higher pH improved the solubility of the PAA (Hill *et al.*

1986) and that future cultures should be maintained above a pH that is higher than 7.6. That said, the high pH in the best performing culture was obtained freely while other cultures had to be maintained with the addition of NaOH, which cannot be explained.
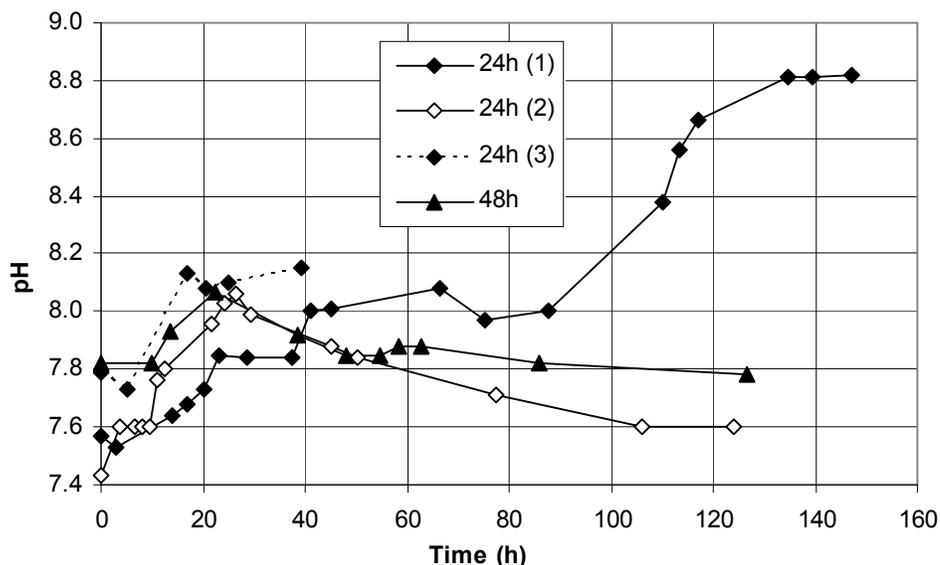
Figure 5.15 Monitored pH during growth of *Y. lipolytica* WT grown on 3.2% (wt/v) glucose in bioreactor culture. 1% (v/v) Hexylbenzene added at 24h or 48h.

The PAA formation by *Y. lipolytica* WT culture with hexylbenzene addition at 11h and the best performing culture, of one the triplicate cultures with hexylbenzene addition at 24h, are compared further. The PAA formation started to increase substantially at approximately 45h and 75h for cultures with hexylbenzene addition at 11h and 24h, respectively (Figure 5.13). This corresponded to glucose concentrations of approximately 1.8% (wt/v) and 2.8% (wt/v) (Figure 5.6). These results prove that bioconversion was not inhibited at glucose concentrations of up to 2.8% (wt/v). Furthermore, at the time of glucose exhaustion (70h and 115h for cultures with hexylbenzene addition at 11h and 24h, respectively) the increase in PAA ceased, even if 100% conversion of hexylbenzene was not achieved. This verifies the importance of the presence of an adequate amount of carbon for cell growth and/or maintenance.

The hexylbenzene concentration was measured in *Y. lipolytica* WT bioreactor culture. It was observed that hexylbenzene was exhausted in the cultures with hexylbenzene addition at 11h and the best performing 24h (from the triplicate

cultivations) (Figure 5.16). This did not correspond to PAA formation of either culture because a 100% conversion of PAA was not measured. It was suggested that the disappearance of hexylbenzene or PAA product could be due to 1) PAA utilisation or 2) the formation of metabolic intermediates, which were not measured with GC analysis.
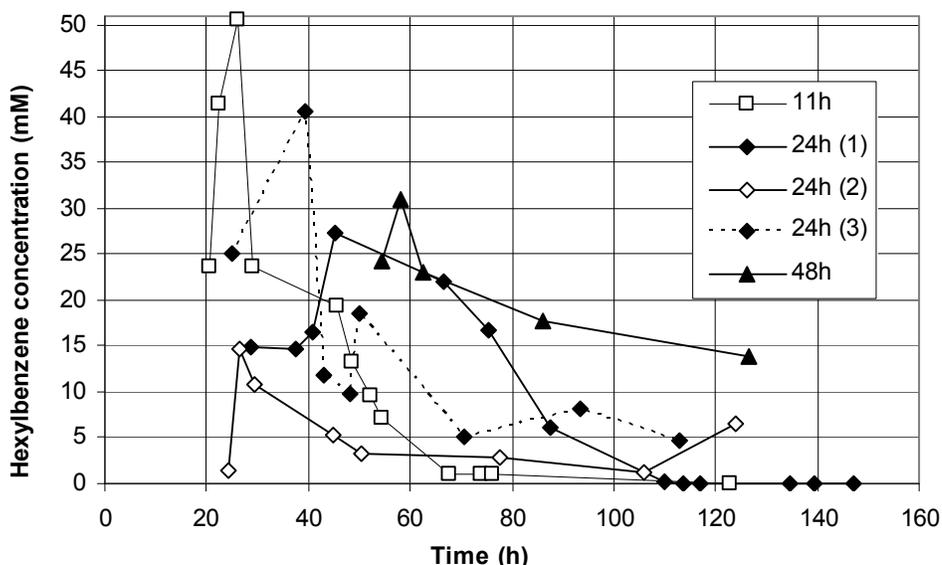
Figure 5.16 Determination of the effect of time of hexylbenzene addition on hexylbenzene utilisation by *Y. lipolytica* WT grown on 3.2% (wt/v) glucose in bioreactor culture. 1% (v/v) Hexylbenzene added at 11h, 24h or 48h.

To determine whether PAA was utilisable, an investigation was carried out where PAA was fed to *Y. lipolytica* WT culture at 24h and 48h in YP_2D_2 medium (data not shown). 0.38% (v/v) and 0.36% (v/v) PAA were added at 24h and 48h to reduce possible toxicity. Prior to addition PAA was dissolved in a NaOH solution to neutralise the pH. The final pH in the culture was above 8.3. Analysis of the residual PAA in culture confirmed that all PAA was accounted for and that PAA was not metabolised by *Y. lipolytica* WT.

To determine whether metabolic intermediates were formed, NMR analyses were carried out on both the aqueous and hydrophobic extract. This was done at the end of cultivation on the best performing culture with hexylbenzene addition at 24h. It was found that no other metabolic intermediate had been formed in either extract.

These results suggest that the apparent absence of PAA in some of the batches could have been caused by an inaccuracy in the analytical procedure. One possibility is an incomplete extraction of the PAA. Modification of the method to increase the amount of 1M hydrochloric acid from 50µl to 500µl showed some improvement (Smit, personal communication). This modified extraction procedure was carried out on the best performing culture with hexylbenzene addition at 24h. Nevertheless, using this modified method still only 80% conversion was obtained after depletion of hexylbenzene.

Although the highest amount of accumulated PAA was obtained in one of the triplicate cultures with hexylbenzene addition at 24h, the highest specific PAA formation was obtained in the culture with hexylbenzene added at 11h. It would seem that the potential for bioconversion was maximised when hexylbenzene was added during active growth, however this had a negative effect on cell growth. However, the poor reproducibility between 24h cultures makes it difficult to draw any solid conclusion. These results suggest that the time of hexylbenzene addition, which will maximise bioconversion, may lie somewhere between 11h and 24h.

5.2.2.2 Influence of glucose concentration on bioconversion

The influence of 3.2% (wt/v) and 6.4% (wt/v) glucose on the production of PAA by *Y. lipolytica* WT was assessed. In these cultures 1% (v/v) hexylbenzene was added at 24h. The growth curves on 3.2% (wt/v) glucose described in subsection 5.2.1.2 relate to the same cultivations described in this section.

The PAA formed by *Y. lipolytica* WT cultures (at approximately 120h) was 41.4mM PAA, 5.4mM PAA, 4.3mM PAA and 15.2mM PAA for the triplicate 3.2% (wt/v) glucose cultures and 6.4% (wt/v) glucose culture, respectively (Figure 5.17). Similarly, the specific PAA formations were 1.9×10^{-11}mmol PAA/cell, 1.1×10^{-11}mmol PAA/cell, 1.1×10^{-11}mmol PAA/cell and 2.0×10^{-11}mmol PAA/cell (Figure 5.18).
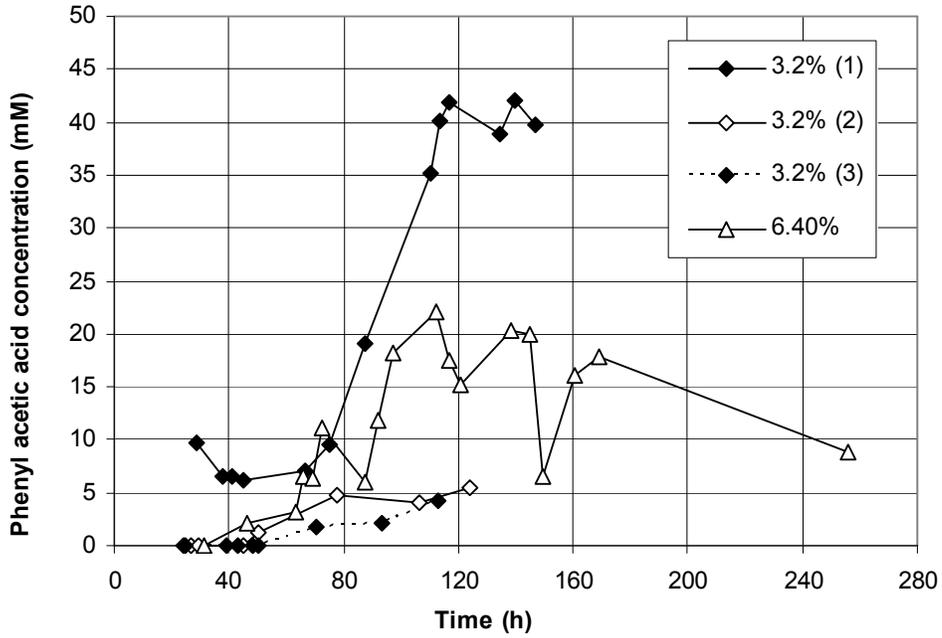
Figure 5.17 Determination of the effect of glucose concentration on PAA formation by *Y. lipolytica* WT grown on 3.2% or 6.4% (wt/v) glucose in bioreactor culture. 1% (v/v) Hexylbenzene added at 24h.
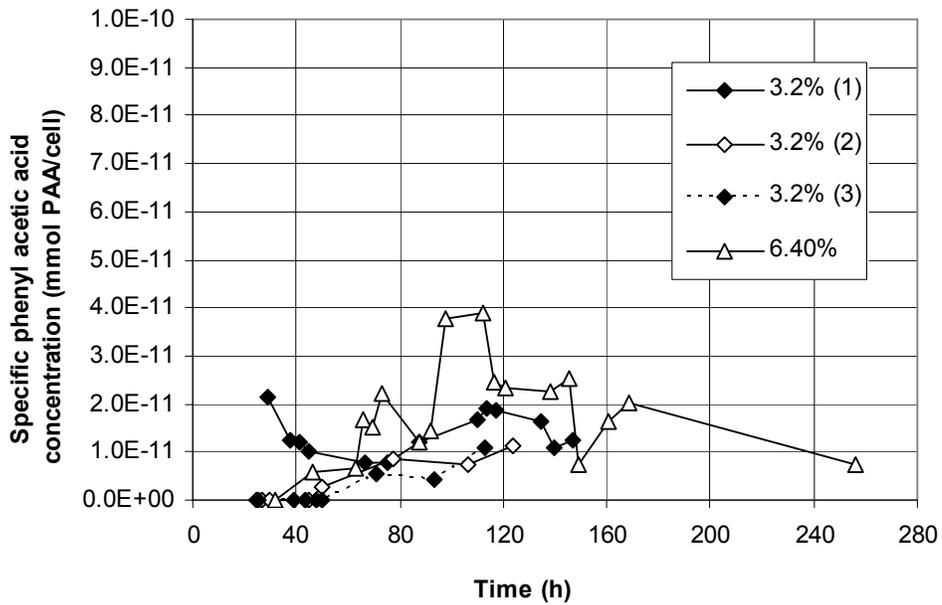
Figure 5.18 Determination of the effect of glucose concentration on specific PAA formation by *Y. lipolytica* WT grown on 3.2% or 6.4% (wt/v) glucose in bioreactor culture. 1% (v/v) Hexylbenzene added at 24h.

The PAA formation in the 6.4% (wt/v) glucose culture started to increase substantially at approximately 60h (Figure 5.17). This corresponds to a glucose concentration of approximately 5.0% (wt/v) (Figure 5.10). These results confirm that the bioconversion of hexylbenzene is not inhibited at glucose concentrations as high as 5.0% (wt/v).

The PAA accumulated by *Y. lipolytica* WT culture was increased in the 6.4% (wt/v) glucose culture when compared to two of the triplicate 3.2% (wt/v) glucose cultures. That said, the specific product formations in 3.2% (wt/v) and 6.4% (wt/v) glucose cultures were in the same order of magnitude when hexylbenzene was added at 24h (Figure 5.18).

Analysis of the residual hexylbenzene concentrations reveal that hexylbenzene was exhausted after 140h in the 6.4% (wt/v) glucose culture (Figure 5.19). This did also not relate to the corresponding PAA formation because a 100% conversion of PAA was not measured. Further steps for solving this problem have already been described in subsection 5.2.2.1.
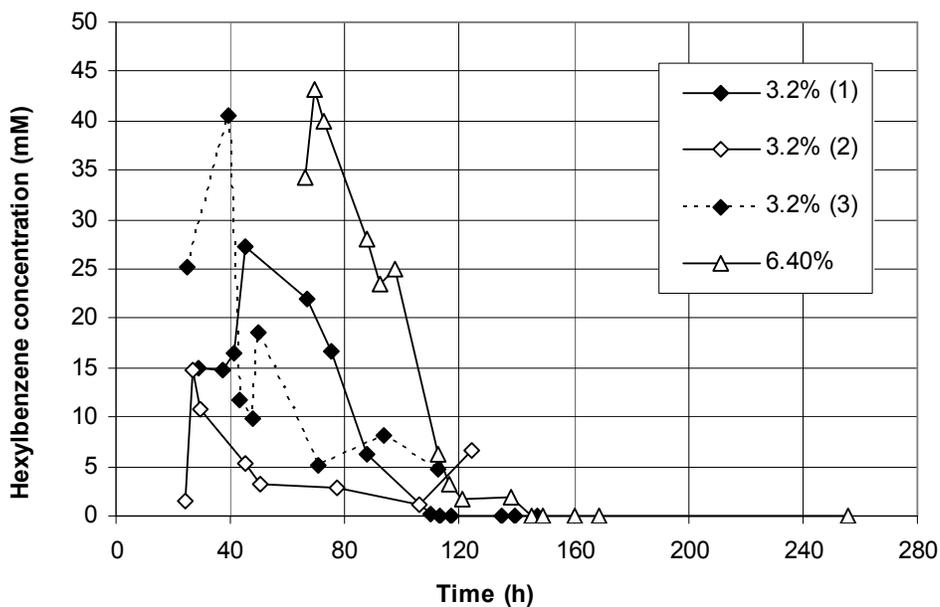
Figure 5.19 Determination of the effect of glucose concentration on hexylbenzene utilisation by *Y. lipolytica* WT grown on 3.2% or 6.4% (wt/v) glucose in bioreactor culture. 1% (v/v) Hexylbenzene added at 24h.

In summary, the specific product formation of PAA by *Y. lipolytica* WT was at its maximum (7.4×10^{-11} mmol PAA/cell) when hexylbenzene was added at 11h.

The specific PAA formation for the rest of the cultures were in the same order of magnitude and at least 3.6-fold lower (1.1×10^{-11} to 2.0×10^{-11} mmol PAA/cell), regardless of the time of hexylbenzene addition (24h or 48h) or change in glucose concentration. This suggests that, under controlled conditions where adequate oxygen is supplied, the bioconversion of hexylbenzene is maximised when it is added during the active growth phase of *Y. lipolytica*. It is suggested that an extension of the active growth phase would further improve the bioconversion. That said, the highest accumulated PAA in bioreactor culture (41.4mM) was not obtained in the *Y. lipolytica* WT culture with hexylbenzene addition at 11h (20.8mM PAA). The lower accumulated PAA was due to the cell growth obtained (2.82×10^{8} cells/ml) which was substantially lower when compared to one of the triplicate cultures with hexylbenzene addition at 24h (2.23×10^{9} cells/ml) and the culture with hexylbenzene addition at 48h (8.90×10^{8} cells/ml). This indicates that, although the bioconversion was maximised when hexylbenzene was added at 11h, cell growth was negatively affected. Furthermore, since an increase in the rate of PAA formation was observed in cultures when residual glucose concentrations of up to 5% (wt/v) were measured, it is not expected that PAA formation will be inhibited by the glucose concentration, but rather that PAA formation is dependent on an adequate amount of glucose supply.

6. Conclusions and recommendations

The parameters of the model system (described in 2.6) were defined (chapter 4) in order to maximise cell growth and bioconversion in shake flasks.

Trace elements, phosphate buffering and added nitrogen did not significantly affect the cell growth of *Y. lipolytica* TVN348. These cultivations, however, were carried out in 150ml culture volumes, which were subsequently found to negatively affect both cell growth and bioconversion due to a low oxygen supply.

Oxygen supply was increased by decreasing the working volume from 150ml to 70ml and further to 50ml for *Y. lipolytica* TVN348 and TVN493 cultures, and for *Y. lipolytica* WT cultures by changing from 50ml cultures in non-baffled flasks to 50ml cultures in baffled flasks. The cell concentration of *Y. lipolytica* TVN348 and TVN493 was increased by 65% and 43% respectively for a decrease in working volume (150ml to 50ml). The cell concentration of *Y. lipolytica* WT was increased by 41% when oxygen supply was increased by switching from non-baffled to baffled flasks. Bioconversion was also increased for an increase in oxygen supply: 2.4mM to 29.0mM PAA (*Y. lipolytica* TVN348) and 1.2mM to 21.7mM PAA (*Y. lipolytica* TVN493) for a decrease in working volume; 10.5mM to 46.6mM PAA (*Y. lipolytica* WT) when switching from non-baffled to baffled flasks. These results indicated that adequate oxygen supply is crucial to both growth and bioconversion, and that further study should be conducted in 50ml working volumes.

The effect of 1.6% (wt/v) and 3.2% (wt/v) glucose on the cell growth of *Y. lipolytica* TVN348 was assessed in 50ml shake flask cultures. Cell concentrations obtained (3.95×10^8cells/ml for 1.6% glucose, 4.03×10^8cells/ml for 3.2% glucose) indicated that the effect of the different glucose concentrations was insignificant. It was suggested that cell growth was neither enhanced nor inhibited by 3.2% (wt/v) glucose.

A range of substrates were examined for their influence on the bioconversion by *Y. lipolytica*, *viz* propylbenzene, butylbenzene, *sec*-butylbenzene, hexylbenzene, ethyltoluene and *tert*-butyltoluene for *Y. lipolytica* TVN348 and TVN493, and hexylbenzene, octylbenzene and decylbenzene for *Y. lipolytica* WT. When the bioconversion by *Y. lipolytica* TVN348 and TVN493 was assessed, hexylbenzene was the only substrate to form a product (14.7mM and 14.1mM PAA for TVN348 and TVN493 respectively). When the bioconversion by *Y. lipolytica* WT was assessed, all the three substrates (hexylbenzene, octylbenzene, decylbenzene) formed PAA (42.6mM, 9.0mM

and 7.0mM PAA for hexylbenzene, octylbenzene and decylbenzene respectively). However, the highest amount of PAA was obtained when using hexylbenzene, and therefore hexylbenzene was regarded as the best substrate for bioconversion by *Y. lipolytica* TVN348, TVN493 and WT.

The requirement for an inducer was assessed by examining the effect of 1% (v/v) ethanol, 1% (v/v) oleic acid or 1% (wt/v) added glucose on the bioconversion by *Y. lipolytica* TVN348. When hexylbenzene was used as substrate, it was found that the addition of ethanol or oleic acid was not necessary for induction of the enzymes, because PAA was formed in all three cases (27.5mM, 31.5mM and 25.0mM PAA for additions of ethanol, oleic acid or added glucose respectively). It was suggested rather that a sufficient amount of carbon was necessary for bioconversion. It was also suggested that hexylbenzene acted as its own inducer of enzymes involved in hydroxylation and further degradation.

