INVESTIGATION OF THE BIOCONVERSION OF CONSTITUENTS OF OLIVE EFFLUENTS FOR THE PRODUCTION OF VALUABLE CHEMICAL COMPOUNDS

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By

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ABSTRACT

Olive mill wastewater is produced in large quantities during the production of olive oil and olive production effluents are produced during the processing of olives. This project was planned to find a use for constituents found in olive production wastewater. The task was carried out by first characterizing the olive effluents, then screening microorganisms for growth in the effluents and reduction of the pollutant properties of the effluents. An investigation into the biotransformation of aromatic compounds present in the effluents into useful chemicals, was carried out.

The olive production effluents were collected from different stages in the process for treating olive wastewater, viz, a fermentation tank (FB), the surface of a digester (LV) and an evaporation pond (SO). The three effluents were characterized by investigating their phenolic composition. Protocatechuic acid, vanillic acid, syringic acid, hydroxyphenyl acetic acid, coumaric acid and ferulic acid were identified in an olive effluent, FB, using thin layer chromatography (TLC) and High perfomance liquid chromatography (HPLC). Hydroxyphenyl acetic acid constitutes almost 60% of the organics in olive effluent FB.

Five bacteria, namely RU-LV1; RU-FB1 and RU-FB2; RU-SO1 and RU-SO2, were isolated from the olive effluents LV, FB and SO respectively. These isolates were found to be halotolerant and were able to grow over a broad temperature and pH range, with the maximum temperature and pH for growth being 28 °C and pH 7 respectively. A range of microorganisms were evaluated for their ability to grow and reduce the total phenolic content of the olive effluents. Among these *Neurospora* crassa showed the highest potential for the biological reduction of total phenolics in olive effluents. Approximately 70% of the total phenolic content was removed by *N. crassa*. *Trametes verscilor*, *Pseudomonas putida* strains, RU-KM1 and RU-KM3$_s$, and the bacteria isolated from olive effluents could also degrade the total phenolic content of olive effluents, but to a lesser extent.

The ability of the five bacterial isolates to grow and degrade aromatic compounds was assessed by growing them in medium with standard aromatic compounds. RU-LV1 degraded 96%, 100%, 73% and 100% of caffeic acid, protocatechuic acid, *p*-coumaric acid and vanillic acid respectively. The other isolates degraded caffeic acid and protocatechuic acid, but their ability to degraded *p*-coumaric acid and vanillic acid was found to be lesser than the ability of RU-LV1 to degrade the same aromatic compounds.

Whole cells of RU-LV1 degraded vanillic acid but no metabolic products were observed on HPLC analysis. Resting cells, French pressed extract, cell free extracts and cell debris from RU-LV1 cells induced with vanillic acid degraded vanillic acid, ferulic acid and vanillin at rates higher than those obtained from non-induced cultures. No products were observed during the degradation of vanillic acid. Ferulic acid was converted into vanillic acid by French pressed extract, cell free extract and cell debris of RU-LV1. The maximum yield of vanillic acid as a product (0.23 mM, 50 %yield) was obtained when cell free extracts of RU-LV1, grown in glucose and induced by vanillic acid, were used for the degradation of 0.4 mM ferulic acid. Vanillin was rapidly converted into vanillic acid by resting cells, cell free extracts and French pressed extract of RU-LV1. Using molecular techniques, the similarity ranking of the RU-LV1 16S rRNA gene and its clone showed a high similarity to *Corynebacterium glutamicum* and *Corynebacterium* ***acedophilum.***

The rapid degradation of vanillin to vanillic acid suggests that extracts from RU-LV1 degrade ferulic acid into vanillin which is immediately oxidized to vanillic acid. Vanillic acid is also considered as a high value chemical. This project has a potential of producing useful chemicals from cheap substrates that can be found in olive effluents.

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List of Abbreviations

COD	Chemical oxygen demand
HPLC	High pressure liquid chromatography
MM	Minimal medium
OMW	Olive mill wastewater
R_f	Retention factor
TLC	Thin layer chromatography

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CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

This thesis reports a study on the olive effluents produced in South Africa, their characterization and an investigation into a useful aspect of the effluent. This chapter describes how olive wastewater is produced, its toxic effects and the beneficial aspects of olive effluents. The potential bioconversion of aromatic compounds by microorganisms into high value compounds, and the enzymes involved in the bioproduction of these fine chemicals are also discussed.

1.2 OLIVE MILL WASTEWATERS: AN INTRODUCTION

Olives are consumed as preserved fruit and as olive oil. Processing of olives usually involves keeping them in pickle or a similar saline for a variable time (from two months to over a year). Prior to packaging, the olives are darkened by a series of successive treatments (between three and five) with sodium hydroxide (lye) followed by intermediate washing for removal of excess alkali and during each treatment, air is bubbled through the suspension. Further washing is used to bring the pulp pH to 8.00 which usually takes two or three days. At this point, the olives are quite dark and immersed in a 1 % solution of ferrous gluconate or lactate in order to fix the colour, and after 24 hours the olives are finally washed and sterilized. This process produces the largest volumes of wastewater among olive manufacturing operations even though recycling the lye solutions and using neutralizing treatments in the washings can decrease the volume of wastewater produced per kilogram of olive by six to eight litres (Borja *et al.*, 1993).

Olive oil processing is carried out by means of the traditional discontinuous press or by the more recent continuous solid/liquid centrifugation system. Both

processes produce two by-product streams : residual solid (husk), which could contain oil to be recovered by means of solvent extraction, and olive mill wastewaters (OMW) (Boari *et al.*, 1984). OMW is composed of washing and processing waters and also contains soft tissues from olive pulp and oil in the form of a very stable emulsion (Borja *et al.*, 1993). OMW are one of the most complex of vegetable manufacturing effluents which makes them resistant to degradation (Chakchouk *et al.*, 1994).

OMW is produced in large quantities during the processing, and considerable pollution occurs as a result of the seasonal OMW production (Hamdi & Ellouz, 1992). This waste is recalcitrant, slightly acidic, pH 4.5 - 5 (Martin *et al.*, 1991; Flouri *et al.*, 1996) and is a persistent pollutant (Nieto *et al.*, 1993). The colour of OMW varies from dark red to black depending on age and type of olives processed (Hamdi, 1993; Yesilada *et al.*, 1995), the characteristic dark colour of these effluents being due to the polymerization of low molecular weight phenolics (Flouri *et al.*, 1996). The maximum biological oxygen demand (BOD) and chemical oxygen demand (COD) concentrations reach 100 and 220 g/dm^3 respectively (Hamdi, 1993; Borja *et al.*, 1993). OMW usually possesses a high antibacterial and phytotoxic effect exerted by different phenolic compounds which are directly toxic to animals and plants whether terrestrial or aquatic (Moreno *et al.*, 1987; Gonzalez *et al.*, 1994; Rodriguez *et al.*, 1988).

1.3 MICROBIAL CHARACTERIZATION OF OLIVE MILL WASTEWATER

Little research has been carried out on the characterization of OMW with respect to the presence of microbial activity. The variety of organic components in OMW favors the development of microorganisms that provide a wide range of metabolic capabilities that could be useful in the bioremediation process of OMW (Ramos-Cormenzana *et al.*, 1995). The prolonged evaporation process in open ponds allows a variety of microbial processes (both aerobic and anaerobic) to develop

(Millan *et al.*, 2000). The concentration of microorganisms in OMW ranges from 10^5 to 10^6 colony forming units (CFU)/mL, comprising several types of bacteria, yeasts and molds (Fiestas Ros de Ursinos and Borja-Padila, 1996; Millan *et al.*, 2000). These microorganisms are capable of breaking down compounds classified as resistant (Fiestas Ros de Ursinos and Borja-Padila, 1996).

Even though the microbial load of OMW is high, potentially hazardous microorganisms are not present at all and the indicator microorganisms such as *Clostridium* and enterococci were generally not found in OMW (Mouncif *et al.*, 1993). This may suggest that OMW are not involved in the microbial pollution of the environment.

Yeasts, molds and lactic acid bacteria are the main microorganisms found in OMW (Mouncif *et al.*, 1993). The yeasts and molds are active in decay of organic matter and they can play a role in the degradation of organic components, leading to natural transformation to minerals. Borja-Padilla *et al.* (1992) used a yeast-like mould *Geotrichum candidum* in aerobic treatment of OMW prior to biomethanization. Nieto *et al.* (1992) showed the activity of *Aspergillus niger* in the biodegradation of polyphenolic compounds. OMW disposal ponds may contain a variety of molds, some of which seem to be distributed among different ponds. These may arrive to the ponds from nearby plant material or soil, therefore reflecting their local incidence (Millan *et al.*, 2000). Yeast and molds strains isolated from OMW, and the distribution of species reported is in Table 1.1.

Table 1.1 Strain distribution of yeasts and molds in OMW (Mouncif *et al.*, 1993; Millan *et al.*, 2000)

Yeasts	%	Molds	%
Pichia sp	27.15	*Penicillium sp.*	30.3
P. carsoni	1.85	*Aspergillus sp.*	9.1
Debaryomyces hansenii	29	*Geotrichum sp.*	60.6
D. castelli	3.7		
Sacharomyces cerevisiae	12.95		
Candida zeylanoides	5.55		
C. oleophila	5.55		
C. versatilis	3.7		
Schizosaccharomyces malidevorans	3.7		
Rhodotorula mucilaginosa	1.85		

1.4 OLIVE MILL WASTEWATER COMPOSITION

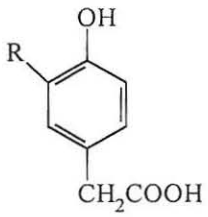
Olive mill wastewater (OMW) composition is highly variable both qualitatively and quantitatively, since OMW is a natural product, processed from a raw material and subject to varying conditions which are difficult to control (Ramos – Cormenzana *et al.*, 1995). The composition of OMW depends on the type of process involved in obtaining the oil, (Borja *et al.*, 1993; Scioli and Vollaro, 1997), climatic conditions, variety of olives, soil and harvesting time (Karapinar and Worgan, 1983).

The fundamental composition is essentially 83 – 96% water, 3.5 – 15% organic substances and 0.5 – 2% minerals (Ramos – Cormenzana *et al.*, 1995, Greco *et al.*, 1999). The organic fraction is composed by sugars (1 – 8%), nitrogen compounds (0.5 – 2.4%), organic acids (0.5 – 1.5%), fats (0.02 – 1%) as well as phenols and pectins (1 – 1.5%) (Greco *et al.*, 1999). All minerals have been identified in OMW but sodium, potassium, calcium and phosphorus are the more plentiful. Sugars such as raffinose, mannose, saccharose, glucose, arabinose and xylose are present in OMW. The organic acids contained in OMW are principally fumaric, glyceric, lactic, malic and malonic acids. All the amino acids have been

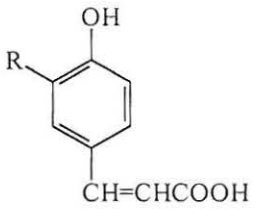
identified in OMW, most plentiful being aspartic and glutamic acid, proline and glycine (Hamdi, 1993).

This type of effluent contains large amounts of organic matter, the average concentration of volatile solids and inorganic matter being 15% and 2% respectively (Chakchouk *et al.*, 1994). OMW contains large concentrations of toxic phenolic compounds (Klibanov *et al.*, 1983; Sanjust *et al.*, 1991). The organic fraction includes sugars, tannins, polyphenols, anthocyanins, mucilages, polyalcohols, pectins and lipids (Boari *et al.*, 1984; Hamdi & Ellouz, 1992; Hamdi, 1993). Tannins concentrations range from 8 to 16 g/L. The phenolic compound concentration exceeds 10 g/L and residual oil depends on the olive oil processing method, but can reach 50 g/L.

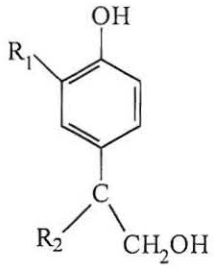
The main simple phenolic acids in OMW include syringic, *p*-hydroxyphenylacetic, vanillic, veratric, caffeic, protocatechuic, *p*-coumaric and cinnamic acids (Balice and Cera, 1984; Hamdi, 1993). Phenolic alcohols include 4-hydroxyphenyl alcohol and 3,4-dihydroxyphenyl ethanol. Other phenolic compounds that have also been detected in OMW include catechol, 4-methylcatechol, tyrosol, hydroxytyrosol, gallic acid, *p*-hydroxybenzoic acid, ferulic acid and oleuropeine glycoside (Capasso *et al.*, 1992; Montedoro *et al.*, 1992). The structures of several phenolic compounds reported to be found in OMW are shown in Figure 1.1. These phenolic compounds have been reported to inhibit the growth of certain microorganisms particularly bacteria (Paredes *et al.*, 1986; Moreno *et al.*, 1987).



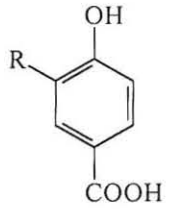
R = H 4-Hydroxyphenyl acetic acid
R = OH 3,4-Dihydroxyphenyl acetic acid



R = H Cumaric acid
R = OH Caffeic acid
R = OCH_3 Ferulic acid



R_1 = H R_2 = H Tyrosol
R_1 = OH R_2 = H Hydroxytyrosol



R = H 4-Hydroxybezoic aci
R = OH Protocatechuic acid
R = OCH_3 Vanillic acid

Figure 1.1 An overview of the phenolic compounds found in OMW (Balice & Cera, 1987; Capasso *et al.*, 1992; Nieto *et al.*, 1992; Perez *et al.*, 1992).

Other features of OMW are a temperature of 25 – 45 °C on dumping and a great ease of fermentation on storage, which gives rise to changes in its composition, yet does not necessarily result in its biodegradation or a diminished ecological toxicity (Gonzalez *et al.*, 1994).

1.5 PROCESSES FOR OMW TREATMENT

The production of industrial wastewaters which are not amenable to conventional biological oxidation has led to the development of several alternative oxidation processes including catalytic wet oxidation, ozonation and UV irradiation to electrochemical treatment. However, these are often considerably more expensive than biological treatment (Mantzarinos *et al.*, 1996).

Most of the treatment processes used for industrial and domestic wastewaters have been tested on OMW (Boari *et al.*, 1984). Several physico-chemical processes including simple evaporation, ultrafiltration and reverse osmosis have been used to detoxify this effluent. These processes require costly investment and maintenance and can be unprofitable for the seasonal oil extraction industry (Nieto *et al.*, 1993; D'Annibale *et al.*, 1998). Evaporation pits, for example only partially reduce the toxicity of OMW (Moreno *et al.*, 1987). Therefore great interest has been focused on biological treatment of OMW, as an alternative to the conventional treatment processes.

1.5.1 Anaerobic digestion of OMW

Most of the studies on the biological treatment of OMW focus on anaerobic treatment of the wastewater which can be useful for decreasing the high BOD and COD, since their well-known advantages include savings in energy and chemicals and low sludge production. Moreover, the seasonal operation of olive oil mills is not a disadvantage for anaerobic processes because the observed methanogen decay rates are low and a digester can be easily restarted after several months of shutdown (Hamdi, 1991). Anaerobic treatment is one of the processes which only partially repays its own expenses via the production of methane (Nieto *et al.*, 1993).

The biological disposal of OMW by anaerobic processes such as anaerobic contact, upflow anaerobic sludge blanket (UASB) and anaerobic filter have been applied to diluted OMW (Hamdi, 1993). However, bacteria particularly methanogens, are sensitive to the high phenolic content (Boari *et al.*, 1984; Hamdi *et al.*, 1992, Sayadi & Ellouz, 1993). Therefore, although anaerobic digestion of this type of residue is feasible, and quite appealing from an energy viewpoint, the presence of phenolic inhibitors slows down the process, hinders removal of part of the COD and detracts from its economic viability (Hamdi *et al.*, 1992; Sayadi & Ellouz, 1993). It has been shown that the removal of a high proportion of

phenolic compounds improves the performance and behaviour of the anaerobic purification process (Hamdi & Garcia, 1991; Borja *et al.*, 1992; Borja *et al.*, 1993; Sayadi & Ellouz, 1992).

1.5.2 Coupling of processes for OMW treatment

Physical pretreatments like filtration, ultrafiltration, distillation, evaporation and freezing were developed for reducing the toxicity and the consumption of oxygen for biooxidation of OMW with an aerated mixed culture of aerobic bacteria and active fungi (Hamdi, 1993). Differential distillation was also proposed in order to carry out anaerobic digestion of OMW without dilution and minimal inhibition of methanogenic bacteria, (Chakchouk *et al.*, 1994).

Coupling chemical and biological oxidations steps represents an attractive alternative solution in order to solve problems of toxicity and poor biodegradability of the olive mill wastewaters. Such coupled processes induce a completely decolorised treated OMW with a low COD concentration. Compounds remaining after chemical treatment are easily biodegraded, and adding a biological treatment step after chemical oxidation enables the treatment to be taken to completion, offering a good option for OMW remediation (Chakchouk *et al.*, 1994).

To solve the problem of inhibition of microorganisms for biomethanization during anaerobic digestion, a combination of aerobic and anaerobic processes has been used with a certain degree of success (Millan *et al.*, 2000). Certain fungal species such as *Aspergillus terreus* and *Geotrichum candidum* have been used during the first stage of treatment to reduce the COD of OMW and to eliminate the phenolic inhibitors (Borja *et al.*, 1990; 1992; 1993; Borja and Gonzalez, 1994).

1.6 BENEFICIAL EFFECTS OF OLIVE MILL WASTEWATERS (OMW)

However, not all the effects of OMW are adverse and there are some important beneficial effects. From the standpoint of an environmental and energy recovery policy, OMW are a valuable resource for their high agronomic value (Tomati *et al.*, 1996). OMW contain valuable substances which could be recovered. Moreover, olive mill waste products could be used as substrates in biotechnological processes, for the production of other valuable metabolites (Ramos-Cormenzana *et al.*, 1995). Therefore increasing attention has been paid to discovering and developing a use for OMW.

1.6.1 Bioremediation of OMW for use as a fertilizer

The aqueous wastes from olive mill processing represent a valuable fertilizing material and a good source of organic compounds (Ramos-Cormenzana *et al.*, 1995). Among technologies aimed at bioremediating OMW and realising a market for such waste as a fertilizer, composting is the most suitable alternative which, permits the return to croplands of the nutrients taken up by olive tree cultivation. Moreover, composting avoids some of the drawbacks, i.e. phytotoxicity, leaching of nutrients, inhibition of microflora, often recorded when OMW are supplied directly to landfields (Tomati *et al.*, 1996). When applied to soil OMW acts as a broad-spectrum herbicide, where its half-life depends on the amounts applied to soils and the frequency of application in a given period on the soil aeration (Ramos-Cormenzana *et al.*, 1995).

The use of OMW as a fertilizer is an inexpensive method of disposal and important advantages may be derived for soil fertility: the effective use of plant nutrients contained in the wastes, a low cost of water taking into account the increasing scarcity of hydraulic resources for irrigation, and supply of organic matter which enhances microbial activity and improves the physical and chemical properties of soil (Cegarra *et al.*, 1996). Chaptjipavlidis *et al.* (1986) reported that

repeated applications of OMW to the soil resulted in an enhancement of the microbiota which can degrade the phytotoxic components of the liquid wastes. Investigations have shown that the compost obtained by composting olive-mill wastes with other organic wastes can be used as fertilizer and soil conditioner (Ramos-Cormenzana *et al.*, 1995). Some reports suggest that nitrogen-fixing microorganisms increased after soil treatment with olive-mill wastes (Garcia-Barrionuevo *et al.*, 1992; 1993). The final goal of OMW composting is to obtain a high quality fertilizer, so particular interest must be paid to the evolution of the process (Tomati *et al.*, 1996).

Although this approach is ecologically interesting to solve the OMW problem, it does not allow the recovery of the valuable substances contained in the liquid wastes and could be also limited by the possible phytotoxic effects of OMW. There are also serious concerns that the use of unremediated OMW as a fertilizer will cause pollution of water courses.

1.6.2 Use as food and in biomass and single cell protein food industry

In recent years particular interest has been given to the production of biomass in submerged cultures as this opens up new possibilities for recycling of organic wastes. The production of microbial biomass for use as 'single cell protein' will be economically viable if the costs are low for large scale production. Using *Torulopsis utilis* it has been shown that the yeasts could grow sufficiently well in chemostat on OMW to use the process for industrial *Torulopsis* yeast production (Ramos-Cormenzana *et al.*, 1995). Edible mushrooms are promising for obtaining valuable food biomass since mycelia can develop in aerated liquid cultures (Setti *et al.*, 1998). Many kinds of edible mushrooms are growing in composts fed with OMW as substrate. Natural pigments, anthocyanins, contained in OMW have been reported by several authors and they can be used as natural food colouring agents (Ramos-Cormenzana *et al.*, 1995).

1.6.3 Utilisation as growth medium for algae

Within the residual slurry from olive oil extraction industry, some components are useful as nutrients for growing microalgae (Villacslaras *et al.*, 1996). In a study on the microbial characterisation of OMW (Ramos-Cormenzana, 1986) it was found that a large spectrum of microorganisms could grow on agar solidified medium made with OMW as culture medium. It is thus suggested that lagoons containing OMW could be used to grow algae.

1.6.4 Uses of Biopolymeric substance production from OMW

Olive liquid wastes were shown to provide good quality organic substrate for pullulan production fermentation (Ramos-Cormenzana *et al.*, 1995). In a study on the effect of OMW on *Azotobacter*, it was observed that it stimulates poly-hydroxy-β-butyric acid (PHB) production by *Azotobacter chroococcum* cells (Garcia-Barrionuevo, 1991). Therefore, OMW could serve as an inexpensive substrate for PHB production, increasing the possibility of producing bioplastics at competitive prices.

1.6.5 Use as a bioenergetic source (or for biogas production)

1.6.5.1 Biogas

OMW, when used as a fertilizer, retains its phytotoxicity practically unchanged. However, OMW is a good potential energy source for anaerobic fermentation because of its high organic content and methane is a potential bioproduct, although the production costs for biogases are often rather high (Ramos-Cormenzana *et al.*, 1995). The advantage of biogases is their further potential use as energy sources. Research on biogas produced from OMW, under anaerobic fermentation, gives high yields in the bioreactors (Dalis, 1989). However, although substantial investments were made to try to optmise this process, one of the major problems is related to the inhibitory effect of OMW on microorganisms

both under aerobic and anaerobic conditions (Ramos-Cormenzana *et al.*, 1995). Pretreatments of OMW to reduce the phenolic compounds, using aerobic biodegradation processes using *Aspergillus terreus* (Nieto *et al.*, 1993) or *Azotobacter chroococcum* (Borja *et al.*, 1993), were shown to substantially improve methane production, when compared to methane production during anaerobic digestion of untreated OMW.

1.6.5.2 Production of ethanol

Production of other bioenergetic compounds from OMW has also been evaluated with butanol, butanediol (Wachner *et al.*, 1988) and ethanol (Bambalov *et al.*, 1989) being among the compounds tested. The microbial strains involved in these processes have been isolated from such effluents and they were tentatively identified as *Candida wickerhamii*, *C. molishiana* and *Saccharomyces cerevisiae*. The product yields (g of ethanol per g of sugar) reached 87% of the theoretical yield, but the final alcohol concentration was low about 1.3% at best (Bambalov *et al.*, 1989). The recovery costs for such low concerntration of ethanol from the fermentation broth are likely to be rather high if the traditional extraction methods are used. However, the process may be less expensive if other methods such as membrane separation could be used for extraction.

1.6.6 The employment of OMW as source of biologically active compounds

It is known that a number of compounds found in OMW are inhibitory to microorganisms (Gonzalez *et al.*, 1990). Although the use of such compounds as antimicrobials in pharmacological applications has not yet been exploited, it is certainly to be considered. Various OMW components could be extracted for specific applications, for example, polyphenols represent a class of compounds with interesting pharmaceutical applications. Antibiotic production is one of the more feasible applications, based on the known antimicrobial activity of OMW including antifungal activity (Ramos-Cormenzana *et al.*, 1995).

Ramos-Cormenzana *et al.* (1995) suggested that it would be worthwhile to study the possible application of oligomers from OMW as biological response factors. Similar investigations on polysaccharides obtained by OMW biotransformation would be interesting to initiate. The organic acids should also be studied as 'biological response modifiers'(Ramos-Cormenzana *et al.*, 1995). Furthermore, some of the phenolic compounds such as ferulic and vanillic acids found in OMW could be used as starting materials in the biosynthesis of fine chemicals.

1.7 BIOCONVERSION OF AROMATIC COMPOUNDS FOUND IN OLIVE MILL WASTEWATERS

There is growing interest in the use of whole cell bioconversion processes in the synthesis of fine chemicals (Perestelo *et al.*, 1989). For nearly every type of reaction known in chemistry (oxidative, reductive, hydrolytic and conjugative) there is a biocatalytic equivalent. The key advantage of biocatalysis is that enzymes and microorganisms catalyse stereospecific and regiospecific reactions under mild conditions, thereby saving energy (Cheetham, 1993; Barghini *et al.*, 1998). This ability is especially important when only one isomer, specifically one stereoisomer, has the required biological activity and the other(s) are either inactive or have an undesirable activity, so that existing chemical processes, that produce only a mixture of isomers, are not suitable. By comparison chemical processes are usually non selective and much less environmentally friendly, particularly when reagents are used in stoichiometric amounts or require heavy metal catalysis (Cheetham, 1993).

Chemically synthesized flavor chemicals are artificial and can thus not be used as natural flavors (Falconnier *et al.*, 1994; Muheim and Lerch, 1999). The trend and demand for natural and healthy food in the past years has also had an impact on the flavoring producing industries, due to public demand for flavors to be natural. Physical processes such as extraction and distillations are used to yield the natural

flavor compounds (Falconnier *et al.*, 1994; Muheim and Lerch, 1999), but often key flavor chemicals cannot be obtained from nature via these routes at reasonable prices. As an alternative, fermentation and enzymatic reactions can be employed for the production of so called "natural aroma chemicals" (Muheim and Lerch, 1999). Today, contemporary microbiological techniques are being applied increasingly to enhance the efficiency of microbial biocatalysis, for the production of specific flavor and fragrance chemicals (Hagerdorn and Kaphammer, 1994). Bulk flavoring chemicals such as citric acid, high fructose corn syrup, and glutamic acid are produced in millions of kilograms annually, using microbial processes (Falconnier *et al.*, 1994).

The use of microbial batch fermentations for the production of specific chemicals is often faster, more economical and cleaner than chemical synthesis. This has been demonstrated in the production of amino acids and other chemicals (Pometto III and Crawford, 1983). The use of enzymes and microorganisms in biotransformations has been also successful in the production of sweet, fruit-flavour molecules and it has proved relatively easy to stimulate the enzyme processes that produce flavor chemicals in plants, using microorganisms or isolated enzymes (Cheetham, 1993).

1.7.1 Aroma chemicals

Aroma chemicals are the volatile organic compounds found in foods and perfumes that provide their distinctive organoleptic character (Hagerdorn and Kaphammer, 1994). Vanillin is one of the most widely used aroma chemicals in food, followed by benzaldeyhde (Krings and Berger, 1998).

1.7.1.1 Vanillin – a valuable chemical compound

At the present time, vanillin (3-methoxy-4-hydroxybenzaldehyde) is the flavouring most widely used in the agri-foodstuffs industries. However, the

production of natural vanillin from vanilla pods covers only 20% of the market requirements (Lesage-Messen *et al.*, 1999). Vanilla is extracted from the cured pods of the flowers of the vanilla vine. During the curing process, a large number of flavor compounds are formed which impact to extract the pleasing aroma and balance organoleptic properties characteristic of vanilla. While vanilla is highly prized for use in flavoring of foodstuffs, its use is restricted by high cost, stemming from the complex, low yielding methods associated with its manufacture. Vanillin is one of the principal components responsible for the characteristic aroma and flavor of vanilla extract (Labuda *et al.*, 1994).

Synthetic vanillin, most often produced by treatment of sulfite waste liquors from paper mills, is typically used as a low cost substitute for vanilla and indeed may even be present as an adulterant in vanilla extract. Because of its origin and method of manufacture, vanillin derived from sulfite waste liquor is not considered to a natural food component (Labuda *et al.*, 1994). Vanillin which is obtained by chemical synthesis maybe suitable for the manufacture of perfumes and cosmetics, but it may give rise to legislative problems in the agri-foodstuffs industries (Lesage – Messen *et al.*, 1999).

The very high price of natural vanilla flavour, isolated from vanilla pods, and the consumer demand for naturally produced foods have stimulated the search for alternative means of vanillin production. A very promising way to obtain natural flavours is the use of microorganisms, since the EEC legislation incorporates under the term "natural products" those produced from biological sources by living cells, or their components, including enzymes (Falconnier *et al.*, 1994). A process for the production of natural vanillin via bioconversion of natural vanillin precursors by a low cost process would therefore have great value and utility.

More than 12 000 tons of synthetic vanillin is produced each year, mostly from petrochemicals such as guaiacol, but also from lignin, a by-product of wood pulping (Hagerdorn and Kaphammer, 1994). Vanillin is a metabolic intermediate

in the biodegradation of a variety of natural products including eugenol, ferulic acid, vanillic acid and lignin (Hagerdorn and Kaphammer, 1994; Tadasa and Kayanara, 1983; Toms and Wood, 1970). The catabolism of these compounds is an important aspect for the mineralization of plant wastes because they are released during the breakdown of lignin and cell wall materials by white rot fungi (Civolani *et al.*, 2000). Recently glucose has been used as a starting material for the synthesis of vanillin (Li and Frost, 1998).

1.7.1.1.1 Use of eugenol for vanillin production

Eugenol is the principal constituent of clove oil, an inexpensive, commercially available raw material (Hagerdorn and Kaphammer, 1994). Eugenol is a cheap substrate for the production of natural aroma chemicals of the methoxy phenol type such as vanillin, coniferylaldehyde and coniferly alcohol, and eugenol is used in many perfume and aroma compositions because of its oriental and spicy clove odour (Rabenhorst, 1996). Some of the earliest work on eugenol biotransformation to vanillin used *Corynebacterium* which was isolated for growth on eugenol and found to accumulate low levels of both ferulic acid and vanillin (8 – 18 milligrams per liter) (Tadasa, 1977). In 1983, Tadasa and Kayanara identified vanillin, ferulic acid and vanillic acid as some of the early intermediates in eugenol metabolism by a strain of *Pseudomonas*.

Labuda *et al.* (1992; 1993) have reported the biotransformation of eugenol to vanillin. However, thiol reagents were required to enhance the levels of accumulation and the levels were very low (3.6 milligrams per liter). *Serratia marcescens* (DSM 30126) has been used to oxidize isoeugenol to vanillin. The organism was first grown on glycerol and then suspended in a minimal medium containing isoeugenol, but the rate of isoeugenol biotransformation was very low (0.018 grams per liter per hour). Eugenol was also metabolized to vanillin by this organism, but high levels of meat and yeast extract were used, and the yield was very low (18 milligrams per liter) (Hagerdorn and Kaphammer, 1994).

Vanillin was found as a product of biotransformation of isoeugenol using freely suspended cells and immobilized cell cultures of *Capsicum frutescens* (Rao and Ravishankar, 1999). The mechanism of the formation of vanillin from isoeugenol is shown in Figure 1.2. Eugenol is known to be rather toxic to microorganisms, inhibiting growth and metabolism (Muheim and Lerch, 1999) and the production yield of vanillin from eugenol is very poor. Alternatively, isoeugenol can be used, as it provides better production rates. However, natural isoeugenol is not available in large volumes, which would limit its use for vanillin production (Feron *et al.*, 1996).

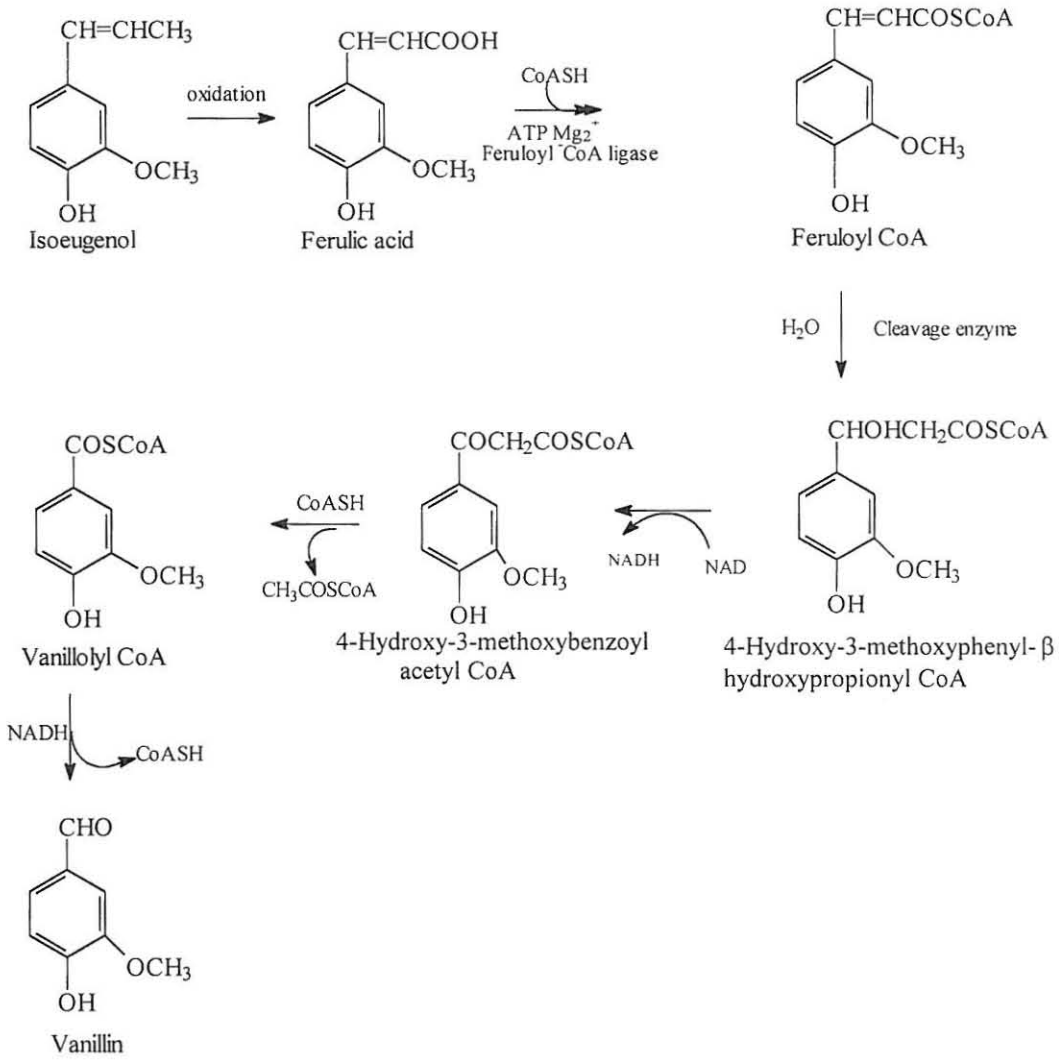


Figure 1.2 Possible biosynthetic pathway of vanillin formation from isoeugenol (Rao and Ravishankar, 1999).

