A CUSTOMISED BIOREACTOR FOR BENEFICIATION AND BIOREMEDIATION OF EFFLUENTS CONTAINING HIGH VALUE ORGANIC CHEMICALS

SG Burton • DA Cowan • C Garcin •
A van Schalkwyk • C Werner

WRC Report No. 1361/1/06

Water Research Commission

A CUSTOMISED BIOREACTOR FOR BENEFICIATION AND BIOREMEDIATION OF EFFLUENTS CONTAINING HIGH VALUE ORGANIC CHEMICALS

by

S G Burton[1], D A Cowan[2], C Garcin[1], A van Schalkwyk[2] and C Werner[1]
[1]Department of Chemical Engineering, University of Cape Town
[2]Department of Biotechnology, University of the Western Cape

Report to the Water Research Commission on the project

"Development of a customised bioreactor for bioremediation of organic-containing effluents and conversion of constituents to high value chemicals"

WATER RESEARCH COMMISSION PROJECT K5/1361

Head of Department : Professor E van Steen

Project leader : Associate Professor S G Burton

WRC Report No: 1361/1/06

ISBN No: 1-77005-380-8

FEBRUARY 2006

Disclaimer

This report emanates from a project financed by the Water Research Commission (WRC) and is approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the WRC or the members of the project steering committee, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

Printed by Silowa Printers: 012 804 1164

EXECUTIVE SUMMARY

Introduction to the problem

In the agricultural industry, the processing of food materials often results in problematic organic-polluted wastewaters. As a prime example, the process of table olive production involves several water-intensive washing steps and fermentation (Fig (i)), resulting in a complex, darkly coloured effluent containing high concentrations of lipids, polysaccharides and a range of phenolic compounds. The phenolics exhibit antimicrobial and phytotoxic properties, and this, together with a high chemical oxygen demand of up to 200g/L, makes the process wastewaters resistant to biodegradation. Hence the olive process industry has a disposal problem. Current practice of using preliminary anaerobic fermentation followed by ponding has not solved the environmental problem, largely because of the presence of the phenolic components. The olive industry in South Africa is expanding rapidly and a means of conserving water and protecting the local environment will be valuable.

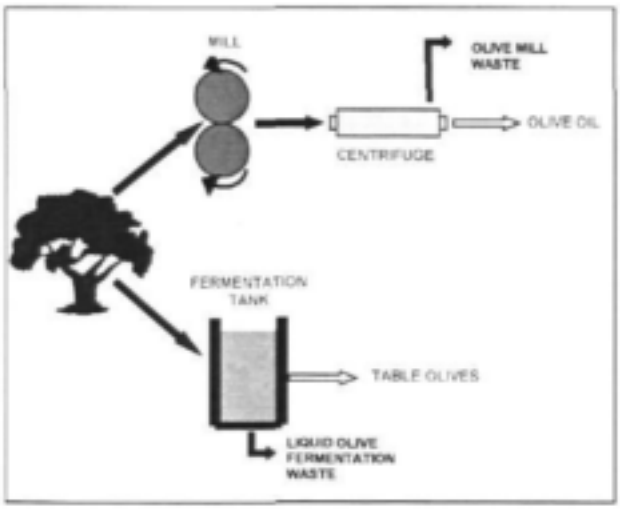


Figure (i) The olive process and waste production

Project aims

The main aim of the project was to design, construct and demonstrate a biocatalytic bioreactor for the bioremediation of specific organic-containing wastewaters, with concomitant recovery of useful organic components, via their biotransformation to high-value products. The development of the bioreactor would be based on our existing understanding of

bioremediation of polyphenolic wastewaters, with the additional design and assembly of the components required for recovery of phenolic derivatives from treated effluents using hydrophobic membranes. The next objective was the application of the bioreactor in treatment of olive production wastewaters with concomitant production and recovery of phenolic-related derivatives. It was also an objective to adapt this system for treatment on wine production waste waters and production and recovery of resveratrole-related derivatives. However, preliminary investigations showed that winery wastewaters were too dilute to yield sufficient amounts of high-value phenolics to make processing economically viable. Based on outcomes of the objectives listed above, we aimed to adapt the bioreactor design for application using alternative wastewaters to demonstrate generic utility as wells as to produce other high-value products. Such technology is considered to be applicable with several food industry wastestreams, eg from fruit processing.

A high value product from the waste

It is well documented that the low molecular weight phenolics in olives and olive oil, which include hydroxytyrosol, are powerful antioxidants and have broad biological activity including health benefits (contributing to the Mediterranean diet effect; Robards 2003, Ryan et al. 2002, 1999a&b, McDonald et al. 2001). These phenolic compounds are also present in olive production wastewaters. In particular, the simple o-diphenol, hydroxytyrosol (3,4-dihydroxyphenylethanol) is present as the glycoside oleuropein (Fig (ii)), the main phenolic compound in green olives responsible for their bitter taste. In processing of green olives, hydroxytyrosol is released into solution. Hydroxytyrosol has only recently become commercially available despite widespread commercial, pharmaceutical and medicinal interest, and it is prohibitively expensive (~US$1000-2000/g). Its recovery and purification from olive *mill* wastewater (from olive oil production) has previously been investigated (Capasso *et al.*, 1999, 1994). However, fermentation brines represent a more viable source of extractable hydroxytyrosol because they are aqueous streams and have low concentrations of suspended solids (as compared with olive mill waste which contains emulsified oil and olive pulp solids). Thus the extraction of hydroxytyrosol has been a primary focus in the course of our study. Compounds such as vanillin and resveratrole wer also deteceted but at concentrations too low to warrant immediate attention.

In this project we have developed technology to facilitate the extraction of hydroxytyrosol from table olive wastewaters produced in the Western Cape, and then to bioremediate the residual extracted wastewater. The first objective achieved was a complete examination of the properties and composition of olive fermentation brines, both local and those reported in the literature, with particular focus on the phenolic fraction and its antioxidant activity (see table below).

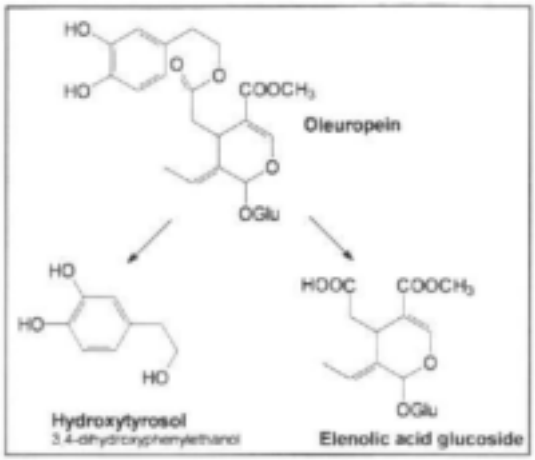


Figure (ii) Hydroxytyrosol from oleuropein

Table 2.1: Physico-chemical analysis of olive brines and mill wastewaters

	pH	Cond. ($mS.cm^{-1}$)	TS ($g.L^{-1}$)	DS ($g.L^{-1}$)	SS ($g.L^{-1}$)	COD ($g.L^{-1}$)	TOC ($g.L^{-1}$)	N ($g.L^{-1}$)	Sugar ($g.L^{-1}$)	Lipids ($g.L^{-1}$)	TP ($g.L^{-1}$)	SP ($g.L^{-1}$)	K^+ ($g.L^{-1}$)
Black olive brine													
OW-04	4.5	83.1	114.2	113.9	0.30	75	18.35	0.20	0.26	1.83	4.91	2.89	3.71
OW-08	5.2	76.1	129.2	128.8	0.38	81	16.50	0.19	0.31	0.56	5.28	3.31	
OW-13	4.6	97.7	92.1	91.2	0.82	55	6.25	0.03	0.22	9.36	1.78	1.04	
OW-15	5.2	81.8	139.0	137.9	1.07	82	15.55	0.18	0.34	0.89	4.45	2.92	
Benitez et al (2001)	12.6		8.16		0.34	3					0.24		
Beltran-Heredia et al (2000)			5.72	5.52	0.20	6		0.03			0.18		
NaOH wasetwater													
OW-09	9.9	16.3	59.8	58.5	1.30	81	19.90	0.43	9.31	1.39	2.21	1.80	
Kotsou et al (2004)	12.3	34.0	20.3	16.8	3.54	10					0.32	0.03	
Kyriacou et al (2004)	11.0					22		0.002					2.5
Green olive brine													
OW-10	4.1	79.0	114.3	113.9	0.40	57	12.70	0.02	1.18	1.08	2.61	1.65	
Owen *et al* (2003)												1.36	
Mill wastewater (OMW)													
OW-06	4.9	9.6	119.1	58.8	60.3	202	15.40	0.46	12.6	74.0	4.07		3.42
OW-17	5.1	11.10	88.9	61.8	27.1	101	19.80	0.39	14.7	58.3	3.78	2.92	
Fadil et al (2003)	5.2		92.4	6.2	86.2	124		0.15	12.8		8.2		
Assas et al (2002)	4.9		109.0	94.0	15.0	120		0.08					
Borja *et al* (2003)	5.1		124.0	15.8	106.6	113					1.83		
D'Annibale et al (2004)	5.3				38.0	85		1.3	8.75		5.50		0.78
Raposo et al (2003)	4.9		16.7	2.7		22	6.28	0.21			0.06		

TS = total solids, SS = suspended solids, DS = dissolved solids, COD = chemical oxygen demand,
TOC = total organic carbon, N = nitrogen, TP = total phenols, SP = simple phenols, K = potassium

There is wide variation in concentration levels between the different types of wastewater and also between wastewater from different sources, but in all cases the organic load (measured as Chemical Oxygen Demand, COD) exceeds the discharge limits stated in Section 39 of the National Water Act (>75mg.L^{-1}). Olive wastewaters are naturally acidic due to the presence of phenolic and other organic acids including elanolic, lactic and acetic acids. However, in some cases high pH arises because of the addition of NaOH during processing, particularly of

green olives. The brines are also highly saline (as indicated by conductivity and dissolved solids) from the salt used during processing. This increases the resistance of these wastes to biological degradation, as both phenol- and halotolerant microorganisms are required. Suspended solids in brines are low compared to OMW which contain large amounts of pulped olive solids. The residual oil of OMW is wasted primary product and is indicative of inefficient extraction arising from the formation of an oil-water emulsion that is not readily separated by the centrifuges used for oil extraction. It is to be expected, however, that mill wastewaters would be easier to degrade as they do not have the high salinity of the brines. The brines have minimal lipids; this is because the olive cells are not disrupted in the fermentation process as they are during the milling process for oil extraction.

All the wastewaters have low nitrogen concentrations. For biological degradation a COD to nitrogen ratio of around 20:1 is desirable, since microbial growth requires nitrogen as a nutrient. In this project it was necessary to determine whether nitrogen supplementation was necessary for biodegradation, since this may lead to increased costs. The wastes also have high concentrations of potassium which is, incidentally, the reason why residual olive pulp and solids from oil extraction cannot be used as animal feed.

Comparison of brine wastewater data (in Table 2.1 above) showed that there is a higher organic load in our local wastewaters than others report; this is generally because these wastewaters reported by others come from storage facilities that are mixed with rinsing and other wastewaters, whereas our data related to wastes directly from fermentation tanks. This is relevant in terms of our objective of extracting value-added by-products (see next section).

A process to extract hydroxytyrosol

In order to obtain hydroxytyrosol from local olive production brines, a novel supported-solvent membrane extractor was developed. Membrane-supported solvent extraction was successfully demonstrated at laboratory scale (0.1m^2 membrane module) and was found to be effective for the extraction of hydroxytyrosol from olive wastewaters, as verified by high performance liquid chromatography. Pertinent parameters such as flow and extraction rates have been measured and mathematically modelled, which allows for the calculation of necessary plant size and optimisation of operating conditions. The most cost-intensive parts of the suggested process are the evaporation and condensation steps, due to the energy requirements for heating and cooling. To this end the membrane extraction process should be over-designed to allow for maximum stripping of product from the wastewater, with capacity based on the maximum anticipated wastewater production volumes. This results in the minimum required equipment size for evaporation and condensation. Over-design of the membrane system will have only a small impact on overall capital requirements.

Economic benefits of hydroxytyrosol extraction

This investigation revealed that approximately 1 g of hydroxytyrosol could be obtained per litre of olive brine, using the membrane extractor. This compound has recently become commercially available (in limited amounts) internationally, for use as a natural antioxidant, and is priced at a high market value (US $1000 – 2000 per gram). Pure hydroxytyrosol has a current market value of US1200$ per gram, and one local table olive processor produces at least 150 000L per annum of this high strength wastewater i.e. 150kg total, worth, potentially, 1800M US$. The total South African production potential for hydroxytyrosol is at least five times this amount. At present the world production of hydroxytyrosol is not well known, but the potential uses are numerous; for example, one is as a healthy replacement for synthetic (and toxic) food additives such as BHT and BHA which are used at approximately 1ppm in fats and oils. Thus, the scope of the commercial possibilities of extracting hydroxytyrosol is clear, and this is a very significant opportunity to add value to the bioremediation process.

Extraction rates essentially depend on the amount of membrane surface area used, and this would be dictated by the wastewater production volumes. The extraction resulted in the wastewaster being converted to an effluent containing significantly reduced (by approximately 50%) low molecular weight phenolics. Since these are the components which are generally antimicrobial, the extracted wastewater becomes more amenable to biodegradative treatment.

Bioremediation of olive production wastewaters

The project included an investigation of how microbial degradation of the extracted effluent might best be achieved. Microorganisms capable of growing in the presence of the olive waste were isolated from the evaporation ponds at local olive production plants, and cultured in media where the only available carbon nutrient was the olive waste. On this basis, the organisms that grew could be assumed to be producing enzymes required to degrade the phenolic compounds which would otherwise be toxic to microbial metabolism Such enzymes are key to bioremediation of the toxic wastewaters. In particular, one of the isolated strains, AS-35, showed considerable potential for olive waste biodegradation. This organism was selected for more detailed investigation in which it was subjected to chemically mediated mutation, producing mutants with improved tolerance to the olive phenolics, to the point where it could tolerate 4 times the concentration that the native strain could. The profiles of the protein (enzyme) composition of the native and mutant strains were analysed by modern 2-dimensional electrophoresis (proteomic) techniques, and results indicated that the mutant had enhanced ability resist to the chemical stress imposed by the waste.

Initial results of bioremediation studies using strain AS-35 in shake flask experiments (at 250mL scale) using a 25% dilution of the brine wastewater showed a 56% reduction of

phenols, occurring predominantly in the first 48 hours. The maximum substrate utilization rate with respect to total phenols, was determined to be 0.23 $g.L^{-1}$/day. A separate set of experiments were conducted in the waste to correlate growth and phenol removal, and to compare effectiveness in shake flask, airlift and stirred tank reactors. The maximum specific growth rates (μ_{max}) using 10% raw waste were determined as 0.161, 0.102 and 0.123 hr^{-1} for the shake flask, airlift and stirred tank reactors respectively, with a significantly longer lag phase observed for the stirred tank reactor. In terms of bacterial growth on alternative carbon sources these values compare favourably with those achieved by others (Abuhamed et al. 2004). In these experiments, a 45% reduction of the total phenolic fraction was achieved in shake flask culture and 25% in the airlift and stirred tank reactors. The maximum specific degradation rates of total phenols were 0.096, 0.051 and 0.112 $g.L^{-1}$/ day for the shake flask, airlift and stirred tank reactor respectively. Concomitant COD reductions of 32 (1.8 – 1.2 g/L) and 47% (2,1 – 1.1 g/L) for the airlift reactor and shake flask experiments show appreciable detoxification of the waste. Later experiments, using waste from which hydroxytyrosol had already been extracted, showed superior results (see next paragraph).

Various metabolic compounds produced by several the isolates as by-products of the phenol biodegradation process, such as vanillin, were identified. However, in comparison to the levels of hydroxytyrosol which could be extracted, these compounds were of much less significance and would require more down stream processing. Nevertheless, this remains an avenue which should be pursued at a later stage.

A variety of other microorganisms including yeast, fungi, and mixed cultures are also known to be able to biodegrade olive waste. For comparison with the bacterial isolates, we investigated a well-known white rot fungus, *Trametes pubescens* and also mixed acclimatised cultures for their ability to remove phenolics and COD from the olive waste. In the case of bacterial cultures, while organic acids and some simple phenolics were consumed, the formation of high molecular weight humic substances and subsequent darkening of the waste was a common trend. *T. pubescens* was the only organism capable of decolourising the waste. Coupled with an excellent removal of phenols and ability to produce extremely high titres of laccase in starter culture, this organism could potentially be used in a treatment process. *T. pubescens* was also able to efficiently remove phenols from extracted waste and an additional nitrogen source was not required; this is important in maintaining low costs in a bioremediation process. The total phenols removal from previously extracted waste showed a similar overall degradation trend of more than 50% total phenols removed. The maximum degradation rates ($g.L^{-1}$/day), 0.23 and 0.25 for supplemented and non-supplemented media respectively, are almost identical. This data shows that the larger polyphenolic fraction can still be biodegraded to a certain extent and that the added nitrogen source is not necessary to achieve satisfactory total phenols removal with *T. pubescens* in an extracted waste medium.

A generic bioreactor and the two-stage process

In the course of the project a bioreactor system was developed to treat the high strength wastewater by a two-stage process (Figure (iii)) comprising initial extraction followed by bioremediation in a novel membrane bioreactor (Figure (iv)).

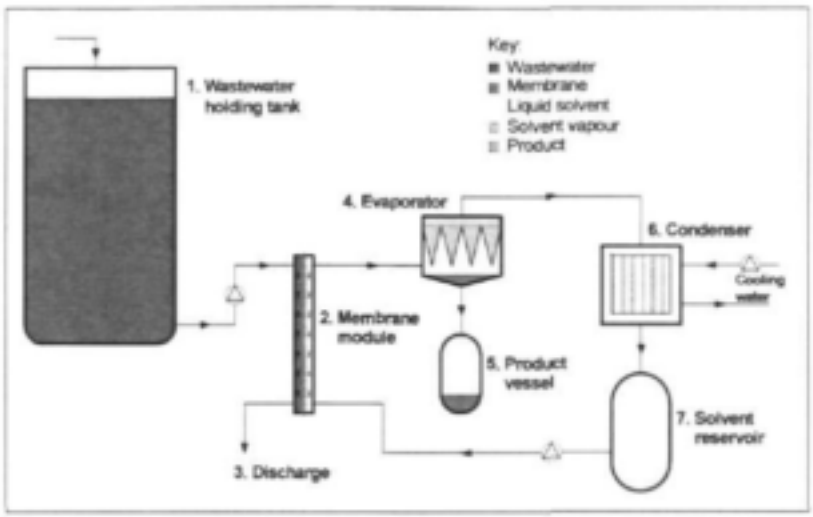


Figure (iii) Schematic diagram of proposed complete extraction system

A submerged-membrane bioreactor was designed for biological treatment of high strength wastewaters (Figure (iv)). The reactor was designed so as to enable the investigation of hydrodynamic effects upon membrane performance through the use of different sized draught tubes. The hydrodynamics in the reactor were modeled allowing for the prediction of gas-induced recirculation velocity past the membrane, based on superficial aeration intensity and reactor geometry. The physical effects of the hydrodynamic regime upon membrane fouling characteristics were then investigated through a critical flux experiment, illustrating the importance of high cross-flow velocities. The implications are that such reactors can be designed for optimal membrane performance and reactor operation. The optimal reactor configuration was demonstrated by batch degradation experiments. The performance of the reactor was satisfactory in terms of both membrane fouling characteristics and overall degradation rates.

Degradation experiments using fungi (*Aspergillus*), bacteria (*Bacillus*), yeast (*Candida*) and mixed microbial cultures were all successfully performed in the submerged membrane bioreactor, demonstrating the versatility and generic nature of the system. The degradation rates in the reactor were significantly better than parallel shake flask experiments. Using *Candida* as an example, total phenol removal levels of approximately 67% were achieved from solutions with initial concentrations of 960mg.L^{-1}, and COD reduction achieved was 57% from initial concentrations of around 16g.L^{-1}. The maximum degradation rates for phenol and

COD were 0.48 and 4.3g.$L^{-1}day^{-1}$ respectively. These were almost double the degradation rates obtained in shake flask cultures, and the percentage total removals were also improved by a factor of ~1.5.

The implication for submerged membrane bioreactors is that the reactor performance can be greatly improved by careful design of a draught tube located around the membrane unit. The draught tube serves two purposes: firstly, it confines the air bubbles tightly around the membrane which improves the bubble scouring effect that removes deposited material from the membrane surface, and secondly, it induces a recirculating cross flow liquid velocity past the membrane which also reduces membrane fouling.

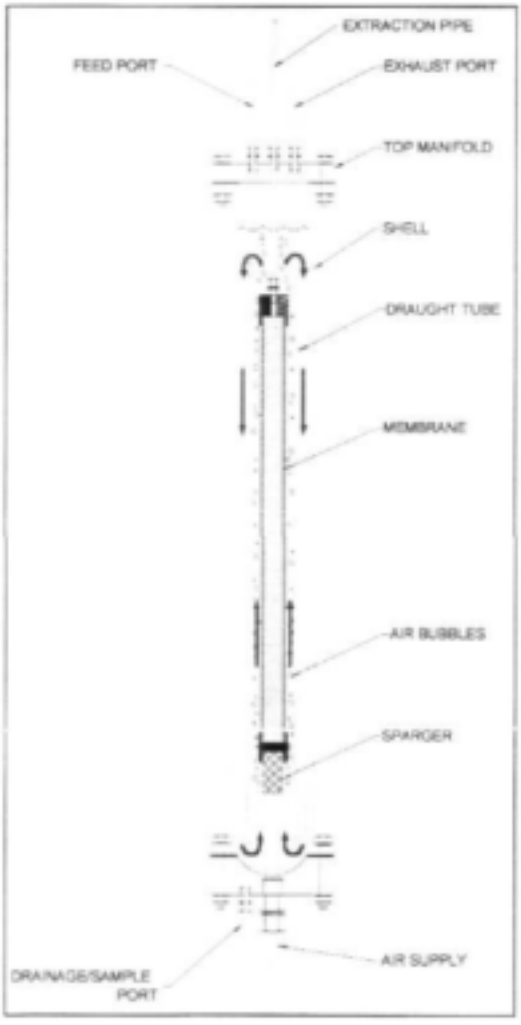


Figure (iv) Schematic of submerged membrane airlift reactor

For the purpose of further system development and experimentation it was decided to design a scaled-up reactor system (Fig (v)), which is currently in construction. A larger reactor is required for fitting instruments like DO meter, pH probe and level sensors, as well as allowing for larger sample volumes. This will aid the modeling of microbial biomass evolution and substrate utilization kinetics. The basic reactor design is shown below. The reactor was designed such that the riser to downcomer ratio gives predicted liquid velocity in the riser of $0.34 m.s^{-1}$ at an aeration intensity of 1volume/volume/minute (vvm).

The membrane extraction system could be incorporated into a complete extraction process as illustrated in Figure (iii). Using a membrane based extraction system allows for considerable scaling-down of equipment (compared to a conventional counter-current liquid-liquid extraction process), which means that such extraction systems could feasibly be installed at small-scale production facilities. This concept was submitted as a business plan for the National Innovation Competition. Patenting of the process is being investigated, and scaled-up (continuous) system development is under way.

On the basis of the above, a scaled-up reactor was designed for further studies and complete system development. This work would be a valuable extension of the present project.

Figure 6.8 Scaled-up 4L airlift membrane bioreactor

7.2 RECOMMENDATIONS

7.2.1 Business development

There is enormous potential for developing a process in which hydroxytyrosol is extracted and marketed as an antioxidant. The research team conducting this project have now written a business plan for such a process, and it is recommended that a patent should now be established, to support this. This plan presents a novel and cost-effective procedure for the extraction of the valuable antioxidant hydroxytyrosol from olive production wastewaters in relatively pure form (>90%). There are several potential markets for this crude extract, including incorporation into olive extract antioxidant products, fine chemical producers can further refine it for research and medicinal purposes, beneficial food and drink preservatives, and a cosmetics preservative. The projected sales price for the crude extract is R10 000/kg. [If a nutraceutical manufacturer were to incorporate 20mg of extract into a tablet, this will cost R0.20/tablet for the active ingredient and estimated extract production quantities would allow for the manufacture of approximately 7 million such pills annually. The total net profit after three years of operation could be at least R1m]. A membrane-based extraction process also allows for scaling-down of capital equipment, which could make small-scale stand-alone systems feasible. This should be considered, particularly in South Africa, for the establishment of SMME activities associated with, or satellite to, large olive production plants. The scale of a hydroxytyrosol production facility should be determined by the market, rather than an effort to treat the full volume of waste from olive production in the region.

Market research and market development are also required, because hydroxytyrosol is not widely available and therefore is not yet widely known in the public domain. An experimental programme should be implemented to demonstrate its biochemical benefits, in terms of antioxidant capacity, is feasible using a selected range of standard chemical antioxidant assays and cell culture-based studies. Such methodologies are available in the UCT research group labs and should be used to support a market development plan.

7.2.2 Technical development

The reactor design which has been achieved requires refinement and then a phase of optimisation. The observations from this project indicate that for submerged membrane bioreactors, the reactor performance can be greatly improved by careful design of a draught tube located around the membrane unit. The research team suggests that support for a phase of optimisation should be proposed and that a pilot plant system might then be implemented at a local production facility. Using the scaled-up reactor designed for further studies, and complete system development would be a valuable extension of the present project.

ACKNOWLEDGEMENTS

The research in this report emanated from a project funded by the Water Research Commission and entitled:

"Development of a customised bioreactor for bioremediation of organic-containing effluents and conversion of constituents to high value chemicals"

The Steering Committee responsible for this project consisted of the following persons:

Dr G Offringa	Water Research Commission (Chairman)
Professor S Burton	University of Cape Town
Professor D Cowan	University of the Western Cape
Ms R Mulbauer	BHP Billiton
Ms L van der Meer	Olivesinfact
Dr Len Dekker	

The financing of the project by the Water Research Commission and the Royal Society, UK, and the contribution of the members of the Steering Committee are acknowledged gratefully.

This project was only possible with the cooperation of many individuals and institutions. The authors therefore wish to record their sincere thanks to the following:

The National Research Foundation

The staff of the UCT Chemical Engineering Department and Workshop

Buffet Olives (Pty) Ltd

Vesuvio Olives (Pty) Ltd

PROJECT OUTPUTS

Patent in preparation:

The Use of Novel Extraction Technology for the Recovery of a Highly Valuable Antioxidant from Olive Processing Wastewaters Resulting in a Product for the Nutraceutical Industry.
C Garcin and S Burton.

Business plan for National Innovation Competition 2004:
Novel Extraction Technology for the Recovery of Valuable Components from Agro-industrial Wastewater Effluents.
C Garcin and S Burton.

Papers in preparation

1. Garcin, D. Cowan and S. Burton. Quantification, Extraction and Antioxidant Activity Assessment of Hydroxytyrosol (3,4-dihydroxyphenylethanol) from Olive Fermentation Brines (submitted).

2. Garcin, D. Cowan and S. Burton. Extraction of antioxidants from olive wastewaters using membrane-assisted solvent extraction.

3. Garcin, D. Cowan and S. Burton. Development of a submerged membrane bioreactor for high strength wastewater treatment.

4. Garcin, D. Cowan and S. Burton. Towards a comprehensive treatment and beneficiation system for olive wastewaters.

5. Werner C, Garcin C and Burton S. Development of a bioreactor system for bioremediation of wasterwater from olive production.

6. Van Schalkwyk, A, Ndimba BK, Burton SG and Cowan DA (2004) Proteome analysis of a novel olive waste degrading *Bacillus sp.* mutant.

Conference presentations

International presentations:

1. Burton S. Biocatalysis using oxidases. Keynote. International Biotechnology Symposium, Santiago, Chile, 2004

2. Burton S. "Biotransformations with polyphenol oxidase and laccase" Department of Chemical Engineering, Georgia State University, Atlanta, November 2003

3. Burton S., Biotransformations of phenolics. Keynote lecture, Enzyme Engineering XVII, Santa Fe, 2003

4. Van Schalkwyk, A. Proteome analysis of a novel olive waste degrading Bacillus sp. Mutant", at the NRF/ Royal Society, Pretoria, August 2004.

National presentations:

1. Burton S. Biocatalysis, Biotransformations and Bioremediation. Presentation for **Chemical Engineering Day**, Cape Town, Feb 2002

2. van Schalkwyk A, Garcin C, Burton S G, Cowan D A. UV-mutagenesis as a means of improving the biotransformation potential of bacteria isolated from olive oil wastewater. SASBMB National conference, Pretoria, 2003.

3. Garcin C, Burton S, Cowan D. Identification, extraction and quantification of polyphenolic antioxidants from olive fermentation brines. SASBMB National conference, Pretoria, 2003.

4. van Schalkwyk A, Garcin C, Burton S G, Cowan D A. UV-mutagenesis as a means of improving the biotransformation potential of bacteria isolated from olive oil wastewater. Cape Biotech, 2003.

5. C Garcin, S Burton, D Cowan. Identification, extraction and quantification of polyphenolic antioxidants from olive fermentation brines. Cape Biotech, 2003

6. C Werner, C Garcin, D Cowan and S Burton. The biodegradation of the constituents of olive fermentation wastewaters using locally isolated microorganisms. Cape Biotech, 2003.

7. C. Garcin and S. Burton. Selective recovery of low molecular weight phenolic antioxidants from olive production wastewaters using membrane-assisted liquid extraction. Water Institute of South Africa (WISA) Biennial conference and exhibition, 2004, Cape Town, South Africa

8. A van Schalkwyk, S Burton and D Cowan. Improving the biotransformation potential of bacteria isolated from olive wastewater", at the 18th Congress of the South African Genetics society, Stellenbosch, April 2004.

9. A van Schalkwyk, S Burton and D Cowan . Proteome analysis of a novel olive waste degrading Bacillus sp. Mutant", at the 19th Congress of the South African Society of Biochemistry and Molecular Biology, Stellenbosch, January 2005

10. C Werner, C Garcin, D Cowan and S Burton. The biodegradation of the constituents of olive fermentation wastewaters using fungal and bacterial systems. SASBMB National conference, Stellenbosch, 2005

THESES

Masters thesis:

C Werner An investigation into the Biodegradability of Black Olive Brine Wastewater. (in preparation)

PhD theses:

C Garcin Investigation into the beneficiation and treatment of wastewaters from the olive industry: application of membrane technology (in preparation)

A van Schalkwyk Engineering microorganisms for degradation of phenolic pollutants (in preparation)

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION AND BACKGROUND: OLIVE PROCESSING WASTE WATERS AND THEIR BIOREMEDIATION

1.1 INTRODUCTION

Industrial activity leads to the generation of many by-products and waste products as a result of manufacturing or processing operations. Such xenobiotic organic containing effluents are problematic as they have an adverse effect on the environment, and therefore require treatment before discharge or preferably reuse. Phenolic-containing effluents in particular are generated by many industries, including petroleum refining, coal gasification, pulp and paper, pharmaceutical, resin and pesticide. Phenolic compounds have phytotoxic and antimicrobial properties, and therefore they are resistant to biodegradation and tend to persist in the environment.

Treatment of such effluents is thus important, and is achieved through physical, chemical or biological processes, or combinations thereof. These effluents generally have high organic loads which makes them unsuitable for conventional biological wastewater treatment processes. Physical treatments which are used include incineration, distillation, filtration or solvent extraction, while chemical treatments are predominantly oxidation or precipitation processes.

Despite the general antimicrobial properties of phenolic compounds, there are numerous microorganisms that can metabolise them. Degradation of lignin is one example of such biological metabolism of phenolic compounds. Many biologically-based processes have been developed for treatment of high strength wastewaters, as this often results in the most cost-effective solution, compared to physical or chemical processes. Biological treatment processes are often also combined with physical or chemical processes to obtain a high quality effluent.

The context of this project was to develop a generic bioreactor for biological treatment of phenolic-containing effluents. Authentic agro-industrial phenolic effluents arising from the production of olives were investigated as a model pollutant during the course of the project.

1.2 OLIVES AND ASSOCIATED WASTEWATERS

Olives are seasonal fruit and they spoil (ferment) quickly; thus they are processed as soon as possible after harvest, generally within a few hours. Olives, oil and the associated wastes are thus also produced seasonally which is problematic because waste treatment plants either need to be designed and scaled to meet large, but temporary, waste flow requirements. The plants then stand idle for several months. Alternatively, a smaller scale system can be used, but then large storage facilities are required. In addition, wastewater treatment facilities are expensive and are often not economically feasible for small producers. However, increasingly stringent wastewater legislation is forcing producers to confront the issue. This problem has led to the development of regional treatment facilities in the Mediterranean regions where critical densities of producers permit economy of scale. Such facilities take a holistic approach to waste treatment, and if not profitable (through by-products) are at least cost-effective (Gurbuz et al. 2004, Vlyssides et al. 2004).

Specifically, the problem is that large quantities of darkly coloured phenolic-rich wastewater are generated during the production of table olives and olive oil, up to 1.5m^3/ton of olives for oil, and more for table olives. These wastewaters constitute a serious environmental pollution and disposal problem, due to their high organic load, high phenolic content and acidity, which make the wastes recalcitrant to biodegradation. The quantity produced has been estimated to be of the order 3 x 10^7 $m^3.year^{-1}$ in the Mediterranean regions alone. Considering that 1 m^3 of this waste is organically equivalent to 100-200 m^3 of municipal wastewater, the scale of the degradation and disposal problem becomes apparent (Sayadi et al. 2000, Hamdi 1993). In South Africa, local table olive processors produce an estimated 500 000 L per annum of this high strength wastewater.

The olive production wastewater (OW) has a very high polluting organic load, expressed as Chemical Oxygen Demand (COD) or Biochemical Oxygen Demand (BOD). The COD and BOD reach 250 and 100 $kgO_2.m^{-3}$ respectively, depending on processing system (Lesage-Meesen et al. 2001, Hamdi 1993). The main organic constituents responsible for these high values are sugars, volatile acids and polyalcohols, pectins, fats and particularly ortho-, di- and polyphenols (Rozzi and Malpei 1996).

The total concentration of phenols can reach up to 10 g/L in olive wastewaters; this can be broadly separated into a low molecular weight (M_w) monomeric fraction (< 8 kDa) and a higher M_w fraction consisting of monomer-free polyphenols (> 8 kDa). It is the low M_w fraction that is predominantly responsible for the phytotoxic and antimicrobial effects of OW (Sayadi et al. 2000, Ramos-Cormenzana et al. 1998, Garcia et al. 1997). The higher molecular mass fraction

contains polyphenols, tannins and humic substances that are responsible for the dark colour, and are particularly resistant to degradation (Sayadi et al. 2000). The phenolic composition of OW is also variable, depending on olive cultivar and region, as well as processing conditions. The low molecular weight fraction is of much interest, as these phenolics are known to be biologically active compounds with strong antioxidant activity. More than 50 natural low molecular weight phenolic compounds have been identified in OW; this is discussed in more detail in section 1.4.

The phenolic component of the waste has antimicrobial and phytotoxic properties (Martin et al. 2002, Borja et al. 1996, 1995 &1992, Monteoliva-Sanchez et al. 1996, Ramos-Cormenzana et al. 1996, Hamdi 1992, Rodriguez et al. 1988), and is detrimental and toxic to municipal sewage treatment systems; these are capable of dealing with only low concentrations of feed (in the order of 1 kg $COD.m^{-3}$). Mixed culture aerobic treatment systems such as activated sludge and trickling filters are unsuitable for the treatment of OW, as these systems can also only tolerate low concentrations of feed. This means unacceptably long hydraulic retention times and the necessity for dilution (Rozzi and Malpei 1996). Disposal of olive wastewaters in sewers is thus discouraged and, in most cases, is illegal.

OW is traditionally disposed of in evaporation ponds, but this does little to reduce the organic load, merely serving to concentrate the waste. There are additional problems associated with this approach, which include leaching into groundwater, overspill from flooding and offensive smells. Thick, phenolic-rich sludge accumulates in these ponds. In fact, autooxidation and subsequent polymerization can make stored wastewaters more toxic and resistant to degradation than the fresh wastes (Assas et al. 2002). As a result, most wastewater treatment systems have been tried with greater or lesser success for the treatment of OMW. Initially studies were directed solely towards the degradation (removal of COD and colour) of the waste. However, the cost of such treatment and the abundance of the waste has spurned the investigation of the production of value-added products, *i.e.* utilising the waste as a resource for production of value-added by-products.

1.3 TREATMENT OF OLIVE WASTEWATERS

Wastewater treatment processes which have been tested on OW, with varied success, can be broadly classified into physico-chemical processes, biological processes, or a combination of the two. Biological processes can be further divided into enzymatic, aerobic or anaerobic treatments. Initially, treatment processes were directed towards the bioremediation/detoxification of the waste, however, because of its abundance and the cost of treatment, later research has been

directed towards its utilisation as a substrate for the production of some type of value-added product.

Physico-chemical processes for treating OW are reasonably widely employed but mostly unsatisfactory. These include thermal processes such as distillation, evaporation and incineration. The drawbacks of these processes are related to post treatment and disposal of produced emissions. Distillation and evaporation serve to concentrate the waste, but the concentrated product is still problematic. The distillate is not pure; it contains volatile alcohols and acids. Evaporation ponds have very little effect on the organic load of the waste. The concentrated paste cannot be used to provide the heat required for distillation as the combustion thereof produces toxic atmospheric pollution.

Chemical processes such as flocculation and clarification are not very efficient because most of the organic pollutants are difficult to precipitate. Liming is extensively employed; however, the efficiency of even heavy liming is in the order of 40-50%. Liming as post-treatment to biological processes has however proved useful in the removal of residual pollutants and suspended solids. Other chemical processes that have been investigated are treatment with aluminium sulphate, gelatine, bentonite or hydrogen peroxide. Ozonation has also been attempted as pre-treatment to a biological stage with some success. These have all proved to be beneficial but not spectacular and rather costly for the incremental improvements achieved. Membrane concentration processes such as ultrafiltration have been suggested but not investigated; problems arise from the high suspended solids concentration and the cost of such installations (Aktas et al. 2001, Flouri et al. 1996, Rozzi and Malpei 1996, Hamdi 1993, Federici et al. 1991).

Land treatment of olive mill wastewaters (OMW) has been utilised quite extensively; the waste is spread as biofertilizer for agricultural lands. This is an inexpensive and beneficial method of disposing of OMW, and involves simply irrigating agricultural land with OMW. Natural microorganisms within the top 2 m of soil can almost completely remove the organic and inorganic components of OMW at a limited annual dosage (~5000-10000 $m^3.ha^{-1}.year^{-1}$). This can be beneficial to soil fertility by increasing the soil organic matter content, which in turn stimulates microbial activity, as well as increasing available nitrogen, potassium and phosphorus. There is a concomitant increase in soil electrical conductivity, but generally below values indicating salinization (Caberra et al. 1996). However, care must taken not to irrigate around the bases of trees or too soon before sowing, and it is not recommended to irrigate growing crops due to the phytotoxicity of the raw effluent. The relatively low concentration of nitrogenous organic compounds in OMW and richness in carbon sources provides favourable conditions for free-living dinitrogen fixing microorganisms, predominantly members of *Azotobacter*. This can be

particularly beneficial to soil fertility (Balis et al. 1996). However, brined olive wastewaters are unsuitable for such an application due to the high quantities of salt present in the waste streams.

As pre-treatment to biofertilization, a recent study has shown that polyphenol oxidase enzymes occur naturally in olive husk (solid residue from pressing). These enzymes which originate in the seeds, are effective in significantly reducing phenolic content of OW, and incorporating a simple 'filtration' type process of OW through the husk in order to detoxify it makes subsequent biofertilization particularly attractive (Greco et al. 1999). In another study, OW was fermented with *Azotobacter vinelandii* to produce a high quality, nitrogen rich liquid fertilizer that could be applied directly to crops and showed very promising results (Chatjipavlidis et al. 1996).

Composting of OW with additional agricultural waste substrates is another method of recycling the waste with beneficial effects on soil fertility. The composting process eliminates the toxicity and degrades the OW, and experiments have shown that yields with these composts are similar to and sometimes even better than results achieved with (costly) mineral fertilizers (Cegarra et al. 1996, Monteoliva-Sanchez et a. 1996, Tomati et al. 1996).

In terms of a bioprocess, detoxification of OW means reducing phenolic content, colour and COD. The most effective species appear to be the lignolytic microorganisms. Primary amongst these are the white-rot fungi, which produce enzymes such as the peroxidases (*e.g.* lignin peroxidase and manganese peroxidase), and polyphenol oxidases (laccase and tyrosinase). Various moulds, yeasts and bacteria produce similar enzymes and are also capable of biodegrading OW. White-rot fungi are primary decomposers of wood: the irregular and recalcitrant nature of lignin and the fact that it contains substructures found in many primary pollutants (like phenols) means that these fungi can oxidatively degrade these pollutants *i.e.* the enzyme systems are non-specific. Species that have been used include: *Lentinula edodes*, *Phanerochaete chrysosporium*, *Phanerochaete flavido-alba*, *Coriolus* (*Trametes*) *versicolor*, and *Pleurotus* species (*P. ostreatus*, *P. pulmonarius*, *P. eryngii*, *P. cornucopias*). Experiments with these organisms have achieved between 45-75% colour removal, 60-90% reduction in phenols and up to 80% reduction in COD. Most of these organisms are able to utilise OW as sole carbon source and require no other additives. However, they do require dilution of OW for optimal efficiency; the undiluted waste is inhibitory towards growth. Whole-cell treatment has to date been more effective than crude or purified enzymatic treatment. Processes involving these fungi are generally submerged liquid fermentations, sometimes with immobilisation of the organisms. Only small pilot-scale experiments have so far been conducted due to the cost and batch nature of such processes. It is interesting to note that two of the white-rot fungi are very popular edible species: *Lentinula edodes* is commonly known as Shiitake mushroom (very popular in the East) and *Pleurotus*

ostreatus is the common oyster mushroom. In fact, several papers have been published on the production of edible biomass from OW (Blanquez et al. 2002, Sayadi et al. 2000, D'Annibale et al. 1998, Flouri et al. 1996, Martirani et al. 1996, Sayadi and Ellouz 1994 & 1993 & 1992, Vinciguerra et al. 1995 & 1993).

Other organisms that have been used effectively to detoxify OW are: *Aspergillus* species (*A. niger, A. terreus*), *Bacillus* species, *Penicillium* species, *Geotrichium candidum*, *Azotobacter*, *Ralstonia* and *Pseudomonas* species, and several others (Di Gioia et al. 2001, Garcia Garcia et al. 2000 & 1997, Robles et al. 2000, Ramos-Cormenzana et al. 1998, Fiorelli et al. 1996, Hamdi and Ellouz 1992, Hamdi et al. 1991, Perez et al. 1990). Pseudomonads are the most common species isolated from OMW ponds and composting.

Instead of considering OW as a costly waste problem, researchers have focused on its utilisation as an inexpensive and abundant substrate for biotransformation into some type of useful product. The list of products produced at laboratory scale from OW (with simultaneous detoxification) is considerable and continually increasing.

Anaerobic fermentations for the production of methane are an attractive idea for olive producers, as this will allow the recovery of some of the energy consumed in the oil production process. OW (with high COD) is particularly well suited to anaerobic fermentation when compared to other (more dilute) food industrial wastewaters. Types of process include anaerobic filters, anaerobic contact and upflow anaerobic sludge blankets. Other advantages include less sludge production (than aerobic fermentations), and the fact that digesters can be easily restarted without re-inoculation after several months of standing idle. The energy required for anaerobic fermentation is generally lower than for aerobic fermentations. The disadvantages are that the phenols and lipids inhibit the action of activated sludge microbial consortia; there is danger of organic overloading and long hydraulic retention times are required. In this regard, the above aerobic biological treatment processes have been investigated as pre-treatment for anaerobic fermentation, with significant improvement of performance. Influent concentrations for anaerobic digestion range from 15-70 kg $COD.m^{-3}$, loading rates vary from 2-4 kg $COD.m^{-3}.day^{-1}$ and hydraulic retention times are of the order of 20-25 days. Yields for untreated OMW range from 250-400 ml CH_4/g COD, and for pre-treated OW this improves to around 350-1000 ml CH_4/g COD. In effect, enough methane can be produced to cover the energy needs of both an olive oil production plant and the anaerobic reactor. Various pilot plants are in operation in the Mediterranean regions (Bertin et al. 2001, Erguder et al. 2000, Borja et al. 1996 &1995, Fiestas Ros de Ursinos and Borja-Padilla 1996, Hamdi 1996 & 1992, Fiestas et al. 1990). OMW has also

been anaerobically fermented for butanol production using a solventogenic *Clostridium* species (Waehner et al. 1988).

In the case of aerobic OW fermentations, there are many that have useful by-products in conjunction with detoxification and biodegradation. The white-rot fungi mentioned above produce enzymes capable of degrading OW as well as significant quantities of biomass, thus OW can be considered as a substrate for the production (and possible commercial exploitation) of these enzymes or biomass. *Pleurotus* sp. and *Lentinula edodes* have been extensively studied in this regard due to the impressive suite of enzymes produced when they are grown on OW, and the fact that they produce edible biomass. These fungi produce extracellular activity while also producing manganese peroxidase, manganese independent peroxidase and phenol oxidase, as well as xylanases, proteases and cellulases (D'Annibale et al. 2000 & 1999, Setti et al. 1998, Zervakis et al. 1996, Sanjust et al. 1991). Of these the peroxidases and phenol oxidases are of particular interest to enzymatic industrial bioremediation of phenolics and other aromatic compounds.

These enzymes can be easily produced in submerged fermentations, but there is also growing interest in solid-state fermentations (SSF). Recent studies have shown that it is possible to achieve about 75% reduction in toxicity and 80-90% removal of phenolics from OW within only two days of SSF. Significantly higher laccase production was reported in SSF, compared to parallel submerged fermentations with the same organisms (Setti et al. 1998, Zervakis et al. 1996, Sanjust et al. 1991). One method employed was immobilisation of mycelia on expanded clay or perlite, with sparging of recycled OW over the surface; another was inoculating an olive husk press-cake supplemented with liquid OMW. Both these methods produced *Pleurotus* fruiting bodies of high quality and yield after 15 days, *Letinula edodes* taking about twice this time for fruiting. Comparisons to commercial mushrooms showed no appreciable difference in taste, texture or form, and phenolic accumulation was not detected in the fruit. Laccase from *L. edodes* has been extensively characterised, and methods for the production thereof in SSF have been well documented (D'Annibale et al 2000 & 1999).

