Characterization and engineering of *Bacillus megaterium* AS-35, for use in biodegradation of processed olive wastewater.

Antoinette van Schalkwyk

A thesis submitted in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Department of Biotechnology,
University of the Western Cape
Bellville

Supervisor: Prof. D.A. Cowan

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SUMMARY

Characterization and engineering of *Bacillus megaterium* AS-35, for use in biodegradation of processed olive wastewater.

ANTOINETTE VAN SCHALKWYK

PhD thesis, Department Biotechnology,
University of the Western Cape

The popularization and health benefits associated with the "Mediterranean diet" saw a world wide increase in the production and consumption of processed olives and olive oil. During the brining of table olives large quantities of processed olive waste water is seasonally generated. This blackish-brown, malodours liquid is rich in organic and phenolic compounds, which cause environmental problems upon discarding. Currently, processed wastewater is discarded into large evaporation ponds where it poses serious environmental risks. The biodegradation of organic substrates present in the olive wastewater is inhibited by the high concentrations of phenolic compounds.

In order to identify organisms which could potentially be used in the bioremediation of olive wastewater, 36 microbial strains were isolated from evaporation ponds in the Boland region of South Africa. Twenty five isolates were capable of growth on 50% olive wastewater and their bioremediation potential as well as their ability to produce valuable intermediate compounds were subsequently characterized. Based on the RP-HPLC results, which showed that a number of chemical intermediates were produced in fermentation of olive wastewater, isolate AS-35 was selected for further analysis. Strain AS-35, identified as a *Bacillus megaterium,* was significantly influenced by the exposure to olive waste. The total cellular protein profile, generation time and cellular morphology of this isolate were dramatically affected by the introduction of olive waste.

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This study investigated the differential gene display of *B. megaterium* following exposure to olive wastewater. Proteomic and transcriptomic differences of the organism cultured in nutrient rich LB and olive wastewater were compared. These results indicated that AS-35 expressed genes involved in glycolysis, tryptophan and nucleotide synthesis as well as the chaperones GroEL and DnaK during its growth in LB. In contrast, genes induced following the abolishment of glucose dependent catabolite repression, genes involved in biotin synthesis and ß-oxidation of fatty or organic acids as well as a gene whose expression is regulated by stress induced σ^B-dependent regulon were expressed during olive waste growth.

Classical chemical mutagenesis performed on strain AS-35, produced a hyper-resistant mutant (strain DF4) capable of growth on 80% olive waste. Protein profiles of mutant DF4 cultured in both nutrient rich (LB) media and olive waste were compared to those of the wild type AS-35. In contrast to AS-35 that exhibited distinctive protein profiles when cultured in olive waste and LB broth respectively, the profiles observed for DF4 appeared to be similar irrespective of the culture media. The phenotype of mutant DF4 unchanged by the different culturing conditions, resembled that of wild type cultured in olive wastewater. This suggested that the mutant, in contrast to the wild type, was incapable of altering its gene display in order to reflect the culturing conditions and subsequently suggesting that a mutation(s) occurred in a regulatory pathway(s).

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The results showed that the chemically mutated strain DF4 is capable of growth on a higher olive waste concentration. The mutant reduces more of the total phenol content when grown in the absence of alternative carbon sources than the wild type AS-35. This enhanced bioremediation phenotype supported the unchanged protein profiles of mutant DF4 irrespective of the concentration of olive waste.

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DECLARATION

I declare that *Characterization and engineering of Bacillus megaterium AS-35, for use in biodegradation of processed olive wastewater* is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Antoinette van Schalkwyk 14 November 2005

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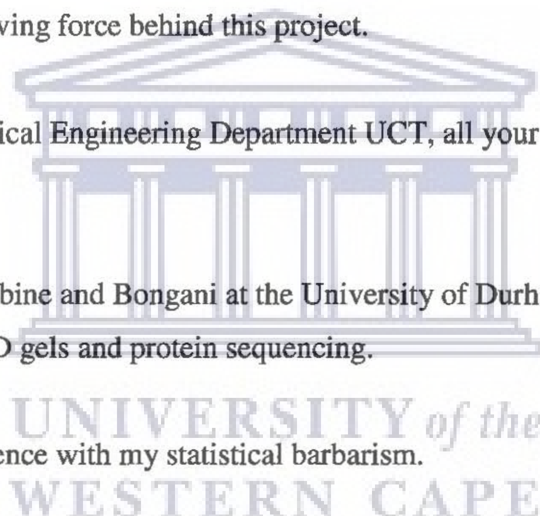

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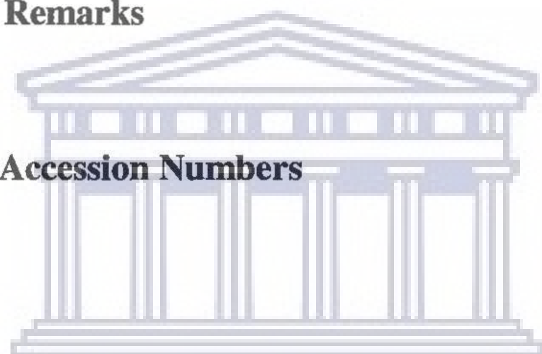

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Chapter 1

Literature Review

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1.1 Introduction

In recent years the "Mediterranean diet", which promotes the consumption of fruits, vegetables, olives and olive oil, has become a popular world wide trend. Such diets have been associated with a reduction in the risk of coronary heart diseases and prostate and colon cancers (Tuck and Hayball, 2002). The majority of the beneficial effects displayed by this diet have been credited to the potent antioxidants present, especially in olives and olive oil (McDonald *et al.,* 2001). The antioxidant activity in olive oil is due to the presence of more than 30 phenolic compounds, with oleuropein, hydroxytyrosol and tyrosol being present at the highest concentrations (Tuck and Hayball, 2002). Other important phenolic compounds present in olive products include *p*-coumaric, caffeic, ferulic and vanillic acids (Lesage-Meessen *et al.,* 2001). The concentration and composition of phenolic compounds present in processed olives depends on the olive cultivar, irrigation of the trees, processing method and storage (Romero *et al.,* 2002a; Mulinacci *et al.,* 2001; Garcia *et al.,* 2001). Oleuropein, one of the most abundant phenolic compounds in olives, consists of hydroxytyrosol esterified with elenolic acid. This compound is also responsible for the bitter taste of unprocessed olives (Romero *et al.,* 2002b; Bianco *et al.,* 1999). Different cultivars of olives are processed using different methods in order to remove the bitter taste as well as to modify the colour of the olive. The debittering of table olives along with the production of olive oil generates large quantities of processed olive wastewater.

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1.2 Processed olive wastewater

Processed olive wastewater is the water used during either the processing of table olives or the production of olive oil. The latter is known as olive mill wastewater (OMW). Although the two types of wastewater differ in the nature of generation, quantity produced and composition, both have chemical and physical similarities (Section 1.2.2).

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1.2.1 Table olives processing

The three most important table olive styles are Spanish-style green olives in brine, Greek-style naturally black olives in brine and Californian-style ripe black olives. Other production methods, such as the treatment of naturally black Thassos olives with layers of table salt, are practiced only in selected regions (Blekas e*t al.,* 2002). The principal aim during table olive processing is to remove the bitter taste associated with unprocessed olive fruits.

1.2.1.1 Spanish-style green olives.

Olives of cultivars such as Manzilla, Hoijiblanca and Gordal are harvested when they reach a green-straw colour. Post-harvest washing and sorting is followed by lye treatment (ca 1.8-2.5 % w/v NaOH) (Sanchez *et al.*, 2000). During the alkaline treatment, hydrolysis of ester bonds occurs, which resulted in olive debittering. Oleuropein is hydrolyzed to hydroxytyrosol and elenolic acid glucoside while verbascoside is hydrolyzed to caffeic acid and hydroxytyrosol 1-O-rhamnosylglucoside (Blekas *et al.*, 2002).

The sodium hydroxide is removed through repeated washing and the olives are placed in brine and left to ferment. Natural fermentation implies the involvement of native micro-organisms during the process. *Lactobacillus,* especially *Lactobacillus plantarum* and *L. casei,* have been identified as the dominant species during fermentation (Randazzo *et al.,* 2004). These micro-organisms promote a reduction in the pH, effectively eliminating competing bacteria (Sanchez *et al.*, 2000). The phenolic composition is not modified during the lactic acid fermentation, but decreases due to diffusion into the brine (Romero *et al.*, 2004b., Blekas e*t al.,* 2002). The rate of diffusion is regulated by the salt concentration of the brine (Sanchez *et al.*, 2000).

Further processing involves the pitting and stuffing of olives. Subjecting the olives to these processes further decreases the total phenol concentration present in the final product. The subsequent decrease in phenol content is due to diffusion either during the floating separation of pitted and unpitted olives or the repeated washing steps prior to olive stuffing (Romero *et al.,* 2004b). The final packaged product contains the third

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highest phenol content of the commercial Greek table olives examined, with the major compounds being hydroxytyrosol, tyrosol and luteolin (Blekas *et al.*, 2002).

1.2.1.2 Greek-style naturally black.

The production of Greek-style naturally black olives involved a milder treatment compared to the processing of Spanish-style green olives. Fully ripe black olives are harvested, washed and stored in liquid brine where yeast fermentation occurs. The change in olive colour from green to black during olive maturation is due to the increase of monomeric anthocyanins, especially cyanidin 3-glucoside and cyanidin 3-rutinoside. Other major changes occurring during olive maturation are the decrease in oleuropein together with the increase in hydroxytyrosol-4-β-glucoside (Romero *et al.*, 2004b.). Following harvesting and washing, the olives are placed in 6-10% salt brine where natural fermentation occurs during the following months (Nychas *et al.*, 2002). Except for diffusion of phenolic compounds into the brine, the major phenolic changes that occur during fermentation are the polymerization of monomeric anthocyanins which aid in colour stabilization (Blekas *et al.*, 2002).

Greek-Style naturally black olives have the highest phenolic content of the different olive products. This is significant since ripe olives contain the lowest phenolic concentration due to the decrease of oleuropein during olive maturation. Hydroxytyrosol, tyrosol, salidroside and verbascoside constitute the majority of the phenolic content present in the final product (Blekas *et al.*, 2002).

A special method of processing Greek-style naturally black olives, performed on Nychati Kalamata olives, produces "Kalamata olives in brine". During this process, ripe black Kalamata olives are harvested and brined for 5-8 days, with the water changed 2 to 3 times a day. Following brining, the olives are immersed in wine vinegar for 1-3 days. This popular method of processing olives delivers an olive which contains the highest concentration of phenolic compounds (Blekas *et al.*, 2002). The majority of the wastewater used in this study was generated using this processing method of Nychati Kalamata fruits (Figure 1.1).

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Figure 1.1. Nyatchi Kalamata olive orchard in the Boland district of South Africa.

1.2.1.3 Californian-style black.

The production of Californian-style black olives, referred to as "turning colour in brine", starts with the harvesting of yellow to purple olives. The olives are preserved in acidified brine, where hydrolysis of oleuropein occurs along with the extractive diffusion of phenolic compounds. Following the brining the olives are darkened in air under alkaline conditions. The oxidization and polymerization of o-diphenols is mainly responsible for the darkening of the olive colour. In the final product, hydroxytyrosol, tyrosol, salidroside and verbascoside constitute the major phenolic compounds present (Romero *et al.*, 2004a.).

1.2.2 Olive oil

Annually, over 2.5 million tons of olive oil is produced, with Spain, Italy, Greece and the Maghrebian countries being the major producers (Brenes *et al.*, 1999). The most popular form of olive oil used in the Mediterranean diet is virgin olive oil (Perez *et al.*, 2003). Extra-virgin olive oil is unrefined oil which contains phenolic compounds usually removed from other virgin olive oils during refining stages (Tuck and Hayball, 2002).

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These phenolic compounds are responsible for the bitter taste, flavor, aroma and resistance to oxidation associated with virgin olive oil (Brenes *et al.,* 1999; Romero *et al.,* 2002a). The major phenolic compounds in olive oil are hydroxytyrosol, tyrosol, cinammic acid, homovanillic alcohol, homovanillic acid, p-coumaric acid, oleuropein and elenolic acid (Tuck and Hayball, 2002).

Olive oil is produced using one of three methods: the traditional press method, the 3-phase centrifugation process and the continuous process. During the traditional press method olives are washed in cold water, followed by crushing and malaxing using warm water. The mixture is then further pressed producing a solid press-cake and an oil-water liquid phase. Olive oil is separated from wastewater by either gravitation or centrifugation during decanting of the liquid sludge (Vlyssides *et al.,* 2004). This method is responsible for generating 40-55 l wastewater per 100 kg of olives (Fiorentino *et al.,* 2004).

The 3-phase centrifugation method washes olives in cold water followed by crushing and malaxing in warm water. The olive mixture is then decanted generating a solid cake, liquid and sludge. Both the liquid and sludge phases are centrifuged, separating the olive oil from the wastewater (Vlyssides *et al.,* 2004).

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A 2-phase centrifugation method has recently become popular. This method washes olives in cold water, but then re-uses the washing water during crushing and malaxing and subsequent centrifugation where olive oil is separated from the waste sludge (Vlyssides *et al.,* 2004). The two-phase centrifugation system produces 80-120 l of wastewater per 100 kg of olives processed (Fiorentino *et al.,* 2004).

1.2.3 Composition of olive wastewater

It is estimated that 3.0×10^{10} l of wastewater is produced annually by the global olive oil processing industry (Lanciotti *et al.,* 2005). In addition, 8.5×10^{9} l of wastewater is generated during the processing of table olives (Beltran-Heredia *et al.,* 2000a). The composition of olive wastewater is dependent on various factors: the olive cultivar used

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(Romero *et al.*, 2004a.; Esti *et al.*, 1998), differences in irrigation strategies (Romero *et al.*, 2002b) and the different methods of processing. Even though there are substantial differences between the various olive wastewaters, all have certain underlying similarities. Most olive wastewater is acidic (pH 4-5.5), has a blackish-brown colour, is malodorous, contains potassium and phosphate salts, and is rich in organic substrates such as fats, sugars, protein, organic acids and polyphenols (Mulinacci *et al.*, 2001). The composition of olive wastewater generated during the processing of Kalamata olives in the Boland district of South Africa is shown in Table 1.1 (Burton *et al.*, 2005).

Table 1.1. Results of olive effluent obtained from table olives processing water in the Boland district of South Africa.

*All units in g/l unless otherwise stated	Kalamata olives
pH	4.53
Conductivity ($mS.cm^{-1}$)	83.1
Total solids	114.2
COD	58.7
TOC	18.5
Reducing sugars	0.26
Lipids	1.83
Total phenol	5.53
Hydroxytyrosol	1.6
Tyrosol	0.81
Na^+	22.35
K^+	3.71
Cl^-	30.00
NO_2^-	0.00
PO_4^-	0.62
SO_4^-	1.04

* The results in this table are courtesy of Mr. C. Garcin, Chemical Engineering Department, University of Cape Town

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1.2.4 Disposal of olive wastewater

Disposal of processed olive wastewater poses certain environmental concerns. The main problem is the high organic content present in the wastewater. Chemical oxygen demand of 80-200 g/l and biological oxygen demand of 50-100 g/l is on average 200-400 times higher than typical municipal sewage water (Fiorentino *et al.*, 2004). Biological treatment of this organic load is inhibited by antibacterial and phytotoxic constituents, mainly due to the high phenol content (Kotsou *et al.*, 2004b). The application of olive wastewater as fertilizer has been investigated and the Italian government allows the spreading of 50 -80 m^3/ha depending on the method of olive processing used (Fiorentino *et al.*, 2004). The phytotoxicity of processed olive wastewater is especially inhibitory during seed germination, but various grain varieties can tolerate wastewater during early growth stages (Casa *et al.*, 2003; Rinaldi *et al.*, 2003).

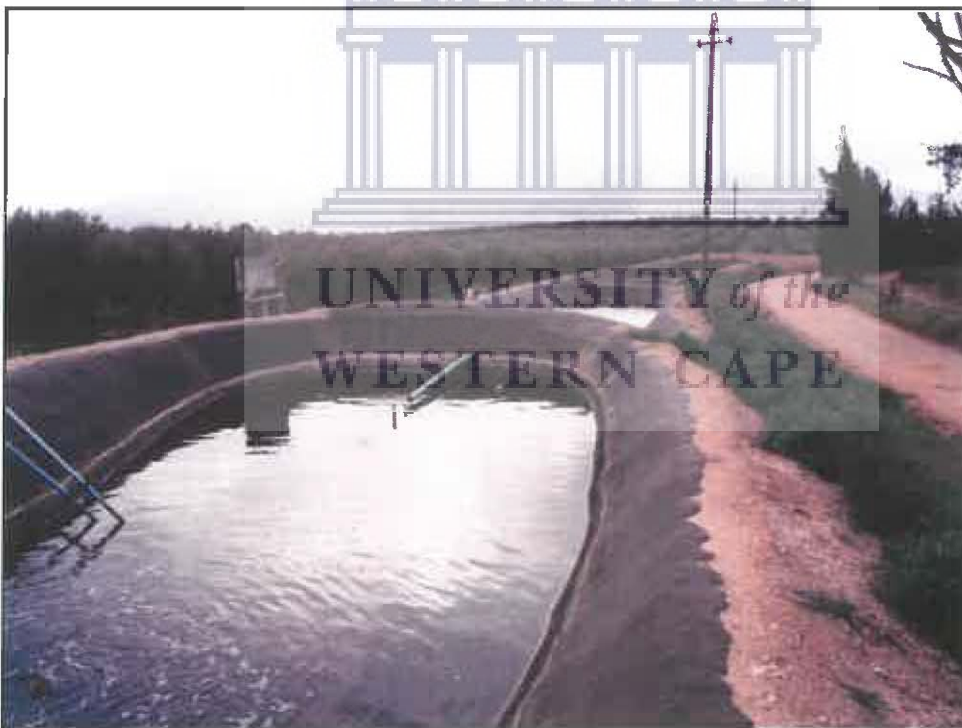

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Figure 1.2. Evaporation disposal ponds located in Boland district of South Africa.

In all the Mediterranean countries it is illegal to dispose of untreated processed olive wastewater into the sewage systems. Unfortunately, untreated olive wastewater is

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discarded into rivers, streams and the sea. The majority of industries discard the untreated processes olive wastewater into evaporation ponds (Figure 1.2). These ponds pose serious risks of surface and ground water contamination through leakage or occasional heavy rain falls, along with the risks to human health associated with the breeding of insects (Kyriacou *et al.*, 2005; Spandre and Dellomonaco, 1996).

1.2.5 Remediation of olive wastewater

Processed olive wastewater has economic potential. It is rich in valuable antioxidants (Tuck and Hayball, 2002), and has a high organic load. The possibility of recovering the polyphenol content of wastewater has been investigated, but resulted in limited success (Mulinacci *et al.*, 2001). Processed olive wastewater has been used as a substrate during the production of secondary products. These applications include the production of hydrogen by fermentation with *Rhodobacter sphaeroides* (Eroğlu *et al.*, 2004), the production of polyhydroxylalkoates from *Azotobacter chroococcum* H23 (Pozo *et al.*, 2002) and the production of laccase and Mn-peroxidase by *Panus tigrinus* (Fenice *et al.*, 2003). Various treatment methods concerned with the remediation of olive wastewater have been suggested and are listed in Table 1.2. An advantage of non-biological treatment is that the process is not inhibited by the high phenolic content.

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Table 1.2. Various treatment methods applied during the remediation of olive wastewater.

Treatment	Literature cited
Electrocoagulation of phenolic compounds	Adhoum and Monser, 2004; Meyssami and Kasaeian, 2005
Ultrasonic irradiation of wastewater	Atanassova *et al.*, 2005
Photochemical treatment of the phenolic content	Cermola *et al.*, 2004
Chemical treatment using lime	Aktas *et al.*, 2001
Combining chemical treatment using Fenton's reagent and biological treatment	Kotsou. *et al.*, 2004a
Combining other chemical treatments with biological remediation	Benitez *et al.*, 2003; Fiorentino *et al.*, 2004
Detoxification using super absorbent polymers	Davies *et al.*, 2004
Using biological treatment in conjunction with ozonation	Beltran-Heredia *et al.*, 2000a & b
Oxidative enzymatic treatment of the phenolic content	Gianfreda, *et al.*, 2003; Toscano *et al.*, 2003

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1.2.6 Biological treatment of processed olive wastewater

Biological agents used in the treatment of olive wastewater includes microalgae (Pinto *et al.*, 2003), and various fungal and bacterial isolates. Both aerobic and anaerobic treatment methods have been investigated (Aggelis *et al.*, 2001).

1.2.6.1 Fungal treatment of processed olive wastewater.

Bioremediation of processed olive wastewater with fungal isolates typically results in partial removal of the phenolic content as well as reduction in the COD. Isolates used in bioremediation studies are listed in Table 1.3. The removal of phenolic compounds using fungal isolates has been associated with their ability to produce laccase, peroxidase and manganese-peroxidase enzymes (Aggelis *et al.*, 2002; Perez *et al.*, 1998).

Table 1.3. Fungal strains used in the bioremediation of processed olive wastewater.

Fungal culture	Literature cited	COD removed	Reduction in Phenolic content
Abortiporus biennis	Aggelis *et al.*, 2002		55%
Aspergillus niger	Cereti *et al.*, 2004	64%	1.96%
	Kyriacou *et al.* 2005	86%	65%
Aspergillus sp.	Fadil *et al.*, 2003	52.5%	44.3%
Aspergillus terreus	Hoyos *et al.*, 2002	65.8%	
Candida tropicalis	Ettayebi *et al.*, 2003	69.7%	69.2 %
	Fadil *et al.*, 2003	62.8%	51.7%
Dichomitus squalens	Aggelis *et al.*, 2002		36%
Geotrichum candidum	Assas *et al.*, 2002	50%	
Geotrichum sp.	Fadil *et al.*, 2003	55.0%	46.6%
Lentinula edodes	D'Annibale *et al.*, 1998		88.5%
Panellus stipticus	Aggelis *et al.*, 2002		42%
Panus tigrinus	D'Annibale *et al.*, 2004b	62.3%	88%
Phanerochaete chrysosporium	Dias *et al.*, 2004	45%	90%
Pleurotus ostreatus	Aggelis *et al.*, 2002		52%
Yarrowia lipolytica	Lanciotti *et al.*, 2005	41.2%	21.7%

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1.2.6.2 Bacterial bioremediation of processed olive wastewater

Bacteria have been used less frequently in treatment of olive wastewater. Isolates that have been used include: *Comamonas* strain AV1A, *Ralstonia* strain AV5BG, *Ralstonia* sp. LD35, *Pseudomonas* strain AV2A, *Pseudomonas putida, Sphingomonas* strain AV6C (Di Gioia *et al.,* 2001; Bertin *et al.,* 2001; Di Gioia *et al.,* 2002;), *Propionibacterium microaerophilum.* (Koussemon *et al.*, 2001), *Lactobacillus plantarum* (Ayed and Hamdi, 2003), *Clostridium bifermentans* (Chamkha *et al.*, 2001) and *Azotobacter vinelandii* (Ehaliotis *et al.,*1999). The majority of these investigations have concentrated on the conversion of specific aromatic compounds, rather than the removal of total phenol content as with the fungal fermentations.

One of the major problems associated with the implementation of bioremediation is cost. The cost of operating a bioremediation plant includes the purchase of a bioreactor, supplementation with additional nitrogen sources and, in most cases, the need for significant dilution of processed olive wastewater (Bertin *et al.,* 2001). The requirement for dilution is particularly significant with the use of bacterial bioremediation systems. For example, a ten fold dilution and addition of 0.1% yeast extract was necessary for the use of *Lactobacillus plantarum* in the fermentation of olive mill waste (Ayed and Hamdi, 2003).

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Olive waste is a harsh environment with a number of factors thought to contribute to "stress" constraints on microbial growth (Ayed and Hamdi, 2003). The potential stress factors in the processed olive wastewater used in this study include salt (3%), phenolics (0.55%) and low pH (4.5) (Table 1.1).

1.3 Cellular regulation in bacteria under stress conditions

Bacteria pre-adapted to a specific stress condition have the ability to adapt faster to additional stress conditions than wild type organisms. For example, *Salmonella enterica* serovar Typhimurium which was adapted to acid stress, was found to show resistance against salt and heat stress (Wonderling *et al.,* 2004). Methods used by bacteria to adapt to these stresses include the uptake or *de novo* synthesis of small molecules (compatible solutes) in order to maintain turgor pressure (Wonderling *et al.,* 2004) and the use of ABC transporter pumps to export excess substances (Bourdineaud *et al.,* 2004).

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These functional changes often occur in association with morphological changes. Elongated cell morphology has been described for *Bacillus subtilis* under high temperature and salt stress conditions (Krüger *et al.*, 1994). Similarly, an average cell elongation from 1.22 μm to 1.57 μm was observed during the acid adaptation (pH 5.0) of *Propionibacterium freudenreichii* (Jan *et al.*, 2001). Changes in cell morphology are possibly a secondary effect due to an alteration in protein synthesis or accumulation of small molecules. An increase in protein content in the cell membrane following stress application has been observed (Bourdineaud *et al.*, 2004). Various studies have also described the *de novo* synthesis of stress-related proteins. One such study on *Lactococcus lactis* identified 12 new proteins that were produced during osmotic stress, and 33 produced during low pH stress. In addition, the expression levels of 14 proteins increased during starvation (Sanders *et al.*, 1999). The synthesis of novel proteins is not the sole mechanism responsible for changes in the proteome. Protein mis-folding is a regular occurrence during cell growth under stress conditions (Mason *et al.*, 1999). Under such conditions, the chaperon proteins GroEL, GroES and dnaK are typically expressed at high levels (Petersohn *et al.*, 2001). Changes in the proteome are, however, generally a direct reflection of transcriptional regulation.

1.3.1 Stress response in Bacillus sp.

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Bacillus megaterium is found in various habitats including, soil, seawater, sediments, rice paddies, dried food, honey and milk. The genome of this spore-forming Gram-positive bacterium has not yet been sequenced, and limited proteomic data is available. However, it is known as a suitable system to express foreign genes due to the low protease activity and high secretion capability (Scholle *et al.*, 2003). The organism can acquire and maintain up to seven indigenous plasmids. It is estimated that up to 11 % of *Bacillus megaterium* QM B1551 cellular DNA is present on these plasmids. Genes identified on these plasmids encode proteins involved in cell division (FtsZ and FtsK), germination (CwlJ), styrene degradation (StyA), and heavy metal resistance. One of these megaplasmids pBM400 contains rRNA genes including 5S rRNA, 23S rRNA and 16S rRNA genes (Scholle *et al.*, 2003).

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In the presence of rapidly metabolizing sugars such as glucose and fructose, *B. megaterium* tightly controlled carbon catabolite repression on the genes coding for the utilization of less favorable carbon sources (Küster-Schöck *et al.*, 1999). Recently, 169 proteins of *B. megaterium* cultured in media containing glucose as sole carbon source or in conjunction with xylose, were sequenced and identified by comparing the sequences to the closely-related *Bacillus subtilis* genome data. Glucose was not only utilized through glycolysis and TCA cycle, but also stored as polyhydroxybutyrate (PHB) as carbon and energy source (Wang *et al.*, 2005).

In contrast to the well-studied *B. subtilis,* little information is available on the stress response of *B. megaterium.* However, the close phylogenetic relationship between these two species suggests that many of their mechanistic systems may be similar. σ^B is one of 17 sigma factors present in *B. subtlis,* and responsible for the transcription of the general stress regulon and genes involved during glucose and phosphate starvation (Rollenhagen *et al.,* 2003). The gene encoding the sigma factor (*sig*B) is located in an operon along with seven other genes (Pa – *rsb*R – *rsb*S – *rsb*T – *rsb*U – Pb – *rsb*V- *rsb*W –*sig*B - *rsb*X). The operon has two promoter recognition sequences, one dependent on σ^A (Pa) and the second on σ^B (Pb). Transcription of this operon occurs at basal levels using the σ^A-dependent promoter, but during σ^B response, four genes are up-regulated through the σ^B-dependent promoter. RsbW, an anti-sigma factor, binds to the σ^B produced at basal levels thus inhibiting the sigma factor from binding to core RNA polymerases. During glucose, phosphate or oxygen starvation(s), conditions similar to cellular entry into stationary phase, low levels of ATP are present in the cell. This leads to the activation of RsbP, thought the hydrolase or acyltransferase function of RsbQ. Activated RsbP dephosphorylate the phosphorylated-RsbV. In this un-phosphorylated state, the RsbV protein is capable of competitive binding to RsbW, thus releasing σ^B to bind RNA polymerase and trigger the subsequent transcription of its own operon and the genes involved in glucose, phosphate or oxygen starvation. The second method of activating σ^B-dependent transcription occurs when the cell is exposed to various stress conditions such as heat shock, acid, salt and ethanol treatment. The specific signals involved during this response are not yet known. During unstressed conditions, the kinase RsbT is trapped in a complex consisting of RsbS and the complex-stabilizer RsbR. After exposure to

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stress, RsbT phosphorylate RsbS which subsequently disassemble the RsbR-RsbS-RsbT complex. In its free form RsbT can phosphorylate RsbU. Similar to the active RsbP, phosphorylated-RsbU can phosphorylate RsbV, which subsequently interact with RsbW releasing σ^B to initiate the transcription of general stress response genes (Voelker *et al.*, 1995., Hecker and Völker, 1998., Chen *et al.*, 2004., Holtmann, *et al.*, 2004). There is a counter activity to the release of σ^B, which involves the phosphatase activity of RsbX. RsbX dephosphorylates RsbS-P, in order for it to form an inhibiting complex with RsbR and RsbT (Chen *et al.*, 2004). In an attempt to identify mediators of the stress signaling, Obg, a GTP binding protein was shown to interact with RsbT, RsbW and RsbX (Scott and Haldenwang, 1999). The accumulation of ppGpp, during amino acid starvation, does not induce the transcription of *sig*B. In contrast to controlling the stress σ factors in *E. coli*, σ^B activation is not dependent on the activity of GroEL and DnaK chaperones (Scott *et al.*, 1999). The induction of *sig*B and subsequent transcription of genes involved in the general stress response leads to a growth restriction on cells that are resistant to multiple stresses, in anticipation of future stress (Hecker and Völker, 1998). It is estimated that about 125 genes are σ^B-dependently transcribed during general stress responses, including glucose and phosphate starvation (Petersohn *et al.*, 2001). A number of σ^B-independent stress regulation events have been identified. One such system is the σ^W-dependent regulation of a salt shock regulon containing approximately 44 genes (Petersohn *et al.*, 2001).

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1.3.2 Techniques used to identify differences in gene expression

Gene regulation during specific cellular responses could be identified either on nucleotide, "genomic or transcriptomic" or protein "proteomic" levels. DNA and RNA analysis include techniques such as microarrays, transcriptional analysis (representative difference analysis, RDA), transposon mutagenesis and consensus promoter based screening (Petersohn *et al.*, 2001., Felske, 2002). Microarrays are commonly used to identify differences in transcriptomes, sustaining a $527 million industry (Tomlinson and Holt, 2001). This technique is based on having a microchip which could contain over 10 000 single stranded oligonucleotides, each representing a sequenced subsection of the target genome. Total RNA of the organism during various cellular responses are

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extracted and hybridized to the oligonucleotides on the microchip. Hybridization of each transcriptome is assigned a different colour with positive controls for both transcriptomes indicated by a third colour. This is a relatively simple approach, generating large quantities of information. A limitation of this technique is the availability of microchips containing the genome of a specific organism (Whiteley *et al.*, 2001).

The limitation imposed by microchip availability is surmounted by a second technique, Representative Difference Analysis (RDA). This PCR-based technique identifies different nucleic acid fragments in two similar genomes or transcriptomes. RDA relies on the ligation of two unique adaptor sets, each to the specific transcriptomes in the comparison (Figure 1.3). The short primer (short-P) in one adaptor set is 5'-phosphorylated and could thus be used in order to generate a complimentary primer recognition sequence. Once this site is generated, multiple copies of the transcriptome can be amplified by PCR. This amplified transcriptome is called the "driver". The second transcriptome ligated to the un-phosphorylated adaptor set is called the "tester". Subtractive hybridization is performed with 100-fold more DNA from the "driver" than "tester". Only where two complimentary "tester" DNA strands anneal will the primer recognition sequence be created and amplicons obtained during the subsequent PCR reaction. The amplicons obtained from "tester" specific PCR represent genes unique to the specific transcriptome. Genes which are up- or down-regulated, but still transcribed in both transcriptomes, will not be detected with this method, due to the 100:1 ratio of "driver" to "tester" DNA (Felske, 2002).

