Effect of cultivation conditions on heterologous expression of oxidoreductases by the yeast *Blastobotrys adeninivorans*

By

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Chapter 1

Introduction to the study

1. Applications of oxidoreductases, P450s and VAO, and expression in *Blastobotrys adeninivorans*

1.1. Hydroxylation

Hydroxylation reactions have been studied in microorganisms, plants and animals for many years (van Beilen & Enrico, 2007). These reactions are described as "the conversion of a carbon-hydrogen to a carbon-hydroxyl bond" according to Herbert & Hedda (2000). The product of hydroxylation is a hydroxylated compound that results from addition of one or more molecules of oxygen to an organic compound. In living organisms, these kinds of reactions serve many purposes including, among many, the detoxification of compounds in the body, degradation of environmental pollutants by microorganisms and the synthesis of secondary metabolites. They also find applications in the pharmaceutical, fine chemical, bioremediation and food industries.

Examples of applications of biological hydroxylation include production of indigo (Pathak & Madamwar, 2010), chiral pharmaceutical intermediates (Ramesh, 2008), chiral cis-diols (McIver *et al*., 2008) as well as oxyfunctionalization of unactivated carbons (Carballeira *et al*., 2009). The chemical counterparts of some of these processes pose serious health and environmental hazards due to toxic materials and catalysts used as well as the toxic by-products and wastewater released. As a result, these processes need to be replaced by processes that are more environmentally- and producer- friendly. Microorganisms and enzymes usually serve this purpose well. The enzymes usually involved in biological hydroxylation include monooxygenases, dioxygenases, peroxidases and vanillyl alcohol oxidase (VAO). For the purpose of this research, we will focus on cytochrome P450 monooxygenases (CYPs) and VAO.

1.2. Cytochrome P450 monooxygenases

Monooxygenases catalyze the addition of one atom from molecular oxygen to organic compounds to yield alcohols and epoxides. These products are further oxidized to other oxygenated compounds such as aldehydes, ketones and carboxylic acids. Monooxygenases include cytochrome P450-dependent monooxygenases (CYP450s), non-heme iron monooxygenases, copper-dependent monooxygenases and flavin-monooxygenases. Urlacher *et al*., (2004) extensively reviewed all of these.

CYP450s are widely distributed in nature and are found in bacteria, fungi, insects, animals and plants. These enzymes play a vital role in biosynthesis of prostaglandins and steroids as well as many secondary metabolites in plants and Actinomycetes. They also detoxify hydrophobic xenobiotic compounds such as drugs or chemical pollutants. Their catalytic reactions include epoxidation, sulfoxidation, dealkylation and hydroxylation. Hydroxylation reactions involve introduction of an oxygen atom into allylic positions of double bonds or even into unactivated aromatic and aliphatic hydrocarbons. The CYP450s are characterized by a heme iron group and during these hydroxylation reactions, one oxygen atom, activated by a reduced heme iron, is added to the substrate. The second oxygen atom is reduced to water by accepting electrons from NAD(P)H *via* a redox partner such as a flavoprotein or ferredoxin (Urlacher *et al*., 2004).

In self-sufficient cytochrome P450s, the P450 and reductase domains are naturally fused. Examples of this class of proteins are CYP102A1 from *Bacillus megaterium* (Narhi and Fulco, 1987) and CYP505A1 from *Fusarium oxysporum* (Nakayama *et al*., 1996).

1.2.1. Issues relating to heterologous expression of CYP450s

Heterologous expression of CYP450s presents several problems in both bacteria and yeasts. Using strong promoters, in *Escherichia coli*, results in misfolded CYP450s due to the higher demand placed on the folding machinery (Waegeman and Soetaert, 2011). As a result, protein processing steps stall and stress responses are activated, leading to protein degradation or formation of inclusion bodies (Waegeman and Soetaert, 2011). *E. coli* also lacks a suitable electron transfer system for CYP450s and therefore requires either coexpression of appropriate reductase partners or addition of purified preparations to CYP450 preparations for restoring

activity (Barnes *et al*., 1991; Blake *et al*., 1996). As *E. coli* lacks its own CYP450s, it can be a good host for CYP450 expression. However, because *E. coli* does not accumulate more heme unnecessarily due to its toxicity, it cannot cope with a higher demand for heme by CYP450 expression (Harnastai *et al*., 2006). Thus, 5-aminolevulinic acid (5-ALA) is usually added to CYP450-expressing strains to improve expression levels or glutamyl-tRNA reductase (*hemA*) can be coexpressed with CYP450s (Richardson *et al*., 1995; Harnastai *et al*., 2006). Alternatively, yeasts are better equipped for CYP450 expression, since they harbor their own CYP450s, and thus have suitable reductase systems and endoplasmic reticulum (ER) membrane environment, as well as adequate heme available. They also do not need sequence modifications (e.g. N-terminal modifications) to express eukaryotic CYP450s (Purnapatre *et al*., 2008; Zöllner *et al*., 2010). Having own CYP450s nonetheless can interfere with heterologous expression of other CYP450s, especially during biotransformations where by-products might form due to activity by native CYP450s. Despite all these, both bacteria and yeasts have their advantages and disadvantages, and both can be used successfully depending on the specific CYP450 being expressed.

1.3. Vanillyl-alcohol Oxidase

Vanillyl-alcohol oxidase (VAO; EC 1.2.3.38), from *Penicillium simplicissimum*, is a flavoprotein with the flavin adenine dinucleotide (FAD) as covalently bound prosthetic group (Benen *et al*., 1998; Fraaije *et al*., 1998). The enzyme catalyzes a broad spectrum of reactions including oxidation, demethylation, deamination, hydroxylation, and dehydrogenation of a number of phenolic compounds (aromatic alcohols, ethers, amines, allylphenols and alkylphenols; Fraaije *et al*., 1995; van den Heuvel *et al*., 1998).

VAO was initially investigated because it catalyzed the oxygen-dependent conversion of vanillyl alcohol to the flavour compound vanillin (de Jong *et al*., 1992). The enzyme also catalyzes the hydroxylation of eugenol, obtained from clove oil, into coniferyl alcohol (van Berkel *et al*., 1997). Coniferyl alcohol is one of the intermediates in the eugenol degradation pathway and is usually further oxidized to ferulic acid used for vanillin production (Tadasa and Kayahara, 1983).

Other potential applications of VAO include production of: i) 4-hydroxybenzaldehyde and 4-hydroxybenzyl alcohol (vanilla flavour components; Fraaije *et al*., 1995); ii) optically pure

aromatic compounds (van den Heuvel *et al*., 1998); iii) 4-vinylphenol (present in wine and orange juice; Chatonnet *et al*., 1992); and iv) *N*-methyl-D-aspartate receptor antagonists such as ifenprodil and ninhydrin (Williams *et al*., 1993; Whittemore *et al*., 1997; Tamiz *et al*., 1998).

VAO expression in *E. coli* has been poor (Benen *et al*., 1998; Overhage *et al*., 2003; Van Rooyen, 2012) but excellent expression has was shown in *Aspergillus niger* NW156-T10 and *Amycolatopsis* sp. HR167 (Benen *et al*., 1998; Overhage *et al*., 2006). Surprisingly, Overhage *et al*., (2006) also reported the best expression of *VAO* in *E*. *coli*. However, when *E*. *coli* was directly compared to *Blastobotrys adeninivorans* UOFS Y-1220 for expression of *VAO*, *B*. *adeninivorans* showed better enzyme activity even though it was less than that of previous research (Van Rooyen, 2012). Lastly, to the best of my knowledge, expression of *VAO* has not been reported in yeast until recently (Smit *et al*., 2012a; Van Rooyen, 2012).

1.4. Expression of two oxidoreductases, CYP505A1 and VAO, in *B. adeninivorans*

A wide-range vector that allows the expression and/or co-expression of cloned genes in a number of different yeasts has been developed in our department (Smit *et al*., 2012a, b). This vector has the following important features: (i) a kanamycin resistance gene for propagation in *E. coli*; (ii) 18S rDNA fragments from *Kluyveromyces marxianus* flanking the "yeast cassette" for genomic integration; (iii) hygromycin resistance gene under control of the *Saccharomyces cerevisiae TEF* (translation elongation factor) promoter for selecting yeast transformants; and (iv) *Yarrowia lipolytica TEF* promoter for driving expression of the gene under investigation, for example *PsVAO* (Figure 1).

Two CYP450s, including CYP505A1, a self-sufficient fatty acid hydroxylase from *Fusarium oxysporum*, as well as the VAO from *P. simplicissimum* (*PsVAO*) were used to evaluate different yeasts as hosts for heterologous expression of oxidoreductases. The yeasts used in this comparative study were *S. cerevisiae, Hansenula polymorpha, Kluyveromyces lactis, B. adeninivorans* (syn. *Arxula adeninivorans*)*, Y. lipolytica, Candida deformans* and *K. marxianus.*

Among the yeasts tested for expression of *PsVAO* and *CYP505A1 B. adeninivorans* UOFS Y-1220 performed the best for both these genes, when whole cell biotransformations of,

respectively, eugenol and 4-hexylbenzoic acid were used to evaluate expression. Results obtained in these studies will be discussed in more detail in Chapter 2.

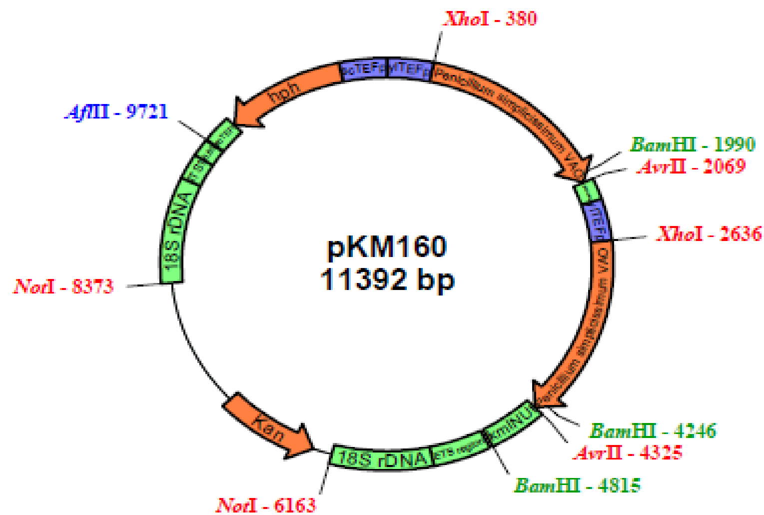


Figure 1 The wide range vector developed at University of the Free State. This vector shows an example of a vector containing two copies of *PsVAO*. Figure adapted from Smit *et al.*, (2012a).

1.5. Aims of the study

Promising expression of *PsVAO* and *CYP505A1* in the yeast *B. adeninivorans* UOFS Y-1220 motivated further evaluation of this organism as a host for these two oxidoreductases. In this research, we set out to study the effect of cultivation conditions on heterologous expression of *PsVAO* and *CYP505A1* in *B. adeninivorans* UOFS Y-1220 as well as on biomass production.

The objectives of this project were:

- To develop assays to monitor *VAO* and *CYP505A1* expression;
- To determine the effect of media composition on biomass production and the heterologous expression of both enzymes in shake-flasks; and
- To determine the effect of dissolved oxygen tension (DOT) on biomass production and the heterologous expression of *VAO* in bioreactors.

Chapter 2

Literature Review

2. Recombinant protein expression and fermentation strategies in *B. adeninivorans*

2.1. The non-conventional yeast *B. adeninivorans*

2.1.1. Discovery and Classification

In 1984, Middelhoven and co-workers reported a yeast species which they named *Trichosporon adeninivorans* after isolation from soil through enrichment cultures (Middelhoven *et al*., 1984). The type strain CBS8244T exhibited exotic biochemical activities, most notably assimilation of a range of amines and purine compounds, including adenine, as sole sources of carbon and energy.

In 1990, Gienow *et al*., (1990) isolated the second strain, LS3 (PAR-4) from wood hydrolysates in Siberia (Kapultsevich, Institute of Genetics and Selection of Industrial Microorganisms, Moscow, Russia). This strain was also able to utilize a wide array of compounds as sources of nitrogen and carbon.

In the same year, seven additional strains were discovered; three from chopped maize herbage ensiled at 25 or 30 °C in The Netherlands and four from humus-rich soil in South Africa (van der Walt *et al*., 1990). A new genus *Arxula* van der Walt, M. T. Smith & Yamada (Candidaceae) comprising two species, i.e. *A. terrestre*, the type species (van der Walt and Johanssen) van der Walt, M. T. Smith & Yamada, nov. comb., and *A. adeninivorans* (Middelhoven, Hoogkamer Te-Niet and Kreger van Rij) van der Walt, M. T. Smith and Yamada, nov. comb. was proposed. All the strains in this genus are haploid, ascomycetous, arthroconidial, xerotolerant, anamorphic, non-pathogenic and can assimilate nitrate like *H. polymorpha* (Middelhoven *et al*., 1984; van der Walt *et al*., 1990). After extensive phylogenetic

studies done recently by Kurtzmann and Robnett (2007), the authors renamed the yeast *Blastobotrys adeninivorans*. This was based on taxonomic priorities since the genus *Blastobotrys* has taxonomic priority over *Arxula*. Throughout this thesis, the genus *Arxula* will be referred to as *Blastobotrys*.

2.1.2. Physiological characteristics

Studies by Middelhoven *et al*., (1984, 1991, and 1992), Gienow *et al*., (1990) and Van der Walt *et al*., (1990) provide elaborate physiological descriptions of *B. adeninivorans*. The yeast can utilize a wide range of compounds including starch, tannic acid, n-alkanes, xylose and purines as sole sources of carbon and energy providing many alternatives for substrate-based fermentation control and bioprocess design. Additionally, halo-tolerance (Tag *et al*., 1998; Stoltenburg *et al*., 1999; Yang *et al*., 2000), thermo-tolerance and temperature-dependent dimorphism (Wartmann *et al*., 1995) particularly evident in the Siberian wild-type strain LS3 are of high biotechnological interest.

Yang and co-workers reported growth of *B. adeninivorans* LS3 at NaCl concentrations of up to 20 % (w/v) in yeast minimal medium described by Tanaka *et al*., (1967). This feature allows the development of highly concentrated media for fed-batch cultivation in high cell density fermentations (HCDF), and the use of concentrated buffer for pH control in shake-flask cultures (Hellwig *et al*., 2005).

Wartmann *et al*., (1995) reported the growth of *B. adeninivorans* LS3 at temperatures up to 48 °C and its survival for several hours at 55 °C, without previous adaptation to increased temperatures. They observed morphology change from budding to mycelial cells (Figure 2 A and B, respectively) at temperatures above 42 °C, with reversion—where cell budding was restored—occurring at temperatures below 42 °C.

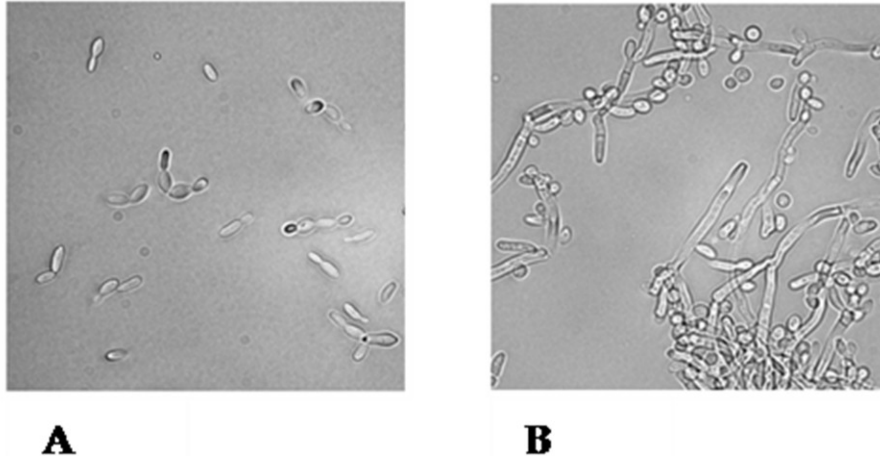


Figure 2 Temperature-dependent dimorphism of *B. adeninivorans*. The figure shows (A) cells grown at 37 °C and (B) cells grown at 42 °C. The pictures were taken from Stöckmann *et al.*, (2009)

2.1.3. Applications

Since its first isolation, *B. adeninivorans* LS3 (PAR-4) has been used for the following:

i) Production of single cell proteins (SCP) (Böttcher *et al*., 1988) due to its thermo-tolerance compared to established SCP organisms (Hellwig *et al*., 2005);

ii) Gene donor for glucoamylase production using *K. lactis*- and *S. cerevisiae*-based expression platforms (Bui *et al*., 1996a; b);

iii) A model organism to study degradation pathways of hydroxylated aromatic acids, e.g. tannin (Sietmann *et al*., 2010);

iv) A microbial biosensor for measurement of biodegradable substances (Chan *et al*., 1999);

v) A biosensor for detection of estrogenic activities in wastewater (Hahn *et al*., 2006);

vi) A host for heterologous expression of a number of genes (Böer *et al*., 2005).

2.2. Development of expression platforms for *B. adeninivorans*

The first *B. adeninivorans*-based transformation platform was established using the *B. adeninivorans* and *S. cerevisiae*–derived *LYS2* as marker genes for selection (Kunze *et al*., 1990; Kunze and Kunze, 1996). The *LYS2* gene codes for α-amino-adipate reductase that reduces α-amino-adipate to an aldehyde in the biosynthesis of lysine and employs an auxotrophic strain. Transformation vectors derived in this manner were either unstably integrated into the chromosomal DNA in low copy numbers or were of episomal fate.

Thus, a system based on stable integration of foreign DNA into the ribosomal DNA (rDNA) was developed using vector pAL-HPH1 (Figure 3a; Rösel and Kunze, 1998). It uses the *B. adeninivorans*-derived 25S rDNA fragment for rDNA targeting with the strong constitutive *B. adeninivorans TEF1* promoter to drive expression. Furthermore, the *E. coli*-derived *hph* gene conferring resistance to hygromycin B or the *ALEU2* and *AILV1* genes (from *B. adeninivorans*) for complementation of auxotrophic strains serve as selective markers (Rösel and Kunze, 1998; Steinborn *et al*., 2005; Wartmann *et al*., 1998, 2003b). *ALEU2* is a gene that encodes β–isopropylmalate dehydrogenase responsible for leucine biosynthesis in several yeast species (Satyanarayana *et al*., 1968; Keogh *et al*., 1998; Lu *et al*., 1998; Hisatomi *et al*., 1995; Rodrigues *et al*., 2001; De la Rosa *et al*., 2001). *AILV1* encodes the enzyme threonine deaminase. *B. adeninivorans* auxotrophic strains, *aleu2* and *ailv1,* were reported by Samsonova *et al*., (1989; 1996).

Strains transformed under hygromycin B contained 2–10 plasmid copies stably integrated into the rDNA by homologous recombination (Rösel and Kunze, 1998). When *ALEU2* and *AILV1* served as selection markers, only one to three plasmid copies were present in the transformants (Wartmann *et al*., 1998; 2003a). Although *ALEU2* and *AILV1* yields low plasmid copy numbers when used as selection markers, they are more favored over dominant selection markers that require the use of toxic compounds or antibiotics.

A new and appealing host–vector system based on *atrp1* complementation, under control of the defective *ALEU2* promoter, yields transformants with up to 20 plasmid copies (Steinborn *et al*., 2007b). Another *B. adeninivorans* mutant strain (G1212 [*aleu2 atrp1::ALEU2*]) was generated for this purpose. The *ATRP1* gene encodes for a phosphoribosyl anthranilate isomerase that catalyze the third step in tryptophan biosynthesis. Improvements in vectors (pAL-ATRP1) using *atrp1* led to plasmids lacking all initially included bacterial sequences upon

integration, favoring commercial/biotechnological applications (Madzak *et al*., 2004). A range of transformation elements and physical maps of vectors for *B. adeninivorans* are shown in Figure 3 and summarized in Table 1.

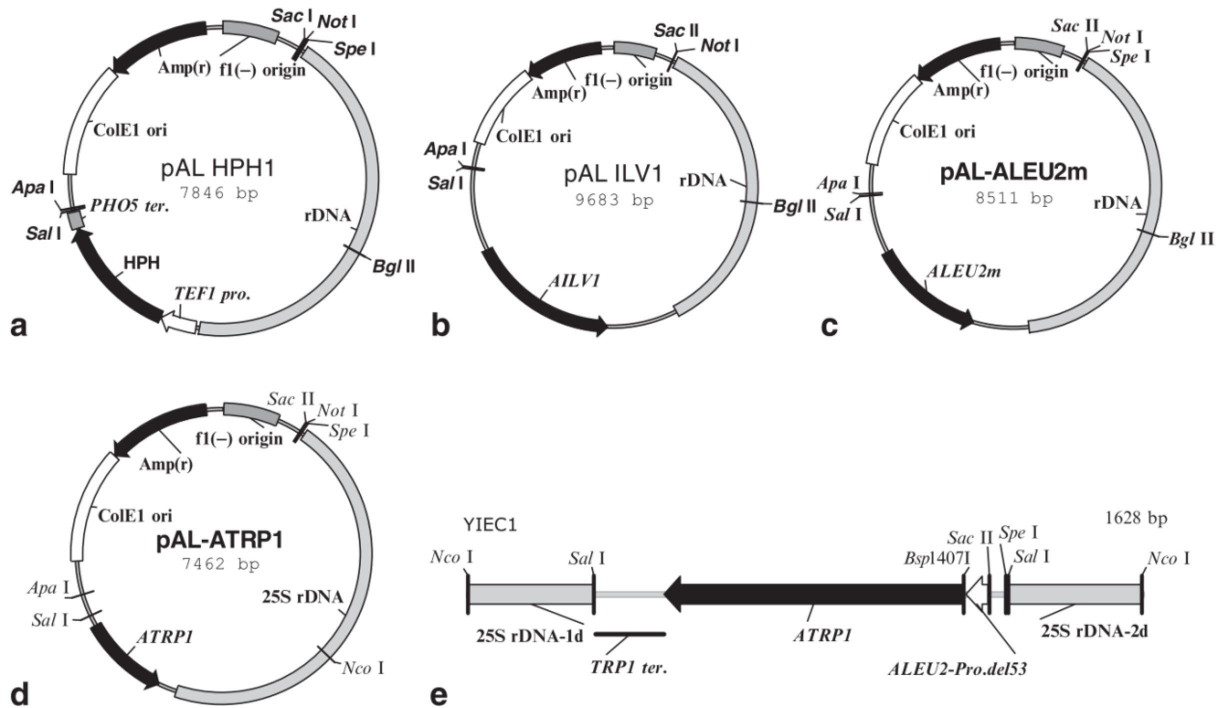


Figure 3 Vector maps for *B*. *adeninivorans*-based expression. (a) Vector pAL-HPH1 comprises the following elements: 25S rDNA sequence (rDNA) for chromosomal targeting, an expression cassette for *E*. *coli*-derived *hph* gene in the order *B*. *adeninivorans*-derived *TEF1* promoter (*TEF1* pro.), the *hph*-coding sequence (HPH), *S. cerevisiae*-derived *PHO5* terminator (PHO5 ter.), unique *Apa*I and *Sal*I restriction sites for insertion of expression cassettes and unique *Bgl*II site within the rDNA sequence for linearization. The vectors (b) pAL-AILV1, (c) pAL-ALEU2m and (d) pAL-ATRP1 harbor the selection markers *AILV1*, *ALEU2m* or *ATRP1* instead of the expression cassette for *E. Coli*-derived *hph* gene. (e) Yeast integration-expression cassettes (YIEC1), a novel vector type for multicopy transformation of *B. adeninivorans* lacking an *E. coli* part. It's flanked by *Nco*I sites and comprises the 25S rDNA sequences and the selection marker *ATRP1* fused to the 58 bp deleted *ALEU2* promoter. Figure adapted from Böer *et al*., (2009).