Another interesting application for the recycling of OW is the production of enzymes for use during the oil extraction process. Pectinase was produced by *Cryptococcus albidus* var. *albidus* grown on OMW, supplemented with sunflower calathide meal (to increase pectin concentration). The enzymes so produced were added to olives during the malaxation (softening) stage of the oil production process, with a resultant significant improvement in oil yield and quality (Montedoro et al. 1993, Federici et al. 1991, Federici 1988). More recently a study of a similar nature was performed using commercially available enzymes with positive results (Garcia et al. 2001).

OW has been tested as a substrate for a wide range of other bioprocesses, *e.g.* production of single cell protein for animal feed supplementation using *Saccharomyces chevaliere* and *S. rouxii* (Gharsallah 1993); production of microalgae *Chlorella pyrenoidosa* and *Scenedesmus obliquus* (Villasclaras et al. 1996). Several recent processes are of interest because of their novelty: Xanthan has been produced using OW as substrate by the organism *Xanthomonas campestris*. Xanthan is the most commercially accepted microbial polysaccharide, and is used for food and non-food purposes as thickener or viscosifier. *X. campestris* can grow on OW as sole nutrient source, although yields were improved by the addition of nitrogen and salts (Lopez et al. 2001, Lopez and Ramos-Cormenzana 1996).

Azotobacter vinelandii was cultivated in diluted OW as sole nutrient source, and produced large titres of capsular polysaccharide. This was entrapped in polyvinyl alcohol membranes and used for heavy metal recovery. Cadmium and lead ions were adsorbed from a liquid waste stream using these membranes (Pasetti et al. 1996). In similar work, *Azotobacter chroococcum* was used to produce polyhydroxyalkanoates (PHA's, 'bioplastics'), in particular poly-β-hydroxybutyrate (PHB). PHB has physical properties very similar to polypropylene, but is completely biodegradable to CO_2 and energy by many bacteria, fungi and algae. There is much interest in degradable 'bioplastics', but they are not currently competitively priced due to the use of glucose as feedstock. Alternative inexpensive feedstock (such as OW) might alleviate this problem. In addition, yields were high when OW was used as substrate: (intracellular) PHA's were produced up to 50% of cell dry weight after 24 hrs. This is ascribed to the natural high carbon, low nitrogen ratio of OW (Gonzalez-Lopez et al. 1996).

1.4 PHENOLIC ANTIOXIDANTS FROM OLIVES

Reports on the beneficial aspects of the "Mediterranean diet" and olive oil in particular have increased dramatically in recent years. In Greece, Italy and Spain, for example, the incidence of cardio-vascular disease and cancer are significantly lower than in other Western countries (Rose et al. 1986). The Mediterranean diet is largely vegetarian in nature and olive oil is the principle source of fat. Olive oil has a unique fatty acid profile consisting of predominantly monounsaturated fats (56-84% oleic acid, 3-21% linoleic acid). However, this alone cannot account for the beneficial aspects because oleic acid is also one of the predominant fatty acids in animal foods like poultry and pork, and the percentage oleic acid in the Mediterranean diet is only slightly higher than in other Western diets such as the American diet (Visioli and Galli 1998).

It has become apparent that it is more likely to be the polyphenolic components of olive oil (rather than the fatty acid profile) that are responsible for its beneficial qualities (Tuck and Hayball 2002, Kris-Etherton et al. 2002, Visioli and Galli 1998). Phenolic compounds occur in all plants as secondary metabolites, and embrace a considerable range of substances possessing an aromatic ring bearing one or more hydroxy substituents. Some are involved in metabolism, others protect vulnerable cell constituents against photooxidation by virtue of their strong UV absorption. They also play a role in disease resistance. However, intense interest in plant phenolics is due to their antioxidant activity *i.e.* their ability to scavenge reactive oxygen species. Olives have particularly high quantities of such phenolic compounds (Robards 2003, Antolovich et al. 2000, Ryan et al. 1999a). Despite the antimicrobial and phytotoxic properties of these phenolics, their effect on humans is deemed to be beneficial, cytotoxicity only occurring at levels far exceeding those attainable after habitual consumption (Babich and Visioli 2003).

The phenolics in olives can be conveniently classified into two major subclasses, simple and complex (hydrolysable). The main complex phenolic compound occurring in the green olive fruit is oleuropein (See Figure 1.1). This is the compound responsible for the bitter taste of green olives. The concentration thereof declines as the olives mature from green to black, and the oleuropein is hydrolysed to elenolic acid glucoside and predominantly hydroxytyrosol (3,4-dihydroxyphenylethanol), as well as several other simple phenolic compounds like tyrosol and phenolic acids (mostly hydroxytyrosol derivatives). Other complex phenols include ligostride and verbascoside (Tuck and Hayball 2002, Lesage-Meesen et al. 2001, Mc Donald et al. 2001, Ryan et al. 1999b, Visioli and Galli 1998, Brenes et al. 1995). These phenolics end up in the oil and are responsible for its unique organoleptic properties, as well as the remarkable stability of the oil to oxidation.

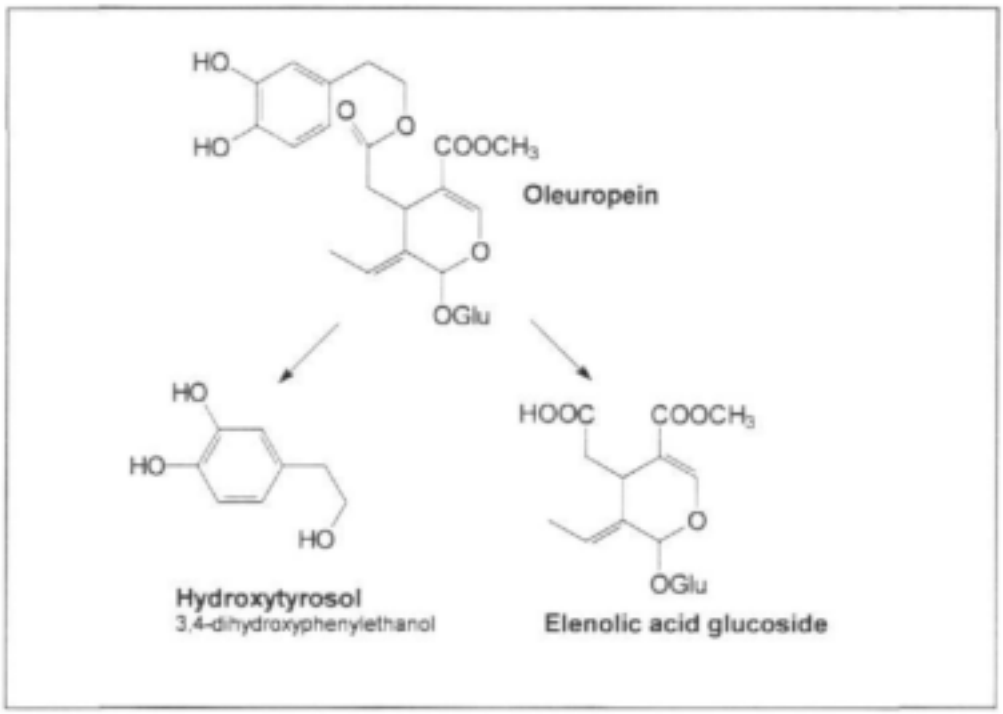


Figure 1.1: Hydrolysis of oleuropein

The antioxidant and other biological properties (particularly of hydroxytyrosol) have been the subject of intensive research in recent years. Various *in vivo* and *vitro* studies have shown:

- Antioxidant activity and free radical scavenging (Hayball and Tuck 2002, Mc Donald et al. 2001, Espin et al. 2000, Visioli et al. 1998, Baldioli et al. 1996)
- Prevention of low density lipoprotein (LDL) oxidation (Aruoma et al. 1998), Covas et al. 2000)
- Prevention of oxidative DNA damage (Armstrong et al. 1997, Aruoma et al. 1998)
- Anti-proliferative properties (Nicolaiew et al. 1998, Szaefer et al. 2003)
- Prevention of platelet aggregation (Petroni et al. 1995)
- Antibacterial (mycoplasmal) activity (Capasso et al. 1995, Furneri et al. 2002)
- Inhibition of 5- and 12-lipoxgenases (Kohyama et al. 1997, De la Puerta et al. 1999)
- Anti-inflammatory properties (Middleton & Kandaswami 1994)

The beneficial phenolics in the olives and oil are also known to occur in olive wastewaters, often at significantly high concentrations (Mulinacci et al. 2001, Ramos-Cormenzana et al. 1998, Capasso et al. 1992, Rodriguez et al. 1988). The wastes thus represent a possible source of valuable antioxidants that could be extracted for commercial use. One study reports on the commercial viability of such a process (Capasso et al. 1994), concluding that it is more

economically viable to chemically synthesize hydroxytyrosol than extract it (but environmentally unsound). However, these authors did not investigate OFW as source of phenolics, and recent advances in extraction technology (*e.g.* solid phase extraction and supported liquid membrane extraction) warrant an investigation.

1.5 BIOREACTORS FOR WASTEWATER TREATMENT

A review of bioreactor technology was presented in WRC project K5/1129. That project detailed the use of draught tube airlift reactors for the remediation of organic-containing effluents. Advantages of airlift bioreactors include simplicity of design (no moving parts), good mass transfer in gentle mixing conditions, small footprint and low energy requirements (Chisti, Chisti and Moo-Young , Ryan D. PhD thesis, 2004). In this work the airlift reactor concept was further developed by incorporating a membrane unit, effectively making a submerged membrane bioreactor (sMBR). sMBR's are becoming increasingly popular for wastewater treatment as they have several unique advantages: recycle of biomass, separation of solids and hydraulic residence times, low sludge discharge, high quality treated effluent and low energy requirements (Stephenson et al 2000, van der Roest et al 2002). A drawback of sMBR's is that the membranes are susceptible to fouling, which increases energy requirements and necessitates periodic backflushing and membrane cleaning. Nevertheless, sMBR's have been investigated for a variety of applications, commercial and industrial installations are becoming increasingly common (Schoeberl et al. 2005, Wen et al. 2004, Dhouib et al. 2005, Holler and Trosch 2001, Katayon et al. 2004, Konovalova et al. 2003, Cote et al. 1997). In this work physical, hydrodynamic and operational parameters were experimentally investigated inside the reactor, which led to the development of a design strategy for optimal operation. Bioreactors are discussed in more detail in the relevant section.

1.6 PROJECT OVERVIEW

1.6.1 Project Aims

At the outset of the project, the objectives were set as follows:

1. To design, construct and demonstrate a Biocatalytic Bioreactor (BB) for the bioremediation of specific organic-containing wastewaters, with concomitant recovery of useful organic components via their biotransformation to high-value products.

2. Development of a Biocatalytic Bioreactor (BB) based on our existing understanding of bioremediation of polyphenolic wastewaters, with the additional design and assembly of the components required for recovery of phenolic derivatives from treated effluents using hydrophobic membranes

3. Application of the BB in treatment of olive production wastewaters with production and recovery of vanillin-related derivatives

4. Adaptation of this BB system for treatment on wine production waste waters and production and recovery of resveratrole-related derivatives

5. Based on outcomes of the objectives listed above, adaptation of the BB design for application with a selected alternative effluent, to demonstrate generic utility.

The primary goal of the research project was bioreactor development, but this was necessarily preceded by a thorough analysis of the available wastewaters, which included olive wasteqater and winery wastewater. This was followed by an investigation into microbiological degradation of the wastewater.

The total phenolic content and the analysis of the phenols present in winery wastewater and olive wastewater were compared. Green olive wastewaters and mill wastewaters were analysed for comparative purposes. The winery wastewater was found to contain, as the major organics, ethanol and acetic acid, and only very small concentrations of phenolics. Even the principle phenolics, vanillin and resveratrole, were barely detectable. Thus, extraction of these would require very large volumetric throughput to yield useful amounts of antioxidants, and these levels would be too low to support an extraction and recovery process.

It was therefore decided to concentrate on olive wastewaters in this project, as these contained much higher levels of phenolics. In particular, brined black olive production wastewater is the least well studied of all the olive-derived wastewaters, while green olive wastewaters and mill wastewaters from the production of olive oil, have been studied extensively. Mill wastewater in particular has been the subject of intensive research, because globally, most olives are used for the production of oil. Analysis of wastewaters is the subject of Chapter 2 of this report.

Valuable phenolic antioxidants were found in the olive wastewaters in high concentrations. These are a possible valuable by-product, therefore novel extraction technology was investigated for the recovery of these compounds (reported in Chapter 3). The main phenolic antioxidant in the

wastewaters is hydroxytyrosol, a compound of much biomedical interest and of high market value in its pure form. A crude wastewater extract containing hydroxytyrosol could be used as a natural antioxidant preservative for food or in cosmetics, or could be incorporated into a nutraceutical product. Such an extract is of significant commercial interest. This work is described in Chapter 3.

For investigation of microbial degradation of the olive wastewater a three pronged approach was adopted: firstly, wild-type microbial strains were isolated from several wastewater sources and evaluated in terms of their degradative capacity. One of these strains was chosen for detailed genetic studies and mutagenesis in order to attempt to improve degradative ability. This is presented in Chapter 4. Secondly, this strain, together with other commercially available pure cultures, were evaluated for their degradative ability and results were compared with results reported in the literature. Lastly, mixed microbial cultures were obtained, enriched and acclimatised, and these were also then subject to degradation experiments. This work is presented in Chapter 5.

Finally, chapter 6 presents the design, characterisation and operation of a novel submerged membrane bioreactor. The reactor was first subjected to hydronamic characterisation and modelling followed by an investigation into critical factors affecting the membrane performance. The reactor was then tested as a generic bioreactor in its applicability to degrade olve wastewater using a range of different microbial species. The bioreactor can also be adapted for treatment of alternative effluents.

The final outcome of the project is a feasible process design that incorporates extraction of the high-value antioxidant content of the waste, followed by a bioremediation step in a membrane bioreactor system. Such systems have applicability particularly for small-scale producers, as they have low energy requirements (and hence operating costs) and have potential to produce secondary income. The membrane extraction system was submitted as a business plan to the NRF National innovation Competition (and was awarded Honours).

CHAPTER 2

ANALYSIS OF OLIVE WASTEWATERS

2.1 INTRODUCTION

2.1.1 Wastewater Sources

Wastewaters were periodically collected from various local olive processors over a period of three years. These included one large (commercial) and one small (traditional) table olive producing facilities, and two producers of olive oil. Analyses and most subsequent work was focused on the black table olive brines from the large commercial facility, while the other wastewaters were analysed for comparison and seasonal variation. Wastewaters were stored at –20°C for long term, otherwise at 4°C for short term or during analyses.

2.2 METHODS AND MATERIALS

2.2.1 General procedures

Standard compounds and reagents were all of analytical or HPLC grade as required, and were obtained from Merck, Fluka, Sigma-Aldrich or BDH chemicals companies. Distilled and de-ionized reagent water was obtained from a Millipore Elix 3 purification system. All analyses and experiments were performed at ambient laboratory temperature (25±2°C), unless otherwise stated. Analytical determinations and experiments were performed in triplicate where possible, with results presented as the mean. Standard deviation was in general less than 5% and as such is not stated with results. Common and routine analyses were performed according to the American Public Health Association's (APHA) Standard Methods for the Examination of Water and Wastewater (1998)

2.2.2 Analytical Equipment

A Lutron CD-4301 conductivity meter was used to measure salinity, and a Cyberscan 1000 was used for pH measurements. All spectrophotometric analyses were performed on a Unicam

Helios α UV/Vis spectrophotometer. A Buchi R200 Rotavapor was used for *in vacuo* solvent reduction at 35°C. Total organic carbon was measured using a SGE Anatoc Series II analyser.

2.2.3 Total, suspended and dissolved solids

Total solids were determined by evaporating a well mixed sample in an oven at 103-105°C, until constant weight was achieved. The samples were cooled to room temperature in a dessicator before weighing. Dissolved solids were determined in the same manner after a sample had been passed through a filter (Millipore 0.45μm nitrocellulose). Suspended solids are the portion retained by the filter; these were also measured gravimetrically after drying by subtracting the initial weight of the filter.

2.2.4 Chemical oxygen demand (COD)

COD is defined as the amount of a specified oxidant that reacts with a sample under controlled conditions. It is used as a measure of pollutants in wastewater and natural water. Both organic and inorganic components of a sample are subject to oxidation, but in most cases it is the organic component that predominates and is of the greater interest. Most types of organic matter are oxidised by a boiling mixture of chromic and sulphuric acids. A sample is refluxed in a strongly acid solution with a known excess of potassium dichromate ($K_2Cr_2O_7$). After digestion the remaining $K_2Cr_2O_7$ is titrated with ferrous ammonium sulphate to determine the amount consumed, and the oxidizable matter is calculated in terms of oxygen equivalent. COD measurement was originally performed according to the reflux method described in APHA standard methods, however this is a time-consuming and tedious process thus the Merck COD reagent set (500 – 10 000mg.L^{-1}) was subsequently used in conjunction with a digestion block and a Nova Spectroquant 60 photometer. Potassium hydrogen phthalate (KHP, Merck) was used as a standard at concentrations 500 and 1000mg.L^{-1} (COD).

2.2.5 Total organic carbon

Total organic carbon (TOC) is a convenient and direct expression of total organic content, however it does not measure other organically bound elements such as nitrogen and hydrogen. It is independent of the oxidation state of the organic matter, and as such is normally performed in conjunction with BOD or COD. TOC was measured using a SGE Anatoc Series II analyser.

2.2.6 Total nitrogen

NH_4-N was determined by both ion chromatography and colorimetric assay according to Chaney and Norbach (1962). Total nitrogen was measured using a Hanna reagent set (HI 93767 low range) in conjunction with a Hanna C214 bench photometer.

2.2.7 Sugar

Reducing sugars (readily available carbon sources) were determined using the dinitrosalicylic acid colorimetric method according to Miller (1959). Glucose was used as standard from 0 – 1g.L^{-1}.

2.2.8 Lipids

Lipids were determined by extraction with chloroform: methanol (2:1) using the Folch general procedure (Folch *et al.* 1957).

2.2.9 Total phenolics

These were determined colorimetrically using the Folin-Ciocalteau reagent according to the procedure of Garcia *et al.* (2000). Gallic acid at 0 – 100mg.L^{-1} was used as standard and all total phenolic assays are thus reported as gallic acid equivalents (GAE).

2.2.10 Liquid/liquid solvent extraction of simple phenolics

Simple phenolics were extracted from raw waste samples using 3 x 1 v/v ethyl acetate. The separated organic phases were pooled, dried over anhydrous Na_2SO_4, and concentrated to dryness in a rotary evaporator. The residue was resuspended in 0.1vol methanol. The phenolic extract was subject to UV scans, COD determination (to asses the portion of total COD attributable to phenols), and was used for high performance liquid chromatography (HPLC) to determine individual phenolic components and concentrations thereof.

2.2.11 High performance liquid chromatography (HPLC)

Reversed phase HPLC was used for identification and quantification of individual phenolic compounds. This was performed on a Merck Hitachi L-7000 series machine equipped with auto sampler. Monophenolics were separated by isocratic elution using a mobile phase of 80:20:2.5 H_2O:Methanol:Acetic acid at a flow rate of 1ml.min^{-1}. For separating more complex phenolics and glucosides gradient elution was performed. The mobile phase was 5% formic acid (solvent A) and methanol (solvent B) in ratios described elsewhere (Vinha et al 2004), at 1mL.min^{-1}. The column was a Waters Spherisorb® S5 ODS1 4.6x250mm, with a Phenomenex guard column. UV detection was at 280nm. Individual compounds were identified by comparison of retention times against both internal and external standards. Standard curves of concentration vs. peak area were used to quantify components.

2.2.12 Size exclusion chromatography

Size exclusion chromatography and fractionation was performed to obtain the molecular weight distribution of phenolic compounds using an AKTA Prime fraction collector. The stationary phase was Sephadex G-50 medium in a 70x26mm column (Amersham); the mobile phase was water at 1mL.min^{-1}. UV detection was at 280nm. 2ml sample was loaded onto the column and was collected in 10ml fractions after elution. Dextran blue (M_w = 2000kDa), dextran sulphate (M_w = 5kDa) and gallic acid (M_w = 170Da) were used as retention time calibration standards. Humic acid (2g.L^{-1}) was also run through the column as a comparison for high molecular weight polyphenolics in the wastewaters.

2.2.13 Ion chromatography

This was performed courtesy of the Department of Geology, UCT.

2.2.14 Organic acids

Organic acids were analysed by the Analytical laboratory, Chemical Engineering Department, UCT.

2.2.15 Synthesis of hydroxytyrosol

Several protocols are available for the synthesis of hydroxytyrosol, including: chemical synthesis from 3,4-dihydroxyphenylacetic acid (Capasso et al. 1999), acid or alkaline hydrolysis of oleuropein (Litridou et al. 1997) or biocatalytic synthesis using tyrosinase (Espin et al. 2001). Alkali hydrolysis was performed according to Garcia et al. (1996) as follows: 100mg Oleuropein (Extrasynthese, Gamay, France) was added to 6M NaOH (20ml) and placed in the dark for 5h under N_2. The resulting solution was adjusted to pH 3 with HCl (conc.) and then extracted 3x with diethyl ether (1v/v). The combined extracts were mixed with 10ml 0.1M HCl, and the organic solvent was removed *in vacuo*. The remaining aqueous solution was treated with 30mg activated carbon for 20min., and filtered to give a purified and colourless hydroxytyrosol solution at a concentration of 752mg.L^{-1}.

2.3 RESULTS AND DISCUSSION

2.3.1 General discussion

All the olive-derived wastewaters can be considered high strength wastewaters unsuitable for discharge into sewers, watercourses or the ocean. Table 2.1 presents some of the data collected over 3 years in comparison to other published data from Mediterranean regions. There is large variation in values between the different types of wastewater and also between wastewater from different sources, however organic load (measured as COD) in all cases exceeds the discharge limits stated in Section 39 of the National Water Act (>75mg.L^{-1}).

Olive wastewaters are naturally acidic due to the presence of phenolic and other organic acids (e.g. elanolic, lactic and acetic). In the case of high pH this is because of the addition of NaOH during processing. The brines are also highly saline (as indicated by conductivity and dissolved solids) from the salt used during processing. This increases the resistance of these wastes to biological degradation, as both phenol- and halotolerant microorganisms are required. Suspended solids in brines are low compared to OMW, as these contain large amounts of pulped olive solids.

The residual oil of OMW is wasted primary product and indicative of inefficient extraction. This is due to the formation of an oil-water emulsion that is not readily separated by the centrifuges used for oil extraction. Adjustments to the processing stage (centrifuge feed rate and recycle) could possibly help to minimise the quantity of oil in the final waste. High lipid concentration could be

inhibitory to the biological process, as well as being detrimental to physical aspects (like filtration) of a proposed system. It is to be expected, however, that mill wastewaters would be easier to degrade as they do not have the high salinity of the brines.

The brines have minimal lipids; this is because the olive cells are not disrupted in the fermentation process as they are during the milling process for oil extraction. The exception is the sample OW-13; this was wastewater from the traditional brining process that was deliberately covered with a layer of oil during fermentation.

Table 2.1: Physico-chemical analysis of olive brines and mill wastewaters

	pH	Cond. ($mS.cm^{-1}$)	TS ($g.L^{-1}$)	DS ($g.L^{-1}$)	SS ($g.L^{-1}$)	COD ($g.L^{-1}$)	TOC ($g.L^{-1}$)	N ($g.L^{-1}$)	Sugar ($g.L^{-1}$)	Lipids ($g.L^{-1}$)	TP ($g.L^{-1}$)	SP ($g.L^{-1}$)	K^+ ($g.L^{-1}$)
Black olive brine													
OW-04	4.5	83.1	114.2	113.9	0.30	75	18.35	0.20	0.26	1.83	4.91	2.89	3.71
OW-08	5.2	76.1	129.2	128.8	0.38	81	16.50	0.19	0.31	0.56	5.28	3.31	
OW-13	4.6	97.7	92.1	91.2	0.82	55	6.25	0.03	0.22	9.36	1.78	1.04	
OW-15	5.2	81.8	139.0	137.9	1.07	82	15.55	0.18	0.34	0.89	4.45	2.92	
Benitez et al (2001)	12.6		8.16		0.34	3					0.24		
Beltran-Heredia et al (2000)			5.72	5.52	0.20	6		0.03			0.18		
NaOH wasetwater													
OW-09	9.9	16.3	59.8	58.5	1.30	81	19.90	0.43	9.31	1.39	2.21	1.60	
Kotsou et al (2004)	12.3	34.0	20.3	16.8	3.54	10					0.32	0.03	
Kyriacou et al (2004)	11.0					22		0.002					2.5
Green olive brine													
OW-10	4.1	79.0	114.3	113.9	0.40	57	12.70	0.02	1.18	1.08	2.61	1.65	
Owen *et al* (2003)												1.36	
Mill wastewater (OMW)													
OW-06	4.9	9.6	119.1	58.8	60.3	202	15.40	0.46	12.6	74.0	4.07		3.42
OW-17	5.1	11.10	88.9	61.8	27.1	101	19.80	0.39	14.7	58.3	3.78	2.92	
Fadil et al (2003)	5.2		92.4	6.2	86.2	124		0.15	12.8		8.2		
Assas et al (2002)	4.9		109.0	94.0	15.0	120		0.08					
Borja *et al* (2003)	5.1		124.0	15.8	106.6	113					1.83		
D'Annibale et al (2004)	5.3				38.0	85		1.3	8.75		5.50		0.78
Raposo et al (2003)	4.9		16.7	2.7		22	6.28	0.21			0.06		

TS = total solids, SS = suspended solids, DS = dissolved solids, COD = chemical oxygen demand, TOC = total organic carbon, N = nitrogen, TP = total phenols, SP = simple phenols, K = potassium

OMW has a relatively high concentration of sugar compared to brines (also probably due to the lack of cell disruption). Sugar is a readily assimilated carbon source for most microorganisms and as such is beneficial in terms of biological degradation.

All the wastewaters have low nitrogen concentrations. For biological degradation a COD to nitrogen ratio of around 20:1 is desirable. Whether the wastes will need nitrogen

supplementation or not will have to be determined by experiment. The wastes also have high concentrations of potassium, which is incidentally why residual olive pulp and solids from oil extraction cannot be used as animal feed.

Comparative brine wastewater data (in Table 2.1) from other researchers shows lower organic load than our local wastewaters; this is generally because these wastewaters come from storage facilities that are mixed with rinsing and other wastewaters.

Organic acids detected in the brine wastewaters were lactic, acetic, citric and elenolic. Lactic acid concentrations were around $2g.L^{-1}$, acetic acid around $0.5g.L^{-1}$ and citric acid concentrations were around $1g.L^{-1}$. Elenolic acid concentrations were not determined due to the unavailability of a pure standard. This peak was identified as elenolic acid as its peak size increased dramatically after acid hydrolysis of the NaOH wastewater. This is because of the degradation of elenolic acid glucoside into elenolic acid and glucose (see Chapter 1). High concentrations of lactic acid are to be expected in the brines as the microbial consortium responsible for olive fermentation contains predominantly *Lactobacillus* species (Quintana et al. 1997).

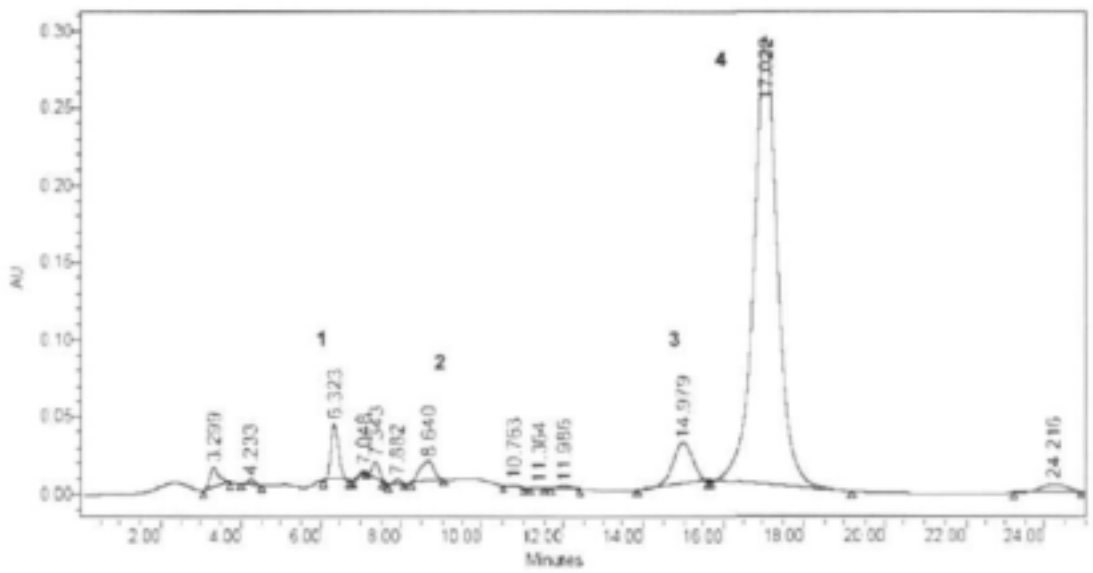


Figure 2.1: Organic acid profile of black olive brine. (1) Lactic acid, (2) acetic acid, (3) citric acid and (4) elanolic acid

2.3.2 Analysis of phenolic components

Total phenol concentrations in the brines are of the same order as in OMW and can be considered to be highly concentrated (in the order of $g.L^{-1}$), equal to or greater than many industrially produced phenolic waste streams. The US EPA classifies phenols as priority pollutants; EU legislation (80/778/EC) limits maximum concentration in drinking water to be $0.5\mu g.L^{-1}$ (less for chlorinated water). It is worth noting however that natural simple phenolic antioxidants that occur in olives and oil are only toxic to human cells at levels far exceeding those obtainable by habitual consumption of olives and olive oil (Babich and Visioli 2003). In olive wastewaters however, phenolic compounds occur at much higher concentrations, exceeding $700mg.L^{-1}$ at which phytotoxic and antimicrobial effects are observable (Visioli et al 1999).

Figure 2.2 shows the apparent molecular weight distribution of phenolic compounds in the black olive brine. There is a wide range of phenolics, from monomeric phenolics (~150Da) to higher molecular weight condensed tannins (<8kDa) and humic and lignin like substances (up to 2000kDa).

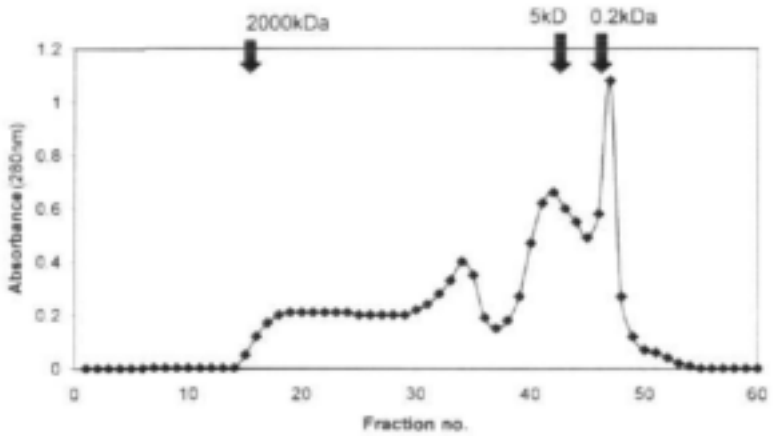
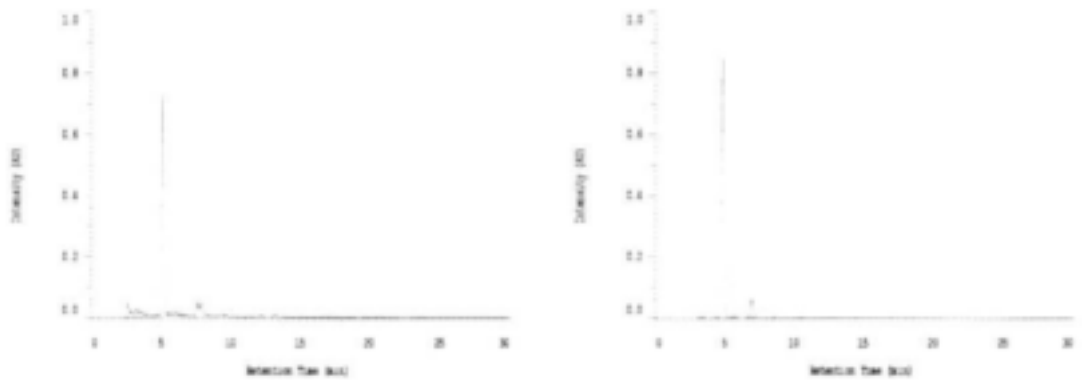


Figure 2.2: Molecular weight distribution of phenolic compounds from black olive brine

The low molecular weight compounds are of much interest as antioxidants, as discussed in Chapter 1. The low molecular weight compounds were identified and quantified by HPLC in both raw wastewater and extracts. The HPLC guard column excludes compounds of high molecular weight, only small monomeric and diphenols are detected.

Figures 2.3a & b show the HPLC profile of simple phenolics that occur in the black olive brine, and in the extract of that brine. Hydroxytyrosol is the main low M_w component in the brine,

occurring at concentration of 1.29g.L^{-1} (~70% of total low M_w phenolics), followed by tyrosol (0.22g.L^{-1}) and 3,4-dihydroxyphenylacetic acid (0.08g.L^{-1}).



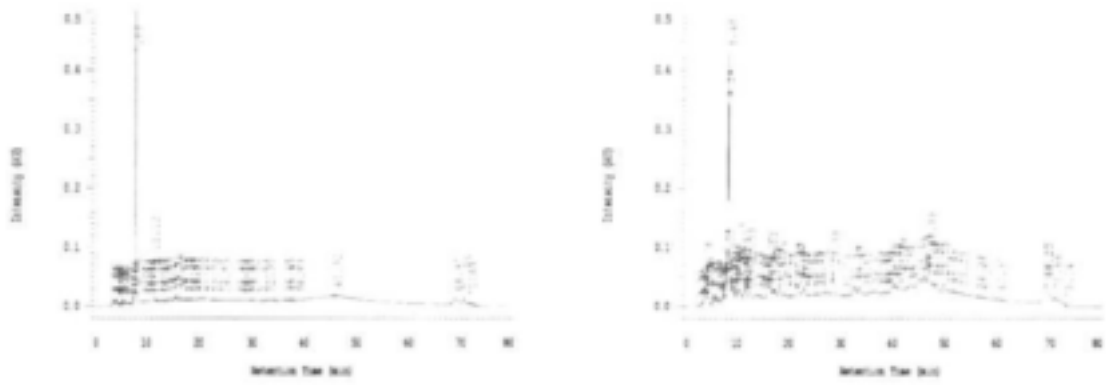
a b

Figure 2.3: Isocratic elution HPLC chromatograms of black olive brine (a) and extract (b)

The extract was diluted to approximately the same concentration of phenolics as in the brine to allow for comparison of profiles. The profile of the extract is much cleaner than that of the brine, where there are many other trace compounds. The extract contains only hydroxytyrosol (large peak) and tyrosol (small peak).

The low M_w phenolics in the brine samples account for a significant percentage of the total phenols, and are in the same range as those reported by other researchers (Owen et al. 2003, de Castro and Brenes 2001, Brenes and de Castro 1998). Black olive brine contained the highest concentration, followed by green olive brine and NaOH waste. The green olive brine, however, showed the highest percentage of simple phenolics with respect to total phenols (~90%).

Other compounds identified in trace quantities were gallic acid, protocatechuic acid and 4-hydroxyphenol. Hydroxytyrosol was also the main component of both the green olive brine and NaOH wastewater extracts. It is interesting to note that the phenolic profiles of these wastes are much less complex than those from OMW, which generally contain numerous phenolics and their glycosides. Figure 2.4 shows gradient elution profiles (for detection of phenolics and glycosides) for a black olive brine sample compared to an OMW sample.



a b

Figure 2.4: Phenolic profiles of black olive brine (a) and olive mill wastewater (b)

Although hydroxytyrosol is the major component of both waste sources, the profile of the mill wastewater is much more complex than that of the brine, indicating the greater diversity of phenolic components and glucosides, which elute at higher retention times. After acid hydrolysis the profile of the brine remained unchanged, whereas for the OMW several peaks disappeared and new ones appeared, indicating hydrolysis of glucosidic bonds (data not shown).

Hydroxytyrosol was recovered by liquid/liquid solvent extraction from the brine wastes at a minimum yield of 0.92g.L^{-1}. This is an order of magnitude higher than reported extraction yields from OMW. Mulinacci et al. (2001) report a maximum hydroxytyrosol concentration in OMW of 0.131g.L^{-1}, while other extraction yields range from 0.065-0.091g.L^{-1} (Capasso et al. 1999 &1994). Hydroxytyrosol concentrations generally appear to be higher in fermentation brines than mill wastewaters; values ranging from 0.60-4.84g.L^{-1} have been reported (Owen et al. 2003, de Castro and Brenes 2001, Brenes and de Castro 1998), while in this work hydroxytyrosol concentrations varied between 1 – 3g.L^{-1} in the brine wastewaters.

2.3.3 Radical scavenging activity of phenolic wastewater extracts

The black olive brine extract was investigated for its antioxidant activity, as it contained the highest quantity of simple phenolics. Antioxidant activity was determined using the DPPH$^{\cdot}$ (2,2-diphenyl-1-picrylhydrazil) stable free radical scavenging method according to Sanchez-Moreno et al. (1998). DPPH$^{\cdot}$ stock solutions were 25mg/L. 3.9mL of this solution was reacted with 0.1mL of various concentrations of antioxidant standards (ascorbic and gallic acids) and extracts at ranges

from 10-1000mg(antioxidant).g^{-1}(DPPH$^{\cdot}$). Extract concentrations were determined using the total phenols assay as above. Radical scavenging was measured as a decrease in absorbance at 515nm, using a DPPH$^{\cdot}$ standard curve of 0-50mg/L for calibration. The ratios of antioxidant to DPPH$^{\cdot}$ were plotted against the DPPH$^{\cdot}$ concentration remaining at steady state after the reaction. From this plot it was possible to determine the ratio of antioxidant to DPPH$^{\cdot}$ that caused the initial DPPH$^{\cdot}$ concentration to decrease by half, which is referred to as the EC_{50} (effective concentration) and gives a comparative measure of the antioxidant activity of various compounds (Brand-Williams et al. 1995).

Figure 2.5 shows the final DPPH$^{\cdot}$ concentrations for various ratios of antioxidant to DPPH$^{\cdot}$. From this plot it is possible to estimate the amount of antioxidant necessary to reduce the initial DPPH$^{\cdot}$ concentration by half (EC_{50}). Estimates of EC_{50} from Figure 4 are ~12g.kg^{-1} for gallic acid, ~14g.kg^{-1} for the extract and ~98g.kg^{-1} for ascorbic acid.

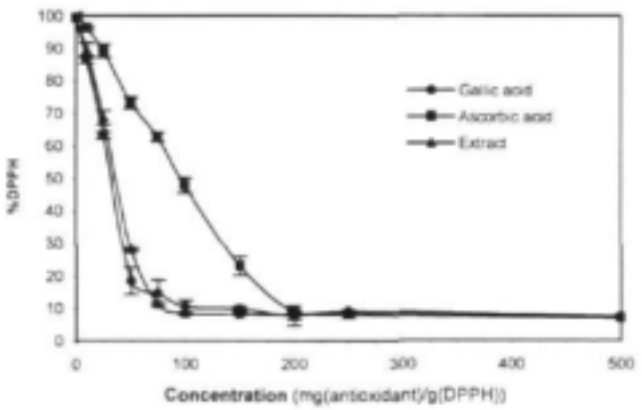


Figure 2.5: Final DPPH concentrations after reaction with antioxidants

The antioxidant activity of the extract is thus very close to that of gallic acid, which is a powerful antioxidant (Brand-Williams et al. 1995). This activity is mostly attributable to the hydroxytyrosol content of the extract, as it is known that hydroxytyrosol is a more potent antioxidant than tyrosol (Tuck and Hayball 2002). It is notable that the radical scavenging activity of hydroxytyrosol is much higher than commercially used lipid food preservatives such as butylated hydroxyanisole (BHA, EC_{50}=93g.kg^{-1}; Sanchez Moreno et al. 1998).

2.4 DISCUSSION

The wastewaters were shown to have a very high organic load, due to phenolic and other organic components. In addition the brines have high salinity, which is problematic for biodegradation purposes as this creates osmotic stress and thus unfavorable growth conditions. Wastewaters with these conditions necessarily need dilution before biological treatment is possible. Fortunately, washing and rinsing waters from production facilities are generally pooled together with brines and this serves to significantly dilute the wastewaters. However subsequent treatment of the wastewaters is not only desirable but in fact essential.

The phenolic components of the brine wastewaters were shown to be highly diverse, from low molecular weight monomers all the way through to high molecular weight tannins and humic substances (*i.e.* from ~150Da to 2000kDa). The higher molecular weight polyphenolics are responsible for the dark colour of the wastewaters.

The brined olive fermentation wastewaters were shown to contain considerable amounts of simple phenolics (more than OMW and much more than occur in olive oil), particularly hydroxytyrosol. A crude phenolic extract of fermentation brine which contained ~90% of this compound was shown to have powerful antioxidant activity. The hydroxytyrosol is extractable from the wastewaters at levels around 1g.L^{-1}. Considering that pure hydroxytyrosol has a market value of US1000 – 2000$ per gram, the scope of the commercial possibilities of extracting it from the wastewaters becomes clear.

These waste streams could thus serve as a source of valuable phenolic antioxidants, subject to the development of an efficient extraction system. The fermentation brines are more attractive than OMW for the recovery of phenolics, largely due to their higher hydroxytyrosol concentration, aqueous nature and lower suspended solids content. In crude form, such extracts could be used as natural food or cosmetics preservative or in a nutraceutical product, or they could be further processed to yield hydroxytyrosol in pure form, which has a high market value.

2.5 CONCLUSIONS

- The phenolic components of the brine wastewaters are highly diverse, containing low molecular weight monomers, high molecular weight tannins and humic substances (*i.e.* from ~150Da to 2000kDa).
- The higher molecular weight polyphenolics are responsible for the dark colour of the wastewaters.
- The brined olive fermentation wastewaters were shown to more simple phenolics than OMW and much more than occur in olive oil).
- An extract of fermentation brine gave hydroxytyrosol at levels around 1g.L^{-1}.
- The fermentation brines are an attractive source of simple phenolics largely due to their higher hydroxytyrosol concentration, aqueous nature and lower suspended solids content.

CHAPTER 3

MEMBRANE-ASSISTED SOLVENT EXTRACTION (MSE) OF PHENOLIC ANTIOXIDANTS

3.1 INTRODUCTION

For recovery of phenolic components in aqueous waste streams at concentrations greater than 50mg.L^{-1} it is known that liquid-liquid solvent extraction (LLSE) is the most cost-effective process (Doherty and Malone 2001). In this project, the technology investigated for recovery of the product hydroxytyrosol from olive wastewater is of relatively recent origin, and arises due to rapid advances in polymer science and associated membrane technology. This technology, called membrane-assisted solvent extraction (MSE), can be considered an advance in LLSE. It has several unique advantages over conventional solvent extraction methods. In LLSE, an organic solvent phase of different density and/or hydrophobicity is vigorously mixed in a tank with the aqueous phase (e.g. the wastewater), allowing the extraction of the desired product into the organic solvent due to higher affinity or solubility. The mixture is then allowed to settle and the two phases separate due to their immiscibility. The aqueous phase is then decanted and discarded, and the (volatile) solvent phase is evaporated off leaving a residue containing the desired compounds.

MSE is based on a similar principle to LLSE except that the two phases are not mixed; they are separated by a hydrophobic (water resistant) membrane. The compounds to be extracted diffuse through this membrane from the aqueous phase into the solvent phase in a process called membrane contacting, however the liquid phases do not pass through the membrane because of the hydrophobicity. This technique has been investigated by others for several applications, such as the separation-concentration of phenol (Urtiaga *et al.*, 1992a, 1992b) and cresol (Ferreira *et al.*, 2005) from aqueous waste streams, recovery of valeric acid (Rodriguez *et al.*, 1997) and the extraction of aroma compounds from fermentation broths (Baudot *et al.*, 2001). However, as yet it has not been used as a method for the "stripping" of valuable antioxidants from olive and similar organic-loaded wastewaters. The membrane is a contained in a module consisting of many long thin hollow fibres; thus there is a large surface area for mass transfer incorporated into a small volume, which results in good extraction rates, as well as compact plant size (small footprint) compared to LLSE. The process is also known as membrane contacting.

The main advantage of MSE is that since there is no mixing, subsequent separation and decanting of the aqueous and organic phases are not required. This makes the system design very simple and saves on plant capital. The formation of emulsions is avoided; such emulsions take long times to separate and significantly affect the processing time in LLSE-based processes. In addition, MSE lends itself readily to continuous processes, which means that post-extraction processing equipment such as evaporators and condensers can be considerably scaled-down compared to equivalent (batch) LLSE plant requirements. A further benefit is that no pre-treatment of wastewater is required as in LLSE, which also reduces processing time. Because the extraction is a diffusion-based process, there are no significant pressure requirements, and thus energy costs are kept to a minimum; only small pumps are required. A disadvantage of MSE is that the membrane offers additional resistance to mass transfer, but this can be overcome by use of membrane modules with large surface to volume ratios.

The aim of the work described in this chapter was to evaluate the overall mass transfer coefficient for a membrane contactor, which then informs the design and scaling of an extraction system to obtain hydroxytyrosol from olive wastewaters, based on production volumes, flow rates, concentrations and membrane area.

3.2 METHOD AND MATERIALS

Batch extraction experiments were performed to determine overall mass transfer coefficient. A Microdyne polypropylene hollow fibre membrane module (MD020CP2N, 0.2 µm pore size) was used as contactor (details in section 3.4). Reservoirs of aqueous (waste) and organic (extracting) phases were placed on magnetic stirrers and supplied to the lumen and shell sides respectively by peristaltic pump. The time course of changes in phenolic concentrations in the two phases were monitored by periodic sampling, until constant concentrations had been reached. Concentrations of hydroxytyrosol, total phenols and total simple phenols were evaluated as described in Chapter 2. This data, together with the distribution coefficient, allowed for the calculation of the overall mass transfer coefficient. A mercury manometer was used to measure trans-membrane pressure, while flow rates (and corresponding Reynolds numbers) were set so as to ensure minimal boundary layer resistance to mass transfer in the aqueous and organic phases. Figure 3.1 shows the experimental system used for extraction experiments.