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Differences in mRNA level are not proportional to differences in protein level. This is due to variation in protein translation and degradation rates as well as post-translational modifications (Tomlinson and Holt, 2001). Changes in the proteomes can be visualized by 2D gel electrophoresis, followed by the identification of individual proteins (Wang *et al.*, 2005). A complex mixture of proteins, such as the total protein content of a cell, is first separated in a gel containing a pH gradient, based on the isoelectric point of the individual proteins. In the second dimension, the proteins are separated based on their molecular weights. The proteins are subsequently stained in order to visualize them and

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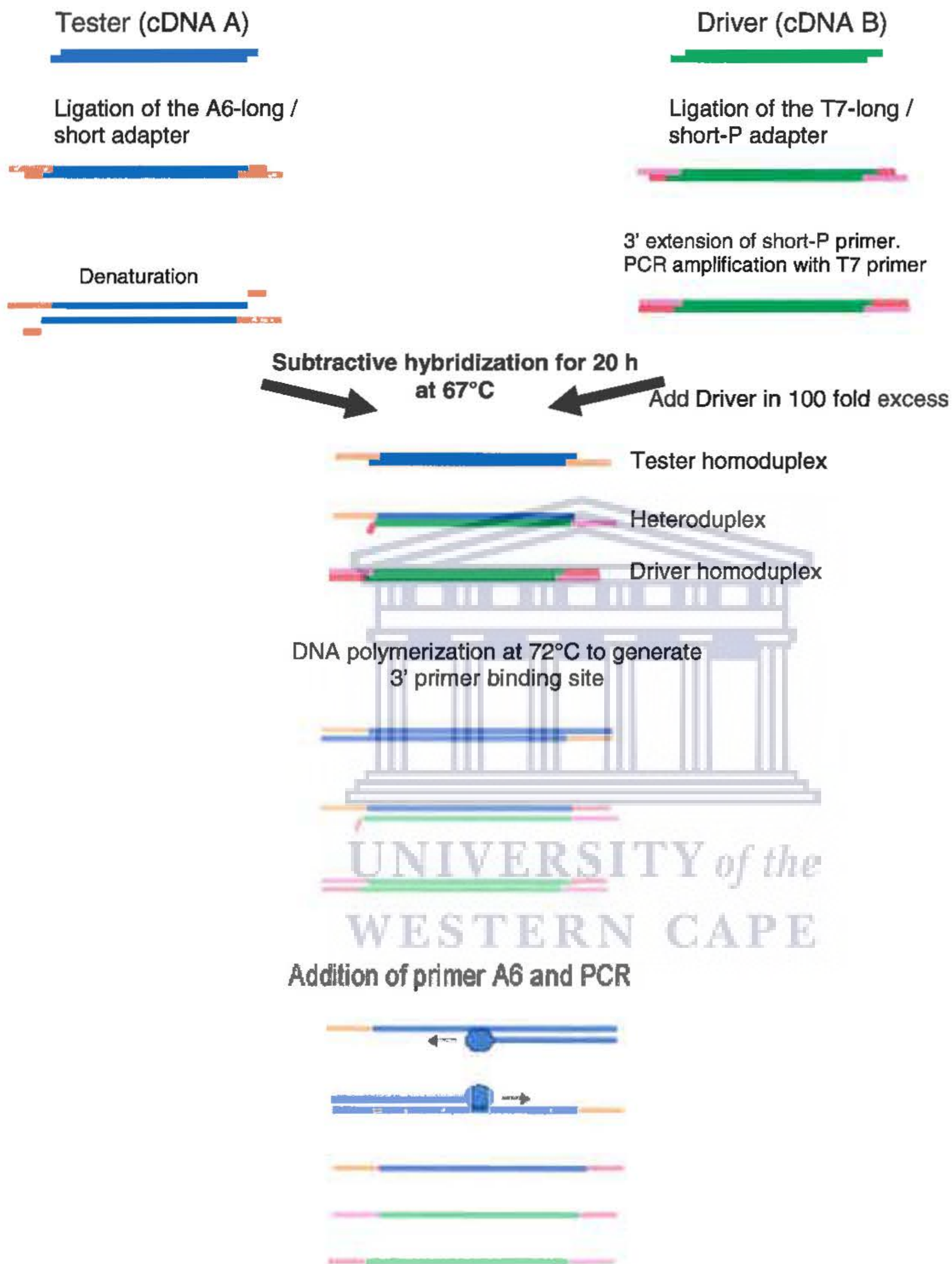

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Figure 1.3. Schematic outline for construction of RNA subtraction libraries. Total RNA of two different culturing conditions is isolated and cDNA synthesized (Felske, 2000).

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the image acquired with specialized software, which is capable of estimating spot intensity. It is then assumed that the intensity of a single spot is directly related to the concentration of that protein in the sample, suggesting that an increase or decrease in intensity relates to up- or down-regulation of the protein, respectively (Meleth *et al.*, 2005). Various problems are encountered during the analysis of protein regulation using 2D PAGE. First, the image obtained from a 2D PAGE is only representative of a single time point during cellular response. There is thus no indication if the protein is newly induced but not yet accumulated, synthesized and accumulated or still present but no longer synthesized (Bernhardt *et al.*, 2003). This problem was subsequently addressed by sampling regularly and analyzing overlays of the different protein profiles (Bernhardt *et al.*, 2003). The second problem concerns the reproducibility of replicates from a single biological sample. Effects generated by differential loading, running and staining are normalized by various software programs (Meleth *et al.*, 2005). A third difficulty is in the identification of individual proteins. A pre-existing knowledge of the organism's genome sequence, or related organisms, is required in order to positively identify the protein sequence (Bernhardt *et al.*, 2003).

Even though RDA and 2D gel electrophoresis have advantages and disadvantage, both are highly valuable techniques for the investigation of differential gene expression in organisms cultured in olive wastewater and nutrient rich media.

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1.3.3 Enhancing bioremediation by means of mutagenesis

A previous study showed that the capability of *Bacillus megaterium* ATCC 33085 to germinate and sporulate was inhibited following the exposure of the organism to 5.6 mmol/l of total phenolic compounds extracted from olive mill wastewater (Rodriguez *et al.*, 1988). The high phenol content present in processed olive wastewater has been implicated in the toxicity of the wastewater on various biological systems (Fiorentino *et al.*, 2003). Limitations such as these inhibits the usage of biological systems during the bioremediation of the wastewater. This could be overcome through genetic manipulations, generating organisms with a higher tolerance towards the phenolic compounds. The potential of mutagenesis in order to generate hyper-tolerant organisms is

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nowhere more evident than in the biodegradation of xenobiotics (van der Meer *et al.*, 1992). However, as a general objective, mutation of *B. megaterium* in order to enhance its tolerance of olive wastewater poses certain difficulties. The genome of the organism is not yet sequenced, eliminating the possibility of targeting the specific genes involved in bioremediation, such as genes located in the *phh* operon of *P. putida* (Ng *et al.*, 1995). Random mutagenesis may be performed in such cases, using classical techniques such as UV or chemical mutagenesis. *B. megaterium* has proved to be more resistant to UV and gamma-radiation than the closely-related *B. subtilis*, rendering chemical mutagenesis a more viable option (English and Vary, 1986). Chemicals previously used in mutagenesis studies include: N-methyl-N'-nitro-N-nitrosguanidine (MNNG), ethyl methanesulfonate (EMS), 4-nitroquinoline-1-oxide, 1,2,-dimethylhydrazine, bleomycin, hydrogen peroxide and 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) (Ibrahim and O'Sullivan, 2000., Ferguson *et al.*, 2003). The first problem selecting a suitable mutagenic agent against *B. megaterium* involved the organism's tolerance towards several of these chemicals. Certain *Lactobacillus* and *Bacillus* sp., including *B. megaterium*, were previously shown to be tolerant against 4-nitroquinoline-1-oxide, N-methyl-N'-nitro-N-nitrosguanidine and 1,2,-dimethylhydrazine (Caldini *et al.*, 2002). The second problem relates to the anti-mutagenesis activity of certain phenolic compounds present in olive wastewater. Ferulic, p-coumaric, and 5-5-dehydrodoferulic acid were implicated in protecting bacteria against chemical mutagenesis from 2-amino-3-methylimidazo[4,5-f]quinoline, bleomycin and hydrogen peroxide (Ferguson *et al.*, 2003). In order to circumvent this problem, it was decided to culture the bacteria in nutrient rich, or media containing low concentrations of phenolic concentration prior to chemical treatment.

Formamide (CH_3NO) is commonly used to stabilize single stranded DNA or RNA (Sambrook *et al.*, 1989). This chemical is also known to direct the incorporation of mismatched base pairs, with a preference for guanine. The carbon of formamide undergoes hydrogen bonding with the nitrogen atom of the base involved in glycosidic bonding of the base to the deoxyribose. The presence of the formamide forms a distorted B-DNA helix, with the formamide on the outside. Since the formamide is incapable of hydrogen bonds with neighboring bases, an abasic lesion forms. During DNA replication,

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the DNA polymerase may not incorporate a nucleotide opposite the lost base, thus inducing frameshift mutations (Maufrais *et al.*, 2003).

1.4 Aims of this study

It is evident that micro-organisms may be effectively applied in the bioremediation of olive wastewater. Even though this wastewater is bactericidal and constitutes a high stress environment for bacteria, various species have been shown to survive under these conditions (Bertin *et al.*, 2001). The broad objectives of this project were to identify bacteria capable of growth and bioremediation of olive wastewater. Even though these organisms are adapted to this toxic environment, using them in a fermentative bioremediation process could potentially be improved by future engineering of the selected strains for the removal of phenolic compounds as well as reduction of COD.

Therefore, the specific aims of this investigation were the following:

* To isolate microorganisms capable of growth in and bioremediation of processed olive wastewater. The bioremediation potential of each isolate would be measured by their abilities to remove the phenol content present in the processed olive wastewater and to produce polyphenol oxidases.

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* To identify differences in gene expression of the selected isolate(s) under different culturing conditions. Differences in both the transcriptome and proteome would be identified.

* To subject selected isolate(s) to chemical mutagenesis in order to enhance their bioremediation potential, with special emphasis on their ability to reduce the total phenol concentration present in olive wastewater. Differences in the phenotype, proteome and transcriptome of wild type and mutants would be identified.

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Chapter 2

Methods and Materials

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2.1 Microbial isolates

2.1.1 Origin of strains

Olive waste samples were collected from various evaporation ponds on farms in the Stellenbosch area (Western Cape, South Africa). One to two milliliter aliquots were plated onto 25% [v/v] olive waste agar (containing 0.5% [w/v] yeast extract and 1.5% [w/v] agar) and incubated at 30 or 37°C for 24-96 hours. Isolates with unique colony morphology and colour were selected and repeatedly subcultured on 25% olive waste agar in order to obtain pure cultures.

2.1.2 Bacterial strains and culture conditions

Wild type and mutant isolates were cultured in either Luria Broth LB (1% tryptone, 0.5% yeast extract and 1% NaCl, pH 7.0) or 25% [v/v] olive wastewater broth containing 0.5% [w/v] yeast extract and incubated at 30 °C with shaking at 250 r.p.m.. The cultures were maintained on olive waste agar (25% [v/v] olive waste, 0.5% [w/v] yeast extract, 1.5% [w/v] agar, pH 6.0) or LB agar (1% [v/v] tryptone, 0.5% [w/v] yeast extract and 1% NaCl, 1.5% [w/v] agar, pH 7.0). Stocks were stored in 20% glycerol at -80°C.

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2.2 Morphological characterization of isolates from olive waste

Fifteen microliter volumes of culture were heat fixed on a microscope slides and Gram stained (Gerhardt *et al.,* 1994). Morphological properties of all isolates were determined by light microscopy using a Laborlux 11 (Leitz) PL Fluotar fluorescent microscope (Carl Zeiss) and images were captured using an Axiocam (Zeiss) charge-coupled device (CCD) camera and analyzed using Axiovision 2.0 (Zeiss).

2.3 Polyphenol oxidase assay

All isolates were tested for their ability to produce polyphenol oxidases after 16 hours growth in 25% olive waste. One milliliter of each culture was collected in an eppendorf

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tube and centrifuged for 3 minutes at 14 000 x g. The cell pellets were resuspended in 0.1M sodium phosphate buffer pH 7.0 and sonicated for 30 seconds at 60% power using a Bandelin Sonopuls (Germany). Cellular extracts were used to test for peroxidase and tyrosinase activities while the supernatants were used to determine the total phenol reduction and laccase activity and its monophenolic composition was determined on reverse phase HPLC. Reverse phase HPLC was performed by Mr. C. Garcin at the Chemical Engineering Department, University of Cape Town. The monophenolics were separated using a mobile phase of 80:20:2.5 H_2O: methanol: acetic acid at a flow rate of 1 $ml.min^{-1}$ on a Merck Hitachi L-7000 series. A Waters Spherisorb® S5 ODS1 4.6 x 250nm with Phenomenex guard column was used and the monophenolic compounds detected at 280nm. The RP-HPLC profiles were compared to standards of model compounds previously described in olive waste (Blekas *et al.*, 2002).

2.2.1 Peroxidase activity

Peroxidase activity was determined spectrophotometrically at 460nm by monitoring the oxidation of 0.05 ml 15 mg/ml (w/v) guaicol (Fluka, Switzerland). The substrate was added to 1 ml sodium-phosphate buffer (0.1 M, pH 5.6) containing 0.1 ml 0.39M H_2O_2. The absorbance was measured immediately after adding 0.2 ml sample and again after 4 minutes (ε_{460} = 43.6 M^{-1} cm^{-1}) (Putter 1975). Horse radish peroxidase (Roche) enzyme was used as a positive control. One unit of peroxidase activity was defined as the amount of enzyme oxidizing 1μmol substrate per minute. All enzyme assays were performed in triplicate.

2.2.2 Tyrosinase activity

Tyrosinase activity was determined using 0.181 mg L-tyrosine (Sigma, Germany) as substrate in 2.8 ml sodium-phosphate buffer (0.1M, pH 6.5) (Ikehata and Nicell, 2000). The increase in absorbance at 280nm was measured after 3 minutes, following the addition of 0.1ml substrate. One unit of enzyme was defined as an increase in absorbance at 280nm of 0.001 AU per minute. All enzyme assays were performed in triplicate.

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2.2.3 Laccase activity

The oxidation of 0.05 ml 10.9 mg/ml ABTS (2,2-azinobis-3-ethylbenzthiazoline-6-sulfonate) (Roche, Germany) in 1 ml sodium-phosphate buffer (0.1 M, pH 5.6) was monitored spectrophotometrically at 420nm (Robles *et al.*, 2000). Absorbances after the addition of 0.2 ml supernatant were measured after 3 minutes (extinction coefficient of $3.6x10^4$ $M^{-1}.cm^{-1}$). Purified *Trametes versicolor* laccase enzyme was used as positive control. One unit of laccase activity was defined as the amount of enzyme that oxidized 1 μmol of substrate per minute. All enzyme assays were performed in triplicate.

2.4 Total phenol reduction

The concentration of total phenolics was determined by the Folin-Ciocalteu method (Aggelis *et al.*, 2002). The reduction by 0.1 ml olive wastewater supernatant of 0.5 ml Folin-Ciocalteu reagent (Merck, Gemany) was determined spectrophotometrically by measuring the absorbance at 765nm after 30 minutes incubation at 20°C, after addition of 0.4 ml 7.5% (w/v) sodium carbonate. The phenolic content of the sample was expressed as a gallic acid equivalent (mM). The total reduction of phenol was presented as a percentage of the initial phenol concentration (mM). All the phenol concentrations were performed in triplicate.

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2.5 Ferulic acid decarboxylase assay

Ferulic acid decarboxylase activity was determined as described by Van Beek and Priest (2000). Cells were collected by centrifugation at 10 000 x g for 6 minutes and resuspended in an equal volume 0.1 M sodium phosphate buffer, pH 5.8 containing 100 μl / ml ferulic acid (Fluka). Samples were incubated at 30 °C for 12 hours, and a 1 ml aliquot recovered at hourly intervals. Two hundred microliters of supernatant was diluted with 0.8 ml ddH_2O and an absorbance spectrum from 200nm to 350nm obtained. Ferulic

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acid has absorption peaks at 284 and 312nm (Van Beek and Priest 2000). All enzyme assays were performed in triplicate.

2.6 16S rDNA analysis of selected isolates

Chromosomal DNA was extracted from isolates AS-9, AS-15, AS-18 and AS-35 using cetyltrimethylammonium bromide (CTAB) as previously described (Jansen, 1995). The precipitated DNA was resuspended in 20 µl ddH_2O and a 1µl aliquot was analyzed by electrophoresis on a 1% (w/v) agarose gel in order to estimate the integrity and concentration of the sample.

A 1.5 kb region of the 16S rDNA gene was amplified using universal primers E9F and U1510R (Weisburg *et al.*, 1991) (Table 2.1). The PCR reaction mixture (50 µl) contained 1 x PCR buffer (10 mM KCl, 20 mM Tris-HCl (pH 8.8), 0.1% [v/v] TritonX-100, 2 mM $MgCl_2$, and 10 mM $(NH_4)_2SO_4$), 0.2 mM of each dNTP, 25 pmol of each primer and 1 U of *Taq* DNA polymerase (Southern Cross Biotechnology). Following an initial denaturation at 94°C for 3 min, the samples were subjected to 30 cycles of amplification in a Perkin-Elmer GeneAmp 2700 thermal cycler using the following cycle conditions: denaturation at 94°C for 45 s, primer annealing at 55°C for 30 s and extension at 72°C for 1 min followed by a final extension step at 72°C for 4 min. As a control, a reaction mixture containing distilled water and all other reagents but no template DNA was included in the analysis. Following PCR amplification, 5 µl aliquots of the reaction mixtures were analyzed in the presence of an appropriate molecular size marker by 1% (w/v) agarose gel electrophoresis.

The 1.5 kb PCR amplicons were gel purified using either Gene-clean or the GFX PCR DNA purification kit (Amersham Biosciences). Purified products were cloned using InsT/A clone (Fermentas), according to the manufacturer's instructions. The ligation mixture was electroporated into electro-competent *E. coli* DH5α cells (Sambrook *et al.*, 1989) using a BioRad gene Pulser™. The transformed cells were selected by plating

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aliquots of 100-200 µl onto LB-agar plates supplemented with 100mg/ml Ampicillin (Sigma-Aldrich). The cells were plated together with 10 µl IPTG (100 mM stock solution) and 50 µl X-gal (2% [w/v] stock solution) to allow for blue/white colour selection, based on insertional inactivation of the lacZ' marker gene in the vector. The plates were incubated overnight at 37°C and investigated for the presence of recombinant transformants.

Plasmid DNA was extracted using Qiagen plasmid purification kit and analyzed on a 1% agarose gel. Recombinant plasmids were sequenced using the DYEnamic ET Dye terminator kit and analysed on the MegaBACE 500 automated sequencer (Amersham Biosciences) with primers M13F, M13R, F2, F3 and R4 (Table 2.1). The identities of the strains were determined by searching known sequences in the GenBank Database using a BLASTN 114 homology search (Altschul *et al.*, 1997) available at the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/).

Table 2.1 Primers used in this study

Oligonucleotide primer	*Nucleic acid sequence*
E9 Forward	5' – GAGTTTGATCCTTCCTCAG – 3'
U1510 Reverse	5' – GGTTACCTTGTTACGACTT – 3'
F2 Forward	5' – ACTCCTACGGGAGGCAGCAG – 3'
F3 Forward	5' – GCCAGCAGCCGCGGTAATAC – 3'
R4 Reverse	5' – CCATTGTAGTACGTGTGTAGCCC – 3'
M13 Forward	5' - GTTTCCCAGTCACGAC - 3'
M13 Reverse	5' - GTAAAACGACGGCCAGT - 3'
T7-long	5' – TTTCTAATACGACTCACTATAGGCCGCCAGT -3'
T7	5' – AATACGACTCACTATAGG – 3'
A6-long	5' – TTTATTTAGGTGACACTATAGATTAGGCCGCCAGT – 3'
A6	5' – ATTTAGGTGACACTATAGA – 3'
Short	5' – CTGGCGGCCTACCA – 3'
Short-P	5' - P - CTGGCGGCCTACCA – 3'

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2.7 Growth analysis

Growth kinetics of isolates AS-9, AS-15, AS-18, AS-24 and AS-35 were determined in 25% olive waste batch cultures over 136 hours. Samples were collected every 2 hours and the growth rates were determined by measuring the turbidity of the culture at 600 nm. The samples were centrifuged and the supernatants used to analyze the monophenolic content by reverse phase HPLC as described in section 2.3.

2.8 Model compound biotransformations

AS-35 was cultured in M9 minimal medium at 30°C, containing 5 mM NH_4NO_3 and 1 mM model compound as sole carbon source (Sambrook *et al.*, 1989). The following model compounds were used: Ferulic acid, tyrosol, vanillic acid, gallic acid and p-hydroxyphenyl acetic acid. All model compounds except for ferulic acid were purchased from Merck, Germany. Aliquots were taken at hourly intervals and analyzed by scanning from 200 to 350nm.

2.9 Chemical mutagenesis

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Wild type isolate AS-35 was subjected to chemical mutagenesis based on the method described by Gasson *et al.* (1998). The isolate was cultured overnight in 100 ml LB. Cultures were harvested by centrifugation and the cells resuspended in 10 ml sterile 0.1 M KH_2PO_4 pH 7.0 containing 100 µl/ml formamide (Merck, Germany). These cell suspensions were incubated at 37°C for 1 hour with gentle agitation. The cells were subsequently pelleted and resuspended in 20 ml LB, followed by overnight recovery incubation at 30°C. Overnight cultures were harvested by centrifugation, resuspended in 2 ml sterile 0.1 M KH_2PO_4 pH 7 and plated onto 2.5% (w/v) agar containing 70% (v/v) olive wastewater supplemented with 0.5% (w/v) yeast extract and cultured at 30°C overnight.

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2.10 Characterization of putative mutants

Eight putative mutant isolates were cultured in 50% olive wastewater. One ml aliquots of each culture were sampled after 16, 28 and 40 hours. The cells were pelleted and resuspended in 0.1M sodium phosphate buffer pH 5.0, while the supernatant was used to determine total phenol reduction and decolourization. The cellular fraction was sonicated for 30 seconds at 60% power and used in subsequent peroxidase activity assay. Total phenol determinations and peroxidase activity assays were preformed as previously described (Section 2.4 and Section 2.3.1). Decolourization of the media was determined by measuring the absorbance of the supernatant at 525nm.

2.11 Growth rate analysis of wild type and mutant strains

The growth kinetics of isolates AS-35 and DF4 were determined by measuring the optical density (OD) of batch cultures at 600 nm. Each isolate was cultured overnight at 30°C in LB and 25% olive waste broth. These pre-cultures were subsequently used to inoculate 20 ml LB and broth containing either 25% (v/v) or 80% (v/v) olive processing wastewater to $OD_{600\ nm}$ of 0.05. Optical density was measured every 2 hours over a 15 hour period.

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The growth rate and ability to reduce total phenol content of wild type AS-35 and mutant DF4 was investigated in 25% olive wastewater media containing various nitrogen sources or with different initial pHs. Different nitrogen sources that were investigated included 0.5% yeast extract, 0.5% $Ca(NO_3)_2$ and 25% olive wastewater with no additions. The influence of initial pH on growth and total phenol removal was investigated by inoculating the mutant and wild type into 25% olive wastewater containing 0.5% yeast extract with an initial pH ranging from 4 to 8.

2.12 Morphological analysis

Fifteen microliter volumes of 16 hour cultures in both LB and 25% olive wastewater broth were transferred to clean microscope slides before heat fixation. The samples were stained with Safranin and observed as described previously (Section 2.2).

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2.13 Biochemical analysis

AS-35 cultured in LB and olive waste was used to inoculate CHB medium (bioMerieux, France) to an OD_{600nm} of 0.1. These inocula were applied to API 50 CH (bioMerieux, France) biochemical strips and incubated at 30°C for 24 and 48 hours.

2.14 Total cellular proteome analysis

Isolates were cultured at 30°C in LB and olive waste broth containing 25% olive waste to an OD_{600nm} of 1.0. Cells were harvested by centrifugation at 14 000 x g for 5 minutes, the supernatant discarded and the pellets resuspended in 300 µl 10% TCA. Cell suspensions were sonicated for 5 x 30s at 60% power. The precipitated proteins together with cellular debris were collected by centrifuging for 5 minutes at 16 000 r.p.m at 4°C, and the supernatants discarded. The pellets were washed with 500 µl ice cold 80% acetone, air dried and subsequently resuspended in 200 µl lysis buffer (9 M urea, 2 M thiourea, 4% CHAPS). Following solubilisation, protein extracts were separated from insoluble cellular debris by centrifuging at 14 000 x g. for 5 minutes at room temperature. Supernatants were transferred to clean microfuge tubes. The concentration of extracted proteins was estimated using a modified Bradford protocol (Ramagli and Rodriguez, 1985). Five microliter samples were added to 10µl 0.1M HCl, 85 µl lysis buffer and 900 µl Bradford reagent (BioRad). The samples were incubated for 5 minutes and the absorbance measured at 595nm. Protein concentrations were determined against BSA standards (BioRad).

Fifty mg of total cellular proteins were analyzed on a 12% polyacrylamide gel (0.375 M Tris-Cl; 0.1% SDS, pH 8.8), with 4% stacking gel (0.125M Tris-Cl; 0.1% SDS, pH 6.8) at 100 V using a standard SDS buffer (25 mM Tris-HCl, 250 mM glycine, 0.1% [w/v] SDS; pH 8.3) (Laemmli, 1970). The proteins were visualized using Coomassie Brilliant Blue R250 (Sigma) and de-stained using 10% glacial acetic acid, 1% glycerol.

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2.15 2D protein analysis

To resolve differences in protein expression profiles, total protein extracts were analyzed by 2D protein separation. Large format (18 cm) isoelectric focusing dry strips (Amersham Bioscience) in pH ranges of 3–10 and pH 6–11 were rehydrated overnight using 350 µl of the respective sample preparations (Berkelman and Stenstedt 1998). Each sample contained 400 µg of protein, 1% (w/v) DTT and 2% (w/v) of the appropriate ampholytes dissolved in urea lysis buffer. The rehydrated strips were focused for a total of 70 000 Vh using a Multiphor II System (Amersham Bioscience). Small format (7cm) isoelectric focusing drystrips (Amersham Bioscience) pH 3-10, were rehydrated in 125µl sample and focused for a total of 6 500 Vh. Focused strips were equilibrated in 2 ml equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 4% SDS and trace amounts of bromophenol blue (Sigma)) containing 100 mg/ml DTT followed by equilibration buffer containing 48 mg/ml iodoacetamide (Chivasa *et al.,* 2000). Second dimension separation was performed on 12% polyacrylamide gels. The separated proteins were stained with Coomassie Brilliant Blue R250 (Sigma).
Protein identifications were performed on a Maldi TOF as previously described (Chivasa *et al.,* 2000).

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2.16 RNA subtraction library analysis

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Differential gene expression of AS-35 cultured in LB was compared to AS-35 cultured in 25% olive wastewater broth by constructing RNA subtraction libraries (Felske, 2002) (Figure 1.3).

2.16.1 RNA isolation and cDNA synthesis.

Total RNA was extracted from AS-35 cultured in LB and 25% olive waste broth using the hot phenol method with slight modifications (Krumlauf, 1996). The cells of an overnight culture were collected by centrifugation and washed in cold 0.1M sodium phosphate buffer, pH 7.0. Cells were resuspended in 11 ml 50 mM sodium acetate, pH 5.2 containing 1% SDS and lysed by sonification at 60% power for 25 seconds. An equal

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volume of pre-warmed phenol saturated with 50 mM sodium acetate, pH 5.2 was added and the samples incubated for 15 minutes at 60°C, followed by 5 minutes at –10 °C. The samples were centrifuged at 5000 x g for 10 minutes at 2 °C in order to separate the phenol phase from the aqueous phase. The phenol treatment was repeated and the RNA containing upper phase was transferred to a clean tube. Total RNA was precipitated by the addition of 0.1 volumes of 5M NaCl and 2 volumes of 95% ethanol, followed by incubation at –20°C for 30 minutes. The RNA pellet was transferred to a clean eppendorf tube and washed with 70% ethanol. The RNA was resuspended in 50 μl DEPC treated ddH_2O and 1 μl was analyzed on a 1% agarose gel.

cDNA was synthesized in a two step reaction using 2 μl hexa-oligonucleotides (Roche) as random primers. First strand synthesis was performed at 42°C for 2 hours using 200U Revert Aid^{TM} HMinus M-MuLV reverse transcriptase (Fermentas) in a 32μl reaction containing 1 x PCR buffer (50 mM Tris-HCl, pH 8.3, 50 mM KCl, 4 mM $MgCl_2$, 10 mM DTT), 0.2 mM of each dNTP and 50 ng RNA. Second strand synthesis was performed using 1U *Taq* polymerase in a 50 μl reaction containing 32μl from first strand synthesis, 1 x PCR buffer (10 mM KCl, 20 mM Tris-HCl, pH 8.8, 0.1% [v/v] Triton X-100, 2 mM $MgCl_2$, and 10mM $(NH_4)_2SO_4$), 0.2 mM of each dNTP and 1μl hexamer primers. Complementary strands were generated during an initial extension of 25 minutes at 72°C. Copies of the cDNA were made during 25 cycles consisting of a 30 second 94°C denaturing, 30 seconds 60°C annealing and 1 minute 72°C extension. The latter was increased with 5 seconds per cycle. A final extension at 72°C for 7 minutes was followed by incubation at 4°C. One microliter cDNA was analyzed on a 1% agarose gel. The remaining cDNA product was precipitated using 0.3 volumes of 7.5 M ammonium acetate, pH 5.0 and an equal volume of 96% ethanol at -20°C for 30 minutes. The samples were centrifuged for 10 minutes at 14 000 x g. to pellet the DNA. The pellet was washed in 70% EtOH, air dried and resuspended in 20 μl ddH_2O.

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2.16.2 Driver synthesis

A diagrammatical outline of the RNA subtraction experimental procedure is shown in Figure 1.3. Primer T7-long was annealed to primer Short-P in order to create the driver adaptors, while the tester adaptors consisted of annealed primers A6-long and Short (Table 2.1). Both adaptor sets were ligated to the cDNA using 1U T4 DNA ligase (Fermentas) and 1μl Peg4000 overnight at 20°C. The drivers were amplified by using 2μl ligation mix (T7-long / short-P ligated to cDNA) in a 50μl reaction containing 1 x PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% [v/v] Triton X-100), 0.2 mM of each dNTP and 1 U of *Taq* DNA polymerase (Southern Cross Biotechnology). *Taq* DNA Polymerase was used at 72°C for 25 minutes to fill in the gaps at the end of the adaptors in order to generate T7 primer annealing site. Twenty-five pmol T7 primer was added to the reaction mixture and the DNA was denatured for 5 minutes at 94°C. Forty cycles PCR was performed, with each cycle consisting of a 30 second denaturation step at 94°C, 30 second annealing at 54°C and 1 minute extension at 72°C. The extension time was increased by 5 seconds per cycle and was followed by a final extension of 7 minutes. A 1 μl aliquot of the driver amplicons was analyzed on a 1% agarose gel before precipitating the remaining DNA as described in section 2.16.1.

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2.16.3 Subtractive hybridization

Two microliters of driver and tester DNA were added to a 10 μl reaction mixture containing 1x TE and 2μl 5M NaCl. The DNA was denatured for 5 minutes at 95°C followed by 16 hours of hybridization at 67°C. A PCR reaction similar to that described in driver synthesis was performed using A6 as primer. This reaction amplified dsDNA fragments unique to the tester cDNA. The whole PCR mixture was analyzed on a 1% agarose gel. Unique bands were purified using the Qiagen Gel purification kit and ligated into InsT/A clone (Fermentas) according to the manufacturer's instructions. The ligation mixture was used to transform competent *E. coli* DH5α cells. Colony PCR using primers M13F and M13R was used to screen for recombinant clones (Section 2.17.4). Selected recombinant clones were sequenced using M13F as previously described (Section 2.6).

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2.17 cDNA hybridization libraries

2.17.1 Genomic library construction

In order to obtain full length gene sequences containing the flanking regulation sites, AS-35 cDNA hybridization libraries were constructed. AS-35 genomic DNA was extracted as previously described (Jansen, 1995) and partially digested using *Bam*HI (Fermentas). At the same time, pUC18 DNA was completely digested using the same enzyme. These digestions were performed by incubating 500 ng of DNA with 1 U of *Bam*HI enzyme at 37°C for 1 hour and 2 hours respectively. The linear plasmid was dephosphorylized using 1 U of Shrimp Alkaline Phosphatase (Fermentas) for 30 minutes at 37°C. Both digested plasmid and genomic DNA were precipitated using 7.5 M ammonium acetate as described in section 2.18.1. Plasmid and genomic DNA fragments, in a ratio of 1:2 were ligated overnight at 16°C using 1 U T4 ligase (Fermentas). Half of the ligation reaction was used to transform competent *E. coli* DH5α using electroporation as described in section 2.6. Cells were plated onto LB agar containing 100 mg/ml ampicillin, 10 μl IPTG (100 mM stock solution) and 50 μl X-gal (2% [w/v] stock solution). Nine hundred white colonies were selected and subcultured onto LB agar in plates containing 100 mg/ml ampicillin.