1.7.1.1.2 Use of ferulic acid as a substrate for vanillin production

Most studies on the biological production of vanillin have investigated the biotransformation of ferulic acid [3-(4-hydroxy-3-methoxyphenyl)-propenoic acid] using various microorganisms and enzymes. Ferulic acid is of interest as a renewable resource for the production of useful chemicals (Huang *et* al., 1993) and it is well recognized for its antioxidant properties (Graf, 1992). Ferulic acid is a readily available and inexpensive starting material. It is present, for example, in the cell wall fraction of agricultural by products such as beet-pulps (0.9% of the dry weight) or cereal brans (2.0% of the dry weight in maize brans) (Lesage-Meesen *et al.*, 1999). Ferulic acid is one of the major phenolic lignin monomers found in woods, various grasses and corn hulls (Huang *et al.*, 1993) and is a product of the microbial oxidation of lignin (Hagerdorn and Kaphammer, 1994). Ferulic acid occurs in a free or combined form, in many monocotyledons and dicotyledons (Falconnier *et al.*, 1994). The use of enzymes and microbial transformations as means of generating value-added products from ferulic acid has been exploited. Ferulic acid may be used in free form or alternatively in bound form; bound ferulic acid is understood to mean an ester of a sugar or of an oligosaccharhide with ferulic acid (Lesage-Meesen *et al.*, 1999).

Ferulic acid is sequentially degraded to vanillin, vanillic acid and protocatechuic acid by bacteria (Toms and Wood, 1970; Sutherland *et al.*, 1983) and fungi (Huang *et al.*, 1993). Vanillin and vanillic acid have long been known to be intermediates in ferulic acid metabolism (Turner and Rice, 1975; Henderson and Fammer, 1995), but only trace amounts of vanillin accumulation have been reported. One of the earliest reports of vanillin as an intermediate of ferulic acid metabolism by microorganisms appeared in 1955 (Henderson and Fammer, 1955). Cartwright and Buswell (1967) reported both vanillin and vanillic acid as metabolic intermediates of *Pseudomonas fluorescens* (Strain T) grown on ferulic acid. Toms and Wood (1970) reported that a strain of *Pseudomonas acidovorans* oxidized vanillic acid and protocatechuic acid when grown on ferulic acid.

Several laboraties have reported the isolation of mutants that accumulate products from ferulic acid, but as in other studies, none of these reports describe the accumulation of commercially significant amounts of vanillin (Hagerdorn and Kaphammer, 1994). Otuk *et al.* (1985) found that a strain of *Escherichia coli* isolated from decaying wood accumulated vanillin, vanillic acid and protocatechuic acid from ferulic acid, but these authors did not quantify the amounts of products accumulated. *Streptomyces setonii* has been reported to metabolize ferulic acid via vanillin and protocatechuic acid (Sutherland *et al.*, 1983).

In a study on ferulic acid metabolism by *Pycnoporus cinnabarinus*, it was established that vanillic acid underwent oxidative decarboxylation into methoxyquinone, as well as reductive conversions into vanillin and vanillyl alcohol (Falconnier *et al.*, 1994). On the basis of this study, a process was developed for producing vanillin with *P. cinnabarinus*, at the optimum 64 milligrams per liter vanillin was obtained from ferulic acid (Gross *et al.*, 1991) which was too low for realistic industrial applications. In order to improve the vanillin yield from ferulic acid, another strategy, in which the complementary bioconversions abilities of two filamentous fungi were combined, was developed. In the first step, the micromycete *Aspergillus niger* transforms ferulic acid into vanillic acid, while in the second step, vanillic acid is metabolized into vanillin by the white rot fungus, *P. cinnabarinus*. In this way, more than 200 milligrams per liter vanillin was obtained with maltose as a carbon source, but high levels of methoxyquinone considerably limited the vanillin production (Lesage-Meesen *et al.*, 1996).

In order to by-pass the pathway leading to methoxyquinone and thus to favour the reductive pathway leading to vanillin, Lesage-Meesen *et al.* (1997) investigated the metabolism of vanillic acid by *P. cinnabarinus* strains in the presence of cellobiose. Decarboxylation and formation of methoxyquinone

occurred when maltose was used as a carbon source in *P. cinnabarinus* cultures, whereas cellobiose channeled the transformation of vanillic acid into vanillin, giving a yield of more than 500 milligrams per liter. However, the behaviour of the strains studied in the study differed depending on how cellobiose was applied (Lesage-Meesen *et al.*, 1997). The mechanism of synthesis of vanillin from ferulic acid is shown in figure 1.3.

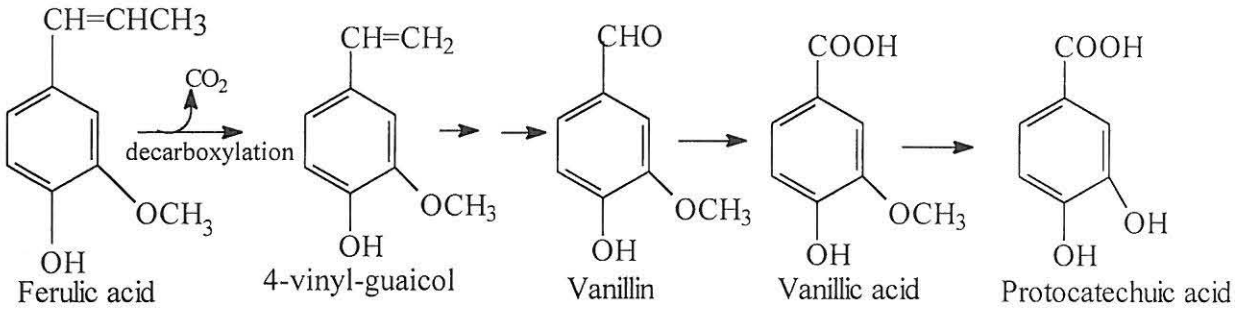


Figure 1.3 Possible pathway of ferulic acid metabolism by *Bacillus coagulans* BK 07 (Karmakar *et al.*, 2000)

1.7.1.1.3 Use of vanillic acid as a substrate for vanillin production

Vanillic acid is also important for biotechnological applications since it is used as a starting material in the chemical synthesis of oxygenated aromatic chemicals such as vanillin (Cheetham, 1993; Rosazza, 1995). Vanillic acid is a well known product of the degradation of lignin and lignin-related substances by white rot fungi (Lesage-Messen *et al.*, 1997) and other microorganisms (Sutherland *et al.*, 1983). The metabolism of vanillic acid has been thoroughly studied in *Sporotrichum pulverulentum* and it has been established that vanillic acid is either oxidatively decarboxylated into methoxyquinone or reduced into vanillin and vanillyl alcohol (Ander *et al.*, 1980; Buswell *et al.*, 1982; Ander *et al.*, 1982).

The metabolism of vanillic acid has been studied in wild type *S. pulverulentum* and in three different mutants, and was found to depend on culture conditions. The reducing pathway was not expressed until the fungus had access to an easily metabolized carbon source such as glucose or cellobiose, while decarboxylation took place in the cultures when only vanillic acid was present (Ander *et al.*, 1980).

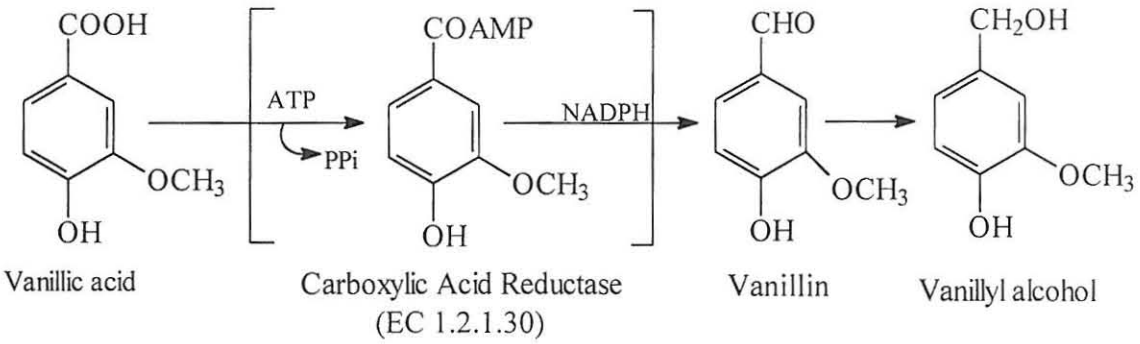


Figure 1.4 Bioconversion of vanillic acid into vanillin (Ander *et al.*, 1980).

1.7.1.1.4 Lignin as a substrate for vanillin production

Although vanillin is produced commercially via chemical oxidation of lignin, few reports have emerged on the microbial accumulation of significant quantities of vanillin from lignin. Vanillin and vanillic acid have been detected in trace amounts as a product of the fungal degradation of lignin, but the rates and levels of accumulation are very low (Hagerdorn and Kaphammer, 1994).

Though white rot fungi are responsible for delignification of wood, the precise biochemical fate of the lignin fragments is not well understood (Kirk, 1984; Kirk and Farrell, 1987). A purified lignin peroxidase from *Phanerochaete chrysosporium* was shown to degrade lignin into smaller molecular weight

compounds (Hammel *et al.*, 1993; Tien and Kirk, 1983). However, complete depolymerization of lignin to lignin monomers at commercially significant levels has not been demonstrated (Hagerdorn and Kaphammer, 1994) and the microbial oxidation of lignin to vanillin remains an elusive commercial target.

1.7.1.1.5 Glucose as a substrate for vanillin production

The starting material for the chemical synthesis of vanillin is phenol which is methylated with dimethylsulfate (which is carcinogenic) leading to guaiacol with subsequent reactions leading to vanillin (Liese and Filho, 1999). An alternative route to vanillin based on biocatalysis and starting from glucose has been developed and in the first step glucose is converted to vanillic acid by a recombinant *Escherichia coli* biocatalyst (Li and Frost, 1998). The intermediate is then reduced to vanillin by aryl aldehyde dehydrogenase isolated from *Neurospora crassa* (Figure 1.4). This new biocatalytic route exhibits advantages over the synthethic vanillin production as it does not involve carcinogenic or toxic intermediates and it is not based on the use of the non-renewable petroleum (Li and Frost, 1998).

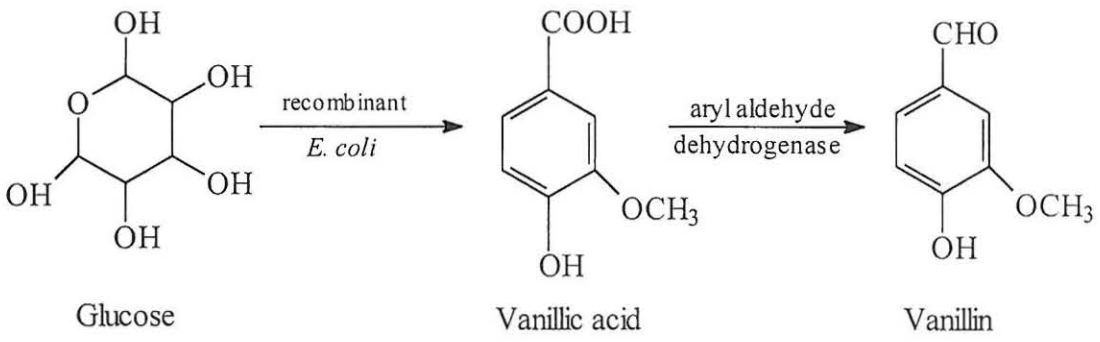


Figure 1.5 Bioconversion of glucose to vanillin (Li and Frost, 1998).

1.7.1.2 Benzaldehyde - the second most important flavor compound

In quantity, benzaldehyde is the second most important flavor molecule after vanillin (Krings and Berger, 1998). Natural benzaldehyde is usually liberated from amygdalin, a cyanogenic glycoside present in fruit kernels, and is used as a key ingredient in cherry and other natural fruit flavours (Cheethan, 1996). However, hydrocyanic is an undesirable, toxic byproduct of the extraction of natural benzaldehyde from such sources as apricot kernels. Currently, the fermentation of natural substrates such as phenylalanine offers an attractive route for the biosynthesis of natural benzaldehyde without the production of such toxic byproducts and with the benefits of a 'natural' label (Hagerdorn and Kaphammer, 1994, Cheetham, 1996; Feron *et al.*, 1996). This process is aided by a plentiful cheap supply of natural L-phenylalanine, which has become available as an intermediate of the synthesis of the high density sweetener, aspartame (Cheetham, 1996).

Pseudomonas putida ATCC 55012 catabolizes L-phenylalanine through phenylpyruvate and phenylacetaldehyde to phenylacetate. Phenylacetate is then converted to mandelate, which is further catabolized to benzoylformate. The benzoylformate is then converted to benzaldehyde in a cell free reaction using benzoylformate decarboxylase purified from the wild type strain of *P. putida* ATCC 55012 or any bacterial strain that can grow on L-phenylalanine and mandelate (Hagerdorn and Kaphammer, 1994; Feron *et al.*, 1996). Direct accumulation of benzaldehyde is not practical with this microorganism because of the toxicity of the product. The two stage process – accumulation of benzoylformate followed by an enzymatic decarboxylation to benzaldehyde – appears to circumvent the toxicity problems associated with direct accumulation of benzaldehyde (Hagerdorn and Kaphammer, 1996).

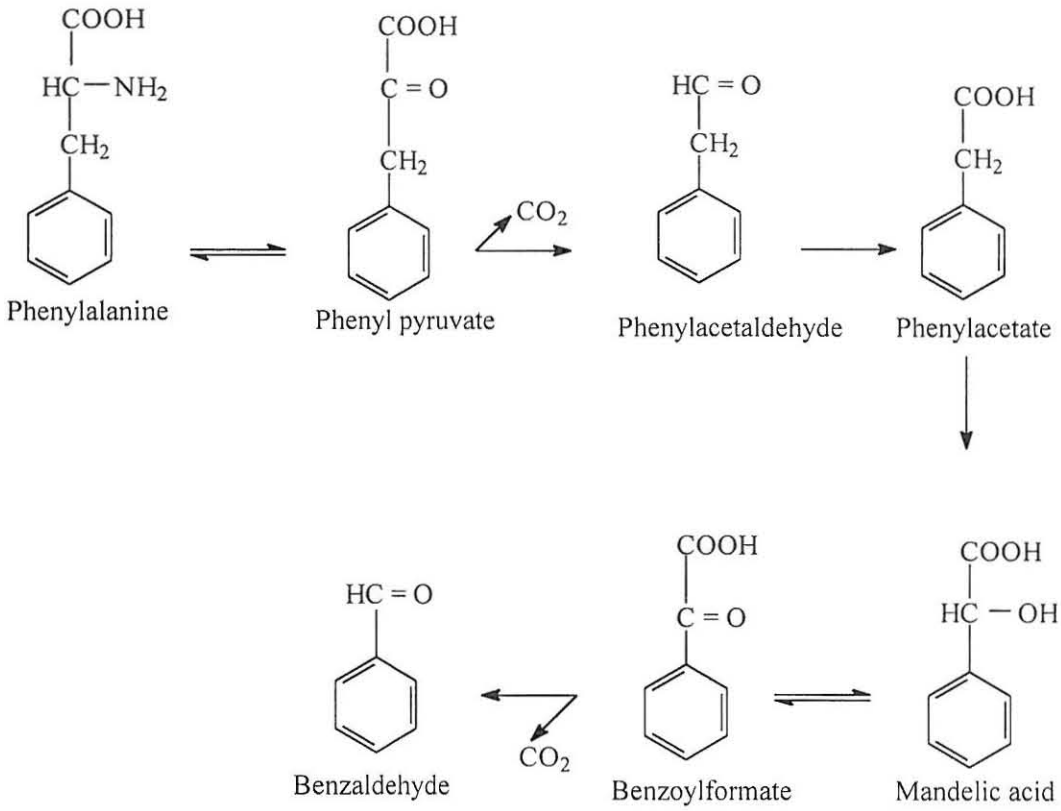


Figure 1. 6 Biotransformation of phenylalanine to benzaldehyde (Hagerdorn and Kaphammer, 1994).

1.8 ENZYMES INVOLVED IN THE BIOCONVERSION OF AROMATIC COMPOUNDS TO USEFUL AND VALUE ADDED CHEMICALS

Most of the reported processes for the conversion of isoeugenol, ferulic or vanillic acids to vanillin used intact cells, with a little emphasis on the enzyme systems responsible for this conversion. However an understanding of the biochemical and enzymatic processes involved in aromatic acids biotransformations is required as a theoretical basis for the ultimate development of biocatalytic processes for the production of large amounts of ferulic acid-derived aromatic chemicals (Huang *et al.*, 1994).

Huang *et al* (1994) reported the characterisation of ferulate decarboxylase, produced by *Pseudomonas fluorescens* UI-670. The enzyme appeared to be constitutive in nature because enzyme production was not affected by the inclusion of ferulic acid in the culture medium, was colorless, stable in the cold (4 °C), and did not require exogenously added cofactors or metal ions for full enzyme activity. Ferulate decarboxylase exhibited optima at pH 7.3 and 27 °C in 20 mM phosphate buffer (Huang *et al.*, 1994). These properties are comparable to those reported for *p*-coumarate decarboxylase from *Cladosporium phlei* (Harada and Mino, 1976). SDS-PAGE analysis showed that purified ferulate decarboxylase displayed a single band with a molecular mass of 20 400 D. The molecular mass of the native enzyme was about 40 400 D, suggesting that ferulate decarboxylase was a homodimer in solution (Huang *et al.*, 1994). By UV-visible spectroscopy, ferulate decarboxylase contained no apparent flavin component and the decarboxylation reaction was not enhanced by addition of FMN, FAD, NAD^+, $NADP^+$, NADH H^+ or NADPH H^+. Since EDTA had no effect on the enzyme, there is no apparent requirement for metals during ferulic acid decarboxylation (Huang *et al.*, 1994).

Bacillus pumilus PS213, isolated from bovine ruminal fluid, produces ferulic acid and *p*-coumarate decarboxylase only in the presence of the substrates; therefore this enzyme is induced by these two cinnamic acids (Degrassi *et al.*, 1995). Separation of the purified ferulate and *p*-coumarate decarboxylase by isoeletric focusing and HPLC suggested the presence of three isoforms with very similar pIs (4.6) and identical molecular masses. The discrepancy between the molecular masses determined by SDS-PAGE and size exclusion chromatography (23 and 45 kDa, respectively) indicates that the enzyme could be a homodimer. This is also in agreement with the N-terminal sequence homogeneity of the band excised from the SDS-PAGE gel. Ferulate and *p*-coumarate decarboxylase loses activity above 37 °C, is stable in the acidic range and its activity is completely or partially inactivated by Cu^{2+}, Ag^{2+}, Zn^{2+} and Fe^{2+} (Degrassi *et al.*, 1995).

Vanillate-*O*-demethylase catalyses the first step of vanillate degradation (Ruzzi *et al.*, 1997). This is a two-component enzyme classified as a IA oxygenase. It comprises a reductase containing both a flavin and a [2Fe-2S] redox center, and an oxygenase containing a substrate-binding site, an iron-binding site and a Rieske-type [2Fe-2S] cluster (Morawski *et al.*, 2000*a*). Direct measurement of the activity of this enzyme in cell free extracts is difficult to achieve, since the enzyme is extremely air-sensitive and unstable (Morawaski *et al.*, 2000*b*).

Vanillyl-alcohol oxidase (VAO; EC 1.1.3.38) is a flavoprotein from the ascomycete *Penicillium simplicissimum* which was shown to catalyse the oxidation of vanillyl alcohol to the flavour compound vanillin. This relatively stable enzyme is a homooctamer of 509 kDa with each 64 kDa monomer containing a flavin adenine dinucleotide (FAD) as covalently bound prosthetic group (van den Heuvel *et al.*, 2000; 2001). The cap domain covers the active site and the larger domain creates a binding site for the ADP part of the FAD prosthetic group. The phenolic substrate is bound almost parallel to the flavin ring (van den Heuvel *et al.*, 2000).

1.9 OBJECTIVES

Olive effluents from different parts of the world, Italy, Spain and Morocco, have been reported to contain similar phenolic acids. However the overall composition of each effluent is different depending on the region. No characterization of olive production effluents produced in South Africa has been reported in the literature, and thus the first objective of this study was to characterize olive effluents produced in the Western Cape, South Africa.

Olive mill wastewater has been reported to have antimicrobial effects which make it difficult to bioremediate the effluent. However microbial growth has been reported by other researchers and a screening method was required to investigate microorganisms found in olive effluents, and which would be able to utilize

polyphenols as carbon sources and thus eliminate olive effluents' inhibitory action. The second aim of this study was to isolate, characterize and identify bacteria found in olive effluents obtained from the Western Cape.

A related objective was to screen bacteria isolated from the effluents and selected alternative microorganisms for growth and phenolic content removal of the olive effluents. *Pseudomonas putida* KM1 and *Pseudomonas putida* KM_{3s} and the white rot fungi, *Trametes versicolor* and *Neurospora crassa*, were available from our culture collection and these organisms are known to have the ability to degrade extensively a diverse range of very persistent toxic environmental pollutants.

Aromatic acids found in olive effluents (for example ferulic and vanillic acids) have been used in the bioproduction of vanillin, a flavour compound. Vanillin produced from natural substrates is a high value compound and methods to produce natural vanillin are attracting much interest. Thus, the fourth objective was to investigate the biodegradation of vanillic and ferulic acids for vanillin production by bacteria isolated from the olive effluents.

CHAPTER 2

MATERIALS AND METHODS

2.1 CHARACTERISATION OF OLIVE EFFLUENTS

2.1.1 Effluents used

Three types of olive effluents were obtained from an olive processing plant in the Western Cape, labelled as follows:

FB- collected from the fermentation tank.

LV- collected from the surface of the digester tank.

SO- collected from the evaporation pond

2.1.2 Extraction of organic compounds from olive effluents

Olive effluent (200 mL) and olive effluent adjusted to pH 1 with H_2SO_4 (200 mL) were each extracted three times with an equal volume of ethyl acetate. The three organic extracts were combined and dried over anhydrous Na_2SO_4, filtered and evaporated using a Buchi Rotavapor R-114. The residue was re-dissolved in 5 ml of water/acetonitrile (60:40) or methanol and analysed by HPLC (see below).

UV analysis was carried out to determine the wavelength at which maximum absorbance for the extracted effluents occurred in the range 200 - 800 nm using Shimadzu UV-160A spectrophotometer.

2.1.3 Analyses of phenols

Olive effluents and olive effluents acidified to pH 2 with hydrochloric acid were extracted with ethyl acetate (v/v) at room temperature. The organic fraction was dried with anhydrous sodium sulphate for 30 – 40 minutes. The extract was concentrated to dryness using a Buchi Rotavapor R-114 and re-dissolved with a

mixture methanol/water (60 : 40). The total phenolic content was determined by the Folin – Ciocalteau assay (Appendix C).

2.1.4 Standard compounds

Caffeic acid, ferulic acid, *p*-coumaric acid, *m*-coumaric acid, protocatechiuc acid, *p*-hydroxyphenylacetic acid, *p*-hydroxybenzaldehyde, syringic acid, vanillic acid, 4-chlorophenol, 2,4-dichlorophenol, *p*-cresol, 4-methoxyphenol, *m*-cresol and phenol were pure products obtained from Sigma Aldrich.

2.1.5 Thin layer chromatography analysis of standard compounds and effluent extracts

The standard compounds and extracted effluents were dissolved in chloroform/ methanol (1 : 1) and analysed by elution on 20 x 20cm Silica gel 60 F_{254} (Merck) plates. The mobile phase was 10% acetic acid in chloroform. The constituents were visualised by exposure to UV light.

2.1.6 High performance liquid chromatography (HPLC) analysis of standard compounds and effluent extracts

HPLC analysis was conducted using a LaChrom L-7400 (Merck, Hitachi, Germany) HPLC system, equipped with a variable UV/VIS detector and an 80 position auto sampler/auto injector (20µl), with a 4.6 x 250 mm Spherisorb S5ODS1 Waters (U.S.A.) C_{18} Reverse phase column and guard column. The eluates were detected at 280 nm, the flow rate was 1 mL/min, the mobile phases used were water/acetonitrile (60 : 40), 2% acetic acid in water and water : methanol : acetic acid (68 : 30 : 2) and running time was 40 minutes. Extracted effluents were dissolved in methanol and 10mM of the standard compounds were prepared in methanol. A 1 in 10 dilution of each standard compound in methanol : HPLC mobile phase was prepared and analysed by HPLC. The retention times of

standard compounds were compared with those of extracted organic compounds of olive effluents. To confirm the identity of the peaks in the olive effluent extract (FB) after comparison of retention times of the standard compounds, the extract was spiked with the standard compounds one at a time and re-analysed.

2.2 ISOLATION AND CHARACTERISATION OF BACTERIA FROM OLIVE

2.2.1 Isolation of microorganisms from olive effluents

100 μL of each type of effluent was spread on nutrient agar (NA) plates and incubated at 28 °C. The different colonies obtained were streaked on NA plates until a pure culture of each colony was found. The pure cultures were streaked on respective olive effluent based agar plates (10 - 100%) and incubated at 28 °C. The isolates that grew on 10% to 100% olive effluent are kept on NA at 4 °C and transferred every four weeks.

2.2.2 Characterisation of bacterial isolates from olive effluents

The isolates were characterised physiologically by their growth temperature, growth pH and their ability to grow in the presence of NaCl. These bacterial isolates were also checked by their Gram staining and their ability to degrade some of the phenolic compounds that are reported to be found in olive effluents.

2.3 SCREENING OF MICROORGANISMS FOR GROWTH IN OLIVE EFFLUENTS

2.3.1 Effluent preparation

For this study FB and LV olive effluents were used. Each effluent was diluted to 50% using distilled water. The effluents were centrifuged and filtered through a

Whatman filter paper to remove suspended solids, then autoclaved and allowed to cool to room temperature before use. The 50% solutions were used as the stock solutions for all further analysis.

2.3.4 Preliminary plate study

Plate experiments were conducted to determine the ability of microorganisms to grow on olive effluents, using the microorganisms listed below. The solid media for each microorganism was supplemented with the respective olive effluent, in a series of plates made with the final effluent concentrations of : 10, 20, 30, 40 and 50%. The plates were inoculated with the different microorganisms and incubated at a temperature suitable for the microbe used. All experiments were conducted in triplicate. Microorganisms used were as follows:

Bacterial isolates: RU-LV1, RU-FB1, RU-FB2, RU-SO1, RU-SO2

Pseudomonas putida strains: RU-KM1 and RU-KM3$_s$

Fungi: *Trametes versicolor*, *Neurospora crassa*

2.3.5 Flask culture study

2.3.5.1 Bacteria

A series of 250 mL flasks were prepared containing 100 mL of culture medium and inoculated on the basis of the results of the plate experiments. Media were prepared in the same way as for the plate study except that the agar was replaced with broth, and only 10 and 20% effluent final concentrations were used. The flasks were inoculated with the selected bacterial isolate and incubated with shaking (200 rpm) at a temperature suitable for that microorganism. All experiments were conducted in duplicate.

2.3.5.2 Fungi

(a) Trametes versicolor from a malt extract agar was homogenized under sterile conditions and 5 mL of the homogenate was transferred to 250 mL flasks each containing 45 mL of Trametes defined media (TDM) (Appendix) supplemented with 10% or 20% of LV and 5% or 10% FB effluent final concentration. These were then incubated at 30° C with (200 rpm) or without shaking for 7d.

Trametes versicolor was also grown statically in TDM or TDM supplemented with olive effluent. After seven days, the fungi was transferred to fresh media in the presence or absence of the olive effluent, and incubated at 30 °C, without shaking for 7d. All experiments were conducted in duplicate.

(b) Neurospora crassa – 50 mL of half strength Vogel's medium N (Appendix) was inoculated with *Neurospora crassa* and incubated at 28 °C for 48 hours with shaking (130 rpm). The culture was homogenized under sterile conditions and 5 mL of the homogenate was transferred to 250 mL flasks each containing 45 mL of half strength Vogel's medium N, supplemented with 10% or 20% LV and 5%, 10% or 20% FB final concentration. These were then incubated at 28 °C with (130 rpm) or without shaking for 7d.

2.3.5.3 Analysis of flask cultures

The flask cultures were centrifuged at 10000 rpm for 10 minutes and the supernatants were used to determine the total phenol content as described in Section 2.1.3.

2.4 DEGRADATION OF MODEL COMPOUNDS BY BACTERIAL ISOLATES FROM OLIVE

Growth and substrate utilization by the five bacterial isolates was measured in 250 mL Erlenmeyer flasks containing 100 mL of nutrient broth and 1 mM or 5 mM of the model compounds. The flasks were inoculated with each of the bacteria and incubated at 28 °C with shaking at 200 rpm. Every day, from day 0 (the day of inoculation) to day 5, one mL of the culture medium was removed. Growth was measured as optical density at 600 nm, the sample was then centrifuged at 13000 rpm for 5 minutes and the supernatants were kept in the freezer at – 20 °C until they were analysed by HPLC.

2.4.1 HPLC quantitative analysis of degradation of model compounds by bacterial isolates

The supernatants were analysed with a LaChrom HPLC system equipped with a UV detector and a 80 position autosampler autoinjector. Separation was achieved on a Wakosil II C 18 Reverse phase column with a guard column positioned before the analytical column. The detector wavelength was set at 280 nm. The mobile phase, at a flow rate of 1 mL per minute, was water : methanol : acetic acid (68:30:2).

2.5 INVESTIGATION INTO THE BIOCONVERSION OF VANILLIC ACID AND FERULIC ACID BY RU-LV1

2.5.1 Degradation of vanillic and ferulic acid by RU-LV1 in minimal media

RU-LV1 was grown in 250 mL flasks containing 100 mL of minimal media (MM) (Appendix A) with the substitution of glucose by 1 mM ferulic acid or vanillic acid. In other flasks RU-LV1 was grown in MM with 0.1 % glucose and

after 24 hours of growth, 1 mM ferulic acid or vanillic acid was added to the culture medium. The flasks were incubated at 28° C at 200rpm. Each day, from the day of inoculation for cells grown in MM with the phenolic acids as a carbon source, or the day of addition of the aromatic compounds after 24 hours of growth of RU-LV1, a sample (1 mL) was taken from the culture medium, centrifuged at 13000 rpm for 5 minutes and stored in the freezer at – 20 C until analysed by HPLC.

2.5.2 Degradation of vanillic and ferulic acids using resting cells, french pressed extract, cell free extract or cell debris of the bacterial isolate RU-LV1

Resting cells – Microorganisms are grown and harvested by centrifugation, the cell pellet is suspended in buffer and then used as resting cells.
French pressed extract – The cell suspension in buffer is passed through a French press and the extract is used.
Cell free extract – After passing the cell suspension through the French press, the French pressed extract is centrifuged and the supernatant is used as a cell free extract.
Cell membrane fractions – After passing the cell suspension through the french press and centrifuging, the cell pellet is used as the cell debris.

2.5.2.1 Culture conditions for resting cells

RU-LV1 was grown in minimal media with glucose as a carbon source, or minimal media supplemented with 0.01% vanillic acid as a carbon source or minimal media with 0.1% glucose and 0.01% vanillic acid added after 12 hours of growth. The culture medium was incubated at 28 °C at 200 rpm for 24 hours.

2.5.2.2 Resting cells

Cells cultivated in 250 mL shake flasks containing 100 mL medium (as above) were harvested when they reached 1.00 – 1.50. The cells were collected by

centrifugation at 10000 rpm for 10 minutes, washed in sterile 0.1 M sodium phosphate buffer (pH 7) and then resuspended in the same buffer to a concentration of 100mg of cells per mL of buffer. The cell suspension was used immediately, as described in section 2.4.2.4.

2.5.2.3 French pressed extract, cell free extract and cell membrane fractions

The cell suspension prepared as described above was passed through a French press. The French pressed extracts were used as they were or were centrifuged at 10000 rpm for 10 minutes to remove cell debris. The cell free extracts were used immediately. The cell debris was kept at – 20 °C freezer until further use.

2.5.2.4 Biotransformation by resting cells, French pressed extract, cell free extracts or cell debris

One mL of vanillic acid or ferulic acid in sterile sodium phosphate buffer (0.1 M, pH 7) was mixed with 1 mL resting cell suspension, French pressed extract, cell free extract or cell debris in buffer. The mixture was incubated with shaking at 200 rpm, at 28 °C. Samples were taken every 2 hours for 24 hours. The samples were centrifuged at 13000 rpm to remove cell debris and 500 µL 12% tricarboxylic acetic acid was added to stop the reaction. The supernatants were analysed by HPLC as described in Section 2.2.4.

2.6 IDENTIFICATION OF RU-LV1 USING MOLECULAR TECHNIQUES

2.6.1 Extraction and purification of genomic DNA

DNA was extracted in a similar manner to that outlined in "Current protocols in Molecular Biology" (Ausubel *et al.*, 1983). The chromosomal DNA extracted from RU-LV1 contained contaminant RNA. Removal of this RNA was therefore required. RNase enzyme was utilized in order to degrade the RNA present in the sample. A final concentration of 10 µg/mL was used and the reaction was then

incubated at 37 °C for half an hour. Once the RNA had been degraded, the RNase protein was removed so that a PCR reaction could be carried out. Removal of the RNase was achieved using a phenol DNA extraction and DNA precipitation protocol (Appendix D).

2.6.2 Analysis of the DNA

The concentration of the extracted and purified DNA was estimated by comparison to λ DNA of a known concentration eletrophoresed at 80V on a 1% agarose gel. The DNA was visualized using ethidium bromide which interchelates between base-pairs of the DNA and is fluorescent when exposed to ultra-violet light (Sambrook *et al.*, 1989). Computerised photographic analysis of the gel was then done using Kodak Digital Imaging System KDS1D 2.0. The concentration of the extracted and purified DNA was also estimated by using the GeneQuant RNA/DNA Calculator (Pharmacia/Biotech).

2.6.3 Amplification of the 16S rRNA gene

The primers utilized in the amplification of the 16S rRNA gene were the universal primers GM5F and 907RG. Approximately 500 ng of genomic DNA and 0.5 units of Taq polymerase (Promega) was used in a 50 µL PCR reaction mixture. Polymerase buffer (X10), 5 µM GM5F and 5 µM 907RG primers were used along with 0.2 mM of each of the dNTPs. The reaction mixture was made up to 50 µL with sterile $dddH_2O$ and subject to thermal cycling (Table 2.1). The PCR product was purified using the High Pure PCR Product Purification Kit (Roche).

Table 2.1 Thermal cycling program utilized for PCR reaction

STEP	TEMPERATURE	TIME	NO OF CYCLES
INITIAL DENATURATION	95 °C	60 seconds	1
DENATURATION	94 °C	30 seconds	4
ANNEALING	68 °C	45 seconds	
EXTENSION	72 °C	3 minutes	
DENATURATION	94 °C	30 seconds	4
ANNEALING	66 °C	45 seconds	
EXTENSION	72 °C	3 minutes	
DENATURATION	94 °C	30 seconds	4
ANNEALING	64 °C	45 seconds	
EXTENSION	72 °C	3 minutes	
DENATURATION	94 °C	30 seconds	4
ANNEALING	62 °C	45 seconds	
EXTENSION	72 °C	3 minutes	
DENATURATION	94 °C	30 seconds	12
ANNEALING	60 °C	45seconds	
EXTENSION	72 °C	3 minutes	
FINAL EXTENSION	72 °C	4 minutes	1

2.6.4 Cloning and sequencing of the amplified 16S rRNA gene of RU-LV1

The sequence of RU-LV1 16S rRNA gene was determined by cloning the PCR fragments generated previously into pGEM-T Easy Vector and transforming the recombinant vector into *Escherichia coli* DH5ά cells.

i) Ligation

The 16S rRNA PCR product from RU-LV1 was inserted into appropriate vector using the pGEM-T Easy vector kit (Promega)

ii) Transformation

The ligation reaction was incubated at 4 °C overnight after which 10 µL of the ligated DNA was transformed into competent *E. coli* DH5ά. Preparation of competent *E. coli* DH5ά cells was carried out by the method described in Appendix E.