Details for the experimental system were as follows:

Number of membranes $n = 40$

ID = 1.8mm

OD = 2.0 mm

Pore size = 0.2 μ

Total membrane surface area $A_m = 0.1m^2$

Lumen cross sectional area $A_l = 2.5x10^{-6}m^2$

Wastewater volume = solvent volume = 500ml

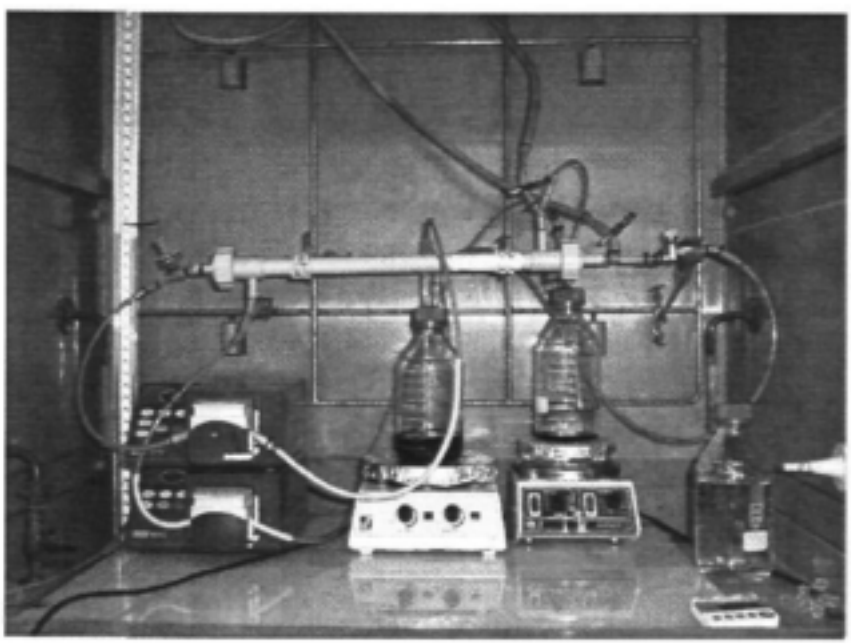


Figure 3.1: Experimental membrane extraction system

3.3 EXTRACTION THEORY

Calculation of the overall mass transfer coefficient is a complex mathematical problem due to the variation of concentration driving force along the axial length of the membrane, which is affected by flow rates and distribution coefficients. Several mathematical models for determination of the overall mass transfer coefficient have been proposed and evaluated. These include models based on fundamental principles (Urtiaga *et al.*, 1992a, 1992b), Wilson-plot methodology (Viegas *et al.*, 1998) and the Olander and Hatta models (Ferreira *et al.*, 2005). In this work mass transfer

within the contactor was modeled according to Gonzalez-Munoz *et al.* (2003), where a relatively simple analytical solution was proposed as a solution to second-order differential mass balance equations.

The equation for mass transfer can be described by

$$J_s = K_o A_m (c_a - c_a^*) \qquad (3.1)$$

where J_s = solute flux ($g.s^{-1}$)

K_o = overall mass transfer coefficient ($m.s^{-1}$)

A_m = membrane surface area (m^2)

c_a = solute concentration in the aqueous phase at time t ($mg.L^{-1}$)

c_a^* = solute concentration in aqueous phase in equilibrium with organic phase at time t ($mg.L^{-1}$)

A mass balance can be performed on the flux J_s in relation to depletion of the solute of the aqueous phase. For co-current flow:

$$c_a = \frac{V c_a^0}{1+V} + \frac{c_a^0}{1+V}\exp(-ct) \qquad (3.2)$$

where

$$c = \frac{Q_a}{V_a}\frac{(1+V)}{(1+Q)}\left\{1-\exp\left[-\frac{A_m K_o}{Q_a}(1+Q)\right]\right\} \qquad (3.3)$$

and

$$V = \frac{V_a}{V_o D} \qquad (3.4)$$

$$Q = \frac{Q_a}{Q_o D} \qquad (3.5)$$

The initial phenol concentration is c_a^0 ($mg.L^{-1}$), V_a and V_o are the volumes of the aqueous and organic phases (m^3), Q_a and Q_o ($m^3.s^{-1}$) are the flow rates in the aqueous and organic phases, and D is the distribution coefficient of solute between the aqueous and organic phases at steady state. From equation (3.2), the time dependence of solute in the aqueous phase can be expressed in the form

$$c_a = a + b\exp(-ct) \qquad (3.6)$$

Here a and b depend only on known parameters, and the value of c can obtained from a straight line plot of $\ln(c_a - a)$ against time where the slope is c. K_a can then be calculated from equation (3.3). For the complete mathematical treatment of the problem, the reader is referred to the paper by Gonzalez-Munoz *et al.* (2003).

3.4 RESULTS AND DISCUSSION

3.4.1 Calculation of distribution coefficients

The distribution coefficients of hydroxytyrosol, low M_w phenols and total phenols between the aqueous and organic phase were first determined. Ethyl acetate was used as organic solvent, and this was mixed with wastewater at various dilutions. Concentrations in the two phases at steady state were then plotted against each other to obtain distribution coefficients. These results are shown in Figure 3.2, where the slope of the straight line obtained is the effective distribution coefficient. Batch contacting experiments were then conducted.

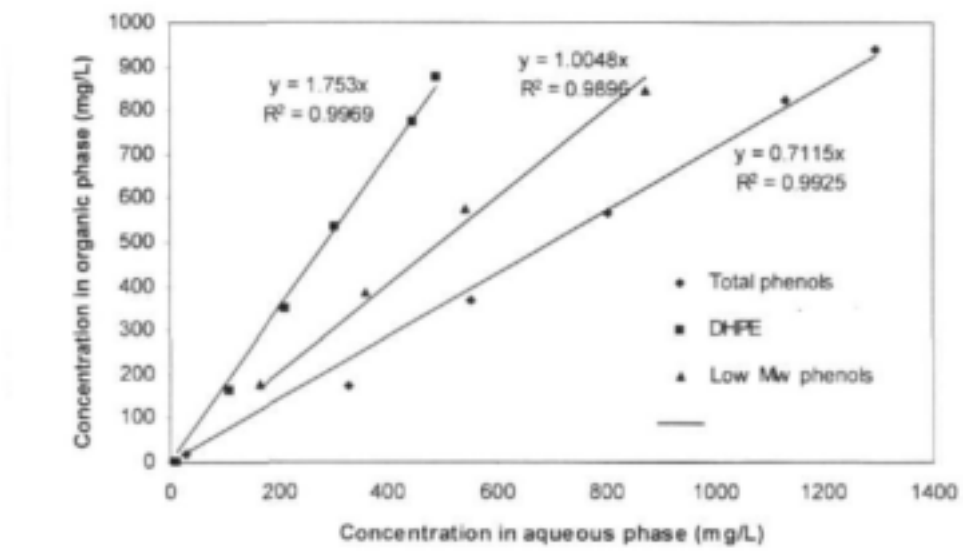


Figure 3.2: Distribution coefficients of phenolic components between the aqueous and organic phases

3.4.2 Calculation of mass transfer coefficient for hydroxytyrosol

Reynolds number for the aqueous phase in the lumen side of the membrane:

$$\mathrm{Re}_a = \frac{d_i v \rho}{\mu} = 4.32$$

where d_i = lumen diameter = 1.8×10^{-3}m

v = average cross-sectional velocity = 19×10^{-3}m.s^{-1}

ρ = aqueous phase density = 1.1×10^{3}kg.m^{-3}

μ = viscosity = 8.7×10^{-3}kg.m^{-1}.s^{-1}

Figure 3.3 shows typical time-dependent concentrations of hydroxytyrosol in the aqueous and organic phases over time during a typical batch contacting experiment..

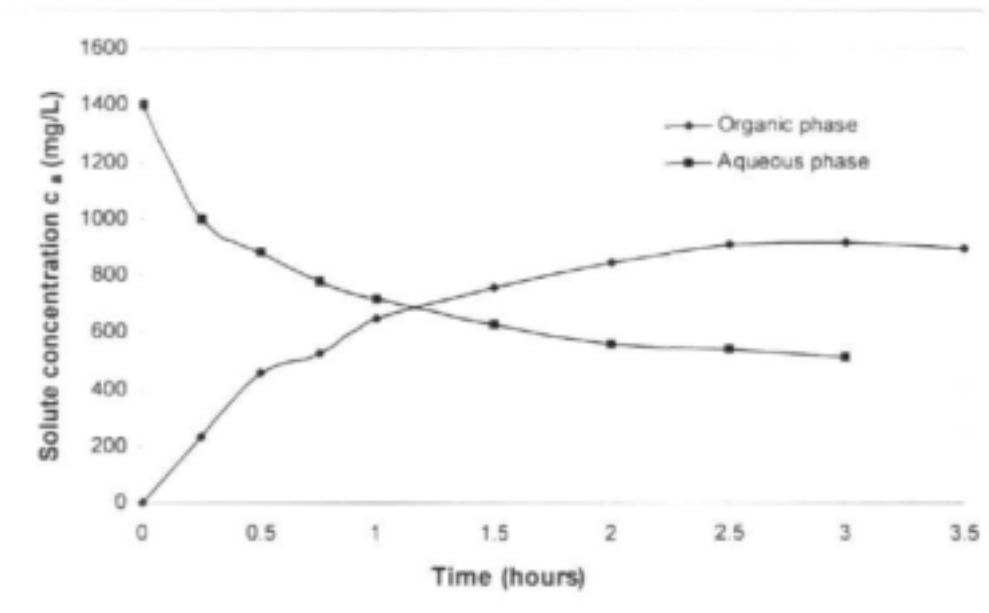


Figure 3.3. Concentrations of hydroxytyrosol in the aqueous and organic phases over time during membrane extraction

Similar plots were obtained for total phenols and simple phenols.

Taking the above data and plotting it as the natural log of the concentration less coefficient *a* vs. time results in the graph of figure 3.4, where the slope is the coefficient *c* in equation (6). Equation (3) can then be rearranged to solve for K_a:

$$K_a = \frac{-Q_a}{A_m(1+Q)} \ln\left[1 - c\left(\frac{V_a}{Q_a}\right)\left(\frac{1+Q}{1+V}\right)\right] = 8.14\times10^{-5} m.s^{-1} \quad (7)$$

Similarly, for total phenols $K_a = 5.15\text{x}10^{-5} m.s^{-1}$

For simple phenols $K_a = 6.18\text{x}10^{-5} m.s^{-1}$

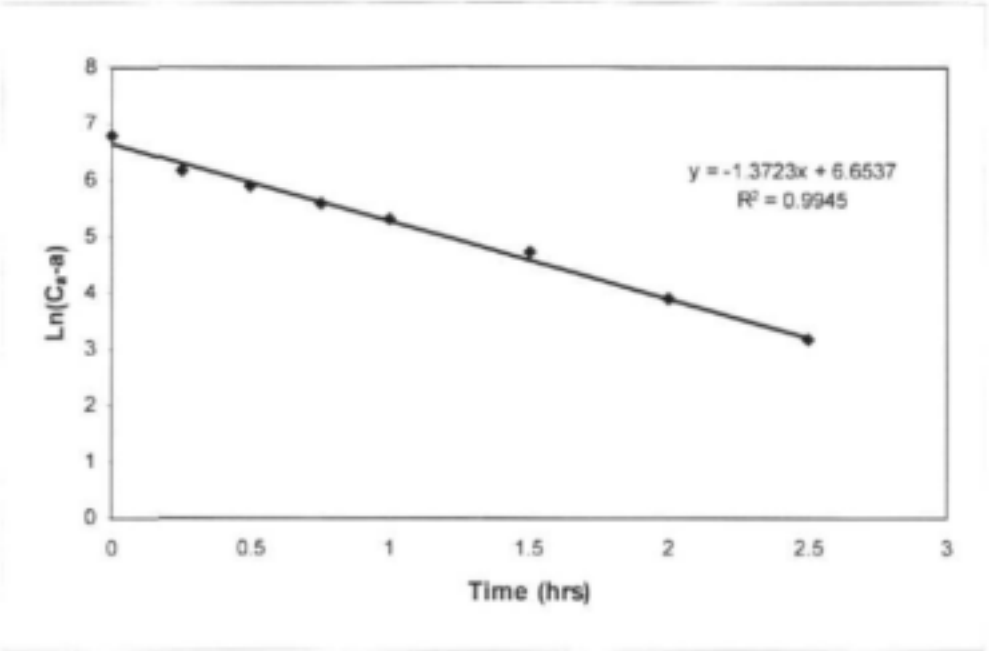


Figure 3.4: Linearised plot of mass transfer over time

A problem of aqueous phase breaking through into the organic phase was encountered during some experiments. This can be ascribed to the high porosity of the membranes (~70%) and large pore size (0.2μm), compared to modules used by other researchers. This also resulted in large mass transfer coefficients, an order of magnitude larger than those determined by other researchers (Gonzalez-Munoz *et al.*, 2003; Urtiaga *et al.*, 1992a). Using a different membrane module with different specifications, particularly with high hydrophobicity, could rectify this problem. A new membrane extraction module (Liqui-cel) has been obtained with a membrane surface area of $1m^2$, this is to be employed for further research and system development.

The mass transfer coefficient for hydroxytyrosol was greater than that for total phenols. This is because ethyl acetate is selective for low molecular weight phenolics, and the higher molecular weight polyphenolics and tannins remained in the aqueous phase. Thus, the system is capable of selectively extracting the hydroxytyrosol (and traces of other low M_w compounds).

3.4.3 HPLC analysis of olive wastewater extracts

Figures 3.5a-c show HPLC profiles of the aqueous and organic phases before and after extraction experiments. The large peak is hydroxytyrosol.

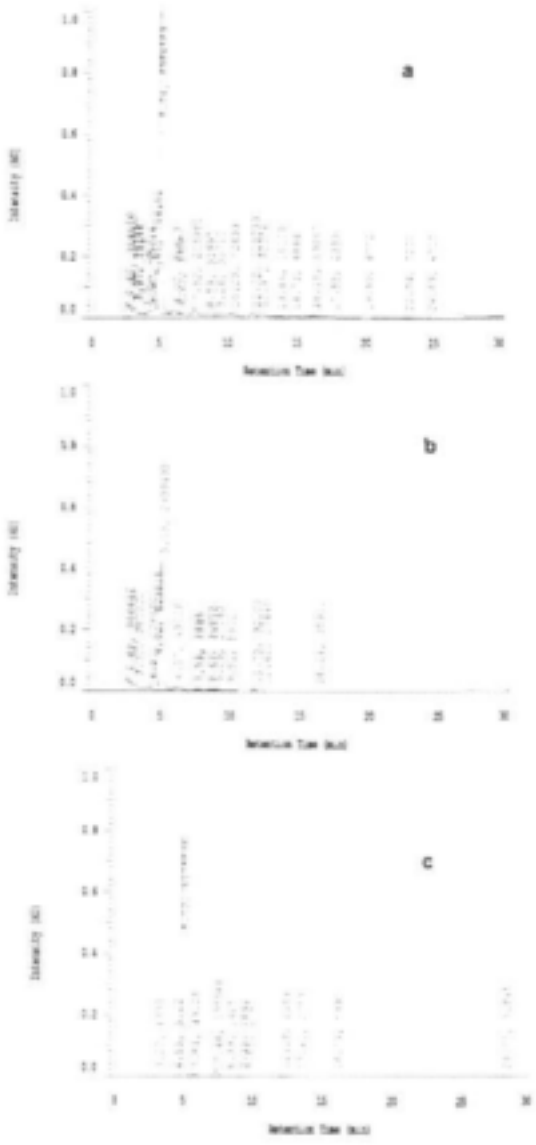


Figure 3.5: HPLC chromatograms of low M_w phenolics from the membrane extraction experiment. (a) and (b) initial final concentrations in the wastewater, (c) extracted concentration in the solvent

As in previous liquid/liquid solvent extraction experiments, the extract contains only hydroxytyrosol and tyrosol.

The amount of residual unextracted hydroxytyrosol remaining in the aqueous phase is a function of the distribution coefficient. However, this is relatively inconsequential as vast quantities of wastewater are available from which hydroxytyrosol can be extracted; solvent processing is much more likely to be the limiting factor in terms of system design for a hydroxytyrosol production process.

Given that hydroxytyrosol concentration are of the order 1 – 3g.L^{-1} in the wastewaters and there is a virtually limitless supply, it can be expected that maximum concentrations in the solvent wilbe of the order 1.8 – 5.3g.L^{-1}. The mass transfer coefficient in conjunction with membrane surface area and (minimum) solvent flow rate then dictate the rate at which hydroxytyrosol can be extracted from the wastewater streams.

3.5 A PROTOTYPE EXTRACTION SYSTEM

The membrane extraction system could be incorporated into a complete extraction process as illustrated in Figure 3.6. Using a membrane based extraction system allows for considerable scaling-down of equipment (compared to a conventional counter-current liquid-liquid extraction process), which means that such extraction systems could feasibly be installed at small-scale production facilities. This concept was submitted as a business plan for the National Innovation Competition. Patenting of the process is being investigated, and scaled-up (continuous) system development is under way.

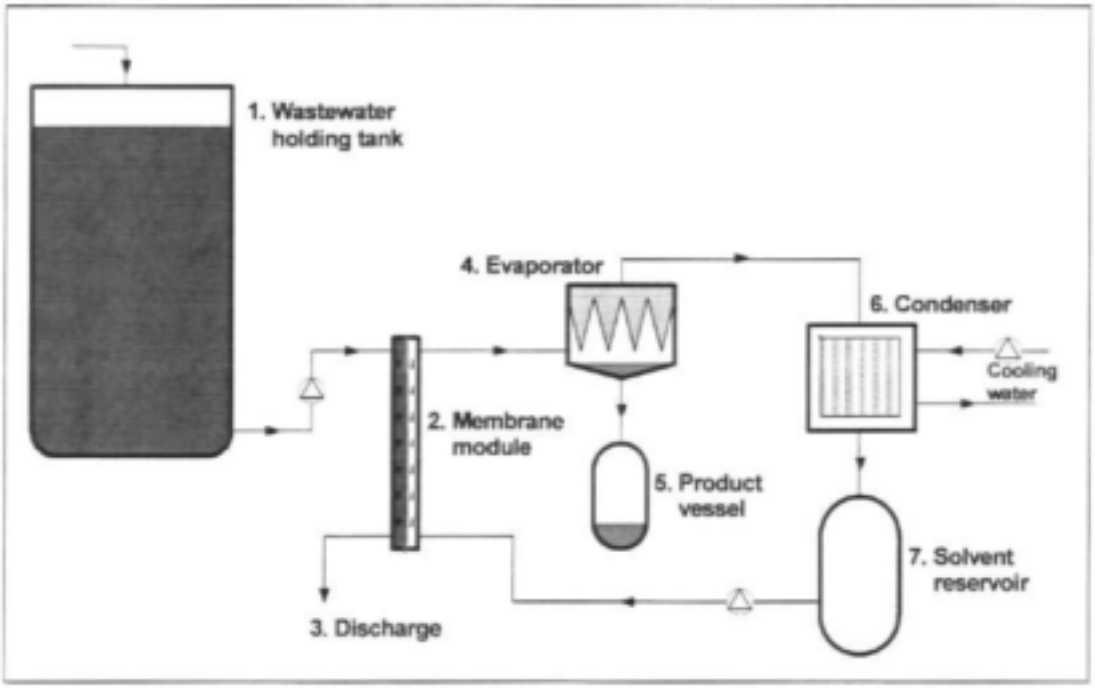


Figure 3.6: Schematic diagram of proposed complete extraction system

3.6 CONCLUSIONS

- Membrane solvent extraction was demonstrated at laboratory scale (0.1m^2 membrane module) and was found to be effective for the extraction of hydroxytyrosol from olive wastewaters.
- Parameters such as flow and extraction rates were measured and mathematically modelled, allowing for the calculation of necessary plant size and optimisation of operating conditions.
- The most cost-intensive parts of the suggested process are the evaporation and condensation steps, due to the energy requirements for heating and cooling. To this end the membrane extraction process should be over-designed to allow for maximum stripping of product from the wastewater, with capacity based on the maximum anticipated wastewater production volumes. This results in the minimum required equipment size for evaporation and condensation. Over-design of the membrane system will have only a small impact on overall capital requirements.
- It is possible to extract ~1$g.L^{-1}$ from the average olive brine wastewater. Extraction *rates* essentially depend on the amount of membrane surface area employed, and this will be dictated by wastewater production volumes.

CHAPTER 4

ISOLATION, IDENTIFICATION AND EVALUATION OF MICROORGANISMS FOR DEGRADATION AND BIOTRANSFORMATION OF OLIVE WASTEWATERS

4.1 INTRODUCTION

A key component of any bioremediation system is a highly active biological agent capable of (a) withstanding the conditions pertaining in the waste, and (b) converting all, or at least a significant portion, of the pollutants present to less harmful products. Since olive wastewaters are recognized to be toxic to many microorganisms (Fiorentino et al, 2003; Adhoum and Monser, 2004) it cannot be assumed that the mixed microbial consortia present in many water treatment processes will necessarily fulfill either of these criteria, nor that they will maintain a constant population balance over time. This variability would likely result in losses of bioremediation activity over time which may be unpredictable and could lead to inconsistent performance of the bioremediation system. Thus, in this project, it was necessary to seek microbial strains which were tolerant of the olive production waste. In order to facilitate development of the bioreactor in this investigation, it was desirable to use a single strain in the bioreactor.

On the basis that olive waste-tolerant microorganisms would be capable of living in the presence of the polluting phenolic compounds, phenol-metabolizing microorganisms were isolated from within and around olive wastewater evaporation ponds. Selective isolation was performed by using olive wastewater as the sole carbon source in the first isolation cultures, since microorganisms capable of growing on the waste would be utilizing the waste as a nutrient source and thus would be producing the enzymes required to breakdown the carbon compounds in the waste.

The enzyme activities of the isolates were investigated and evaluated in order to gain understanding of the nature of their phenol metabolism and also to investigate any other biotransformation capability they might show. Four strains were chosen for further investigation based on the enzyme activities they exhibited and their capability in degrading the phenolics in the waste. Biotransformation experiments were then performed in which the olive wastewater and a range of model compounds were used as substrates. The reactant and product profiles were monitored over the course of these experiments using HPLC.

The strains of interest were identified by molecular methods (using 16s rDNA analysis). The most promising, strain AS-35, was identified as *Bacillus megaterium*. This strain was subjected to conditions under which genetic mutation could occur, with the aim of isolating a mutant with improved tolerance for the olive waste or better capacity to detoxify it.

Proteomics presents a method for investigating the differences in the protein profiles of microorganisms subjected to varying conditions. This can be used in developing understanding of the effects of, for instance, stress-inducing or toxic compounds on the metabolism of the organisms. Under specific (stress) conditions, the cells of the microorganism will produce specific proteins, eg., enzymes required to allow the cells to survive. In the presence of toxic compounds, therefore, the cells would be expected to produce the enzymes they can produce to degrade the compounds and hence reduce the environmental threat to the cells. Profiling of cells subjected to such stress conditions would thus differ from those not being stressed, and analysis of the different states can provide information on the enzymes involved in the degradation. In this study, a proteomics analysis of strain AS-35 and its mutant, DF4, was conducted.

4.2 METHODS AND MATERIALS

4.2.1 Bacterial strains and culturing conditions

4.2.1.1 Isolation of wild-type strains from olive wastes

Bacterial strains and culture conditions

Aliquots of olive waste samples collected from various evaporation ponds 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 and 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. The cultures were maintained and cultured on olive waste agar (25% [v/v] olive waste, 0.5% [w/v] yeast extract, 1.5% [w/v] Agar, pH 6.0) or olive waste broth (25% [v/v] olive waste, 0.5% [w/v] yeast extract, pH 6.0) at 30°C with shaking at 250 rpm. Twenty percent glycerol stocks are stored at -80°C. In later experiments, the selected isolate AS-35 and putative mutant DF4 were routinely cultivated in Luria- Bertani (LB) broth (0.5% [w/v] yeast extract, 1% [w/v] tryptone, 1% [w/v] NaCl; pH 7.4) at 30°C.

Morphological analysis

Gram staining was performed on all isolates as described by Gerhardt *et al.* (1994). Briefly, 15 µl of the cultures were transferred to clean microscope slides and spread evenly over the surface of the slides prior to heat fixation. The slide were stained for 1 min with crystal violet and then thoroughly rinsed under running water. The procedure was repeated once more using an iodide solution before destaining the cells by incubating the fixed cells for 30 s with acetone-alcohol. After removal of the acetone-alcohol, the cells were counter-stained for 2 min with safranin, rinsed under running water, air-dried and then examined 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).

4.2.1.2 Enzyme activities in strains isolated from olive waste ponds

All Isolates were tested for ability to produce polyphenol oxidases after 16 hours growth in 25% olive waste. One ml culture were pelleted and resuspended in 0.1M sodium phosphate buffer pH 7.0 before sonicating for 30 seconds at 60% power. Cellular extracts were used to test for peroxidase and tyrosinase enzyme activity. The supernatant was used to determine the total phenol reduction, laccase enzyme activity and its monophenolic composition was determined on reverse phase HPLC.

Peroxidase activity

Peroxidase enzyme activity was determined using the method of Putter 1975. Fifty microliters of 15mg/ml (w/v) guaicol (aq), together with 1ml 0.1M sodium phosphate buffer pH 5.6, 200 µl sample and 100µl 0.8M H2O2 were added to the reaction. Absorbance at 460nm was measured spectrophotometrically () after 4 minutes (Σ=25,6 cm2/µmole). One unit of peroxidase (Roche) enzyme was incorporated as a positive control.

Tyrosinase activity

Tyrosinase enzyme activity was determined using the method of Ikehata and Nicell 2000. In brief, 0.181mg of L-tyrosine was combined with 2.8ml 0.1M sodium phosphate buffer pH 6.5 and 100 µl substrate. The conversion of L-tyrosine was observed at 280nm.

Laccase activity

The oxidation of ABTS was determined spectrophometrically at OD420nm (Robles et al, 2000). Two hundred microliters supernatant was added to a 1 ml reaction mixture consisting of 50µl 10.9mg/ml (w/v) substrate in 0.1M Sodium phosphate buffer pH 5.6. The enzymatic reaction was

observed for 3 minutes with an extinction coefficient of 3.6x104 M-1.cm-1. One unit of Trametes versicolor laccase enzyme was used as positive control.

Total phenol reduction capability

Foli-Ciocalteu reagent was used to measure the total phenol concentration in the olive waste (Aggelis, et al, 2002). Five hundred microliters of reagent was mixed with 400 µl 7.5% (w/v) Sodium carbonate and 100 µl supernatant. The reaction was incubated in the dark for 30 minutes at 20°C before reading the absorbency at 765nm. A gallic acid standard was used. The total reduction of phenol was represented as a percentage of the initial phenol concentration (mM).

Ferulic acid decarboxylase

Ferulic acid decarboxylase activity of the isolates was determined based on the method described by Van Beek and Priest (2000). This implies that the isolates were cultured in 25 % olive waste to an OD600nm of 1.0. The cells were collected through centrifugation at 6 000 r.p.m. for 6 minutes and resuspended in an equal volume 0.1 M Sodium phosphate buffer, pH 5.8 containing 100 µl / ml ferulic acid. Samples were incubated at 30 °C for 5 hours, collecting 1ml of sample every hour. Two hundred microliters of supernatant was analyzed together with 800 µl ddH2O on uv scans from 200nm to 350nm. Ferulic acid has absorption peaks at 284 and 312nm.

Ferulic acid esterase activity:

Agar plates containing 0.1% ethyl ferulate were stab inoculated with 100µl volumes of culture supernatant and cellular extracts and incubated at 30°C for 4 days.

4.2.2 Characterization of selected isolates

4.2.2.1 Growth analysis of selected strains

Growth kinetics of isolates AS-9, AS-15, AS-18, AS-24 and AS-35 were performed in 25% olive waste batch cultures for 136 hours. Samples were collected every 2 hours and the growth rates were observed by measuring optical density (OD) of the culture at 600 nm. The samples were centrifuged and the supernatant use to analyze the monophenolic content on reverse phase HPLC.

4.2.2.2 16S rDNA analysis of selected isolates

Chromosomal DNA isolation

Chromosomal DNA of isolates AS-9, AS-15, AS-18, AS-24 and AS-35 were extracted using cetyltrimethylammonium bromide (CTAB) as described by Jansen (1995). Briefly, the cells from 500 µl of an overnight culture were collected by centrifugation at 10 000 rpm for 3 min and

suspended in 567 µl of 1 x TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). The cells were lysed by the addition of SDS to a final concentration of 0.5% (v/v) and the proteins were digested by addition of proteinase K to a final concentration of 100 µg/ml in a total volume of 600 µl. Following incubation at 37°C for 1 h, 100 µl of 5 M NaCl and 80 µl of a CTAB/NaCl solution was added and incubation was continued for 10 min at 65°C. The CTAB-protein/polysaccharide complexes were removed by extraction with an equal volume of chloroform:isoamyl alcohol (24:1) followed by centrifugation at 10 000 rpm for 5 min. The supernatant, containing the genomic DNA, was recovered and transferred to a new microfuge tube. The remaining CTAB was removed by addition of an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) followed by centrifugation (10 000 rpm, 5 min). The chromosomal DNA was precipitated from the recovered supernatant by addition of 0.6 volume isopropanol. The precipitated chromosomal DNA was pelleted by brief centrifugation, rinsed with 70% ethanol, dried under vacuum and resuspended in 20 µl UHQ water. An aliquot of the DNA was analyzed by electrophoresis on a 1% (w/v) agarose gel.

PCR amplification of the 16S rDNA gene

A 1.5 kb region of the 16S rDNA gene was then amplified using universal primers E9F and U1510R (Weisburg *et al.*, 1991) (Table 4.1). The PCR reaction mixture (50 µl) contained 1 x PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% [v/v] TritonX-100), 1.5 mM $MgCl_2$, 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 2400 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, aliquots of the reaction mixtures were analyzed in the presence of an appropriate molecular size marker by 1% (w/v) agarose gel electrophoresis.

Table 4.1 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'
pUC/M13 Forward	5' – GTTTCCCAGTCACGAC – 3'
pUC/M13 Reverse	5' – GTAAAACGACGGCCAGT – 3'
T7 Long	5' – TTTCTATACGACTCACTATAGGCCGCCAGT – 3'
T7	5' – AATACGACTCACTATAGG – 3'
A6 Long	5' – TTTATTTAGGTGACACTATAGATTAGGCCGCCAGT – 3'
A6	5' – ATTTAGGTGACACTATAGA – 3'
Short	5' – CTGGCGGCCTACCA – 3'
Short-P	5'P – CTGGCGGCCTACCA – 3'

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 into InsT/A clone (Fermentas), according to the manufactures instructions. The ligation mixture was transformed into competent *E. coli* DH5α cells.

Preparation of competent E. coli DH5α cells

Competent E. coli DH5α cells were prepared according to the procedures described by Chung and Miller (1988). An overnight culture was prepared by inoculating 10 ml of LB-broth with a colony from a freshly streaked culture of E. coli DH5α. After overnight incubation at 37°C with shaking, 1 ml of the culture was inoculated into 100 ml preheated (at 37°C) sterile LB broth and grown to an OD540 of 0.3 to 0.4. The cells from 30 ml of the culture were pelleted in a polypropylene tube by centrifugation at 5 000 rpm for 10 min at 4°C. The pellet was suspended in 3 ml ice-cold TSS (0.1 M $MgCl_2$, 0.1 M $MgSO_4$, 10% [w/v] PEG 8000 prepared in 93 ml LB-broth, 5% [v/v] DMSO; pH 6.5). Following incubation on ice for 15 min, the cell suspension was aliquoted into microfuge tubes and stored at -70°C until use.

Transformation of competent E. coli DH5α cells

After allowing the competent *E. coli* DH5α cells to thaw on ice, the cells were transformed using the method described by Chung and Miller (1988). An aliquot of the cells (100 µl) and the ligation reaction mixture (10 µl) were mixed in a pre-cooled microfuge tube and incubated on ice for 1 h. After addition of 500 µl LB-broth containing 20 mM glucose, the transformation mixtures were incubated with shaking at 37°C for 3 h. The transformed cells were selected by plating the cells in aliquots of 100-200 µl onto LB-agar plates supplemented with the appropriate antibiotic. The plates were incubated overnight at 37°C and investigated for the presence of recombinant transformants. When appropriate, 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.

Plasmid DNA was extracted using Quiagen 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. 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/).

4.2.2.3 Biotransformations of model compounds by selected strain AS-35

Resting cells

AS-35 was grown in 100ml 5% olive waste to an OD600 of 1.5, late-exponential phase and centrifuged to pellet 20 ml of cells. The cells were resuspended in 20 ml 0.1M Sodium phosphate buffer pH 5.8, containing 1mM model compound and incubated at 30°C. 1.4 ml Sample was collected hourly, centrifuged and the supernatant was kept for HPLC analysis. The resting cell experiments were performed in duplicate, with one sample being subjected to 3 minutes sonication prior to incubation.

Culturing AS-35 with model compounds as sole carbon source

AS-35 was selected for reaction with model phenolic compounds. The strain was cultured in M9 minimal medium at 30°C, containing 5mM NH_4NO_3 and 1mM model compound as sole carbon source. The following model compounds were used in separate experiments: Ferulic acid, tyrosol, vanillic acid, gallic acid and p-hydroxyphenyl acetic acid.

4.2.3 Mutagenesis of wild-type strain AS-35

4.2.3.1 Classical mutagenesis

UV mutagenesis

Culturing AS-35 to late-exponential phase in 25% olive waste. Plate 50µl culture on agar containing 80 % olive waste and subject the inverted plate to uv for various time periods, 30, 35 and 40 seconds. Incubate the plates over night at 30 °C and subculture colonies to new 80 % olive waste plates.

Chemical mutagenesis

Isolate AS-35 was chemically mutated using classical mutagenesis (Gasson *et al* 1998). The isolate was cultured overnight in 100 ml LB. Culture suspensions 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. 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 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 processing wastewater supplemented with 0.5% (w/v) yeast extract and cultured at 30°C overnight.

4.2.3.2 Screening of putative mutants

Eight putative mutant isolates were cultured in 50% olive wastewater. One ml of cells was 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 enzyme activity assay. Total phenol determination and peroxidase activity assays were preformed as previously described. Decolourization of the media was investigated by measuring the absorbency of 1ml supernatant at 525nm.

4.2.4 Comparisons between wild type AS-35 and mutant DF4

4.2.4.1 Growth kinetics of selected mutant and wild type

The growth kinetics in batch cultures of isolates AS-35 and DF4 were observed by measuring optical density (OD) of the culture 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.

The growth rate and ability to reduce total phenol content of wild type AS-35 and mutant DF4 were investigated in 25% olive wastewater media containing various differences. Different nitrogen sources that were investigated include 0.5% yeast extract, 0.5% Ca(NO3)2 and 25% olive wastewater containing no additional sources. In 25% olive wastewater containing 0.5% yeast extract the influence of different initial pH was investigated with a range consisting of pH 4, 6 and 8.

4.2.4.2 Isolate identification

16S rDNA analysis.

Wild type AS-35 and mutant DF4 were cultured in LB and olive waste prior to genome isolation and 16S rDNA identification, as previously described. The 16S rDNA sequences of AS-35 and DF4, cultured in LB have been deposited in the GenBank database with the following accession numbers: AS-35, AY764132 and DF4, AY764133.

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 inoculums were applied to API 50 CH (bioMerieux, France) biochemical strips and incubated at 30°C for 24 and 48 hours.

4.2.4.3 Morphological characteristics

Fifteen microliters of a 16 hour AS-35 and DF4 culture in both LB and 25% olive wastewater broth was transferred to a clean microscope slide before heat fixation. The samples were stained using Safranien and observed as describe previously.

In order to investigate a time dependent change in cellular morphology observed between AS-35 when cultured in LB and 25% olive waste, we used over night cultures of both as inoculums. Twenty milliliter LB was inoculated with an over night olive waste culture and sampled every 4 hours. The reciprocal was also performed and microscopically analyzed.

4.2.4.4 Total cellular proteome analysis

The isolates were cultured at 30°C in LB and olive waste broth containing 25% olive waste to an $OD_{600\ nm}$ of 1.0. The cells were harvested by centrifugation at 16 000 x g, the supernatant discarded and the pellets resuspended in 300 µl 10% TCA. The cell suspensions were sonicated for 5 x 30s at 60% power. The protein extract together with cellular debris were collected by centrifuging for 5 minutes at 16 000 x g at 4°C, and supernatants were discarded. The pellets were washed with 500 µl ice cold 80% acetone. The pellets were air dried and subsequently resuspended in 200 µl lysis buffer (9 M urea, 2 M thio-urea, 4% CHAPS). Following

solubilisation, the protein extracts were separated from the insoluble cellular debris by centrifuging at 16 000 x g for 5 minutes at room temperature and the supernatants were transferred to clean eppendorf tubes. The concentrations of extracted proteins were estimated using a modified Bradford protocol (Ramagli and Rodriguez 1985). In short 5µl sample was added to 10µl 0.1M HCl in a 100 µl reaction and mixed with 900 µl Bradford reagent (BioRad). Following a 5 minute incubations were the absorbency observed at 595nm. Protein concentrations were determined using a BSA standard.

Samples each of 50 mg of total cellular proteins were analyzed by SDS-PAGE. Proteins were separated on 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. The buffer used was the standard Laemmli buffer used for SDS-PAGE (25 mM Tris-HCl, 250 mM glycine, 0.1% [w/v] SDS; pH 8.3) (Laemmli, 1970). The proteins were visualized using Coomassie R250 and de-stained using 10% glacial acetic acid and 1% glycerol.

2D protein analysis

To resolve differences in the protein expression profiles, we analysed the total protein extracts by 2D protein separation. Large format (18 cm) isoelectric focusing dry strips (Amersham Bioscience) in a pH range 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) containing 100 mg/ml DTT followed by equilibration buffer containing 48 mg/ml iodoacetamide (Chivas *et al* 2000). Second dimension separation was performed on 12% polyacrylamide gels. The separated proteins were stained with Coomassie brilliant blue R250 (Sigma).

4.2.4.5 Protein induction studies

To investigate a time dependent change in protein profiles observed between AS-35 when cultured in LB and 25% olive waste, we used over night cultures of both as starting cultures. We inoculated LB with an over night olive waste culture and sample every 4 hours. We performed the reciprocal and analyzed the proteins on two dimensional PAGE.

4.2.4.6 RNA subtraction library analysis

RNA isolation and cDNA synthesis.

Total RNA was extracted using the hot phenol method as described by Krumlauf, 1996. The cells of an overnight culture was collected through centrifugation and washed in cold 0.1M Sodium phosphate buffer pH 7.0. The cells were resuspended in 11 ml 50 mM sodium acetate, pH 5.2 containing 1% SDS and lysed through sonification at 60% power for 25 seconds. Equal volume pre-warmed phenol saturated in 50 mM sodium acetate, pH 5.2 was added and the samples were incubated for 15 minutes at 60°C, followed by 5 minutes incubation at –10 °C. Centrifuging at 3000 r.p.m for 10 minutes at 2 °C separated the phases, followed by the removal of the bottom phase. The phenol treatment was repeated and the supernatant was transferred to a clean tube. Total RNA was precipitated after the addition of 0.1volume 5M NaCl and 2 volumes 95% ethanol and incubation at –20°C for 30 minutes. The RNA was collected and washed in 70% ethanol. The RNA was resuspended in 50 µl DEPC treated UHQ and 4 µl was analyzed on an agarose gel.
CDNA was synthesized using Revert Aid™ Hminus M-MuLV reverse transcriptase (Fermentas). Two microliters hexanucleotides (Roche) was used in a 50 µl reaction according to the manufactures instructions. Total cDNA was analyzed on a 1% agarose gel and purified using Qiagen PCR purification kit.

Driver synthesis

Felske 2002 described the construction of RNA subtraction libraries. Primer T7-long was annealed to primer short-P in order to create the driver adaptors, while the tester adaptors consisted of primer A6-long and short. Both adaptor sets were ligated to cDNA using 1U T4 ligase (Fermentas) and 1µl Peg400 over night at 20°C. The drivers were amplified by using 2µl ligation mix (T7-short-P) in a 50µl reaction containing 1 x PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% [v/v] TritonX-100), 0.2 mM of each dNTP and 1 U of *Taq* DNA polymerase (Southern Cross Biotechnology). The Taq DNA Polymerase was used to fill in the gaps at the end of the adaptors at 72°C for 50 minutes followed by the addition of 25pmol primer T7. A 30 cycle reaction then followed a 5 minute denaturation at 94°C. The cycle was similar to that described in 16S rDNA amplification. Driver amplicons were analyzed on a 1% agarose gel before purification using the Qiagen PCR purification kit

Subtractive hybridization and PCR

Two microliters of driver and tester were added to a 10 µl reaction 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 of fragments unique to the tester cDNA. Unique bands were purified using the Qiagen Gel purification kit and ligated into InsT/A clone (Fermentas), according to the manufactures instructions. The ligation mixture was transformed into competent *E. coli* DH5α cells and screened for recombinant clones using M13F and M13R in a colony PCR reaction. Recombinant clones were sequenced using M13F as previously described.

4.3 RESULTS

4.3.1 Isolation of wild-type strains from olive wastes

In an attempt to identify organisms which can potentially be used in the biodegradation of polyphenolic compounds, we isolated micro-organisms from the bioflora native to olive wastewater. Samples taken from various evaporation ponds and the surrounding soil were plated on selective media. Thirty-six colonies, each exhibiting an unique colony morphology, were subcultured to obtain pure cultures. The source of the isolates and morphological characteristics are presented in Table 4.2.

The cell morphology of each of these cultures was analyses by bright-filed microscopy (Figure 4.1). Of the 36 cultures 8 were putatively identified as fungi, whilst the remaining 26 cultures exhibited morphologies typically associated with bacteria. All but one of the bacterial cultures appeared to be rod-shaped. Interestingly, the majority of these cultures were Gram negative. This is in contrast to the composition of the bioflore associated with naturally fermented green olives, which predominantly consist of Lactobacillis spp. (Randazzo et al. 2004).

Table 4.2 - Distribution of isolates from various sampling points at a local olive production plant

Sampling points	Number of isolates
Liquid from top evaporation pond	11
Soil from top evaporation pond	3
Liquid from middle evaporation pond	3
Soil from middle evaporation pond	6
Liquid from bottom evaporation pond	1
Soil from bottom evaporation pond	4
Soil from dried evaporation pond	2
Soil from dried compost heap	5

4.3.2 Biodegradation capability of olive waste isolates

4.3.2.1 Enzyme activities in strains isolated from olive wastewater

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

The biodegradation capacity of the isolates was partly assessed through their ability to produce polyphenol oxidases. We were particularly interested in laccase, peroxidase and tyrosinase activity, since these enzymes have been implicated in the depolymerisation of phenolic compounds. Significant levels of peroxidase activity were observed for most of the isolates, using guaicol as substrate (Figure 4.2). Cellular extracts of AS-30 and AS-34 showed the highest activity with substrate conversion rates approaching 2.5 pmol/min. This is comparable to the activity of 1U of commercially available peroxidase enzyme (2.7 pmol/min). No significant tyrosinase or laccase activity was detected after 16 hours of growth in olive waste.

In addition to screening for polyphenol oxidase, their ability 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% after 16 hours, whilst AS-16 removed approximately 43% (Figure 4. 3).

Part of our investigation into the isolates biodegradation capabilities was to investigate their ability to produce economically valuable compounds such as vanillin. Ferulic acid [3-(4-hydroxy-3-methoxyphenyl)-propenoic acid] is an extremely abundant plant product, whose biotransformation to vanillin via the decarboxylation to 4-vinyl guaiacol is widely reported in various bacterial and fungal isolates (Rosazza et al 1995). Sixteen isolates showed significant decrease in ferulic acid concentrations coupled with the formation of. 4-vinyl guaiacol (Figure 4.4). Isolates AS-9, AS-24 and AS-35 were found to be most effective in decarboxylating ferulic acid. Only isolate AS-1 showed ferulic acid esterase activity after 4 days of incubation.

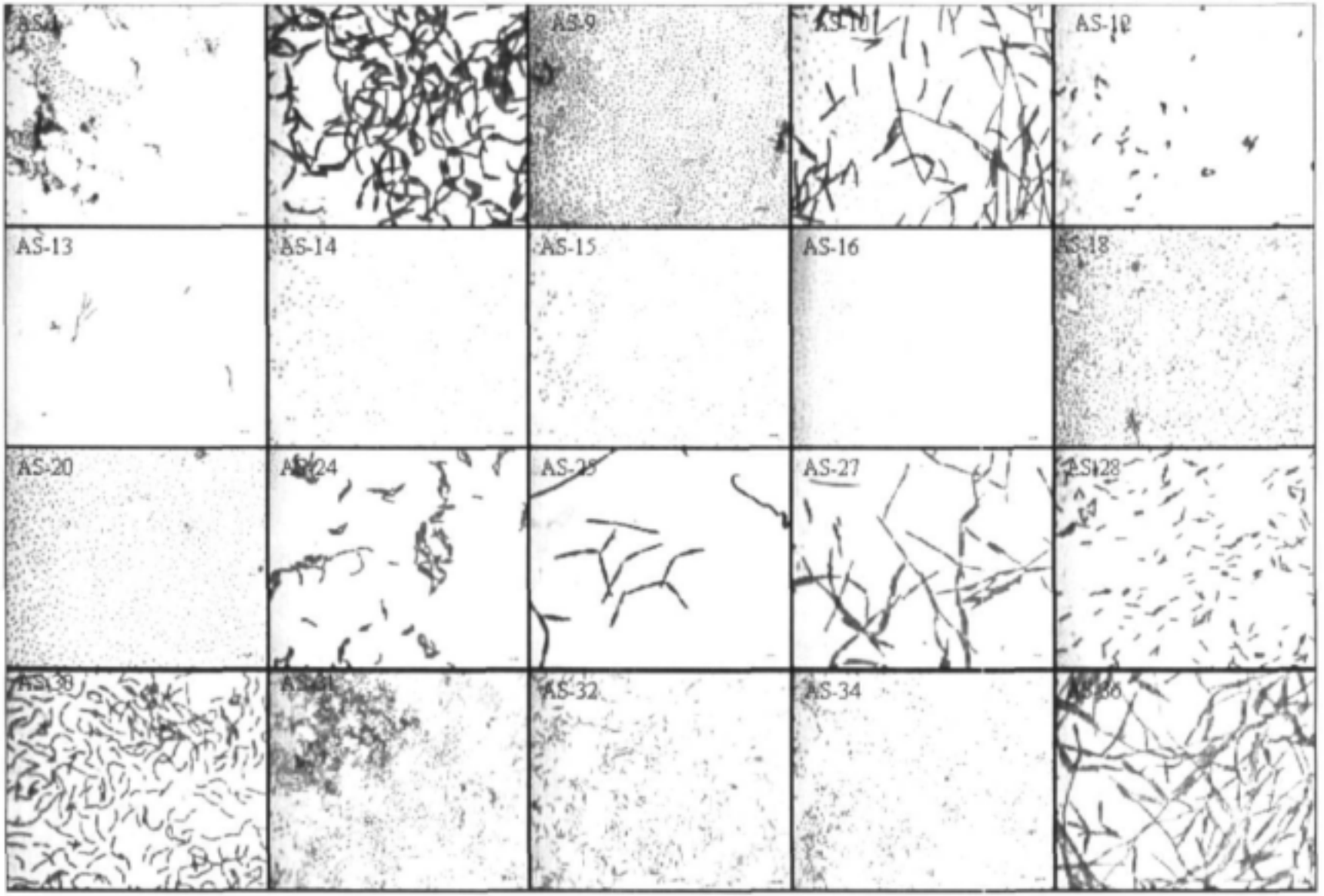


Figure 4.1. Photomicrographs showing the distinct morphologies of 20 different isolates. Scale: --- = 0.02mm.