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2.17.2 cDNA probe synthesis

cDNA of AS-35 cultured in LB and 25% olive waste was synthesized as described in section 2.18.1. Each 25 μl PCR reaction contained 150 ng of cDNA, 1U *Taq* polymerase, 1 x PCR buffer, 2 μl hexanucleotides, 2 μl dNTP's and 2 μl DIG labeled dNTP mixture (Roche). The cDNA was denatured for 2 minutes at 94°C followed by 10 cycles of denaturing at 94°C for 30 seconds, annealing at 49°C for 30 seconds and extension at 72°C for 1 minute. This was followed by 20 cycles of denaturing at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 1 minute. A final extension was performed at 72°C for 5 minutes. The cDNA probe was precipitated using 7.5 M

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ammonium acetate as described in section 2.16.1. The probes were tested according to the manufacturer's specifications.

2.17.3 Colony blots

Fourteen nylon membranes were cut to fit onto the 7 square petri dishes. The membranes were carefully placed on the perti dishes and the selected recombinant colonies were transferred. Genomic DNA from strain AS-35 cultured in LB or olive waste and pUC18 plasmid DNA was also spotted on the membranes as controls. The membranes were allowed to dry, placed for 10 minutes with the DNA side up on filter paper soaked in denaturation solution (1.5 M NaCl, 0.5 M NaOH), followed by 10 minutes on filter paper soaked in neutralization solution (1 M Tris-HCl (pH 7.2), 1.5 M NaCl, 10 mM EDTA). The membranes were washed in 2 x SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0), and allowed to dry. The DNA was fixed to the membranes by incubating for 2 hours at 80°C.

Each membrane was sealed in a separate plastic bag together with DIG-Easy Hyb buffer (Roche) and prehybridized for 30 min at 42°C. The prehybridization buffer was then removed from the plastic bags and replaced with hybridization buffer so as to just cover the membranes, after which 25 ng/ml of the denatured labeled probes (Section 2.17.2) were added to the buffer and the bags resealed. Hybridization was allowed to proceed at 42°C for 16 h. After hybridization, the membranes were recovered and washed twice for 5 min in 2 x SSC; 0.1% SDS at room temperature, followed by 0.5 x SSC, 0.1% SDS at 65°C. The hybridized probes were detected by rinsing the membranes in washing buffer (0.1 M maleic acid, 0.15 M NaCl, 0.3% [v/v] Tween-20; pH 7.5), followed by incubation for 30 min in 1 x blocking solution (prepared by diluting the supplied blocking solution 10-fold in maleic acid buffer [0.1 M maleic acid, 0.15 M NaCl; pH 7.5]). The membranes were incubated for 30 min at room temperature in antibody solution (alkaline phosphatase-conjugated anti-digoxigenin, diluted 1:5000 in fresh blocking solution). The unbound primary antibodies were removed by washing the membranes twice for 15 min in washing buffer and equilibrated for 5 min in detection buffer (0.1 M Tris-HCl, 0.1 M

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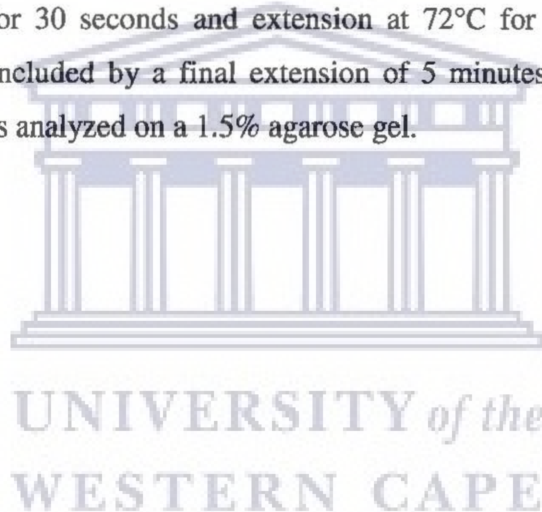
NaCl; pH 9.5). The membranes were then immersed in the alkaline phosphatase enzyme substrate (NBT/BCIP stock diluted 1:50 in detection buffer) until the dots became visible.

2.17.4 Colony PCR

Colony PCR was performed in 96 well PCR plates on selected colonies using M13F and M13R primers (Table 2.1). Each 25 µl reaction contained 0.5U *Taq* polymerase enzyme, 1 x PCR buffer, 0.4 pmol primer and 0.2 mM of each dNTP. An eppendorf pipette tip was used to transfer cells from each bacterial colony to the PCR reaction. The cells were first lysed at 95° for 10 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 1 minute. The amplification reaction was concluded by a final extension of 5 minutes at 72°C. Five microliters of PCR product was analyzed on a 1.5% agarose gel.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Chapter 3

Isolation and characterization of putative olive waste biodegrading micro-organisms

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3.1 Introduction

The production of Kalamata table olives is essentially a two step procedure. First, the olives are placed in fresh water for a few days, where the principal aim is to remove hydrosoluble oleuropein. This secoiridoid is composed of elenolic acid esterified to hydroxytyrosol, and responsible for the bitter taste of olives (Bianco *et al.,* 1999; Romero *et al.,* 2002a). During this time the water is changed frequently. The olives are then brined in 5-10% NaCl solution (Randazzo *et al.,* 2004). Processed wastewater released from this debittering procedure is rich in polyphenols, with a total phenol content of approximately 1.4 g/l, and organic acids (Aggelis *et al.*, 2002). The low molecular weight phenol compounds are responsible for the toxicity to biological systems, including plants, crustaceans, fungi and bacteria (Fiorentino *et al.,* 2003; Adhoum and Monser, 2004). As with the wastewater produced during the process of olive oil production (olive mill wastewater), processed olive wastewater has a brownish-black colour and foul odor. These wastewaters are currently disposed of in evaporation ponds, where they cause serious odor emission problems and have potential for local ecological disasters either from leakage into underground water tables or from occasional heavy rainfalls (Spandre and Dellomonaco, 1996).

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Treatment of olive wastewater is not only beneficial for the environment, but potentially offers economic returns, for example the purification of valuable antioxidants (Hamdi, 1993). The majority of the biodegradation processes aim to remove the phenol content present in wastewater in order to solve secondary problems such as toxicity and dark colour (Benitez *et al.*, 1997; Bertin *et al.,* 2001; Aggelis *et al.*, 2002; D'Annibale *et al.*, 2004a).

The preliminary aim of this project was to isolate organisms from processed olive wastewater in order to identify suitable candidates for degradation of wastewater phenolics.

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3.2 Results

3.2.1 Isolation and morphological characterization of strains native to olive wastewater

In an attempt to identify organisms which could potentially be used in the biodegradation of polyphenolic compounds, micro-organisms were isolated from the communities native to olive wastewater. Samples taken from various evaporation ponds and the surrounding soil were plated on selective 25% olive waste media. Thirty-six colonies, each exhibiting an unique colony morphology, were subcultured to obtain pure cultures. The source of the isolates and their morphological characteristics are presented in Table 3.1.

Table 3.1 Morphological characteristics of olive waste isolates.

Isolate	*Gram*	*Morphology*	**Sample*
AS 1	-	Rods	Ss
AS 2	-	Small rods	Tl
AS 3	-	Small rods	Tl
AS 4	Fungus	Long rods	Tl
AS 5	-	Small rods	Ms
AS 6	-	Small rods	Ms
AS 7	-	Small rods	Tl
AS 8	-	Small rods	Tl
AS 9	-	Small rods	Tl
AS 10	Fungus	Long rods	Ss
AS 11	Fungus	Long rods	Ss
AS 12	Fungus	Rods	Bs
AS 13	Fungus	Rods	Ss
AS 14	-	Rods	Tl
AS 15	-	Rods	Bs
AS 16	-	Small rods	Bs
AS 17	-	Rods	Ds
AS 18	-	Rods	Tl
AS 19	-	Rods	Tl
AS 20	-	Rods	Ds
AS 21	-	Rods	Tl
AS 22	-	Rods	Ms
AS 23	+	Cocci	Tl
AS 24	-	Rods	Ml
AS 25	Fungus	Rods	Ml
AS 26	+	Rods	Tl
AS 27	Fungus		Tl
AS 28	-	Rods	Ms
AS 29	+	Rods	Ts
AS 30	-	Rods	Ms
AS 31	-	Small rods	Ts

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AS 32	-	Rods	Ml
AS 33	-	Rods	Bl
AS 34	-	Small rods	Ms
AS 35	+	Rods	Ms
AS 36	Fungus	Long rods with conidia	Ts

*Tl Liquid from the top evaporation pond
Ts Soil from around the top evaporation pond
Ml Liquid from the middle evaporation pond
Ms Soil from around the middle evaporation pond
Bl Liquid from the bottom evaporation pond
Bs Soil from the bottom of evaporation pond
Ds Soil from around a dry evaporation pond
Ss Soil from a dry compost heap
Dl Liquid from a digester

The cell morphology of each culture was analyzed by bright-field microscopy. Of the 36 cultures, 8 were putatively identified as fungi, based on their unique cellular morphology and reaction towards (Caldwell, 1995). Gram stain. The remaining 26 cultures exhibited morphologies typically associated with bacteria. All but one of the bacterial cultures appeared to be rod-shaped. The majority of these cultures were Gram negative. The morphological characteristics of the 25 cultures selected for use in subsequent biodegradation studies are shown in Figure 3.1. All the isolates, except the Gram positive isolate AS-35, were stained with safranin in order to visualize cellular morphology. AS-35 was stained with crystal violet, a dye that binds to the peptidoglycan layer of Gram positive organisms (Caldwell, 1995).

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3.2.2 Biodegradion of olive waste by selected isolates

To test the tolerance of the 36 isolates to elevated phenol concentrations, each was subcultured on media containing 50% and 75% (v/v) olive wastewater. None of the isolates was capable of growth on 75% olive waste, while 25 showed growth on 50% olive waste. This criterion was used as a basis for initial selection and all isolates that were not capable of growth under these conditions were excluded from further analyses.

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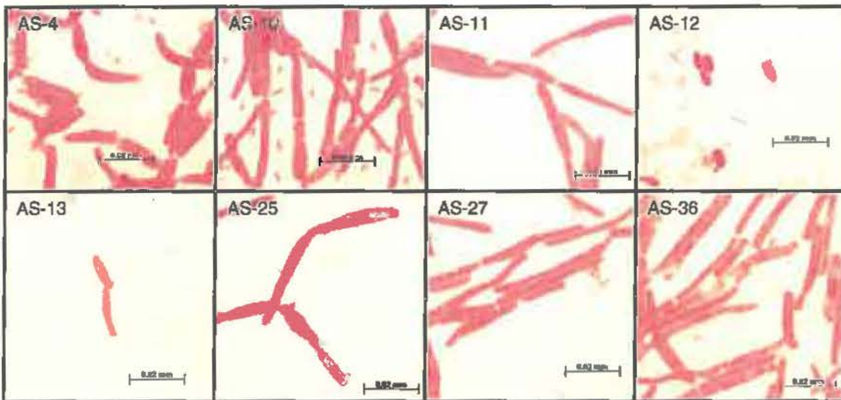
Section A: Putative fungal isolates.



Section B: Putative bacterial isolates.

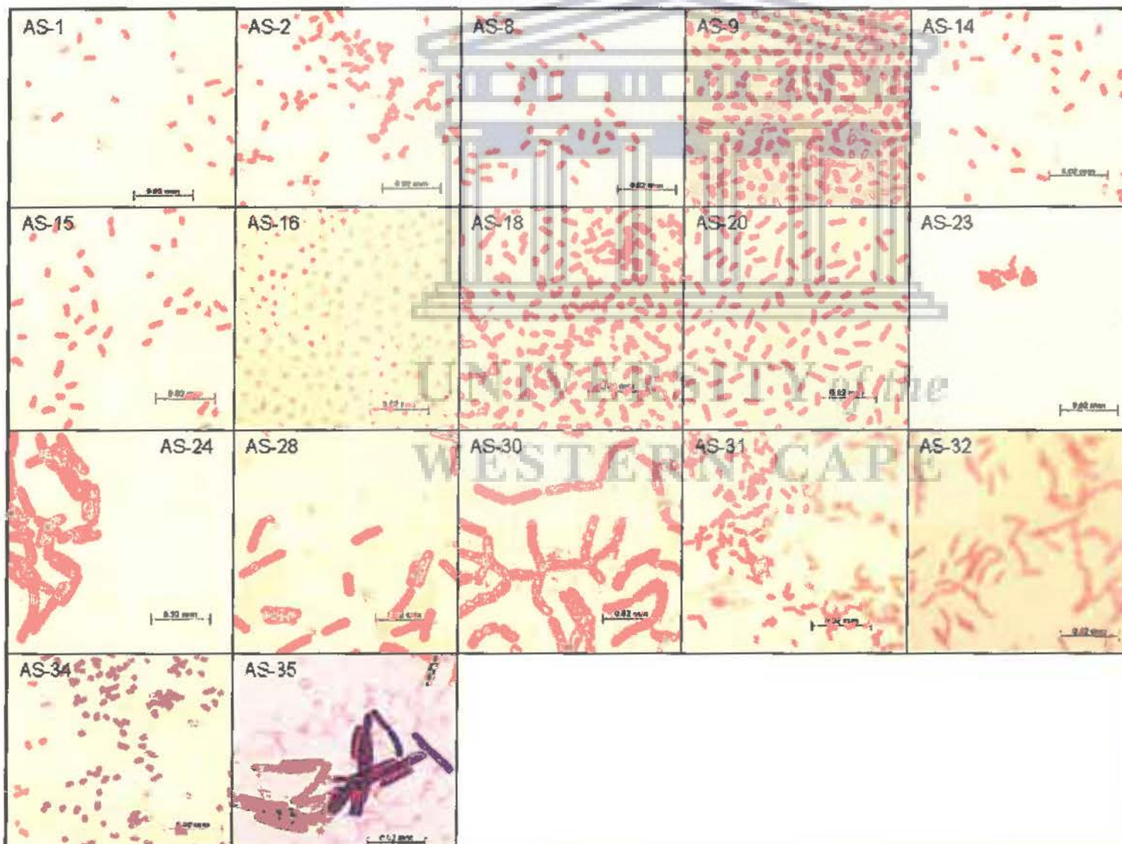


Figure 3.1. Photomicrographs showing the distinct morphologies of the 25 different olive wastewater isolates. The scale bars represent 0.02mm.

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The biodegradation capacity of the isolates was partly assessed through their ability to produce polyphenol oxidases. Laccase, peroxidase and tyrosinase activities have been implicated in the depolymerisation of phenolic compounds (Perez *et al.*, 1998). Significant levels of peroxidase activity, using guaicol as substrate, were observed in most isolates (Figure 3.2) as well as in their extracellular environment (results not shown). Cellular extracts of AS-30 and AS-34 showed the highest activity with substrate conversion rates approaching 2.6 pmol/min/μg. This is comparable to the activity of 1U of commercially available peroxidase enzyme (2.9 pmol/min/μg). No significant tyrosinase or laccase activity was detected after 16 hours of growth in olive waste.

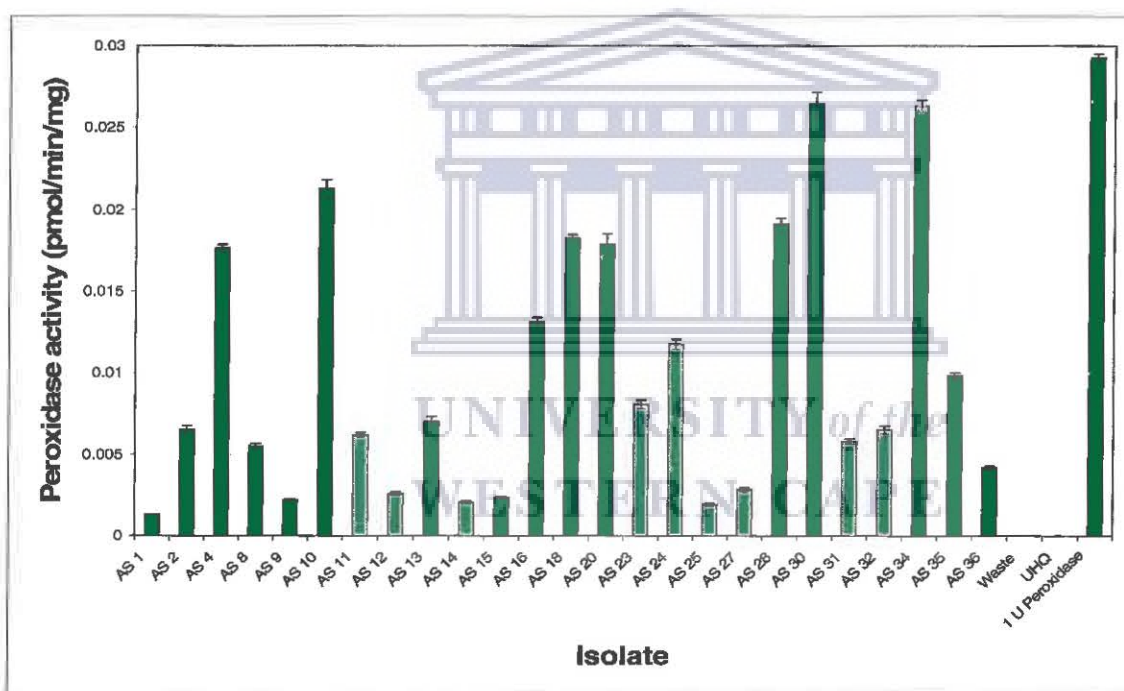

Figure 3.2. Peroxidase activity of the 25 selected isolates measured after 16h of culturing in 25% olive wastewater. The conversion of guaicol (pmol/min/mg) was measured after 4 minutes.

The ability of the isolates to degrade the phenolic compounds present in olive wastewater was analyzed by measuring the reduction in total phenol concentration. All isolates reduced the phenol content by at least 13% of the original concentration (7.6 mM) after 16 hours, whilst AS-16 showed the highest level of phenol reduction (approximately 43%) (Figure 3.3).

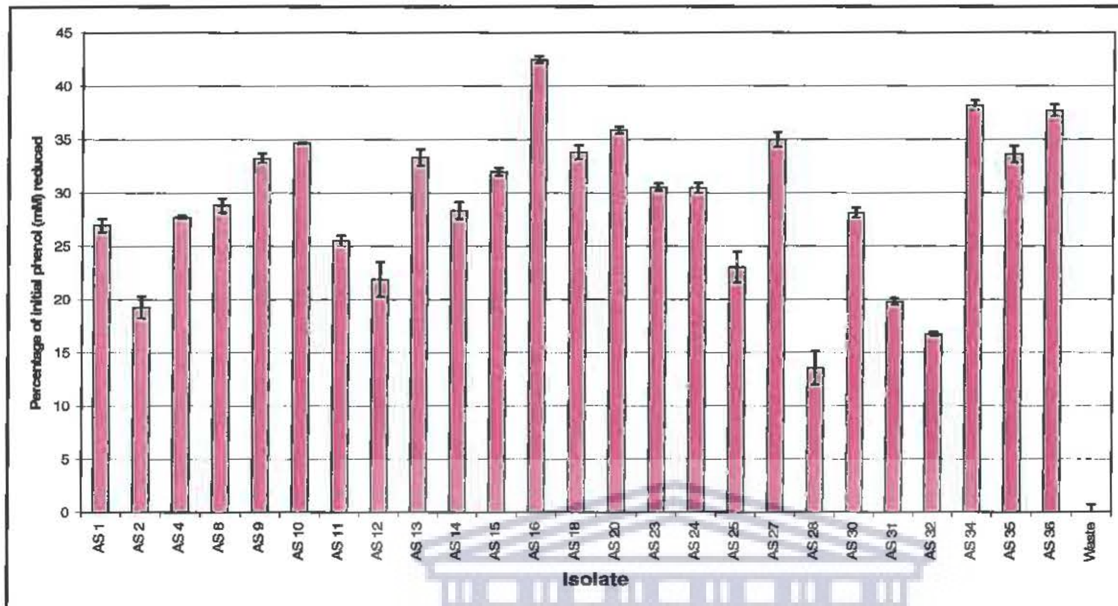

Figure 3.3. Ability of selected isolates to reduce the total phenol content present in olive waste supernatant after 16 hours of incubation. The reduction is shown as a percentage of the initial phenol concentration.

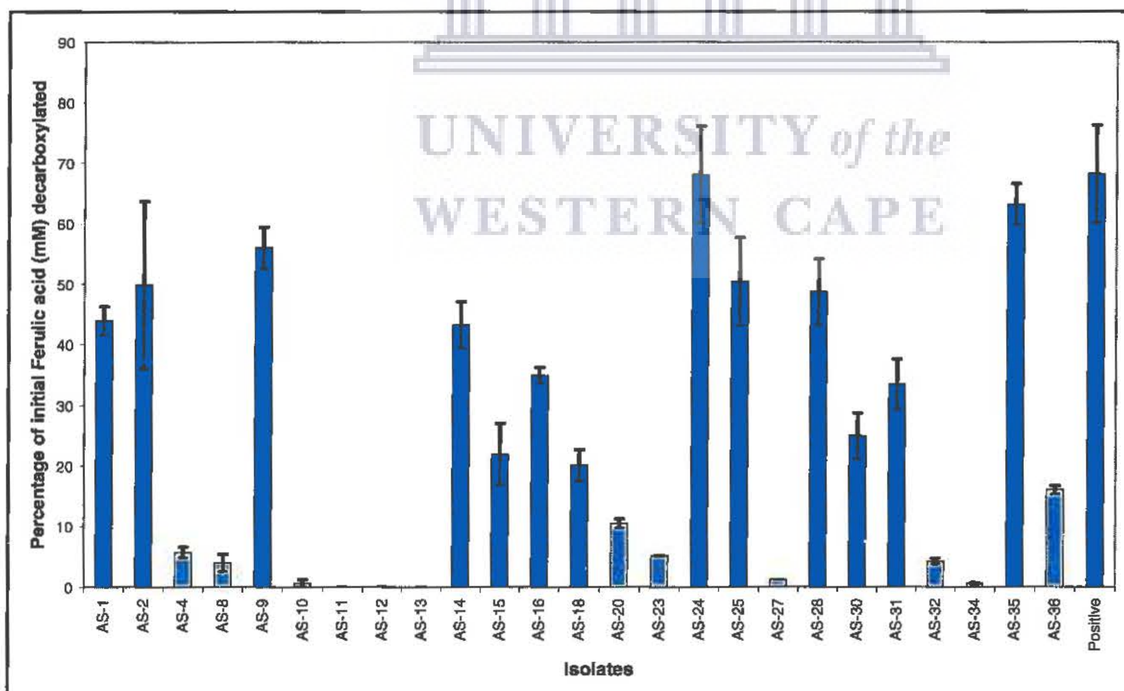

Figure 3.4. Decarboxylation of ferulic acid to 4-vinyl guaiacol for 25 selected isolates. Activity is presented as percentage reduction of initial ferulic acid concentration.

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All isolates were tested for their capacity to produce economically valuable compounds such as 4-vinyl guaiacol from ferulic acid. Ferulic acid [3-(4-hydroxy-3-methoxyphenyl)-propenoic acid] is an extremely abundant plant product, whose biotransformation to vanillin via decarboxylation to 4-vinyl guaiacol is widely reported in various bacterial and fungal isolates (Rosazza *et al.,* 1995). Sixteen isolates showed a significant decrease in the original ferulic acid concentrations (100 µg/ml) coupled with the formation of 4-vinyl guaiacol (Figure 3.4). Isolates AS-9, AS-24 and AS-35 were found to be most effective in decarboxylating ferulic acid.

3.2.3 The composition of low molecular weight phenolic compounds produced during the treatment of olive wastewater

The composition of low molecular weight phenolic compounds produced by the isolates was analyzed by RP-HPLC over 64 hour fermentations on olive waste media. Profiles of these fractions were compared to a set of model compounds, previously identified in olive waste (Bertin *et al.,* 2001). These included benzoic acid, catechol, galic acid, p-hydroxyphenyl acetic acid, hydroxytyrosol, protocatechuic acid and syringic acid. A variety of novel compounds are possible, including *de novo* synthesized secondary metabolites and intermediate products formed during aromatic degradation. Several of these compounds, such as hydroxytyrosol, catechol, protocatechuic acid, p-hydroxyphenyl acetic acid and syringic acid, were observed in fermentations of isolate AS-35. Based on these results, 4 isolates (AS-9, AS-15, AS-18 and AS-35) were selected for further investigation (Figure 3.5 and Figure 3.6).

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3.2.4 Identification of selected isolates

The four isolates selected for further characterization were identified by 16S rRNA gene sequencing. Three of the isolates, AS-9, AS-15 and AS-18, were identified as *Klebsiella oxytoca* (99%, 99% and 98% sequence homology, respectively) (Appendix 1), while AS-35 was identified as a *Bacillus* sp. (87% sequence homology). The BLAST results are shown in Table 3.2.

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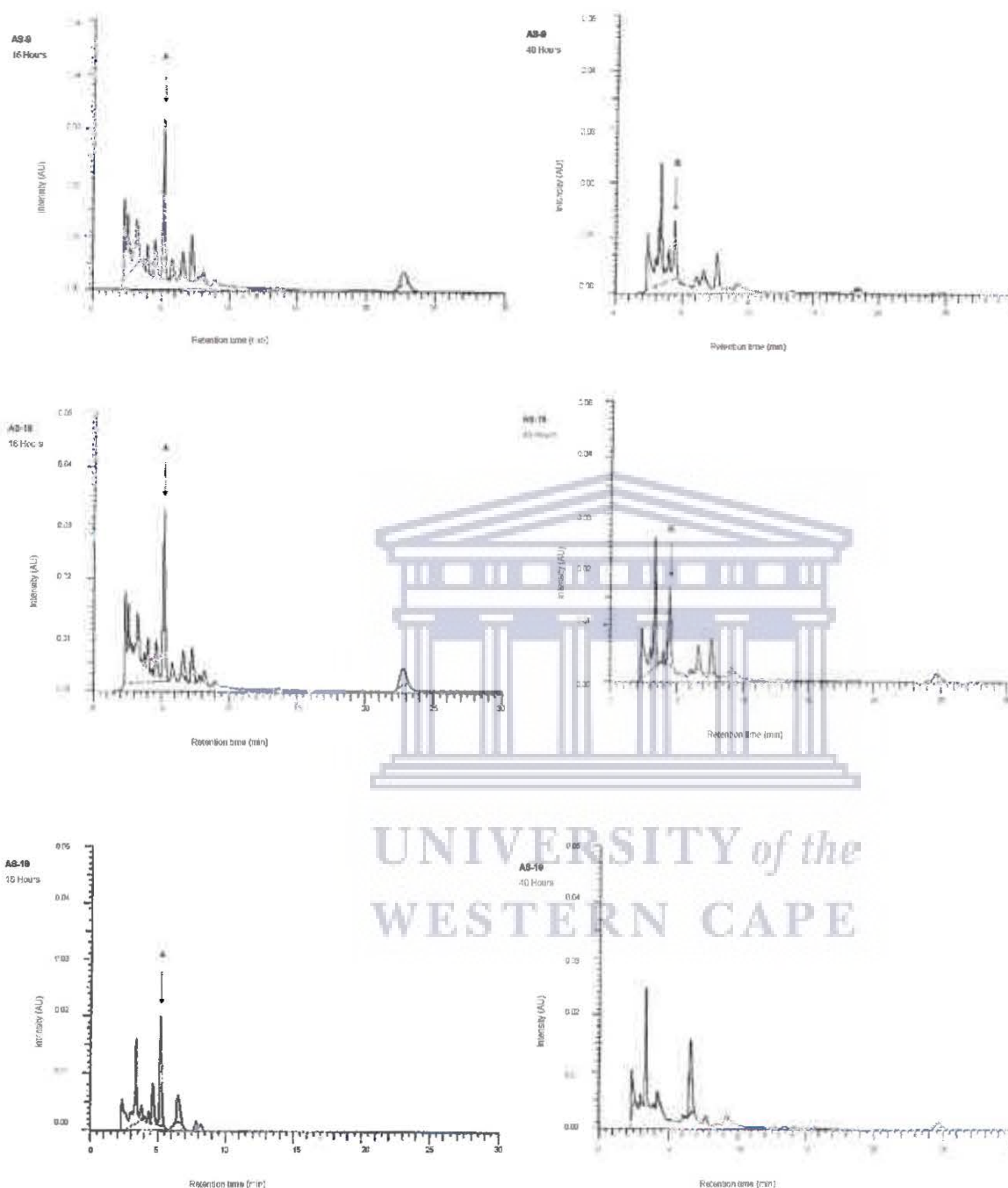

Figure 3.5. Reverse phase HPLC traces showing low molecular weight phenolic compounds present in the culture supernatants of isolates AS-9, AS-15 and AS-18 grown in olive wastewater media for 16 and 40 hours, respectively. Hydroxytyrosol, the most abundant low molecular weight aromatic compound, is indicated by arrow A and gallic acid, a commercially important aromatic compound, is indicated by arrow B.

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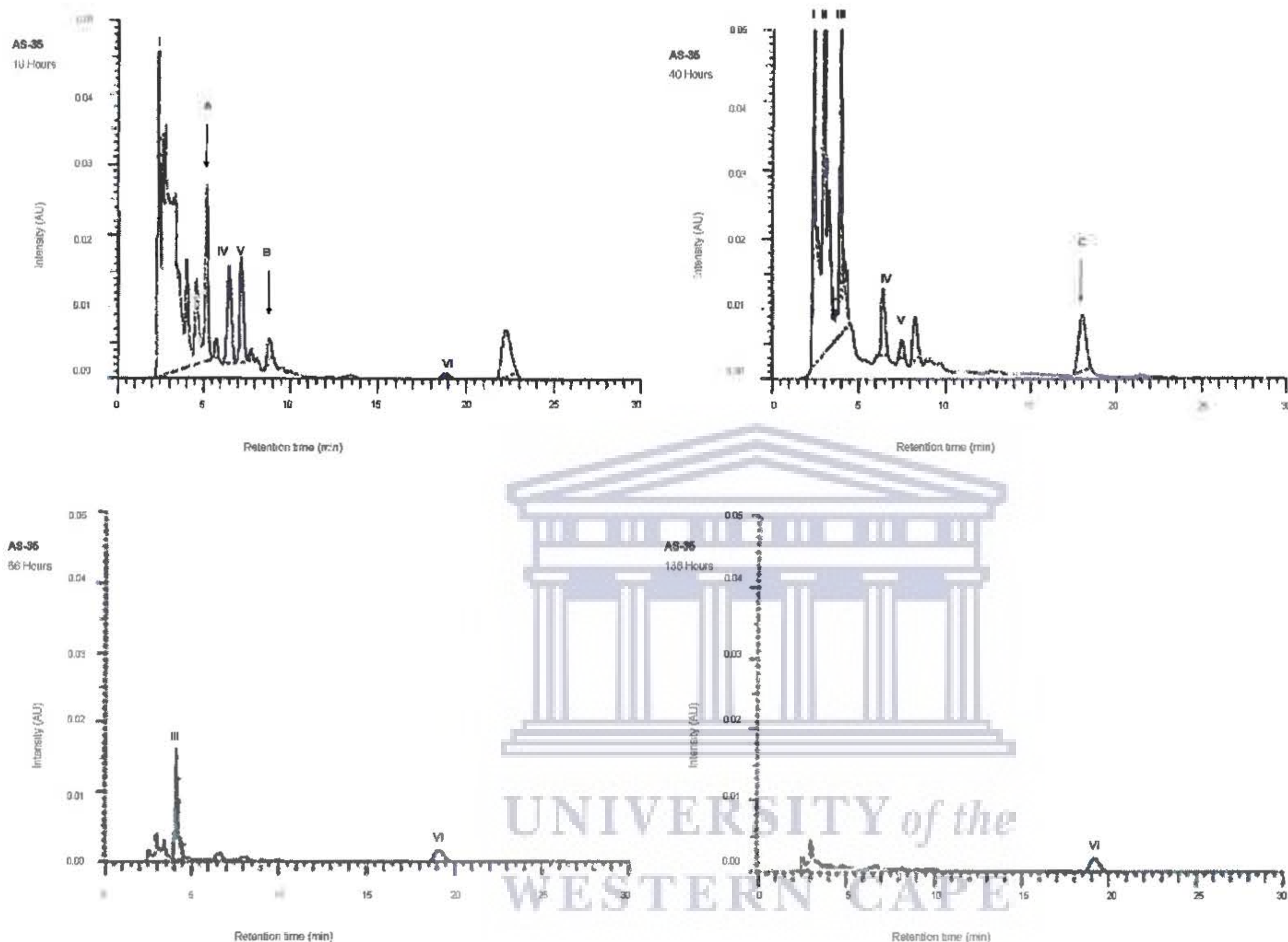

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Figure 3.6. Reverse phase HPLC traces showing low molecular weight phenolic compounds present in the culture supernatant of isolate AS-35 grown in olive wastewater media for 16, 40, 66 and 136 hours respectively. Hydroxytyrosol is indicated by arrow A, p-hydroxyphenyl acetic acid by arrow B and syringic acid by arrow C. The letters I – VI indicate unidentified compounds produced and / or degraded by AS-35. Figures 3.5 and 3.6 are courtesy of C. Garcin, Chemical Engineering Department, University of Cape Town.

3.2.5 Growth analysis of selected isolates

Stationary phase for all of the 4 isolates were reached after 8-9 hours and no major deviation from stationary phase were observed over the following 25 hours of incubation

(Figure 3.7 and Table 3.3).

