THE DEVELOPMENT OF THE EMERGING TECHNOLOGIES SUSTAINABILITY ASSESSMENT (ETSA) AND ITS APPLICATION IN THE DESIGN OF A BIOPROCESS FOR THE TREATMENT OF WINE DISTILLERY EFFLUENT

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ABSTRACT

Emerging Technologies Sustainability Assessment (ETSA) is a new technology assessment tool that was developed in order to compare emerging processes or technologies to existing alternatives. It utilizes information modules, with the minimum use of resources such as time and money, in order to determine if the process under development is comparatively favourable and should be developed beyond the early conceptual phase. The preliminary ETSA is vital in order to identify the gaps in the existing information and the specific methodologies to be used for data capture and analysis. The use of experimental design tools, such as Design-Expert, can facilitate rapid and efficient collection of necessary data and fits in well with the rationale for the ETSA.

Wine distillery effluent (vinasse) is the residue left after alcohol has been distilled from fermented grape juice. It is an acidic, darkly coloured effluent, with a high COD and polyphenol content. The most popular method of disposal of this effluent, land application, is no longer viable due to stricter legislation and pressure on the industry to better manage its wastes. Although the ability of white-rot fungi to degrade a number of pollutants is well-known, fungal treatment of wine distillery effluent is still in the conceptual phase.

The performance of the fungal remediation system was assessed experimentally in terms of COD removal and laccase production using Design-Expert. Although *Pycnoporus sanguineus* was found to be most efficient at COD removal (85%) from 30% vinasse, laccase production was low (0.021U/l). The optimum design for economically viable fungal treatment used *Trametes pubescens*. This fungus was able to remove over 50% of the COD from undiluted vinasse while producing almost 800U/l of the valuable laccase enzyme within three days. Since the effluent from

the fungal system did not meet the legal limits for wastewater disposal, a two-stage aerobic-anaerobic system is suggested to improve the quality of the effluent prior to disposal.

The ETSA was used to assess the fungal technology in relation to the two current methods of vinasse treatment and disposal, namely land application and anaerobic digestion. Based on the ETSA, which considered environmental, social and economic impacts, the fungal system proved to be potentially competitive and further development of the technology is suggested.

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LIST OF ABBREVIATIONS

ABR – anaerobic baffled reactor

ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonate)

AF – anaerobic filter

AS – activated sludge

BOD – biological oxygen demand

BSW – biomethanated spentwash

COD – chemical oxygen demand

CTSA – Cleaner Technologies Substitute Assessment

DSP – downstream processing

EDTA – ethylenediaminetetraacetic acid

EIA – Environmental Impact Assessment

EPA – Environmental Protection Agency

ETSA – Emerging Technologies Sustainability Assessment

HRT – hydraulic retention time

LiP – Lignin peroxidase

LMEs – lignin-modifying enzymes

LS – lagoon sludge

MEB – malt extract broth

MnP – Manganese peroxidase

MSB – mineral salts broth

MSB-MEB – mineral salts malt extract broth

NEMA – National Environmental Management Act

NPK+FYM – nitrogen, phosphate, potassium and farm yard manure

OLR – organic loading rate

OMW – olive mill wastewater

OTA – Office of Technology Assessment

PAHs – polycyclic aromatic hydrocarbons

PCBs – polychlorinated biphenyls

PVPP - polyvinylpolypyrrolidone

RSM – Response Surface Methodology

RSW – raw spentwash

SAPPI – South African Paper and Pulp Industry

SRT – solids retention time

TA – Technology Assessment

TKN – Total Kjeldahl Nitrogen

UASB – Upflow Anaerobic Sludge Blanket

CHAPTER I: LITERATURE REVIEW

1.1. Bioremediation and biotechnology:

Until a decade ago, man's greatest challenge was to speed up the industrialization process. Today, we attempt to find ways to deal with growing industrialization and the problems it causes, including the production of waste. Although the White Paper on Integrated Pollution and Waste Management for South Africa (2000) advocates a hierarchical approach to waste management, prevention and minimization is not always viable. Bioremediation is a general concept encompassing all those processes and actions that occur in order to biotransform an environment that is already altered by contaminants, to its original status. The exact nature of the processes that can be used to achieve the desired results vary, but they still have the same principle: the use of microorganisms or their products (enzymes) to remove contamination (Thassitou & Arvanitoyannis, 2001).

Aside from the areas usually associated with environmental biotechnology such as environmental protection and restoration, agricultural and industrial practices (Sayler, 1997), bioremediation and waste treatment technology are gaining increasing interest. Environmental policy, legislation and economics have been important drivers of research and the application of these technologies (Sayler, 1997). The world outlook regarding environmental biotechnology is undergoing a radical change, where the technology is no longer viewed only as a "way to reduce cost and liabilities associated with past environmental problems" but as a method of treating the environmental problems while simultaneously gaining financial profits, for example from value added products (Sayler, 1997).

The global market for environmental technologies is estimated to approach about $500 billion dollars annually early in the 21st century (Sayler, 1997). If the potential of environmental biotechnology is fully realized, the proportionate share of such a market would be significant (Sayler, 1997). Despite the paradigm shift towards pollution minimization and prevention and environmental sustainability (Sayler, 1997), environmental contamination will undoubtedly occur in the future, although the incidence and magnitude of pollution will hopefully decline. Even so, prospects for biotechnology in the remediation of environmental contamination will continue to exist, and will probably be driven by cost and risk factors (Sayler, 1997). A "resurgence and growth in research and applications of biotechnology" is anticipated regarding the treatment of a broad

range of wastes, pollution minimization strategies and the development of "niche-specific biotechnology" for specific waste streams among others (Sayler, 1997).

However, success and progress in the development of environmental technology will be slowed if the trends in the social and political situation are not understood (Miller, 1997). The public in industrialized countries for example, expect continuous advancement towards achieving environmental sustainability. Despite political and social issues such as the economy and unemployment, the majority of people declare concern for the environment and do not submit to the suggestion that "environmental protection must be traded-off for economic gain" (Miller, 1997). This appears to be due mostly to anxiety regarding the impacts of pollution on the health of present and future generations and the uncertainty of the viability of global economic and ecological systems (Miller, 1997). These concerns will doubtlessly continue to pressure both governments and industries, which have to respond to the demand for continuous environmental upliftment and management to ensure sustainability (Miller, 1997).

There are numerous technologies developed to satisfy the requirements of society; however the development of new technologies has been rapid and methods had to be developed in order to assess the potential effects (both positive and negative) of technologies, despite their original intent.

1.2. Technology Assessment (TA):

Technology Assessment (TA) began in the late 1960s due to an increasing appreciation of the vital role of technology in modern society and its potential for unintended and sometimes harmful, consequences. Technological developments are primarily driven by market forces and governmental support, and although most were developed with the intention to change the quality of life for the better, questions arose about the secondary and higher order negative effects of these new technologies. TA was usually carried out as a component of an Environmental Impact Assessment (EIA), but although TA was implemented in most Western countries, it did not have the legal basis and structural framework which made EIA a success (Porter, 1995).

Coates (1976, as quoted by Vanclay & Bronstein, 1995) defined TA as being: "the systematic study of the effects on society that may occur when a technology is introduced, extended or modified, with emphasis on the impacts that are unintended, indirect or delayed." This indicates that TA is an ordered approach to investigating the effects of a certain technology development on society in its broadest sense, across all cultural and geographical barriers (Vanclay & Bronstein, 1995).

Daddario (the founder of the U.S. Office of Technology Assessment [OTA]) described TA in the following way: "Technology Assessment is a form of policy research which provides a balanced appraisal to the policy maker. Ideally, it is a system to ask the right questions and obtain correct and timely answers. It identifies policy issues, assesses the impact of alternative courses of action and presents findings. It is a method of analysis that systematically appraises the nature, significance, status and merit of a technological program" (Internet Reference 1).

The process of TA requires that it envisions future scenarios and evaluates the risks on society and the environment. The dynamic relationship between technology and society dictates that it would be impossible to predict future scenarios with certainty. The actual aim of TA is to eliminate scenarios which are deemed implausible by the assessment (Vlachos & Hendriks, 1977). This means that technologies that can be detrimental in some way can be eliminated or altered so as to no longer present unacceptable risks.

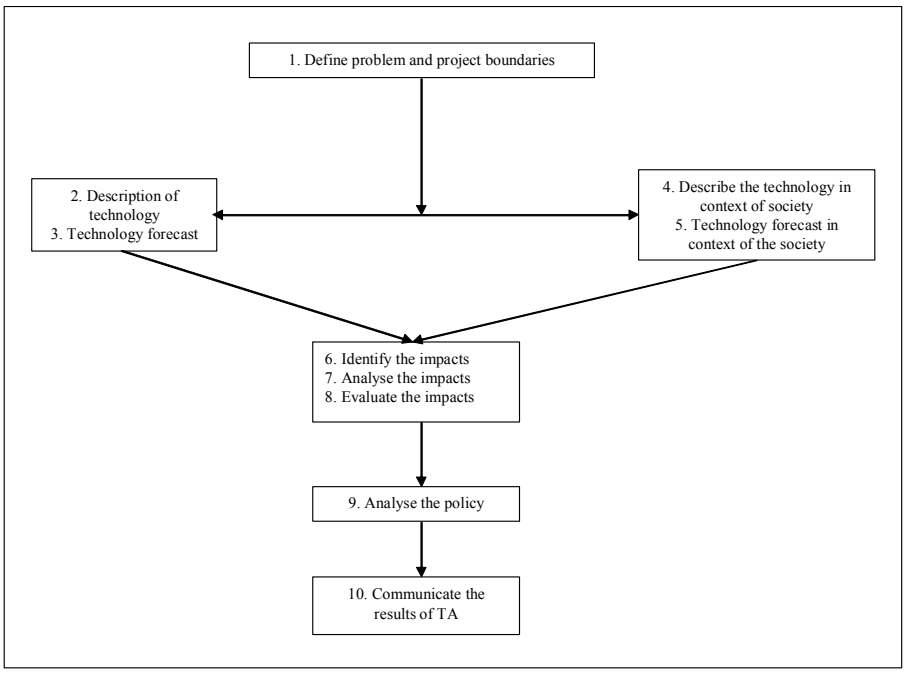

Figure 1.1: Method for TA (modified from Vanclay & Bronstein, 1995)

There is no one specific model for carrying out TA. The methodology depends on the organization and their specific focus. One simple process that can be applied consists of 10 main steps (Figure 1.1) as described by Vanclay and Bronstein (1995).

Firstly, the problem or project should be clearly defined. It is then necessary to set clear boundaries for the project in order to ensure that the main focus is not lost. Step 2 involves defining the technology as it is, while Step 3 involves describing the place of this technology in the future. Steps 4 and 5 consider the society into which the technology will be introduced and its acceptance. Steps

6, 7 and 8 look at the possible impacts of the technology, with respect to whom or what is affected and the degree of impact. Current policies pertaining to this technology are analysed in step 9, so that the decision options and their consequences can be evaluated. The final step involves communication i.e. reporting the results of the TA to all the interested and affected parties (Vanclay & Bronstein, 1995).

This method is best applied for the assessment of a single technology at a particular time (Vanclay & Bronstein, 1995) whereas other TA methodologies such as Cleaner Technologies Substitute Assessment (CTSA) comparatively assess more than one process at a time.

1.3. Cleaner Technologies Substitute Assessment (CTSA):

CTSA is another method of TA that was developed by the U.S. Environmental Protection Agency (EPA) as a systematic method for analyzing technologies. It is a comparative assessment of a number of processes and encompasses factors such as comparative risk, competitiveness such as performance and costs, and contributions to resource conservation (Internet Reference 1). Figure 1.2 shows the steps involved in CTSA.

Since TA is a multi-disciplinary activity, it requires varied expertize if accurate assessment of the economic, social and environmental impacts are to be made. Therefore the first step is to assemble a project team. A scoping document gives an initial impression of the industry status, which gives a brief indication of the task and possible solutions. The 'use cluster' is a list of alternative processes or technologies that can be used to solve the problem faced and those that offer environmentally beneficial opportunities could be selected as substitutes. The substitutes would thereafter be narrowed down to the best and most feasible (subset). The 'project baseline' is a list of the technologies that are the current standard practice in the industry and the most familiar. Before commencement of the CTSA, the boundaries of evaluation are determined, i.e. what aspect of technologies are to be considered? These include human health, costs, etc. (Internet Reference 1).

CTSA starts with data collection including process details, risk data, competitiveness and conservation data (Figure 1.3). This data is analysed in the second stage and comparisons and trade-offs negotiated based on the comparisons using information gathered in each of the information modules (Internet Reference 2).

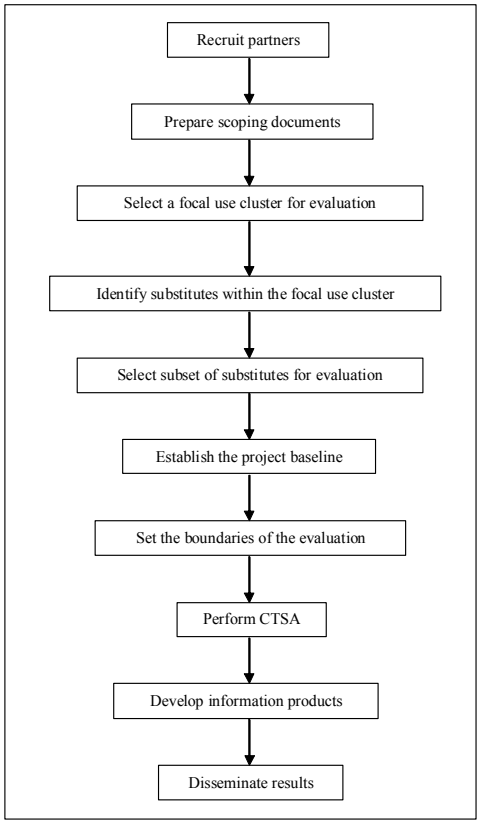

Figure 1.2: Steps in conducting a CTSA (Internet Reference 1)

It must be noted that the goal of a CTSA is not to recommend specific alternatives, but to present the trade-offs related to risk, competitiveness and conservation in such a way as to allow decision-makers to select the alternative that best suits their own goals, values and requirements. Throughout the CTSA process, data is collected on additional environmental improvement opportunities that could be implemented regardless of which alternative or substitute is used. Although the CTSA process is depicted as a linear, step-wise process the data collection and data analysis components are frequently worked on simultaneously (Internet Reference 2).

The CTSA methodology therefore offers industry sectors a systematic approach for evaluating risks to human health and the environment, as well as the performance, costs and natural resource use of traditional and alternative technologies. Once the CTSA is completed, the results must then be communicated to all the interested and affected parties in an attempt to encourage the use of cleaner processes (Internet Reference 1).

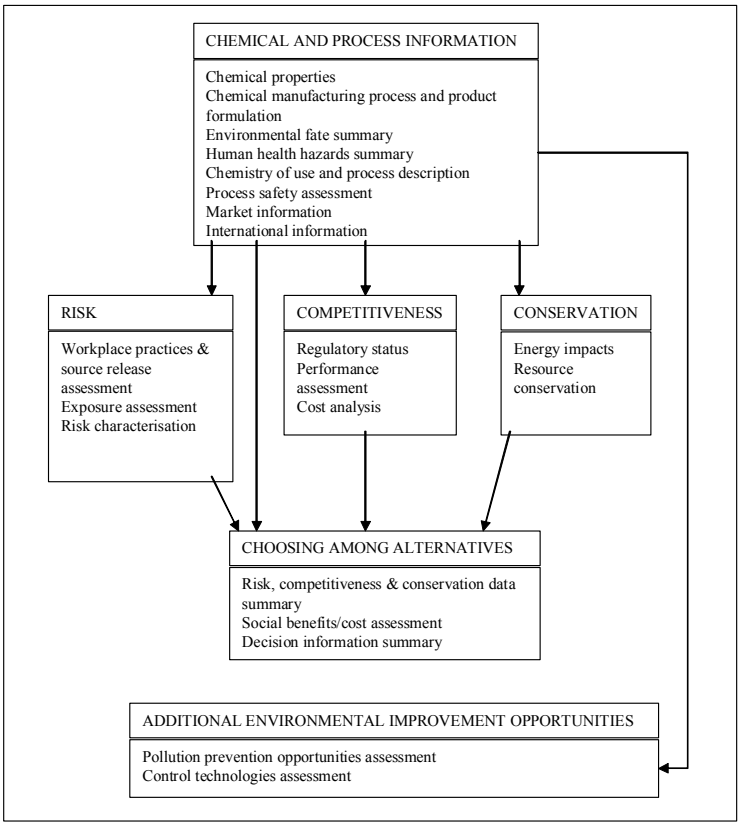

Figure 1.3: CTSA information flows illustrating the different components and modules involved (Internet Reference 1)

In modern times, the environment has become a worldwide concern due to greater awareness, thus pressure on industries to evaluate their processes and adopt 'cleaner' processes has increased. Methods of TA will surely change with time, but the primary objectives of comparing technologies in order to make a well-informed decision will remain. Even in situations where productivity is the main concern, a TA is invaluable since environmental well-being and productivity are virtually inseparable. For example, not only does the environment benefit from waste reduction and optimization of energy utilization, but costs are also reduced. Furthermore companies that make changes in their processes to prevent pollution may enjoy increased acceptance and support from environmentally aware consumers (Internet Reference 1).

There exists greater potential for the CTSA approach. This model, with some adaptation, can be used to assess the theoretical viability of a conceptual process or technology and to compare it to the available alternatives. The Emerging Technologies Sustainability Assessment (ETSA) was developed for the specific purpose of assessing the potential of alternative, emerging technologies relative to existing options.

1.4. Emerging Technologies Sustainability Assessment (ETSA):

ETSA was developed by Khan (2002) in order to assess the future viability of a technology currently under development. It is essentially derived from the CTSA model, however the major difference lies in the purpose of the assessment tool. As discussed previously, CTSA compares existing processes in order to inform companies of the sustainability of available processes. The ETSA on the other hand focuses on emerging processes or technologies, which it compares to existing alternatives in order to determine if the process under development is comparatively favourable and should be developed beyond the early conceptual phase. Users of this new tool would primarily be researchers in any field of technology development, including biotechnology, but could also include venture capitalists, banks and other investment agencies that would use the ETSA in order to assess new prospects for investment.

Research constitutes one of the major costs of process development and, in addition, it is a time consuming procedure. The aim of the ETSA is to evaluate a process in the early stages of development in order to ascertain the likely viability in terms of sustainability (economic, social and environmental) and competitiveness of the emerging process. In this way, expensive research need not be conducted, nor will valuable time and effort be directed towards development of a process that will ultimately not be competitive from the perspective of sustainability.

The ETSA is a flexible model that, as with the CTSA, requires the use of information modules. Modules can be added or removed from the model as required by the scope of a particular project. For example, a project may only focus on the performance characteristics of a technology; therefore modules such as market size may be unnecessary. Extensive literature reviews are required in order to collect the information for the modules regarding the various comparative technologies, each of which must be currently in use in the same context as the emerging technology.

The technologies are compared with regard to the risks involved, effectiveness or performance of the systems, conservation and environmental impacts, social impacts and regulatory status among others. As the tool sets out to evaluate an emerging technology, all the information required for each of the information modules is often not available. The preliminary ETSA step regarding the technology being assessed is therefore vital in order to determine what information is missing and how best to approach the acquisition of that data. Unlike with established technologies, one of the gaps expected when assessing an emerging technology is the performance of the system. This data would most likely be obtained through laboratory-scale experimental systems and this often

involves long time periods and high cost. The ETSA would assist in reducing these costs and the timeframe by focusing the research on obtaining only the data necessary to complete the ETSA.

The purpose of the current study was to evaluate the use of ETSA in the context of environmental biotechnology. More specifically, the ETSA was used to assess an alternative technology for the remediation of wine distillery effluent.

1.5. Wine and the wine industry:

In the current marketplace, a "successful wine" is the result of a combination of the artistic and economic aspects of wine production. A thorough understanding of human behaviour and the intrinsic and extrinsic factors influencing product purchase is essential. Wine itself is a unique commodity and is historically associated with the elite of society (Bisson *et al.*, 2002). In developed countries, wine consumers are still typically prosperous, although it is also drunk in impoverished regions of the world where it is safer to drink wine than the local water supply (Bisson *et al.*, 2002).

In the past, the definition of quality and wine standards were set by the wine producer and consumers were often made to feel uncultured if they did not like a certain type of wine. Today, however, the roles have changed with quality defined and standards set by the consumer due to rapid globalization and access to information. The modern consumer is knowledgeable, with an advanced perception of product value and a decisive demand for quality (Bisson *et al.*, 2002). Moreover, the wine consumer is motivated by more than the intrinsic taste and aroma of a wine. Extrinsic factors such as bottle and label design, health benefits and environmentally sustainable production are as important (Bisson *et al.*, 2002). Sustainable production is becoming an increasingly important economic driver of profitability in many sectors and the wine industry is no different (Bisson *et al.*, 2002).

Grapes can be grown in a diverse range of soil and climate conditions. Indeed, the concept of *terroir* states that the local environment influences the composition of the grapes and thus the wine from that region (Bisson *et al.*, 2002). The wine is consequently expected to possess unique qualities gained from the *terroir*, differentiating it from the same type of wine from other regions (Bisson *et al.*, 2002). This signifies that high quality wine can be, and is, produced on all six continents by both affluent and developing nations (Bisson *et al.*, 2002). Furthermore, one of the 'value-added' economic benefits is tourism. Wine production areas are known for their beauty and the income from tourism to wine producing areas is economically important to many countries. This

is also one of the major reasons for strong governmental support in the research and development of the wine industry (Bisson *et al.*, 2002).

Currently and increasingly in the near future, the perception of the wine producer as being environmentally conscious and conscientious will strongly motivate the purchasing decision of the consumer. The typical wine consumer is both affluent and well educated, and as awareness concerning the global environment increases, so too will the demand for environmentally sound agricultural and disposal processes (Bisson *et al.*, 2002). In order for a wine producer to remain competitive in the global arena, they would have to produce a high quality wine while maintaining efficiency and processes that have the least possible environmental impacts. Furthermore, large settling and evaporation ponds (which can emit offensive odours) for disposal of effluent from the wine-making process would destroy the aesthetic value of the vineyards and decrease tourism benefits.

The entire vineyard harvest is not always fermented to form wine as the final product. Some wines, wine lees or fermented grape juice of the quality required by brandy and spirit makers are distilled to remove the ethanol and some flavour components which are used to manufacture brandy and other spirits.

1.6. Wine distillery effluent:

Distillation is a process that separates chemicals by the difference in their vaporization potential or boiling point. The beverage industry is one of the oldest users of distillation (Internet Reference 3) and includes the manufacture of brandy and other spirits from wine (Lalov *et al.*, 2000). Grape wine distillery effluent is the residue left after alcohol has been distilled from fermented grape juice and is also referred to as distillery spentwash, wine vinasses, distillery slops and wine distillery wastewater. Wine distillery effluent often includes wastewater originating from other winery and distillery operations such as rinsing, washing, sanitizing, etc. These wastewaters are often mixed and treated as a single effluent stream.

There are other raw materials that are commonly fermented and distilled for the production of alcohol for spirits or other uses (Basu, 1975). These include: cane sugar molasses, beet sugar molasses, rye, barley and wheat among others (Basu, 1975; Blonskaja *et al.*, 2003). These industries produce large volumes of high organic strength effluents similar to those produced by wine distillation, and are also a major source of soil and water pollution in many countries. Between 1987

and 1989, for example, the production of ethyl alcohol from sugar cane molasses in Mexico reached 115 million litres. This was accompanied by the generation of 1500 million litres of wastewater with a high organic content (Zedillo, 1990).

The composition of vinasse is variable and also dependent on the type of raw material distilled, e.g. wines, lees, pressed grapes, etc. (Sales *et al.*, 1987). Wine distillery effluent is acidic in character, with high organic matter content and it varies in colour from brown to dark red. It contains organic acids, salts, yeast cells, soluble proteins, carbohydrates and phenolic compounds together with various inorganic compounds which are normal constituents of wine (Table 1.1). Wine vinasse is also characterized by high temperature (as it leaves the plant), high biological oxygen demand (BOD) and chemical oxygen demand (COD) measurements and a high concentration of polyphenols (Table 1.1). Due to the nature of these effluents, and the possible environmental hazards posed, it is vital to treat the effluent before discharge.

Treatment before discharge is, however, not always practiced as a simple/uncomplicated, cheap, successful and robust treatment system is yet to be developed. In North Estonia, for example, the Rakvere plant produces $20m^3$ of spirits per day, and this is accompanied by the production of 500 – $600m^3$ of wastewater (Blonskaja *et al.*, 2003). This wastewater is currently discharged into the sewage system without prior treatment (Blonskaja *et al.*, 2003). In Spain, wine distilleries are plentiful. Their wastewaters are seasonally variable and present environmental problems when discharged into surface waters (Beltran *et al.*, 2001). Here, the most common disposal route for distillery effluent is dilution with domestic wastewater, followed by treatment at municipal wastewater treatment plants (Beltran *et al.*, 2001).

As mentioned in the example above, the volume of effluent produced, together with the flow rates and quality of effluent, is often seasonal and dependent on the various winery production periods throughout the year. This is illustrated by Table 1.2, which summarizes the typical production periods at wineries in South Australia (EPA Technical Bulletin, 1996).

Table 1.1: Comparison of the characteristics of common distillery effluents (from literature)

Parameters	Basu, 1975	Ramana *et al.*, 2002b	Borja *et al.*, 1993	Robertiello, 1982	Dekker, 2003	Khan, this study
Type	Beet sugar molasses distillery vinasse	Molasses distillery vinasse	Wine distillery vinasse	Wine distillery vinasse	Wine distillery effluent	Wine distillery effluent
pH	5.1 – 5.7	4.0 – 4.1	3.8	3.2	4.0	3.5 – 4
Temperature (ºC)	98 – 99	ND	ND	ND	ND	ND
Electrical conductivity (EC) (dSm^{-1})	ND	25.3	ND	ND	ND	ND
Total solids	109275 – 145200	2820	32000	24800	18000	15505
Total suspended Solids	5390 – 8120	ND	3700	ND	ND	722.5
Total dissolved solids	ND	ND	ND	ND	ND	10155
Potassium	9300 – 18750	13000	ND	ND	3000	ND
Sodium	1070 – 1920	ND	ND	ND	75	ND
Calcium	900 – 2100	ND	ND	ND	410	ND
Magnesium	400 – 700	ND	ND	ND	160	ND
Manganese	ND	ND	ND	ND	2.3	ND
Phosphate	355 – 1030	240	130	220	150	ND
Orthophosphate	ND	139	ND	ND	130	315 - 369
Sulphates	5440 – 6600	1310	ND	ND	150	ND
Chlorine	2494 – 2925	4050	ND	ND	160	2.35 – 2.445
Total Iron	0.24 – 26.2	ND	ND	ND	15	1.011 – 1.171
Copper	4.4 – 29.6	ND	ND	ND	2	1.295 – 1.807
Nickel	ND	ND	ND	ND	0.4	ND
Aluminium	ND	ND	ND	ND	3	ND
Lead	0.6 – 1.7	ND	ND	ND	ND	ND
Zinc	27 – 225.5	ND	ND	ND	ND	ND
Total Nitrogen	1050 – 8900	2.02	ND	660	350	ND
Ammonium-N	114 – 380	125.4	140	ND	10	0.2 – 0.25
Organic-N	670 – 8759	ND	ND	ND	ND	ND
Nitrate-N	ND	Trace	ND	ND	1	310.5 – 473.75
COD	115200 – 176344	ND	40000	27400	15000 – 35000	25000 - 30000
BOD	61250 – 95750	ND	ND	14700	ND	ND
Total polyphenols	ND	ND	290	ND	ND	470 - 1200

Note: All values are in milligrams per litre, unless stated otherwise.
Note: ND = not determined

Table 1.2: Typical production periods at wineries in South Australia (from EPA Technical Bulletin, 1996)

Period	Typical months of the year	Typical production activities
Pre-vintage	Jan – Feb	Washing of equipment in readiness for vintage and some caustic washing of tanks.
Early vintage	Feb – March	Wastewater production rapidly rising to peak vintage flow and has reached 30% of maximum weekly flow; vintage operations dominated by white wine production.
Peak vintage	March – May	Wastewater production has reached 70% of peak weekly volume; impact of vintage-only operations at maximum.
Late vintage	April – June	Wastewater production rapidly falling towards non-vintage flows and has decreased to 30% of the maximum weekly flow; vintage operations dominated by production of red and 'sticky' wines; *distillation of ethanol spirit may also coincide with this period.*
Post-vintage	May – Sept	Pre-fermentation operations have ceased; impact of caustic cleaning is at its greatest.
Non-vintage	June – Dec	Wastewater generation at lowest and generally less than 10% of maximum weekly flows during vintage; wastewater quality highly dependent on day-to-day activities.

The effect of the discharge of untreated distillery effluent into water sources has been relatively poorly studied (Sheehan & Greenfield, 1980) and remains so. Accordingly, toxicity data for wine distillery effluent is unavailable. An early study by Verma & Dalala (1976) however shows the toxic effect of molasses distillery effluent on fish. The survival of two fish species were observed when subjected to undiluted distillery effluent at different temperature and pH values. It was clear from the study data that the disposal of untreated distillery effluent into natural water sources is unacceptable as it is toxic to fish even at concentrations as low as 11.15% effluent.

Large quantities of effluent are produced by the industry as roughly 10-15l vinasse are generated by distillery units for each litre of ethanol produced, according to Decloux *et al.* (2002) and this makes economic treatment difficult. However an efficient treatment method is needed as this effluent may result in the disruption of natural processes in waters and soils due to the problematically high levels of contaminants in the waste (Hayward *et al.*, 2000).

1.7. Disposal and remediation of wine distillery effluent:

Enormous pressure has been placed on distilleries from authorities and an increasingly environmentally aware public to reduce and/or treat their wastewater. A number of technologies

exist that can and have been applied to the disposal of wine distillery wastewater, with land application and anaerobic digestion being the most popular.

1.7.1. Land application/irrigation:

Currently, land application/irrigation is the most commonly practised method for disposal of wine effluent in South Africa (Mulidzi *et al.*, 2002). This method does not include any effective treatment of the effluent. Wastewater is first screened and settled in ponds, then distributed over land containing trees, grass or other crops using flood (channels) or spray irrigation (sprinklers) systems.

Distillery effluent was thought to have a beneficial impact on crop yields, however some believe the high salt concentrations in distillery effluent may result in severe inhibitory effects on seed germination. In investigations by Ramana *et al.* (2002b), a large difference in response to varying concentrations of distillery effluent was observed with regards to germination success (percentage germination) and speed of germination. At low concentrations all crops tested, except tomato, showed no inhibition to seed germination, however the percentage germination as well as speed of germination decreased with increasing effluent concentration (Ramana *et al.*, 2002b). It was deduced that pH was not responsible for the results as the pH remained around 4 despite dilution. Instead, the reduction in percentage germination and speed of germination was due to the excessive quantities of organic salts (and thus high electrical conductivity) present in distillery effluent (Pandey & Sony, 1994). This leads to increasing osmotic pressure with increasing concentrations of effluent, which makes the uptake of water more difficult and retards germination (Ramana *et al.*, 2002b). The study also found that at higher effluent concentrations (>25%), there was significant fungal growth on the seeds which could also have been responsible for poor germination at high concentrations (Ramana *et al.*, 2002b). In conclusion, the authors proposed caution when using distillery effluent for pre-sowing irrigation purposes as the effects are crop specific and governed by effluent concentration (Ramana *et al.*, 2002b).