4.3.2.2 Low molecular weight phenolic profiles

The low molecular weight phenolic content produced by the isolates were analyzed over a 64 hour period, by analyzing a fraction of the olive waste culturing media supernatant by reverse phase HPLC. Profiles of these fractions were compared to a set of model compounds, previously described to occur in olive waste Bertin et al 2001, for example: benzoic acid 22.74 min, catechol 6.45 min, galic acid 4.42 min, p-hydroxyphenyl acetic acid 8.83 min, hydroxy tyrosol 5.12 min, protocatechuic acid 7.77min and syringic acid 17.99 min. Several of these compounds were observed in the profiles of AS-35 for example, hydroxytyrosol, catechol, protocatechuic acid, p-hydroxyphenyl acetic acid and syringic acid in AS-35. Based on these results, 4 isolates (AS-9, AS-15, AS-18 and AS-35) were selected for further investigation (Appendix 4.1).

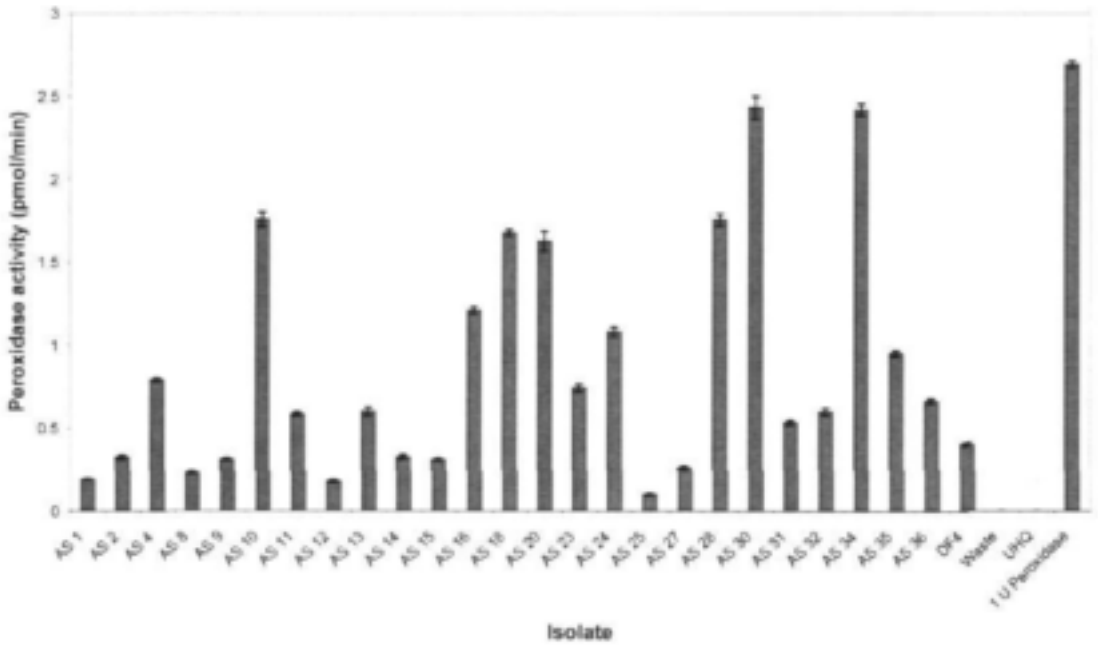


Figure 4.2: Peroxidase activity (pmol/min) of isolates measured after 16h of culturing in 25% olive waste. (Data shown are means of triplicate readings).

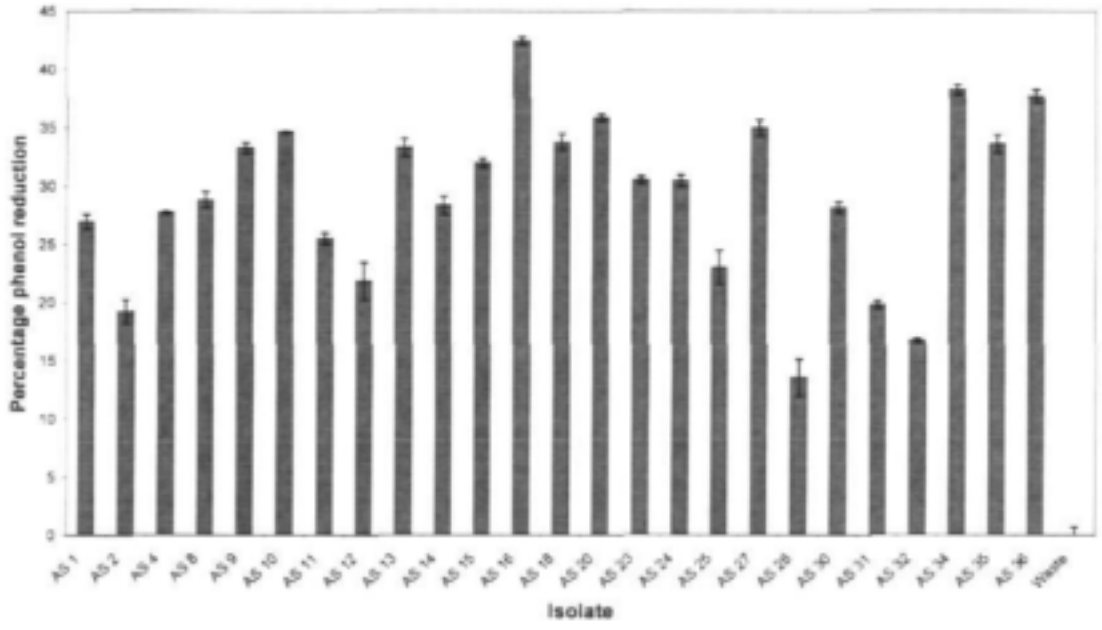


Figure 4.3: **Reduction in total phenol content (mM) of the olive waste supernatant after 16 hours cultivation in 25% olive waste. The reduction is illustrated as a percentage of the initial phenol concentration**. (Data shown are means of triplicate readings).

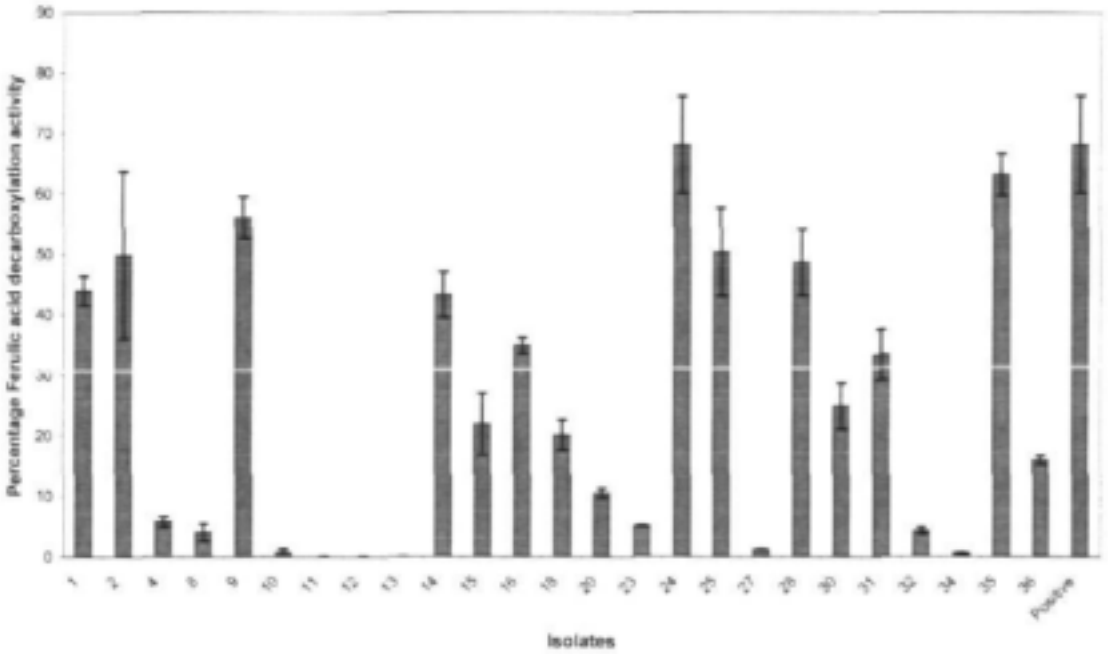


Figure 4.4: **Reduction in ferulic acid concentration (mM) after 8 hours. The reduction is illustrated as a percentage of the initial ferulic acid concentration.** (Data shown are means of triplicate readings).

4.3.3 Characterization of selected isolates

4.3.3.1 Molecular identification of selected isolates

Based on these results, 5 isolates (AS-9, AS-15, AS-18, AS-24 and AS-35) were selected for further investigation. From 16S rDNA analysis, 3 of them, AS-9, AS-15 and AS-18 showed 99%, 99% and 98% homology with *Klebsiella oxytoca*. Figure 4.5 shows a phylogenetic rooted tree, which is a means of representing the related-ness of isolates by the horizontal distance of the branches. 16S rDNA analysis shows the homology between AS-9, AS-15, AS-18, *Klebsiella oxytoca* type strain, UWC-10 and *E. coli*. AS-35 was identified as a *Bacillus sp*. We were unable to conclusively identify AS-24.

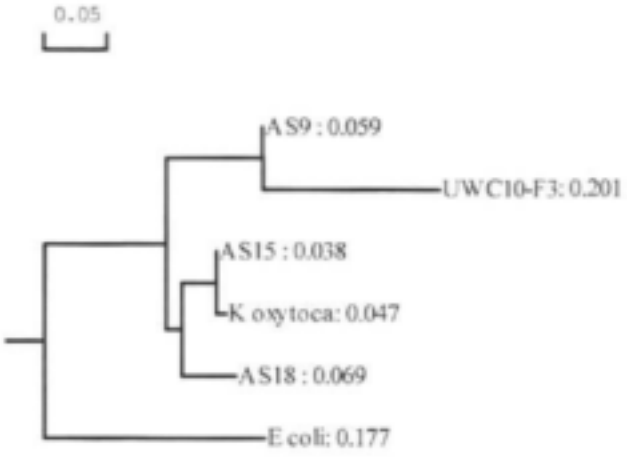


Figure 4.5: Schematic representation of phylogenetic homology between AS-9, AS-15, AS-18 with *Klebsiella oxytoca*.

4.3.3.2 Further biodegradation analysis

Similar growth rates were observed for selected isolates AS-9, AS-15, AS-18, AS-24 and AS-35 with stationary phase reached after 8-9 hours and no major deviation in OD600nm for the rest of the 36 hours. We used supernatant aliquots from this experiment to again analyze the changes in their low molecular phenol profile, using reverse phase HPLC. AS-35 was selected due to the multitude of new peaks observed in its low molecular weight profile for subsequent classical chemical mutagenesis, to enhance its biodegradation capability (Appendix 4.2).

4.3.3.3 Model compound degradation of AS-35

The biodegradation capabilities of AS-35 were further characterized by culturing it in various olive waste media and by measuring its enzymatic activities in the presence of model substrates. It was shown to be capable of utilizing ferulic acid and vanillic acid, but not tyrosol, p-hydroxyphenyl acetic acid or gallic acid (Fig 4.6).

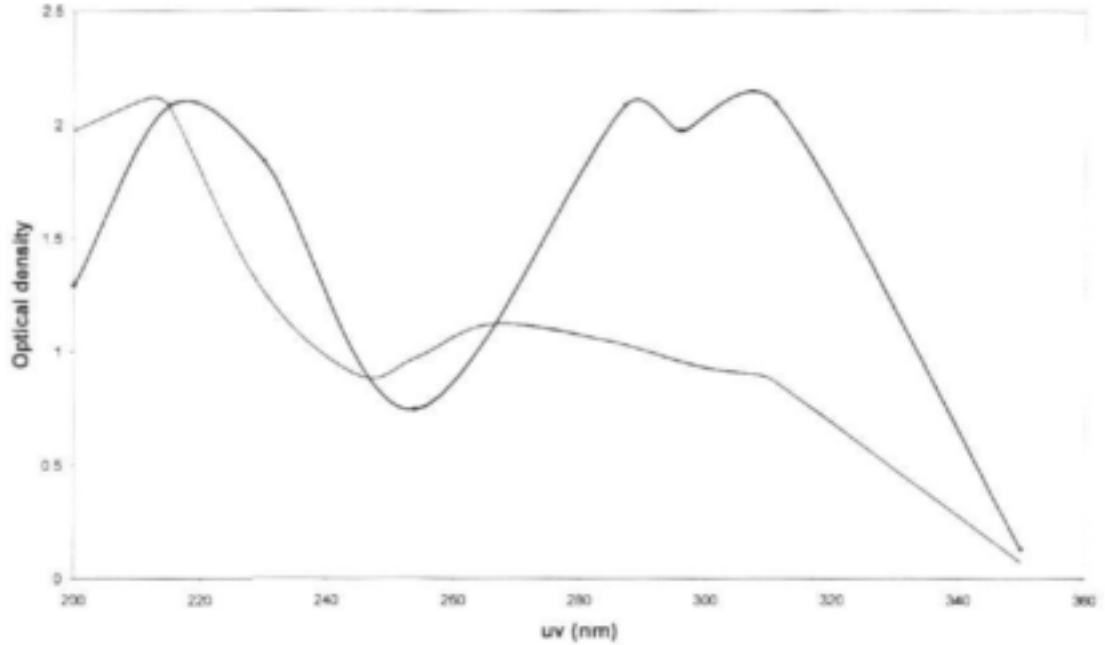


Figure 4.6: Spectrophometric analysis of AS-35 ability to decarboxylate ferulic acid over a 10 hour incubation period. Ferulic acid has absorbency peaks at 284 and 312 nm, while 4-vinyl guaiacol has a peak at 264 nm.

4.3.4 Production of mutant strains with improved activities

4.3.4.1 Mutant isolation and characterisation

In order to enhance the biodegradation capability of AS-35, we subjected the wild type isolate to uv, but unsuitable quantity of putative mutants were obtained, thus we performed classical chemical mutagenesis using formamide. This is a chemical shown to induce frameshift mutations and to promote the mis-incorporation of guanine through shift-base interactions with opposing amino groups (Maufrais *et al.*, 2003). We exposed growing cells to the mutagen before culturing them on agar containing 80% olive waste, a concentration at which the environmental isolate AS-35 showed no growth. Eight hyper-resistant mutants capable of growth on 80% olive waste were identified. The one dimensional profile of the total intracellular proteins of the mutants, cultured in 50% olive waste, was analyzed on SDS-Page and protein profiles similar to the wild type AS-35, cultured under the same conditions, were observed (Figure 4.7). Separating proteins based on

size alone has limitations and because of the absence of differences in the protein profiles of the mutants, we analyzed the olive waste biodegradation capability of the mutants in order to select a suitable mutant for further 2D protein analysis.

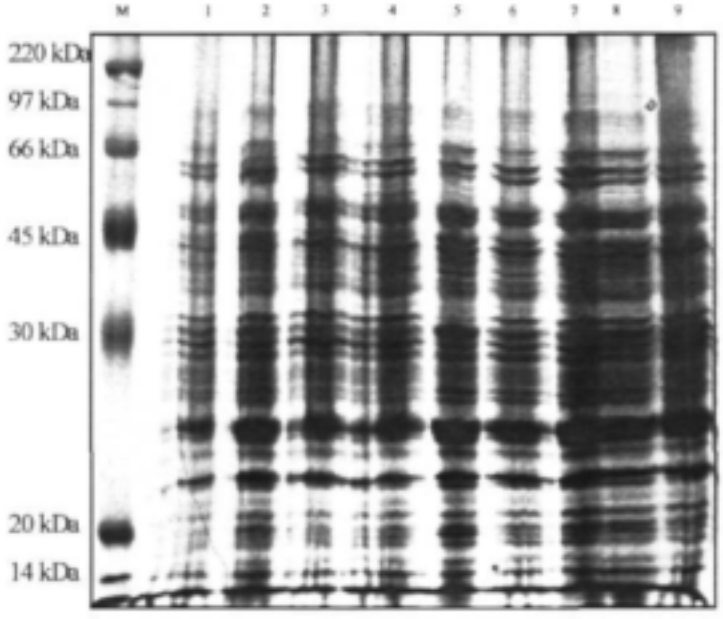


Figure 4.7: Total protein profiles of wild type AS-35 and putative mutants. Wild type AS-35 is in lane 1, while the rest of the lanes represent mutants AF1, AF5, BF1, BF2, BF5, DF4, DF5 and DF6 respectively.

4.3.4.2 Characterization selection for a suitable mutant

Isolate AS-35, capable of growth on 50% olive wastewater, was selected in the previous chapter to undergo chemical mutagenesis. This was in order to enhance its biodegradative capabilities. It was subjected to growth on 75% olive wastewater after treatment with formamide. Various hyper resistant putative mutants were obtained, from which 8 was selected for further characterization. They were evaluated based on their ability to produce peroxidase, to reduce both the total phenol content and brown-black colour of the olive wastewater (Table 4.3). 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, 3 of them did remove an equal percentage of the total phenol content. Based on these results, we selected DF4 for further characterization.

Table 4.3 Biodegradation characteristics of wild type AS-35 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 the mutants.

Sample	Peroxidase activity (μM/min)	Total phenol reduction %	Total Decolorization %
AS-35	0.27	28.91	12.45
AF 1	0.93	4.01	3.64
AF 5	1.68	34.44	9.45
BF 1	2.39	29.05	8.18
BF 2	2.9	2.49	7.05
BF 5	1.81	14.38	2.95
DF 4	2.7	28.49	7.77
DF 5	1.95	10.24	6.14
DF 6	0.62	6.92	3.64

4.3.5 Comparative characterization of wild type AS-35 and mutant DF4

4.3.5.1 Growth kinetics of AS-35 and DF4

The wild type strain AS-35 and its mutant, DF 4, were cultured in LB (nutrient rich) broth and in 25% olive waste medium in order to investigate the differences in protein induction due to the absence and presence of phenolics. Analysis of the growth kinetics of wild type AS-35 and mutant DF4, showed that AS-35 exhibited faster growth rate when cultured in LB compared to the mutant DF4, but similar rates were observed for AS-35 and DF4 when cultured in 25% olive waste (Figure 4.8). From the growth kinetics studies we can conclude that both isolates reach a similar OD_{600nm} after 16 hours.

The growth rate of AS-35 and DF4 and their abilities to reduce total phenol content were characterized further, by culturing it in olive wastewater media containing different nitrogen sources and at different initial pH. Although both isolates were capable of growth in 25% olive wastewater with our supplementary nitrogen sources, DF4 had a faster adaptation to the more stringent environment (Figure 4.9). This longer lag phase of AS-35 could indicate that the wild type needs to activate genes important in the survival of the organism, while the mutant had already activated these genes. During this experiment we also observed that even though the concentration of total phenol reduced by both wild type and mutant are similar in 25% olive

wastewater containing 0.5% yeast extract, the mutant reduced significant more phenol in more stringent media (Figure 4.10).

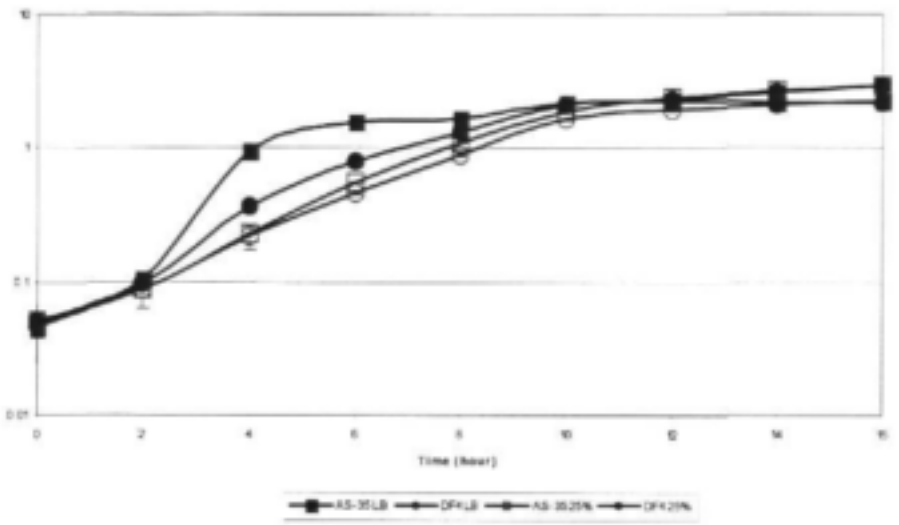


Figure 4.8: Growth kinetics (Absorbance at 600nm on vertical axis *vs.* time) of wild type AS-35 ■ and mutant DF4 ● cultured in LB compared to AS-35 □ and DF4 ○ cultured in 25% olive waste.

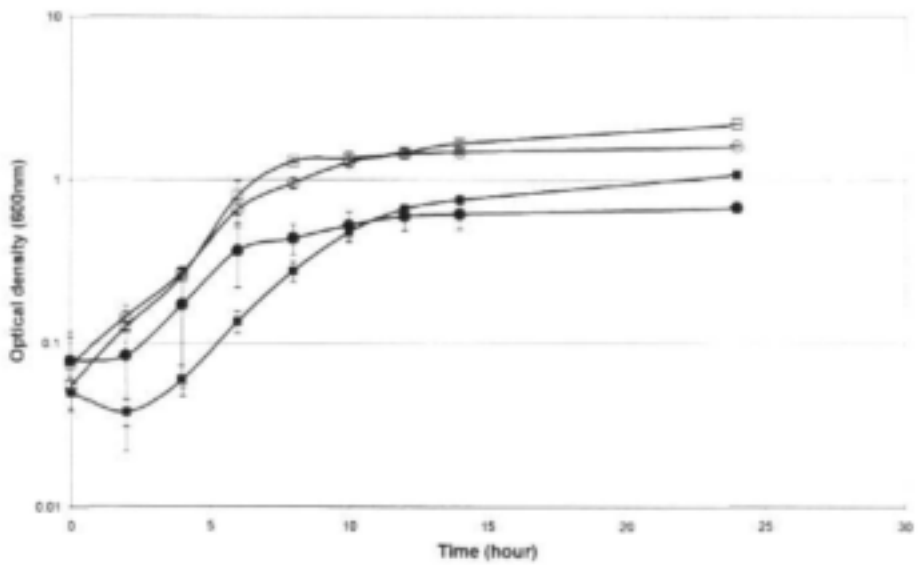


Figure 4.9: Growth kinetics (Absorbance at 600nm *vs.* time) of wild type AS-35 ■ and mutant DF4 ● cultured in 25% olive wastewater compared to AS-35 □ and DF4 ○ cultured in 25% olive waste containing 0.5% yeast extract.

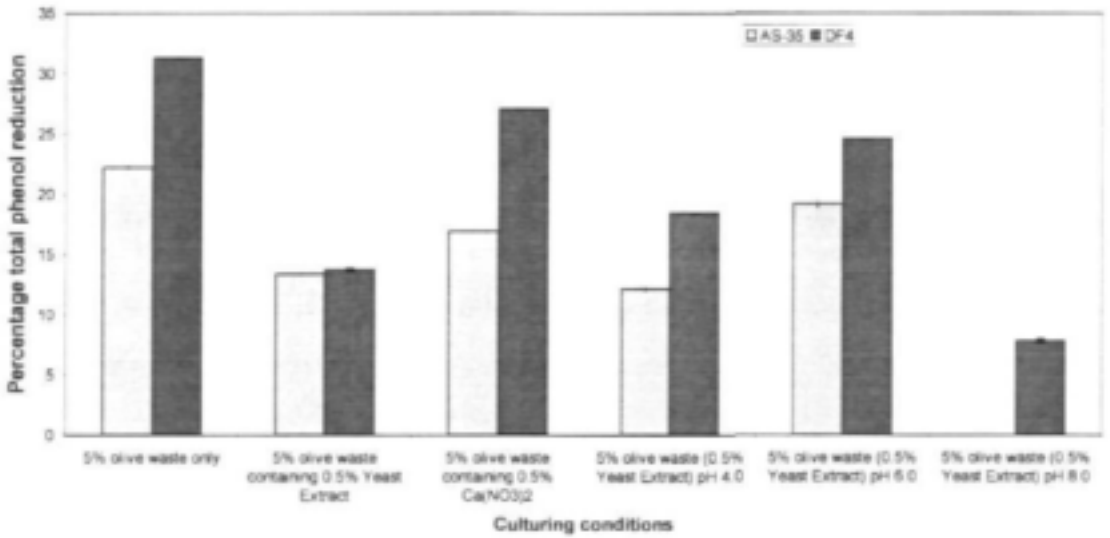


Figure 4.10. Total phenol reduction of AS-35 and DF4 cultured in 25% olive waste containing either various nitrogen source or different initial pH levels. Total phenol reduction after 14 hours is represented as a percentage of the original level of phenol.

4.3.5.2 Isolate identification

16S rDNA gene analysis was performed on AS-35 and DF4, cultured both in LB and olive waste. Bi-directional sequence analyses of the complete 1.5 kb gene region indicated that they are identical pure cultures and were identified as *Bacillus sp.* We performed API 50 CHB biochemical test on AS-35, cultured in LB and olive waste, and identified the isolate as *Bacillus Megaterium.*

4.3.5.3 Morphological characterization of AS-35 and DF4

Microscopic analysis clearly revealed enlarged cell morphology in the case of mutant DF4 as compared with 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 4.11). These differences in cell morphology correlate with the differences observed in growth rates, where there is a clear distinction between AS-35 cultured in LB and olive waste, but not with the mutant under the same conditions.

We investigated AS-35 ability to reverse this morphology by inoculating olive waste media with a culture that was grown in LB and the reciprocal. The culture was investigated 4, 8 and 16 hours post inoculation (Figure 4.12). It appears that the isolate previously cultured in LB and then transferred into olive waste increase in size before the reciprocal decrease.

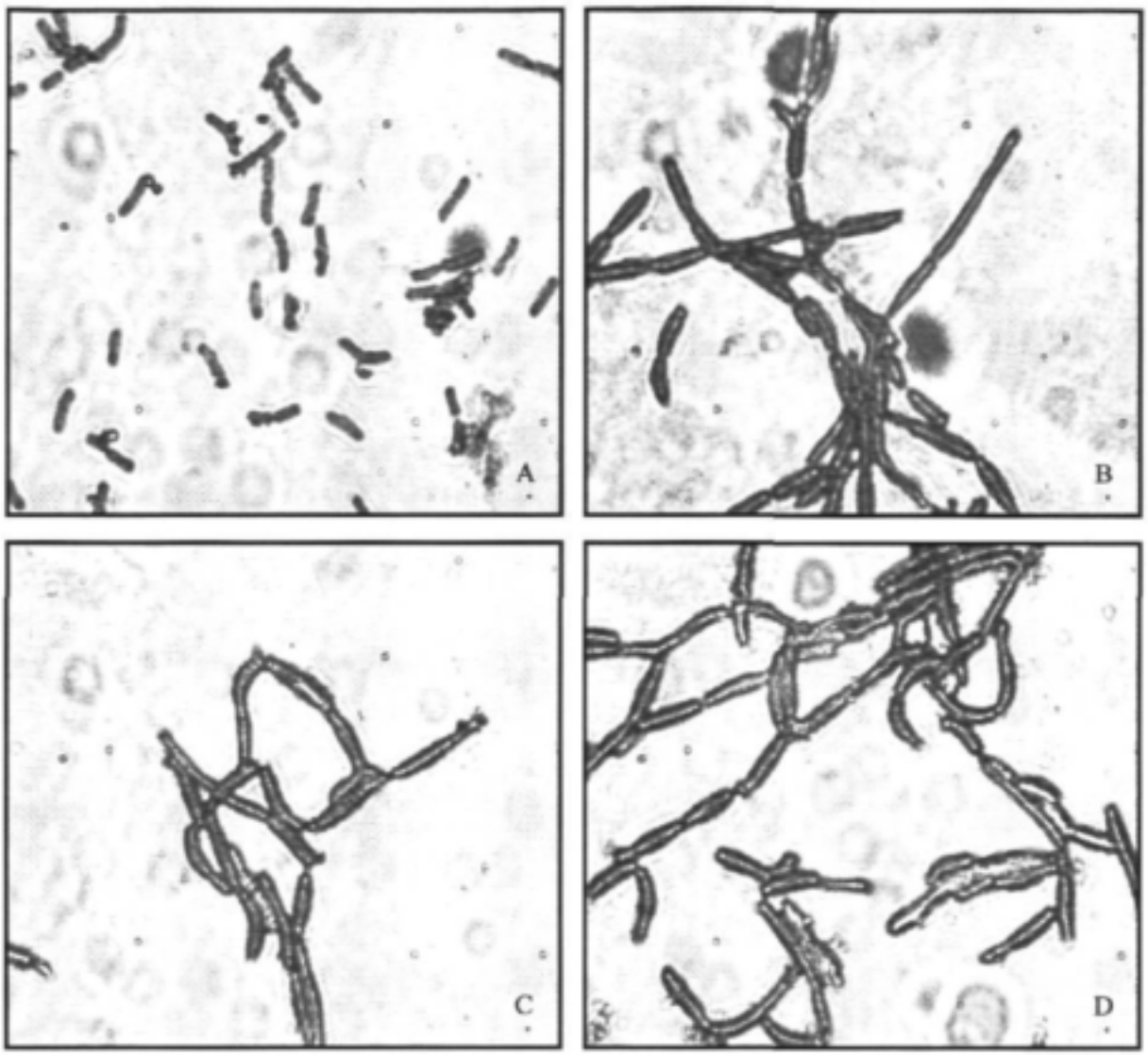


Figure 4.11. Photomicrographs showing the phenotypes of the wild-type AS-35 (A,B) and the mutant DF4 (C,D). A and C represent the cells cultured in LB, while B and D represent olive waste cultured cells. [Approximate scale: ——— = 0.02 mm]

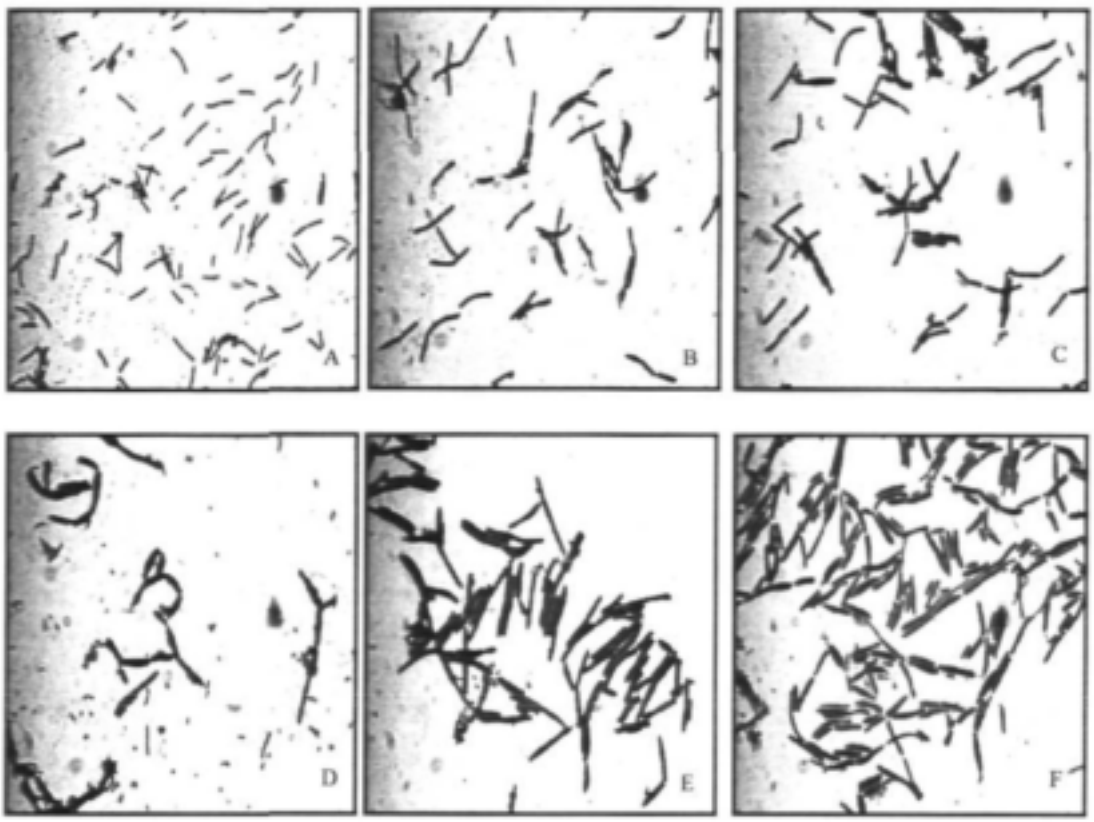


Figure 4.12. Photomicrographs showing the phenotypes of the wild-type AS-35. Figure A is AS-35 cultured overnight in LB and Figure D is in olive waste. Figure B and C are respectively 4 and 8 hours after a LB culture was inoculated into olive waste. Figure E and F represent 4 and 8 hours after inoculating LB with an olive waste culture. [Approximate scale: —— = 0.02 mm]

4.3.5.4 Proteomic analysis of AS-35 and DF4 protein expression

Total protein extracts were prepared for AS–35 and DF4 at 4 and 8 hours post-inoculation respectively, corresponding to mid-exponential phase (OD_{600nm} 1.0) for both isolates. The total cellular protein profile of wild type AS–35 and mutant DF4 cultured in LB and 25% olive waste was analyzed on SDS-PAGE (Figure 4.13). Analyses of the respective profiles revealed that the expression profile of AS–35 grown in standard LB broth differed significantly from the profile of the same isolate grown in the presence of 25% olive waste. In contrast the expression profiles of DF4 remained unchanged irrespective of the culturing media, resembling that of AS–35 grown in the presence of olive waste.

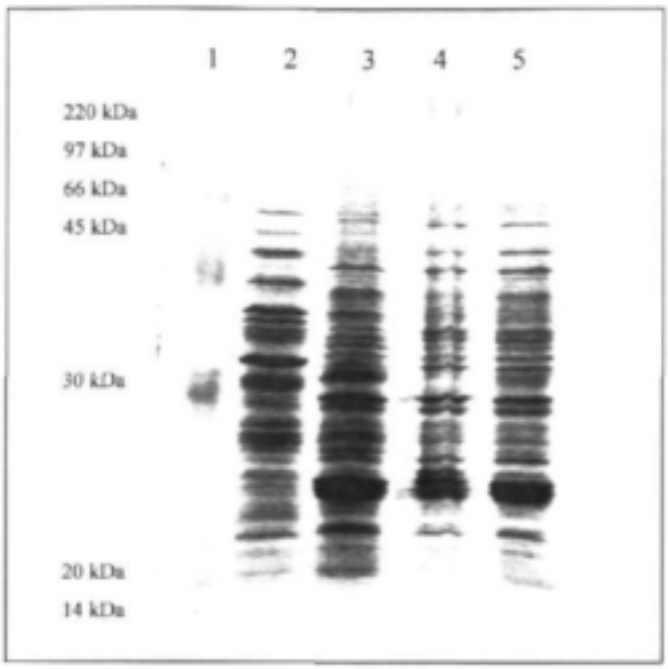


Figure 4.13: Total protein profiles of wild type AS-35 and mutant DF4 cultured in LB and olive waste. Lane 1 and 2 represent AS-35 protein profile cultured in LB and 25% olive waste respectively. Protein profile of mutant DF4 is indicated in lanes 3-4, with lane 3 representing the mutant cultured in LB, lane 4 in 25 % olive waste.

Based on these findings we investigated the difference in protein profile of AS-35 and DF4 using 2D analysis. Two dimensional analyses of AS-35 and DF4 total cellular proteins were performed on a pH range of 3-10 (Figure 4.14). It is noted that in the case of AS-35 cultured in LB, the majority of proteins appear to be acidic in nature. This is in contrast with the profile of mutant DF4 and AS-35 cultured in olive waste, where the majority of the proteins display a neutral pI. To narrow the field of our investigation, we performed the same experiment on a pH range of 6-11 (Figure 4.15). These gels were not that informative, due to the pI variations observed and we decided to continue using only pH 3-10 strips. Although 25 proteins common to both AS-35 and DF4 were identified, the majority of proteins were unique to either the mutant or wild type.

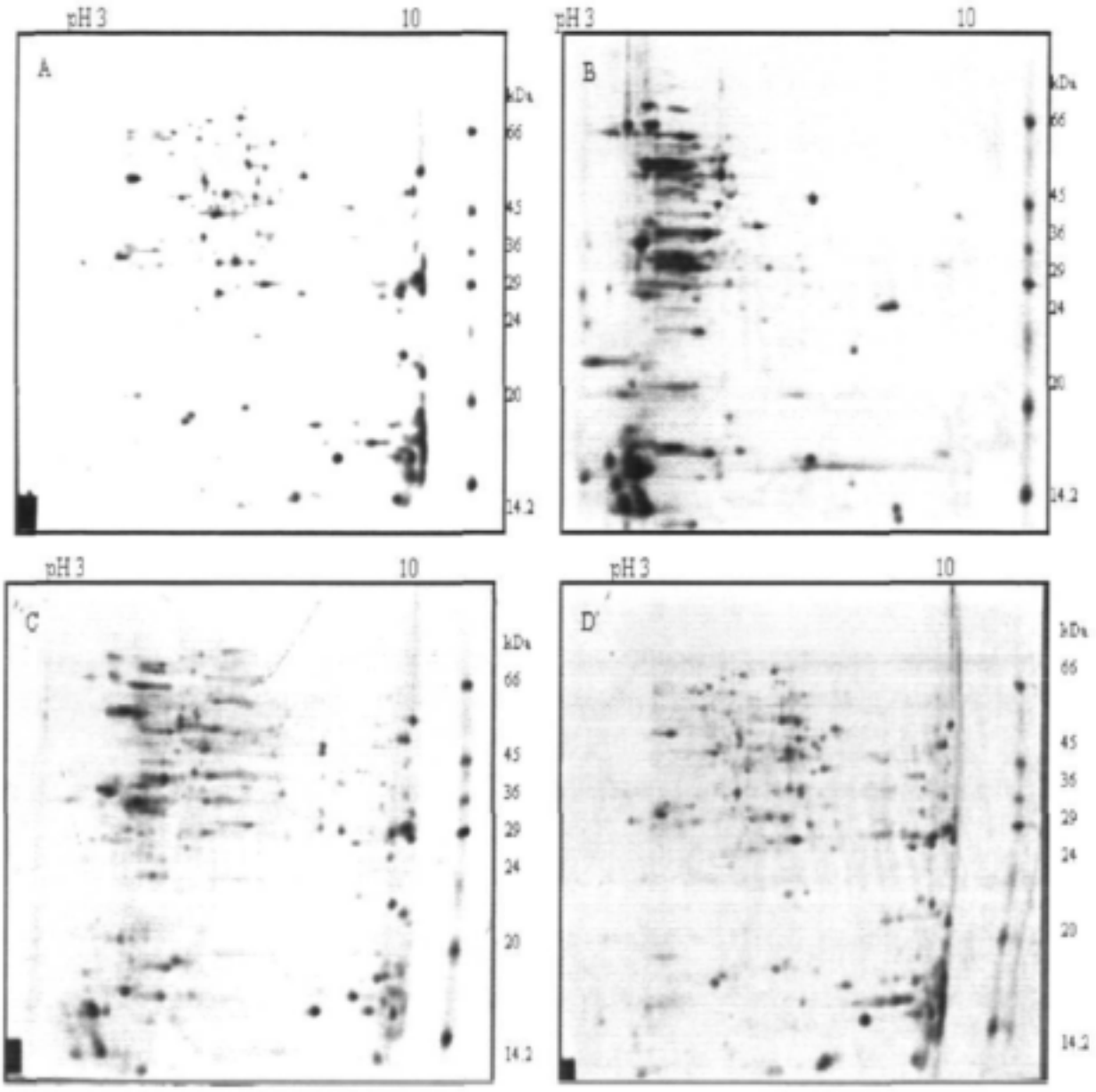


Figure 4.14: Large format two-dimensional gel analysis of wild type AS-35 and mutant DF4 pH 3-10. Figure A and B represent wild type AS-35 cultured in 25% olive waste and LB respectively, while C and D represent mutant DF 4 cultured in olive waste and LB respectively.

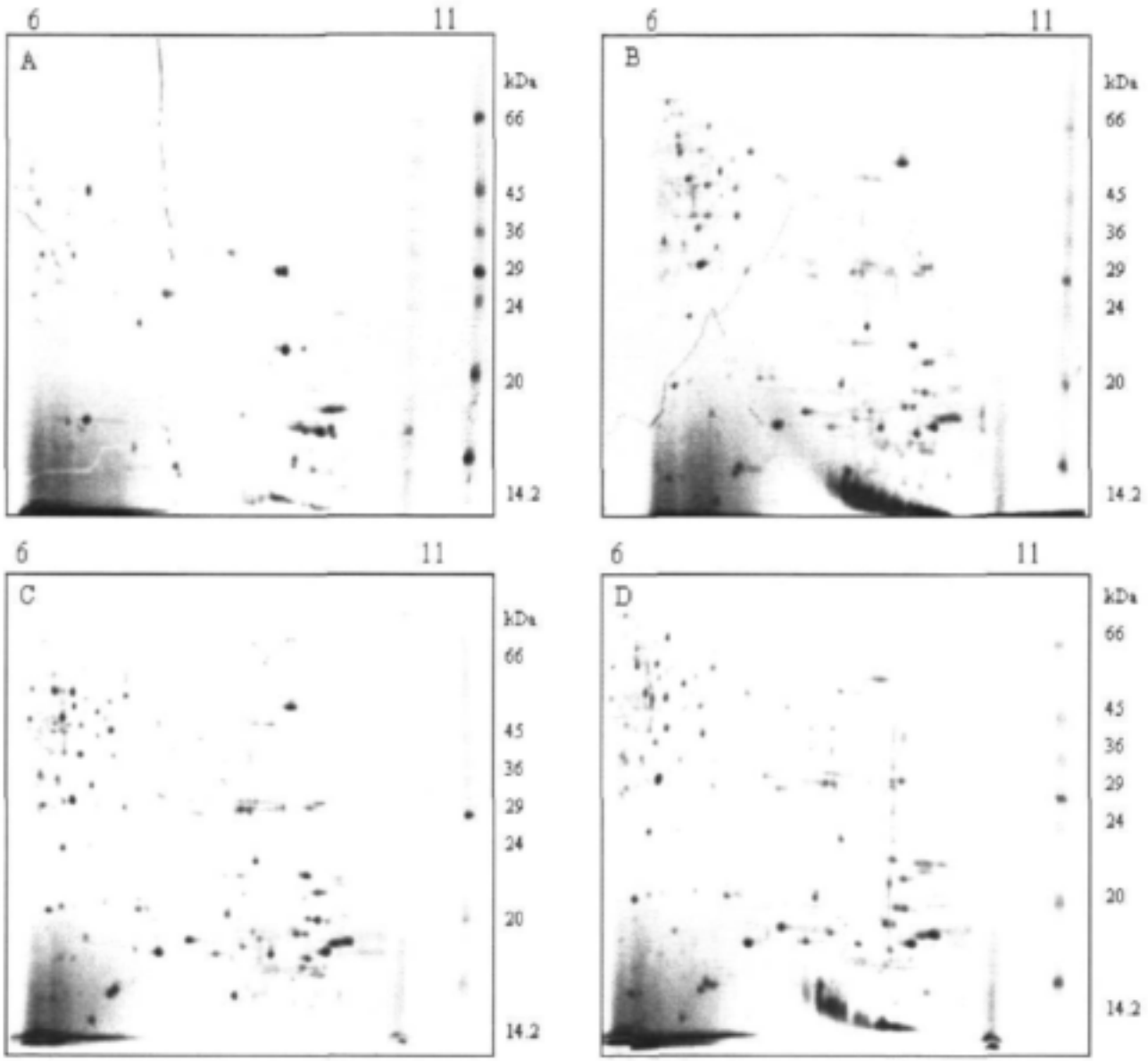


Figure 4.15: Large format two-dimensional gel analysis of wild type AS-35 and mutant DF4 pH 6-11. Figure A and B represent wild type AS-35 cultured in LB and 25% olive waste respectively, while C and D represent mutant DF 4 cultured in LB and olive waste respectively.

4.3.5.6 Induction studies

In order to investigate protein activation during wild type AS-35 adaptation to environmental changes, we observed the changes that occurred in wild type AS-35 protein profile when an overnight LB culture was inoculated into 25% olive waste media (similar to the microscopy experiment) and protein extracted analyzed 4 and 8 hours post inoculation (Figure 4.16). The reciprocal, inoculating AS-35 cultured for 16 hours in olive waste into LB media and observing its protein profile after 4 and 8 hours, were also performed. Four hours after introducing a LB culture to olive waste there was still a predominant LB profile, with 13 olive waste proteins and 7 unique ones. Eight hours post inoculation belonged the majority of the proteins to the olive waste profile, while 19 LB proteins still remain. The olive waste culture transferred to LB showed a predominant LB profile after 4 hours of incubation. This implies that AS-35 takes longer to adapt to an olive waste environment than the LB.