Table 1 *B. adeninivorans* vector elements

Component	Characteristics
Selection marker	
Amp(r), *E. coli hph*	Antibiotic resistance (ampicillin; hygromycin B)
LYS2, *AILV1*, *ALEU2m*, *ATRP1*	Auxotrophy complementation
Promoters	
TEF1, *AHSB4*	Constitutively expressed
GAA, *AHOG1*, *AILV1*, *AINV1*, *ALIP*, *AXDH*, *ATAL*	Inducible by specific carbon sources
Terminator (s)	
S. cerevisiae PHO5	
Cloning into yeast cells	
25S rDNA	Homologous integration into chromosomal DNA

2.3. Recombinant protein production

2.3.1. Examples

Development of *B. adeninivorans* as a host for expressing recombinant proteins has been widely studied (Böer *et al*., 2005). There is a history of successful expression of several proteins—covering different phylogenetic origins, e.g. bacteria, fungi, mammals and humans (Table 2)—in this yeast. Most of the proteins expressed employed the wild-type strain *B. adeninivorans* LS3 (Gienow *et al*., 1990) and the mutant strain 135 (Wartmann *et al*., 2000). Others used the leucine–auxotrophic LS3–derived strains G1211 (Samsonova *et al*., 1989, 1996) and G1212 (Steinborn *et al*., 2007)—which contain disrupted *atrp1* gene (see Table 2 for references).

The proteins expressed were either secreted or intracellular localized depending on the availability of a secretion signal. In most cases, activity of the protein was used to indicate the amount of protein expressed, with some excreted proteins produced on a milligram per liter scale, for example human serum albumin (HSA) and interleukine-6 (Wartmann *et al*., 2002, 2003a, b; Böer *et al*., 2007).

Most of the studies done so far employed shake-flask cultures. Only few recent cases show scale-up to batch or fed-batch cultivation in bioreactors, which had remarkable improvements in volumetric productivities (Hellwig *et al*., 2005; Knoll *et al*., 2007; Böer *et al*., 2011). However, these bioreactor studies only deal with strains expressing recombinant extracellular proteins. Thus, it will be interesting to observe these improvements for intracellular proteins as well.

For the most part, integrative expression vectors allowed stable integration of recombinant DNA into the host's genome, using *B. adeninivorans*-derived 25S rDNA. Although this resulted in low copy numbers, the *TEF1* promoter, in most cases, ensured strong constitutive expression (Rösel and Kunze, 1995). However, constitutive promoters are not always favorable as there is possibility of generating cells with reduced heterologous gene expression (Romanos, 1998).

Table 2 Expression of recombinant proteins in *B. adeninivorans*

Protein	Promoter/Selection marker	Vector type[a]/Production[b]	Reference
Bacterial			
***Pseudomonas putida* Catechol 2,3-dioxygenase[c]**	*AILV1/ALYS2*	Int. and Rep./Flask: 0.4 pkat mg^{-1}	Kunze and Kunze (1996)
	AINV/ALEU2m	Int. and Rep./Flask: 4.5 pkat mg^{-1}	Böer *et al*., (2004a)
***E. coli* β-galactosidase[c]**	*GAA/HPH1*	Int./Flask: 350 kU mg^{-1}	Wartmann and Kunze (2000)
	AHOG1/HPH1	Int./Flask: 350 U mg^{-1}	Böer *et al*., (2004b)
***Klebsiella sp.ASR1* Extracellular phytase[d]**	*GAA/ALEU2m*	Int./Flask: 75 mkat L^{-1}	Hahn *et al*., (2006)
***Ralstonia eutropha* β-ketothiolase**	*TEF1/HPH1*	Int./Flask or Fed-batch: 2.2 % PHA[e]	Terentiev *et al*., (2004a)
***R. eutropha* Acetoacetyl CoA reductase**	*TEF1/HPH1*	Int./Flask or Fed-batch: 2.2 % PHA[e]	Terentiev *et al*., (2004a)
***R. eutropha* PHA synthase**	*TEF1/ALEU2m*	Int./Flask or Fed-batch: 2.2 % PHA[e]	Terentiev *et al*., (2004a)
	TEF1/HPH1	Int./Flask or Fed-batch: 2.2 % PHA[e]	Terentiev *et al*., (2004a)
***Bacillus amyloliquefaciens*α-Amylase**	*TEF1/ALEU2m*	Int./Flask: 150 µkat L^{-1}	Steinborn *et al*., (2005)
***B. amyloliquefaciens*α-Amylase**	*TEF1/ATRP1*	Int./Flask: 300 µkat L^{-1}	Steinborn *et al*., (2007)
Fungal			
***B. adeninivorans* Invertase[d]**	*TEF1/ALEU2m*	Int./Flask: 500 nkat mL^{-1}	Böer *et al*., (2004a)
***B. adeninivorans* Phytase[d]**	*TEF1/ALEU2m*	Int./Flask: 13 FTU mL^{-1}	Hellwig *et al*., (2005)
		Int./Fed-batch: 900 FTU mL^{-1}	Hellwig *et al*., (2005)

		Int./Fed-batch: 10 X 10^6 FTU L^{-1}	Knoll *et al.*, (2007)
***B. adeninivorans* Tannase**[d]	*TEF1/ ALEU2-ATRP1m*	Int./Flask: 400 U L^{-1}	Böer *et al.*, (2009)
		Int./Flask: 1 642 U L^{-1}	Böer *et al.*, (2011)
		Int./Fed-batch: 51 900 U L^{-1}	Böer *et al.*, (2011)
		Int./Fed-batch: 31 300 U L^{-1}	Böer *et al.*, (2011)
***B. adeninivorans* Acid phosphatase**[d]	*TEF1/ALEU2m*	Int./Flask: 17 054 U g^{-1}	Minocha *et al.*, (2007)
		Int./Batch: 18 465 U g^{-1}	Minocha *et al.*, (2007)
***B. adeninivorans* Xylitol dehydrogenase**[c]	*TEF1/ALEU2m*	Int./Flask: 600 mkat L^{-1}	Böer *et al.*, (2005c)
***B. adeninivorans* Lipase**[d]	*TEF1/ALEU2m*	Int./Flask: 3 300 U L^{-1}	Böer *et al.*, (2005b)
***B. adeninivorans* Transaldolase**[c]	*TEF1/ALEU2m*	Int./Flask: 35 mkat L^{-1}	El Fiki *et al.*, (2007)
Mammalian (non-human)			
***Aequorea victoria* Green fluorescent protein**[c]	*TEF1/HPH1*	Int./Flask: n.d.	Wartmann *et al.*, (2002b)
	TEF1/ALEU2m	Int./Flask: n.d.	Wartmann *et al.*, (2003a)
	AHSB4/ALEU2m	Int./Flask: n.d.	Wartmann *et al.*, (2003b)
	AXDH/HPH1	Int./Flask: n.d.	Böer *et al.*, (2005c)
Human			
***Homo sapiens* human serum albumin**[d]	*TEF1/HPH1*	Int./Flask: 50 mg L^{-1}	Wartmann *et al.*, (2002b)
	TEF1/ALEU2m	Int./Flask: 50 mg L^{-1}	Wartmann *et al.*, (2003a)

	AHSB4/ALEU2m	Int./Flask: 50 mg L^{-1}	Wartmann *et al*., (2003b)
	ATAL/ALEU2m	Int./Flask: 0.6 mg L^{-1}	El Fiki *et al*., (2007)
***H. sapiens* Estrogene receptor α**	*TEF1/HPH1*	Int./Flask: n.d.	Hahn *et al*., (2006)
***H. sapiens* Interleukin-6[d]**	*TEF1/ALEU2m*	Int./Flask: 220 mg L^{-1} (b.c.)	Böer *et al*., (2007)
		Int./Flask: 145 mg L^{-1} (m.)	Böer *et al*., (2007)

[a] Int.: Integrative vectors

[b] Cultures were performed in shaken (flask) or stirred bioreactors (batch or fed-batch cultivation).

[c] These proteins were produced intracellularly and the exact location was not reported in most cases

[d] These proteins were excreted into the medium; acid phosphatase was an exception since its cell-wall bound

[e] % final product per dry weight

Abbreviations: n.d. – not determined, b.c. – budding cells, m. – mycelia

In some cases increased copy number did not improve recombinant protein production. For example, cloning of two *ATAN1* expression modules in recombinant strains did not result in increased protein yields; it instead proved detrimental to the cells as indicated by low biomass yields (Böer *et al*., 2011). Although this did not affect the product yield, as it remained the same, it showed the significance of metabolic burden placed on yeast cells by heterologous expression of some genes (Romanos, 1998).

Gellissen *et al*., (2005) compared the efficiency of heterologous expression by *B. adeninivorans* with other yeast expression platforms. The authors acknowledged, in their review, the difficulty of comparing yeast-based expression platforms. However, one study by Böer *et al*., (2007) compared *B. adeninivorans*, *H. polymorpha* and *S. cerevisiae* for the production of human IL-6. Only *B. adeninivorans* correctly processed the MF1-IL6 precursor even though all the yeasts showed high production of the recombinant protein. In an indirect comparison, cells of *B. adeninivorans* G1212 secreted more than seven–fold tannase as compared to *P. pastoris* (Böer *et al*., 2011). These observations may not mean that *B. adeninivorans* is a better platform in general but shows the potential of this yeast as a host for recombinant protein production. Again there is no “one size fits all” when it comes to heterologous expression. Therefore, it is necessary to screen different yeasts to find suitable expression platform for a particular protein.

2.3.2. Comparison of different yeasts for CYP450 and VAO expression

In trying to find suitable expression platforms for CYP505A1, CYP53B1 and VAO, Smit *et al*., (2012a, b) developed a broad range vector to test different yeasts simultaneously. This showed, for the first time, expression of CYP450s (CYP505A1 and CYP53B1) in *B. adeninivorans*. The following yeasts—*B. adeninivorans*, *H. polymorpha*, *K. lactis*, *K. marxianus*, *S. cerevisiae*, *Y. lipolytica* and *C. deformans*—were compared for the expression of PsVAO. *B. adeninivorans* showed the best expression of PsVAO as suggested by biotransformation of eugenol (Figure 4). This yeast achieved more than 95 % conversion of eugenol after 48 h. *K. marxianus, Y. lipolytica* and *C. deformans* also showed significant activity, with activity descending in this order.

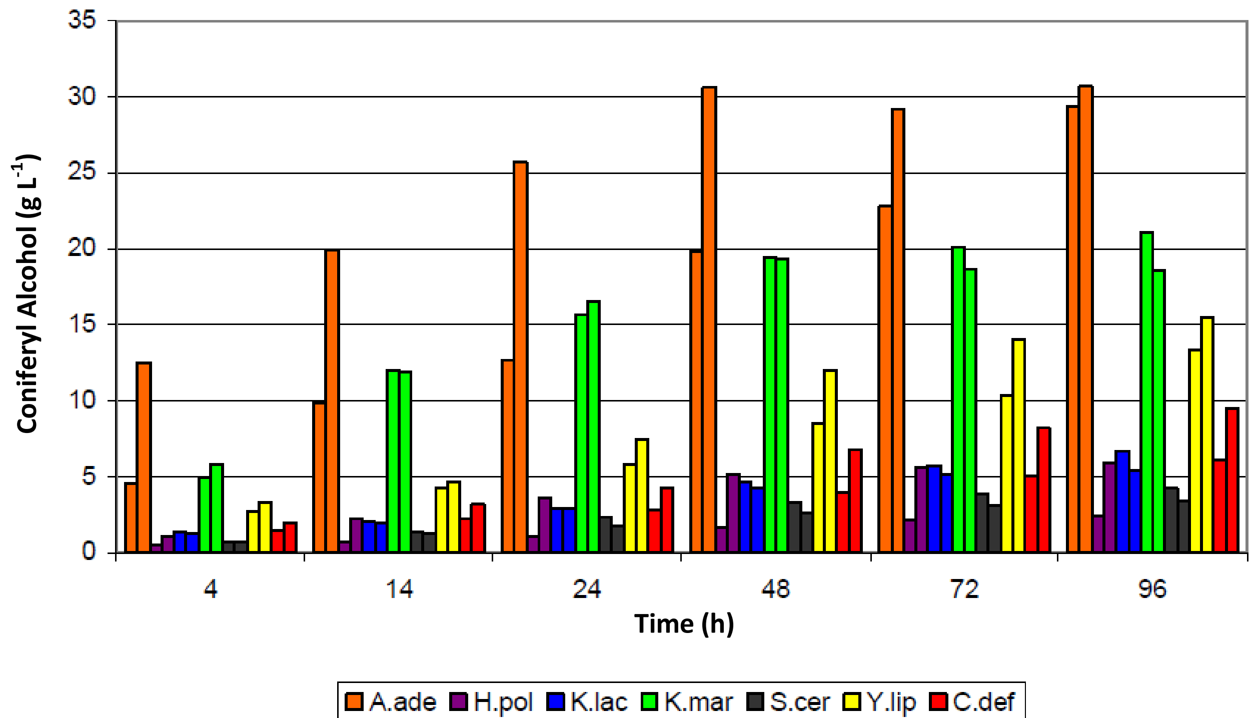


Figure 4 Coniferyl alcohol production by yeasts transformed with pKM130 expressing the *P. simplicissimum* VAO gene under transcriptional control of the *Y. lipolytica TEF* promoter. Figure adapted from Smit *et al*., (2012a).

In other studies, *B. adeninivorans* again showed the highest gene expression, in this case for CYP505A1 and CYP53B1 (Theron, 2012; Theron *et al*., 2014). Biotransformation of the substrates 4-hexylbenzoic acid and *p*-hydroxybenzoic acid by CYP505A1 and CYP53B1, respectively, showed highest conversion with *B. adeninivorans*. Again, *B. adeninivorans* outperformed the yeasts *K. marxianus*, *S. cerevisiae*, *Y. lipolytica,* and *H. polymorpha*.

2.4. Cultivation conditions and process parameters for recombinant protein production

Only few systematic studies define cultivation conditions and process parameters for recombinant protein production in *B. adeninivorans*. Two case studies, which feature the wild-type strain LS3 and its leucine-auxotrophic mutant G1211 (G1212), will be discussed at the end

of this section. Direct application of these studies to other *B. adeninivorans* strains will not necessarily be applicable (Kunze and Kunze, 1994). There is also not yet an industrial process based on *B. adeninivorans* (Stöckmann *et al*., 2009).

2.4.1. Issues regarding cultivation media

Cultivation media plays a very significant role in the development of yeast based processes for industrial applications (Hahn-Hägerdal, 2005). Hahn-Hägerdal (2005) noted that fermentation performance and physiological phenotype of the yeast strain is largely a reflection of the composition of cultivation medium. Generally, complex media allow vigorous growth of microorganisms as compared to mineral media. This is due to the availability of biosynthetic precursors in complex media that reduce the need for microorganisms to produce them and waste their metabolic energy in the process.

In order to develop a yeast-based process for large-scale production of heterologous proteins and other metabolites successfully, choice of medium composition must be done with care. This is because of the role played by different media in the production of heterologous proteins. For example, heterologous production of laccase in *Y. lipolytica* increased three fold when cultivation switched from yeast nitrogen base (YNB) to a complex medium (Madzak *et al*., 2005). Another example showed a 20-fold increase in production of a potent thrombin-specific inhibitor, hirudin, when recombinant *S. cerevisiae* was cultivated in a complex medium (Choi *et al*., 1994). In addition, *S. cerevisiae* autoselective strains expressing heterologous xylanase or α-L-arabinofuranosidase genes showed 24- and up to 70-fold higher enzyme levels, respectively, when grown in complex medium (La Grange, 1996; Crous *et al*., 1996).

The examples above show a positive effect on the production of the heterologous proteins investigated when the yeasts *Y. lipolytica* and *S. cerevisiae* were cultivated in complex media. However, in some cases a chemically defined medium would be preferable, especially for protein purification. For example, switching medium composition during cultivation allowed a single-step purification of recombinant cysteine proteinase (NsCys) produced by *P. pastoris* (Aoki *et al*., 2003) - the authors first grew the yeast in complex medium to obtain biomass in short time and then transferred it to a minimal medium that facilitated protein secretion and purification. This of course might be crucial for proteins secreted into the medium.

The examples above show how critical medium composition is on the production of heterologous proteins. The effect will not be the same for different proteins though. Therefore, different classical as well as statistical techniques are employed for optimization of medium composition for heterologous protein production (Rao and Satyanarayana, 2003; Chen, 1996).

2.4.2. Cultivation media for *B. adeninivorans*

Hellwig *et al*., (2005) used *B. adeninivorans* wild-type strain LS3 and mutant strain 135 to investigate synthetic media and operating conditions for non-limited growth in shake-flask cultures and stirred tank bioreactors (STRs). Respiration rates in shake-flasks were monitored by online measurement of oxygen transfer rate (OTR) with a RAMOS device (Anderlei and Büchs, 2001; Anderlei *et al*., 2004). A RAMOS device uses an oxygen sensor to analyze oxygen concentration in the gas headspace of the shake flask (Losen *et al*., 2004). This is done in two phases, i. e. measuring and rinsing. Measuring of oxygen depletion, with slope corresponding to OTR, takes place in a completely sealed flask during the measuring phase. After, in the rinsing phase, controlled airflow passes through the shake flasks to restore the original gas equilibrium. Throughout this process, proper airflow control allows the average headspace gas concentration of the measuring and normal shake flasks (with cotton plug) to be equivalent. This measuring and rinsing cycle repeats continuously until cultivation finishes. Successful application of the RAMOS device has been reported elsewhere (Silberbach *et al*., 2003; Stöckmann *et al*., 2003 a, b; Losen *et al*., 2004).

Two synthetic media (Table 3), i.e. yeast minimal media (YMM) originally described for *Candida albicans* (Tanaka *et al*., 1967) and the SYN6 medium described for *H. polymorpha* (Jenzelewski, 2002), were assessed by Hellwig *et al*., (2005). As shown in Table 3, there are higher ammonium and magnesium concentrations in SYN6 medium compared to YMM medium. The iron content is very limited in YMM and there are five to more than 80 times increased concentrations of microelements in SYN6 relative to YMM. The vitamin content of YMM is also low with only thiamine at very low concentrations compared to SYN6 that has biotin in addition to thiamine. Thus, it is clear that the SYN6 medium is richer in nutrients compared to YMM and thus allows non-limited supply of nutrients to the yeast.

Table 3 Compositions of synthetic minimal media YMM and SYN6 (Gellissen, Ed 2005)

Basal Salts	Concentration $g\ L^{-1}$		Micro Elements	Concentration $mg\ L^{-1}$		Vitamins	Concentration $mg\ L^{-1}$	
	YMM	SYN6		YMM	SYN6		YMM	SYN6
$(NH_4)_2SO_4$	6	7.66	H_3BO_3	0.5	0.66	Biotin	0.04	0.4
KH_2PO_4	1	1	$CuSO_4{\cdot}5H_2O$	0.107	5.5	Thiamine-HCl	2	133.5
$MgSO_4{\cdot}7H_2O$	2.04	3	KI	0.1	0.66			
$CaCl_2{\cdot}2H_2O$	1	1	$ZnSO_4{\cdot}7H_2O$	0.4	20			
NaCl		0.33	$MnSO_4{\cdot}H_2O$	0.303	26.5			
KCl		0.3	$Na_2MoO_4{\cdot}2H_2O$	0.234	0.66			
$FeCl_3{\cdot}6H_2O$	0.01		$CoCl_2{\cdot}6H_2O$	0.183	0.66			
MES	27.3	27.3	EDTA		66.5			
Glucose	20	20	$(NH_4)FeSO_4{\cdot}6H_2O$		66.5			
			$NiSO_4.6H_2O$		0.66			

B. adeninivorans cells grown in shake flasks containing YMM showed limited growth compared to SYN6 as shown by respiration curves (Figure 5). The original YMM gave poor culture respiration rates below 10 mmol L^{-1} h^{-1} due to low calcium and iron content. Increase of the concentration of these compounds in YMM improved the respiration rates (up to 25 mmol L^{-1} h^{-1}) even though the growth of *B. adeninivorans* remained limited. To prevent the limitations SYN6 was employed which allowed high respiration rates (44 mmol L^{-1} h^{-1}). This was due to high nutrient concentrations in the media. In all media glucose was used as the carbon source. Low respiration rates were undesirable as they lead to poor biomass yields and long cultivation times (Hellwig *et al*., 2005).

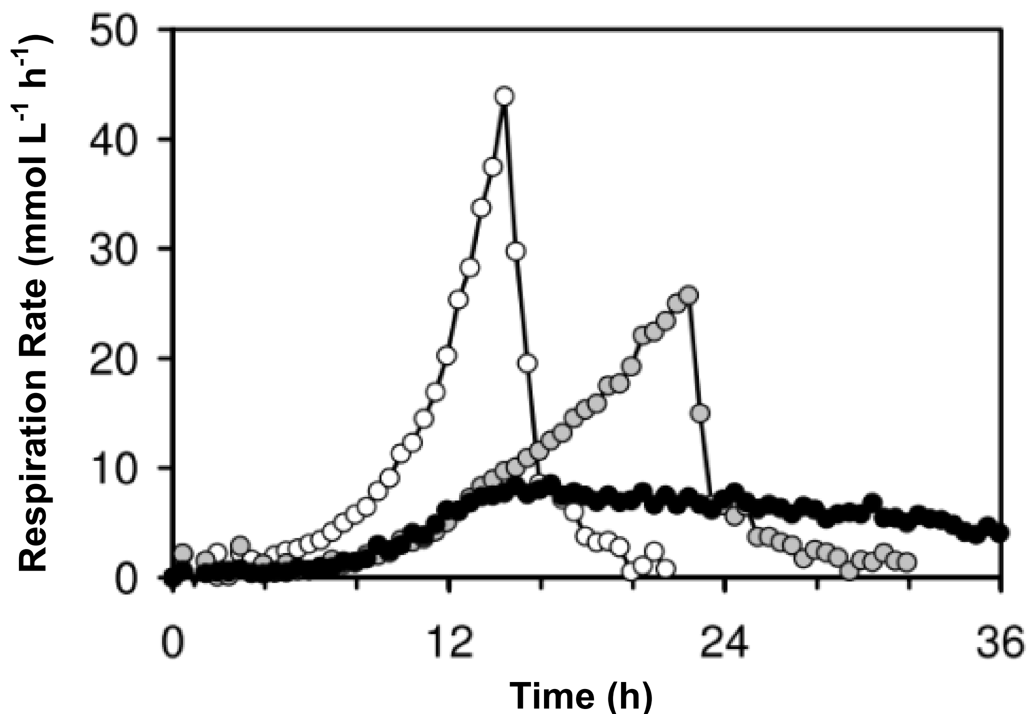


Figure 5 Online measurement of respiration rates for assessment of minimal medium for *B. adeninivorans* shake-flask cultivation. Original YMM (black circles), Modified YMM* (grey circles) and SYN6 (open circles). Cells were cultivated at 30 °C and initial pH of 6.4 using MES buffer. Figure adapted from Stöckmann *et al.*, (2009)

Table 4 shows operational conditions that allowed OTR_{max} of 36 and 58 mmol L^{-1} h^{-1} for MES buffered modified YMM* and SYN6 shake flask cultures, respectively. The optimal pH values observed for growth of *B. adeninivorans* ranged from 2.8 to 6.5, allowing maximal growth rates of 0.32 ± 0.01 h^{-1} (strain LS3) and 0.31 ± 0.01 h^{-1} (strain 135) (Gellissen, ed 2005). In further growth experiments with strain LS3 using fed-batch STR, batch phase cultures showed respiration rate, maximum specific growth rate and biomass yield values similar to shake flask experiments. The conditions and results for SYN6 and YMM medium for fed-batch STR are shown in Table 4. A high OTR_{max} of 150 mmol L^{-1} h^{-1} was only achieved during glucose-limited feeding.

Cases 1 and 2 below shows examples of application of cultivation conditions described for *B. adeninivorans* LS3 in the production of two industrially important enzymes, phytase and tannase, respectively. These conditions can be used, with modification where needed, for large-

scale production of other enzymes of industrial or biotechnological importance. In the case of intracellular enzymes, the resulting yeast can serve as a biocatalyst if isolation of pure enzyme is not required.

Table 4 Cultivation conditions and example of results obtained for *B. adeninivorans*

Conditions[#]	Shake-flasks		Fed- batch STR	
	YMM*	SYN6-MES	SYN6[a]	YMM
Flask/Reactor size (mL)	250	250	2000	1 250
Working volume (mL)	20	10	2000	n.a[b]
Buffer / pH control	MES (0.14 M)	MES (0.14 M)	Ammonia[c] (12.5 % w/w)	NaOH (4 – 5 N)
Temperature (°C)	30	30	30	30
pH	6.4 – 5.3	6.4 – 5.3	6	5
Shaking/Stirrer speed (rpm)	350	350	400 – 2000	500 – 1000
Aeration rate (vvm)	n.a	n.a	0.4 - .1.5	1 – 2
Dissolved oxygen tension (DOT, %)	n.a	n.a	40	20 – 50
OTR_{max} (mmol L^{-1} h^{-1})	36	58	150	n.a
Maximum specific growth rate (μ_{max}, h^{-1})	0.32 (LS3) 0.31 (135)	0.32 (LS3)[d] 0.31 (135)	n.a	n.a
Biomass (g L^{-1})	11	11	112	162
Biomass yield ($Y_{x/s}$)	0.55	0.55	0.57	0.81

[#]Böer *et al*., 2011, Knoll *et al*., 2007, and Hellwig *et al*., 2005.