Ramana *et al.* (2002c) showed that distillery effluent had a high manurial potential as its application to maize crops increased the total chlorophyll content and dry matter production of the maize. The researchers also observed the same results for groundnut (Ramana *et al.*, 2002a). An increase in grain yield of maize, associated with larger cob sizes, higher numbers of seeds per cob and increased 100-grain weight, was found in crops irrigated with distillery effluent. However, due to its poor and imbalanced supply of nutrients, distillery effluent still could not produce grain yield equivalent to the recommended NPK+FYM (nitrogen, phosphate, potassium and farm yard manure) (Ramana *et al.*, 2002c). Moreover, the amount of effluent added could not be increased to enhance

yield, as this would have resulted in problems of soil salinity, so it was recommended that some fertilizer should be used to supplement distillery effluent for increased grain yields (Ramana *et al.*, 2002c).

It must be noted, however that the types of crops and soils are very important if choosing to irrigate land with distillery effluent since the effects of distillery effluent is crop specific (Ramana *et al.*, 2002b) and soil dependent (Mulidzi *et al*., 2002). Mulidzi *et al.* (2002) investigated the irrigation of kikuyu grass on sandy soil with winery wastewater. They found that the organic components of the effluent leached through the sandy soil, and reached the groundwater table. Lateral subsurface water movement also caused these pollutants to be carried away from the point of origin to other surface waters (Mulidzi *et al.*, 2002). The results of the investigation showed that:

- the organic component of winery effluent did not benefit the soil as it was not retained by the sand and posed a serious pollution hazard to any water body into which it may be carried;
- disposal of high strength effluents should not occur on sites with sandy soils which have a low nutrient retention, low water storage capabilities and high permeabilities; and
- large volumes of effluent irrigating small areas of land aggravated leaching.

The disposal of wine distillery effluent occurs in all the wine producing countries, however the problems of effluent disposal on unsuitable soil is exacerbated in developing countries. For example, many South African wineries often have little choice or few alternatives regarding the area of land available to them, the type of soil on the land or even the location of the land (Mulidzi *et al*., 2002). It is therefore becoming more and more essential, especially in South Africa, for a cheap and simple yet effective method of dealing with high organic strength effluents to be found as this once popular method of irrigation with effluent can no longer be sustained. The land application process and the associated hazards will be discussed in detail in Chapter III.

1.7.2. Anaerobic digestion:

Until recently, anaerobic digestion was only applied for the stabilization of concentrated organic slurries such as sewage sludge and animal manure. It was believed that the process was slow, did not remove more than 50% of the organic load, required high temperatures and was unreliable (Vandevivere & Verstraete, 2001). This view has altered over the last 20 years and anaerobic digestion is now an established, high-rate wastewater treatment technology (Vandevivere & Verstraete, 2001).

Unlike industrial fermentation, anaerobic digestion is not a unique man-made process. The objective here has been to confine the natural organisms in a man-made system and to optimize the rates and extents of the natural reactions so that polluting substances will be destroyed (Hobson & Wheatley, 1993).

The microorganisms that carry out reactions of anaerobic digestion are organisms known as anaerobes (i.e. they live without oxygen and may, indeed, be killed by oxygen). While there is a gradation in tolerance to oxygen among anaerobic organisms, many of those found in anaerobic digesters are amongst the least oxygen tolerant. The dominant microbial population and the reactions of the digester are determined by the nature of the feedstock. In an anaerobic digester, there is a series of linked reactions that provide energy and substrates for microbial growth and leads to the final formation of methane, carbon dioxide and microbial cells (Hobson & Wheatley, 1993). Vandevivere & Verstraete (2001) described the stepwise process of conversion of organic compounds to biogas anaerobically, wherein different groups of bacteria operate sequentially to effect full degradation of the substrates as:

- acidogenesis by hydrolytic acidogens where polymers are cleaved into short-chain fatty acids;
- acetogenesis by syntrophic acetogens which degrade the fatty acids into acetate and H_2; and
- methanogenesis by methanogens which transform acetate and hydrogen into methane and carbon dioxide (biogas).

Under anaerobic conditions, the process of oxidative phosphorylation cannot occur. The cells are thus deprived of their principle method of generating energy. Therefore, under anaerobic conditions, the energy must be provided from the very process of degrading the original substrate (Ratledge, 2001). This process is called substrate level phosphorylation and yields only about 8% of the energy that can be produced under aerobic conditions. Thus, to produce a given weight of cells, much more substrate must be degraded under anaerobic than under aerobic conditions (Ratledge, 2001). In terms of biotechnology, anaerobic organisms are very important due to the fact that they consume only a small portion of the potentially available energy in the substrate to bring about the conversion (and thereby the treatment) of the substrate. They act, therefore, as true catalysts that change the substrate but not themselves; a very important principle in sustainable development (Verstraete *et al.*, 1997).

High biomass concentrations (50g/l) can be attained in reactors using granular sludge, allowing very high volumetric loading rates to be used. According to Verstraete *et al*. (1997) the understanding of

the fundamentals of the anaerobic process has overcome some of the major drawbacks. These include:

- *low stability of anaerobic processes* – proper design, operation and control are vital in order for anaerobic digestion systems to be highly stable;
- *slow start-up times of anaerobic reactors* – greater knowledge of the growth conditions of anaerobic bacteria and the use of active sludge from an existing process as the inoculum have decreased start-up times to the point where full-scale operations can be started up within a few weeks, sometimes even days;
- *odour problems resulting from anaerobic processes* – physico-chemical methods or biofilters can be used to prevent such problems; and
- *the relatively high susceptibility of methanogens and acetogens to toxic compounds* – this can be true, however, anaerobic consortia have a higher potential to adapt and to degrade undesirable compounds than previously thought.

Some major advantages of anaerobic wastewater treatment over aerobic treatment include (Vandevivere & Verstraete, 2001):

- small sludge production (0.1kg per kg BOD) and
- low energy consumption since no aeration is required and energy is recovered in the form of biogas (theoretically 0.35l CH_4 per g BOD)

The development of high-rate anaerobic reactors for the treatment of high strength wastewaters, including wine distillery effluent, will be discussed in detail in Chapter IV.

1.7.3. Integrated chemical and biological processes:

In most treatment plants, biological oxidation is usually the unit process where most of the organic load of the wastewater is removed (Beltran *et al.*, 2001). The wastewater itself cannot be toxic to the cultures involved in the biodegradation of the organics nor should it have a large amount of refractory compounds, or the process will fail. Scott and Ollis (1995) suggested that an integration of chemical and biological processes could be used to overcome the difficulties facing either process alone, as each process has innate limitations with regard to applicability, effectiveness and cost. A combination of available processes for wastewater treatment could be arranged to utilize the strengths of each process to achieve the desired effluent quality, within reasonable economic constraints (Scott & Ollis, 1995).

Beltran *et al.* (2001) investigated the use of ozone to possibly overcome the problems involved in the biological treatment of wine distillery wastewaters. Ozone, a powerful oxidant and disinfectant in water treatment, can improve the biodegradability of effluents by removing refractory compounds or compounds toxic to microorganisms such as polyphenols. The action of ozone on organic matter is pH dependent, therefore the levels of degradation achieved using ozone as a chemical oxidizing agent is also strongly pH dependent (Beltran *et al.*, 2001). It was found that pH sequential ozonation using 2 x 10 minute acidic periods and 2 x 50 minute alkaline periods was necessary for optimal oxidation rates, and it improved the biodegradability of wastewater by increasing the BOD:COD ratio (Beltran *et al.*, 2001). However, as ozone is an effective disinfectant, it could be detrimental to the biological system if toxic residual amounts remained from the ozonation step, and this must be avoided. Ozonation is also a tremendously expensive process and its use for the oxidative treatment of wine distillery wastewater can only be realistic if it is accompanied by a cheaper biological oxidation of major biodegradable pollutants (Beltran *et al.*, 2001).

1.7.4. Treatment with chitosan:

Chitosan is a modified natural carbohydrate polymer that is non-toxic and biodegradable and is derived from the chitin component of the exoskeleton of crustaceans (Lalov *et al.*, 2000). It is a cheap and accessible biopolymer, with a positive charge and can be used as an ion exchanger for the treatment of wine vinasse as it adsorbs the organic components (Lalov *et al.*, 2000). Lalov *et al.* (2000) found the optimal chitosan concentration for the most efficient purification and best saturation was 10g/l, with a contact time of 30 minutes and an initial COD of 2800 mg/l (10% vinasse solution). The chitosan column in these conditions results in high levels of organic acid adsorption to the column, and therefore an increase in vinasse pH. The organic components were then extracted from the surface of the chitosan by direct methanation. A reactor volume of 300ml containing 100ml active mixed methanogenic culture was required to treat 1g chitosan saturated with organic acids at pH 7 and 37ºC (Lalov *et al.*, 2000). Under these strictly anaerobic conditions, biogas formation reached 67% methane content after 21 days. Chitosan is a cheap and available biopolymer that can be efficiently used to adsorb the acidic component of vinasse although high dilution rates are required (Lalov *et al.*, 2000).

1.7.5. Activated sludge treatment:

Activated sludge (AS) treatment has been used to treat wine distillery effluent. Sales *et al.* (1987) found that an activated sludge digester treating vinasse required 28 days for its start-up i.e. it took 4 weeks for the pH to stabilize at pH 8. Once stable, the optimal retention time for vinasse in the

digester was found to be 8 days as the pH was found to decrease as retention time was increased. During this time, the pH ranged from 6.5 – 8, with a final COD removal of 78% and BOD removal of 88%. pH neutralization resulted in an undesirable increase in the total solids content of vinasse and was found to be unnecessary as it did not improve the purification process (Sales *et al.*, 1987). This resulted in simplification of the process and a reduction in operating costs. The residual COD was attributed to the presence of refractory compounds such as polyphenols which are difficult for aerobic microbes to degrade (Sales *et al.*, 1987). Furthermore, the treatment capacity of the activated sludge digester was found to cease if the organic load was too high or the retention time too short. The phenomenon of wash-out, whereby microbe regeneration equals microbe evacuation, was also found to be problematic under certain conditions (Sales *et al.*, 1987).

1.8. Fungal degradation of recalcitrant pollutants:

Although fungi have shown potential for the treatment of various specific pollutants and mixed effluents, literature regarding the use of fungi for the treatment of wine distillery effluent could not be found. Fungi have, however, been used to effectively treat other dark coloured phenolic effluents such as molasses wastewater (Jimenez *et al.*, 2004) and olive mill wastewater (Hamdi & Garcia, 1993; Perez *et al.*, 1998; Ruiz *et al.*, 2002; Aggelis *et al.*, 2003; Fenice *et al.*, 2003) although fungal treatment was used as a pre-treatment for anaerobic digestion in those cases. This implies however, that fungi have the potential to treat wine distillery effluent, which has high polyphenol content (Table 1.1).

The traditional mainstay of fungal biotechnology is the fermentation industry; however, researchers are now looking beyond this. Fungi are increasingly being evaluated for industrial uses that are outside the scope of the fermentation industry. Since environmental issues are of increasing concern, research is now being directed towards applying microbial technology to ameliorate the effects of environmental pollution (bioremediation). The approaches to bioremediation vary depending on the type of environment, either solid, liquid or air, the nature of the pollutant and the immediacy of the threat. There are two main ways in which fungi can be used to degrade effluents or pollutants in a liquid environment (Wainwright, 1992):

- first, bioreactors can be used, in which a liquid effluent is exposed to the specific, live fungus which must be capable of degrading a single pollutant or, preferably, a mixture of pollutants; while
- the second approach involves the use of a product derived from a fungus, such as an enzyme, to treat the polluted liquid.

Many industries produce effluent wastes containing a range of recalcitrant pollutants. The wastewater from the paper industry, for example, is one of the major unsolved environmental problems. The effluent contains recalcitrant lignin derivatives, which give an intense dark colour (Calvo *et al*., 1998; Nagarathnamma *et al.*, 1999). Yet another large problem is that of the dyes used as colorants in many industries. These are synthetically produced and their environmental fate is not well understood (Chivukula & Renganathan, 1995; Pointing & Vrijmoed, 2000; Wesenberg *et al.*, 2003). There has been considerable global scientific effort to research the use of fungi, especially the lignin degrading white rot fungi, to decompose these and similar environmental pollutants. Some of the most extensively researched pollutant degradation by white-rot fungi includes:

- munitions waste including 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) (Fernando *et al.*, 1990; Spiker *et al.*, 1992);
- polychlorinated biphenyls (PCBs) (Zeddel *et al.*, 1993; Novotny *et al.*, 1997);
- polycyclic aromatic hydrocarbons (PAHs) (Muncnerova & Augustin, 1994; Pickard *et al.*, 1999);
- polychlorinated Kraft bleaching effluents (Bajpai, 1999; Nagarathnamma *et al.*, 1999); and
- synthetic dyes (e.g. Pointing & Vrijmoed, 2000; Tekere *et al.*, 2001; Wesenberg *et al.*, 2003).

Many of the white rot fungi, such as *Phanerochaete chrysosporium*, are exceptionally versatile at the task of degrading recalcitrant compounds (Wainwright, 1992; Nyanhongo *et al.*, 2002a). *P. chrysosporium*, for example, produces a variety of non-specific ligninases (Wainwright, 1992; Tien & Kirk, 1988), which act upon organic molecules other than those present in lignin. Therefore, although these enzymes are used in natural ecosystems to degrade lignin, they can be employed in biotechnology to degrade a range of complex phenol-containing compounds such as pesticides (e.g. Bumpus & Aust, 1987; Bumpus *et al.*, 1993), chlorinated lignin wastes (e.g. Nagarathnamma *et al.*, 1999), and phenolic azo and other synthetic dyes (e.g. Pointing & Vrijmoed, 2000) among others. The advantages of using white rot fungi are that (Wainwright, 1992):

- they can be grown on cheap substrates;
- given effective engineering systems, these fungi can be used to treat different effluents, even using different effluent as the growth media (e.g. distillery effluent); and
- the enzymes produced may be used directly in the process at hand or they may be extracted and sold.

There are, however, constraints to using biological processes for effluent treatment since these processes are generally most efficient at low concentrations of the contaminant (Peralta-Zamora *et*

al., 2003). Furthermore, organisms can require long retention times, be sensitive to shock loads and produce large amounts of solid residue (Peralta-Zamora *et al.*, 2003).

During the past 2 to 3 decades, there has been considerable research to investigate the possibilities offered by the use of enzymes in waste treatment. This, according to Karam & Nicell (1997), has been due to:

- an increase in the rate of introduction of xenobiotics and recalcitrant organic pollutants into the environment. Simpler, faster, cheaper, more reliable and more effective alternative methods are required since conventional chemical and biological processes cannot achieve the necessary degree of pollutant removal;
- enzymes can be used to target specific pollutants for treatment; and
- recent advances in biotechnology such as improved isolation and purification procedures have resulted in the production of cheaper and more readily available enzymes.

Enzymatic treatment falls between the two traditional categories of physico-chemical and biological processes. It involves chemical processes based on the action of biological catalysts and offers many potential advantages when compared to traditional physico-chemical and biological treatment. According to Karam & Nicell (1997), these include:

- its applicability to biorefractory compounds;
- operation over broad ranges of contaminant concentrations;
- operation over a wide range of pH, temperature and salinity conditions;
- the absence of shock loading effects;
- delays associated with the acclimatization of biomass are eliminated;
- easier and simpler process control;
- a reduction in sludge volume since no biomass is generated; and
- enzymes are less likely to be inhibited by substances that may be toxic to living organisms.

As illustrated, there is indeed a niche to be found for enzymes. This in turn leads to the obvious conclusion that viable, sustainable and cost effective processes need to be developed for the production of useful, reliable and readily available enzymes. Some of the most valuable broad substrate enzymes for bioremediation and other processes are produced by the white-rot or ligninolytic fungi.

1.9. Ligninolytic (white-rot) fungi and their enzymes:

One of the major classes of pollutants and one of the most heavily regulated in many countries, is the aromatic compounds, which include phenols and aromatic amines. They are found in a variety of industrial wastewaters, including the wastewater from petroleum refining, coal conversion, resins and plastics, wood preservation, metal coating, dyes and other chemicals, textiles, mining and pulp and paper. Most aromatic compounds are toxic and consequently must be removed before the wastewater can be discharged into the environment (Karam & Nicell, 1997).

Lignin is the most abundant renewable aromatic polymer in the biosphere and is a significant component of plant materials. It is also known to be one of the most recalcitrant biomaterials on earth. It is a complex aromatic macromolecule, which acts as a type of glue between polysaccharide filaments and fibres giving strength and rigidity to cell walls and tissues of all vascular plants (Hofrichter, 2002). Due to the several types of covalent bonds between the phenylpropanoid units of the lignin polymer as well as their heterogeneity, lignin cannot be cleaved by hydrolytic enzymes as is the case with most other natural polymers (Hofrichter, 2002). Lignin degradation plays a key role in the cycling of carbon in the biosphere (Ichinose *et al*., 1999) and white-rot fungi are the most efficient known degraders of this compound (Kirk & Farrell, 1987; Eriksson *et al.*, 1990; Heinzkill *et al*., 1998).

Once the ligninolytic ability of white-rot fungi was discovered, it was proposed that due to their non-specificity, the fungi and their enzymes would be ideal candidates for bioremediation. The ligninolytic system of some white-rot fungi, in addition to non-specificity, is not necessarily induced by lignin or lignin-related compounds (Mester & Tien, 2000). As a result it is possible to degrade pollutants at relatively low concentrations. Furthermore, the ligninolytic enzymes are extracellularly excreted and since lignin degradation is non-specific and a radical-based oxidation, these lignin-degrading fungi are capable of degrading a mixed variety of pollutants (Mester & Tien, 2000). This is particularly advantageous in the area of bioremediation where a mixture of different pollutants can be found in most contaminated sites. As research continues, the number of compounds known to be susceptible to degradation by ligninolytic fungi is likely to increase.

The generally accepted theory is that lignin itself is not the target substrate in primary metabolism of fungi, as its oxidation does not result in any energy gain (Jeffries, 1990). Instead, the extracellular ligninolytic enzymes make the initial attack on the non-phenolic aromatic nuclei present (Mester & Tien, 2000). The enzymes perform a one-electron oxidation of non-phenolic

aromatic nuclei contained in lignin or the pollutant, which generates cation radicals (Mester & Tien, 2000; Pointing, 2001). Spontaneous chemical reactions such as C-C cleavage or hydroxylation of the cation radicals results in more hydrophilic (aromatic and aliphatic) products (Mester & Tien, 2000; Pointing, 2001). These products are then taken up by the fungal cells and co-metabolized in the presence of a suitable carbon source to carbon dioxide (Mester & Tien, 2000). Lignin is thus mineralized during secondary metabolism in order to access wood polysaccharides that are present in lignin-carbohydrate complexes, and it is by this means that white-rot fungi are able to access an energy source which other organisms cannot (Jeffries, 1990).

White-rot fungi generally secrete one or more of three extracellular enzymes that are required for lignin degradation. These enzymes, called lignin-modifying enzymes (LMEs), are lignin peroxidase and manganese peroxidase, both of which are glycosylated heme-containing peroxidases and laccase, which is a copper-containing polyphenoloxidase (Youn *et al.*, 1995; Nagarathnamma *et al.*, 1999; Cameron *et al.*, 2000; Ferraz *et al.*, 2003). These fungi are more efficient than bacteria at the degradation of toxic or insoluble chemicals as the enzymes are extracellular (Cameron *et al.*, 2000). Bacteria would require endocytosis of the chemicals, which could be fatal to the bacterial cell. Although bioremediation is an established technology, most of the treatment methods currently employed use prokaryotes. White-rot fungi show great potential for use in bioremediation due to the production of extracellular enzymes and their ability to degrade a wide range of pollutants including toxic or insoluble substances.

Studies have shown that laccase plays a significant role in the degradation of phenolic groups in lignin mineralization (Ander & Eriksson, 1976). It was found that laccase-deficient fungi were unable to degrade lignin, while laccase-positive species were able to degrade lignin and other wood components (Ander & Eriksson, 1976). The subject of the current study, wine distillery effluent, generally contains high levels of polyphenols (Table 1.1). Since it is believed that laccase is involved in the removal of potentially toxic phenols that are generated during lignin degradation, laccase-producing fungi are likely to possess the potential to treat the effluent. Moreover, laccase can be a valuable by-product of the fungal treatment system, as it has a multitude of applications (Section 1.11).

1.10. Laccase:

Laccase (EC 1.10.3.3; benzenediol:oxygen oxidoreductase) is a major component of the lignin degrading system of white-rot fungi (Youn *et al.*, 1995; Heinzkill *et al.*, 1998) and is produced

constitutively by some fungi during primary metabolism (Cameron *et al.*, 2000). Laccases are mostly extracellular glycoproteins with molecular weights between 60 and 80 kDa (Heinzkill *et al*., 1998). It is part of a group called the multicopper enzymes, which include ascorbic acid oxidase and ceruloplasmin (Mayer & Staples, 2002). There are three different classes of oxidases: blue, yellow and white with laccase belonging to the blue class (Heinzkill *et al*., 1998; Galhaup *et al.*, 2002). They usually contain 4 copper ions per molecule distributed in 3 different copper binding sites and each copper ion is apparently involved in the catalytic mechanism (Malmstrom *et al.*, 1968; Heinzkill *et al*., 1998). Laccase functions by a one-electron oxidation of the substrate which is coupled to the simultaneous reduction of molecular oxygen to water (Palmieri *et al*., 1997; Herpoel *et al.*, 2000; Galhaup *et al*., 2002).

While laccases of some white-rot fungi have been purified and characterized biochemically, there are not many systematic and comparative studies available on quantitative laccase production. Since laccase is important for various biotechnological applications, there is an underlying need to expand the spectrum of laccase-producing organisms and to enhance the potential of their laccase-producing ability (Arora & Gill, 2001; Herpoel *et al.*, 2000).

1.11. Some applications of laccase:

1.11.1. The paper and pulp industry:

The world-wide production of pulp raw materials is approximately 70 million tons per year corresponding to a market value of paper and pulp chemicals of more than US$ 5 billion (Nedwin, 1997). Presently, large amounts of chlorine and chlorine chemicals are employed in the bleaching of Kraft pulp, producing chlorinated organic substances as by-products, including chloro-lignins, chloro-phenols, chloro-guaiacols, chloro-catechols and chloro-aliphatics (Nielson *et al.*, 1991) which can be toxic, mutagenic, persistent, bioaccumulating and can cause many harmful disturbances in biological systems (Bajpai, 1999). There is a strong desire in the industry to reduce (and eliminate) the use of chlorine and other chlorinated bleaching agents in the manufacture of white paper from pulp and some work using enzymes has been successful (e.g. Monteiro & de Carvalho, 1998). One of the major concerns regarding the reduction of chemical use is to retain the high quality of brightness of the final paper product without losing paper strength (Nedwin, 1997). The use of enzymes was not, until recently, considered technically or economically viable in the paper and pulp industry, due to the unavailability of readily utilisable enzymes. Research by scientific institutions and enzyme producers however, has led to the development of enzyme production systems that offer significant benefits to the industry (Bajpai, 1999), providing an

opportunity to minimize the amount of toxic pollutants generated by the industry and to improve the existing technology in an economically feasible fashion (Christov & Prior, 1998).

This is especially significant in South Africa as the country is a significant producer of pulp and paper products internationally. The industry in this country is based on the harvesting of managed forests and it not only contributes at least 3% to the gross national product but is also a major employer (Christov & Prior, 1998). South African Pulp and Paper Industry (SAPPI) supplies half of the country's paper requirements and is the largest papermaker in Africa. The pulp mills in South Africa are more often than not located in areas with a high population density. Therefore the implementation of this new enzyme technology could result in significant environmental benefits to the local residents (Christov & Prior, 1998).

Laccase can be used in the bleaching process (Crestin & Argyropolous, 1998; Monteiro & de Carvalho, 1998; Nagarathnamma *et al.*, 1999) and for the treatment of the bleaching by-products by polymerizing and thereby rendering less soluble many of the low molecular weight non-chlorinated and chlorinated phenolics. Esposito *et al.* (1993) for example found that *Pycnoporus sanguineus* cultured on alkaline Kraft effluent produced mainly laccase and this biological treatment resulted in a 51% decrease in colour, a 41% decrease in COD and a 55.2% decrease in BOD.

1.11.2. Bonding of fibreboards:

Oxidoreductases such as laccase have recently been found to be successful in the bonding of fibreboards, particle boards, paper boards and Kraft-liner boards (Felby *et al.*, 2002). Felby *et al.* (2002) tested laccase for use in the bonding of fibreboards and found that currently, the technology may not be economically feasible due to a combination of higher pressing temperatures and longer pressing times required for laccase-catalyzed bonding. However, laccase treatment has shown much promise in the field as laccase-treated boards exhibited an increase in strength and dimensional stability (Felby *et al.*, 2002). Also, the mechanical properties of the laccase-treated boards were at the same level as those treated in the traditional manner (i.e. treatment with urea-formaldehyde resin and wax) (Felby *et al.*, 2002). The study by Felby *et al.* (2002) concluded that fibreboards bonded by laccase-catalyzed oxidation could be produced in a continuous pilot-scale process, and that the strength properties of the boards produced were comparable to conventionally treated boards.

1.11.3. Degradation of textile dyes:

Azo dyes constitute the largest group of colorants used in industry. They are produced only through chemical synthesis and their fate in the environment is not well understood. These dyes are resistant

to aerobic bacterial degradation, but white rot fungi have been found to be efficient in complete degradation of a number of azo dyes (Chivukula & Renganathan, 1995). More specifically, it has been shown that the laccases produced by these fungi have the ability to oxidize phenolic azo dyes that have an electron rich phenolic ring (Chivukula & Renganathan, 1995).

Nyanhongo *et al.* (2002a) found laccase to be a very efficient decolourizer of the synthetic dyes anthraquinone, azo, indigo and triarylmethane but that the rate of decolourization depended on the source of the enzyme and the structure of the dye. Of the laccase from three *Trametes* species tested, the laccase produced by *T. modesta* was found to be most efficient at dye degradation and this efficiency was significantly increased upon the addition of mediators like 1-hydroxybenzotriazole. It was also found that the optimum pH for decolourization varied for each individual dye and that the rate of decolourization by the laccase from *T. modesta* increased with an increase in temperature from 50°C to 60°C (Nyanhongo *et al.*, 2002a).

The decolourization of industrial effluents is a field of increasing research due to the increasing stringency of government legislation regarding the release of contaminated effluents. Existing processes for treatment of dye-wastewater produce large amounts of sludge and are not economical. They also fail in the degradation of dye mixtures. Since laccase is able to degrade dyes of diverse chemical structures, the development of processes to remove dyes from industrial effluent using laccase, is very promising (Lorenzo *et al.*, 2002).

1.11.4. Denim finishing:

Around 1.8 billion pairs of denim jeans are sold annually (Internet Reference 4). Since most people do not prefer these to look new, denim garments are normally subjected to a wash treatment to give them a slightly worn look. Traditionally, denim is faded by the abrasive action of lightweight pumice stones on the fabric surface, removing some of the dye. Excess abrasion can, however, damage the fabric. In contrast, enzymes age clothes gently and have revealed new possibilities in denim finishing by increasing the variety of finishes available. Laccase can be and is used for the purpose of fading denim, for example, DeniLite II is produced by Novozymes for this purpose (Internet Reference 4).

Laccase and other cellulases are the most widely used fading process today due to the advantages of new looks, lower costs, shorter treatment times and less solid waste. A small dose of laccase can replace several kilograms of stones. The use of fewer stones results in less damage to garments, less wear on machines, less pumice dust in the working environment and more room in the machines for

garments. Furthermore, the wastewater is free of sediment that can clog drains and it is no longer necessary to remove small stones and dust from the finished material (Internet Reference 4).

1.11.5. Biosensors:

A mediated reagentless enzyme inhibition electrode was developed and tested by Daigle *et al.* (1998). The system is based on the mediated reduction of oxygen by an enzyme co-immobilized with an "osmium redox polymer hydrogel on glassy carbon electrode surfaces" (Daigle *et al*., 1998). This sensor can be used for the detection of low levels of respiratory toxins such as azide and other modulators of enzyme activity (Daigle *et al*., 1998). Of the enzymes tested, interactions between the mediators and laccase was found to be three orders of magnitude faster than with ceruloplasmin and one order of magnitude faster than that for tyrosinase (Daigle *et al*., 1998).

The use of fungal laccases in the construction of biosensors for the determination of phenols is increasing. Freire *et al.* (2002), successfully developed a laccase-based flow injection electrochemical biosensor for the detection of phenolic compounds. The biosensor was found to have high sensitivity, presenting selective measurements to micromolar concentrations of phenol, *p*-chlorophenol, guaiacol and chloro-guaiacol. The laccase-based biosensor was also shown to be highly stable over the long term and can be accurately used for measurements for more than 3 months (Freire *et al.*, 2002). Kulys & Vidziunaite (2003) have also developed biosensors containing recombinant fungal laccase for the detection of phenols.

1.11.6. Other uses:

Besides those uses of laccase stated above, there are many other ways in which this enzyme can be utilized. These include:

- As a bleaching agent in hair products (Onuki *et al.*, 2000; Pruche *et al.*, 2000)
- In batteries (Barton *et al.*, 2002)
- In chemical conversions
  - e.g. laccase catalyses the conversion of 4-methyl-3-hydroxyanthranilic acid to 2-amino-4,6-imethyl-3-phenoxazinone-1,9-carbozylic acid, i.e. actinocin (Osiadacz *et al.*, 1999); and
  - e.g. benzyl alcohols can be converted to the corresponding benzaldehydes by laccase-catalyzed oxidation (Potthast *et al.*, 1996)
- As a probe in medical diagnostics for example the construction of an enzyme sensor, based on laccase and glucose dehydrogenase, to simultaneously distinguish between morphine and codeine in a drug sample. Laccase oxidizes morphine in the presence of oxygen and glucose dehydrogenase then regenerates morphine. Codeine is not oxidized by laccase therefore the

sensor selectively detects morphine. It is a simple and rapid method allowing for the differentiation of morphine from codeine in less than 1 minute (Bauer *et al.*, 1999).

- Various applications of laccase in the food industry, including remediation of food industry wastewater, beer and wine stabilization, fruit juice processing and baking among others, as reviewed by Minussi *et al*. (2002).

The laccase utilized in bioremediation can be used in a crude form, while other more specific uses like those above generally require a greater degree of purity of the enzyme. It is clear that a number of potential applications for laccase exist; however in order for the enzyme to be used on a large scale, efficient laccase production systems must first be established.

1.12. Laccase production in white-rot fungi:

White-rot fungi that produce laccase, in general produce them as multiple isoenzymes (Bollag & Leonowicz, 1984). Production of laccase in large amounts is essential in order for a commercial venture to be successful and although laccase is produced constitutively in some fungi (Bollag & Leonowicz, 1984), the production of laccase is inducible and can be significantly increased by a variety of substances as can be seen in the following cases. Furthermore, production may be influenced by a range of factors including the type of support matrix used, carbon and nitrogen sources and the presence of inducers.