Care was taken to compare parameters within the same set of experiments and controls were used to allow cross-experiment comparison. Therefore since duplicate or triplicate flasks in each experiment exhibited satisfactory reproducibility and all conclusions were based on 95% confidence intervals, these findings were considered reliable for making recommendations on the defined model system.

The parameters of the model system were therefore defined in shake flasks for the maximisation of cell growth and bioconversion:

- Trace elements, phosphate buffering and added nitrogen had negligible effects on cell growth

- Adequate oxygen supply was crucial for both cell growth and bioconversion

- Glucose concentration was not inhibitory at 3.2%

- Hexylbenzene acted as its own substrate inducer and no additional inducers were required

The defined model system was used in chapter 5 to identify the most promising strain out of *Y. lipolytica* TVN348, TVN493 and WT for further examination. Using this defined model system the cell growth of and bioconversion by *Y. lipolytica* TVN348, TVN493 and WT were evaluated. It was found that there was no significant difference in either cell growth (4.00×10^8cells/ml, 4.11×10^8cells/ml and 4.31×10^8cells/ml for TVN348, TVN493 and WT respectively) or bioconversion (44.6mM, 46.6mM and 47.9mM PAA

for TVN348, TVN493 and WT respectively) between the three strains. Under the condition of these experiments, it would appear that the genetic modification of *Y. lipolytica* TVN348 and TVN493 had not been successful, and therefore it was decided to continue further investigation with *Y. lipolytica* WT until an appropriate modified strain became available.

Under controlled conditions, the influence of time of substrate addition and glucose concentration on the cell growth and bioconversion of *Y. lipolytica* WT was assessed. It was found that cell growth was increased when hexylbenzene was added at 48h (8.90×10^8cells/ml) when compared to two of the triplicate cultures with hexylbenzene addition at 24h (4.74×10^8cells/ml and 3.92×10^8cells/ml) and the culture with hexylbenzene addition at 11h (2.82×10^8cells/ml). The poor reproducibility between the triplicate cultures with hexylbenzene addition as 24h makes it difficult to determine whether hexylbenzene addition at 24h or 48h maximised cell growth.

The cell growth was not significantly improved when the glucose concentration was increased from 3.2% (wt/v) to 6.4% (wt/v) (3.92×10^8cells/ml, 4.74×10^8cells/ml and 2.23×10^9cells/ml for 3.2% glucose cultures to 7.47×10^8cells/ml for 6.4% glucose culture); however, it was also not inhibited.

The specific PAA formation by *Y. lipolytica* WT was at its maximum when hexylbenzene was added at 11h (7.4×10^{-11}mmol PAA/cell) and was at least 3.2-fold higher than any of the other cultures assessed in bioreactor studies. This suggests that the bioconversion of hexylbenzene is maximised when it is added during the active growth phase. However, the highest accumulated PAA was not obtained in the culture with hexylbenzene addition at 11h (20.8mM PAA) due to a lower cell growth. The highest accumulated PAA was obtained in the best performing 24h hexylbenzene addition culture (41.4mM PAA). Accumulated PAA was increased in 6.4% (wt/v) glucose culture (15.2mM PAA) when compared with two of the 3.2% (wt/v) glucose cultures (5.4mM and 4.3mM PAA). These results indicate that the increased glucose concentration did not inhibit bioconversion by *Y. lipolytica* WT. PAA started to increase substantially in the 6.4% (wt/v) glucose culture when 5% (wt/v) residual glucose was observed, suggesting that the bioconversion of hexylbenzene is not inhibited at glucose concentrations as high as 5.0% (wt/v).

During bioconversion by *Y. lipolytica*, the PAA measured after hexylbenzene exhaustion did not, however, correspond to 100% conversion. Further, poor reproducibility was found in the bioreactor cultures. The disappearance of hexylbenzene without a corresponding accumulation of PAA and poor

reproducibility was investigated by determining whether other metabolic intermediates were being formed and accumulated from the hexylbenzene or alternatively, whether PAA was further degraded. However, NMR analyses of both the aqueous and organic phases of *Y. lipolytica* WT culture with hexylbenzene addition at 24h did not identify the formation and accumulation of other metabolic intermediates. In addition, substitution of hexylbenzene with PAA as substrate confirmed that PAA could not be metabolised. In view of the poor reproducibility of PAA formation, care should be exercised when interpreting the results from these data.

It is proposed that future work be conducted in bioreactor culture where glucose is added in fed-batch operation. The continuous addition of glucose is expected to extend the active growth phase, thus continuing to maximise the potential for bioconversion. The low cell growth factor is also expected to become inconsequential since actively growing cells will continue to increase in cell concentration, and eventually improve the accumulated PAA. In so doing, both growth and bioconversion of *Y. lipolytica* are expected to be maximised.

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Appendix A: Chemical Suppliers

Chemical	Supplier
3,5-dinitrosalicylic acid	Fluka
Ammonium sulphate	Saarchem
Antifoam A	Fluka
Bacteriological agar	Biolab
Butylbenzene	Aldrich
Decylbenzene	Aldrich
di-Potassium hydrogen orthophosphate anhydrous	Saarchem
Ethanol (99.5% purity)	Saarchem
Ethyltoluene	Aldrich
Ferrous sulphate	Saarchem
Glucose anhydrous	Saarchem
Glycerol	Saarchem
Hexylbenzene	Aldrich
Hexylbenzene	Aldrich
Hydrochloric acid	Saarchem
Magnesium sulphate	Saarchem
Manganese sulphate	Saarchem
Octylbenzene	Aldrich
Oleic acid	Aldrich
Peptone	Biolab
Phenol	Saarchem
Potassium dihydrogen orthophosphate	Saarchem
Propylbenzene	Aldrich
Rochelle salts	Saarchem
sec-butylbenzene	Aldrich
Sodium chloride	Saarchem
Sodium hydroxide	Saarchem
Sodium-metabisulphite	Saarchem
tert-Butyl-methyl-ether	Fluka
tert-butyltoluene	Aldrich
Trimethylsulfonium hydroxide	UFS
Undecanol	Fluka
Yeast extract	Biolab
Zinc sulphate	Saarchem

Appendix B: Calibrations

B.1 Glucose (DNS) calibrations

DNS Calibration 1 : 14 March 2006

Glucose concentration (g/l)	Absorbance (@ 510nm)
1	0.127
2	0.168
5	0.231
10	0.399
20	0.591
30	0.722
40	0.754

$$y = 55.344x - 8.2271$$
$$R^2 = 0.9369$$
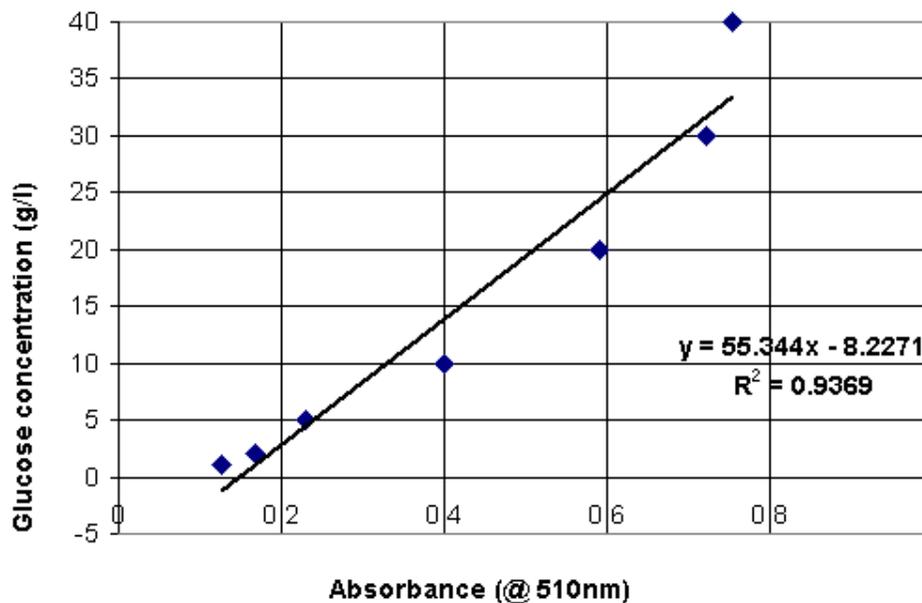
This calibration is based on a DNS glucose assay similar to the assay described in Chapter 3. Details of DNS reagent and method are:

3.5-dinitrosalicylic acid 10g, Phenol 2g, Sodium hydroxide 10g, Sodium metabisulphite 0.75g - reagent prepared by adding melted phenol to remaining reagents dissolved in 100ml make up distilled water. Separate solution of 40g/l Rochelle salts was also prepared. The supernatant of a culture sample was diluted to 10%. 500µl of the dilution was added to 500µl DNS reagent and boiled for 5min and cooled for 5min. After cooling, 200µl of the Rochelle salts solution was added to the mixture, and this mixture was diluted with 10ml distilled water before being read on the spectrophotometer at 510nm.

DNS Calibration 2 : 21 June 2006

Glucose concentration (g/l)	Sample	Absorbance (@ 510nm)
0.1	1	0.083
0.1	2	0.168
0.1	3	0.084
0.2	1	0.172
0.2	2	0.181
0.2	3	0.174
0.4	1	0.402
0.4	2	0.398
0.4	3	0.374
0.8	1	0.617
0.8	2	0.625
0.8	3	0.586
1	1	0.771
1	2	0.662
1	3	0.623

$y = 1.3184x$

$R^2 = 0.9394$
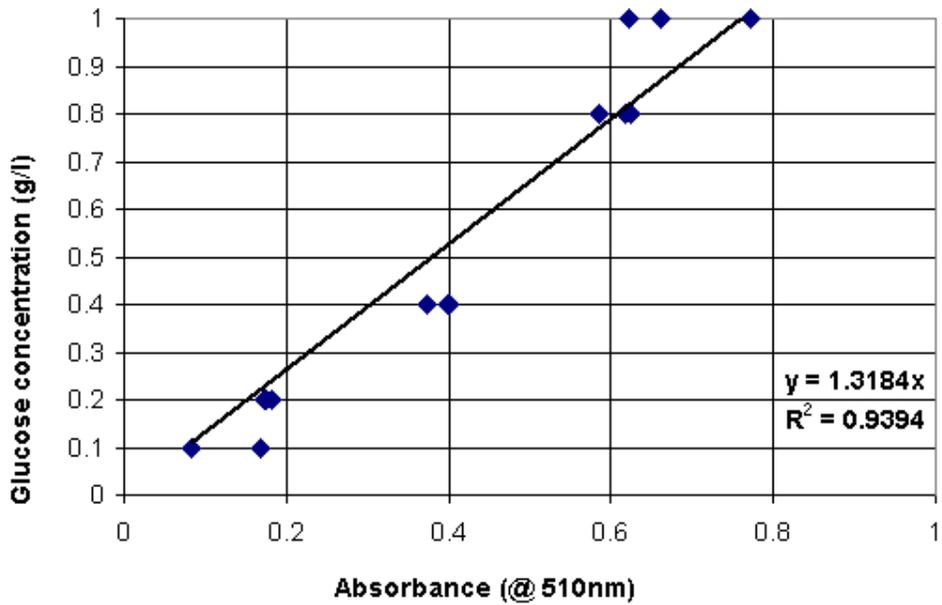
This calibration is based on the DNS glucose assay described in Chapter 3, however absorbance was read at 510 nm and the sample was diluted with 3.2ml distilled water instead of the 8.2ml, as reported in the Materials and Methods chapter.

DNS Calibration 3 : 11 September 2006

Glucose concentration (g/l)	Sample	Absorbance (@ 540nm)
0	-	0.04
0.2	1	0.07
0.2	2	0.069
0.2	3	0.074
0.4	1	0.112
0.4	2	0.115
0.4	3	0.111
0.6	1	0.149
0.6	2	0.148
0.6	3	0.145
0.8	1	0.2
0.8	2	0.201
0.8	3	0.205
1	1	0.243
1	2	0.249
1	3	0.243

$y = 4.6615x - 0.1292$

$R^2 = 0.9927$
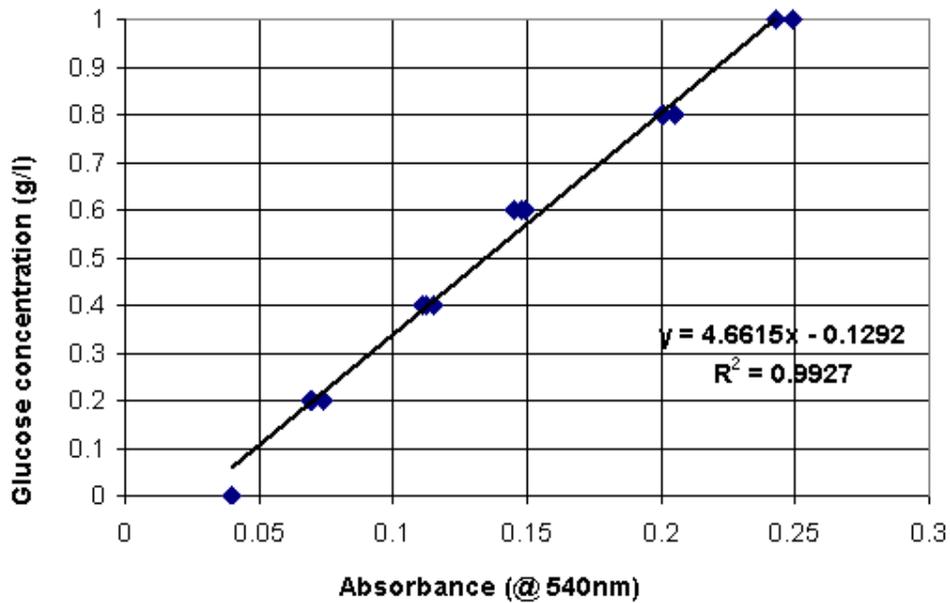
This calibrationsi based on the DNS glucose assay described in Chapter 3, however the absorbance was read at <u>540nm</u>.

DNS Calibration 4: 10 October 2006

Glucose concentration (g/l)	Sample	Absorbance (@ 523nm)
0.2	1	0.057
0.2	2	0.06
0.2	3	0.059
0.4	1	0.155
0.4	2	0.157
0.4	3	0.154
0.6	1	0.261
0.6	2	0.259
0.6	3	0.258
0.8	1	0.312
0.8	2	0.322
0.8	3	0.316
1	1	0.38
1	2	0.357
1	3	0.394

$y = 2.5477x$
$R^2 = 0.9762$
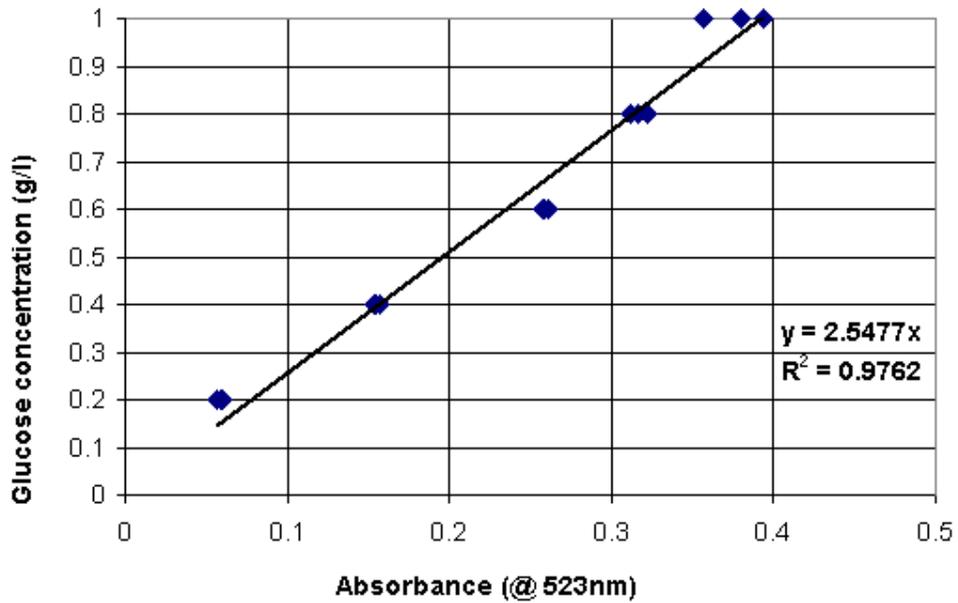
This calibration is based on the DNS glucose assay described in Chapter 3.

B.2 Hexylbenzene (GC) calibration

GC Calibration

HB (%)	HB (mM)	Integrated Areas		(Area HB)/ (Area undecanol)
		HB	Undecanol	
0	0.00	0	n/a	0
0.33	17.72	99178	132697	0.747
0.5	26.58	115698	108687	1.065
1	53.15	310598	117521	2.643
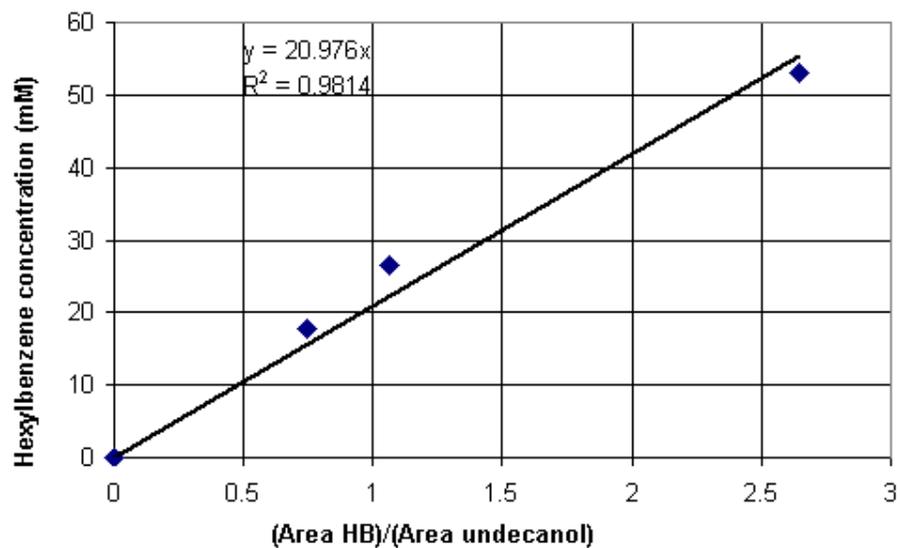
B.3 Phenyl acetic acid (GC) calibrations

<u>GC Calibration 1</u>

Given by University of the Free State as:
PAA conc (mM) = 7.8043 x (area PAA)/(area internal standard=undecanol)

This calibration was only used for data generated from samples that were analysed at the University of the Free State

The samples used with this calibration were methylated with trimeythylsulfonium hydroxide.