[a] This denotes abbreviations for different types of medium used for cultivation studies on *B. adeninivorans* with YMM used for tannase production and SYN6 for phytase.

[b]n.a: information is not available.

[c] The ammonia solution also served as a nitrogen source in addition to pH control.

[d] Abbreviations LS3 and 135 stands for *B. adeninivorans* strains LS3 and 135, respectively

2.4.3. Examples of application of the above cultivation conditions

Case study 1: Recombinant phytase production in shake-flask cultures and high-cell-density fermentation of *B. adeninivorans* using SYN6 medium

Phytases catalyze the hydrolysis of phytic acid to myo-inositol and inorganic phosphates. The secreted phytase from *B. adeninivorans* has optimal temperature and pH of 75 °C and 4.5, respectively (Sano *et al*., 1999). *B. adeninivorans* 1211 (*aleu2*) secreting a homologous phytase under the control of constitutive *TEF* promoter (Wartmann *et al*., 2003a; Rösel and Kunze, 1995), was cultivated in shake-flasks using SYN6-MES medium and in fed-batch STRs under conditions described for wild-type strain LS3 (Hellwig *et al*., 2005; Table 4). As with the wild-type strain LS3, *B. adeninivorans* 1211 (*aleu2*) had growth characteristics of the batch phase similar to those observed in shake-flask experiments implying that the metabolism and growth of the recombinant strain was not affected by transformation. A similar observation was made for growth-coupled phytase production of ca. 13 FTU mL^{-1} (FTU = amount of enzyme releasing 1 µmol of inorganic phosphate per min from sodium phytate at pH 5.5 and 37 °C) with improvement up to ca. 900 FTU mL^{-1} achieved during glucose-limited feeding. Thus, cultivation conditions described by Hellwig *et al*., (2005) proved applicable to phytase production. In addition, Knoll *et al*., (2007) reported phytase activities of up to 10×10^6 FTU L^{-1} obtained under pressurized cultivation conditions (up to 5 bar) using modified SYN6 medium in fed-batch cultivation (Figure 6). These conditions provided non-oxygen limited growth resulting in cell densities of up to 224 g L^{-1} in 42 hours (a 2-fold biomass improvement as compared to Hellwig *et al*., 2005).

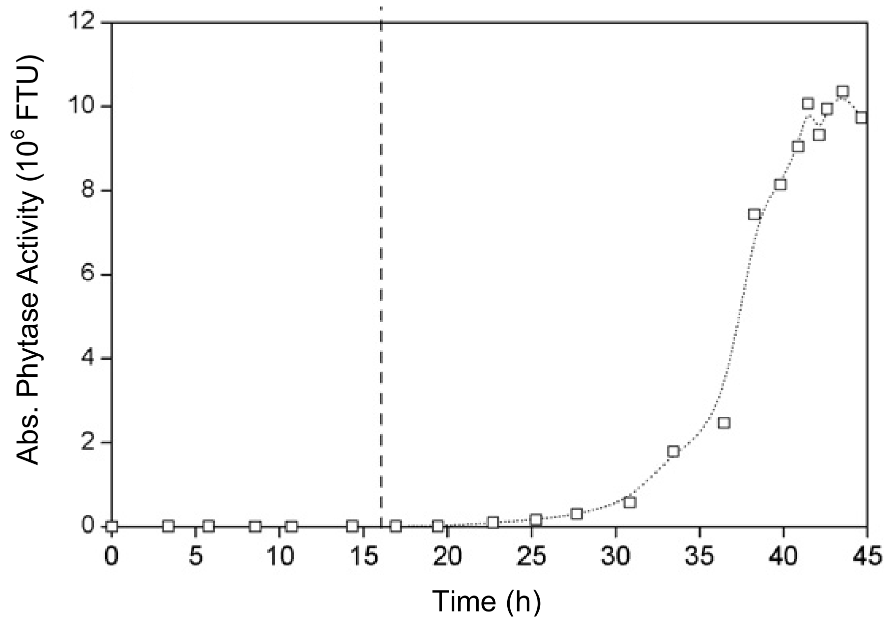


Figure 6 Recombinant phytase production by *B. adeninivorans* G1211 regulated by *TEF* promoter in high cell density cultivation (HCDC). The culture was grown in SYN6 medium at temperature and pH of 30 °C and 6, respectively. Figure adapted from Knoll *et al.*, (2007)

Case study 2: Optimization of tannase production in fed-batch STRs using YMM medium

Tannase plays a central role in production of gallic acid by catalyzing the hydrolysis of ester and depside bonds in hydrolysable tannins with the removal of glucose residues (Deschamps *et al*., 1983; Haslam & Stangroom, 1966). *B. adeninivorans* secretes tannase when grown on tannic or gallic acid (Böer *et al*., 2009). This enzyme shows optimum activity at temperature and pH of 40 °C and six, respectively.

Recently, Böer *et al*., (2011) optimized *B. adeninivorans* strains for homologous tannase production. Several plasmids carrying one or two tannase (*ATAN1*) expression modules in the same or opposite orientations were constructed. Transformants overexpressing *ATAN1* gene under the control of *B. adeninivorans*-derived *TEF1* promoter, cultured in shake-flasks using complex (YEPD) or YMM medium produced up to 1 642 U L^{-1} of tannase regardless of the copy number. Fed-batch cultivations of recombinant strains carried out using YMM medium (see Table 4 for fermentation conditions) achieved highest activity of about 51 900 U L^{-1} in strains

containing one *ATAN1* expression module (G1212/YRC102-ATAN1, Figure 7 B). However, maximum yield coefficients *Y(P/X)* of tannase remained similar. Although the author's interest was to optimize tannase production, they also observed a high cell density of about 162 g L^{-1} after 142 hours of cultivation. The work also shows successful application of fed-batch fermentation in large-scale production of recombinant proteins by *B. adeninivorans*.

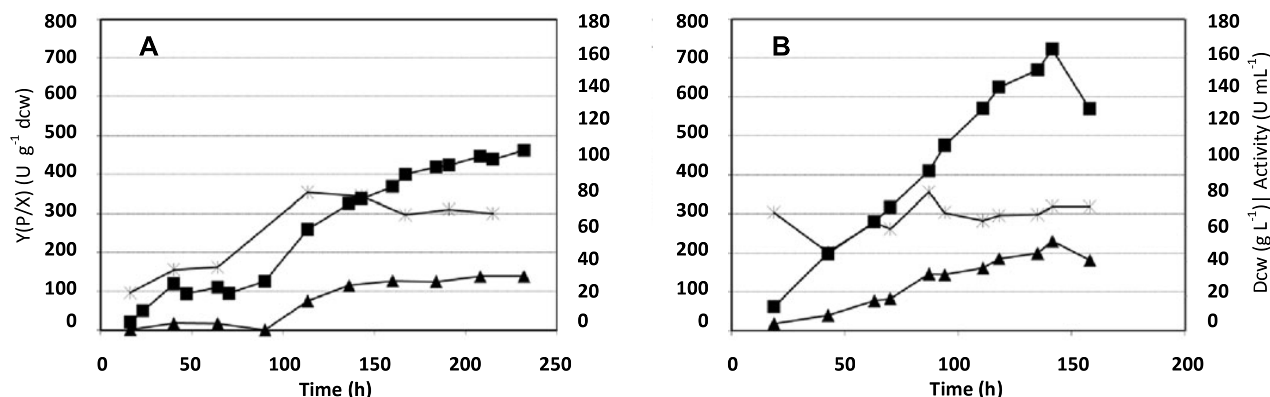


Figure 7 Fed-batch cultivation of A. *B. adeninivorans* G1212/YRC102-2ATAN1v and B. G1212/YRC102-ATAN1 in the bioreactor using YMM with glucose as the carbon source. Time course of Atan1p activity (triangle), output *Y(P/X)* (asterisk) and dcw (square). Figure adapted from Böer *et al.*, (2011)

2.5. Concluding remarks

This literature review highlights some of the major studies on *B. adeninivorans* so far. The focus of these has been to establish this yeast as an expression platform for recombinant gene expression. Cultivation media and culture conditions have also been studied at length. However, a lot still needs to be studied in this organism.

Looking at the literature, the expression of a specific gene has not been studied much in *B. adeninivorans* except researchers showing capability of this yeast in expressing several genes. Production of phytase and optimization of tannase are some of the few studies that attempted to focus on a specific gene. The role of culture conditions on the expression of these and the extent to which they affect expression is significant. These types of studies add another dimension to optimization studies in addition to genetics, especially for protein or biocatalyst production at an industrial scale.

The expression of CYP450s as well as VAO has recently been described for the first time in *B*. *adeninivorans*. These groups of enzymes, which are the focus of this thesis, are both expressed as intracellular proteins and there is not enough background on the factors affecting expression of these proteins in *B*. *adeninivorans*, since most proteins expressed were excreted into the culture medium. Although this makes sense for protein isolation and purification purposes, the CYP450s especially, do not favour this setup due to their requirement for reductase partners that transfer electrons to them. Therefore, it will be interesting to study the factors that affect their expression in *B*. *adeninivorans*.

Overall, this work aims to understand the culture conditions affecting the expression of oxidoreductases, i.e. CYP505A1 and VAO, in *B*. *adeninivorans*. Furthermore, to understand how these conditions affect biomass production and the relation of this to foreign gene expression. These are some of the questions not answered by current literature on *B*. *adeninivorans*.

Chapter 3

Materials and Methods

3. Introduction

This chapter presents all the methodology followed in this thesis. The assay development methods are presented first, followed by studies of medium composition and culture conditions on CYP505A1 then VAO. One of the objectives of this study was to develop simple assays that could be used to analyze large numbers of samples in a short time. The usually employed GC and HPLC, although accurate, take long to analyze large numbers of samples. Therefore, both thin layer chromatography (TLC) and ultra violet light spectroscopy (UV) were explored for simple assay methods and ability to analyze large numbers of samples by using TLC or a microtiter plate reader for UV assays. The last section deals with the effect of dissolved oxygen tension (DOT) on VAO expression and biomass production in bioreactors using results obtained from media and culture condition studies.

Studies of the effect of medium composition and culture conditions on CYP505A1 and VAO was carried out using a type of fractional factorial design known as Plackett-Burman (Plackett and Burman, 1946). Plackett-Burman design is often used for screening several variables of a process at once using minimum resources and time. Studies by Srinivas et al., (2004), Robert *et al*., (2006), Rajendiran *et al*., (2011), Naveena *et al*., (2005), Ahuja *et al*., (2004) and Li *et al*., (2007) are some of the examples that demonstrate the application of this statistical design.

Plackett-Burman allowed the identification of significant nutrients among 19 sources or categories of medium nutrients for their effect on alpha galactosidase production by *A. niger* MRSS 234 using solid state fermentation (Srinivas *et al*., 2004). Urea, corn steep liquor, guar flour and citiric acid were shortlisted for further optimization. Ahuja *et. al*., (2004) successfully identified components limiting aggregated morphology of *Teredinobacter turnirae* (a shipworm bacterium) also using Plackett-Burman design. Increasing the concentrations of the limiting

components (i. e. $MnCl_2 \cdot 4H_2O$, Na_2CO_3 and K_2HPO_4) by 4-fold, 12-fold and 12-fold, respectively the authors were able to obtain exponential growth of the bacterium. Screening 15 parameters for L-(+) lactic acid production from wheat bran in a solid state fermentation by *Lactobacillus amylophilus* GV6 peptone, yeast extract, tri-ammonium citrate, $NaH_2PO_4 \cdot 2H_2O$ and Tween 80 were found to influence L-(+) lactic acid productivity (Naveena *et al*., 2005). Factors significantly affecting yield and compositions of pectins extracted from chicory roots were successfully determined using Plackett-Burman design (Robert *et al*., 2006). The list does not end here.

The Plackett-Burman is a statistical design model that is based on the first order model (Plackett and Burman, 1946):

$$Y = \beta_0 + \sum \beta_i x_i.$$

This model (where β_0, β_i and x_i are intercept, coefficient and variables, respectively) was used in this project to identify variables that significantly affect CYP505A1 and VAO production and biomass production by *B. adeninivorans* UOFS Y-1220. Plackett-Burman allows the researcher to evaluate the effect of k variables in *k* + 1 trials (experiments) where k represents the number of the variables (medium components and culture conditions). Each variable is assigned two levels, high and low, which are shown by (+) and (-) as given in Table 6 and 8.

Most of the variables studied here as well as their levels were arbitrarily chosen as a starting point. Some of the factors were selected from other studies in my honours research. The suitability of the Plackett-Burman design here was mainly due to lack of immediately available variables to study, therefore the design provided a quick identification of the factors that we could focus on for further studies.

3.1. Materials

3.1.1. Chemicals

Coniferyl alcohol, eugenol, 4-hexylbenzoic acid and p-hydroxybenzoic acid were purchased from Sigma-Aldrich. 1,2-Dodecanediol was purchased from Fluka and hygromycin from HyClone.

3.1.2. Yeast strains and Maintenance

In this study, *B. adeninivorans* UOFS Y-1220 transformed with pKM160-PsVAO or pKM177-CYP505A1 was used. The *Y. lipolytica*-derived constitutive *TEF* promoter regulated the expression of the cloned genes. Glycerol stocks (15 % v/v) of the strains were maintained at – 80 °C. These glycerol stocks served to inoculate YPD-agar plates supplemented with hygromycin (0.6 mg L^{-1}) to revive the strains. The inoculated plates were incubated at 30 °C for 24 h.

3.2. Methods

3.2.1. Preparation of YPD medium and YPD-hygromycin B agar plates

YPD is a complex medium that contains yeast extract (Y), peptone (P) and dextrose (D, sugar). The YPD medium (pH ~ 6.5) was prepared by adding, in one litre of distilled water: 10 g yeast extract, 20 g peptone, and 20 g glucose (or 22.1 g glucose monohydrate). The medium was stirred until all the chemicals were fully dissolved. This solution was sterilized by autoclaving at 121 kPa for 15 min, and cooled to room temperature before used for cultivation.

To prepare YPD agar plates, YPD medium was again prepared as shown above, with 20 g of agar added before sterilization. This was followed by cooling the medium to near solidification (ca. 50 °C). After this, hygromycin B solution (50 mg mL^{-1}) was aseptically added up to a final concentration of 600 µg L^{-1}. The final medium was then poured aseptically into sterile Petri dishes that were afterwards left to solidify before being stored at 4 °C.

3.2.2. Growth and harvesting of cells

YPD medium (50 mL), in 500 mL Erlenmeyer flasks, was inoculated with a loop full of 24 h YPD-agar culture. The cultures were incubated on a rotary shaker at 120 rpm and 30 °C. After 48 h, they were removed and transferred to pre-weighed 50 mL centrifuge tubes—for preparation of wet cell biomass—or samples from the cultures were transferred to pre-weighed 1 mL centrifuge tubes (unless specified otherwise)—for determination of dry cell biomass.

3.2.3. Preparation of wet cell biomass

Pre-weighed centrifuge tubes (50 mL) containing the cultures were centrifuged at 4000 x *g* for 10 min. The supernatant was decanted and the pellet used as wet cell biomass. After determining the weight of the wet biomass, the pellet was resuspended in a suitable volume of potassium phosphate buffer (50 mM, pH 8) to give a desired biomass concentration.

3.2.4. Determination of dry cell biomass

For dry cell biomass determination, three pre-weighed centrifuge tubes, each filled with 1 mL culture, were centrifuged at 15 294 x *g* for 10 min. Supernatants were decanted and pellets dried at 80 °C until the mass—weighed with an electronic precision balance (Mettler AJ100, Switzerland)—remained constant.

3.2.5. Assay Development

Section A: CYP505A1

3.2.5.1. Thin layer chromatography

Sample loading

Although this method falls under CYP505A1 section, thin layer chromatography (TLC) was also used qualitatively to analyze VAO biotransformations. Therefore, how TLC was applied for VAO experiments will be discussed as well. The ethyl acetate extracts from the biotransformation experiments were applied to the TLC plates (TLC Silica gel 60F254, Merck LGaA, Germany) at one to 1.5 cm height from the lower edge. Ten microliter samples were spotted for VAO experiments and only two microliter samples for CYP505A1. The lower sample volume for CYP505A1 was chosen to allow good separation of peaks on the ImageJ chromatogram. All the samples were manually spotted using a pipette. The TLC plates were air dried before transfer to the TLC chamber for development.

Development of the chromatogram

The mobile phase used for TLC plate development contained di-n-butyl ether, formic acid and water in the ratio 90:7:3. The TLC chamber was pre-saturated overnight with the mobile phase at room temperature before being used. The sample spotted TLC plates were transferred to the TLC chamber and allowed to stand until the mobile phase had covered up to 80 to 90 % of the plate. These plates were then taken out and air-dried before visualization.

Visualization of the spots

The developed TLC plates were visualized under UV light using a UV lamp held manually towards the plate. The compounds on the plate appeared as black spots on a green background. The plates were photographed using Gel Doc (Bio Rad).

3.2.5.2. Analysis of 4-hexylbenzoic acid and its biotransformation products using ImageJ software

After obtaining the thin-layer chromatography pictures from the Gel-Doc the pictures were saved as TIFF images. To begin the analysis, the image (1) was first loaded onto the ImageJ program (Figure 8). This image is usually grey with black spots (from UV) denoting the products and substrate or internal standard. Each column (represented by series of spots) showing one sample or experiment was highlighted using “Rectangular” (2) tool as shown by the yellow rectangles. After selection, the image background was removed using “Subtract background” under “Process” (3) to achieve a flat base on the chromatogram. All the lanes highlighted by the “Rectangular” tool were plotted using “Plot lanes” in “Gels” under “Analyze” (4). The “Straight” (5) tool was used to draw the baseline to remove all the noise. The level of noise was taken as the space on the plate covered only by the mobile phase. The “Wand” (6) tool was used afterwards to select the peaks on the chromatogram. This tool calculates the area of the peaks based on the pixel intensity of the spots. As each spot was clicked or selected the calculated areas were shown immediately in another window named “Results” (7). The obtained data was saved with an excel extension (.xls) for later use.

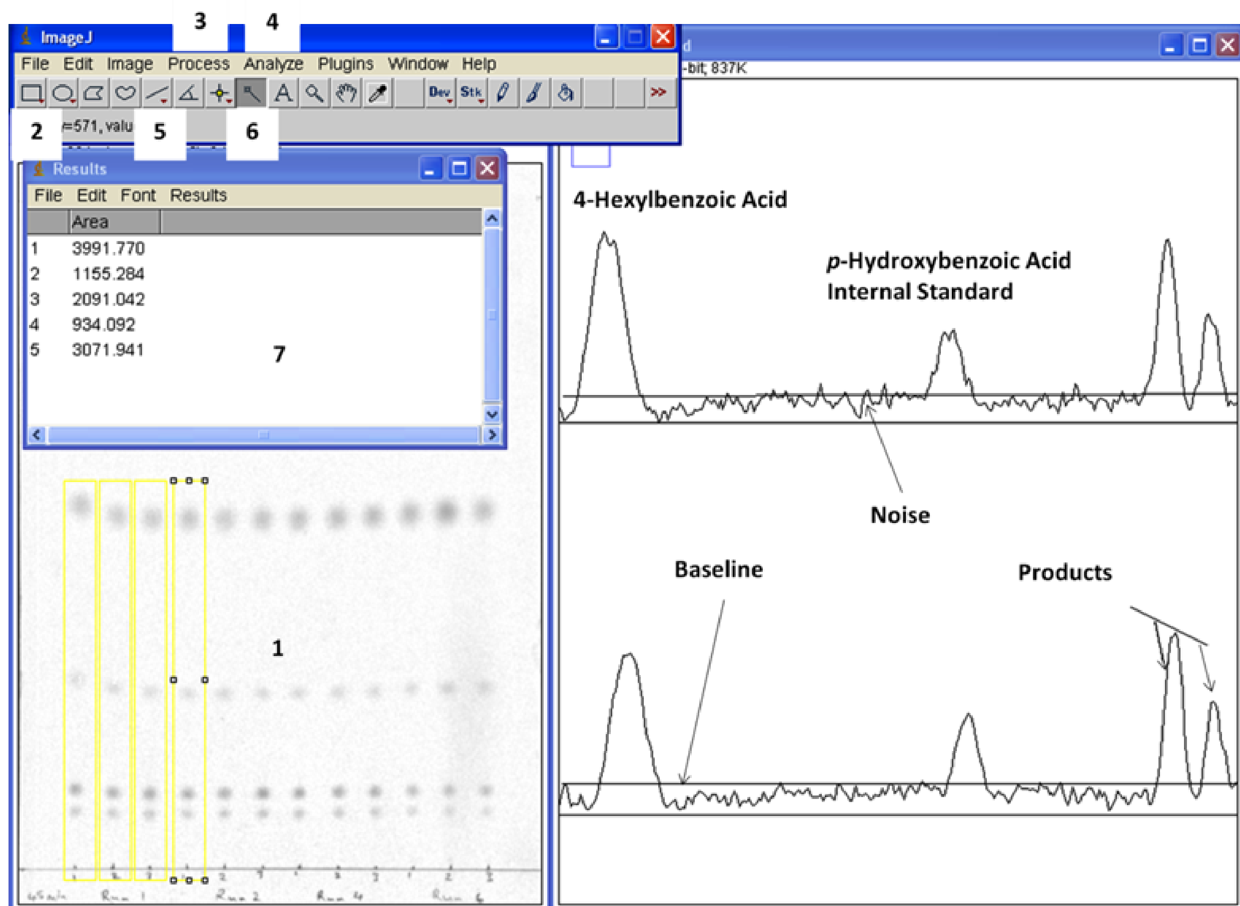


Figure 8 Screenshot of ImageJ as used for the analysis of 4-hexylbenzoic acid biotransformation

3.2.5.3. Determination of linear relationship between 4-hexylbenzoic acid and its biotransformation products from CYP505A1

Cells of *B. adeninivorans* UOFS Y-1220 were revived and grown as shown in 3.1.2. and 3.2.2., respectively. After 48 h, cells were harvested and the concentration of wet cell biomass determined as in 3.2.2. and 3.2.3. These cells were resuspended in potassium phosphate buffer (50 mM, pH 8) to final concentration of 50 g_{wcw} L^{-1}. This cell suspension was transferred to 24 mL amber vials at final volume of 500 µL per vial. The substrate, 4-hexylbenzoic acid (dissolved in dimethyl sulfoxide [DMSO] as co-solvent), was added to the amber vials to final concentrations of 1, 2 and 3 mM to start the biotransformations. The reaction mixtures (in closed amber vials) were incubated on a rotary shaker at 30 °C and 120 rpm until complete

consumption of 4-hexylbenzoic acid was obtained as determined by TLC. Ethyl acetate (1 mL), containing internal standard *p*-hydroxybenzoic acid, was used to extract the biotransformation mixtures. This was followed by vortexing for 10 to 20 min at maximum speed and centrifugation at 15 294 x *g* for 5 min. The TLC plates were developed and photographed according to 3.2.5.1. and chromatograms obtained according to 3.2.5.2. The ratios of the spots to internal standard were used to plot the relationship between the products and 4-hexylbenzoic acid. The experimental assays were done in triplicate and the results represent the averages.

3.2.5.4. Determination of progress curves for biotransformation of 4-hexylbenzoic acid by CYP505A1

Cells of *B. adeninivorans* UOFS Y-1220 were grown and suspended in potassium phosphate buffer as described in 3.2.2. and 3.2.3. The cell suspension (50 g_{wcw} L^{-1} final biomass) was transferred to 24 mL amber vials at final volume of 500 µL per vial. 4-Hexylbenzoic acid was added to the cell suspensions at final concentrations of 5 mM. The vials were sealed and incubated on a rotary shaker at 30 °C and 120 rpm. At 15 min intervals, a single amber vial was removed from the shaker. The reaction mixture was extracted using ethyl acetate (containing 1 mM *p*-hydroxybenzoic acid), and the rest of the procedure was similar to 3.2.5.3. This experiment was done in triplicate and the results are the averages of the three experiments.