1.12.1. Support matrices:

Studies conducted by Lorenzo *et al*. (2002) showed that biowastes could provide some nutrients for fungal growth, so only a low initial concentration of glucose was needed. It was also shown that the addition of lignocellulose residues to submerged cultures of *Trametes versicolor* (a white-rot Basidiomycete) reduced manganese peroxidase (MnP) production. It was suggested that the presence of these lignin residues changed the metabolic pathway of the fungus toward laccase production, since the addition of these residues highly improved laccase activity levels. Furthermore, insignificant levels of lignin peroxidase (LiP) were detected. Therefore, if the fungus was being cultured for the production of laccase, the addition of lignocellulose residues would make commercial purification easier, and the process more economical (Lorenzo *et al*., 2002).

Couto *et al*. (2002) investigated laccase production by *T. versicolor* under semi-solid state conditions. Two main types of solid materials were employed: inert materials (polyurethane foam) and non-inert materials (agricultural residues). While the foam simply acted as an attachment site

for the organism, the agricultural residues also functioned as a source of nutrients. The main advantage of this type of cultivation over liquid culture was a reduction in production costs as agricultural by-products (such as straw and bran) were used, and these reproduced the natural living conditions of the fungus (Couto *et al.*, 2002). The use of either wood shavings or wheat straw as supports resulted in laccase activities 2-fold higher than those obtained for the inert support. Barley straw resulted in activities 3-fold higher and barley bran resulted in laccase activities 4-fold higher than those achieved using the inert support. Therefore non-inert supports clearly induced higher laccase activity than inert supports for *T. versicolor* (Couto *et al*., 2002).

1.12.2. Carbon source:

Galhaup *et al*. (2002) have shown *Trametes pubescens*, a white-rot fungus, to be an excellent producer of extracellular laccase. Laccase formation in *T. pubescens* was subject to glucose repression, where the presence of glucose in the medium above a certain concentration repressed laccase production (Galhaup *et al.*, 2002). Therefore, by continuously feeding a low, non-repressing amount of glucose to a culture of *T. pubescens*, it was found that laccase production could be increased 2-fold (Galhaup *et al.*, 2002). The maximum laccase activity achieved in a fed-batch reaction was 743 U ml^{-1}, where 1 Unit of activity was defined as "the amount of enzyme required to oxidize 1μmol of substrate per minute" (Galhaup *et al*., 2002).

1.12.3. Nitrogen source:

Laccase activity was found to appear earlier and in higher concentrations in nitrogen-rich media (in the form of peptone) than in nitrogen-deficient media for cultures of *Pleurotus ostreatus* and *Lentinula edodes* (Kaal *et al*., 1995). Also, laccase production by *T. pubescens* was found to be optimal in a medium where the only nitrogen source was in the form of peptone from meat, with the total N concentration at 80mM (Galhaup *et al*., 2002). This is considerably higher than the concentration routinely used for laccase production in other fungi (Galhaup *et al*., 2002). An increase in the concentration of this nutrient led to no further increase in laccase activity, but a decrease in concentration led to a reduction in laccase activity (Galhaup *et al*., 2002). Couto *et al*. (2002) found that the onset of laccase activity by *T. versicolor* coincided with the total consumption of ammonium-N, suggesting that laccase production was triggered by nitrogen limitation. However, a low initial ammonium concentration led to lower laccase levels, probably due to limited fungal growth. Although nitrogen-rich media are often required to induce laccase production, some white-rot fungi such as *Pycnoporus sanguineus* (also called *Pycnoporus cinnabarinus*) require a nitrogen-limited environment for enhanced laccase production (Bollag & Leonowicz, 1984; Pointing *et al.*, 2000).

1.12.4. Other laccase inducers:

Research carried out by Arora and Gill (2001) indicated that higher laccase production was obtained from fungi grown in malt extract broth (MEB) or mineral salts malt extract broth (MSB-MEB) in comparison to mineral salts broth (MSB) alone. Their study also showed that the effect of veratryl alcohol on laccase production varied with the organism used and also with the growth medium. Furthermore, guaiacol supplementation enhanced laccase production from 2 to 232 fold in different fungi, although very high concentrations of guaiacol seemed to suppress laccase production (Arora & Gill, 2001).

Laccase production in *Phlebia radiata* increased more than 200-fold upon veratryl alcohol supplementation to MSB (Arora & Gill, 2001). This was a significant increase when compared to the 2 to 6-fold increase in laccase production by *Daedalea flavida*, *Phlebia brevispora* and *Polyporus sanguineus* upon addition of veratryl alcohol to MEB (Arora & Gill, 2001). This showed that the effect of veratryl alcohol supplementation on laccase production varied depending on the organism and the basal medium used.

The optimal conditions for laccase production by *T. pubescens* seem to differ from those typically reported for other fungi. It was found that the presence of Cu in the millimolar range in the culture medium was an important prerequisite for high laccase production by this fungus (Galhaup *et al*., 2002). Furthermore, although the addition of aromatic compounds are routinely used to boost laccase production (by several-fold) in other organisms, the effects of these compounds were negligible (or even decreased production in the case of vanillic acid and p-anisidine) in *T. pubescens* (Galhaup *et al*., 2002).

In contrast, Nyanhongo *et al*. (2002b) found that veratryl alcohol gave more than a 350% increase in laccase production in *Trametes modesta*. Copper sulphate and 2,5 xylidine were also found to increase laccase production by more than 350%, while caffeic acid and syringic acid more than doubled the production of laccase in this fungus (Nyanhongo *et al*., 2002b).

Leonowicz & Trojanowski (1975) and Rogalski *et al.* (1991) showed that either ferulic acid or ligninsulphonate could induce laccase activity, and this was again shown by Hublik & Schinner (2000) with *Pleurotus ostreatus*. With their work on the white-rot fungus *P. cinnabarinus*, Herpoel *et al.* (2000) found that the effect of an inducer (ferulic acid) differed with different strains of the fungus. While ferulic acid significantly enhanced laccase production in some strains, it had no effect on others (Herpoel *et al.*, 2000).

It has been shown that the induction of laccase is possible, however the effect of various inducers differ. The effects of inducers appear to vary according to the fungus and even the fungal strain used.

Based on the information reviewed above, the fungal remediation of wine distillery effluent and the production of valuable by-products could be a feasible alternative. The viability of this alternative could be assessed using the ETSA model.

1.13. Hypotheses:

- The Emerging Technologies Sustainability Assessment model can be used to efficiently compare emerging technologies to existing processes, and to determine the likely sustainability and competitiveness of an emerging technology early in the developmental process.
- Remediation of wine distillery effluent by a white-rot fungus is potentially a sustainable, competitive and cost-effective process.

1.14. Objectives:

- To develop the ETSA model to be used for the comparison of the sustainability and competitiveness of emerging technologies to existing processes.
- To use the ETSA model to identify gaps in the information regarding the fungal degradation of wine distillery effluent.
- To design experiments specifically to acquire the above information and to determine the most likely configuration of the fungal process.
- To incorporate the newly acquired process data into the ETSA and to assess the alternative processes.

CHAPTER II: PRELIMINARY EMERGING TECHNOLOGIES SUSTAINABILITY ASSESSMENT

2.1. Background:

As discussed in Chapter 1, ETSA uses a modular approach to data collection and analysis using information modules. An information module refers to a standard analysis or set of data that is designed to build on or feed into other information modules in order to form an overall assessment of the various technologies being evaluated, for example, the hazards identification and characterization feeds into the exposure and risk assessment module.

ETSA involves four stages: preliminary ETSA, data collection, data analysis and relative technology comparisons. Table 2.1 lists some possible information modules that can be used in terms of the ETSA framework and an overview of what each module entails. As the ETSA can be used to assess a number of different technologies (e.g. waste treatment technologies, manufacturing technologies, bioprocesses, etc.) the definitions for the modules should be adjusted accordingly. Note that this table is adapted from the CTSA framework (Internet Reference 2).

The preliminary ETSA serves as a preparatory step that allows one to:

- determine which information modules are relevant to the study being undertaken and add, remove or combine modules from the framework accordingly;
- determine the gaps in current knowledge; and to
- determine the best approach to gather the information necessary for the ETSA.

Upon completion of the preliminary ETSA, data collection from the sources identified and data analysis can commence.

2.2. Method:

This study focused on the emerging fungal technology for the treatment of wine distillery effluent. This was assessed in comparison to the two alternative processes currently employed for effluent treatment/disposal, namely land application/irrigation and anaerobic digestion.

Table 2.1: The basic ETSA information modules and an overview of each (as adapted from CTSA modules, Internet Reference 2)

Information module	**Overview**
Process description	Description of process operations, equipment and material flows and any modifications. May include process flow diagrams.
Hazards identification and characterization	Identifies and describes potential hazards to the environment or human health and safety posed by the process or its products
Exposure assessment	Quantitative or qualitative evaluation of the contact an organism (human or environmental) may have with hazards described. Describes magnitude, frequency, duration and route of contact.
Risk assessment	This is an integration of hazard and exposure information to quantitatively or qualitatively assess risk. Describes potential risk to human health and the environment. Includes any assumptions, scientific judgements and uncertainties in the process.
Social Impacts	Qualitative assessment of benefits or costs of processes in terms of effects on health, recreation, productivity and other social welfare issues.
Market information	Economic data used to evaluate the value of the technology to the target market and market trends (includes international markets and trade issues).
Workplace practices and source release assessment	Identifies the workplace practices that contribute to environmental release and worker exposure. Also identifies the sources, amounts and characteristics of environmental releases (both on- and off-site).
Regulatory status	Determines the statutes and regulations that govern a particular industrial process and technology and its products.
Performance assessment	Describes the effectiveness of alternative processes in achieving the desired function.
Cost analysis	Includes capital, operating and maintenance costs, indirect costs and any benefits. May include liability costs or less tangible benefits or costs.
Energy impacts	Evaluates the sources and rates of energy consumption in a process.
Resource use/conservation	Determines the types of resources consumed or conserved and evaluates the sources and rates of resource consumption of a process.
Relative technology evaluation	Comparison of the examined processes relative to each other in terms of the information modules. Is the emerging technology competitive and sustainable?

The preliminary ETSA considers each of the alternative processes individually, and for each information module, determines what information is required and where this could be located. At this point, certain assumptions are made regarding the availability of information on the more established alternatives. The assumption made was that all the relevant information on the existing technologies would be readily available in literary sources. The preliminary ETSA then concentrated on acquiring information regarding the emerging alternative technology, i.e. fungal treatment of wine distillery effluent.

There are basically three key means of obtaining the information necessary to perform the ETSA. These are:

- extensive reviews of the literature available regarding the processes under evaluation;
- consultation with experts in the fields being investigated; and
- experimental analysis, which is generally an expensive and time consuming task. It is therefore important to identify the key information which can only be obtained in this manner. With the emerging technology, some of the fundamental information may be available in literature, but it is unlikely that all the necessary information will be available.

The module regarding workplace practices and source release assessment was removed as it fell outside the interest of this project. The exposure assessment and risk assessment modules were combined for the ETSA of alternatives for wine distillery effluent treatment. It was decided that the data collection and analysis would be done separately for each of the alternative technologies and the relative technology evaluation would be done comparatively for the processes thereafter. The modules associated with data collection and analyses for each alternative technology are presented in Chapters 3, 4 and 6, with the relative technology evaluation module forming Chapter 7 of this report.

2.3. Results and Discussion:

As it was assumed that the information on both land irrigation and anaerobic digestion of distillery effluent would be available in literature, Table 2.2 shows the information modules required for analysis of the fungal technology the ETSA and the approach(es) chosen in order to capture and analyze that data efficiently.

From Table 2.2, the gaps in the information for the fungal treatment technology requiring experimental research were found to exist in the actual performance of the proposed fungal treatment system. At this stage of the evaluation, experimental data would most likely be limited to flask or laboratory-scale studies. The performance of the fungal system would need to be assessed in terms of its ability to remove contaminants in the form of COD and polyphenols. The capability of the fungal system for laccase enzyme production would also need to be assessed as this potential value-added product could be recovered from the system.

Although the current ETSA would allow for a decision as to whether further development of the fungal process should take place, scale-up, which refers to “any increase according to a fixed ratio”

(Reisman, 1993), can introduce changes to the performance of a biological system. Thus, it would be appropriate to conduct a follow-up ETSA using data from a larger system should the initial ETSA indicate that the process is competitive. The use of computer programmes to predict the effects of scale-up is an important tool for ETSA as it is the cheapest and easiest method of gaining information regarding the systems' performance without actual physical scale-up of the process. Table 2.3 lists the factors, according to Reisman (1993) that should be considered for scale-up of the fungal process and the potential concerns related to each.

Table 2.2: The ETSA information modules and the proposed method/s of data capture for the novel fungal treatment system

Information module	**Data capture method**
Process description	Literature and consultation with experts
Hazard Identification and characterization	Literature and consultation with experts
Exposure and risk assessment	Literature and consultation with experts
Social impacts	Literature and consultation with experts
Performance assessment	Experimental
Regulatory status	Literature
Financial considerations	Deduced from performance assessment and literature
Market size and information	Literature and consultation with experts
Energy impacts	Literature and consultation with experts
Resource use/conservation	Literature and consultation with experts

Table 2.3: Scale-up considerations for fungal treatment of wine distillery effluent and the potential concerns (adapted from Reisman, 1993)

Factor	**Potential concerns**
Strain selection	Purity, stability, by-product formation, viscosity and mutation
Raw materials (wine distillery effluent)	Availability (as it is a seasonal effluent) and composition (which is seasonally variable)
Inoculum	Culture times, quality control, volumes of inoculum required (related to reactor volume), transfer time, storage parameters
Sterilization (if required)	Method used, volumes, total heat input, fouling, time
Selection of process parameters	Mixing, power and shear effects, homogeneity (mass transfer rates), pH, pressure, temperature, oxygen levels, recycling or dilution parameters
Materials of construction and cleaning	Contaminants, surfactants, corrosion/erosion, construction quality, agents used and released during cleaning
Monitoring	Quality of control, alarms and response times, sensitivity of sensors required and their stability, sampling methods
Downstream processing	Biomass removal method, laccase extraction method, quality control, quality of process control, transfer times, stability, contaminant products, recycling the effluent stream

Following completion of the preliminary ETSA, is the data collection step. The collection of data from experiments, literature and consultation can be run concurrently as each does not depend on the outcome of any of the others. Collection of performance data, even on a small scale, can be extremely costly and time-consuming, and therefore experiments should be carefully designed so that the required data is obtained as efficiently as possible.

2.4. Conclusion:

The preliminary ETSA is necessary in order to determine the project scope and is used to identify the gaps in information and the specific methodologies to be used for data acquisition and analysis. It encompasses a holistic approach, where emphasis is not only given to the steps involved in actually performing an ETSA, but to the preparative and development steps that are essential for the success of the entire assessment project. It also provides a logical approach to the next step in an ETSA, which is to collect the data required.

One of the drawbacks of the method is that the availability of certain data is assumed during the early stages of the ETSA. If the information is later not found or unavailable, the data collection methods and even the information modules themselves may have to be reassessed according to the availability of information.

CHAPTER III: INVESTIGATION OF LAND APPLICATION/IRRIGATION OF WINE DISTILLERY EFFLUENT

3.1. Introduction:

The wastewater generated by distilling operations is predominantly discarded still underflow, which refers to the highly concentrated vinasse that remains after the distillation of wine (Hayward, 2000).The bulk of the wastewater generated in wine cellars, which is generally added to the distillation operation waste stream, is produced during relatively short time periods, primarily during and just after the vintage season (Hayward, 2000). Although the time periods are short, vast quantities of effluent are produced mainly as a result of washing, rinsing and sanitizing operations (Hayward *et al.*, 2000).

There are currently three popular methods employed by wineries/distilleries to handle their wastewater (Hayward, 2000):

- the collection of effluent in storage tanks or dams, followed by irrigation;
- wastewater treatment in ponds, primarily settling of solids and evaporation processes, and application of resultant sludge on land; or
- discharge of the effluent stream to a local municipal treatment facility.

All of the above methods have their own associated problems and environmental risks. Treatment of such a highly concentrated organic wastewater at municipal facilities is very expensive and is often not a feasible, practicable or viable option (Hayward, 2000).

In South Africa, more than 90% of wineries and distillery plants dispose of their effluent by means of application to soil (Mulidzi *et al.*, 2002). This study will therefore concentrate on land application of distillery effluent, and not discharge into municipal wastewater treatment plants. As discussed in Chapter 1, due to the high concentrations of nutrients in the effluent, it can potentially be used as a fertilizer for various crops (Ramana *et al.*, 2002a). This chapter will look at the practice of land irrigation with raw wine distillery effluent in terms of the ETSA information modules discussed in Chapter 2.

3.2. Process description:

The traditional approach of wineries/distilleries in the handling or management of their wastewater has been to collect the effluent streams in evaporation ponds (Internet Reference 5). As a result of changes in legislation and market perceptions over the last 20 or more years, this method is no longer feasible. The majority of distilleries find that the most cost effective method of disposal is the application of the effluent to land planted with trees, grass, vines or other crops (Internet Reference 5; Mulidzi *et al.*, 2002; van Schoor & Roussouw, 2004).

This method relies on the crops chosen to detoxify the effluent by using the high concentrations of nutrients and organics for growth, thereby removing them from the soil. It is, however, necessary for some pre-treatment of the effluent prior to irrigation which may include screening and settling to remove most of the settleable and suspended solids. Once the solids have been removed, the effluent is then piped to the desired location and dispersed on land by either flood or spray irrigation. The type of irrigation used is dependent on the types of crops grown and stages of growth. The solids removed by pre-treatment are generally disposed in a landfill.

3.3. Hazard identification and characterization:

The hazard identification & characterization and exposure & risk assessment modules concern human health and environmental impacts. Impacts on society will be discussed in a separate information module (Section 3.5). The main hazard factors posed by the land application process were identified as the raw effluent and its components and offgases (methane, carbon dioxide and hydrogen sulphide) that are produced under anaerobic conditions in the soil as well as the screening and settling ponds.

Raw effluent and its components:

Mulidzi *et al.* (2002) showed that the organic component of the effluent can move down through the soil to the water table. This occurs mostly during irrigation, but also due to leaks in the pond linings and pipelines. One of the major concerns with land disposal of distillery effluents should be the leaching potential of the pollutants and thus their ability to reach the water table. The origin of pollution and its effects are often far removed from each other. Subsurface, lateral transport of pollutants from the area of disposal to water sources is such an example. This potential pollution could adversely affect communities, especially in rural areas in South Africa where distilleries are located, that have little access to a piped water system, and depend on a particular water source for

consumption and other purposes. Contamination of the communal water source, be it a river or stream, could lead to community upheaval and strife. If leaching occurs, it may constitute an ongoing pollution hazard, which may remain undetected for extended periods.

Once the effluent reaches rivers, streams or other watercourses, it would cause a disturbance to the natural processes of the watercourse. Aside from the odour, the effluent would also increase the COD, BOD and nutrient levels in the water, leading to eutrophication which has a number of adverse effects. These include accelerated lake overgrowth and algal blooms, reduced photosynthesis by aquatic plants due to algal blooms preventing the penetration of sunlight, oxygen deficiency in the water resulting in the death of aquatic species, accelerated silting up of open watercourses due to the accumulation of dead plant and animal matter, and the development of anaerobic conditions resulting in the production of hydrogen sulphide, which is toxic to all higher forms of life (Biamon & Hazen, 1983; Internet Reference 6). These situations favour the growth of a few fish species that feed on algae, while other predator fish are depleted (Internet Reference 6). Eutrophication of water sources is a dire problem that is fatal to many aquatic plant and animal species and must be avoided. The toxicity of wine distillery effluent and its direct impacts have not been extensively studied, however a study on the toxicity of a similar effluent (molasses distillery effluent) is discussed in Section 1.6.

The spatial extent of pollution from distillery effluent can be extensive. Gonzales *et al.* (1979) showed that the effluent plume resulting from the discharge of untreated distillery effluent could extend for many kilometres and that such a plume was normally anoxic and was able to kill fish, crabs, snails and other marine life. It is therefore imperative that distillery effluent not be allowed to reach fresh or marine water sources, as organisms life killed by the toxic effluent could be consumed by humans and may prove hazardous due to bioaccumulation of toxins.

Biamon & Hazen (1983) studied the effects of the effluent plume caused by the discharge of slops from the largest rum distillery in the world, which is located at Ensenad de Boca Vieja, in the Caribbean. In 1979, this distillery was found to pump more than 1.4×10^6 l/day untreated effluent, more than 300 days per year, into the bay (Costle, 1979). The 1000m long effluent plume created along the coastline had several negative effects (Biamon & Hazen, 1983). The concentration of nitrates, phosphates, sulphates, total phosphorous and total organic carbon in the waters all increased significantly (Biamon & Hazen, 1983). Furthermore, there was a decrease in pH, dissolved oxygen and turbidity of the water (Biamon & Hazen, 1983). The effluent had a toxic effect on all marine life, including bacteria at the point of discharge (Biamon & Hazen, 1983).

Further on, after slight dilution had occurred, the presence of the rum distillery effluent was found to have had a decidedly stimulatory effect on the entire bacterial community (Biamon & Hazen, 1983). *A. hydrophila*, a potentially pathogenic anaerobic bacterium common in sewage effluent, flourished in the distillery effluent-contaminated marine waters and it was found that the presence of the effluent significantly increased the densities of this bacterium (Biamon & Hazen, 1983). Reservoirs of this potential pathogen exist not only in salt and fresh water, but soil as well (Internet Reference 7). Therefore, the potential for the increase in soil of this organism due to the addition of distillery wastewater exists. *A. hydrophila* is associated with gastroenteritis, septicaemia, ocular and respiratory tract infections, pneumonia and urinary tract infections, although the infectious dose is unknown (Internet Reference 7; Internet Reference 8). Its host range includes humans, amphibians, fish, reptiles and birds (Internet Reference 7), and it is transmitted via contact with contaminated water and soil and ingestion of contaminated fish or reptiles (among other modes of transmission) (Internet Reference 7).

It can therefore be concluded that the indirect impact of the application of high strength organic wastewaters is the stimulation of growth of some naturally occurring pathogenic organisms such as *A. hydrophila*. The potential exists for the increase of such pathogens in both soils and water if wine distillery effluent is dispersed over land. This poses a major hazard to the health of people and other organisms (frogs, fish, etc.) that are exposed to the irrigated land or the contaminated water.

The addition of this acidic effluent with high salt concentrations would certainly increase the salinity of the soil over time and disturb normal soil processes, affecting both the soil organisms and the vegetation present. Some of the effects of effluent application to soil and vegetation are presented in Section 3.6.3.

Also, if the soil at the disposal site is disturbed, odours indicative of anaerobic decomposition become evident. The rate at which the organic material decomposes under the prevailing anaerobic conditions is extremely slow, as the thick, black, foul-smelling layer remains even several months after irrigation (Mulidzi *et al.*, 2002). These conditions imply that nutrient uptake by the crops irrigated with the wastewater and degradation by normal soil organisms is not always effective. Anaerobic digestion produces potentially hazardous gases, namely methane, carbon dioxide and hydrogen sulphide.

Methane (CH_4):

Anaerobic organisms break down organic matter, producing methane and carbon dioxide. Methane has been identified as a major hazard in anaerobic processes, especially landfill (Shah, 2000). It is a colourless, odourless and tasteless gas and, although the gas is biologically inactive and essentially non-toxic, it is an asphyxiant (Internet Reference 9). If high concentrations of methane are inhaled, adequate oxygen can be excluded resulting in asphyxiation, the symptoms of which include dizziness, nausea, deeper breathing, unconsciousness and death (Internet Reference 9). Methane is not known or expected to cause chronic health problems, aggravate health problems, cause tissue irritation, cause cancers or mutations or affect reproduction (Internet Reference 9).

Methane has been identified as one of the greenhouse gases that enhance the earth's natural greenhouse effect called global warming and accounts for approximately 20% of the total warming effect of greenhouse gases (Miller, 2000). Aside from the ability of methane to remain in the troposphere for 9 – 15 years, each CH_4 molecule is able to store around 20 times the heat stored by a CO_2 molecule (Miller, 2000). Although current climate projection models cannot accurately predict specific climate changes or their effects in various parts of the world, predictions include droughts, increased storms and rainfall, rising sea levels and the loss of biodiversity (Miller, 2000). Therefore processes that increase the amount of greenhouse gases, such as methane, in the atmosphere are likely to have long-term direct impacts on the environment and indirect impacts on humans due to climate change and increased global temperatures.

Methane is also an extremely flammable gas that can be explosive if exposed to heat, flames or other sources of ignition (Internet Reference 9). Methane can also be explosive when mixed with air in a confined space (Shah, 2000), therefore accumulations of this gas is hazardous to human health and safety. Precautionary measures should be taken against static discharges and other sources of ignition.

Carbon dioxide (CO_2):

As mentioned, carbon dioxide is also a product of anaerobic digestion. This is a stable, colourless and odourless gas that is non toxic, but may cause nausea, vasodilation or asphyxiation at sufficiently high concentrations (Internet Reference 10). Precautions such as sufficient ventilation must be taken to ensure that this gas does not accumulate in confined spaces (Internet Reference 10). Carbon dioxide is the most important greenhouse gas produced by human activities, and the rates of production are increasing. Although CO_2 molecules trap less heat than other greenhouse gases, this gas accounts for 50%-60% of the global warming effect due to the total volumes of

carbon dioxide produced (Miller, 2000). As mentioned above, the production of greenhouse gases has long-term direct impacts on the environment and indirect impacts on humans.

Hydrogen sulphide (H_2S):

Hydrogen sulphide is formed under anaerobic conditions when sulphate reducing prokaryotes (SRPs) use sulphate as an electron acceptor resulting in hydrogen sulphide production (Hvitved-Jacobsen & Nielsen, 2000). It is a colourless gas that can be detected at low concentrations by its distinctive smell of rotten eggs (Internet Reference 11). This gas is also highly flammable and may form an explosive mixture with air (Internet Reference 12). It is also incompatible with many metals and strong oxidizing agents and may react violently with metal oxides, copper, fluorine and sodium (Internet Reference 12). Under anaerobic conditions, hydrogen sulphide is the most stable form of sulphur and it is also toxic to plants, animals and microorganisms. Although the exact mechanism of toxicity is unclear, some possible mechanisms have been described. These include changes to the internal cell pH of organisms, denaturation of native protein due to the formation of sulphide and disulphide cross-links between polypeptide chains and interference with coenzymes A and M due to the formation of sulphide linkages (O'Flaherty & Colleran, 2000). The correlation between hydrogen sulphide concentration and toxicity to microorganisms is not proportional, but dependent on other parameters such as pH.

Hydrogen sulphide can also be associated with several human health problems. The toxicity of this gas and its effects increase with its concentration, although it can only be detected by smell at low concentrations (down to 0.1 – 0.2ppm) (Hvitved-Jacobsen & Nielsen, 2000). Table 3.1 lists the effects of hydrogen sulphide on humans, as taken from the US National Research Council concerning the potential effects of hydrogen sulphide in sewer systems (cited in: Hvitved-Jacobsen & Nielsen, 2000).

Table 3.1: Effects of hydrogen sulphide on humans (from US National Research council, cited in: Hvitved-Jacobsen & Nielsen, 2000)

Effect	Atmospheric hydrogen sulphide concentration (ppm)
Odour limit	0.1 – 0.2
Unpleasant smell	3 – 5
Recommended criterion per workday	10
Effect on eyes	50 – 100
Inactivation of smell	150 – 250
Serious water accumulation in lungs	300 – 500
Fatal impact on nervous system	500 – 1000
Immediate cessation of respiration	1000 – 2000

Landfill gas:

The degradation of the organic solids removed during the pre-treatment process and disposed in a landfill, is similar to the anaerobic degradation of the organic material in other environments (Westlake, 1995). Biological activity quickly depletes any oxygen trapped in void spaces and the local environment becomes anaerobic. As a result, anaerobic microorganisms flourish and methane and carbon dioxide are the main gases produced, together with hydrogen sulphide if sulphate is present in the waste (Westlake, 1995). These gases may migrate through the landfill into the atmosphere, the surrounding soil or into groundwater, where they pose health hazards, explosion hazards and hazards associated with atmospheric pollution. Although the escape of landfill gases can be reduced by landfill covers or barriers or venting, many explosion-related incidents have been reported (Westlake, 1995; Shah, 2000). The hazards associated with methane, carbon dioxide and hydrogen sulphide are discussed above. Furthermore, landfill gases contain other toxic trace gases, even if the landfill gas is flared.

Although all of the above hazards may be present in the proximity of areas irrigated with distillery effluent, the likelihood and consequence of exposure need to be assessed in order to determine the overall risk associated with this practice.

3.4. Exposure and risk assessment:

Exposure refers to the contact between humans/the environment and a hazard and will be assessed qualitatively according to the probability and period of exposure. The risk assessment uses the data and information available to estimate the probability of negative impacts on human health or the environment from exposure to a hazard.

The risks involved in all the alternative processes to be assessed will be classified qualitatively in terms of the probability and period of exposure and the severity of human and/environmental hazards. The ratings used in the current study for each category are listed in Table 3.2. The risk value is calculated by multiplying the ratings for each category (Guild *et al.* 2001), i.e.:

RISK = SEVERITY OF HAZARD X PROBABILITY OF EXPOSURE X PERIOD OF EXPOSURE

The severity of the hazard is based on the type of impact a hazard could have on humans or the environment. The probability of exposure refers to the likelihood of humans or the environment being exposed to the hazard. Finally, the period of exposure refers to the amount of time humans or the environment may be exposed to the hazard if exposure did occur. The risk value calculated for each potential hazard posed to human health or the environment is then assessed against the risk classification table (Table 3.3) to determine whether the factor poses a high, medium or low risk. The risk classifications are very conservative, mainly due to the uncertainty of the exact impacts of the effluent on human health, with scores above 18 regarded as high risk.

Table 3.2: Ratings for the risk categories

Severity of the hazard	
1	No human health or environmental impact
2	Short term human health or environmental impact (<1 month)
3	Long term human health or environmental impact (1 month – 5 years)
4	Permanent human health or environmental impact (>5 years)
Probability of exposure to hazard	
1	Impossible
2	Possible but unlikely
3	Possible and likely
4	Definite
Period of exposure to hazard	
1	Very brief (one day per year or less)
2	Short periods of time (a few days/weeks per year)
3	Longer periods of time (a few months or more per year)
4	Continuous

Table 3.3: Risk classification table

Risk Value	Risk Classification
< 12	Low
12 – 18	Medium
> 18	High

The hazards posed by land application of wine distillery effluent were rated according to Table 3.2 and the calculated risk value was then assessed according to Table 3.3. This assessment is indicated in Table 3.4.