<u>GC Calibration 2</u>

PAA (g/ml)	PAA (mM)	Integrated Areas		(Area PAA)/ (Area undecanol)
		PAA	Undecanol	
0	0	0	n/a	0
0.002	14.690	11521	54874	0.209953712
0.004	29.379	463160	995357	0.465320483
0.006	44.069	117981	142157	0.829934509
0.008	58.759	233311	244353	0.954811277
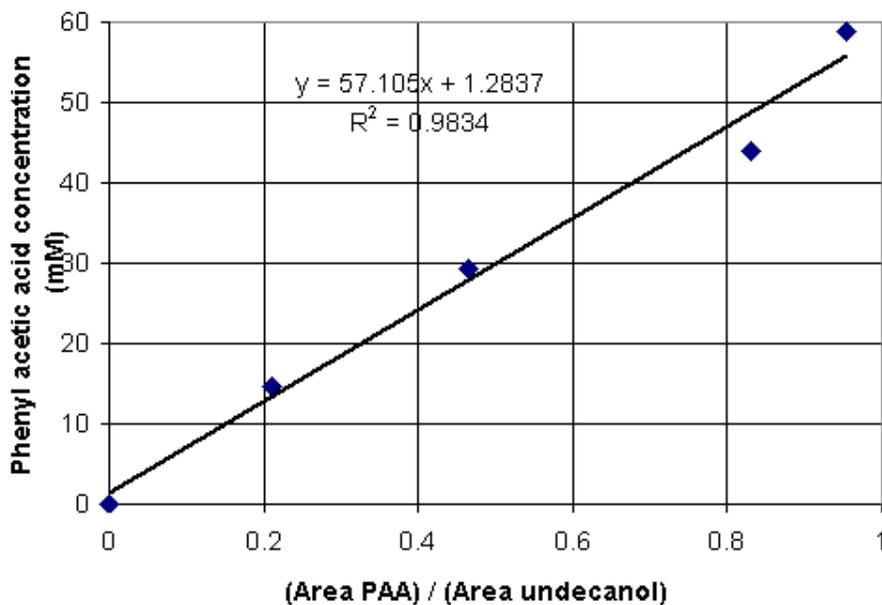
The samples used with this calibration were methylated with trimeythylsulfonium hydroxide

<u>GC Calibration 3</u>

PAA (g/ml)	PAA (mM)	Integrated Areas		(Area PAA)/ (Area undecanol)
		PAA	Undecanol	
0	0	0	0	0
0.0031	22.769	1337.1	6326.3	0.211
0.005	36.724	2274.7	5892.3	0.386
0.0068	49.945	3515.7	6064.9	0.580
0.0092	67.573	4796.1	5637.1	0.851
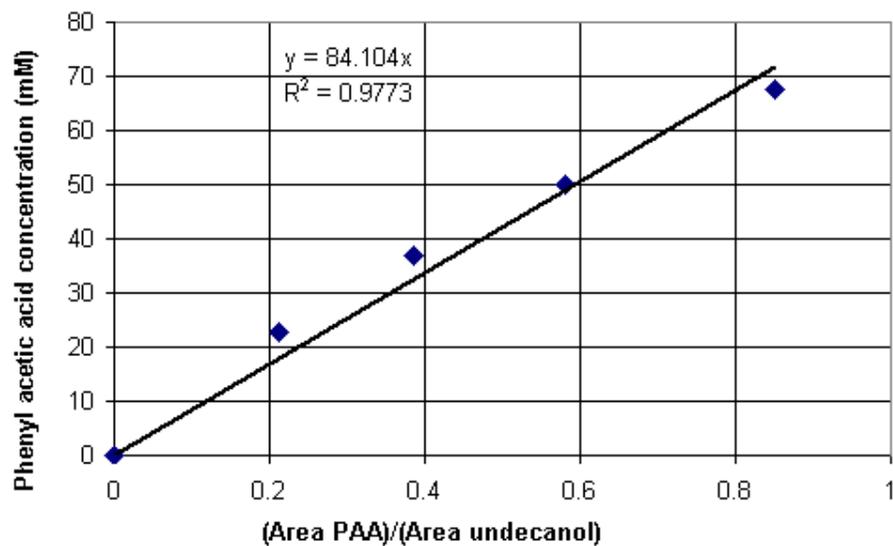
The samples used with this calibration were not methylated and used on a different GC with the same conditions described in Chapter 3

Appendix C: GC

C.1 Extraction efficiency tested

SAMPLE 1							
Component	Mass or vol. (g or µl)	Area 1	Extracted 1 (g or µl)	Area 2	Extracted 2 (g or µl)	Area 3	Extracted 3 (g or µl)
Octylbenzene	10	1548784	11.561	2439564	11.232	1694498	11.629
Undecanol	0.006	755009	0.006	1222256	0.006	821453	0.006
PAA	0.004	548038	0.0061	882443	0.0060	597307	0.0061

Average (g or µl)	Standard deviation	% Std deviation
11.474	2.12E-01	1.85E-02
0.006	1.06E-18	1.77E-16
0.006	2.61E-05	4.30E-03

SAMPLE 2						
Component	Mass or vol. (g or µl)	Area 1	Extracted 1 (g or µl)	Area 2	Extracted 2 (g or µl)	Average (g or µl)
Octylbenzene	10	1744084	11.027	1729825	11.062	11.044
Undecanol	0.006	889252	0.006	879305	0.006	0.006
PAA	0.004	570203	0.0052	601205	0.0057	0.0055

Average (g or µl)	Standard deviation	% Std deviation
11.044	2.50E-02	2.27E-03
0.006	0.00E+00	0.00E+00
0.005	2.92E-04	5.35E-02

C.2 Sample chromatogram

```
*****************************************************************************
*                            PRIMARY                                        *
*  TIME :  2:51 pm     DELTA CHROMATOGRAPHY DATA SYSTEM     DATE :  2/11/ 7  *
*                         AREA PERCENT REPORT                                *
*****************************************************************************

    SAMPLE NAME :  PAA cal sample2          CHROMATOGRAM FILE :    0211PAA2
    SAMPLE TYPE :  SAMP                     CHROMATOGRAM SOURCE : ACQUIRE
    SAMPLE AMOUNT         1.00              METHOD NAME        : AINGY
    DILUTE     :         1.00              VIAL               :  1 OF  1

    Chromatogram 0211PAA2 recorded at  2:24 pm on 02/11/107
    Captured by analyst Hanlie Botha using method : AINGY
```
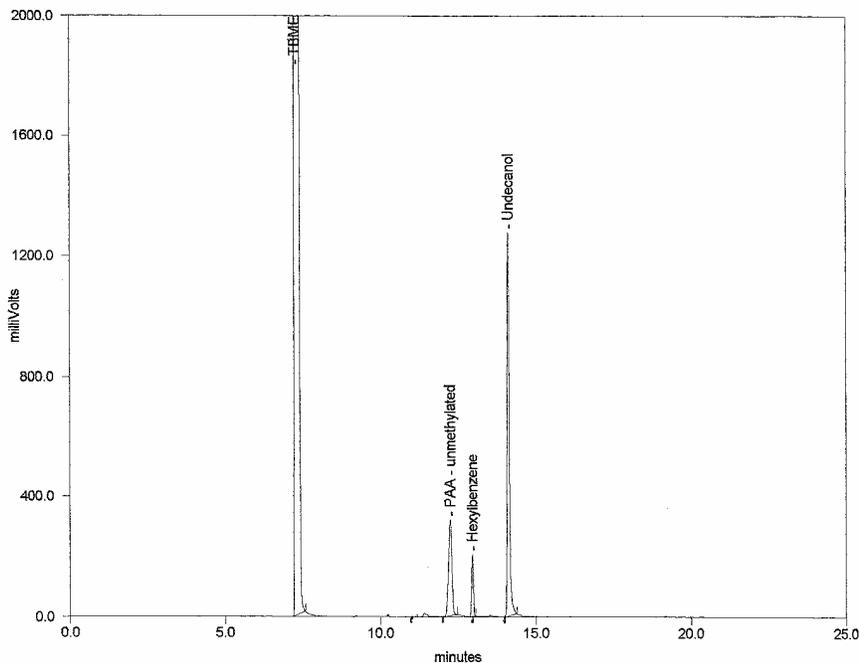


```
============================================================================
PK#  CPT#  COMPONENT NAME      RET. TIME   WIDTH      AREA        RESULT
---  ---   -----------------   ---------  ------    ---------     ---------
 1    1    TBME                  7.25       9.60     98833.3        0.0
      2    PAA/TMSH
 2    3    PAA - unmethylated   ·12.26     ·6.70      2274.7        0.0
 3    4    Hexylbenzene         ⁄12.96     ·3.60       800.3        0.0
 4    5    Undecanol            ·14.13      4.10      5892.3        0.0
----------------------------------------------------------------------------
TOTALS :                                            107800.6       0.0
============================================================================
```

```
          Page  1 : PAA cal sample2 :  2:51 pm Fri Nov 02 2007
```

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Appendix D: Sample calculations

D.1 Confidence intervals

The 95% confidence intervals for data points were calculated by using the formula

$$\bar{x} \pm z\sigma$$

(D.1)

Where

- $\bar{x}$ is either the average measurement of sample readings of identical culture at a specific time

- $\pm z\sigma$ is the confidence interval

- z is dependent on the confidence interval chosen and is equal to 1.96 for a 95% confidence interval

- σ is the standard deviation between identical sample readings at a specific time

E.g. Section 4.2.1

The standard deviation in cell growth of *Y. lipolytica* TVN348 (at 48h) in 50ml cultures was 2.58×10^7 cells/ml (when disregarding the high outlier) and the average cell concentration of the 50ml cultures were 5.32×10^8 cells/ml.

"zσ" is therefore equal to $(1.96)(2.58 \times 10^7) = 5.06 \times 10^7$ cells/ml. If the cell concentration of the other cultures (at 48h) therefore lay within the interval of $5.32 \times 10^8 \pm 5.06 \times 10^7$ cells/ml, it could be said with a 95% confidence interval that they were not significantly different from the 50ml cultures.

D.2 Specific growth rate

The specific growth rates were calculated by the following equation (Bailey & Ollis):

$$\mu = \frac{\ln x_2 - \ln x_1}{t_2 - t_1} \quad at\ t = t_1 + \frac{t_2 - t_1}{2}$$

(D.2)

Where

- μ is the specific growth rate (h^{-1}) at time $t = t_1 + \frac{t_2 - t_1}{2}$

- x_2 is the cell concentration at time t_2

- x_1 is the cell concentration at time t_1

E.g. Section 4.2.1

The cell concentrations of *Y. lipolytica* TVN348 at 0h and 24h were 2.00×10^7 cells/ml and 6.58×10^7 cells/ml respectively. From this data:

$$t_1 = 0h; \quad t_2 = 24h; \quad x_1 = 2.00 \times 10^7; \quad x_2 = 6.58 \times 10^7$$

Therefore,

$$t = \frac{24 - 0}{2} = 12; \quad \mu = \frac{\ln(6.58 \times 10^7) - \ln(2.00 \times 10^7)}{24 - 0} = 0.0496 h^{-1}\ at\ 12h$$

Appendix E: Raw experimental data

E.1 Chapter 4
Chapter 4.1 - Influence of trace elements, buffering and added nitrogen on cell growth (1)

Data description: Cultivation of TVN 348 in Medium 1 to determine the influence of added nutrients

Time (h)	Sample	Cell growth concentration										Glucose concentration (g/l)					Observations/ Comments
		Cell number (cells/ml)					O.D.										
		Cells/ square	Dil. factor	Cell conc.	Std dev.	Average	Absorb. (@510nm)	Dil. factor	Actual OD	Std dev.	Ave.	Absorb. *	Dil. factor	Glucose conc.	Std dev.	Average	
0	*Inoculum*	1.2	1	4.72E+06	n/a	4.72E+06	-	n/a	-	n/a	-	-	n/a	-	n/a	32g/l added	Initial glucose assumed and not measured, OD not measured
3	1	6.1		2.44E+07			0.220		0.220			0.647		27.58			
	2	5.7	1	2.28E+07	7.66E+05	2.34E+07	0.201	1	0.201	0.05	0.171	0.650	1	27.75	0.96	26.85	
	3	5.7		2.28E+07			0.124		0.124			0.623		26.25			
	4	5.9		2.36E+07			0.137		0.137			0.615		25.81			
21	1	9.2		7.36E+07			0.619		0.619			0.631		26.69			pH = 7.59, budding cells
	2	10.8	2	8.64E+07	8.81E+06	7.76E+07	0.592	1	0.592	0.01	0.613	0.610	1	25.53	0.86	26.60	pH = 7.6
	3	10.4		8.32E+07			0.623		0.623			0.648		27.64			pH = 7.58, elongated cells
	4	8.4		6.72E+07			0.618		0.618			0.628		26.53			pH = 7.58
26	1	12.2		1.46E+08			0.714		0.714			-		-			DNS could not be measured
	2	8.3		9.96E+07			0.638		0.638			0.250		5.61			
	3	11.6	3	1.39E+08	2.35E+07	1.23E+08	0.689	1	0.689	0.04	0.672	0.359	1	11.64	4.27	8.63	
	4	8.8		1.06E+08			0.646		0.646			-		-			DNS could not be measured, budding cells
31	1	15.4		1.85E+08			0.750		0.750			-		-			DNS could not be measured
	2	14.0	3	1.68E+08	9.62E+06	1.71E+08	0.784	1	0.784	0.08	0.701	0.234	1	4.72	0.23	4.56	
	3	13.9		1.67E+08			0.635		0.635			0.228		4.39			
	4	13.6		1.63E+08			0.635		0.635			-		-			DNS could not be measured
43.5	1	15.4		1.85E+08			0.751		0.751			0.207		3.23			
	2	13.2	3	1.58E+08	1.19E+07	1.76E+08	0.776	1	0.776	0.01	0.762	0.213	1	3.56	0.18	3.43	
	3	15.1		1.81E+08			0.769		0.769			0.212		3.51			
	4	14.9		1.79E+08			0.750		0.750			-		-			DNS could not be measured

* DNS Calibration 1 used

Chapter 4.1 - Influence of trace elements, buffering and added nitrogen on cell growth (2)

Data description: Cultivation of TVN 348 in Medium 2 to determine the influence of added nutrients

Time (h)	Sample	Cell growth concentration										Glucose concentration (g/l)					Observations/ Comments
		Cell number (cells/ml)					O.D.										
		Cells/ square	Dil. factor	Cell conc.	Std dev.	Average	Absorb. (@510nm)	Dil. factor	Actual OD	Std dev.	Ave.	Absorb.*	Dil. factor	Glucose conc.	Std dev.	Average	
0	*Inoculum*	1.25	1	5.00E+06	n/a	5.00E+06	-	n/a	-	n/a	-	-	n/a	-	n/a	32g/l added	Initial glucose assumed and not measured, OD not measured
3	1	5.3	1	2.12E+07	1.15E+06	1.98E+07	0.120	1	0.120	0.02	0.128	0.654	1	27.97	3.22	30.03	
	2	4.6		1.84E+07			0.131		0.131			0.668		28.74			
	3	5.0		2.00E+07			0.151		0.151			0.778		34.83			
	4	4.9		1.96E+07			0.108		0.108			0.665		28.58			
21	1	9.3	2	7.44E+07	9.56E+06	7.46E+07	0.624	1	0.624	0.04	0.615	0.685	1	29.68	1.28	28.24	pH = 7.59, elongated cells
	2	8.0		6.40E+07			0.575		0.575			0.658		28.19			pH = 7.6
	3	10.9		8.72E+07			0.590		0.590			0.629		26.58			pH = 7.58
	4	9.1		7.28E+07			0.672		0.672			0.664		28.52			pH = 7.58
26	1	6.4	3	7.68E+07	1.90E+07	9.48E+07	0.721	1	0.721	0.05	0.655	-	1	-	1.57	9.48	DNS could not be measured
	2	6.8		8.16E+07			0.640		0.640			0.34		10.59			
	3	8.6		1.03E+08			0.649		0.649			0.3		8.38			
	4	9.8		1.18E+08			0.610		0.610			-		-			DNS could not be measured
31	1	11.5	3	1.38E+08	1.16E+07	1.49E+08	0.738	1	0.738	0.05	0.673	-	1	-	1.25	10.20	DNS could not be measured
	2	13.1		1.57E+08			0.674		0.674			0.317		9.32			
	3	11.7		1.40E+08			0.638		0.638			0.349		11.09			
	4	13.4		1.61E+08			0.641		0.641			-		-			DNS could not be measured
43.5	1	16.3	3	1.96E+08	2.69E+07	2.00E+08	0.736	1	0.736	0.02	0.711	0.282	1	7.38	1.04	7.14	
	2	17.5		2.10E+08			0.680		0.680			0.294		8.04			
	3	13.7		1.64E+08			0.722		0.722			0.257		6.00			
	4	19.0		2.28E+08			0.704		0.704			-		-			DNS could not be measured

* DNS Calibration 1 used

Chapter 4.1 - Influence of trace elements, buffering and added nitrogen on cell growth (3)

Data description: Cultivation of TVN 348 in Medium 3 to determine the influence of added nutrients

Time (h)	Sample	Cells/square	Dil. factor	Cell conc.	Std dev.	Average	Absorb. (@510nm)	Dil. factor	Actual OD	Std dev.	Ave.	Absorb.*	Dil. factor	Glucose conc.	Std dev.	Average	Observations/Comments
						Cell growth concentration								**Glucose concentration (g/l)**			
0	Inoculum	1.32	1	5.28E+06	n/a	5.28E+06	-	n/a	-	n/a	-	-	n/a	-	n/a	32g/l added	Initial glucose assumed and not measured, OD not measured
3	1	5.6	1	2.24E+07	2.62E+06	1.96E+07	0.090	1	0.090	0.01	0.102	0.694	1	30.18	2.58	29.94	
	2	4.3		1.72E+07			0.098		0.098			0.641		27.25			
	3	4.8		1.92E+07			0.118		0.118			0.734		32.40			
21	1	10.2	2	8.16E+07	7.21E+06	8.21E+07	0.656	1	0.656	0.03	0.653	0.653	1	27.91	0.86	27.06	pH = 7.27
	2	9.4		7.52E+07			0.617		0.617			0.622		26.20			pH = 7.30
	3	11.2		8.96E+07			0.685		0.685			0.638		27.08			pH = 7.34
26	1	9.5	3	1.14E+08	1.08E+07	1.25E+08	0.714	1	0.714	0.04	0.664	-	1	-	1.45	9.23	DNS could not be measured
	2	10.5		1.26E+08			0.631		0.631			0.297		8.21			
	3	11.3		1.36E+08			0.647		0.647			0.334		10.26			
31	1	13.1	3	1.57E+08	9.62E+06	1.47E+08	0.667	1	0.667	0.05	0.729	-	1	-	0.82	9.68	DNS could not be measured
	2	12.2		1.46E+08			0.766		0.766			0.313		9.10			
	3	11.5		1.38E+08			0.753		0.753			0.334		10.26			
43.5	1	19.0	3	2.28E+08	2.95E+07	2.06E+08	0.759	1	0.759	0.01	0.757	-	1	-	0.43	8.35	DNS could not be measured
	2	14.4		1.73E+08			0.762		0.762			0.294		8.04			
	3	18.2		2.18E+08			0.749		0.749			0.305		8.65			

* DNS Calibration 1 used

Chapter 4.2 - Influence of oxygen supply on cell growth and bioconversion (1)

Data description: Cultivation of TVN 348 in 50ml and 150ml medium to determine the influence of oxygen supply

Time (h)	Sample	Cells/ square	Dil. factor	Cell conc.	Std dev.	Average	Absorb. (@620nm)	Dil. factor	Actual OD	Std dev.	Ave.	Absorb. *	Dil. factor	Glucose conc.	Std dev.	Average	Observations/ Comments
				Cell number (cells/ml)					O.D.					Glucose concentration (g/l)			
0	*Inoculum*	5.0	1	2.00E+07	n/a	2.00E+07	0.154	1	0.15	n/a	0.15	-	n/a	-	n/a	-	
7	1	-	n/a	-	n/a	-	0.471	1	0.47	0.04	0.45	0.232	20	19.05	3.31	20.46	50 ml culture
	2	-		-			0.486		0.49			0.272		22.77			50 ml culture
	3	-		-			0.463		0.46			0.298		25.20			50 ml culture
	4	-		-			0.475		0.48			0.267		22.31			50 ml culture
	5	-		-			0.376		0.38			0.194		15.50			50 ml culture
	6	-		-			0.472		0.47			0.217		17.65			50 ml culture
	7	-		-			0.424		0.42			0.250		20.72			150 ml culture
24	1	13.2	4	2.11E+08	6.58E+07	1.93E+08	0.557	10	5.57	0.78	4.98	0.132	20	9.72	2.20	11.31	1% (v/v) ethanol added
	2	8.8		1.41E+08			0.574		5.74			0.136		10.10			
	3	7.6		1.22E+08			0.500		5.00			0.138		10.28			
	4	11.9		1.90E+08			0.502		5.02			0.148		11.21			
	5	11.5		1.84E+08			0.512		5.12			0.129		9.44			
	6	19.3		3.09E+08			0.504		5.04			0.165		12.80			
	7	5.6		8.96E+07	n/a	-	0.334		3.34			0.195		15.60			
31.5	1	-	n/a	-	n/a	-	0.667	10	6.67	1.28	5.57	0.081	20	4.97	3.30	6.05	
	2	-		-			0.589		5.89			0.101		6.83			
	3	-		-			-		-			0.083		5.15			
	4	-		-			0.630		6.30			0.082		5.06			
	5	-		-			0.596		5.96			0.072		4.13			
	6	-		-			0.550		5.50			0.061		3.10			
	7	-		-			0.307		3.07			0.168		13.08			
48	1	12.3	10	4.92E+08	1.08E+08	5.32E+08	0.436	16	6.98	0.72	6.37	0	20	-2.58	4.82	-0.03	Analyses done before hexylbenzene addition, *DNS Calibration 3 used*
	2	12.1		4.84E+08			0.459		7.34			0		-2.58			
	3	18.7		7.48E+08			0.327		5.23			0		-2.58			
	4	12.1		4.84E+08			0.416		6.66			0		-2.58			
	5	13.2		5.28E+08			0.372		5.95			0		-2.58			
	6	11.4		4.56E+08			0.371		5.94			0.058		2.82			
	7	8.1		3.24E+08	n/a	-	0.406		6.50			0.134		9.91			

Chapter 4.2 - Influence of oxygen supply on cell growth and bioconversion (2)

Data description: Product formation by TVN 348 in 50ml, 100ml & 150ml cultures to determine the influence of oxygen supply