Olive waste is a complex medium consisting of various stress-inducing factors. Some of these are the low pH, high salt content and high phenol content. We thus decided to investigate each of these factors separately in LB media. We cultured AS-35 in LB containing 3% NaCl, LB containing 3% phenol and LB a pH of 5.0.

4.3.5.7 RNA subtraction libraries

The protein profile observed for each of the isolates under the different culture conditions contained too many differences to be identified individually. We thus investigated the differences in gene expression on RNA level through RNA subtraction libraries. The process is based on the hybridization of identical cDNA fragment and the amplification of unique ones. Sequence data from the cDNA library indicated putative exonuclease V gamma chain, GntR-family transcription factor and anthranilate phosphoribosyltransferase, were uniquely associated with AS-35 cultured in LB. In contrast putative proteins 3-hydroxyacyl-CoA dehydrogenase and short-chain dehydrogenase/reductase were only identified when AS-35 was cultured in 25% olive waste. More sequence data from the library is needed in order to construct a more detailed list of the proteins involved in olive waste biodegradation.

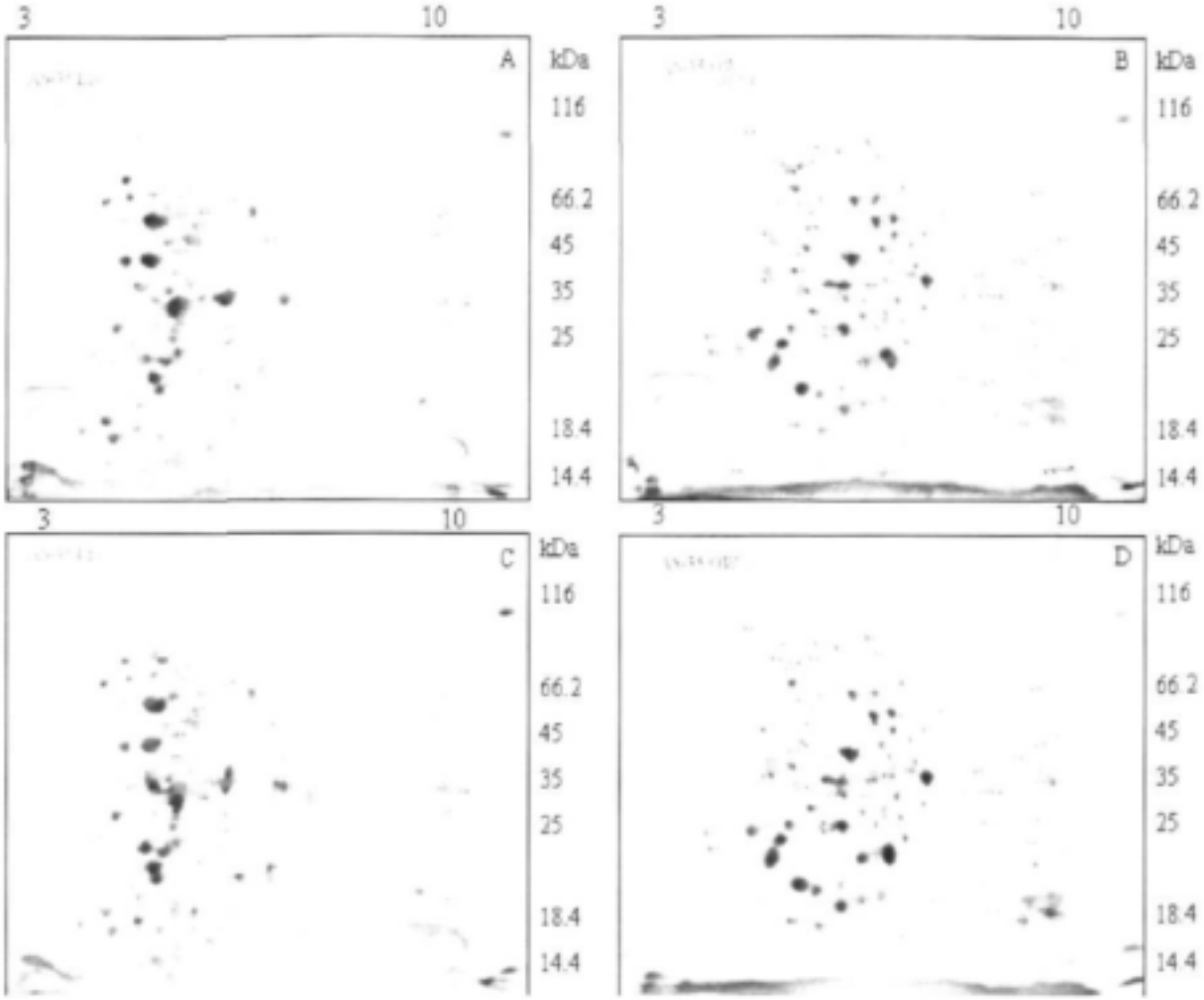


Figure 4.16: Comparative 2D electrograph of wild type AS-35 cultured in LB and 25% olive waste for various time periods. Figure A and C compare a 16 hour AS-35 culture in LB with a AS-35 olive waste culture transferred into LB after 4 (A) and 8hours (C). Proteins uniquely associated with growth in LB are indicated with red, whilst proteins unique for growth in the induction studies are indicated in green. Proteins common to both culturing conditions are indicated in blue.. Figures B and D compare the same experiment to a 16 hour culture of AS-35 in 25% olive waste. Proteins unique to 25% olive waste is represented in red, while the proteins unique to the LB induction study are presented in green. Proteins that occur in both are indicated in blue.

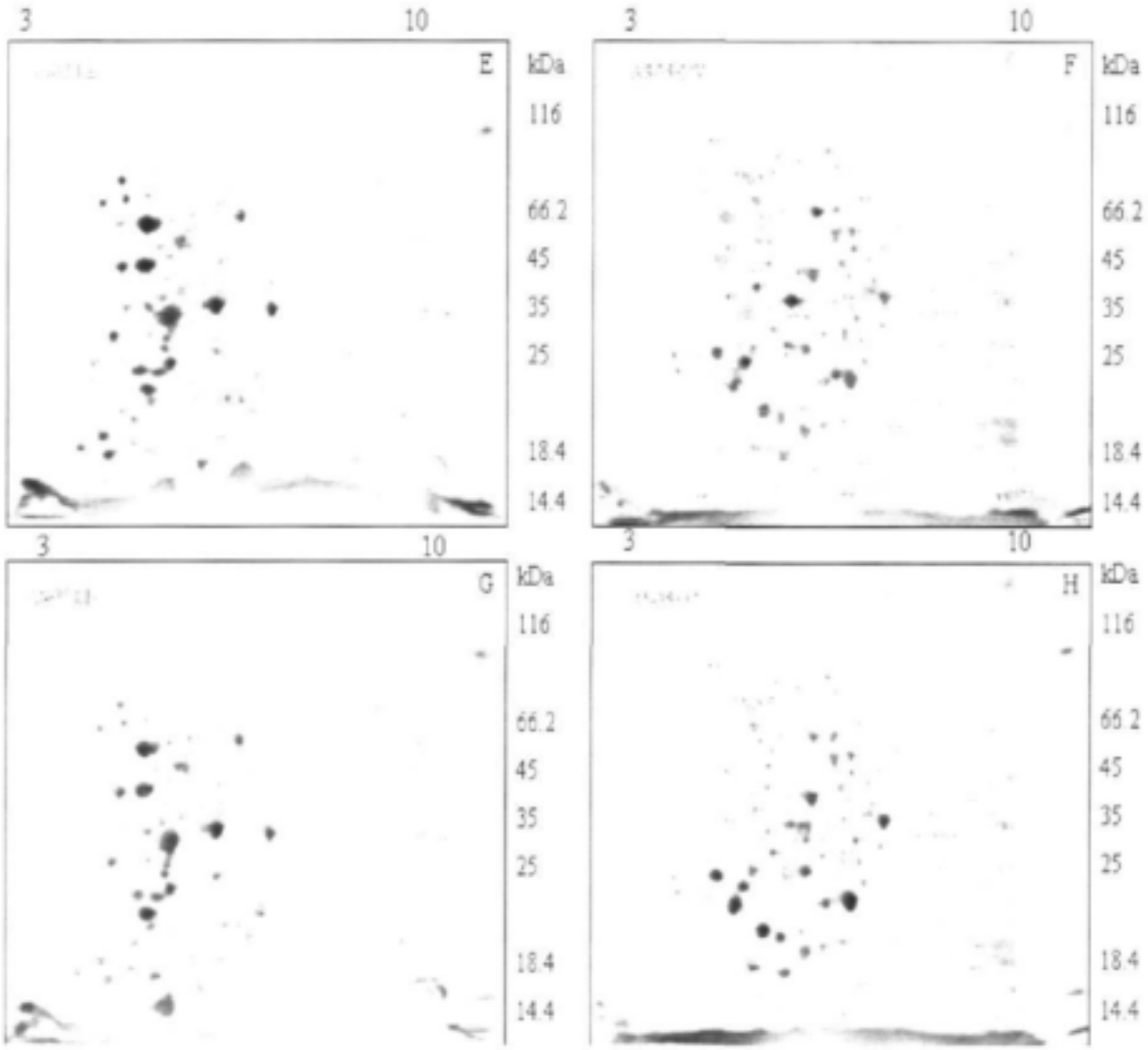


Figure 4.16: cont.. Comparative 2D electrograph of wild type AS-35 cultured in LB and 25% olive waste for various time periods. Figure E and G compare AS-35 culture in LB with a AS-35 culture in LB and then transferred into 25% olive waste after 4 (E) and 8 hours (G). Proteins uniquely associated with growth in LB are indicated with red, whilst proteins unique for growth in the induction studies are indicated in green. Proteins common to both culturing conditions are indicated in blue. Figures F and H compare the same experiment to a 16 hour culture of AS-35 in 25% olive waste. Proteins unique to 25% olive waste is represented in red, while the proteins unique to the olive waste induction study are presented in green. Proteins that occur in both are indicated in blue. This comparisons of 2D gels were performed using Decon Delta 2D.

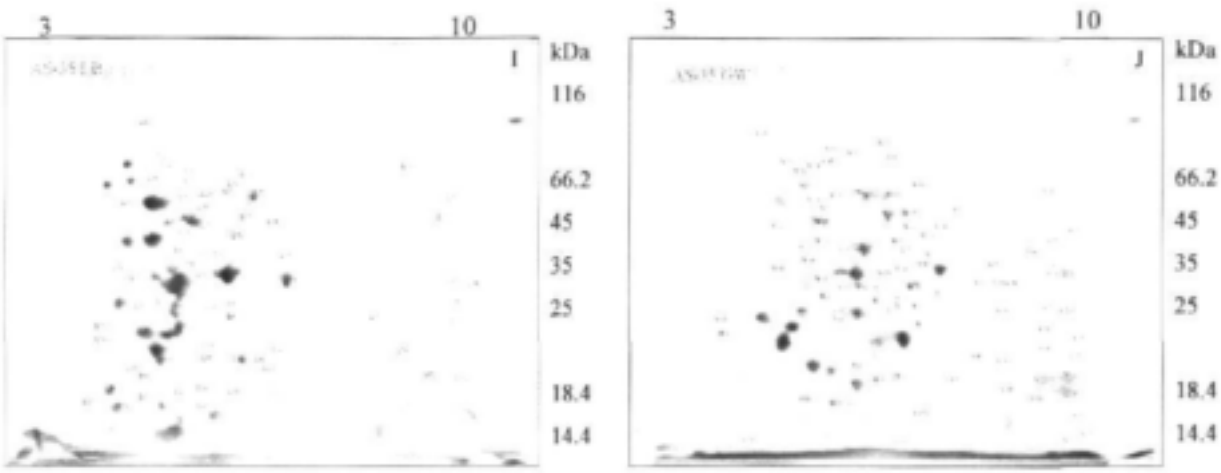


Figure 4.16 cont.. Comparative 2D electrograph of wild type AS-35 cultured in LB containing 3% NaCl. Figure I compare AS-35 cultured in LB (red) with AS-35 cultured in LB containing 3% NaCl (green). Proteins that are found in both are indicated in blue. AS-35 cultured in LB containing 3% NaCl (green) is compared to AS-35 cultured in 25% olive waste (red) in figure J. Common proteins are indicated in blue.

4.4 DISCUSSION

Thirty-five pure cultures were isolated from olive waste evaporation ponds. These organisms were screened for their ability to produce polyphenol oxidases, the enzymes responsible for the depolymerization of polyphenolic compounds and subsequent decolorization of the wastewater. High levels of peroxidase activity were detected, but an insignificant level of tyrosinase and laccase. A possible reason for the absence of detectable levels of enzyme activity could be that the 16 hour incubation period is insufficient for induction of enzyme synthesis. Despite this, total phenol reduction of higher than 10 % was detected for all the isolates. In an attempted to identify economically valuable products formed during biodegradation, extracellular fractions were analyzed by reverse phase HPLC. Various metabolic compounds produced by several the isolates as byproducts of the phenol biodegradation process, such as vanillin were identified in this way. These results suggested that AS-35, identified as *Bacillus megaterium,* could potentially be used in olive waste bioremediation processes and was subsequently selected for further analyses

In an attempt to further enhance the biodegradation capabilities of AS-35 classical mutagenesis was performed on this isolate. Chemical mutations produced 8 putative hyper-resistant mutants that were capable of growth on a higher concentration of olive waste (80%) than the wild type. The mutants' biodegradation capabilities were compared to that of the wild type. Putative mutant DF4 showed significant increases in its ability to adapt to the high polyphenolic content of olive waste water.

It has been proposed that catabolism of aromatic compounds is only advantageous to the host if it does not interfere with the host fitness and that these pathways are repressed until the preferred nutrients are in short supply (Sze *et al.,* 2002). This was observed for AS-35 as well, and to a lesser degree, in DF4. Regulation of catabolism operons such as these, (for example, catabolism of methylphenols or toluene in *Pseudomonas)*, is under the control of a constitutively expressed transcription activator that binds to the RpoN-dependent promoter in the presence of the aromatic effector (Cases and de Lorenzo, 2005). Although both these transcription activators show homology with NtrC two-component regulators, the absence of a sensor protein been shown for both (Parvel *et al., 1994*). Based on this, could it be possible that the degradation of various monophenolics present in the olive waste are controlled within the same operon.

Total protein expression profiles of the wild type AS-35 and mutant DF4 were analyzed on SDS-PAGE and 2D PAGE. The protein expression profile of the wild type was found to vary depending on the particular environment it found itself in. This was particularly evident when the expression

profile of AS-35 cultured in either a nutrient rich environment or 25 % olive waste were compared. In contrast to this, similar profiles were observed for the mutant DF4 irrespective of the culturing conditions. Furthermore, the mutant profile was similar to that observed in wild type cultured in 25% olive waste. This result correlated well with differences in the cell morphology and growth kinetics of the respective isolates.

These results suggest that the mutations introduced into DF4, occurred within regulatory pathways involved in olive waste degradation. Likely candidates include sigma factors, which have been shown to be involved in transcription activation of many different and unrelated genes (Parvel, *et al.*, 1994). A more plausible explanation is changes involving a two-component signal transduction system. These systems are directly responsible for sensing and responding to stress factors in the environment (Martinez-Antonio and Collado-Vides 2003). The involvement of these systems can be examined by measuring the levels of autophosphorylation brought about by the introduction of radio-active labelled phosphates in various time and media dependant studies analysing the protein profiles. This is an area for future work.

4.5 CONCLUSIONS

- Pure cultures were isolated on selective media from olive wastewater, yielding 35 isolates capable of growing on varying concentrations of olive wastewater.
- Partial characterization of the isolates, with special emphasis on their biodegradation capabilities, was completed to determine the best strains for further development.
- Isolate AS-35 was selected for subsequent mutagenesis with the objective of enhancing its biodegradation capabilities.
- Following treatment with the mutagen formamide, mutants were generated that were capable of growth on 80% olive wastewater which was a much higher concentration than the native strain could tolerate.
- Putative mutants were screened based on their biodegradation potential and mutant DF4 were selected for further characterization.
- Wild type AS-35 and mutant DF4 were compared based on their growth kinetics, cellular morphology and total protein expression profiles. DF4 has a higher tolerance for olive waste and thus has successfully been mutated in an advantageous manner.
- Both wild type and the mutant DF4 were identified as *Bacillus megaterium*.
- The results of analysis of the native and mutant strains suggest that the mutations introduced into DF4 occurred within regulatory pathways involved in olive waste degradation, either in sigma factors which are involved in transcription activation of many

genes or involving a two-component signal transduction system responsible for sensing and responding to stress factors in the environment.

- Differential gene expression comparisons based on their RNA are currently being investigated to further develop this work later.

CHAPTER 5

BIOLOGICAL DEGRADATION STUDIES OF BLACK OLIVE BRINES

5.1 INTRODUCTION

Olive wastewater is a complex waste, as discussed in detail in Chapter 2. Not only does it have a high organic load, but this load is comprised of many simple and complex polyphenolics. These polymeric phenolic compounds, similar in structure to lignin, give this waste its characteristic dark colour and are resistant to biodegradation. The simple monophenols are more easily degraded but have been shown to be highly toxic to many microorganisms and plants (Capasso et al. 1992).

There are a wide variety of organisms that are capable of degrading phenolic compounds and these include bacteria, yeast and fungi. Numerous investigations into the biodegradation of wastewater from the olive mill and table olive industries, using a variety of these organisms and mixed consortia, have been conducted and good degradation results have been achieved (Table 5.1). The objective of these microbial studies is to maximise the rate of removal of these undesirable organic components by the breakdown of polymeric phenolics to monomers which can be mineralised as opposed to the formation of humic substances, which still act as pollutants in aqueous environments. The breakdown of the polymeric fraction leads to the subsequent decolourisation of the waste. A significant correlation between decolourisation and phenol removal has been shown (Sayadi and Ellouz, 1995).

Enzymes play a crucial role in the biodegradation of phenolic wastewaters and the key enzymes involved in phenol degradation are phenol hydroxylase and catechol oxygenase. The phenol is first dihydroxylated to form a catechol derivative and ring fission then occurs. Ring fission can occur through either meta or ortho oxidation. The bacterium *Pseudomonas putida* has been shown to degrade phenol via the meta pathway (Sala-Trepat et al. 1973). Fungi are able to effectively degrade lignin through their ligninolytic enzyme system (laccase, manganese peroxidase and lignin peroxidase), and therefore offer a unique advantage to the biodegradation of the lignin-like compounds present in the waste. The decolourisation mentioned earlier has been linked to the presence of some of these enzymes (Vinciguerra et al. 1995, Martiriani et al. 1996, Perez et al. 1998, Sayadi and Ellouz, 1995).

The research reported in this chapter is focused on the biodegradation of extracted and raw olive fermentation wastewaters with a variety of organisms. A locally isolated bacterium (AS 35, *Bacillus megaterium)* obtained from olive plant evaporation ponds (and which should be acclimatized to this waste) was investigated for its ability to decolourise and detoxify olive wastewater. A well known white rot fungus, *Trametes pubescens,* which has been shown to be an excellent laccase producer (Galhaup *et al,* 2002) and may be able to decolourise this waste was also investigated. In addition, mixed cultures were also investigated as they are used in conventional wastewater treatment and could provide a good comparison for degradation efficiencies. Mixed, acclimatised microbial cultures are commonly used to degrade wastewaters in industrial or municipal applications because it is problematic to maintain pure microbial cultures over long time periods. In mixed cultures a stable consortium of microbial species develops, and sterile operation is not necessary because any organism from the external environment that can out-compete an existing dominant species is advantageous. Conventional large-scale wastewater treatment systems typically operate with low influent COD (< 2g.L^{-1}) and mineral solids content .

The studies were carried out in batch mode and a variety of vessel configurations, namely shake flasks, pneumatic and stirred tank reactors, were investigated in order to determine conditions for achieving the best degradation possible. The waste taken directly from the olive fermentation tanks was diluted, in our experiments. This resulted in effluent samples with concentrations close to those normally entering an evaporation pond after dilution with factory washings. The degradation efficiency was measured in terms of total phenols and COD removal, as well as decolourisation and the evolution of low molecular weight phenolic compounds as determined with size exclusion chromatography.

5.2 MATERIALS AND METHODS

5.2.1 Maintenance of organisms and inoculum preparation

5.2.1.1 Bacillus megaterium AS-35

The organism was maintained at 4° C on Nutrient agar (2 % raw waste) and subcultured every two months. All media was sterilized for 20 minutes at 121°C. The inoculum was prepared in a Nutrient broth (2 % waste) medium and inoculated with a single colony from an agar plate under aseptic conditions. The flask was then incubated at 28°C with orbital shaking at 150 rpm for 24 hours.

5.2.1.2 Trametes pubescens

The fungus was maintained on 3% Malt extract agar at 4°C and subcultured every two months. All media was sterilized for 20 minutes at 121°C. A single agar plate was homogenized in 200ml of sterilized dH_2O with a benchtop homogenizer and 50 mL of this homogenate was used to inoculate 500ml of a *Trametes* defined media (Ryan, 2004) with 2 % raw or extracted waste to act as an inducer.

5.2.1.3 Candida maltosa

Candida was maintained on agar Petri plates containing (in $g.L^{-1}$): glucose 10, peptone 5, yeast extract 3, malt extract 3 and agar 15. Plates were subcultured every two months and stored at 4°C. The inoculum was the same composition as above excluding the agar and including 2% (v/v) wastewater to act as an inducer. Starter cultures (200mL) were grown in 500ml Erlenmeyer shake flasks at 150rpm, 25°C for 24hrs.

5.2.1.4 Aspergillus niger

Aspergillus was maintained on Petri plates containing (in $g.L^{-1}$): malt extract 20, glucose 20, peptone 1 and agar 20. Plates were subcultured every 2 months and stored at 4°C. The inoculum was the same composition as above, excluding the agar and including 2% (v/v) wastewater. Starter cultures (200mL) were grown in 500ml Erlenmeyer shake flasks at 150rpm, 25°C for 48hrs.

5.2.1.5 Mixed cultures

For isolation of mixed cultures samples were taken from three sources: green and black olive brines at the end of olive fermentation, and from an aerated discharge lagoon/evaporation pond system. The three isolates were then enriched on nitrogen ($5g.L^{-1}$ YE) supplemented wastewater (25%), and also on MRS broth (Fluka) with (2% wastewater) to select for *Lactobacilli* and yeasts that predominate in the fermentation brines. Enriched and acclimatised cultures of the different isolates (6 of) were used as inoculum for further degradation studies.

5.2.2 Fermentation conditions (*Bacillus megaterium* AS-35, *T. pubescens*, *C. maltosa*, *A. niger*)

Note: Phenol concentrations were found to vary somewhat between different experiments because different wastewater collections were used. However, in all cases the wastewater referred to is black olive fermentation brine. Dilutions of the wastewater were based on volume

and not phenol concentration, because the phenol concentration varied between different fermentations. However, the salt (NaCl) added to make the brine was consistent at 10% (w/v).

5.2.2.1 Shake flask experiments

Experiments were carried out in 500 ml Erlenmeyer flasks with 250ml of varying concentrations of raw or extracted waste media, with added nitrogen source (Yeast extract and ammonium nitrate, 1:1, to 5 g/L), and inoculated with a 10 % inoculum. All media was sterilised at 121°C for 20 min prior to inoculation. The flasks were incubated at 28°C with orbital shaking at 150 rpm for the duration of the experiment. Samples (5 mL) were taken aseptically for analysis. All experiments were carried out in triplicate.

5.2.2.2 Batch bioreactors

Stirred tank reactor

Batch runs were carried out in a New Brunswick BIOFLO 110 stirred tank reactor with a working volume of 5 L at 28°C. The fermentation media and inoculum size was identical to that used in the shake flask experiments. The agitation was set to 250 rpm and the air flow rate at 1VVM. Samples (20 mL) were drawn aseptically every 24 hours for analysis. The pH was monitored for the duration of the experiment.

Airlift reactor

The reactor is the same as was used in a previous study for the bioremediation of a stripped gas liquor effluent (Ryan, 2004). It has a height of 500mm, an aspect ratio (H/D) of 4.5, internal draught tube diameter of 55mm and working volume of 3.8L. Experiments were conducted at 28°C with a constant air flow rate of 1.5 L/min. The media and inoculum was identical to that used in the stirred tank reactor. Samples (10 mL) were taken aseptically for analysis.

5.2.2.3 Mixed cultures

Mixed culture samples were isolated from different olive-derived sources and then enriched on various substrates. Degradation experiments were all performed in shake flasks (250mL Erlenmeyer) with 100mL medium at 150rpm. All wastewaters had previously been extracted (as described earlier) to recover low M_w phenolics. Initially cultures were inoculated (5mL) into 25% extracted OW15 + 0.5%YE and left to grow for 10 days with daily sampling for analysis (total phenols & COD). The six enriched mixed cultures were compared for their degradative ability and the most effective mixed culture was selected and used for further experiments.

The effect of YE supplementation was investigated by repeating the above experiment with 0 and 0.1% YE to determine if the addition of a nitrogen source has a significant affect on the degradation process.

To investigate inhibition effects increasing concentrations of wastewater (25, 50, 75 and 100%) were used as growth medium for the selected mixed culture. Again daily samples were taken for analysis.

5.2.4 Analytical techniques

5.2.4.1 Post fermentation COD and phenols determinations (mixed cultures)

Chemical precipitation of residual wastewater after fermentation was performed by adding increasing concentrations of lime (CaO, Merck). These were mixed by vortexing, allowed to settle and centrifuged to remove solids. Residual (soluble) COD and phenols were determined, and compared to the same lime treatment performed on wastewater that had not been subject to degradation by the mixed culture.

5.2.4.2 Protein determinations

These were done using the Bradford reagent from Sigma Chemical Co. The procedure is based on the formation of a protein-dye (Brilliant Blue G) complex. This complex causes a shift in the absorption maximum of the dye from 465 to 595nm. The reaction mixture contained 0.05 mL of sample or standard (BSA standards, 0 – 1mg/mL) and 1.5 mL reagent. The reaction mixtures were mixed and left to incubate at room temperature for 20 minutes.

5.2.4.3 Enzyme assays (Laccase assay)

Laccase was assayed using the procedure of Roy-Arcand and Archibald (1991). The reaction mixture contained 2.5 mL 0.1 M sodium acetate buffer (pH 5), 0.33 mL 5 mM ABTS (2,2'-azinobis 3-ethylbenzthiazoline-6-sufonic acid) and 0.17 mL sample. Oxidation of ABTS was measured by determining the increase in absorbance of the mixture at 420nm (ε = 36 000 M^{-1}). One unit (U) of enzyme activity was defined as the amount of enzyme required to oxidize 1 µmol of ABTS per min. The assay was performed on the supernatants of centrifuged samples from the inoculum preparations since laccase is an extracellular enzyme.

5.3 RESULTS AND DISCUSSION

5.3.1 Summary of Results

The studies done on aerobic biodegradation of olive wastewaters show relatively good COD and total phenols removal for most organisms investigated and the results from our study compare favourably with those of other researchers (Table 5.1)

Table 5.1: Summary of organisms and degradation results achieved during this study and from literature

Organism	X_{COD} (%)	X_{TP} (%)	Max deg rate (g/L.day^{-1})	μ_{max} (hr^{-1})	Substrate	Configuration	Reference
RU-LV1*			0.147				
AS 1*	nd	56	0.32 (TP)	nd	Brine	Shake flask	
AS 3*	nd	64	0.36 (TP)	nd	Brine	Shake flask	
AS 9*	40	63	0.3 (TP)	nd	Brine	Shake flask	
*B. megaterium**	40	56	0.23 (TP)	0.161	Brine	Shake flask	
*C. maltosa**			0.279		Brine	Shake flask	
C.tropicalis (immobilized)	69.7	36.5	-	0.35	OMW	Fermenter	Ettayebi *et al.* (2003)
L. plantarum	55	46			OMW	Flask (anaerobic)	Ayed and Hamdi (2003)
L. edodes	67	88	-	0.05	OMW	Shake flask	D' Annibale *et al.* (2004)
*A. niger**			0.149 (TP)		Brine	Shake flask	
A. terreus	65.77	-	3.02 (COD)	0.062	OMW (20%)	Fermenter	Garrido Hoyos *et al.* (2002)
*T. pubescens**	46	79		0.0317	Brine	Fermenter	
Mixed cultures*			0.19 (TP) 0.093 (TP)		Brine	Shake flask	
Yarrowia lipolytica	80	-	4.32 (COD) -	-	OMW	Fermenter	Scioli and

	75	92		0.06	OMW	Fermenter	Vollaro (1997)
Ph. Chrysosporium			0.6 (TP)				Garcia Garcia *et al.* (2000)
	-	-		0.051	Phenol	Serum bottles	Abuhamed *et al.* (2004)
P. putida							Martirani *et al.* (1996)
	nd	90	0.065 (phenol)	-	OMW (10%)	Shake flask	Assas *et al.* (2002)
Pleurotus ostreatus	65	nd	-	-	OMW (fresh)	Bioreactor	Robles *et al.* (2000)
Geotrichum candidum	75	68	-		OMW	Shake flask	
Penicillium							

* This study

nd = not determined

OMW = Olive mill wastewater

X_{COD} = Total removal of COD (%)

X_{TP} = Total removal of Total phenols (%)

The results reported by others are predominantly aerobic biodegradation investigations with the exception of that for *Lactobacillus plantarum* (Ayed and Hamdi, 2003). These researchers found that this organism, a facultative anaerobe, was able to decolourise the waste under anaerobic conditions, and that this was improved by the low pH achieved at the end of the fermentation. The depolymerisation was attributed to the action of the tannase enzyme but no enzyme identification was reported. An investigation was done using this organism in anaerobic conditions in our lab but the results do not compare favourably with those achieved by Ayed and Hamdi (2003). The organism was unable to grow on the attempted dilution of olive brine wastewater and as a consequence there was no phenol or COD removal.

As reported by Ayed and Hamdi, decolourisation seems to be pH dependent and this has been highlighted by other researchers. Qin et al. (1997) noticed that the colour of green tea catechins changed from light to dark brown with an increase in pH. Assas et al. (2002) state that the consumption of organic acids by *G. candidum* in stored olive mill waste induced an increase in pH and led to amplified autooxidation of phenolic compounds with higher molecular weight and resistance to biodegradation. The same was obtained by Field and Lettinga (1991) with *A. niger* on spruce bark extracts, when tannins were autooxidised and polymerised at pH in excess of 6 to give recalcitrant, dark polymers. It has also been shown that simple phenolic compounds

polymerise during storage of olive mill wastewater and assume high molecular weight (Hamdi, 1992).

From the studies conducted in our laboratory on bacterial strains RU-LV1, AS 1, AS 3, AS 9, *B. megaterium* AS-35 and yeast (*Candida maltosa*), the waste consistently goes darker after fermentation which would indicate polymerisation, and the pH profiles of fermentations with the *B. megaterium* AS-35 show a rapid increase in pH from approximately 4.6 to 9 (Fig. 5.4). *Candida* showed a high rate of phenol degradation and also grows quickly with the fermentation being complete after 3 days. However, studies conducted with the fungus *T. pubescens* showed that this culture maintained fairly constant pH values, below 6, throughout fermentation, and subsequently achieved good decolourisation. This result compares favourably with that achieved by Ayed and Hamdi (2003) and other researchers. Assas et al. (2002) achieved good decolourisation on fresh olive mill wastewater at pH between 4 and 5. The lower pH values have been reported to be optimal for enzymic decolourisation (Kim and Shoda, 1999). The involvement of the ligninolytic enzymes in decolourisation has been proposed by many researchers (Assas et al. 2002, Sayadi and Ellouz, 1992, Aggelis et al. 2003, Jaouani et al. 2003, D'Annibale et al. 1998) but the extent to which decolourisation can be attributed to these enzymes, however, is a contentious issue among researchers. Dias et al. (2004) reported a significant correlation between laccase production and decolourisation, an encouraging observation for further studies with *T. pubescens*.

5.3.2 Bioremediation using *Bacillus megaterium* AS-35

5.3.2.1 Initial degradation study

Results from the initial shake flask experiments (at 250mL scale) using a 25% dilution of the brine wastewater showed a 56% reduction of phenols (Fig. 5.1) occurring predominantly in the first 48 hours. The maximum substrate utilization rate (dS/dt), with respect to total phenols, was determined to be 0.23 $g.L^{-1}$/day. The HPLC data showed two predominant peaks (Fig.5.2 a), one indicating hydroxytyrosol (second peak) and the other tyrosol. These two compounds are significant in the aromatic fraction of the waste at the start of the fermentation. The hydroxytyrosol peak disappeared over the course of the fermentation as it is readily utilized by the organism but the tyrosol peak was still visible after 72 hours (Fig. 5.2 b). The pH of this system (Fig. 5.2) showed a rapid increase from 4.6 to 8.8 within 54 hours and this is thought to be a direct result of the organism metabolizing organic acids, of which lactic acid, acetic acid and elanolic acid are the most abundant (chapter 2).

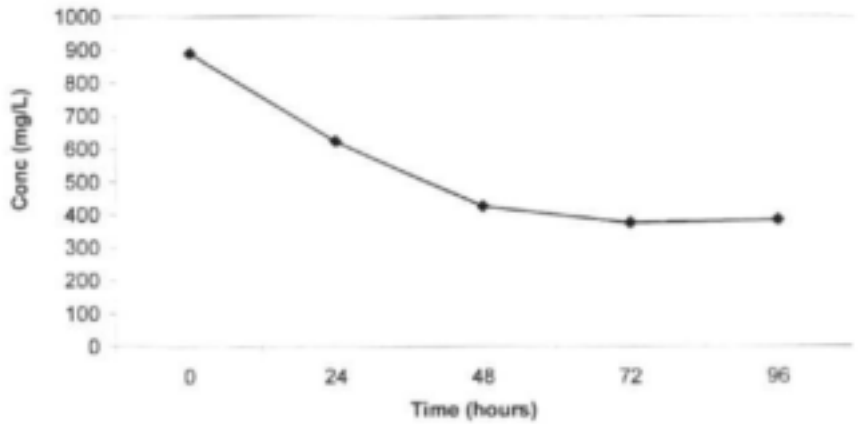


Figure 5.1: Total phenols removal of *B. megaterium* AS-35 in 25% raw waste.

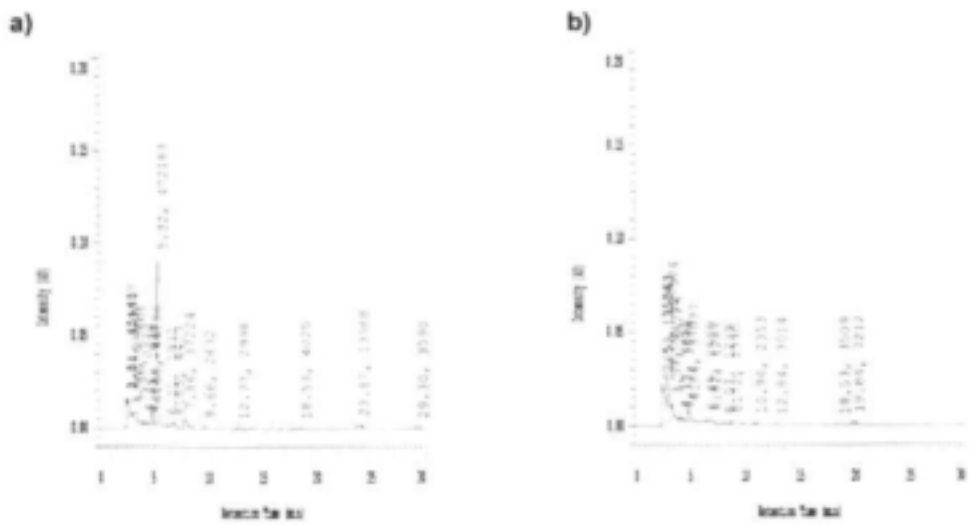


Figure 5.2: HPLC chromatograms at a) t = 0 and b) t = 72 hours of fermentation of 25% raw waste with *B. megaterium* AS-35.

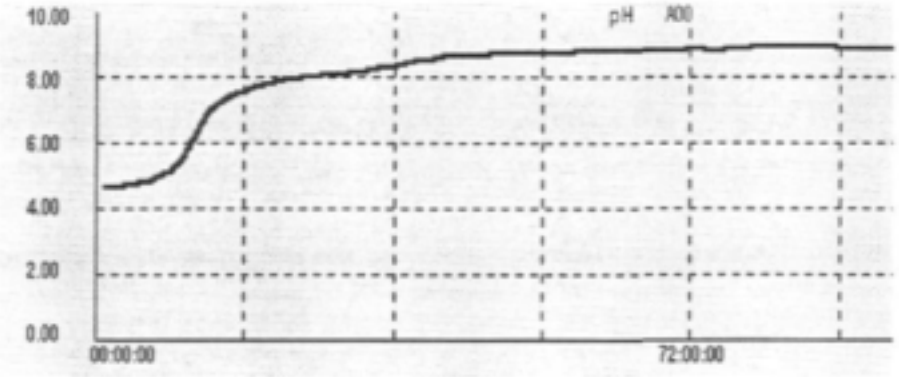


Figure 5.3: pH profiles from the fermentation of *B. megaterium* AS-35 in 25% raw waste.

5.3.2.2 Growth and degradation kinetics

In order to better understand the degradation capabilities of *B. megaterium* AS-35, a separate set of experiments were conducted in the waste to correlate growth and phenol removal. The maximum specific growth rates (μ_{max}) on 10% raw waste were determined as 0.161, 0.102 and 0.123 hr^{-1} for the shake flask, airlift and stirred tank reactors respectively, with a significantly longer lag phase observed for the stirred tank reactor. In terms of bacterial growth on alternative carbon sources these values compare favourably with those achieved by Abuhamed et al. (2004) in which they grew *Pseudomonas putida* on phenol (50 mg/L) as a single substrate. Unfortunately the degradation results obtained much earlier proved difficult to reproduce and in a 10% dilution of the waste, a 45% reduction of the total phenolic fraction was achieved in shake flask culture (Fig. 5.4) and 25% in the airlift and stirred tank reactors (Figs. 5.5 and 5.6). The maximum specific degradation rates, in terms of total phenols, (dS/dt) were 0.096, 0.051 and 0.112 $g.L^{-1}$/ day for the shake flask, airlift and stirred tank reactor respectively. Concomitant COD reductions of 32 (1.8 – 1.2 g/L) and 47% (2.1 – 1.1 g/L) for the airlift reactor and shake flask are fairly good and show reasonable detoxification of the waste. The reduced biodegradation efficiency is not fully understood.

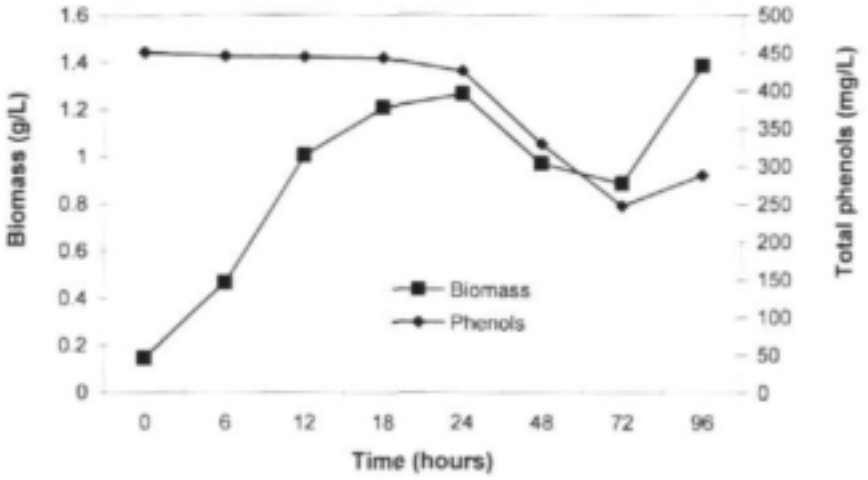


Figure 5.4: Total phenols and biomass determinations of *B. megaterium* AS-35 fermented in 10% raw waste in shake flask culture.

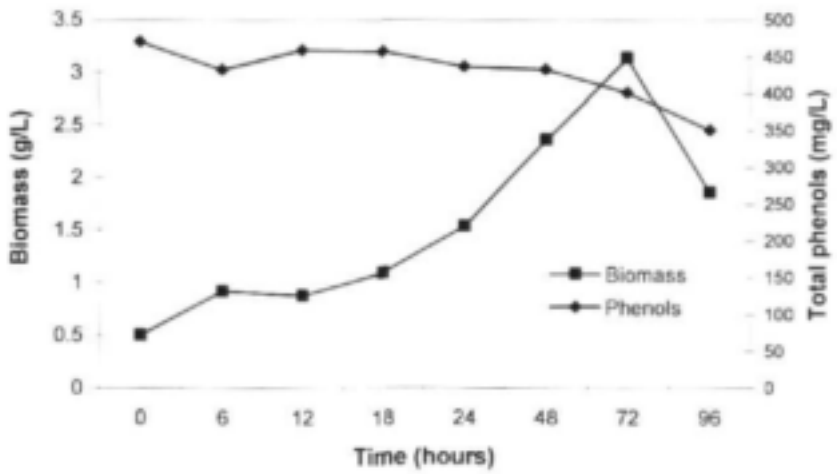


Figure 5.5: Biomass and total phenols of strain AS-35 fermented in 10% waste in the airlift reactor.

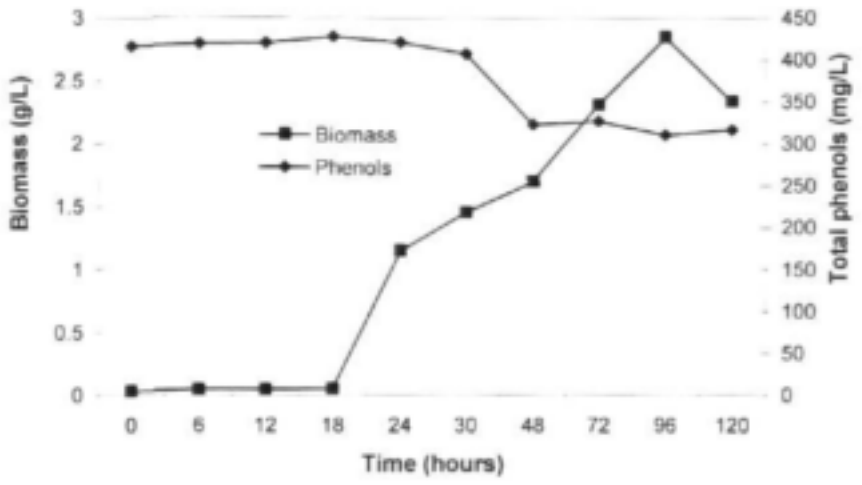


Figure 5.6: Biomass and total phenols of strain AS-35 fermented in 10% waste in the stirred tank reactor.

The molecular weight distribution of the phenolic compounds was assessed using size exclusion chromatography in order to determine their fate after fermentation and the data (Figs. 5.7 and 5.8) show a definite increase in the larger polymeric fraction as well as an increase in the smaller phenolic fraction. This phenomenon is followed by a significant increase in colour (Fig. 5.9). The formation of larger humic, and other polymeric, substances is a common occurrence in aerobic fermentation (as discussed earlier). This is usually coupled to increasing pH, which promotes autooxidation, and this was observed for these fermentations.

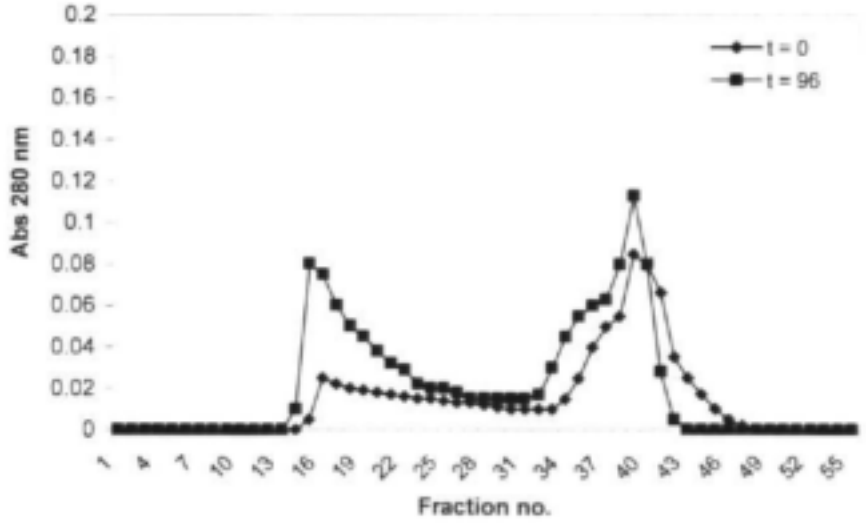


Figure 5.7: Molecular weight distribution profiles of the aromatic compounds before and after the fermentation of 10% waste in shake flask cultures with strain AS-35.

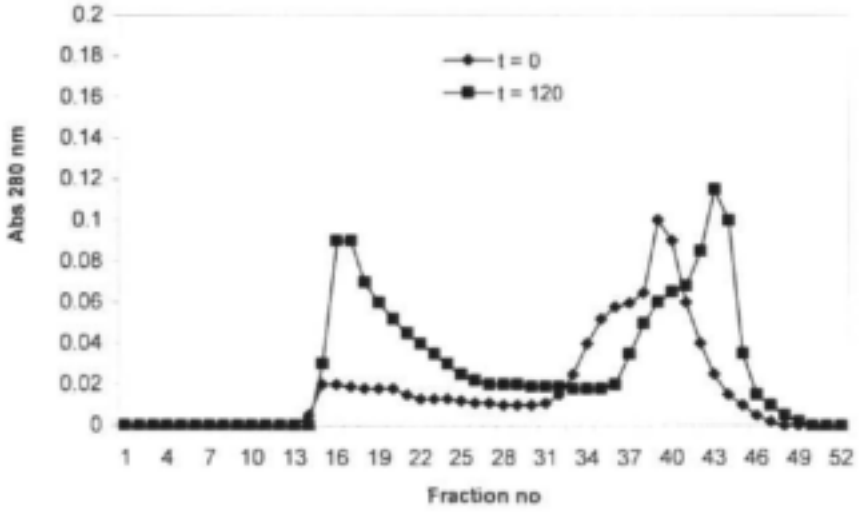


Figure 5.8: Molecular weight distribution profiles of the aromatic compounds before and after the fermentation of 10% waste in airlift cultures with *B. megaterium* AS-35.