Table 3.4: Classification of hazards posed by the application wine distillery effluent to land

Process	Factor	Impact	Rating			Risk Value	Risk classification
			Severity	Probability	Period		
Screening and settling ponds	Effluent	Human health	3	2	2	12	Medium
		Environment	3	2	4	24	High
	Methane and carbon dioxide	Human health	3	2	4	24	High
		Environment	4	4	4	64	High
	Hydrogen sulphide	Human health	4	3	4	48	High
		Environment	1	4	4	16	Medium
	Leachate	Human health	3	2	4	24	High
		Environment	3	2	4	24	High
Landfill	Gas	Human health	4	3	1	12	Medium
		Environment	4	4	1	16	Medium
Transport (pipeline)	Effluent	Human health	3	2	2	12	Medium
		Environment	3	3	2	18	Medium
Irrigation	Effluent	Human health	3	3	3	27	High
		Environment	3	4	3	36	High
	Leachate	Human health	3	3	4	36	High
		Environment	3	3	4	36	High
	Methane and carbon dioxide	Human health	3	2	2	12	Medium
		Environment	4	3	3	36	High
	Hydrogen sulphide	Human health	4	2	2	16	Medium
		Environment	1	3	3	9	Low
	Salinity	Environment	4	4	3	36	High

Screening and settling ponds:

Throughout the process of land application, there could be a high level of exposure of both humans and other environmental organisms to the effluent. The first exposure to the effluent could occur via the open settling ponds/lagoons. These are not usually enclosed to aid evaporation and if the ponds are not suitably lined, some leaching could occur. The leachate presents a high risk to humans and the environment. However, ponds are usually built to strict regulations which include the installation of appropriate long-term lining, which decreases the risks of leaching. Since some anaerobic digestion occurs in the screening and settling ponds (Internet Reference 5; Internet Reference 13) gases such as methane, carbon dioxide and hydrogen sulphide, among others, are produced to which humans and other organisms and the environment are exposed, as filters or gas traps are not used so the gases are simply vented to the atmosphere. The volumes and concentrations of these gases are usually not recorded as a gas collection system is impractical. However, as methane, carbon dioxide and hydrogen sulphide are potentially very hazardous to the environment and humans, this is rated as a high risk process.

Landfill:

The production of solid wastes from settling ponds would be low as large volumes of distillery effluent are only produced seasonally. Furthermore, the settling ponds would probably be very large therefore only requiring infrequent emptying. The hazardous landfill gases produced due to the addition of wastes from this system to a landfill will be infrequent, thus reducing the periods of exposure and presenting a medium risk to humans and the environment (Table 3.4).

Transport (pipelines):

The plantations to be irrigated are usually situated some distance from the distillery. This then means that the effluent has to be pumped to the disposal site. In the case of Berri Estates in Southern Australia for example, the plantation site was 5km away from the lagoons (Internet Reference 5). Anaerobic conditions could occur in the pipeline if the flow rate is not regular, which would lead to the production of hydrogen sulphide that could cause corrosion. Any leaks in the pipeline would result in the soil, organisms and vegetation in the area being exposed to the raw effluent and the gases produced under anaerobic conditions. It is unlikely that humans will be exposed to the effluent from these leaks (as the pipeline is expected to be underground) unless leaching to the level of the water table occurs. If the system is well monitored, leaks in the pipeline would be detected and can be repaired within a short period of time. The transport of the effluent using pipelines is therefore a medium risk process with a rating of 12 regarding human health and 18 regarding environmental impact (Table 3.4).

Irrigation:

Before vinasse is released onto soil in the environment the soils must be carefully chosen and the irrigation systems monitored and regulated (Internet Reference 13). If not, leaching of the organic component through the soil to the level of the water table can occur (Mulidzi *et al.*, 2002). One major concern is the effect of the continued application of acidic wastewater on soil pH in the area (Internet Reference 5). The soil at the Berri Estates woodlot that was irrigated with winery wastewater was analysed at 3 depths (30cm, 60cm and 90cm) at different sample points between 1987 and 1992 (Internet Reference 5). The study concluded that the pH reduction thus far was negligible, and horticultural lime could be used if the pH falls below 6.5 (Internet Reference 5). In South Africa however, the minimum pH for land disposal is 6.0 (Table 3.6), therefore acidic effluents should not be added to land in the first place. Therefore, pH neutralization of distillery effluent before land application is necessary.

Moreover, at the point of disposal, further human and environmental exposure occurs as either flood or spray irrigation is used to disperse the effluent over the land. Humans working in the plantation are likely to be exposed to the effluent and even though the toxicity of the effluent to humans is unknown, some negative effects on health are possible (high risk rating of 27) (Table 3.4). The vegetation on the land is the target of irrigation, as the wastewater serves as a fertilizer. Soil organisms, however would also obviously be exposed to the effluent during the process. Since wine distillery effluent is only produced seasonally, irrigation and hence exposure to the effluent would only occur for a few months in the year.

However, if leaching occurs, leading to the pollution of open, useable water sources, all organisms in or around the water source (high environmental risk of 48) or users of the water source (including humans) will be exposed to the pollutants (high risk rating of 36) (Table 3.4). This exposure would also be continuous as studies have shown that the leachate tends to persist in the environment long after the irrigation season had ended (Mulidzi *et al.*, 2002).

The effluent has a high concentration of salts and salinization of the soil is likely to occur over time (Nguyen, 2003). This would affect the normal soil organisms and processes and crop growth thus the high risk rating of 36. Also, as the effluent infiltrates the soil, a degree of anaerobic digestion occurs (Mulidzi *et al.*, 2002) and again gases such as methane, carbon dioxide and hydrogen sulphide are produced. Soil organisms are exposed to these gas emissions. There is also the potential for further exposure of humans and the atmosphere to gases if the soil is disturbed, thereby

releasing the gases (Mulidzi *et al.*, 2002). Disturbances of the soil are only likely to occur for a few weeks a year during the planting season.

Generally, the direct use of raw effluent on agricultural land is not considered safe due to the high BOD and COD levels (Pathak *et al.*, 1998). It can also be concluded from the above assessment that land application of wine distillery effluent can be classified as a high risk process that is hazardous to both humans and the environment; hence a more effective method of managing wine distillery effluent is required.

3.5. Social Impacts:

The social impacts are often just as vital as the environmental impacts. In fact, these impacts can often be linked. One of the main social impacts is the foul odour produced by the anaerobic degradation of organic components of the effluent in screening, settling and evaporation ponds (Shah, 2000). This could be very unpleasant for the surrounding community. Further odours could potentially be released at the irrigation sites if the soil on the tract of land is disturbed. Moreover, the hydrogen sulphide produced under anaerobic conditions, is a component of the foul odours and can cause corrosion of concrete materials and several types of metal (Boon *et al.*, 1998; Smet *et al.*, 1998). This would cause damage to the local infrastructure, negatively affecting the community. The impacts of these gases could be mitigated by considering the relative location of the ponds, local communities and the prevailing wind direction. The release of greenhouse gases such as methane and carbon dioxide could have long-term negative social impacts due to climate changes and increased global temperatures.

Irrigation with effluent could have a positive effect on society in that it can create employment and training opportunities. The frequency of employment and number of employees needed are dependent on the type of crop grown and the size of the plantations. The potential for job creation was considered under the categories of skilled, unskilled, permanent and temporary labour. The number of potential jobs created during construction and operation of an irrigation system were classified in Table 3.5 as low (<3), medium (3 – 10) and high (>10). Opportunities also exist for capacity building in the form of training of technicians to run the daily operation and monitoring procedures of the system. Skilled contracted consultants may be required to advise the distillery on the land to be purchased, irrigation schedules, monitoring and regulation, potential hazards and relevant legislation.

Table 3.5: The number of potential jobs created during the construction and operation of an irrigation system treating wine distillery effluent (Low = <3, Medium = 3-10, High = >10)

Job category	Number of jobs	
	Construction	Operation
Skilled and permanent	Low	Low
Skilled and temporary	Low	Low
Unskilled and permanent	Low	Medium
Unskilled and temporary	High	Low – High

Another advantage of the process is the aesthetic benefit to the region in terms of the growth of trees or other plants (Internet Reference 5). This relies, however, on the success of the irrigation practices. The sustainability of the practice of irrigation with wastewater is dependent on the effective rates of uptake of water and nutrients by the crops (Internet Reference 13). If the rates of nutrient and water uptake are suitable, trees or other crops could flourish as long as the process is well monitored and regulated. This could have benefits for the local tourism industry and improve the living and working environment of the local community.

3.6. Performance Assessment:

The performance assessment module is used to assess the effectiveness of the process in terms of its ability to treat/dispose of the effluent, the process reliability and the by-products or outputs of the system. Land irrigation with wastewater assumes that the wastewater has fertilizing potential and can be used to grow crops. Therefore the process performance regarding land irrigation with wine distillery effluent would also include the effectiveness of the effluent as a fertilizer.

3.6.1. Ability of the land irrigation process to dispose of effluent:

Although land irrigation is a popular method of disposal of distillery effluent (Mulidzi *et al.*, 2002; Ramana *et al*., 2002c; Bustamante *et al*., 2005; Chrobak & Ryder, 2005; Tano *et al*., 2005), data regarding the crops fertilized with distillery effluent and the irrigation practices for South African distilleries are currently unavailable. One of the most detailed and long-term winery irrigation programmes publicized is that of the Berri Estates Winery situated in Southern Australia's Riverland region. This example illustrates that the irrigation process is capable of handling the disposal of wine distillery effluent with the aid of strict monitoring and regulation practices. The Berri Estates irrigation system is highly developed with precise monitoring and regulation schemes. They implemented their plan to irrigate a tree plantation with the winery wastewater in 1986 (Internet Reference 5). All the wastewater from the winery and distillery operations is collected

from the Estate as a single effluent stream which undergoes primary treatment consisting of screening and settling ponds before being pumped to a 40 hectare tree plantation 5km away (Internet Reference 13). They installed soil moisture probes throughout the plantation (at various depths) in 2001 in order to ascertain if any unexpected drainage occurred which could lead to salinization of the groundwater and displacement of the saline groundwater into open water sources (Internet Reference 5).

Continuous profile monitoring of the soil moisture content allowed for the following conditions (Internet Reference 13):

- Wastewater could be targeted to the depth of the plant root zone. In this manner, the vertical movement of water through the soil profile could be tracked. Also, deep drainage of the water could be detected and controlled, and leaching could be avoided.
- The rate of water and nutrient uptake by the crops could be maximized. This avoided water stress on the plants and water logging of the soil. The upper and lower irrigation thresholds could be adjusted according to the water and nutrient uptake rates during different seasons.

With the presence of the soil moisture probes, the Estate found that (Internet Reference 13):

- the plantation could be utilized more efficiently as better irrigation scheduling could be arranged; and
- the health and vigour of the trees in the plantation were maintained due to improvements in irrigation efficiency which led to optimal and predictable uptake of water and nutrients by the woodlot.

It was concluded that soil moisture probes allowed for the sustainable management of the woodlot with regard to irrigation efficiency and drainage to groundwater (Internet Reference 13). However, storage of effluent may be necessary as irrigation would not be possible during the wet season.

While this high-tech approach was possible for the distillery mentioned above, it is not always possible in other countries. In a country such as South Africa where many distilleries have few alternatives as to the type of soil on which the effluent is disposed, the area of land or even the location of the land available, this method of disposal becomes less than optimal. With few options, monitoring and regulation is often negligent, further increasing the risks of negative environmental impacts, which in turn increases the risk of financial losses, environmental damage and prosecution under the terms of the relevant legislation.

3.6.2. Reliability of the irrigation system:

The irrigation of land with wastewater is a relatively simple process, with little opportunity for mechanical problems provided the necessary skilled personnel are available. Potential problems could include corrosion of pipes, damage to pond linings and saturation of the soil. The latter could lead to contamination of groundwater. Overall, few problems could be expected, provided the system is well-monitored and regulated and the necessary preparations have been made for back-up, for example ample pond space to store effluent during the rainy season.

3.6.3. Use of products of the irrigation system:

The results by research groups using molasses distillery wastewater can be used to draw conclusions as to the potential effectiveness of wine distillery effluent as a fertilizer. Although raw distillery effluent can be used as a fertilizer, high dilution rates and pH adjustment is required for this method to be legal in South Africa (Table 1.1 and Section 3.7). The effectiveness of vinasse as a fertilizer is improved by anaerobic treatment before irrigation (Ramana *et al.*, 2002a). In order to increase the efficiency of raw distillery effluent as a nutrient source, some amount of fertilizer should be supplemented (Ramana *et al.*, 2002c). Also, the crops chosen for irrigation must be carefully chosen as the effects of distillery effluent are crop-specific (Ramana *et al.*, 2002b). Furthermore, the land for irrigation must be carefully chosen and irrigation itself has to be highly controlled (using soil monitoring probes) in order to ensure that the full irrigation potential is achieved and also to make certain that waterlogging and leaching do not occur (Internet Reference 13), as these are potentially hazardous events (Section 3.4). As mentioned before, these options are not always available for South African distilleries. Overall, even though the potential for use of vinasse as an effective fertilizer exists, South African distilleries require a more effective and beneficial method of handling their wastewater.

An important aspect of the performance assessment is the ability of the technology to meet regulatory standards. This is discussed in detail in the following section.

3.7. Regulatory Status:

The last 20 years have seen an increase in public concern for the state of the environment and protection of our natural resources which has led to the development of stricter environmental legislation. A number of key Acts are relevant to the disposal of distillery effluent, and include:

- Constitution of the Republic of South Africa (Act 108 of 1996)
- Environment Conservation Act (Act 73 of 1989)

- National Environmental Management Act (Act 107 of 1998), and
- National Water Act (Act 36 of 1998).

Constitution of the Republic of South Africa (Act 108 of 1996):

Chapter 2, Section 24 of the Bill of Rights in the Constitution of the Republic of South Africa contains the protection of environmental rights. It states that *"everyone has the right – (a) to an environment that is not harmful to their health or well-being; and (b) to have the environment protected, for the benefit of present and future generations, through reasonable legislative and other measures that i) prevent pollution and ecological degradation; ii) promote conservation; and iii) secure ecologically sustainable development and use of natural resources while promoting justifiable economic and social development"*.

Clearly, it is the duty of every citizen (or institution) to ensure that he or she conducts any actions in an environmentally responsible manner (Hayward *et al.*, 2000). Legislation and other measures should be in place to prevent pollution, destruction of the environment and to promote conservation. This section of the Bill of Rights also introduces the First World trend of sustainable development. Distilleries are therefore legally responsible for the management of the potential pollutants they create. As irrigation with distillery effluent could pose high risks to humans and the environment, distilleries may face liability in terms of Section 24 of this Act.

Environment Conservation Act (Act 73 of 1989):

Although this Act is now mostly outdated it is still valid in many areas and is indicative of the times and the past view on environmental protection and legislation. Little attention was given to the origin of the offensive material, neither was there any regard for the ability of organizations to avoid pollution. It focused simply on the polluting action only (Hayward *et al.*, 2000). Law enforcement consisted of warning or prosecuting organizations or people that were caught violating the requirements of the law. This type of approach led to typical end-of-pipe treatment plans, where the environment was only considered when the organization had to address the pollution or had to structure an end-of-pipe treatment plant in order to avoid contravention of the legislation (Hayward *et al.*, 2000). The environment was not considered when production processes were designed; it was only considered when a decision had to be made regarding the pollution produced. Agricultural enterprises and wine cellars (which include distilleries) are listed in terms of Article 21 of this Act as practices that may have a negative impact on the environment (van Schoor, 2001) and therefore an effective effluent management programme must be instated. Furthermore, this Act still covers Environmental Impact Assessment (EIA), meaning that any distillery wanting to irrigate its effluent

would be subject to assessment. In light of the potentially long-term negative impacts of this disposal method on humans and the environment, irrigation of distillery effluent is unlikely to be allowed on completion of an EIA.

National Environmental Management Act (Act 107 of 1998):

This Act has impacted significantly on the legal liability and accountability of industry with regard to environmental matters (Hayward *et al.*, 2000). The general First World trend which incorporates the concept of sustainable development whilst aiming to maintain, or even improve, the natural, biological and social environment is upheld in South Africa by the National Environmental Management Act (NEMA) (Hayward *et al.*, 2000). The Act clearly defines pollution as *"any change in the environment caused by disturbances, including substances, radioactive or other waves, and noise or odours, which have an adverse effect on humans or the ecosystem"*. This comprehensive definition means that industries can now categorize their activities more easily, and the government can more easily identify industries or companies that do not conform to the legal limits and enforce the law (Hayward *et al.*, 2000).

Section 28 of the Act clearly states that every person must take reasonable measures to prevent pollution or degradation from occurring, recurring or continuing. If the polluting activity is unavoidable, it must at least be minimized and its effects rectified (Hayward *et al.*, 2000). The Act imposes an obligation on a person defined as *"an owner of land or premises, a person in control of land or premises or a person who has the right to use the land or premise on which or in which – (a) any activity or process is or was performed or undertaken; or (b) any other situation exists, which causes, has caused or is likely to cause significant pollution or degradation of the environment"* to take reasonable measures to minimize or repair the effects of the polluting activity. The law introduces a shift in the paradigm from a reactive policing system to a pro-active management system (Hayward *et al.*, 2000). According to the Act, companies are obliged to employ the best practicable environmental management and control systems in all their activities, including waste management (Hayward *et al.*, 2000). As irrigation of distillery effluent poses considerable pollution risk to the environment, this system is unacceptable in terms of NEMA. Furthermore, distilleries using this disposal method may be liable for costly remediation of contaminated water (surface and sub-surface) and soil.

National Water Act (Act 36 of 1998):

This Act was implemented in 1999, and it introduced several new concepts (Hayward *et al.*, 2000). It is linked to NEMA, but whereas NEMA includes all natural resources, the National Water Act

deals specifically with the use and pollution of water resources. This Act is vital to South Africa since water is a scarce resource and it is not evenly distributed throughout the country.

A new concept introduced by the Act is that the aquatic ecology forms part of the water resource and should be protected as such (Hayward *et al.*, 2000). A reserve is defined as *"the quantity and quality of water required -...(b) to protect aquatic ecosystems in order to secure ecologically sustainable development and use of the relevant water resource"*. The reserve is the classified according to its ecological sensitivity which determines the minimum water quantity and quality that will have to be maintained (Hayward *et al.*, 2000). The classification of the reserve has to be considered when granting licenses for water extraction or effluent discharge (Hayward *et al.*, 2000). Therefore distilleries that require dilution water for their processes, including effluent disposal or treatment, are affected as the right to water requires a licence that can be revoked if the water abstraction source is not properly maintained or if a more beneficial socio-economic option is presented.

Section 19 of the Act deals with pollution prevention, especially where pollution of a water resource has occurred, is occurring or may occur due to activities on land. It clearly states *"the person who owns, controls, occupies or uses the land in question is responsible for taking measures to prevent pollution of water resources"*. The Catchment Management Agency or Department concerned may itself take measures to prevent the pollution or to remedy its effects if action is not taken by the responsible party. If this occurs, the agency can then recover all reasonable costs from the person/s responsible for the pollution.

Distilleries are often unaware that the practice of irrigation with wastewater is controlled under the National Water Act (Hayward & Trerise, 2000). Table 3.6 shows the standards applicable to the irrigation of wastewater such as wine distillery effluent as set out by the General Authorizations in terms of the National Water Act (Act 36 of 1998) (Hayward & Trerise, 2000).

Table 3.6: Standards Applicable to Irrigation of Wine Distillery Effluent (as per General Authorizations in terms of the National Water Act) (Hayward & Trerise, 2000)

Parameter/Substance	<500m^3/day to be irrigated	<50m^3/day to be irrigated
Electrical Conductivity (EC)	<200mS/m	<200mS/m
COD	400mg/l	5000mg/l
pH	6.0-9.0	6.0-9.0
Faecal coliforms	100 000 per 100ml	100 000 per 100ml
Sodium Adsorption Ratio (NAR)	<5	<5

These General Authorizations (Government Notice No. 1191) were published in the Government Gazette (20526) in 1999 in terms of the National Water Act, and it places the following requirements with regard to irrigation with wastewater (including distillery effluent) (van Schoor, 2001):

- One is required to register as a user if on any given day, that person wants to irrigate >10m^3 of water originating from distillery operations (van Schoor, 2001). Once registered, up to 500m^3 of wastewater may be irrigated per day (for crop production, including grazing) provided that the effluent meets the standards set out in Table 3.6.
- If the COD of the effluent is between 400-5000mg/l, irrigation after registration but without a licence is allowed, but irrigation may be up to 50m^3 only on any given day (van Schoor, 2001). Registration as a user and a permit/licence is required for irrigation of more than 50m^3 of this same effluent per day.
- The registered user is only allowed to irrigate land above the 100 year floodline or further than 100m from the edge of any water source or borehole that is used for drinking water or is an animal water hole (van Schoor, 2001). Furthermore, no surface or groundwater may be contaminated by irrigation (van Schoor, 2001).
- It is also obligatory for the registered user to measure the quantity of effluent irrigated on a weekly basis, and to measure the quality of the effluent on a monthly basis and at the point just prior to irrigation (van Schoor, 2001). Written records of these measurements must be made and kept for inspection by the relevant authority (van Schoor, 2001).
- The user is also required to demarcate the area of irrigation on a 1:50 000 topographic map, and provide a copy to the responsible authority (van Schoor, 2001). Details of the crops receiving the wastewater irrigation, the irrigation techniques used and details of emergency procedures must also be provided to the responsible authority (van Schoor, 2001).
- Measures should be taken to ensure that the following occurrences are prevented at all times (van Schoor, 2001):
    - waterlogging or damaging of the soil;

  - occurrence of flies and mosquitoes;
  - occurrence of bad odours;
  - occurrence of secondary pollution;
  - penetration (by the effluent) of any surface resources; or
  - unauthorized use of water by members of the public.
- Lastly, any stormwater (rain water) run-off originating from the irrigation area must be collected to prevent contamination of pure water sources (van Schoor, 2001).

Current legislation and public views dictate responsibility and liability concerning the disposal of pollutive wastes. Wine distillery effluent is definitely a pollutive waste as it can cause various negative environmental impacts if released irresponsibly and the raw effluent (Table 1.1) does not meet the South African minimum legal requirements for land irrigation. High dilution and pH neutralization of distillery effluent is required to reach legal levels for irrigation. The distillery itself is responsible for its wastes and therefore requires a method of treatment or disposal that is suitable for the environment, meets the legal requirements and is economical and feasible.

3.8. Market size and information:

If the irrigation process can be capably monitored and regulated, some benefits could arise from this method of disposal. The international market welcomes products whose processes result in or encourage environmental conservation or rehabilitation and therefore the use of distillery wastewater as fertilizer for the growth of grass, trees or any indigenous vegetation could result in a positive market response. However, if not properly monitored and controlled, there are a number of significant negative impacts on both human health and the environment which could lower the company image.

The use of the distillery wastewater for irrigation of vines could lead to a self-sustaining process between the vineyard and distillery. Aside from climate, the contents of the soil and irrigation water contribute tremendously to the flavours present in the wines and eventually the brandies resulting from the grape harvest. Also, the high salt content of the effluent would increase soil salinity. Most wineries would therefore, presumably, not welcome the idea of irrigating their vineyards with wastewater of a very seasonal composition.

Although irrigation is currently the most popular method of disposal of distillery effluent, current trends of responsible waste management and sustainability together with stricter legislation indicate

that this process will soon become unacceptable due to the high risks posed to human health and the environment and the potential for an ongoing pollution hazard far removed from the point of disposal.

3.9. Financial considerations:

A deciding factor in the choice of technology that is used is the cost involved and the potential for return on costs. The financial considerations module identifies the cost requirements involved in a process and the possible return on costs.

3.9.1. Design and Construction:

One of the major costs involved in irrigation of wastewater would be the acquisition of appropriate and sufficient land. The land must be carefully chosen according to the soils present, depth to the water table, distance from water sources and distance from the site of effluent production i.e. the distillery. As effective irrigation requires flat land (Nguyen, 2003), level land must be purchased or the distillery will have to bear the costs of levelling.

Nguyen (2003) estimated that the capital costs required for the construction of a spray irrigation system for centrifuged effluent from an ethanol-from-starch waste plant on 730ha of land assumed to be adjacent to the distillery, would be 2 million Australian dollars. This estimate was based on a hypothetical 50Ml/year plant producing centrifuged effluent at a rate of 2.9Ml/day for 345 days per year (Nguyen, 2003). The costs of implementing an irrigation system can be further reduced if less energy-intensive methods of irrigation such as flood/channel irrigation, which use gravity flow, are used.

Other capital costs aside from construction of the irrigation system include equipment such as sieves and/or filters that are needed for the screening ponds to prevent gross solids entering the settling ponds. Settling ponds also have to be constructed if these are not already available. If available, the current evaporative lagoons could be used, but one has to ensure they are suitable. Storage ponds are also required as irrigation is not possible during wet weather periods (Nguyen, 2003).

Furthermore, pipelines have to be constructed from the settling/storage ponds to the irrigation site and pumps have to be installed or costs are incurred for overland transport of the effluent in trucks

or tankers. Either transportation method can prove expensive if the plantation is located some distance away from the distillery.

Soil monitoring probes are also essential for responsible irrigation and regulation. Sufficient probes will have to purchased and installed throughout the plantation to give a representative view of the entire irrigation site. If the system is to be fully automated, costs are incurred in the purchase and installation of the system, and in the energy costs from its operation.

3.9.2. Operation:

Nguyen (2003) estimated the overall annual cost of the hypothetical plant to be A$ 440 000, which included operating costs such as chemicals, labour and power, and the capital charge (A$ 300 000) (Nguyen, 2003). The generation of wine distillery effluent however, is seasonal; therefore the system would only be fully operational for a few months per year. This implies that the annual operating costs should be lower. In South Africa, water costs would also be incurred as dilution of the distillery effluent is necessary in order to meet the legal requirements for irrigation with wastewater.

The operating costs would include the costs for the energy required to pump the effluent to the plantation or fuel costs for overland transport. Also, a suitable crop should be chosen and the seeds/seedlings purchased and planted. The seedlings have to be especially well cared for during the establishment period, and this could involve costs such as water and fertilizer.

3.9.3. Personnel:

Employees will have to be trained to use the irrigation, regulation and monitoring systems. The personnel costs would include the wages and benefits such as medical aid for the permanent staff required. There would be additional labour costs incurred as extra workers would be required during the planting and harvesting periods.

3.9.4. By-products and waste:

The harvest and sale of valuable crops could provide some return on costs. The value recovered from crop sales would be dependent on the value of the crop and the harvest size.

Once the settling ponds are full, they are usually emptied using either a front-end loader to remove sludge or the contents are pumped out as a slurry. The frequency of solids removal depends on the size of the ponds, volumes of effluent and the settleable solids content of the effluent. The general

practice of distilleries for solids and sludge disposal is the deposition in a landfill (Internet Reference 5). This practice, together with the transport of the solids to a suitable landfill can be costly.

As is evident from the estimates above by Nguyen (2003), although land irrigation seems a simple process, it can be very expensive with little, if any, return on the investment. The estimates given for the capital costs are only for construction of the irrigation system and did not include any pre-treatment costs. Furthermore, the cost comparison study concluded that although irrigation is the current and popular option for disposal of distillery effluent, alternatives should be considered as salt and nutrient build-up does occur (Nguyen, 2003). This would imply that more land would have to be purchased when the soil reached a saturation point or if the distillery expanded its operation.

South African wineries and distilleries are almost all situated in the Western Cape region, where the land and climate are optimal for vine growth. The biggest problem in South Africa would therefore probably be that suitable and sufficient land near a distillery would most probably be unavailable or very expensive as there are more profitable means of using the land, i.e. vine growth. Therefore, higher costs for the construction of a pipeline or some other method of transport will be incurred to move the effluent from the point of generation to the disposal point. Therefore distilleries in South Africa would find a small, on-site treatment plant more suited to their needs.

3.10. Energy Impacts:

The amount of energy that will be used, if any, is dependent on the choices made by the distillery regarding how the irrigation system would work. If gravity is used to distribute the effluent on the land, there will be very little, if any, energy use. However, if the effluent has to be actively pumped to the plantation and onto the land, the energy impacts will be greater during the production season than during the off-season. If the effluent is transported to the irrigation site using trucks or other transport, there will be energy impacts in the form of fuel use. A fully automated system would require electricity or fuel for generators if electricity cannot be supplied or if the electricity supply is unreliable. Once the settling ponds are full, the slurry or sludge will have to be removed by pump or front-end loader and then transported to a disposal site. Utilizing either method would have an energy impact in the form of electricity or fuel use.

3.11. Resource Conservation:

The production of effluent is constant even if the volumes are seasonal, therefore even if the irrigation schedule is highly efficient, more land will be needed as the distillery increases its operations to meet market demands. There could also be destruction of natural resources if there is a need for the removal of indigenous vegetation for land. If the pollutants reach water sources, it could result in the destruction of that aquatic ecosystem with far-reaching consequences. The irrigation method may be of benefit to the natural resources in an area by facilitating growth of natural vegetation.

3.12. Conclusions:

Land irrigation of raw wine distillery effluent is illegal in South Africa as the vinasse does not meet the legal requirements for disposal. Pre-treatment such as dilution, pH adjustment and solids removal are required before disposal on land is possible. Furthermore, although raw distillery effluent does have fertilizing potential, it is not as effective as treated effluent or conventional fertilizers. In addition, the effects of distillery effluent are crop-specific therefore research is necessary before a suitable crop can be chosen.

The process also poses some hazards as studies show that the effluent can leach through the soil, reaching the level of the water table. This could result in far-reaching effects on the environment and human health as the leachate may be transported to open water sources. Furthermore, potential hazards exist in the production and release of methane and hydrogen sulphide from screening and settling ponds. The risk assessment method used for this study classified the irrigation of wine distillery effluent as a high risk process.

Although this is the simplest method for vinasse disposal, it can be very expensive with little, if any, return on the investment. Extensive monitoring and precise regulation is necessary in order to reduce the risks of leaching of the effluent. The system can benefit society in that it creates some permanent jobs; however most labourers would only be required seasonally. In addition, the process cannot be sustained in a country like South Africa where distilleries are situated in the highly specific wine regions, where land is likely to be either unavailable or very expensive.

CHAPTER IV: INVESTIGATION OF ANAEROBIC TREATMENT OF WINE DISTILLERY EFFLUENT

4.1. Introduction:

Anaerobic digestion is the degradation of biodegradable matter under anaerobic conditions by a consortium of anaerobic microorganisms and has, over the years, proved to be an attractive option for the treatment of industrial wastewaters (Vijayaraghavan & Ramanujam, 2000; Uzal *et al.*, 2003).

The development of high-rate anaerobic reactors has increased the use of anaerobic processes, especially for the treatment of high-strength wastewaters (Akunna & Clark, 2000). These high-rate anaerobic reactors are advantageous as they achieve separation between hydraulic retention time (HRT) and solids retention time (SRT) (Shivayogimath & Ramanujam, 1999; Akunna & Clark, 2000). This separation prevents washout of the relatively slow-growing anaerobic microorganisms and allows them to remain within the reactor independently of the wastewater flow (Akunna & Clark, 2000). It also allows for the application of higher volumetric loading rates and significantly enhances the treatment efficiency of the process (Shivayogimath & Ramanujam, 1999; Akunna & Clark, 2000).

There are various different types of reactors that can be employed, such as the upflow anaerobic sludge blanket (UASB), fluidized bed, expanded bed, anaerobic baffled reactors (ABR), anaerobic filters (AF) and fixed film reactors. Despite the range of configurations available, the UASB reactor is the most popular high-rate anaerobic reactor configuration in use today for the treatment of high-strength industrial wastewaters including distillery effluents (e.g. Cheng *et al.*, 1990; Laubscher *et al.*, 2001; Sharma & Singh, 2001; Uzal *et al.*, 2003). For this reason, the ETSA of anaerobic digestion for the treatment of distillery effluent will be limited to this technology option.