Time	Sample	Subrate and product analysis								Observations/ Comments
		Integrated areas on GC			Substrate conc. (mM)		Product conc. (mM)			
(h)		Substrate	Product	Internal std	Each	Average	Each	Std dev.	Average	
48	a	-	-	-		-		n/a	0	50 ml, 1% HB added
	b	-	-	-		-		n/a	0	100 ml, 1% HB added
	c	-	-	-		-		n/a	0	150 ml, 1% HB added
	d	-	-	-		-		n/a	0	original 150 ml, 1% HB added
49	a	-	-	-		-		n/a	0	
	b	-	-	-		-		n/a	0	
	c	-	-	-		-		n/a	0	
	d	-	-	-		-		n/a	0	
54	a	-	-	-		-		n/a	0	
	b	-	-	-		-		n/a	0	
	c	-	-	-		-		n/a	0	
	d	-	-	-		-		n/a	0	
72	a	-	38522	49524		-		n/a	6.07	
	b	-	0	n/a		-		n/a	0.00	
	c	-	0	n/a		-		n/a	0.00	
	d	-	0	n/a		-		n/a	0.00	
79	a	-	68394	59661		-		n/a	8.95	
	b	-	19034	56288		-		n/a	2.64	
	c	-	0	n/a		-		n/a	0.00	
	d	-	0	n/a		-		n/a	0.00	
96	a	-	125598	57957		-		n/a	16.91	
	b	-	68131	70575		-		n/a	7.53	
	c	-	7757	54892		-		n/a	1.10	
	d	-	0	n/a		-		n/a	0.00	
120	a	-	196251	54368		-		n/a	28.17	
	b	-	113884	55909		-		n/a	15.90	
	c	-	29323	60502		-		n/a	3.78	
	d	-	19416	63610		-		n/a	2.38	* GC product calibration 1 used

Chapter 4.2 - Influence of oxygen supply on cell growth and bioconversion (3)

Data description: Cultivation of TVN 493 in 50ml and 150ml cultures to determine the influence of oxygen supply

Time (h)	Sample	Cell growth concentration										Glucose concentration (g/l)					Observations/ Comments
		Cell number (cells/ml)					O.D.										
		Cells/ square	Dil. factor	Cell conc.	Std dev.	Average	Absorb. (@620nm)	Dil. factor	Actual OD	Std dev.	Ave.	Absorb. *	Dil. factor	Glucose conc.	Std dev.	Average	
0	*Inoculum*	7.8	1	3.12E+07	n/a	3.12E+07	0.333	1	0.33	n/a	0.333	-	n/a	-	n/a	-	
7	1	-	n/a	-	n/a	-	0.658	1	0.66	0.04	0.71	0.215	20	17.46	1.90	17.47	50 ml culture
	2	-		-			0.738		0.74			0.190		15.13			50 ml culture
	3	-		-			0.756		0.76			0.248		20.54			50 ml culture
	4	-		-			0.736		0.74			0.190		15.13			50 ml culture
	5	-		-			0.742		0.74			0.224		18.30			50 ml culture
	6	-		-			0.696		0.70			0.215		17.46			50 ml culture
	7	-		-			0.661		0.66			0.224		18.30			150 ml culture
24	1	10.8	4	1.73E+08	6.21E+07	1.87E+08	0.714	10	7.14	1.05	6.36	0.128	20	9.35	2.23	8.66	1% (v/v) ethanol added
	2	7.6		1.22E+08			0.663		6.63			0.119		8.51			
	3	8.8		1.41E+08			0.664		6.64			0.105		7.21			
	4	17.9		2.86E+08			0.701		7.01			0.104		7.11			
	5	10.3		1.65E+08			0.654		6.54			0.124		8.98			
	6	14.7		2.35E+08			0.651		6.51			0.096		6.37			
	7	9.1		1.46E+08	n/a	-	0.404		4.04			0.168		13.08			
31.5	1	-	n/a	-	n/a	-	0.793	10	7.93	1.20	7.29	0.076	20	4.50	3.02	6.59	
	2	-		-			0.79		7.90			0.11		7.67			
	3	-		-			0.771		7.71			0.078		4.69			
	4	-		-			0.835		8.35			0.102		6.93			
	5	-		-			0.723		7.23			0.074		4.32			
	6	-		-			0.718		7.18			0.084		5.25			
	7	-		-			0.474		4.74			0.165		12.80			
48	1	13.4	10	5.36E+08	6.02E+07	4.81E+08	0.394	16	6.30	1.13	6.40	0	20	-2.58	3.84	-1.13	Analyses done before hexylbenzene addition, * DNS Calibration 3 used
	2	12.6		5.04E+08			0.408		6.53			0		-2.58			
	3	11.8		4.72E+08			0.339		5.42			0		-2.58			
	4	11.2		4.48E+08			0.441		7.06			0		-2.58			
	5	13.6		5.44E+08			0.495		7.92			0		-2.58			
	6	9.6		3.84E+08			0.439		7.02			0		-2.58			
	7	8.4		3.36E+08	n/a	-	0.565	8	4.52			0.109		7.58			

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Chapter 4.2 - Influence of oxygen supply on cell growth and bioconversion (4)

Time	Sample	Subrate and product analysis								Observations/ Comments
		Integrated areas on GC			Substrate conc. (mM)		Product conc. (mM) *			
(h)	Sample	Substrate	Product	Internal std	Each	Average	Each	Std dev.	Average	Observations/ Comments
48	a	-	-	-		-		n/a	0	50 ml, 1% HB added
	b	-	-	-		-		n/a	0	100 ml, 1% HB added
	c	-	-	-		-		n/a	0	150 ml, 1% HB added
	d	-	-	-		-		n/a	0	original 150 ml, 1% HB added
49	a	-	-	-		-		n/a	0	
	b	-	-	-		-		n/a	0	
	c	-	-	-		-		n/a	0	
	d	-	-	-		-		n/a	0	
54	a	-	-	-		-		n/a	0	
	b	-	-	-		-		n/a	0	
	c	-	-	-		-		n/a	0	
	d	-	-	-		-		n/a	0	
72	a	-	33640	54291		-		n/a	4.84	
	b	-	7389	53423		-		n/a	1.08	
	c	-	0	n/a		-		n/a	0.00	
	d	-	0	n/a		-		n/a	0.00	
79	a	-	42529	54675		-		n/a	6.07	
	b	-	17325	55935		-		n/a	2.42	
	c	-	0	n/a		-		n/a	0.00	
	d	-	0	n/a		-		n/a	0.00	
96	a	-	101503	58111		-		n/a	13.63	
	b	-	53354	54148		-		n/a	7.69	
	c	-	14369	57164		-		n/a	1.96	
	d	-	0	n/a		-		n/a	0.00	
120	a	-	161890	58336		-		n/a	21.66	
	b	-	124052	58628		-		n/a	16.51	
	c	-	49803	52311		-		n/a	7.43	
	d	-	8657	55471		-		n/a	1.22	

Data description: Product formation by TVN 493 in 50ml, 100ml & 150ml cultures to determine the influence of oxygen supply

* GC product calibration 1 used

Chapter 4.2 - Influence of oxygen supply on cell growth and bioconversion (5)

Data description: Cultivation of TVN 348 in 70ml culture to determine the influence of oxygen supply

Time (h)	Sample	Cell growth concentration										Glucose concentration (g/l)					Observations/ Comments
		Cell number (cells/ml)					O.D.										
		Cells/square	Dil. factor	Cell conc.	Std dev.	Average	Absorb. (@620nm)	Dil. factor	Actual OD	Std dev.	Ave.	Absorb.*	Dil. factor	Glucose conc.	Std dev.	Average	
0	*Inoculum*	1.6	5	3.12E+07	n/a	3.12E+07	1.600	1	1.60	n/a	1.60	-	n/a	-	n/a	-	
6	1	4.0	10	1.60E+08	6.23E+07	1.53E+08	0.329	20	6.58	0.22	6.69	0.330	20	16.81	0.47	17.36	
	2	5.3		2.12E+08			0.327		6.54			0.347		17.68			
	3	2.2		8.80E+07			0.347		6.94			0.345		17.58			
16.5	1	8.4	10	3.36E+08	3.45E+07	3.43E+08	0.661	20	13.22	1.28	14.59	0.314	20	16.00	1.01	14.91	
	2	9.5		3.80E+08			0.739		14.78			0.289		14.73			
	3	7.8		3.12E+08			0.788		15.76			0.275		14.01			
24	1	9.2	10	3.68E+08	3.35E+07	3.29E+08	0.658	30	19.74	0.52	19.41	0.243	20	12.38	0.22	12.23	1% (v/v) ethanol added
	2	7.8		3.12E+08			0.656		19.68			0.242		12.33			1% (v/v) ethanol added
	3	7.7		3.08E+08			0.627		18.81			0.235		11.97			
48	1	11.5	10	4.60E+08	3.56E+07	4.48E+08	0.705	40	28.20	1.24	29.43	0.058	20	2.96	0.41	2.53	1% (v/v) hexylbenzene added
	2	11.9		4.76E+08			0.767		30.68			0.042		2.14			1% (v/v) hexylbenzene added
	3	10.2		4.08E+08			0.735		29.40			0.049		2.50			1% (v/v) hexylbenzene added
68	1	8.5	10	3.40E+08	3.01E+08	5.96E+08	0.619	40	24.76	1.17	24.15	0.033	20	1.68	0.42	1.22	
	2	13.0		5.20E+08			0.570		22.80			0.022		1.12			
	3	23.2		9.28E+08			0.622		24.88			0.017		0.87			
75	1	14.4	10	5.76E+08	6.68E+07	6.36E+08	-	n/a	-	n/a	-	-	n/a	-	n/a	-	
	2	17.7		7.08E+08			-		-			-		-			
	3	15.6		6.24E+08			-		-			-		-			
97	1	13.9	10	5.56E+08	1.08E+08	6.65E+08	0.595	40	23.80	2.87	21.28	0.017	20	0.87	0.23	0.63	
	2	16.7		6.68E+08			0.547		21.88			0.012		0.61			
	3	19.3		7.72E+08			0.454		18.16			0.008		0.41			

* DNS Calibration 4 used

Chapter 4.2 - Influence of oxygen supply on cell growth and bioconversion (6)

Data description: Product formation by TVN 348 in 70 ml culture to determine the influence of oxygen supply

| Time | Sample | Subrate and product analysis | | | | | | | | Observations/ Comments |
| | | Integrated areas on GC | | | Substrate conc. (mM) | | Product conc. (mM) * | | | |
(h)	Sample	Substrate	Product	Internal std	Each	Average	Each	Std dev.	Average	
48	1	-	0	n/a	-		0			1% (v/v) hexylbenzene added
	2	-	0	n/a	-	-	0	0	0	1% (v/v) hexylbenzene added
	3	-	0	n/a	-		0			1% (v/v) hexylbenzene added
68	1	-	0	n/a	-		0			
	2	-	0	n/a	-	-	0	0	0	
	3	-	0	n/a	-		0			
75	1	-	0	n/a	-		0			
	2	-	0	n/a	-	-	0	0	0	
	3	-	0	n/a	-		0			
97	1	-	0	n/a	-		0			
	2	-	0	n/a	-	-	0	1.158	0.669	
	3	-	22024	1741237			2.006			
148	1	-	801870	4786682			10.850			
	2	-	167393	1897051		-	6.323	3.301	9.974	
	3	-	630365	3139833			12.748			

GC product calibration 2 used

Chapter 4.2 - Influence of oxygen supply on cell growth and bioconversion (7)

Data description: Cultivation of TVN 493 in 70ml culture to determine the influence of oxygen supply

Time (h)	Sample	Cells/ square	Dil. factor	Cell conc.	Std dev.	Average	Absorb. (@510nm)	Dil. factor	Actual OD	Std dev.	Ave.	Absorb.*	Dil. factor	Glucose conc.	Std dev.	Average	Observations/ Comments
		Cell growth concentration											**Glucose concentration (g/l)**					
		Cell number (cells/ml)					**O.D.**										
0	*Inoculum*	1.8	5	3.66E+07	n/a	3.66E+07	1.72	1	1.72	n/a	1.72	-	n/a	-	n/a	-	
6	1	3.1	10	1.24E+08	4.00E+06	1.20E+08	0.400	20	8.00	1.08	6.77	0.348	20	17.73	0.16	17.60	
	2	3.0		1.20E+08			0.300		6.00			0.346		17.63			
	3	2.9		1.16E+08			0.315		6.30			0.342		17.43			
16.5	1	7.1	10	2.84E+08	1.74E+07	2.76E+08	0.798	20	15.96	0.33	15.85	0.290	20	14.78	0.70	14.66	
	2	7.2		2.88E+08			0.774		15.48			0.273		13.91			
	3	6.4		2.56E+08			0.805		16.10			0.300		15.29			
24	1	10.9	10	4.36E+08	7.41E+07	3.60E+08	0.704	30	21.12	0.98	20.28	0.221	20	11.26	0.16	11.23	1% (v/v) ethanol added
	2	7.2		2.88E+08			0.684		20.52			0.217		11.06			1% (v/v) ethanol added
	3	8.9		3.56E+08			0.640		19.20			0.223		11.36			
48	1	8.8	10	3.52E+08	8.78E+07	3.99E+08	0.726	40	29.04	0.38	29.17	0.025	20	1.27	0.29	1.51	1% (v/v) hexylbenzene added
	2	8.6		3.44E+08			0.722		28.88			0.036		1.83			1% (v/v) hexylbenzene added
	3	12.5		5.00E+08			0.740		29.60			0.028		1.43			1% (v/v) hexylbenzene added
68	1	31.4	10	1.26E+09	2.98E+08	9.12E+08	0.587	40	23.48	3.66	21.91	0.013	20	0.66	0.13	0.71	
	2	18.3		7.32E+08			0.613		24.52			0.017		0.87			
	3	18.7		7.48E+08			0.443		17.72			0.012		0.61			
75	1	16.4	10	6.56E+08	7.21E+07	5.87E+08	-	n/a	-	n/a	-	-	n/a	-	n/a	-	
	2	14.8		5.92E+08			-		-			-		-			
	3	12.8		5.12E+08			-		-			-		-			
97	1	19.6	10	7.84E+08	2.24E+08	7.36E+08	0.743	40	29.72	4.43	25.12	0.009	20	0.46	0.18	0.31	
	2	12.3		4.92E+08			0.619		24.76			0.002		0.10			
	3	23.3		9.32E+08			0.522		20.88			0.007		0.36			

* DNS Calibration 4 used

Chapter 4.2 - Influence of oxygen supply on cell growth and bioconversion (8)

Data description: Product formation by TVN 493 in 70 ml culture to determine the influence of oxygen supply

Time (h)	Sample	Substrate	Product	Internal std	Each	Average	Each	Std dev.	Average	Observations/ Comments
			Integrated areas on GC		Substrate conc. (mM)		Product conc. (mM) *			
48	1	-	0	n/a	-		0			1% (v/v) hexylbenzene added
	2	-	0	n/a	-	-	0	0	0	1% (v/v) hexylbenzene added
	3	-	0	n/a	-		0			1% (v/v) hexylbenzene added
68	1	-	0	n/a	-		0			
	2	-	0	n/a	-	-	0	0	0	
	3	-	0	n/a	-		0			
75	1	-	0	n/a	-		0			
	2	-	0	n/a	-	-	0	0	0	
	3	-	0	n/a	-		0			
97	1	-	0	n/a	-		0			
	2	-	12217	1140816	-	-	1.895	1.094	0.632	
	3	-	0	n/a			0			
148	1	-	-	-			-			
	2	-	399212	1207131		-	20.169	5.810	16.061	
	3	-	548887	2938061			11.952			

* GC product calibration 2 used

Chapter 4.2 - Influence of oxygen supply on cell growth and bioconversion (9)

Data description: Cultivation of WT in 50ml culture in baffled flasks to determine the influence of oxygen supply

Time (h)	Sample	Cell growth concentration										Glucose concentration (g/l)					Observations/ Comments
		Cell number (cells/ml)					O.D.										
		Cells/ square	Dil. factor	Cell conc.	Std dev.	Average	Absorb. (@510nm)	Dil. factor	Actual OD	Std dev.	Ave.	Absorb.*	Dil. factor	Glucose conc.	Std dev.	Average	
0	Inoculum	8.5	1	3.40E+07	n/a	3.40E+07	0.227	6	1.36	n/a	1.36	-	n/a	-	n/a	-	
3	1	4.0	10	1.60E+08	2.83E+06	1.62E+08	0.384		3.84	0.07	3.79	-	n/a	-	n/a	-	
	2	4.1		1.64E+08			0.374	10	3.74			-		-			
6	1	6.3	10	2.52E+08	2.55E+07	2.70E+08	0.598		5.98	0.04	5.95	-	n/a	-	n/a	-	
	2	7.2		2.88E+08			0.592	10	5.92			-		-			
24	1	9.1	10	3.64E+08	2.83E+06	3.62E+08	0.676		33.80	0.74	33.28	-	n/a	-	n/a	-	1% (v/v) hexylbenzene added
	2	9.0		3.60E+08			0.655	50	32.75			-		-			1% (v/v) hexylbenzene added
27	1	9.9	20	7.92E+08	1.13E+07	7.84E+08	0.565		33.90	6.36	29.40	-	n/a	-	n/a	-	
	2	9.7		7.76E+08			0.415	60	24.90			-		-			
30	1	8.3	25	8.30E+08	1.06E+08	9.05E+08	0.377		26.39	4.26	23.38	-	n/a	-	n/a	-	
	2	9.8		9.80E+08			0.291	70	20.37			-		-			
48	1	14.5	30	1.74E+09	3.39E+07	1.72E+09	0.350		35.00	4.60	31.75	-	n/a	-	n/a	-	
	2	14.1		1.69E+09			0.285	100	28.50			-		-			
54	1	11.1	30	1.33E+09	9.33E+07	1.27E+09	0.362		36.20	3.75	33.55	-	n/a	-	n/a	-	
	2	10.0		1.20E+09			0.309	100	30.90			-		-			
72	1	15.2	30	1.82E+09	2.55E+07	1.81E+09	0.401		40.10	3.54	37.60	-	n/a	-	n/a	-	
	2	14.9		1.79E+09			0.351	100	35.10			-		-			
144	1	18.2	30	2.18E+09	2.55E+07	2.17E+09	0.421		42.10	2.33	40.45	-	n/a	-	n/a	-	
	2	17.9		2.15E+09			0.388	100	38.80			-		-			

DNS Calibration 4 used

Chapter 4.2 - Influence of oxygen supply on cell growth and bioconversion (10)

Data description: Product formation by WT in 50 ml culture in baffled flasks to determine the influence of oxygen supply

Time (h)	Sample	Integrated areas on GC Substrate	Product	Internal std	Substrate conc. (mM) Each	Average	Product conc. (mM) * Each	Std dev.	Average	Observations/ Comments
24	1	-	0	n/a	-	-	0	0	0	1% (v/v) hexylbenzene added
	2	-	0	n/a	-		0			1% (v/v) hexylbenzene added
27	1	-	0	n/a	-	-	0	0	0	
	2	-	0	n/a	-		0			
30	1	-	0	n/a	-	-	0	0	0	
	2	-	0	n/a	-		0			
48	1	-	0	n/a	-	-	0	2.393	1.692	
	2	-	81114	2204769	-		3.385			
54	1	-	116567	1911347	-	-	4.766	0.740	4.243	
	2	-	64860	1520703	-		3.719			
72	1	-	220235	967043	-	-	14.289	4.137	11.363	
	2	-	165250	1318992	-		8.438			
144	1	-	2316629	3445074	-	-	39.684	4.166	42.629	
	2	-	1797694	2317784	-		45.575			

* GC product calibration 2 used

Chapter 4.2 - Influence of oxygen supply on cell growth and bioconversion (11)

Data description: Cultivation of WT in 50ml culture in non-baffled flasks to determine the influence of oxygen supply