Table 3.2. BLAST results of isolates AS-9, AS-15, AS-18 and AS-35 16S gene analysis.

Isolate	Bp	The 4 best homology predictions
AS-9	558	gi|26000221|gb|AY150697.1| *Klebsiella oxytoca* 16S ribosomal RNA gene, partial sequence Length = 1434 Score = 841 bits (424), Expect = 0.0 Identities = 445/448 (99%), Gaps = 3/448 (0%) gi|6693806|gb|AF129440.1|AF129440 *Klebsiella oxytoca* 16S ribosomal RNA gene, partial sequence Length = 1436 Score = 841 bits (424), Expect = 0.0 Identities = 445/448 (99%), Gaps = 3/448 (0%)
AS-15	750	gi|14270691|gb|AF319525.3|AF319525 *Klebsiella oxytoca* 16S ribosomal RNA gene, partial sequence Length = 1335 Score = 1390 bits (701), Expect = 0.0 Identities = 731/738 (99%), Gaps = 5/738 (0%) gi|3282034|emb|Y17660.1|KOY17660 *Klebsiella oxytoca* 16S rRNA gene, strain 5725y, partial Length = 1452 Score = 1390 bits (701), Expect = 0.0 Identities = 731/738 (99%), Gaps = 5/738 (0%)
AS-18	830	gi|14270691|gb|AF319525.3|AF319525 *Klebsiella oxytoca* 16S ribosomal RNA gene, partial sequence Length = 1335 Score = 1417 bits (715), Expect = 0.0 Identities = 783/795 (98%), Gaps = 8/795 (1%) gi|3282034|emb|Y17660.1|KOY17660 *Klebsiella oxytoca* 16S rRNA gene, strain 5725y, partial Length = 1452 Score = 1417 bits (715), Expect = 0.0 Identities = 783/795 (98%), Gaps = 8/795 (1%)
AS-35	692	gi|27497630|gb|AY167810.1| *Bacillus* sp. SAFN-020 16S ribosomal RNA gene, partial sequence Length = 1342 Score = 60.0 bits (30), Expect = 5e-06 Identities = 84/96 (87%), Gaps = 7/96 (7%) gi|27497622|gb|AY167802.1| *Bacillus benzoevorans* strain SAFN-025 16S ribosomal RNA gene, partial sequence Length = 1343 Score = 56.0 bits (28), Expect = 8e-05 Identities = 129/152 (84%), Gaps = 15/152 (9%)

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Aliquots of culture supernatant from the growth rate experiment were used to analyze the changes in the low molecular phenol profile. Using RP-HPLC, several new peaks were observed in the low molecular weight profile of AS-35 supernatant (Figure 3.6, I-VI). Most of the low molecular weight compounds, except the unidentified peaks denoted III and VI, were lost after 136 hours of incubation. Based on the capability of AS-35 to remove most of the low molecular weight phenolic compounds, it was subsequently selected for classical chemical mutagenesis to enhance its biodegradation capability.

3.2.6 Biotransformation of model compound by isolate AS-35

The ability of isolate AS-35 to biodegrade various model compounds as sole carbon sources was investigated. The profile of each compound was analyzed at hourly intervals by UV absorption spectrum. AS-35 was capable of decarboxylating vanillic acid

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(absorption peaks at 250 and 291 nm) to vanillin (absorption peak at 278nm) and ferulic acid (absorption peaks at 284 and 312 nm) to 4-vinyl guaiacol (absorption peak at 264 nm) (Figure 3.9). A schematic representation of the ferulic and vanillic acid biodegradation pathway is indicated in Figure 3.8. No change in spectrum was observed for tyrosol, p-hydroxyphenyl acetic acid or gallic acid. Enzyme assays were validated by performing the assays using no cellular extract, boiled cellular extract and double the cellular extract (data not shown).

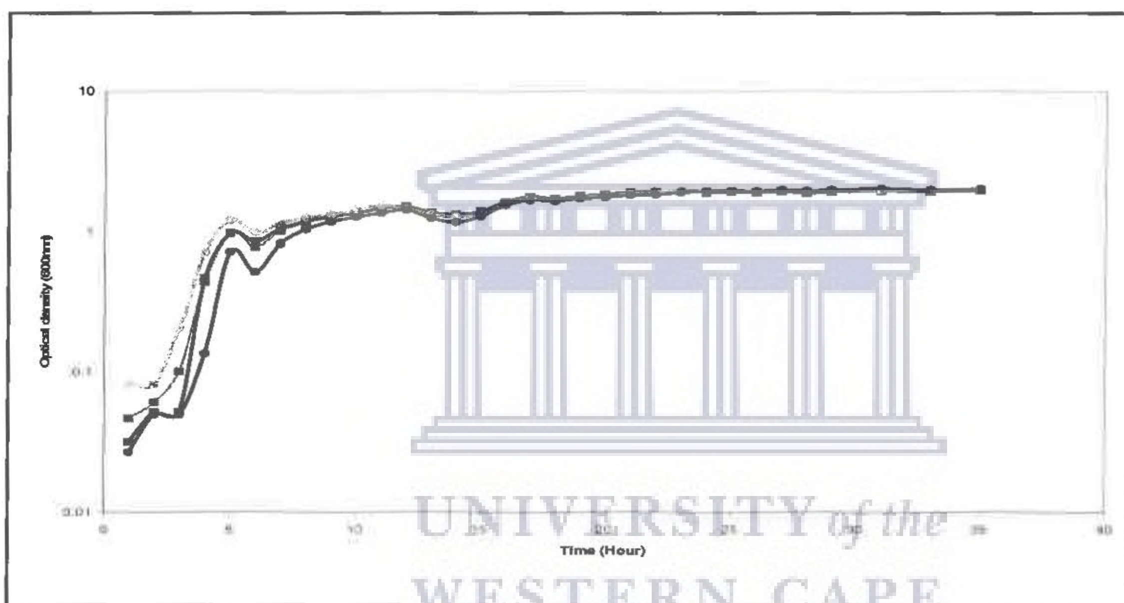

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Figure 3.7. Growth profiles (OD600nm) of selected isolates AS-9, AS-15, AS-18 and AS-35. AS-9 ■, AS-15 ■, AS-18 ● and AS-35 ● were cultured in 25% olive waste.

Table 3.3. Growth kinetics of isolates AS-9, AS-15, AS-18 and AS-35.

Isolate	Lag phase (hours)	μ / hour	g / hour	g / min
AS-9	± 2	0.6	1.2	74.4
AS-15	± 2	0.6	1.2	69.3
AS-18	± 2	0.5	1.3	78.9
AS-35	± 2	0.5	1.4	82.4

μ is Ln(Ys /Ye)/Δt, where Ys is the absorbency at the start of stationary phase and Ye is the absorbency at the start of exponential phase. Δt is the difference in time (hours) of Ys and Ye.
g is the generation time of the organism.

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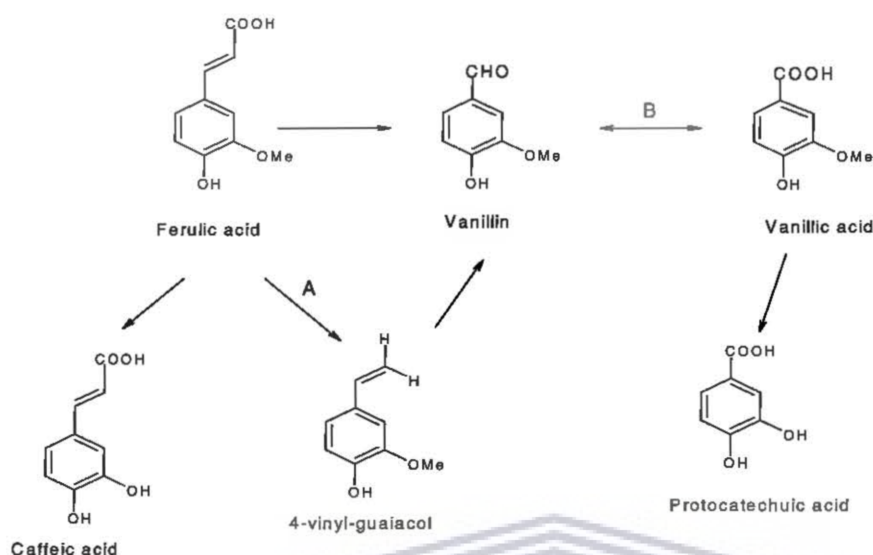

Figure 3.8. Diagrammatic scheme showing the degradation of ferulic acid and vanillin. Ferulic acid is decarboxylated (A) to 4-vinyl-guaiacol, while aldehyde dehydrogenase (B) transform vanillin to vanillic acid.

3.3 Discussion

It is estimated that a million tons of table olives are produced globally each year, with every kg of olives producing 8.5 l of wastewater (Beltran-Heredia *et al.,* 2000b). This is however dwarfed by the 3.0 x 10^7 m^3 of wastewater annually generated in the production of olive oil (Lanciotti *et al.,* 2005). This disparity in the amount of waste produced by the respective processes is partly responsible for the lack of attention afforded to management of table olive wastewater (Aggelis *et al.,* 2001). Compared to the Mediterranean countries that are responsible for 95% of the world's table olives and olive oil production, South Africa is a minor contributor. The first industrial olive cultivation in South Africa started at the beginning of the 20th century and it was only in the 1970's that the production and profitability of table olives surpassed that of olive oil, with approximately 40% of the local production being sold as table olives (www.southafrica.info.proudlysa). However, the ever-increasing volumes of wastewater produced by the local industry necessitate investigations into alternative ways of disposing of the effluent.

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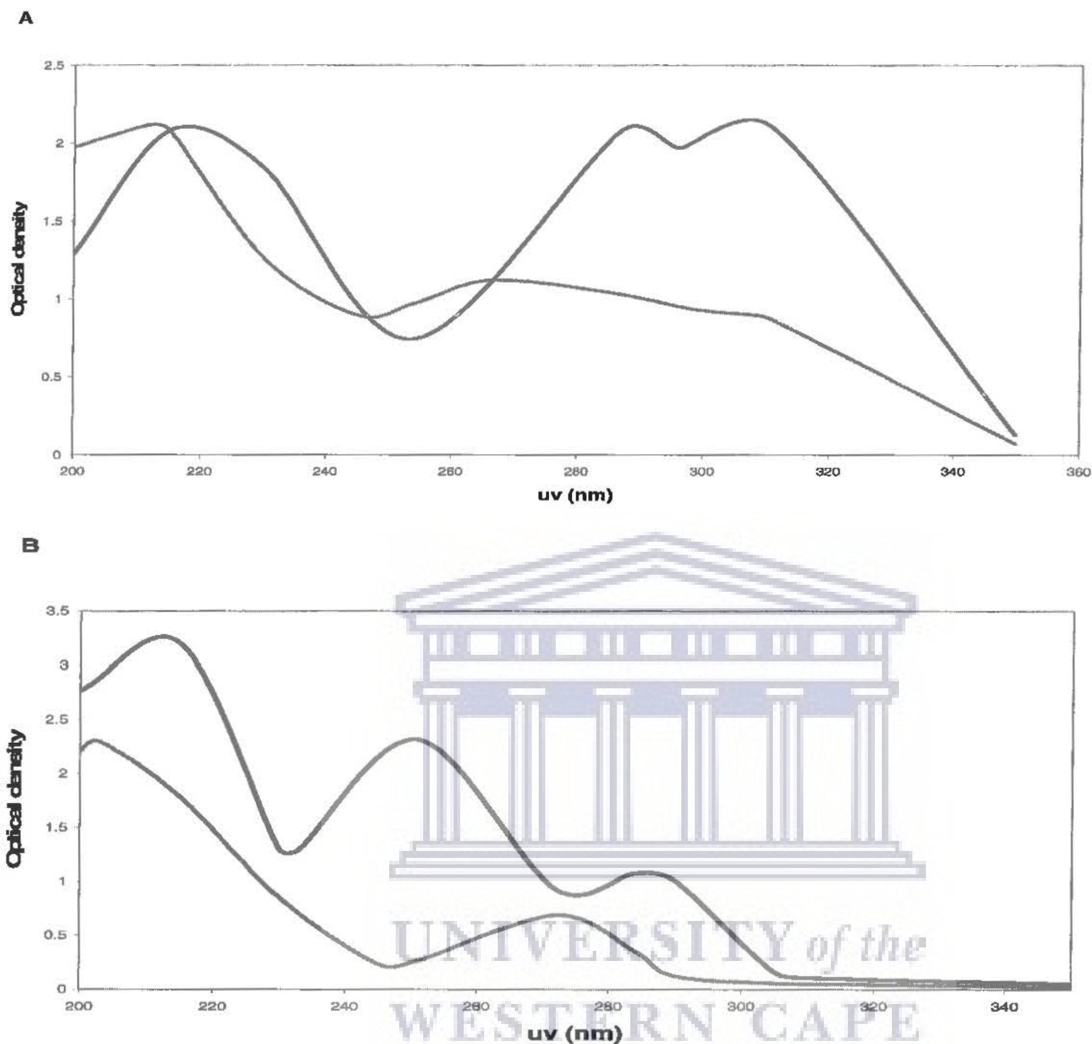

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Figure 3.9. Scanning spectrophotometric analysis of the culture supernatant of AS-35 grown in ferulic acid (A) and vanillic acid (B) as sole carbon source.

A. Ferulic acid concentration at 0 ▬ and 12 ▬ hours post AS-35 inoculation. Ferulic acid has absorption peaks at 284 and 312 nm, while 4-vinyl guaiacol has a peak at 264 nm.

B. Vanillic acid concentration at 0 ▬ and 12 ▬ hours post AS-35 inoculation. Vanillic acid has absorption peaks at 250 and 291nm, while vanillin has a peak at 278nm.

The composition of processed olive wastewater varies from season to season and between producers, but it is generally not suitable to be disposed via municipal sewerage waste systems. As a result, most of the wastewater is discarded into evaporation ponds where it poses an environmental risk. The use of micro-organisms in biodegradation and

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remediation of olive wastewater has previously been explored. The majority of these investigations focus on the use of fungi, especially white-rot fungi. The success with which these organisms accomplish the removal of phenolic compounds, decolourization of waste, and lowering of COD and BOD have been linked to their ability to produce laccases and manganese peroxidases (Archibald and Roy, 1992; Casa *et al.*, 2003; D' Annibale *et al.*, 2004b; Ettayebi *et al.*, 2003; Fadil *et al.*, 2003; Jaouani *et al.*, 2003; Lanciotti *et al.*, 2005).

In an attempt to isolate organisms which could potentially be used for bioremediation of wastewater, 36 microbial strains were isolated from evaporation ponds in the Boland region of South Africa. Using bright field microscopy 78% of these isolates were putatively identified as bacterial species. Of the 36 isolates, 25 were shown to tolerate elevated concentrations of olive wastewater. In all cases treatment of wastewater with these isolates resulted in a significant decrease in the total phenol content. Enzymatic assays revealed that the majority of the isolates produced peroxidases, while neither laccase nor tyrosinase activity could be detected for any of the isolates. All polyphenol oxidase assays were performed with cellular extracts and extracellular medium. The lack of laccase activity is somewhat surprising, since polyphenol oxidases and especially laccases are known to be of particular importance in the biodegradation of phenolic compounds (Aggelis *et al.*, 2002., Assas *et al.*, 2002., D'Annibale *et al.*, 2004a and Perez *et al.*, 1998). Previous studies reported that the carbon and nitrogen sources used to supplement olive wastewater have different effects on the micro-organism's ability to produce peroxidase, thus providing a probable explanation for the failure to detect these enzyme activities is due to supplementing the wastewater with yeast extract (Ayed *et al.*, 2004). Although laccases are predominantly associated with plants and fungi, putative laccase genes analogous to known fungal genes have recently been identified in the genomes of various bacteria (Alexandre and Zhulin, 2000).

Several bacterial species such as *Comamonas* spp., *Ralstonia* spp., *Sphingomonas* spp. and *Pseudomonas* spp. have previously been implicated in the degradation of monocyclic aromatic compounds present in olive waste. These compounds include 4-

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hydroxyphenylacetic acid, 3,4-di-methoxybenzoic acid (veratric acid), 4-hydroxybenzoic acid, 4-hydroxy-3,5-dimethoxybenzoic acid (syringic acid), 3-phenyl-2-propenoic acid (cinnamic acid), 3,4-dihydroxybenzoic acid (caffeic acid) and 4-hydroxy-3-methoxybenzoic acid (vanillic acid) (Di Gioia *et al.*, 2002). In order to establish whether the isolates exhibited similar enzymatic activities, the chemical composition of the low molecular weight fraction of treated olive wastewater were analyzed by reverse phase HPLC. Four isolates (AS-9, AS-15, AS-18 and AS-35) was shown to markedly reduce the levels of previously identified target compounds. In addition, several value-added compounds were produced as by-products of the degradation process. Analysis of the 16S rDNA sequences of these four isolates identified the first three as *Klebsiella oxytoca,* a Gram negative soil organism, while the fourth (AS-35) could only be identified to genus level, that being a *Bacillus* sp.

Few studies have employed bacteria in the bioremediation of olive wastewater. These studies focused specifically on the degradation of hydroxylated and methoxylated monocyclic aromatic compounds (Di Gioia *et al.*, 2004), where a secondary objective was the generation of value-added monocyclic aromatic compounds from the olive waste. A secondary aim of this study was to identify an isolate that could potentially be used to produce economically valuable aromatic compounds during the biodegradation of the olive wastewater. Detailed analysis of the chemical composition of the low molecular weight fraction of treated olive wastewater revealed that isolate AS-35 produced a number of metabolic intermediates of possible economic interest. The ability of AS-35 to grow and utilize vanillic acid, ferulic acid, tyrosol, p-hydroxyphenylacetic acid and gallic acid as sole carbon source was subsequently investigated. The isolate was capable of degrading vanillic and ferulic acid through decarboxylation of the compounds. AS-35 was incapable of utilizing tyrosol, p-hydroxyphenylacetic acid and gallic acid as sole carbon sources even though these substrates were degrade during the organism's growth in olive wastewater. The absence of utilizable carbon and nitrogen sources could be a possible explanation for this phenomenon. Based on these results, AS-35 was selected for subsequent analyses.

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Chapter 4

Differential gene expression of AS-35 under different growth conditions.

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4.1 Introduction

Microorganisms have evolved numerous mechanisms to effectively deal with the stresses induced by the inhospitable environments. The best described mechanism of adaptation involves the regulation of protein expression at a transcriptional level (Shingler, 2003). Other levels of adaptation include the manipulation of translation, of RNA stability, post-translational modifications of the proteins and differential protein degradation (Sanders *et al.*, 1999). In most cases these mechanisms are reflected in changes in the transcriptome and or the proteome of the microorganism.

These cellular changes are prompted by the sensing of effector compounds in the immediate environment and can often be associated with specific environmental conditions (Cases and de Lorenzo, 2005). Moreover, crosstalk between the various stress-response mechanisms is known to occur. This allows organisms to adapted more readily to additional stresses they may subsequently encounter (Wonderling *et al.,* 2004). In *B. subtilis* this is accomplished when environmental or metabolic stress activates the σ^B-dependent general stress response regulon. This regulon consist of more than 150 genes and provides non-growing, non-sporulating *B. subtilis* with multiple, non-specific and pre-emptive resistance to various stresses (Höper *et al.,* 2005). The response to environmental stress is facilitated by the reoccurring presence of chaperones belonging to class I heat shock proteins. GroEL and dnaK are some of the better known examples of this class of protein and are typically expressed at elevated levels when bacteria are exposed to increase heat or during the over-production of insoluble proteins (Sanders *et al.,* 1999., Jürgen *et al.*, 2001). These chaperons play an important role during the activation of stress σ factors in *E. coli*, but are unimportant in *B. subtilis* stress σ^B activation (Scott *et al.*, 1999).

Wastewater generated during the processing of olives is highly toxic to various biological systems. This is not only due to the high aromatic organic content, but also the acidity and high osmolarity of the waste (Fiorentino *et al.*, 2003). Each of these factors is known to contribute to the imposition of stress conditions for organisms growing in this

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environment (Sanders *et al.*, 1999). It is therefore assumed that organism growing in processed olive wastewater will show molecular evidence of adaptation to survival in this stress environment. This chapter describes the changes in the transcriptome and proteome of AS-35 in response to stress conditions associated with growth in olive wastewater.

4.2 Results

4.2.1 Differential protein expression profiles

The total protein profiles of AS-35 cultured under different growth conditions were compared using 2D SDS-PAGE analysis. Cultures were grown in either LB or 25% olive wastewater and sampled at time-points corresponding to late-exponential phase. Triplicate growth profiles showed that AS-35 cultured in LB reached late-exponential phase (OD_{600nm} of 1.0) after 4 hours and after 8 hours when cultured in olive wastewater (Figure 4.1). Distinctive differences were found to exist between the total protein profiles of AS-35 under these conditions (Figure 4.2). These profiles were considered characteristic of each of the respective culturing conditions and are henceforth referred to as the master profiles. Comparisons were performed using Delta2D software.

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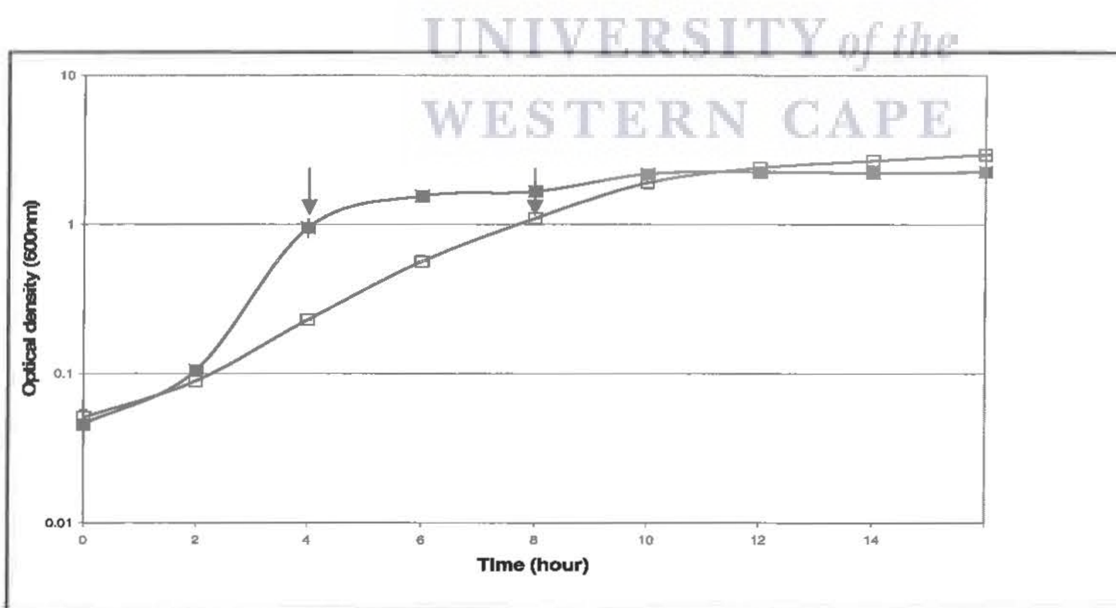

Figure 4.1 Logarithmic representation of the growth profile (OD600nm) of AS-35 cultured in LB ■ and in 25% olive wastewater □. Total protein extraction occurred when indicated by the arrows.

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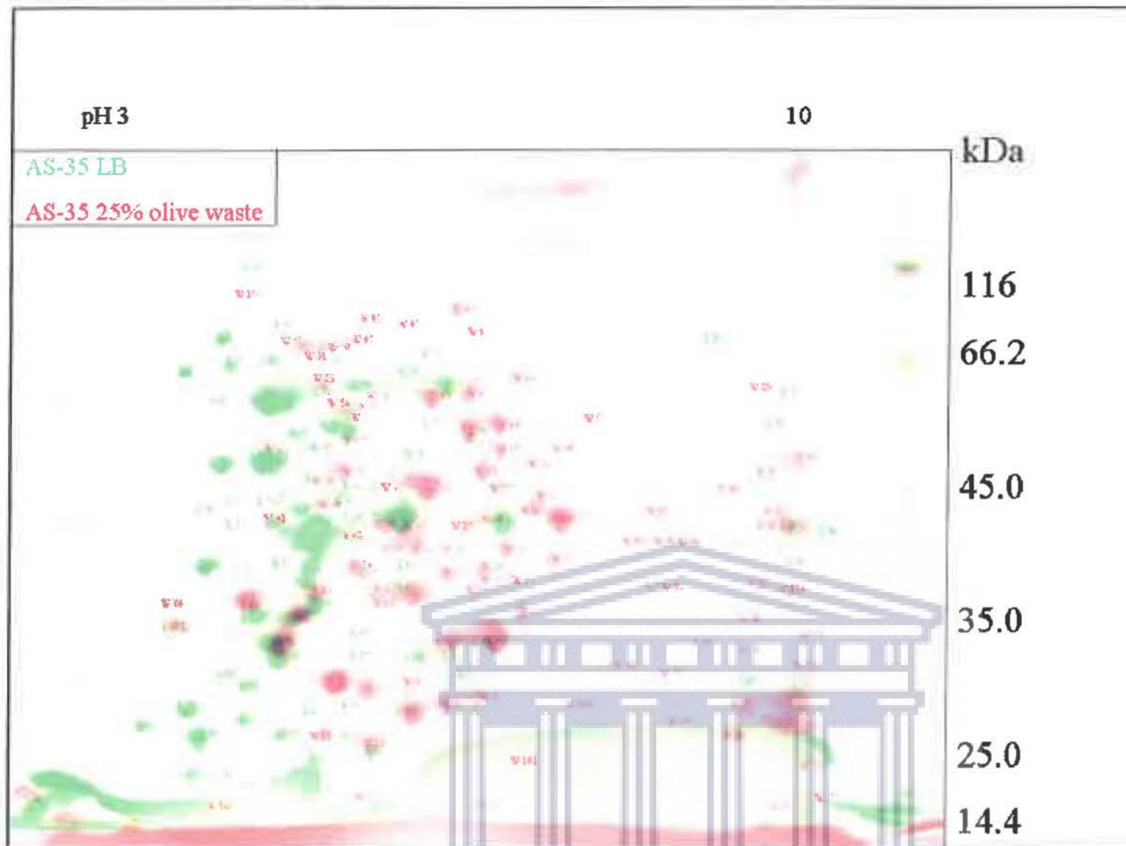

Figure 4.2. Two-dimensional gel electrophoretic analysis comparing the total protein profile of AS-35 cultured in LB (green) and 25% olive wastewater (red), respectively. Cultures were sampled at time-points corresponding to late-exponential phase.

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In order to establish an approximate timeframe in which these adaptive changes occur, the total protein profiles of AS-35 cultured in olive wastewater were analyzed at different time intervals. Broth containing 25% olive wastewater was inoculated with AS-35 cultured overnight in LB. Samples were collected at four-hourly intervals and compared by 2D SDS-PAGE analysis. Results of the analysis are shown in Figure 4.3. Four hours post-inoculation the protein profile still closely resembled that of the LB master profile (Figure 4.3 A). However, a protein distinctively associated with growth in 25% olive wastewater was clearly evident even at this early stage of adaptation (marked as O2 in Figure 4.3). With the continued exposure of the isolate to the olive wastewater, the protein profile evolved to reflect that of the olive wastewater master profile (Figures 4.3 C and D).

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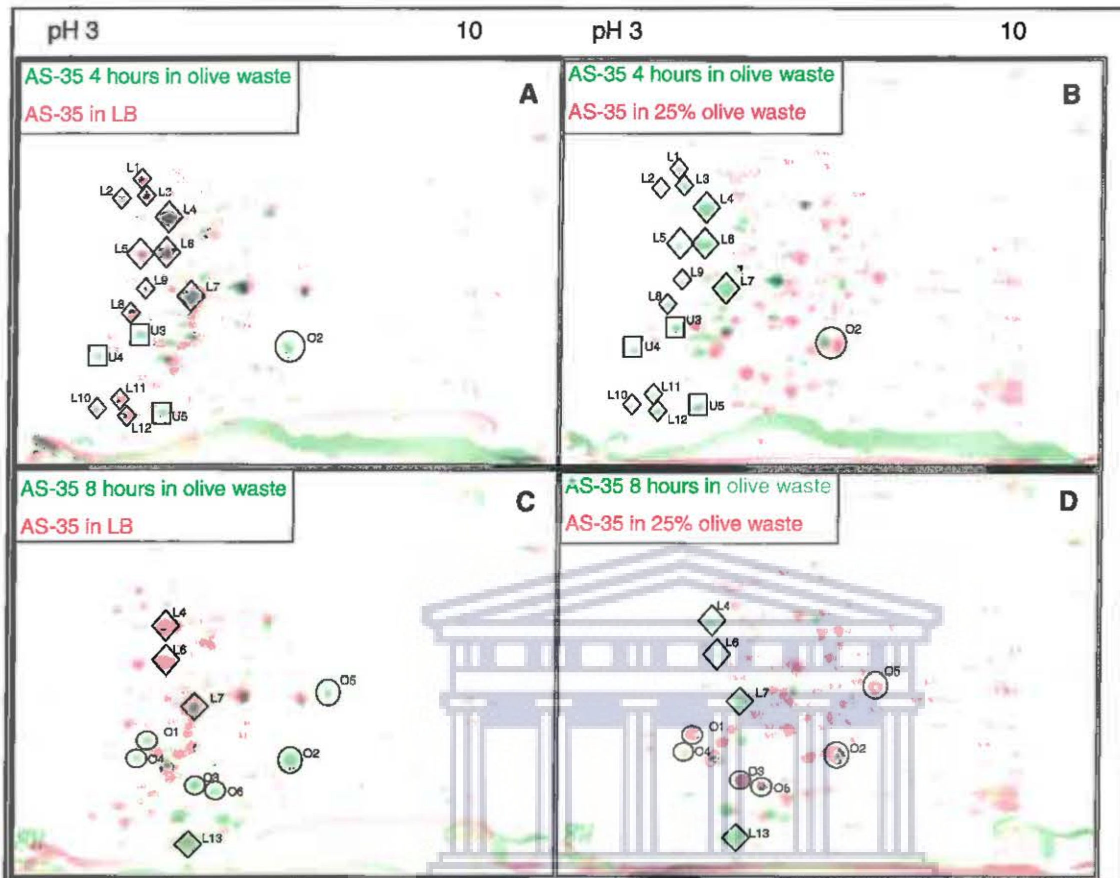

Figure 4.3. Two-dimensional gel electrophoretic analysis of the total protein profile of AS-35 cultured in 25% olive wastewater. Cultures were sampled at four-hourly intervals and compared to the respective master profiles. Panels A and B show the comparisons between the respective master profiles and the sample taken 4 hours post-inoculation. Panels C and D show the comparisons between the respective master profiles and the sample taken 8 hours post-inoculation. Proteins uniquely associated with growth in a nutrient rich environment are indicated by the diamond-shaped boxes (◊) whilst proteins uniquely associated with growth in olive wastewater are encircled (○). Square boxes (□) indicate proteins which appear to be transiently expressed.

At 8 hours post-inoculation several additional proteins distinctively associated with growth in olive wastewater were observed (marked as O1 to 6). Despite this, some remnants of the LB master profile could still be seen with proteins L4, L6, L7 and L13 clearly evident. Interestingly, a number of proteins not associated with either of the master profiles were observed during the initial stages of adaptation (marked as U3 to 5 in Figure 4.3 A and B).

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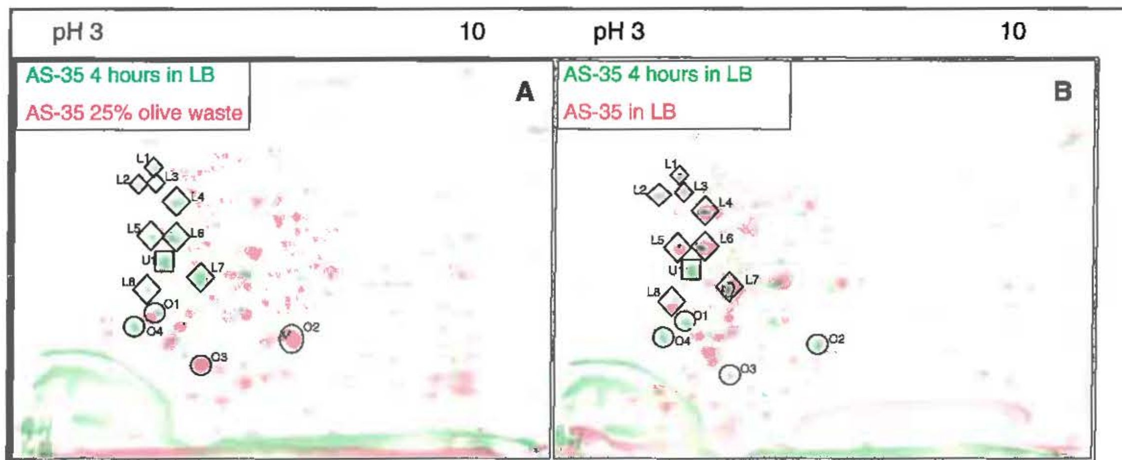

Figure 4.4. Two-dimensional gel electrophoretic analysis of the total protein profile of AS-35 cultured in LB. Cultures were sampled at four hour post-inoculation and compared to the respective master profiles. Proteins uniquely associated with growth in a nutrient rich environment are indicated by the diamond-shaped boxes (◊) whilst proteins uniquely associated with growth in olive wastewater are encircled (○). Square boxes (□) indicate proteins which appear to be transiently expressed.