Section B: VAO

3.2.5.5. Gas chromatography analysis of eugenol biotransformation

A gas chromatograph (HP 5890 II) with an auto sampler was used for GC analysis. The instrument was equipped with a SEE BPX 70 column (30 m x 0.25 mm, i.d.) with 0.25 µm coating. The injector and FID detector were set at 250 and 280 °C, respectively. The temperature program was set as follows: initial temperature of 150 °C held for 1 min and ramped at 10 °C min^{-1} to 250 °C for 11 min. Hydrogen flow rate was maintained at 3 mL min^{-1}. Under these conditions the retention times of eugenol, coniferyl alcohol and the internal

standard 1,2-dodecanediol were approximately 10.55, 22.11 and 14.02 min, respectively (Figure 9).

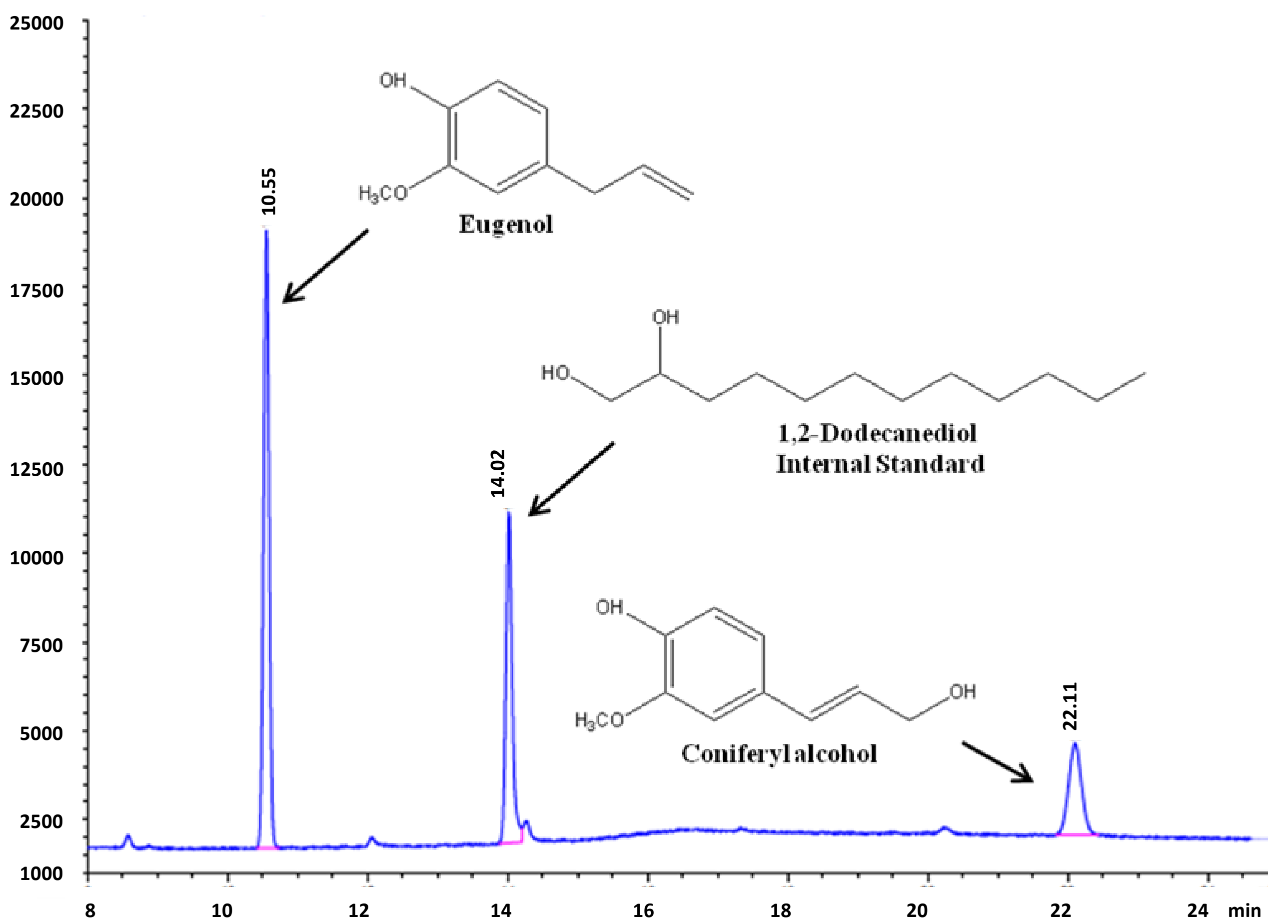


Figure 9 Typical GC chromatogram showing eugenol, coniferyl alcohol and internal standard 1,2-dodecanediol obtained using GC column SEE BPX 70 (30 m x 0.25 mm x 0.25 µm).

3.2.5.6. Standard solutions and calibration curves on GC and UV

GC standards of five different concentrations of coniferyl alcohol and eugenol were prepared in ethyl acetate containing 0.25 % (w/v) 1,2-dodecanediol. These standards (3 µL) were directly injected into the GC (HP 5890 II). The chromatograms generated were used to plot standard curves using linear regression of standard area/internal standard area against concentration. For UV, only coniferyl alcohol standards were prepared and were also in ethyl acetate (without internal standard). Evaporation of ethyl acetate during transfer of the standards

to microtiter plates was minimized by using a multi-pipette. The absorbances of these standards were determined at 320 nm using ethyl acetate as blank. The 320 nm wavelength was selected after separate absorbance spectra of coniferyl alcohol (80 µM) and eugenol (400 µM) were recorded in the 200 – 350 nm wavelength range. Standards were prepared in duplicate.

3.2.5.7. Progress curves for eugenol biotransformation by *B. adeninivorans* UOFS Y-1220 expressing VAO

Cells (100 g_{wcw} L^{-1}) of *B. adeninivorans* UOFS Y-1220 suspended in potassium phosphate buffer (10 mL, 50 mM, pH 8) were used to convert eugenol to coniferyl alcohol. Cell suspension (10 mL) was transferred to 50 mL Erlenmeyer flasks (x 3) and eugenol added to a final concentration of 64.5 mM. The flasks were incubated at 30 °C and 120 rpm on a rotary shaker for 10 h. Samples of 100 µL (x 2) were removed every 2 h and extracted with ethyl acetate (1 mL) containing 1,2-dodecanediol (0.25 % w/v) as internal standard. Ethyl acetate extracts were suitably diluted and divided into two fractions. One fraction was used for UV absorbance readings at 320 nm and the other fraction was analyzed using GC.

3.2.5.8. Determination of initial rates of VAO in whole-cells of *B. adeninivorans* UOFS Y-1220

B. adeninivorans UOFS Y-1220 was grown and suspended in potassium phosphate buffer (50 mM, pH 8) after harvest as described in 3.2.2. and 3.2.3. Wet cell biomass suspension (100 g_{wcw} L^{-1}) was then used for determining initial VAO rates. One milliliter was added into 40 mL amber vials (in duplicate). The reactions were initiated by adding eugenol (50 µL) to the cell suspensions to give a final concentration of 325 mM. The sealed amber vials were incubated at 30 °C and 120 rpm on a rotary shaker. At predetermined time intervals the amber vials were removed from the shaker. The reaction mixtures were extracted twice, using ethyl acetate (500 µL), and transferred to 2 mL centrifuge tubes. This was followed by vortexing for 10 to 20 min at maximum speed and subsequent centrifugation at 15 294 x *g* for 5 min. The extracts were then diluted by adding 2 µL extract to 198 µL of ethyl acetate in UV microtiter plates (costar, Corning, USA). The Spectra Max microtiter plate reader (Molecular Devices,

Switzerland) was set to shake the diluted extracts for 10 s to allow proper mixing before taking a reading. Absorbance was measured at 320 nm and used in subsequent calculations to determine coniferyl alcohol concentration. These coniferyl alcohol concentrations throughout this study were corrected using a GC to UV correlation curve (Figure 17).

3.2.5.9. Relationship between initial rates of whole-cell VAO and biomass concentration of *B. adeninivorans* UOFS Y-1220

Different cell suspensions of wet cell biomass, ranging from 5 to 300 g L^{-1}, were prepared in potassium phosphate buffer (50 mM, pH 8). These cell suspensions (500 μL) were added to triplicate sets of 24 mL amber vials. The reactions were initiated as shown in 3.2.5.8. using appropriate volume of eugenol to give 325 mM starting concentration. The rest of the procedure was similar to 3.2.5.8. except that, after two hours, all the vials were removed from the shaker.

3.2.6. Identification of significant nutrients for CYP505A1 and VAO expression

Section A: CYP505A1 expression

3.2.6.1. Culture media and preparation

Two types of media were used for cultivation, i.e. YPD medium for pre-culture and SYN6-1 medium (Gellissen, ed. 2005; Knabben *et al.*, 2010) for main culture. The SYN6-1 medium contained, in g L^{-1}: Glucose, 20; $(NH_4)_2SO_4$, 7.66; KH_2PO_4, 1; $MgSO_4 \cdot 7H_2O$, 3; $CaCl_2 \cdot 2H_2O$, 0.2; NaCl, 0.3; KCl, 3.3;$(NH_4)_2FeSO_4 \cdot 6H_2O$, 0.0665; EDTA, 0.0665; $MnSO_4 \cdot H_2O$, 0.0265; $NiSO_4 \cdot 6H_2O$, 0.00053; $CuSO_4 \cdot 5H_2O$, 0.0055; $ZnSO_4 \cdot 7H_2O$, 0.02; $CoCl_2 \cdot 6H_2O$, 0.00065; H_3BO_3, 0.00065; $Na_2MoO_2 \cdot 2H_2O$, 0.00065; KI, 0.00065; D (+) Biotin (H), 0.0004; Thiamine - HCl (B_1), 0.134. Two buffers were used with this medium, i.e. MES (2-*N*-morpholinoethanesulfonic acid) and potassium phosphate buffer each at a concentration of 140 mM. When potassium phosphate was used as a buffer, the MES buffer and KH_2PO_4 were omitted from the medium. The preparation of the SYN6-1 medium was carried out as shown in Table 5. This medium was divided into six different stock solutions, i.e. basal salts, potassium phosphate buffer, vitamins,

micro elements, trace elements and $CaCl_2 \cdot 2H_2O$. Unlike other compounds, the basal salts were prepared and autoclaved separately using a volume of distilled water lower than final medium volume. Therefore, the concentrations of basal salts were as shown in Table 5. $CaCl_2 \cdot 2H_2O$ was also prepared separately but added to the basal salts prior autoclaving. All the other solutions were prepared and added the following way just before cultivation: vitamins, micro- and trace-elements were filter-sterilized into the medium and sterile (autoclaved) glucose and potassium phosphate buffer were aseptically added. In the case of MES buffer, MES was already added as part of the basal salts. Correct proportions of the solutions were mixed to make total culture volumes of 50 or 150 mL per 500 mL Erlenmeyer flask and pH adjusted to 6 or 7 for the Plackett-Burman experiments.

Table 5 Stock solutions for SYN6-1 medium for shake-flask (Gellissen, ed. 2005 and Knabben *et al.*, 2010)

Stock Solution	Components		Preparation	Volume
Basal Salts	$(NH_4)_2SO_4$	7.66 g L^{-1}	Dissolve all chemicals in the order one by one in distilled water	Variable
	$MgSO_4 \cdot 7H_2O$	3 g L^{-1}		
	KCl	3.3 g L^{-1}		
	NaCl	0.3 g L^{-1}		
	MES	27.3 g L^{-1}		
	KH_2PO_4	1 g L^{-1}		
Potassium phosphate buffer (2 M)	KH_2PO_4	16.74 g L^{-1}	Dissolve both salts in 1000 mL and adjust the pH to 8 with 5 M NaOH	1000 mL
	K_2HPO_4	13.32 g L^{-1}		
Vitamins (100 x)	D (+) Biotin (H)	0.04 g L^{-1}	Dissolve both vitamins in distilled water and keep refrigerated at 4 °C	100 mL
	Thiamine - HCl	13.35 g L^{-1}		

	(B_1)			
Micro elements (100 x)	EDTA	6.65 g L^{-1}	Dissolve all components in the order in distilled water and keep refrigerated at 4 °C	100 mL each stock solution
	$(NH_4)_2FeSO_4 \cdot 6H_2O$	6.65 g L^{-1}		
	$MnSO_4 \cdot H_2O$	2.65 g L^{-1}		
	$ZnSO_4 \cdot 7H_2O$	2 g L^{-1}		
	$CuSO_4 \cdot 5H_2O$	0.55 g L^{-1}		
Trace elements (100 x)	$NiSO_4 \cdot 6H_2O$	0.066 g L^{-1}		
	$CoCl_2 \cdot 6H_2O$	0.066 g L^{-1}		
	H_3BO_3	0.066 g L^{-1}		
	$Na_2MoO_2 \cdot 2H_2O$	0.066 g L^{-1}		
	KI	0.066 g L^{-1}		
$CaCl_2 \cdot 2H_2O$		100 g L^{-1}	Dissolve in distilled water and autoclave	100 mL
Carbon source	Glucose-monohydrate or glycerol	440 g L^{-1}	Dissolve 440 g in distilled water up to 1000 mL and autoclave for glucose-monohydrate. For glycerol dilute 319 mL in distilled water up to 1000 mL and autoclave	1000 mL for each carbon source

3.2.6.2. Determination of specific activities of CYP505A1 in whole-cells of *B. adeninivorans* UOFS Y-1220

B. adeninivorans UOFS Y-1220 cells (50 g_{wcw} L^{-1}) suspended in potassium phosphate buffer (50 mM, pH 8) were used for determining specific activities of CYP505A1. One milliliter of this cell suspension was added to 24 mL brown amber vials. The reactions were started by adding 4-hexylbenzoic acid (dissolved in DMSO) to the cell suspensions at a final concentration of 5 mM. The amber vials were sealed and incubated at 30 °C and 120 rpm on a rotary shaker. After 45 min, the amber vials were removed from the rotary shaker and reaction mixtures immediately extracted twice using ethyl acetate (containing *p*-hydroxybenzoic as internal standard). This was achieved by vortexing the ethyl acetate extracts for 10 to 20 min at maximum speed followed by centrifugation at 15 294 x *g* for 5 min. When the reaction mixtures could not be extracted immediately, the samples were cooled and stored at – 20 °C. The extracts were spotted on TLC plates using 2 µL per sample. The TLC plates were developed according to 3.2.1. These were used in ImageJ to obtain the areas of the spots to estimate the product concentration and calculate specific and volumetric activities of CYP505A1 in whole-cells. One Unit (U) of whole-cell CYP505A1 was defined as the amount of biomass that catalyzed the formation of 1 µmol of product 1 (ω-2 OH-HBA) from 4-hexylbenzoic acid per min using potassium phosphate buffer (50 mM, pH 8) at 30 °C.

3.2.6.3. Plackett-Burman design and analysis of variance for CYP505A1

Media components and culture conditions significantly affecting biomass production and CYP505A1 expression by *B. adeninivorans* UOFS Y-1220 were identified using Plackett-Burman design of experiments. Seven variables (Table 6) were used to generate eight experimental trials (Table 10) using SPC for excel software (www.spcforexcel.com; achieved 27 August 2012). The Plackett-Burman design gave the media formulation and growth conditions for all the eight experimental trials as shown by the '+' an '-' matrix. All media were prepared as described in 3.2.6.1. Variables included buffer, initial pH, carbon source, substrate concentration, culture volume, δ-aminolevulinic acid and harvest time. Four responses (dependent variables) chosen as output from the eight experiments included biomass, specific activity, volumetric activity and final pH. The difference between the average of measurements obtained at high (+) and low (-) values was used as main effect for each variable.

Table 6 Variables for Plackett-Burman screening and their levels

Variables with designate	Low level (-)	High level (+)
A Buffer*	PO_4	MES
B Initial pH	6	7
C Carbon source	Glucose	Glycerol
D Substrate concentration (g L^{-1})	20	40
E Culture volume (mL)	50	150
F δ-Aminolevulinic acid (g L^{-1})	0	0.8
G Harvest time (h)	48	72

*: PO_4 = Potassium phosphate buffer, MES = 2-(*N*-morpholino)ethanesulfonic acid. Both of these buffers had a final concentration of 140 mM in the culture medium.

Section B: VAO expression

3.2.6.4. Culture media and preparation

Two types of media were used for cultivation, i.e. YPD medium for pre-culture and SYN6-2 medium (Knoll *et al.*, 2007) for main culture. Most of the elements concentrations of this medium were lowered for solubility of the medium. The medium could dissolve better after this even though there were still precipitates forming after autoclaving. Despite this, the medium was used after literature searches showed that this solubility issue is resolved as the cultivation progresses in bioreactors (Knabben *et al.*, 2010). Knabben *et al.*, (2010) measured the concentrations of SO_4^{2-}, NH_4^+, K^+ and PO_4^{3-} and observed an increase in SO_4^{2-}, NH_4^+ and PO_4^{3-} after 16 h of batch cultivation, which suggested that salts that had precipitated after sterilization were redissolving. The preparation of the SYN6-2 medium was carried out as shown in the Table 7 below. Basal salts were dissolved as they appear in the table using a volume that is less than the final volume of the culture medium. The rest of the medium was divided into four different stock solutions, i.e. glucose, potassium phosphate buffer, vitamins and metals.

Appropriate volume of the metals and potassium phosphate buffer were added to the basal salts (depending on the final volume of the culture medium) and autoclaved together. Glucose was sterilized separately and added afterwards. The vitamins were filter-sterilized into the total medium before cultivation.

Table 7 Composition and preparation of SYN6-2 (Modified from Knoll *et al*., 2007 and Knabben *et al*., 2010)

Stock Solution	Components		Preparation	Volume
Salts	$(NH_4)_2SO_4$	5 g L^{-1}	Dissolve all chemicals in the order one by one in distilled water	Variable
	$MgSO_4 \cdot 7H_2O$	0.4 g L^{-1}		
	Na_2SO_4	0.05 g L^{-1}		
	$CaCl_2 \cdot 6H_2O$	0.3 g L^{-1}		
Potassium phosphate buffer (2 M)	KH_2PO_4	16.74 g L^{-1}	Dissolve both salts in 1000 mL and adjust the pH to 8 with 5 M NaOH	1000 mL
	K_2HPO_4	13.32 g L^{-1}		
Vitamins (100 x)	D (+) Biotin (H)	80 mg L^{-1}	Dissolve both vitamins in distilled water and keep refrigerated at 4 °C.	100 mL
	Thiamine - HCl (B_1)	5.3 g L^{-1}		
Trace elements (100 x)	$(NH_4)_2FeSO_4 \cdot 6H_2O$	27 g L^{-1}	Dissolve all components in the order in distilled water and autoclave.	1000 mL
	$MnSO_4 \cdot H_2O$	110 g L^{-1}		
	$NiCl \cdot 6H_2O$	53 mg L^{-1}		
	$CuSO_4 \cdot 5H_2O$	1.1 g L^{-1}		
	$ZnSO_4 \cdot 7H_2O$	8 g L^{-1}		

	$CoCl_2 \cdot 6H_2O$	270 mg L^{-1}		
	H_3BO_3	270 mg L^{-1}		
	$Na_2MoO_2 \cdot 2H_2O$	34 mg L^{-1}		
	KI	135 mg L^{-1}		
Carbon source	Glucose-monohydrate	440 g L^{-1}	Dissolve 440 g in distilled water up to 1000 mL and autoclave	1000 mL

3.2.6.5. Cultivation for Plackett-Burman design

Cells of *B. adeninivorans* UOFS Y-1220 were revived as described in 3.1.2. This was followed by inoculating 50 mL YPD medium with a loop full of 24 h culture in four 500 mL Erlenmeyer flasks. The flasks where incubated at 30 °C and 120 rpm for 24 h on a rotary shaker. The cultures where combined and used to inoculate (with final cell concentration up to 10 % v/v) 12 flasks containing 50 and 150 mL SYN6-2 media containing different compositions (Table 8). These were also incubated on a shaker at 30 °C and 120 rpm for 48 h before whole-cell specific activities for VAO were quantified. The different compositions of the 12 flasks were determined using Plackett-Burman design of experiments in SPC for Excel program. The details are given in 3.2.6.7.

3.2.6.6. Determination of VAO whole-cell activity

Wet cell biomass of culture was prepared by diluting or concentrating cells up to final concentration of 10 % (ww/v) in potassium phosphate buffer (pH 8, 50 mM). The expression of VAO was determined by quantifying the initial whole-cell activities towards eugenol in brown amber vials (40 mL). To the vials was added 2mL 10 % (ww/v) cell suspensions, with eugenol concentration up to 325 mM. The vials were incubated on a rotary shaker at 30 °C and 120 rpm for 2 h. After incubation, the reaction mixture was extracted twice with ethyl acetate, vortexed for

5 min at maximum speed and centrifuged at 15 294 x *g* for 10 min. The supernatant (extract) was suitably diluted with ethyl acetate up to 200 µL in microtiter UV plates (Costa, USA). A microtiter plate reader (Molecular Devices, Switzerland) was used to determine UV absorbance of the extracts. A correlation shown in Figure 13 was used to determine product formation for initial reaction rates calculation.

3.2.6.7. Plackett-Burman design and analysis of variance for VAO

To identify medium components and culture conditions that have significant effect on VAO expression, the Plackett-Burman design was employed. Twelve experimental runs (Table 8) were generated from eight independent variables (Table 8) using the Plackett-Burman design in SPC for Excel program (www.spcforexcel.com; achieved 27 August 2012). The formulation for the media used was generated as shown in Table 8. Three responses (dependent variables), i.e. biomass, specific activity and volumetric activity, were chosen as an output from the 12 experiments. Each variable's main effect was calculated as the difference between the average of measurements obtained at high value (+) and at the low value (-). The eight variables selected for the experiments comprised metals, vitamins, salts, glucose concentration, inoculum size, potassium phosphate buffer concentration, initial medium pH and flask:culture ratio (Table 8). The term 'flask:culture ratio' had similar meaning to 'culture volume' that was used in CYP505A1 experiments. This term means the ratio of flask volume to culture volume. Each of the variables was assigned two values, i.e. high (+) and low (-).The cultures were grown for 48 h. Another two Plackett-Burman designs were conducted the same way with some variables kept constant. These will only be used here for general discussion of VAO Plackett-Burman results.

Table 8 Variables for Plackett-Burman screening and their levels

Variables with designate	Low level (-)	High level (+)
A Metals/Trace elements (mg L^{-1})		
$(NH_4)_2FeSO_4{\cdot}6H_2O$	270	540
$MnSO_4{\cdot}H_2O$	110	220
$NiCl{\cdot}6H_2O$	0.53	1.06
$CuSO_4{\cdot}5H_2O$	11	22
$ZnSO_4{\cdot}7H_2O$	80	160
$CoCl_2{\cdot}6H_2O$	2.7	5.4
H_3BO_3	2.7	5.4
Na_2MoO_2	0.34	0.68
B Vitamins (mg L^{-1})		
D (+) Biotin (H)	0.8	1.6
Thiamine – HCl (B1)	53	106
C Basal Salts (mg L^{-1})		
$(NH_4)_2SO_4$	2500	5000
$MgSO_4{\cdot}7H_2O$	200	400
Na_2SO_4	25	50
$CaCl_2{\cdot}6H_2O$	150	300
KI	0.675	1.35
D Glucose concentration (g L^{-1})	40	80
E Inoculum Size (% v/v)	10	20

F Potassium phosphate buffer concentration (mM)	50	200
G Initial pH	6	8
H Flask:Culture Ratio	3.33	10

3.2.7. Effect of dissolved oxygen tension on VAO expression and biomass production

3.2.7.1. Culture media and preparation

Two types of media were used for cultivation, i.e. YPD and modified SYN6-2 media for pre- and main cultures, respectively. The modified SYN6-2 medium composition is shown in Table 9 below and was selected because it gave a high volumetric productivity with high biomass and whole-cell specific activity during Plackett-Burman screening (experimental trial 7, Table 13). This medium differs from the original SYN6-2 (Table 7) in terms of metal concentrations. The best run from the 12-trial Plackett-Burman experiment was used since the 12-trial was the only one that showed a significant model ($p<0.05$). Each of the categories of elements, i.e. metals, vitamins, salts, glucose and potassium phosphate buffer were prepared separately. Salts, glucose and buffer were sterilized by autoclave while metals and vitamins were filter sterilized into the medium during preparation. Appropriate volumes of the different stock solutions were combined to make a final volume of 225 mL in the bioreactors.