Anaerobic systems are advantageous over aerobic systems as they produce energy in the form of a combustible biogas, while requiring reduced energy input as no aeration is required. Furthermore, they have low sludge production, they achieve high COD removal efficiencies at high loading rates and they have lower volume requirements therefore they require less floor area (Vlissidis & Zouboulis, 1993; Wentzel *et al.*, 1994; Vandevivere & Verstraete, 2001). In addition to the

advantages over aerobic systems, Wentzel *et al.* (1994) suggest that UASB processes offer further advantages over conventional anaerobic systems as:

- UASB systems can withstand far higher loading rates than those for mixed anaerobic systems which implies that much smaller reactor volumes are required when treating a waste stream using a UASB system;
- the UASB is the only anaerobic system that can effectively remove significant concentrations of nitrogen;
- UASB systems do not require separate gravity sedimentation tanks; and
- UASB systems do not require artificial mixing of the contents, which is both expensive and difficult to achieve in completely mixed anaerobic systems.

In spite of the above, anaerobic digestion can be a sensitive process that requires some skill and understanding of the process and its parameters.

4.2. Process Description:

A typical process flow for wine distillery effluent treated with a UASB system is illustrated in Figure 4.1. Since the effluent leaves the distillery at very high temperatures of 70ºC-80ºC for wine distillery effluent (Wolmarans & de Villiers, 2002), it would first have to be collected and cooled (probably by passing through a cooling tower) to approximately 30ºC-37ºC for optimal degradation in mesophilic reactors (Goodwin & Stuart, 1994; Wolmarans & De Villiers, 2002) or to around 51±3ºC for thermophilic reactors (Vlissidis & Zouboulis, 1993). Pre-treatment such as centrifugation would also be required to remove suspended solids from wine distillery wastewater prior to the UASB treatment (Laubscher *et al.*, 2001; Wolmarans & de Villiers, 2002). A conditioning step is required before anaerobic digestion whereby the pH of the effluent is increased from approximately 3.5-4 to > 6.6 which is optimal for the growth of the methanogenic bacteria required for degradation. During start-up, this increase in pH is usually via the addition of sufficient sodium hydroxide or an equivalent alkaline solution. As anaerobic digestion results in an increase in alkalinity of the wastewater being treated (Moosbrugger *et al.*, 1993a, 1993b; Wolmarans & De Villiers, 2002), the treated effluent can then be recycled into the conditioning tank in order to correct the pH of the untreated effluent. However, sodium hydroxide can still be added at any stage if recirculation is insufficient (Sam-Soon *et al.*, 1987; Moosbrugger *et al.*, 1993a, 1993b; Wentzel *et al.*, 1994; Wolmarans & De Villiers, 2002).

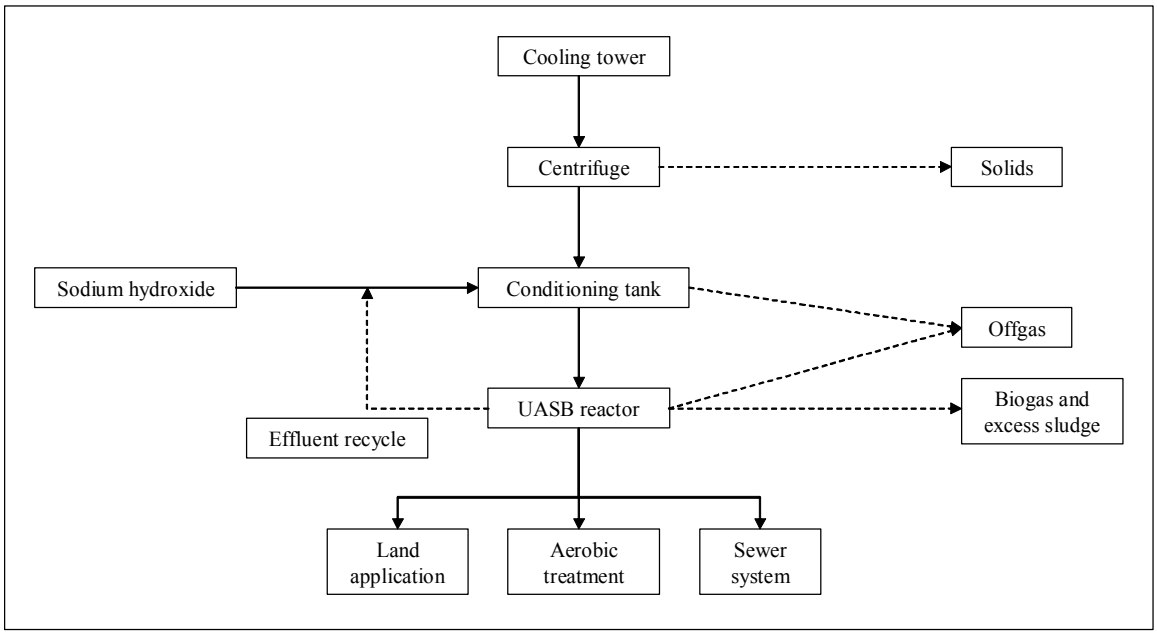

Figure 4.1: Process flow diagram of a typical UASB system for the treatment of wine distillery effluent (modified from Wolmarans & de Villiers, 2002)

The development of the UASB reactor has been a major breakthrough in anaerobic technology in the last decade (Verstraete *et al*., 1997) and several hundred of these reactors are in use worldwide (Akunna & Clark, 2000). UASB reactors require wastewater to be pumped upward at a rate between 0.5 and 1.5 metres per hour through the reactor under strictly anaerobic conditions (Verstraete *et al*., 1997). Anaerobic microbes within the reactor grow in the form of granules or conglomerates varying in size between 0.5-5mm in diameter (Verstraete *et al*., 1997). The biocatalytic activities of the granules degrade the organic matter in the wastewater rapidly and almost completely to biogas. The success of UASB reactors is highly dependent on the ability of the anaerobic microorganisms to form and maintain active, settleable granules (Lettinga *et al.*, 1984; Fang *et al.*, 1994; Akunna & Clark, 2000). The granules consist of anaerobic prokaryotes which have self-immobilized into compact forms (Akunna & Clark, 2000). This granulation enhances the settleability of the biomass, which in turn leads to better retention of the biomass within the reactor (Yan & Tay, 1997). Sam-Soon *et al.* (1987) suggested that 4 conditions were necessary for the development of settleable, active granules:

- the development of a high hydrogen partial pressure (pH_2) environment;
- a limited cysteine source, either from the feed or the action of organisms such as cell death;
- an excess supply of nitrogen in the form of free and saline ammonia; and
- a near neutral substrate (feed) pH.

The influent loading of a typical UASB reactor varies from 10 to 20kg COD per cubic metre reactor volume per day (Verstraete *et al*., 1997). Although the UASB reactor is highly successful in terms

of COD removal, an aerobic process should follow the anaerobic treatment of highly concentrated wastewaters for optimum treatment. An aerobic process following anaerobic digestion will, however, be smaller and will consume less energy than one that receives untreated wastewaters (Verstraete *et al.*, 1997).

A schematic diagram of a typical UASB reactor is shown in Figure 4.2. The influent is fed through the bottom of the reactor, where it infiltrates the granular sludge bed. The feed distribution system influences the liquid upflow velocity (Hobson & Wheatley, 1993) and the feed system can also be used to improve the contact between the sludge and wastewater. The shape of the reactor, the liquid upflow velocity and a phase separator such as a filter or baffles is used to promote gas-solid-liquid separation. A typical UASB plant consists of a biological reaction zone situated below a sedimentation zone (Hickey *et al.*, 1991). As the effluent moves up through the active sludge blanket it passes through the biological zone, where the pH-conditioned wastewater is converted to methane and carbon dioxide by the anaerobic organisms (Hickey *et al.*, 1991).

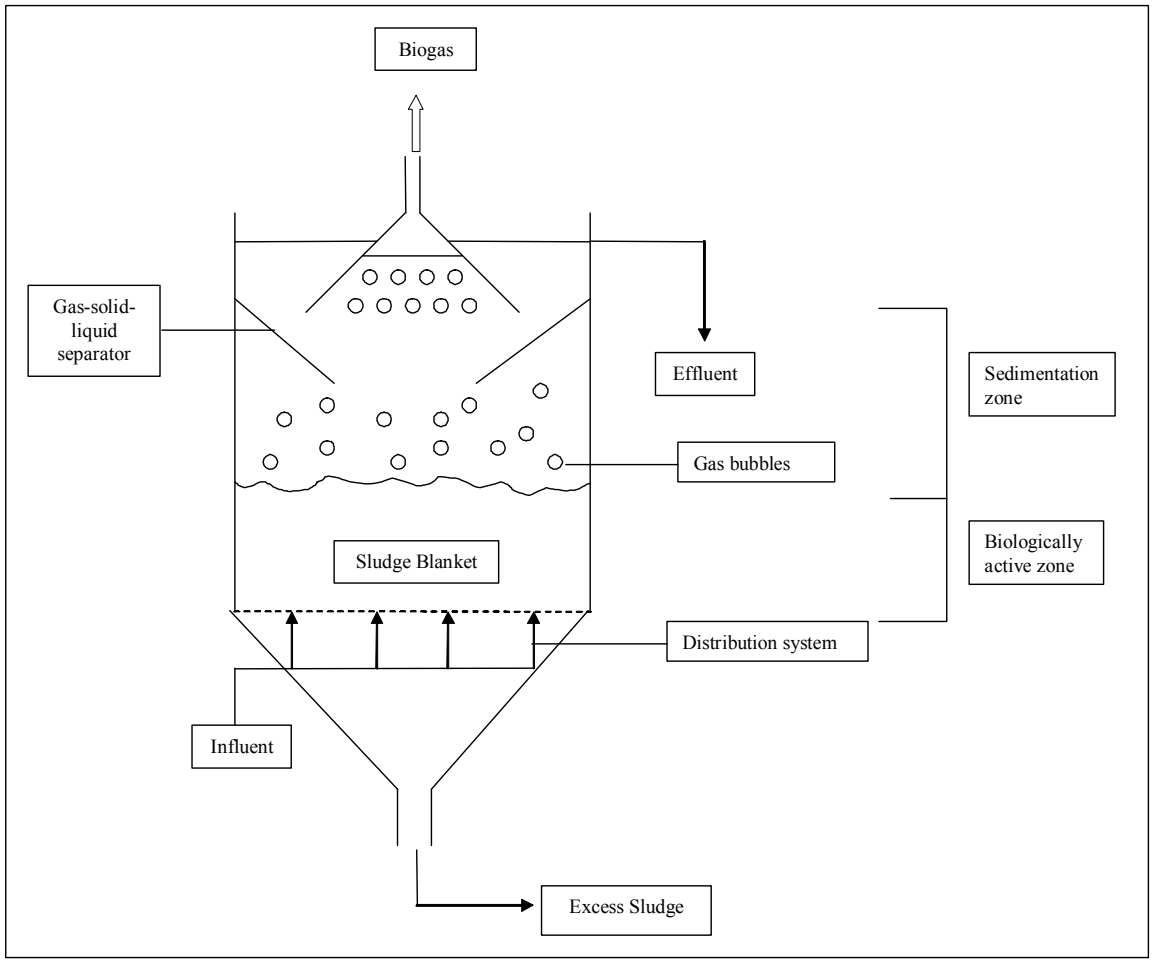

Figure 4.2: A schematic diagram of a typical Upflow Anaerobic Sludge Blanket (UASB) reactor (adapted from Goodwin & Stuart, 1994)

In the sedimentation zone, a gas-solid-liquid phase separator situated at the top of the reactor separates the biogas produced, the sludge lifted by the gas bubbles and the reactor effluent from each other (Hickey *et al.*, 1991). The separator is a key factor in ensuring the retention and accumulation of biomass within the reactor. The separated granular sludge sinks back to the sludge bed by gravity while the gas and treated effluent pass out of the reactor.

The liquid overflow (reactor effluent) is collected and as anaerobic treatment alone cannot satisfactorily treat vinasse to meet standards for direct disposal into water sources (Vlissidis & Zouboulis, 1993), a suitable disposal method is required. Anaerobically treated effluent is usually pumped to the municipal wastewater treatment plant (sewer) for further treatment (Wolmarans & De Villiers, 2002). However, it could also be applied to land (Ramana *et al.*, 2002a; Ramana *et al.*, 2002b; Ramana *et al.*, 2002c) or undergo an aerobic polishing step (Vlissidis & Zouboulis, 1993).

The biogas produced during the process is either collected to be used as an energy source (Lata *et al.*, 2002) or it is flared after passing through a foam tank to remove any residual solids (Wolmarans & De Villiers, 2002). Offgas is also produced in conditioning tanks and other stages and this is usually collected and treated through a biofilter before being vented to the atmosphere (Wolmarans & De Villiers, 2002).

Excess biomass (digested sludge) is removed from the bottom of the reactor and, together with the solids resulting from centrifugation of untreated effluent, is disposed appropriately. Since sludge production in anaerobic systems is very low (typically 0.1kg sludge produced per kg BOD) (Vandevivere & Verstraete, 2001) and the reactor is only active for a few months a year, the quantity of odorous, black/grey sludge requiring removal should be low. The main methods for sludge disposal are landfill, land application and incineration. This sludge should have a low volatile solids content and is thus less likely to putrefy in storage and cause odour problems. However, due to the lower content of volatiles, the calorific value of the sludge is reduced which is of major significance if the sludge is intended for use in energy production such as gasification of thermally dried sludge. The suspended solids removed by centrifugation should however, have a relatively high calorific value, as the volatile fraction of suspended solids from distillery wastewater ranges from 89.5 to 94.6% (Basu, 1975).

Thickening and dewatering of the sludge is necessary prior to disposal in order to reduce the moisture content and hence sludge volume. This process is vital in order to reduce the amount of waste for disposal and the transport costs required to convey the solid waste to a disposal site. One

of the main problems that hinder thickening of digested sludge is that the fresh sludge continues to digest during storage and the thickening process, hence generating considerable carbon dioxide and methane. This digestion can continue for weeks and gas formation gives rise to gas bubbles that disturb the sludge and prevent consolidation. If sulphate and sulphate reducing prokaryotes (SRPs) are present, H_2S can be formed. The hazards associated with the release of methane, carbon dioxide and hydrogen sulphide are discussed in Section 3.3.

Conditioning of sludge prior to dewatering involves chemical or physical treatment in order to enhance water removal and improve solids capture. The choice of conditioning method is vital for economical sludge treatment as conditioning contributes significantly to treatment costs (often exceeding 50% of total cost) (US EPA, 1987) due to the high unit cost and the large quantities of chemicals required. As a centrifugation system would already be employed by the process, it is assumed that this method of dewatering would be optimal. Distilleries generally dispose of these solid wastes in a landfill (Internet Reference 5). The hazards associated with anaerobic digestion and sludge disposal in a landfill will be discussed in the following section.

4.3. Hazard identification and characterization:

A number of hazards to human health and the environment have been associated with the treatment of distillery effluent using an anaerobic system. These are described below and their significance assessed in Section 4.4.

Raw effluent:

The potential hazards that the raw effluent poses to humans and the environment are discussed in Section 3.3.

Alkaline chemicals:

Alkali will have to be stored on-site for the pH conditioning of the effluent before anaerobic treatment, although the amount of alkali needed can be minimized by effluent recycling (Wentzel *et al.*, 1994). Alkaline chemicals such as sodium hydroxide and sodium carbonate can cause chemical burns and irritation on contact with the skin and eyes, burns and irritation to the digestive tract if ingested or burns and irritation to the respiratory tract if inhaled (Internet Reference 14; Internet Internet Reference 15). These chemicals are corrosive and prolonged or repeated contact with the skin can cause dermatitis (Internet Reference 14) and sensitization (Internet Internet Reference 15). Some other effects of human exposure include chemical conjunctivitis, corneal destruction,

coughing and difficulty breathing (Internet Reference 14; Internet Internet Reference 15) and perforation of the digestive tract on ingestion causing pain, nausea, vomiting diarrhoea and shock (Internet Reference 14). Appropriate protective gear including gloves and safety glasses should always be worn when handling these chemicals.

Methane (CH_4) and carbon dioxide (CO_2):

The anaerobic process generates biogas of which a large proportion is methane, if the system is functioning well. The risks associated with methane production are discussed in Section 3.3. The anaerobic process also produces carbon dioxide and flaring of biogas results in further production and release of carbon dioxide into the atmosphere. The hazards associated with this gas are discussed in Section 3.3. These gases are also produced as offgases during the conditioning step.

Hydrogen sulphide (H_2S):

SRPs compete with other anaerobic microorganisms in an anaerobic environment. If high concentrations of sulphate are present, large amounts of hydrogen sulphide may be produced during anaerobic digestion and the conditioning step. The risks associated with hydrogen sulphide production are discussed in Section 3.3.

Solids and excess sludge:

As discussed in Section 4.2, continued digestion of sludge and organic solids occurs during thickening processes resulting in methane production. If sulphate is present, hydrogen sulphide can also be generated by SRPs. Furthermore, the disposal of sludge and solids in landfills increases the organic component in the landfill. The risks associated with methane and hydrogen sulphide production and sludge disposal in a landfill are discussed in Section 3.3.

4.4. Exposure and Risk Assessment:

Table 4.1 rates the hazards associated with anaerobic digestion using the ratings table (Table 3.2). The risk value was calculated and the risk classified as low medium or high according to the classification table (Table 3.3).

Raw effluent:

This system would be a closed process, thus exposure of humans and the environment to the raw effluent would be minimal, unless mishaps such as a pipe burst occurred. If such incidents occur,

they should be repaired quickly, thereby reducing the period of exposure. Overall the raw effluent posed a medium risk to humans and the environment (risk ratings of 12) (Table 4.1).

Conditioning:

Alkali chemicals are usually only necessary during the initial start-up of a UASB system, as effluent recycling can be used to adjust the pH once the system is stable. This reduces the amount of alkali chemicals stored on-site and therefore the period of possible exposure. Although the severity of exposure is high, the probability of human or environmental exposure to such chemicals is unlikely as all chemicals should be stored and handled in a manner that minimizes accidental exposure (medium risk rating of 16) (Table 4.1). Those employees handling the material would be supplied with and trained to use the necessary personal protective equipment.

Offgases such as hydrogen sulphide and methane are produced in the conditioning tanks and other areas such as the weirs in a UASB reactor. Although the concentrations of the offgas is unknown, it is simply collected and vented to the atmosphere (Wolmarans & de Villiers, 2002), thereby exposing both humans and other organisms in the environment to these potentially hazardous gases.

UASB:

The anaerobic process produces methane and carbon dioxide and this biogas is collected and either flared (Wolmarans & de Villiers, 2002) or used as an energy source (Lata *et al.*, 2002). Flaring of biogas converts methane to carbon dioxide and both gases are greenhouse gases, which could result in long-term environmental risk. Furthermore, methane is highly volatile, therefore if released, there is a potential for greater risks to human health and safety to be incurred due to the explosion risk.

Hydrogen sulphide, methane and carbon dioxide are also produced during the conditioning of digested sludge and solids, however the low sludge production rates by anaerobic digestion and the production of solid wastes from pre-treatment would be further reduced as the effluent is seasonally produced hence the plant would only be fully operational for short periods during the year. Therefore the disposal of digested sludge and solids into a landfill and the subsequent exposure to the gases produced due to the addition of the wastes from this system to the landfill would be reduced.

Table 4.1: Assessment of risk associated with anaerobic digestion of wine distillery effluent

Process	Factor	Impact	Rating			Risk Value	Risk classification
			Severity	Probability	Period		
Raw effluent		Human health	3	2	2	12	Medium
		Environment	3	2	2	12	Medium
Conditioning	Alkali storage	Human health	4	2	2	16	Medium
		Environment	3	2	2	16	Medium
	Hydrogen sulphide	Human health	4	4	3	48	High
		Environment	1	4	3	12	Medium
	Methane and carbon dioxide	Human health	3	4	3	36	High
		Environment	4	4	3	48	High
UASB	Hydrogen sulphide	Human health	4	2	1	8	Low
		Environment	1	2	1	2	Low
	Methane and carbon dioxide	Human health	3	2	2	12	Medium
		Environment	4	2	2	16	Medium
Digested sludge and solids conditioning	Hydrogen sulphide	Human health	4	3	1	12	Medium
		Environment	1	3	1	3	Low
	Methane and carbon dioxide	Human health	3	3	1	9	Low
		Environment	4	3	1	12	Medium
Landfill	Gas	Human health	4	3	2	24	High
		Environment	4	3	2	24	High

The impacts of the treated effluent are dependent on the post-anaerobic treatment step employed. The reactor effluent is handled in one of three ways:

- Land application/irrigation (Ramana *et al.*, 2002a; Ramana *et al.*, 2002b; Ramana *et al.*, 2002c) – the risks associated with this process could be reduced from a high (Section 3.4) to a medium risk process due a reduction in the organic load of the effluent before irrigation.
- Aerobic treatment (Vlissidis & Zouboulis, 1993) – If this is used as a polishing step, the risks encountered would depend on the type of aerobic treatment undertaken. The final effluent could only be discharged into an appropriate water source if it met the legal requirements, which implies minimum impact severity. However, this would also result in high levels of exposure of aquatic organisms (and humans) to the treated effluent during the production season. A severity rating of 2, probability rating of 3 and period of exposure rating of 3 gives a risk value of 18 (Tables 3.2). The risk posed to humans and the environment by anaerobic then aerobically treated effluent is thus classified as a medium risk (Table 3.3).

- Disposal into municipal wastewater treatment plants – this would also be classified as above (medium) as the effluent would be treated at the municipal plant then disposed into a water source.

4.5. Social Impacts:

The production of odorous gases such as H_2S, would negatively impact on the surrounding community. The location of the plant in such cases should be considered, as it would be preferable if the local community did not lie in the path of the prevailing winds. The production of greenhouse gases could have long-term social impacts as global warming could cause severe climate changes, droughts, severe storms, changes in rainfall pattern and rising sea levels. This implies many negative social impacts including food shortages, forced abandonment of coastal towns and cities and potential loss of livelihoods.

This process could have some positive social impacts in the form of job creation. This aspect will be assessed according to the method described in Section 3.5. Table 4.2 shows the number of potential jobs created during the construction and operation of an anaerobic facility.

Skilled professionals are required during the design and construction of the anaerobic system. Moreover, as the anaerobic process can be very unstable, highly skilled, permanent workers are needed for the daily operation and monitoring of the system. Some permanent jobs could be available for unskilled labour for sludge removal and general plant maintenance.

Table 4.2: The number of potential jobs created during the construction and operation of an anaerobic treatment system for wine distillery effluent (Low= <3, Medium= 3–10, High= >10)

Job category	Number of jobs	
	Construction	Operation
Skilled and permanent	Low	Medium
Skilled and temporary	Low	Low
Unskilled and permanent	Low	Low
Unskilled and temporary	Low	Low

4.6. Performance Assessment:

4.6.1. Start-up:

Distillery effluent is produced seasonally and therefore the UASB would not be fully operational all year round. This poses a problem in that anaerobic systems generally require long start-up periods, ranging from a few weeks to months, during which time effluent quality may be lower than required.

A suitable source of sludge for the inoculum is essential for UASB start-up, as the sludge should be able to degrade the compounds present in the distillery wastewater. Preferably, sludge from a plant treating the same/similar effluent should be used (Wolmarans & de Villiers, 2002). This would reduce the start-up phase as an initial period for sludge acclimatization will be unnecessary. The sludge should also be able to form granules when using distillery effluent as a substrate. The formulation of active, settleable sludge granules is essential for biomass retention, which is one of the advantages of UASB reactors (Moosbrugger *et al.*, 1993a, 1993b).

During start-up, low organic loading rates (OLR) should be applied (Wolmarans & de Villiers, 2002) and small stepwise increases should only be made once operational stability is reached after each increase i.e. once the COD removal efficiency reaches and remains >90% (Wolmarans & de Villiers, 2002). Furthermore, start-up could take a long time since vinasse has a high COD content which must be diluted to ensure low OLR during start-up. This also implies that storage facilities may be necessary during start-up, especially during the production season when effluent generation rates are high.

4.6.2. Ability of UASB to dispose of effluent:

Moosbrugger *et al.* (1993a, 1993b) assessed the feasibility of wine distillery effluent as a substrate for anaerobic digestion in a UASB based on the prerequisites for pelletization outlined by Sam-Soon *et al.* (1987) and described in Section 4.2. Moosbrugger *et al.* (1993a, 1993b) found that they could not guarantee the suitability of wine distillery effluent for treatment in a UASB system due to the ill-defined organic composition of the waste. However, as comparison of the product formation profiles for wine distillery effluent was found to be similar to that for apple juice wastewater which was used by Sam-Soon *et al.* (1987), a high pH_2 zone, which is suggested, could be inferred. Cysteine deficiency, which was recommended, could not be confirmed as there was no analysis available (Moosbrugger *et al.*, 1993a, 1993b). However, the large number of pellets formed using wine distillery effluent suggested that cysteine was usually deficient (Moosbrugger *et al.*, 1993b).

Nitrogen supplementation was found to be unnecessary as the wastewater contained nitrogen in quantities well in excess of the metabolic requirements of the anaerobic microorganisms (Moosbrugger *et al.*, 1993a, 1993b). Sam-Soon *et al.* (1987) suggested a minimum sludge pH of 6.6, and this should be maintained by either recycling of effluent, alkalinity addition or both if necessary. Wine distillery wastewater was found to generate high amounts of H_2CO_3 alkalinity, and, after a start-up period, recycling of the reactor effluent could be used to maintain the recommended minimum bed pH of 6.6 (Moosbrugger *et al.*, 1993a, 1993b). Although granulation is possible in UASB reactors treating wine distillery effluent, the granules are not as compact and stable as those produced in other wastewaters (Moosbrugger *et al.*, 1993a, 1993b).

Table 4.3 shows the treatment efficiency of distillery wastewaters treated using UASB reactors, adapted from literature.

Table 4.3: Characteristics of distillery effluents treated by UASB (adapted from literature)

Type of Distillery Effluent	Grape wine distillery	Grape wine distillery	Grape wine distillery	Malt whisky distillery pot ale
Wastewater COD (mg/l)	>30 000	12 975 – 35 775	25 000 – 35 000	30 000 – 50 000
Influent COD (mg/l)	5 000 – 15 000	26 669 (average)	-	6 690 – 21 050 (i.e. 20 – 70% wastewater)
Organic Loading Rate (OLR) (kg COD/m^3.day)	15 (max.)	18 (max.)	15	1.36 – 10.02 (5.46 average)
Percentage COD Removal (%)	82	>90%	90 – 95%	70 - 90% (80% average)
Hydraulic Retention Time (HRT)	1.1 days	4 – 11 hours	-	2.1days
Effluent COD (mg/l)	<3 000	875 - 9950	-	<5 000
Start-up	-	25 days	14	-
Reactor temperature (ºC)	-	34 - 36	-	35
Reference	Cheng *et al.*, 1990	Wolmarans & de Villiers, 2002	Driessen *et al.*, 1994	Goodwin *et al.*, 2001

Nutrients such as nitrogen together with some trace elements are necessary for successful anaerobic digestion, especially during start-up (Hickey *et al.*, 1991). Research conducted by Moosbrugger *et al.* (1993a, 1993b) using a lab-scale UASB reactor concluded that wine distillery effluent contained sufficient nitrogen and trace elements and no further supplementation was required. Driessen *et al.* (1994) also concluded that the addition of nutrients was unnecessary. Wine vinasse contains 15mg/l Fe (Table 1.1) which is beneficial as Fe deficiency results in poor settling quality and decreased

activity of the sludge (Balszezyk *et al.*, 1991). Although Ni and Co may not be essential for sludge development, they may be important for activity as these micronutrients form part of co-factors such as F_{430} and corrinoids which are specifically possessed by methanogenic organisms in high concentrations (Sharma & Singh, 2001). Addition of these elements may therefore increase the activity of sludge in UASB reactors treating wine distillery effluent.

4.6.3. Process stability and reliability:

The temperature within anaerobic reactors is very important, especially when treating waste streams that have variable flow rates and high COD concentrations (Wolmarans & de Villiers, 2002). Higher loading rates should not be applied unless the reactor is at the optimal temperature or reactor failure could occur (Wolmarans & de Villiers, 2002). This means that treatment of wine distillery effluent is a temperature sensitive process and a method to retain a stable temperature is required. The UASB reactors in use for the treatment of high strength wastewaters today usually operate between 34ºC to 37ºC i.e. mesophilic conditions. This is usually accomplished by the use of an appropriate heating system such as flow heaters and water jacket systems (Goodwin *et al.*, 2001).

As can be seen from Table 4.3, the UASB process can potentially treat wine distillery wastewater with organic loading rates as high as 18kg COD/m^3.d and COD removal efficiencies of >90%. The performance of UASB reactors for the treatment of vinasse can be directly related to COD removal efficiency (Moosbrugger *et al.*, 1993a). Due to the seasonal nature of wine distillery effluent, the influent COD varies considerably (Wolmarans & de Villiers, 2002). This affects the system most during the start-up period, where erratic influent COD leads to erratic OLR which in turn leads to inconsistent COD removal (Wolmarans & de Villiers, 2002). Once the system has attained stable operation however, the removal efficiency tends to remain consistent even with continued fluctuations in the OLR (Wolmarans & de Villiers, 2002).

The UASB system can be unstable, and sudden reactor failure can occur. Sheehan & Greenfield (1980) suggested that influent dilution is essential as the main cause of reactor failure is the high load of inorganic compounds present in vinasse. Goodwin *et al.* (2001) found that even after long periods of reactor stability (140 days), increased OLR of 50% to 70% vinasse resulted in drastically reduced biogas production and COD removals (<50%) even though the pH remained stable at around 7. Once the OLR was reduced by feeding with 20% to 30% vinasse, the biogas production and COD removal efficiency improved (Goodwin *et al.*, 2001). These results did not compare favourably to previous studies by the same research group (Goodwin & Stuart, 1994), which achieved 90% COD removal efficiencies also using malt whisky distillery vinasse. The reason for

the comparatively poor performance was unknown, however a number of factors are suggested that could have resulted in decreased efficiency such as a different source of the malt whisky distillery vinasse, reduced sludge activity or lack of nutrient supplementation (Goodwin *et al.*, 2001).

Even though low concentrations of sulphate can be beneficial to the growth of methanogens and acetogens (Hickey *et al.*, 1991), problems could be encountered with the treatment of wine distillery effluent in UASB systems as the vinasse contains approximately 150mg/l sulphate (Table 1.1). High sulphate concentrations can cause the growth of a large number of sulphate reducing prokaryotes (SRP) within an anaerobic system (Hickey *et al.*, 1991), and the subsequent production of hydrogen sulphide may eventually inhibit the growth and activity of methanogenic bacteria if it accumulates to high levels (Hickey *et al.*, 1991; Driessen *et al.*, 1994). This could eventually lead to digester failure.

Although low concentrations of calcium appear to be beneficial for granulation in UASB, concentrations greater than 450mg/l leads to increased sludge wash-out (Hulshoff-Pol *et al.*, 1983) which decreases the reactor efficiency. The calcium concentration of 410mg/l (Table 1.1) in wine vinasse is therefore close to the maximum, which could prove problematic.

Another difficulty could be encountered due to the high solids content of wine distillery effluent (15505 – 18000mg/l total solids, Table 1.1). High solids concentrations in a UASB can result in severe biomass wash-out due to attachment of bacteria to fine solid particles and consequently, a decline in COD removal efficiencies (Hickey *et al.*, 1991). Therefore a solids removal pre-treatment system such as centrifugation is essential.