Time (h)	Sample	Cell growth concentration										Glucose concentration (g/l)					Observations/ Comments
		Cell number (cells/ml)					O.D.										
		Cells/ square	Dil. factor	Cell conc.	Std dev.	Average	Absorb. (@510nm)	Dil. factor	Actual OD	Std dev.	Ave.	Absorb.*	Dil. factor	Glucose conc.	Std dev.	Average	
0	Inoculum	4.2	1	1.68E+07	n/a	1.68E+07	0.110	10	1.10	n/a	1.10	-	n/a	-	n/a	-	pH = 7.51
3	1	3.3	10	1.32E+08	8.49E+06	1.38E+08	0.290		2.90	0.11	2.98	0.625	40	63.69	16.14	52.28	
	2	3.6		1.44E+08			0.305	10	3.05			0.401		40.87			
6	1	5.7	10	2.28E+08	0.00E+00	2.28E+08	0.494		4.94	0.24	5.11	0.454	40	46.27	7.71	40.81	
	2	5.7		2.28E+08			0.528	10	5.28			0.347		35.36			
24	1	15.6	10	6.24E+08	8.49E+06	6.30E+08	0.621		31.05	0.81	30.48	0.264	40	26.90	2.81	28.89	1% (v/v) hexylbenzene added
	2	15.9		6.36E+08			0.598	50	29.90			0.303		30.88			1% (v/v) hexylbenzene added
27	1	8.6	20	6.88E+08	1.70E+07	7.00E+08	0.327		32.70	1.56	31.60	0.278	40	28.33	0.36	28.08	
	2	8.9		7.12E+08			0.305	100	30.50			0.273		27.82			
30	1	7.9	25	7.90E+08	1.41E+07	7.80E+08	0.331		33.10	0.35	32.85	0.211	40	21.50	1.51	22.57	pH = 7.68
	2	7.7		7.70E+08			0.326	100	32.60			0.232		23.64			
48	1	7.1	30	8.52E+08	1.70E+07	8.40E+08	0.354		35.40	1.27	34.50	0.210	40	21.40	1.15	20.59	
	2	6.9		8.28E+08			0.336	100	33.60			0.194		19.77			
54	1	7.9	30	9.48E+08	1.70E+07	9.36E+08	0.390		39.00	0.99	38.30	0.184	40	18.75	0.72	19.26	
	2	7.7		9.24E+08			0.376	100	37.60			0.194		19.77			
72	1	10.3	30	1.24E+09	5.09E+07	1.20E+09	0.388		38.80	1.70	37.60	0.068	40	6.93	0.36	7.18	
	2	9.7		1.16E+09			0.364	100	36.40			0.073		7.44			
144	1	13.1	30	1.57E+09	4.24E+07	1.54E+09	0.322		32.20	2.40	33.90	0.000	40	0.00	0.00	0.00	pH = 8.38
	2	12.6		1.51E+09			0.356	100	35.60			0.000		0.00			

* DNS Calibration 4 used

Chapter 4.2 - Influence of oxygen supply on cell growth and bioconversion (12)

Data description: Product formation by WT in 50 ml culture in non-baffled flasks to determine the influence of oxygen supply

Time		Subrate and product analysis								Observations/ Comments
		Integrated areas on GC			Substrate conc. (mM)		Product conc. (mM) *			
(h)	Sample	Substrate	Product	Internal std	Each	Average	Each	Std dev.	Average	
24	1	-	0	n/a	-	-	0	0	0	1% (v/v) hexylbenzene added
	2	-	0	n/a	-		0			1% (v/v) hexylbenzene added
27	1	-	0	n/a	-	-	0	0	0	
	2	-	0	n/a	-		0			
30	1	-	0	n/a	-	-	0	0	0	
	2	-	0	n/a	-		0			
48	1	-	0	n/a	-	-	0	0	0	
	2	-	0	n/a	-		0			
54	1	-	0	n/a	-	-	0	1.562	1.104	
	2	-	15631	965016	-		2.209			
72	1	-	11628	624367	-	-	2.347	2.288	3.965	
	2	-	80680	1071544	-		5.583			
144	1	-	195727	1355952	-	-	9.527	1.359	10.488	
	2	-	184899	1038758	-		11.448			

* GC product calibration 2 used

Chapter 4.3 - Influence of glucose concentration on cell growth (1)

Data description: Cultivation of TVN 348 on 16g/l glucose to determine the influence of glucose concentration

Time (h)	Sample	Cells/ square	Dil. factor	Cell conc.	Std dev.	Average	Absorb. (@510nm)	Dil. factor	Actual OD	Std dev.	Ave.	Absorb.*	Dil. factor	Glucose conc.	Std dev.	Average	Observations/ Comments
0	*Inoculum*	-	n/a	-	n/a	-	-	n/a	-	n/a	-	-	n/a	-	n/a	-	
13.5	1	11.6	5	2.32E+08			-		-			0.266		13.55			
	2	7.5	6	1.80E+08	3.31E+07	1.94E+08	0.627	25	15.68	0.35	15.93	0.249	20	12.69	0.65	13.40	
	3	7.1		1.70E+08			0.647		16.18			0.274		13.96			
17.5	1	8.6	7	2.41E+08			0.740		18.50			0.235		11.97			
	2	9.7		2.72E+08	3.51E+07	2.38E+08	0.710	25	17.75	0.43	18.25	0.237	20	12.08	0.36	11.82	
	3	7.2		2.02E+08			0.740		18.50			0.224		11.41			
21.5	1	8.5	8	2.72E+08			0.538		21.52			0.135		6.88			
	2	9.4		3.01E+08	4.93E+07	3.14E+08	0.389	40	15.56	3.65	17.32	0.159	20	8.10	1.10	8.02	
	3	11.5		3.68E+08			0.372		14.88			0.178		9.07			
24.5	1	10.9	9	3.92E+08			0.389		15.56			0.141		7.18			
	2	11.0		3.96E+08	8.00E+07	3.48E+08	0.541	40	21.64	3.06	18.41	0.167	20	8.51	0.67	7.80	
	3	7.1		2.56E+08			0.451		18.04			0.151		7.69			
37.5	1	8.5	11	3.74E+08			0.410		16.40			0.010		0.51			
	2	11.4		5.02E+08	9.84E+07	3.95E+08	0.526	40	21.04	3.92	20.55	0.015	20	0.76	0.21	0.54	
	3	7.0		3.08E+08			0.605		24.20			0.007		0.36			
43	1	-	n/a	-	n/a	-	0.516		20.64			0.000		0.00			
	2	-	n/a	-	n/a	-	0.487	40	19.48	1.39	20.79	0.002	20	0.10	0.06	0.07	
	3	-	n/a	-	n/a	-	0.556		22.24			0.002		0.10			
47.5	1	-	n/a	-	n/a	-	0.549		21.96			0.000		0.00			
	2	-	n/a	-	n/a	-	0.492	40	19.68	1.20	21.04	0.000	20	0.00	0.00	0.00	
	3	-	n/a	-	n/a	-	0.537		21.48			0.000		0.00			
65	1	-	n/a	-	n/a	-	0.530		21.20			0.000		0.00			
	2	-	n/a	-	n/a	-	0.534	40	21.36	2.63	19.76	0.000	20	0.00	0.00	0.00	
	3	-	n/a	-	n/a	-	0.418		16.72			0.000		0.00			

* DNS Calibration 4 used

Chapter 4.3 - Influence of glucose concentration on cell growth (2)

Data description: Cultivation of TVN 348 on 32g/l glucose to determine the influence of glucose concentration

Time (h)	Sample	Cell growth concentration										Glucose concentration (g/l)					Observations/ Comments
		Cell number (cells/ml)					O.D.										
		Cells/ square	Dil. factor	Cell conc.	Std dev.	Average	Absorb. (@510nm)	Dil. factor	Actual OD	Std dev.	Ave.	Absorb.*	Dil. factor	Glucose conc.	Std dev.	Average	
0	Inoculum	-	n/a	-	n/a	-	-	n/a	-	n/a	-	-	n/a	-	n/a	-	
13.5	1	9.1	6	2.18E+08	3.00E+07	1.84E+08	0.687	25	17.18	0.13	17.25	0.287	40	29.25	1.89	28.06	
	2	6.8		1.63E+08			0.687		17.18			0.254		25.88			
	3	7.1		1.70E+08			0.696		17.40			0.285		29.04			
17.5	1	9.4	7	2.63E+08	1.97E+07	2.84E+08	0.814	25	20.35	4.24	15.46	0.282	40	28.74	2.30	27.62	
	2	10.8		3.02E+08			0.529		13.23			0.245		24.97			
	3	10.2		2.86E+08			0.512		12.80			0.286		29.15			
21.5	1	11.4	8	3.65E+08	2.25E+07	3.63E+08	0.604	40	24.16	0.21	23.93	0.214	40	21.81	2.20	24.12	
	2	10.6		3.39E+08			0.597		23.88			0.239		24.36			
	3	12.0		3.84E+08			0.594		23.76			0.257		26.19			
24.5	1	10.3	9	3.71E+08	2.40E+07	3.44E+08	0.584	40	23.36	0.35	23.75	0.237	40	24.15	1.18	24.05	Elongation occurring
	2	9.0		3.24E+08			0.601		24.04			0.247		25.17			
	3	9.4		3.38E+08			0.596		23.84			0.224		22.83			
37.5	1	10.4	11	4.58E+08	9.78E+07	4.03E+08	0.742	40	29.68	1.57	30.01	0.176	40	17.94	0.82	17.12	
	2	6.6		2.90E+08			0.716		28.64			0.160		16.31			
	3	10.5		4.62E+08			0.793		31.72			0.168		17.12			
43	1	-	n/a	-	n/a	-	0.640	50	32.00	2.75	32.72	0.116	40	11.82	0.31	11.89	
	2	-	n/a	-	n/a	-	0.608		30.40			0.114		11.62			
	3	-	n/a	-	n/a	-	0.715		35.75			0.120		12.23			
47.5	1	-	n/a	-	n/a	-	0.622	50	31.10	1.55	29.53	0.077	40	7.85	1.27	8.87	
	2	-	n/a	-	n/a	-	0.590		29.50			0.101		10.29			
	3	-	n/a	-	n/a	-	0.560		28.00			0.083		8.46			
65	1	-	n/a	-	n/a	-	0.610	50	30.50	1.93	32.60	0.000	40	0.00	0.18	0.20	
	2	-	n/a	-	n/a	-	0.660		33.00			0.003		0.31			
	3	-	n/a	-	n/a	-	0.686		34.30			0.003		0.31			

DNS Calibration 4 used

Chapter 4.4 - Influence of alkylbenzene substrate on bioconversion (1)

Data description: Cultivation of TVN 348 for the bioconversion of a range of alkylbenzenes to determine the influence of substrate type

Time (h)	Sample	Cell growth concentration										Glucose concentration (g/l)					Observations/ Comments
		Cell number (cells/ml)					O.D.										
		Cells/ square	Dil. factor	Cell conc.	Std dev.	Average	Absorb. (@620nm)	Dil. factor	Actual OD	Std dev.	Ave.	Absorb. *	Dil. factor	Glucose conc.	Std dev.	Average	
0	Inoculum	8.1	1	3.24E+07	n/a	8.10E+00	0.469	1	0.47	n/a	0.469	-	n/a	-	n/a	-	
7	1	-	n/a	-	n/a	-	0.508	4	2.03	0.07	2.05	0.183	20	14.48	2.42	18.89	
	2	-		-			0.514		2.06			0.229		18.77			
	3	-		-			0.484		1.94			0.259		21.56			
	4	-		-			0.520		2.08			0.239		19.70			
	5	-		-			0.533		2.13			0.227		18.58			
	6	-		-			-		-			0.245		20.26			
24	1	19.1	5	3.82E+08	4.69E+07	3.58E+08	0.315	16	5.04	0.81	6.66	0.104	20	7.11	1.20	7.10	1% (v/v) ethanol added
	2	18.2		3.64E+08			0.440		7.04			0.107		7.39			
	3	21.5		4.30E+08			0.433		6.93			0.106		7.30			
	4	17.8		3.56E+08			0.432		6.91			0.089		5.71			
	5	15.3		3.06E+08			0.451		7.22			0.092		5.99			
	6	15.4		3.08E+08			0.428		6.85			0.125		9.07			
31.5	1	-	n/a	-	n/a	-	0.540	16	8.64	0.30	8.61	0.067	20	3.66	0.72	3.55	
	2	-		-			0.521		8.34			0.063		3.29			
	3	-		-			0.518		8.29			0.072		4.13			
	4	-		-			0.554		8.86			0.073		4.22			
	5	-		-			0.559		8.94			0.054		2.45			
	6	-		-			-		-			-		-			
48	1	11.4	10	4.56E+08	9.95E+07	4.73E+08	0.576	16	9.22	0.25	9.56	0.042	20	1.33	0.36	1.54	0.5% Propylbenzene added
	2	13.4		5.36E+08			0.604		9.66			0.042		1.33			0.5% Butylbenzene added
	3	14.4		5.76E+08			0.610		9.76			0.051		2.17			0.5% Hexylbenzene added
	4	12		4.80E+08			0.586		9.38			0.043		1.42			0.5% Ethyltoluene added
	5	7.9		3.16E+08			0.611		9.78			0.043		1.42			0.5% tert-butyltoluene added
	6	-		-			-		-			-		-			0.5% sec-butylbenzene added

* DNS Calibration 3 used

Chapter 4.4 - Influence of alkylbenzene substrate on bioconversion (2)

Time (h)	Sample	Subrate and product analysis								Observations/ Comments
		Integrated areas on GC			Substrate conc. (mM)		Product conc. (mM) *			
		Substrate	Product	Internal std	Each	Average	Each	Std dev.	Average	
49	1	-	0	n/a	-	-	0	n/a	n/a	
	2	-	0	n/a	-	-	0	n/a	n/a	
	3	-	0	n/a	-	-	0	n/a	n/a	
	4	-	0	n/a	-	-	0	n/a	n/a	
	5	-	0	n/a	-	-	0	n/a	n/a	
	6	-	0	n/a	-	-	0	n/a	n/a	
54	1	-	0	n/a	-	-	0	n/a	n/a	
	2	-	0	n/a	-	-	0	n/a	n/a	
	3	-	0	n/a	-	-	0	n/a	n/a	
	4	-	0	n/a	-	-	0	n/a	n/a	
	5	-	0	n/a	-	-	0	n/a	n/a	
	6	-	0	n/a	-	-	0	n/a	n/a	
72	1	-	0	n/a	-	-	0	n/a	n/a	0.5% Propylbenzene added
	2	-	0	n/a	-	-	0	n/a	n/a	0.5% Butylbenzene added
	3	-	15585	50461	-	-	2.410	n/a	n/a	0.5% Hexylbenzene added
	4	-	0	n/a	-	-	0	n/a	n/a	0.5% Ethyltoluene added
	5	-	0	n/a	-	-	0	n/a	n/a	0.5% *tert*-butyltoluene added
	6	-	0	n/a	-	-	0	n/a	n/a	0.5% *sec*-butylbenzene added
79	1	-	0	n/a	-	-	0	n/a	n/a	
	2	-	0	n/a	-	-	0	n/a	n/a	
	3	-	26786	53538	-	-	3.905	n/a	n/a	
	4	-	0	n/a	-	-	0	n/a	n/a	
	5	-	0	n/a	-	-	0	n/a	n/a	
	6	-	0	n/a	-	-	0	n/a	n/a	
96	1	-	0	n/a	-	-	0	n/a	n/a	0.5% Propylbenzene added
	2	-	0	n/a	-	-	0	n/a	n/a	0.5% Butylbenzene added
	3	-	86960	54031	-	-	12.561	n/a	n/a	0.5% Hexylbenzene added
	4	-	0	n/a	-	-	0	n/a	n/a	0.5% Ethyltoluene added
	5	-	0	n/a	-	-	0	n/a	n/a	0.5% *tert*-butyltoluene added
	6	-	0	n/a	-	-	0	n/a	n/a	0.5% *sec*-butylbenzene added

Data description: Product formation by TVN 348 for the bioconversion of a range of alkylbenzenes to determine the influence of substrate type

Data description: Product formation by TVN 348 for the bioconversion of a range of alkylbenzenes to determine the influence of substrate type

Time		Subrate and product analysis								Observations/ Comments
		Integrated areas on GC			Substrate conc. (mM)		Product conc. (mM) *			
(h)	**Sample**	**Substrate**	**Product**	**Internal std**	**Each**	**Average**	**Each**	**Std dev.**	**Average**	
120	1	-	0	n/a	-	-	0	n/a	n/a	
	2	-	0	n/a	-	-	0	n/a	n/a	
	3	-	69461	36824	-	-	14.721	n/a	n/a	
	4	-	0	n/a	-	-	0	n/a	n/a	
	5	-	0	n/a	-	-	0	n/a	n/a	
	6	-	0	n/a	-	-	0	n/a	n/a	

* GC product calibration 1 used

Chapter 4.4 - Influence of alkylbenzene substrate on bioconversion (3)

Data description: Cultivation of TVN 493 for the bioconversion of a range of alkylbenzenes to determine the influence of substrate type

Time (h)	Sample	Cell growth concentration										Glucose concentration (g/l)					Observations/ Comments
		Cell number (cells/ml)					O.D.										
		Cells/ square	Dil. factor	Cell conc.	Std dev.	Average	Absorb. (@620nm)	Dil. factor	Actual OD	Std dev.	Ave.	Absorb. *	Dil. factor	Glucose conc.	Std dev.	Average	
0	Inoculum	10.9	1	4.36E+07	n/a	4.36E+07	0.453	1	0.45	n/a	0.453	-	n/a	-	n/a	-	
7	1	-	n/a	-	n/a	-	0.485	4	1.94	0.09	1.87	0.232	20	19.05	1.56	17.07	
	2	-		-			0.450		1.80			0.226		18.49			
	3	-		-			0.444		1.78			0.195		15.60			
	4	-		-			0.496		1.98			0.193		15.41			
	5	-		-			0.446		1.78			0.218		17.74			
	6	-		-			0.481		1.92			0.201		16.16			
24	1	7.7	5	1.54E+08	5.68E+07	2.56E+08	0.411	16	6.58	0.43	7.20	0.107	20	7.39	1.29	6.93	1% (v/v) ethanol added
	2	13.5		2.70E+08			0.441		7.06			0.117		8.32			
	3	12.9		2.58E+08			0.470		7.52			0.097		6.46			
	4	16.0		3.20E+08			0.485		7.76			0.079		4.78			
	5	12.2		2.44E+08			0.434		6.94			0.114		8.04			
	6	14.6		2.92E+08			0.460		7.36			0.098		6.55			
31.5	1	-	n/a	-	n/a	-	0.485	16	7.76	0.62	8.61	0.075	20	4.41	0.88	3.13	
	2	-		-			0.491		7.86			0.070		3.94			
	3	-		-			0.562		8.99			0.056		2.64			
	4	-		-			0.564		9.02			0.056		2.64			
	5	-		-			0.558		8.93			0.061		3.10			
	6	-		-			0.567		9.07			0.050		2.08			
48	1	9.2	10	3.68E+08	8.29E+07	4.33E+08	0.488	16	7.81	0.57	8.67	0.043	20	1.42	0.13	1.56	Analyses done before substrate addition
	2	10.7		4.28E+08			0.572		9.15			0.046		1.70			
	3	13.8		5.52E+08			0.559		8.94			0.043		1.42			
	4	12.9		5.16E+08			0.511		8.18			0.046		1.70			
	5	9.3		3.72E+08			0.577		9.23			0.045		1.61			
	6	9		3.60E+08			0.546		8.74			0.044		1.52			

* DNS Calibration 3 used

Chapter 4.4 - Influence of alkylbenzene substrate on bioconversion (4)

Time		Subrate and product analysis								Observations/ Comments
		Integrated areas on GC			Substrate conc. (mM)		Product conc. (mM) *			
(h)	Sample	Substrate	Product	Internal std	Each	Average	Each	Std dev.	Average	

Data description: Product formation by TVN 493 for the bioconversion of a range of alkylbenzenes to determine the influence of substrate type

Time (h)	Sample	Substrate	Product	Internal std	Each	Average	Each	Std dev.	Average	Observations/ Comments
49	1	-	0	n/a	-	-	0	n/a	n/a	
	2	-	0	n/a	-	-	0	n/a	n/a	
	3	-	0	n/a	-	-	0	n/a	n/a	
	4	-	0	n/a	-	-	0	n/a	n/a	
	5	-	0	n/a	-	-	0	n/a	n/a	
	6	-	0	n/a	-	-	0	n/a	n/a	
54	1	-	0	n/a	-	-	0	n/a	n/a	
	2	-	0	n/a	-	-	0	n/a	n/a	
	3	-	0	n/a	-	-	0	n/a	n/a	
	4	-	0	n/a	-	-	0	n/a	n/a	
	5	-	0	n/a	-	-	0	n/a	n/a	
	6	-	0	n/a	-	-	0	n/a	n/a	
72	1	-	0	n/a	-	-	0	n/a	n/a	0.5% Propylbenzene added
	2	-	0	n/a	-	-	0	n/a	n/a	0.5% Butylbenzene added
	3	-	17257	50651	-	-	2.659	n/a	n/a	0.5% Hexylbenzene added
	4	-	0	n/a	-	-	0	n/a	n/a	0.5% Ethyltoluene added
	5	-	0	n/a	-	-	0	n/a	n/a	0.5% tert-butyltoluene added
	6	-	0	n/a	-	-	0	n/a	n/a	0.5% sec-butylbenzene added
79	1	-	0	n/a	-	-	0	n/a	n/a	
	2	-	0	n/a	-	-	0	n/a	n/a	
	3	-	35510	49395	-	-	5.611	n/a	n/a	
	4	-	0	n/a	-	-	0	n/a	n/a	
	5	-	0	n/a	-	-	0	n/a	n/a	
	6	-	0	n/a	-	-	0	n/a	n/a	
96	1	-	0	n/a	-	-	0	n/a	n/a	0.5% Propylbenzene added
	2	-	0	n/a	-	-	0	n/a	n/a	0.5% Butylbenzene added
	3	-	81661	48365	-	-	13.177	n/a	n/a	0.5% Hexylbenzene added
	4	-	0	n/a	-	-	0	n/a	n/a	0.5% Ethyltoluene added
	5	-	0	n/a	-	-	0	n/a	n/a	0.5% tert-butyltoluene added
	6	-	0	n/a	-	-	0	n/a	n/a	0.5% sec-butylbenzene added