3.2.7.2. Batch cultivation of *B. adeninivorans* UOFS Y-1220 in bioreactors

Cells of *B. adeninivorans* UOFS Y-1220 were revived as described in 3.1.2. The revived cells were used to inoculate 500 mL Erlenmeyer flasks containing 50 mL YPD media. These flasks were incubated for 24 h on a rotary shaker at 30 °C and 120 rpm for preparation of pre-cultures. The cultures were pooled after 24 h and used to inoculate (25 mL) the SYN6-2 medium up to final concentration of 10 % (v/v) in a bioreactor (Infors, Switzerland). The bioreactor flask size was 300 mL with a working volume of 250 mL.

Table 9 Medium selected from Plackett-Burman experiments

Stock Solution	Components		Preparation	Volume
Salts	$(NH_4)_2SO_4$	5 g L^{-1}	Dissolve all chemicals in the order one by one in distilled water	Variable
	$MgSO_4 \cdot 7H_2O$	0.4 g L^{-1}		
	Na_2SO_4	0.05 g L^{-1}		
	$CaCl_2 \cdot 6H_2O$	0.3 g L^{-1}		
Potassium phosphate buffer (2 M)	KH_2PO_4	16.74 g L^{-1}	Dissolve both salts in 1000 mL and adjust the pH to 8 with 5 M NaOH	1000 mL
	K_2HPO_4	13.32 g L^{-1}		
Vitamins (100 x)	D (+) Biotin (H)	80 mg L^{-1}	Dissolve both vitamins in distilled water and keep refrigerated at 4 °C	100 mL
	Thiamine - HCl (B_1)	5.3 g L^{-1}		
Trace elements (100 x)	$(NH_4)_2FeSO_4 \cdot 6H_2O$	54 g L^{-1}	Dissolve all components in the order in distilled water and autoclave	1000 mL
	$MnSO_4 \cdot H_2O$	22 g L^{-1}		
	$NiCl \cdot 6H_2O$	106 mg L^{-1}		
	$CuSO_4 \cdot 5H_2O$	2.2 g L^{-1}		
	$ZnSO_4 \cdot 7H_2O$	16 g L^{-1}		
	$CoCl_2 \cdot 6H_2O$	540 mg L^{-1}		
	H_3BO_3	540 mg L^{-1}		
	$Na_2MoO_2 \cdot 2H_2O$	68 mg L^{-1}		
	KI	135 mg L^{-1}		

Carbon source	Glucose-monohydrate	440 g L^{-1}	Dissolve 440 g in distilled water up to 1000 mL and autoclave	1000 mL

3.2.7.3. The effect of dissolved oxygen tension on VAO expression and biomass production

To determine the effect of dissolved oxygen tension (DOT) on VAO expression and biomass production, *B. adeninivorans* UOFS Y-1220 was cultivated at 10, 30 and 60 % DOT for 48 h. Before cultivation was started, the pH was at approximately 7–7.3 units. This was due to potassium phosphate buffer present in the medium. The gas flow rate was initially kept at 15 NL/h (equivalent to 1 vvm) and was increased stepwise to a maximum of 45 NL/h during cultivation. This was necessary to achieve DOT's of 30 and 60 %. To prevent the stirrer from stopping or stirring very slowly at the beginning of cultivation due to high DOT (ca. 97 - 100 %), the stirrer was kept off until the DOT levels dropped below the required levels. Both the stirrer speed and gas flow were used for controlling DOT. Samples (4 mL) were taken from the reactors at certain intervals throughout the cultivation. These samples were used for biomass and specific activity determination. The SYN6-2 medium is highly concentrated. Therefore, with production of high biomass it became challenging to maintain the correct DOT (especially with fresh cells). The reactor had to run at maximum stirrer speed and flow rate (at some point) to keep up with increasing biomass.

Chapter 4

Results and discussion

4. Introduction

As stated previously, this study was conducted to investigate the effects of medium components and DOT on biomass production and heterologous expression of CYP505A1 and VAO by *B. adeninivorans* UOFS Y-1220. The organization of this chapter follows the sequence of the questions posed in Chapter 1 under the aims. TLC and UV assays to monitor expression of CYP505A1 and VAO were developed, which made it easy to compare large numbers of samples for enzyme production by measuring specific whole-cell activities. The effects of medium components on biomass production as well as CYP505A1 and VAO activity were then investigated in shake-flasks using the Plackett-Burman experimental design. From this, the medium that gave the highest volumetric activity (or productivity) was selected and used for evaluating the effect of DOT in bioreactors. The discussion of the results is provided as the results are presented.

4.1. Assay Development

Section A: CYP505A1 Activity

4.1.1. Determination of linear relationship between 4-hexylbenzoic acid and conversion products produced by CYP505A1

A linear relationship was determined between the concentration of 4-hexylbenzoic acid and that of its hydroxylation products produced by CYP505A1 upon complete conversion of the substrate. This was to estimate the amounts of products produced, since the standards of these products were not available. The two products formed from 4-hexylbenzoic acid by CYP505A1 have been identified by Theron *et al.*, (2014) as the ω-1 OH-HBA (Product 2) and ω-2 OH-HBA

(Product 1) hydroxylated products. P 1 was used to plot a standard curve of product against 4-hexylbenzoic acid. P 1 was more stable than P 2 that is overoxidised further by CYP505A1 as hypothesized by Theron *et al.*, (2014). This standard curve, which provides the estimated concentrations of the product, was used to calculate specific and volumetric activities of intracellular CYP505A1 in the Plackett-Burman experiments. Figure 10 shows the linear plots of products P1 (ω-2 OH-HBA) and P2 (ω-1 OH-HBA) as well as their sum.

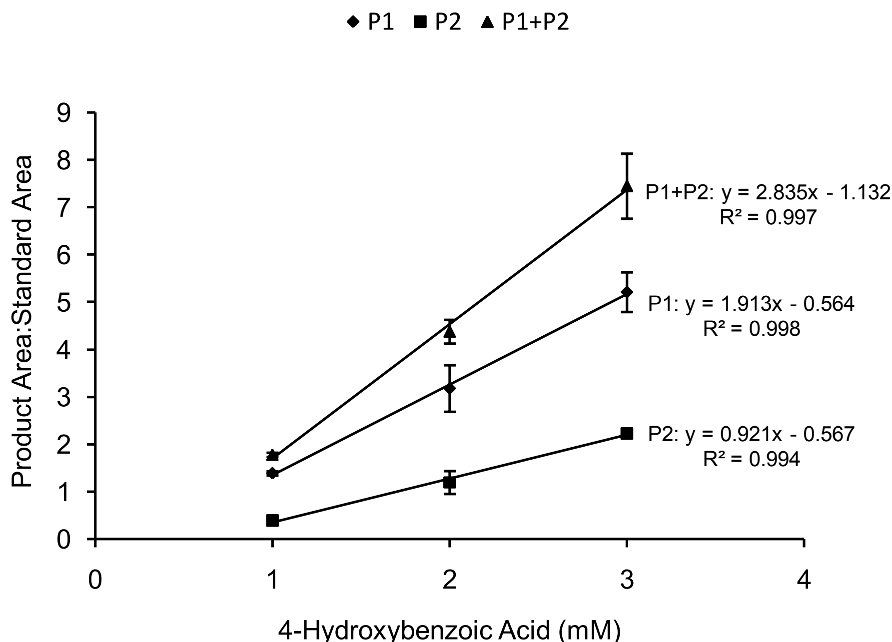


Figure 10 Linear relationship of products and 4-hexylbenzoic acid consumed. The reactions were done in triplicate (standard deviations are shown) and the analysis of reaction mixtures was performed only after complete consumption of 4-hexylbenzoic acid at 1, 2 and 3 mM concentrations.

4.1.2. Determination of progress curves for biotransformation of 4-hexylbenzoic acid by CYP505A1

Time course biotransformations of 4-hexylbenzoic acid were performed. The purpose was to obtain progress curves which would enable the identification of the linear range needed for quantification of initial activities. As shown in Figure 11, formation of biotransformation

products only showed the linear relationship with time up to 60 min. After that time it seemed like the activity leveled off. From this figure it was clear that determination of initial activities of intracellular CYP505A1 would be accurately performed when the CYP505A1 assay had incubation time less than 60 min. Therefore, for all the assays involving the CYP505A1, an assay time of 45 min was chosen to avoid any side reactions that would lead to erroneous initial activities.

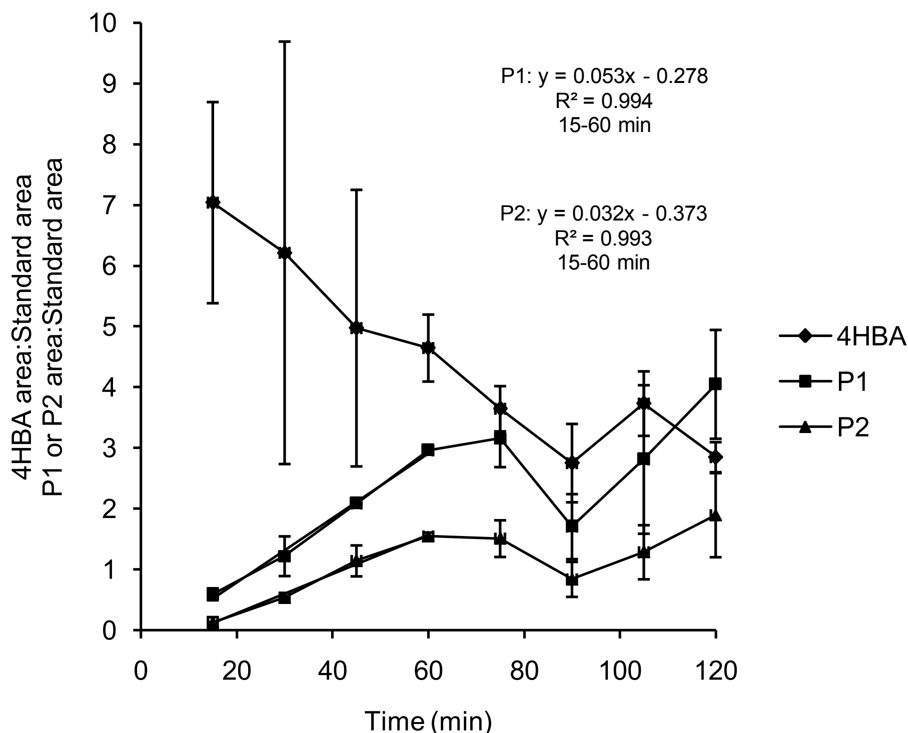


Figure 11 Biotransformation of 4-hexylbenzoic acid by intracellular CYP505A1 in cells of *B. adeninivorans* UOFS Y-1220. Abbreviations: 4HBA – 4-hexylbenzoic acid, P1 – ω-2 OH-HBA and P2 – ω-1 OH-HBA.

Section B: VAO Activity

4.1.3. Construction of standard curves for quantification of coniferyl alcohol and eugenol

Authentic standards of eugenol and coniferyl alcohol were used to determine the wavelength to follow production of coniferyl alcohol in the presence of eugenol. This was achieved by determining the absorbance spectra of eugenol and coniferyl alcohol between 200 and 800 nm. The two compounds showed absorbance between 200 and 350 nm (Figure 12).

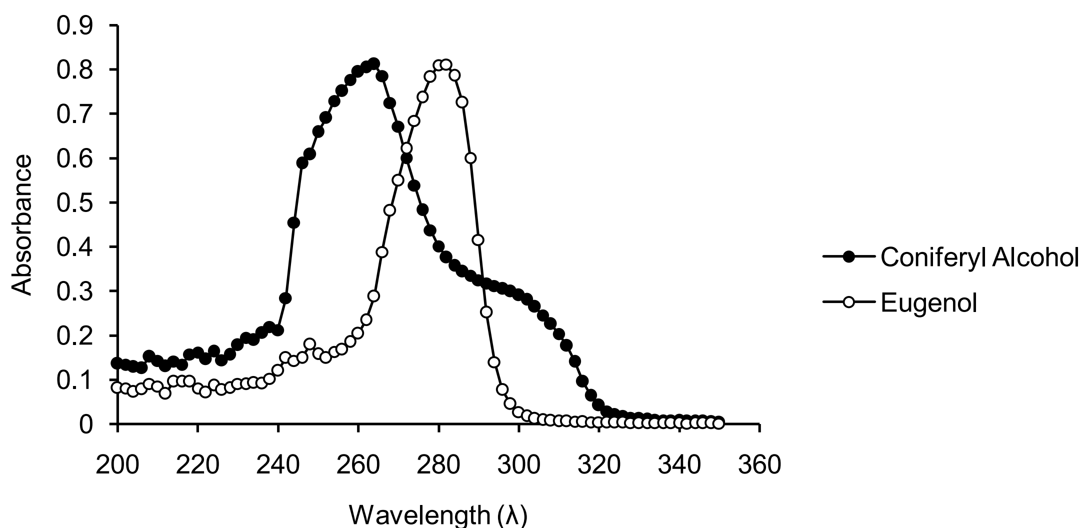


Figure 12 Wavelength scan of coniferyl alcohol (80 µM; closed circles) and eugenol (400 µM; open circles). These scans were recorded using a microtiter plate reader at 25 °C.

Between 305 and 320 nm coniferyl alcohol still showed absorbance, while eugenol did not. Standard procedure would have been to assay at 305 nm, because the absorbance of coniferyl alcohol at 305 nm is the highest giving the highest sensitivity. However, because the VAO activity and starting eugenol concentrations were very high, assaying at 305 nm necessitated several dilution steps if coniferyl alcohol absorbance were to be measured using

microtiter plates with 200 µL per well. Therefore, it was decided to measure at 320 nm where sensitivity was low.

Increasing concentrations of coniferyl alcohol ranging from 0.01 to 0.1 g L^{-1} prepared in ethyl acetate showed linear relationship to absorbance at 320 nm (Figure 13). Linear regression analysis of this standard curve gave a correlation factor (R^2) of 0.994. To validate the response from the standard curve obtained using the microtiter plate reader similar standard curves of coniferyl alcohol and eugenol were prepared using GC (Figure 14). Linear regression analysis of these also gave good correlation factors of 0.984 and 0.998 for coniferyl alcohol and eugenol, respectively. The response from UV was compared to GC by analysis of eugenol biotransformation samples from the same experiment.

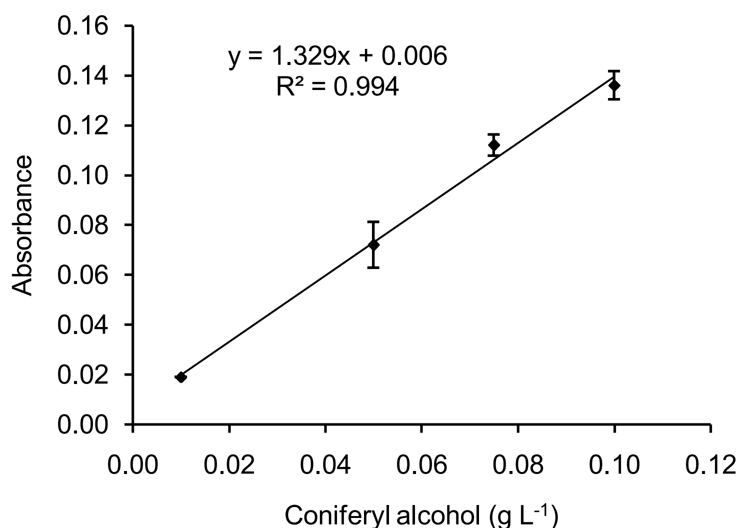


Figure 13 Standard curve of coniferyl alcohol (n=3) determined spectrophotometrically at 320 nm using a microtiter plate reader. Different concentrations of coniferyl alcohol were prepared in ethyl acetate using an authentic standard. This was done in triplicate (standard deviations are shown).

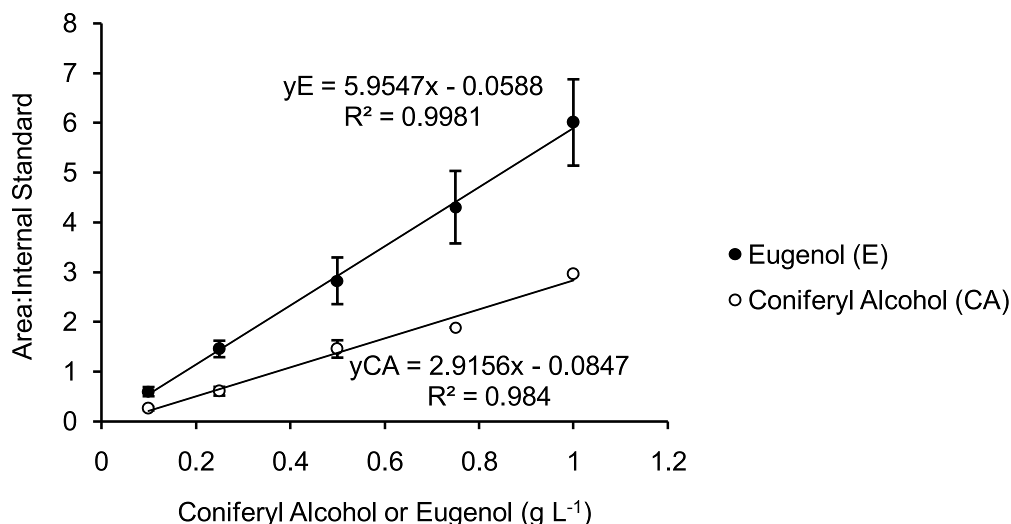


Figure 14 Standard curves of coniferyl alcohol (n=2) and eugenol (n=2) determined by gas chromatography. Standards of both aromatic compounds were prepared in ethyl acetate containing 0.25 % w/v 1,2-dodecanediol as internal standard. This was done in duplicate (range bars are shown).

4.1.4. VAO whole-cell progress curves using UV and GC for quantification of coniferyl alcohol production

Coniferyl alcohol production and eugenol consumption by VAO expressing whole-cells of *B. adeninivorans* UOFS Y-1220 suspended in potassium phosphate buffer (50 mM, pH 8) is shown in Figure 15. VAO activity leveled off just after four hours due to substrate depletion. Coniferyl alcohol concentrations obtained with UV were not comparable to those obtained with GC. As seen in Figure 15, the final coniferyl alcohol concentrations from UV were approximately twice those determined with GC. TLC analysis (A to C in Figure 16) of similar biotransformation samples revealed occurrence of other intermediates in addition to coniferyl alcohol. Already after two hours, intermediate A—which may be coniferyl aldehyde resulting from coniferyl alcohol oxidation—was formed. Other intermediates, B and C, were present after 10 hours of biotransformation. This suggests that the UV could not distinguish between these compounds and consequently detected them as coniferyl alcohol. The fact that UV showed ca. 90 % yield of coniferyl alcohol from eugenol while only ca. 62 % was observed from GC supports this hypothesis.

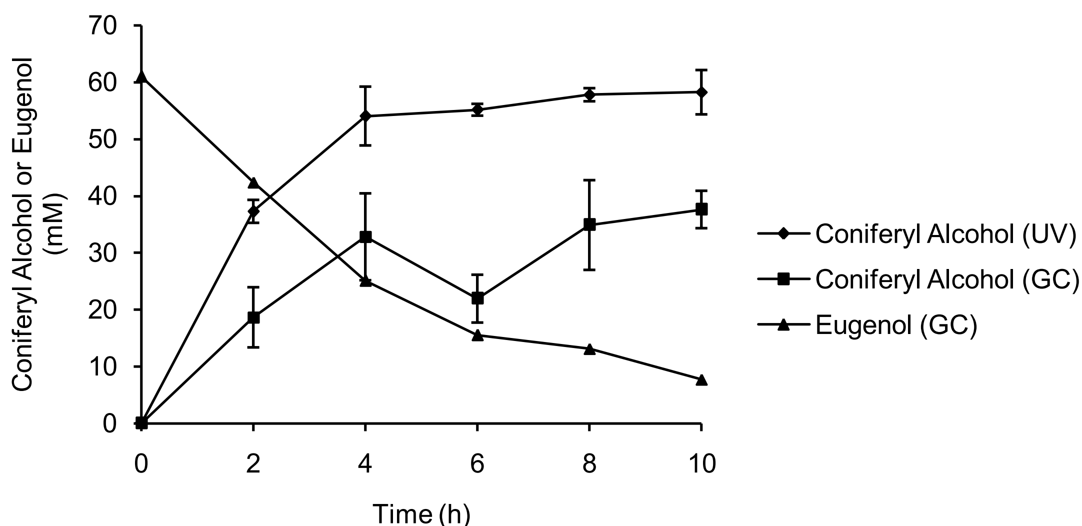


Figure 15 Progress curve of VAO activity showing coniferyl alcohol (n=3) production from eugenol (n=1) biotransformation. *B. adeninivorans* (100 g_{wcw} L^{-1}) suspended in potassium phosphate buffer (10 mL, 50 mM, pH 8) were incubated with eugenol (64.5 mM) in a 50 ml Erlenmeyer flask at 30 °C and 120 rpm on a rotary shaker. Time course samples were removed from the flasks and extracted with ethyl acetate containing internal standard followed by centrifugation. The supernatants were used for coniferyl alcohol and eugenol determinations after suitable dilutions were made. Coniferyl alcohol concentrations were determined by both UV and GC while eugenol was only determined by GC. The coniferyl alcohol was analyzed in triplicate (standard deviations are shown) and eugenol once.

Since a UV spectrophotometric assay was our main aim a correlation curve between data obtained using UV and GC was drawn (Figure 17). A linear relationship was observed for coniferyl alcohol concentrations below 100 mM (GC) and 150 mM (UV) with an R^2 of 0.996. These concentrations are far higher than the concentrations expected from determining initial rates of VAO in whole cells. Therefore, using this correlation, accurate concentration of coniferyl alcohol can be estimated from UV data. As a result, in further experiments coniferyl alcohol concentrations measured by UV were corrected using this correlation curve.

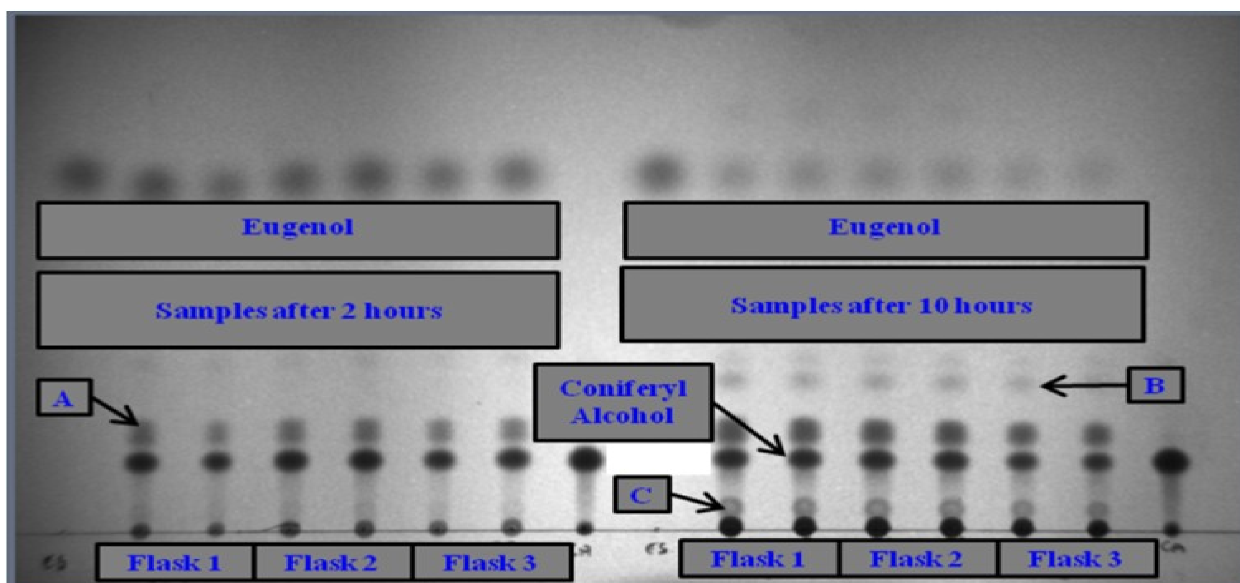


Figure 16 TLC plate showing different unknown products formed when *B. adeninivorans* UOFS Y-1220, expressing VAO, were incubated with eugenol. From the same samples used for VAO progress curve (Figure 15), 2 and 10 h samples were chosen for TLC analysis. Ten microlitre of each sample was spotted on the TLC plate and dried. The mobile phase used to develop this plate contained di-n-butyl ether, formic acid and water in the ratio 90: 7: 3.