White colonies, indicating the presence of an insert in the vector, were picked off the agar plate using sterile toothpicks and place into sterile test tubes containing 5 mL of Luria broth (Appendix F) containing ampicillin. The test tubes were then placed on a rotation shaker at 37 °C overnight. Once grown, 1.5 mL of each of the cultures were placed into eppendorfs and microfuged for 1 minute to pellet the cells. The supernatant was removed and the cell pellet was resuspended in 50 µL Smart buffer (Appendix G) (Berghammer and Auer, 1993). The reaction mix was incubated at room temperature for 10 minutes. The lysed cells were placed in boiling water bath for precisely 1 minute, in order to inactivate the enzyme present in the Smart buffer, after which they were immediately placed on ice. The eppendorfs were microfuged for 10 minutes to precipitate the cell debris and chromosomal DNA whilst leaving the plasmid DNA in solution. 10 µL of the supernatant was used per 20 µL restriction digest in order to verify the presence of the correct insert in the plasmid isolated. The restriction endonucleases utilized were Sac1 and Ksp1 (Roche) (1 unit of each). The reaction mixture was made up to 20 µL with sterile dddH_2O and incubated at 37 °C for approximately 2 hours. After digestion, 20 µL of each reaction was eletrophoresed on a 1% agarose gel at 80 V and the separated DNA fragments visualized using Kodak Digital Imaging System KDS1D 2.0.

Restriction digests of transformants which resulted in the correct size inserts were then recultured in 5 mL Luria broth with ampicillin at 37 °C. A High Pure Plasmid Isolation Kit (Boehringer Mannheim, Germany) was used to isolate the recombinant clone containing the correct size.

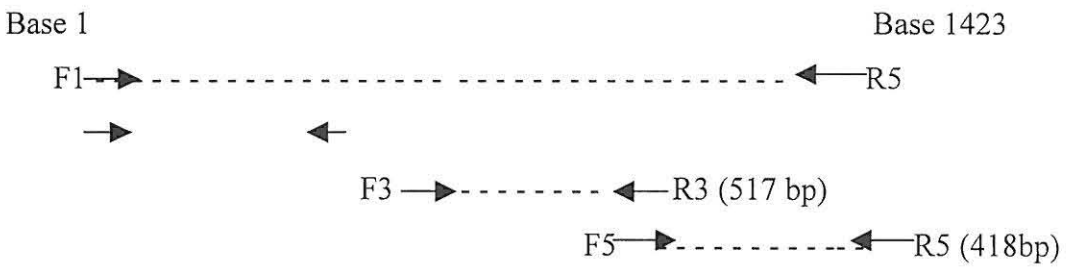
To verify that the purified vector plasmid contains the 16S rRNA gene as its insert, restriction digests with Sac1 and Ksp1 were carried out.

The RU – LV1 16S rRNA gene cloned into pUC18 was then sequenced using Big Dye. The sequencing reaction contained big dye terminator ready mix (4 μL), DNA (2.5 μL), primer (2 μL), dilution buffer (2 μL) and deionozed water (9.5 μL). The primers used were puCF (1.5 pmol/μL) and puCR (1.5 pmol/μL).

2.6.5 Sequence analysis of the 16S rRNA region of bacteria

DNA was also sent to UCT and direct sequencing of PCR products was done by Di James (UCT) and the following method was followed.

Primers used for amplification (PCR) of the 16s sequence were designed so that the 5' end sequences were the same as the Cy5 end-labelled (fluorescent) primers used for sequencing. The length of the 16S rRNA sequence is approximately 1423bp using universal primers 16S rRNA primers (F1; F3; F5; forward and R1; R2; R3; reverse). Three forward and three reverse PCR primers were used to cover the entire length. Four PCR reactions were performed on each genomic DNA. In the first one the two outer most primers (F1 and R5) were used. The other three reactions used combinations of primers to produce products of approximately 500 bp each, for example:



R1/F3 and R3/F5 primers do not overlap, the junctions were checked by sequencing the amplified products of the F1R5 primers across them.

DYEnamic ET Dye terminator cycle sequencing kit for MegaBase was used.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 CHARACTERIZATION OF OLIVE EFFLUENTS

The phenolic composition of olive mill wastewater (OMW) has been studied by several authors (Balice and Cera, 1984; Hamdi, 1993; Montedoro *et al.*, 1992; Capasso *et al.*, 1992). However, no characterisation of olive production wastes has been reported in the literature and this study reports the work done to characterise olive production effluents obtained from an olive processing plant in the Western Cape, South Africa.

Three types of olive effluents used in this study were labelled as follows:

FB- collected from the fermentation tank.

LV- collected from the surface of the digester tank.

SO- collected from the evaporation pond

The olive effluents FB, LV and SO were reddish-brown, black and light green respectively. The chemical analysis of the olive effluents is shown in Table 3.1 and FB contained higher conductivity, COD and chlorides as compared to LV and SO. However the COD was lower than that of OMW, which has been reported to be 220 g/L (Hamdi, 1993; Borja *et al.*, 1993). The lower COD suggest that olive production effluents are less of an environmental problem than OMW.

Table 3. 1 Chemical analysis of the olive effluents FB, LV and SO.

Effluent	Conductivity (mS/m)	COD (mg/mL)	Chlorides
FB	7160	32758	37071
LV	1288	2238	4890
SO	567	562	3624

3.1.1 Total phenolic content of olive effluents

In experiments to determine their phenolic content, samples of the olive effluents, and samples acidified to pH 2 with hydrochloric acid, were extracted three times with ethyl acetate at room temperature. The three organic extracts were combined and dried with anhydrous sodium sulphate. The residue was concentrated to dryness in a rotary evaporator and re-dissolved in methanol/water. The total phenolic content of the effluent extracts was determined by the Folin-Ciocalteau assay (Appendix B) (Garcia *et al.*, 2000).

The total phenolic content of the olive effluents FB, LV and SO and that of the same olive effluents adjusted to pH 2 is shown in Figure 3.1. Effluent FB, LV and SO were found to contain 1361.6, 675.4 and 30 mg/L total phenolics respectively. When the extracts were made after acidification of the effluents, the total phenolic content of FB, LV and SO were found to be 1572.9, 335.4 and 1.7 mg/L respectively. The phenolic content of the extract obtained from FB was higher after adjustment to pH 2, whereas the phenolic content of the other two acidified effluents was lower. The pH of the olive effluents FB, LV and SO when sampled were found to be 3.51, 7.86 and 7.70 respectively. FB contained a higher phenolic content than the other two effluents. A possible explanation for the reduced phenolic content in LV and SO as compared to FB might be the partial natural biodegradation of the phenolic compounds prior to sampling. FB is collected from the fermentation tank which is the first effluent after the processing of olives. From the fermentation tank the effluent goes to the digester and then to the evaporation pond. The storage of olive mill wastes in evaporation ponds partially reduces contamination of OMW with phenolic compounds (Moreno *et al.*, 1987). This is observed in this study whereby SO collected from the evaporation pond had the lowest total phenolic content as compared to the other olive production effluents.

The total phenolic content of FB is in the same range as the total phenolic content of OMW which has been reported to be between 1.2 and 16.8 g/L (Hamdi 1991; Nieto *et al.*, 1993; Garcia *et al.*, 2000). The lower phenolic content in the olive effluents LV and SO suggest that these two olive production wastes are less harmful pollutants as compared to FB.

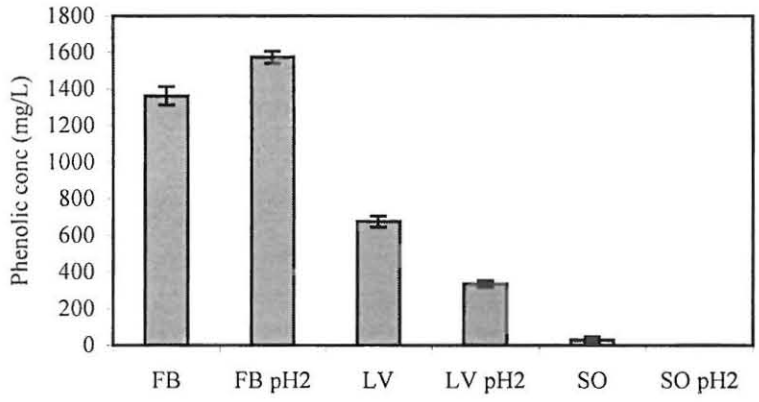


Figure 3.1 Total phenolic content of olive effluents before and after adjusting to pH 2. FB, LV and SO olive effluents were extracted using ethyl acetate, the organic extract was dried over anhydrous sodium sulphate and evaporated. Then the extracts were dissolved in methanol/water and the total phenolic content was measured by the Folin-Ciocalteau's method (Appendix B).

3.1.2 UV analysis of effluent extracts dissolved in different solvents

The effluents were extracted as explained above but the residue obtained was dissolved in methanol, ethanol and water-acetonitrile (60:40) and UV analysis in the range 200-800nm was carried out to determine the wavelength at which the effluent extracts absorbed and hence to investigate the nature of the compounds present in the effluent extracts.

The olive effluents LV and FB were used in UV analysis and the wavelengths at which the effluent extracts absorbed are shown in Table 3.2. In all the solvents used to dissolve the effluent extracts, methanol, ethanol and water-acetonitrile, the compounds in the effluent extracts absorb at almost the same wavelength. The wavelength at which the effluent extracts absorbed was found to be between 270 and 310 nm. These wavelengths indicate the presence of aromatic compounds in the effluent extracts.

Table 3.2 Wavelengths at which effluent extracts absorbed when UV analysis was carried. The extracts were dissolved in methanol, ethanol and water-acetonitrile (60:40).

Effluent type	Methanol	Ethanol	Water-Acetonitrile (60:40)
LV	275 nm	280 nm	299 nm
FB	278 nm	293 nm	301 nm
LV (pH1)	277 nm	288 nm	302 nm
FB (pH1)		309 nm	301 nm

3.1.3 Thin layer chromatography (TLC) analysis of standard compounds and effluent extracts

TLC is a useful technique because it is relatively quick and requires small quantities of materials. As a preliminary test TLC was used to investigate the number of components in the olive effluents LV and FB and to obtain an indication of their polarity. TLC analysis of the effluent extracts and standard compounds was carried out on Silica plates eluting with 10% acetic acid in chloroform.

The retention factor (R_f) values of effluent extracts and standard compounds are listed in Table 3.3. The results indicated that FB separated into three components, LV into two components, FB (pH 1) into four components and LV (pH 1) into two components when they were analysed using UV light (Figure 3.2). The colour of FB and FB (pH 1) components was observed to be light and dark brown respectively. Protocatechuic acid was found to be dark brown in colour and it migrated on the TLC plate with approximately the same distance as one of the components in FB and FB (pH 1) effluent extracts. *p*-Coumaric acid was yellow in colour and it migrated the same distance as the third component of the FB pH 1 extract. Ferulic acid, syringic acid and vanillic acid were found to have the same R_f value which corresponded to the R_f of the fourth component of the effluent extract of FB pH 1. Caffeic acid and 4-hydrobenzaldehyde separated into two and three components on the TLC plate respectively. The migration of the effluent extract components in FB pH 1 with approximately the same R_f values as those of the standard compounds suggest that protocatechuic acid, ferulic acid, syringic acid and vanillic acid are present in the olive effluent FB. However these results needed to be confirmed by separating and analysing the effluent components in the extracts using different mobile phases for TLC or different analytical methods. Therefore HPLC analysis of the effluents was carried out.

Table 3.3 R_f values of effluent extract and standard compounds on TLC plates.

Compound	R_f values
p-Coumaric acid	0.75
Protocatechuic acid	0.26
FB pH 1Component 1	0.17
FB pH 1Component 2	0.25
FB pH 1Component 3	0.67
FB pH 1 Component 4	0.92
4-Hydrobenzaldehyde	0.17, 0.28, 0.97
Syringic acid	0.91
LV Component 1	0.17
LV Component 2	0.92
Caffeic acid	0.25, 0.89
Ferulic acid	0.91
LV pH 1 Component 1	0.17
LV pH 1 Component 2	0.91
Vanillic acid	0.9
4-Hydroxyphenyl acetic	0.58
FB Component 1	0.17
FB Component 2	0.3
FB Component 3	0.42

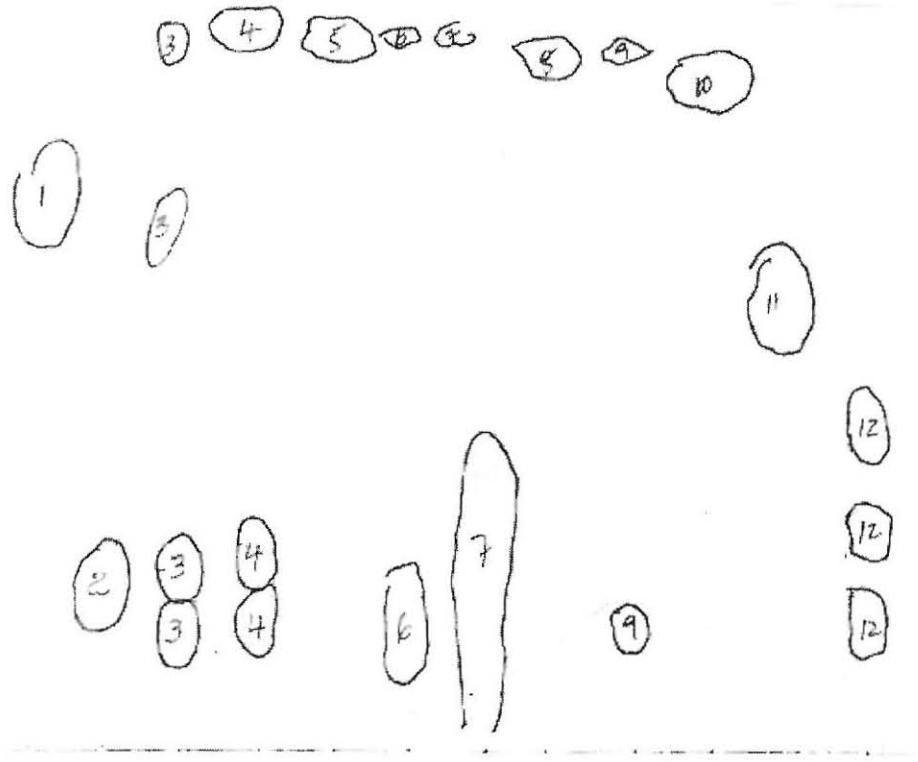


Figure 3.2 TLC of standard compounds and effluent extracts detected under UV analysis. The extracts and model compounds were dissolved in methanol and the mobile phase was 10% acetic acid in chloroform. 1= *p*-Coumaric acid, 2 = Protocatechuic acid, 3 = FB pH 1 extract, 4 = Hydroxybenzaldehyde, 5 = Syringic acid, 6 = LV extract, 7 = Caffeic acid, 8 = Ferulic acid, 9 = LV pH 1 extract, 10 = vanillic acid, 11 = 4-Hydroxyphenyl acetic acid, 12 = FB extract

3.1.4 High performance liquid chromatography (HPLC) analysis of standard compounds and effluent extracts

HPLC analysis was carried out to characterize the compounds in the effluents. The effluents FB and LV were centrifuged, filtered and analysed by a La Chrom HPLC system. The mobile phase used was water/methanol/acetic acid (68:30:2). When the effluents were analysed by HPLC it was observed that LV components

were polar as compared to FB components since LV components eluted earlier than FB components. But on the overall the components in the effluents are polar because they eluted before 20 minutes. FB contained more aromatics than LV as it can be noticed by the number of component peaks and the higher absorbance in the FB HPLC profile (Figure 3.3 and 3.4). A better separation of components was observed in FB.

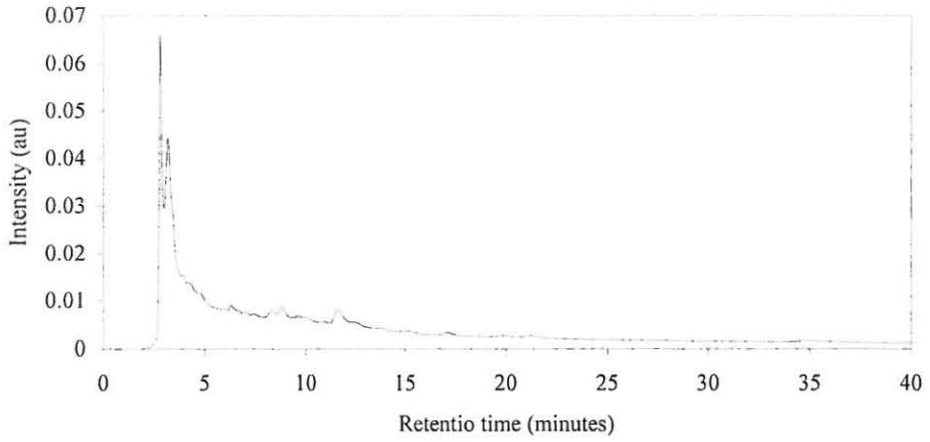


Figure 3.3 HPLC profile of the olive production effluent, LV. LV was centrifuged, filtered and analysed by HPLC. Mobile phase used was water/methanol/acetic acid (68:30:2).

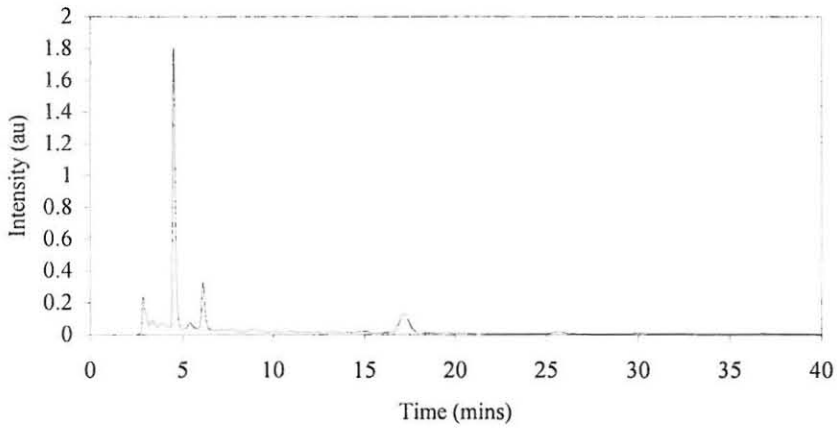


Figure 3.4 HPLC fingerprint of the olive production waste, FB. The effluent FB was centrifuged, filtered and analysed by HPLC. Mobile phase used was water/methanol/acetic acid (68:30:2).

Ethyl acetate has been reported to be the most effective solvent for recovering phenolic compounds from wastes (Perez *et al.*, 1992). Therefore the effluents were extracted three times with an equal volume of ethyl acetate, dried over anhydrous sodium sulphate and evaporated. The residue was dissolved in methanol and analysed by HPLC using a variety of mobile phases which include acetonitrile-water (40:60), water-acetic acid (98:2) and water-methanol-acetic acid (68:30:2).

The HPLC results obtained showed that separation of the effluents was achieved using a mobile phase of water-methanol-acetic acid (68:30:2). A better separation was observed when the LV extract was analysed by HPLC (Figure 3.5). The HPLC fingerprint of the FB organic extract showed more component peaks as compared the fingerprint of FB effluent (Figure 3.6). The effluent extracts were concentrated (20x) and this can be observed in the higher absorbance of the components in the effluent extract HPLC profiles. The FB extract had more

detectable components at a higher concentration as indicated by the higher absorbance as compared to that of the LV extract.

To facilitate the extraction procedure, the effluents were adjusted to pH 1 and the acidified effluents were extracted with ethyl acetate as explained above. The effluent extract was analysed by HPLC. The basic HPLC fingerprint of the FB extract did not change much on acidification. However, the component peaks at retention time 7.6, 10.68 and 13.99 increased in the FB extract adjusted to pH 1 (Figure 3.8). For LV pH 1 extract the absorbance of the HPLC profile doubled upon acidification and the component peaks at 4.35 and 5.85 minutes increased (Figure 3.9).

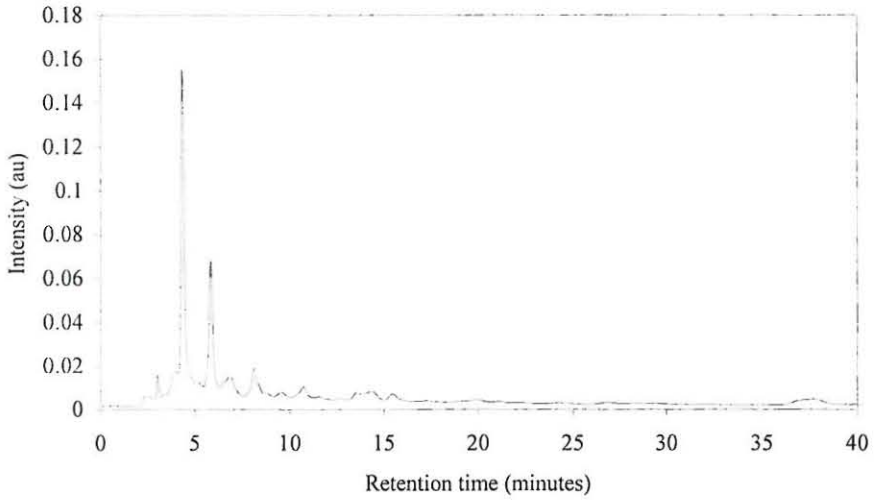


Figure 3.5 HPLC fingerprint of LV extract. LV was extracted with ethyl acetate, dried and evaporated. The residue was dissolved in methanol and analysed by HPLC. Mobile phase used was water/methanol/acetic acid (68:30:2).

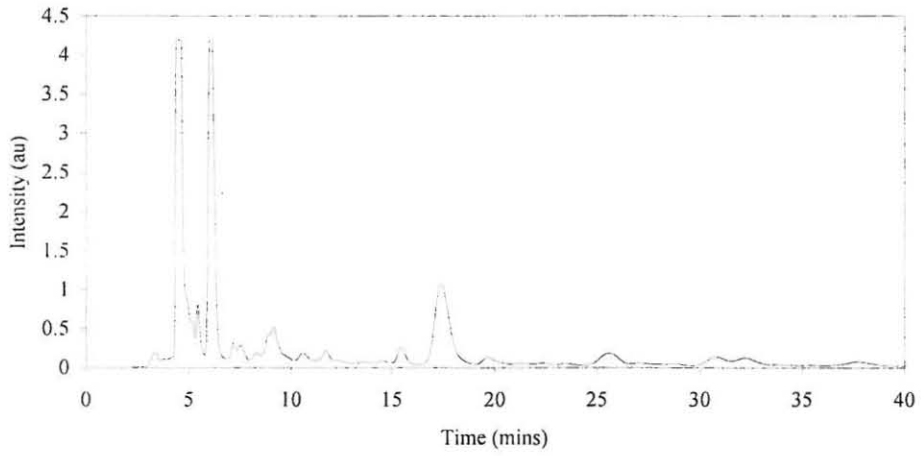


Figure 3.6 HPLC profile of FB extract. FB was extracted with ethyl acetate, dried and evaporated. The residue was dissolved in methanol and analysed by HPLC. Mobile phase used was water/methanol/acetic acid (68:30:2).

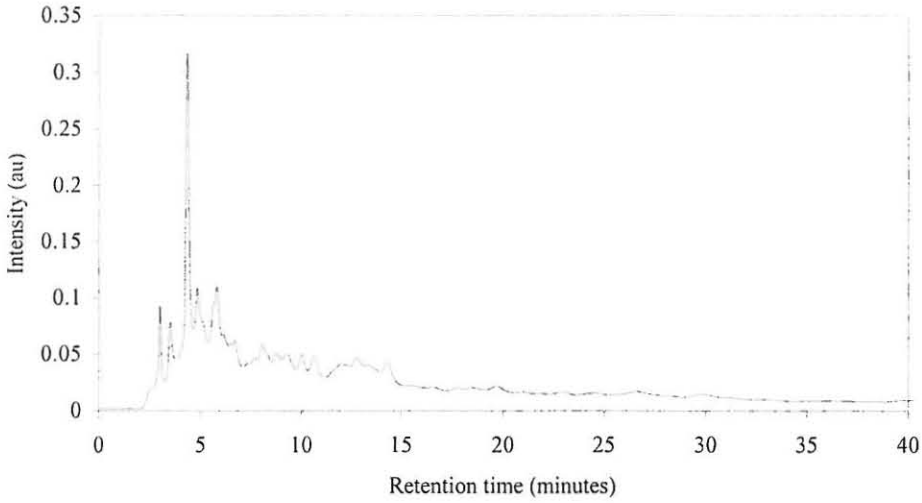


Figure 3.7 HPLC fingerprint of LV pH 1 extract. LV was adjusted to pH 1, extracted with ethyl acetate, dried and evaporated. The residue was dissolved in methanol and analysed by HPLC. Mobile phase used was water/methanol/acetic acid (68:30:2).

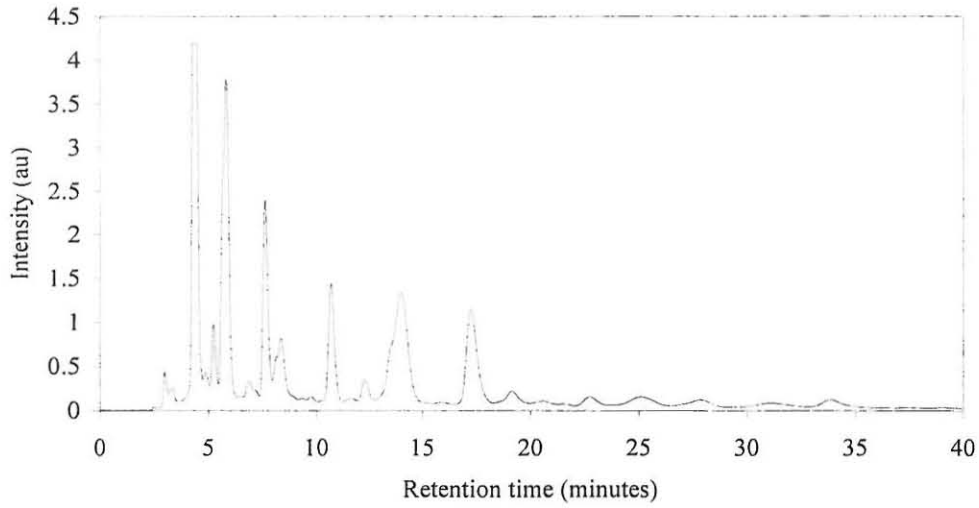


Figure 3.8 HPLC profile of FB pH 1 extract. FB was adjusted to pH 1, extracted with ethyl acetate, dried and evaporated. The residue was dissolved in methanol and analysed by HPLC. Mobile phase used was water/methanol/acetic acid (68:30:2).

When analysing OMW using HPLC, a first idea of the compound can be obtained by comparing retention times in the HPLC chromatogram with those of standard compounds (Knupp *et al.*, 1996). Therefore model compounds known to be found in OMW were dissolved in methanol and HPLC analysis was carried out, using water-acetonitrile (60:40) and water-methanol-acetic acid (68:30:2) as mobile phases, to compare their retention times with the retention times of the components in the effluent extract.

The effluent extract FB pH 1 was chosen for identification of the components observed in its HPLC "fingerprint". The retention times of the standard compounds were comparable to those of the extracted organic compounds in the

effluent FB adjusted to pH 1. When the mobile phase of water-acetonitrile (60:40) was used, some of the standard compounds were eluted with almost the same retention time (Table 3.4). The mobile phase that did give better separation of components in olive effluents and the reference compounds eluted at different times was water-methanol-acetic acid (68:30:2), and therefore this mobile phase was used in all the other HPLC analysis.

To confirm the identity of the peaks in the olive effluent extract (FB pH 1) after comparison of retention times of the standard compounds, additional identification was carried out using "spiking experiments". This involved the addition of each individual model compound to the FB pH 1 effluent extract and analysis by HPLC. If the peak area of the component of interest increased in proportion with the added standard compound, it can be confirmed that the model compound is contained in the effluent.

Ten standard compounds, ferulic acid, protocatechuic acid, *p*- and *m*- coumaric acid, syringic acid, hydroxy phenyl acetic acid, vanillic acid, caffeic acid, hydroxybenzaldehyde and catechol, were added to FB pH 1 extract at 1 mM concentration during spiking experiments. In this extract, six model compounds were identified by HPLC "spiking experiments": protocatechuic acid, hydroxy phenyl acetic acid, vanillic acid, syringic acid, coumaric acid and ferulic acid (Figure 3.9). Five of the standard compounds also had approximately the same R_f values as the components found in FB pH 1 extract when analysed by TLC (Section 3.1.3). Vanillic acid, *p*-hydroxy phenyl acetic acid, syringic acid, coumaric acid, ferulic acid and protocatechuic acid have been identified in OMW using gas chromatography, TLC or HPLC (Balice and Cera, 1984; Perez *et al.*, 1992; Knupp *et al.*, 1996). These results suggest that olive production effluent FB pH 1 has the same phenolic composition as OMW.

Table 3.4 HPLC retention times of standard compounds found in methanol when eluted using acetonitrile-water (60:40) and water-methanol-acetic acid (68:30:2)

Standard compound	Water-acetonitrile (60:40)	Water-methanol-acetic acid (68:30:2)
Caffeic acid	1.825	8.41
Ferulic acid	1.900	17.14
p-Coumaric acid	1.875	13.24
m-Coumaric acid	1.866	13.53
Protocatechuic acid	1.891	4.85
p-Hydroxyphenylacetic acid	1.916	5.91
Syringic acid	1.958	10.68
Vanillic acid	1.991	7.60
p-Hydroxybenzaldehyde	3.433	8.15
4-Chlorophenol	7.416	ND
2,4-Dichlorophenol	9.525	ND
p-Cresol	6.225	ND
4-Methoxyphenol	4.633	ND
m-Cresol	6.225	ND
Phenol	5.016	ND
Catechol	ND	5.25

ND = Not done

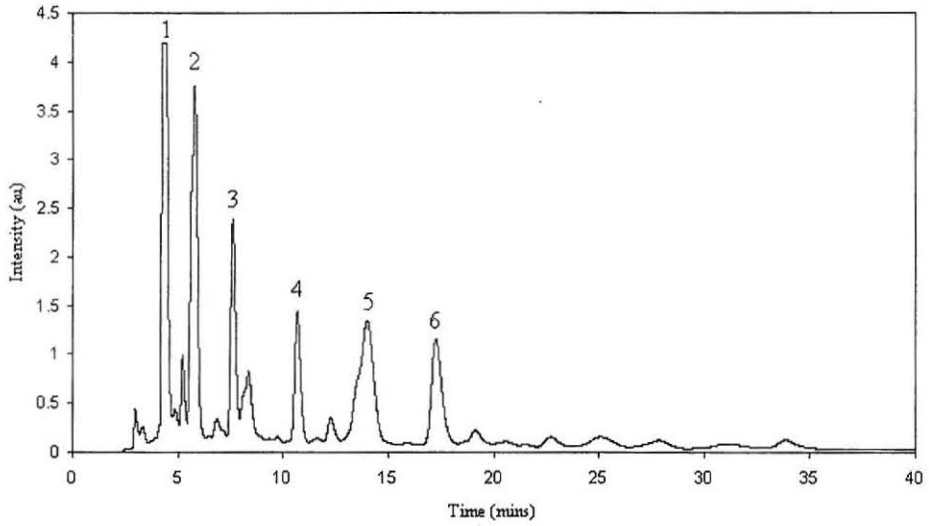


Figure 3.9 HPLC fingerprint of components extracted from FB pH 1. FB was acidified to pH 1, extracted with ethyl acetate, dried and evaporated. The residue was dissolved in methanol and analysed by HPLC. Mobile phase used was water/methanol/acetic acid (68:30:2). Aromatic compounds were added one at a time to FB pH 1 extract and the increase of the peak area of interest in proportion to the concentration of the added model compound was noticed. 1 = Protocatechuic acid, 2 = Hydroxy phenyl acetic acid, 3 = Vanillic acid, 4 = Syringic acid, 5 = Coumaric acid, 6 = Ferulic acid.

Quantitative results of compounds found in the FB pH 1 extract have been evaluated by comparing their peak areas with calibration curve areas (Appendix C). The concentrations of the identified model compounds in the effluent extract FB adjusted to pH 1 are shown in Table 3.1.4. Syringic and 4-hydroxyphenyl acetic acid have been reported to constitute almost 50% of the total phenolic acids identified in OMW (Balice and Cera, 1984). In this study 4-hydroxyphenyl acetic acid constitute over 60% of the aromatic compounds identified in the extract of the olive production waste FB adjusted to pH 1 and this indicate a difference between olive production wastes and OMW.

Table 3.5 Concentration of the standard compounds found in FB adjusted to pH 1

Compound	Concentration (mM)
Protocatechuic acid	25.12
Vanillic acid	6.7
Syringic acid	3.1
Coumaric acid	1.28
Ferulic acid	4.45
p-Hydroxyphenylacetic acid	67.8

The results of the characterisation of olive production effluents suggest that the first effluent after olive processing, FB, is similar in composition to OMW since its COD, total phenolic content and aromatic compounds identified in it are similar to those that have been reported in OMW. This should not be surprising since the phenolics in OMW have been attributed to phenolics found in the cell walls of olives (Capasso *et al.*, 1995). However since these effluents are produced during olive processing as compared to olive oil extraction the pollution problem is less than the problem of OMW.

3.2 ISOLATION OF MICROORGANIMS FROM WESTERN CAPE OLIVE EFFLUENTS

Olive mill wastewater (OMW) is known to contain a high antimicrobial activity due to its high phenolic content. But microorganisms have been isolated from OMW and soil treated with OMW (Mouncif *et al.*, 1993; Mechichi *et al.*, 1999 a and b; Millan *et al.*, 2000, Koussemon *et al.*, 2001). To our knowledge no research has been done on the microbial activity of olive production wastewater from the Western Cape in South Africa. This study deals with the isolation of bacteria from olive production effluents from the Western Cape.

Five bacterial isolates were cultured from the three olive production effluents used. The bacteria were named according to the effluent they were isolated from and the place the isolation was done, RU for Rhodes University. RU-LV1, RU-FB1 and RU-FB2, and RU-SO1 and RU-SO2, were isolated from LV, FB and SO respectively. These isolates were observed to grow in 10% to 100% of the respective olive effluents in agar plates. Since these isolates grew on up to 100% of the respective olive effluents with no nutrient addition, it can be deduced that these microbes can derive all necessary nutrients from the effluents.

All these isolates were Gram negative and rod shaped. The isolates were found to tolerate a wide pH and temperature range (Table 3.6, Figure 3.10 and 3.11) which could be important for treating wastewaters under conditions of pH and temperature fluctuations. The optimum growth temperature for all these bacterial isolates was 28 °C, which is not surprising since these effluents are normally stored at temperatures not exceeding 30 °C. Accordingly in all subsequent experiments the bacterial isolates were grown at 28 °C. During a study of the microbiological characterization of olive mill wastewaters in Morocco, all the microorganisms were also cultured at 28 °C (Mouncif *et al.*, 1993).

The isolated bacteria tolerated high salt concentrations (Table 3.6 and Figure 3.12.) and their growth under high salt concentrations should not be surprising since the olive effluents contain very high salt concentrations (Section 3.1). The growth of the bacteria isolated from the olive effluents in nutrient broth containing high NaCl were lower than in nutrient broth with no addition of NaCl (Figure 3.2.3). The RU-LV1 isolate grew in medium with high NaCl concentration (Figure 3.2.3) and this correlates with a report of a yeast strain, *Debaryomyces*, isolated from olive mill wastewater which showed growth in high NaCl concentrations (12 – 15%) at 37 °C (Mouncif *et al.*, 1993). High salt concentrations of some effluents are a problem in microbial waste treatment

(Yanase *et al.*, 1992). Microbes tolerating high salt concentrations may be useful for the treatment of wastewater with high salt concentrations.

Table 3.6 Physiological characteristics of bacterial isolates from Western Cape olive effluents.