One of the major problems aside from the costs and process instability associated with full-scale UASB systems, is that of floating or unsettleable granules (Hickey *et al.*, 1991). This usually occurs when a shock load is received by the reactor, indicating that UASB reactors are sensitive to shock loads even though they are capable of handling high OLRs (Samson *et al.*, 1984; Hickey *et al.*, 1991). As the composition and volumes of vinasse are highly seasonal, a UASB system treating this effluent is bound to be subjected to shock loads. Thus, in order to avoid a reduction in performance a storage system needs to be in place in order for a constant loading rate to be achieved.

It can be concluded that although anaerobic digestion can remove >90% COD from distillery effluent, the system is prone to instability. A solids removal step is required before anaerobic treatment, but no supplementation is required. Changes in the process parameters or influent

composition could result in reactor failure. Considering the above, and that the composition of wine distillery effluent is seasonal, anaerobic digestion of this effluent could prove problematic and unreliable. According to literature, the performance of the UASB is highly dependent on a range of factors and even slight deviations from these optimal conditions can result in process failure (Hulshoff-Pol *et al.*, 1983; Samson *et al.*, 1984; Hickey *et al.*, 1991; Goodwin *et al.*, 2001; Wolmarans & de Villiers, 2002), which implies that process conditions have to be optimal at all times or reactor failure is likely to occur. This, in turn, requires skilled operators, which may be in short supply in developing countries such as South Africa.

4.6.4. Use of end-products:

This method of treatment results in the production of methane, carbon dioxide and some sulphide gases, the latter of which is associated with foul odours. The biogas produced can be collected and used to fulfil at least some of the energy requirements of the treatment plant. The potential for energy generation is discussed in Section 4.10.

As sludge production rates are low for anaerobic systems, little excess sludge could be expected. The digested sludge produced would require conditioning and dewatering prior to appropriate disposal. Furthermore, anaerobically treated distillery effluent has greater fertilizer value than raw effluent according to Ramana *et al.* (2002a) and could be used, together with an effective monitoring and regulation scheme, to effectively irrigate land.

4.7. Regulatory Status:

The regulatory requirements pertaining to wastewater production and the liability of the company producing the effluent are provided in Section 3.7. The applicable regulations depend on the final fate/use of the anaerobically treated wastewater. If the reactor effluent is used to irrigate land, the regulations governing irrigation with wastewater are applicable (Section 3.7). As anaerobic treatment is capable of removing up to 90% of the influent COD, the effluent could meet the standards for irrigation of $<50 m^3$/day, depending on the influent COD. The reactor effluent could also be discharged to the sewer system, however it would have to meet the standards set by the particular municipal treatment works which vary according to capacity and capability. In South Africa however, it is unlikely that a suitable municipal treatment system is available in the rural wine regions.

If the wastewater is discharged into a water source, the General Authorizations in terms of Section 39 of the National Water Act (1998), which includes the regulations regarding the discharge of wastewater into water resources, would apply. In terms of these authorizations, which applies to surface water resources but not groundwater sources (van Schoor, 2001), one must register with the department before discharging any treated wastewater into a water source. Certain water resources are identified as "listed", which means that the treated wastewater disposed into these resources have to comply with stricter requirements i.e. the list of special limits in Table 4.4 (van Schoor, 2001). The registered person must ensure that the quantity of wastewater discharged is determined on a weekly basis, while the quality of the wastewater must be tested on a monthly basis (van Schoor, 2001). The general authorizations state that a registered person may discharge up to 2000m^3 of wastewater on any given day into a water resource, provided that:

- the treated effluent complies with the general limit or special limit values if discharge into a listed water resource is intended, set out in Table 4.4;
- the discharge does not change the natural, ambient temperature of the receiving water by more than 3ºC and no more than 2ºC for listed water resources; and
- the discharge is not considered a complex industrial wastewater i.e. the industrial effluent must be suitably treated prior to discharge.

Table 4.4 below lists the general and special limits for wastewater discharge into a water resource as set out by the General Authorizations in terms of Section 39 of the National Water Act. When considered with Table 4.3 it is clear that distillery effluent treated with UASB system would not meet the legal requirements for wastewater discharge into a water resource in terms of COD content (Table 4.4). Analysis of the various other elements stipulated by the regulations were not available, although Moosbrugger *et al.* (1993a, 1993b) found that effluent Total Kjeldahl Nitrogen (TKN) was 45mg/l and inorganic-N concentrations were 30mg/l from a UASB system treating wine distillery wastewater. This indicates that the anaerobically treated effluent is also unlikely to meet the requirements for disposal to a water source in terms of nitrogen content. Further treatment is therefore essential in order for the treated water to meet the requirements for disposal to receiving water bodies.

Table 4.4: General and special limits applicable to the discharge of wastewater into a water resource as specified by the General Authorizations in terms of Section 39 of the National Water Act (National Water Act, 1998)

Substance/Parameter	General Limit	Special Limit
Faecal coliforms (per 100ml)	1 000	0
COD	75*	30*
pH	5.5 – 9.5	5.5 – 7.5
Ammonia-N (ionized and unionized)	3	2
Nitrate/Nitrite-N	15	1.5
Free chlorine	0.25	0
Suspended solids	25	10
Electrical conductivity (mS/m)	70mS/m above intake to a maximum of 150mS/m	50mS/m above background receiving water, to a maximum of 100mS/m
Orthophosphate (PO_4-P)	10	1 (median) and 2.5 (maximum)
Fluoride	1	1
Soap, oil or grease	2.5	0
Dissolved arsenic	0.02	0.01
Dissolved cadmium	0.005	0.001
Dissolved chromium	0.05	0.02
Dissolved copper	0.01	0.002
Dissolved cyanide	0.02	0.01
Dissolved iron	0.3	0.3
Dissolved lead	0.01	0.006
Dissolved manganese	0.1	0.1
Mercury and its compounds	0.005	0.001
Dissolved selenium	0.02	0.02
Dissolved zinc	0.1	0.04
Boron	1	0.5

Note: All substances/parameters in mg/l unless otherwise stated.
*After removal of algae

4.8. Market Size and Information:

As mentioned in Section 4.7, anaerobic treatment of wine distillery effluent is unlikely to meet discharge standards in South Africa. Despite this, high-rate reactors like UASBs still have a high market potential as they are a well-studied, proven technology that has been effective in the treatment of other high-strength industrial wastewaters (Driessen *et al.*, 1994; Lata *et al.*, 2002). Although they are also used extensively worldwide for the treatment of wastewaters from distilleries, breweries, the paper and pulp industry and chemical industries (Cheng *et al.*, 1990;

Driessen *et al.*, 1994), polishing steps are always required. The production of a valuable resource in the form of biogas is an additional benefit of the system and the financial implications are considered below.

4.9. Financial Considerations:

Aside from process instability, the cost involved has always been a major drawback to the installation and operation of anaerobic systems. Although full-scale UASB systems have been used for the treatment of wine distillery effluent in South Africa (Wolmarans & de Villiers, 2002), the financial data was unavailable.

4.9.1. Design and construction:

Nguyen (2003) attempted to analyse the costs involved in the establishment of a hypothetical 50Ml/year anaerobic plant treating ethanol-from-starch wastewater. This example assumed a constant wastewater production of 2.9Ml/day for 345 days a year and that the treated effluent would be irrigated onto land using spray irrigation (Nguyen, 2003). The cost of the irrigation system is discussed in Section 3.9, and the capital cost of the digester itself would be A$5.6 million (Nguyen, 2003), with a total capital required of A$7.6 million (Nguyen, 2003). Additional facilities that would improve the effectiveness of UASB systems such as biofilters would increase the capital costs.

4.9.2. Operation:

The total annual cost of operation of the theoretical digester and spray irrigation system would be A$1.576 million including annual capital charge at 15% of A$1.14 million (Nguyen, 2003). The annual costs involved would include labour, maintenance and chemical costs (such as sodium hydroxide/sodium carbonate for pH conditioning requirements).

4.9.3. Personnel:

The personnel costs would also be higher for a process such as anaerobic digestion as the process is a complex and unstable one that requires the employment of skilled personnel. The labour costs would be increased with the increased skill of the workers employed, and would include benefits such as medical aid for the permanent staff.

4.9.4. By-products and waste:

Anaerobic digestion can be very attractive as it provides a renewable source of energy in the form of combustible biogas as a by-product which can be used to meet the energy demands of the treatment plant (Lata *et al.*, 2002). One drawback of this treatment is that due to the seasonal character of the effluent, the plant will not be used for long time periods in the year and would thus be unproductive. Table 4.5 shows the periods of operation (over three seasons) of a full-scale UASB plant treating vinasse from a distillery in Wellington, South Africa (Wolmarans & de Villiers, 2002). As can be seen, the plant was only operational for a few months a year, therefore it could potentially extend the time for the investment costs to be recovered if this includes revenue or savings from sale or utilization of the biogas.

Table 4.5: Operation periods of a full-scale UASB plant treating wine distillery effluent (from Wolmarans & de Villiers, 2002)

Season	Period of operation
1998	17 February to 30 March
1999	3 February to 19 March 8 to 16 April 28 April to 8 May
2000	2 February to 7 April 9 to 18 May

Although Nguyen (2003) estimated the total annual cost of a hypothetical anaerobic system would be offset by the sale of the biogas produced (estimated to be worth A$430 000 per year), this reduction was minimal and still resulted in a high annual cost of A$1.146 million. Furthermore, biogas treatment such as lime scrubbing would be required to remove any hydrogen sulphide present if the biogas was intended for power generation (Lata *et al.*, 2002). Such additional treatments would add to both the capital costs and annual operating costs.

Moreover, the conditioning and dewatering of excess sludge and its disposal is a costly initiative. These costs would be reduced as sludge production is slow, high-rate reactors such as UASB reactors are designed to retain the biomass and the reactor would only be fully functional for a few months per year (Table 4.5), further reducing sludge production.

It was concluded that although anaerobic treatment of distillery effluent could result in as much as 90% COD removal, the high capital and operating costs required made this alternative an unattractive one (Nguyen, 2003).

4.10. Energy Impacts:

UASB systems require limited energy inputs as mechanical mixing is not required. However, energy is necessary for pumping of the effluent into the conditioning tanks and UASB reactor.

The anaerobic process produces a readily combustible biogas, which can be used to meet at least part of the energy demand of the treatment plant (Lata *et al.*, 2002). This implies that the biogas produced by a UASB reactor could be used to meet the energy demands of a plant treating wine distillery effluent.

In India for example, there are over 200 distilleries, which generate 12 – 16 litres effluent per litre of alcohol produced (Lata *et al.*, 2002). During the 1994-1995 production season, a total of 1165 x 10^6l alcohol was produced (Lata *et al.*, 2002) and it was estimated that the potential of these distilleries for biogas production, based on their performance if all the distilleries chose to employ high rate anaerobic systems such as UASB for effluent treatment, would be 560 x 10^6 m^3 per year. If the calorific value of the biogas is assumed to be 5300kcal/m^3 then this amounts to 158MW of power (Lata *et al.*, 2002). The potential therefore, for high energy returns from anaerobic digestion exists.

4.11. Resource Use/Conservation:

As anaerobic digestion produces a renewable source of energy in the form of biogas, there is reduced use of other sources of energy such as non-renewable fossil fuels. However, dilution rates of as high as 20-30% effluent (Goodwin et al., 2001) are required in most cases if reactor failure is to be avoided. Recycling of the treated effluent could be used for pH conditioning and dilution in order to reduce the amount of fresh water consumed by the process. The overall drain on resources would therefore be reduced, although if the final effluent is irrigated onto land, the issues discussed in Section 3.11 would need to be considered.

4.12. Conclusions:

Once a UASB system has been successfully started up and is in stable operation, it can be considered an effective process for the treatment of wine distillery effluent (Wolmarans & de Villiers, 2002). It is, however, only effective as a treatment step in a larger treatment system as a polishing step may be necessary because the treated effluent would still not meet legal requirements

for disposal to water sources, although it could be disposed to the sewer or used for irrigation. The start-up phase is crucial and a low OLR must be applied with small stepwise increases until a stable state is reached (Wolmarans & de Villiers, 2002). The COD removal efficiency should also be carefully monitored during this period (Wolmarans & de Villiers, 2002), as reactor failure could occur very quickly. Overall, anaerobic digestion of wine distillery effluent can be successful given optimal operating conditions, and useful biogas is produced. Although the system can produce good quality effluent, it is still a comparatively unstable and expensive process that requires long start-up periods, skilled technicians to operate the system and high capital and operating costs. Furthermore, wine distillery effluent generation and composition is seasonal and this could prove problematic for an anaerobic system as the process is sensitive to shock loads and variable influent composition. Also, high dilution rates are required to ensure an effective and stable system, therefore large amounts of water is necessary, although the consumption of this resource can be reduced by effluent recycling. A primary aerobic system could be used to reduce the organic load of vinasse in order to reduce the dilution rates required for anaerobic treatment (Hamdi & Garcia, 1993), which could in turn improve the stability of the anaerobic system (Jimenez *et al.*, 2003).

CHAPTER V: TREATMENT OF WINE DISTILLERY EFFLUENT AND THE PRODUCTION OF LACCASE BY WHITE ROT FUNGI

5.1. Introduction:

The use of white-rot fungi for the treatment of wine distillery effluent was considered as these fungi are known to degrade a wide variety of substrates and produce valuable enzymes as a by-product. Fungi have been used to treat other darkly coloured phenolic wastewaters such as molasses and olive mill wastewater (Hamdi & Garcia, 1993; Perez *et al.*, 1998; Ruiz *et al.*, 2002; Aggelis *et al.*, 2003; Fenice *et al.*, 2003; Jimenez *et al.*, 2004). The preliminary ETSA identified that information regarding the performance of the novel fungal system for treatment of distillery effluent did not exist and that it would therefore need to be acquired via experimental laboratory studies. The performance of the system was evaluated on its ability to reduce the COD and polyphenol content of the wastewater. The production of the valuable laccase enzyme was also assessed, as the production of valuable by-products could be a significant advantage of the fungal treatment system.

The experimental approach had to be carefully planned as the rationale of the ETSA would be defeated if laborious experimental work and large sums of money were spent gathering the missing data for completion of the ETSA. Statistical experimental design using new software tools such as Design-Expert can be used to facilitate the design of experiments and further reduce the use of resources such as time and money. The use of response surface methodology (RSM) is a common practice in modern biological research (e.g. Farrera *et al.*, 1998; Chakravarti & Sahai, 2002; Lai *et al.*, 2003; Zhao *et al.*, 2005), especially as it is not always possible to determine the most important factors in multivariable processes such as biological systems (Kalil *et al.*, 2000). RSM involves a number of statistical methods that can be used for designing experiments, evaluating the effect/s of factors, building models and determining the optimum conditions or levels of factors for desirable responses. This study utilized the Box-Behnken design, which is a three-level factorial design that was suitable for the evaluation of the effects of selected variables on the responses. This design could also be used to optimize a process while utilizing a small number of experimental runs.

As the vinasse will always be variable, it was not considered a factor to be tested as the fungal system should be able to survive and treat the variable effluent. As the ETSA is limited to only the basic information required to determine if a system should be further developed, the factors to be

tested should be carefully chosen. Some assumptions had to be made prior to deciding which factors would be chosen for experimentation. These assumptions were that:

- a polyphenol recovery system could be possible upstream of the vinasse treatment system as the tannins/polyphenol could have commercial value and such a system could possibly reduce the COD content of the influent to the fungal system. It was decided that the maximum amount of polyphenols would simply be precipitated out of the vinasse using insoluble polyvinylpolypyrrolidone (PVPP) (D'Alvise *et al.*, 2000) to observe how the removal of polyphenol altered the effluent and the response of the fungal system;
- the fungi would not require pH adjustment of the vinasse, and would be able to effectively reduce the COD content of the effluent at the original pH;
- the fungi would not be able to withstand high effluent concentrations, therefore the effluent was diluted to 30% of its original strength;
- the fungi may require nutrient supplementation for effective growth, degradation and laccase production, therefore combinations of carbon, phosphate and nitrogen sources were tested.

One of the problems associated with designing a process for the treatment of wine distillery effluent was that the effluent was seasonally variable. This was further complicated by the fact that it was not always possible to use the same batch of raw material for an experiment and it was only possible to do a limited number of runs at a time due to laboratory constraints. Design-Expert could be programmed to take these factors into account by using the 'blocking' technique when designing the experimental set-up. As the influent vinasse was not a factor, this experimental design technique allowed one to "remove" the variations from the analysis expected due to different batches of influent and due to the experiments being run at different times. This removed the effect of raw material variability on the responses and allowed for the identification of the effects of the other chosen factors.

Fungi had to be screened for their ability to treat wine distillery effluent. Aside from the wild fungal isolates screened, two well-known lignin degraders and laccase producers namely *Trametes pubescens* and *Ceriporiopsis subvermispora*, were also tested.

T. pubescens is a white-rot fungus that has been identified as having the capability for high laccase production. The extracellular laccase production by this fungus can be greatly enhanced using optimal Cu concentrations in a basal medium and in this way, to achieve production of up to 65 U/ml (Galhaup & Haltrich, 2001). It was also found that easily utilized carbon sources such as

glucose yielded higher laccase activity than more slowly consumed sources such as glycerol (Galhaup *et al.*, 2002). The study also showed that high glucose levels of up to 40 g/l increased laccase production, but that any further increase in the glucose concentration resulted in repression of laccase production (Galhaup *et al.*, 2002). Laccase production by this fungus was also affected by the nature and concentration of nitrogen present in the medium. Optimum laccase production (319 U/ml) occurred with 10 g/l peptone from meat as the nitrogen source, while some other complex nitrogen sources reduced fungal growth and enzyme production (Galhaup *et al.*, 2002). Laccase production by *T. pubescens* differs from studies of laccase production by other white-rot fungi as the addition of aromatic compounds, which are used to induce laccase production in other fungi, has been shown to be unnecessary for high levels of laccase production by this fungus (Galhaup *et al.*, 2002). Under optimal conditions, using a synthetic medium, *T. pubescens* was capable of producing up to 743 U/ml in a fed batch fermentation process (Galhaup *et al.*, 2002).

C. subvermispora is also a white-rot fungus that is able to selectively degrade lignin and produce laccase (Karahanian *et al.*, 1998; Ferraz *et al.*, 2003; Guerra *et al.*, 2003). Ferraz *et al.* (2003) showed that laccase activity by this fungus peaked at 12.6 units per 200ml culture medium after 30 days of degradation of *Eucalyptus grandis* wood chips.

The white-rot fungus *Pycnoporus sanguineus* was isolated from a decaying forest tree in Hogsback, South Africa. It was identified using the morphology of its fruiting body. This fungus is known to produce laccase as the only polyphenoloxidase (Pointing & Vrijmoed, 2000). *P. sanguineus* was also shown to effectively decolourize a number of synthetic dyes and produce more than 1200U/l laccase within 7 days (Pointing & Vrijmoed, 2000) although this fungus can produce even higher amounts of laccase. For example, studies by Muzariri *et al.* (2001) showed that a wild isolate of *P. sanguineus* found in hot, dry regions of Zimbabwe produced up to 3610U/l laccase in a synthetic medium within 7 days.

While *T. pubescens* and *P. sanguineus* were specifically chosen for this study due to their laccase production capabilities, *C. subvermispora* was chosen for its ability to degrade lignin and lignin-like compounds which would be important in the degradation of the polyphenol-rich wine distillery effluent.

The purpose of this study was to determine which of the 3 candidate fungal species would be most suitable for treatment of distillery effluent. Furthermore, it was necessary to determine the growth

conditions required and the performance of the treatment process in terms of COD and polyphenol removal as well as laccase production.

5.2. Materials and methods:

5.2.1. Wine distillery effluent:

Wine distillery effluent was collected during the peak production season from the Distell company situated in Worcester, South Africa, and stored in plastic containers at 4ºC until use. The containers were always well shaken before removing effluent for the experiments. The raw effluent was characterized according to the analytical methods below (Section 5.2.7).

5.2.2. Screening for suitable fungal strains:

The first phase was to identify candidate fungal strains capable of degrading some of the compounds within the effluent. Wild fungal isolates collected in Hogsback, South Africa during March 2003, were screened for their ability to treat wine distillery effluent by culturing them on effluent plates (described below). The fungal isolates, which were found on dead wood and identified according to the morphology of their fruiting bodies, were first cultured to pure culture on malt extract agar (MEA) plates and then sub-cultured on distillery effluent plates. Pure cultures were obtained by plating small fragments of the inner basidiocarp onto MEA. The plates were incubated at 28ºC and the resultant mycelial growth was repeatedly sub-cultured on MEA until the cultures were pure. The best wild isolate chosen was *P. sanguineus*, which is known to produce laccase and degrade a number of synthetic dyes (Pointing & Vrijmoed, 2000). *T. pubescens* CBS 696.94 and *C. subvermispora* CBS 347.63, purchased from the Centraalbureau voor Schimmelcultures, were also sub-cultured on effluent plates as both are white-rot fungi known to produce laccase and are capable of degrading lignin and polyphenolic compounds. The effluent plates contained 100% effluent and 2% agar and were adjusted to pH 5 in order to allow the agar to set. A 1cm x 1cm plug was removed from each fungal culture originally grown on MEA and each placed centrally on individual effluent plates. The plates were incubated at 28ºC for 5-7 days as these were all mesophilic fungi. The effectiveness of effluent degradation was based on the clearing of the dark colour of the solid medium. The clearing produced on the effluent plates, thought to be due to polyphenol degradation (Tekere *et al.*, 2001), was assessed visually against an uninocculated control and scored on a scale from 1 to 5, with 5 being total clearing of the plate and 1 being no clearing. This was used as a crude screening method to determine if the isolates were capable of degrading wine distillery effluent.

5.2.3. Polyphenol removal experiments:

As discussed, it was assumed that a polyphenol removal process could be in place upstream of the treatment facility, therefore it was important to determine the effects of polyphenol removal on the treatment system. Experiments were conducted in duplicate to determine the minimum amount of polyvinylpolypyrrolidone (PVPP) required for maximum polyphenol removal from wine distillery effluent. PVPP was purchased from Sigma Chemical Co., South Africa. As it was necessary to filter and centrifuge the PVPP-treated effluent in order to remove the PVPP-polyphenol complex that formed, total polyphenol analysis (Section 5.2.7) was also performed for duplicate samples of raw effluent, effluent filtered through Whatman #1 filter paper, effluent centrifuged using a bench-top centrifuge (Eppendorf) @ 10 000rpm for 3 minutes, and effluent that had been subjected to both filtration and centrifugation in order to determine the amount of polyphenols removed by these various physico-chemical processes. The amounts of PVPP addition tested were 1g, 5g, 10g and 15g per 100ml raw effluent. After addition of PVPP, the effluent was stirred slowly for one hour at room temperature then centrifuged. The COD concentration of the effluent after optimal polyphenol extraction using PVPP was also determined to determine the contribution of polyphenols to the total COD content of the effluent.

5.2.4. Box-Behnken experiment:

Laboratory flask experiments were used to obtain the performance data identified during the preliminary ETSA. The specific objectives of this experiment were to screen the three fungi chosen (Section 5.2.2) and to optimize the nutrient variables discussed below for maximum COD removal. Box-Behnken plots were used to design the experiments, which was desirable as they allow for optimization of responses, but with only a fraction of the experiments required for other factorial designs.

A computer software programme, Design-Expert version 6.0.10 (Stat-Ease Inc., Minneapolis, USA), was used to design batch experiments incorporating the following experimental variables/factors:

- Fungal Strain: *T. pubescens, P. sanguineus* or *C. subvermispora*.
- Total polyphenol content: 100% effluent with polyphenols extracted using PVPP or 30% effluent without polyphenol removal.
- Glucose supplementation: no supplementation, high supplementation (10g/l) or low supplementation (1g/l).
- Organic nitrogen supplementation: no supplementation in the form of yeast extract, high supplementation (10g/l) or low supplementation (2g/l).

- Inorganic nitrogen supplementation: no supplementation in the form of ammonium tartrate, high supplementation (10g/l) or low supplementation (2g/l).
- Phosphate supplementation: no supplementation in the form of potassium dihydrogen orthophosphate (KH_2PO_4), high supplementation (10g/l) or low supplementation (2g/l).

The design matrix is shown in Appendix 1. Factor coding was very important in the response surface design as it reduced the range of each numerical factor to a common scale of -1 to +1 regardless of the relative magnitude of the factors. Typically, -1 is the lower level of the factor (e.g. 0g/l glucose), 0 is the middle level (e.g. 1g/l glucose) and +1 is the upper level (e.g. 10g/l glucose). In this study, -1 indicated no supplementation, 0 signified low supplementation and +1 signified high supplementation. 180 combinations of the 6 factors above were generated (3 blocks of 60) (Appendix 1) and removed the raw effluent composition as a factor. The experimental runs were conducted in batch flask cultures.

The fungal biomass required was cultured on MEA plates which comprised 50g/l malt extract and 5-6g/l agar, both of which were purchased from Merck, South Africa. For each strain, three 1 x 1cm plugs were removed and used to inoculate 50g/l malt extract broth (MEB) (50g/l). After 10 days, the liquid culture was briefly homogenized using a blender (Ultraturrex) in order to break up the fungal pellets that had formed and 5ml of this mixture was used as the inoculum for the Box-Behnken experiments. 500ml conical flasks containing the required amount of medium and supplements (Appendix 1) containing a final volume of 200ml were inoculated with the appropriate fungus and incubated for 12 days at 28ºC on a bench-top shaker (Labcon) at 150rpm. Responses measured for each flask were: pH, COD concentration, total polyphenol concentration, laccase concentration and biomass (dry weight). The methods for these analyses are discussed in Section 5.2.7.

Once the responses were analyzed statistically by Design-Expert, the optimization option was used to search for the combination of factors and their levels to satisfy the requirements placed on each of the responses and factors for an optimal process. The desirability function was used to measure the desirability of each optimization option. The desirability range was from 0 to 1 with 0 being the least desirable and 1 the most desirable. Once the programme had calculated the combination of factors that produced the optimal responses as dictated, it mathematically calculated the desirability of the option. One could optimize a single response regardless of others, or single responses subject to upper and/lower constraints on other responses. For the optimization and desirability function, each response can be assigned an importance relative to the other responses. Since COD removal

was considered the most important factor in terms of effluent treatment, it was given the highest importance level of 5, while all other factors were assigned no importance (importance rating = 0). The most desirable outcome of the software optimization which gave a desirability rating of 1 (Table 5.1) was then tested experimentally as described below (Section 5.2.5) in order to compare the predicted and actual outcome of a fungal system optimized for maximum COD removal.

5.2.5. Evaluation of predicted performance:

According to the predicted solutions generated by Design-Expert (see Section 5.2.4), the maximum amount of COD removal from 30% effluent (no polyphenol extraction with PVPP) could be achieved using 0g/l glucose, 0.06g/l phosphate, 0.24g/l ammonium tartrate (inorganic nitrogen) and 0g/l yeast extract (organic nitrogen) and *P. sanguineus*. These values were calculated from the predicted optimal factor levels (Table 5.1) as follows:

e.g. Phosphate: -1 = 0g/l

0 = 2g/l

+1 = 10g/l

Therefore a factor level of -0.97 = 0.6g/l.

The prediction was tested using two experimental approaches:

Test 1:

This was conducted exactly as for the Box-Behnken experiment, in order to confirm the accuracy of the predicted optimal responses.

According to the predicted factor levels for high COD removal efficiency (Table 5.1), 4 x 500ml flasks were set up each containing 195ml 30% wine distillery effluent, 0.012g dihydrogen orthophosphate (0.06g/l) and 0.048g ammonium tartrate (0.24g/l), no glucose and no yeast extract. Three flasks were inoculated with 5ml *P. sanguineus* while 1 flask was left un-inoculated as the control. Once initial samples were taken (t = 0), the flasks were incubated at 28ºC on a desktop shaker (Labcon) at 150rpm for 12 days. Final samples were collected on day 12 and subjected to analysis of pH, COD concentration, total polyphenol concentration and laccase assays as described in Section 5.2.7.

Test 2:

This test was performed in order to determine if/when a plateau of COD removal was reached in order to ascertain if the hydraulic retention time (HRT) for maximum COD removal could be

decreased from the 12-day period used in Test 1. The flasks were set up as described above (Test 1), with triplicate experimental flasks and an un-inoculated control. The experiment was run over 12 days as for the Box-Behnken experiment and 3ml samples were taken daily and analysed for COD concentration, polyphenol concentration and laccase production. The analytical methods are described in Section 5.2.7.

5.2.6. pH adjustment experiments:

Results from parallel work (unpublished) in the research group showed that pH adjustment of the wine distillery effluent to 5.3 with Na_2CO_3 resulted in increased laccase production by *T. pubescens* in undiluted effluent (Pers. Comm., P.J. Strong, 2004).

As the Box-Behnken experiment did not include pH as a factor, it was decided that the impact of this optimal pH on fungal treatment would be investigated in an independent study. The aim of this experiment was to determine how pH adjustment affected COD removal by *T. pubescens* and to determine whether there was an effect on effluent treatment and laccase production when NaOH was used to adjust the pH instead of Na_2CO_3. NaOH is cheaper than Na_2CO_3 and therefore preferable. 500ml conical flasks with 20ml *T. pubescens* liquid inoculum (grown in 50g/l MEB) and 180ml undiluted vinasse with pH adjusted to 5.3 using NaOH or Na_2CO_3 were used, and the experiment was conducted in triplicate. Samples were taken and analysed daily for COD concentration, polyphenol concentration and laccase concentration (see Section 5.2.7). The experiment was halted once a plateau of COD and polyphenol reduction was reached. The flask contents were then centrifuged to remove the biomass and analysed for: biomass production (dry weight), COD concentration, polyphenol concentration, pH and laccase concentration (see Section 5.2.7).

As the Box-Behnken experiment predicted that the optimal fungal strain for COD removal was *P. sanguineus*, it was decided to determine if pH adjustment of the effluent allowed for fungal growth, effluent degradation and increased laccase production in 100% effluent when inoculated with this species. The pH of the effluent was adjusted to 5.3 using Na_2CO_3 as this chemical showed some advantage over NaOH regarding COD removal and laccase production by *T. pubescens*. The experiment was conducted as described above for *T. pubescens*.

5.2.7. Analytical techniques:

COD:

The Spectroquant® Kit and Novaquant spectrophotometer (Merck) (Test number 023) were used to determine COD concentrations. The percentage COD removal (COD removal efficiency) was used as the primary indicator of the effectiveness of the fungal treatment.

Total polyphenol concentration:

Total polyphenol concentrations were determined using the Folin-Ciocalteau reagent method described by Box (1983). The percent total polyphenol removal was also used as an indicator of the removal of potentially hazardous components of the effluent.

Biomass production (dry weight):

The mass of fungal biomass in the various experiments was determined using the dry weight method as for suspended solids analysis (APHA *et al.*, 1998).

pH:

The pH of the supernatant after removal of biomass was determined directly after centrifugation, at room temperature, using a standard pH meter (Cyberscan 2500). The pH meter was calibrated before each use.

Laccase assay:

The assay chemicals were obtained from Sigma Chemical Company, South Africa. After centrifugation, the supernatant was used for the assays, which were carried out in microtitre plates in triplicate. 270µl aliquots of assay reagent and 30µl sample was added to each well before performing a kinetic read for 1 minute. The assay method employed was the oxidation of the substrate 2,2´-azino-bis(3-ethylbenz-thiazoline-6-sulphonate) (ABTS) (Jordaan & Leukes, 2003). The laccase assay reagent consisted 0.1mM ABTS, 0.3mM ethylenediaminetetraacetic acid (EDTA) and 1M succinate-lactate buffer (pH4.5). The absorbances of the samples were measured at 420nm. Since the assay is temperature sensitive, an incubation setpoint of 25°C was set on the Powerwave microtitre plate reader (Jordaan & Leukes, 2003).