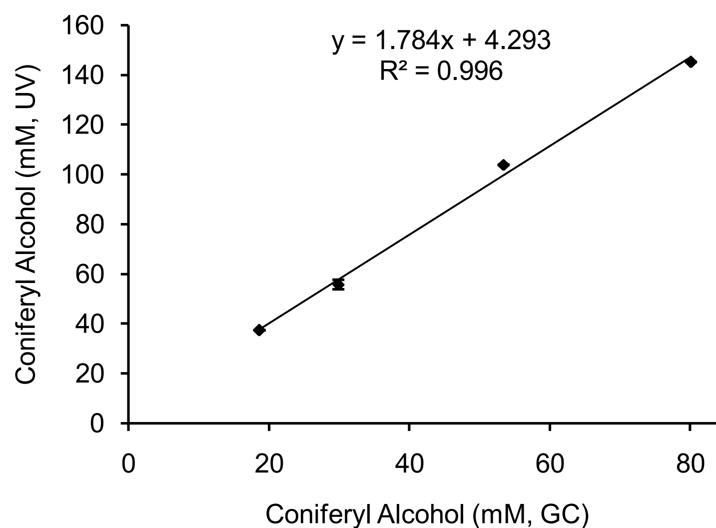


Figure 17 Linear relationship between coniferyl alcohol concentrations determined using UV and GC. This was done in duplicate (range bars are shown).

4.1.5. Initial rates of VAO in whole-cells of *B. adeninivorans* UOFS Y-1220

From the VAO progress curves it was observed that the linear rate of the enzyme lasted for two to four hours before leveling off when using 64.5 mM eugenol. Therefore, the eugenol concentration was increased five-fold at same biomass concentration to determine a linear rate that lasted longer. This would allow a longer time interval to work with. The initial rate also had to be at least within 10 to 20 % of substrate consumption to avoid depletion of eugenol as observed after six hours on the progress curves determined from GC (Figure 15). Conversion of coniferyl alcohol to other metabolites also had to be avoided. Thus, initial rates of VAO in whole-cells of *B. adeninivorans* UFS Y-1220 were determined by monitoring coniferyl alcohol production from eugenol over a period of at least six hours. A linear initial rate was observed for VAO over a period of 360 min (Figure 18). This showed the stability of intracellular VAO activity for that period.

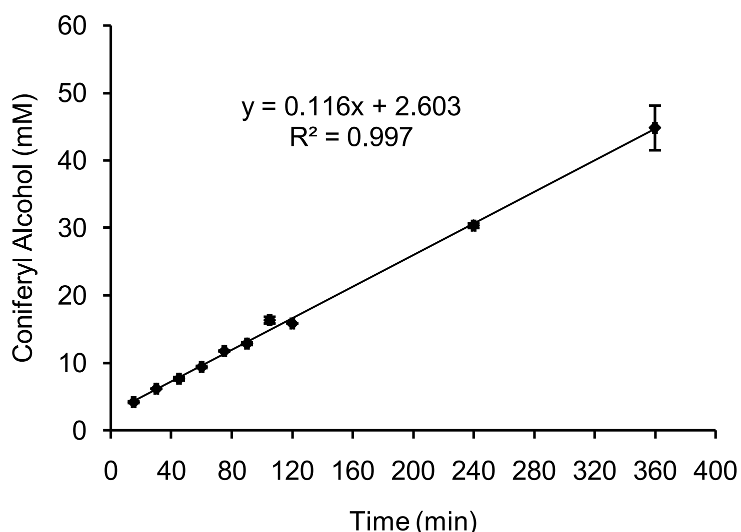


Figure 18 Initial rate of VAO in whole-cells of *B. adeninivorans* UOFS Y-1220 obtained by measuring the accumulation of coniferyl alcohol over time by UV microtiter plate reader (320 nm). Whole-cells of *B. adeninivorans* (100 g_{wcw} L^{-1}) suspended in potassium phosphate buffer (1 mL, 50 mM, pH 8) were incubated with eugenol (325 mM) in 40 mL sealed amber vials at 30 °C and 120 rpm on a rotary shaker. One vial was removed from the shaker over time and the reaction mixture extracted twice with ethyl acetate (2 mL). After centrifugation, the supernatants were used for coniferyl alcohol quantification using a microtiter plate reader. This was done duplicate (range bars are shown).

4.1.6. Relationship between initial rates of whole-cell VAO and biomass concentration in *B. adeninivorans* UOFS Y-1220

To select a suitable range of biomass at which activity assays would be carried out, different concentrations of biomass were used to perform biotransformations of eugenol (325 mM) over two hours. Results from these biotransformations showed linear relationship between initial rates and biomass concentration up to 200 g L^{-1} wet cell biomass (Figure 19). In this concentration range, there were no limiting factors such as eugenol depletion and oxygen limitation, which would cause the initial rates to level off. The oxygen limitation might explain why the activity leveled off at high biomass concentrations (more than 200 g_{wcw} L^{-1}) even though there was still enough eugenol (data not shown). This is because VAO requires oxygen to function (de Jong *et al.*, 1992) and the amber vials were sealed during the biotransformations, which limited oxygen supply especially at high biomass concentrations.

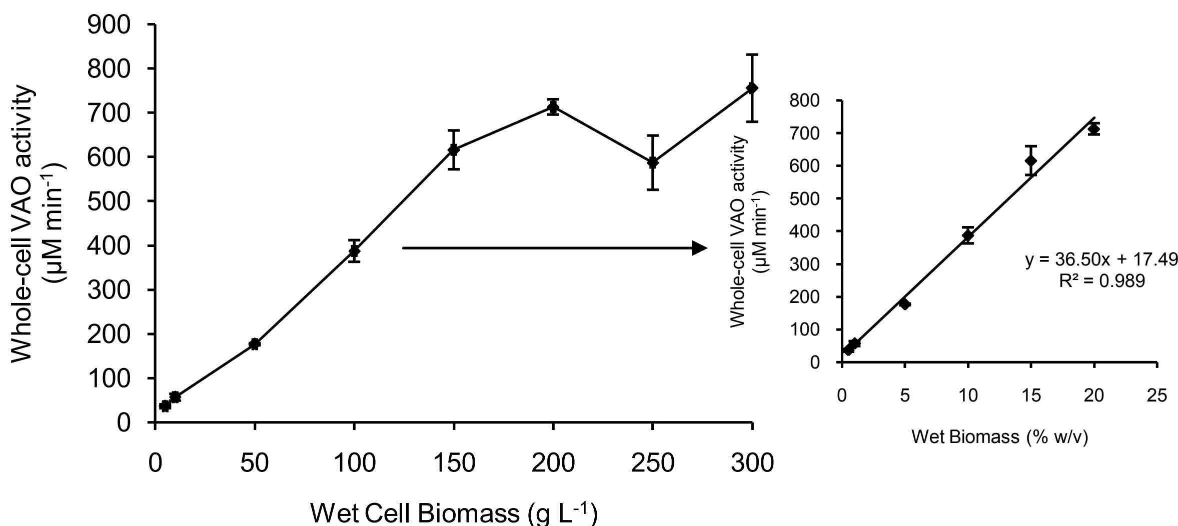


Figure 19 Relationship between initial rates (n=3) of whole-cell VAO and biomass by *B. adeninivorans* UOFS Y-1220. Whole-cells of *B. adeninivorans* (5 to 300 g L^{-1} wet cell weights) suspended in potassium phosphate buffer (0.5 mL, 50 mM, pH 8) were incubated with eugenol (325 mM) in 24 mL sealed amber vials at 30 °C and 120 rpm on a rotary shaker. After two hours, all the vials were removed from the shaker and the reaction mixture extracted twice with ethyl acetate (1 mL). After centrifugation, the supernatants were used for coniferyl alcohol quantification on microtiter plate reader (at 320 nm). This was done in triplicate (standard deviations are shown).

Therefore, based on these findings, a simple and rapid assay for quantification of VAO activity in whole-cells of *B. adeninivorans* UOFS Y-1220 was developed. According to this, *B. adeninivorans* UOFS Y-1220 can be incubated with eugenol in amber vials for 90 - 120 min for coniferyl alcohol production. The concentration of the cell suspension must be in the range of five to 150 g_{wcw} L^{-1} preferably 50 to 100 g_{wcw} L^{-1}. Using this assay, many samples could be assayed simultaneously for quantification of VAO activities. As a result, subsequent experiments for the determination of initial rates of VAO in *B. adeninivorans* UOFS Y-1220 were performed this way. Triplicate assays of one specific time point, e.g. 120 min, within linear range were carried out in amber vials. One unit (U) of whole-cell VAO activity was described as the amount of enzyme that catalyzes the formation of 1 µmol of coniferyl alcohol from eugenol per min using potassium phosphate buffer (50 mM, pH 8) at 30 °C.

4.2. Medium components and culture conditions that significantly affect biomass production and CYP505A1 expression in *B. adeninivorans* UOFS Y-1220

Plackett-Burman design was used to evaluate factors that play a significant role in expression of CYP505A1 (indicated by whole-cell CYP505A1 activity) and biomass production by *B. adeninivorans* UOFS Y-1220 expressing CYP505A1. Seven variables were selected for the Plackett-Burman experiments (Table 6). These comprised buffer, initial pH, carbon source, substrate concentration, culture volume (in 500 mL Erlenmeyer flask), δ-aminolevulinic acid addition and harvest time. A Plackett-Burman experimental design of eight trials with biomass, specific activity of CYP505A1, volumetric activity and final pH as responses is shown in Table 10. Eight flasks containing media with different compositions as calculated from the Plackett-Burman design (Table 10) were prepared. Half of these had a culture volume of 50 mL and the other 150 mL. The media were inoculated with *B. adeninivorans* UOFS Y-1220 at final concentration of 10 % (v/v). Four flasks were incubated for 48 h on a rotary shaker at 120 rpm and 30 °C. The other four were incubated for 72 h. At harvest, samples were taken from the cultures, pH was measured, biomass concentrations were determined, and other samples were used to determine CYP505A1 whole-cell activities.

Specific whole-cell activities of CYP505A1 ranged between 2.1 and 3.7 U g^{-1}. Final pH did not change much, while biomass and volumetric activity also showed large variations. Final

biomass concentrations ranged from 5.9 to 27.8 g L^{-1} while volumetric activity had a range between 21.3 to 98.2 U L^{-1} and pH from 3.58 to 6.42. Regression coefficients and main effects of the variables for all responses were calculated using SPC for excel software (Tables 11 and 12). SPC for excel did not find any significant factors and therefore no F or p values were calculated. However, the main effects of the variables on biomass production and CYP505A1 specific activities will be discussed here.

Regression analysis of the seven variables for biomass (Table 11; Figure 20A1, 2) showed that glycerol was preferred over glucose by *B*. *adeninivorans* UOFS Y-1220. Higher initial pH (7), higher carbon source concentration (40 g L^{-1}) and longer harvest time (72 h) improved biomass production while δ-aminolevulinic acid had a negative effect on biomass production. Potassium phosphate buffer and lower culture volume (50 mL) also benefitted biomass production.

In the case of specific whole-cell CYP505A1 activity (Table 12; Figure 20B1, 2), enzyme activity was improved by MES buffer, glycerol and higher culture volume (150 mL). Lower initial pH (6), lower carbon source concentration (20 g L^{-1}) and shorter harvest time (48 h) lead to higher CYP505A1 expression. Surprisingly, δ-aminolevulinic acid did not improve CYP505A1 expression as expected (addition of 5-aminolevulic acid improves CYP450 expression in *E*. *coli*; Richardson *et al*., 1995).

In general, pH dropped during growth of *B*. *adeninivorans* UOFS Y-1220 (Table 10). The average pH of approximately 5.78 and 4.67 were calculated when the starting pH were seven and six, respectively. Initial higher pH in shake-flasks resulted in a slightly higher pH although still lower than the starting pH. Strangely, MES buffer, glycerol and higher culture volume (150 mL), factors that enhanced CYP505A1 activity, lead to higher final pH while CYP505A1 activity effect graphs show the enzyme activity benefitted when the medium pH started at six (Table 13; Figure 20C1, 2). Low substrate concentration (20 g L^{-1}) and shorter harvest time (48 h) favoured lower final pH (both factors at low-level increased whole-cell (CYP505A1) specific activity).

Table 10 Plackett-Burman design for screening of variables significant for CYP505A1 and biomass production by *B. adeninivorans* UOFS Y-1220 [Factors: 7, Replicates: 0, Design: 8, Runs: 8, Centre Points: 0]

Trial Number	A	B	C	D	E	F	G	Biomass* (g L^{-1})	CYP505A1[#] Specific Activity (U g^{-1})	CYP505A1 Volumetric Activity (U L^{-1})	Final pH
1	-	+	-	+	+	+	-	8.1	2.3±0.08	18.4±0.62	6.4
2	+	-	-	+	-	+	+	15.8	2.1±0.03	32.7±0.48	3.58
3	+	+	+	-	-	+	-	14.2	3.1±0.01	43.6±0.1	6.2
4	-	-	-	-	-	-	-	9.7	2.9±0.06	28.3±0.56	4.03
5	-	+	+	+	-	-	+	27.8	2.8±0.53	78.0±14.59	4.08
6	-	-	+	-	+	+	+	9.4	2.9±0.11	26.9±0.99	5.24
7	+	-	+	+	+	-	-	5.9	3.7±0.21	21.6±1.22	5.84
8	+	+	-	-	+	-	+	8.2	2.8±0.1	22.6±0.8	6.42

Symbols: * = Dry cell weight, the standard deviation was 1-2 %[#] = this represents whole-cell specific activity of CYP505A1 since the enzyme is produced intracellularly. Each trial number represents a single experiment and the columns marked A – G represents the variables used in this study. Each variable has two levels, high (+) and low (-). Biomass, final pH, CYP505A1 specific and volumetric activities were selected as responses from the model.

Table 11 Main effects and β-coefficients of each variable on biomass production by *B. adeninivorans* UOFS Y-1220

Variables with designate	Lower level (-)	Higher level (+)	Main effect	β-coefficients
Model				
A Buffer*	PO_4	MES	-2.725	-1.368
B Initial pH	6	7	4.375	2.175
C Carbon source	Glucose	Glycerol	3.875	1.943
D Substrate concentration (g L^{-1})	20	40	4.025	2.018
E Culture volume (mL)	50	150	-8.975	-4.493
F δ-Aminolevulinic acid (g L^{-1})	0	0.8	-1.025	-0.500
G Harvest time (h)	48	72	5.825	2.900

*: PO_4 = Potassium phosphate buffer, MES = 2-(*N*-morpholino)ethanesulfonic acid. Both of these buffers had a final concentration of 140 mM in the culture medium.

Table 12 Main effects and β–coefficients of each variable on specific whole-cell CYP505A1 activity by *B. adeninivorans* UOFS Y-1220

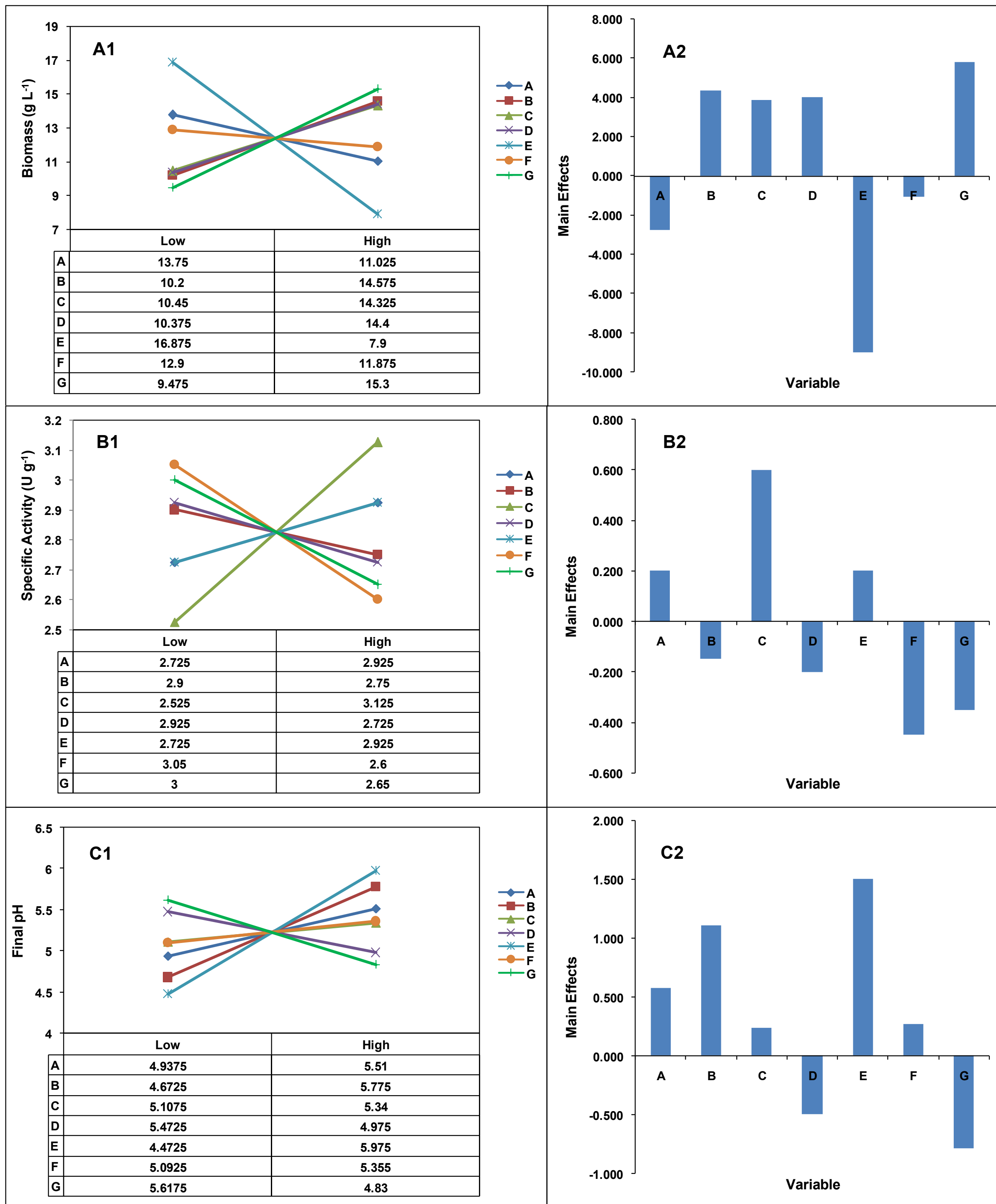
Variables with designate	Lower level (-)	Higher level (+)	Main effect	β-coefficients
Model				
A Buffer*	PO_4	MES	0.200	0.100
B Initial pH	6	7	-0.150	-0.075.
C Carbon source	Glucose	Glycerol	0.600	0.3
D Substrate concentration (g L^{-1})	20	40	-0.200	-0.100
E Culture volume (mL)	50	150	0.200	0.100
F δ-Aminolevulinic acid (g L^{-1})	0	0.8	-0.450	-0.225
G Harvest time (h)	48	72	-0.350	-0.175

*: PO_4 = Potassium phosphate buffer, MES = 2-(*N*-morpholino)ethanesulfonic acid. Both of these buffers had a final concentration of 140 mM in the culture medium.

Table 13 Main effects and β–coefficients of each variable on pH by *B. adeninivorans* UOFS Y-1220

Variables with designate	Lower level (-)	Higher level (+)	Main effect	β-coefficients
Model				
A Buffer*	PO_4	MES	0.573	0.286
B Initial pH	6	7	1.103	0.551.
C Carbon source	Glucose	Glycerol	0.233	0.116
D Substrate concentration (g L^{-1})	20	40	-0.498	-0.249
E Culture volume (mL)	50	150	1.503	0.751
F δ-Aminolevulinic acid (g L^{-1})	0	0.8	0.263	0.131
G Harvest time (h)	48	72	-0.788	-0.394

*: PO_4 = Potassium phosphate buffer, MES = 2-(*N*-morpholino)ethanesulfonic acid. Both of these buffers had a final concentration of 140 mM in the culture medium.



	Low	High
A	13.75	11.025
B	10.2	14.575
C	10.45	14.325
D	10.375	14.4
E	16.875	7.9
F	12.9	11.875
G	9.475	15.3

	Low	High
A	2.725	2.925
B	2.9	2.75
C	2.525	3.125
D	2.925	2.725
E	2.725	2.925
F	3.05	2.6
G	3	2.65

	Low	High
A	4.9375	5.51
B	4.6725	5.775
C	5.1075	5.34
D	5.4725	4.975
E	4.4725	5.975
F	5.0925	5.355
G	5.6175	4.83

Figure 20 Main effect graphs of biomass (A), CYP505A1 specific activity (B) and Final pH (C). A1, B1 and C1: The estimated effect of variables at their low and high levels. A2, B2 and C2: The effect of variables. Legend: A, Buffer; B, Initial pH; C, Carbon source; D, Substrate concentration; E; Volume of culture; F, δ-aminolevulinic acid; G, Harvest time.

4.3. Medium components and culture conditions that significantly affect biomass production and VAO expression in *B. adeninivorans* UOFS Y-1220

Plackett-Burman design was also used to identify factors that have significant effects on heterologous VAO expression (indicated by whole-cell VAO activity) and biomass production by *B. adeninivorans* UOFS Y-1220. Eight variables, as set out in Table 8, were selected for Plackett-Burman experiments. These included metals, vitamins, basal salts, glucose concentration, inoculum size, potassium phosphate buffer concentration, initial pH and flask:culture ratio. A Plackett-Burman experimental design of 12 trials with corresponding responses, i. e. biomass, specific activity of VAO and volumetric activity, is shown in Table 14. Twelve flasks (500 mL) containing different media compositions as determined by the Plackett-Burman design matrix were prepared. Six flasks contained 50 mL of media (Flask:Culture ratio of 10) and another six contained 150 mL (Flask:Culture ratio of 3.33). The media were inoculated with *B. adeninivorans* UOFS Y-1220 at final concentrations of 10 and 20 % (v/v). All the flasks were incubated on a rotary shaker at 120 rpm and 30 °C for 48 h. At harvest, samples were taken from the cultures, for biomass determinations and to determine VAO whole-cell activities. The results for these experiments are shown in Figure 21 and Table 14.

Statistical analysis was conducted for the Plackett-Burman design experiment comprising 12 trials. The SPC for excel software was used for calculating regression coefficients, F values and p values of the factors for all responses (Tables 15–17). Specific activities of VAO varied from 1.18 to 26.17 U g^{-1} for the 12 trials conducted (Table 14). This large variation showed that the variables tested had strong influence on VAO expression. The same observation was made for biomass and volumetric activity. Biomass production ranged from 11.47 to 24.03 g L^{-1} and volumetric activity from 13.55 to 504.98 U L^{-1}. Analysis of variance (ANOVA) showed that the PB models for biomass and specific whole-cell VAO activity were significant with p-values less than 0.05 (confidence interval greater than 95 %).

Table 15 and Figure 21 (A1,2) show that, of the eight variables evaluated, glucose concentration, inoculum size, potassium phosphate buffer concentration, initial pH, vitamins and flask:culture ratio had significant effect on biomass production ($p<0.05$). Higher glucose concentration (80 g L^{-1}), inoculum size (20 % v/v), potassium phosphate buffer (200 mM), initial pH 8 and flask:culture ratio (10) were preferred for yeast growth. The flask:culture ratio of 10

meant a working volume of 50 mL per 500 mL flask. Only additional vitamins showed a significant negative effect on biomass production.

Regression analysis of the results for specific whole-cell VAO activity (Table 16 and Figure 21 B1, 2) showed that buffer concentration and initial pH had significant effect on specific whole-cell VAO activities with higher buffer concentration and initial pH improving VAO activity. The same observations were made for volumetric activity (Table 17). Higher concentration of metals, vitamins, basal salts, inoculum size (20 % v/v) and larger flask:culture ratio (10) also apparently improved specific whole-cell VAO activity. Higher glucose concentration (80 g L^{-1}) decreased specific whole-cell VAO activity. A similar observation was made for CYP505A1.