	Isolate				
Physiological characteristic	RU – LV1	RU – FB1	RU – FB2	RU – SO1	RU – SO2
Growth temp	20 – 40 °C	15 – 45 °C	15 – 45 °C	15 – 45 °C	15 – 40 °C
Growth pH	6 - 10	5 - 9	5 - 9	5 - 9	5 – 10
Growth in NaCl	0.015 – 9.00%	0.015 – 6.00%	0.015 – 6.00%	0.015 – 4.00%	0.015 – 5.00%

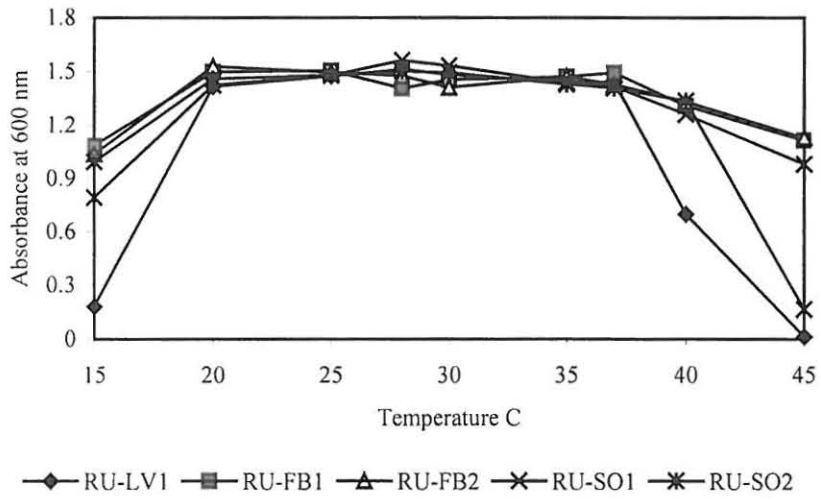


Figure 3.10 Growth, measured as absorbance at 600 nm, of the bacterial isolates at different temperatures. The inoculated liquid medium was incubated at different temperatures with agitation at 200 rpm and after 24 hours of incubation growth was measured.

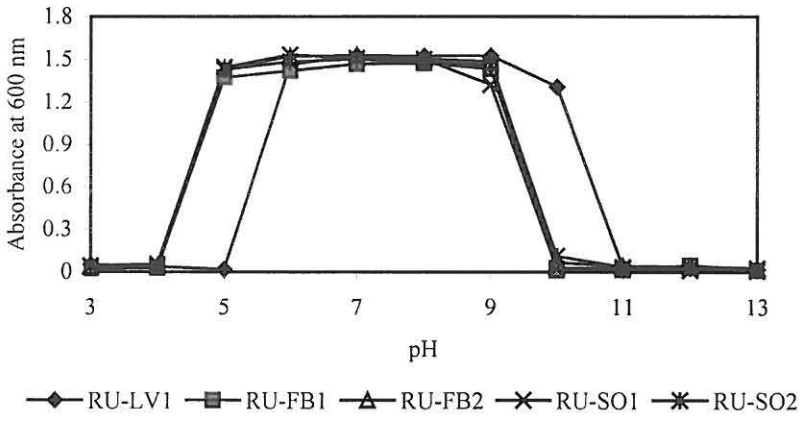


Figure 3.11 Growth, measured as absorbance at 600 nm, of the isolates in liquid medium adjusted to different pH values. The adjusted medium was inoculated with the selected isolate and incubated for 24 hours at 28 °C with shaking at 200 rpm.

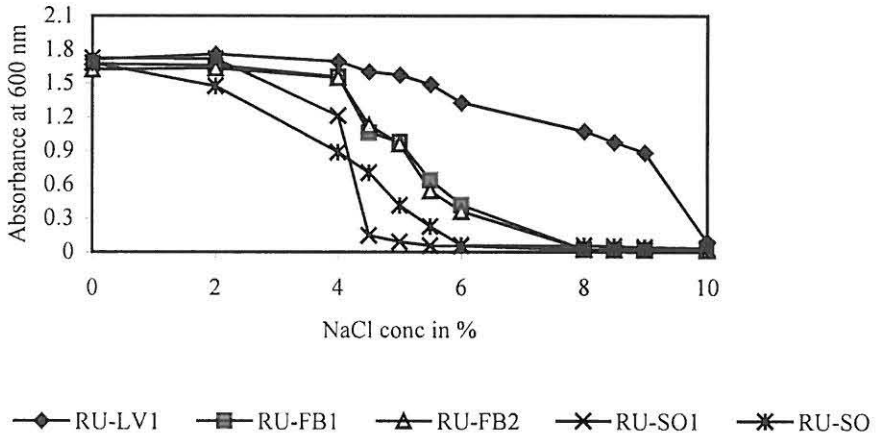


Figure 3.12 Growth, measured as absorbance at 600 nm, of the isolates in nutrient broth with different concentrations of NaCl. The inoculated nutrient broths with varying NaCl concentrations were incubated at 28 °C with shaking (200 rpm) and after 24 hours growth was determined.

Further studies are needed for the identification and taxonomy of the microorganisms which inhabit olive effluents. The microbes isolated from olive effluents can be active in organic matter decay and they can play a role in the degradation of organic components leading to a natural transformation of organic components to minerals.

3.3 SCREENING OF MICROORGANISMS FOR GROWTH IN OLIVE EFFLUENTS

OMW has been reported to have a high antimicrobial activity due to its phenolic content. However, other microorganisms can be screened for growth in OMW. Microbes such as fungi have been studied for their ability to degrade OMW which resulted in reduction of the phenolic content of the waste and sometimes the removal of the dark colour (Sayadi and Ellouz, 1993; Vinciguerra *et al.*, 1993; Yesilada *et al.*, 1995; Scioli *et al.*, 1997; Setti *et al.*, 1998).

3.3.1 Growth of microorganisms on agar plates supplemented with olive effluents

The olive effluents FB and LV were used for this study, since these olive effluents were found to contain higher concentrations of phenolics as compared to SO. Plate experiments were conducted in order to determine the ability of the selected microbes to grow on olive effluents. The selected microorganisms were grown in their respective solid medium (Appendix A) supplemented with FB and LV one at a time, to a final effluent concentration of 10, 20, 30, 40 and 50% in plates.

The growth of microorganisms on agar plates supplemented with olive effluents is shown in Table 3.7 – 3.10. There was nothing added to the agar plates except the growth medium of the microbe and the effluent even though OMW is known to contain very low concentrations of sulphate and nitrogen (Hamdi *et al.*, 1991).

This suggests that the selected microbes are able to grow under conditions of low sulphate and nitrogen concentrations found in the olive effluents.

RU-FB1 and RU-FB2 grew on agar plates supplemented with low concentrations of FB (10 and 20%) and growth inhibition of the other isolated bacteria occurred on plates supplemented with FB (Table 3.7). The isolate RU-LV1 grew well on plates containing up to 50% of LV (Table 3.8). This was expected since this microbe was isolated from the LV olive effluent. The other bacteria isolated from olive effluents grew on agar plates supplemented with LV, but less growth was observed at 40 and 50% concentrations for RU-SO1 and RU-SO2 (Table 3.8). RU-SO1 and RU-SO2 were isolated from the SO olive effluent which contained a lower phenolic content as compared to LV and the weaker growth of these isolates suggests that they were not adaptable to the higher phenolic content of LV. FB is more acidic (pH 3.51) than LV (pH 7.86) and in general bacteria prefer a neutral pH to an acidic one, which may explain why these bacteria grew well on plates supplemented with LV as compared to plates supplemented with FB.

Pseudomonas putida strains RU-KM1 and RU-KM3$_s$ were isolated from Chemistry garden of the Rhodes University (Burton and Dorrington, personal communication) and since the Pseudomonads were isolated from soil which contained aromatic compounds, they might be expected to have the ability to grow in effluents which contain aromatic compounds. These soil isolates have been tested for the treatment of wastewaters but no work has been done using *P. putida* KM1 and KM3$_s$ for growth in olive effluents. The *Pseudomonas putida* strains grew on both of the olive effluents. There was less growth for KM1 at higher concentrations of LV (40 – 50%) and neither of the *P. putida* strains grew at high concentrations of FB (40 – 50%) (Table 3.9).

White rot fungi are known to have the ability to degrade lignin monomers and therefore the ability of *Neurospora crassa* and *Trametes versicolor* to grow on olive production effluents was investigated. Both *N. crassa* and *T. versicolor* grew

on 10 to 50% of each of the olive effluents used (Table 3.10). There was less growth of *T. versicolor* at higher concentrations (40 – 50%) of FB, possibly due to a decrease in easily assimilatable nutrients. Increasing the concentrations of FB in agar plates decreased the amount of solid medium in the plates and thereby decreasing the amount of easily assimilatable nutrients that are found in the growth medium. These results correlate with results found by Setti *et al.* (1998) where these authors reported that the amount of biomass from *Pleurotus ostreatus* decreased proportionally with the decrease of the malt extract concentration when the microbe was grown in 10 –100% OMW.

Table 3.7 Growth of bacterial isolates from olive effluents on agar plates supplemented with the olive effluent, FB.

Isolate	10%	20%	30%	40%	50%
RU-LV1	-	-	-	-	-
RU-FB1	++	+	-	-	-
RU-FB2	+	++	-	-	-
RU-SO1	-	-	-	-	-
RU-SO2	-	-	-	-	-

++ = Growth, + = Poor growth, - = No growth

Table 3.8 Growth of bacteria isolated from olive effluents on agar plates supplemented with the olive effluent, LV.

Isolate	10%	20%	30%	40%	50%
RU-LV1	+++	+++	+++	+++	+++
RU-FB1	++	+	++	++	++
RU-FB2	+	+	++	++	+
RU-SO1	++	++	++	+	+
RU-SO2	++	+	++	+	+

+++ = Significant growth, ++ = Growth, + = Poor growth

Table 3.9 Growth of *Pseudomonas putida* strains, RU-KM1 and RU-KM3$_s$ on agar plates supplemented with olive effluents.

Effluent	Strain	10%	20%	30%	40%	50%
LV	KM1	+ + +	+ + +	+ +	+	+
	KM3$_s$	+ + +	+ + +	+ + +	+ +	+ +
FB	KM1	+ +	+ +	+	-	-
	KM3$_s$	+ +	+ +	+ +	-	-

+ + + = Significant growth, + + = Growth, + = Poor growth, - = No growth

Table 3.10 Growth of the white rot fungi, *Trametes versicolor* and *Neurospora crassa*, on agar plates supplemented with olive effluents, LV and FB.

Effluent	Fungus	10%	20%	30%	40%	50%
LV	*T. versicolor*	+ + +	+ + +	+ +	+ +	+
	N. crassa	+ + +	+ + +	+ + +	+ + +	+ +
FB	*T. versicolor*	+ +	+ +	+ +	+	+
	N. crassa	+ + +	+ + +	+ +	+ +	+

+ + + = Significant growth, + + = Growth, + = Poor growth

3.3.2 Reduction of the phenolic content of olive effluents FB and LV in flask culture experiments

Fungi isolated from OMW have been used to detoxify OMW (Millan *et al.*, 2000) and therefore the reduction of the phenolic content of olive production waste by bacteria isolated from such effluents was investigated in this study. For comparison the removal of phenolics in olive effluents by *Pseudomonas putida* RU-KM1 and RU-KM3$_s$ and the fungi *Neurospora crassa* and *Trametes versicolor* was also explored.

The selected microorganisms were grown in their respective liquid medium (Appendix A) supplemented with the olive effluents, FB and LV, one at a time, to a final effluent concentration of 20% for the bacteria and 5, 10 and 20% for the fungi. The flasks were incubated at ± 28 °C and phenolic content determinations were performed after prior removal of the microbe by centrifugation at 10000 rpm for 10 minutes.

Since no growth was observed when the isolated bacteria were inoculated into nutrient broth supplemented with 20, 10 or 5% FB, no phenolic content removal was determined and these results correlate with the plate experiments where the isolates were not able to grown on nutrient agar plates supplemented with FB. The bacteria isolated from olive effluents were able to grow in nutrient broth supplemented with 20% LV. Degradation of the phenolics in LV by these microorganisms was observed after three days but growing the microorganisms for seven days did not increase the phenolic content removal, and therefore, phenolic content decreases are reported from three days cultures in liquid medium supplemented with 20% LV. In terms of the highest phenol removal, these bacterial isolates can be ordered in the sequence RU-SO1 > RU-LV1 > RU-FB1 > RU-FB2 > RU-SO2 (Figure 3.13). RU-SO1 removed 47.2% of the total phenol content whereas RU-LV1, which was isolated from LV degraded 39.4% of the phenolics found in LV, this is surprising since RU-SO1 was isolated from an olive effluent with a lower phenolic content than LV. The reason for RU-SO1 showing better efficiency in reducing the phenolic content of 20% LV as compared to the other isolates might be because when RU-SO1 was grown in 20%LV there was induction of enzymes which are responsible for the removal of phenols.

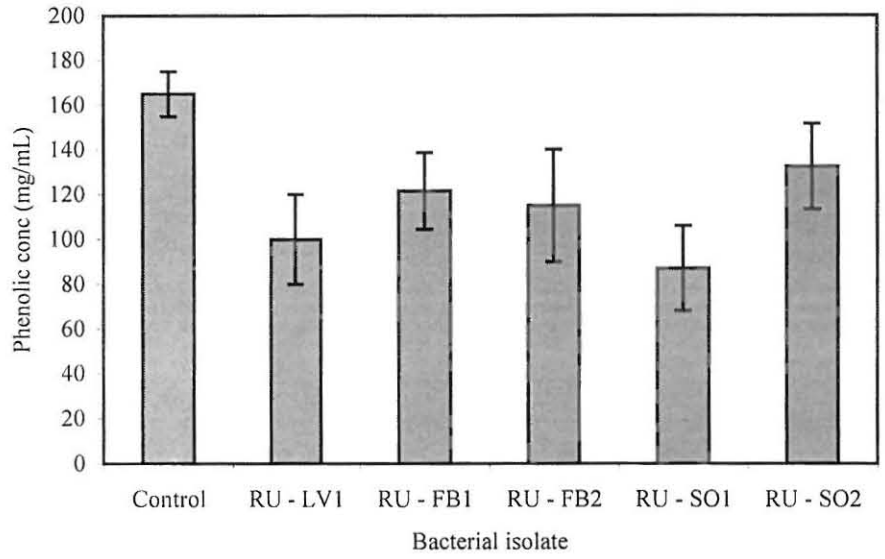


Figure 3.13 The total phenolic content of 20% LV after three days of treatment with bacteria isolated from olive effluents. 20% LV in nutrient broth was not inoculated and was incubated under the same conditions as the inoculated flasks and was used as a control. Standard deviations are shown.

The decrease in the phenolic content of olive effluents by the *Pseudomonas putida* strains was also determined after three days. Both the *Pseudomonas* strains grew in liquid medium supplemented with 20% FB or 20% LV. The RU-KM1 culture removed 47% and 41% of the total phenolic content of 20% FB and 20% LV respectively, and RU-KM3$_s$ removed 40% and 33% of the phenolic content of 20% FB and 20% LV respectively (Figure 3.14). The *Pseudomonas* strains were isolated from soil and soil isolates have been used in the treatment of wastewaters.

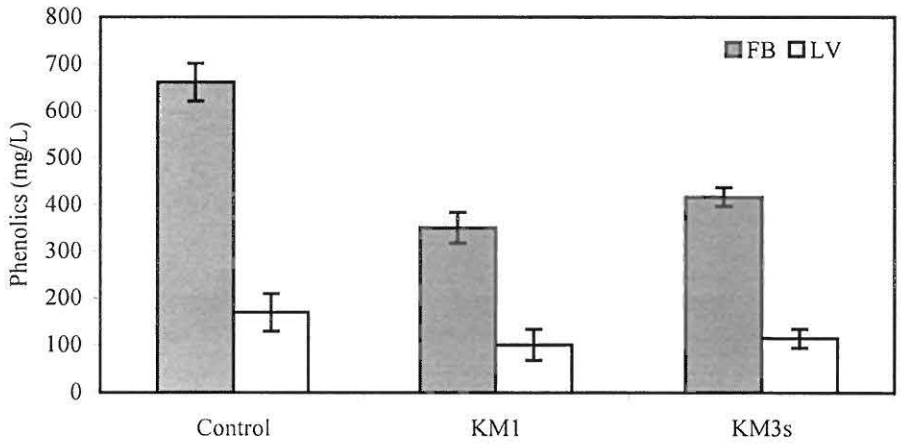


Figure 3.14 Reduction of the total phenolic content of 20% FB and 20% LV by *Pseudomonas putida* RU-KM1 and RU-KM3$_s$. 20% FB and 20% LV in liquid medium without any inoculation was incubated under the same conditions to be used as controls.

Since *T. versicolor* normally takes seven days to grow in its liquid growth medium, TDM (Appendix A), the reduction in total phenolic content of olive effluents by the fungus was determined after seven days. This fungus did not grow in TDM (Appendix A) supplemented with 20% LV, therefore 10% of LV and 10% FB and 5% FB effluent were used, these lower concentrations of FB were used because this effluent contains a higher phenolic content than LV.

T. versicolor removed 71% and 78% of the total phenolic content of 10% LV without and with shaking (at 200rpm) respectively (Figure 3.15). This fungus reduced 51% and 30% of the phenolic content of 5% FB with and without agitation respectively (Figure 3.16). Approximately 50% and 87% of the phenolics in 10% FB were removed under static and shaking conditions respectively (Figure 3.17).

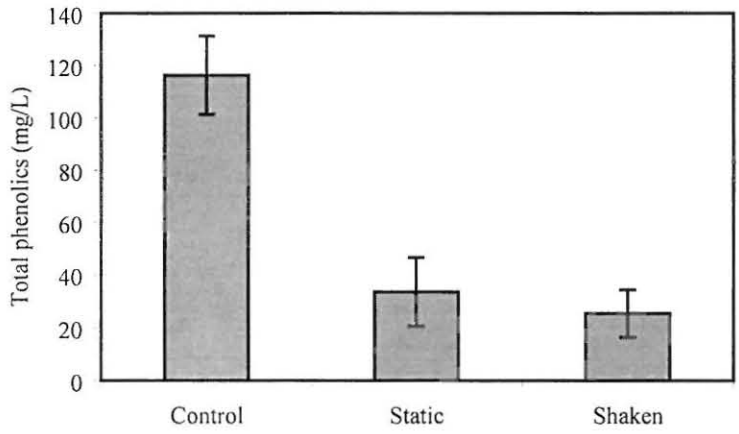


Figure 3.15 The reduction of the total phenolic content of 10% LV by *Trametes versicolor* with (200rpm) or without shaking for 7d. 10% LV in liquid medium without any inoculation was incubated under the same conditions and used as a control.

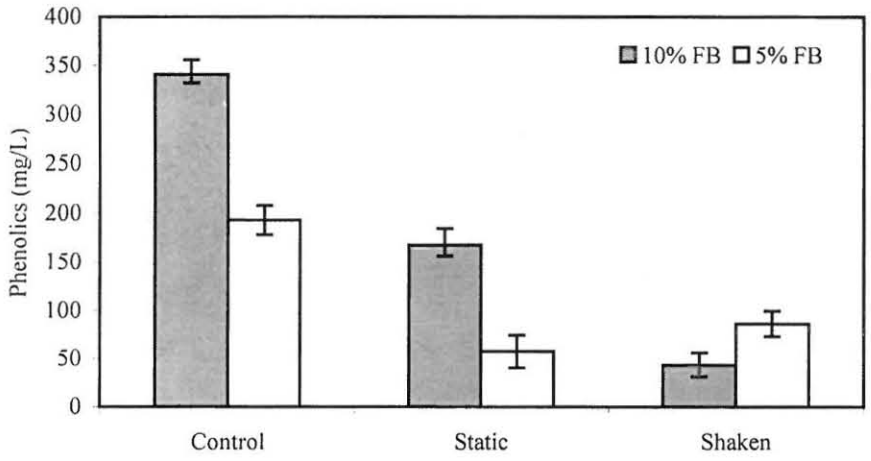


Figure 3.16 Reduction of the total phenolic content of 5% and 10 % FB by *Trametes versicolor*. 5% and 10% FB in liquid medium without any inoculation were incubated under the same conditions and used as controls.

In other flask cultures *T. versicolor* was grown in TDM or TDM (Appendix A) supplemented with FB or LV under static conditions for 7d, then the medium was aseptically removed and replaced with TDM supplemented with 10% LV, 10% FB or 5 % FB and incubated under static conditions for 7d. Statically growing the fungus first, and then using it to reduce the total phenolic content of the effluents under static conditions, led to decreases in the reduction of the phenolic content for the LV effluents, whereas this lead to an increase in the removal of phenolics in FB (Figure 3.17 – 3.19). Under static conditions *T. versicolor* in TDM removed more phenolics than when grown in TDM supplemented with the olive effluents. It has been reported than adaptation of fungi to increasing concentrations of olive effluents can lead to phenol removals up to 90% of the total phenolic content of olive effluents (Martirani *et al.*, 1996). However these authors did not compare the reduction of the total phenolic content with the microbe grown in a defined media and that of the adapted mycelia. These results indicate that *T. versicolor* does not have to be adapted in order to degrade the phenolics of the olive effluents FB and LV, which would save time in a bioremediation process.

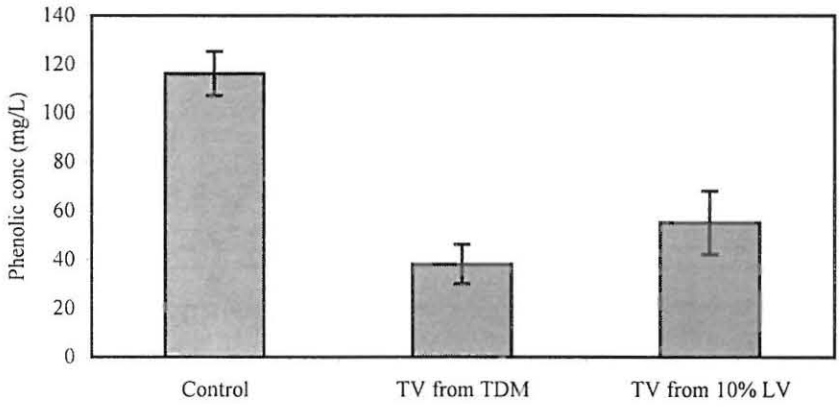


Figure 3.17 Degradation of the total phenolic content of 10% LV by *Trametes versicolor* (TV) grown in Trametes defined media (TDM) or in TDM supplemented with 10% LV. *T. versicolor* was grown in TDM or TDM supplemented with 10% LV under static conditions for 7d and then the liquid medium was removed and replaced with sterile TDM supplemented with 10% LV and incubated for 7d. The control was treated the same way but there was no inoculation.

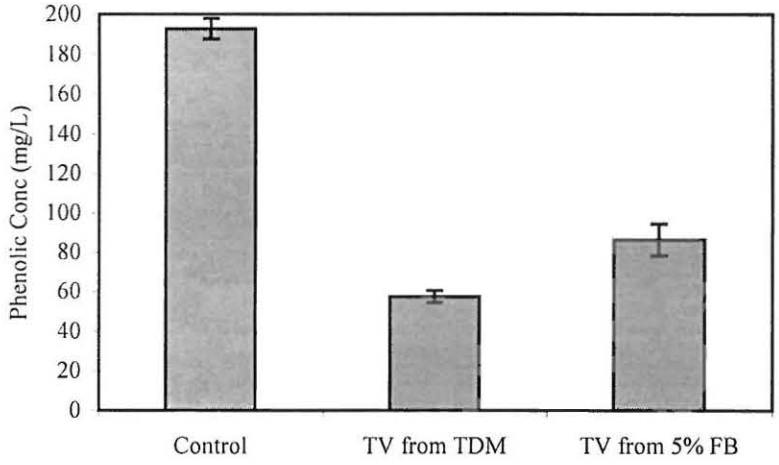


Figure 3.18 Total phenolic content of 5% FB treated with *Trametes versicolor* grown in TDM or in TDM supplemented with 5% FB. *T. versicolor* was grown in TDM or TDM supplemented with 5% FB under static conditions for 7d and then the liquid medium was removed and replaced with TDM supplemented with 5% FB and incubated for 7d. The control was treated the same way but there was no inoculation

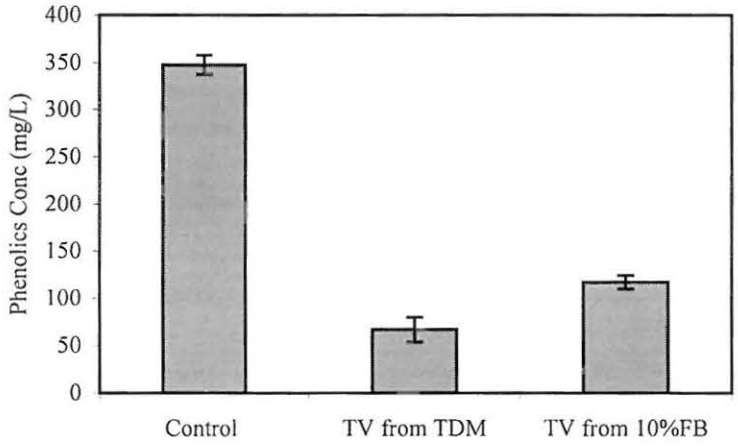


Figure 3.19 Total phenolic content of 10% FB treated with *Trametes versicolor* grown in TDM or in TDM supplemented with 10% FB. *T versicolor* was grown in TDM or TDM supplemented with 10% FB under static conditions for 7d and then the liquid medium was removed and replaced with sterile TDM supplemented with 10% FB and incubated for 7d. The control was treated the same way but there was no inoculation.

N. crassa takes approximately two or three days to grow (Horowitz *et al.*, 1970) but it required a week for this fungus to reduce the phenolics found in olive effluents, FB and LV. The fungus was able to grow in 20% FB and 20% LV, and for comparison, *N. crassa* was also grown in 10% LV, 10% FB and 5% FB. *N. crassa* removed 70% and 72% of the total phenolic content of 10% LV without and with (130 rpm) shaking respectively (Figure 3.20). The fungus also reduced 63% and 84% of the total phenolic content of 20% LV without and with agitation respectively (Figure 3.20). Approximately 45% and 53% of the phenolics were removed by *N. crassa* from 5% FB without and with shaking respectively (Figure 3.21). *N. crassa* removed 55% and 69% of the total phenolic content of 10% FB without and with shaking (at 130 rpm) respectively (Figure 3.21). This fungus reduced 51% and 30% of the *N. crassa* reduced 61% and 83% of the total phenolic content of 20% FB without and with shaking respectively (Figure 3.21). Increasing the final effluent concentration increased the ability of *N. crassa* to reduce the total phenolic content of the effluents FB and LV.

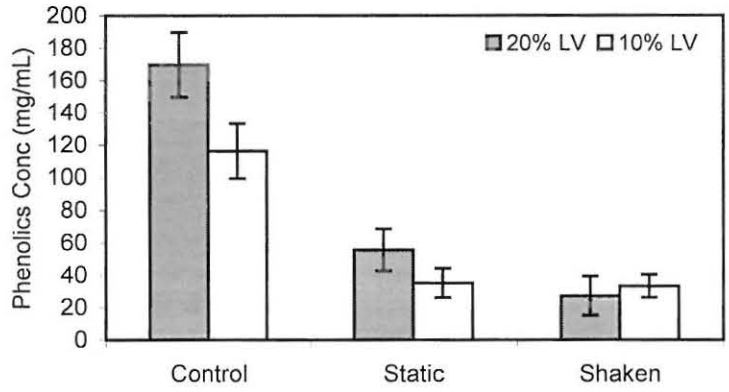


Figure 3.20 Reduction of the phenolic content of 20% LV and 10% LV by *N. crassa* with and without shaking. 20% LV and 10% LV in liquid medium without inoculation were incubated under the same conditions and were used as controls.

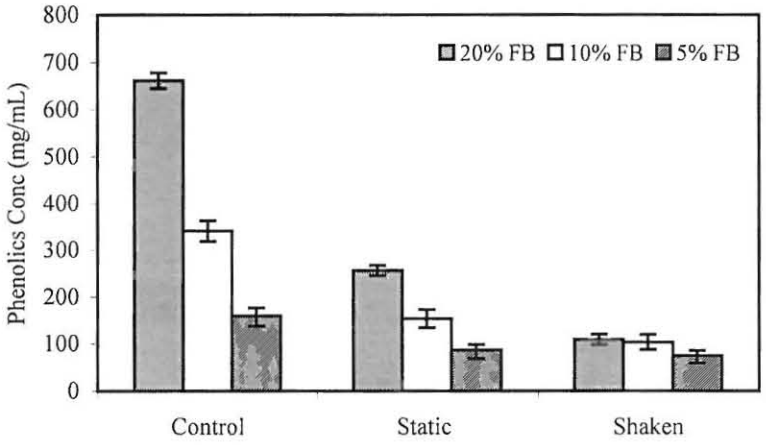


Figure 3.21 Reduction of the phenolic content of 20%, 10% and 5% FB by *N. crassa* with and without shaking. 20%, 10% and 5% FB in liquid medium without inoculation were incubated under the same conditions and were used as controls.

This study compared the capabilities of the bacterial isolates from olive effluents for the removal of the total phenolic content of the waste, and also compared the reduction of the phenolics from olive effluents by the isolated bacterial strains, the *Pseudomonas putida* strains isolated from soil and the white rot fungi, *Trametes versicolor* and *Neurospora crassa*. When comparing the bacteria, the Pseudomonas strains indicated better efficiency in reducing the total phenolic content of the olive effluents as compared to the bacteria isolated from the olive effluents since these *P. putida* strains also grew and reduced the total phenolic content of 20% FB (Table 3.10 and 3.11).

N. crassa reduced the phenolic content of 20% FB whereas *T. versicolor* did not grow in media supplemented with 20% LV which has a lower phenolic content than 20% FB (Table 3.14). Both *T. versicolor* and *N. crassa* showed a high phenolic content removal activity when grown under agitation than under static conditions for both LV and FB olive effluents (Table 3.12 and 3.14). The two fungi had a similar pattern in reducing the total phenolic content of the other concentrations of the olive effluents, FB and LV. When comparing the reduction of phenolics by bacteria and fungi, the results obtained indicated better efficiency of the white rot fungi in reducing the total phenolic content of olive effluents. This is not surprising since white rot fungi have been studied in degrading lignin related compounds (Aust, 1990; Field *et al.*, 1993). Fungi have also been studied for their ability to degrade olive wastes which sometimes resulted in the removal of the dark colour of these wastes (Borja *et al.*, 1993; Hamdi & Garcia, 1993; Flouri *et al.*, 1996; D'Annibale *et al.*, 1998; Garcia *et al.*, 2000). When a bacterium was used to bioremediate olive wastes, an increase in the total phenolic content of the waste was observed due to the biotransformation of the phenolics in the effluent by the bacterial strain (Martinez *et al.*, 1993). Therefore the lower rate of the bacteria in reducing the phenolic content of the effluents might be due to the transformation of some phenolics in the effluents. A possible explanation for the lower phenolic content of the effluents after treatment with the fungi might be the mineralization of the phenolic compounds in the effluents to carbon dioxide

and water, since the fungi are known to mineralize lignin related compounds to carbon dioxide and water (Aust, 1990) and aromatic compounds known to be found in olive mill waste are known to be lignin related (Sanjust *et al.*, 1991).

Table 3.10 Removal of the phenolic content of 20% LV by the bacterial isolates from olive effluents.

Isolate	% Removal
RU – SO1	47%
RU – LV1	40%
RU – FB2	30%
RU – FB1	26%
RU – SO2	20%

Table 3.11 Removal of the phenolic content of the olive effluents, FB and LV by *Pseudomonas putida* RU-KM1 and RU-KM3$_s$.

Effluent conc	RU-KM1	RUKM3$_s$
20%FB	47%	40%
20%LV	41%	33%

Table 3.12 Removal of the phenolic content of FB and LV by *Trametes versicolor*.

Effluent conc	With shaking	Without shaking
5% FB	51%	30%
10% FB	87%	50%
10% LV	78%	71%

Table 3.13 Removal of the phenolic content of FB and LV by *Trametes versicolor* which was grown for 7d in TDM or TDM supplemented with the effluent and then the liquid medium was removed and replaced with TDM supplemented with the olive effluents FB and LV.

Effluent conc	TV from TDM	TV from effluent
10% FB	81%	66%
5% FB	70%	55%
10% LV	67%	53%

Table 3.14 Removal of the phenolic content of the olive effluents FB and LV by *Neurospora crassa*

Effluent conc	Static	Shaken
5% FB	45%	53%
10% FB	55%	69%
20% FB	61%	83%
10% LV	70%	72%
20% LV	63%	84%

3.4 DEGRADATION OF MODEL COMPOUNDS BY BACTERIA ISOLATED FROM OLIVE EFFLUENTS.

It is important to describe degraders of lower molecular weight lignin-related compounds in order to understand fully the natural lignin degradation process (Betts and Dart, 1988), and correspondingly, it is also important to investigate the degradation of phenolic compounds found in olive effluents by microorganisms inhabiting these effluents, in order to understand the natural degradation processes of olive effluents. Microorganisms isolated from OMW have been used to

degrade aromatic compounds known to be found in OMW. *Sporobacterium olearium* isolated from an OMW digester utilized syringic acid, vanillic acid and ferulic acid as carbon and energy sources (Mechichi *et al.*, 1999).

3.4.1 Growth characteristics and degradation of 1 mM model compounds

The five bacterial isolates from Western Cape olive production effluents were grown in flasks containing nutrient broth supplemented with 1 mM of the model compounds, one at a time. The flask cultures were incubated at 28 °C with shaking and every day, from day 0 (the day of inoculation) to day 6, samples of the culture medium were removed. Growth was measured as optical density at 600 nm, the sample was then centrifuged and the supernatants were kept in the freezer at – 20 °C until they were analysed by HPLC.

All five isolates were able to grow in nutrient broth supplemented with caffeic acid, p-coumaric acid, protocatechuic acid, syringic acid or vanillic acid at 1 mM concentration. However, less growth was observed for RU-SO1 in nutrient broth supplemented with 1 mM p-coumaric acid. No lag phase was observed during the growth of these isolates in all the phenolic compounds at 1 mM. The growth and degradation of the model compounds by the bacterial isolates is shown in Figure 3.22 – 3.26.

In case of isolate RU-LV1, one day after inoculation no vanillic acid was detected in the culture medium. Protocatechuic acid was degraded completely after three days of growth and RU-LV1 degraded 96% of caffeic acid and 73% of p-coumaric acid after three and five days respectively. No degradation of syringic acid was observed.

The isolate RU-FB1 degraded 96% of the caffeic acid after two days. During its growth in media with protocatechuic and vanillic acids RU-FB1 degraded 50 and 76 % of these model compounds respectively. Syringic and p-coumaric acids

were not degraded by this isolate. In culture of RU-FB2, 96% of caffeic acid was degraded after two days of growth, 59% of protocatechuic acid was degraded, and no degradation was observed for p-coumaric, syringic and vanillic acids.

Two days after inoculation of RU-SO1, 96% of caffeic acid was removed from the culture medium. Protocatechuic and p-coumaric acid were degraded by about 97 and 68% and there was no degradation of syringic and vanillic acids. Both caffeic and protocatechuic acids were degraded to approximately 97% by RU-SO2, vanillic, syringic and p-coumaric were not degraded by RU-SO2.