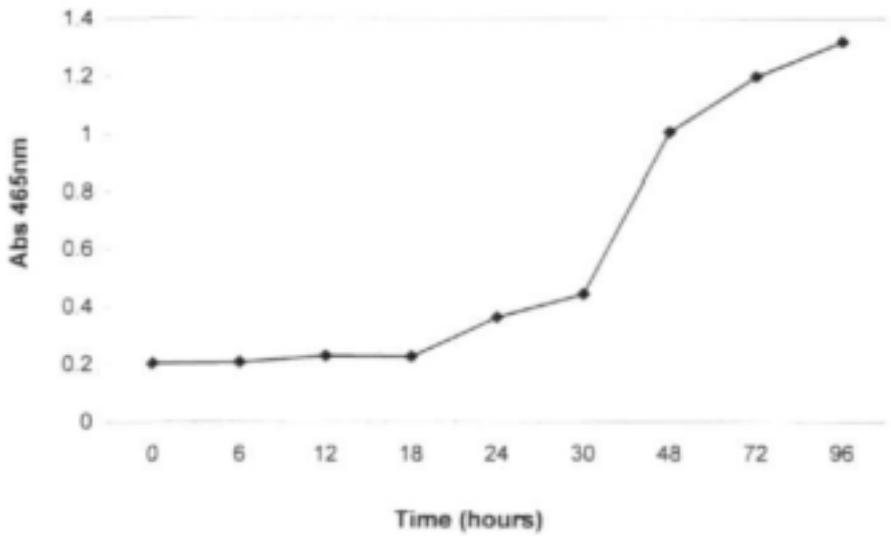


Figure 5.9: Colour evolution during the fermentation of 25% raw waste with *B. megaterium* AS-35.

5.3.3 Growth and degradation kinetics of *Trametes pubescens*

The results show a decrease of total phenols in excess of 70% (figures 5.10, 5.11 and 5.12) with the use of this fungus in the three different configurations used and the growth achieved indicates that this organism is not inhibited at this concentration. The maximum specific growth rates (μ_{max}) for the fungus are 0.0492 h^{-1}, 0.0429 h^{-1} and 0.0317 h^{-1} for the shake flask, airlift and stirred tank reactors respectively which are very similar to those obtained by Garcia Garcia et al (1997) for *Aspergillus terreus* (0.06 hr^{-1}) and *Geotrichum candidum* (0.047 hr^{-1}) used in the biodegradation of vinasse. The maximum degradation rates, with respect to total phenols, are 0.098, 0.085 and 0.191 $g.L^{-1}$/day for the shake flask, airlift and stirred tank reactors respectively. These determinations indicate that the shake flasks produced better growth results and phenols biodegradation compared to that of the airlift and stirred tank reactors, which is surprising. It would be expected that the best results, especially for a fungus, would have come from the airlift reactor as the oxygen mass transfer is more efficient compared to the shake flask.

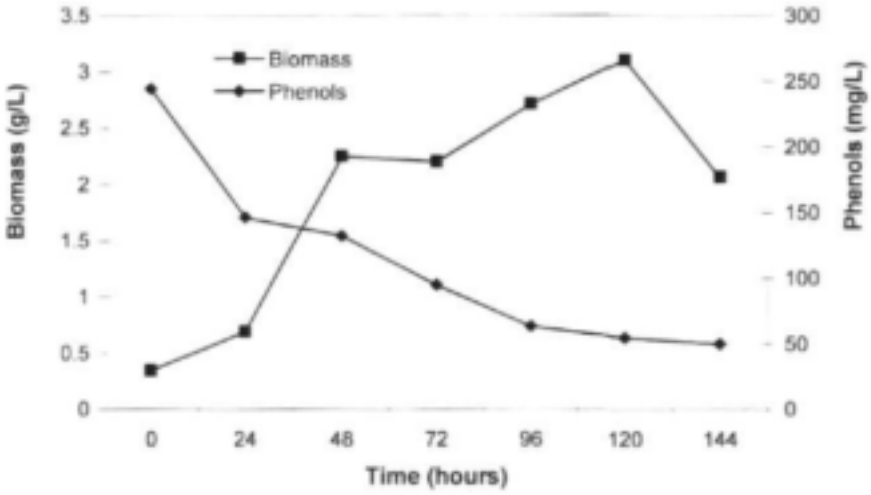


Figure 5.10: Biomass and total phenols determinations of *T. pubescens* fermented in 10% waste in shake flask culture.

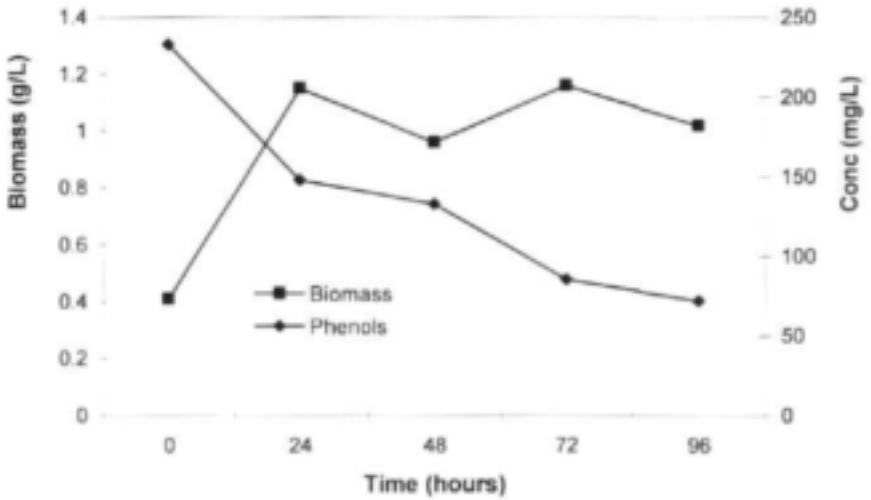


Figure 5.11: Biomass and total phenols determinations of *T. pubescens* fermented in 10% waste in airlift reactor.

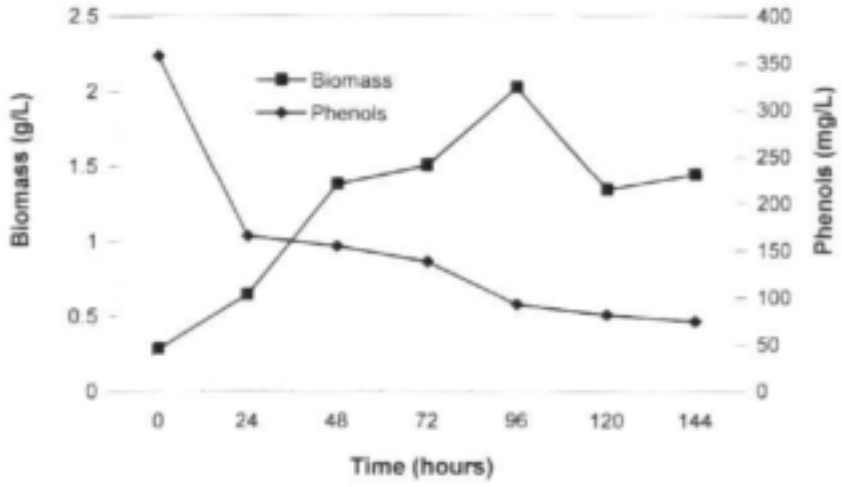


Figure 5.12: Biomass and total phenols determinations of *T. pubescens* fermented in 10% waste in the CSTR.

In contrast to that of the bacterial fermentation, the results from the colour analysis of *Trametes* reactions (Fig. 5.13) showed a decrease in colour of up to 42%. The molecular weight distribution profiles (Figures 5.14, 5.15 and 5.16) from these experiments also indicate an increase in the larger polymeric fraction, towards the formation of humic acid type compounds as seen for the *Bacillus*, but the smaller fraction is consistently reduced, unlike that for the *Bacillus* experiments. The humic substances formed are likely to be at a high enough concentration to contribute substantially to colour formation but compounds such as condensed tannins and other lignin-like compounds, which are slightly smaller and wolud contribute largely to the dark colour were reduced, which resulted in the decrease in colour. A possible explanation for this occurrence is the action of the laccase enzyme as it is a lignin degrading enzyme. *T. pubescens* produces high titres (3 U/mL) after four days of culture of the experimental inoculum. The fact that the pH remains low throughout fermentation creating a favourable environment for enzymic decolourisation (Kim and Shoda, 1999) makes this a feasible hypothesis. *T. pubescens* has been employed in previous bioremediation investigations and showed effective removals of phenol, *p*-cresol, *m*-cresol and *o*-cresol (WRC, 2003). Unfortunately no laccase activity was detected and this could be due to the adsorption of the remaining polymers to the enzymes as has been reported previously (Field and Lettinga, 1991). The laccase enzyme is frequently used in the paper and pulp industry as it is a very effective bleaching agent. Laccases are essential to the breakdown of lignin and thus it would make good sense to utilize this ligninolytic system since the compounds present in the waste are lignin like in structure.

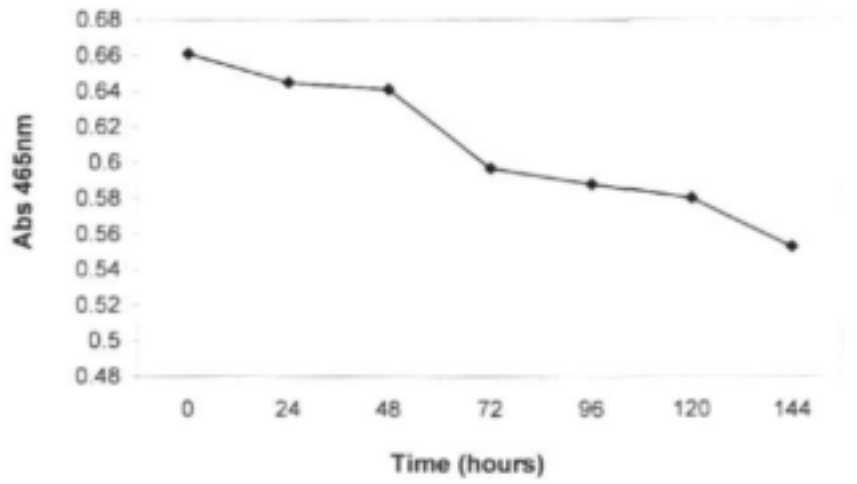


Figure 5.13: Monitoring of the colour evolution in the fermentation by *T. pubescens* of 10% raw waste.

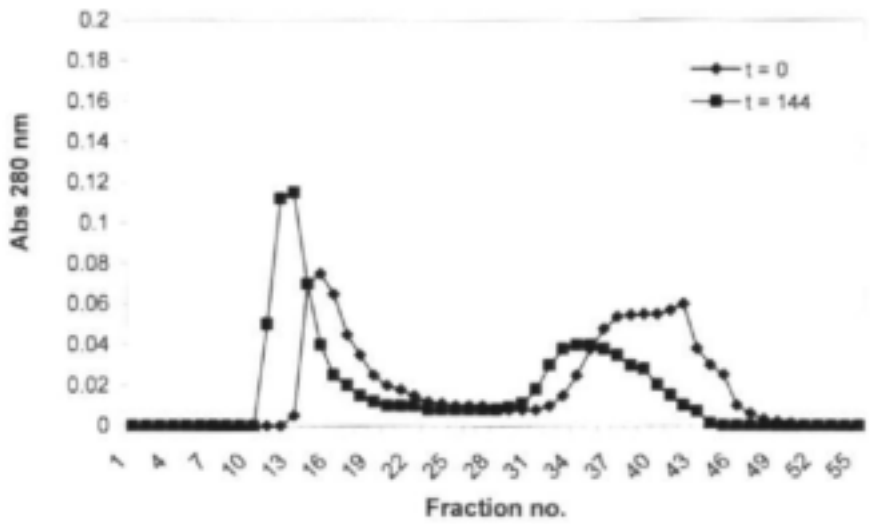


Figure 5.14: Molecular weight distribution profiles of the aromatic compounds before and after fermentation in shake flask culture with *T. pubescens*.

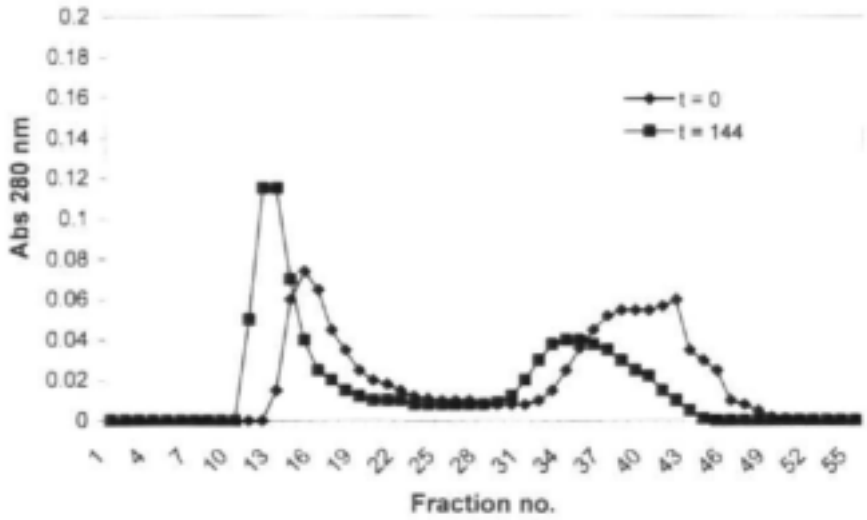


Figure 5.15: Molecular weight distribution profiles of the aromatic compounds before and after fermentation in airlift culture with *T. pubescens*.

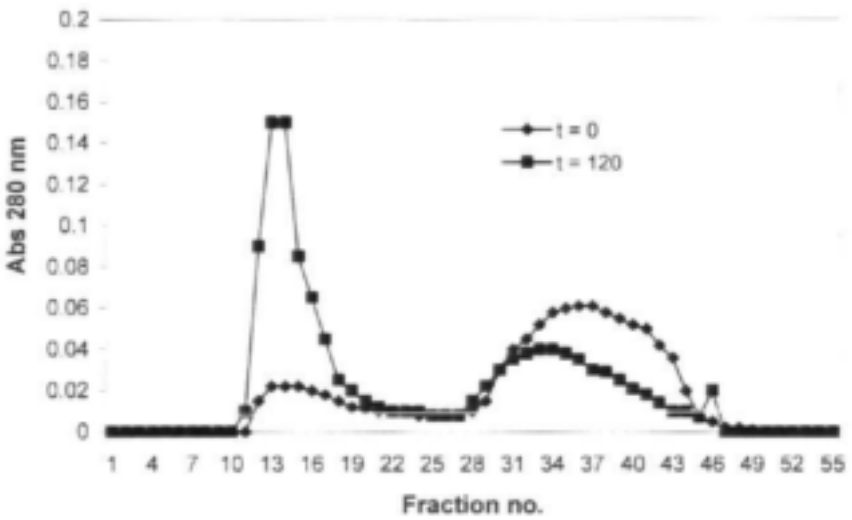


Figure 5.16: Molecular weight distribution profiles of the aromatic compounds before and after fermentation in stirred tank culture with *T. pubescens*.

Extracted waste degradation studies – impact of the additional nitrogen source

Extraction of the waste with ethyl acetate removes the smaller monomeric phenols, of which Hydroxytyrosol is the most abundant. As discussed in previous chapters, this compound is a valuable antioxidant and its recovery prior to degradation of the waste would be of economic benefit. Therefore, the biodegradability of the higher M_w polymeric phenolics remaining after extraction was investigated. Two experiments were conducted with a 25% dilution of this waste, one supplemented with an additional nitrogen source (yeast extract) and one without. The total phenols removal for this experiment (Fig. 5.17) indicates a similar overall degradation trend for both and more than 50% total phenols reduction. The maximum degradation rates ($g.L^{-1}$/day), 0.23 and 0.25 for supplemented and non-supplemented media respectively, are almost identical. This data shows that the larger polyphenolic fraction can still be biodegraded to a certain extent and that the added nitrogen source is not necessary to achieve satisfactory total phenols removal with *T. pubescens* in an extracted waste medium. The fact that nitrogen does not need to be added has an important economic impact as it would reduce costs of a potential future treatment process.

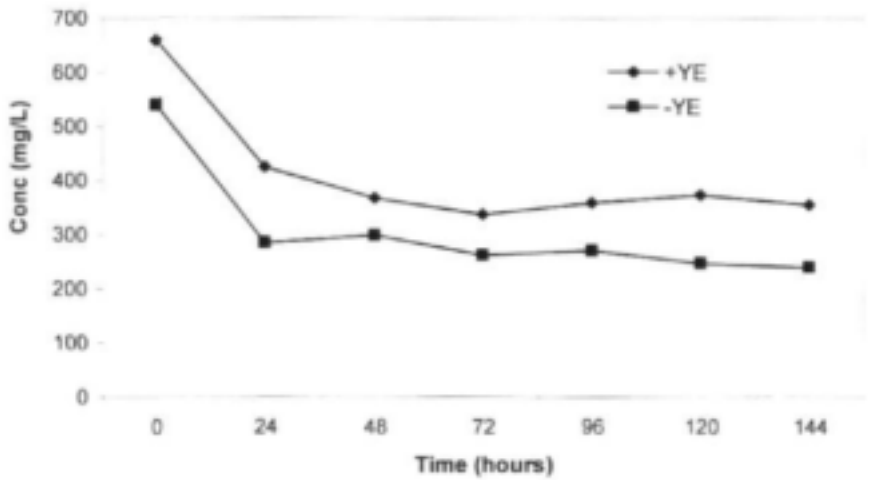


Figure 5.17: Total phenols removal of 25% extracted waste with and without additional nitrogen with *T. pubescens*.

5.3.4 Mixed culture degradation experiments

The aim of this work was to develop and acclimatize a mixed culture capable of aerobically degrading (predominantly) extracted wastewater i.e. mainly high M_w polyphenolics and tannins. The mixed culture that gave the best degradation results was subject to further experiments to determine degradation rates and inhibition effects when exposed to increasing concentrations of wastewater. Figure 5.18 shows the initial (control) and final phenol concentrations (after 240 hrs. of fermentation) of samples isolated from various sources and enriched on either MRS broth or wastewater (OW). Figure 5.19 shows COD for the same. The sample taken from the evaporation pond and enriched on wastewater-based medium (OW-EVAP) showed the best degradation in terms of total phenol and COD removal and was thus analysed in more detail.

The OW-EVAP culture achieved results of 52% total phenols removal and 79% COD removal at initial concentrations of 430mg.L^{-1} and 17.3g.L^{-1} respectively. All the cultures resulted in a darkening of the wastewater medium due to the formation of high M_w humic substances. Figure 5.20 shows the time course of total phenol and COD removal for the OW-EVAP mixed culture.

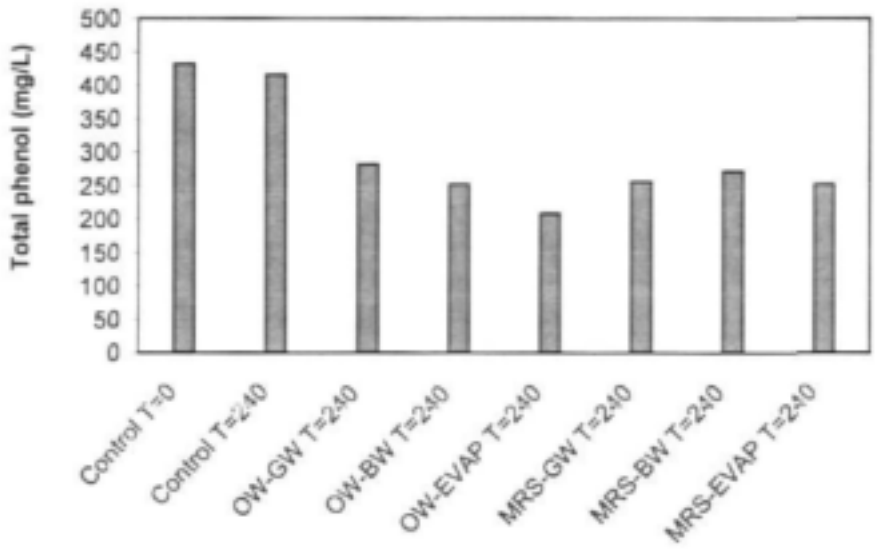


Figure 5.18: Phenol degradation of 25% OW by different enrichment cultures. OW-GW, OW-BW and OW-EVAP refer to cultures enriched in OW and isolated from green olives, black olives and evaporation pond respectively. Similarly for cultures enriched in MRS broth.

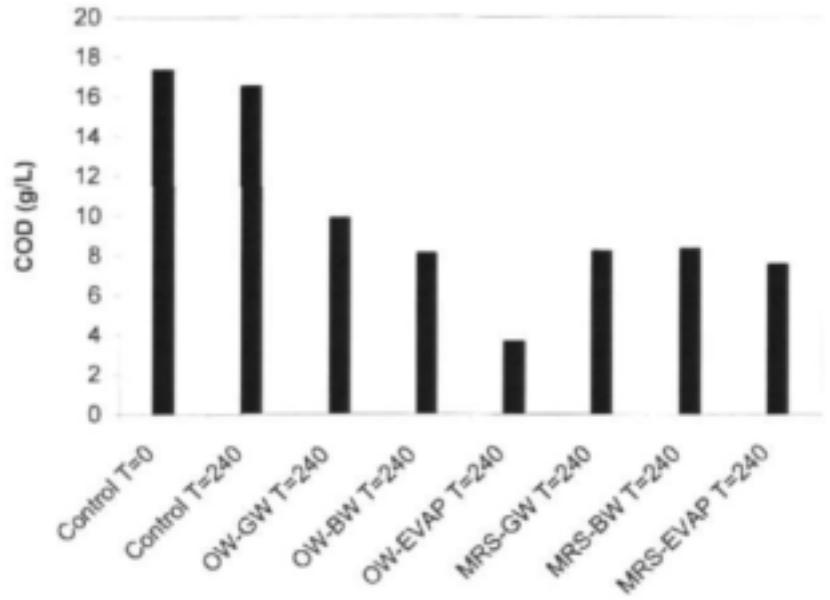


Figure 5.19: COD removal from 25% OW by different enrichment cultures. OW-GW, OW-BW and OW-EVAP refer to cultures enriched in OW and isolated from green olives, black olives and evaporation pond respectively.

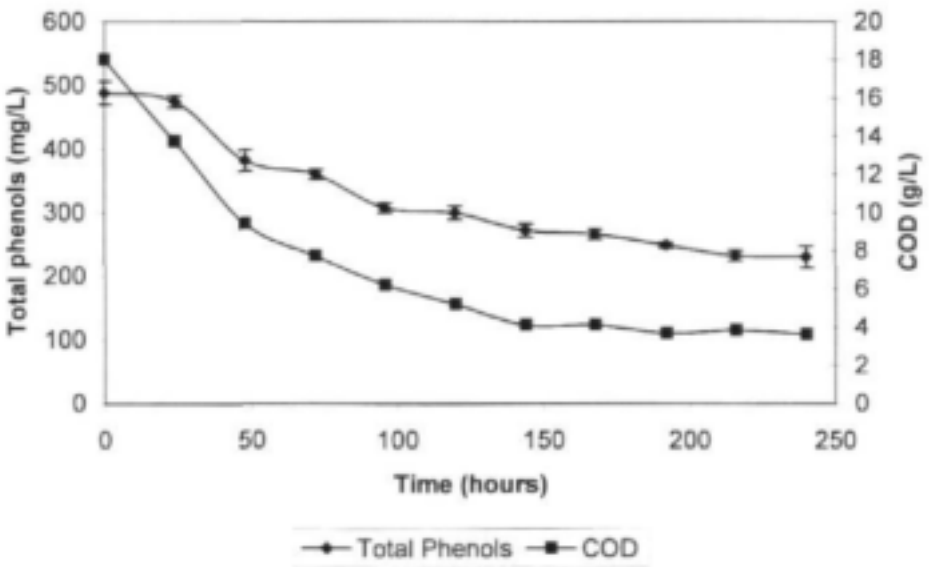


Figure 5.20: Reduction over time of total phenols and COD for the OW-EVAP mixed culture.

From the above it can be seen that COD remained reasonably constant after 150hrs. Maximum and average COD removal rates were 4.32 and 1.44g.L^{-1}.day^{-1} respectively, while for total phenols these values were 93 and 26mg. L^{-1}.day^{-1}. The reduction in COD is very good for the mixed culture compared to pure cultures studied above and that of other researchers. However, the residual phenol value (230mg.L^{-1}) remains high, which is not ideal. Next, the effect of yeast extract as supplementary nitrogen source was investigated. Ideally one would not want to use any additives (or pH correction) for degradation purposes, as this increases cost of treatment. However, the wastewater is known to be deficient in nitrogen, for ideal degradation purposes (ideally COD:N:P = 100:5:1). Total nitrogen in 25% wastewater was 34mg/L which gives COD:N of 100:0.2. 0 and 0.1% YE supplementation was used in experiments repeated as above. Results for these experiments were nearly identical (data not shown), indicating that nitrogen supplementation is not necessary for the mixed culture.

The effect of increasing wastewater concentration was investigated. Again the high M_w extracted waste was used, made up at concentrations of 25, 50, 75 and 100%, in duplicate, no yeast extract. Reduction in total phenol concentrations are shown in figure 4.21. Based on percentage, the 25% wastewater showed the most phenol removal, however in terms of degradation rate (mg.L^{-1}.day^{-1}) the 50 and 75% OW concentrations showed the best results. Average values of 26 and 33 mg.L^{-1}.day^{-1} phenol removal were measured for the 50 and 75% concentrations respectively, which was better than the 25% concentration. The 100% concentration was clearly inhibitory, as only marginal degradation was observed.

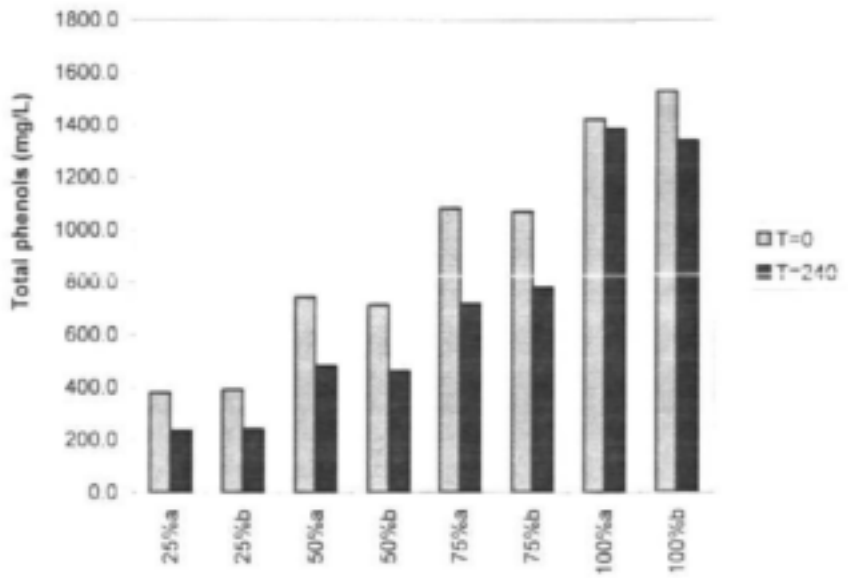


Figure 5.21: Total phenol concentrations before and after fermentation for increasing concentration of wastewater

The pH in the above experiment increased from around 5 initially to around 9 for all concentrations. All flasks also showed an increase in colour, indicating the formation of higher MW polyphenolics (humic acids) through polymerisation.

An advantage of the formation of such compounds is that they are readily removed through precipitation. A precipitation experiment was thus performed on the 50% wastewater sample after mixed culture fermentation, and also on raw 50% wastewater for comparison. CaO was used for precipitation at concentrations of 0, 25, 50, 100 and 200g/L. Samples were vortexed, allowed to settle and then centrifuged. Results are shown in figures 5.22 a-c.

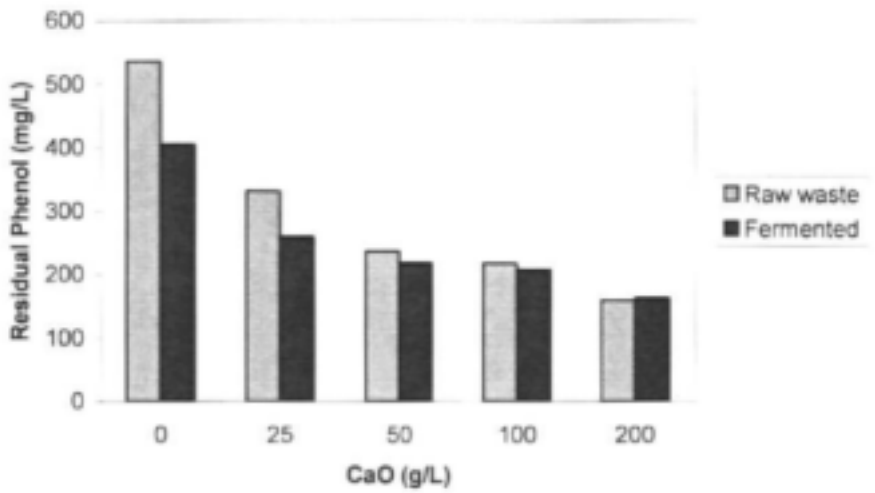


Figure 5.22a: Effect of increasing concentration of CaO on residual total phenol

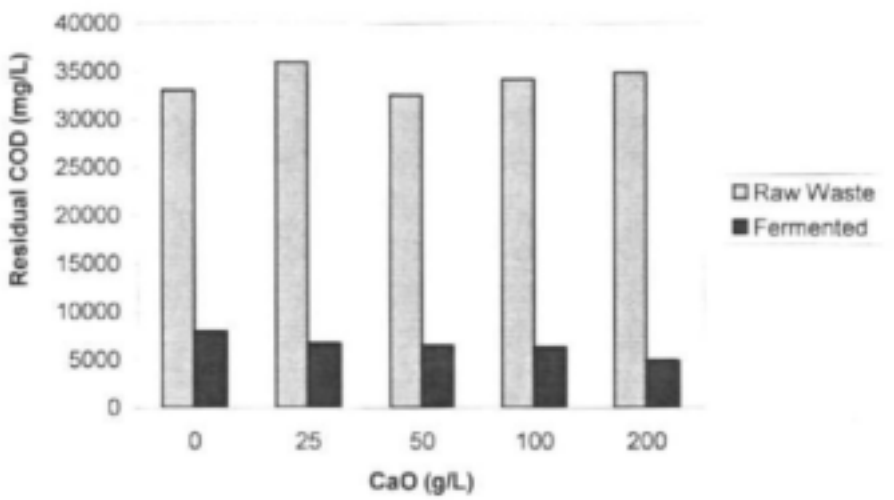


Figure 5.22b: Effect of increasing concentration of CaO on residual COD

Precipitation with CaO

Precipitation of raw waste with CaO had a significant effect in terms of phenol removal: more than 50% reduction using 5% (w/v) CaO. However, the residual phenol concentration in the fermented waste was only marginally better than in the raw waste, indicating that if this approach was to be used for phenol removal, mixed culture fermentation is essentially unnecessary. The mixed culture fermentation did remove significant amount of other organics, however, which significantly reduced the organic load in terms of COD. The precipitation, even at high CaO concentrations, had little effect on reducing residual COD of unfermented wastewater, as can be seen from Figure 6b. The polyphenolics precipitated reduced the colour of the wastewater (as measured by absorbance at 550nm) as shown in figure 5.22c.

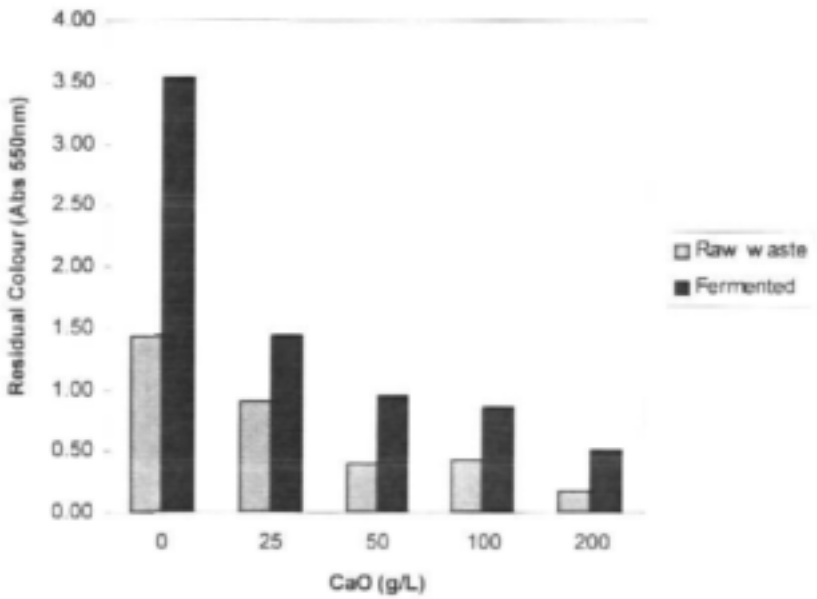


Figure 5.22c: Effect of increasing CaO concentration on residual colour

In all cases tested using mixed cultures, the final colour was darker in the fermented waste than the raw waste, indicating the polymerisation that takes place in mixed culture aerobic fermentations. Colour was significantly reduced with increasing concentrations of CaO.

There are several drawbacks to using precipitation as a treatment method for olive wastewaters, irrespective of whether the waste is fermented first or not. Firstly, at a nominal dose of say 50g/L, several hundred kilograms of CaO would be required for annual quantities of wastewater produced, which will have a negative impact on the cost of treatment. Secondly, the precipitate

itself will require subsequent disposal (possibly composting), which is an additional treatment process that is undesirable. Thirdly, adding 25g/L or more CaO immediately causes the pH of the treated water to rise to ~12, which is problematic in terms of subsequent disposal or reuse. Any type of lime-based treatment will have similar impact, and as such the method is perceived to be unsuitable for olive wastewater treatment.

Molecular weight distribution determination

Size exclusion chromatography molecular weight distributions of the fermented waste confirmed the formation of high molecular weight products (see Figure 5.23), which is an undesirable consequence and indicates that mixed culture fermentation of the waste is possibly not the best option in terms of biological treatment. Depolymerisation and subsequent mineralisation of the waste is a much more desirable outcome.

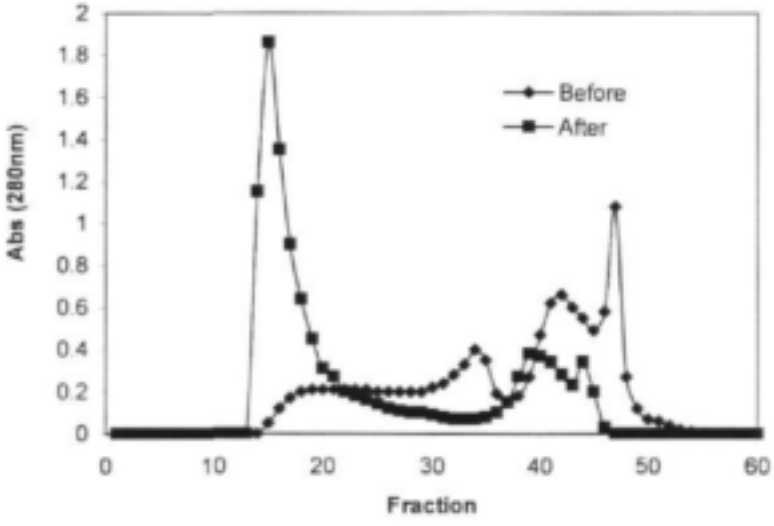


Figure 5.23: Molecular weight distribution of phenolics before and after fermentation with mixed microbial culture

These studies have tested the capabilities of a bacterium, fungus and mixed cultures for their ability to bioremediate black olive wastewater, measured in total phenols and COD removal. The results achieved showed good reductions of total phenols, the toxic fraction of this waste. The bacterium *B. megaterium* reduced this parameter from 849 mg/L to 383 mg/L in a 25% dilution of the waste, and from 426 mg/L to 247 mg/L in a 10% dilution. *T. pubescens* reduced total phenols

from 357 mg/L to 74 mg/L in a 10% dilution. The COD removal for both of these organisms was approximately 50%, from 2g/L to 1g/L in a 10% dilution. The mixed cultures reduced the total phenols from approximately 480 mg/L to 230 mg/L and achieved the best COD removal of 17.3 g/L to 3.6 g/L. This data illustrates that the fungus achieves the best total phenols removal but at a high dilution. The mixed cultures and *B. megaterium* achieve relatively similar total phenols removal but the mixed cultures have an excellent COD reducing capacity. *T. pubescens* is the only organism to reduce the colour, however, which is a very desirable result.

5.4 CONCLUSIONS

- Black olive brine wastewater is a high strength phenolic waste and quite resistant to degradation due to high phenolic content and high COD. However, a variety of microorganisms including yeast, fungi, bacteria and mixed cultures are able to biodegrade this waste. The degradation results achieved in this study, in terms of COD and Total phenols, show that *T. pubescens* is the most efficient at phenols removal. Nevertheless, at a high dilution mixed cultures achieved excellent COD removal.
- Other components of the waste that contribute to the COD, e.g organic acids, were readily consumed.
- Despite the reduction in total phenols content, formation of high molecular weight humic substances and subsequent darkening of the waste was a common trend for all the organisms besides *T. pubescens*. These compounds are more resistant to biodegradation.
- Increased pH promotes autooxidation of phenolics to polymeric substances.
- *T. pubescens* was the only organism capable of decolourising the waste. Coupled with an excellent removal of phenols and ability to produce extremely high titres of laccase in starter culture, this organism could potentially be used in a treatment process. Relatively high dilution of the waste is required, however, as the fungus seems to be inhibited at higher concentrations.
- *T. pubescens* was also able to efficiently remove phenols from extracted waste and an additional nitrogen source was not required.

CHAPTER 6

DEVELOPMENT OF A GENERIC BIOREACTOR FOR TREATMENT OF HIGH-STRENGTH WASTEWATERS

6.1 INTRODUCTION

Biological degradation has been shown to be a cost-effective and environmentally friendly method of wastewater treatment. However, conventional wastewater treatment plants have several disadvantages such as large surface area requirements, low influent concentrations (dilution requirements) and transport to remote locations due to offensive odours. Conventional treatment systems would include technologies like activated sludge sequencing batch reactor type systems. Thus there is a need for small-scale intensive bioreactor systems that can be operated as stand-alone "end of pipe" treatment systems, before suitable discharge of effluent.

There are several promising technological options for such small-scale systems, including airlift reactors, jet loop reactors, packed or fluidized beds, and increasingly, membrane bioreactors. Airlift devices operate on the principle of mixing and mass transfer due to an introduced gaseous phase, e.g. bubble columns and draught tube devices. Convective (even turbulent) mass transfer regimes are possible within such reactors with minimum associated shear stress. These are good conditions for optimal microbial growth and metabolism. Other advantages of airlift devices include their simplicity (no moving parts) and low energy requirements (compared to mechanical agitation). The versatility and efficacy of airlift devices has been extensively studied and demonstrated (Chisti 1989 & 1992, Chisti and Moo-Young 1987 & 1993, Jin and Lant 2004, Quan et al. 2003, Couvert et al. 2004).

Membrane bioreactors have recently been receiving considerable attention as a promising technology for both small- and large-scale wastewater treatment. There are several well-established commercially available systems e.g. Liqui-cel®, Kubota® and Zenon®. These reactors incorporate a membrane separation system which can be used for biodegradation for or withdrawal of product from the reactor. This also allows for recycle of biomass, effectively separating hydraulic and solids residence times. A membrane separation unit can be located externally to the reactor, or preferably, submerged within the reactor vessel, which negates the need for a recirculation pump as the product water is withdrawn by suction. Submerged membrane bioreactors (sMBR's) rely on sparged air bubbles to provide a scouring effect that helps to keep the external surfaces of the membranes clean, as well as providing the oxygen

necessary for microbial metabolism. Such systems are capable of producing high quality treated effluent with minimal sludge discharge in a single unit operation, resulting in small plant footprint and high volumetric productivity. sMBR's are also more energy efficient than conventional treatment systems (Stephenson et al. 2000, van der Roest et al. 2002). Publications proliferate in the recent literature regarding the development and application of sMBR's, which could now reasonably be called an established technology (Holler and Trosch 2001, Dhouib et al. 2005, Wen et al. 2004, Schoeberl et al. 2005, Katayon et al. 2004, Konovalova et al. 2003, Cote et al. 1997)

Hydrodynamics within an sMBR have an important effect on membrane performance, as the gas-induced liquid velocity past the membrane surface directly affects concentration boundary layers and thus membrane fouling. High crossflow velocities are desirable as they reduce the phenomena known as concentration polarization (of suspended solids), which results in cake layer formation and concomitant reduction in membrane flux. Hydrodynamics, aeration effects and other parameters like solids concentration, hydraulic residence time and suction pressure affect operational performance of the sMBR. These have been extensively studied (Fane et al. 2002, Sofia et al. 2004, Liu et al. 2000 & 2003, Ueda et al. 1997, Gui et al. 2002, Le-Clech et al. 2003). Molecular weight cut-off (MWCO) of the membranes used in a sMBR depend on the application. For high quality treated domestic wastewater effluents where water reuse is required, pore size of 0.2 μm are common, while for a high strength wastewater where a single biological unit operation is unlikely to capable of complete treatment (as is the case for olive wastewaters), larger pore size membranes can be used. This improves permeate flux and thus reduces required membrane surface area and cost.

In this work a microfiltration ceramic membrane bioreactor was designed for treatment of high strength wastewaters, using olive wastewaters as a model agro-industrial polluting effluent. The membrane used was kindly donated by V. Linkov, University of the Western Cape. The ceramic membrane served to support the biofilm while proviing the large flux required. The reactor was designed so as to enable the investigation of hydrodynamic effects upon membrane fouling.

6.2 DESIGN AND MANUFACTURING OF AIRLIFT-MEMBRANE BIOREACTOR

A laboratory bench top reactor was designed as a standard bubble column with a centrally located single tubular ceramic membrane, and a working volume of 1L. One of two different sized draught tubes could be inserted into the reactor, creating a rising (and recirculating) liquid flow regime past the membrane. This allowed for investigation of hydrodynamic effects with

different riser to downcomer cross-sectional area ratios (A_R:A_d), and also for comparison of draught-tube to bubble column configuration. The reactor was constructed from clear Perspex to allow for visual observation. Figure 6.1 shows a schematic cross-section of the reactor with a draught tube in place (see Appendix 6.1 for full design details).

Reactor details

Total height	= 600mm
External diameter	= 60mm
Internal diameter	= 54mm
Total volume	= 1.3L
Wet working volume	= 1L

Draught tube 1

External diameter	= 30mm
Internal Diameter	= 26mm

Riser cross-sectional area = $A_r = 4.36 \times 10^{-4} m^2$

Downcomer cross-sectional area $A_d = 1.58 \times 10^{-3} m^2$

Area below draught tube $A_b = 2.20 \times 10^{-3} m^2$

A_R:A_d	1 : 3.6

Draught tube 2

External diameter	= 40mm
Internal daiameter	= 34mm
A_R:A_d	1 : 1

Membrane

Active length	= 193mm
Diameter	= 12mm
Membrane area	$A_m = 7.276 \times 10^{-3} m^2$
Nominal pore size	= 1μm
Material:	SiO/Al_2O_3 ceramic complex

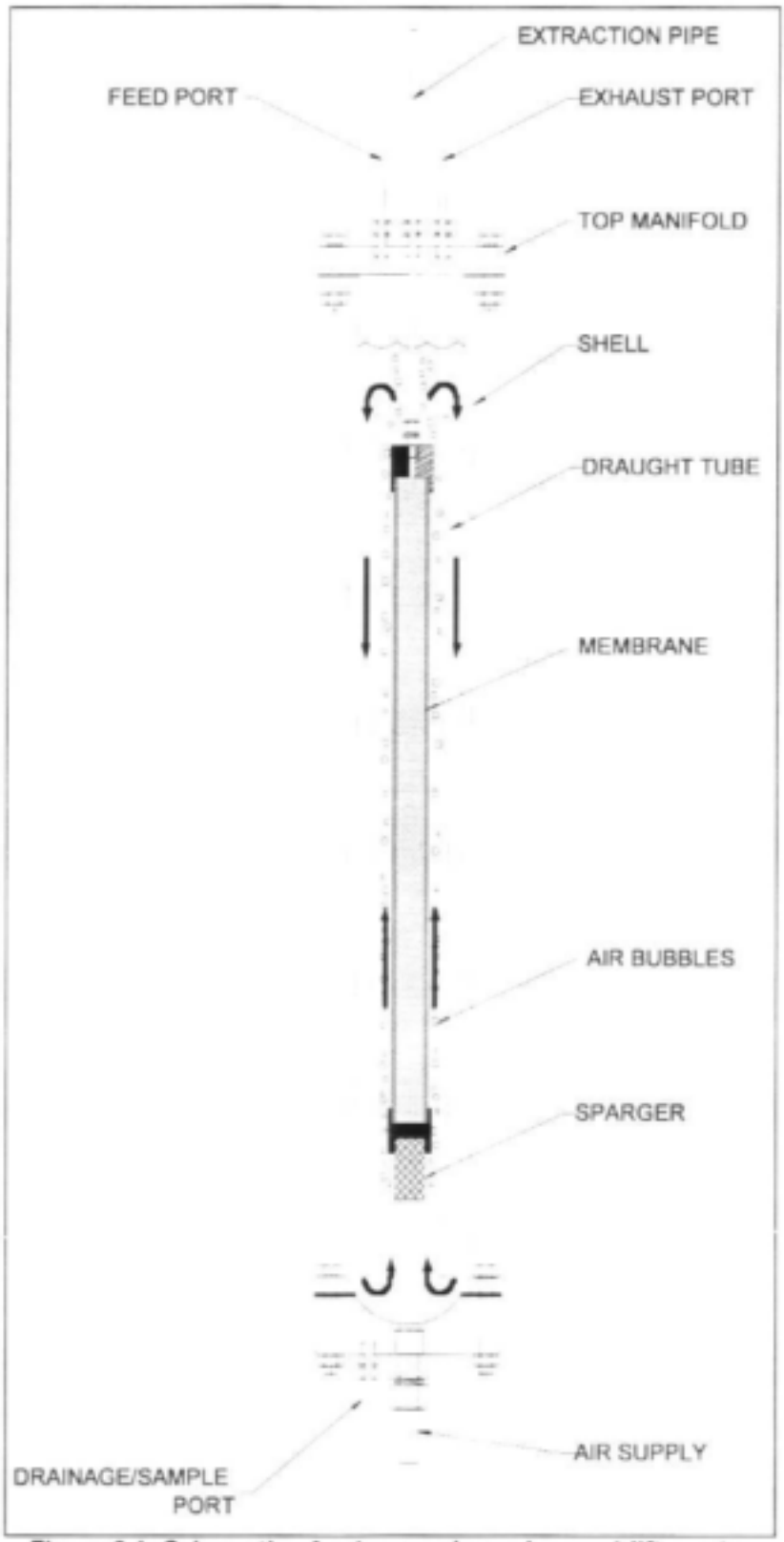


Figure 6.1: Schematic of submerged membrane airlift reactor

The oxygen sparger beneath the membrane is made from sintered steel and can be considered a fine bubble diffuser. Airflow to the reactor came from a compressor and was monitored and controlled using a rotameter. Figure 6.2 shows a schematic diagram of the entire reactor system.