In a reciprocal approach to the experiment describe above, LB broth was inoculated with AS-35 cultured overnight in 25% wastewater. Under these conditions, the protein profile of AS-35 regressed to a state similar to the LB master profile as early as 4 hours post-inoculation (Figure 4.4). Some remnants of the olive wastewater master profile prevailed (marked O1 to 4 in Figure 4.4). It is interesting to note that the protein marked O2 was also expressed during the initial adaptation of the isolate to olive wastewater. Volumes of each spot indicated in Figures 4.3 and 4.4 are presented in Table 4.1. Comparison of the protein profile of the isolate at 8 hours post-inoculation and the LB master profile revealed no obvious difference between the proteomes, suggesting that the profile fully regressed prior to this time point (data not shown). Proteins identified through significant statistical analysis were indicated on the comparative figures.

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Table 4.1. Volumes based on Gaussian images for each spot marked in Figures 4.3 and 4.4.

Spot	LB	25% OW	4H in LB	4H in OW	8H in LB	8H in OW
L1	121					
L2	111					
L3	121					
L4	185					
L5	162					
L6	175					
L7	195					
L8	137					
L9	130					
L10	104					
L11	114					
L12	148					
O1			146			
O2		187				
O3		172				
O4		140				
O5		165				
O6		137				

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4.2.2 cDNA hybridization libraries

Two-dimensional SDS-PAGE analysis revealed that the proteome of AS-35 was significantly influenced by specific culturing conditions. In order to establish whether these differences were similarly reflected at a transcriptional level, a genomic library was constructed. The genome of AS-35 was digested with *Sau*3A and ligated into *Bam*HI-digested pUC18 plasmid. Seven hundred and seven recombinant colonies were selected for further analysis. Colony hybridizations were performed using DIG-labeled cDNA prepared from AS-35 cultured in LB and 25% olive wastewater to late-exponential phase, respectively. Results indicated that 164 colonies contained genomic DNA homologous to the cDNA prepared from AS-35 cultured in 25% olive waste. An additional 150 colonies hybridized to cDNA prepared from AS-35 cultured in LB. Of the 314 colonies shown to contain actively transcribed genomic regions, 52 were present in both transcriptomes. The genome library clones contain not only the reading frame of genes present in the transcriptome, but flanking genomic regions as well. In order to avoid sequencing partial genes flanking the reading frame of interest, RNA subtraction libraries were constructed. These libraries use the transcriptome, thus avoiding sequence non-transcriptional DNA.

4.2.3 Subtractive RNA libraries

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A RNA subtraction library (Felske, 2002) was constructed in an attempt to identify genes uniquely associated with specific culturing conditions. A graphical representation of the methodology used is given in Figure 1.3. Total RNA was extracted from AS-35 cultured in LB and 25% olive wastewater (Figure 4.5). cDNA was synthesized using random hexameric oligonucleotide primers (Figure 4.6) and ligated to both combinations of adaptor sets, A6-long / Short-P and T7-long / Short. The ligations involving the A6-long / Short-P adaptor set were used to generate multiple driver amplicons (Figure 4.7). The driver DNA from AS-35 cultured in LB was hybridized to tester DNA from AS-35 cultured in 25% olive wastewater, in order to identify genes uniquely transcribed during growth in olive wastewater. The reciprocal driver (A6-long / Short-P – cDNA from AS-35 cultured in olive waste) and tester (T7-long / Short – cDNA from AS-35 cultured in LB) combination was used to identify genes uniquely transcribed during LB culturing. Hybridization mixtures were used as template DNA during PCR reactions in order to

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amplify, using primer T7, amplicons of tester-tester DNA hybrids (Figure 4.8). Amplicons were purified, cloned and sequenced. Inserts lengths ranging from 1500 bp to 300 bp were sequenced, but is was not practically possible to sequence all the clones generated in the library. The gene products were putatively identified using online Blast and Interpro sequence identification software. The search results are summarized in Table 4.2.

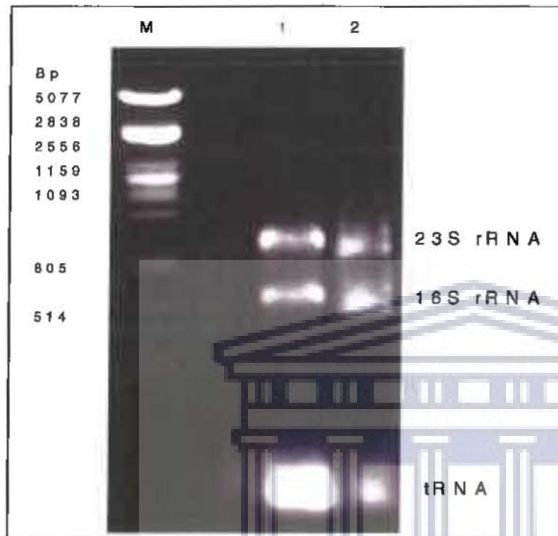

Figure 4.5. Agarose gel showing total RNA isolation from AS-35. RNA from AS-35 cultured in LB is in lane 1, while total RNA of AS-35 cultured in 25% olive wastewater is indicated in lane 2. The sizes of the DNA molecular weight marker, phage λ DNA digested with PstI, (lane M) are indicated.

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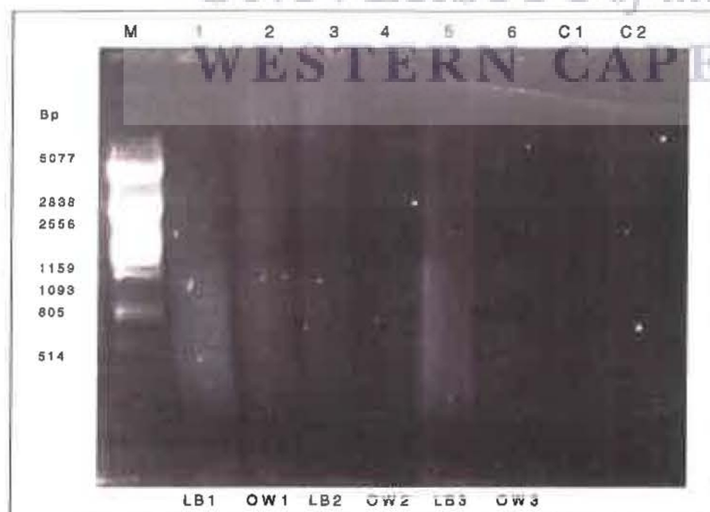

Figure 4.6. Agarose gel showing synthesized cDNA. Lanes 1-6 indicate AS-35 cDNA synthesized using total RNA from 3 different RNA extraction experiments. C1 is the negative control

containing no RNA, while C2 is the negative control where Taq DNA polymerase was used instead of reverse transcriptase.

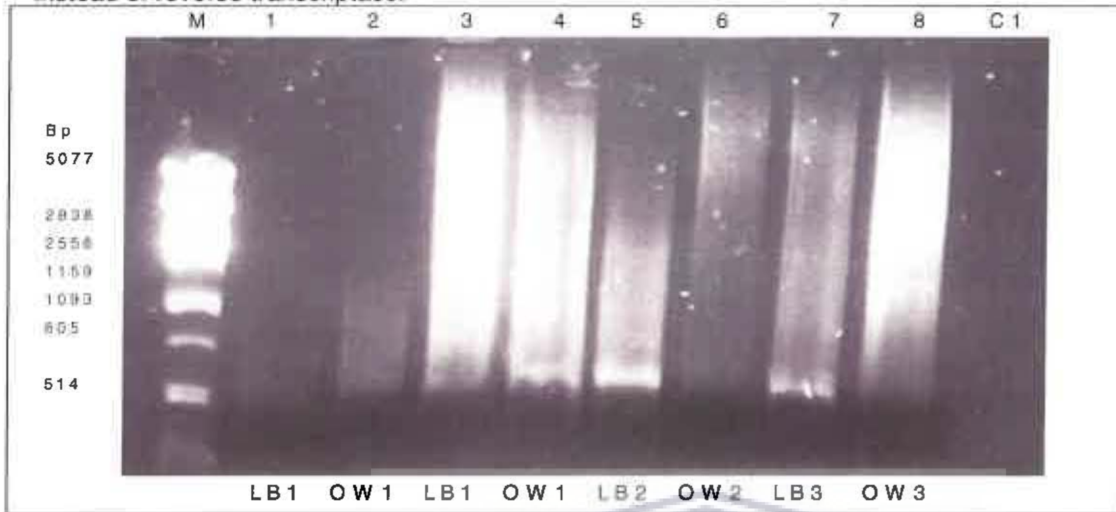

Figure 4.7. Agarose gel showing driver synthesis using AS-35 cDNA and primer A6. The sizes of the DNA molecular weight marker, phage λ DNA digested with PstI (lane M) are indicated. Lanes 1-2 represent driver synthesized from cDNA LB1 and OW1 (figure 3.4) using 1 µl ligation mixture. Lanes 3-8 represent driver amplicons of cDNA LB1, OW1, LB2, OW2, LB3 and OW3 where 2 µl of ligation mixture was used. C1 is a negative control performed with no DNA.

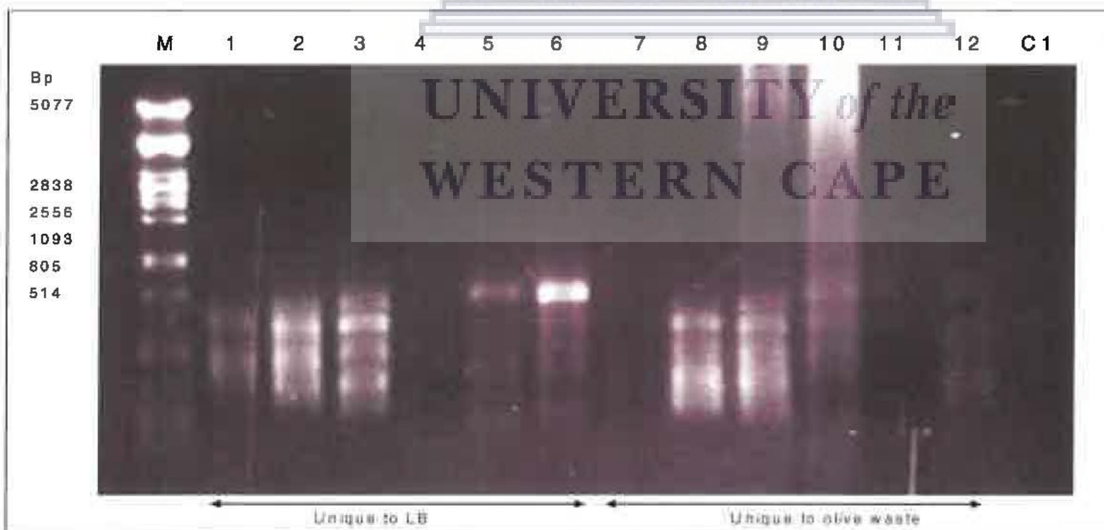

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Figure 4.8. Agarose gel showing the products of the hybridization PCR. The molecular weight sizes of lambda DNA digested with PstI is indicated left of the figure. Lanes 1-6 indicate hybridization PCR amplicons of AS-35 unique to LB, while lanes 7-12 represent hybridized PCR amplicons of AS-34 unique to olive waste media. Lane C1 is a DNA-minus control PCR reaction.

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4.2.3.1 Gene expression unique to growth in LB.

LB1 shared significant homology to specific subunits of several proteins including exodeoxyribonucleases, helicases or hydrolases. These proteins exhibit a wide variety of catalytic activities including ATP-dependent exonuclease, DNA-dependent ATPase activities, ATP-stimulated endonuclease and ATP-dependent helicase. In the presence of ATP, exonucleolytic catabolism occurs in either 5' to 3' or 3' to 5' to yield 5'-phosphooligonucleotides.

LB4 was identified as a putative phosphoribosyltransferase, glycosyltransferase or anthranilate transferase, all enzymes involved in aromatic amino acid biosynthesis. These proteins are actively involved in the synthesis of L-tryptophan by catalyzing N-(5-phospho-D-ribosyl)-antranilate and diphosphate to anthranilate and 5-phospho-alpha-D-ribose 1-diphosphate. This results in the redirection of the aromatic amino acid biosynthesis pathway in favor of tryptophan synthesis.

D1.1 and D1.4 were both putatively identified as transporter proteins. D1.4 was found to be homologous to pbuX, a transport protein involved in the uptake of xanthine or uracil. D1.1 shares significant homology with LysE, a protein involved in the secretion of L-lysine. During growth in media containing peptides rich in L-lysine, accumulation of the amino acid in the cell occurs, which is potentially toxic to the cell and are thus secreted (Simic *et al.,* 2001).

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L6G and L10H shared a high degree of homology. Both these proteins are homologous to *fla*R, a putative DNA modulation kinase. Proteins belonging to this family are involved in gene regulation during amino acid synthesis or carbohydrate metabolism.

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Table 4.2. Homology prediction for clones present in RNA library.

Sample	Unique to	Amino acids	Highest homology prediction
LB1	LB	180	gi|50083653|ref|YP_045163.1| exonuclease V, gamma chain [Ac... 228 6e-59
LB 3A	LB	63	gi|50086411|ref|YP_047921.1| putative transcriptional regul... 68 7e-11
LB 3B	LB	52	gi|11356712|pir||T44462 DNA-binding protein [imported] - St... 31 10.0
LB 4	LB	54	gi|50085544|ref|YP_047054.1| anthranilate phosphoribosyltra... 97 1e-19
D1.1	LB	47	gi|50084171|ref|YP_045681.1| putative amino acid transport ... 57 2e-07
D1.4	LB	164	gi|50083925|ref|YP_045435.1| putative transporter [Acinetob... 232 2e-60
L6G	LB	79	gi|23098537|ref|NP_692003.1| DNA topology modulation protein ... 62.4 1e-08
L10H	LB	173	gi|56962068|ref|YP_173791.1| DNA topology modulation protein ... 145 2e-33
OW 1	OW	170	gi|15812039|gb|AAL09091.1| DcaH [Acinetobacter sp. ADP1] >g... 223 2e-57
OW 4	OW	142	gi|52078952|ref|YP_077743.1| putative Short-chain dehydroge... 246 9e-65
A3.3	OW	93	gi|15419053|gb|AAK96892.1| biotin synthase [Acinetobacter c... 176 2e-43
D3.2	OW	77	gi|56420424|ref|YP_147742.1| myo-inositol catabolism protei... 174 8e-43
B6	OW	178	gi|56421324|ref|YP_148642.1| hypothetical protein GK2789 [Geo... 134 9e-31
D8	OW	84	gi|52078952|ref|YP_077743.1| putative Short-chain dehydrogena... 137 7e-32
F6	OW	143	gi|29897493|gb|AAP10769.1| Ribonuclease III [Bacillus cereus ... 179 7e-44
G6	OW	200	gi|23126820|ref|ZP_00108704.1| COG3391: Uncharacterized conse... 108 9e-23
G10	OW	240	gi|52078952|ref|YP_077743.1| putative Short-chain dehydrogena... 419 5e-116

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4.2.3.2 Gene expression unique to growth in 25% olive wastewater.

Putative protein OW1 was identified as a probable dehydrogenase with significant homology to the C-terminal sequence of 3-hydroxyacyl-CoA dehydrogenase. The enzyme 3-hydroxyacyl-CoA dehydrogenase is responsible for the reduction of 3-hydroxyl-CoA to 3-oxoacyl-CoA, during ß-oxidation of fatty acids or organic acid catabolism.

OW4, D8 and G10 appeared to be different parts of the same protein (see Appendices 3 and 4). This enzyme belongs to the family of short-chain dehydrogenases and reductases and shares significant homology with a hypothetical oxidoreductase ydaD and yhdF of *B. subtilis.* The function of ydaD is still unknown, but expression of this protein has repeatedly been associated with σ^B-dependent induction of the global stress response (Petersohn *et al.,* 2001., Höper *et al.,* 2005).

A3.3 was found to be homologous to BioB, an iron binding enzyme involved in the final steps of the biotin synthesis pathway (Cosper *et al.*, 2003) while D3.2, was homologous to the IolB protein involved in the myo-inositol catabolism.

F6 was homologous to a ribonuclease III, which is involved in the processing of ribosomal RNA precursors by the digestion of double stranded RNA.

G6 was homologous to the integrin alpha chain. These transmembrane proteins are receptors for cell adhesion to extracellular matrices.

4.3 Discussion

The composition of olive wastewater is of such a nature that it could be considered as an environment incapable of supporting abundant life (Rodriguez *et al.*, 1988). Microorganisms capable of surviving under these conditions are therefore likely to have developed specific adaptations in response to these stresses. However, such adaptations require the expenditure of limited resources and are thus tightly regulated (Shingler,

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2003). In line with this, the proteome of AS-35 was found to be influenced by specific culturing conditions. Proteome analyses were performed during late-exponential phase, when simple carbons, such as glucose, exhaustion occur. Proteins induced during glucose starvation belong to either the σ^B-dependent general stress regulon or to glucose-starvation-specific responses, which entails the activation of alternative carbon catabolism pathways. In *B. subtilus,* the onset of glucose starvation accompanied the *de novo* synthesis of 150 proteins, and the synthesis termination of 400 other proteins (Bernhardt *et al.*, 2003). During environmental stresses such as heat, salt, ethanol and peroxide, *Bacillus subtilis* general stress response transcription factor, σ^B, has been implicated in the transcriptional activation of 125 genes while 118 genes are σ^B-independently activated and 44 genes involved in the adaptation to salt and ethanol stress are known to be depend on the σ^W regulon (Petersohn *et al.*, 2001).

The changes in the proteome were closely mirrored at the transcriptional level with approximately a third of transcriptome unaffected by the nutrient states. RNA subtractive hybridizations were used to identify genes that were uniquely transcribed in AS-35 during growth in 25% olive wastewater. OW4, D8 and G10 were homologous to *yda*D of *B. subtilis*, a putative oxidoreductase, with unknown function. The transcription of *yda*D is known to be regulated during stress response by σ^B (Petersohn *et al.*, 2001). Mutational analysis revealed that this putative NAD dehydrogenase, are potentially involved during oxidative stress resistance. It is hypothesized that short-chain dehydrogenases act as generators of oxygen radicals, which protect the cytosolic membrane against stresses such as ethanol, salt and low temperatures (Höper *et al.*, 2005). Confirmation for this hypothesis was presented when a *yda*D mutant displayed significant sensitivity towards oxidative stress in growth-arrested *Clostridium perfringens* (Briolat and Reysset, 2002).

Several additional genes were identified that could be associated with the specific nutrient composition of the different culturing conditions. D1.4 was the only gene identified as uniquely expressed during culturing in LB that could be directly associated with abundance of a specific chemical compound. It was found to be homologous to the protein pbuX. In *B. subtilis* the *pbu*x gene forms part of the *xpt-pbuX* operon responsible

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for the usages of extracellular purines during nucleotide synthesis. pbuX is responsible for xanthine acquisition and is strictly controlled by the translation of xanthine phosphoribosyltransferase (*xpt*). When nitrogen is in excess, conditions similar to growth in LB, the purine catabolic pathway is repressed and the *xpt-pbuX* operon is induced. Conversely during nitrogen limitation (as in olive wastewater, Table 1.1), cells utilize purines as a nitrogen source and the purine catabolic pathway is induced while the expression of the *xpt-pbuX* operon is repressed (Christiansen *et al.,* 1996).

Putative genes identified as being unique to the transcriptome of AS-35 cultured in olive waste included a 3-hydroxyacyl-CoA dehydrogenase (OW1) and a biotin synthase (A3.3). The putative 3-hydroxyacyl-CoA dehydrogenase is involved in the ß-oxidation catabolism of fatty or organic acids. Organic acids are a major constituent of olive wastewater and could potentially be used as the predominant carbon and energy source under these conditions (Mulinacci *et al.,* 2001). BioB, the biotin synthase, is the last enzyme involved in the synthesis of biotin and responsible for the conversion of dethlobiotin to biotin. Biotin is important co-factor required during certain carboxyl transfer reactions or carbon dioxide fixation and is synthesized by gene products of the *bio*WAFDBI operon in *B. subtilis*. The *bio*WAFDBI operon is negatively regulated by the presence of biotin and the bifunctional protein BirA. Fatty acids and pimelic acid in particular, are used as starting materials for the synthesis of biotin (Rodionov *et al.*, 2002). Thus, two putative proteins (OW1 and A3.3) involved in different metabolic pathways, which both uses fatty or organic acids as starting materials were identified in AS-35 cultured in olive wastewater.

In addition, D3.2 was found to be homologous to IolB, a protein with unknown function. IolB forms part of the *iol*ABCDEFGHIJ operon of *B. subtilis* involved in the catabolism of myo-inositol. The operon is negatively repressed during the presence of glucose by catabolite repressor CcpA. The latter recognizes and binds to one of two catabolite-responsive elements (*cre*'s), of which one is located in *iol*B. In the absence of glucose and presence of inositol, the operon is induced by the IolR induction system located on a second operon *iol*RS (Miwa and Fujita, 2001). The identification of IolB in AS-35

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cultured in olive wastewater implies that negative-regulation generally associated with the presence of glucose is abolished and that alternative carbons sources such as inositol were utilized.

Two putative proteins, L6G and L10H, identified in LB were homologous to a DNA modulating regulator FlaR. This transcriptional regulator activates transcription by modulation of the superhelicity of the template DNA. FlaR is the best known regulator in this family, which also includes the transcriptional control of *pro*U in *E. coli*. FlaR is thought to play a role in flagellal regulation during chemotaxis (Sleator *et al.*, 2003). Due to the glucose starvation associated with stationary phase, chemotaxis could be a method for the cells to find available nutrients.

In contrast to the gradual adaptation of the isolate to environmental conditions associated with olive waste wastewater, adaptation to a nutrient rich environment appeared to occur more readily. The efficiency of bacteria to adapt to changing nutrient conditions is not solely determined by the organisms' ability to activate novel genes or biochemical pathways, but more so in its ability to judge the impact these transcriptional activations would have on the its physiological and metabolic state. Bacteria exposed to olive wastewater are confronted with various signals, which include nutrient availability, osmolarity, chaotropic agents, and interaction with surfaces or other cells. It is vitally important for the organism to direct its response in order to sustain energy and cell viability. In contrast, when bacteria are exposed to a nutrient rich environment the initial response is to determine preferential carbon catabolism of nutrients prior to rapid growth (Cases and de Lorenzo, 2001).

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Chapter 5

Chemical mutagenesis of AS-35

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5.1 Introduction

Fermentation using micro-organisms naturally found in olive wastewater has been suggested as an alternative method of bioremediation of the wastewater. Even though these organisms are adapted to this toxic environment, the bioremediation process can be time-consuming and inefficient for the removal of phenolic compounds and reduction of COD (Ayed and Hamdi, 2003). However, it has previously been shown that these limitations can be overcome by the genetic manipulation of micro-organisms resulting in the enhanced degradation of xenobiotic compounds (van der Meer *et al.*, 1992).

The physical effects of genetic manipulation or mutation can be broadly classified as either microlesions, where only one or two base changes occur, or macrolesions which involves large sections of DNA. Microlesions effect changes in biochemical pathways through the introduction of nonsense mutations, insertions or deletions resulting in the inactivation of affected genes. In the case of macrolesions, large pieces of DNA can be deleted, duplicated or inverted, each producing mutations with various degrees of success (Caldwell, 1995).

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Generally, artificial mutations to genes known to be involved in specific biochemical pathways are accomplished by knockout or site directed mutagenesis (Egland *et al.*, 1997). Both approaches require a preexisting knowledge of the target sequence and often involve the introduction of foreign DNA. Even with the recent advances in genomics, the genetic sequence of interest is not always known, nor is it always desirable to introduce foreign DNA to a system. Random mutagenesis can be applied in these situations. Techniques included under this broad category are DNA shuffling, error-prone PCR, and classical UV or chemical mutagenesis. These methods require no previous knowledge of the organism's genome, and produces mutants that can safely be used in industrial applications.

Chapter 3 describes the isolation of several micro-organisms capable of the bioremediation of olive wastewater. Even though several of these isolates were shown to

be capable of removing low molecular weight phenolic compounds, the process kinetics coupled with their inability to tolerate high concentrations of olive wastewater might limit their application in bioprocesses. In an attempt to enhance the biodegradation capabilities of isolate AS-35, this organism was subjected to chemical mutagenesis. The biodegradation characteristics of putative mutant isolates were subsequently compared to that of the wild type.

5.2 Results

5.2.1 Selection of a putative chemically-induced mutant isolate

Isolate AS-35 was incapable of growth on olive wastewater agar at concentrations exceeding 50%. Cell viability against formamide was tested and 100 µl/ml of the mutagen was found to be suitable for mutagenesis. Cell suspensions were treated with formamide and plated on selective medium containing 75% olive wastewater. Various hyper-resistant putative mutants were obtained, 8 of which were selected for further characterization. Putative mutants were compared to the wild type based on peroxidase production, their ability to reduce the total phenol content and decolourization of olive wastewater (Table 5.1). All the putative mutants showed an increase in peroxidase activity when compared to the wild type. While none of the putative mutants reduced the dark colour of the waste to the same extent as the wild type, three mutant isolates (AF5, BF1 and DF4) exhibited levels of total phenol reduction similar to that of the wild type. Based on the results in Table 5.1 as well as the mutant's growth capabilities, mutant DF4 was subsequently selected for further analysis.

5.2.2 Isolate identification

Analysis of the 16S rRNA gene was performed on AS-35 and DF4, cultured in both LB and 25% olive wastewater, in order to verify the identity of the mutant (Appendix 2). Bi-directional sequence analyses of the complete 1.5 kb gene region indicated that they were identical pure cultures and were identified as a *Bacillus* sp. (Table 5.2). API 50 CHB biochemical tests identified the isolate as *Bacillus megaterium.* The results of the API

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tests also indicated a range of chemicals that could be metabolized as sole carbon sources (Table 5.3).

Table 5.1. Biodegradation characteristics of wild type AS-35 were compared to the putative mutants. The peroxidase activity (µM/min), total phenol reduction (%/day) and total decolorization of the olive waste (%/day) of wild type AS-35 is compared to that of the 8 mutant isolates. Both the reduction in total phenol content and decolorization is represented as a percentage of the initial concentration (mM) or initial optical density (OD525nm).

Sample	Peroxidase activity (µM/min)	Total phenol reduction %	Total Decolorization %
AS-35	0.3	28.9	12. 5
AF 1	0.9	4.0	3.6
AF 5	1.7	34.4	9.5
BF 1	2.4	29.1	8.2
BF 2	2.9	2.5	7.1
BF 5	1.8	14.4	3.0
DF 4	2.7	28.5	7.8
DF 5	2.0	10.2	6.1
DF 6	0.6	6.9	3.6

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Table 5.2. BLAST results of wild type AS-35 and mutant DF4 16S rDNA gene analysis.

Isolate	bp	
AS-35 LB	1350	gi|46367731|dbj|AB126771.1| Bacillus sp. TW4 gene for 16S r... 1279 0.0 gi|15076638|dbj|AB066347.1| Bacillus sp. No.49 gene for 16S... 1277 0.0
AS-35 OW	1310	gi|46367731|dbj|AB126771.1| Bacillus sp. TW4 gene for 16S r... 1283 0.0 gi|15076638|dbj|AB066347.1| Bacillus sp. No.49 gene for 16S... 1281 0.0
DF 4 LB	1516	gi|15076638|dbj|AB066347.1| Bacillus sp. No.49 gene for 16S... 2960 0.0 gi|15076636|dbj|AB066345.1| Bacillus sp. No.54 gene for 16S... 2960 0.0
DF 4 OW	1553	gi|15076638|dbj|AB066347.1| Bacillus sp. No.49 gene for 16S... 2930 0.0 gi|15076636|dbj|AB066345.1| Bacillus sp. No.54 gene for 16S... 2930 0.0

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Table 5.3. Tests for growth utilizing sole carbon sources.

Active ingredients	Reaction	Active ingredients	Reaction
Glycerol	+	Salicin	+
Erythriol	-	D-Cellobiose	+
D-Arabinose	-	D-Maltose	+
L-Arabinose	+	D-Lactose	+
D-Ribose	+	D-Melibiose	+
D-Xylose	+	D-Saccharose	+
L-Xylose	-	D-Trehalose	+
D-Adonitol	-	Inulin	-
Methyl-ß-D-xylopyranoside	-	D-Melezitose	+
D-Galactose	+	D-Raffinose	+
D-Glucose	+	Amidon	+
D-Fructose	+	Glycogen	+
D-Mannose	-	Xylitol	-
L-Sorbose	-	Gentiobiose	+
L-Rhamnose	-	D-Turanose	+
Dulcitol	-	D-Xylose	-
Inositol	+	D-Tagatose	-
D-Mannitol	+	D-Fucose	-
D-Sorbitol	-	L-Fucose	-
Methyl-α-D-mannopyranoside	-	D-Arabitol	-
Methyl-α-D-glucopyranoside	-	L-Arabitol	-
N-Acetyl-glucosamine	+	Potassium gluconate	-
Amygdalin	+	Potassium 2-ketogluconate	-
Arbutin	+	Potassium 5-ketogluconate	-
Esculin ferric citracte	+		

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5.2.3 Growth kinetics of wild type AS-35 compared to mutant DF4

Several reports have noted that mutagenesis may influence the growth rate of mutant isolates (Kadurugamuwa *et al.*, 1993; Hoang *et al.*, 2000). It is possible that the phenotypic effect following mutagenesis may be due to growth impairment of the strain rather than alteration of specific genes. Thus, to investigate whether the introduced mutations influenced the growth properties of the mutant, the wild-type AS-35 as well as mutant DF 4 strain were cultured in LB-broth and 25% olive wastewater and their growth assessed. A higher concentration of olive waste could not be used due to the turbidity of olive wastewater. Analysis of the growth kinetics showed that AS-35 exhibited almost 2.5 times faster generation time when cultured in LB compared to the mutant DF4, but similar rates, calculated based on generation time, were observed for the isolates when cultured in 25% olive waste (Figure 5.1 and Table 5.4).

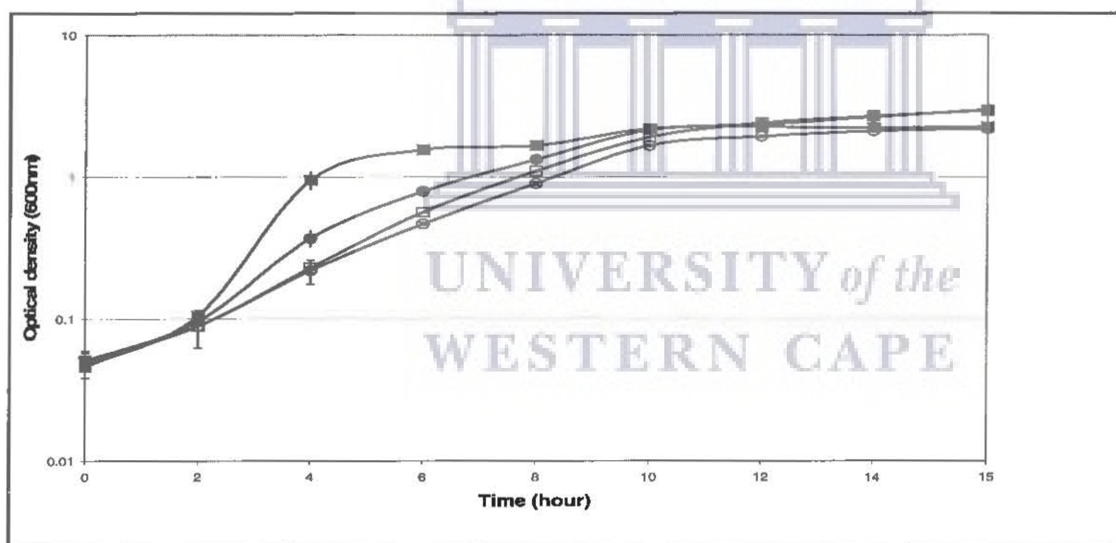

Figure 5.1. A logarithmic representation of the growth profiles (OD600nm) of wild type AS-35 and mutant DF4. AS-35 ■ and DF4 ● cultured in LB were compared to AS-35 □ and DF4 ○ cultured in 25% olive waste.

Previous studies have highlighted the necessity for nitrogen supplementation of olive waste to sustain microbial growth (Nganwa, 2000). To access the influence of the availability of nitrogen on the growth rates of AS-35 and DF4, both isolates were cultured in the absence of additional nitrogen or in the presence of an alternative nitrogen

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source (0.5% $Ca(NO_3)_2$). In addition, the influence of initial pH on the growth rate of the cultures was investigated by culturing in 25% olive waste containing 0.5% yeast extract at pH 4, 6, and 8, respectively.