Using HPLC analysis no metabolic product peaks were observed in the culture liquids, for all model compounds used in this study. These results indicate that the bacteria isolated from olive effluents were able to degrade some of aromatic compounds (1 mM) found in olive effluents. Even though all the isolated bacteria grew well in nutrient broth supplemented with 1 mM syringic acid, none of the bacterial isolates degraded 1 mM syringic acid (Table 3.15) and this correlates with the work of Delneri *et al.* (1995) where these authors reported that syringic acid was not metabolised by *Acinetobacter calcoaceticus* strains DSM 586 and DSM 590. Perez *et al.*, (1990) reported that vanillic acid was needed to induce the degradation of syringic acid when Pseudomonas spp. were used for degrading phenolic acids. Therefore it is possible that the isolates used in this work need induction in order to degrade syringic acid.

The bacterial isolates degraded model compounds with one (vanillic acid) or no (protocatehuic acid, caffeic acid and *p*-coumaric acid) methoxyl group and no degradation was observed for syringic acid which has two methoxyl groups. Andreoni *et al.* (1995) reported that no growth and therefore no degradation was observed when *Pseudomonas fluorescens* strain FE2 was grown in medium supplemented with aromatic compounds that have two methoxyl groups such as syringic acid and veratric acid.

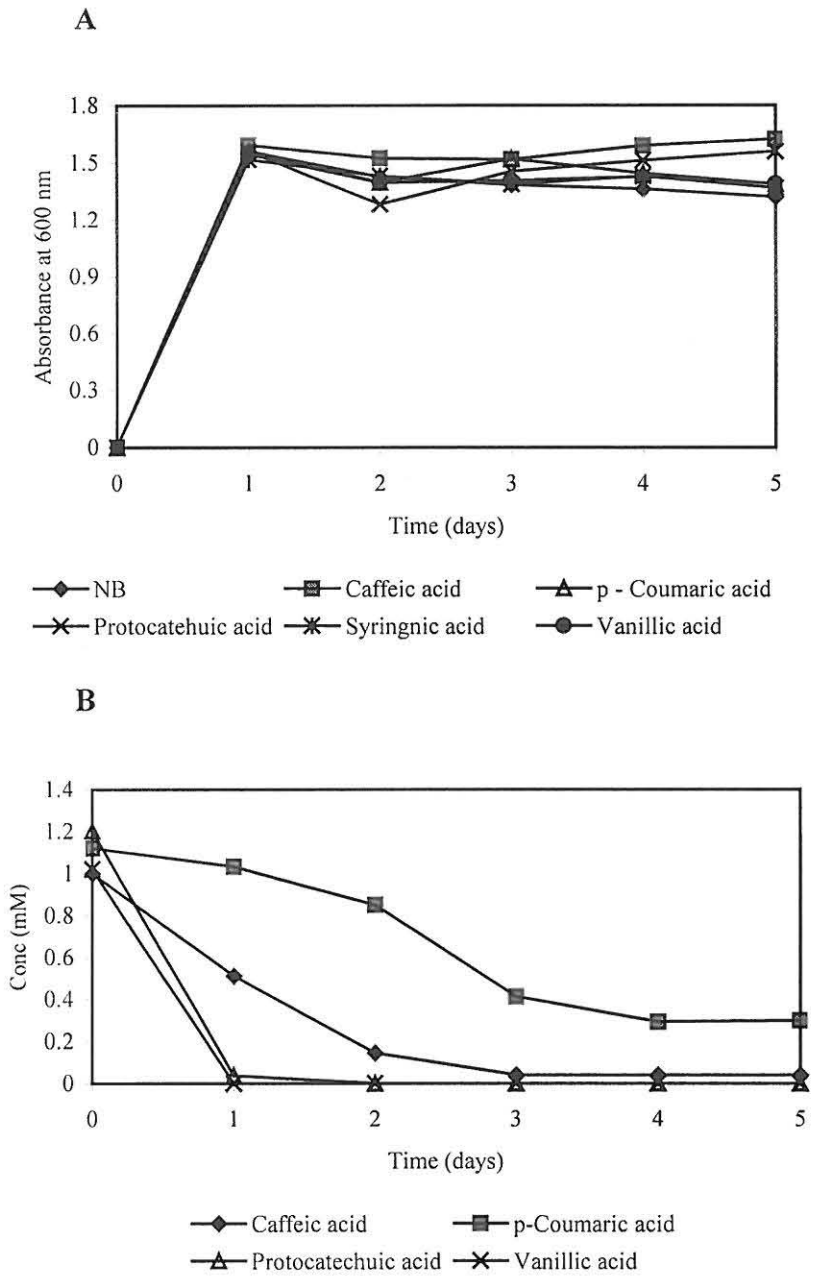
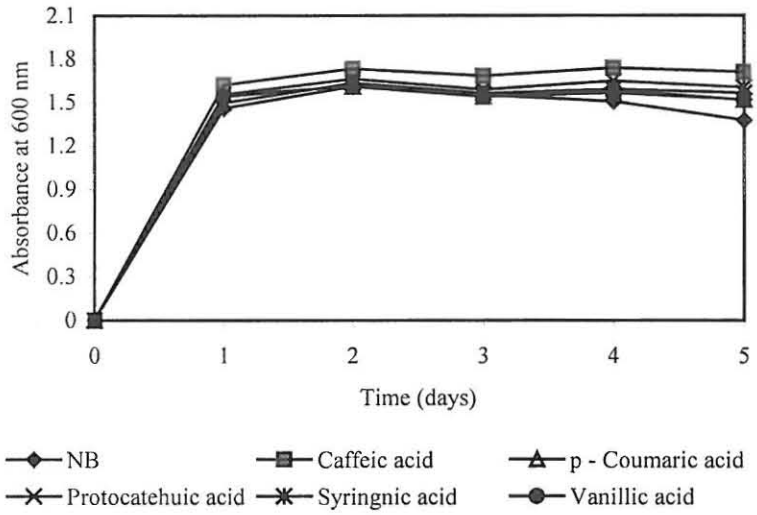


Figure 3.22 Growth of RU-LV1 determined as absorbance at 600 nm (A) and degradation of model compounds (1mM) by RU-LV1 (B). RU-LV1 was grown in nutrient broth supplemented with the aromatic compounds and incubated at 28 °C at 200 rpm. Samples were taken daily, OD 600 was measured, samples were centrifuged prior storage in the freezer and then samples were analysed by HPLC.

A



B

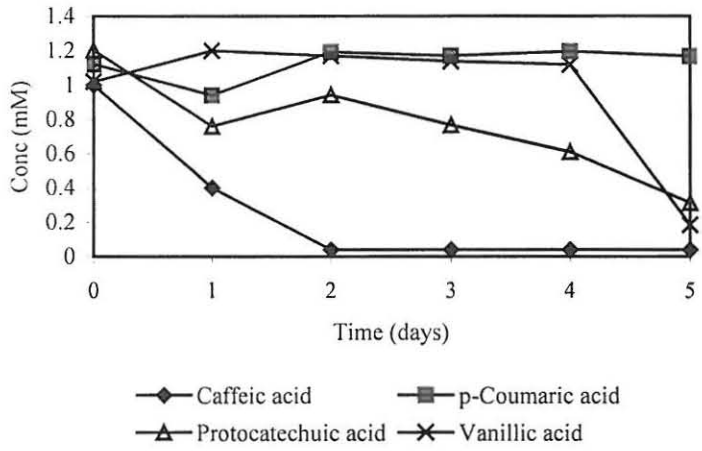
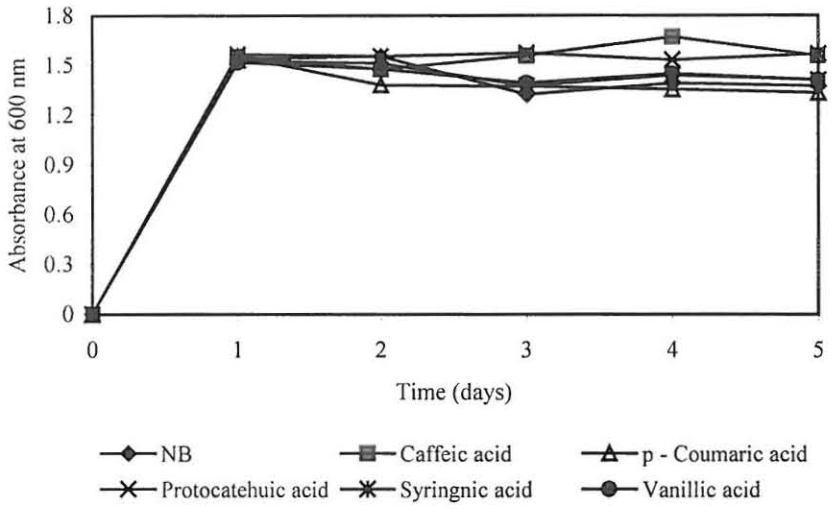


Figure 3.23 Growth of RU-FB1 determined as absorbance at 600 nm (A) and degradation of model compounds (1mM) by RU-FB1 (B). RU-FB1 was grown in nutrient broth supplemented with the aromatic compounds and incubated at 28 °C at 200 rpm. Samples were taken daily, OD 600 was measured, samples were centrifuged prior storage in the freezer and then samples were analysed by HPLC.

A



B

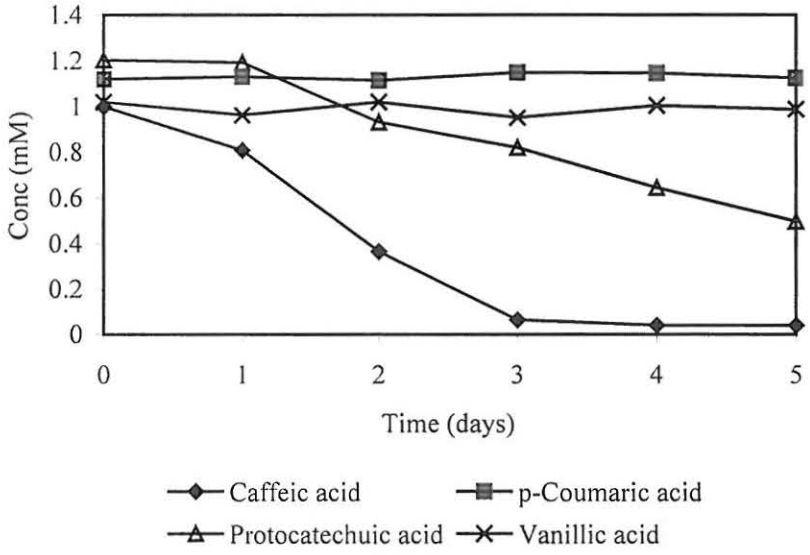
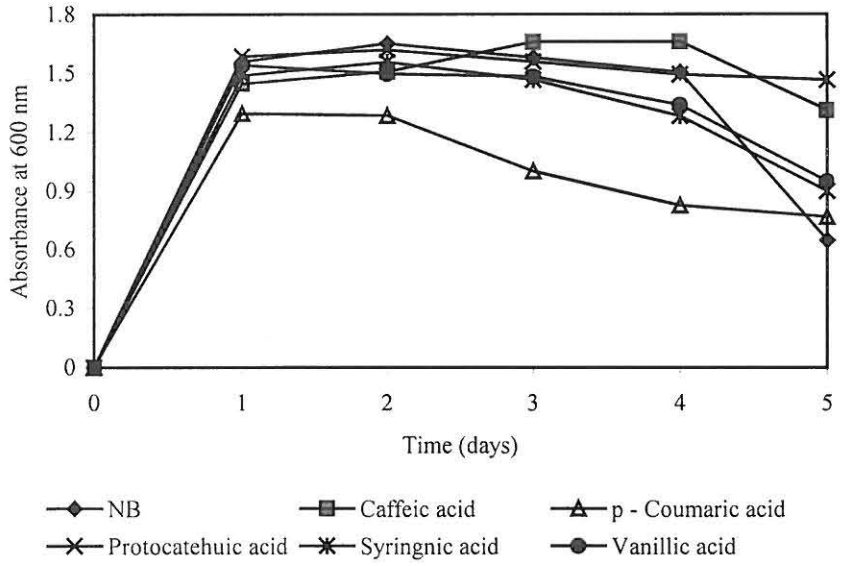


Figure 3.24 Growth of RU-FB2 determined as absorbance at 600 nm (A) and degradation of model compounds (1mM) by RU-FB2 (B). RU-FB2 was grown in nutrient broth supplemented with the aromatic compounds and incubated at 28 °C at 200 rpm. Samples were taken daily, OD 600 was measured, samples were centrifuged prior storage in the freezer and then samples were analysed by HPLC.

A



B

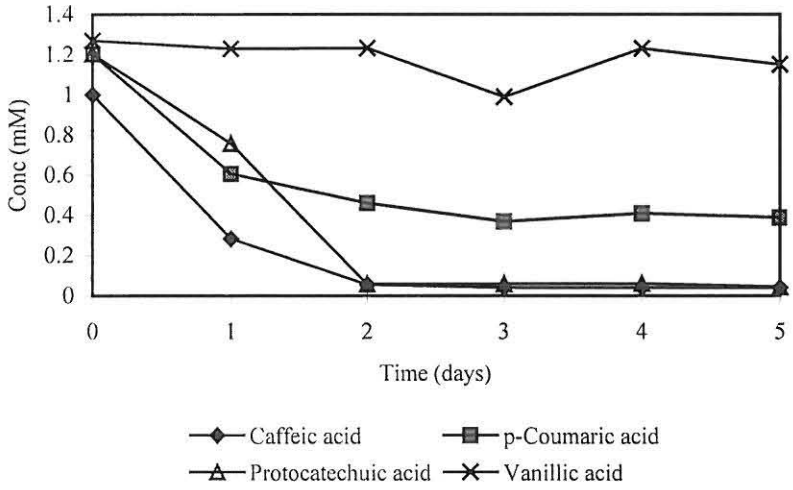


Figure 3.25 Growth of RU-SO1 determined as absorbance at 600 nm (A) and degradation of model compounds (1mM) by RU-SO1 (B). RU-SO1 was grown in nutrient broth supplemented with the aromatic compounds and incubated at 28 °C at 200 rpm. Samples were taken daily, OD 600 was measured, samples were centrifuged prior storage in the freezer and then samples were analysed by HPLC.

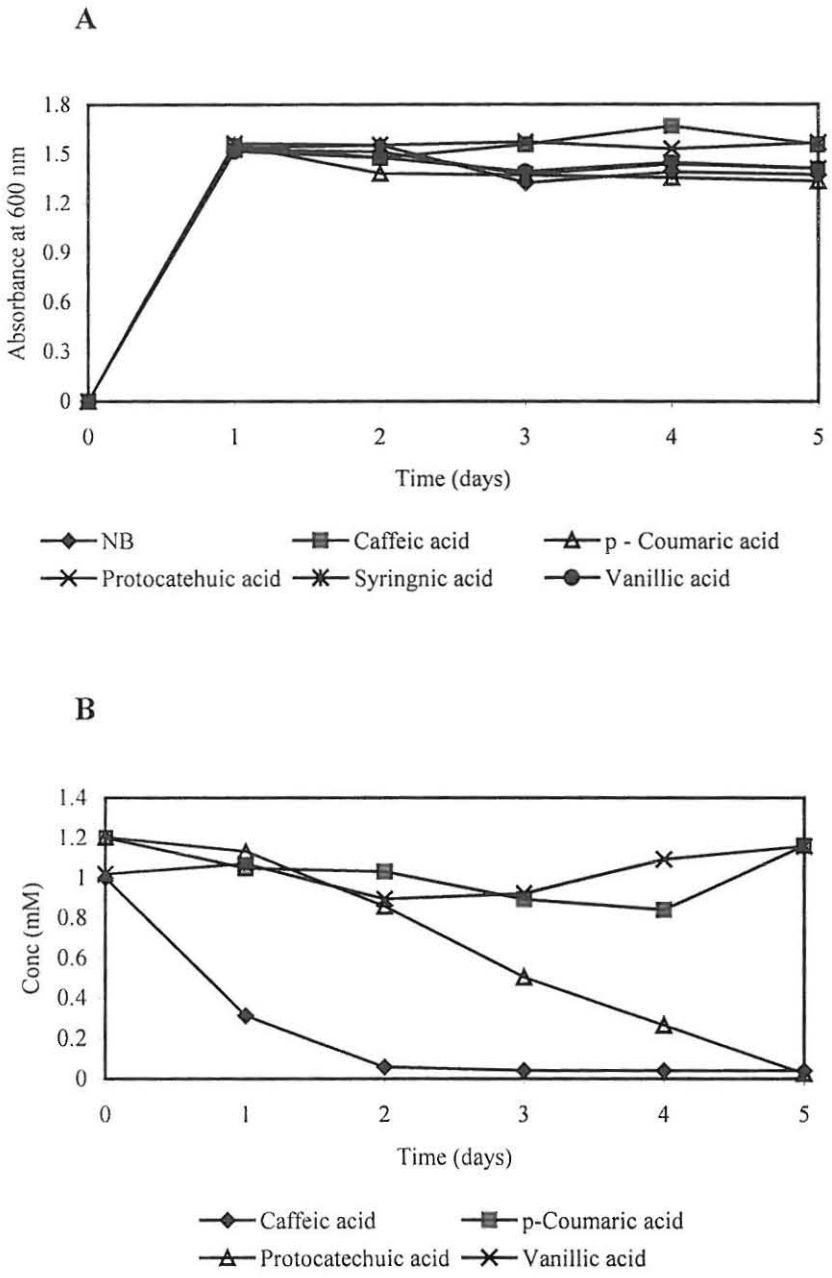


Figure 3.26 Growth of RU-SO2 determined as absorbance at 600 nm (A) and degradation of model compounds (1mM) by RU-SO2 (B). RU-SO2 was grown in nutrient broth supplemented with the aromatic compounds and incubated at 28 °C at 200 rpm. Samples were taken, OD 600 was measured, samples were centrifuged prior storage in the freezer and then samples were analysed by HPLC.

Table 3.15 Degradation of model compounds at 1 mM by bacteria isolated from the olive effluents.

Isolate	% Degradation of model compounds at 1 mM				
	Caffeic	*p*-Coumaric	Protocatechuic	Syringic	Vanillic
RU-LV1	96%	73%	100%	-	100%
RU-FB1	96%	-	50%	-	76%
RU-FB2	96%	-	59%	-	-
RU-SO1	96%	68%	97%	-	-
RU-SO2	97%	-	97%	-	-

- = No degradation was observed

3.4.2 Growth characteristics and degradation of 5 mM model compounds

Since all the isolated bacterial isolates degraded caffeic and protocatechuic acid at 1mM and RU-LV1 completely degraded vanillic acid at 1 mM, the concentration of these three model compounds was increased to 5 mM and the degradation of these aromatic acids at this higher concentration by all the bacterial strains was investigated.

Growth was observed for the five bacterial isolates in nutrient broth supplemented with caffeic, protocatechuic and vanillic acids at 5 mM. RU-LV1 showed a lag phase of 24 hours during its growth in media with 5 mM caffeic acid and 5 mM vanillic acid which corresponded to a lag phase in degradation (Figure 3.27 to 3.31).

All the bacterial isolates degraded 99% of the caffeic acid, with RU-LV1 removing the phenolic compound in the culture medium after three days of growth. Protocatechuic acid was completely degraded by RU-SO1 within three days. Approximately 97, 88, 93% and 98% of protocatechuic acid was degraded

by RU-LV1, RU-FB1, RU-FB2 and RU-SO2 respectively. Vanillic acid was completely degraded within three days by RU-LV1 (Figure 3.27 to 3.31).

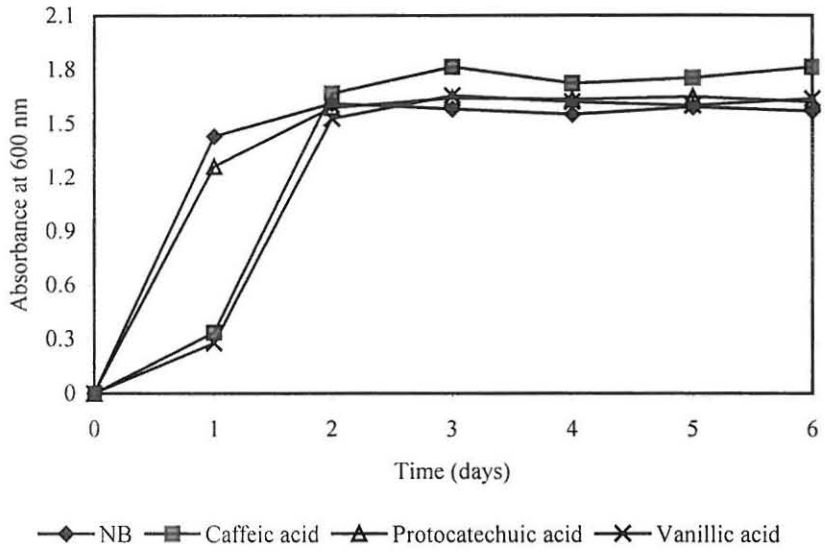
Despite the fast growth rate of strains RU-FB1, RU-FB2, RU-SO1 and RU-SO2 in media supplemented with 5 mM vanillic acid, no degradation of this phenolic compound was observed by HPLC analysis (Figure 3.27 to 3.31). Higher concentrations (10 mM) of caffeic, protocatechuic and vanillic acids proved to be toxic to the bacteria isolated from olive effluents, as indicated by the lack of growth.

Table 3.16 Degradation of model compounds at 5 mM by bacteria isolated from the olive effluents.

Isolate	**% Degradation of model compounds at 5 mM**		
	Caffeic	Protocatechuic	Vanillic
RU-LV1	99%	97%	100%
RU-FB1	99%	88%	-
RU-FB2	99%	93%	-
RU-SO1	99%	100%	-
RU-SO2	99%	98%	-

- = No degradation observed

A



B

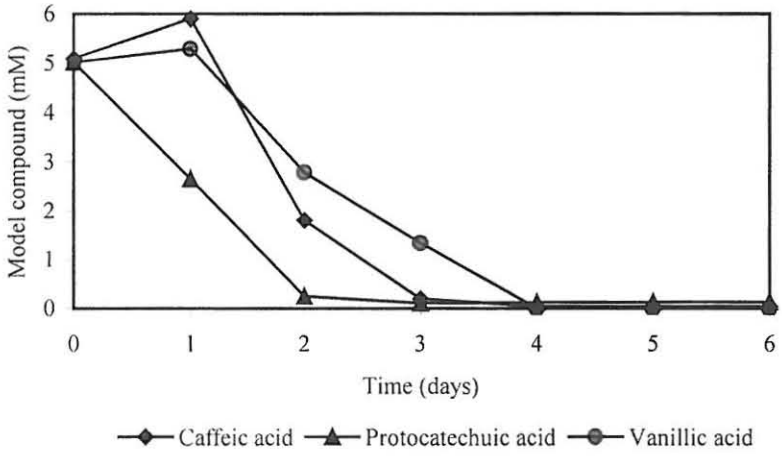
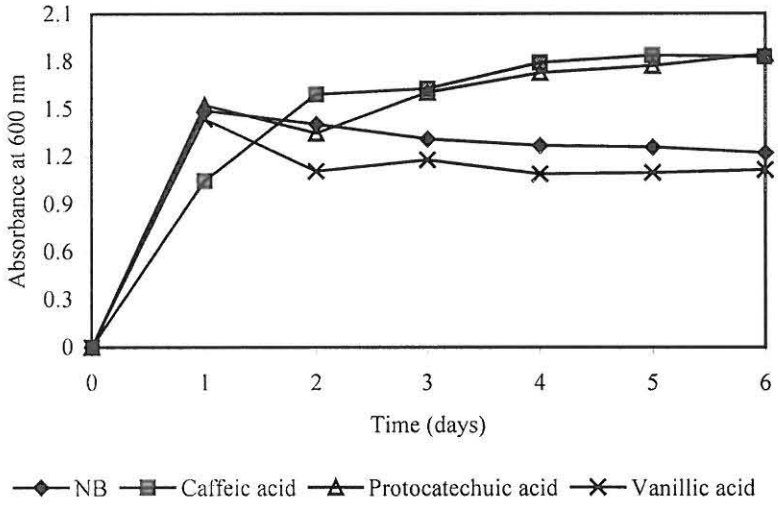


Figure 3.27 Growth of RU-LV1 determined as absorbance at 600 nm (A) and degradation of 5 mM model compounds by RU-LV1 (B). RU-LV1 was grown in nutrient broth supplemented with caffeic acid, protocatechuic acid or vanillic acid at 5 mM and incubated at 28 °C at 200 rpm. Samples were taken daily, OD at 600 nm was measured and samples were centrifuged prior storage in the freezer and then samples were analysed by HPLC.

A



B

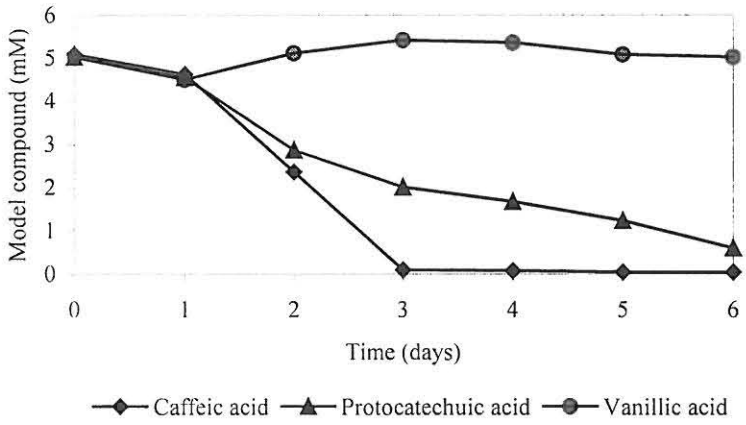
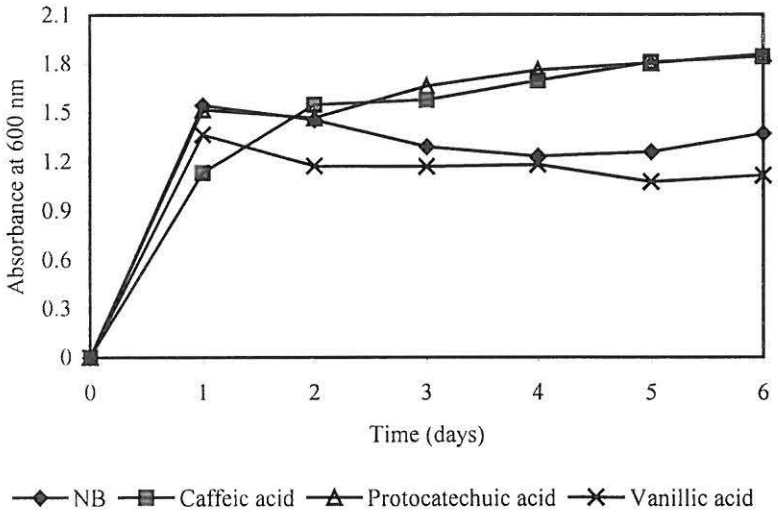


Figure 3.28 Growth of RU-FB1 determined as absorbance at 600 nm (A) and degradation of 5 mM model compounds by RU-FB1 (B). RU-FB1 was grown in nutrient broth supplemented with caffeic acid, protocatechuic acid or vanillic acid at 5 mM and incubated at 28 °C at 200 rpm. Samples were taken daily, OD at 600 nm was measured and samples were centrifuged prior storage in the freezer and then samples were analysed by HPLC.

A



B

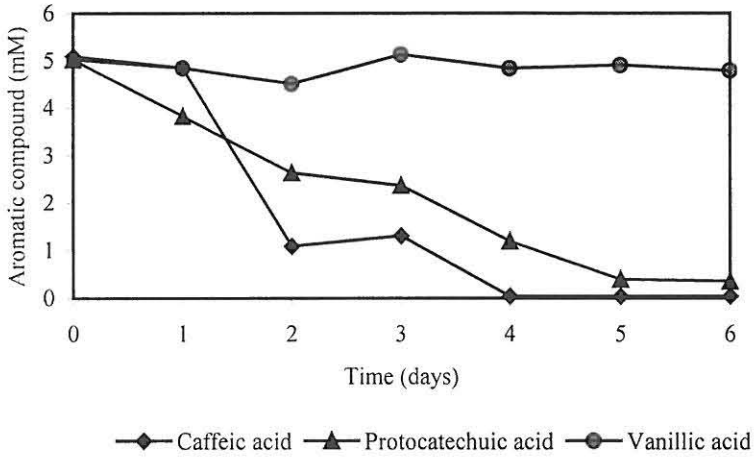
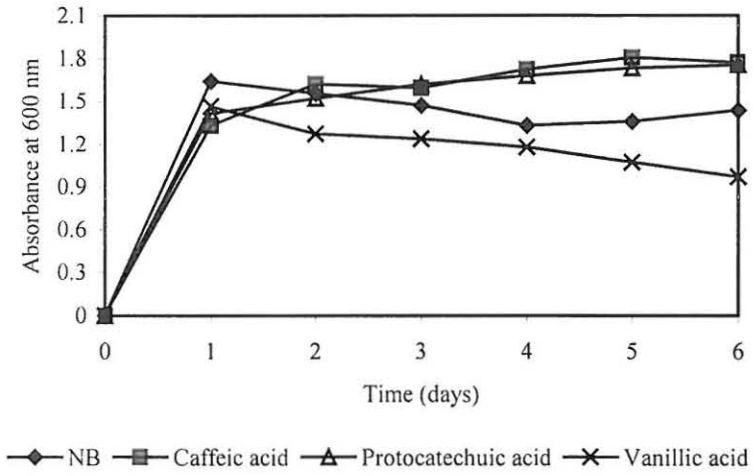


Figure 3.29 Growth of RU-FB2 determined as absorbance at 600 nm (A) and degradation of 5 mM model compounds by RU-FB2 (B). RU-FB2 was grown in nutrient broth supplemented with caffeic acid, protocatechuic acid or vanillic acid at 5 mM and incubated at 28 °C at 200 rpm. Samples were taken daily, OD at 600 nm was measured and samples were centrifuged prior storage in the freezer and then samples were analysed by HPLC.

A



B

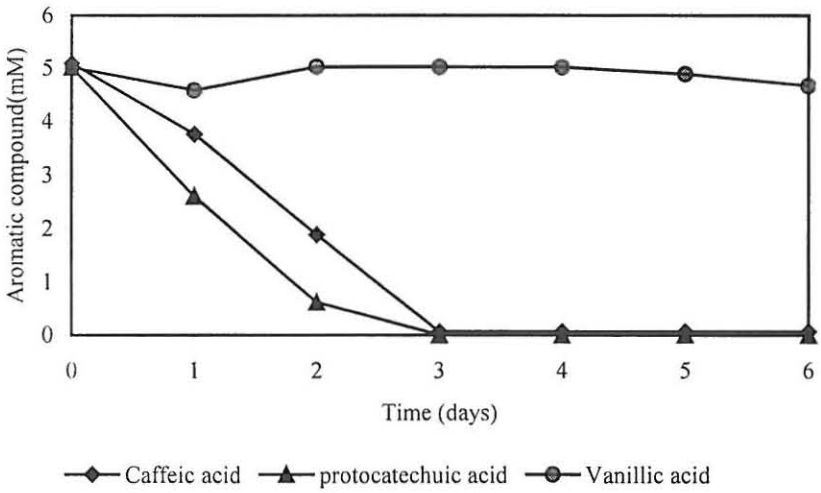
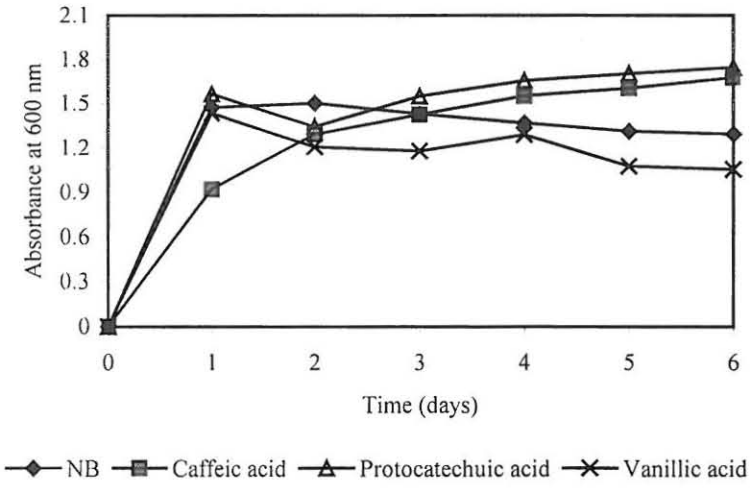


Figure 3.30 Growth of RU-SO1 determined as absorbance at 600 nm (A) and degradation of 5 mM model compounds by RU-SO1 (B). RU-SO1 was grown in nutrient broth supplemented with caffeic acid, protocatechuic acid or vanillic acid at 5 mM and incubated at 28 °C at 200 rpm. Samples were taken daily, OD at 600 nm was measured and samples were centrifuged prior storage in the freezer and then samples were analysed by HPLC.

A



B

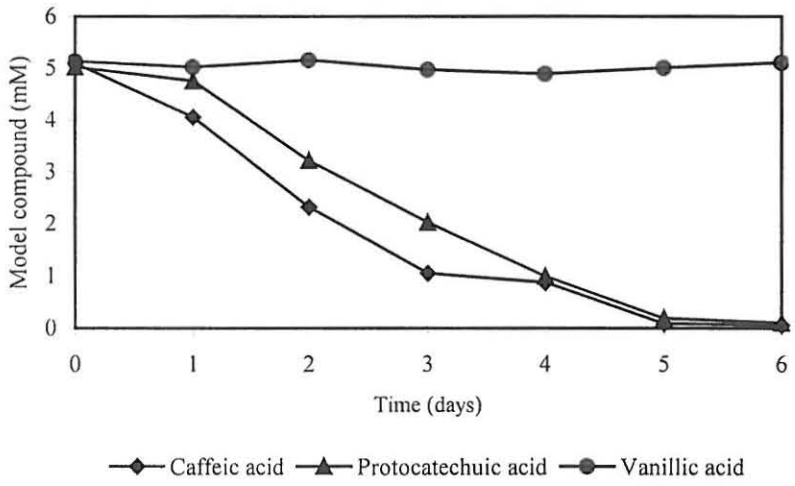


Figure 3.31 Growth of RU-SO2 determined as absorbance at 600 nm (A) and degradation of 5 mM model compounds by RU-SO2 (B). RU-SO2 was grown in nutrient broth supplemented with caffeic acid, protocatechuic acid or vanillic acid at 5 mM and incubated at 28 °C at 200 rpm. Samples were taken daily, OD at 600 nm was measured and samples were centrifuged prior storage in the freezer and then samples were analysed by HPLC.

During the growth of all the isolates in nutrient broth supplemented with caffeic and protocatechuic acid, the medium became black and dark brown respectively. The cause of the colour change may be because of the production of intermediates during the degradation of these aromatic compounds. Yanase *et al.*, (1992) reported that the medium became black during phenol degradation by a bacterium belonging to the family Rhizobiaceae and these authors suggested that the colour change might be caused by accumulation of catechol as a degradation intermediate.

RU-LV1 showed better efficiency in degrading the model compounds used in this study than all the other isolates. The degradation of model compounds found in olive effluents by bacteria isolated from the effluents suggests that the microorganisms inhabiting olive effluents are bioremediating the effluents during storage. Microorganisms degrading a large number of organic acids in efficiently could prevent undesirable properties in wastewater from olive mills which are a result of its phenolic content.

The presence of micoorganisms able to degrade aromatic compounds in OMW is not surprising as the wastewater contains a wide range of aromatic compounds that are released from olive cell walls during the oil extraction process (Capasso *et al.*, 1995). Correspondingly, the presence of bacteria able to degrade phenolic acids in olive production effluents should not be surprising as these effluents were found to contain some of the aromatic compounds found in OMW (Section 3.1.).