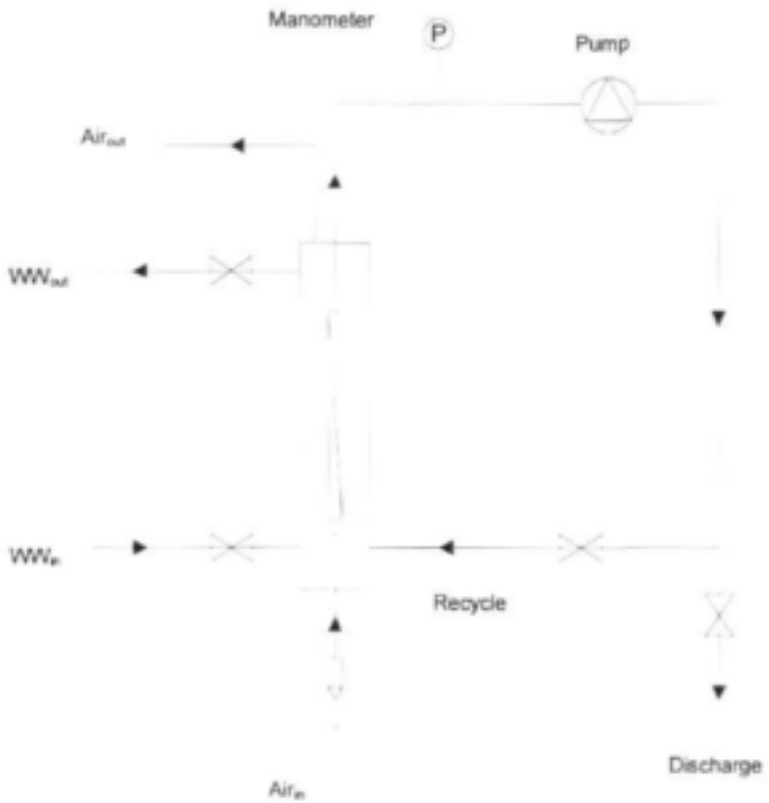


Figure 6.2: Schematic of bioreactor system

6.3 HYDRODYNAMIC ANALYSIS AND MODELING

Hydrodynamics in the reactor were modelled according to the work of Chisti and Moo-Young (1993). This is an empirical model that predicts the gas-induced liquid circulation velocity based on aeration intensity, reactor geometry and liquid column height. It has been found to be applicable for a wide range of reactor designs and operating conditions, including draught tube and annulus sparged internal loop reactors, split cylinder reactors and external loop reactors, of both round and rectangular cross-sectional areas.

The main frictional loss occurs as the recirculating liquid passes underneath the draught tube, as flow is constrained here. It is much larger than the frictional loss at the membrane walls or at the top (open channel) of the reactor. The latter two can be neglected, then the empirical equation for (superficial) velocity of the liquid in the riser of an internal loop is given by:

$$U_{Lr} = \left[\frac{2gh_D(\varepsilon_r - \varepsilon_d)}{K_b\left(\frac{A_r}{A_d}\right)^2 \frac{1}{(1-\varepsilon_d)^2}} \right]^{0.5} \qquad (6.1)$$

Where U_{Lr} = liquid velocity in the riser ($m.s^{-1}$)

g = gravitational acceleration = $9.81 m.s^{-2}$

h_D = gas-liquid dispersion height (m)

ε_r, ε_d = fractional gas holdup in riser and downcomer respectively

K_b = frictional loss coefficient for the bottom of the reactor

A_r, A_d = cross-sectional area of riser and downcomer respectively (m^2)

Solving this equation is an iterative process, as the parameters are all mutually dependent. One iteration is shown below for the reactor with draught tube 1 inserted, at an airflow rate of $1.2 m.s^{-1}$. Firstly, a value is assumed for the liquid riser velocity, and then the corresponding fractional gas hold-up in the riser is calculated.

$$\varepsilon_r = \frac{U_{Gr}}{0.24 + 1.35(U_{Gr} + U_{Lr})^{0.93}} \qquad (6.2)$$

where U_{Gr} = superficial gas velocity in the riser ($m.s^{-1}$).

This equation was developed by Hills (1976) for independently controlled flow of air and water (U_{Lr} + U_{Gr}) of less than $1.3 m.s^{-1}$.

$$U_{Gr} = \frac{\phi_G}{A_r} \qquad (6.3)$$

where ϕ_G = gas flow rate = $1.2 \times 10^{-2} m^3.min^{-1} = 2 \times 10^{-5} m^3.s^{-1}$

A_r = riser cross-sectional area = $4.36 \times 10^{-4} m^2$

therefore $U_{Gr} = 0.0459 m.s^{-1}$

and $U_{Lr} = 2 m.s^{-1}$ (assumed value)

The above values are substituted into equation (6.2), resulting in

$\varepsilon_r = 0.016$

For annulus-sparged draught tube reactors without gas-liquid separators, there is the following empirical relationship between riser and downcomer for gas hold-up (Chisti 1989):

$$\varepsilon_d \approx 0.9\varepsilon_r \tag{6.4}$$

therefore $\varepsilon_d = 0.014$

The overall gas hold-up is then calculated by

$$\varepsilon = \frac{\varepsilon_r A_r + \varepsilon_d A_d}{A_r + A_d} \tag{6.5}$$

therefore $\varepsilon = 0.014$

Next, the gas-liquid dispersion height is calculated:

$$h_D = \frac{h_L}{1-\varepsilon} \tag{6.6}$$

where h_D = dispersed height (m)

h_L = ungassed liquid height = 0.43m

therefore $h_D = 0.436$

The frictional loss coefficient for the bottom of the reactor is given by:

$$K_B = 11.402\left(\frac{A_d}{A_b}\right)^{0.789} \tag{6.7}$$

therefore $K_B = 8.794$

Equation (6.7) applies to all types of internal airlift geometries and operational modes (Chisti 1989, Chisti *et al* 1988). Finally, all these values are substituted back into equation (6.1) to obtain a new value for riser liquid velocity:

$U_L = 0.05 m.s^{-1}$

The calculation is then repeated from equation (6.2) onwards. The process converges within 4 to 5 iterations. One can perform iterations for different superficial airflow rates (using a spreadsheet); this allows for the prediction of theoretical riser velocity based on increasing airflow rates. The model is shown in figure 6.3, in conjunction with actual measured values. The model is robust: an initial assumption of riser velocity of $U_L = 2 m.s^{-1}$ is somewhat unrealistic considering that the superficial gas velocity is only $0.04 m.s^{-1}$, nevertheless it still converges quickly.

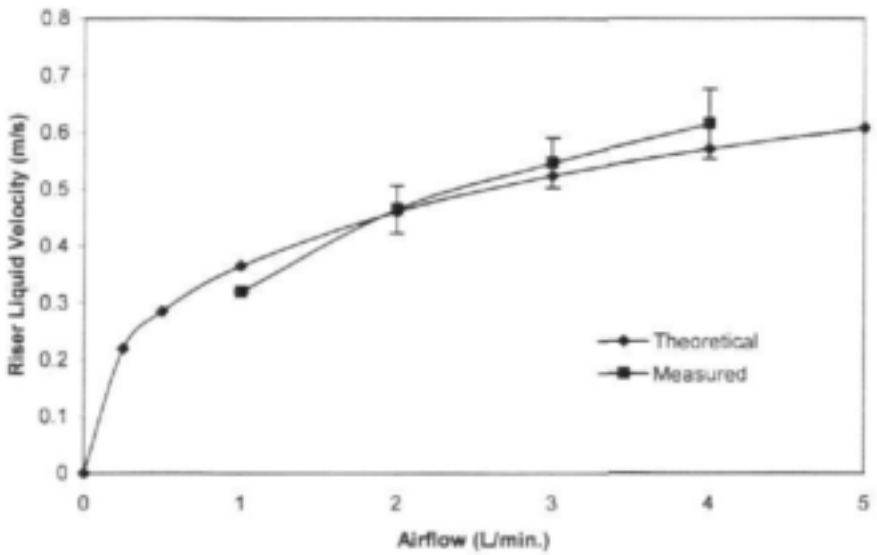


Figure 6.3: Theoretical and measured liquid riser velocity as a function of airflow rate

To measure the actual riser velocity tracer studies were employed. Black rubber pellets were used as tracers; these were filmed using a digital camera. The passage of the pellets past the membrane gave a length measurement, while frame-by-frame analysis gave a time scale, allowing for calculation of average velocity in the riser.

Possible sources of error between the modelled and measured values are incorrect friction coefficients and end effects (due to small scale of the reactor), and slight negative buoyancy of the tracers.

The model closely approximated the real situation for both draught tubes. It can be used as an estimate for design of reactor geometry based on required volume and flow rates (see section 6.6). The model is, however, limited to near Newtonian liquids (viscosity not considered).

6.4 CRITICAL FLUX EXPERIMENTS

6.4.1 Introduction

Critical flux can be defined as the rate of flow through a membrane (per unit area) above which the rate of deposition of solid (or colloidal) material onto the membrane surface exceeds the rate of removal due to hydrodynamic factors. Therefore, when critical flux is exceeded rapid fouling of

the membrane occurs; membrane flux declines and transmembrane pressure (driving force) increases (Sofia *et al* 2004, Fane *et al* 2002, Wu *et al* 1999, Bouhabila *et al* 2001, Stephenson *et al* 2000). Below the critical value flux is directly proportional to transmembrane pressure. The critical flux is significantly enhanced with increased liquid crossflow past the membrane surface (U_{Lf}). It is thus desirable to operate membrane bioreactors below the critical flux as this extends operational time, and minimizes energy and cleaning requirements.

Critical flux is generally determined by performing a flux-step experiment. Here flux is increased in a step-wise fashion until transmembrane pressure starts to increase exponentially, which then corresponds to the critical value.

The aim of this work was to investigate the effect of draught tube-reactor geometry on critical flux for a model colloidal system. A narrow draught tube increases liquid velocity past membrane (as well as constraining air bubbles to enhance the scouring effect), which is expected to improve the critical flux.

6.4.2 Theory of critical flux analysis

Flux of a fluid through a membrane can be described by the D'Arcy equation:

$$J = K\Delta P \qquad (6.8)$$

where J = permeate flux ($L.m^{-2}.hr^{-1}$)

ΔP = transmembrane pressure (kPa)

and $$K = \frac{A_m k_m}{\mu}$$

where A_m = membrane area (m^2)

k_m = intrinsic membrane permeability (m^{-1})

μ = liquid viscosity (mPa.s)

The system permeability K ($L.m^{-2}.hr^{-1}.kPa^{-1}$ or $m^2.s.kg^{-1}$) is a dynamic coefficient that includes permeating liquid properties, whereas k_m is a function only of membrane properties (e.g. pore size, pore tortuosity, membrane material, hydrophobicity). K is the inverse of total resistance to flow of liquid through the membrane for the system, and as such can be used to measure fouling occurring on or within the membrane, as the viscosity in this case remains constant. Through measurement of flux and transmembrane pressure it was possible to calculate the value of K (as a function of time) for given hydrodynamic conditions. At some stage during the flux-step increase there comes a point at which the resistance to flow starts to increase exponentially, the critical

flux. In this case it is measured as a corresponding exponential decline of the membrane permeability *K*.

6.4.3 Materials and methods

Sterilised (heat inactivated) *Candida maltosa* cell broth at suspended solids concentration of 7.55g.L^{-1} was used as model colloidal solution for fouling experiments. Sterilisation removes biological factors (like increasing viscosity and cell density) from the experiment. Viscosity of the broth was 0.95mPa.s. Permeate was withdrawn continuously through the membrane by suction using a peristaltic pump. Permeate flow rate and membrane flux are directly related through membrane surface area, therefore a step increase in flux was obtained by increasing the suction pump flow rate. After a flux-step increase the system was monitored until steady state was reached (~30 minutes), after which the next flux-step increase was made.

Transmembrane pressure (TMP) was measured with mercury manometer. Flow rates were measured using a volumetric flask and a stopwatch. Air was supplied at 1L.min^{-1} in all experiments. Permeate withdrawn from the membrane was returned to the reactor to maintain a constant volume.

The flux step experiment was repeated for the three different configurations (bubble column, narrow and wide draught tube). Pure water flux measurements were made between experiments to monitor long-term irreversible fouling. After each experiment the membrane was soaked overnight in a 5N NaOH solution to clean the membrane.

6.4.4 Results

Figure 6.4 shows the effect of increasing membrane flux (flow rate) on the membrane permeability. For the bubble column and wide draught tube (36/40 DT) the membrane permeability starts to decline as soon as the permeate flow rate is increased above a value of 1.4ml.min^{-1}, indicating that critical flux in these conditions has been exceeded. For the narrow draught tube (26/30 DT) the situation is much improved, here the critical flux is only reached at a flow rate of 2.6ml.min^{-1}, or almost double the flux compared to the other two configurations.

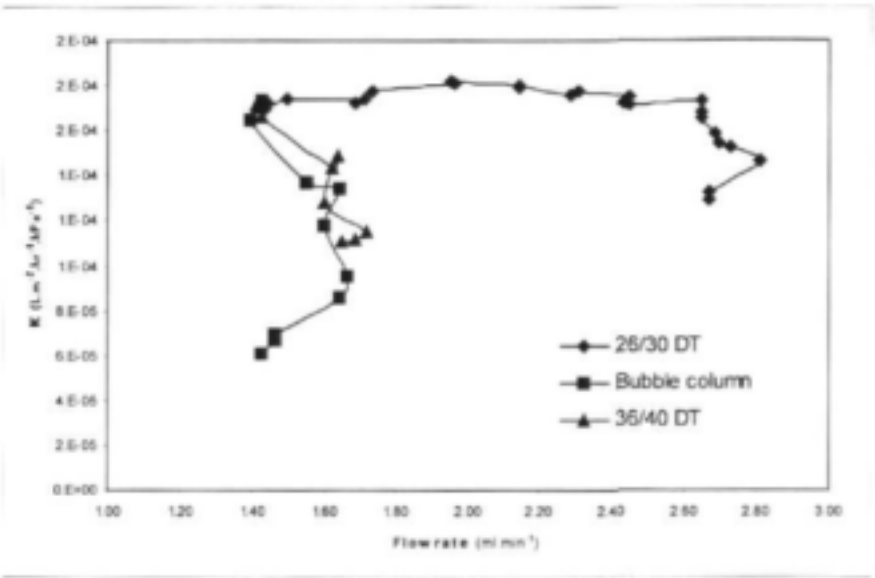


Figure 6.4 – Membrane permeability as a function of increasing flow rate

Figure 6.5 shows the membrane permeability as a function of time for the same experiment, illustrating the same trend: rapidly declining permeability for the bubble column and wide draught tube compared to the relatively stable permeability of the narrow draught tube.

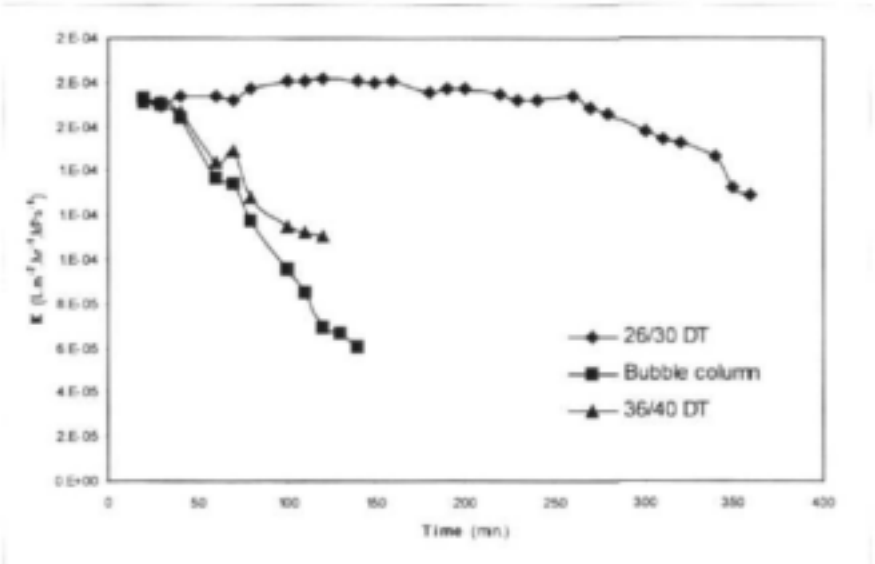


Figure 6.5 – Membrane permeability as a function of time

The data occurs in groups of 3 measurements for each flux-step increase. The critical flux is clearly improved at higher crossflow velocity, which was achieved by using a narrow draught tube around the membrane while keeping all other parameters constant. Theoretical riser velocity for the narrow draught tube at the given conditions was 0.34m.s^{-1}, while for the wider draught tube it

is 0.19 $m.s^{-1}$. These results agree very well with those of Liu et al. (2000), who observed a "critical crossflow velocity" of approximately 0.3$m.s^{-1}$. When crossflow velocity was below this value, they observed rapid membrane fouling irrespective of flux conditions or suspended solids concentrations.

The implications of the above experiment are that membrane bioreactors should be carefully designed (in a geometric sense) to maximize the hydraulic situation. Cross flow velocity past the membrane has a fundamental effect on membrane fouling, and thus also on operational time, cleaning costs, and downtime.

6.5 BATCH DEGRADATION STUDIES

6.5.1 Introduction

Taking the above work into consideration, the next step was to evaluate the bioreactor for degradative ability. Batch degradation studies were thus performed using several microbial species investigated in Chapter 5. Parameters of interest were degradation rates (in terms of COD and total phenol), and membrane performance, well as the versatility of the bioreactor in terms of growing different types of microorganisms (bacteria, fungi, yeast and mixed cultures). Degradation rates, as well as membrane fouling, were investigated for the different reactor configurations.

6.5.2 Materials and methods

Wastewater

This was black olive brine as described previously. Various dilutions were used for microbial degradation, and wastewaters were supplemented with yeast extract (nitrogen source) as required. Wastewaters were autoclaved for sterilization before use.

Experimental system

The experimental system was set up as illustrated in Figure 6.2. Before operation the system was sterilised using a 10% bleach solution, after which the reactor was rinsed with sterile tap

water. After inoculation, the supply air was switched on and set to a flow rate of 1L.min^{-1} (1vvm). Air was filter sterilised and humidified before introduction into the reactor. The suction pump was then switched on, and permeate withdrawal through the membrane was set to approximately 0.75ml.min^{-1}, which was well below the critical flux determined above. Samples were taken (daily) from the permeate withdrawn from the reactor, while the remainder of the permeate was returned to the reactor so as to maintain consistent hydrodynamic conditions. Membrane fouling was monitored throughout experiments by measuring suction pressure and permeate flux as described above. Experiments with each organism were repeated under identical conditions but with different reactor configurations (bubble column or draught tube), and results were then compared.

Organisms

These were *B. megaterium, A. niger, C. maltosa,* and mixed cultures as described in Chapter 5. A 10% inoculum was used in all cases. Initial and steady state biomass concentrations were measured as mixed liquor suspended solids (MLSS).

6.5.3 Results

Degradation experiments using fungi (*Aspergillus n.*), bacteria (*Bacillus m.*), yeast (*Candida m.*) and mixed microbial cultures were all successfully performed in the sMBR, demonstrating the versatility and generic nature of the system.

Degradation rates were generally found to be better than in shake flask cultures, most likely due to better oxygen mass transfer coefficients. Results are shown here for two degradation experiments using *Candida m.*, the same organism as used in the flux step experiments (section 6.4). In these experiments however, the cells were in an active metabolic state (not heat inactivated as in section 5.4), which allows for the evaluation of metabolic effects on membrane fouling (increasing biomass and viscosity). Results are presented for two experiments where the only difference was the presence or absence of a draught tube in the reactor, i.e. bubble column or draught tube configuration. Figure 6.6 shows reduction in total phenols and COD over time for the two configurations. There is not much difference between the two configurations in terms of degradation. Total phenol removal was around 67% from initial concentrations of around 960mg.L^{-1}, COD reduction was 57% from initial concentrations of around 16g.L^{-1}. Maximum degradation rates for phenol and COD were 0.48 and 4.3g.L^{-1}day^{-1} respectively. These were

almost double the degradation rates obtained in shake flask cultures, and percentage total removals were also improved by a factor of ~1.5.

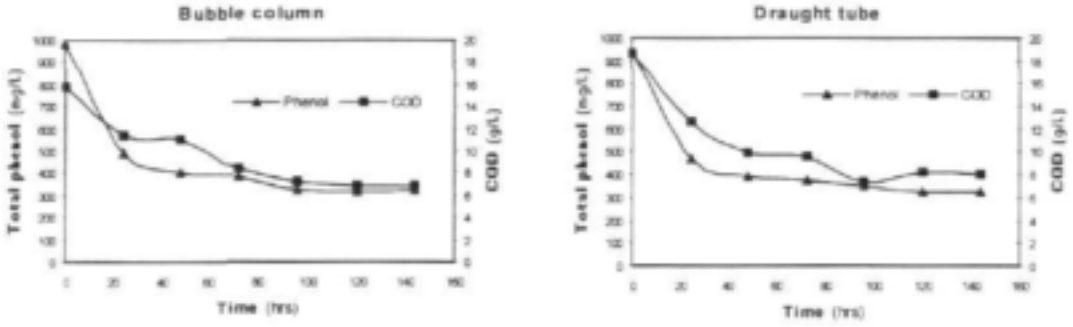


Figure 6.6: Phenol and COD degradation rates for different reactor configurations

Figure 6.7 shows membrane permeability over time for the two different configurations. Permeate withdrawal was set at 0.75ml.min^{-1} for both experiments, well below the critical flux determined in section 6.4. Once again, permeate withdrawal was continuous, with no backwashing or membrane cleaning. 0.75ml.min^{-1} equates to a theoretical residence time of 22hrs for wastewater in the reactor. Despite operation below critical flux, gradual fouling of the membrane was observed in both cases, as is expected. However, the fouling in the bubble column configuration was significantly more severe than that which occurred in the draught tube reactor. These results were consistent for all degradation experiments with different organisms. This demonstrates the importance of gas-induced cross flow velocity past the membrane surface in terms of fouling.

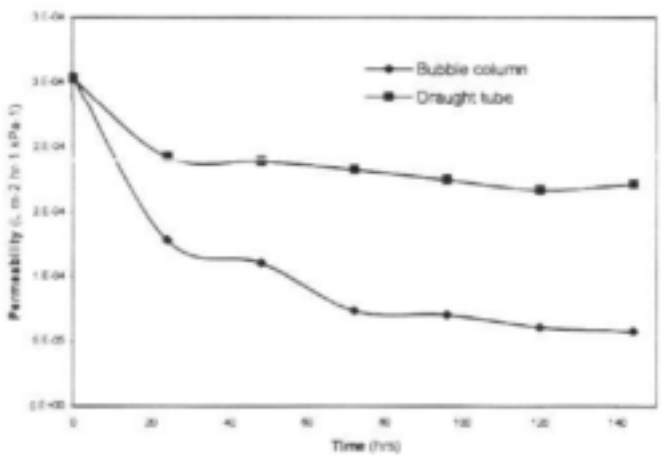


Figure 6.7: Membrane permeability for different reactor configurations

The implications for submerged membrane bioreactors is that the reactor performance can be greatly improved by careful design of a draught tube located around the membrane unit. The draught tube serves two purposes: firstly, it confines the air bubbles tightly around the membrane which improves the bubble scouring effect that removes deposited material from the membrane surface, and secondly, it induces a recirculating cross flow liquid velocity past the membrane which also reduces membrane fouling as discussed in previous sections.

6.6 SCALE-UP OF BIOREACTOR AND SYSTEM DESIGN

For the purpose of further system development and experimentation it was decided to design a scaled-up reactor system, which is currently in construction. A larger reactor is required for fitting instruments such as DO meter, pH probe and level sensors, as well as allowing for larger sample volumes. This will aid the modeling of microbial biomass evolution and substrate utilization kinetics. The basic reactor design is shown in Figure 6.8. The reactor was designed such that the riser to downcomer ratio gives predicted liquid velocity in the riser of $0.34 m.s^{-1}$ at an aeration intensity of 1vvm. It will be useful, at a later stage, to compare this with a small commercial unit, to determine which is more applicable for industrial use.

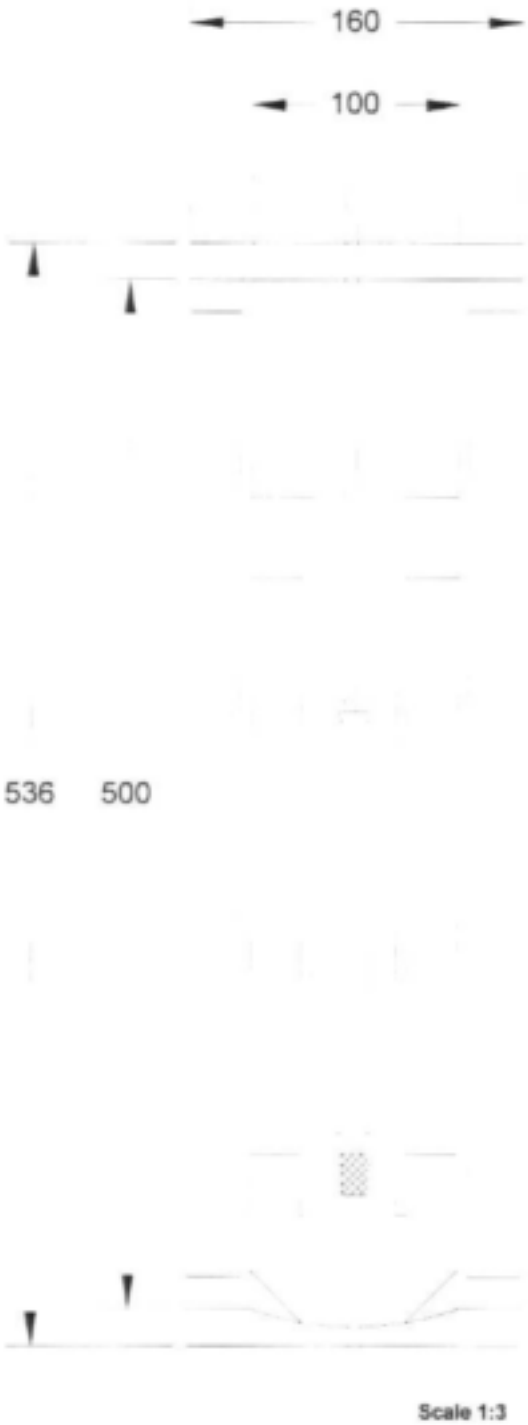


Figure 6.8 Scaled-up 4L airlift membrane bioreactor

Total internal volume	$V_{tot} = 3.93x10^{-3}$ m^3
Liquid volume	$V = 3.02x10^{-3}$ m^3
Riser cross sectional area	$A_R = 1.61x10^{-3}$ m^2
Downcomer cross-sectional area	$A_D = 5.89x10^{-3}$ m^2
Sectional area below draught tube	$A_B = 4.24x10^{-3}$ m^2
Riser to downcomer ratio	$A_R/A_D = 0.196$
Aspect ratio (h:w)	5:1
Aeration rate at 1vvm	$0.05x10^{-3}$ m^3.s^{-1} (3 L.min^{-1})
Superficial gas velocity in riser	$U_{GR} = 0.043$ m.s^{-1}
Predicted liquid riser velocity	$U_{LR} = 0.34$ m.s^{-1}
Membrane details:	
Active length	0.210 m
Diameter	0.012 m
Surface area	$A_M = 7.540x10^{-3}$ m^2
Nominal pore size	1 μm or smaller (0.2μm)

6.7 CONCLUSIONS

- A submerged-membrane bioreactor was designed for biological treatment of high strength wastewaters. The reactor was designed so as to enable the investigation of hydrodynamic effects upon membrane performance through the use of different sized draught tubes.
- The hydrodynamics in the reactor were modeled allowing for the prediction gas-induced recirculation velocity past the membrane, based on superficial aeration intensity and reactor geometry.
- The physical effects of the hydrodynamic regime upon membrane fouling characteristics were investigated through a critical flux experiment, illustrating the importance of high cross-flow velocities. The implications are that such reactors can be designed for optimal membrane performance and reactor operation.
- The optimal reactor configuration was demonstrated by batch degradation experiments. The performance of the reactor was satisfactory in terms of both membrane fouling characteristics and overall degradation rates. Degradation rates in the reactor were significantly better than parallel shake flask experiments.

- The versatility of the reactor was demonstrated through the cultivation of both pure cultures (fungi, yeast and bacteria) and mixed, acclimatized cultures for wastewater degradation purposes.
- On the basis of the above, a scaled-up reactor was designed for further studies and complete system development. This should be compared with a commercial sMBR in terms of prcactical feasibility. This work would be a valuable extension of the present project.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

7.1 CONCLUSIONS

7.1.1 Olive production wastewaters

The physical and chemical characteristics of table olive derived wastewaters were analysed and compared to wastewaters from the production of olive oil. Analysis was performed to identify potentially valuable by-products from the wastewaters, and also for the purpose of microbial degradation studies. Like olive mill wastewaters, the brines have a high organic load, in part due to high concentrations of phenolic compounds. The brines are unsuitable for disposal and require some type of treatment.

The phenolic fraction of the wastewaters is comprised of simple low molecular weight monophenolics, a wide range of larger molecular weight condensed tannins, and high molecular weight humic substances. The higher molecular weight compounds are responsible for the dark colour of the wastewaters and are not readily degradable, while the low molecular weight phenolics are more toxic but biodegradable. The low molecular weight phenolic compounds in the waste are known to be powerful natural antioxidants, with many beneficial effects upon human physiology

Compounds extracted by organic solvent extraction of the wastewaters were shown to have powerful antioxidant activity as determined by the DPPH free radical scavenging method. Low molecular weight phenolic compounds in wastewaters and extracts were identified and quantified using HPLC. The most abundant compound was shown to be hydroxytyrosol. This compound has recently become commercially available (in limited amounts) internationally, for use as a natural antioxidant, and is priced at a high market value (US $1000 – 2000 per gram). This then, is a significant opportunity for value addition to a bioremediation process.

7.1.2 Extraction of a high-value product from the waste

A valuable antioxidant (hydroxytyrosol) has been recovered from the olive wastewaters using a membrane-assisted solvent extraction process. Membrane solvent extraction was shown to be an effective methodology for the extraction of hydroxytyrosol. Investigations were carried out at lab-scale and verified by high performance liquid chromatography. The overall mass transfer

coefficient was calculated to facilitate for system design. Pertinent parameters such as flow and extraction rates were measured and mathematically modelled, also to allow for the design of a plant in terms of calculation of the required plant size and optimisation of operating conditions. In addition, local wastewater volumes and composition from both oil and table olive producers were investigated to estimate potential yields. The most cost-intensive parts of the process would be the evaporation and condensation steps, due to their energy requirements for heating and cooling.

7.1.3 Suitable microorganisms for olive wastewater bioremediation

Bacteria isolated from the environment

With the objective of identifying suitable bacterial strains for olive waste water purification, 35 environmental isolates were screened for their ability to produce phenol oxidases, the group of enzymes considered likely to be responsible for the depolymerization of polyphenolic compounds, in the expectation that their activity would result in decolorization of the wastewater. High levels of peroxidase activity were detected, but only insignificant levels of tyrosinase and laccase were found. Nevertheless, total phenolic concentration reductions of higher than 10 % were detected for all the isolates.

In order to detect and identify any economically valuable products formed during biodegradation, extracellular fractions from liquid culture of the strains were analyzed by reverse phase HPLC. Various metabolic compounds produced by several the isolates as by-products of the phenol biodegradation process, such as vanillin, were identified in this way. However, in comparison to the levels of hydroxytyrosol which could be extracted, these compounds were of much less significance and would require more down stream processing. Nevertheless, this remains an avenue which should be pursued at a later stage.

The preliminary results suggested that strain AS-35, identified as *Bacillus megaterium*, could potentially be used in olive waste bioremediation processes and it was subsequently selected for further analyses. To further enhance the biodegradation capabilities of AS-35, classical mutagenesis was performed on this isolate. Chemical mutations produced 8 putative hyper-resistant mutants that were capable of growth on a higher concentration of olive waste (80%) than the wild type. The mutants' biodegradation capabilities were compared to that of the wild type, and putative mutant DF4 showed significant increases in its ability to adapt to the high polyphenolic content of olive waste water.

Total protein expression profiles of the wild type AS-35 and mutant DF4 were analyzed by a proteomics approach, using SDS-PAGE and 2D PAGE. The protein expression profile of the wild type was found to vary depending on the particular environment in which it was grown. This was particularly evident when the expression profile of AS-35 cultured in either a nutrient rich environment or 25 % olive waste were compared. In contrast to this, similar profiles were observed for the mutant DF4 irrespective of the culturing conditions. Furthermore, the mutant profile was similar to that observed in wild type cultured in 25%olive waste. These results suggest that the mutations introduced into DF4 occurred within regulatory pathways involved in olive waste degradation. Likely candidates include sigma factors which are cellular systems are directly responsible for sensing and responding to stress factors in the environment The involvement of these systems can be examined by measuring the levels of autophosphorylation brought about by the introduction of radio-active labelled phosphates in various time- and media-dependent studies analysing the protein profiles. This is an area for future work.

Determination of bioremediation potential of selected microorganisms

Black olive brine wastewater is a high strength phenolic waste and it is relatively resistant to microbial degradation due to high phenolic content and high COD. However, certain microorganisms including yeast, fungi, bacteria and mixed cultures are able to biodegrade this waste. We investigated a range of likely strains selected on the basis of our experience in previous research, and the literature. These strains were *Trametes pubescens*, *Aspergillus niger*, *Candida maltosa* and *Lactobacillus plantarum* and a mixed consortium isolated from the disposal ponds.

The selected strains were then subjected to investigations in which we monitored their degradation capacity in terms of removal of COD and total phenols concentration. *Trametes pubescens* was able to reduce total phenols by 280 mg/L (from an initial concentration of 460 mg/L) in 10% diluted waste, *Aspergillus niger* by 50mg/l, *Candida maltosa* by 80mg/L and the mixed cultures achieved 250 mg/L removal as well as a COD reduction from 17.3 g/L to 3.6 g/L, the most efficient compared to the pure strains.

Components of the waste other than phenols (which contribute to the COD, e.g organic acids) were observed to be readily consumed. This is to be expected as they are readily assimilated by most microorganisms.

In most cases, successful reduction in total phenols content was achieved, at removal levels varying from 180 up to 460 mg/L from initial concentrations of approximately 460 mg/L. However,

formation of high molecular weight humic substances and subsequent darkening of the waste was a common trend for all the organisms apart from *T. pubescens*. This darkening is problematic not only because of the aesthetic issues of disposal of dark waters, but also because these compounds are more resistant to biodegradation. We also determined that increased pH promotes autooxidation of phenolics to polymeric substances. *T. pubescens* was the only organism capable of decolourising the waste. Coupled with an excellent removal of phenols and ability to produce extremely high titres of laccase in starter culture, this organism could potentially be used in a treatment process. Relatively high dilution of the waste is required, however, as the fungus seems to be inhibited at higher concentrations. *T. pubescens* was also able to efficiently remove phenols from the wastewater after its extraction to obtain hydroxytyrosol and an additional nitrogen source was not required.

In terms of the results achieved, the fungus has a higher capacity for the removal of total phenols as compared to the bacterium. The literature reports an abundance of aerobic biodegradation studies and these researchers use, predominantly, fungal species for investigation. Bacteria are known to be excellent phenol degraders but their application in such a complex waste seems to be limited. The most attractive aspect for using the fungus is its ability to produce laccase in high concentrations. Laccase and the other ligninolytic enzymes have the capacity to degrade lignin like compounds, of which there are many in this waste. Black olive wastewaters have been shown to act as an inducer for laccase production and perhaps, if optimised, could be used as a substrate.

7.1.4 The generic bioreactor system

A key concept in the project was the development of a generic bioreactor which could be used in different situations, for different applications. Initially it was considered that the generic nature would come from application of the technology to various wastewaters. However it has become clear that the real constraints arise from the variations imposed by different microbial strains, in the bioreactor. This is due to their wide variability in terms of tolerance of medium composition (and hence effluent concentration) and growth characteristics, both in terms of morphology and growth kinetics. Thus, the generic nature of the bioreactor has been demonstrated on the basis of application with a range of microorganisms.

A submerged-membrane bioreactor was designed for biological treatment of high strength wastewaters. The reactor was designed so as to enable the investigation of hydrodynamic effects upon membrane performance through the use of different sized draught tubes and the

hydrodynamics were then modeled to facilitate the prediction gas-induced recirculation velocity past the membrane, based on superficial aeration intensity and reactor geometry.

The physical effects of the hydrodynamic regime upon membrane fouling characteristics were investigated through a critical flux experiment, illustrating the importance of high cross-flow velocities. The implications are that such reactors can be designed for optimal membrane performance and reactor operation.

The optimal reactor configuration was demonstrated by batch degradation experiments. The performance of the reactor was satisfactory in terms of both membrane fouling characteristics and overall degradation rates. Degradation rates in the reactor were significantly better than parallel shake flask experiments.

The versatility of the reactor was demonstrated through the cultivation of both pure cultures (fungi, yeast and bacteria) and mixed, acclimatized cultures for wastewater degradation purposes On the basis of the above, a scaled-up reactor was designed for further studies and complete system development, as well as for comparison with reactors already commercially available.

7.2 RECOMMENDATIONS

7.2.1 Business development

There is enormous potential for developing a process in which hydroxytyrosol is extracted and marketed as an antioxidant. The research team conducting this project have now written a business plan for such a process, and it is recommended that a patent should now be established, to support this.

A membrane-based extraction process also allows for scaling-down of capital equipment, which could make small-scale stand-alone systems feasible. This should be considered, particularly in South Africa, for the establishment of SMME activities associated with, or satellite to, large olive production plants. The scale of a hydroxytyrosol production facility should be determined by the market, rather than an effort to treat the full volume of waste from olive production in the region.

Market research and market development are also required, because hydroxytyrosol is not widely available and therefore is not yet widely known in the public domain. An experimental programme should be implemented to demonstrate its biochemical benefits, in terms of

antioxidant capacity, is feasible using a selected range of standard chemical antioxidant assays and cell culture-based studies. Such methodologies are available in the UCT research group labs and should be used to support a market development plan.

7.2.1 Technical development

The reactor design which has been achieved requires refinement and then a phase of optimisation. The research team suggests that support for such a phase should be proposed and that a pilot plant system might be implemented at a local production facility.

In particular, in the extraction of hydroxytyrosol, the membrane extraction process should be over-designed to allow for maximum stripping of product from the wastewater, with capacity based on the maximum anticipated wastewater production volumes. Over-design of the membrane system will have only a small impact on overall capital requirements. Also, Liqui-cel® membrane contactor modules should be investigated, as these modules have been shown to be effective by other researchers.

REFERENCES

Abuhamed, T., Bayraktar, E., Mehmetoglu, T and Mehmetoglu, U. *Kinetics model for growth of Pseudomonas putida F1 during benzene, toluene and phenol biodegradation*. Proc. Biochem. 39 (2004) 983-988.

Aggelis G, Iconomou D, Christou M, Bokas D, Kotzailias S, Christo G, Tsagou A and Papanikolaou, *Phenolic removal in a model olive oil mill wastewater using Pleurotus ostreatus in bioreactor cultures and biological evaluation of the process*, Wat. Res. 37 (2003) 3897-3904

Aggelis GG, Gavala HN and Lyberatos G, *Combined and separate aerobic and anaerobic biotreatment of green olive debittering wastewater*, J. Agric. Eng. Res. 80 (2001) 283-292

Aggelis, G., Ehaliotis, C., Nerud, F., Stoychev, I., Lyberatos, G. and Zervakis, G.I. (2002). Evaluation of white-rot fungi for detoxification end decolorization of effluents from the green olive debittering process. *Appl. Microbiol Biotechnol.* 59. 353-360

Ahn KH, Song KG, Cho E, Cho J, Yun H, Lee S and Kim J, *Enhanced biological phosphorus and nitrogen removal using a sequencing anoxic/anaerobic membrane bioreactor process*, Desalination 157 (2003) 345-352

Aktas ES, Imre S and Ersoy L, *Characterisation and lime treatment of olive mill wastewater*, Wat. Res. 35.9 (2001) 2336-40

Alburquerque JA, Gonzalvez J, Garcia D and Cegarra J, *Agrochemical characterisation of "alperujo", a solid by-product of the two-phase centrifugation method for olive oil extraction*, Bioresource Technol. 91 (2004) 195-200

Alexievaa Z, Gerginova M, Zlateva P and Peneva N, *Comparison of growth kinetics and phenol metabolizing enzymes of* Trichosporon cutaneum *R57 and mutants with modified degradation abilities*, Enzyme Microb. Technol (2004)

Al-Malah K, Azzam MOJ and Abu-Lail NI, *Olive mills effluent (OME) wastewater post-treatment using activated clay*, Separation and purification technology 20 (2000) 225-234

Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. (1997). Gapped Blast and PSI-BLAST: a new generation of protein data base search programs. *Nucleic Acids Res.* 25. 3389-3402
amplification for phylogenetic study. *J. Bacteriol.* 175: 697-703.

Annibale, A.D., Casa, R., Pieruccetti, F., Ricci, M, and Marabottini, R. (2004). *Lentinula edodes* removes phenols from olive-mill wastewater: Impact on durum wheat (*Triticum durum* Desf.) germinability. Chemosphere 45. 887-895.

Antolovich M, Prenzler P, Robards K and Ryan D, *Sample Preparation in the Determination of Phenolic Compounds in Fruits*, The Analyst 125 (2000) 989-1009

Assas, N., Ayed, L., Marouani, L and Hamdi, M. *Decolorization of fresh and stored-black olive mill wastewaters by Geotrichum candidum*. Proc. Biochem. 38 (2002) 361-365.

Ayed, L and Hamdi, M. *Fermenttive decolorization of olive mill wastewater by Lactobacillus plantarum*. Proc. Biochem. 39 (2003) 59-65.

Babich H and Visioli F, *In vitro cytotoxicity to humans cells in culture of some phenolics from olive oil*, Il Farmaco 58 (2003) 403-407

bacteriophage T4. *Nature* 227: 680-685.

Bae TH, Han SS and Tak TM, *Membrane sequencing batch reactor system for the treatment of diary industry wastewater*, Proc. Biochem. 39 (2003) 221-231

Baldioli M, Servilli M, Perretti G and Montedoro GF, *Antioxidant activity of tocopherols and phenolic compounds of virgin olive oil*, JAOCS 73 (1996) 1589-1593

Balice V and Cera O, *Acidic phenolic fraction of the olive vegetation wastewater determined by a gas chromatographic method*, Grasas y Aceitas 35:3 (1984) 178-180

Balis C, Chatzipavlidis J and Flouri F, *Olive mill waste as substrate for nitrogen fixation*, Int. Biodet. Biodeg. (1996)169-178

Benitez FJ, Beltran-Heredia J, Torregrosa J and Dominguez JR, *Aerobic treatment of black olive wastewater and the effect of an ozonation stage*, Bioproc. Eng. 20 (1999) 355-361

Benitez J, Beltran-Heredia J, Torregrosa J, Acero JL and Cercas V, *Aerobic degradation of olive mill wastewaters*, Appl. Microbiol. Biotechnol. 47 (1997) 185-188

Berkelman, T., Stenstedt, T. (1998). 2-D Electrophoresis Amersham Biotech Instruction Manual. 80-6429-60, p.18.

Bertin L, Majone M, Di Gioia D and Fava F, *An aerobic fixed-phase biofilm reactor system for the degradation of the low-molecular weight aromatic compounds occurring in the effluents of anaerobic digestors treating olive mill wastewaters*, J. Biotechnol. 87 (2001) 161-177

Bertin, L., Majone, M., Di Gioia, D. and Fava, F. (2001). An aerobic fixed-phase biofilm reactor system for the degradation of the low-molecular weight aromatic compounds occurring in the effluents of anaerobic digestors treating olive mill wastewaters. *J. Biotechnol.* 87: 161-171.