The extinction coefficient (ε) for laccase activity at 420nm is 36 000$M^{-1}cm^{-1}$.The slope of the kinetic graph indicated activity and 1 unit of activity (U) is defined as the amount of enzyme necessary to oxidize 1µmol of substrate (ABTS) to product in 1 minute at 25ºC.

Ammonia-N, Nitrate-N, Chlorine (total) and Orthophosphate (PO_4):

The concentrations of these compounds were measured using the relevant Spectroquant® Kits available from Merck.

Total, dissolved and suspended solids:

All the solids analyses were performed according to the methods set out in APHA *et al.* (1998).

5.3. Results and Discussion:

5.3.1. Screening for suitable fungal strains:

The most effective wild isolate in terms of its ability to remove colour from test plates, was *P. sanguineus* (identified according to the morphology of the fruiting body) with a score of 4. The two other fungal strains known to degrade lignin and produce laccase that were also assessed were found to produce clearing on the effluent plates. These were *T. pubescens* (Galhaup & Haltrich, 2001; Galhaup *et al.*, 2002) with a score of 5 and *C. subvermispora* (Karahanian *et al.*, 1998; Ferraz *et al.*, 2003) with a score of 3.

These three white rot fungi were then used throughout the research to assess their ability to treat wine distillery effluent in terms of COD and total polyphenol reduction, and their ability to produce the laccase enzyme.

5.3.2. Polyphenol removal experiments:

Figure 5.1 indicated that filtering through Whatman #1 filters and centrifugation removed a small amount of the polyphenolic compounds, and that PVPP (5g/100ml) was efficient at removing over 75% of the polyphenols present in wine distillery effluent. The addition of greater than 5g PVPP per 100ml effluent did not significantly increase the removal of polyphenols, indicating the possible saturation point, where the maximum amount of polyphenols that could bind to PVPP was reached. Therefore, it was decided that the optimum amount of PVPP required to remove the maximum quantity of polyphenols was 5g/100ml and this would be added in future experiments when extraction of a large portion of the polyphenols was required.

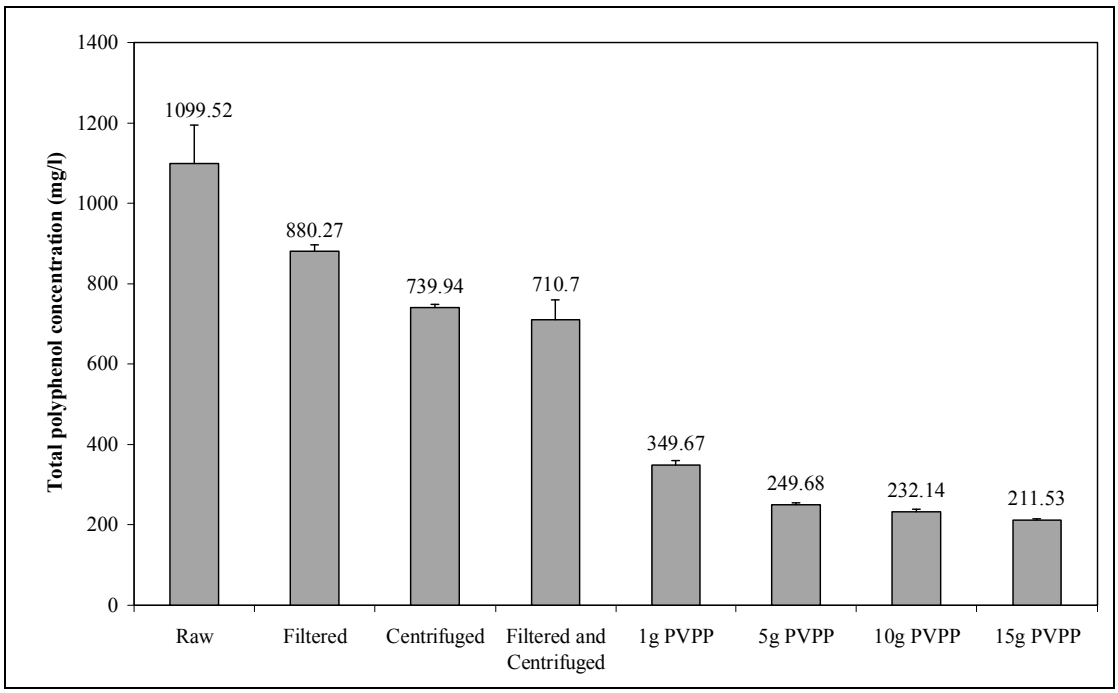

Figure 5.1: Results of polyphenol extraction from wine distillery effluent using PVPP

Although up to 75% polyphenol removal was achieved using the optimal amount of PVPP (5g/100ml), Figure 5.2 shows that very little of the COD was removed by the extraction process (5.26%). This implied that the polyphenols present were only accountable for a very small percentage of the total COD present in the effluent. Therefore, even if a polyphenol/tannin extraction process occurred before fungal treatment, dilution of the resultant effluent could be necessary.

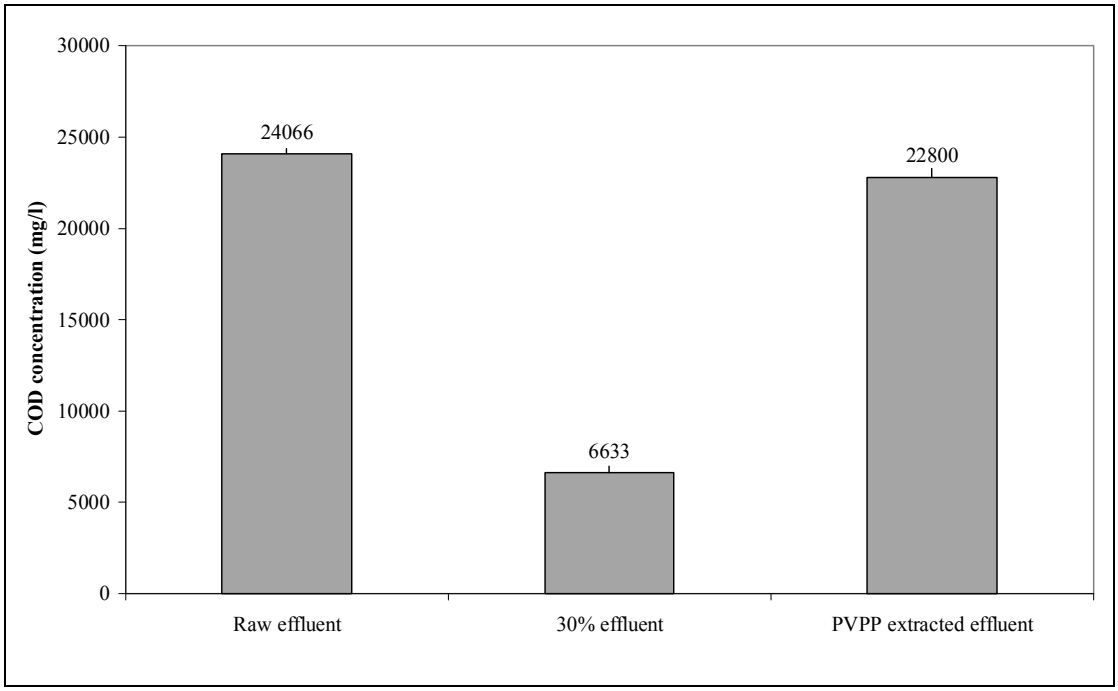

Figure 5.2: Typical COD content of raw wine distillery effluent, 30% effluent and PVPP treated effluent

5.3.3. Box-Behnken experiment:

The data resulting from the flask experiments was captured on the Design-Expert programme and analysed in terms of the various responses measured. The RSM models for all the responses were significant as the adequate precision value which measures the signal to noise ratio, were greater than 4 (the lowest desirable limit) for all responses. The programme was then used to optimize for the highest COD removal efficiency (importance of 5) regardless of all the other factors and responses. COD removal was considered the most important factor considering that the fungal treatment technology was being evaluated primarily for its ability to treat the effluent to a level where it was suitable for discharge. The criteria placed on all the factors and responses other than percent COD removal were kept in range. The solutions predicted (Table 5.1) that *P. sanguineus* was the most effective of the 3 fungal strains tested in terms of COD removal efficiency (86.5% COD removal). It also showed that polyphenol extraction was unnecessary as higher COD removal efficiencies are achieved in effluent where polyphenols were not artificially removed using PVPP.

It is hypothesized that PVPP probably removes the lower molecular mass polyphenols leaving behind high molecular mass polyphenols. This could explain the higher COD removals in effluent without polyphenol removal as high molecular mass polyphenols are recalcitrant compounds that inhibit fungi and tend to decrease their levels of COD removal (Sayadi *et al.*, 2000). Sayadi *et al.* (2000) found that polyphenols with a molecular mass >60kDa did not affect the growth of the white-rot fungus *Phanerochaete chrysosporium* but rather its degradative system, resulting in decreased polyphenol (colour) and COD removal. The desirability rating of 1 (most desirable) for solutions 1 and 2 indicated that these two sets of conditions were optimal for high COD removal.

The model predicted that no carbon or organic nitrogen supplementation would be required for high COD removal as factor levels of -1 indicate 0g/l. Furthermore, very little inorganic nitrogen (0.24g/l according to the predicted factor level of -0.88) and phosphate (0.06g/l according to the predicted factor level of -0.97) were necessary for high levels of COD removal. This indicated that these macronutrients were present in sufficient quantities in the effluent and implied that additional costs for supplementation of these nutrients could be omitted or decreased. It must be noted that the experiment utilized a 30% concentration of effluent (approximately 8500 – 9500mg/l) as it was assumed that growth of the fungi on undiluted raw effluent (approximately 25000 – 30000mg/l) would be unsatisfactory.

Table 5.1: Solutions generated when the process was optimized for maximum COD removal

Solutions												
Number	**Glucose**	**Phosphorous**	**Ammonium tartrate**	**Yeast extract**	**Polyphenol**	**Strain**	**Biomass**	**pH**	**Laccase**	**% Polyphenol removal**	**% COD removal**	**Desirability**
1	-1.00	-0.97	-0.88	-1.00	no extraction	*P. sanguineus*	0.251365	8.18791	0.526041	67.9625	86.503	1
2	-0.95	-0.77	-0.99	-0.99	no extraction	*P. sanguineus*	0.265323	8.01352	0.522919	68.3503	86.5346	1
3	-1.00	-0.36	-1.00	-0.98	no extraction	*P. sanguineus*	0.2847	7.76277	0.522281	69.0106	86.46	0.998597
4	-1.00	-1.00	-0.82	-0.99	no extraction	*P. sanguineus*	0.248266	8.21013	0.533358	67.7794	86.1504	0.987909
5	-1.00	0.95	-1.00	-1.00	no extraction	*P. sanguineus*	0.4319	6.84569	0.543798	71.0473	85.8721	0.978574

5.3.4. Evaluation of predicted performance:

The first predicted solution with a desirability of 1 was tested experimentally to determine the accuracy of the predicted results. As described in Section 5.2.5, two separate tests were conducted.

Test 1:

The results after the initial and final analysis (day 12) were compared to the results predicted by the RSM model based on the data obtained from the Box-Behnken experiment. As is evident from Table 5.2, the experimental values for COD removal (85%) and polyphenol removal (61.77%) compared favourably to the predicted values (86.5% and 67.96% respectively). The optimization criteria did not take the laccase, pH and biomass responses into consideration therefore these could not be more accurately predicted.

The low actual (0.021U/l) and predicted (0.526U/l) values for laccase activity indicated that a system optimized for high COD removal resulted in low laccase production. If laccase production was required to recover costs of the treatment process, a compromise would have to be made between COD removal efficiency and laccase production. Optimization analyses giving both laccase production and COD removal high importance would have to be performed and tested. The experimental laccase value reported could be overestimated, as earlier experiments in this laboratory have indicated that the presence of polyphenolic compounds could interfere with the laccase assay and this is further supported by the detection of laccase in the uninoculated control flask (data not shown). It can therefore be concluded that there is little, if any laccase production by *P. sanguineus* under conditions optimal for COD removal. Furthermore, although the pH of the medium did increase over the 12 days from pH 3.79 to 5.62, this increase was not as much as predicted by the RSM model (pH 8.18).

Table 5.2: The average results of Test 1 for the determination of the accuracy of the results predicted by the RSM model for optimized COD removal (n = 3)

Parameter	Predicted value		Experimental value		
	t = 12	% removal	t = 0	t = 12	% removal
COD	-	***86.5***	10 744 (3668)	1 632 (76)	***85***
Polyphenol	-	***67.9***	250.3 (7.6)	95.7 (6.3)	***61.77***
Laccase	0.526U/l	-	0.0	0.021U/l (0.062)	-
pH	8.18	-	3.79 (0.01)	5.62 (0.06)	-
Biomass	1.255g/l	-	-	2.813g/l (0.144)	-

Note: Standard deviation indicated in ( ).

Test 2:

In this study, samples were taken daily over 12 days in order to determine if/when a plateau of COD removal was reached. Figure 5.3 shows the COD removal efficiency of *P. sanguineus* in wine distillery effluent over the 12-day experimental period. Although a high COD removal of up to 70% occurred by day 7, there was surprisingly, a sudden drop in removal to around 30% i.e. the COD content in the system increased, between day 8 and 9. This could have been due to cell death and lysis, which could have increased the COD levels. The standard deviation between triplicate samples between day 8 and day 12 were also high, indicating some inaccuracy of the measured concentrations. The removal efficiency did recover, and reached a maximum of almost 80% by day 12. Again, this value is slightly lower than the experimental values (85%) achieved in Test 1 and the predicted values (86.5%), but it is comparable. Furthermore, Figure 5.3 shows that the retention time of the system could be reduced to 8 days, where there was a steady increase up to 70% removal by day 8, as a further 4 days removes only a further 10% of the remaining COD.

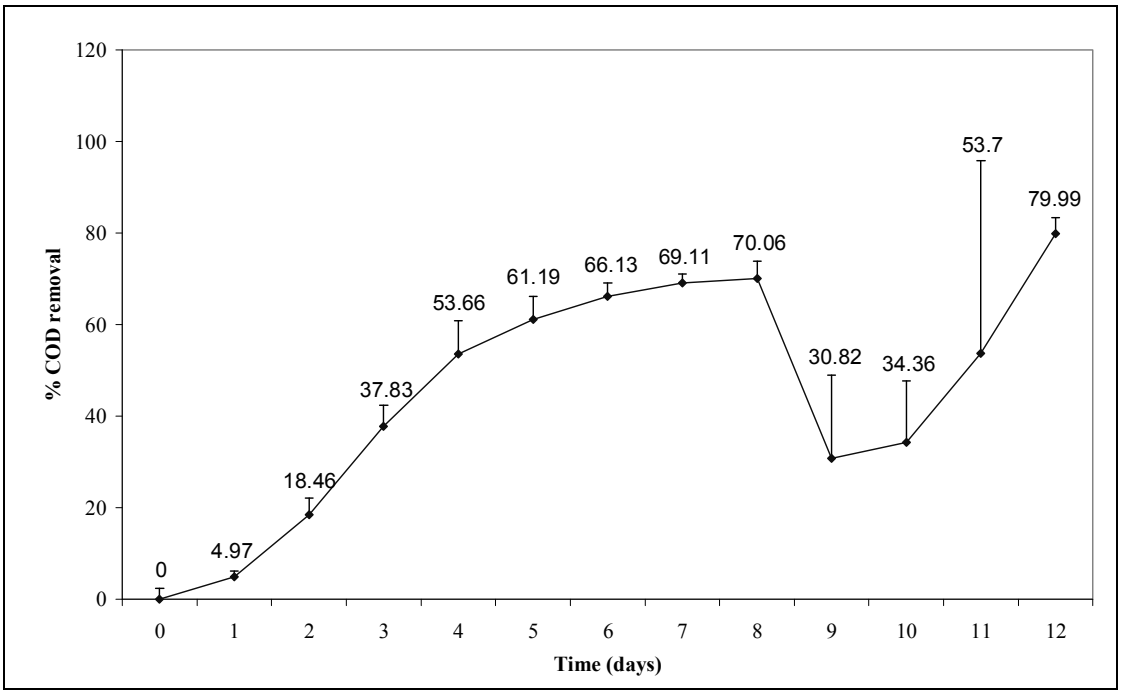

Figure 5.3: Cumulative COD removal from wine distillery effluent by *P. sanguineus* over 12 days (n = 3)

Figure 5.4 shows the percentage polyphenol removal over the 12 days of the test. The maximum polyphenol removal occurred by day 10 (55.96%), and although this was still lower than the 12 day experimental value (61.77%) achieved in the first test and the predicted value (67.9%), it indicated that the retention time of the fungal treatment could be decreased from 12 to 10 days. The lower polyphenol removal could have been due to contamination as the flasks had to be opened daily for collection of samples. It must be noted that some increases in the polyphenol concentration did occur, for example on days 2, 5 and 11, but the cause is unknown.

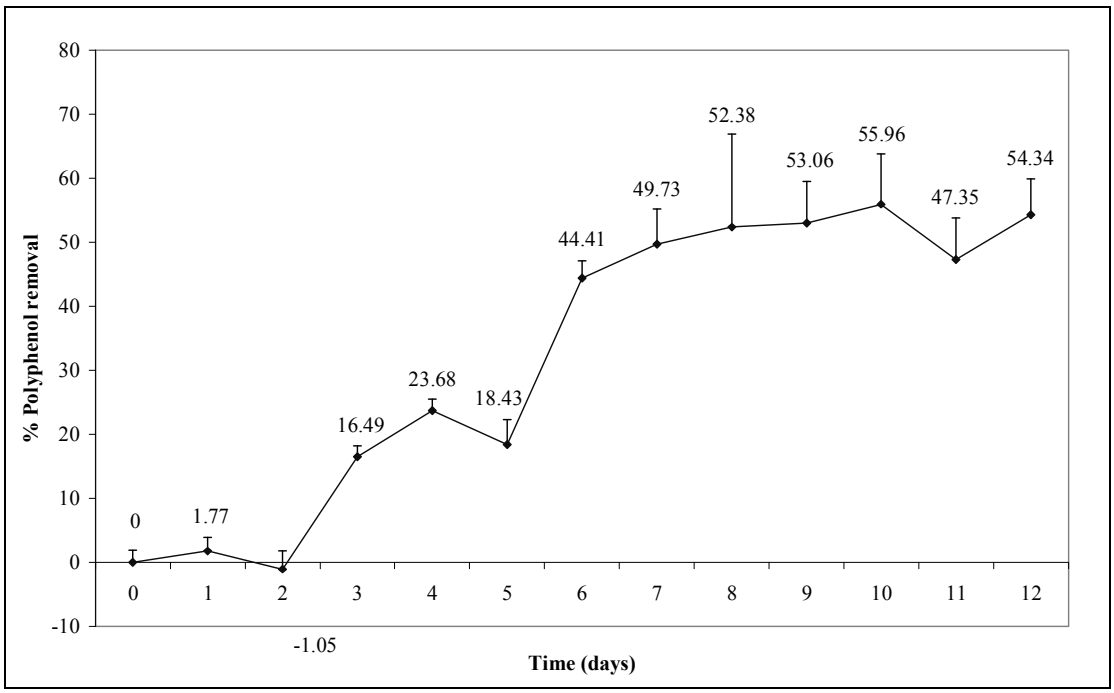

Figure 5.4: Cumulative polyphenol removal from wine distillery effluent by *P. sanguineus* over 12 days (n = 3)

It was found that laccase production by *P. sanguineus* under these test conditions was highly erratic (data not shown). As explained earlier, since laccase was also detected in the uninoculated control experiment, it was thought that polyphenols present in the effluent interfered with the assay when laccase was either not present or present in very small amounts. Laccase was detected at a maximum of 1.12U/l (standard deviation of 1.20) on day 10, however 1.06U/l was detected in the control experiment on the same day (standard deviation of 0.29). Although this fungus is known to produce more than 1200U/l laccase as its sole polyphenoloxidase when grown in other industrial wastewaters such as those from the textile dye industry (Pointing & Vrijmoed, 2000), it can be concluded that there was little to no laccase production by *P. sanguineus* in 30% wine distillery effluent, under the conditions stated.

5.3.5. pH adjustment experiments:

As mentioned in Section 5.2.6, parallel research in this laboratory showed that the optimal pH for laccase production by *T. pubescens* in undiluted effluent was 5.3 (Pers. Comm., P.J. Strong, unpublished data). Although the Box-Behnken experiment did not identify this fungus as the best candidate for COD removal, it was decided that the effect of the pH change on COD removal by this fungus would be investigated, especially as pH adjustment allowed for the fungus to grow in undiluted effluent without nutrient supplementation. This could have implications for the cost of the treatment process.

Although the three fungi tested all tended to increase the pH in the Box-Behnken experiment, *T. pubescens* decreased the pH when grown in undiluted effluent adjusted to pH 5.3 (Figure 5.5). As the difference between the final pH of the two pH adjusted systems was minimal (0.01 pH units), it was concluded that the action of the fungus on pH was not dependent on the chemical used to adjust the pH of the effluent initially.

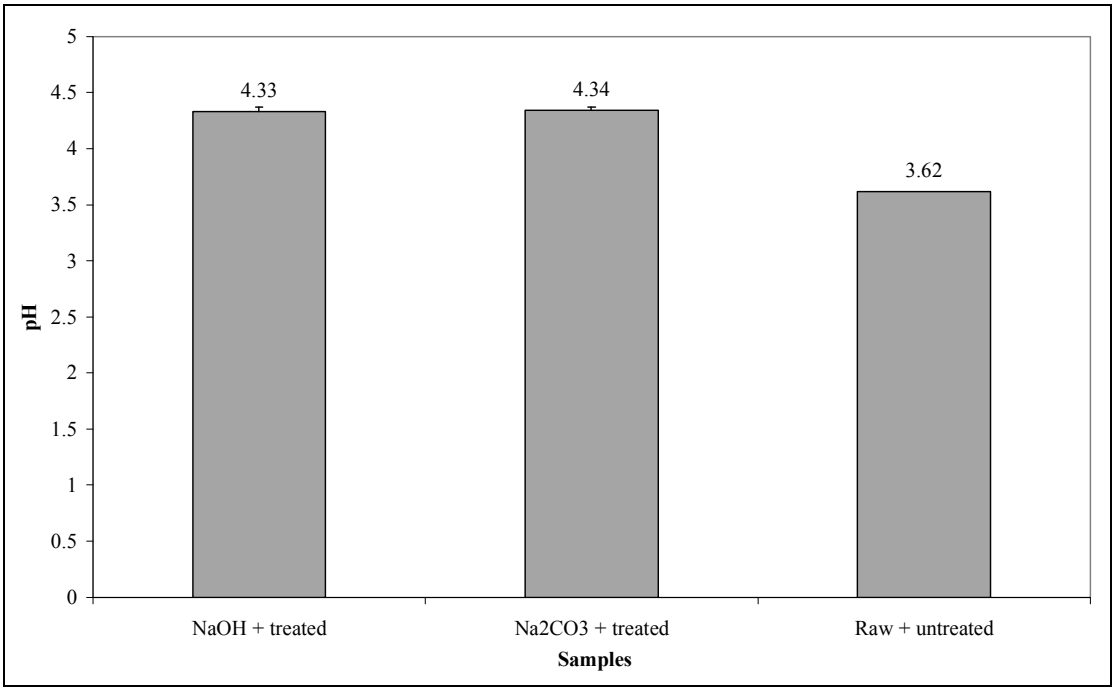

Figure 5.5: pH measurements after 7 days of pH adjusted effluent treated with *T. pubescens* (n = 3)

The COD removal efficiency (52.5%) was lower than achieved by *P. sanguineus* in the Box-Behnken experiment (85%). This implied that higher initial COD concentrations could decrease the COD removal efficiency. There was also little difference in the removal efficiency when the initial effluent pH was adjusted with NaOH or Na_2CO_3, however the highest COD removal was reached in effluent with pH adjusted using Na_2CO_3 (52.5%) (Figure 5.6). For effluent with pH adjusted using NaOH, COD removal occurred rapidly with up to 30% of the COD removed in the first day. Removal increased up to 48% by day 4 then decreased again before reaching a maximum of 51% by day 7. For effluent with the initial pH adjusted to 5.3 using Na_2CO_3, the onset of COD removal was slightly slower. However, by day 2 there was an increase in removal efficiency, which reached a maximum of 49% by day 3. COD concentrations fluctuated before reaching a maximum COD removal of 52.5% by day 7 (Figure 5.6). The COD removal efficiency from wine distillery wastewater in this experiment was comparable to that achieved by white-rot fungi for the degradation of another darkly coloured, phenolic effluent, olive mill wastewater (OMW). The study by Jaouani *et al.* (2003) found that for OMW with 50g/l COD, the four best fungi for COD removal were all white-rot fungi, with removal efficiencies of 49.9% for *Pleurotus sajor caju*, 47.1% for

Pycnoporus coccineus, 41.4% for *Coriolopsis polyzona* and 39.2% for *Lentinus tigrinus*. Furthermore, Figure 5.6 shows that the minimum retention time needed for the highest COD removal efficiency by *T. pubescens* was only 3 days in 100% effluent with pH adjusted to 5.3 using Na_2CO_3. Although the COD removal by *T. pubescens* was lower than that achieved by *Aspergillus niger* on undiluted OMW (61.6% COD removal), the OMW was supplemented with nutrients, whereas the wine vinasse in this study was not supplemented (Hamdi & Garcia, 1993). The COD removal efficiency of 52.5% by *T. pubescens* was found to be comparable to the maximum COD removal of 52.1% from a 50% dilution of beet molasses vinasse by a *Penicillium* sp. within 5 days of incubation (Jimenez *et al.*, 2003). The final COD concentration in the current study, of approximately 14000mg/l obtained after 3 days, did not meet the legal requirements of 75mg/l for disposal (Table 4.4). Effluent recycling or possibly a further polishing step would therefore be required to achieve better effluent quality prior to discharge.

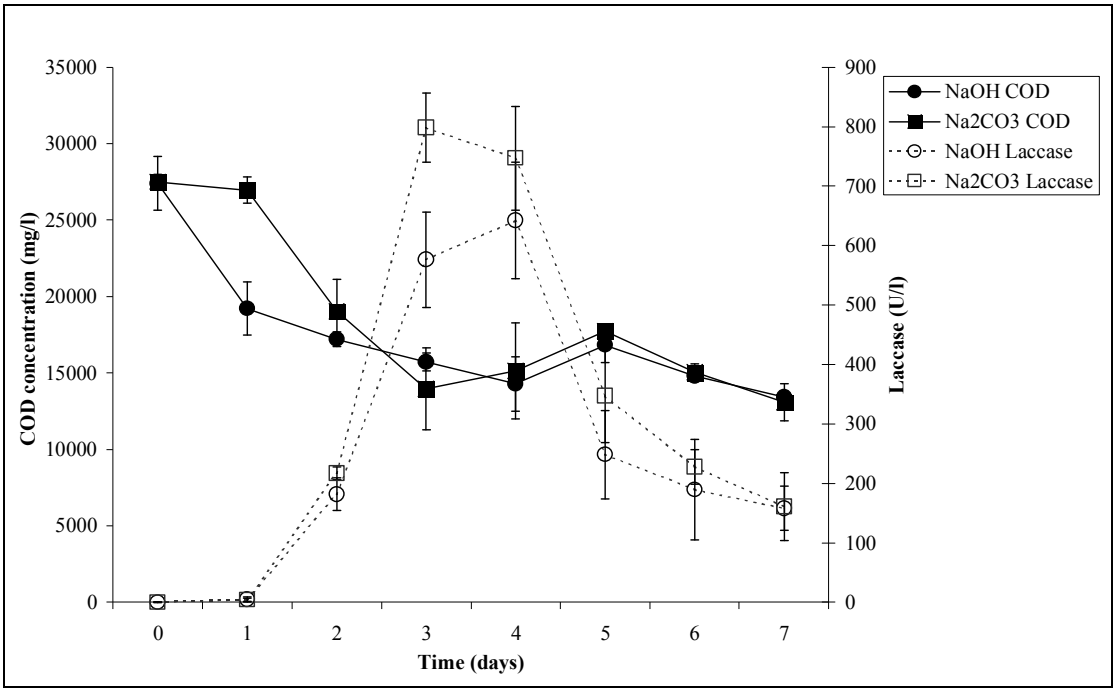

Figure 5.6: COD concentration vs. laccase production by *T. pubescens* over 7 days in wine distillery effluent with pH adjusted to 5.3 using NaOH or Na_2CO_3 (n = 3)

Laccase production was higher in effluent with pH adjusted to 5.3 using Na_2CO_3 than in effluent with the pH adjusted using NaOH (Figure 5.6). Furthermore, the maximum laccase concentration for Na_2CO_3-adjusted effluent (798.5 U/l) was reached on day 3, whereas the maximum reached in NaOH-adjusted effluent (692.5 U/l) was reached later, on day 4 (Figure 5.6). This production exceeded the laccase production by *Panus tigrinus* in an airlift reactor fed with diluted OMW supplemented with sucrose and yeast extract within 7 days (410U/l) (Fenice *et al.*, 2003). The laccase production by *P. tigrinus* was increased to 4600U/l in a stirred tank reactor with a retention

time of 13 days (Fenice *et al.*, 2003). The study determined that impeller speed and aeration rate affected laccase production (Fenice *et al.*, 2003), which implies that laccase production by *T. pubescens* could be improved with optimal reactor design. Furthermore, *T. pubescens* in this study produced maximum laccase by day 3, whereas *P. tigrinus* produced no enzyme before day 5 regardless of the reactor used or the conditions imposed (Fenice *et al.*, 2003). Laccase production can also be affected by immobilization. Free mycelia of a *Trametes* sp. in an optimal medium were found to produce up to 14600U/l, while immobilized mycelia in the same medium achieved 7800U/l laccase activity (Jang *et al.*, 2002). Also, laccase production could be further increased by repeated batch cultures of free mycelia (Jang *et al.*, 2002). As is evident, the laccase production by *T. pubescens* in this study is not optimal, and activity could possibly be increased several-fold using a number of techniques discussed above.

Although the correlation for both NaOH laccase : NaOH COD and Na_2CO_3 laccase : Na_2CO_3 COD was not significant ($r = -0.562$ and $r = -0.659$ respectively, $P > 0.05$, Statistica 7), there did appear to be a modest negative correlation between laccase concentration and COD concentration. The lowest COD measurements occurred on the days of highest laccase production. This was possibly due to the fact that laccase is a broad range enzyme and is therefore capable of acting on and degrading a large number of compounds. As the laccase activity increased, a greater number of compounds that contributed to the COD concentration may have been degraded, resulting in higher COD removal. COD removal was still not as efficient as achieved in the Box-Behnken experiments, but this was most probably due to the higher initial COD concentration as undiluted effluent was used in this instance while 30% effluent was used in the Box-Behnken experiment. Further investigation into the relationship between laccase production and COD removal is required.