The degradation of model compounds by the bacterial isolates was not intended to investigate in detail the production intermediates during the degradation process but a simple investigation into the degradation of aromatic compounds found in olive effluents by bacteria isolated from such effluents. Because they are found in olive wastes their catabolism by bacteria found in olive effluents is essential to the biodegradation of olive effluents.

3.5. INVESTIGATION INTO THE BIOCONVERSION OF VANILLIC ACID AND FERULIC ACID BY RU-LV1

Vanillic and ferulic acids have been used as starting materials in enzymatic synthesis of vanillin, a high value flavour compound (Huang *et al.*, 1993; Rosazza *et al.*, 1995; Mulheim & Lerch, 1999; Li & Rosazza, 2000). These two acids have been reported to be found in OMW (Balice and Cera, 1984; Perez *et al.*, 1992; Hamdi, 1993) and they have also been identified as constituents in FB adjusted to pH 1, an olive production effluent used in this study (Section 3.1). The ability to degrade model compounds by the isolate RU-LV1 was greater than that of all the other isolates from olive effluents (Section 3.4). Therefore RU-LV1 was selected to investigate the potential bioconversion of vanillic and ferulic acids into vanillin.

3.5.1 Growth characteristics and degradation by whole cells in minimal media

RU-LV1 was grown in minimal media (MM) (Appendix A) with the substitution of glucose by 1 mM ferulic acid or vanillic acid. In other flasks RU-LV1 was grown in MM with 0.1 % glucose and after 24 hours of growth, 1 mM ferulic acid or vanillic acid was added to the culture medium. The flasks were incubated at 28° C at 200rpm. Each day, from the day of inoculation for cells grown in MM with the phenolic acids as a carbon source, or the day of addition of the aromatic compounds after 24 hours of growth of RU-LV1, a sample (1 mL) was taken from the culture medium, growth was measured as OD at 600 nm and the sample was stored in the freezer after centrifugation. Samples were then analysed by HPLC.

RU-LV1 grew in media with the phenolic acids present at 1 mM as the carbon source (Figure 3.32). The maximum OD at 600 nm reached was 0.778 with ferulic acid as carbon source after two days, after which the stationary phase of growth was reached. With vanillic acid as a carbon source the maximum OD at 600 nm

was 0.94 after three days. The optimum growth (OD at 600 nm was 1.170) of RU-LV1, observed in MM with glucose as a carbon source. There was less growth in cultures where glucose was substituted with the aromatic compounds, indicating that glucose is easily assimilatable by RU-LV1. More growth was observed when the aromatic acids were added after 24 hours of growth, RU-LV1 in these flasks had two carbon sources, glucose and the standard compounds. Although RU-LV1 grew in MM (Appendix A) with ferulic acid as a carbon source and also in flasks where 1 mM ferulic acid was added after 24 hours of RU-LV1 culture growth, no apparent degradation of ferulic acid was observed by HPLC analysis.

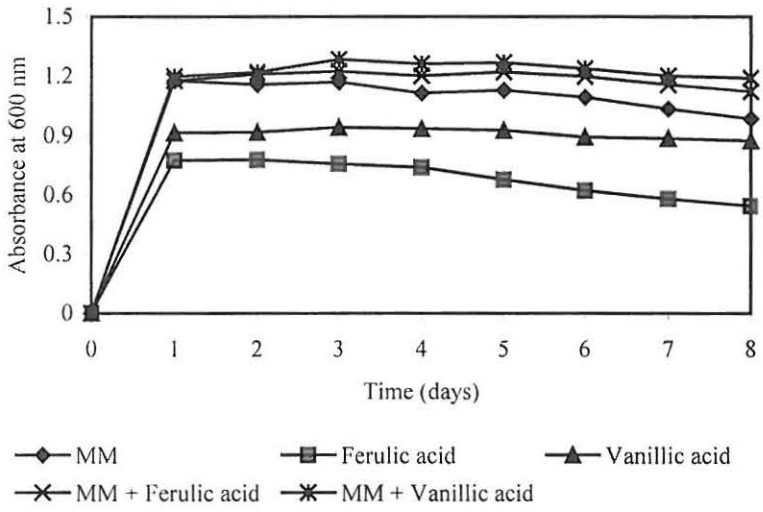


Figure 3.32 Growth, monitored as absorbance at 600 nm, of RU-LV1 in MM, MM with the substitution of glucose by 1 mM ferulic acid or vanillic acid. RU-LV1 was also grown in MM and then after 24 hours, 1 mM ferulic or vanillic acid was added to the growth medium.

When RU-LV1 was grown in MM which contained glucose and 1 mM vanillic acid added after 24 hours of growth to the medium, approximately 96% of 1 mM vanillic acid was degraded 5 days after the addition of the aromatic acid (Figure

3.33). The cells presumably used glucose as a carbon source until the glucose was depleted in the medium, and then used the vanillic acid as a carbon source. About 96% of 1 mM vanillic acid was removed by RU-LV1 cells per day after inoculation of MM with 1 mM vanillic acid instead of glucose (Figure 3.33).

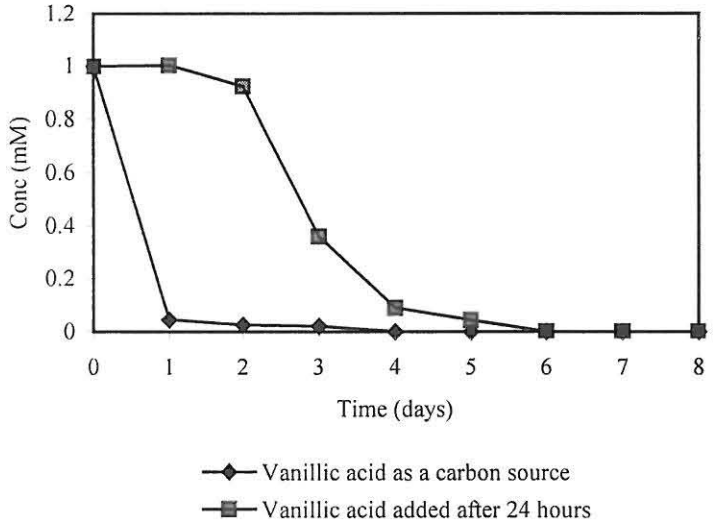


Figure 3.33 Degradation of 1 mM vanillic acid by whole cells of RU-LV1. RU-LV1 was grown in medium with 1 mM vanillic acid as a carbon source and in other flasks 1 mM vanillic acid was added after 24 hours of growth of the culture in MM.

Since it was noticed that only 3.4% of the 1 mM vanillic acid remained in the culture medium after 24 hours of incubation in samples where vanillic acid was used as a carbon source (Figure 3.33), the experiment was repeated and samples were taken at hourly intervals for 24 hours. In the experiment, RU-LV1 was inoculated in flasks containing MM, MM with 1 mM vanillic acid instead of glucose, MM without glucose and yeast extract, and MM without glucose and yeast extract but with 1 mM vanillic acid. The variation in the medium mixtures was to find out what is the source of nutrients when glucose was omitted in the growth medium.

The maximum OD at 600 nm reached by RU-LV1 in flasks without glucose was 0.945 after 20 hours, and in flasks with 1 mM vanillic acid as a carbon source the OD 600 was 1.264 after 24 hours. The OD measured in flasks without glucose and yeast extract was 0.062 after 15 hours and OD without glucose and yeast extract but with the addition of 1 mM vanillic acid was 0.037 after 15 hours (Figure 3.34). These results suggest that yeast extract may be the source of essential assumable nutrients in the absence of glucose since even with vanillic acid only an OD600 of 0.037 was reached.

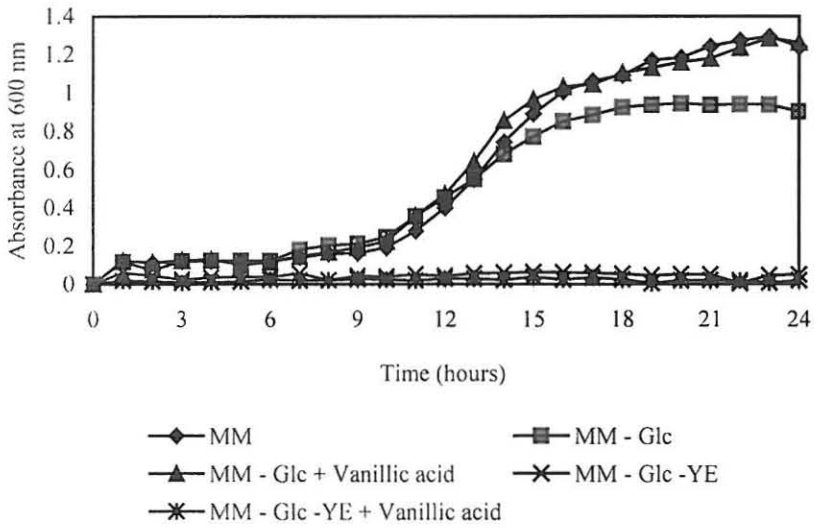


Figure 3.34 Growth of RU-LV1 in minimal medium (MM), MM with the substitution of glucose by 1 mM vanillic acid (MM – Glc + Vanillic acid), MM without glucose and yeast extract (MM – Glc – YE), and MM without glucose and yeast extract but with 1 mM vanillic acid (MM – Glc – YE + Vanillic acid). Samples were taken at hourly intervals for 24 hours and growth was monitored as absorbance at 600 nm.

There was no degradation of vanillic acid in media without glucose and yeast extract, these results correspond with the growth results where no growth was observed for RU-LV1 in flasks without glucose and yeast extract. There was a

lag phase in the degradation of 1 mM vanillic acid in flasks with the substitution of glucose with 1 mM vanillic acid and this lag phase corresponded to a lag phase in the growth of RU-LV1 culture in the same flasks. Approximately 48% of 1 mM vanillic acid was removed in the culture media with vanillic acid and yeast extract after 15 hours of growth, with vanillic acid gradually disappearing to 0.052 mM after 24 hours (Figure 3.35). However no metabolic products were observed in the culture medium, on analysis by HPLC. Karmakar *et al.* (2000) found yeast extract to be essential for rapid growth and ferulic acid degradation by *Bacillus coagulans* BK07 and in this study there was no growth and degradation of vanillic acid by RU-LV1 observed in the absence of glucose and yeast extract. This may suggest that yeast extract may be important for growth and the degradation of vanillic acid by RU-LV1 when glucose is absent in the growth medium.

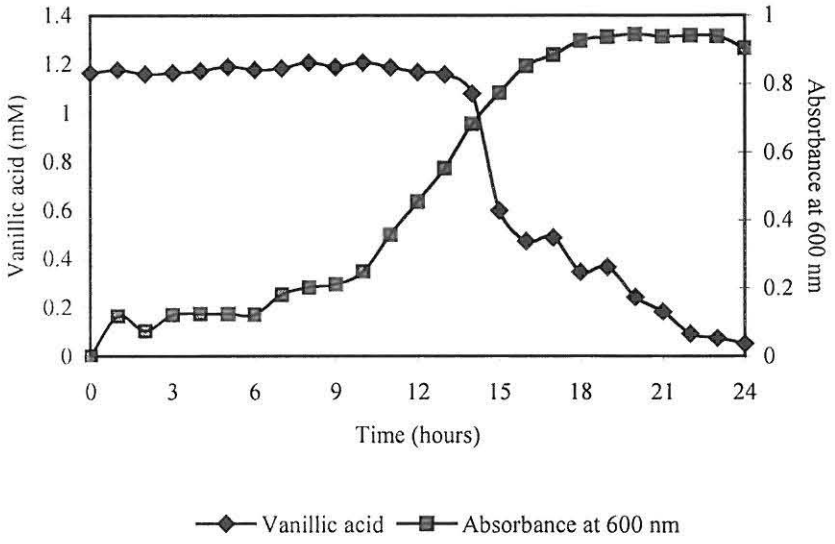


Figure 3.35 Growth of RU-LV1, monitored as absorbance at 600 nm and hourly degradation of vanillic acid by RU-LV1. RU-LV1 was grown in MM with vanillic acid as a carbon source and samples were taken hourly, OD 600 was measured, samples were centrifuged and analysed by HPLC.

3.5.2 Degradation of vanillic acid by resting cells, french pressed extract, cell free extract and cell membrane fractions of the isolate RU-LV1

To find out whether products were formed during vanillic acid degradation, further studies were carried out. RU-LV1 was grown in minimal media (MM) (Appendix A) with glucose as a carbon source, or MM supplemented with 0.01% vanillic acid as a carbon source or MM with 0.1% glucose and 0.01% vanillic acid added after 12 hours of growth. The flasks were incubated at 28 °C at 200 rpm for 24 hours. Cells were then harvested by centrifugation at 10000 rpm for 10 minutes, washed in sterile 0.1 M sodium phosphate buffer (pH 7) and then resuspended in the same buffer to a concentration of 100 mg of cells per mL of buffer. This cell suspension was used as resting cells and was used to study the degradation of vanillic acid. In other experiments, the resting cell suspension was also passed through a French press and the French pressed extract was obtained

and used, or the extract was centrifuged and the supernatant used as the cell free extract. When the French pressed extract was centrifuged the pelleted cells were used as the cell membrane fractions. Barghini *et al.* (1998) reported that the use of resting cells increased the formation of products even though there was no biomass duplication. These experiments involving vanillic acid and resting cells or extracts were carried out to investigate if products would be formed during the degradation of vanillic acid.

The growth of RU-LV1 in MM (Appendix A) with glucose and MM (Appendix A) with glucose and 0.01% vanillic acid reached the OD of 1.30 – 1.50 whereas the growth of RU-LV1 in MM (Appendix A) with 0.01% vanillic acid as a carbon source reached the OD of 1.00 – 1.20 after 24 hours. These results indicate that glucose was readily used for growth by RU-LV1.

0.01% vanillic acid in MM was added as an inducer for the degradation of aromatic acids, and therefore resting cells or extracts obtained from RU-LV1 cells grown in the presence of 0.01% vanillic acid are termed induced in this study. Induced resting cells of RU-LV1 completely degraded 0.6 mM vanillic acid after two hours as compared to non-induced resting cells which degraded 61% of vanillic acid after 24 hours (Figure 3.36). However no metabolic products were detected in the supernatant of the reaction mixture. Higher concentrations (1 mM) of vanillic acid were not degraded by resting cells of RU-LV1.

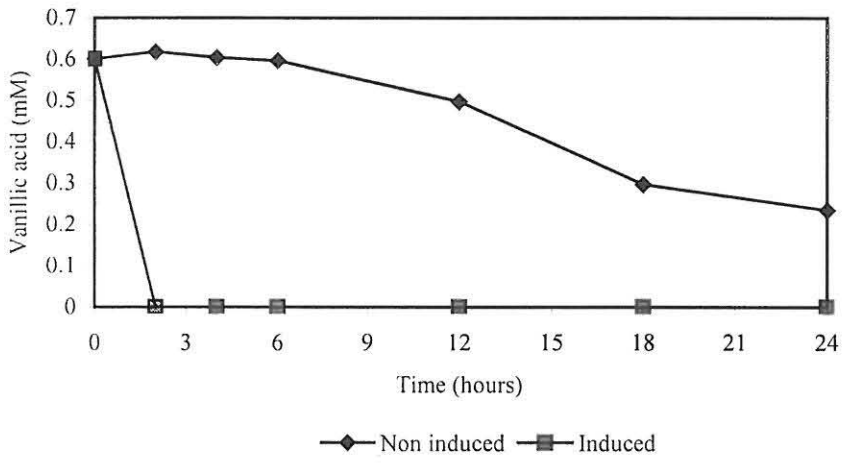


Figure 3.36 Degradation of 0.5 mM vanillic acid by induced and non-induced resting cells of RU-LV1. RU-LV1 was grown in flasks with glucose or vanillic acid as a carbon source. Cells were harvested and suspended in buffer, and the cell suspension was used for vanillic acid degradation.

Cell free extracts of induced and non-induced RU-LV1 cells completely degraded 0.5 mM vanillic acid after 11 and 14 hours respectively (Figure 3.37). Cell membrane fractions of induced and non-induced cells completely degraded 0.5 mM vanillic acid after 18 and 24 hours respectively (Figure 3.38).

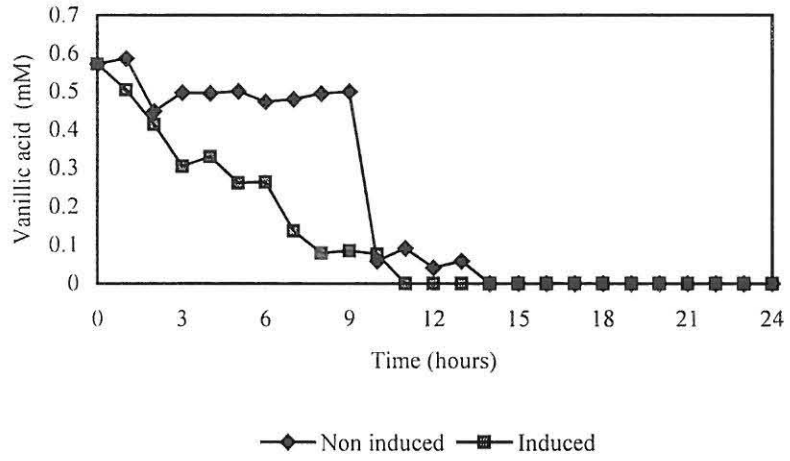


Figure 3.37 Degradation of 0.5 mM vanillic acid with the cell free extracts of RU-LV1. The cell free extracts were obtained by centrifuging French pressed extract and the supernatant used as a cell free extract.

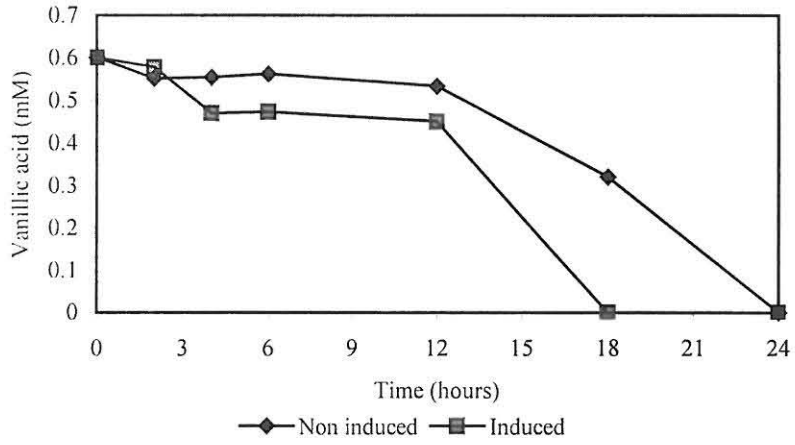


Figure 3.38 Degradation of 0.5 mM vanillic acid by the cell membrane fractions of RU-LV1. RU-LV1 was grown in medium with glucose (for non induced) or vanillic acid (for the induced) as a carbon source. Cells were harvested, French pressed and then centrifuged. The pellet obtained after centrifugation was used as cell debris.

Increasing the concentration of vanillic acid from 0.5 mM to 1 mM only delayed degradation of vanillic acid by the cell free extracts and the cell membrane fractions obtained from RU-LV1. The results obtained using 1 mM vanillic acid are comparable to those of 0.5 mM whereby cell free extracts and cell debris from induced RU-LV1 degraded 1 mM vanillic acid faster than those from non-induced cells. The French pressed extract of induced RU-LV1 completely removed 1 mM vanillic acid after 4 hours and the French pressed extract of non-induced cells took 18 hours to completely degrade all the vanillic acid (1 mM) in the reaction mixture (Figure 3.39). Cell free extracts of non-induced cells did not degrade 1 mM vanillic acid whereas cell free extract of induced cells degraded 93% of 1 mM vanillic acid after 24 hours (Figure 3.40). The cell membrane fractions of induced cells completely removed all the vanillic acid (1 mM) after 4 hours and the cell membrane fractions of non-induced cells took 24 hours to completely degrade 1 mM vanillic acid (Figure 3.41). The results of treating 1 mM vanillic acid by the French pressed extract of induced cells and those obtained using the cell membrane fractions indicate that the enzymes used for the degradation of vanillic acid are largely in the cell membrane fractions, since cell free extracts took a longer time to degrade vanillic acid.

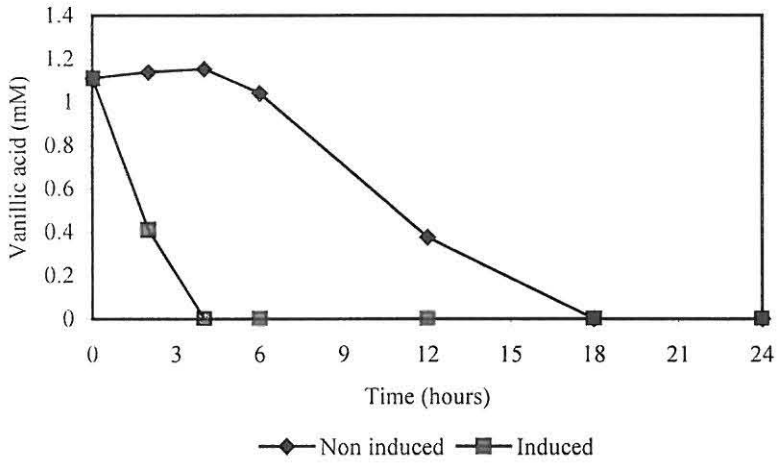


Figure 3.39 Degradation of 1 mM vanillic acid by French pressed extracts of RU-LV1. RU-LV1 was grown in non-induced and induced cultures, the cells were harvested, suspended in buffer, the cell suspension passed through a French press and the extract was used for vanillic acid degradation.

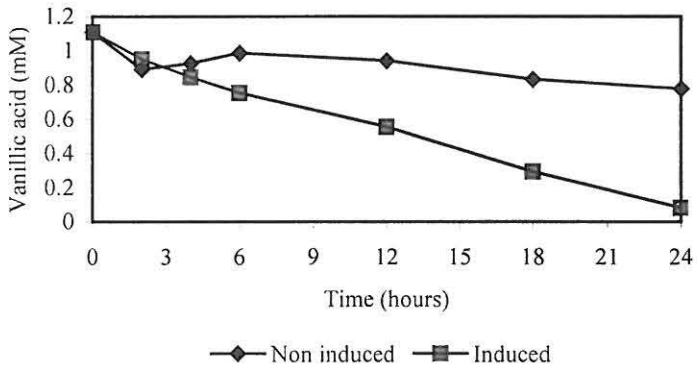


Figure 3.40 Degradation of 1 mM vanillic acid by cell free extracts of RU-LV1. RU-LV1 was grown in non-induced and induced cultures, the cells were harvested, suspended in buffer, the cell suspension passed through a French press and the extract was centrifuged and the supernatant was used as the cell free extract for vanillic acid degradation.

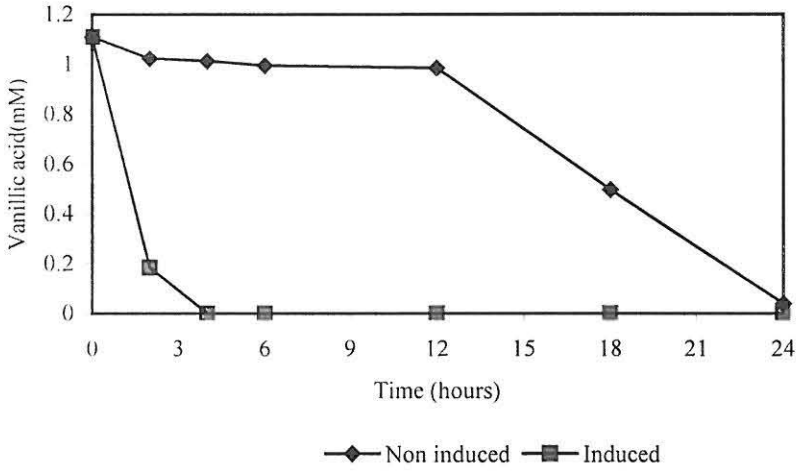


Figure 3.41 Degradation of 1 mM vanillic acid by the cell membrane fractions of RU-LV1. RU-LV1 was grown in non-induced and induced cultures, the cells were harvested, suspended in buffer, the cell suspension passed through a French press and the extract was centrifuged and the pellet was used as the cell debris for vanillic acid degradation.

RU-LV1 was also grown in MM (Appendix A) with glucose for 12 hours after which 0.01% vanillic acid was added and the culture was grown for another 24 hours. Then resting cells, French pressed extract, cell free extracts and cell debris of this culture were used in degrading 1 mM vanillic acid. The French pressed extract and the cell membrane fractions completely degraded vanillic acid after 24 hours. Resting cells and cell free extracts degraded 76 and 60 % of 1 mM vanillic acid after 24 hours (Figure 3.42).

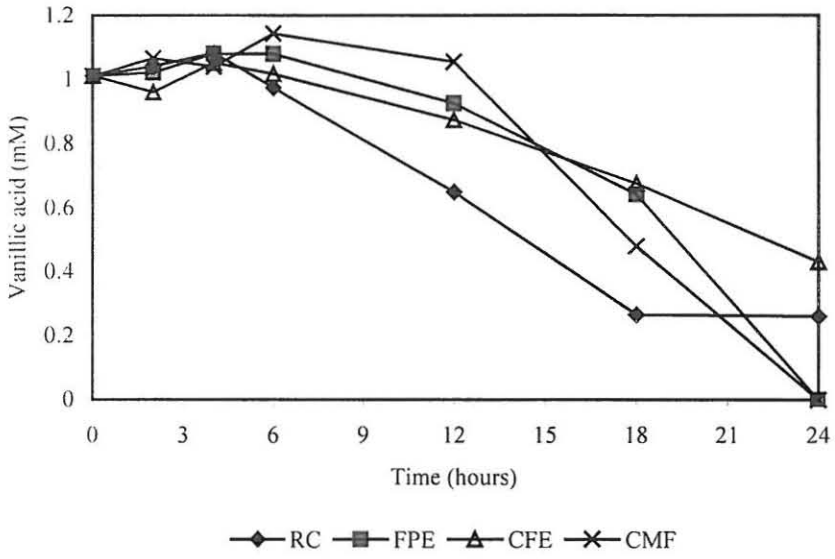


Figure 3.42 Degradation of 1 mM vanillic acid with resting cells (RC), French pressed extract (FPE), cell free extract (CFE) and cell membrane fractions (CMF) of RU-LV1 grown in glucose and 0.01% vanillic acid added after 12 hours of growth in a medium with glucose.

The results of the degradation of vanillic acid by resting cells, French pressed extract, cell free extracts and cell membrane fractions indicate the induction of an enzyme for the degradation of vanillic acid since suspensions obtained from cells grown in 0.01% vanillic acid (used an inducer) always degraded vanillic acid faster than the cells grown in a medium with glucose as a carbon source. In all the reactions for the degradation of vanillic acid no metabolic products were observed on HPLC analysis. Andreoni *et al.* (1995) reported that *Pseudomonas fluorescens* strain FE2 completely metabolized vanillic acid within 24 hours without the appearance of any metabolite. Vanillate-*O*-demethylase is the enzyme responsible for the demethylation of vanillic acid (Civolani *et al.*, 2000). It has been reported that direct measurement of the activity of this enzyme in cell free extracts is difficult to achieve, since the enzyme is extremely air-sensitive and unstable (Morawaki *et al.*, 2000b).

3.5.3 Conversion of ferulic acid by resting cells, French pressed extract, cell free extract and cell membrane fractions of RU-LV1

There was no degradation of 1 mM ferulic acid using whole cells of RU-LV1, and therefore the possibility of degradation and bioconversion of ferulic acid by cell suspensions at high density or extracts with soluble enzymes was studied. One advantage of using resting cells is the possibility of operating biotransformation experiments in the presence of ferulic acid at concentrations that inhibited microbial growth (Barghini *et al.*, 1998).

RU-LV1 was grown in minimal media (MM) (Appendix A) with glucose as a carbon source, or MM supplemented with 0.01% vanillic acid as a carbon source or MM with 0.1% glucose and 0.01% vanillic acid added after 12 hours of growth. The flasks were incubated at 28°C at 200 rpm. When cells reached the absorbance of 1.00 – 1.50 at 600 nm, they were harvested by centrifugation at 10000 rpm for 10 minutes, washed in sterile sodium phosphate buffer (pH 7) and then resuspended in the same buffer to a concentration of 100 mg of cells per mL of buffer. This cell suspension was used as resting cells and was used to study the degradation of ferulic acid. In other experiments the resting cell suspension was also passed through a French press and the French pressed extract was obtained and used, or the extract was centrifuged and the supernatant used as the cell free extract for bioconversion of ferulic acid. When the French pressed extract was centrifuged the pelleted cells were used as the cell membrane fractions. Resting cells or extracts obtained from cells grown in flasks with 0.01% vanillic acid instead of glucose are taken as induced suspensions in this study.

Resting cells of both induced and non-induced RU-LV1 did not degrade 0.4 mM ferulic acid even after 24 hours of incubation. French pressed extract of both induced and non-induced RU-LV1 completely removed 0.4 mM of ferulic acid in the reaction after 18 hours (Figure 3.43). Vanillic acid was observed as a product

of ferulic acid degradation. Vanillic acid accumulated from 10 hours to 16 hours and after that was degraded. These results indicate that the cells first used ferulic acid and when it was completely degraded they used vanillic acid because vanillic acid increased from 10 hours to 16 hours and then decreased until it was completely removed from the reaction mixture. The highest yield of vanillic acid from 0.4 mM ferulic acid was found to be 0.13 mM after 16 hours using the French extract of both induced and non-induced cells (Figure 3.43).

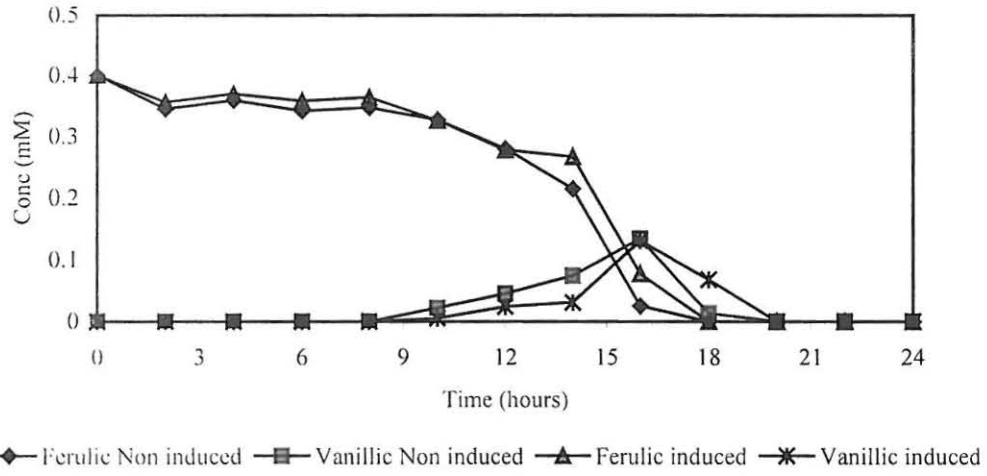


Figure 3.43 Bioconversion of 0.4 mM ferulic acid by French pressed extract of RU-LV1. RU-LV1 was grown in non-induced and induced cultures, the cells were harvested, suspended in buffer, the cell suspension was passed through a French press and the extract used for ferulic acid degradation.

When RU-LV1 was grown in flasks with glucose for 12 hours and then 0.01% vanillic acid added as an inducer, the French pressed extract degraded 100% of 0.4 mM ferulic acid after 20 hours. The vanillic acid yield was 0.1 mM after 16 hours and then the concentration of vanillic acid gradually decreased until it was completely removed from the reaction mixture (Figure 3.44).

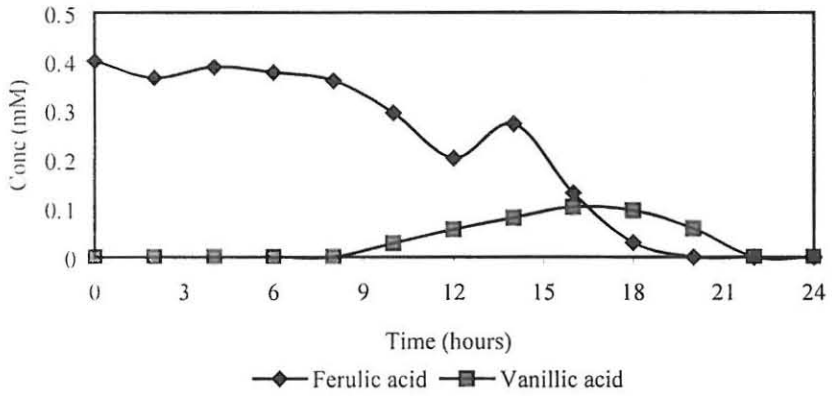


Figure 3.44 Bioconversion of 0.4 mM ferulic acid by the French pressed extract of RU-LV1. RU-LV1 was grown in glucose and 0.01% vanillic acid, the cells were harvested, suspended in buffer, the cell suspension passed through a French press and the extract was used for ferulic acid degradation.

In view of the disappearance of vanillic acid from the reaction mixture when the French pressed extract was used, cell free extracts, obtained by centrifuging the French pressed extract and using the supernatant, were used for the biotransformation of ferulic acid. The cell free extracts of non-induced cells degraded 82% of ferulic acid (0.4 mM) and the vanillic acid accumulated from 16 hours with a gradual increase from 0.014 mM to 0.076 mM after 24 hours. The cell free extracts from induced cells degraded 80 % of 0.4 mM ferulic acid after 16 hours, but only 0.036 mM vanillic acid was observed in the reaction after 16 hours. The vanillic acid gradually disappeared from the reaction mixture. Trace amounts of vanillin were observed in the reaction supernatant (Figure 3.45).

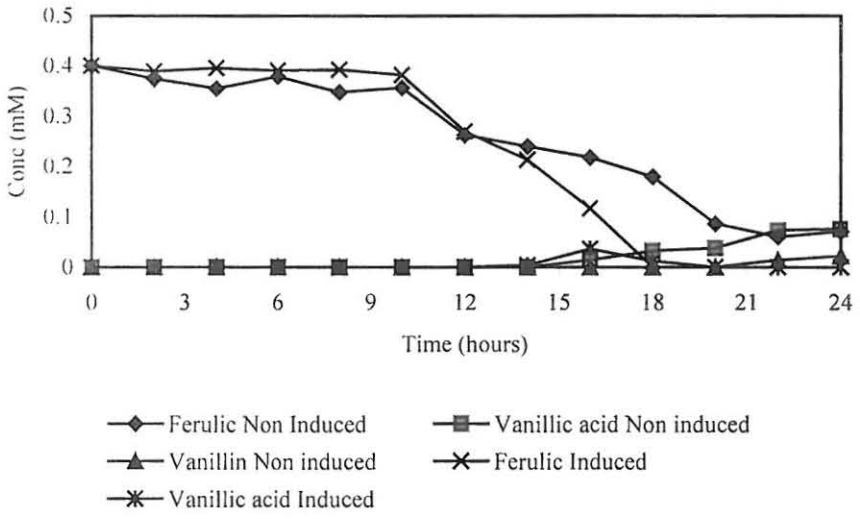


Figure 3.45 Bioconversion of 0.4 mM ferulic acid by the cell free extract of RU-LV1. RU-LV1 was grown in glucose and 0.01% vanillic acid, the cells were harvested, suspended in buffer, the cell suspension passed through a French press and the extract was used for ferulic acid degradation.

Cell free extracts from cells grown on glucose but induced with 0.01% vanillic acid degraded 88% of ferulic acid at 0.4 mM after 24 hours. Vanillic acid was observed from 14 hours and it reached a maximum concentration of 0.14 mM after 22 hours and traces of vanillin were observed from 20 hours to 24 hours (Figure 3.46).