Data description: Product formation by TVN 493 for the bioconversion of a range of alkylbenzenes to determine the influence of substrate type

Time		Subrate and product analysis								Observations/ Comments
		Integrated areas on GC			Substrate conc. (mM)		Product conc. (mM) *			
(h)	Sample	Substrate	Product	Internal std	Each	Average	Each	Std dev.	Average	
120	1	-	0	n/a	-	-	0	n/a	n/a	
	2	-	0	n/a	-	-	0	n/a	n/a	
	3	-	63128	34943	-	-	14.099	n/a	n/a	
	4	-	0	n/a	-	-	0	n/a	n/a	
	5	-	0	n/a	-	-	0	n/a	n/a	
	6	-	0	n/a	-	-	0	n/a	n/a	

GC product calibration 1 used

Chapter 4.4 - Influence of alkylbenzene substrate on bioconversion (5)

Data description: Cultivation of WT for the bioconversion of hexylbenzene to determine the influence of substrate type

Time	Sample	Cell growth concentration										Glucose concentration (g/l)					Observations/ Comments
		Cell number (cells/ml)					O.D.										
(h)		Cells/ square	Dil. factor	Cell conc.	Std dev.	Average	Absorb. (@510nm)	Dil. factor	Actual OD	Std dev.	Ave.	Absorb.*	Dil. factor	Glucose conc.	Std dev.	Average	
0	Inoculum	8.5	1	3.40E+07	n/a	3.40E+07	0.227	6	1.36	n/a	1.36	-	n/a	-	n/a	-	
3	1	4.0	10	1.60E+08	2.83E+06	1.62E+08	0.384	10	3.84	0.07	3.79	-	n/a	-	n/a	-	
	2	4.1		1.64E+08			0.374		3.74			-		-			
6	1	6.3	10	2.52E+08	2.55E+07	2.70E+08	0.598	10	5.98	0.04	5.95	-	n/a	-	n/a	-	
	2	7.2		2.88E+08			0.592		5.92			-		-			
24	1	9.1	10	3.64E+08	2.83E+06	3.62E+08	0.676	50	33.80	0.74	33.28	-	n/a	-	n/a	-	1% (v/v) hexylbenzene added
	2	9.0		3.60E+08			0.655		32.75			-		-			
27	1	9.8	20	7.84E+08	5.66E+06	7.80E+08	0.565	60	33.90	6.36	29.40	-	n/a	-	n/a	-	
	2	9.7		7.76E+08			0.415		24.90			-		-			
30	1	9.9	25	9.90E+08	7.07E+06	9.85E+08	0.377	70	26.39	4.26	23.38	-	n/a	-	n/a	-	
	2	9.8		9.80E+08			0.291		20.37			-		-			
48	1	14.5	30	1.74E+09	3.39E+07	1.72E+09	0.350	100	35.00	4.60	31.75	-	n/a	-	n/a	-	
	2	14.1		1.69E+09			0.285		28.50			-		-			
54	1	11.1	30	1.33E+09	9.33E+07	1.27E+09	0.362	100	36.20	3.75	33.55	0.224	40	22.83	4.90	19.36	
	2	10.0		1.20E+09			0.309		30.90			0.156		15.90			
72	1	15.2	30	1.82E+09	2.55E+07	1.81E+09	0.401	100	40.10	3.54	37.60	0.118	40	12.03	0.86	11.41	
	2	14.9		1.79E+09			0.351		35.10			0.106		10.80			
144	1	18.2	30	2.18E+09	2.55E+07	2.17E+09	0.421	100	42.10	2.33	40.45	0.001	40	0.10	0.79	0.66	
	2	17.9		2.15E+09			0.388		38.80			0.012		1.22			

DNS Calibration 4 used

Chapter 4.4 - Influence of alkylbenzene substrate on bioconversion (6)

Data description: Product formation by WT for the bioconversion of hexylbenzene to determine the influence of substrate type

Time (h)	Sample	Integrated areas on GC Substrate	Integrated areas on GC Product	Integrated areas on GC Internal std	Substrate conc. (mM) Each	Substrate conc. (mM) Average	Product conc. (mM) * Each	Product conc. (mM) * Std dev.	Product conc. (mM) * Average	Observations/ Comments
24	1	-	0	n/a	-	-	0	0	0	1% (v/v) hexylbenzene added
	2	-	0	n/a	-		0			
27	1	-	0	n/a	-	-	0	0	0	
	2	-	0	n/a	-		0			
30	1	-	0	n/a	-	-	0	0	0	
	2	-	0	n/a	-		0			
48	1	-	0	n/a	-	-	0	2.393	1.692	
	2	-	81114	2204769	-		3.385			
54	1	-	116567	1911347	-	-	4.766	0.740	4.243	
	2	-	64860	1520703	-		3.719			
72	1	-	220235	967043	-	-	14.289	4.137	11.363	
	2	-	165250	1318992	-		8.438			
144	1	-	2316629	3445074	-	-	39.684	4.166	42.629	
	2	-	1797694	2317784	-		45.575			

* GC product calibration 2 used

Chapter 4.4 - Influence of alkylbenzene substrate on bioconversion (7)

Data description: Cultivation of WT for the bioconversion of octylbenzene to determine the influence of substrate type

Time	Sample	Cell growth concentration										Glucose concentration (g/l)					Observations/ Comments
		Cell number (cells/ml)					O.D.										
(h)		Cells/ square	Dil. factor	Cell conc.	Std dev.	Average	Absorb. (@510nm)	Dil. factor	Actual OD	Std dev.	Ave.	Absorb.*	Dil. factor	Glucose conc.	Std dev.	Average	
0	Inoculum	6.5	1	2.60E+07	n/a	2.60E+07	0.221	6	1.33	n/a	1.33	0.320	40	32.61	n/a	32.61	
3	1	4.2	10	1.68E+08	1.41E+07	1.58E+08	0.287		2.87	0.06	2.91	0.311	40	31.69	n/a	31.80	
	2	3.7		1.48E+08			0.295	10	2.95			0.313		31.90			
6	1	4.6	10	1.84E+08	1.13E+07	1.76E+08	0.421		4.21	0.29	4.42	0.298	40	30.37	n/a	30.52	
	2	4.2		1.68E+08			0.462	10	4.62			0.301		30.67			
24	1	11.0	10	4.40E+08	5.66E+06	4.44E+08	0.514		25.70	2.79	27.68	0.243	40	24.76	n/a	24.81	1% (v/v) octylbenzene added
	2	11.2		4.48E+08			0.593	50	29.65			0.244		24.87			
27	1	5.9	20	4.72E+08	1.13E+07	4.80E+08	0.255		15.30	1.87	16.62	0.215	40	21.91	n/a	22.17	
	2	6.1		4.88E+08			0.299	60	17.94			0.220		22.42			
30	1	6.4	25	6.40E+08	4.24E+07	6.70E+08	0.301		21.07	0.20	21.21	0.197	40	20.08	n/a	20.53	
	2	7.0		7.00E+08			0.305	70	21.35			0.206		20.99			
48	1	6.7	30	8.04E+08	2.55E+07	7.86E+08	0.403		40.30	0.92	40.95	0.072	40	7.34	n/a	7.03	
	2	6.4		7.68E+08			0.416	100	41.60			0.066		6.73			
54	1	7.4	30	8.88E+08	1.70E+07	9.00E+08	0.454		45.40	1.27	46.30	0.039	40	3.97	n/a	3.87	
	2	7.6		9.12E+08			0.472	100	47.20			0.037		3.77			
72	1	9.8	30	1.18E+09	8.49E+06	1.18E+09	0.515		51.50	0.49	51.15	0.011	40	1.12	n/a	1.07	
	2	9.9		1.19E+09			0.508	100	50.80			0.010		1.02			
144	1	9.4	30	1.13E+09	8.49E+06	1.12E+09	0.488		48.80	0.64	48.35	0.000	40	0.00	n/a	0.00	
	2	9.3		1.12E+09			0.479	100	47.90			0.000		0.00			

* DNS Calibration 4 used

Chapter 4.4 - Influence of alkylbenzene substrate on bioconversion (8)

Data description: Product formation by WT for the bioconversion of octylbenzene to determine the influence of substrate type

Time	Sample	Subrate and product analysis								Observations/ Comments
		Integrated areas on GC			Substrate conc. (mM)		Product conc. (mM) *			
(h)		Substrate	Product	Internal std	Each	Average	Each	Std dev.	Average	
24	1	-	0	n/a	-	-	0	0	0	1% (v/v) octylbenzene added
	2	-	0	n/a	-		0			
27	1	-	0	n/a	-	-	0	0	0	
	2	-	0	n/a	-		0			
30	1	-	0	n/a	-	-	0	0	0	
	2	-	0	n/a	-		0			
48	1	-	0	n/a	-	-	0	0	0	
	2	-	0	n/a	-		0			
54	1	-	106.0	13721.0	-	-	0.650	0.123	0.563	
	2	-	41.3	7297.8	-		0.476			
72	1	-	280.6	8504.8	-	-	2.775	0.181	2.647	
	2	-	386.0	12889.0	-		2.519			
144	1	-	859.1	7499.4	-	-	9.635	0.807	9.064	
	2	-	846.1	8377.9	-		8.494			

* GC product calibration 3 used

Chapter 4.4 - Influence of alkylbenzene substrate on bioconversion (9)

Data description: Cultivation of WT for the bioconversion of decylbenzene to determine the influence of substrate type

Time (h)	Sample	Cell growth concentration										Glucose concentration (g/l)					Observations/ Comments
		Cell number (cells/ml)					O.D.										
		Cells/ square	Dil. factor	Cell conc.	Std dev.	Average	Absorb. (@510nm)	Dil. factor	Actual OD	Std dev.	Ave.	Absorb.*	Dil. factor	Glucose conc.	Std dev.	Average	
0	*Inoculum*	6.5	1	2.60E+07	n/a	2.60E+07	0.227	6	1.36	n/a	1.36	0.320	40	32.61	n/a	32.61	
3	1	4.2	10	1.68E+08	8.49E+06	1.62E+08	0.279		2.79	0.17	2.67	0.314	40	32.00	0.29	32.20	
	2	3.9		1.56E+08			0.255	10	2.55			0.318		32.41			
6	1	4.3	10	1.72E+08	8.49E+06	1.66E+08	0.446		4.46	0.23	4.30	0.273	40	27.82	0.58	28.23	
	2	4.0		1.60E+08			0.413	10	4.13			0.281		28.64			
24	1	12.0	10	4.80E+08	5.66E+06	4.76E+08	0.554		27.70	0.78	28.25	0.218	40	22.22	0.22	22.37	1% (v/v) decylbenzene added
	2	11.8		4.72E+08			0.576	50	28.80			0.221		22.52			
27	1	6.4	20	5.12E+08	5.66E+06	5.16E+08	0.299		17.94	1.61	16.80	0.201	40	20.48	0.29	20.69	
	2	6.5		5.20E+08			0.261	60	15.66			0.205		20.89			
30	1	7.1	25	7.10E+08	1.41E+07	7.20E+08	0.303		21.21	0.40	21.49	0.171	40	17.43	0.79	17.99	
	2	7.3		7.30E+08			0.311	70	21.77			0.182		18.55			
48	1	6.6	30	7.92E+08	4.24E+07	8.22E+08	0.444		44.40	0.07	44.45	0.031	40	3.16	0.43	3.46	
	2	7.1		8.52E+08			0.445	100	44.50			0.037		3.77			
54	1	6.4	30	7.68E+08	2.55E+07	7.86E+08	0.490		49.00	1.13	48.20	0.011	40	1.12	0.07	1.07	
	2	6.7		8.04E+08			0.474	100	47.40			0.010		1.02			
72	1	8.3	30	9.96E+08	3.39E+07	1.02E+09	0.494		49.40	0.42	49.10	0.000	40	0.00	0.00	0.00	
	2	8.7		1.04E+09			0.488	100	48.80			0.000		0.00			
144	1	8.5	30	1.02E+09	8.49E+06	1.03E+09	0.501		50.10	0.35	49.85	0.000	40	0.00	0.00	0.00	
	2	8.6		1.03E+09			0.496	100	49.60			0.000		0.00			

* DNS Calibration 4 used

<u>Chapter 4.4 - Influence of alkylbenzene substrate on bioconversion (10)</u>

Data description: Product formation by WT for the bioconversion of decylbenzene to determine the influence of substrate type

Time	Sample	Subrate and product analysis								Observations/ Comments
		Integrated areas on GC			Substrate conc. (mM)		Product conc. (mM) *			
(h)	Sample	Substrate	Product	Internal std	Each	Average	Each	Std dev.	Average	Observations/ Comments
24	1	-	0	n/a	-	-	0	0	0	1% (v/v) decylbenzene added
	2	-	0	n/a	-		0			
27	1	-	0	n/a	-	-	0	0	0	
	2	-	0	n/a	-		0			
30	1	-	0	n/a	-	-	0	0	0	
	2	-	0	n/a	-		0			
48	1	-	0	n/a	-	-	0	0	0	
	2	-	0	n/a	-		0			
54	1	-	-	-	-	-	-	0	0	
	2	-	-	-	-		-			
72	1	-	-	-	-	-	-	-	1.989	Only final data provided
	2	-	-	-	-		-			
144	1	-	-	-	-	-	-	-	7.021	
	2	-	-	-	-		-			

* GC product calibration 3 used

Chapter 4.5 - Inducer requirement (1)

Data description: Cultivation of TVN 348 using a range of possible inducers to determine inducer requirements

Time (h)	Sample	Cells/ square	Dil. factor	Cell conc.	Std dev.	Average	Absorb. (@620nm)	Dil. factor	Actual OD	Std dev.	Ave.	Absorb. *	Dil. factor	Glucose conc.	Std dev.	Average	Observations/ Comments
0	*Inoculum*	-	n/a	-	n/a	-	0.471	1	0.47	n/a	0.471	-	n/a	-	n/a	-	
6	1	-		-			0.303		1.21			0.119		8.51			
	2	-	n/a	-	n/a	-	0.300	4	1.20	0.01	1.22	0.118	20	8.42	1.16	7.80	
	3	-		-			0.313		1.25			0.097		6.46			
24	1	-		-			0.405		6.48			0.078		4.69			1% (v/v) Ethanol added
	2	-	n/a	-	n/a	-	0.452	16	7.23	n/a	n/a	0.068	20	3.76	n/a	n/a	1% (v/v) Oleic acid added
	3	-		-			0.440		7.04			0.091		5.90			1% (v/v) Glucose added
48	1	-		-			0.593		9.49			0.057		2.73			
	2	-	n/a	-	n/a	-	0.785	16	12.56	n/a	n/a	0.045	20	1.61	n/a	n/a	
	3	-		-			0.610		9.76			0.047		1.80			

Cell growth concentration spans the Cell number and O.D. groups; *Cell number (cells/ml)* spans Cells/square through Average; *O.D.* spans Absorb. through Ave.; *Glucose concentration (g/l)* spans Absorb.* through Average.

* DNS Calibration 3 used

Chapter 4.5 - Inducer requirement (2)

Data description: Product formation byTVN 348 using a range of possible inducers to determine inducer requirements

Time	Sample	Subrate and product analysis								Observations/ Comments
		Integrated areas on GC			Substrate conc. (mM)		Product conc. (mM) *			
(h)	Sample	Substrate	Product	Internal std	Each	Average	Each	Std dev.	Average	
52	1	-	0	n/a	-		0		n/a	(1% ethanol)
	2	-	0	n/a	-	-	0	n/a	n/a	(1% oleic acid)
	3	-	0	n/a	-		0		n/a	(1% glucose)
77	1	-	0	n/a	-		0		n/a	
	2	-	0	n/a	-	-	0	n/a	n/a	
	3	-	0	n/a	-		0		n/a	
96	1	-	48893	26349	-		14.482		n/a	
	2	-	0	0	-	-	0	n/a	n/a	
	3	-	0	0	-		0		n/a	
120	1	-	87670	24898	-		27.480		n/a	
	2	-	104706	25957	-	-	31.481	n/a	n/a	
	3	-	83353	25993	-		25.026		n/a	

* GC product calibration 1 used

E.2 Chapter 5
Chapter 5.1 - Identification of the most suitable strain for cell growth and bioconversion of hexylbenzene (1)

Data description: Cultivation of TVN 348 using the model system for strain selection

Time (h)	Sample	Cells/ square	Dil. factor	Cell conc.	Std dev.	Average	Absorb. (@620nm)	Dil. factor	Actual OD	Std dev.	Ave.	Absorb. *	Dil. factor	Glucose conc.	Std dev.	Average	Observations/ Comments
0	Inoculum	10.6	1	4.24E+07	n/a	4.24E+07	0.42	4	1.68	n/a	1.68	-	n/a	-	n/a	-	
4	1	6.5	2	5.20E+07	6.40E+06	5.20E+07	0.229	20	4.58	0.45	4.25	-	n/a	-	n/a	-	
	2	7.3		5.84E+07			0.222		4.44			-		-			
	3	5.7		4.56E+07			0.187		3.74			-		-			
15	1	11.3	5	2.26E+08	1.22E+07	2.13E+08	0.62	30	18.60	0.70	18.37	-	n/a	-	n/a	-	
	2	10.6		2.12E+08			0.631		18.93			-		-			
	3	8.4	6	2.02E+08			0.586		17.58			-		-			
20	1	9.1	8	2.91E+08	1.29E+07	2.89E+08	0.728	30	21.84	0.45	21.78	-	n/a	-	n/a	-	
	2	8.6		2.75E+08			0.74		22.20			-		-			
	3	9.4		3.01E+08			0.71		21.30			-		-			
24	1	6.8	9	2.45E+08	7.69E+07	3.01E+08	0.583	40	23.32	0.59	24.00	-	n/a	-	n/a	-	1% (v/v) Hexylbenzene added
	2	7.5		2.70E+08			0.607		24.28			-		-			
	3	10.8		3.89E+08			0.61		24.40			-		-			Lots of dividing cells
42	1	10.4	11	4.58E+08	1.00E+08	4.87E+08	0.487	50	24.35	0.10	24.38	-	n/a	-	n/a	-	
	2	9.2		4.05E+08			0.49		24.50			-		-			
	3	13.6		5.98E+08			0.486		24.30			-		-			
50	1	8.5	13	4.42E+08	7.72E+07	4.61E+08	0.592	40	23.68	0.58	23.08	-	n/a	-	n/a	-	
	2	10.5		5.46E+08			0.576		23.04			-		-			
	3	7.6		3.95E+08			0.563		22.52			-		-			
66	1	8.3	13	4.32E+08	7.66E+07	5.15E+08	0.783	40	31.32	1.20	30.88	-	n/a	-	n/a	-	
	2	11.2		5.82E+08			0.738		29.52			-		-			
	3	10.2		5.30E+08			0.795		31.80			-		-			
145	1	11.8	11	5.19E+08	6.05E+07	4.66E+08	0.581	40	23.24	0.34	23.20	-	n/a	-	n/a	-	
	2	10.9		4.80E+08			0.588		23.52			-		-			
	3	9.1		4.00E+08			0.571		22.84			-		-			
161	1	8.2	11	3.61E+08	3.96E+07	4.00E+08	0.557	40	22.28	0.24	22.33	-	n/a	-	n/a	-	
	2	10		4.40E+08			0.565		22.60			-		-			
	3	9.1		4.00E+08			0.553		22.12			-		-			*DNS calibration 4 used

Chapter 5.1 - Identification of the most suitable strain for cell growth and bioconversion of hexylbenzene (2)