Table 5. 4. Growth Kinetics of wild type AS-35 and mutant DF4

	Wild type AS-35				Mutant DF4			
Growth media	Lag phase (hours)	μ / hour	g / hour	g / min	Lag phase (hours)	μ / hour	g / hour	g / min
LB	>2	1.1	0.6	37.9	2	0.4	1.6	96.8
25% olive waste + 0.5% yeast extract	>2	0.6	2.0	118.4	>2	0.3	2.0	120.6
25% olive waste	>4	0.3	2.4	145.3	±2	0.4	1.9	112.7
25% olive waste + 0.5% $Ca(NO_3)_2$	±2	0.1	4.8	293.4	±1	0.3	2.1	123.1
25% olive waste + 0.5% yeast extract (pH 4.0)	±2	0.2	3.3	196.4	±2	0.2	3.2	192.2
25% olive waste + 0.5% yeast extract (pH 6.0)	±4	0.4	1.7	104.1	±2	0.3	2.2	133.3
25% olive waste + 0.5% yeast extract (pH 8.0)	±2	0.3	2.3	139.1	±2	0.2	3.0	182.0

μ is $Ln(Ys/Ye)/\Delta t$, where Ys is the absorbency at the start of stationary phase and Ye is the absorbency at the start of exponential phase. Δt is the difference in time (hours) of Ys and Ye.
g is the generation time of the organism.

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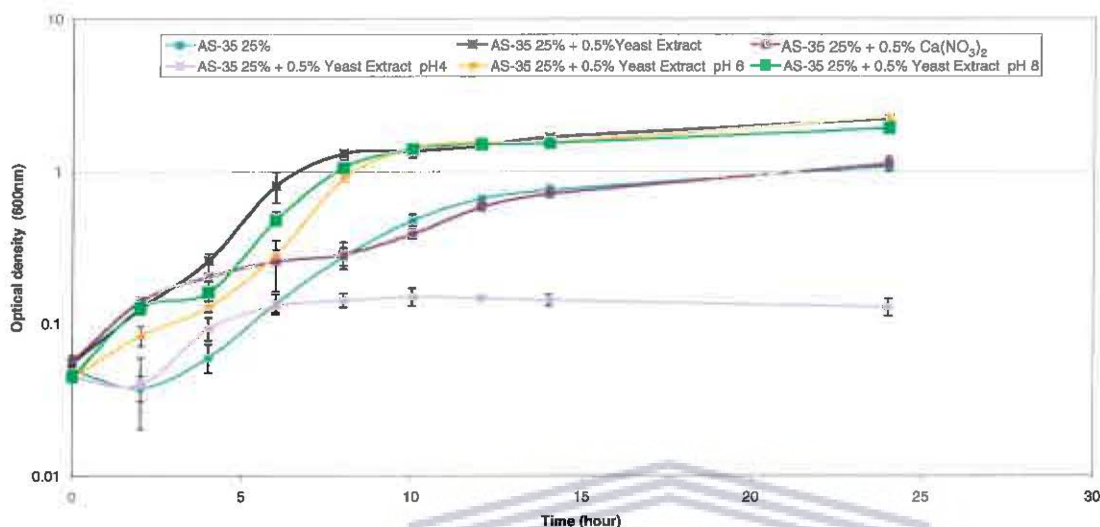

Figure 5.2. Growth profiles of wild type AS-35. The wild type was repeatedly cultured in 25% olive waste containing different nitrogen sources or 25% olive waste containing 0.5% yeast extract with different initial pH values.

Although AS-35 was capable of growth in 25% olive waste without any additional nitrogen sources, its growth rate increased by 20% in the presence of 0.5% yeast extract (Figure 5.2 and Table 5.4). The growth rate was not restored by the addition of an alternative nitrogen source, $Ca(NO_3)_2$, suggesting that yeast extract provide the organism with other nutrients (Figure 5.3 and Table 5.4). The growth profiles of DF4 closely mirrored that of AS-35 even though the mutant does not exhibit a decrease in growth rate in the absence of yeast extract (Figure 5.4 and Table 5.4). However, DF4 appeared to adjust more rapidly to olive waste constituents. This is indicated by the shorter lag phase compared to wild type AS-35 under these conditions (Figure 5.4 and Table 5.4). The growth rates of the isolates were found to be dependent on the initial pH of the culture media. In both cases, growth was significantly impaired in an acidic environment (Figures 5.2 and 5.3).

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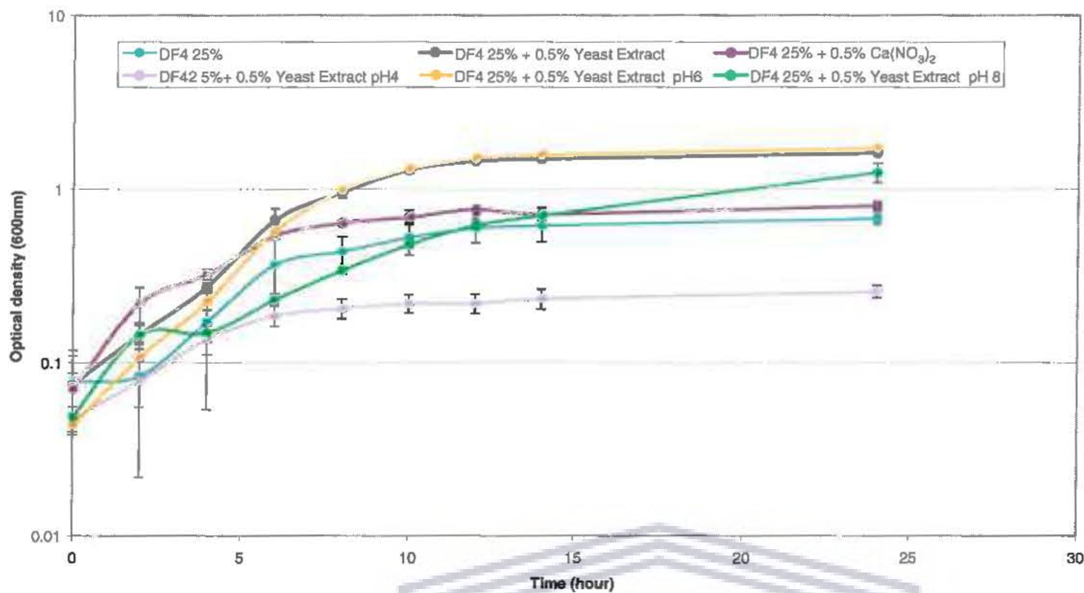

Figure 5.3. Growth profiles of mutant DF4. Mutant DF4 was cultured in 25% olive waste containing different nitrogen sources or 25% olive waste containing 0.5% yeast extract with different initial pH values.

During the previous comparison of phenol reduction in 25% olive waste by AS-35 and DF4, no significant difference in the level of total phenol reduction was observed. However, the biodegradation capacity of DF4 appeared to be enhanced in the presence of additional stress factors, such as nutrient limitation and acidity (Figure 5.5).

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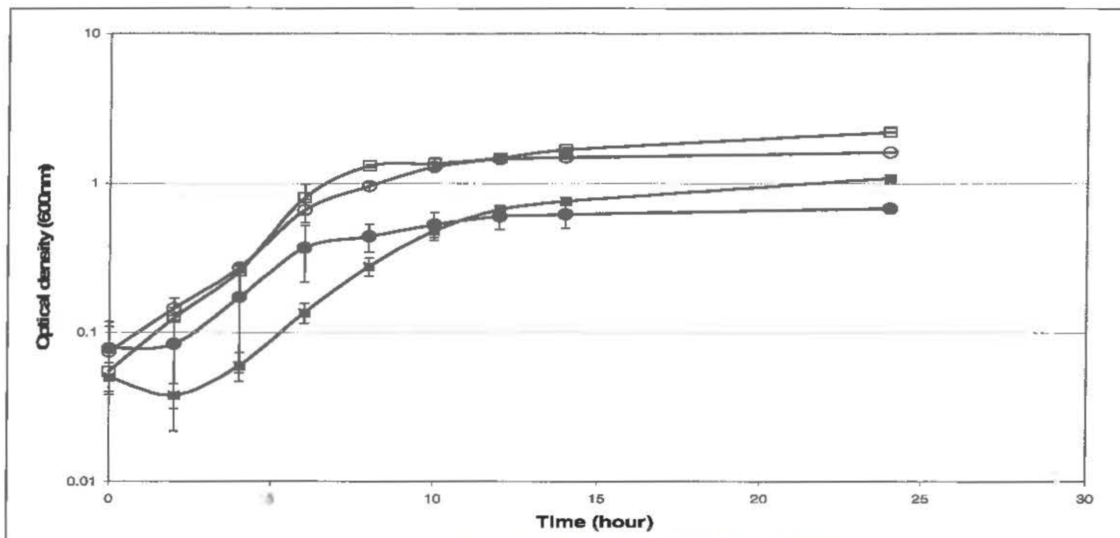

Figure 5.4. Growth profiles of wild type AS-35 and mutant DF4. AS-35 ■ and DF4 ● cultured in 25% olive wastewater, with no additional nitrogen source, were compared to AS-35 □ and DF4 ○ cultured in 25% olive waste containing 0.5% yeast extract. The 0.5% yeast extract served both as an additional nitrogen and carbon source.

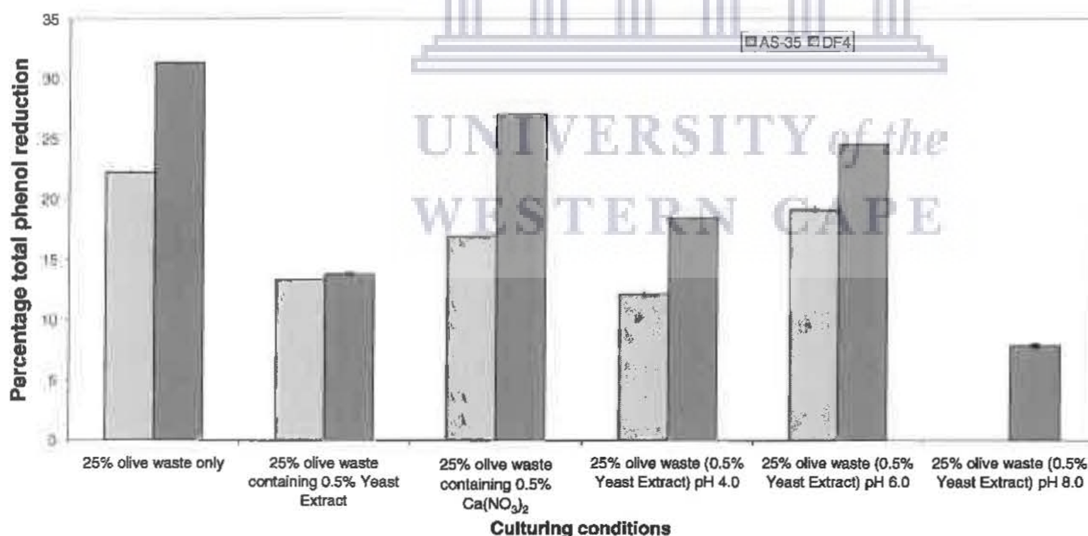

Figure 5.5. Total phenol reduction of AS-35 and DF4 cultured in 25% olive waste containing either various nitrogen sources or 0.5% yeast extract with different initial pH values. Total phenol reduction after 14 hours is represented as a percentage of the initial phenol concentration (mM).

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5.2.4 Morphological characterization of AS-35 and DF4

Microscopic analysis clearly revealed enlarged cell morphology in the case of mutant DF4 as compared to the wild type AS-35 when cultured in LB. A similar enlarged morphology was observed for the wild type when cultured in 25% olive waste (Figure 5.6). These differences in cell morphology correlated with the differences observed in growth rates.

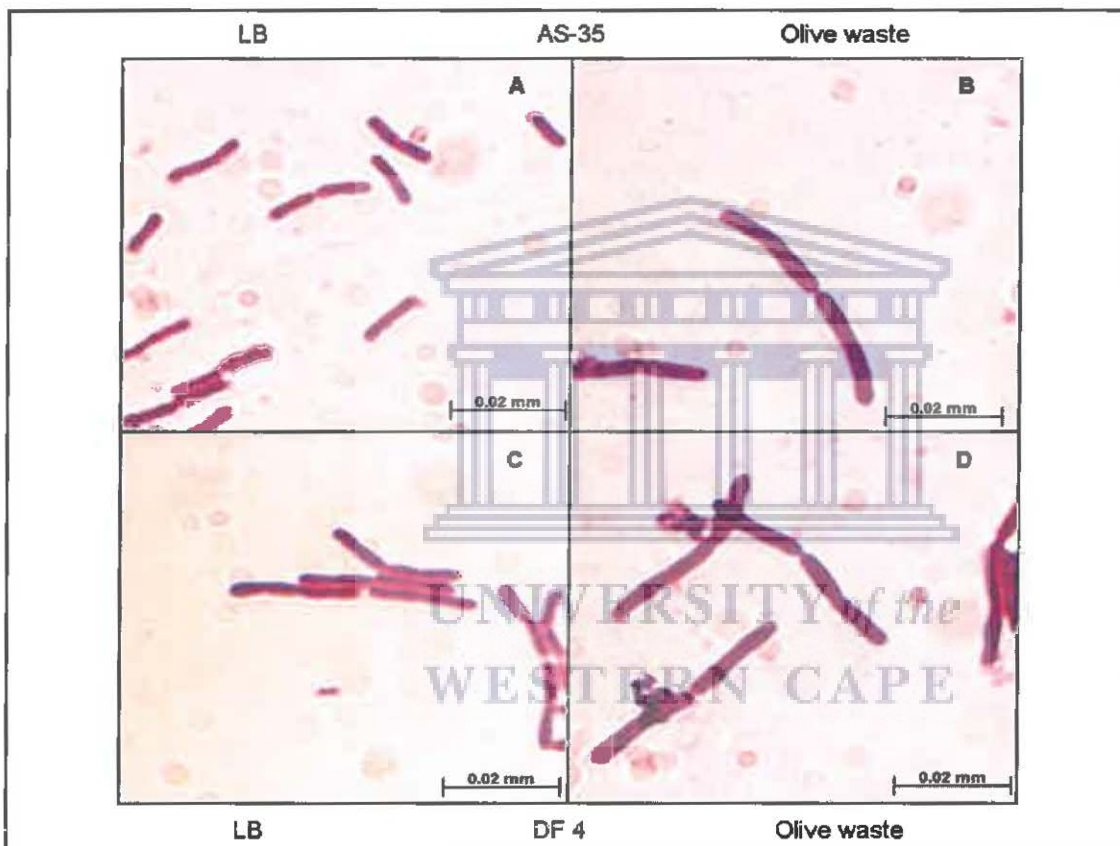

Figure 5.6. Photomicrographs showing the cellular morphological phenotypes of the wild-type AS-35 and mutant DF4 after culturing in different media. Wild type AS-35 is shown in Figures A and B, while the mutant DF4 is shown in Figures C and D. Figures A and C show the different isolates cultured in LB, while Figures B and D show the isolates after culturing in 25% olive waste media. All the cells were stained with crystal violet. The scale bar represents 0.02mm.

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5.2.5 Proteomic analysis of AS-35 and DF4 protein expression

The total cellular protein profile of wild type AS–35 and mutant DF4 cultured in LB and 25% olive waste was analyzed by SDS-PAGE (Figure 5.7). Cultures were sampled at time-points corresponding to late-exponential phase (OD600nm 1.0). Analyses of the respective protein profiles revealed that the expression profile of AS–35 grown in LB differed noticeably from the profile of the same isolate grown in the presence of 25% olive waste. In contrast, the expression profile of DF4 remained unchanged irrespective of the culturing media, and closely resembled that of AS–35 grown in the presence of olive waste.

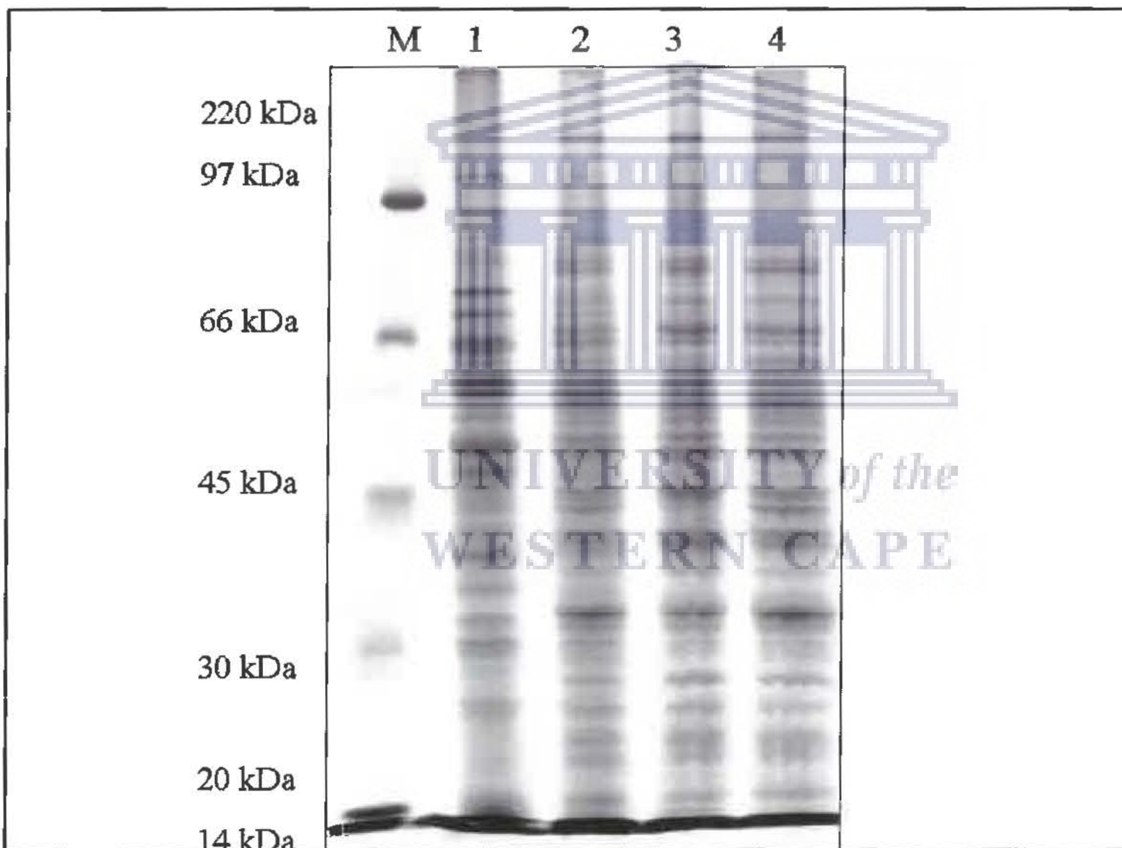

Figure 5.7. Total protein profiles of wild type AS-35 and mutant DF4 cultured in LB and 25% olive waste. Lanes 1 and 2 represent AS-35 protein profile cultured in LB and 25% olive waste, respectively. Protein profiles of mutant DF4 are indicated in lanes 3 and 4, with lane 3 representing the mutant cultured in LB and lane 4 in 25 % olive waste

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Differences in protein profiles of AS-35 and DF4 were further delineated using 2D PAGE analysis in a pH 3 to 10 range. Images representing each of the relevant profiles are shown in Figure 5.8. Duplicates of each gel were analyzed using PD Quest™ 7.4.0 gel analysis software (Bio-Rad). This software creates a three-dimensional Gaussian image of every spot and compares these images between the master template and the other gels in the analysis group. This generates a matched set consisting of corresponding spots. The results of the comparative analysis using DF4 cultured in 25% olive wastewater as master template are summarized in Table 5.5.

Table 5.5. Protein comparisons using PD Quest of two-dimensional gels depicted in Figure 5.8.

Gel	Number of spots	Number of spots matched	Match rate
AS-35 olive waste	221	69	31%
AS-35 LB	184	45	30%
DF 4 olive waste*	246	246	100%
DF 4 LB	207	52	25%

* Master template.

The comparative match set of the 8 gels were exported from PD Quest and further analyzed using the Ludesi freeware service (www.ludesi.com). Normalized amalgamations of the duplicate gels were used for inter-group comparisons. The levels of significance of the results were assessed using ANOVA. The principle of ANOVA is to compare multiple samples based on their means (Samuels, 1989). Probabilities of less than 0.05 were considered to be significant.

The protein profile of AS-35 cultured in LB shared more similarities to the mutant DF4 cultured in 25% olive waste that the wild type cultured in olive waste (Figure 5.8 and Table 5.5). Although the profiles of DF4 cultured in both LB and 25% olive waste closely resembled that of AS-35 cultured in 25% olive waste, a number of distinct differences between these profiles were identified. A total of 16 proteins were putatively identified using Maldi-TOF mass spectrometry (Figure 5.8). Differences in expression of these proteins are clearly reflected in the spot volumes associated with each of the

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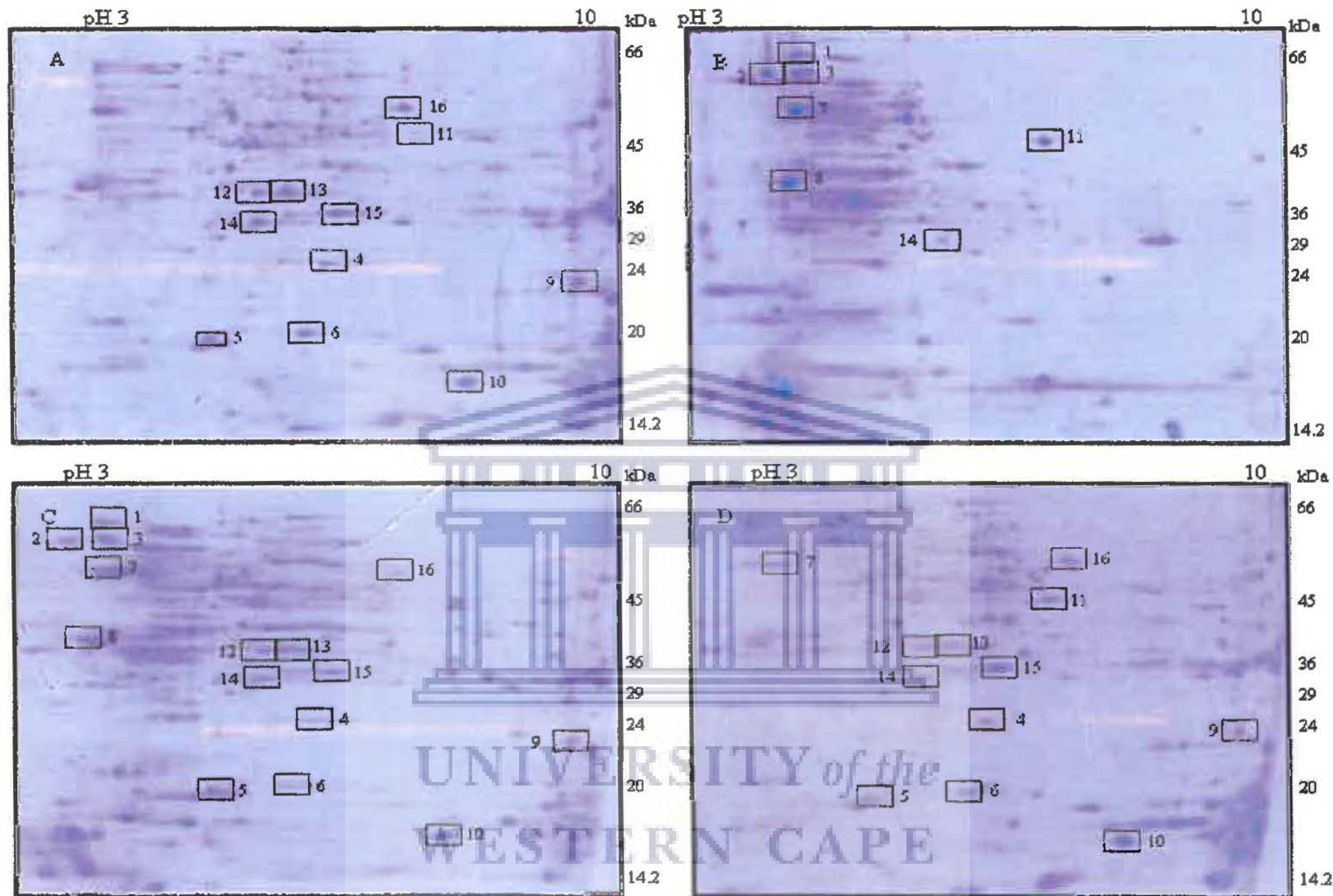

Figure 5.8. Two-dimensional images of total protein profiles of AS-35 (A and B) and DF 4 (C and D). Figures A and C represent the isolates cultured in 25% olive waste, while figures B and D represent the isolates cultured in LB.

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proteins (Table 5.6). Three proteins (1, 3 and 7) have a significant p-value of less than 0.05, while another 3 proteins 9, 15 and 16 have a p-value of less than 0.1. The remaining 10 proteins could not be positively identified, due to a lack in sequence availability for *B. megaterium* (Table 5.7).

Table 5.6. Volumes based on Gaussian images for each spot marked in Figure 4.8. Both gel duplicates are presented. The protein with the highest density and subsequently highest Gaussian volume is indicated next to the bar representing its volume.

Spot number	AS-35 LB	AS-35 25%	DF4 LB	DF4 25%
1 / L1	11454			
2 / L2	36441			
3 / L3	66317			
4 / O2				15924
5 / O3		31514		
6 / O7			26885	
7	89429			
8	79999			
9		22959		
10		68462		
11	62849			
12		50161		
13		28449		
14			32370	
15			45746	
16			28531	

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Table 5.7. Mascot homology prediction of protein identification using Maldi-TOF.

Spot number	p	pI	Mw (kDa)	Highest homology predicted
1 / L1	<0.05	4.42	65.3	[gi:39629] Putative DnaK, *Bacillus megaterium*
2 / L2	<1.0			No significant homology
3 / L3	<0.05	4.62	57.3	[gi:6526961] GroEL, *Bacillus* sp. MS
4 / O2	<1.0			No significant homology
5 / O3	<1.0			No significant homology
6 / O7	<1.0			No significant homology
7	<0.05	4.4	46.4	[gi:47567341] Enolase, *Bacillus cereus* G9241
8	<1.0			No significant homology
9	<0.1	8.37	26.4	[gi:68192672] GntR, *Mesorhizobium* sp. BNC1
10	<1.0			No significant homology
11	<1.0			No significant homology
12	<1.0			No significant homology
13	<1.0			No significant homology
14	<1.0			No significant homology
15	<0.1	5.02	36.3	Bsu1596 FtsY, *Bacillus subtilis* 168
16	<0.1	7.67	55.0	[gi:13473458] Putative ATP synthase, *Mesorhizobium loti* MAFF303099

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A similar approach was applied for samples analyzed on gels with a pI range of 6 to 11. However, no additional significant differences were observed.

5.2.6 Subtractive RNA libraries

A RNA subtraction library (Felske, 2002) was constructed as described in section 4.2.3, in an attempt to identify genes uniquely associated with specific culturing conditions in wild type and mutant strains. Total RNA was extracted from late-exponential phase AS-35 and DF4 cultured in LB and 25% olive wastewater. Subtractive hybridization analysis

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of combinations between mutant and wild type were investigated. Mutant DF4 cultured in 25% olive waste was compared to mutant DF4 cultured in LB. Mutant and wild type cultured in 25% olive waste and LB were compared with their respective counterparts. The gene products were putatively identified using online Blast and Interpro sequence identification software (Appendix 5). The search results are summarized in Table 5.8.

Table 5.8. Homology prediction for clones present in RNA library.

Sample	Unique to	Compared to	Amino acids	Highest homology prediction
1 A 1	DF4 LB	DF4 OW	77	gi|29894471|gb|AAP07761.1| Oxidoreductase [*Bacillus cereus* ATCC 14579], Expect = 4e-22 Identities = 52/77 (67%), Positives = 63/77 (81%),
1 A 3	DF4 LB	DF4 OW	54	gi|48853958|ref|ZP_00308123.1| 3-oxoacyl-[acyl-carrier-protein] synthase III [*Cytophaga hutchinsonii*], Expect = 3e-08 Identities = 26/33 (78%), Positives = 32/33 (96%),
1 A 8	DF4 LB	DF4 OW	45	gi|28894910|ref|NP_799509.1| hypothetical protein [*Bacillus megaterium*], Expect = 3e-18 Identities = 45/46 (97%), Positives = 45/46 (97%),
1 B 2	DF4 OW	DF4 LB	85	gi|42781884|ref|NP_979131.1| DNA topology modulation protein FlaR, putative [*Bacillus cereus* ATCC 10987] Expect = 2e-10 Identities = 30/68 (44%), Positives = 48/68 (70%),
2 A 1	DF4 LB	AS-35 LB	292	gi|2632719|emb|CAB12226.1| ydaD [*Bacillus subtilis* subsp. subtilis str. 168] gi|3123232|sp|P80873|GS39_BACSU General stress protein 39 (GSP39) gi|1881230|dbj|BAA19257.1| BELONGS TO THE INSECT-TYPE ALCOHOL DEHYDROGENASE / RIBITOL DEHYDROGENASE FAMILY. [*Bacillus subtilis*] Expect = 3e-126 Identities = 221/272 (81%), Positives = 242/272 (88%),
2 A 6	DF4 LB	AS-35 LB	102	gi|23129019|ref|ZP_00110853.1| COG3293: Transposase and inactivated derivatives [*Nostoc punctiforme* PCC 73102] Expect = 5e-31 Identities = 84/176 (47%), Positives = 118/176 (67%),
2 B 2	AS-35 LB	DF4 LB	119	gi|28894909|ref|NP_799507.1| hypothetical protein [*Bacillus megaterium*], Expect = 2e-40 Identities = 78/82 (95%), Positives = 81/82 (98%),
3 A 3	DF4 OW	AS-35 OW	123	gi|52142716|ref|YP_084113.1| DNA topology modulation protein [*Bacillus cereus* E33L], Expect = 2e-20 Identities = 51/112 (45%), Positives = 76/112 (67%)
3 B 1	AS-35 OW	DF4 OW	170	gi|33348045|gb|AAQ15446.1| PseT polynucleotide 5'-kinase and 3'-phosphatase [*Enterobacteria* phage RB49], Expect = 4e-31 Identities = 73/167 (43%), Positives = 102/167 (61%)

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5.2.6.1 Comparing the transcriptomes of mutant DF4 cultured in 25% olive waste and LB

No significant differences were observed when comparing the morphology and protein profiles of mutant DF4 cultured in 25% olive waste and LB (see Sections 5.2.4 and 5.2.5). Comparative subtraction hybridization of the transciptome of mutant DF4 cultured under these conditions identified a putative 3-oxoacyl-synthase III, an oxidoruductase and a DNA topology modulator.

1A3 is a putative gene uniquely expressed in DF4 cultured in LB. 1A3 showed significant homology with FabH a 3-oxoacyl-[acyl-carrier-protein] from *B. subtilis*. It initiates fatty acid synthesis by catalyzing the condensation of acetyl-coenzyme A with an acyl carrier protein. The substrate specificity of FabH is the determining factor for branch chain fatty acid synthesis by type II fatty acid synthase. Fatty acid synthesis is important for membrane, spore and poly-B-hydroxybutyric acid formation (Choi *et al*., 2000).

One gene uniquely expressed in DF4 when cultured in olive waste was 1B2, a protein with homology to DNA topology modulation protein, FlaR. This protein is homologous to L6G and L10H, identified during the comparison of AS-35 cultured in LB to the wild type cultured in olive wastewater (see Section 4.2.3.1). This transcription factor regulates transcription by modulating the superhelix of the template DNA. It has been associated with chemotaxsis in various enterobacteria (Goyard and Bertin, 1997).

5.2.6.2 Comparing the transcriptomes of wild type AS-35 and mutant DF4 cultured in LB

Significant phenotypic and proteomic differences were observed when comparing wild type AS-35 and mutant DF4 cultured in LB. Putative gene 2A1 uniquely expressed in mutant DF4 showed significant homology to ydaD short-chain dehydrogenases of *B. subtilis*. Similar homologues, OW4, D8 and G10, were identified during the comparison of AS-35 cultured in 25% olive waste to the wild type cultured in LB (see Section 4.2.3.2) (Appendix 3). The function of ydaD in *B. subtilis* is still unknown, but

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indications are that expression of the protein is under the control of the σB- dependent general stress response. These NAD(P)-dependent dehydrogenases are thought to play a role in the intracellular balance of the redox potential during stress conditions (Petersohn et al., 1999).
The second putative protein (2A6) uniquely expressed in mutant DF4 was homologous to a phage transposase.

Gene 2B2, unique to wild type AS-35, was homologous to a group of diverse lipocalins. These proteins are involved in various processes, including ligand-bound transporting of nutrients, control of cell regulation, pheromone transport, cryptic colouration, and the enzymatic synthesis of prostaglandins.

5.2.6.3 Comparing the transcriptomes of wild type AS-35 and mutant DF4 cultured in 25% olive waste

Gene 3A3, unique to mutant DF4 cultured in olive wastewater, shows significant homology with previously identified proteins 1B2, L6G and L10H (see Sections 4.2.3.1 and 5.2.6.1) (Appendix 4).