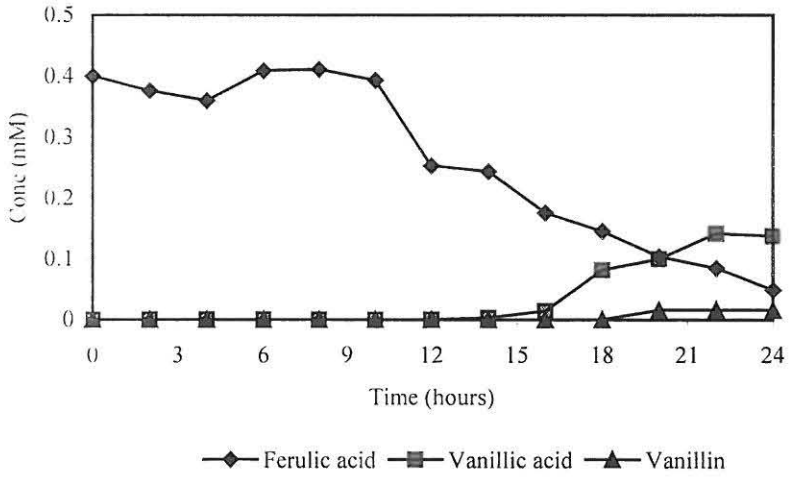


Figure 3.46 Conversion of ferulic acid by cell free extract of RU-LV1 grown on glucose and induced 0.01% vanillic acid. The cells were French pressed and centrifuged, and then the clear supernatant was used as the cell free extract.

When using cell free extracts the highest yield of vanillic acid was achieved using an extract from cells grown in glucose and vanillic acid. Trace amounts of vanillin were also observed with cell free extracts from non-induced cells and cells grown in glucose and vanillic acid. The vanillin appeared from 20 hours, and it accumulated until 24 hours (Figure 3.46).

In order to improve enzyme solubilisation, the French pressed extract was disrupted with an ultrasonicator (5 x 10 seconds bursts with 20 seconds breaks in-between), and the suspension was then centrifuged. The clear supernatant, taken as a source of soluble enzymes, was used as a cell free extract for ferulic acid bioconversion. The protein concentration of the cell free extract obtained by French pressing and the cell free extract obtained by French pressing and sonication was determined using the Bradford's assay (Appendix B). Adding sonication after French pressing increased protein concentration of the cell free extract (Figure 3.47).

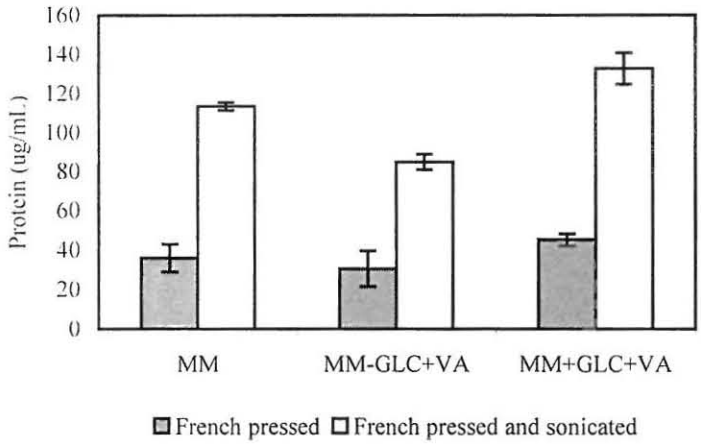


Figure 3.47 Protein concentration of the cell free extract. RU-LV1 was grown in MM, MM-GLC+VA and MM+GLC+VA, the cells were harvested, French pressed, other RU-LV1 cells were sonicated after French pressing. The French pressed extract and the sonicated French pressed extract were centrifuged and the clear supernatant was used to determine protein concentration using the Bradford's assay (Appendix B)

The sonicated French pressed cell free extract of non-induced cells degraded 75% of 0.4 mM ferulic acid after 24 hours. Vanillic acid was detected from 12 hour's reaction and its concentration increased from 0.025 mM (after 12 hours) to 0.142 mM after 24 hours. Trace amounts of vanillin were observed after 24 hours of the reaction. Ferulic acid was completely removed from the reaction mixture after 14 hours when sonicated french pressed cell free extracts of induced cells were used. Vanillic acid appeared after 12 hours at a concentration of 0.057 mM. However, no vanillic acid was observed after 14 hours (3.48).

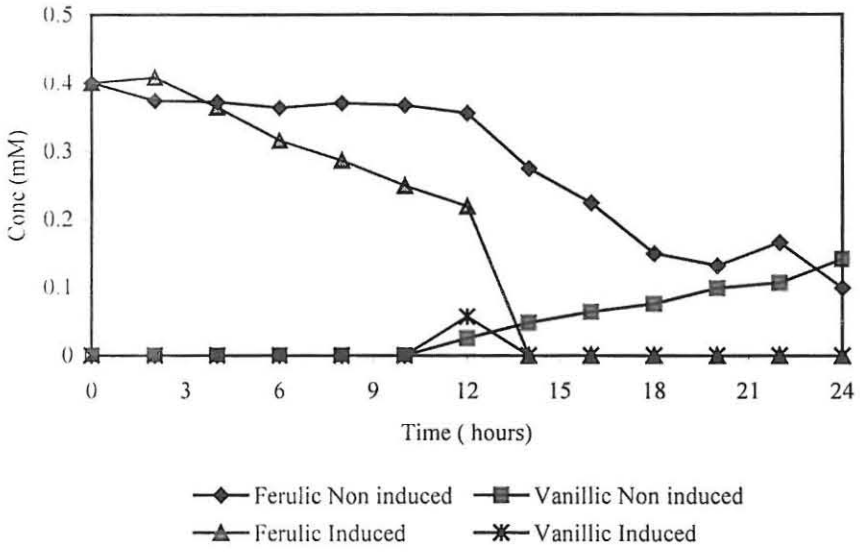


Figure 3.48 Bioconversion of ferulic acid by cell free extracts of RU-LV1. Non-induced and induced cells of RU-LV1 were French pressed, then sonicated and centrifuged. The clear supernatant was used as a cell free extract.

Cell free extracts which were French pressed and sonicated, obtained from cells grown in glucose and induced with 0.01% vanillic acid, degraded 74 % of ferulic acid after 24 hours. The yield of vanillic acid reached 0.23 mM after 24 hours and this was the maximum yield of vanillic acid with 0.4 mM ferulic acid (Figure 3.49). RU-LV1 in these experiments was grown in two carbon sources, glucose and vanillic acid, and in Section 3.5.1 the highest growth of the culture was observed in flasks with glucose and vanillic acid. When RU-LV1 culture is grown in glucose and vanillic acid, more vanillic acid is produced suggesting that more enzymes are produced and French pressing and sonicating release the enzymes into the supernatant.

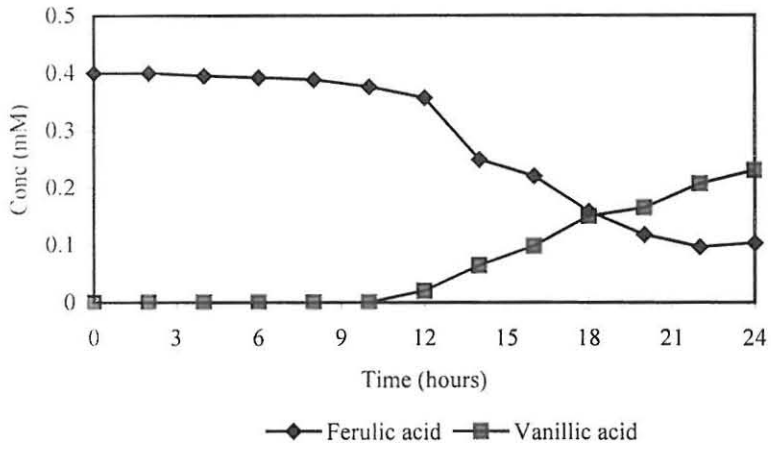


Figure 3.49 Bioconversion of ferulic acid by cell free extracts that have been obtained by French pressing and sonicating RU-LV1 cells grown on glucose and induced with 0.01% vanillic acid.

When using the cell membrane fractions of non-induced and induced cells after French pressing and sonicating cells, 100 % of 0.4 mM ferulic acid was degraded after 14 hours of the reaction. Vanillic acid accumulated from 8 hours and the concentration reached 0.1 mM after 12 hours, then disappeared from the reaction mixture (Figure 3.50). The cell debris of French pressed sonicated cells grown on glucose and induced with vanillic acid completely degraded ferulic acid after 14 hours, with the accumulation of vanillic acid from 8 hours gradually increasing to 0.092 mM after 12 hours, then vanillic acid was completely degraded from the reaction mixture (Figure 3.51).

The results of the ferulic acid reactions with the cell membrane fractions indicate that these cells use ferulic acid until it is completely removed from the reaction. When ferulic acid is degraded, it is converted into vanillic acid. After all the ferulic acid has disappeared from the reaction mixture, the cells use vanillic acid. Another possibility is that the enzyme responsible for vanillic acid degradation is

present in the reaction mixture since the cell membrane fractions of RU-LV1 also degraded vanillic acid (Section 3.5.2).

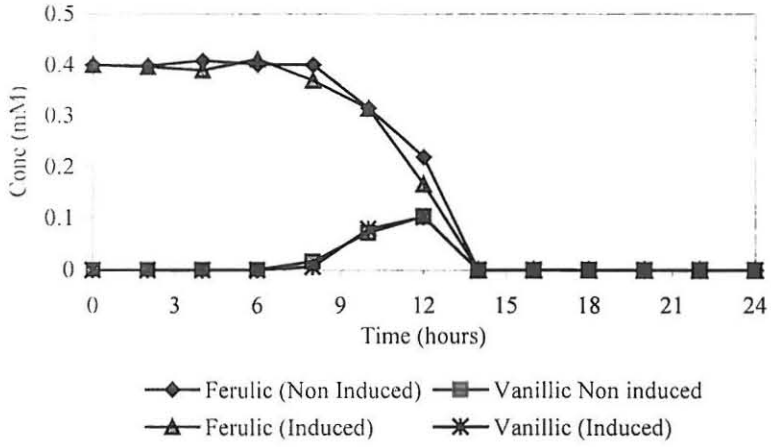


Figure 3.50 Conversion of 0.4 mM ferulic acid by cell membrane fractions from French pressed and sonicated RU-LV1 cells.

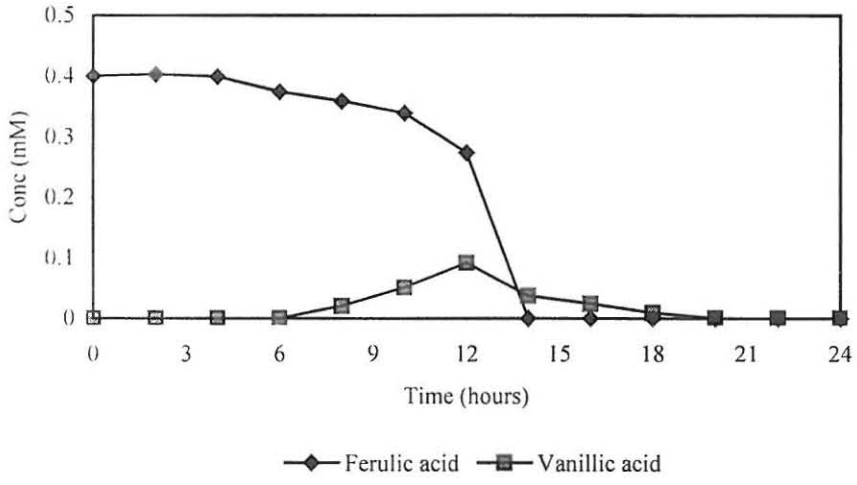


Figure 3.51 Biotransformation of 0.4 mM ferulic acid by cell membrane fractions from French pressed and sonicated RU-LV1 cells grown in glucose and 0.01% vanillic acid.

Even though vanillic acid appeared earlier when the cell debris was used, as compared to when the cell free extracts were used, it also disappeared quickly. The lower yields of vanillic acid from the cell debris reactions as compared to the cell free extracts from French pressed sonicated cells indicate that the enzymes for the conversion of ferulic acid to vanillic acid are released into the cell free extract.

Increasing the time of sonication from 5 x 10 seconds bursts to 10 x, 15 x or 20 x 10 second bursts to obtain cell free extracts did not improve the conversion of ferulic acid to vanillic acid (Figure 3.52). However, the rate of conversion of ferulic acid was slower when the sonication time was increased suggesting that the enzyme(s) responsible for ferulic acid degradation may have been denatured during extended sonication.

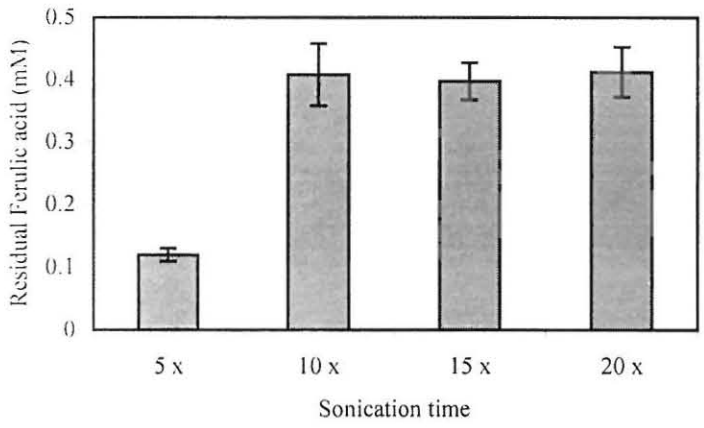


Figure 3.52 Degradation of ferulic acid by cell free extracts obtained by French pressing and sonication (for different intervals) RU-LV1 cells grown in glucose and vanillic acid. After French pressing, the extract was sonicated for 5 x, 10 x, 15 x or 20 x 10 second bursts, then centrifuged and the supernatant was used as cell free extract.

In an attempt to increase the rate of conversion of ferulic acid and to increase the production of vanillic acid from ferulic acid, NAD^+, ATP and CoA were added to the reaction mixture of ferulic acid and cell free extracts (method adapted from Huang *et al.*, 1993). Huang *et al.* (1993) reported that cell free extracts of *Rhodotorula rubra* did not transform ferulic acid into vanillic acid without the addition of cofactors. Cell free extracts of *Streptomyces setonii* demonstrated that the bioconversion from ferulic acid to vanillic acid is dependant on CoA/ATP and NAD^+ (Sutherland *et al.*, 1983). The cell free extracts were obtained by French pressing and sonicating (5 x 10 second bursts) of RU-LV1 cells grown on glucose and induced with 0.01% vanillic acid. Addition of the three cofactors partially inhibited ferulic acid conversion and hence vanillic acid production was decreased (Figure 3.53)

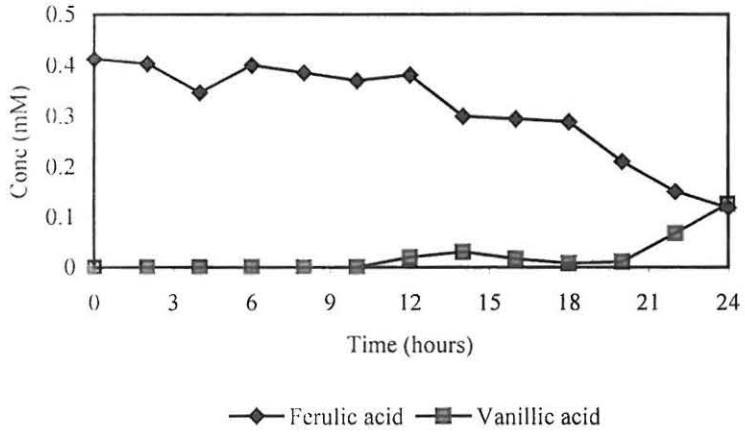


Figure 3.53 Conversion of 0.4 mM ferulic acid by cell free extract obtained by french pressing and sonicating of RU-LV1 cells grown in glucose and 0.01% vanillic acid, NAD^+, CoA and ATP were added in the reaction mixture.

In other experiments the concentration of ferulic acid in the reaction mixture was increased in order to investigate whether higher ferulic acid concentrations produce a higher yield of vanillic acid. Cell free extracts obtained by French

pressing and sonicating (5 x 10 second bursts) RU-LV1 cells grown on glucose and induced with 0.01% vanillic acid were used in reactions using the higher concentrations of ferulic acid. Increasing the concentration of ferulic acid resulted in an increased production of vanillic acid. When the concentration of ferulic acid was increased to 0.5 mM, 0.4 mM vanillic acid was observed after 14 hours of the reaction and 0.64 mM vanillic acid was produced after 18 hours when 0.8 mM ferulic acid was used (Figure 3.54). Higher concentrations (1 and 1.2 mM) of ferulic acid were not degraded by the cell free extracts and therefore no vanillic acid was observed in the reaction even after 36 hours of incubation.

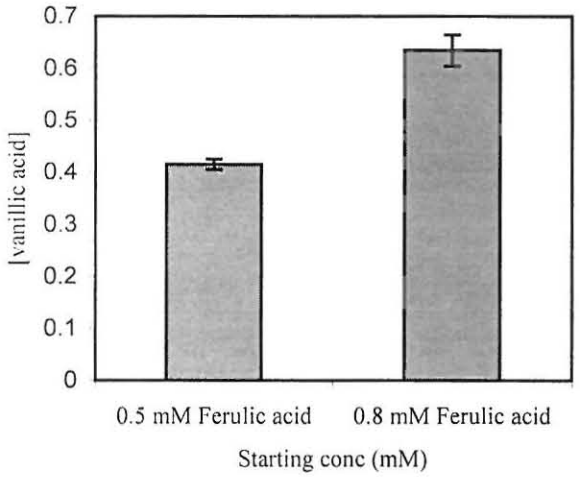


Figure 3.54 Conversion of different concentrations of ferulic acid by cell free extracts obtained by french pressing and sonicating RU-LV1 cells grown on glucose and induced with 0.01% vanillic acid.

Even though vanillic acid was found as a conversion product of ferulic acid in the reaction supernatant, little or no accumulation of vanillin was observed. Vanillic acid has been reported to be a prominent intermediate in the degradation of ferulic acid by many bacteria (Toms and Wood, 1970; Sutherland *et al.*, 1983; Andreoni *et al.*, 1984; Huang *et al.*, 1993; Andreoni *et al.*, 1995). An *Escherichia coli* strain

isolated from decaying bark, produced vanillin, vanillic acid and protocatechuic acid from ferulic acid and vanillic acid yielded protocatechuic acid by demethylation (Otuk, 1985).

Ferulic acid decarboxylase, the enzyme responsible for the degradation of ferulic acid was reported to be colourless, constitutive in nature and did not require cofactors or metal ions for full enzyme activity (Huang *et al.*, 1994). The enzyme involved in the degradation of ferulic acid in this study appears to be similar to ferulic acid decarboxylase in that the cell free extract was colourless and did not need cofactors for the degradation of ferulic acid. It differs from ferulic acid decarboxylase in that the enzyme was induced by the addition of 0.01% vanillic acid in the medium.

3.5.5 Bioconversion of vanillin by resting cells, French pressed extract, cell free extract and cell membrane fractions of RU-LV1

To determine whether vanillic acid was formed by the oxidation of vanillin, resting cells, French pressed extract, cell free extract and cell debris of RU-LV1 were used in the degradation of 0.5 mM vanillin. RU-LV1 was grown in MM (Appendix A) and MM with 0.01% vanillic acid instead of glucose. When RU-LV1 culture reached the absorbance of 1.00 – 1.50 at 600 nm, the cells were harvested by centrifugation at 10000 rpm for 10 minutes, washed in sterile 0.1 M sodium phosphate buffer (pH 7) and then resuspended in the same buffer to a concentration of 100 mg of cells per mL of buffer. This cell suspension was used as resting cells and was used to study the degradation of vanillin. In other experiments the resting cell suspension was also passed through a French press and the French pressed extract was centrifuged and the clear supernatant used as the cell free extract for biotransformation of vanillin. When the French pressed extract was centrifuged the pelleted cells were used as the cell membrane fractions.

Resting cells of both non-induced and induced RU-LV1 completely degraded 0.5 mM vanillin within 4 hours, resting cells of the non-induced culture transformed vanillin into vanillic acid (Figure 3.55). In reactions where resting cells were obtained from induced cultures, no vanillic acid was observed in the reaction supernatants perhaps because the vanillic acid was degraded as soon as vanillin was converted to it. In the reactions where vanillic acid was used as a substrate, there was no product detected in the reaction supernatants (Section 3.4.2).

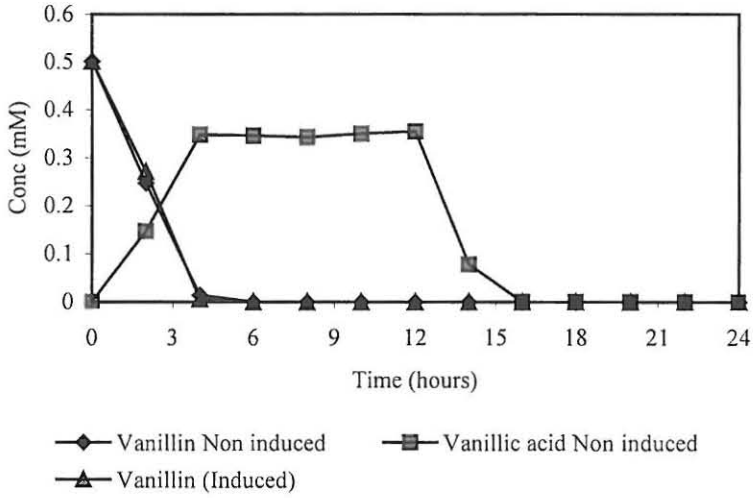


Figure 3.55 Bioconversion of 0.5 mM vanillin by resting cells of RU-LV1. RU-LV1 was grown in flasks with glucose or vanillic acid as a carbon source, after 24 hours the cells were harvested and suspended in buffer. The cell suspension was used for the degradation of vanillin.

The conversion of vanillin by the cell free extracts was delayed as compared with conversion using resting cells and complete degradation occurred after 14 hours and 10 hours using extracts from non-induced and induced cells respectively. In reactions where cell free extracts were obtained from non-induced cells, 0.157 mM vanillic acid was observed after 12 hours of the reaction, thereafter the vanillic acid decreased until it disappeared from the reaction. 0.079 mM vanillic

acid after 8 hours was observed in vanillin reactions where induced cell free extracts were used and after 8 hours the vanillic acid disappeared from the reaction mixture (Figure 3.56).

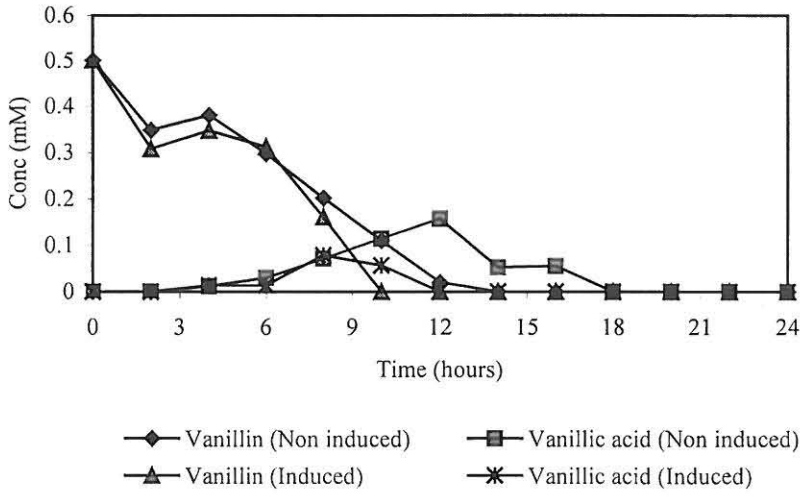


Figure 3.56 Bioconversion of vanillin by cell free extracts of RU-LV1. Non-induced and induced cells of RU-LV1 were French pressed and centrifuged, then the clear supernatant was used as a cell free extract.

Cell membrane fractions obtained from centrifuging both the non-induced and induced French pressed extract converted 0.5 mM vanillin within two hours of the reaction and 0.332 mM of vanillic acid was observed in the reaction (Figure 3.57).

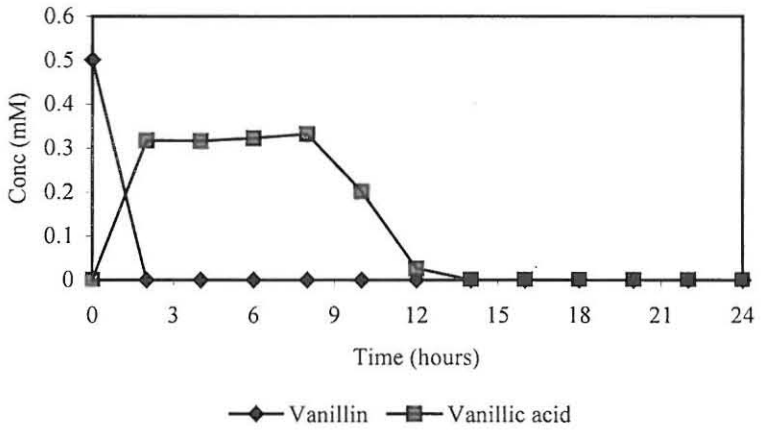


Figure 3.57 Bioconversion of vanillin by cell membrane fractions of RU-LV1. Non-induced and induced cells of RU-LV1 were French pressed and centrifuged, the pellet supernatant was used as the cell debris.

Resting cells, cell free extracts obtained by French pressing and cell membrane fractions of the French pressed RU – LV1 grown in glucose or cells induced with 0.01% vanillic acid degraded vanillin into vanillic acid. RU-LV1 was also grown in glucose and after 12 hours of growth induced with 0.01% vanillic acid, then resting cells and cell free extract obtained by French pressing and the centrifuging the French pressed extract, were used to investigate the bioconversion of vanillin. Resting cells completely degraded vanillin in 4 hours whereas cell free extracts took 10 hours to complete the degradation of vanillin. In both cases vanillic acid was found as the bioconversion product. However, more vanillic acid (0.293 mM) was found in the reaction where resting cells were used as compared to 0.152 mM when cell free extracts degraded vanillin (Figure 3.58 and Figure 3.59). The cell membrane fractions completely degraded vanillin within 2 hours and 0.408 mM vanillic acid was observed within 4 hours of the reaction (Figure 3.60).

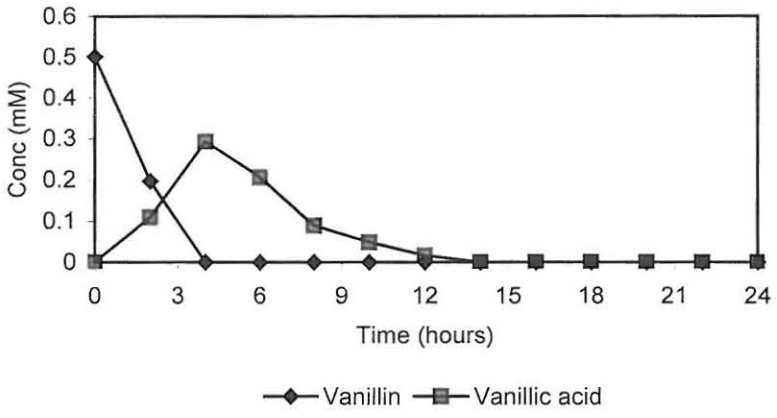


Figure 3.58 Bioconversion of vanillin into vanillic acid by resting cells of the culture grown in glucose and 0.01% vanillic acid. RU-LV1 was grown on glucose and induced with 0.01% vanillic acid, the cells were harvested and suspended in buffer, then the cell suspension was used for the degradation of vanillin.

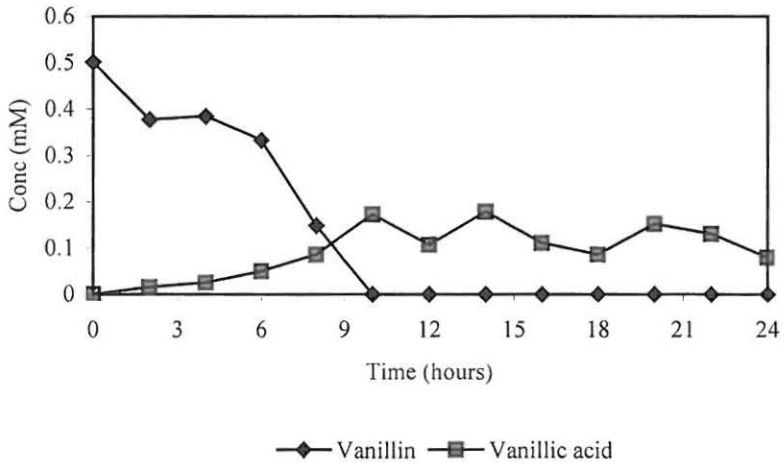


Figure 3.59 Conversion of vanillin into vanillic acid by cell free extracts obtained by French pressing and centrifuging RU-LV1 cells grown in glucose and induced with 0.01% vanillic acid.

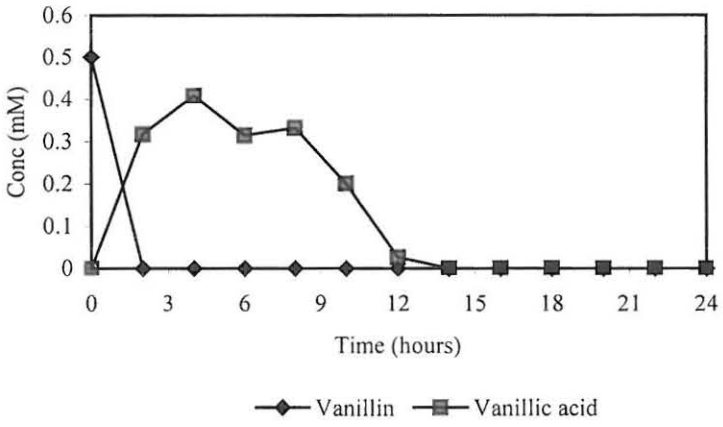


Figure 3.60 Biotransformation of vanillin by cell membrane fractions of RU-LV1 culture grown in glucose and 0.01% vanillic acid. RU-LV1 was grown on glucose and induced with 0.01% vanillic acid, the cells were harvested and French pressed, the French pressed extract was centrifuged and the pellet was used as cell debris.

The higher conversion of vanillin as compared to that of ferulic acid might be the reason for not being able to detect vanillin in the reaction supernatants during the biotransformation of ferulic acid. Karmakar *et al.* (2000) found vanillic acid as a product of ferulic acid degradation by *Bacillus coagulans*. The French pressed extract, cell free extract and the cell membrane fractions of RU-LV1 degraded ferulic acid with the accumulation of a high value aromatic compound (vanillin). Vanillin was presumably then rapidly oxidized to vanillic acid and the aromatic ring of vanillic acid was disrupted to form non-aromatic compounds. Karmakar *et al.* (2000) also suggested that vanillic acid was produced by the oxidation of vanillin, when *Bacillus coagulans* was used for the degradation of ferulic acid.

The proposed pathway for ferulic acid degradation by French pressed extract, cell free extracts and cell membrane fractions of RU-LV1 is shown in Figure 3.61.

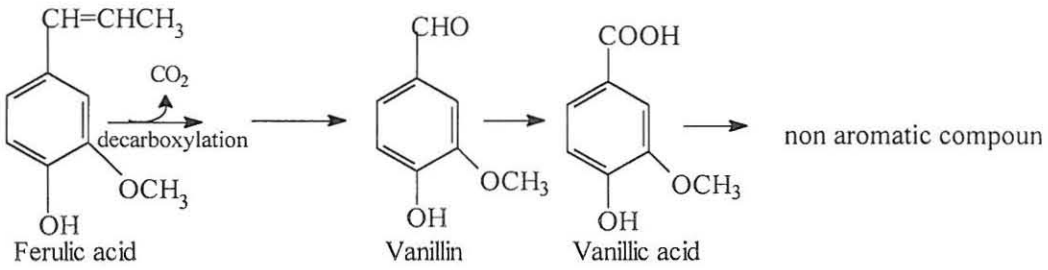


Figure 3.61 Proposed pathway for ferulic acid degradation by French pressed extracts, cell free extracts and cell membrane fractions of RU-LV1.

Vanillic acid has also been reported to be a high value chemical (Pometto and Crawford, 1983; Karmakar *et al.*, 2000). Pometto and Crawford (1983) reported that vanillic acid as a specialty chemical has been found to be more expensive about 10 times than vanillin, it has been used as a monomer in the synthesis of polyesters, vanillic acid diethyl amide is widely used in Europe as an analeptic medicine, and 5-nitrovanillic acid and 5-aminovanillic acid have antibacterial activity. Extracts obtained from RU-LV1 cells grown in glucose and vanillic acid convert a readily available and inexpensive ferulic acid into a useful chemical.

3.5.6 Stability of the cell free extract

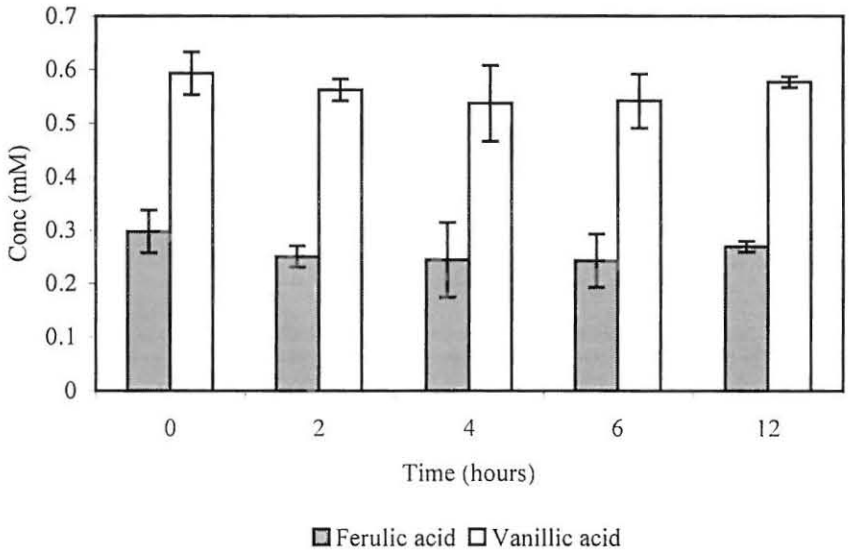
Most of the research on the bioconversion of ferulic acid to vanillin and vanillic acid has been done using whole cells with little emphasis on enzyme systems responsible for the conversion process (Huang *et al.*, 1994). In this study French pressed extracts, cell free extracts and cell membrane fractions have been used in the conversion of ferulic acid to vanillic acid. The ability of the cell free extract, obtained by French pressing and sonicating RU-LV1 cells grown in flasks with glucose and induced with 0.01% vanillic acid, in the bioconversion of ferulic acid to vanillic acid was found to be greater than all the other extracts used (Section

3.5.4). The stability of this cell free extract in the bioconversion of ferulic acid to vanillic acid after storage of the cell free extract was therefore investigated.

RU-LV1 was grown in MM with glucose and 0.01% vanillic acid as an inducer after 12 hours of culture growth. The cells were harvested by centrifugation, suspended in sterile sodium phosphate buffer (pH 7), French pressed and then sonicated (5 x 10 second bursts). The sonicated French pressed extract was centrifuged and the clear supernatant, taken as a source of soluble enzymes, was used a cell free extract. The cell free extract was stored at 4 °C and – 20 °C for 0, 2, 4, 6 and 12 hours, then it was used in the bioconversion of 0.8 mM ferulic acid. An equal volume of the cell free extract was added to 1 mL ferulic acid and the reaction mixture was incubated at 28 °C at 200 rpm for 22 hours. After 22 hours the reaction was stopped by adding 500 mL of 12% trichloroacetic acid and then analysed by HPLC.