Data description: Product formation by TVN 348 using the model system for strain selection

Time (h)	Sample	Substrate	Product	Internal std	Each	Average	Each	Std dev.	Average	Observations/ Comments
			Integrated areas on GC		Substrate conc. (mM)		Product conc. (mM) *			
24	1	-	0	n/a	-		0			
	2	-	0	n/a	-	-	0	n/a	0	1 % (v/v) Hexylbenzene added
	3	-	0	n/a	-		0			
42	1	-	0	n/a	-		0			
	2	-	0	n/a	-	-	0	n/a	0	
	3	-	0	n/a	-		0			
50	1	-	0	n/a	-		0			
	2	-	0	n/a	-	-	0	n/a	0	
	3	-	0	n/a	-		0			
66	1	-	536625	1892376	-		17.48			
	2	-	-	-	-	-	-	-	17.47708	
	3	-	-	-	-		-			
145	1	-	543464	1076496	-		30.11			
	2	-	1488709	1754260	-	-	49.74	12.680	44.563	
	3	-	1531559	1664356	-		53.83			
161	1	-	969482	1297467	-		43.95			
	2	-	667001	1801163	-	-	22.43	11.015	34.548	
	3	-	899210	1427283	-		37.26			

* GC product calibration 2 used

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Chapter 5.1 - Identification of the most suitable strain for cell growth and bioconversion of hexylbenzene (3)

Data description: Cultivation of TVN 493 using the model system for strain selection

Time (h)	Sample	Cell growth concentration										Glucose concentration (g/l)					Observations/ Comments
		Cell number (cells/ml)					O.D.										
		Cells/ square	Dil. factor	Cell conc.	Std dev.	Average	Absorb. (@620nm)	Dil. factor	Actual OD	Std dev.	Ave.	Absorb. *	Dil. factor	Glucose conc.	Std dev.	Average	
0	*Inoculum*	9.7	1	3.88E+07	n/a	3.88E+07	0.472	4	1.89	n/a	1.89	-	n/a	-	n/a	-	
4	1	6.2	2	4.96E+07	1.17E+07	5.09E+07	0.263	20	5.26	0.46	4.73	-	n/a	-	n/a	-	
	2	7.9		6.32E+07			0.22		4.40			-		-			
	3	5		4.00E+07			0.227		4.54			-		-			
15	1	9.6	6	2.30E+08	9.70E+06	2.25E+08	0.631	30	18.93	0.14	19.06	-	n/a	-	n/a	-	
	2	8.9		2.14E+08			0.635		19.05			-		-			
	3	9.6		2.30E+08			0.64		19.20			-		-			
20	1	13.1	8	4.19E+08	1.21E+08	2.82E+08	0.728	30	21.84	0.33	22.03	-	n/a	-	n/a	-	
	2	7.3		2.34E+08			0.747		22.41			-		-			
	3	6		1.92E+08			0.728		21.84			-		-			
24	1	8.2	9	2.95E+08	1.28E+08	3.82E+08	0.625	40	25.00	n/a	25.00	-	n/a	-	n/a	-	1% (v/v) Hexylbenzene added
	2	14.7		5.29E+08			-		-			-		-			
	3	8.9		3.20E+08			-		-			-		-			
42	1	15.5	11	6.82E+08	1.14E+08	5.57E+08	0.478	50	23.90	0.46	23.77	-	n/a	-	n/a	-	
	2	10.4		4.58E+08			0.483		24.15			-		-			
	3	12.1		5.32E+08			0.465		23.25			-		-			
50	1	7.7	13	4.00E+08	3.18E+07	4.35E+08	0.619	40	24.76	1.76	24.33	-	n/a	-	n/a	-	
	2	8.9		4.63E+08			0.646		25.84			-		-			
	3	8.5		4.42E+08			0.56		22.40			-		-			
66	1	9.9	13	5.15E+08	3.64E+07	4.89E+08	0.816	40	32.64	0.57	32.29	-	n/a	-	n/a	-	
	2	8.6		4.47E+08			0.815		32.60			-		-			
	3	9.7		5.04E+08			0.791		31.64			-		-			
145	1	9.5	11	4.18E+08	1.21E+08	4.62E+08	0.619	40	24.76	1.49	23.16	-	n/a	-	n/a	-	
	2	8.4		3.70E+08			0.545		21.80			-		-			
	3	13.6		5.98E+08			0.573		22.92			-		-			
161	1	8.6	11	3.78E+08	3.84E+07	4.11E+08	0.628	40	25.12	2.02	23.17	-	n/a	-	n/a	-	
	2	9.1		4.00E+08			0.527		21.08			-		-			
	3	10.3		4.53E+08			0.583		23.32			-		-			*DNS calibration 4 used*

Chapter 5.1 - Identification of the most suitable strain for cell growth and bioconversion of hexylbenzene (4)

Data description: Product formation by TVN 493 using the model system for strain selection

Time (h)	Sample	Substrate and product analysis								Observations/ Comments
		Integrated areas on GC			Substrate conc. (mM)		Product conc. (mM) *			
		Substrate	Product	Internal std	Each	Average	Each	Std dev.	Average	
24	1	-	0	n/a	-		0			
	2	-	0	n/a	-	-	0	n/a	0	1 % (v/v) Hexylbenzene added
	3	-	0	n/a	-		0			
42	1	-	0	n/a	-		0			
	2	-	0	n/a	-	-	0	n/a	0	
	3	-	0	n/a	-		0			
50	1	-	0	n/a	-		0			
	2	-	0	n/a	-	-	0	n/a	0	
	3	-	0	n/a	-		0			
66	1	-	-	-	-		-			
	2	-	-	-	-	-	-	-	-	
	3	-	-	-	-		-			
145	1	-	651009	1055895	-		36.49			
	2	-	985102	810747	-	-	70.67	20.919	46.614	
	3	-	698823	1270981	-		32.68			
161	1	-	841934	1002731	-		49.23			
	2	-	812628	1342102	-	-	35.86	7.804	40.222	
	3	-	609948	1015792	-		35.57			

* GC product calibration 2 used

Chapter 5.1 - Identification of the most suitable strain for cell growth and bioconversion of hexylbenzene (5)

Data description: Cultivation of WT using the model system for strain selection

Time (h)	Sample	Cells/ square	Dil. factor	Cell conc.	Std dev.	Average	Absorb. (@620nm)	Dil. factor	Actual OD	Std dev.	Ave.	Absorb. *	Dil. factor	Glucose conc.	Std dev.	Average	Observations/ Comments
				Cell growth concentration								Glucose concentration (g/l)					
				Cell number (cells/ml)			O.D.										
0	Inoculum	9.8	1	3.92E+07	n/a	3.92E+07	0.445	4	1.78	n/a	1.78	-	n/a	-	n/a	-	
4	1	4.6	2	3.68E+07	5.25E+06	4.16E+07	0.201	20	4.02	0.49	4.54	-	n/a	-	n/a	-	
	2	5.9		4.72E+07			0.23		4.60			-		-			
	3	5.1		4.08E+07			0.25		5.00			-		-			
15	1	7.5	6	1.80E+08	2.77E+07	2.02E+08	0.593	30	17.79	0.20	17.59	-	n/a	-	n/a	-	
	2	9.7		2.33E+08			0.58		17.40			-		-			
	3	8		1.92E+08			0.586		17.58			-		-			
20	1	7.9	8	2.53E+08	2.08E+07	2.54E+08	0.66	30	19.80	0.93	20.56	-	n/a	-	n/a	-	
	2	8.6		2.75E+08			0.676		20.28			-		-			
	3	7.3		2.34E+08			0.72		21.60			-		-			
24	1	8.9	9	3.20E+08	1.45E+07	3.23E+08	-	40	-	0.14	23.22	-	n/a	-	n/a	-	1% (v/v) Hexylbenzene added
	2	9.4		3.38E+08			0.583		23.32			-		-			
	3	8.6		3.10E+08			0.578		23.12			-		-			
42	1	12.7	11	5.59E+08	5.62E+07	5.10E+08	0.436	50	21.80	1.46	23.47	-	n/a	-	n/a	-	
	2	11.9		5.24E+08			0.481		24.05			-		-			
	3	10.2		4.49E+08			0.491		24.55			-		-			
50	1	6.9	13	3.59E+08	2.38E+07	3.64E+08	0.565	40	22.60	1.24	23.93	-	n/a	-	n/a	-	Smaller elongated cells
	2	7.5		3.90E+08			0.604		24.16			-		-			
	3	6.6		3.43E+08			0.626		25.04			-		-			
66	1	6.4	13	3.33E+08	3.54E+07	3.61E+08	0.712	40	28.48	0.80	28.56	-	n/a	-	n/a	-	Smaller elongated cells still occuring
	2	7.7		4.00E+08			0.735		29.40			-		-			
	3	6.7		3.48E+08			0.695		27.80			-		-			
145	1	8.2	11	3.61E+08	5.00E+07	3.96E+08	0.621	40	24.84	0.71	25.65	-	n/a	-	n/a	-	
	2	10.3		4.53E+08			0.653		26.12			-		-			
	3	8.5		3.74E+08			0.65		26.00			-		-			
161	1	9.3	11	4.09E+08	2.45E+07	4.31E+08	0.588	40	23.52	1.50	25.19	-	n/a	-	n/a	-	
	2	10.4		4.58E+08			0.64		25.60			-		-			
	3	9.7		4.27E+08			0.661		26.44			-		-			*DNS calibration 4 used

Chapter 5.1 - Identification of the most suitable strain for cell growth and bioconversion of hexylbenzene (6)

Data description: Product formation by WT using the model system for strain selection

Time (h)	Sample	Integrated areas on GC - Substrate	Integrated areas on GC - Product	Integrated areas on GC - Internal std	Substrate conc. (mM) Each	Substrate conc. (mM) Average	Product conc. (mM) * Each	Product conc. (mM) * Std dev.	Product conc. (mM) * Average	Observations/ Comments
24	1	-	0	n/a	-		0			
	2	-	0	n/a	-	-	0	n/a	0	1 % (v/v) Hexylbenzene added
	3	-	0	n/a	-		0			
42	1	-	0	n/a	-		0			
	2	-	0	n/a	-	-	0	n/a	0	
	3	-	0	n/a	-		0			
50	1	-	0	n/a	-		0			
	2	-	0	n/a	-	-	0	n/a	0	
	3	-	0	n/a	-		0			
66	1	-	-	-	-		-			
	2	-	-	-	-	-	-	-	-	
	3	-	-	-	-		-			
145	1	-	1011102	1404509	-		42.39			
	2	-	1536922	1328821	-	-	67.33	17.36	47.89	
	3	-	862502	1508580	-		33.93			
161	1	-	1201154	1322869	-		53.13			
	2	-	1256215	1017694	-	-	71.77	16.22	54.79	
	3	-	690743	1033318	-		39.46			

* GC product calibration 2 used

Chapter 5.2 – Bioreactor studies (1)

Data description: Cultivation of WT in a bioreactor - 3.2% initial glucose, no Hexylbenzene added

Time	Cell growth concentration						Glucose concentration (g/l)			Process conditions			
	Cell number (cells/ml)			O.D.									
	Cells/ square	Dil. factor	Cell conc.	Absorb. (@620nm)	Dil. factor	Actual OD	Absorb. *	Dil. factor	Glucose conc.	pH	Agitation (rpm)	DO (%)	Observations/ Comments
(h)													
0.00	7.7	1	3.08E+07	0.104	20	2.08	0.306	40	31.18	7.83	400	60	
2.00	10.9	1	4.36E+07	0.143	20	2.86	0.289	40	29.45	7.75	400	70	
4.17	6.9	2	5.52E+07	0.244	20	4.88	0.301	40	30.67	7.72	400	64	
6.00	6.9	3	8.28E+07	0.518	20	10.36	0.299	40	30.47	7.74	400	68	
22.08	7.6	10	3.04E+08	0.568	40	22.72	0.280	40	28.53	8.09	400	77	
24.17	4.9	11	2.16E+08	0.532	40	21.28	0.276	40	28.13	8.07	400	71	
26.00	9.5	11	4.18E+08	0.578	40	23.12	0.255	40	25.99	8.08	400	78	
28.17	7.8	12	3.74E+08	0.487	50	24.35	0.261	40	26.60	8.09	400	69	
30.00	7.6	13	3.95E+08	0.534	50	26.70	0.256	40	26.09	8.10	400	40	Agitation increased to 450 (56% DO)
47.17	9.2	16	5.89E+08	0.661	60	39.66	0.166	40	16.92	8.05	450	7	Agitation increased to 500 rpm (40% DO)
50.08	12.6	16	8.06E+08	0.688	60	41.28	0.135	40	13.76	8.02	500	30	
53.00	11.2	18	8.06E+08	0.667	70	46.69	0.120	40	12.23	7.96	500	32	
55.67	10.6	20	8.48E+08	0.663	70	46.41	0.104	40	10.60	7.92	500	32	
70.33	16.6	20	1.33E+09	0.685	80	54.80	0	40	0	7.94	500	74	Agitation decreased to 400 rpm (57% DO)
78.33	11.4	22	1.00E+09	0.664	90	59.76	0	40	0	8.08	400	72	
103.83	11.5	22	1.01E+09	0.541	90	48.69	0	40	0	8.47	400	82	

* DNS Calibration 4 used
Dissolved oxygen controlled by manually adjusting the agitation rate

Chapter 5.2 - Bioreactor studies (2)

Data description: Cultivation of WT in a bioreactor - 3.2% initial glucose, 1% Hexylbenzene added at 24h (Batch 1)

Time	Cell growth concentration						Glucose concentration (g/l)			Process conditions			
	Cell number (cells/ml)			O.D.									
	Cells/ square	Dil. factor	Cell conc.	Absorb. (@620nm)	Dil. factor	Actual OD	Absorb. *	Dil. factor	Glucose conc.	pH	Agitation (rpm)	DO (%)	Observations/ Comments
(h)													
0.00	7.6	1	3.04E+07	0.141	20	2.82	0.331	40	33.73	7.57	200	55.0	
3.00	6.1	3	7.32E+07	0.147	20	2.94	0.325	40	33.12	7.53	200	40.0	
14.00	4.6	7	1.29E+08	0.128	40	5.12	0.327	40	33.32	7.64	215	45.0	Elongation starting
17.00	5.6	8	1.79E+08	0.136	40	5.44	0.319	40	32.51	7.68	200	49.0	
20.00	8.9	9	3.20E+08	0.168	40	6.72	0.32	40	32.61	7.73	277	8.0	
23.17	7.1	10	2.84E+08	0.218	40	8.72	0.314	40	32.00	7.85	450	20.0	1% Hexylbenzene added at 24h (O$_2$ switched off for 2 min)
28.67	9.4	12	4.51E+08	0.256	40	10.24	0.324	40	33.02	7.84	223	58.0	
37.58	8.2	16	5.25E+08	0.247	60	14.82	0.323	40	32.92	7.84	207	25.0	
41.00	7.6	18	5.47E+08	0.278	60	16.68	0.321	40	32.71	8.00	230	19.0	
45.22	7.5	20	6.00E+08	0.283	60	16.98	0.319	40	32.51	8.01	200	35.0	
66.43	9.1	25	9.10E+08	0.286	65	18.59	0.295	40	30.06	8.08	390	12.0	Agitation limit ↑ to 650rpm to ↑ DO
75.30	10.2	30	1.22E+09	0.403	65	26.20	0.269	40	27.41	7.97	630	22.0	
87.63	13.2	30	1.58E+09	0.451	65	29.32	0.198	40	20.18	8.00	750	25.0	Agitation limit ↑ to 750rpm to ↑ DO
110.08	16.5	32	2.11E+09	0.582	80	46.56	0.002	40	0.20	8.38	633	22.5	
113.50	13.1	40	2.10E+09	0.534	80	42.72	0	40	0	8.56	586	22.4	
117.00	13.9	40	2.22E+09	0.535	80	42.80	0	40	0	8.66	545	22.7	
134.50	14.9	40	2.38E+09	0.503	80	40.24	0	40	0	8.81	339	23.0	Cells changing colour and rupturing
139.50	24.3	40	3.89E+09	0.489	80	39.12	0	40	0	8.81	316	22.0	
147.00	16.1	50	3.22E+09	0.403	80	32.24	0	40	0	8.82	294	20.0	

DNS Calibration 4 used

0.025% Silicone antifoam added to YP$_2$D$_4$ medium

Dissolved oxygen controlled automatically at 22.5 % by agitation rate (limits: 200rpm to 600 rpm)

Chapter 5.2 - Bioreactor studies (3)

Data description: Product formation by WT in a bioreactor - 3.2% initial glucose, 1% Hexylbenzene added at 24h (Batch 1)

Time	Subrate and product analysis					Observations/ Comments
	Integrated areas on GC			Substrate conc. (mM)	Product conc. (mM) *	
(h)	Substrate	Product	Internal std			
28.7	2243.3	244.5	2127.8	14.886	9.664	1% (v/v) Hexylbenzene added at 24h
37.6	2497.5	185.1	2389.8	14.756	6.514	
41.0	2516	166.7	2154.7	16.488	6.507	
45.2	4447.7	168.5	2295.1	27.363	6.175	
66.4	3443.3	187.9	2215.9	21.941	7.132	Impurity in extraction
75.3	2735	263.3	2319.8	16.647	9.546	
87.6	1092.3	566.3	2488.7	6.197	19.138	
110.1	24.9	952	2273.5	0.155	35.218	
113.5	0	1073.1	2245.7	0	40.189	
117.0	0	1165.3	2340.4	0	41.876	
134.5	0	1139.6	2465.1	0	38.881	
139.5	0	1174.1	2348.1	0	42.054	
147.0	0	1101.1	2331.6	0	39.718	

GC product calibration 3 used; GC calibration 5 used for hexylbenzene

Chapter 5.2 - Bioreactor studies (4)

Data description: Cultivation of WT in a bioreactor - 3.2% initial glucose, 1% Hexylbenzene added at 24h (Batch 2)

| Time | Cell growth concentration | | | | | | Glucose concentration (g/l) | | | Process conditions | | | |
| | Cell number (cells/ml) | | | O.D. | | | | | | | | | |
(h)	Cells/ square	Dil. factor	Cell conc.	Absorb. (@620nm)	Dil. factor	Actual OD	Absorb. *	Dil. factor	Glucose conc.	pH	Agitation (rpm)	DO (%)	Observations/ Comments
0.00	8.4	1	3.36E+07	0.312	20	6.24	-	40	-	7.43	400	75.7	
3.50	8.6	2	6.88E+07	0.306	20	6.12	-	40	-	7.60	400	77.0	28.9 g 2M NaOH solution added
6.50	6.9	3	8.28E+07	0.410	20	8.20	-	40	-	7.60	400	64.0	0.37 g 2M NaOH solution added
8.00	7.7	4	1.23E+08	0.435	20	8.70	-	40	-	7.60	400	70.6	
9.50	6.2	5	1.24E+08	0.419	20	8.38	-	40	-	7.60	400	71.0	
11.00	6.0	6	1.44E+08	0.306	30	9.18	-	40	-	7.76	400	68.0	
12.50	7.6	7	2.13E+08	0.345	30	10.35	-	40	-	7.80	400	64.0	
21.75	8.1	10	3.24E+08	0.372	40	14.88	-	40	-	7.96	400	60.2	
24.25	8.8	11	3.87E+08	0.207	40	8.28	-	40	-	8.03	400	67.0	1% Hexylbenzene added, antifoam added
26.58	7.8	12	3.74E+08	0.297	40	11.88	-	40	-	8.06	400	62.0	
29.50	7.0	15	4.20E+08	0.313	40	12.52	-	40	-	7.99	400	44.0	
45.00	8.4	15	5.04E+08	0.301	50	15.05	-	40	-	7.88	400	45.0	0.20 g 2M NaOH solution added
50.25	8.1	15	4.86E+08	0.290	50	14.50	-	40	-	7.84	400	39.0	0.09 g 2M NaOH solution added
77.50	9.2	15	5.52E+08	0.311	50	15.55	-	40	-	7.71	400	37.0	0.92 g 2M NaOH solution added
106.08	9.5	15	5.70E+08	0.354	50	17.70	0	40	0	7.60	400	36.0	2.14 g 2M NaOH solution added
124.00	7.9	15	4.74E+08	0.294	50	14.70	0	40	0	7.60	400	50.0	18.34 g 2M NaOH solution added

* DNS Calibration 4 used

0.025% Silicone antifoam added to YP$_2$D$_4$ medium, and 2% aqeuouse antifoam solution added manually

Dissolved oxygen not controlled; agitation maintained at 400 rpm

2M Sodium hydroxide solution added when necessary

Chapter 5.2 - Bioreactor studies (5)