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The last protein 3B1, uniquely expressed in wild type AS-35 was homologous to PseT, a polynucleotide 5'-kinase and 3'-phosphatase.

5.3 Discussion

Chapter 3 describes the isolation and selection of organisms capable of growth in and bioremediation of olive wastewater. Based on its ability to metabolize phenolic compounds present in olive wastewater, isolate AS-35 was selected for further analysis. AS-35 was subjected to genetic manipulation by chemical mutagenesis in an attempt to enhance its biodegradation capacity. Chemicals that have been shown to act as effective mutagenic agents include ethyl methanesulfonate (EMS) and N-methyl-N'-nitro-N-nitrosguanidine (MNNG) (Ibrahim and O'Sullivan, 2000). Certain of these mutagens

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have previously been shown to be ineffective in mutating *Bacillus* sp. (4-nitroquinoline-1-oxide) and *Lactobacillus* sp. (4-nitroquinoline-1-oxide, MNNG and 1,2-dimethylhydrazine) (Caldini *et al*, 2002). For *Bacillus megaterium* AS-35, a common DNA denaturing mutagen, formamide, was used in excess. Formamide is known to direct the incorporation of mismatch base pairs with a preference for guanine. This is achieved when the carbon of formamide (CH_3NO) undergoes hydrogen bonding in either *cis* or *trans* with the nitrogen atom of the base involved in the glycosidic bond to the deoxyribose. Even though this nitrogen is not directly involved in base pairing, it does form a fragmentation base which is bypassed by Klenow at a frequency of 33% and *Taq* polymerase at a frequency of 11%, giving rise to frameshift mutations (Maufrais *et al.,* 2003).

After subjecting the wild type strain AS-35 to formamide treatment, mutants were selected based on their ability to grow on concentrations of olive waste shown to be lethal to the wild type organism. The putative mutants' ability to reduce the total phenol content of the waste, as well as their ability to decolourize olive wastewater was investigated. No significant differences were observed between the putative mutants and the wild type. However, all the mutants showed an increase peroxidase activity when compared to the wild type. Putative mutant DF4 was selected, based on all the experimental data collected, and used in subsequent characterization studies.

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The growth rate of wild type AS-35 was compared to mutant DF4 in a batch system containing 25% olive waste. No significant difference between the growth rates of the isolates was observed under these conditions. This was unexpected since most relevant literature reports decreased growth rates for mutants (Cases and de Lorenzo, 2005). The comparative growth rate study was repeated, this time in a batch system containing LB, a nutrient rich media. Wild type AS-35 showed a growth rate two-fold higher than that observed in 25% olive wastewater. This is consistent with the behavior of an organism subjected to stress conditions compared to the same organism in nutrient rich (i.e., non-stressed) conditions (Sanders *et al.*, 1999). Under identical conditions, the mutant showed no significant increase in growth rate in nutrient rich media, and was shown to be similar

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irrespective of the nutrient conditions. AS-35 and DF 4 were cultured in batch systems containing 25% olive waste supplemented with different nitrogen sources. AS-35 grew significantly better in the presence of yeast extract. However, the higher growth rate seemed to negatively influence the organism's ability to reduce the total phenol content. This is in contrast to the response of *G. condidum* which showed enhanced decolourization of olive wastewater when, cultured in the presence of easily biodegradable carbon sources such as glucose (Ayed *et al.*, 2004). Mutant DF 4 grew better than the wild type in the absence of nitrogen supplementation and was more effective in reducing the total phenol content. Based on the differences in growth rate and phenol reduction, it is possible to conclude that AS-35 is subject to strict catabolic repression with respect to phenol degradation. Mutant DF 4 appears to be unaffected by this catabolic repression, and will thus degrade the phenolic compounds irrespective of the presence of simpler carbons present in yeast extract.

AS-35 showed enlarged cell morphology when cultured in olive wastewater. A similarly enlarged morphology was observed for mutant DF4 cultured in olive wastewater. Surprisingly, mutant DF 4 cultured in nutrient rich LB had the same morphology as the cells cultured in olive wastewater. Changes in cell morphology have been previously described for cells under stressed conditions. For example, acid-adapted *P. freudenreichii* cells showed an enlarged morphology compared to untreated cells or cells briefly exposed to acid (Jan *et al.*, 2001). One possible explanation for the increased cell size is an increase in the protein content in the cell membrane associated with hyper-expression of efflux pump proteins (Bourdineaud *et al.*, 2004).

The proteome of AS-35 cultured in olive waste were found to differ significantly from AS-35 cultured in LB (see Chapter 4). In contrast, DF 4 when cultured in olive waste showed a similar proteome profile to that of AS-35 cultured under the same conditions. Surprisingly, the proteome profile of DF 4 cultured in LB shows a higher similarity to that of AS-35 and DF4 cultured in olive waste than AS-35 cultured in LB. Based on its morphology and protein profile, DF 4 appeared to exhibit a constant stress-adapted phenotype, irrespective of the nutrient environment.

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Based on the MS identification of 3 protein spots and the putative genes identified during the subtractive hybridization assay, it appears that AS-35 cultured in LB at an OD_{600nm} of 1.0, still displays an exponentially growing phenotype characterized by glucose catabolite repression. Protein 1, also named L1 in Chapter 4, and protein 3 (named L3) were identified as the chaperones DnaK and GroEL. Even though they are classified as heat shock 1 proteins, elevated expression levels are also known to occur during the over expression of insoluble proteins in *B. subtilis* (Jürgen *et al.*, 2001). In *B. megaterium,* no up regulation of these already induced chaperone proteins were found during the over expression of recombinant proteins, indicating that they are important during exponential growth of this organism (Wang *et al.,* 2005). Unlike *E. coli, B. subtilis* does not depend on GroEL for the activation of it general stress response regulon σ^B, nor are the induction of GroEL or DnaK σ^B-dependent (Scott *et al.*, 1999., Petersohn *et al.*, 2001). The third protein was identified using MS in AS-35 cultured in LB as an enolase. Enolases are glycolytic enzymes responsible for the interconversion of 2-phospho-D-glycerate and phosphoenolpyruvate. In *E.* coli, the enzyme together with RNaseE, polynucleotide phosphorylase (PNPase) and helicase (RhlB) forms part of a degradosome responsible for the degradation of *pts*G mRNA, encoding a glucose transporter, in response to the accumulation of glucose (Morita *et al.*, 2004., Callaghan *et al.*, 2004).

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It is possible that the protein profile of AS-35 cultured in LB displays proteins that were essential for exponential growth, were not synthesized in stationary phase but had not been degraded. This problem is addressed by analyzing the mRNA levels in the subtractive hybridization assay. Based on the results obtained in Chapter 4 as well as in this chapter, it is suggested that proteins still actively involved in nutrient rich, exponential growth were synthesized in AS-35 cultured in LB at OD_{600nm} of 1.0. This is based on the presence of a L-lysine exporter protein, anthranilate phosphoribosyl-transferase, and the xanthine permease. Lysine is an important amino acid during sporulation of *B. megaterium*, which occurs during glucose starved stationary phase (Pitel and Gilvarg, 1970). Anthranilate phosphoribosyl-transferase functions in the synthesis of tryptophan, a process which would also be inhibited by the relA-dependent stringent response at the onset of stationary phase in *B. subtilis* (Bernhardt *et al.*, 2003). The

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expression of a xanthine permease indicates that there is still an abundance of extracellular purines that could be used during the synthesis of nucleotides (Christiansen *et al.,* 1996).

In contrast, the protein profiles and transcriptome analysis of DF4 and AS-35 cultured in olive waste show cellular expression similar to cells that have entered glucose-exhausted stationary phase. In *B. subtilis,* entry into stationary phase is marked by the exhaustion of glucose, the subsequent lowering of intracellular ATP levels and the activation of two distinct pathways. The first is the glucose, phosphate or oxygen starvation induction of the σ^B-dependent general stress response. This provides non-growing, non-sporulating cells with transient, multiple resistances to non-specific stresses. Induction of the σ^B-dependent regulon is soon annulled by the increase in ATP levels due to the utilization of alternative carbon sources. Concurrent with the general stress response is the activation of the relA-dependent stringent response. The stringent response entails the inhibition of glycolytic enzymes, amino acid anabolism and synthesis of translational machinery due to an increase in cellular ppGpp alarmone. During the general stress and stringent responses, σ^H-dependent induction of sporulation occurs. The second distinctive pathway is the glucose starvation specific response. These responses include the inhibition of glycolysis, the induction of alternative carbon utilization pathways and the removal of CcpA-dependent catabolite repression (Berhardt *et al.*, 2003., Miwa and Fujita, 2001).

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The gene product of the first gene of the *gnt* operon, gntR, was identified by MS analysis from the protein profiles of AS-35 cultured in olive waste. The *gnt* operon is responsible for gluconate catabolism and under catabolite repression from glycolytic intermediates (Fujita and Miwa, 1994). Similarly, the *iol*B gene was identified from the transcriptome of AS-35 cultured in olive waste (Chapter 4.2.3.2). The expression of the *iol*ABCDEFGHIJ operon is also subject to catabolite repression in the presence of glucose (Miwa and Fujita, 2001). Thus, based on proteomic and transciptomic data, it appears as if glucose dependent catabolite repression is annulled in AS-35 cultured in olive waste at an OD_{600nm} of 1.0. Protein levels of GntR were significantly expressed in AS-35 cultured in olive waste and DF4 (Table 5.6). Genes similar to the 3-hydroxyacyl-

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CoA dehydrogenase identified in AS-35 when cultured in olive waste were expressed during sporulation in *B. subtilis*. The *mmg* genes which are homologous to the ß-oxidasion pathway are expressed in the absence of glucose during sporulation (Bryan *et al.*, 1996).

Homologues of the putative NAD-dependent dehydrogenase, YdaD, were identified in the transcriptome of both AS-35 cultured in olive waste as well as DF4 cultured in LB during their comparisons to AS-35 cultured in LB. Expression of y*da*D in *B. subtilis* (Petersohn *et al.*, 2001), *C. perfringens* (Briolat and Reysset, 2002) and *S. aureus* (Bischoff *et al.*, 2004) has been suggested to be under the control of σ^B during growth arrested stress conditions. Activation of the σ^B-dependent general stress regulon due to glucose or phosphate starvation does not induce the expression of YdaD. This protein was induced in response to salt and oxidative stress (Petersohn *et al.*, 2001). This could account for the absence of *yda*D mRNA in late-exponential phase AS-35 cultured in LB (Bischoff *et al.*, 2004). This supports the hypothesis that mutant DF4 in LB express a stress induced response, similar to that observed in AS-35 cultured in olive wastewater.

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Chapter 6

Concluding Remarks

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The incorporation of table olives in daily diet became a popular world wide trend, following the discovery of the health benefits associate with this fruit and its oil (Tuck and Hayball, 2002). This translated directly to an increase in the production and processing of tables olives, which subsequently led to an increase in the production of olive wastewater. An increase in social awareness of waste disposal has meant that increasing levels of research and resources have been invested in the safe disposal or bioremediation of toxic olive wastewater (Hamdi, 1993). None of the removal processes are currently in use due to their inability to effectively remove the phenolic compounds. In addition the expense of these processes is not covered by the revenue generated from the olive industry (Ayed *et al.*, 2004). The typical objective for bioremediation studies concerns the removal of total phenol content and reduction of COD.

During this study, micro-organisms capable of olive wastewater bioremediation were isolated from an evaporation pond in the Boland district of South Africa. These isolates' bioremediation capabilities were evaluated by measuring the reduction in the total phenol concentration following fermentation, as well as the organisms' ability to produce polyphenol oxidases. Reduction of phenolic compounds was also analyzed on RP-HPLC, in order to screen for the possible production of valuable intermediate compounds. RP-HPLC results of isolate AS-35 showed that a number of chemical intermediates were produced during the first 40 hours of olive wastewater fermentation followed by the removal of the majority of the phenolic compounds present in the wastewater during the last 94 hours of fermentation. Based on the RP-HPLC results isolate AS-35 was identified as a candidate for further analysis.

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AS-35 was subsequently identified by 16S rDNA sequence analysis and API biochemical test as *Bacillus megaterium*. In order to investigate the influence of olive waste on the cellular adaptation of the organism, the differential gene expression of AS-35 cultured under nutrient rich (LB) and olive waste was compared. AS-35 was capable of partial bioremediation of 50% olive wastewater, but with dramatic phenotypic changes observed in generation time and cellular morphology. This is the first study to investigate differential gene expression of *B. megaterium* in response to olive wastewater exposure.

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Differential gene expression was examined using 2D-gel electrophoresis, subtractive RNA and cDNA hybridization to genomic libraries. Two-dimensional electrophoresis is a reproducible approach to compare hundreds of proteins simultaneously

It is difficult to compile a complete perspective of the pathways involved based on the limited transcriptome and proteome identifications. However, based on the results that were obtained from proteomic and transcriptomic investigations, certain metabolic pathways induced during the different growth conditions could be depicted. The most significant interactions deduced from sequence identifications are illustrated in schematic diagrams in figures 6.1 and 6.2. Figure 6.1 illustrated how glycolysis utilizes glucose or fructose-6-phosphate to produce glyceraldehydes -3-phosphate or pyruvate. This pathway involves the activity of enolase, MS identified in this study, to produce phosphoenolpyruvate which is one of the starting blocks used in phenylalanine, tyrosine and tryptophan synthesis. Anthranilate phosphoribosyl transferase, identified using a RNA library, binds anthranilate to phosphoribosylpyrophosphate (PRPP) during tryptophan synthesis (Campbell, 1995). PRPP is also involved in the conversion of xanthine to guanosine-5-phosphate. The latter reaction only occurs when xanthine permease, identified using the RNA library, collects xanthine from the environment during nitrogen rich conditions (Christiansen *et al.*, 1996).

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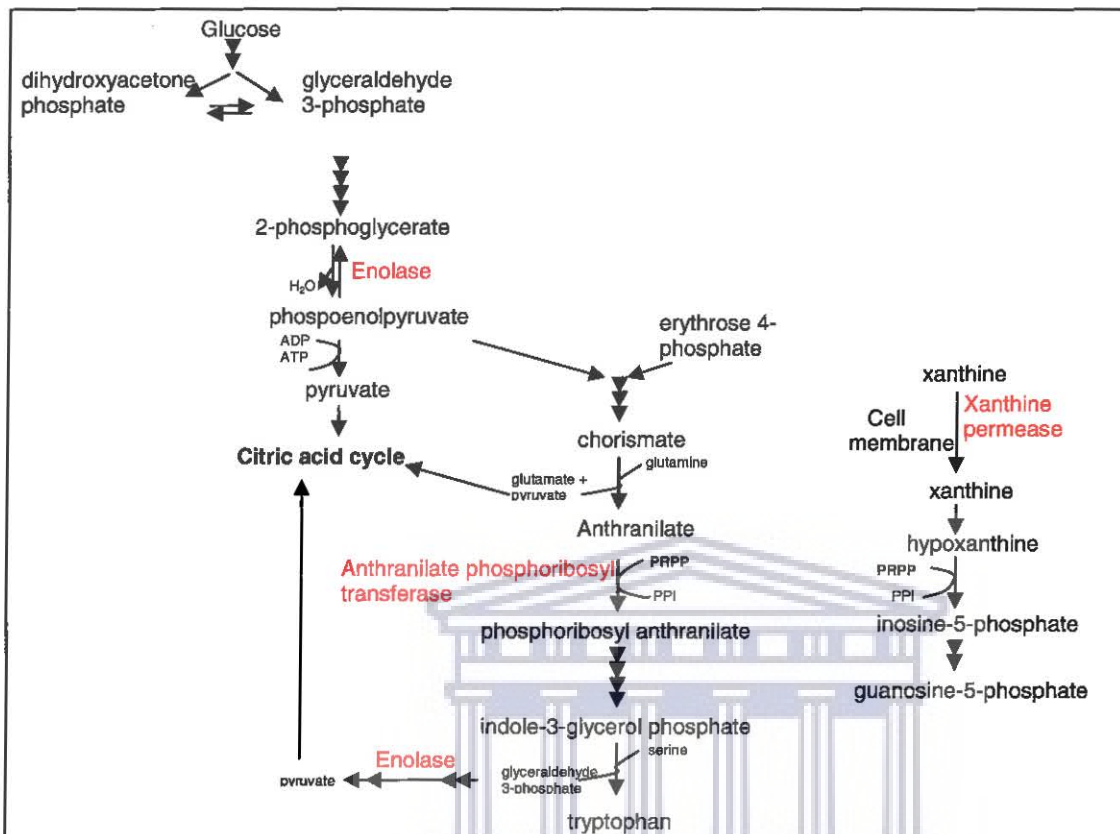

Figure 6.1. Schematic representation of nutrients utilization based on the proteins identified in AS-35 when cultured in LB. The proteins identified in this study are indicated in red.

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Based on these results it is suggested that AS-35 cultured in LB expressed a profile consistent with exponential growth in nutrient rich media (Wang *et al.*, 2005). Presented in figure 6.2 is the pathways identified following exposure to olive waste. From the diagram, it is evident that transcription activation of 2 of these genes, *iol*B and *gnt*R would not occur in the presence of glucose or other easily utilizable sugars (Miwa and Fujita, 2001). The expression of *B. subtilis* homolog *yda*D during olive waste growth, indicate σ^B-dependent activation of the general stress regulon (Petersohn *et al.*, 2001).

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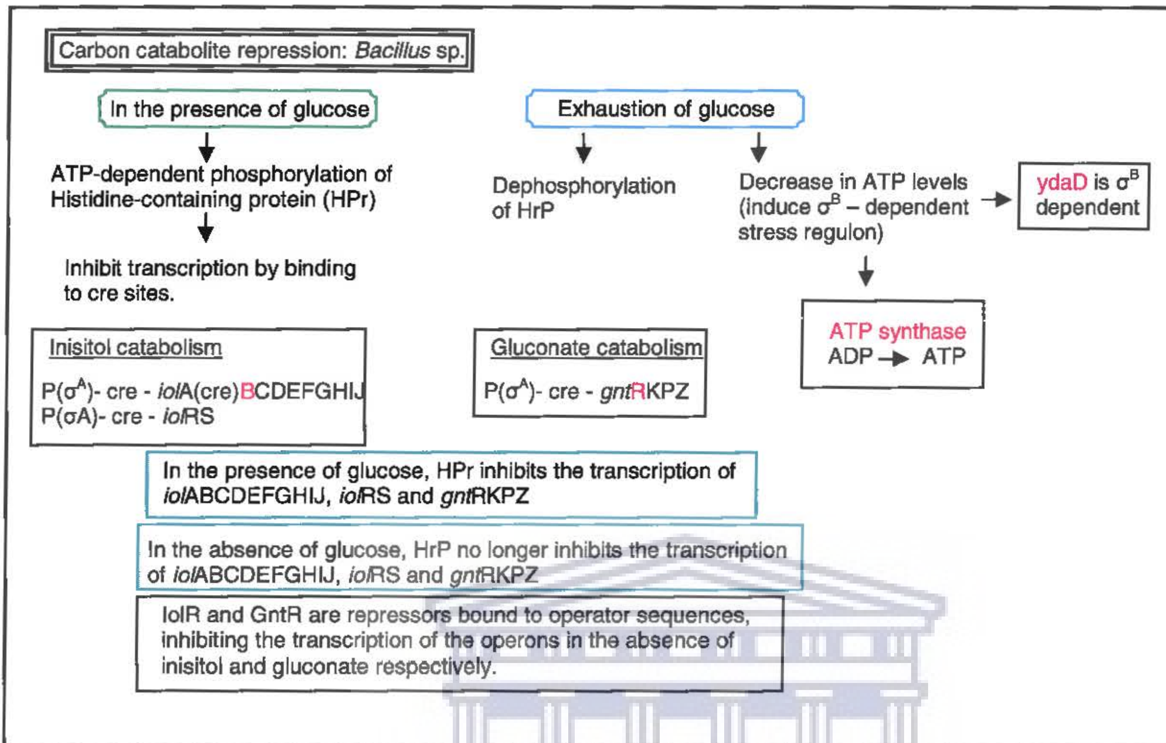

Figure 6.2. Schematic diagram indicating the involvement of proteins identified in AS-35 cultured in olive waste. Based on B. subtilis comparisons, this growth condition resemble glucose starvation. The proteins identified in this study are indicated in pink.

The σ^B-dependent stress response is activated either through glucose, phosphate or oxygen starvation or due to presence of various environmental stresses (Voelker *et al.*, 1995., Hecker and Volker, 1998). Bernhardt *et al.*, 2003, recently indicated that *yda*D is not expressed following the starvation response of σ^B-dependent stress activation, suggesting exclusivity to environmental stresses response. The profile of AS-35 cultured in olive waste resembled that of an organism in stationary phase; i.e., post glucose exhaustion (Bernhardt *et al.*, 2003).

Future investigations could involve the sequencing of more proteins and transcriptomes, which would facilitate a better understanding of the regulation, adaptation and metabolism of *B. megaterium*. Sequencing of the complete *B. megaterium* genome would aid future research, since the majority of this study's homology predictions and functional assumptions were based on data available from *B. subtilis*.

Classical chemical mutagenesis performed on AS-35, using formamide, produced a hyper-resistant mutant, DF4, capable of growth on 80% olive waste. Phenotypically

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mutant DF4 resembled wild type cultured in olive waste supplemented with yeast extract. In contrast to the phenotypic differences observed in wild type analysis, mutant cultured in LB and olive wastewater displayed no significant differences. The mutant grew better than the wild type in the absence of nitrogen supplementation and was more effective in reducing the total phenol content in the absence of readily utilizable carbons found in yeast extract. This is the first study to investigate the application of classical random mutagenesis in order to enhance an organism bioremediation potential of olive wastewater. Previous studies performed on olive waste bioremediations concerned the introduction of single genes or pathways into the genome of suitable organisms (Pignède *et al.*, 2000).

Wild type AS-35 exhibited distinctive protein profiles when cultured in olive waste and LB broth respectively, while the profiles observed for DF4 appeared to be similar irrespective of the culture media. This implies that, while the wild type AS-35 adapted between the different culturing conditions, mutant DF4 only expressed proteins induced during the adaptation to growth in olive waste. The identification of *yda*D in DF4 using RNA subtractive hybridization comparison to AS-35 cultured in LB, suggest the constitutive expression of the σ^B-dependent stress response in the mutant (Peterson *et al.*, 2002., Bernhardt *et al.*, 2003). Based on the proteomic and transcriptomic results obtained from AS-35, it is apparent that cascades of genes are involved in the adaptive response. This is in accordance with the description of regulons involved in the adaptation of *B. subtilis* to changing environments, σ^B-dependent stress response, and metabolic conditions, catabolite repression (Petersohn, *et al.*, 2002., Miwa and Fujita, 2001). Thus the approached followed in this study to enhance the bioremediation potential of AS-35 by random mutagenesis appeared to be more effective than altering a specific regulator or enzyme.

Following the success of the mutagenesis in generating an isolate that was capable of enhanced bioremediation of olive wastewater constituents, it would be possible to employ the mutant in an up-scaled bioreactor. Up-scaled fermentation investigations would provide vital information concerning the mutant's ability to bioremediate olive wastewater in an industrial application.

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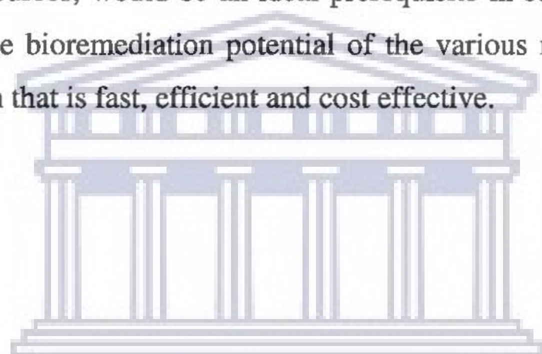
A future area of research would be to subject the mutant to further chemical mutagenesis in order to generate an organism that could potentially grow on undiluted olive wastewater. Subsequent mutagenesis could be performed using the same chemical mutagen, since formamide induced mutagenesis is a random event (Maufrais *et al.*, 2003). Selected isolates such as AS-9 and AS-34 could be subjected to chemical mutagenesis in order to enhance their bioremediation potential. This could possibly provide an additional advantage, since these organisms putatively possess different biochemical pathways to degrade the phenolic compounds and /or adapt to the environmental conditions. Growth on a stringent selective media, containing no additional nitrogen or carbon sources, would be an ideal prerequisite in selecting future mutants. Synergism between the bioremediation potential of the various mutants could provide a bioremediation system that is fast, efficient and cost effective.



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AS9_16S_SEQUENCE    .........................GCAAGCGTT.ATCGGAAT.ACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGT     53
AS15_16S_SEQUENC    .............................GAGCGTATCGGAAT..CTGGGCGTAA.GCGCACGCAGGCGGTCTGTCAAGT     48
AS18_16S_SEQUENC    .........................GGTGCAGCGTATCGGAAT.NCTGGGCGTAA.GCGCACGCAGGCGGTCTGTCAAGT     53
K_oxytoca 16S      CAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGT    560

AS9_16S_SEQUENCE    TCGGA.TTGTGAAATCCC.GGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTGGAGTCTTGTAGAGGGGGGTAGA    131
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AS18_16S_SEQUENC    CGGA...TGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTGGAGTCTTGTAGAGGGGGGTAGA    130
K_oxytoca 16S      CGGA...TGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTGGAGTCTTGTAGAGGGGGGTAGA    637

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K_oxytoca 16S      CTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGACTTGGAGGTT    797

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AS15_16S_SEQUENC    GTTCCCTTGAGGAGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAA    367
AS18_16S_SEQUENC    GTTCCCTTGAGGAGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAA    370
K_oxytoca 16S      GTTCCCTTGAGGAGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAA    877

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AS15_16S_SEQUENC    TGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGAC    447
AS18_16S_SEQUENC    TGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGAC    450
K_oxytoca 16S      TGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGAC    957
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AS9_16S_SEQUENCE     ATCCAGAGAACTTAGCAGAGATGCT.......................................................    476
AS15_16S_SEQUENC     ATCCAGAGAACTTAGCAGAGATGCTTTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGT    527
AS18_16S_SEQUENC     ATCCAGAGAACTTAGCAGAGATGCTTTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGT    530
K_oxytoca 16S        ATCCAGAGAACTTAGCAGAGATGCTTTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGT   1037

AS9_16S_SEQUENCE     ................................................................................    476
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AS18_16S_SEQUENC     TGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAGGA    610
K_oxytoca 16S        TGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACTCAAAGGA   1117

AS9_16S_SEQUENCE     ................................................................................    476
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AS18_16S_SEQUENC     GACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCT    690
K_oxytoca 16S        GACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCT   1197

AS9_16S_SEQUENCE     ................................................................................    476
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AS18_16S_SEQUENC     ACACTGGCATATACAAAGAGAAGCGAACTCGCGAGAGCAAGCGGACCTTCATACAAGTATGTCGATAGTCCGGATTTGGA    770
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AS9_16S_SEQUENCE     ................................................................................    476
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K_oxytoca 16S        GTCTGCAACTCGACTCC..ATGAAG.TCGGAATCGCTAGTAATC..GTGGATCAGAATGCCACGGTG.............   1335

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AS15_16S_SEQUENC     GCCT                                                                                842
AS18_16S_SEQUENC     ....                                                                                830
K_oxytoca 16S        ....                                                                               1335
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Appendix 1. Homology between 16S rDNA gene analysis of AS-9, AS-15, AS-18 and *Klebsiella oxytoca*.

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AS-35_25%_olive_    ....AGATT.GAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACTGATTAG    75
AS-35_LB            ....AGATT.GAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACTGATTAG    75
DF4_25%_olive_wa    ....AGATT.GAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACTGATTAG    75
DF4_LB              ....AGATT.GAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACTGATTAG    75
B._megateruim       TCGACGATTAGAGTTTGATCCTGGCTCAGAACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACTGATTAG    80

AS-35_25%_olive_    AAGCTTGCTTCTATGACGTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACTTCGGG    155
AS-35_LB            AAGCTTGCTTCTATGACGTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACTTCGGG    155
DF4_25%_olive_wa    AAGCTTGCTTCTATGACGTTAGCGGCGGACGGGTGAGTAACACGTGGGCGACCTGCCTGTAAGACTGGGATAACTTCGGG    155
DF4_LB              AAGCTTGCTTCTATGACGTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACTTCGGG    155
B._megateruim       AAGCTTGCTTCTATGACGTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACTTCGGG    160

AS-35_25%_olive_    AAACCGAAGCTAATACCGGATAGGATCTTCTCCTTCATGGGAGATGATTGAAAGATGGTTTCGGCTATCACTTACAGATG    235
AS-35_LB            AAACCGAAGCTAATACCGGATAGGATCTTCTCCTTCATGGGAGATGATTGAAAGATGGTTTCGGCTATCACTTACAGATG    235
DF4_25%_olive_wa    AAACCGAAGCTAATACCGGATAGGATCTTCTCCTTCATGGGAGATGATTGAAAGATGGTTTCGGCTATCACTTACAGATG    235
DF4_LB              AAACCGAAGCTAATACCGGATAGGATCTTCTCCTTCATGGGAGATGATTGAAAGATGGTTTCGGCTATCACTTACAGATG    235
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AS-35_LB            GGCCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCATAGCCGACCTGAGAGGGTGATCGG    315
DF4_25%_olive_wa    GGCCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCCACGATGCATAGCCGACCTGAGAGGGTGATCGG    315
DF4_LB              GGCCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCCACGATGCATAGCCGACCTGAGAGGGTGATCGG    315
B._megateruim       GACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCATAGCCGACCTGAGAGGGTGATCGG    320

AS-35_25%_olive_    CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGAC    395
AS-35_LB            CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGAC    395
DF4_25%_olive_wa    CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGAC    395
DF4_LB              CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGAC    395
B._megateruim       CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGAC    400
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AS-35_25%_olive_     GGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACAAGAGTAACTG   475
AS-35_LB             GGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACAAGAGTAACTG   475
DF4_25%_olive_wa     GGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACAAGAGTAACTG   475
DF4_LB               GGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACAAGAGTAACTG   475
B._megateruim        GGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACGAGAGTAACTG   480

AS-35_25%_olive_     CTTGTACCTTGACGGTACCTAACCAGAAAGCCACGG.CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGC   554
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DF4_25%_olive_wa     CTTGTACCTTGACGGTACCTAACCAGAAAGCCACGG.CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGC   554
DF4_LB               CTTGTACCTTGACGGTACCTAACCAGAAAGCCACGG.CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGC   554
B._megateruim        CTCGTACCTTGACGGTACCTAACCAGAAAGCCACGGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGC   560

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DF4_25%_olive_wa     GTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGA   634
DF4_LB               GTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGA   634
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DF4_25%_olive_wa     GGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGAAAAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGT   714
DF4_LB               GGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGAAAAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGT   714
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AS-35_25%_olive_     GGAGGAACACCAGTGGCGAAGGCGGCTTTTTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATT   794
AS-35_LB             GGAGGAACACCAGTGGCGAAGGCGGCTTTTTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATT   794
DF4_25%_olive_wa     GGAGGAACACCAGTGGCGAAGGCGGCTTTTTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATT   794
DF4_LB               GGAGGAACACCAGTGGCGAAGGCGGCTTTTTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATT   794
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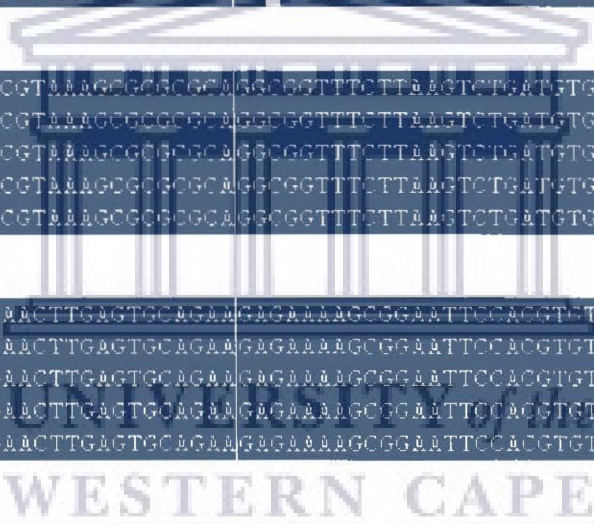
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AS-35_LB           AGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTTGCTGCAGCTAACG    874
DF4_25%_olive_wa   AGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTTGCTGCAGCTAACG    874
DF4_LB             AGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTTGCTGCAGCTAACG    874
B._megateruim      AGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGT.GCTGCAGCTAACG    879

AS-35_25%_olive_   CATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAG    954
AS-35_LB           CATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAG    954
DF4_25%_olive_wa   CATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAG    954
DF4_LB             CATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAG    954
B._megateruim      CATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAG    959