Bianco A, Passacantilli P and Righi G, *Improved procedure for the reduction of esters to alcohols by sodium borohydride*, Synth. Comm. 18:15 (1988) 1765-1771

Blanquez P, Caminal G, Sarra M, Vicent MT and Gabarrell X, *Olive oil mill waste waters decoloration and detoxification in a bioreactor by the white rot fungus* Phanerochaete flavido-alba, Biotecnol. Prog. 18 (2002) 660-662

Bondet V, Brand-Williams W and Berset C, *Kinetics and mechanisms of antioxidant activity using the DPPH free radical method*, Lebensm.-Wiss. u.-Technol. 30 (1997) 609-615

Borja R, Banks CJ, Maestro-Duran R and Alba J, *The effects of the most important phenolic constituents of olive mill wastewater on batch anaerobic methanogenisis*, Env. Technol. 17 (1996) 167-174

Borja R, Martin A, Alonso V, Garcia I and Banks CJ, *Influence of different aerobic pretreatments on the kinetics of anaerobic digestion of olive mill wastewater*, Wat. Res. 29,2 (1995) 489-495

Borja R, Martin A, Maestro R, Alba J and Fiestas JA, *Enhancement of the anaerobic digestion of olive mill wastewater by the removal of phenolic inhibitors*, Proc. Biochem. 27 (1992) 231-237

Borsani R and Ferrando B, *Ultrafiltration plant for olive vegetation waters by polymeric membrane batteries*, Desal. 108 (1996) 281-286

Box JD, *Investigation of the Folin-Ciocalteau phenol reagent for the determination of polyphenolic substances in natural wastewaters*, Water Res. 17 (1983) 511-525

Bramucci M, Kane H, Chen M and Nagarajan V, *Bacterial diversity in an industrial wastewater bioreactor*, Appl. Microbiol. Biotechnol. 62 (2003) 594-600

Brenes M and de Castro A, *Transformation of Oleuropein and its hydrolysis products during Spanish-style green olive processing*, J. Sci. Food Agric. 77 (1998) 353-358

Brenes M, Rejano L, Garcia P, Sanchez AH and Garrido A, *Biochemical changes in phenolic compounds during Spanish-style green olive processing*, J. Agric. Food Chem. 43 (1995) 2702-2706

Bressler E, Pines O, Goldberg I and Braun S, *Conversion of Fumaric acid to L-Malic by sol-gel immobilised* Saccharomyces cerevisiae *in a supported liquid membrane bioreactor*, Biotecnol. Prog. 18 (2002) 445-450

Brookes PR and Livingston AG, *Aqueous-aqueous extraction of organic pollutants through tubular silicone rubber membranes*, J. Mem. Sci. 104 (1995) 119-137

Brookes PR and Livingston AG, *Biological detoxification of a 3-chloronitrobenzene manufacture wastewater in an extractive membrane bioreactor*, Wat. Res. 28:6 (1994) 1347-1354

Brookes PR and Livingston AG, *Biotreatment of a point –source industrial wastewater arising in 3,4-Dichloroaniline manufacture using an extractive membrane bioreactor*, Biotechnol. Prog. 10 (1994) 65-75

Cabrera F, Lopez R, Martinez-Bordiu A, Dupuy de Lome E and Murillo JM, *Land treatment of olive oil mill wastewater*, Int. Biodet. Biodeg. (1996) 215-225

Cabrera F, Lopez R, Martinez-Bordiu A, Dupuy de Lome E and Murillo JM, *Land treatment of olive oil mill wastewater*, Int. Biodet. Biodeg. (1996) 215-225

Capasso R, Evidente A and Visca C, *Production of hydroxytyrosol from olive oil vegetation waters*, Agrochimica 38 (1994) 1-2 pp 165-171

Capasso R, Evidente A, Avolio S and Solla F, *A highly convenient synthesis of hydroxytyrosol and its recovery from agricultural waste waters*, J. Agric. Food Chem. 47 (1999) 1745-1748

Capasso, R., Cristinzio, G., Evidente, A and Scognamiglio, F. *Isolation, spectroscopy and selective phytotoxic effects of polyphenols from vegetable wastewaters*. Phytochem. 31(2002) 4125-4128.

Casa R, D'Annibale A, Pieruccetti F, Stazi SR, Giovannozzi Sermanni G and Lo Casio B, *Reduction of the phenolic components in olive-mill wastewater by an enzymatic treatment and its impact on durum wheat germinability*, Chemosphere 50 (2003) 959-966

Casalino E, Calzaretti G, Sblano C, Landriscina V, Tecce MF and Landriscina C, *Antioxidant effect of hydroxytyrosol (DPE) and* Mn^{2+} *in liver of cadmium-intoxicated rats*, Comparative Biochemistry and Physiology Part C (2002)

Cases, I. and de Lorenzo, V. (2005). Promoters in the environment: Transcriptional regulation in its natural context. *Nature Reviews Microbiology* 3: 105-118

Casey E, Glennon B and Hamer G, *Biofilm development in a membrane-aerated biofilm reactor: Effect of flow velocity on performance*, Biotech. Bioeng. 67:4 (2000) 476-486

Casey E, Glennon B and Hamer G, *Oxygen mass transfer characteristics in a membrane-aerated Biofilm reactor*, Biotech. Bioeng. 62.2 (1999) 183-192

Cegarra J, Paredes C, Roig A, Bernal MP and Garcia D, *Use of olive mill wastewater compost for crop production*, Int. Biodet. Biodeg. (1996) 193-203

Cereti CF, Rossini F, Federici F, Quaratino D, Vassilev N and Fenice M, *Reuse of microbially treated olive mill wastewater as fertiliser for wheat (*Triticum durum *Desf.)*, Bioresource Technol. 91 (2004) 135-140

Chatjipavlidis I, Antonakou M, Demou D, Flouri F and Balis C, *Bio-fertilisation of olive oil mills liquid wastes. The pilot plant in Messinia, Greece*, Int. Biodet. Biodeg. (1996) 183-187

Cheung LM, Cheung PCK and Ooi VEC, *Antioxidant activity and total phenolics of edible mushroom extracts*, Food Chem. 81 (2003) 249-255

Chivasa S, Ndimba BK, Simon WJ, Robertson D, Yu XL, Knox JP, Bolwell P, Slabas AR. (2000). Proteomic analysis of the Arabidopsis thaliana cell wall. *Electrophoresis*. 23(11):1754-65

Chung TP, Tseng HY and Juang RS, *Mass transfer effect and intermediate detection for phenol degradation in immobilized* Pseudomonas putida *systems*, Proc. Biochem. 38 (2003) 1497-1507

Ciafardini G and Zull BA, *Survival of micro-organisms in extra virgin olive oil during storage*, Food Microbiol. 19 (2002) 105-109

Civolani C, Barghini P, Roncetti AR, Ruzzi M and Schiesser A, *Bioconversion of ferulic acid into vanillic acid by means of a vanillate-negative mutant of* Pseudomonas fluorescens *strain BF13*, App. Environ. Microbiol. 66,6 (2000) 2311-2317

Correia PFFM and de Carvalho JMR, *Recovery of phenol from resin plant effluents by emulsion liquid membranes*, J. Mem. Sci. (2003)

D' Annibale, A., Crestini, C., Vinciguerra, V and Sermanni, G. G. *The biodegradation of recalcitrant effluents from an olive mill by a white-rot fungus*. J. Biotechnol. 61(2004) 209-218.

D'Annibale A, Stazi SR, Vinciguerra V, and Giovannozzi Sermani G, *Oxirane-immobilised* Lentinula edodes *laccase: stability and phenolics removal efficiency in olive mill wastewater*, J. Biotechnol. 77 (2000) 265-273

D'Annibale A, Stazi SR, Vinciguerra V, Di Mattia E and Giovannozzi Sermani G, *Characterization of immobilized laccase from* Lentinula edodes *and its use in olive-mill wastewater treatment*, Proc. Biochem. 34 (1999) 697-706

De Castro A and Brenes M, *Fermentation of washing waters of Spanish-style green olive processing*, Proc. Biochem. 36 (2001) 797-802

Dec J and Bollag JM, *Detoxification of substituted phenols by oxidoreductive enzymes through polymerization reactions*, Arch. Environ. Contam. Toxicol. 19 (1990) 543-550

Della Ragione F, Cucciolla V, Borriello A, Della Pietra V, Pontoni G, Racioppi L, Manna C, Galleti P and Zappia V, *Hydroxytyrosol, a natural molecule occurring in olive oil, induces cytochrome c-dependent apoptosis*, Biochem. Biophys. Res. Comm. 278 (2000) 733-739

Dhouib A, Hamad N, Hassairi I and Sayadi S, *Degradation of anionic surfactants by* Citrobacter braaki, Proc. Biochem. 38 (2003) 1245-1250

Di Gioia D, Bertin L, Fava F and Marchetti L, *Biodegradation of hydroxylated and methoxylated benzoic, phenylacetic and phenylpropenoic acids present in olive mill wastewaters by two bacterial strains*, Res. Microbiol. 152 (2001) 83-93

Dias, AA., Bezerra, RM and Pereira, AN. *Activity and elution profile of laccase during biological decolorization and dephenolization of olive mill wastewaters.* Bioresource Technol. 92 (2004) 7-13.

Duran N and Esposito E, *Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment: a review*, Appl. Catal. B: Environmental 28 (2000) 83-90

Ehaliotis C, Papadopoulou K, Kotsou M, Mari I and Balis C, *Adaptation and population dynamics of* Azotobacter vinelandii *during aerobic biological treatment of olive-mill wastewater*, FEMS Microbiol. Ecol. 30 (1999) 301-311

El-Sayed WS, Ibrahim MK, Abu-shady M, El-beih F, Ohmura N, Saiki H and Ando A, *Isolation and identification of a novel strain of the genus Ochrobactrum with phenol-degrading activity*, J. Biosci. Bioeng. 96 (2003) 310-312

Emanuelson EAC, Arcangeli JP and Livingston AG, *The anoxic extractive membrane bioreactor*, Wat. Res. 37 (2003) 1231-1238

Erguder TH, Guven E and Demirer GN, *Anaerobic treatment of olive mill wastes in batch reactors*, Proc. Biochem. 36 (2000) 243-248

Eroglu E, Gunduz U, Yucel M, Turker L and Eroglu I, *Photobiological hydrogen production by using olive mill wastewater as a sole substrate source*, Int. J. Hydrogen Energy (2003)

Espin JC, Soler-Rivas C, Cantos E, Tomas-Barberan FA and Wichers HJ, *Synthesis of the antioxidant hydroxytyrosol using tyrosinase as biocatalyst*, J. Agric. Food Chem. 49 (2001) 1187-1193

Ettayebi, K., Errachidi, F., Jamai, L., Ali Tahri-Jouti, M., Sendide, K and Ettayebi, M. *Biodegradation of polyphenols with immobilised Candida tropicalis under metabolic induction.* FEMS Microbiology Letters 223 (2003) 215-219.

Fadil K, Chahlaoui A, Ouahbi A, Zaid A and Borja R, *Aerobic biodegradation and detoxification of wastewaters from the olive oil industry*, Int. Biodet. Biodeg. 51 (2003) 37-41

Fane AG, Chang S and Chardon E, *Submerged hollow fibre membrane module – design options and operational considerations*, Desal. 146 (2002) 231-236

Farizoglu B, Keskinler B, Yildiz E and Nuhoglu A, *Cheese whey treatment performance of an aerobic jet loop bioreactor*, Proc. Biochem. (2004)

Federici F, *Pectic enzyme production by* Cryptococcus albidus *var.* albidus *on olive vegetation waters enriched with sunflower calathide meal*, Biol. Wastes 25 (1988) 291-301

Federici F, Petruccioli M, Montedoro GF, Begliomini AL and Servili M, *Combined physico-chemical and biological treatment of olive vegetation waters in pilot-plant scale*, Med. Fac. Landbouww Gent 56 (4a) (1991) 1573-1577

Felske, A. (2000). Streamlined representational difference analysis for comprehensive studies of numerous genomes. *Journal of Microbiological Methods*. 50 305– 311

Fenice M, Sermani GG, Federici F and D'Annibale A, *Submerged and solid-state production of laccase and Mn-peroxidase by* Panus tigrinus *on olive mill wastewater-based media*, J. Biotechnol. 100 (2003) 77-85

Feron G, Bonnarme P and Durand A, *Prospects for the microbial production of food flavours*, Trends Food Sci. Technol. 7,9 (1996) 285-293

Ferreira Jorge RM and Livingston AG, *Biological treatment of an alternating source of organic compounds in a single tube extractive membrane bioreactor*, J. Chem. Technol. Biotechnol. 75 (2000) 1174-1182

Ferreira Jorge RM and Livingston AG, *Microbial dynamics in an extractive membrane bioreactor exposed to an alternating sequence of organic compounds*, Biotech. Bioeng. 70:3 (2000) 313-322

Field, J.A., Lettinga, G. (1991). *Treatment and detoxification of aqueous spruce bark extracts by Aspergillus niger.* Wat. Sci. Technol 24. 127 (1991)

Fiestas JA, Martin A and Borja R, *Influence of immobilization supports on the kinetic constants of anaerobic purification of olive mill wastewater*, Biological Wastes 33 (1990) 131-142

Fiestas Ros de Ursinos JA and Borja-Padilla R, *Biomethanization*, Int. Biodet. Biodeg. (1996) 145-153

Fiorelli F, Pasetti L and Galli E, *Fertility-promoting metabolites produced by* Azotobacter vinelandii *grown on olive-mill wastewaters*, Int. Biodet. Biodeg. (1996) 165-167

Fiorentino, A., Gentili, A., Isidori, M., Monaco, P., Nardelli, A., Parrella, A. and Temussi, F. (2003). Environmental effects caused by olive mill wastewater: Toxicity comparison of low molecular weight phenol components. *J. Agric Food Chem.* 51. 1005-1009.

Flouri F, Sotirchos D, Ioannidou S and Balis C, *Decolorization of olive mill liquid wastes by chemical and biological means*, Int. Biodet. Biodeg. (1996) 189-192

Fountoulakis MS, Dokianakis SN, Kornaros ME, Aggelis GG and Lyberatos G, *Removal of phenolics from olive mill wastewater using the white-rot fungus* Pleurotus ostreatus, Water Res. 36 (2002) 4735-4744

Frost and Sullivan, *Market report: membrane technology*, Filtration Industry Analyst, July 2003, p.14

Furneri PM, Marino A, Saija A, Uccella N and Bisignano G, *In vitro antimycoplasmal activity of oleuropein*, Int. J. Antimicrob. Agents 20 (2002) 293-296

Galhaup, C., Wagner, H., Hinterstoisser, B and Haltrich, D. (2002). *Increased production of laccase by the wood-degrading basidiomycete Trametes pubescens.* Enz. Microb. Technol 30 (2002) 529-536.

Galiatsatou P, Metaxas M, Arapoglou D and Kasselouri-Rigopoulou, *Treatment of olive mill waste water with activated carbons from agricultural by-products*, Waste Management 22 (2002) 803-812

Gallego A, Fortunato Ms, Foglia J, Rossi S, Gemini V, Gomez L, Gomez CE, Higa LE and Korol SE, *Biodegradation and detoxification of phenolic compounds by pure and mixed indigenous cultures in aerobic reactors*, Int. Bioget. Biodeg. (2003)

Galli E, Tomati U, Grappelli A and Buffone R, *Recycle of olive oil waste waters for* Pleurotus *mycelium production in submerged culture*, Agrochimica 32 (1988) 451-455

Garcia A, Brenes M, Jose Moyano M, Alba J, Garcia P and Garrido A, *Improvement of phenolic compound content in virgin olive oils by using enzymes during malaxation*, J. Food Eng. 48 (2001) 189-194

Garcia Garcia I, Bonilla Venceslada JL, Jimenez Pena PR and Ramos Gomez E, *Biodegradation of phenol compounds in vinasse using* Aspergillus terreus *and* Geotrichum candidum, Wat. Res. 31:8 (1997) 2005-2011

Garcia Garcia, I., Jimenez Pena, P. R., Bonilla Venceslada, J. L., Martin Martin, A., Martin Santos, M. A and Ramos Gomez, E. *Removal of phenol compounds from olive mill wastewater using Phenarochaete chrysosporium, Aspergillus niger, Aspergillus terrreus and Geotrichum candidum*. Proc. Biochem. 35 (2000) 751-758.

Garcia P, Romero C, Brenes M and Garrido A, *Effect of metal cations on the chemical oxidation of olive o-diphenols in model systems*, J. Agric. Food Chem. 44 (1996) 2101-2105

Garrido Hoyos, S. E., Martinez Nieto, L., Camacho Rubio, F and Ramos Cormenzana, A. *Kinetics of aerobic treatment of olive-mill wastewater (OMW) with Aspergillus terreus*. Proc. Biochem. 37 (2002) 1169-1176.

Gasson, M.J., Kitamura, Y., McLauchlan, W.R., Narbad, A., Parr, A.J., Parsons, E.L., Payne, J., Rhodes, M.J. and Walton, N.J. (1998). Metabolism of Ferulic Acid to Vanillin. *J Biol Chem*, 273, 4163-4170

Gharsallah N, *Production of single cell protein from olive mill wastewaters by yeasts*, Env. Technol. 14 (1993) 391-5

Godia F, Albiol J, Montesinos JL, Perez J, Creus N, Cabello F, Mengual X, Montras A and Lasseur C, *MELISSA: a loop of interconnected bioreactors to develop life support in Space*, J. Biotechnol. 99 (2002) 319-330

Gold SK, *Extending South Africa's olive branch*, Mail and Gaurdian 18,29 (2002)

Gonzalez G, Herrera MG, Garcia MT and Pena MM, *Biodegradation of phenol in a continuous process: comparative study of stirred tank and fluidized bed bioreactors*, Biores. Technol. 76 (2001) 245-251

Gonzalez-Lopez J, Pozo C, Martinez-Toledo MV, Rodelas B and Salmeron V, *Production of polyhydroxyalkanoates by* Azotobacter chroococcum *H23 in wastewater from olive oil mills (alpechin)*, Int. Biodet. Biodeg. (1996) 271-276

Gonzalez-Munoz MJ, Luque S, Alvarez JR and Coca J, *Recovery of phenol from aqueous solutions using hollow fibre contactors*, J. Mem. Sci. 213 (2003) 181-193

Gora L, Jansen JC and Maschmeyer T, *Controlling the performance of Silicalite-1 membranes*, Chem. Eur. J. 6:14 (2000) 2537-2543

Gora L, Nishiyama N, Jansen JC, Kapteijn F, Teplyakov V and Maschmeyer T, *Highly reproducible high-flux silicalite-1 membranes: optimization of silicalite-1 membrane production*, Sep. Purif. Technol. 22-23 (2001) 223-229

Gorinstein S, Martin-Belloso O, Katrich E, Lojek A, Ciz M, Gligelmo-Miguel N, Haruenkit R, Park YS, Jung ST and Trakhtenberg S, *Comparison of the contents of the main biochemical compounds and the antioxidant activity of some Spanish olive oils as determined by four different radical scavenging tests*, J. Nutr. Biochem. 14 (2003) 154-159

Greco G, Toscanoa G, Cioffi M, Gianfreda L and Sannino F, *Dephenolisation of olive mill waste-waters by olive husk*, Wat.Res. 33,13 (1999) 3046-3050

Guo CJ and De Kee D, *Effect of molecular structure on diffusion coefficients of organic solvents in rubbers*, Chem. Eng. Sci. 47-7 (1992) 1525-1532

Gupta T, Pradhan NC and Adhikari B, *Separation of phenol from aqueous solution by pervaporation using HTPB-based polyurethanurea membrane*, J. Mem. Sci. (2003)

Gutfinger T, *Polyphenols in olive oils*, J. Am. Oil Chem. Soc. 11 (1981) 966-968

Hamdi M and Ellouz R, *Bubble column fermentation of olive mill wastewaters by* Aspergillus niger, J. Chem. Tech. Biotechnol. 54 (1992) 331-335

Hamdi M, *Anaerobic digestion of olive mill wastewaters*, Proc. Biochem. 31:2 (1996) 105-110

Hamdi M, *Effects of agitation and pretreatment on the batch anaerobic digestion of olive mill wastewater*, Biores. Technol. 36 (1991) 173-178

Hamdi M, *Future prospects and constraints of olive mill wastewaters use and treatment: A review*, Bioproc. Eng. 8 (1993) 209-214

Hamdi M, Khadir A and Garcia JL, *The use of* Aspergillus niger *for the bioconversion of olive mill waste-waters*, Appl. Microbiol. Biotechnol. 34 (1991) 828-831

Hamdi M, *Toxicity and biodegradibility of olive mill waste waters in batch anaerobic digestion*, Appl. Biochem. Biotechnol. 37 (1992) 155-163

Hasar H, Kinaci C and Unlu A, *Production of non-biodegradable compounds based on biomass activity in a submerged ultrafiltration hollow fibre membrane bioreactor treating raw whey*, Proc. Biochem. (2003)

Hashimoto T, Ibi M, Matsuno K, Nakashima S, Tanigawa T, Yoshikawa T and Yabe-Nishimura C, *An endogenous metabolite of Dopamine, 3,4-dihydroxyphenylethanol, acts as a unique cytoprotective agent against oxidative stress-induced injury*, Free Radical Biology and Medicine (2003)

Hoff KA, Poplsteinova J, Jakobsen HA, Falk-Pedersen O, Juliussen O and Svendsen HF, *Modeling of a membrane reactor*, Int. J. Chem. Reactor Eng. 1 (2003)

Ikehata, K. and Nicell, J.A. 2000. Characterization of tyrosinase for the treatment of aqueous phenols. *Bioresource Technol.* 74: 191-199.

Jansen, K. (ed). 1995. Current Protocols in Molecular Biology. John Willey & Sons Inc., USA.

Jaouani A, Sayadi S, Vanthournhout M and Penninckx MJ, *Potent fungi for the decolourization of olive oil mill wastewaters*, Enzyme Microb. Technol. (2003)

Jaouani, A., Guillen, F., Penninckx, M.J., Martinez, A.T and Martinez, M.J. *Role of Pycnoporus coccineus laccase in the degradation of aromatic compounds in olive mill wastewater.* Enz. Microb. Technol 36 (2005) 478-486.

Jianping W, Wei PLH, Liping D and Guozhu M, *The denitrification of nitrate contained in a gas-liquid-solid three-phase flow airlift loop bioreactor*, Biochem. Eng. J. 15 (2003) 153-157

Jun B-H, Miyanaga K, Tanji Y and Unno H, *Removal of nitrogenous and carbonaceous substances by a porous carrier-membrane hybrid process for wastewater treatment*, Biochem. Eng. J. 14 (2003) 37-44

Kachouri F and Hamdi M, *Enhancement of polyphenols in olive oil by contact with fermented olive mill wastewater*, Proc. Biochem. (2003)

Karmakar B, Vohra RM, Nandanwar H, Sharma P, Gupta KG and Sobti RC, *Rapid degradation of Ferulic acid* via *4-vinylguaiacol and vanillin by a newly isolated strain of* Bacillus coagulans, J. Biotechnol. 80 (2000) 195-202

Kim JH, Hosobuchi M, Kishimoto M, Seki T, Yoshida T, Taguchi H and Ryu DD, *Cellulase production by a solid state fermentation system*, Biotech. Bioeng. 27 (1985) 1445-1450

Kim, J.K and Shoda, M. *Purification and characterisation of a novel peroxidase from Geotrichum candidum Dec 1 involved in decolourisation of dyes.* Appl. Environ. Microbiol. 65, 1029 (1999)

Knupp G, Rucker G, Ramos-Cormenzana A, Garrido Hoyos S, Neugebauer M and Ossenkop T, *Problems of identifying phenolic compounds during the microbial degradation of olive mill wastewater*, Int. Biodet. Biodeg. (1996) PII: S0964-8305(96)00062-5 pp. 277-282

Korbahti BK and Tanyolac A, *Continuous electrochemical treatment of phenolic wastewater in a tubular reactor*, Wat. Res. 37 (2003) 1505-1514

Korpijarvi J, Oinas P and Reunanen J, *Hydrodynamics and mass transfer in an airlift reactor*, Chem. Eng. Sci. 54 (1999) 2255-2262

Koutny M, Ruzicka J and Chlachula J, *Screening for phenol-degrading bacteria in the pristine soils of south Siberia*, Appl. Soil Ecol. 655 (2003) 1-5

Krings U and Berger RG, *Biotechnological Production of Flavours and Fragrances*, Appl. Microbiol. Biotechnol. 49 (1998) 1-8

Kris-Etherton PM, Hecker KD, Bonanome A, Coval SM, Binkoski AE, Hilpert KF and Griel AE, *Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer*, Am. J. Med. 113 (2002) 71-88

Krumlauf, R. (1996). Northern blot analysis. P. 113- 128. Harwood, A. J. (ed). Methods in Molecular Biology. Basic DNA and RNA protocols. Volume 58. Humana Press Inc. New Jersey,

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of

Lara MA, Rodriguez-Malaver AJ, Rojas OJ, Holmquist O, Gonzalez AM, Bullon J, Penaloza N and Araujo E, *Black liquor lignin biodegradation by* Trametes elegans, Int. Biodet. Biodeg. 52 (2003) 167-173

Le-Clech P, Jefferson B and Judd SJ, *Impact of aeration, solids concentration and membrane characteristics on the hydraulic performance of a membrane bioreactor*, J. Mem. Sci. (2003)

Lee-Huang S, Zhang Li, Huang PL, Chang YT and Huang PL, *Anti-HIV activity of olive leaf extract (OLE) and modulation of host cell gene expression by HIV-1 infection and OLE treatment*, Biochem. Biophys. Res. Comm. 307 (2003) 1029-1037

Lesage-Meessen L, Delattre M, Haon M, Thibault JF, Ceccaldi BC, Brunerie P and Asther M, *A two-step bioconversion process for vanillin production from ferulic acid combining* Aspergillus niger *and* Pycnoporus cinnabarinus, J. Biotechnol. 50 (1996) 107-113

Lesage-Meessen L, Navarro D, Maunier S, Sigoillot J-C, Lorquin J, Delattre M, Simon J-L, Asther M and Labat M, *Simple phenolic content in olive oil residues as a function of extraction systems*, Food Chem. 75 (2001) 501-507

Li X and Chu HP, *Membrane bioreactor for the drinking water treatment of polluted surface water supplies*, Water Res. (2003)

Libratore L, Procida G, d'Alessandro N and Cichelli A, *Solid-phase extraction and gas chromatographic analysis of phenolic compounds in virgin olive oil*, Food Chem. 73 (2001) 119-124

Litridou M, Linssen J, Schols H, Bergmans M, Posthumus M, Tsimidou M and Boskou D, *Phenolic compounds in virgin olive oils: Fractionation by solid phase extraction and antioxidant activity assessment*, J. Sci. Food Agric. 74 (1997) 169-174

Liu R, Huang X, Sun YF and Qian Y, *Hydrodynamic effect of sludge accumulation over membrane surfaces in a submerged membrane bioreactor*, Proc. Biochem. 39 (2003) 157-163

Liu SH, Luo GS, Wang Y and Wang YJ, *Preparation of coiled hollow-fibre membrane and mass transfer performance in membrane extraction*, J. Mem. Sci. 215 (2003) 203-211

Livingston AG, *A novel membrane reactor for detoxifying industrial wastewater: II. Biodegreadation of 3-chloronitrobenzene in an industrially produced wastewater*, Biotech. Bioeng. 41 (1992) 927-936

Lopez MJ and Ramos-Cormenzana A, *Xanthan production from olive mill wastewaters*, Int. Biodet. Biodeg. (1996) 263-270

Lopez MJ, Moreno J and Ramos-Cormenzana, Xanthomonas campestris *strain selection for xanthan production from olive mill wastewaters*, Wat. Res. 35.7 (2001) 1828-1830

Marques IP, *Anaerobic digestion treatment of olive mill wastewater for effluent re-use in irrigation*, Desalination 137 (2001) 233-239

Martinez Nieto L, Garrido Hoyos SE, Camacho Rubio F, Garcia Pareja MP and Ramos Cormenzana A, *The biological purification of waste products from olive oil extraction*, Biores. Technol. 43 (1993) 215-219

Martinez-Antonio, A. and Collado-Vides, J. (2003). Identifying global regulators in transcriptional regulatory networks in bacteria. *Cur. Opin. Microbiol.* 6. 482-489.

Martirani, L., Giardina, P., Marzullo, L and Sannia G. *Reduction of phenol content and toxicity in olive mill waste waters with the ligninolytic fungus Pleurotus ostreatus*. Wat. Res. 30 (1996) 1914-1918

Mathias AL, Lopretti MI and Rodrigues AE, *Chemical and biological oxidation of* Pinus pinaster *lignin for the production of vanillin*, J. Chem. Tech. Biotechnol. 64 (1995) 225-234

Maufrais, C., Fazakerley, G.V., Cadet, J. and Boulard, Y. (2003) Structural study of DNA duplex containing an N-(2-deoxy-beta-D-erythro-pentofuranosyl) formamide frameshift by NMR and restrained molecular dynamics. *Nucleic Acids Res.* 15. 5930-40.

McDonald S, Prenzler PD, Antolovich M and Robards K, *Phenolic content and antioxidant activity of olive extracts*, Food Chem. 73 (2001) 73-84

Miranda MA, Marin ML, Amat AM, Arques A and Segui S, *Pyrylium salt-photosensitized degradation of phenolic contaminants present in olive mill wastewater with solar light Part III. Tyrosol and p-hydroxyphenylacetic acid*, App.Catal. B: Environ. 35 (2002) 167-174

Miro-Casas E, Albaladejo MF, Covas MI, Rodriguez JO, Colomer EM, Raventos RML and de la Torre R, *Capillary gas chromatography-mass spectrometry quantitative determination of hydroxytyrosol and tyrosol in human urine after olive oil intake*, Anal. Biochem. 294 (2001) 63-72 Molecular Bacteriology. ASM Press, Washington D.C., USA.

Montedoro GF, Begliomini AL, Servili M, Petruccioli M and Federici F, *Pectinase production from olive vegetation waters and its use in the mechanical extraction process to increase oil yield and improve quality*, Ital. J. Food Sci. 4 (1993) 355-362

Monteoliva-Sanchez M, Incerti C, Ramos-Cormenzana A, Paredes C, Roig A and Cegarra J, *The study of the aerobic bacterial microbiota and the biotoxicity in various samples of olive mill wastewaters (alpechin) during their composting process*, Int. Biodet. Biodeg. (1996) 211-214

Morello JR, Motilva MJ, Tovar MJ and Romero MP, *Changes in commercial olive oil (cv Arbequina) during storage with special emphasis on the phenolic fraction*, Food Chem. (2003)

Moreno JJ, *Effect of olive oil minor components on oxidative stress and arachidonic acid mobilization and metabolism by macrophages raw 264.7*, Free Radical Biology and Medicine 35 (2003)1073-1081

Mouncif M, Tamoh S, Faid M and Achhkari-Begdouri A, *A study of chemical and microbiological characteristics of olive mill waste water in Morocco*, Grasas y Aceitaes 44.6 (1993) 335-38

Muheim A and Lerch K, *Towards a high-yield bioconversion of ferulic acid to vanillin*, Appl. Microbiol. Biotechnol. 51 (1999) 456-461

Mulinacci N, Romani A, Galardi C, Pinelli P, Giaccherini C and Vincieri FF, *Polyphenolic content in olive oil waste waters and related olive samples*, J. Agric. Food Chem. 49,8 (2001)

Negro C, Tommasi L and Miceli A, *Phenolic compounds and antioxidant activity from red grape marc extracts*, Bioresource Technology 87 (2003) 41-44

Noble RD, Way D and Bunge AL, *Liquid membranes*, Ion Exch. Solv. Extr. 10 (1988) 63-103 Ollero P, Serrera A, Arjona R and Alcantarilla S, *The CO_2 gasification kinetics of olive residue*, Biomass and Bioenergy 24 (2003) 151-161

Oukili Q, Chaouch M, Rafiq M, Hadji M, Hamdi M and Benlemlih M, *Bleaching of olive mill wastewater by clay in the presence of hydrogen peroxide*, Ann. Chim. Sci. Mat. 26 (2001) 45-53

Owen RW, Haubner R, Mier W, Giacosa A, Hull WE, Spiegelhalder B and Bartsch H, *Isolation, structure elucidation and antioxidant potential of the major phenolic compounds in brined olive drupes*, Food Chem. Toxicol. 41 (2003)

Pagnanelli F, Mainelli S, Veglio F and Toro L, *Heavy metal removal by olive pomace: biosorbent characterisation and equilibrium modelling*, Chem. Eng. Sci. 58 (2003) 4709-4717

Paredes C, Bernal MP, Roig A, Cegarra J and Sanchez-Monedero MA, *Influence of the bulking agent on the degradation of olive-mill wastewater sludge during composting*, Int. Biodet. Biodeg. (1996) 205-210

Pasetti L, Fiorelli F and Tomati U, Azotobacter vinelandii *biomass production from olive-mill wastewaters for heavy metal recovery*, Int. Biodet. Biodeg. (1996) 163-164

Pavasant P, Freitas dos Santos LM, Pistikopoulos EN and Livingston AG, *Prediction of optimal Biofilm thickness for membrane-attached biofilms growing in an extractive membrane bioreactor*, Biotech. Bioeng. 52 (1996) 373-286

Pavel, H., Forsman, M. and Shingler, V. (1994). An aromatic effector specificity mutant of the transcriptional regulator DmpR overcomes the growth constraints of *Pseudomonas sp.* Strain CF600 on *para*-substituted methylphenols. *J. Bacteriol.* 176: 7550-7557.

Perez J, Ramos-Cormenzana A and Martinez J, *Bacteria degrading phenolic acids isolated on a polymeric pigment*, J. Appl. Bact. 69 (1990) 38-42

Perez, J., De La Rubia, T., Hamman, B and Martinez, J. *Phanerochaete flavido-alba laccase induction and modification of manganese peroxidae isoenzyme pattern in decolourised olive mill wastewaters*. Appl. Environ. Microbiol. 64, 7 (1998) 2726-2729.

Peri C and Pompei C, *Estimation of Different Phenolic Groups in Vegetable Extracts*, Phytochemistry 10 (1971) 2187-89

Perron N and Welander U, *Degradation of phenol and cresols at low temperatures using a suspended-carrier, biofilm process*, Chemosphere 55 (2004) 45-50

Petruccioli M, Servili M, Montedoro GF and Federici F, *Development of a recycle procedure for the utilization of vegetation waters in the olive-oil extraction process*, Biotech. Letters 10:1 (1988) 55-60

Pinto G, Pollio A, Previtera L, Stanzione M and Temussi F, *Removal of low molecular weight phenols from olive oil mill wastewater using microalgae*, Biotechnol. Letters 25 (2003)1657-1659

Polzonetti V, Egidi D, Vita A, Vincenzetti S and Natalini P, *Involvement of oleuropein in (some) digestive metabolic pathways*, Food Chem. (2004)

Putter, J. (1975). Peroxidases. In. Methods of enzymatic analysis. Bergmeyer, H.U. (Ed). Verlag, Chemie, Weinheim. 685-670.

Qin, Y.Z., Anqi, Z., David, T., Yu, H and Zhen-Yu, C. *Stability of green tea catechins*. J. Food Chem. 45, 4624, (1997)

Quintana MCD, Barranco CR, Garcia PG, Balbuena MB and Fernandez AG, *Lactic acid bacteria in table olive fermentations*, Grasas Aceites 48 (1997) 297-311

Ramachandra Rao S and Ravishankar GA, *Biotransformation of isoeugenol to vanilla flavour metabolites and capsasican in suspended and immobilised cell cultures of* Capsicum frutescens: *study of the influence of β-cyclodextrin and fungal elicitor*, Proc. Biochem. 35 (1999) 341-348

Ramagli, L.S., Rodriguez, L.V. (1985). Quantification of microgram amounts of protein in two-dimensional polyacrylamide gel electrophoresis sample buffer. *Electrophoresis*. 6. 559-563.

Ramos-Cormenzana A, Juarez-Jimenez B and Garcia-Pareja MP, *Antimicrobial activity of olive mill wastewaters (Alpechin) and biotransformed olive mill wastewater*, Int. Biodet. Biodeg. (1998) 283-290

Randazzo CL, Restuccia C, Romano D and Caggia C, Lactobacillus casei, *dominant species in naturally fermented Sicilian green olives*, Int. J. Food Microbiol. 90 (2004) 9-14

Rishell S, Casey E, Glennon B and Hamer G, *Mass transfer analysis of a membrane aerated reactor*, Biochem. Eng. J. (2003)

Robards K, Prenzler PD, Tucker G, Swatsitang P and Glover W, *Phenolic compounds and their role in oxidative processes in fruits*, Food Chemistry 66 (1999) 402-436

Robards K, *Strategies for the determination of bioactive phenols in plants, fruit and vegetables*, J. Chrom. A 1000 (2003) 657-691

Robles A, Lucas R, Alvarez de Cienfuegos G and Galvez A, *Phenol-oxidase (laccase) activity in strains of the hyphomycete* Chalara paradoxa *isolated from olive mill wastewater disposal ponds*, Enzyme Microb. Technol. 26 (2000) 484-490

Robles, A., Lucas, R., Alvarez de Cienfuegos, G and Galvez, A. *Biomass production and detoxification of wastewaters from the olive oil industry by strains of Penicillium isolated from wastewater disposal ponds*. Bioresource Technol. 74 (2000) 217-221.

Rodriguez I, Llompart MP and Cela R, *Solid-phase extraction of phenols*, J. Chromatography A 885 (2000) 291-304

Rodriguez M, Viegas RMC, Luque S, Coelhoso IM, Crespo JPSG and Alvarez JR, *Removal of valeric acid from wastewaters by membrane contactors*, J. Mem. Sci. 137 (1997) 45-53

Rodriguez MM, Perez J, Ramos-Cormenzana A and Martinez J, *Effect of extracts obtained from olive oil mill waste waters on* Bacillus Megaterium *ATCC 33085*, J. Appl. Bact. 64 (1988) 219-26

Rozzi A and Malpei F, *Treatment and disposal of olive mill effluents*, Int. Biodet. Biodeg. (1996) 135-144

Ryan D, Antolovich M, Prenzler P, Robards K and Lavee S, *Biotransformations of Phenolic Compounds in* Olea europea *L.*, Scientia Horticulturae 92 (2002) 147-179

Ryan D, Robards K and Lavee S, *Changes in phenolic content of olive during maturation*, Int. J. Food Sci. Technol. 34 (1999b) 265-274

Ryan D, Robards K, Prenzler P, Jardine D, Herlt T and Antolovich M, *Liquid chromatography with electrospray ionisation mass spectrometric detection of phenolic compounds from* Olea europea, J. Chrom. A 855 (1999a) 529-537

Salah N, Miller NJ, Pagana G, Tijburg L, Bolwell GP and Rice-Evans C, *Polyphenolic Flavanols as Scavengers of Aqueous Phase Radicals and as Chain-Breaking Antioxidants*, Arch. Biochem. Biophys. 322:2 (1995) 339-346

Sala-Trepat, J.M., Murray, K., Williams, P.A. J. of Biochem. 28, 347 (1973)
Sanchez-Moreno C, Larrauri JA and Saura-Calixto F, *A procedure to measure the antiradical efficiency of polyphenols*, J. Sci. Food Agric. 76 (1998) 270-276

Sanchez-Villasclaras S, Martinez Sancho ME, Espejo Caballero MT and Delgado Perez A, *Production of microalgae from olive mill wastwater*, Int. Biodet. Biodeg. (1996) 245-247

Sanjust E, Pompei R, Rescigno A, Rinaldi A and Ballero M, *Olive milling wastewater as a medium for growth of four* Pleurotus *species*, Appl. Biochem. Biotechnol. 31 (1991) 223-235

Sannia G, Giardina P, Luna M, Rossi M and Buonocore V, *Laccase from* Pleurotus ostreatus, Biotechnol. Letters 8,11 (1986) 797-800

Sayadi S and Ellouz R, *Decolourization of olive mill waste-waters by the white-rot fungus* Phanerochaete chrysosporium: *involvement of the lignin-degrading system*, Appl. Microbiol. Biotechnol. 37 (1992) 813-817

Sayadi S and Ellouz R, *Screening of white rot fungi for the treatment of olive mill waste-waters*, J. Chem. Tech. Biotech. 57 (1993) 141-146

Sayadi S, Allouche N, Jaouna M and Aloui F, *Detrimental effects of high molecular-mass polyphenols on olive mill wastewater biotreatment*, Proc. Biochem. 35 (2000) 725-735

Sayadi, S and Ellouz, R. *Roles of lignin peroxidase and manganese peroxidase from Phanerochaete chrysosporium in the decolorisation of olive mill wastewaters.* Appl. Environ. Microbiol. 61(1995) 1098-1103

Scioli, C and Vollaro, L. *The use of Yarrowia lipolytica to reduce pollution in olive mill wastewaters.* Wat. Res. 31(10) (1997) 2520-2524.

Segers PAM, Heida JF, de Vries I, Boogaart AJ and Eilander S, *Clinical evaluation of nine hollow fibre membrane oxygenators*, NeSECC Journal 26 (2001) 10-17

Semmens MJ, Dahm K, Shanahan J and Christianson A, *COD and nitrogen removal by biofilms growing on gas permeable membranes*, Wat. Res. (2003)

Setti L, Maly S, Iacondini A, Spinozzi G and Pifferi PG, *Biological treatment of olive milling waste waters by* Pleurotus ostreatus, Annali di Chimica 88 (1998) 201-210

Severn R, *Long termoperating experience with submerged plate MBRs*, Filtration and Separation (2003) Sep.

Sheehan D (Ed.), *Bioremediation Protocols*, Humana Press, New Jersey U.S.A. (1997) ISBN 0-89603-437-2

Smits JP, Rinzema A, Tramper J, Schlosser EE and Knol W, *Accurate Determination of Process Variables in a Solid-State Fermentation System*, Proc. Biochem. 31:7 (1996) 669-78

Sze, C.C., Bernardo, L.M.D. and Shingler, V. (2002). Integration of global regulation of two aromatic-responsive σ_{54} dependent systems: A common phenotype by different mechanisms. *J. Bacteriol.* 184: 760-770

Tasioula-Margari M and Okogeri O, *Simultaneous determination of phenolic compounds and tocopherols in virgin olive oil using HPLC and UV detection*, Food Chem. 74 (2001) 377-383

Terral JF, *Exploitation and management of the olive tree during prehistoric times in Mediterranean France and Spain*, J. Archae. Sci. 27 (2000) 127-133

Tomati U, Galli E, Fiorelli F and Pasetti L, *Fertilizers from composting of olive-mill wastewaters*, Int. Biodet. Biodeg. (1996) 155-162

Toscano G, Colarieti ML and Greco G, *Oxidative polymerization of phenols by a phenol oxidase from green olives*, Enzyme Microb. Technol. (2003)

Tsioulpas A, Dimou D, Iconomou D and Aggelis G, *Phenolic removal in olive oil wastewater by strains of* Pleurotus *spp. In respect to their phenol oxidase (laccase) activity*, Biores. Technol. (accepted 29 Jan. 2002).

Tuck KL and Hayball PJ, *Major phenolic compounds in olive oil: metabolism and health effects*, J. Nutr. Biochem. 13 (2002) 636-644

Urtiaga AM, Ortiz MI, Salazar E and Irabien JA, *Supported liquid membranes for the separation-conentration of phenol. 1.Viability and mass-transfer evaluation*, Ind. Eng. Chem. Res. 31 (1992) 877-886

Urtiaga AM, Ortiz MI, Salazar E and Irabien JA, *Supported liquid membranes for the separation-conentration of phenol. 2.Mass-transfer evaluation according to fundamental equations*, Ind. Eng. Chem. Res. 31 (1992) 1745-1753
USA.

Van Beek, S. and Priest, F.G. (2000). Decarboxylation of substituted cinnamic acids by Lactic acid bacteria isolated during Malt whisky fermentation. *Applied and Environmental Microbiol.* 66. 5322-5328.

van den Heuvel RHH, Fraaije MW, Mattevi A, Laane C and van Berkel WJH, *Vanillyl-alcohol oxidase, a tasteful biocatalyst*, J. Molec. Catal. B: Enzymatic 11 (2001) 185-188

Viegas RMC, Rodriguez M, Luque S, Alvarez JR, Coelhoso IM and Crespo JPSG, *Mass transfer correlations in membrane extraction: analysis of Wilson-plot methodology*, J. Mem. Sci. 145 (1998) 129-142

Vinciguerra V, D'Annibale A, Della Monache G and Giovanni-Sermanni G, *Degradation and biotransformation of phenolic compounds of the waste olive waters by the white-rot fungus* Lentinus edodes, Med. Fac. Landbouww Univ. Gent, 58/4a (1993) 1811-14

Vinciguerra, V., D'Annibale, A., Delle Monache, G and Sermanni, G.G. *Correlated effects during the bioconversion of waste olive waters by Lentinus edodes.* Bioresource Technol. 51(1995) 221-226.

Visioli F and Galli C, *The effect of minor constituents of olive oil on cardiovascular disease: new findings*, Nutrition Reviews 56 (1998) 142-147

Visioli F, Romani A, Mulinacci N, Zarini S, Conte D, Vincieri FF and Galli C, *Antioxidant and other biological activities of olive mill waste waters*, J. Agric. Food Chem. 47 (1999) 3397-3401

Vitaglione P and Fogliano V, *Use of antioxidants to minimise the human health risk associated to mutagenic/carcinogenic heterocyclic amines in food*, J. Chrom. B (2003)

Vogna D, Pezzella A, Panzella L, Napolitano A and d'Ischia M, *Oxidative chemistry of hydroxytyrosol: isolation and characterisation of novel methanooxocinobenzodioxinone derivatives*, Tetrahedron Letters 44 (2003) 8289-8292

Vyas BRM, Volc J and Sasek V, *Lignolytic Enzymes of Selected White Rot Fungi Cultivated on Wheat Straw*, Folia Microbiol. 39:3 (1994) 235-40

Waehner RS, Mendez BA and Giulietti AM, *Olive black water as raw material for butanol production*, Biological wastes 23 (1988) 215-220

Weisburg, W.G., Barns, S.M., Pelletier, D.A. and Lane, D.J. 1991. 16S Ribosomal DNA

Xiangchun Q, Hanchang S, Yongming Z, Jianlong W and Yi Q, *Biodegradation of 2,4-dichlorophenol in an air-lift honeycomb-like ceramic reactor*, Proc. Biochem. (2003)

Xu N, Xing W, Xu N and Shi J, *Study on ceramic membrane bioreactor with turbulence promoter*, Sep. Pur. Technol. ? (2003)

Yoon S-H, *Important operational parameters of membrane bioreactor-sludge disintegration system for zero excess sludge production*, Wat. Res. 37 (2003) 1921-31

Yoon S-H, Kim H-S and Yeom I-T, *The optimum operational condition of membrane bioreactor: cost estimation of aeration and sludge treatment*, Wat. Res. (2003)

Zervakis G, Yiatras P and Balis C, *Edible mushrooms from olive oil mill wastes*, Int. Biodet. Biodeg. (1996) 237-243

Zhang S, van Houten R, Eikelboom DH, Doddema H, Jiang Z, Fan Y and Wang J, *Sewage treatment by a low energy membrane reactor*, Biores. Technol. 90 (2003) 185-192

Zhang SF, Splendiani A, Freitas dos Santos LM and Livingston AG, *Determination of pollutant diffusion coefficients in naturally formed biofilms using a single tube extractive membrane bioreactor*, Biotech. Bioeng. 50:1 (1998) 80-89

Other related WRC reports available:

Bioreactor systems using the white rot fungus Trametes for bioremediation of industrial wastewater.

Burton SG; Ryan DR; van Wyk L

The use of a white-rot fungus in combination with a membrane is being investigated for the breakdown of phenolic organics in petroleum and pulp-and-paper effluents. These effluents are difficult to treat with conventional methods. The scale-up of a laboratory unit and technology transfer to the relevant industries are included in the scope of the project.

Report Number: 1129/1/04 **ISBN No: 1 77005 200 3**

TO ORDER: Contact **Publications** - Telephone No: 012 330 0340
Fax Number: 012 331 2565
E-mail: publications@wrc.org.za

1770053808

Water Research Commission
Private Bag X03, Gezina, 0031, South Africa
Tel: +27 12 330 0340, Fax: +27 12 331 2565
Web: http://www.wrc.org.za