Data description: Product formation by WT in a bioreactor - 3.2% initial glucose, 1% Hexylbenzene added at 24h (Batch 2)

Time	Subrate and product analysis					
	Integrated areas on GC			Substrate conc. (mM)	Product conc. (mM) *	Observations/ Comments
(h)	Substrate	Product	Internal std			
24.25	1608206	0	22622694	1.478	0	1% (v/v) Hexylbenzene added at 24h
26.58	1459391	0	2065556	14.693	0	
29.50	1262116	0	2410550	10.888	0	
45.00	553974	0	2182454	5.279	0	
50.25	218507	0	1422917	3.193	0	
77.50	306504	136738	2208787	2.886	4.819	
106.08	160392	141155	2828058	1.179	4.134	
124.00	70746	16207	223373	6.586	5.427	

* GC product calibration 2 used

Chapter 5.2 - Bioreactor studies (6)

Data description: Cultivation of WT in a bioreactor - 3.2% initial glucose, 1% Hexylbenzene added at 24h (Batch 3)

Time	Cell growth concentration						Glucose concentration (g/l)			Process conditions			
	Cell number (cells/ml)			O.D.									
	Cells/ square	Dil. factor	Cell conc.	Absorb. (@620nm)	Dil. factor	Actual OD	Absorb. *	Dil. factor	Glucose conc.	pH	Agitation (rpm)	DO (%)	Observations/ Comments
(h)													
0.00	4.2	3	5.04E+07	0.172	20	3.44	0.278	40	28.33	7.79	400	64.0	
5.00	9.1	5	1.82E+08	0.370	20	7.40	0.280	40	28.53	7.73	400	19.0	Agitation increased to 450 rpm (33% DO)
17.00	7.0	10	2.80E+08	0.410	40	16.40	0.253	40	25.78	8.13	450	1.0	Agitation increased to 540 rpm (20 DO)
20.50	8.4	12	4.03E+08	0.599	40	23.96	0.221	40	22.52	8.08	540	<10	Agitation increased to 580 rpm (19% DO)
25.00	11.6	15	6.96E+08	0.533	100	53.30	0.191	40	19.46	8.10	-	-	
39.33	7.7	18	5.54E+08	0.625	60	37.50	0.167	40	17.02	8.15	-	-	
43.08	7.0	18	5.04E+08	0.397	60	23.82	0.195	40	19.87	-	-	-	
48.00	5.0	20	4.00E+08	0.334	50	16.70	0.178	40	18.14	-	-	-	DO controlled automatically at 20% by agitation rate (200 rpm to 400 rpm)
50.00	6.9	20	5.52E+08	0.408	60	24.48	0.175	40	17.83	-	-	-	
70.75	4.0	20	3.20E+08	0.219	60	13.14	0.146	40	14.88	-	-	-	
93.33	6.4	20	5.12E+08	0.430	60	25.80	0.141	40	14.37	-	-	-	
112.75	4.9	20	3.92E+08	0.485	60	29.10	0.101	40	10.29	-	-	-	

* DNS Calibration 4 used

0.025% Silicone antifoam added to YP_2D_4 medium, and 1% aqeuouse antifoam solution added manually

Dissolved oxygen controlled manually for the first 24h, then automatically controlled at 20% by agitation rate (200rpm to 600rpm)

Chapter 5.2 - Bioreactor studies (7)

Data description: Product formation by WT in a bioreactor - 3.2% initial glucose, 1% Hexylbenzene added at 24h (Batch 3)

Time	Subrate and product analysis					Observations/ Comments
	Integrated areas on GC			Substrate conc. (mM)	Product conc. (mM) *	
(h)	Substrate	Product	Internal std			
25.00	1332219	0	1100873	25.166	0	1% (v/v) Hexylbenzene added at 24h
39.33	2933904	0	1506706	40.495	0	
43.08	623136	0	1097581	11.807	0	
48.00	571254	0	1217902	9.754	0	
50.00	1139297	0	1272305	18.622	0	
70.75	310621	10216	1259816	5.127	1.747	
93.33	536259	20767	1359984	8.200	2.156	
112.75	309826	71874	1351833	4.766	4.320	

* GC product calibration 2 used

Chapter 5.2 - Bioreactor studies (8)

Data description: Cultivation of WT in a bioreactor - 3.2% initial glucose, 1% Hexylbenzene added at 11h

Time	Cell growth concentration						Glucose concentration (g/l)			Process conditions			Observations/ Comments
	Cell number (cells/ml)			O.D.									
	Cells/	Dil.	Cell	Absorb.	Dil.	Actual	Absorb.		Glucose	pH	Agitation	DO	
(h)	square	factor	conc.	(@620nm)	factor	OD	*	Dil. factor	conc.		(rpm)	(%)	
0.0	7.6	1	3.04E+07	0.397	20	7.94	0.307	40	31.29	-	400	>40	
1.0	9.4	1	3.76E+07	0.275	20	5.50	0.291	40	29.66	-	400	>40	
2.0	5.9	2	4.72E+07	0.229	20	4.58	0.295	40	30.06	-	400	>40	
3.0	7.7	2	6.16E+07	0.235	20	4.70	0.276	40	28.13	-	400	>40	
4.2	6.5	3	7.80E+07	0.308	20	6.16	0.296	40	30.16	-	400	>40	
5.0	8.3	3	9.96E+07	0.400	20	8.00	0.290	40	29.55	-	400	>40	
6.0	5.8	4	9.28E+07	0.446	20	8.92	0.280	40	28.53	-	400	>40	
7.0	9.9	4	1.58E+08	0.569	20	11.38	0.295	40	30.06	-	400	>40	
10.1	11	6	2.64E+08	0.521	30	15.63	0.269	40	27.41	-	400	>40	
11.0	13.8	6	3.31E+08	0.465	30	13.95	0.279	40	28.43	-	400	37.0	Agitation increased to 440rpm overnight/ antifoam added
20.4	8.2	10	3.28E+08	0.570	40	22.80	0.259	40	26.39	-	400	-	
22.5	8.9	10	3.56E+08	0.542	40	21.68	0.244	40	24.87	-	400	-	
26.3	9.7	11	4.27E+08	0.565	40	22.60	0.241	40	24.56	-	400	-	
29.0	9.8	11	4.31E+08	0.549	40	21.96	0.237	40	24.15	-	400	-	
45.6	9.1	15	5.46E+08	0.521	50	26.05	0.170	40	17.32	-	400	-	
48.5	10.2	15	6.12E+08	0.543	50	27.15	0.156	40	15.90	-	400	-	
52.2	9.2	15	5.52E+08	0.535	50	26.75	0.138	40	14.06	-	400	-	
54.5	12.4	15	7.44E+08	0.593	50	29.65	0.141	40	14.37	-	400	-	
67.5	9.6	15	5.76E+08	0.522	60	31.32	0.033	40	3.36	-	400	-	Agitation increased to 550rpm (44% DO)
74.0	6.5	15	3.90E+08	0.617	60	37.02	0.004	40	0.41	-	550	-	
76.0	7.2	15	4.32E+08	0.605	60	36.30	0.004	40	0.41	-	550	-	
123.0	4.7	15	2.82E+08	0.487	60	29.22	0	40	0.00	-	550	-	

DNS Calibration 4 used

0.025% Silicone antifoam added to YP$_2$D$_4$ medium, and 2% aqeuouse antifoam solution added manually

Dissolved oxygen controlled manually for the first 24h, then automatically controlled at 20% by agitation rate (200rpm to 600rpm)

Chapter 5.2 - Bioreactor studies (9)

Data description: Product formation by WT in a bioreactor - 3.2% initial glucose, 1% Hexylbenzene added at 11h						
	Subrate and product analysis					
Time	**Integrated areas on GC**			**Substrate conc. (mM)**	**Product conc. (mM) ***	**Observations/ Comments**
(h)	**Substrate**	**Product**	**Internal std**			
20.4	2653764	50489	2324687	23.740	2.524	1% (v/v) Hexylbenzene added at 11h
22.5	2675723	46932	1341928	41.466	3.281	
26.3	3250394	65406	1336761	50.566	4.078	
29.0	1539669	95849	1352865	23.668	5.330	
45.6	1116212	185606	1202098	19.310	10.101	
48.5	885267	355429	1384282	13.299	15.946	
52.2	648134	436359	1405271	9.591	19.016	
54.5	550395	608379	1586291	7.216	23.185	
67.5	52388	428523	1145121	0.951	22.653	
74.0	55833	367432	1109732	1.046	20.191	
76.0	61730	567822	1165131	1.102	29.114	
123.0	0	549440	1611445	0	20.754	

* GC product calibration 2 used

Chapter 5.2 - Bioreactor studies (10)

Data description: Cultivation of WT in a bioreactor - 3.2% initial glucose, 1% Hexylbenzene added at 48h

| Time | Cell growth concentration | | | | | | Glucose concentration (g/l) | | | Process conditions | | | |
| | Cell number (cells/ml) | | | O.D. | | | | | | | | | |
(h)	Cells/ square	Dil. factor	Cell conc.	Absorb. (@620nm)	Dil. factor	Actual OD	Absorb. *	Dil. factor	Glucose conc.	pH	Agitation (rpm)	DO (%)	Observations/ Comments
0.0	5.8	2	4.64E+07	0.090	20	1.80	0.309	40	31.49	7.82	400	66.0	
10.0	8	4	1.28E+08	0.420	20	8.40	0.284	40	28.94	7.82	400	37.0	
13.5	9.2	5	1.84E+08	0.593	20	11.86	0.286	40	29.15	7.93	400	23.0	Agitation increased to 500rpm (36% DO)
22.3	8.9	11	3.92E+08	0.540	40	21.60	0.246	40	25.07	8.07	500	17.0	Agitation increased to 550rpm (40% DO)
38.7	8.5	15	5.10E+08	0.773	50	38.65	0.123	40	12.53	7.92	550	12.0	Agitation increased to 600rpm (31% DO)
48.0	12	20	9.60E+08	0.719	70	50.33	0.051	40	5.20	7.85	600	>50	Agitation decreased to 550rpm (50% DO)
54.5	9.8	20	7.84E+08	0.724	70	50.68	0.051	40	5.20	7.85	550	72.0	Agitation decreased to 450rpm (40% DO)
58.3	12.1	22	1.06E+09	0.726	80	58.08	0.039	40	3.97	7.88	450	43.0	
62.7	10.3	25	1.03E+09	0.481	100	48.10	0.037	40	3.77	7.88	450	40.0	
86.0	10.5	25	1.05E+09	0.455	100	45.50	0.014	40	1.43	7.82	450	90.0	Agitation decreased to 350 rpm (40% DO)
126.5	8.9	25	8.90E+08	0.453	100	45.30	0.007	40	0.71	7.78	350	95.0	

* DNS Calibration 4 used

0.025% Silicone antifoam added to YP$_2$D$_4$ medium, and 2% aqeuouse antifoam solution added manually

Dissolved oxygen controlled manually for the first 24h, then automatically controlled at 20% by agitation rate (200rpm to 600rpm)

<u>Chapter 5.2 - Bioreactor studies (11)</u>

Data description: Product formation by WT in a bioreactor - 3.2% initial glucose, 1% Hexylbenzene added at 48h

Time	Subrate and product analysis					Observations/ Comments
	Integrated areas on GC			Substrate conc. (mM)	Product conc. (mM) *	
(h)	Substrate	Product	Internal std			
54.5	1108020	0	947936	24.308	0	1% (v/v) Hexylbenzene added at 48h
58.3	1206966	0	811400	30.934	0	
62.7	1373639	0	1241651	23.007	0	
86.0	1058494	0	1237734	17.784	0	
126.5	954789	28071	1429273	13.892	2.405	

* GC product calibration 2 used

Chapter 5.2 - Bioreactor studies (12)

Data description: Cultivation of WT in a bioreactor - 6.4% initial glucose, 1% Hexylbenzene added at 24h

Time	Cell growth concentration						Glucose concentration (g/l)			Process conditions			
	Cell number (cells/ml)			O.D.									
	Cells/ square	Dil. factor	Cell conc.	Absorb. (@620nm)	Dil. factor	Actual OD	Absorb. *	Dil. factor	Glucose conc.	pH	Agitation (rpm)	DO (%)	Observations/ Comments
(h)													
0.0	7.3	1	2.92E+07	0.103	20	2.06	0.311	80	63.39	7.40	400	84	
1.8	10.9	1	4.36E+07	0.162	20	3.24	0.316	80	64.41	7.30	400	72	
9.3	9.6	3	1.15E+08	0.191	30	5.73	0.291	80	59.31	7.60	400	65	
17.5	9.8	5	1.96E+08	0.336	40	13.44	0.291	80	59.31	8.16	400	56	
21.5	7.8	10	3.12E+08	0.439	40	17.56	0.284	80	57.88	8.21	400	64	
24.5	8.2	10	3.28E+08	0.565	40	22.60	0.281	80	57.27	8.17	400	20	
25.5	7.5	12	3.60E+08	0.627	40	25.08	0.273	80	55.64	8.24	400	66	
31.5	7.7	15	4.62E+08	0.486	50	24.30	0.264	80	53.81	8.31	400	50	
46.5	5.8	15	3.48E+08	0.500	50	25.00	0.261	80	53.20	8.18	400	60	
63.0	7.8	15	4.68E+08	0.633	50	31.65	0.248	80	50.55	8.09	400	60	
66.0	6.5	15	3.90E+08	0.376	60	22.56	0.243	80	49.53	8.07	400	62	
69.5	7.0	15	4.20E+08	0.379	60	22.74	0.227	80	46.27	8.04	400	71	
72.8	8.4	15	5.04E+08	0.391	60	23.46	0.239	80	48.71	8.01	400	65	
87.5	6.3	20	5.04E+08	0.397	60	23.82	0.226	80	46.06	7.83	400	55	
92.0	10.4	20	8.32E+08	0.414	60	24.84	0.221	80	45.04	7.78	400	58	
97.5	6.0	20	4.80E+08	0.443	60	26.58	0.219	80	44.64	7.69	400	57	
112.5	5.7	25	5.70E+08	0.509	60	30.54	0.189	80	38.52	7.60	400	44	10.35 g 2M NaOH solution added
116.7	7.2	25	7.20E+08	0.505	60	30.30	0.183	80	37.30	7.60	400	48	4.46 g 2M NaOH solution added
121.0	10.5	25	1.05E+09	0.534	60	32.04	0.170	80	34.65	7.60	410	34.5	-
138.2	8.3	27	8.96E+08	0.741	60	44.46	0.110	80	22.42	7.60	410	10	11.45 g 2M NaOH solution added, Agit ↑ to 500rpm (34%)
145.2	7.3	27	7.88E+08	0.848	60	50.88	0.075	80	15.29	7.60	500	46	9.06 g 2M NaOH solution added
149.2	8.4	27	9.07E+08	0.689	70	48.23	0.053	80	10.80	7.60	500	47	8.08 g 2M NaOH solution added
160.3	9.2	27	9.94E+08	0.773	70	54.11	0.003	80	0.61	7.56	500	51	7.88 g 2M NaOH solution added
168.8	8.1	27	8.75E+08	0.791	70	55.37	0	80	0.00	7.99	500	80	2.63 g 2M NaOH solution added
256.0	11.3	27	1.22E+09	0.644	70	45.08	0	80	0.00	8.83	500	100	0

* DNS Calibration 4 used

0.025% Silicone antifoam added to YP$_2$D$_4$ medium, and 2% aqeuouse antifoam solution added manually; 2M sodium hyrdoxide solution added when necessary

Dissolved oxygen controlled by manually adjusting agitation rate

Chapter 5.2 - Bioreactor studies (13)

Time	Subrate and product analysis					Observations/ Comments
	Integrated areas on GC			Substrate conc. (mM)	Product conc. (mM) *	
(h)	**Substrate**	**Product**	**Internal std**			

Data description: Product formation by WT in a bioreactor - 6.4% initial glucose, 1% Hexylbenzene added at 24h

Time (h)	Substrate	Product	Internal std	Substrate conc. (mM)	Product conc. (mM) *	Observations/ Comments
31.50	2284230	0	1562655	30.399	0	1% (v/v) Hexylbenzene added at 24h
46.50	-	20479	1534854	44.557	2.046	
63.00	6826163	55874	1745510	81.327	3.112	
66.00	3077861	170268	1871912	34.193	6.478	
69.50	4009140	173966	1934175	43.106	6.420	
72.75	4281208	385555	2232286	39.884	11.147	
87.50	1995544	124785	1482476	27.993	6.090	
92.00	2795953	456719	2468411	23.555	11.850	
97.50	1968308	485665	1639076	24.973	18.204	
112.50	659346	793207	2169256	6.321	22.165	
116.67	292759	536162	1886172	3.228	17.516	
121.00	100126	313504	1283918	1.622	15.227	
138.17	94520	339542	1017957	1.931	20.331	
145.17	0	658851	2013874	0	19.966	
149.17	0	155336	1663638	0	6.616	
160.33	0	440975	1694077	0	16.148	
168.75	0	507278	1755744	0	17.783	
256.00	0	227095	1700818	0	8.908	

* GC product calibration 2 used

Chapter 5.2 - Bioreactor studies (14)

Data description: Cultivation of WT in a bioreactor - 3.2% initial glucose, 0.73% Phenyl acetic acid added

Time	Cell growth concentration						Glucose concentration (g/l)			Process conditions			Observations/ Comments
	Cell number (cells/ml)			O.D.									
	Cells/	Dil.	Cell	Absorb.	Dil.	Actual	Absorb.		Glucose		Agitation	DO	
(h)	square	factor	conc.	(@620nm)	factor	OD	*	Dil. factor	conc.	pH	(rpm)	(%)	
0.00	3.9	3	4.68E+07	0.134	20	2.68	0.367	40	37.40	7.56	-	-	
15.17	5.2	7	1.46E+08	0.206	40	8.24	0.367	40	37.40	7.68	-	-	
25.08	4.5	10	1.80E+08	0.231	50	11.55	0.367	40	37.40	7.82	-	-	0.375% (19.9mM) PAA added (in 75ml) at 24h
29.00	4.4	10	1.76E+08	0.213	50	10.65	0.341	40	34.75	9.1	-	-	
48.33	5.1	13	2.65E+08	0.239	60	14.34	0.345	40	35.16	8.55	-	-	0.355% (18.84mM) PAA added (in 100ml) at 48h
64.17	3.9	15	2.34E+08	0.241	60	14.46	0.310	40	31.59	8.56	-	-	
86.83	4.7	15	2.82E+08	0.401	60	24.06	0.296	40	30.16	8.56	-	-	
92.17	5.4	15	3.24E+08	0.785	60	47.10	0.303	40	30.88	8.56	-	-	
100.75	6.5	15	3.90E+08	0.368	60	22.08	0.289	40	29.45	8.56	-	-	
113.67	7.0	15	4.20E+08	0.373	70	26.11	0.280	40	28.53	8.54	-	-	
119.33	5.4	17	3.67E+08	0.411	70	28.77	0.268	40	27.31	8.53	-	-	
137.33	7.2	17	4.90E+08	0.493	70	34.51	0.232	40	23.64	8.37	-	-	
145.83	6.5	17	4.42E+08	0.495	70	34.65	0.218	40	22.22	8.35	-	-	

* DNS Calibration 4 used

Dissolved oxygen controlled automatically controlled at 22.5% by agitation rate (200rpm to 600rpm)

Initial volume = 1.7L, 175ml PAA solution added so that final volume = 1.875L

Chapter 5.2 - Bioreactor studies (15)

Data description: Product formation by WT in a bioreactor - 3.2% initial glucose, 0.74% Phenyl acetic acid added

Time	Subrate and product analysis					Observations/ Comments
	Integrated areas on GC			Substrate conc. (mM)	Product conc. (mM) *	
(h)	Substrate	Product	Internal std			
0	-	0	0	-	0	
15.17	-	0	0	-	0	
25.08	-	0	0	-	0	0.375% (19.9mM) PAA added (in 75ml) at 24h
29	-	3937.5	11761.7	-	20.401	
48.33	-	3344.9	11115.3	-	18.468	0.355% (18.84mM) PAA added (in 100ml) at 48h (total = 38.75mM)
64.17	-	6342.2	10753.2	-	34.964	
86.83	-	6656.4	10847.2	-	36.326	
92.17	-	5671.9	9187	-	36.539	
100.75	-	5138.2	7540.3	-	40.197	
113.67	-	5322.5	7940.5	-	39.561	
119.33	-	6948.8	9850.4	-	41.567	
137.33	-	7506.3	9435	-	46.715	
145.83	-	7156.9	9477.1	-	44.408	

GC product calibration 2 used