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DF4_LB             CATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACTCTAGAGATAGAGCGTTCC    1034
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AS-35_25%_olive_   CCTTCGGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGA    1114
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AS-35_LB           GCGCAACCCTTGATCTTAGTTGCCAGCATTTAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGG    1194
DF4_25%_olive_wa   GCGCAACCCTTGATCTTAGTTGCCAGCATTTAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGG    1194
DF4_LB             GCGCAACCCTTGATCTTAGTTGCCAGCATTTAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGG    1194
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AS-35_25%_olive_    CGAGGTCAAGCCAATCCCATAAAACCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTA   1354
AS-35_LB            CGAGGTCAAGCCAATCCCATAAAACCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTA   1354
DF4_25%_olive_wa    CGAGGTCAAGCCAATCCCATAAAACCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTA   1354
DF4_LB              CGAGGTCAAGCCAATCCCATAAAACCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTA   1354
B._megateruim       CGAGGTCAAGCCAATCCCATAAAACCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTA   1359

AS-35_25%_olive_    GTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAA   1434
AS-35_LB            GTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAA   1434
DF4_25%_olive_wa    GTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAA   1434
DF4_LB              GTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAA   1434
B._megateruim       GTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAA   1439

AS-35_25%_olive_    CACCCGAAGTCGGTGGAGTAACCGTAAGGAGCTAGCCGCCTAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAAGGT   1514
AS-35_LB            CACCCGAAGTCGGTGGAGTAACCGTAAGGAGCTAGCCGCCTAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAAGGT   1514
DF4_25%_olive_wa    CACCCGAAGTCGGTGGAGTAACCGTAAGGAGCTAGCCGCCTAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAAGGT   1514
DF4_LB              CACCCGAAGTCGGTGGAGTAACCGTAAGGAGCTAGCCGCCTAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAAGGT   1514
B._megateruim       CACCCGAAGTCGGTGGAGTAACCGTAAGGAGCTAGCCGCCTAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAAGGT   1519

AS-35_25%_olive_    AACCAATCGGATCCCGGGCCCGTCGACTGCAGAGGCCTGCATGCAAGCTTTCCCTATAG...                     1573
AS-35_LB            AACCAATCGGATCCCGGGCCG.........................................                     1536
DF4_25%_olive_wa    AACCAATCGGATCCCGGGCCCGTCGACTGCAGAGGCCT........................                     1552
DF4_LB              AA............................................................                     1516
B._megateruim       AGCCGTA.......................................................                     1526
```

Appendix 2. Representation of the homology between 16S rDNA gene analysis of AS-35, DF4 cultured in LB and 25% olive wastewater and *Bacillus megateruim.*

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```
D 8     ....................RQSDIESEMNPLPIYEDDNYIGTGKLKGKVALVTGGDSGIGRAVSIAYAEEGADVAIVYL     60
G10     ....................................................LQWGDSGIGRAVSIAYAKEGADVAIVYL     28
OW4     ................................................................................      0
2 A1    FELGTSRMHLDPYVIRLXXGRQSDIESEMNPLPIYEDDNYIGTGKLKGKVALVTGGDSGIGRAVSIAYAEEGADVAIVYL     80
YdaD    ...MNPMDRQTEGQEPQHQDRQPGIESKMNPLPLSEDEDYRGSGKLKGKVAIITGGDSGIGRAAAIAFAKEGADISILYL     77

D 8     NEHSDAEETKARIEEEGVRCLLIA........................................................     84
G10     NEHSDAEETKARIEEEGVRCLPIAGVVGDESFCNEAVEKTVSELGKLDILVNNAGEQHPKESIKEITSEQLERTFKTNFY    108
OW4     TEHSDAEETKARIEEEGVRCLLIAGDVGDESFCNEAVEKTVSELGKLDILVNNAGEQHPKESIKEITSEQLERTFKTNFY     80
2 A1    NEHSDAEETKARIEXEGVRCLLIAGDVGDESFCNEAVEKTVSELGKLDILVNNAGEQHPKESIKEITREQLERTFKTNFY    160
YdaD    DEHSDAEETRKRIEKENVRCLLIPGDVGDENHCEQAVQQTVDHFGKLDILVNNAAEQHPQDSILNISTEQLEKTFRTNIF    157

D 8     ................................................................................     84
G10     SYFYFTKKALDYLGKGSAIINTTSINPYRGNPQLIDYTATKGAINGFTRSMAQALVKDGIRVNAVAPGPIWTPLIPSTFS    188
OW4     SYFYFTKKALDYLGKGSAIINTTSINPYRGNPQLIDYTATKGAINGFTRSMAQALVKDGIRV..................    142
2 A1    SYFYFTKKALDYLGKGSAIINTTSINPYRGNPQLIDYTATKGAINGFTRSMAQALVKDGIRVNAVAPGPIWTPLIPSTFS    240
YdaD    SMFHMTKKALPHLQEGCAIINTTSITAYEGDTALIDYSSTKGAIVSFTRSMAKSLADKGIRVNAVAPGPIWTPLIPATFP    237

D 8     ...................................................     84
G10     GDKVGEFGTDTPMGRPGHPVEHVGCYVLLASNDSSYMTGQTLHVNGGDFMN    239
OW4     ...................................................    142
2 A1    GDKVGEFGTDTPMGRPGHPVEHVGCYVLLASNDSSYMTGQTLHVNGGDFMN    291
YdaD    EEKVKQHGLDTPMGRPGQPVEHAGAYVLLASDESSYMTGQTIHVNGGRFIS    288
```

Appendix 3. Protein sequence homology between D8, G10, OW 4, 2A1 and *yda*D of *B. subtilis*.

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```
L 6G     QXFIMNIYRLPENHXNKNPNMKSFYKEARSEYTECAESAINSCLLFNQLYFLKRGDKMKRVLVMGVSPGVGKSTFAKKLG    80
3 A3     ......................................................GDKMKRVLVMGVSPGVGKSTFAEKL[illegible]    26
L 10H    ..............................................................VFGIRS...LTEFGNPL[illegible]    15
1 B2     ..............................................................VFGIRS...LTEFGNPL[illegible]    15
FlaR     .........................................................MKKNIHILGASG.VGTSTLGAALS    23

L 6G     EKLDLEVYHLDSLYWQPGWTERDNDSFRE..LQEHIVXKDSWIIDGNYSNS.YDLRVKRADTVIYLE[illegible]PLIVCMYRVIKR   157
3 A3     EKLDPEVYRLDSLYWQPGWTERDNDSSRE..LQEHIVKKDSWIIDGNYSNS.YDLRVKRADTVIYLE[illegible]PLIVCMYRVIRR   103
L 10H    PLVQSVLYLQDSHNMRLALK.....LFR....REPAISXFDWNFTATHTSS.PHFSTCVGSGLHSVL[illegible]ELHPG.HG....    80
1 B2     PLVQSVLYLQDSHNMRLALK.....LFR....REPAISRFDWNFTATHTSS.PHFSTCVGSGLHSVL[illegible]ELHPG.HGQITW    84
FlaR     KRLPHTHLDTDNYYWLDKFTKKREIPERRKLLEKDLTINEKWILSGAVCGWGDNLKSYFDLVVFLWI[illegible]QDIRLERLRHRE   103

L 6G     RIVYARKTREDMAKGCPEKVDWSFFTFILTTYRERKKKMRNRFKKLKNDEDYKVFMLMNRKDIENYIQNL......   227
3 A3     RIVYARKTREDMAKDALKK.........................................................   122
L 10H    ............................................................................    80
1 B2     FRVYNHILNALFRLALATAPPYQLNLAW................................................   112
FlaR     FQRYGNEVLAGGSKYEQSKTFLEWASLYDN..AGMEVRSRALHEHWMADLSCPVLKIEDDCSVNERVDRVLDYLSS   177
```

Appendix 4. Protein sequence homology between L6G, L10H, 1B2, 3A3 and *yqaC* of *B. subtilis*.

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Appendix 5. Sequence results obtained from transcriptome analysis.

LB 1 DNA sequence:

```
1     ACAGCTGATT CACTGGCTGG CTCAAGATAC GAATGACGTG CCGCGTCAGC CTAGTGATAT
61    TCTGGTGCTG ACGCCAAGTC TGAAAGAACT CGAACCGGCA ATTCGCAGTG TCTTTGCCCC
121   GCCACCGCGT GAGCGCGAGA CTGGTAGCAA ACGCTTGCAG AAAGATAACC TGTATTTACC
181   GATCCAGATT GCCGGGGTAT CACGTCTGGA TGTCAGCAAT GCCTGGCGCG CGGTACTGGG
241   CCGTATTCAG TGGGTACGGC GCCGTTCCAG TATTGAGGAC TTTGCCGACT GGCTTAGCCT
301   GAATGCGACC CAGCAACGTT ATGGTCCGGA CTATAGTCAG GTAGAACGGA TGTTGCAGTT
361   GCTGACAGAT GCTGGCTTCA AGCGTGGATT GGACCAGCAG CATTTACAGC AAAGTCTGAG
421   CGCAGGCGAT GAGGATTACC GGTTTAGCTT TAAATTCGCG CTGGACCGTC TGGCGTTGGG
481   TCTGGCGATT CCTGAACATA CCCTGTTTCA GGACACCTTG AGTTATGCCA AGGTTCTGGC
541   CAGTGATTTT CCCTTGATAT CCACCCTGAT CCGGATTTAT CAGAATCTGG TAGAGCGCCG
601   TG
```

Translated sequence: Reading frame 2.

```
1     QLIHWLAQDT NDVPRQPSDI LVLTPSLKEL EPAIRSVFAP PPRERETGSK RLQKDNLYLP
61    IQIAGVSRLD VSNAWRAVLG RIQWVRRRSS IEDFADWLSL NATQQRYGPD YSQVERMLQL
121   LTDAGFKRGL DQQHLQQSLS AGDEDYRFSF KFALDRLALG LAIPEHTLFQ DTLSYAKVLA
181   SDFPLISTLI RIYQNLVERR
```

LB 3 DNA sequence:

```
1     CAGGATCACA CTTTGATTAA AGTACGGTTA CATGCCGTTC ATGTATGCGG TTAATTTCTC
61    TTTTTGTTGT AAAGTGACGA GAGCCATTTT TGAGCAATTC AAAGGAGCCA ATTGGTTAAG
121   AGATAAAAAA TATAAGCAGG CCATATTTAC AGAAAAATAA AGCTTACCTA AAATATCAGG
181   CTAAAAGCAG GTTTACGTTC CCTTTAAAAA TAAATTAAAA GCTTTTTGAT TTTAAAAATA
241   TAATTGTATT GATCCTTAAG TTTTACTAAG TTAAAGTGCG GATCCGATGC TCGCGCAAGC
301   ACTTTAACAA TATCCCGAAA GCCACCAGAA TTTCCTGTTC ACTCACACAG GCAAAGCCCA
361   GTAATAATCC TTTCTGCCGG GTGGAATAAT GTTTGGTATA GTATTGCGAT AAGGCTCCGA
421   CCTTGATTCC CAGTTCAAAG CGCTTTGGAT GCCAATCT
```

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Translated sequence: Reading frame 5.

```
1     DWHPKRFELG IKVGALSQYY TKHYSTRQKG LLLGFACVSE QEILVAFGLL KCLREHRIR
61    TLTNLRINTI IFLKSKSFFI FKGNVNLLLA YFRALFFCKY GLLIFFISPI GSFELLKNGS
121   RHFTTKREIN RIHERHVTVL SKCDP
```

LB 4 DNA sequence:

```
1     CCGGGTAGAA CCACGCACAG GTGATGCACC TTGTGCCAGT TCTTCCAGAT CTTTATAGCT
61    TTTCTGCTTT AAACGTAGGG CAAATTCTTC TTTTTTACGG TCAACAATTT TGCCTAAAAT
121   TGTATTTGCG ATATCCACCA TGATCAAAAT CCCTGACCGA ATTAAGCTTC GTAATGTTTA
181   ATAGTTTTGG TAAATTCAGA CAGTACACTC ATTTTCTCAA GTGCATGACC GCCATAGATA
241   ATGTCATGTG CCAGCGCAAC ACCTTGCTTA TAGCTGCTGG TCAAACCGGA GACATAAATA
301   CCTGCGCCGG CATTCAGCGC GATCAT
```

Translated sequence: Reading frame 6.

```
1     DRAECRRRYL CLRFDQQLAR CCAGTHYLWR SCTENECTVI YQNYTLRSLI RSGILIMVDI
61    ANTILGKIVD RKKEEFALRL KQKSYKDLEE LAQGASPVRG STR
```

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D 1.1 DNA sequence:

```
1    CCCAAAACAC ATGCTGCTGT TTTAAACCTT GTTTTAATAC AAAAGCATTC TGTGCACCGA
61   TACTGACAAT CAGGCTCATT CCCACAGCAA AACCACTCAT CATATAACTG AACATCACAT
121  TCCCCTTTTT CGAATAAATG AATTATTGAA CAAAATGAGT GCGATTAATT AAAACTAAGT
181  TAATATCAAT TTAACTAGGT TATTTTTAGG TAATAGAAAT GCTGAATGCG AAACAATGTG
241  ATGCTTTTTA TGCAGTTGCA AAAACGGGAA GTTTCGATCT GGCTGCCGAG CAGCTAAATA
301  TTACTGCTTC GGCTGGCGGC CTAA
```

Translated sequence: Reading frame 6.

```
1     PPAEAVIFS CSAARSKLPV FATAKASHCF AFSISITKPS IDINLVLINR THFVQFIYSK
61   KGNVMFSYMM SGFAVGMSLI VSIGAQNAFV LKQGLKQQHV FW
```

D 1.4 DNA sequence:

```
1    AGCGGTTGGT GATATCACTG CGACTTCAAA GCTTTCCAAC CAGCCAGTTG ATGGTCCAAA
61   ATGGATGCAG CGCATTAAAG GCGATGTACT GGTAAATGGC GCGAATTCAT TCCTTGCCGG
121  TATTTTTAAC ACCTCCCCGA GTTCCGTATT TGCACAAAAT AATGGCGTAA TCCAGTTAAC
181  GGGCGTAGCA AGTCGTTATG TCGGTATCTG GATTGCTGCT TTGCTGGTTA TTCTTGGTTT
241  GCTGCCTGCA GTTGCTGGTA TTATTCAGGC CGTTCCGCAA GCGGTACTGG GTGGTGCAGT
301  GATGGTGATG TTTGGNGCAG TCGCAGCATC TGGTATTAAC NTTCTGTCTG GTGTCCATCT
361  GGACCGTCGT GCCCTGCTGA TTATTGCGAT TTCACTGGCG CTGNGTTTAG GTGTTGCACA
421  AGTTCCACAA ATTCTTG
```

Translated sequence: Reading frame 2.

```
1    VGDITATSK LSNQPVDGPK WMQRIKGDVL VNGANSFLAG IFNTSPSSVF AQNNGVIQLT
61   VASRYVGIW IAALLVILGL LPAVAGIIQA VPQAVLGGAV MVMFGAVAAS GINXLSGVHL
121  DRRALLIIAI SLALXLGVAQ VPQILEHLPE LFRNIFSSGV ATGGL
```

OW 1 DNA sequence:

```
1    GTGGCAATCA GGATTAAAGC CTCTGCTGAA GGTGTCTCAG TTTGCTCAAT CTGAATATTG
61   AACTGTTGCA GATATTGCTC AAGTGCCTGA CGGTCTTCAT CATATTCGGT GCCTAGCCAG
121  ACTGATGGAT ATTGACTTAA CTTTTCAACC AATGCTGGAG CACCATCACC TTCTTTGCTA
181  CCCGAAGTGT AATCATAGAA ACCTTTACCG GTTTTACGAC CTAACTGCTT GGCAATAAAA
241  CGCTGACGGG TAATCACGCT TGGACGGTAA CGTGCTTCTT CATAATACTG ATGATAAATC
301  GATTCACTCA CCGGGTGCGA GACATCCATA CCGGTCAGAT CACCTAATTC AAACGGCCCC
361  ATCTTGAAAC CGATTCCGTC ACGGTAGATC CTGTCAATAT CATAAAATGG TGTGACATTT
421  TCATTCAGAA TTGCATAGGC TTCGCTACCA AAGGCGCGGC CGGCATGGTT AATGATAAAC
481  CCTGGAGTAT CTTTAGCTTT GACACTGGCG GCCTATAGTG AGTCGTAT
```

Translated sequence: Reading frame 5.

```
1    YDSLAASVKA KDTPGFIINH AGRAFGSEAY AILNENVTPF YDIDRIYRDG IGFKMGPFEL
61   GDLTGMDVSH PVSESIYHQY YEEARYRPSV ITRQRFIAKQ LGRKTGKGFY DYTSGSKEGD
121  GAPALVEKLS QYPSVWLGTE YDEDRQALEQ YLQQFNIQIE QTETPSAEAL ILIA
```

A 3.3 DNA sequence:

```
1    TGGTAGGCCG CGAGTTGGGG GCTGCATGGC GCAACCCGCA TGAACGCGAT ATGCCTTATG
61   TGCTGGAAAT GGTACGTGAA GTTAAAGCAC TCGGTCTGGA AACCTGCGTG ACACTGGGGA
121  TGCTAAATGG ATCACAGGCT GAACGCTTGA AAGATGCAGG ACTGGATTAT TACAACCATA
181  ATCTGGACAC TTCTCGTGAA TACTATTCAC ATATCATCAG CACGCGTAGC TTTGATGATC
241  GTCTGGATAC TCTGGATCAT GTGCGTCAAG CTGGTATGAA AG
```

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Translated sequence: Reading frame 3.

```
1    VGRELGAAWR NPHERDMPYV LEMVREVKAL GLETCVTLGM LNGSQAERLK DAGLDYYNHN
61   LDTSREYYSH IISTRSFDDR LDTLDHVRQA GMK
```

D 3.2 DNA sequence:

```
1    TAGGCCGCCA GTGGGAAAGA AGACCCTGTT GAGCTTTACT CTAGTTTGGT GTTGTGAAGA
61   GACATGGTAG GCCGCCAGTG GGAAAGAAGA CCCTGTTGAG CTTTACTCTA GTTTGGTGTT
121  GTGAAGAGAC ATGGTAGACC GCCAGATGTA GGCGTAGAGC ATCGCGGAGC AGGAAATATC
181  GCGAGACAAG TCCATAATAT TCTTCCTGAA CAAAAGCCGG CCGGTAGTCT GCTGGTAGTG
241  GAAGTGTTTA CGCCGGAAGG AAACCGGTCA AGCTATCCTC CTCATAAGCA CGATCAAGAC
301  AACTTGCCGC ATGAATCGTA TTTAGAGGAA ACGTATTATC GTTAAGTCAA CCCAGGACAT
361  GGATTTGCGA TTCAACGAGT ATATACGGAC GATCAATCTC TAGATGAAAC AATGGTCCTC
421  AAAGATGGAG ATGCAG
```

Translated sequence: Reading frame 1.

```
1    AASGKEDPVE LYSSLVLRDM VGRQWERRPC ALLFGVVKRH GRPPDVGVEH RGAGNIARQV
61   HNILPEQKPA GSLLVVEVFT PEGNRSSYPP HKHDQDNLPH ESYLEETYYR VNPGHGFAIQ
121  RVYTDDQSLD ETMVLKDGDA
```

B 6 DNA sequence:

```
1    TTTAGGTGAC ACTATAGATT AGGATTATTA CAGAGTGAGA CTCAGGGCTT GGTTTGGTCT
61   TATTCGCTAT ACATATGAAA TTCCGGTAGT GAAAGTAAGA AAAGATTCAC CGCGTTTGCT
121  TGTTGAAAAG AAGAAAGGAA TGGGTGATCA AGGAAAAGAA GATGAGAATT CATGGAGTGA
181  TTATTCTGTA GAAGACGGGC TAAGCATGTT AGAAAATGCG AAGCAATTTC TTGGACAGGT
241  AGTCGGTTTT CATAAAATTT TCCGTCGTTT CTTACAGAAA GTAACGGTAA GTGATATTAG
301  ATGGCACAGC CGTTTCGGCT TAGGTGACGC TGCGTTAACA GGCGTACTTA CCGGGGCTGT
361  ATGGTCTGCA AAGGGGAGCA TAGTTGGTAT AATCAGCCGT TATATGAAAT TAAAAGATAT
421  GCCTGTCATG AGCGTTACTC CTGTATTTCA ACATCTTTGG TCAGAAACGG TATTTGAGTG
481  CATAATTTCT CTTCGAGTTG GTCAAGCTAT TCTAGTAGGT TTACGGACCT TGAGATACTG
541  GCGGCCTACC ACTGGCGG
```

Translated sequence: Reading frame 2.

```
1    LGDTID*DYY RVRLRAWFGL IRYTYEIPVV KVRKDSPRLL VEKKKGMGDQ GKEDENSWSD
61   YSVEDGLSML ENAKQFLGQV VGFHKIFRRF LQKVTVSDIR WHSRFGLGDA ALTGVLTGAV
121  WSAKGSIVGI ISRYMKLKDM PVMSVTPVFQ HLWSETVFEC IISLRVGQAI LVGLRTLRYW
181  RPTTG
```

F 6 DNA sequence:

```
1    ATTGGGCACT ATAGACGTAG TTGAATTTGT AATGGAGCTY GAGAGGAACT TGATACCGAA
61   ATTTCTGACG AAGAAGCTGA AAAAATTTCA ACCGTAGGAG ATGCAGCTAA TTACATACAG
121  AGCCAAATTT AATCTGTAGT ATATGTATGA AAAGCCCCGT TAAACAGCGG GGCCTTCTCT
181  TCTTTTTAAG AATTTCAAAA AAAGACCTCT TTTTTTTGAA AAGGTAGTGT ATCTTTTAGG
241  ATACCTCGTT TTATAATGAG AGATGTTAGG AAAAAACTAC GAAATAATTG AAGCAGGTAA
301  GCGGAGGTAT TTTATGCCTA AACAATATTC AAATCGAGAT CGAAAGATGA ACGTGAAATT
361  CAAGCAAAAT TTTGCAGAGT TTCAAAAGGA AATTGGTATT CAGTTTACAA ATGAAGCGCT
421  TCTTTTTCAA GCATTTACAC ATTCATCCTA TGTGAATGAG CATCGCAGAA GACCGTATGA
481  AGACAACGAG CGTCTTGAGT CTTTAGGAGA TGCCGTATTA GAGCTAACCG TATCACAATT
541  TTTATTTAAA AAATATCCAA CGATGATTGA AGGTGAGTTA ACGAAGCTTC GTGCAGCAGT
601  TGTGAGCGAA CCGTCGCTTG TTACTTTTGC AAATGAAATG GATTTTGGTA AACTAGTGTT
661  ACTTGGAAAA GGTGGAGAGA TGACTGGCGG
```

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Translated sequence: Reading frame 1.

```
1     NLIPKFLTKK LKKFQPEMQL ITYRAKFNLY MYEKPRTAGP SLLFKNFKKR PLFFEKVVYL
61    LGYLVLEMLG KNYEIIEAGK RRYFMPKQYS NRDRKMNVKF KQNFAEFQKE IGIQFTNEAL
121   LFQAFTHSSY VNEHRRRPYE DNERLESLGD AVLELTVSQF LFKKYPTMIE GELTKLRAAV
181   VSEPSLVTFA NEMDFGKLVL LGKGGEMTG
```

G 6 DNA sequence:

```
1     CCGCCAGTTR GACCTGGCGG TTGCAAACTC GAATAGCAGT AATGTATCGG TGTTACAAAA
61    TACCAGTAGT ACCGGCTCAA TATCTTTTGC TGCAAAAATA GATTACCCCA TTAATAGCGG
121   AGCGTACGAT ATAAAAATAA CTGATTTGGA AGGGGATGGT AAGCCTGATA TTATAGTCGC
181   TAATTCAGCG TCTAATAATT TTTCAATTCT TAAAAATAAC GGTGCAAACG GAGCTATTGG
241   CTTTGCAACG AAGGTAGATT ATAATACAGG TGCTACCCCG TATGGTATAT CGATCGGGGA
301   TGTAAATGGA GACGGCAAGG CTGATATAGC AGTTGCCAAT AATGACAATA ATACGGTGTG
361   GATATTTGTG AACACAAGCA ATAACGGTAA TATAACCCTT GCACCTAAGG TCAGTTTTTC
421   AACCGGCAAC CAGCCACGAA CCGTTTCACT GGGAGACTTT TATGGTGATG GAAGACCGGA
481   GTTGGTAACA CCAAATTGGG TGGCTTATAC AGCATCAGTT TTAGAAAACC GGGTAGGGGT
541   TGCTATACCT GCATATGTTT GTCCACCAGT TGGAAATACC TCTATAGTGT CACCTAAAT
```

Translated sequence: Reading frame 3.

```
1     ASXDLAVANS NSSNVSVLQN TSSTGSISFA AKIDYPINSG AYDIKITDLE GDGKPDIIVA
61    NSASNNFSIL KNNGANGAIG FATKVDYNTG ATPYGISIGD VNGDGKADIA VANNDNNTVW
121   IFVNTSNNGN ITLAPKVSFS TGNQPRTVSL GDFYGDGRPE LVTPNWVAYT ASVLENRVGV
181   AIPAYVCPPV GNTSIVSPK
```

1 A1 DNA sequence:

```
1     CCGCCAGCCG GGTATCGAAA GTGAGATGAA TCCTCTCCCT ATTTATGAGG ATGACGATTA
61    CATTGGTACA GGAAAATTAA AAGGAAAGGT AGCGCTAGTT ACCGGGGNAG ANAGCGGAAT
121   TGGACGAGCT GTATCCATTG CTTATGCGAA AGAAGGAGCG GACGTAGCCA TCGTTTACTT
181   AAACGAACAT AGCGACGCCG AGGAAACAAA AGCCCGCATT GAAGAAGAAG GTG
```

Translated sequence: Reading frame 2.

```
1     RQPGIESEMN PLPIYEDDDY IGTGKLKGKV ALVTGXXSGI GRAVSIAYAK EGADVAIVYL
61    NEHSDAEETK ARIEEEG
```

1 A3 DNA sequence:

```
1     CCGCCAGTTG CATTTATTGA AAGCGGACGT TATAAAAAAG TTGTAGTGGT GGGTGCCGAT
61    AAAATGAGCG CTATTGTTGA CTATAGTGAT CGCTCAACCT GCATACTTTT TGGTGGTGCT
121   GCAGGCGCCN TTNTGCTGGA GCCTGGTGAG GAAGGTAATG GT
```

Translated sequence: Reading frame 1.

```
1     PPVAFIESGR YKKVVVVGAD KMSAIVDYSD RSTCILFGGA AGAXXLEPGE EGNG
```

2 A6 DNA sequence:

```
1     GAAAGCTTGC ATGCAGGCCT CTGCAGTCGA CGGGCCCGGG ATCCGATCCA TATGTAATAC
61    GACTCACTAT AGGCCGCCAG TCTACACCCA GTGCAGGATG TATTGACAGT CAATCAACTA
121   AAATTGCTAG TCGATTAGCA TCCCCAGAAG CGGTTGGTAT TGATGGAAAC AAATTAGTCA
181   ATGGTCACAA ACGTAATCTT CTTGTGGATA CTCTGGGACT AATGATGATG GTGGTAGTTA
241   CTGCGGCAAA TGTTAATGAC CGTAAGGGTG CAACCATGAT TTTGGAAAAG CTTAACCAAA
```

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```
301  TGCGTCACTG ATTCCCACGT CTCTCAACGA TTTGGGTGGA TCGTGGTTAT AGTGGCAAAG
361  CTTTCGTTCT TGCTATTCTC CATACTTTTT ACTGGTGTTT ACAAGTTGTT GTTCCTCCNG
421  TGGGGTAAAA AGGCTTTGTT GTCCAACAGA AGCGCTGGGT TGTTGAACGC ACTTTTGCTT
481  GGCTTTCTCG CTTTCGTCGT TTGACTAGAG AGTACGAATT TTTACCTGAA ACTTCTGAGG
541  CTTTTTTNTA TGTTGACATG ATTCAGATTC TTATCAAACG CCTCGCTTAA CTTCTCAAAC
601  ACGCTCTAAT GCAACTTATG TGGGAATCGC CGATGGAGCA GAANCAAATT GGACATTCTT
661  GAAGAAACTT ACAGAAGAAC AGATACTGGA CTTTTATCAC GCATCAGGTT NNTTGGGAGC
721  AGTCGCCGAG AGTCTATAGT GTCACCTAAA TCCTTTGTAG TCGTATTACA TATGGATCTA
781  GATGCATTCG CGAGGTACCG AGCTCGAATC ACTNGCCGTC NTANNCC
```

Translated sequence: Reading frame 3.

```
1    KLACRPLQST GPGSDPYVIR LTIGRQSTPS AGCIDSQSTK IASRLASPEA VGIDGNKLVN
61   GHKRNLLVDT LGLMMMVVVT AANVNDRKGA TMILEKLNQM RH*FPRLSTI WVDRGYSGKA
121  FVLAILHTFY WCLQVVVPPV G*KGFVVQQK RWVVERTFAW LSRFRRLTRE YEFLPETSEA
181  FXYVDMIQIL IKRLA
```

2 B2 DNA sequence:

```
1    GAAAGCTTGN ATGCAGGCCT CTGCAGTCGA CGGGCCCGGG ATCCGATCCA TATGTAATAC
61   GACTCACTAT AGGTTAGGGC TAGCTTCGGT GGAGGCGTCG GTGGGGTACT ACCCTGGCTG
121  TATTGAAATT CTAACCCGCG GCCCTGATCG GGCCGGGAGA CAGTGTCAGG TGGGCAGTTT
181  GACTGGGGCG GTCGCCTCCT AAAATGTGAC GGAGGCGCCC TAAGGTTCCC TCAGAATGGT
241  TGGAAATCAT TCGTAGAGTG TAAAGGCACA AGGGAGCTTG ACTGCGAGAC CTACAAGTCG
301  AGCAGGGACG AAAGTCGGGC TTAGTGATCC GGTGGTTCCG CATGGAAGGG CCATCGCTCA
361  ACGGATAAAA GCTACCCCGG GGATAACAGG CTTATCTCCC CCAAAAGTCC ACATCGACGG
421  GGAGGTTTGG CACCTCGATG TCGGCGCATC GCATCCTGGG GCTGTAGTCG GTCCCAAGGG
481  TTGGGCTGTT CGCCCATTAA AGCGGTACGC GAGCTGGGTT CAGAACGTCG TGAGACNNNT
541  TCGGTCCCTA TCCGTCGTGG GCGTAGGAAA TTTGAGAGGA GACTGGCGGC CTATAGTGAG
601  TCGACATAGG ATCTAGAGCA TCGCGAGGTN CGAGCTCGAA TCACTGCCNC NANC
```

Translated sequence: Reading frame 2.

```
1    KAXMQASAVD GPGIRSICNT THYRLGLASV EASVGYYPGC IEILTRGPDR AGRQCQVGSL
61   TGAVAS*NVT EAP*GSLRMV GNHS*SVKAQ GSLTARPTSR AGTKVGLSDP VVPHGRAIAQ
121  RIKATPGITG LSPPKVHIDG EVWHLDVGAS HPGAVVGPKG WAVRPLKRYA SWVQNVVRXX
181  RSLSVVGVGN LRGDWRPIVS RHRI*SIARX ELESLPX
```

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3 B1 DNA sequence:

```
1    TAGTGTACCA TCAACATCAA AAATAACTGC TTTTGGTTTA CCAGGAGTCC CATTATATAC
61   TGGAAGACCG AGATACTCTC GCATGCTTTT ATACATTGAA CGTAAAACAT CAATTGGTAC
121  TGCTTTAGTT CCGCGTTTTG AGTTACGTTT AACCAATTCA GTCCAAGGAA CATCAAACAC
181  TTTATGTTCA ACTTTCCAGC CGTATTCTTT GGCAAAAGTT TCCCATGCTA GGCGACGTTC
241  AGGATTCAGG TTAGTATCTG AAATGATTAC TCCCTTAACA GAATCGCCAC CGTACAGAAT
301  ACTTTTAGCT GTATCAAACT GCATACCAGT TACGATACCT CCTTTCTTTT TGGTATACTT
361  GTACTCATCG CGTTCTTCAT GCGCCATAAT AGATTGGCGA TAGTCATCAC GATTGATATT
421  ATAAAACCCG GGATTCTTAG CAATAAATTC ACGAGCCCAA GTACTCTTAC CAGAACCAGG
481  ACAGCCAATA GTCAAAATAA TCTTTTTCAT TTATTTTTTC TCAANTAANG ATTGAATATC
541  CGGATGTAAT CTAGATGCAT TCGCGAGGTA CCGAGCTCGA ATCACTGCCN CGAACCC
```

Translated sequence: Reading frame 2.

```
1    GFXAVIRARY LANASRLHPD IQSLXEKK*M KKIILTIGCP GSGKSTWARE FIAKNPGFYN
61   INRDDYRQSI MAHEERDEYK YTKKKGGIVT GMQFDTAKSI LYGGDSVKGV IISDTNLNPE
121  RRLAWETFAK EYGWKVEHKV FDVPWTELVK RNSKRGTKAV PIDVLRSMYK SMREYLGLPV
181  YNGTPGKPKA VIFDVDGTL
```