Further Plackett-Burman design experiments PB 8 Run 1 and Run 2 were subsequently conducted (Figure 22). The conditions for PB 8 Run 1 were based on PB 12 Run results and those of PB 8 Run 2 on PB 8 Run 1. Inoculum size and flask:culture ratio were both kept constant in PB 8 Run 1 and this further included pH in PB 8 Run 2. The statistical analysis of PB8 Run 1 and Run 2 did not reveal significant effects. However, the overall results of the three Plackett-Burman experiments will also be discussed.

Concentration of metals and vitamins was lowered when conducting both PB 8 experiments (Figure 22). This seemed to contribute to increase in VAO expression as the whole-cell VAO activity increased and became more stable towards the last Plackett-Burman experiment. Simultaneously, the concentration of salts was increased. On average, biomass production was not affected noticeably by these changes.

Initial pH above pH 8 significantly reduced biomass production by *B. adeninivorans* UOFS Y-1220 (Figure 22, PB 8 Run 1: Runs 1, 3, 6 and 7). This suggests that this yeast does not grow at pH above pH 8. In the bioreactor studies conducted initially after the Plackett-Burman experiments, *B. adeninivorans* UOFS Y-1220 did not grow when the pH was set at 8 (data not shown). The yeast started showing growth when the pH was lowered to 6–7. This shows the preferred pH is even lower than pH 8. The preference for pH close to eight though is surprising. Although VAO activity itself is favoured by high pH (de Jong, 1995; Appendix: A), one would not expect production of intracellular enzyme to be affected by external pH as it seems to be the case here.

Table 14 Plackett-Burman design for screening of variables significant for VAO and biomass production by *B. adeninivorans* UOFS Y-1220 [Factors: 8, Replicates: 0, Design: 12, Runs: 12, Centre Points: 0]

Trial Number	A	B	C	D	E	F	G	H	Biomass* ($g\ L^{-1}$)	VAO# Specific Activity ($U\ g^{-1}$)	VAO Volumetric Activity ($U\ L^{-1}$)
1	-	-	-	-	-	-	-	-	11.47±0.15	1.18±0.00	13.55±0.00
2	-	-	+	-	+	+	-	+	19.40±0.40	15.58±0.73	302.25±14.23
3	-	-	-	+	-	+	+	-	18.43±0.35	13.63±0.31	251.22±5.72
4	-	+	+	-	+	+	+	-	16.05±0.07	26.17±0.95	348.09±12.67
5	+	+	-	+	+	+	-	-	16.17±0.15	10.80±0.47	174.58±7.58
6	+	+	+	-	-	-	+	-	11.87±0.15	15.14±0.59	179.61±7.00
7	+	-	+	+	-	+	+	+	24.03±0.25	21.01±0.94	504.98±22.50
8	-	+	-	+	+	-	+	+	22.77±0.21	10.82±1.32	246.34±30.12
9	+	+	-	-	-	+	-	+	15.70±0.10	8.10±0.92	127.15±14.48
10	+	-	-	-	+	-	+	+	20.13±0.25	16.48±1.19	331.79±24.01
11	+	-	+	+	+	-	-	-	15.50±0.20	1.25±0.00	19.31±0.00
12	-	+	+	+	-	-	-	+	19.13±0.15	1.2±0.00	22.98±0.00

Symbols: * = Dry cell weight, # = this represents whole-cell specific activity of VAO since the enzyme is produced intracellularly. Each trial number represents a single experiment and the columns marked A – H represents the variables used in this study. Each variable has two levels, high (+) and low (-). Biomass, VAO specific and volumetric activities were selected as responses from the model.

Table 15 Regression analysis and ANOVA of each variable for biomass production by *B. adeninivorans* UOFS Y-1220

Variables with designate	Lower level (-)	Higher level (+)	Main Effect	β-Coefficients	F- value	P-value	Confidence level (%)
Model					62.541	0.003	99.7
A Metals#	1	2	-0.642	-0.321	3.800	0.146	85.4
B Vitamins#	1	2	-1.212	-0.606	13.551	0.035	96.5
C Basal Salts#	0.25	0.5	0.218	0.109	0.440	0.555	44.5
D Glucose Concentration (g L^{-1})	40	80	3.568	1.784	117.524	0.002	99.8
E Inoculums size (% v/v)	10	20	1.565	0.782	22.606	0.018	98.2
F Buffer* concentration (mM)	50	200	1.485	0.743	20.354	0.020	98.0
G Initial pH	6	8	2.652	1.326	64.899	0.004	99.6
H Flask:Culture Ratio	3.33	10	5.278	2.639	257.152	0.001	99.9

Symbols: # = This table only shows the concentration factors of the metals, vitamins and basal salts as compared to the concentration in the starting medium. The actual concentrations and composition of these are shown in Table 8. The metals here refer to trace elements;* = potassium phosphate buffer was used in these experiments.

Table 16 Regression analysis and ANOVA of each variable for specific whole-cell VAO activity by *B. adeninivorans* UOFS Y-1220

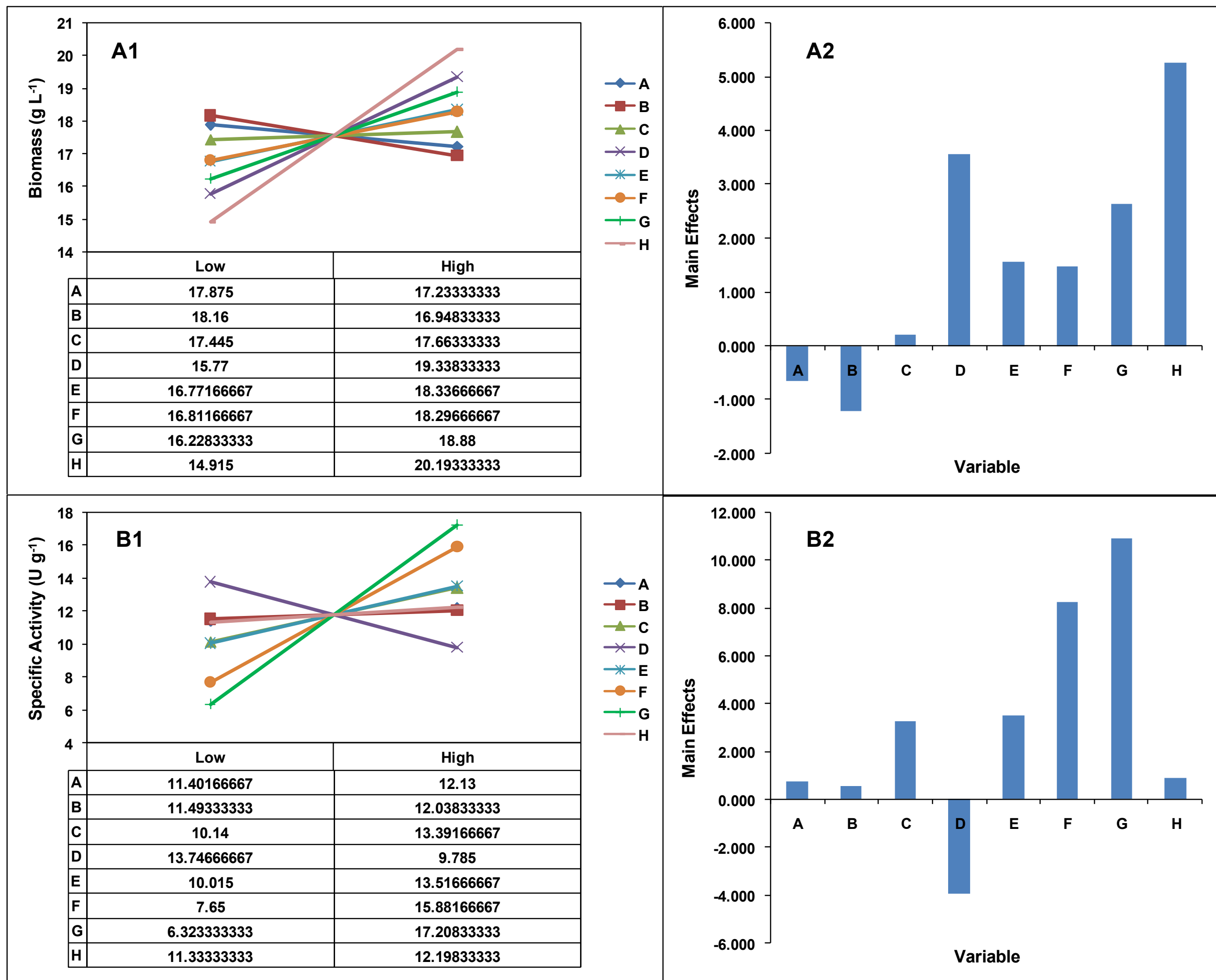
Variables with designate	Lower level (-)	Higher level (+)	Main Effect	β-Coefficients	F value	P-value	Confidence level (%)
Model					13.623	0.027	97.3
A Metals#	1	2	0.728	0.364	0.255	0.648	35.2
B Vitamins#	1	2	0.545	0.273	0.143	0.731	26.9
C Basal Salts#	0.25	0.5	3.252	1.626	5.091	0.109	89.1
D Glucose Concentration (g L^{-1})	40	80	-3.962	-1.981	7.557	0.071	92.9
E Inoculums size (% v/v)	10	20	3.502	1.751	5.904	0.093	90.7
F Buffer* concentration (mM)	50	200	8.232	4.116	32.626	0.011	98.9
G Initial pH	6	8	10.885	5.443	57.048	0.005	99.5
H Flask:Culture Ratio	3.33	10	0.865	0.432	0.360	0.591	40.9

Symbols: # = This table only shows the concentration factors of the metals, vitamins and basal salts as compared to the concentration in the starting medium. The actual concentrations and composition of these are shown in Table 8. The metals here refer to trace elements; * = potassium phosphate buffer was used in these experiments.

Table 17 Regression analysis and ANOVA of each variable for volumetric whole-cell VAO activity by *B. adeninivorans* UOFS Y-1220

Variables with designate	Lower level (-)	Higher level (+)	Main Effect	β-Coefficients	F value	P-value	Confidence level (%)
Model					6.059	0.110	91.7
A Metals#	1	2	25.498	12.749	0.399	0.572	42.8
B Vitamins#	1	2	-54.958	-27.029	1.795	0.273	72.7
C Basal Salts#	0.25	0.5	38.765	19.383	0.923	0.408	59.2
D Glucose Concentration (g L^{-1})	40	80	-13.838	-6.919	0.118	0.754	24.6
E Inoculums size (% v/v)	10	20	53.812	-26.909	1.778	0.275	72.5
F Buffer* concentration (mM)	50	200	149.115	74.558	13.656	0.034	96.6
G Initial pH	6	8	200.368	100.184	24.657	0.016	98.4
H Flask:Culture Ratio	3.33	10	91.522	45.761	5.144	0.108	89.2

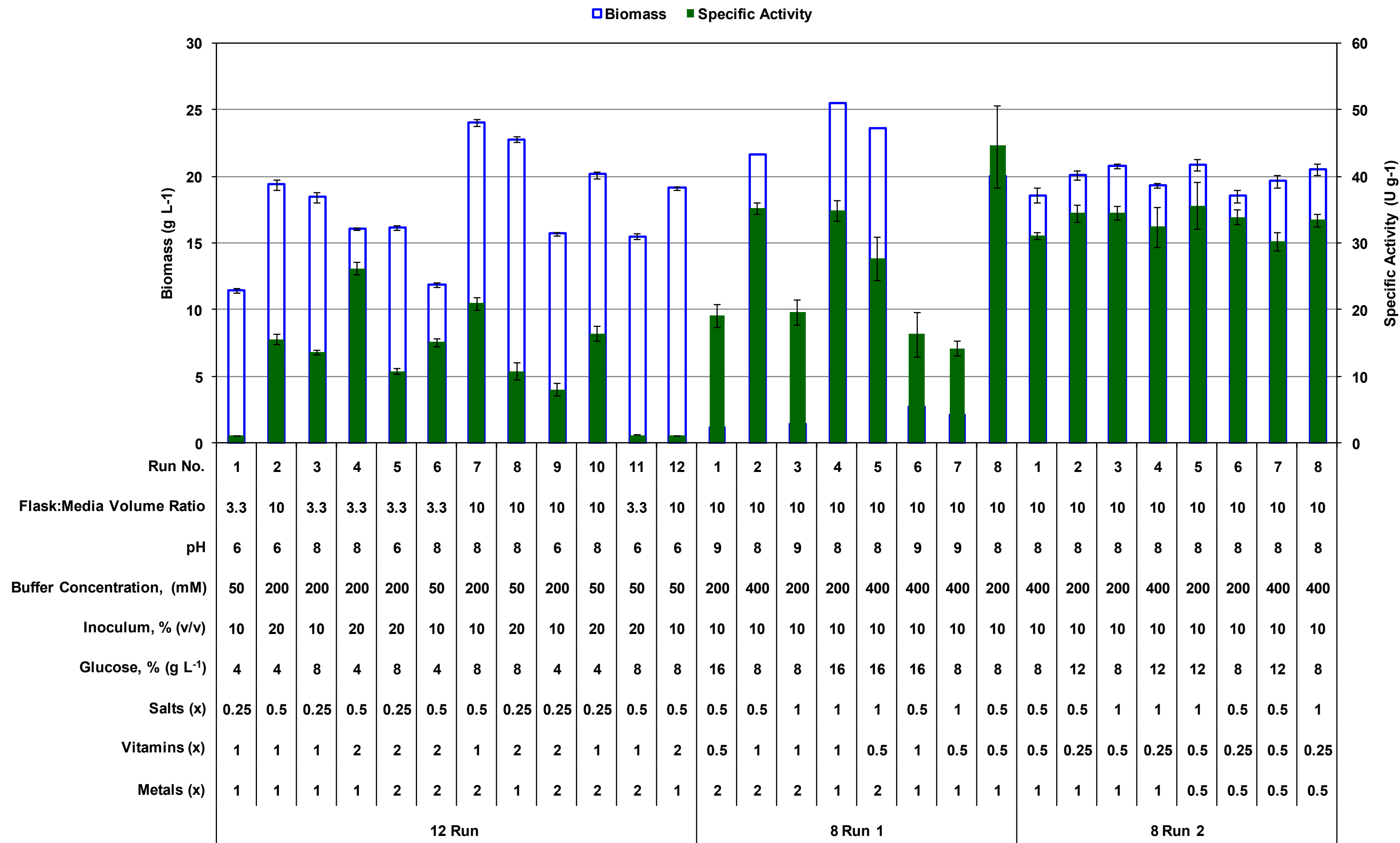
Symbols: # = This table only shows the concentration factors of the metals, vitamins and basal salts as compared to the concentration in the starting medium. The actual concentrations and composition of these are shown in Table 8. The metals here refer to trace elements; * = potassium phosphate buffer was used in these experiments.



	Low	High
A	17.875	17.23333333
B	18.16	16.94833333
C	17.445	17.66333333
D	15.77	19.33833333
E	16.77166667	18.33666667
F	16.81166667	18.29666667
G	16.22833333	18.88
H	14.915	20.19333333

	Low	High
A	11.40166667	12.13
B	11.49333333	12.03833333
C	10.14	13.39166667
D	13.74666667	9.785
E	10.015	13.51666667
F	7.65	15.88166667
G	6.323333333	17.20833333
H	11.33333333	12.19833333

Figure 21 Main effect graphs of biomass (A) and VAO specific activity (B). A1 and B1: The estimated effect of variables at their low and high levels. A2 and B2: The effect of variables. Legend: A, Metals; B, Vitamins; C, Salts; D, Glucose concentration; E; Inoculum size; F, Buffer concentration; G, Initial pH; H, Flask:Culture ratio.



12 Run

Run No.	1	2	3	4	5	6	7	8	9	10	11	12
Flask:Media Volume Ratio	3.3	10	3.3	3.3	3.3	3.3	10	10	10	10	3.3	10
pH	6	6	8	8	6	8	8	8	6	8	6	6
Buffer Concentration, (mM)	50	200	200	200	200	50	200	50	200	50	50	50
Inoculum, % (v/v)	10	20	10	20	20	10	10	20	10	20	20	10
Glucose, % (g L^{-1})	4	4	8	4	8	4	8	8	4	4	8	8
Salts (x)	0.25	0.5	0.25	0.5	0.25	0.5	0.5	0.25	0.25	0.25	0.5	0.5
Vitamins (x)	1	1	1	2	2	2	1	2	2	1	1	2
Metals (x)	1	1	1	1	2	2	2	1	2	2	2	1

8 Run 1

Run No.	1	2	3	4	5	6	7	8
Flask:Media Volume Ratio	10	10	10	10	10	10	10	10
pH	9	8	9	8	8	9	9	8
Buffer Concentration, (mM)	200	400	200	200	400	400	400	200
Inoculum, % (v/v)	10	10	10	10	10	10	10	10
Glucose, % (g L^{-1})	16	8	8	16	16	16	8	8
Salts (x)	0.5	0.5	1	1	1	0.5	1	0.5
Vitamins (x)	0.5	1	1	1	0.5	1	0.5	0.5
Metals (x)	2	2	2	1	2	1	1	1

8 Run 2

Run No.	1	2	3	4	5	6	7	8
Flask:Media Volume Ratio	10	10	10	10	10	10	10	10
pH	8	8	8	8	8	8	8	8
Buffer Concentration, (mM)	400	200	200	400	200	200	400	400
Inoculum, % (v/v)	10	10	10	10	10	10	10	10
Glucose, % (g L^{-1})	8	12	8	12	12	8	12	8
Salts (x)	0.5	0.5	1	1	1	0.5	0.5	1
Vitamins (x)	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.25
Metals (x)	1	1	1	1	0.5	0.5	0.5	0.5

Figure 22 Plackett-Burman experimental designs for factors affecting VAO expression and biomass production by *B. adeninivorans* UOFS Y-1220. The contents of metals, salts and vitamins are given in Table 8, Chapter 3. The biomass concentration and specific activities were done in triplicate (standard deviations are shown).

4.4. Effect of dissolved oxygen tension on VAO expression and biomass production under batch cultivation of *B. adeninivorans* UOFS Y-1220

The effect of dissolved oxygen tension (DOT) on growth and VAO production by *B. adeninivorans* UOFS Y-1220 was investigated in Sixfors bioreactors (Infors, Switzerland) by culturing the yeast under DOT of 10, 30 and 60 % in a batch mode (Figure 23). Sixfors is a multireactor system that has six reactors that can run in parallel. The three DOT batch cultivations were run concurrently with each other. The yeast was cultivated in a SYN6-2 medium with pH range 7-7.3 and a working volume of 250 mL. The DOT was controlled using a combination of air-flow rate and stirrer speed. The cultures were grown for 42-48 h, after which samples were taken to measure dry cell biomass, glucose depletion, specific whole-cell VAO activity and volumetric activity. Bioreactor conditions are shown in Appendix: B, C and D.

The lowest DOT of 10 % is near but not lower than the critical DOT level previously reported for *B. adeninivorans* (Jansen, 2007) and therefore normal growth that is independent of DOT was expected (Pirt, 1975). However, increase in DOT from 10 to 60 % was accompanied by increase in maximum specific growth rate (μ_{max}) from 0.3 to 0.4 h^{-1} (Figure 23; Table 18). Biomass production under 10 and 30 % DOT assumed a linear trend from 10 h up to the end of cultivation. This behavior suggests presence of oxygen limitation because at 60 % DOT *B. adeninivorans* UOFS Y-1220 continued with normal growth until it reached stationery phase after 25 h. The need for high level of DOT for efficient growth by *B. adeninivorans* UOFS Y-1220 is also evident from other studies that showed high cell density cultivation of this genus under fed-batch cultivation using high pressure at 20-40 % DOT (Knoll *et al*., 2007; Knabben *et al*., 2010). Despite the assumed oxygen limitation at 30 % DOT, final biomass concentration after 48 h was nearly the same as that achieved at 60 % DOT conditions. These observations suggest that a change in DOT only affect μ_{max} but not the final biomass concentration.

Tremendous increase of specific whole-cell VAO activity was observed during the first 10 h of cultivation (Figure 23). This implies that VAO production under this expression system is growth associated. The *Y. lipolytica TEF* promoter used for driving the expression of VAO in this expression system can explain this, since it is itself expressed during the growth phase (Rösel and Kunze, 1995). Maximum VAO activities of 89.12, 131.56 and 353.54 U g^{-1} were observed for 60, 10 and 30 % DOT, respectively. After 10 h, specific whole-cell activities of VAO dropped lower than 100 U g^{-1} and further below 50 U g^{-1} with activity at 60 % DOT being the lowest. This type of behavior was unexpected and the reasons behind it were not investigated.

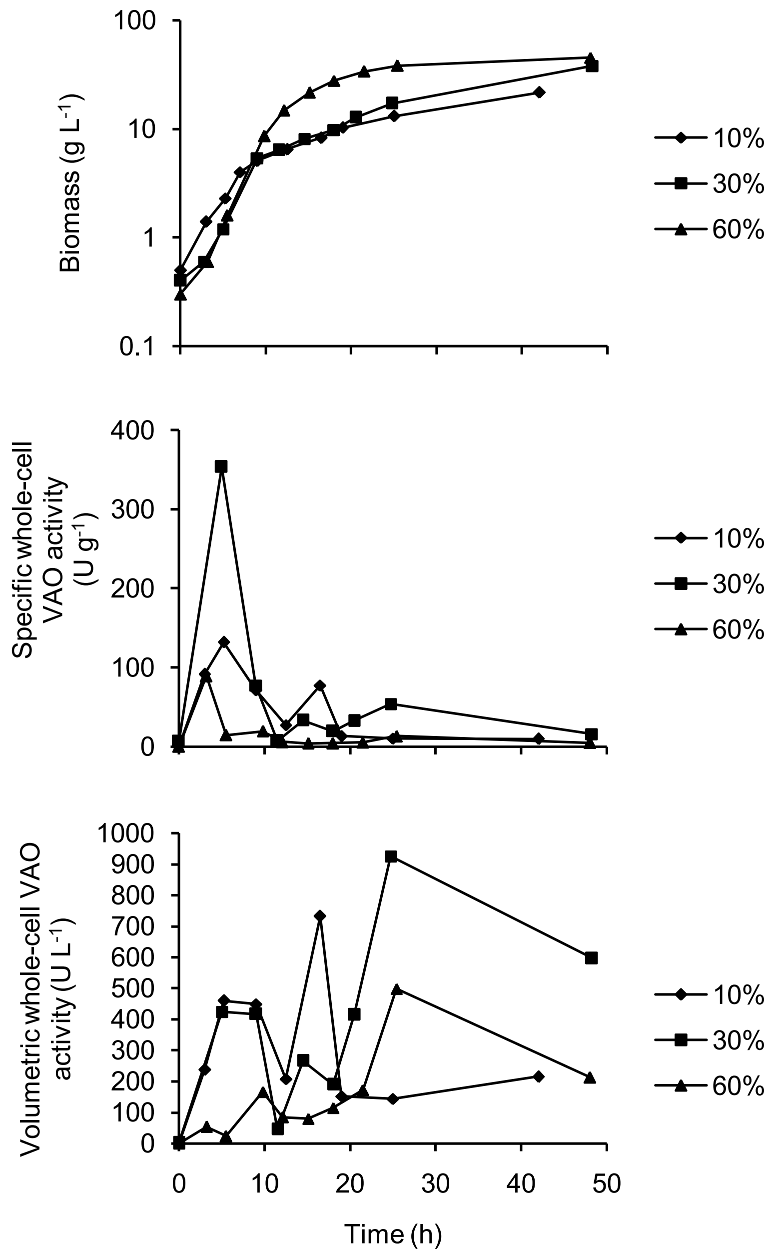


Figure 23 Cultivation profiles of *B. adeninivorans* UOFS Y-1220 at different DOT (10, 30 and 60 %) for biomass (n=3) and VAO production (n=3). Pre-culture of the yeast was grown in YPD using shake-flasks. Batch cultivations were conducted in Sixfors bioreactors using modified SYN6 medium, with 300 mL flasks and 250 mL working volume. Temperature was kept at 30 °C and stirrer speed ranged between 400 and 1200 rpm. The pH was controlled using potassium phosphate buffer (200 mM) and had a range of 4.5-7.5.