Cell free extract stored for 2 to 12 hours at 4 and – 20 °C converted 0.8 mM ferulic acid after 22 hours with almost the same activity as the cell free extract which has been used immediately (Figure 3.62 A). However, there was a lower yield of vanillic acid produced from ferulic acid when the cell free extract was stored at – 20 °C (Figure 3.62 B) and this suggest that the enzyme responsible for vanillic acid production from ferulic acid is affected by the freezing and thawing of the cell free extract. These results correlate with the work by Huang *et al.* (1994) where these authors reported that ferulic acid decarboxylase (the enzyme responsible for ferulic acid degradation) is stable at 4 °C.

A



B

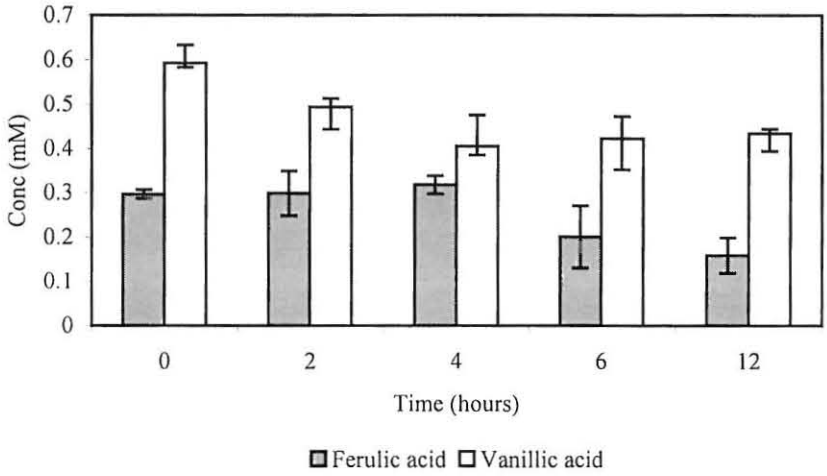


Figure 3.62 Stability of the cell free extract in the bioconversion of ferulic acid to vanillic acid. The cell free extract was obtained by French pressing and sonicating RU-LV1 cells grown in glucose and induced with 0.01% vanillic acid, then it was stored at 4 °C and – 20 °C for 0, 2, 4, 6 and 12 hours. After storage at 4 and – 20 °C, the cell free extract was incubated with 0.8 mM ferulic acid for 22 hours with agitation. A – storage at 4 °C and B – storage at 20 °C.

Ferulic acid was not degraded by whole cells of RU-LV1 but cell free extracts of RU-LV1 cells degraded ferulic acid. Both whole cells and cell free extracts of RU-LV1 degraded vanillic acid. It is possible that active transport is required for the uptake of these acids and that a system for vanillic acid is present but not for ferulic acid. Further investigations are required to find out whether these reactions are carried out by dedicated enzymes or are they fortuitous activities of enzymes with other functions.

3.6 IDENTIFICATION OF RU-LV1 USING MOLECULAR TECHNIQUES

Since extracts obtained from RU-LV1 cells were used for the bioconversion of ferulic acid to vanillic acid and for the degradation of vanillic acid, this isolate was identified using molecular techiniques.

Total genomic DNA was extracted using the method described in Ausubel *et al.* (1983) and the universal primers GM5F and 907RG were used for 16S rRNA gene amplification (Figure 3.63)

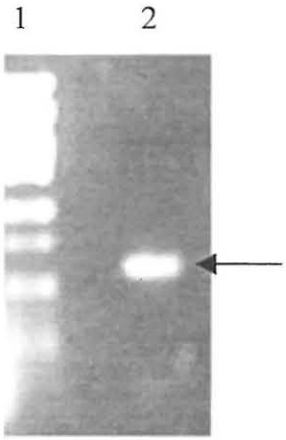


Figure 3.63 16s rDNA amplification of RU-LV1 DNA using primers GM5F and 907RG. 1 = λ DNA digest with *Pst* 1, 2 = PCR product of FU-LV1 DNA which is approximately 586 bp.

This PCR product was inserted into the pGEMT-Easy vector and transformed into *E. coli* DH5ά. The resultant clone was then sequenced via Big Dye and the 16S rRNA gene sequence is shown in Figure 3.64. The sequence of the 16S rRNA gene from RU-LV1 was entered into the Ribosomal Database project (RDP) website similarity rank and aligned with the 10 most similar 16S rRNA sequences in the RDP database (Table 3.17).

```
  1 TCCTACGGGA GGCAGCAGTG GGGAATATTG CACAATGGGC GCAAGCCTGA
    AGGATGCCCT CCGTCGTCAC  CCCTTATAAC GTGTTACCCG  CGTTCGGACT

 51 TGCGGCGACG CCGCGTGGGG GATGACGGCC TTCGGGTTGT AAACTCCTTT
    ACGCCGCTGC GGCGCACCCC CTACTGCCGG AAGCCCAACA TTTGAGGAAA

101 CGCTAGGGAC GAAGCCTTTT GGTGACGGTA CCTGGAGAAG AAGCACCGGC
    GCGATCCCTG  CTTCGGAAAA CCACTGCCAT GGACCTCTTC  TTCGTGGCCG

151 TAACTACGTG CCAGCGGCCG CGGTAATACG TAGGGTGCGA GCGTTGTCCG
    ATTGATGCAC GGTCGCCGGC GCCATTATGC  ATCCCACGCT  CGCAACAGGC

201 GAATTACTGG GCGTAAAGAG CTCGTAGGTG GTTTGTCGCG TCGTCTGTGA
    CTTAATGACC CGCATTTCTC  GAGCATCCAC CAAACAGCGC AGCAGACACT

251 AATCCCGGGG CTTAACTTCG GGCGTGCAGG CGATACGGGC ATAACTTGAG
     TTAGGGCCCC GAATTGAAGCCCGCACGTCC GCTATGCCCG  TATTGAACTC

301 TGCTGTAGGG GAGACTGGAA TTCCTGGTGT  AGCGGTGAAA TGCGCAGATA
    ACGACATCCC CTCTGACCTT  AAGGACCACA TCGCCACTTT  ACGCGTCTAT

351 TCAGGAGGAA CACCAATGGC GAAGGCAGGT CTCTGGGCAG TAACTGACGC
    AGTCCTCCTT  GTGGTTACCG  CTTCCGTCCA  GAGACCCGTC ATTGACTGCG

401 TGAGGAGCGA AAGCATGGGT  AGCGAACAGG ATTAGATACC CTGGTAGTCC
     ACTCCTCGCT  TTCGTACCCA  TCGCTTGTCC  TAATCTATGG GACCATCAGG

451 ATGCCGTAAA CGGTGGGCGC TAGGTGTAGG GGTCTTCCAC GACTTCTGTG
     TACGGCATTT GCCACCCGCG ATCCACATCC CCAGAAGGTG CTGAAGACAC

501 CCGCAGCTAA CGCATTAAGC GCCCCGCCTG GGGAGTACGG CCGCAAGGCT
    GGCGTCGATT GCGTAATTCG CGGGGCGGAC CCCTCATGCC  GGCGTTCCGA

551 AAAACTCAAA GGAATTGACG G
     TTTTGAGTTT  CCTTAACTGC  C
```

Figure 3.64 DNA sequence for RU-LV1 16S rRNA gene cloned into pGEM-T Easy vector.

Table 3.17 Similarity rank of the RU-LV1 16S rRNA gene sequence.

Identities	Similarity %	Organism
567/571	99%	*Corynebacterium glutamicum*
568/572	99%	*Corynebacterium acetoacidophilum*
564/571	98%	*Corynebacterium* sp T144
562/571	98%	*Rhodococcus globerulus*
561/571	98%	*Corynebacterium efficiens* strain YS-155
561/571	98%	*Corynebacterium efficiens* strain YS 52
560/571	98%	*Corynebacterium efficiens* strain YS-314
558/571	97%	*Corynebacterium callunase*
560/575	97%	*Corynebacterium flavascens*
556/571	97%	*Corynebacterium* spp

The similarity ranking of RU-LV1 16S rRNA gene clone indicates a high degree of similarity to *Corynebacterium glutamicum* and *Corynebacterium acetoacidophilum*.

Using six primers (F1; F3; F5 forward and R1; R3; R5 reverse) a 16S rRNA gene of RU-LV1 was sequenced by Di James in UCT and the sequence is shown in Appendix H. This sequence was also entered into RDP website similarity rank and aligned with the 10 most similar 16S rRNA sequences in the RDP database. The results also showed a high similarity to *Corynebacterium glutamicum* (100% similarity) and *Corynebacterium acedophilum* (100% similarity).

Bacteria have been isolated from OMW. *Sporobacterium olearium* gen. nov., sp. nov., *Clostridium methoxybenzovorans* sp. nov. and *Propionibacterium microaerophilum* sp. nov. have been isolated from an OMW treatment digester (Mechichi *et al.*, 1999 a and b, Koussemon *et al.*, 2001). *Sporobacterium olearium* has been used in the degradation of aromatic compounds that are known to be found in OMW (Mechichi *et al.*, 1999b). To our knowledge no research has been done in the bioproduction of high value chemicals using microorganisms

that have been isolated from OMW. However, Tadasa (1977) reported that a strain of *Corynebacterium* was isolated for growth on eugenol and found to accumulate low levels of vanillin. Therefore future work is needed on the enzymology, characterization of RU-LV1 and the biochemistry of the bioconversion of ferulic acid to vanillic acid.

CHAPTER 4

CONCLUSION

This work has been the first step of a research project focused on the development of a biotechnological process for the removal or biotransformation of phenolics in olive production effluents. One of the objectives of this study was to characterize effluents from an olive processing plant. The olive production effluents were collected from different stages in the process for treating olive wastewater, viz, a fermentation tank (FB), the surface of a digester (LV) and an evaporation pond (SO). FB, LV and SO were found to be similar in composition with olive mill wastewater (OMW) in that the first effluent after the processing of olives, FB, had a higher phenolic content and COD than the other two olive effluents, LV and SO. UV analysis of the olive effluents FB and LV clearly indicated that the components of the effluents were aromatic in nature, since these effluents absorbed between 270 and 310 nm. TLC and HPLC results for the identification of components in the effluent extract of FB acidified to pH 1 showed that vanillic acid, *p*-coumaric acid, 4-hydroxyphenyl acetic acid, syringic acid, coumaric acid, ferulic acid and protocatechuic acid are contained in the effluent FB. Quantitative analysis of the phenolic compounds by HPLC demonstrated that hydroxyphenyl acetic acid constitutes approximately 60% of the phenolic acid identified in FB pH 1.

The other aim of this study was to isolate bacteria from olive effluents capable of degrading aromatic compounds that could be present in olive effluents. The use of microorganisms isolated from an industrial effluent can be successful in detoxifying and probably biotransforming that effluent since the microbes are adapted to the toxicity of the effluent. Five bacteria were isolated from three olive production effluents. The isolated bacteria were found to tolerate high salt concentrations and a wide pH and temperature range. These isolates were found

to degrade approximately 96% of caffeic acid and protocatechuic acid at 1 mM concentrations. One isolate RU-LV1 also degraded 73% *p*-coumaric acid and 100% vanillic acid (1 mM). RU-LV1 also degraded 99, 97 and 100 % of 5 mM caffeic acid, 5 mM protocatechuic acid and 5 mM vanillic acid respectively.

OMW usually possesses a high antimicrobial effect, another objective of this work was to screen microrganisms which are able to grow and thereby reduce the total phenolic content of the olive production effluents. The bacteria isolated from the wastes reduced the phenolic content of LV, with RU-SO1 degrading about 47% of the total phenolic content of 20% LV. *Pseudomonas putida* strains RU-KM1 and RU-KM3$_s$ indicated better efficiency in reducing the phenolic content of the olive effluents as compared to the bacteria isolated from the effluents. The treatment of the olive effluents FB and LV with the white rot fungi, *Trametes versicolor* and *Neurospora crassa* produced an appreciable decrease in the phenolic content of the effluents.

The isolate RU-LV1 was chosen for investigating the bioconversion of vanillic acid and ferulic acid. Whole cells, resting cells, French pressed extracts, cell free extracts and cell debris of RU-LV1 degraded vanillic acid. No reaction products were observed from vanillic acid on analysis by HPLC. Whole cells and resting cells of RU-LV1 did not degrade ferulic acid, however French pressed extracts, cell free extracts and cell membrane fractions of RU-LV1 degraded ferulic acid. Vanillic acid was found to be the bioconversion product in the conversion of ferulic acid. The maximum yield of vanillic acid was obtained when cell free extracts, obtained by French pressing and sonicating RU-LV1 cells grown in glucose and induced with 0.01% vanillic acid, was used. The cell free extract was found to be stable at 4 °C for 2 to 12 hours for the conversion of ferulic acid. It was found that the decarboxylation of ferulic acid and the oxidation of vanillin did not require induction while the degradation of vanillic acid required induction.

Even though RU-LV1 was found to be Gram negative in preliminary test, use of molecular techniques indicated that the similarity ranking of RU-LV1 16S rRNA gene and its clone showed a high similarity to *Corynebacterium glutamicum* and *Corynebacterium acedophilum*. *Corynebacterium* belong to Gram positive bacteria, however, and the preliminary Gram stain should be repeated to clarify this issue.

This study has achieved the preliminary steps in a project for the utilization of olive processing waters. Microorganisms have been shown to reduce phenolic pollution in olive effluents. Bacteria isolated from olive wastes degraded aromatic compounds found in the effluents. The study has also shown that a microorganism isolated from these effluents have the ability of converting an inexpensive substrate, ferulic acid which is found in the effluents, into a high value chemical.

4.1 Future work

The olive production waste FB has been shown to contain aromatic compounds (ferulic acid and vanillic acid) that can be used as starting materials in the production of high value compounds, and studies are needed in the extraction of these aromatic acids from the olive effluents. Future work would involve complete characterization and identification of the five bacteria isolated from the olive effluents. The nature of the products during the degradation of model compounds and the olive effluents should be investigated. Should these products be non-toxic they may prove to be acceptable end products for a biological treatment process or may serve as substrates for other microorganisms in the environment. However, there is a risk that the metabolites may represent a metabolic dead end, be of elevated toxicity or be of enhanced mobility in the environment. These potential problems should be considered prior to application of these microorganims for the biotechnological treatment of olive effluents. Optimal conditions and the use of immobilized microorganims for the degradation of olive effluents should be investigated. The isolated bacteria can be genetically

engineered into powerful biological agents for the treatment of olive production effluents and therefore further research studies are needed in this area.

The relationship between the conversion of ferulic acid and (1) growth state of the culture utilized for biomass, (2) the concentration of biomass for cell free extracts and (3) the effect of the carbon source on which the biomass was grown, should be investigated in order to find optimal conditions for the production of vanillic. Further work could also entail the development of the production of vanillin from ferulic acid, and therefore methods such as the use of inhibitors, are needed to stop the oxidation of vanillin to vanillic acid during ferulic acid bioconversion. An understanding of the biochemical and enzymatic processes involved in ferulic acid biotransformations is required as a theoretical basis for the ultimate development of biocatalytic processes for the production of large amounts of ferulic acid-derived aromatic chemicals. Further work would also focus on the isolation, purification and characterization of the enzymes which catalyse the degradation of ferulic acid. The reduction of vanillic acid to vanillin with the addition of cofactors can also be investigated. Shortening the time for vanillic acid production from ferulic acid should be one of the objectives for further studies.

When all the work is done, a scale up method for the utilisation of olive processing effluents can be developed. This would lead to the production of useful chemicals from olive processing waste.

APPENDIX A

MEDIA

A. BACTERIA

A.1 Bacteria isolated from olive effluents, RU – LV1; RU-FB1; RU-FB2; RU- SO1 and RU-SO2

1. Solid medium

RU – LV1 stock cultures were stored at 4 °C on nutrient agar (Biolab chemicals, Merck (SA) plates.

2. Liquid medium

The isolates were grown in nutrient broth or in minimal media (MM). The recipe for MM was obtained from Muheim and Lerch (1999).
The chemicals were purchased from Biolab chemicals, Merck (SA).

MM contained per litre:

Glucose	1 g
Disodium hydrogen orthophosphate	4 g
Potassium dihydrogen orthophosphate	1 g
Yeast extract powder	1 g
Sodium chloride	0.2 g
Magnesium sulphate $7H_2O$	0.2 g**
Calcium chloride -2-hydrate	0.05 g

Variation of minimal media, magnesium sulphate $7H_2O$ was replaced by 0.1 of magnesium sulphate

A2. *Pseudomonas putida* RU-KM1 and RU-KM3$_s$

1. Solid medium

Pseudomona putida strains were stored in nutrient agar at 4 °C.

2. Liquid medium

RU-KM1 and RU-KM3$_s$ were grown in nutrient broth (Biolab chemicals, Merck SA).

B. FUNGI

B1. *Trametes versicolor*

1. Solid media

Trametes versicolor cultures were stored on 3% Malt agar (Biolab chemicals, Merck SA) plates.

2. Liquid media

T. versicolor was cultured in *Trametes* defined medium, the recipe was obtained from Addleman, 1993. The chemicals were purchased from Biolab chemicals, Merck SA.

Trametes Defined Media (TDM)

Contained per litre:

Glucose	10 g
Peptone	5.23 g
Potassium dihydrogen orthophosphate	2 g

Magnesium sulphate	0.5 g
Calcium chloride -2- hydrate	0.1 g
Veratryl alcohol	0.35 mL
Thiamine**	2 mg/L
Succinic acid dimethyl ester	1.3 mL
Sodium chloride	0.29 g
Trace elements	10 mL

Thiamine added after autoclaving.

Thiamine stock solution

0.8 mg/mL

Make up a solution of 100 mL

Thus 0.08 g thiamine made up to 100 mL with dH_2O

Trace elements (100x concentration)

Iron sulphate	0.28 g
Copper sulphate	0.016 g
Zinc chloride	0.034 g
Manganese sulphate	0.169 g
Cobalt chloride	0.095 g
Nickel chloride	0.0012 g
Ammonium molybdate	0.309 g

Made up to 500 mL with sterile dH_2O

B2. *Neurospora crassa*

1.Solid media

Neurospora crassa stock cultures were stored at 4 ^{0}C on *Neurospora crassa*

agar. The chemicals were purchased from Biolab chemicals, Merck SA.

***Neurospora crassa* agar**

Contained per litre:	
Potassium tartate	5g
Sodium nitrate	4g
Magnesium sulphate.$7H_2O$	0.5g
Sodium chloride	0.1g
Calcium chloride	0.1g
Glycerol	20g
Hydrolysed casein	0.25g
Yeast extract	5g
Malt extract	5g
Agar	15g

2. Liquid media

N. crassa was cultured in half – strength Vogel's medium N, the recipe was obtained from Vogel (1964). The chemicals were purchased from Biolab chemicals, Merck SA.

Vogel's medium N

The following components were added successively with stirring at room temperature:

Na_3 citrate	125g
KH_2PO_4 (anhydrous)	250g
NH_4NO_3 (anhydrous)	100g
$MgSO_4.7H_2O$	10g
$CaCl_2.2H_2O$	5g

Then add with stirring:

Trace element solution	5 mL
Biotin solution	2.5 mL

Trace element solution

In 95 mL distilled water, dissolve successively with stirring at room temperature:

Citric acid.$1H_2O$	5g
$ZnSO_4.7H_2O$	5g
$Fe(NH_4)_2(SO_4)_2.6H_2O$	1g
$CuSO_4.5H_2O$	0.25g
$MnSO_4.1H_2O$	0.05g
H_3BO_3 (anhydrous)	0.05g
$Na_2MoO_4.2H_2O$	0.05g

Biotin solution

The biotin solution is a 0.01% aqueous solution stored at –15 ^{0}C.
The resulting total volume is approximately 1000 mL. Chloroform (2 mL) is added as a preservative, and the 50 – times strength medium obtained is stored at room temperature. For use the medium is diluted 50 – fold (or 100 – fold as was the case in this investigation) with deionised water. The single strength medium is designated as N. It has a pH of approximately 5.8. Medium N is then supplemented with a suitable carbon source, and the thus supplemented medium is then sterilized by autoclaving.

APPENDIX B

ANALYTICAL METHODS

1. Folin Ciocalteus's total phenol determination assay (Garcia *et al.*, 2000)

Reagents:

Folin Ciocalteus reagent

Sodium carbonate solution (100 g/L of H_2O)

Method:

Prepare calibration standards ranging from 0 – 500 mg/L of a standard compound.

Pipette 1 mL of each standard into a series of 100 mL volumetric flasks.

Add 16 mL of distilled water to each flask, mix well.

Add 2.5 mL Folin Ciocalteus reagent, mix well for 30 seconds.

Add 15 mL of sodium carbonate solution, mix and bring to mark 100 mL with distilled water.

Stand for 1 hour at room temperature.

Measure absorbance at 765 nm against a reagent blank.

For samples follow the same procedure.

Calculate the total phenol concentration in mg/L equivalents from the standard curve.

2.Bradfords Assay (Bradford, 1976)

Reagents:

Comassie brilliant blue solution (CBB) (100 mg CBB, G-250, in 50 mL 95% ethanol, 100 mL 85% phosphoric acid and 850 mL water)

Bovine serum albumin (100 µg/mL)

0.15 M NaCl

Method:

Aliquote duplicates amount of 0, 10, 20, 30, 40 and 50 μL of bovine serum albumin into microfuge tubes.

Adjust the volume in each tube to 100 μL with 0.15 M NaCl.

Add 1 mL of CCB.

Stand for 2 minutes at room temperature.

Measure absorbance at 595 nm against a reagent blank.

For samples follow the same procedure, but use 50 μL sample.

Calculate protein concentrations in μg/mL equivalents from the standard curve.

APPENDIX C

CALIBRATION CURVES

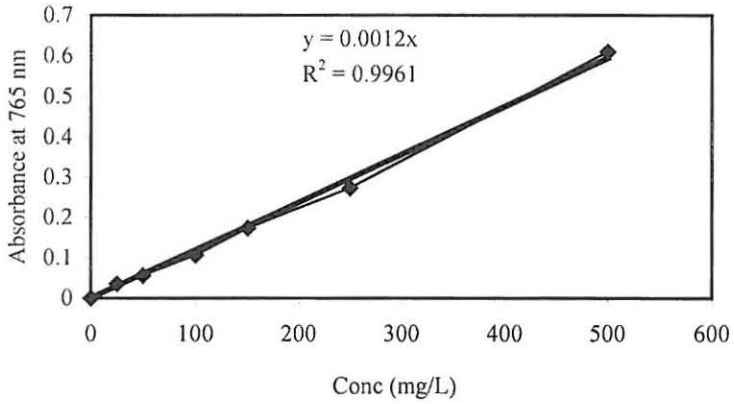


Figure C.1 Calibration curve for total phenolic content assay

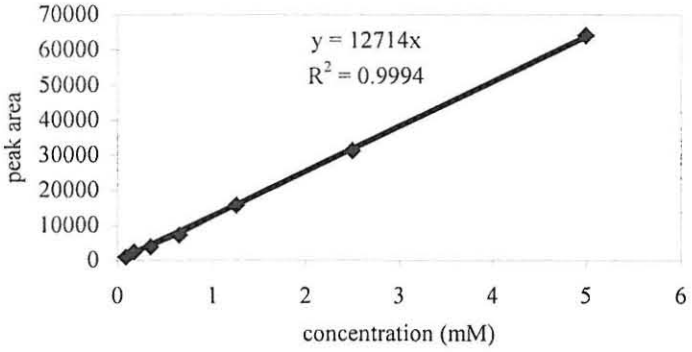


Figure C.2 Calibration curve for *p*-coumaric acid

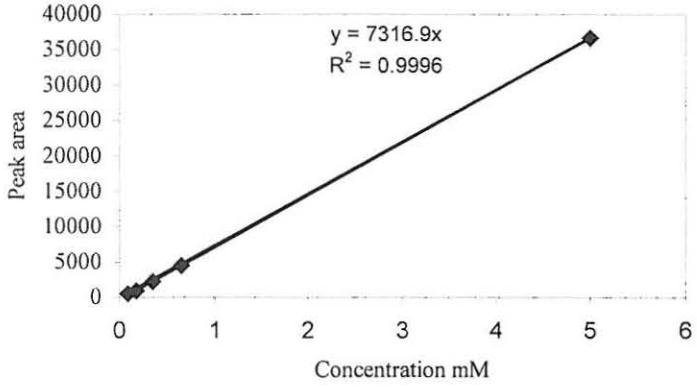


Figure C.3 Calibration curve for caffeic acid

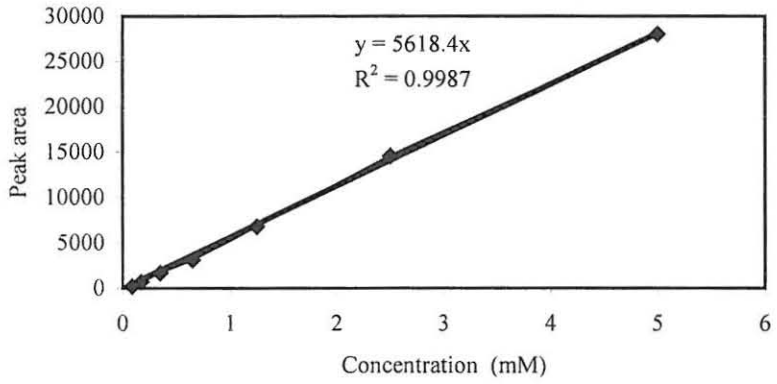


Figure C.4 Calibration curve for protocatechuic acid

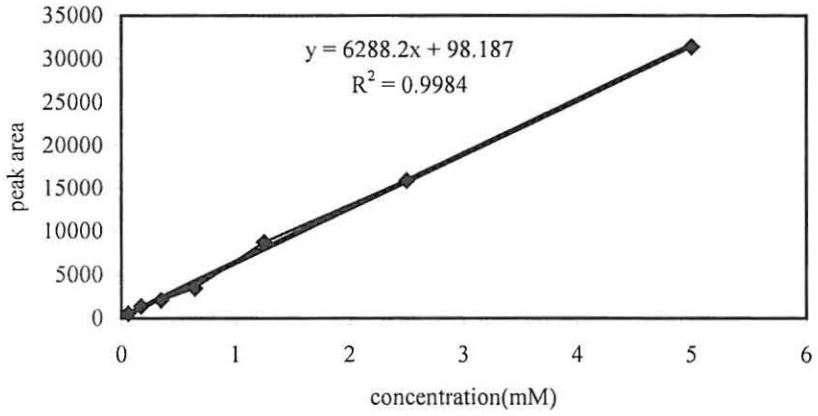


Figure C.5 Calibration curve for vanillic acid

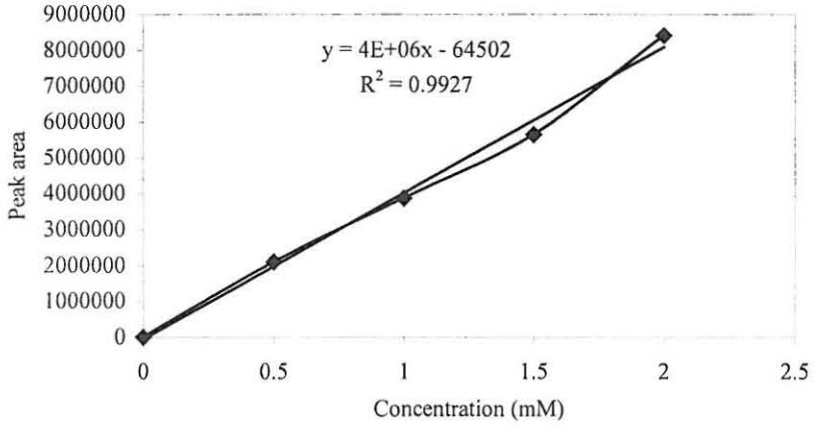


Figure C.5 Calibration curve for ferulic acid

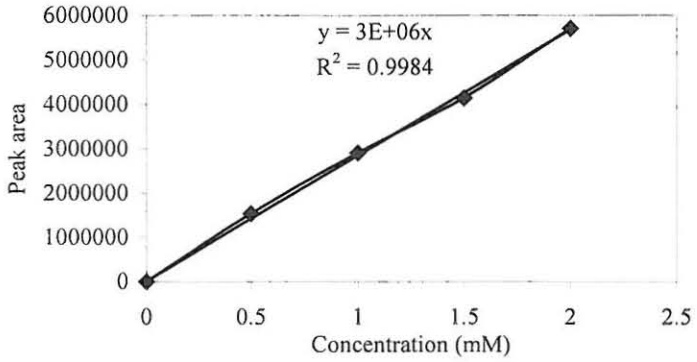


Figure C.6 Calibration curve for vanillin

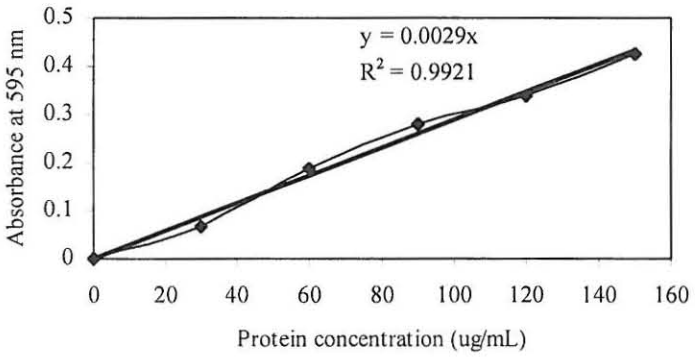


Figure C.7 Calibration curve for the Bradfords protein determination assay

APPENDIX D

1. Phenol DNA extraction and DNA precipitation protocol

1) An equal volume of Phenol : Chloroform : Isoamyl (25 : 24 : 1) was added to the sample and mixed thoroughly.
2) The sample was microfuged for 6 minutes.
3) The aqueous supernatant was carefully removed. Care was taken not to include any of the organic phase or cell debris found at the interface between the two phases.
4) $1/10^{th}$ the volume of the sample of 3 M Sodium Acetate and 2 times the volume of cold 96% ethanol (stored at – 4 °C) was then added.
5) The solution was left at – 4 °C overnight and then microfuged for a further 10 minutes.
6) The supernatant was once again carefully removed whilst being careful not to remove the pellet (The DNA may not be visible however, if the supernatant is removed slowly with a pipette then the DNA should remain in the eppendorf).
7) 100 μL cold 70% ethanol (stored at – 4 °C) was then added to wash the DNA and the eppendorf was microfuged for a further 2 minutes.
8) The supernatant was again carefully removed and the sample was then allowed to air dry (i.e. the ethanol to evaporate)
9) Once dry, the DNA was resuspended in TE buffer (X1).

APPENDIX E

Transformation-compotent *Escherichia coli* were prepared as follow:

A 5 mL test tube was inoculated with DH5ά or Top 10 the previous evening, and a 100 μL aliquot was plated onto the surface of an LB agar plate. The cells, which had grown to confluence the following morning, were scraped off the agar surface and were added to the LB broth. Four LB Erlenmeyer flasks (100 mL) were inoculated with 1.5, 1.0, 0.7 and 0.3 mL of the pre-inoculum, and were incubated on the orbital shaker at 37 °C for approximately 2 hours. The absorbance (A_{600}) of the Erlenmeyer flask incoculated with 1.5 mL pre-inoculum was assessed with LB medium as the blank, until a density of 0.6-0.8 was reached. The Erlenmeyer flasks were cooled for 5-10 minutes in an ice-waterbath, and the flask contents were processed separately until the final step. Following centrifugation (Beckman centrifuge, JA 14 rotor, 5000rpm, 10 min, 4 °C), the supernatant was decanted and the pellet was carefully resuspended in 50 mL RF1 (100 mM KCl, 50 mM $MnCl_2$, 30 mM CH_3COOK, 10 mM $CaCl_2$, 15 % m/v glycerol pH 5.8), followed by a further 20 minutes incubation on ice. The cells were pelleted by a 10 minute centrifugation as described above. The supernatant was decanted and the four pellets were resuspended in 4 mL RF2 (10 mM (MOPS, 10mM KCl, 75 mM $CaCl_2$, 15 % m/v glycerol pH 6.8), and the flask contents were pooled. Aliquots in multiples of 500 μL were pipetted into sterile eppendorf tubes and were stored at –80 °C until used.

Transformation procedure

Add 150 μL of thawed compotent *E. coli* to DNA. Mix

Leave on ice for 20 minutes.

Heat shock at 42 °C for 45 seconds. Cool on ice for 5 minutes.

Add 1 mL of cold LB (containing ampicillin) to the eppendorf, incubate at 37 °C for 1 hour.

Plate 100 μL on LB plates with ampicillin.

APPENDIX F

Luria Broth

Tryptone	10g
Yeast extract	5g
NaCl	5g

Made up to a volume of 1000 mL with ddH_2O.

NOTE : For the preparation of ampicillin plates for plating out of the transformation, add 20g of agar to the Luria broth and after autoclaving and allowing the agar to approximately 50 °C add 1 µL/mL of ampicillin.

Transformation and Storage Buffer (TSB)

Luria broth containing	10% PEG (MW = 3350)
	5% DMSO
	20 mM Mg^{2+} (10 mM $MgCl_2$ + 10 mM $MgSO_4$)

NOTE : The 10% PEG is autoclaved in the Luria broth separately from the 5% DMSO and 20 mM Mg^{2+}.

: Once cool the solutions are mixed and stored in an opaque bottle as TSB is light sensitive.

APPENDIX G

Smart Buffer

Tris (pH8)	10 mM
EDTA	1 mM
Sucrose	15%
Dnase Free Rnase	200 μg/mL
Bovine Serum Albumin	100 μg/mL
Lysozyme	2 mg/mL

APPENDIX H

16S rRNA sequence

TCGGGCGTCAGGCGATACGGGCATAATAGTTTAGGGAGACTGGAATTNCGTGGTGTAgCG
GTGAAATGCGCAGATATCAGGAGGAACACCAATGGCGAAGGCAGGTCTCTGGGCAGTAAC
TGACGCTGAGGAGCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGC
CGTAAACGGTGGGCGCTAGGTGTATGGGTCTTCCACGACTTCTGTGCCGCAGCTAACGCA
TTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGG
GCCCGCACAAGCGGCGGAGCATGTGGATTAAGTTCGATGCAACAGCGAAGAACCTTACCT
GGGCTTGACATGGACCGGATCGGCGTAGAGATACGTTTTCCCTTGTGGTCGGTTCACAGG
TGGTGCATGGTTGTCAGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCAGCAACGAG
CGCAACCCTTGNTACTTATGTTGCCANCACATTGTGGTGGAGTACTCATGAGAGACTGCG
GGGTTAACTCGGAGGACNGGTGGGGATGACGTCAAATCAATCATGCCCCTTATGTCCAGG
GCTTCCACACCATGCTACAATGGTCGGGTACAGCGAGTTGCCACACCGGNGAGGTGGAGC
TAACTCCTCTTAAAAGCCGGCTCAGTTCGGATTGGGGTCTGNAATCGACCCCATNAAGTC
GGAGTCGCTAGTACTCGCAGATCAGCAACGCTAGNGGTGAATACGTTCCCGNGCCTTTGT
ACACACCGACCGTACAGTCATGAAAGTTGGTAACACCGAAGCCATTGCCAACCTTGTTAG
GGNGAACCTNGACGANAAGGTGGNAACCGGGCGAAAAGGACACGCAATCNTACACNGGGA
ACCCGCCTAAAAGAANAAAANCACAAGAANNCACACACAACAAAACACAAACAAGACAGA
GCACACACACANCACAACNCCAACCGAACAGACACAAGAGAACACCACAGCAACCNACNG
CAAGACAGNNGACACCACAAGAACACGANACCGCACAAGANNCAACAGCANACAAAAAAA
ANAAAACAGAAGACACA

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