At the end of cultivation (after 48 h) specific whole-cell activities dropped to 9.41, 15.71 and 4.7 U g^{-1} under 10, 30 and 60 % DOT, respectively. These levels are lower than 21.01 U g^{-1} (Table 14: Run 7) that was obtained in shake-flask using similar medium.

A pattern similar to specific whole-cell VAO activity was observed for volumetric activity (Figure 23) except that volumetric activities reached maximum levels after 25 h when the cells entered stationery growth phase since it is also a function of biomass concentration. At the end of cultivation, the highest final volumetric activity of 597.07 U L^{-1} was achieved at 30 % DOT conditions. This volumetric activity is comparable to 504.98 U L^{-1} observed in Run 7 of the PB12 experiment (Table 18). The low volumetric activity that was observed under 60 % DOT was due to low specific whole-cell VAO activities, since cultivation at 60 % DOT yielded more biomass than that at 30 %.

Table 18 Comparison of cultivation yields for shake-flask Plackett-Burman (PB) 12 Run 7 and bioreactor studies varying dissolved oxygen tension.

Parameter	PB 12 Run 7	DOT 10 %	DOT 30 %	DOT 60 %
μ_{max} (h^{-1})	n. d.	0.3	0.35	0.4
Final Biomass (g_{dcw} L^{-1})/ Time (h)	24.03/48	21.7/42	38/48.25	45.4/48
Biomass Yield (g g^{-1})	0.3	0.27	0.48	0.57
Maximum Specific VAO activity (U g^{-1})/Time (h)	n. d.	131.56/5.25	353.54/5	89.12/3.2
Final Specific VAO activity (U g^{-1})/Time (h)	21.01/48	9.41./42	15.71/48.25	4.70/48
Maximum Volumetric VAO Activity (U L^{-1})*	n. d.	151.7	926.16	498.41
Final Volumetric VAO Activity (U L^{-1})*	504.98	215.46	597.07	213.24

DOT: Dissolved oxygen tension; n. d.: not determined; * = obtained at same time as variables above

4.5. Other observations made

Comparison of the results from all three Plackett-Burman experiments and the bioreactor runs made some additional observations possible. Under the shake-flask cultivation conditions employed in this study, *B. adeninivorans* UOFS Y-1220 could not attain more than 25 g L^{-1} of biomass concentration even though glucose concentrations of up to 160 g L^{-1} were used (Figure 22). Taking the averages, biomass yield coefficient ($Y_{x/s}$) decreased with increasing glucose concentration (Figure 24). This shows that, under the shake-flask conditions employed glucose concentrations beyond 40 g L^{-1} did not yield high biomass concentration.

Under the shake-flask conditions, large quantities of ethanol (maximum ca. 18 g L^{-1}) and glycerol (maximum ca. 10 g L^{-1}) were produced by *B. adeninivorans* UOFS Y-1220 (Appendix: E). Ethanol production under shake-flask conditions due to oxygen limitation was also observed by Hellwig *et al.*, (2005).The production of these by-products could also explain the inability to obtain normal $Y_{x/s}$ of 0.5 in most cases. This could be solved by increasing shaking speed of the rotary shaker as observed in literature (Hellwig *et al.*, 2005). In the bioreactor, using proper DOT control, this situation was resolved and glucose concentration up to 80 g L^{-1} could be utilized efficiently by *B. adeninivorans* UOFS Y-1220 in batch culture (Appendix: F). There was clear consumption of ethanol and glycerol under these conditions.

Interestingly, despite the inefficient use of glucose in shake-flasks, the average specific whole-cell activity of VAO reached maximum at 80 g L^{-1} glucose concentration (Figure 24). The activity was lowest at 40 and 160 g L^{-1}, and dropped slightly at 120 g L^{-1} glucose concentration. Glucose concentration beyond 80 g L^{-1} was apparently detrimental to VAO expression. The results from Figure 24, however, should not be considered in isolation, as the effect of other factors also has to be accounted for.

The average $Y_{x/s}$ at 40 g L^{-1} was higher for VAO Plackett-Burman design experiments than for CYP505A1 ones. This could have been for two reasons. The one reason could be the difference in the compositions of the media employed. The other reason could be the difference in metabolic load placed by enzyme production on growth of the yeast. CYP505A1 could be demanding more energy than VAO for production, thus reducing the growth rate and $Y_{x/s}$ of *B. adeninivorans* UOFS Y-1220. Glick (1995) extensively reviewed the topic of metabolic load by heterologous gene expression. To investigate this aspect in more detail, empty vector control strains should also be included in experiments.

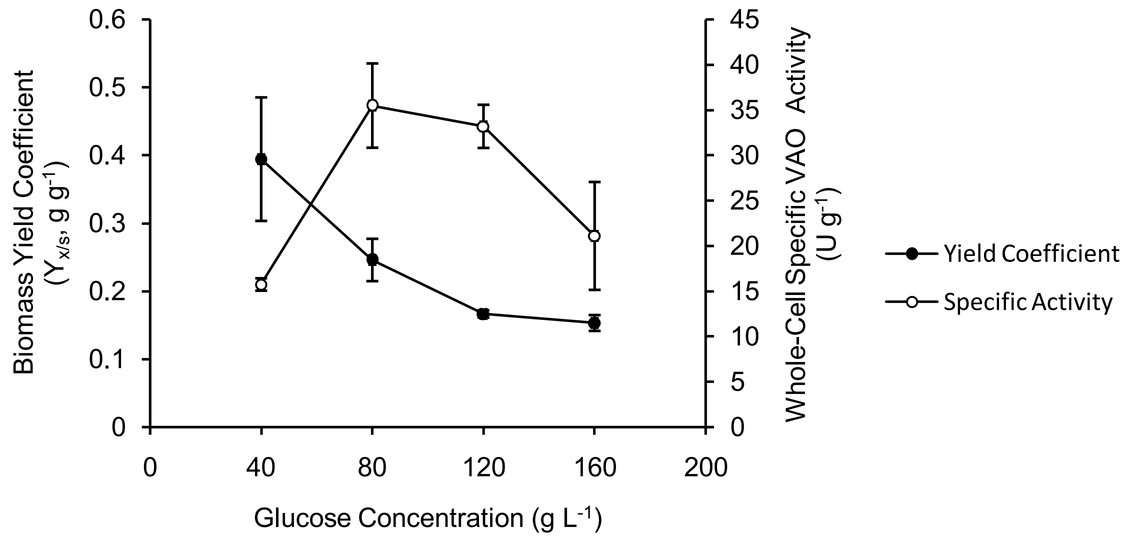


Figure 24 Influence of glucose concentration on average biomass yield coefficient and whole-cell VAO specific activity by *B. adeninivorans* UOFS Y-1220. The averages of the biomass and specific activity were calculated from the Plackett-Burman experiments at different glucose concentrations.

Chapter 5

General conclusions

5. General conclusions

CYP505A1 and VAO were recently, for the first time, expressed in *B. adeninivorans* in our group (Theron *et al*., 2014; Smit *et al*., 2012a, b). This thesis was therefore motivated by the excellent whole-cell activities of intracellular CYP505A1 and VAO achieved in *B. adeninivorans* UOFS Y-1220 when compared to several yeasts expressing the same genes under similar conditions. The current study then set out to study the effects of cultivation conditions and medium composition on biomass production and heterologous expression of CYP505A1 and VAO by *B. adeninivorans* UOFS Y-1220. This was preceded by the development of UV spectroscopy and TLC assays to follow whole-cell activity of CYP505A1 and VAO.

Despite limitations, the thesis has demonstrated that UV spectroscopy and TLC could successfully quantify whole-cell activities of VAO and CYP505A1. In cases where many samples need to be assayed, these methods could replace HPLC and GC that are usually employed. In addition, with necessary improvements, the UV and TLC assays could yield results that are comparable to HPLC and GC.

The Plackett-Burman factorial design proved useful in identifying factors that significantly affect gene expression and biomass production. This was a good starting point, since no literature (to the best of our knowledge) on how culture conditions affect CYP505A1 and VAO expression, or for that matter expression of other oxidoreductases, was available. The design identified several significant factors affecting biomass production and expression of VAO. Although the experiments with CYP505A1 did not reveal statistically significant factors, the results obtained still greatly improved our understanding of CYP505A1 expression in *B. adeninivorans* UOFS Y-1220.

Analyzing byproducts of cultivation such as ethanol and glycerol showed that oxygen availability plays a crucial role for efficient cultivation of *B. adeninivorans* UOFS Y-1220. If the

purpose is only to achieve optimal biomass concentration per substrate utilized then dissolved oxygen tension (DOT) up to 60 % will be necessary. This could also favour volumetric production of a biocatalyst should *B. adeninivorans* UOFS Y-1220 be used as such. However, in our case where production of a recombinant enzyme was also our focus, an optimum level of DOT such as 30 % will suffice. Plackett-Burman design identified vitamins, glucose concentration, inoculum size, phosphate buffer concentration, initial pH and oxygen availability as significant for biomass production in *B. adeninivorans* UOFS Y-1220. Only phosphate buffer concentration and initial pH were identified as significant for VAO expression. Lower concentration of vitamins and starting pH of 8 as well as lower volume of culture (50 mL per 500 mL flask) favoured biomass production. Higher concentrations of glucose (80 g L^{-1}), inoculum (20 % v/v) and potassium phosphate buffer (200 mM) also increased biomass production. The VAO activities benefitted from higher starting pH and concentration of phosphate buffer.

Expression of VAO under the *TEF* promoter is growth associated. This presents a problem to this type of study as the factors that affect heterologous gene expression cannot be determined without strong influence from cell growth. Using an inducible promoter for regulation of expression could improve this situation because it would allow the researcher to induce gene expression when the cells have reached optimum growth at stationery growth phase. This way cell growth cannot interfere with heterologous gene expression. An apparent disadvantage of the *TEF* promoter is that activity of the recombinant enzyme drops towards the end of cultivation, when maximum biomass has been reached.

Although the Plackett-Burman design shows how the factors investigated affect gene expression or biomass production, it only does so based on two factor levels. The extent and limits of the effects remain unknown. Therefore, one factor at a time should next be investigated. When assessing medium composition—because of many elements—metals, vitamins and salts were studied as groups. The effects of the underlying constituents were not determined individually. Therefore, it will be profitable, based on the results of this study, to also focus on this area going forward.

To conclude, this thesis contributes to the understanding of heterologous gene expression of oxidoreductases in *B. adeninivorans*. The literature showed that there is lack of research in this area for *B. adeninivorans*. Furthermore, this thesis lays the basis for optimization studies (in terms of culture conditions and medium composition) of CYP505A1 and VAO expression in *B. adeninivorans* UOFS Y-1220 and possibly other microorganisms.

Summary

Different oxidoreductases are being investigated for hydroxylation reactions to satisfy the global need for environmentally friendly industrial processes. These enzymes are found in microorganisms, animals and plants. Comparative study of different yeasts has shown the best heterologous expression of the fatty-acid hydroxylase (*CYP505A1*) and vanillyl-alcohol oxidase (*VAO*) in *B. adeninivorans* UOFS Y-1220. In an attempt to improve this, we studied the effect of cultivation conditions on biomass production and the expression of both genes. Medium constituents and bioreactor conditions that play significant roles and the nature of those roles on *CYP505A1* and *VAO* expressions were investigated. Plackett-Burman shake-flask studies revealed that vitamins, substrate concentration, inoculum size, buffer concentration, initial pH and culture volume had significant effects on biomass production, while factors significant for *VAO* expression were buffer concentration and initial pH. Higher culture volume and vitamins had a negative effect on biomass production while substrate concentration, inoculum size, buffer concentration and initial pH showed a positive effect. The yeast preferred potassium phosphate buffer, glycerol as carbon source and longer cultivation times but not δ-aminolevulinic acid although these were statistically insignificant. *CYP505A1* expression was better when using MES buffer, glycerol and shorter cultivation times but not δ-aminolevulinic acid. In addition, we observed that substrate concentration and culture volume had negative and positive effect, respectively. Higher buffer concentration and initial pH had a positive effect on *VAO* expression. Moreover, though not statistically significant, substrate concentration and culture volume had negative effect while vitamins, metals, basal salts and inoculum size showed positive effect. Lastly, high DOT, in the bioreactors, had positive impact on biomass production while it decreased the level of VAO expression. In conclusion, the importance of cultivation conditions for gene expression has been highlighted and thus, based on this study, further research on medium composition and bioreactor cultivation can be pursued for optimization purposes.

Keywords: Oxidoreductases, Cultivation conditions, Medium composition, Plackett-Burman factorial design, *Blastobotrys adeninivorans*, Vanillyl-alcohol oxidase, Self-sufficient fatty acid hydroxylase, heterologous gene expression, Dissolved oxygen tension, Translation elongation factor

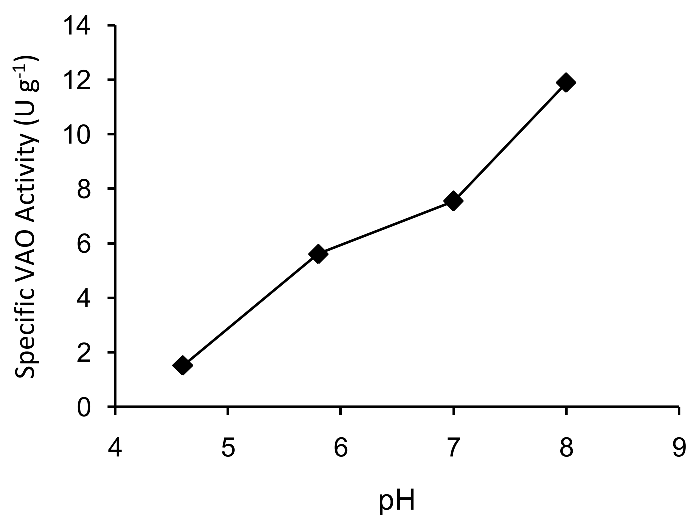
Opsomming

Verskillende oksidoreduktases word ondersoek vir hidroksileringsreaksies om die globale behoefte vir omgewingsvriendelike industriële prosesse te bevredig. Hierdie ensieme is teenwoordig in mikroörganismes, diere en plante. Vergelykende studies van verskillende giste het die beste heteroloë uitdrukking van die vetsuur hidroksilase (CYP505A1) en vanilliel-alkohol oksidase (VAO) in *Blastobotrys adeninivorans* UOVS Y-1220 gelewer. In 'n poging om dit te verbeter, het ons die effek van kweektoestande op biomassa produksie en die uitdrukking van beide gene ondersoek. Medium bestanddele en bioreaktor toestande wat 'n belangrike rol speel en die aard van hierdie rolle op CYP505A1 en VAO uitdrukking is ondersoek. Plackett-Burman skud-vles studies het getoon dat vitamiene, substraatkonsentrasie, inokulum grootte, buffer konsentrasie, aanvanklike pH en kultuur volume bepalend was vir biomassa produksie, terwyl buffer konsentrasie en aanvanklike pH bepalend was vir VAO uitdrukking. Hoër kultuur volume en vitamiene konsentrasie het 'n negatiewe effek op biomassa produksie gehad, terwyl substraatkonsentrasie, inokulum grootte, buffer konsentrasie en aanvanklike pH 'n positiewe effek gehad het. Alhoewel nie statisties betekenisvol nie, wou dit voorkom asof biomassaproduksie bevorder is deur kaliumfosfaat buffer, gliserol as koolstofbron en langer inkubasietyd, maar benadeel is deur δ-aminolevuliniese suur. CYP505A1 uitdrukking was beter met MES buffer, gliserol en korter verbouing, maar nie met δ-aminolevuliniese suur nie. Daarbenewens, het ons opgemerk dat substraatkonsentrasie en kultuur volume onderskeidelik 'n negatiewe en positiewe uitwerking gehad het. Hoër buffer konsentrasie en aanvanklike pH het 'n positiewe uitwerking op VAO uitdrukking gehad. Verder, hoewel nie statisties beduidend nie, het substraatkonsentrasie en kultuur volume 'n negatiewe effek gehad terwyl vitamiene, metale, basale soute en inokulum grootte'n positiewe effek gehad het. Laastens, het 'n hoë DOT in die bioreaktore, 'n positiewe effek op die biomassa produksie gehad, terwyl dit die vlak van VAO uitdrukking verlaag het. Ten slotte, is die belangrikheid van groeitoestande vir die uitdrukking van gene uitgelig en dus, gebaseer op hierdie studie, kan verdere navorsing oor medium samestelling en bioreaktor verbouing ondersoek word vir optimisering.

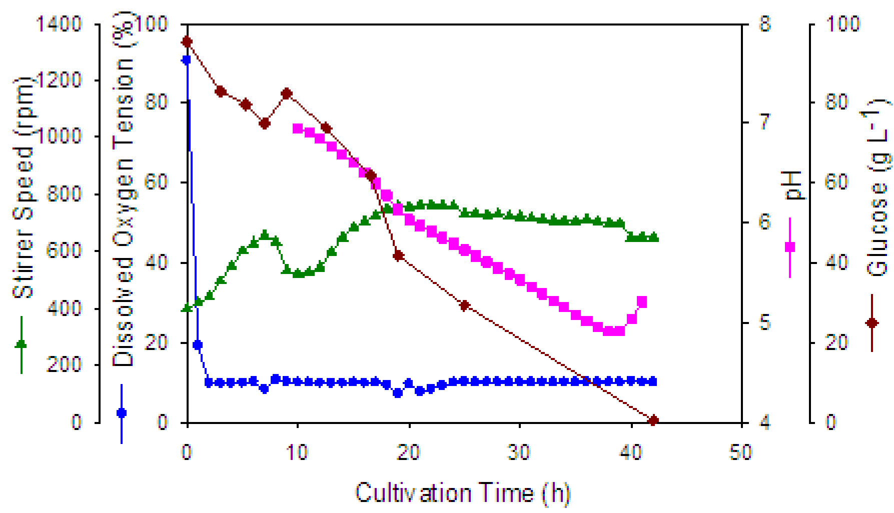
Appendix

A: Increase in pH increases whole-cell activity of VAO by *B. adeninivorans* UOFS Y-1220

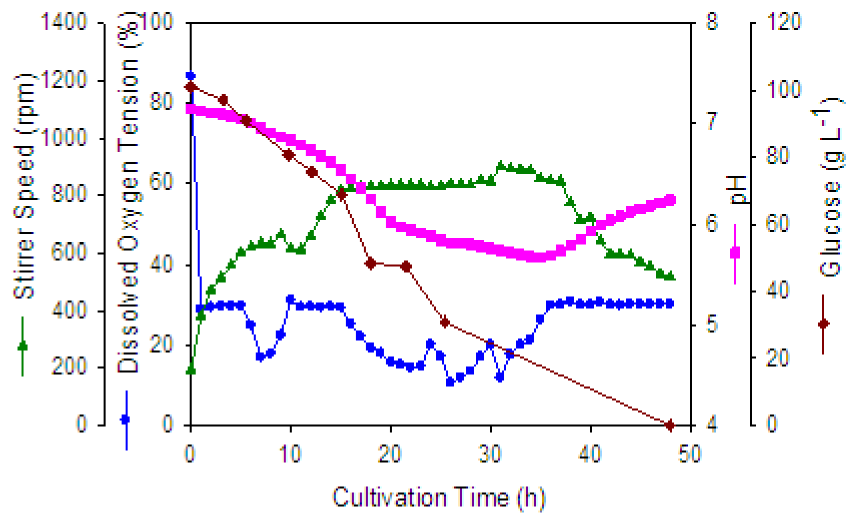
This experiment was done in potassium phosphate buffer (50 mM) at 30 °C and 120 rpm. The reaction medium (10 mL in 100 mL Erlenmeyer flask) contained 100 g_{wcw} L^{-1} and 50 mM eugenol. The reactions were incubated for 120 min.



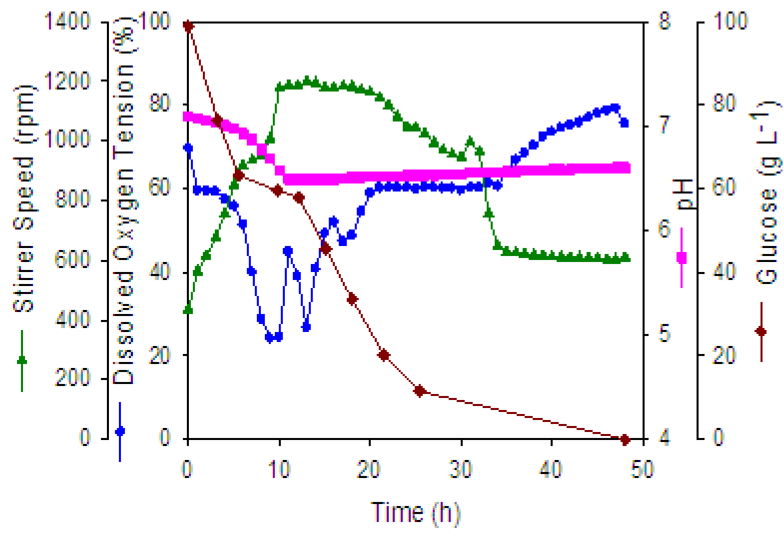
B: Batch cultivation conditions of *B. adeninivorans* UOFS Y-1220 at 10 % DOT



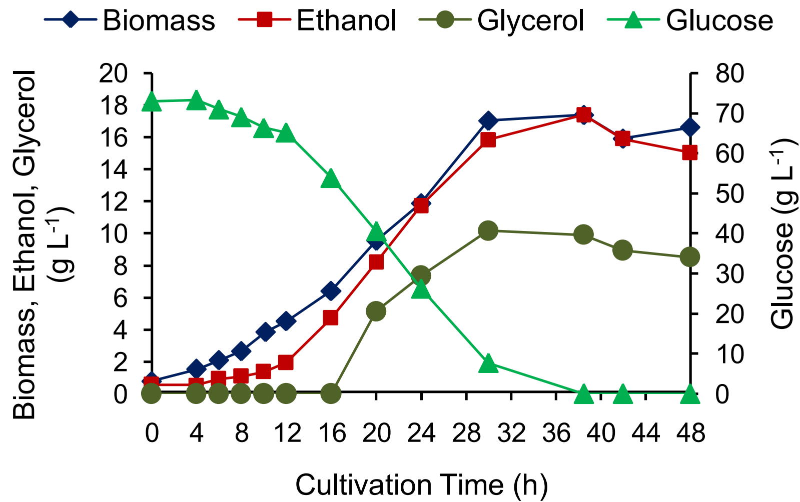
C: Batch cultivation conditions of *B. adeninivorans* UOFS Y-1220 at 30 % DOT



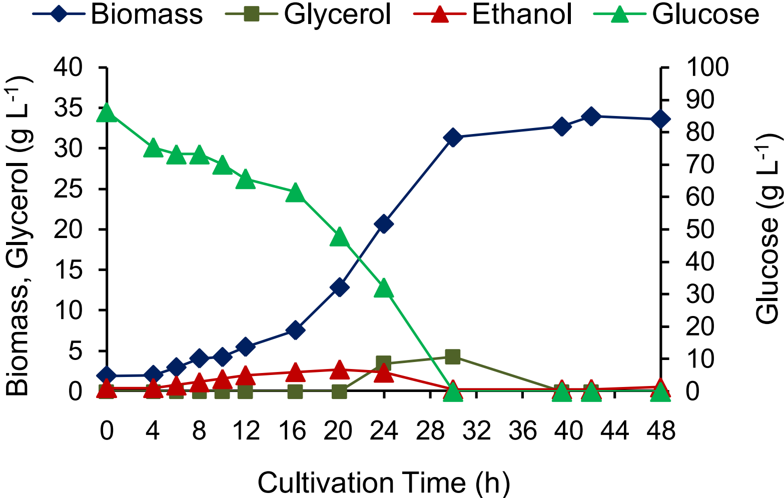
D: Batch cultivation conditions of *B. adeninivorans* UOFS Y-1220 at 60 % DOT



E: Production of side-products by *B. adeninivorans* UOFS Y-1220 under the shake-flask conditions employed in this study.



F: Cultivation by-products, ethanol and glycerol, are depleted during bioreactor cultivation of *B. adeninivorans* UOFS Y-1220.



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Declaration

I declare that the dissertation/thesis hereby handed in for the qualification Magister Scientiae at the University of the Free State is my own independent work and that I have not previously submitted the same work for a qualification at/in another University/faculty.

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