As can be seen from Figure 5.7, most of the polyphenol degradation (75.8%) occurred within the first 3 days of inoculation of the effluent (pH 5.3) with *T. pubescens*. Polyphenol removal efficiencies remained high (75% – 80% removal) with the increased initial polyphenol concentrations as undiluted vinasse was used. This is an improvement on the removal of up to 74% of the phenols present in a 50% solution of beet molasses vinasse by *P. decumbens* in 3 days (Jimenez *et al.*, 2003) and 67.8% polyphenol removal by the same fungus from undiluted molasses vinasse (Jimenez *et al.*, 2004). As can be seen, there was little difference in polyphenol removal by the fungus in effluent with the pH adjusted using NaOH and effluent with pH adjusted using Na_2CO_3, although polyphenol degradation was slightly higher in the latter. The treatment of darkly coloured phenolic wastewaters with fungi results in decolourization of the effluent due to the degradation of polyphenols and the adsorption of polyphenols and tannins on the fungal mycelia

(Hamdi & Garcia, 1993; Sayadi *et al.*, 2000). Further adsorption of polyphenols on proteins or mycelial chitin/extracellular polysaccharides due to hydrogen bonds also decreases the polyphenol content of the effluent (Seng, 1988; Sayadi *et al.*, 2000).

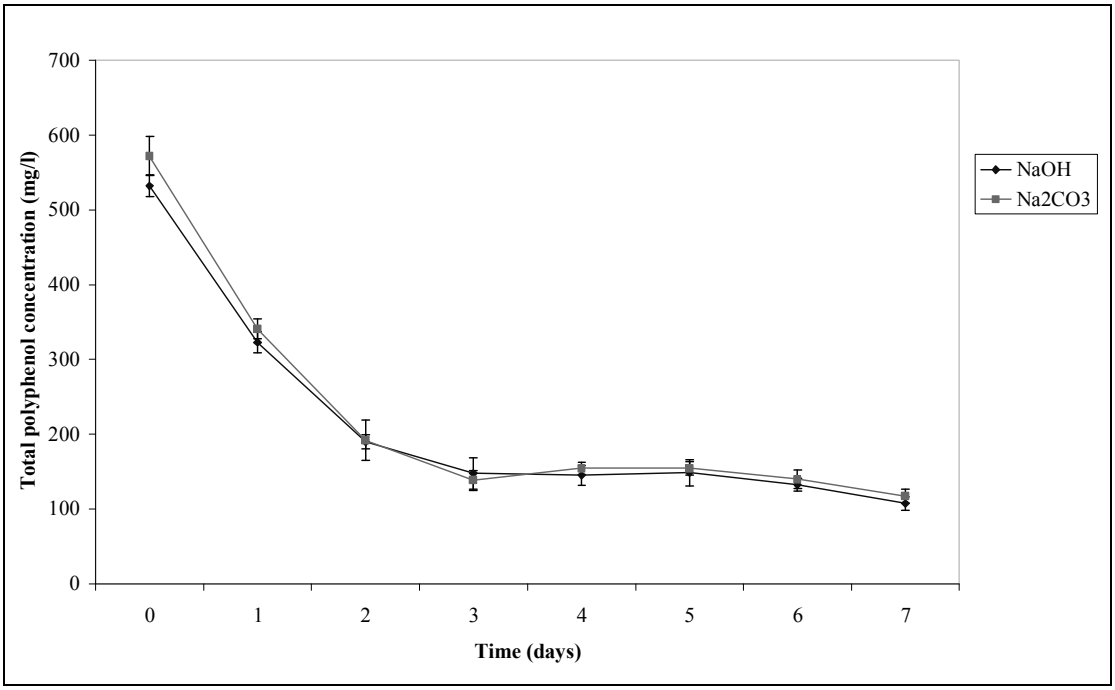

Figure 5.7: Total polyphenol concentration in wine distillery effluent treated with *T. pubescens* over 7 days with initial pH adjusted to 5.3 using NaOH or Na_2CO_3 (n = 3)

Table 5.3: Ammonium concentration in untreated wine distillery effluent and effluent treated with the fungus *T. pubescens* for 7 days (n = 3)

Sample	Average NH_4 concentration (mg/l)
NaOH + treated	<0.3
Na_2CO_3 + treated	<0.3
Untreated	0.2
NaOH + untreated	0.3
Na_2CO_3 + untreated	0.3

A 1 in 5 dilution of the treated samples was the lowest dilution that could be used for the ammonium test without interference. The ammonium concentration in the treated samples were well below the level of detection of the test kit, however less dilute samples could not be used as samples tended to solidify, probably due to the extracellular polysaccharides produced by the fungus. The ammonium concentration of the raw effluent (pH 3.62) was only slightly elevated from 0.2mg/l to 0.3mg/l with pH adjustment to pH 5.3. Although the initial concentration of ammonium in the untreated effluent was low (0.3mg/l), treatment did decrease the ammonium concentration further (Table 5.3). As ammonium-N is considered a N-nutrient source, it can cause eutrophication of water sources and the increase of other organisms at the site of disposal, therefore although the

ammonium concentration in raw effluent is lower than the legal limits for disposal (Table 4.4), the lowest possible ammonium concentration in the process effluent was desirable.

According to Standard Methods (APHA *et al.*, 1998), the determination of nitrate concentration is often difficult due to the high probability that interfering constituents will be present and the limited concentration ranges of the techniques. This was most probably the case in the current study as the variation (standard deviation) even between duplicate samples was very high (data not shown). Conclusive statements regarding the effect of treatment of wine distillery effluent with *T. pubescens* on nitrate concentration could therefore not be made.

The orthophosphate concentration in the untreated effluent was very high (369mg/l) and well beyond the legal limit of 10mg/l for discharge into a water source as set out by the National Water Act (Table 4.4). As is evident from Table 5.4, the treatment of wine distillery effluent by *T. pubescens* was very effective at reducing the levels of orthophosphate (by over 99%) to below the legal requirement within 7 days. This analysis also showed why supplementation with little phosphate was necessary for growth (refer to Table 5.1), as there was sufficient phosphate available in the effluent. pH adjustment did not greatly affect the orthophosphate concentration.

Table 5.4: Orthophosphate concentration in untreated wine distillery effluent and effluent treated with the fungus *T. pubescens* for 7 days (n = 3)

Sample	Average PO_4^{3-} concentration (mg/l)	Standard Deviation
NaOH + treated	2.33	0.41
Na_2CO_3 + treated	2.5	0.45
Untreated	369	1.41
NaOH + untreated	370	2.83
Na_2CO_3 + untreated	392	0

Although the initial total chlorine content of wine distillery effluent was low (2.35mg/l), it was still higher than the legal limits for discharge (0.25mg/l) as set out by the National Water Act (Table 4.4). An unexpected result of the fungal treatment process with *T. pubescens* was that, not only was the fungus unsuccessful at decreasing the total chlorine content, it in fact increased it after 7 days of treatment from 2.4-2.45mg/l to 19.2-26.08mg/l (Table 5.5). Although it is still unclear at this point as to why this would occur, the dechlorination of chlorinated polyphenols is a possibility.

Table 5.5: Total chlorine concentration in untreated wine distillery effluent and effluent treated with the fungus *T. pubescens* for 7 days (n = 3)

Sample	Average Cl_2 concentration (mg/l)	Standard Deviation
NaOH + treated	19.2	2.51
Na_2CO_3 + treated	26.08	4.04
Untreated	2.35	0.07
NaOH + untreated	2.4	0
Na_2CO_3 + untreated	2.45	0.07

As the Box-Behnken experiment predicted that *P. sanguineus* was the best candidate for COD removal, it was decided that the effect of pH adjustment of the effluent to 5.3 on COD removal and laccase production by this fungus would also be investigated. The adjustment of the pH of the initial effluent to 5.3 was accomplished with Na_2CO_3 as the results from the same experiment using *T. pubescens* showed that pH adjustment with Na_2CO_3 produced the most desirable results, especially in terms of higher and more rapid laccase production (Figure 5.6).

The effluent pH exhibited a very slight increase between day 0 and day 2 and after decreasing slightly until day 4, increased steadily to 7.81 by day 7 (Figure 5.8). This increase in pH would be advantageous if the effluent from the fungal treatment process was to undergo anaerobic digestion prior to disposal as anaerobic processes require a more neutral influent pH (Moosbrugger *et al.*, 1993a, 1993b; Wolmarans & de Villiers, 2002).

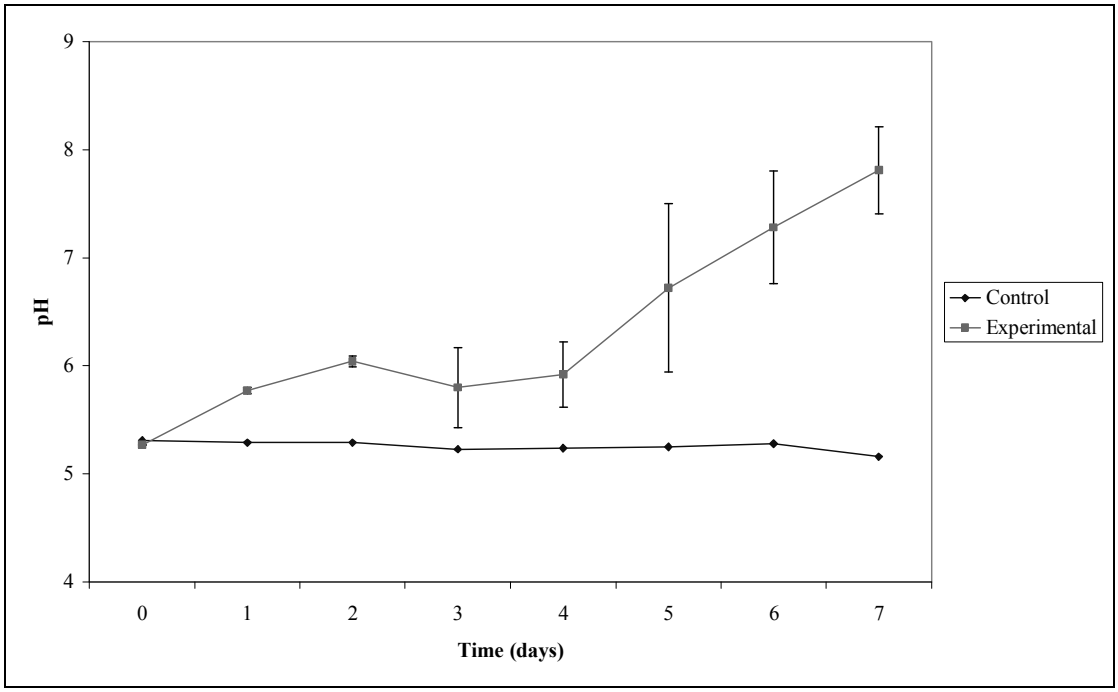

Figure 5.8: Change in pH over 7 days of wine distillery effluent (initial pH 5.3) treated with *P. sanguineus* (n = 2)

The maximum COD removal efficiency (54.26%) was very low compared to the results from the evaluation of predicted performance experiment (Test 2) where approximately 70% removal was achieved by the same fungus by day 7 (Figure 5.3). The current experiment was, however conducted using undiluted effluent whereas the Box-Behnken experiment utilized 30% effluent. Therefore there was a large difference in the initial COD concentration. This decrease in COD removal efficiency can be expected as Jaouani *et al.* (2003) found that the COD removal efficiency of white-rot fungi in OMW decreased between 20-28% for a 50% increase in influent COD. In undiluted effluent (pH 5.3), the COD removal reached 36% by day 5 and a maximum of 54.26% by day 7 (Figure 5.9). The COD removal efficiency by *P. sanguineus* under these conditions was only slightly better than that by *T. pubescens* under the same conditions (52.5%) (Figure 5.6). The decreased COD removal efficiencies were comparable to the decreases in COD removal efficiency due to increased influent COD found by Jaouani *et al.* (2003). Even though the COD removal by both these fungi were lower than that achieved for sugar cane vinasse using an aerobic rotating biological reactor (64% COD removal), the latter was achieved after 23 days whereas the fungi achieved over 50% COD removal within a week. Although the COD concentration decreased by 54.26% in the presence of *P. sanguineus*, the effluent COD remained 11 800mg/l after 7 days, which was far higher than the legal disposal limits of 75mg/l (Table 4.4). Either the influent would have to be diluted or the process effluent will have to undergo a polishing step in order to further decrease the COD concentrations.

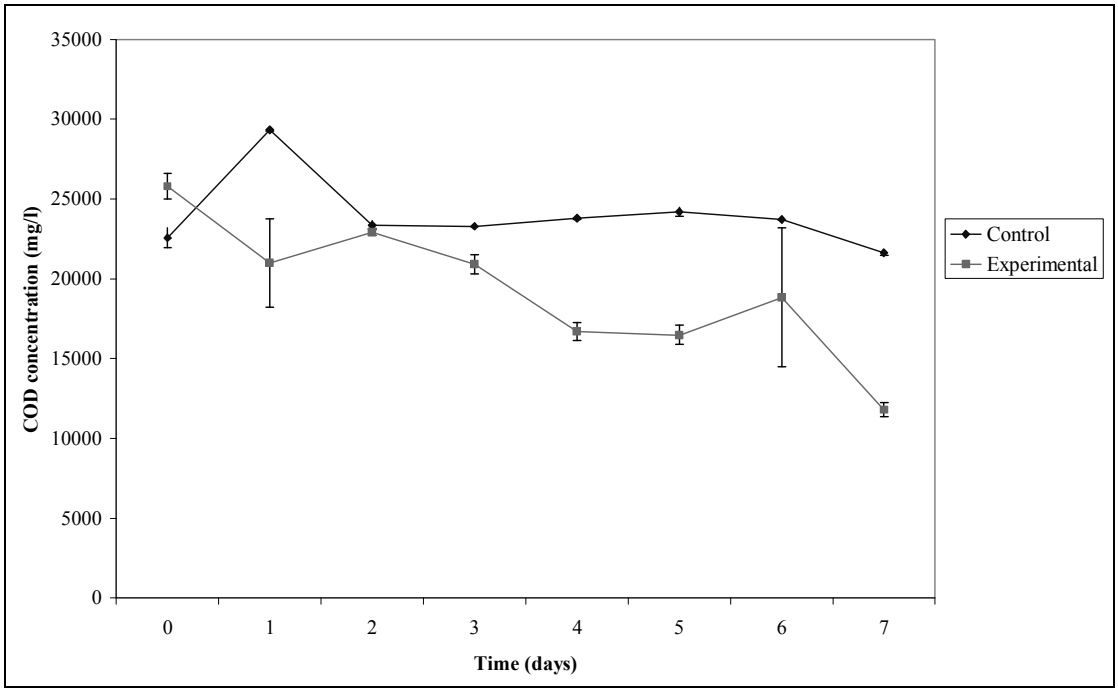

Figure 5.9: COD concentration in wine distillery effluent (initial pH 5.3) treated by *P. sanguineus* over 7 days (n = 2)

As can be seen from Figure 5.10, the percentage polyphenol removal (20.64%) was very low when compared to removal by the same fungus during the Box-Behnken experiment (61.77%) (Table 5.2). This could be expected as the extent of phenol degradation by fungi and the time required for degradation is known to vary as a function of the initial phenol concentration of the medium (Santos & Linardi, 2004). For example, the polyphenol removal efficiency of *Pleurotus ostreatus* treating OMW decreased from 80% removal with 150mg/l influent polyphenol to 50% removal with increased influent polyphenol concentrations (Aggelis *et al.*, 2003). Furthermore, the polyphenol removal in this study was erratic, with the highest polyphenol removal after 7 days at just 20.64% compared to the 80% removal after the same time in the previous experiment using *T. pubescens* (Figure 5.6). This fungus was therefore incapable of achieving high polyphenol removals when the initial concentrations were high. Effluent dilution or recycling could improve the polyphenol removal efficiency of the fungus. In addition, the starting inoculum, i.e. the growth phase of the inoculum and the amount of biomass, could also be a very important parameter affecting phenol degradation, especially when the fungus is cultivated in a complex phenolic medium (Santos & Linardi, 2004). Therefore, optimizing the inoculum could also improve the polyphenol removal efficiency of *P. sanguineus*.

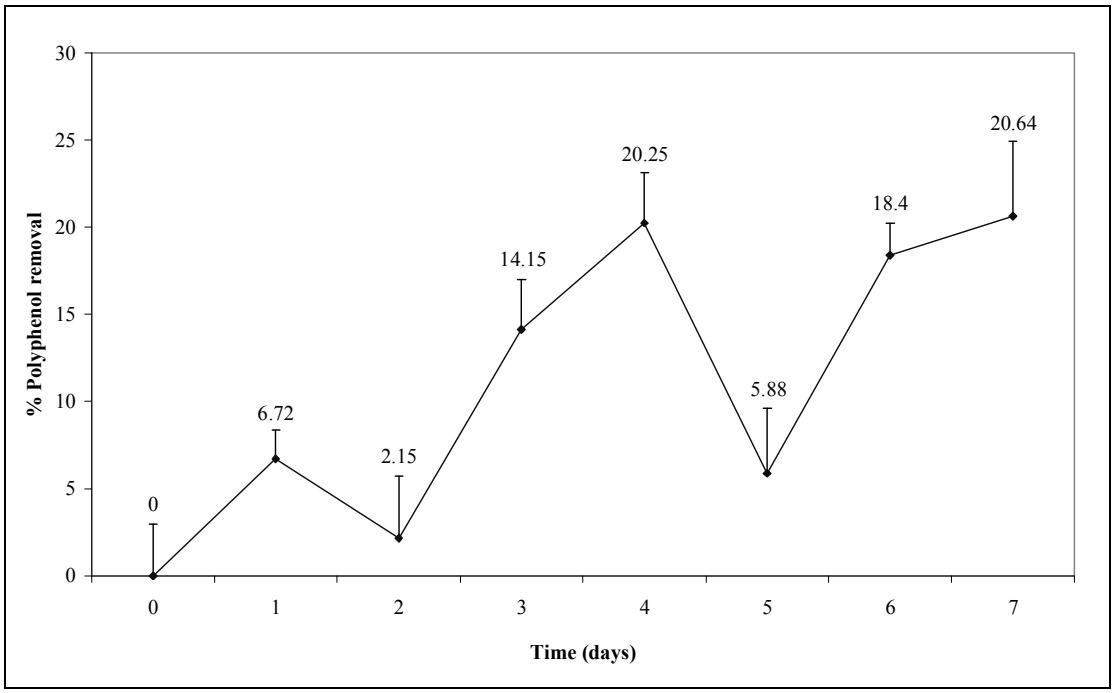

Figure 5.10: Cumulative polyphenol removal from wine distillery effluent (initial pH 5.3) by *P. sanguineus* over 7 days (n = 2)

After 7 days, the laccase produced (6.87U/l, Table 5.6) was still much lower than that achieved by *T. pubescens* after 3 days (798.5U/l) (Figure 5.6). The standard deviation within sample duplicates was, however, large (Table 5.6), indicating that the values were unreliable. Longer retention times

could improve the laccase production by the fungus, as the laccase activity had started to increase by day 7. The low laccase production by *P. sanguineus* was unexpected as this fungus is known to produce laccase as its sole polyphenoloxidase with production >1200U/l in defined liquid media with dye pollutants present (Pointing & Vrijmoed, 2000). Other studies show that this fungus is able to produce as much as 9500U/l in liquid media supplemented with ferulic acid (Herpoel *et al.*, 2000). However laccase activity can differ even within different strains of a fungus (Herpoel *et al.*, 2000). The relationship between COD removal and laccase production was not obvious for this fungus, although the highest COD removal was achieved on the same day as the highest laccase production, i.e. day 7.

Table 5.6: Analysis of wine distillery effluent (initial pH 5.3) treated with *P. sanguineus* for 7 days (n = 2)

Parameter	Control	Experimental
Laccase (U/l)	0	6.87 (8.80)
Ammonium (mg/l)	4.06 (0.03)	1.72 (0.62)
Nitrate (mg/l)	102.75 (5.30)	65.5 (4.60)
Orthophosphate (mg/l)	156.3 (3.39)	76.6 (17.39)
Chlorine (mg/l)	19.41 (0.10)	12.78 (2.28)

Note: Standard deviations given in ( ).

The ammonium concentration in the initial undiluted effluent (pH 5.3) was higher (4.06mg/l) than it was when *T. pubescens* was used for treatment (0.3mg/l). This illustrated the variability in the wine distillery effluent batches. *P. sanguineus* was effective at removing 57.76% of the initial ammonium concentration over 7 days to 1.715mg/l. This removal was sufficient to meet the general ammonium discharge limit of 3mg/l.

Unlike with the previous experiment, there appeared to be few constituents present in this batch of effluent that interfered with the nitrate test as the standard deviation among samples were low (Table 5.6). *P. sanguineus* removed 36.25% of the initial nitrate concentration over 7 days but the final nitrate content (65.5mg/l) was still above the general discharge limit of 15mg/l (Table 4.4). Therefore nitrate removal by *P. sanguineus* from undiluted effluent (pH 5.3) was insufficient and a longer retention time or polishing step is required to decrease the nitrate content.

Compared to *T. pubescens* which achieved >99% orthophosphate removal (Table 5.4), *P. sanguineus* was very inefficient at orthophosphate removal and exhibited only 51% removal (Table 5.6). The initial concentration of orthophosphate was lower than that of the effluent used for *T. pubescens* treatment, which again indicated the variability in composition of wine distillery effluent

between batches. The orthophosphate removal by *P. sanguineus* was insufficient as the resultant concentration (76.6mg/l) was still much higher than the discharge limits (10mg/l) as set out by the general authorizations in terms of the National Water Act (Table 4.4).

Although the reduction in total chlorine concentration was small, the chlorine concentration did not increase as was observed with *T. pubescens* (Table 5.5). The discharge limits for free chlorine set out in terms of the National Water Act is low (0.25mg/l) (Table 4.4), so although *P. sanguineus* did remove some chlorine, this removal was insufficient as the resultant effluent chlorine content (12.78mg/l) (Table 5.6) remained above the legal disposal limit.

5.4. Conclusions:

The purpose of the ETSA, which is to save resources such as time and money, would be defeated if the methods used to obtain the relevant missing data are lengthy and expensive. The use of software such as Design-Expert fits in well with the philosophy of the ETSA as it is an efficient means of obtaining the relevant data rapidly and without unnecessary repetition. The confirmatory studies described in this chapter showed that Design-Expert could be used to design experiments with the minimum number of runs and that the models generated accurately predicted the responses of the fungal treatment system. According to the data obtained during the current study, a fungal treatment system optimized for COD removal required the fungus *P. sanguineus* and very little, if any, additional nutrients.

When the optimal solution for COD removal was tested, the experimental results proved to be similar to the predicted values. The optimal solution resulted in high COD removal (85%) and polyphenol removal of >60% (Table 5.2). The conditions for this response however, required a 30% effluent concentration, which implied that water costs would be incurred in the process for dilution requirements. Furthermore, these conditions produced little, if any, laccase enzyme. Therefore, in a large scale process, cost recovery by laccase extraction and sale would not be likely.

Undiluted effluent with the pH adjusted to 5.3 resulted in highly increased levels of laccase production by *T. pubescens* relative to *P. sanguineus*. The COD removal under these conditions remained just over 50% for this fungus, even though polyphenol removal was high (75% - 80%). Although a slight increase in laccase production was noted for *P. sanguineus* under these conditions, the increase was minimal. Furthermore, the value for laccase activity may have been overestimated as the assay is inaccurate for very low concentrations of the enzyme due to

interference from the polyphenols present. These conditions also drastically reduced the COD and polyphenol removal activity of *P. sanguineus* when compared to its performance under the optimal solution generated by Design-Expert. It can be concluded that if the process is to be used as a treatment system for wine distillery effluent with the aim of recovering costs by laccase production, a compromise will have to be reached between conditions optimal for COD removal and those for laccase production.

Whichever conditions are used, the COD removal was never sufficient to reduce the COD content to below the discharge limits according to the National Water Act. Furthermore, although *T. pubescens* was able to reduce the ammonium and orthophosphate concentration below the legal disposal limit, all the other parameters measured remained too high for discharge. Moreover, *T. pubescens* seemed to increase the total chlorine content of the effluent, although the reason for this increase is unknown. *P. sanguineus* was only able to reduce the ammonium content to below the legal discharge limits. Although the other parameters were also reduced, the reductions in concentrations were insufficient for the resultant effluent concentrations to obtain levels below the legal limits.

At this point it was decided that the best option for fungal treatment would utilize *T. pubescens* in undiluted wine distillery effluent with the pH adjusted to 5.3. Although the COD removal efficiency by this fungus was lower (52.5%) under these conditions (Figure 5.6), the COD removal was still comparable to other fungal processes treating similar wastewaters (Jaouani *et al.*, 2003; Jimenez *et al.*, 2003). Furthermore, this system would possess the added advantages of treating raw, undiluted effluent and commercially viable production of valuable laccase (Figure 5.6). This decision was therefore based on the economic advantages of the treatment system using *T. pubescens*. Moreover, it must be noted that this was not an optimized process and higher COD removals and laccase production could potentially be achieved with optimal reactor design and effluent recycling or repeated cultures.

In the following chapter, the results of the laboratory experiments are used to assess the likely sustainability and competitiveness of the novel fungal treatment system, using *T. pubescens*.

CHAPTER VI: ETSA FOR FUNGAL TREATMENT OF WINE DISTILLERY EFFLUENT AND LACCASE PRODUCTION

6.1. Introduction:

Environmental pollution is a worldwide phenomenon that can be addressed by cleaner production practices, environmental management policies, environmental legislation and treatment of process effluents prior to safe disposal. Even with implementation of the above strategies, industrial effluents, for example from the process of wine distillation, still need to be treated before discharge into the environment. Remediation of industrial effluents can be expensive and complex and it is therefore imperative that simple, efficient, cost-effective and low-risk treatment options be developed.

The preliminary ETSA identified that certain information required to conduct an ETSA on fungal-based treatment of distillery effluent was not available. Chapter 5 described how experimental design was used to gather this data efficiently. It is now possible to complete the ETSA for the novel process for the treatment of wine distillery effluent. The optimal outcome of the fungal process would be the treatment of vinasse while simultaneously producing by-products with some commercial value. As concluded in Chapter 5, the best option for this system is the treatment of undiluted, pH adjusted (5.3) effluent with *T. pubescens*, as this treatment system obtained COD removals comparable to other fungal treatment systems while retaining high laccase production. The ETSA is based on this design.

6.2. Process Description:

As the fungal treatment of wine distillery effluent is still an emerging technology, the description of the process must be based on a conceptual design (Figure 6.1). Further experimental research is still necessary in order to finalize the process design. A two-stage aerobic-anaerobic process may improve the degradation of distillery effluent (Hamdi & Garcia, 1993; Jimenez *et al.*, 2003; Jimenez *et al.*, 2004).

The effluent leaves the distillation plant at very high temperatures of approximately 70–80ºC (Wolmarans & de Villiers, 2002) and as the fungal process is mesophilic (28ºC) it would have to

pass into holding tanks or through a cooling tower to decrease the temperature. If future studies indicate that polyphenol/tannin removal is cost-effective and a suitable market is found for the products, this process may occur upstream of the fungal system. It is not, however necessary, as polyphenol removal does not significantly reduce the COD content of vinasse, and the fungus is capable of treating undiluted effluent, without the removal of any components.

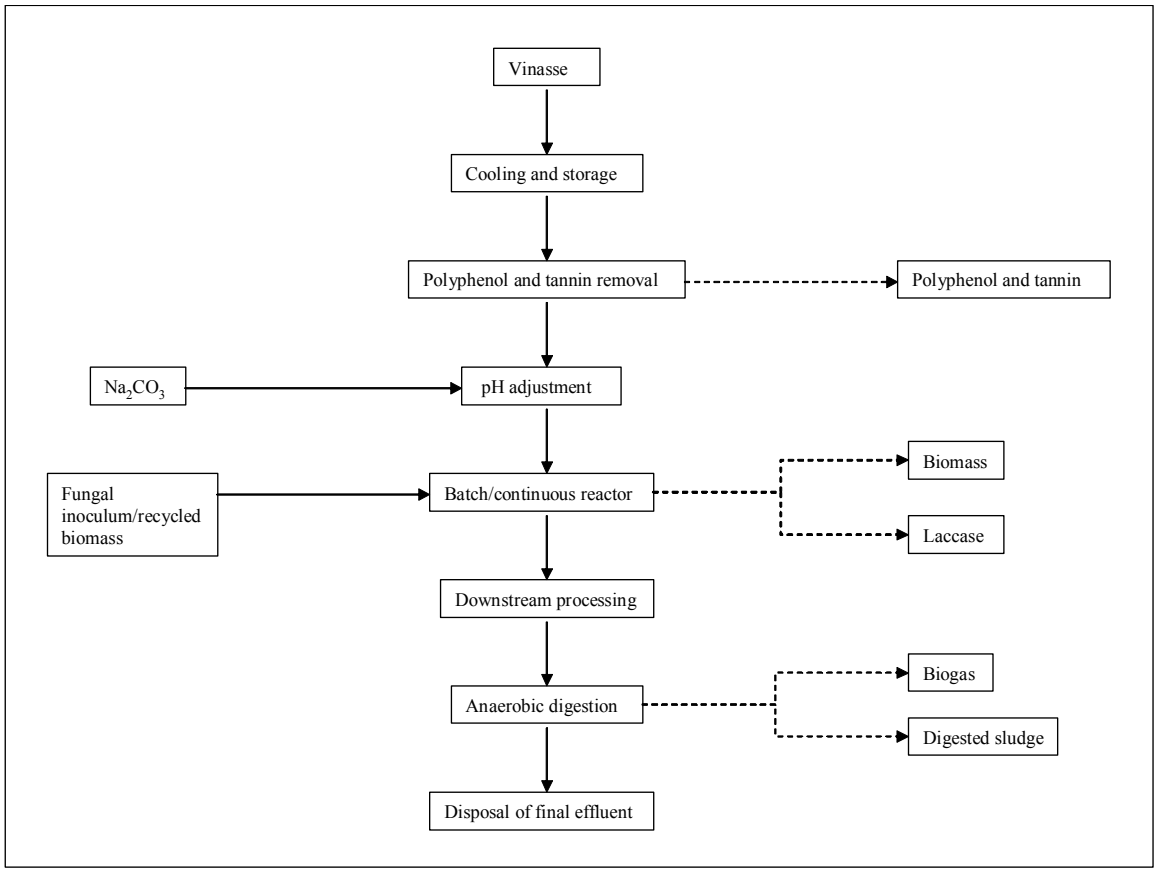

Figure 6.1: Conceptual design of the system for the treatment of wine distillery effluent, indicating the key steps as well as the inputs and outputs of the system

The pH would then need to be adjusted to 5.3 for maximum laccase production (Section 5.3.5). Research still needs to be conducted into the optimal reactor configuration for aerobic treatment with white-rot fungi. The downstream processing (DSP) of the reactor effluent would include biomass removal and laccase extraction. The biomass removed may be recycled to the reactor. According to the studies described in Chapter 5, the resultant effluent does not meet the legal requirements for disposal, and will have to undergo a further treatment step. This step would most likely be anaerobic digestion as previous studies by Hamdi & Garcia (1993), Jimenez *et al.* (2003) and Jimenez *et al.* (2004) have shown that fungal pre-treatment of organic effluents:

- increases the biodegradability of the effluent in the anaerobic system;
- reduces the amount of dilution water required for anaerobic digestion;
- reduces the retention time of the anaerobic system;

- enhances the performance of the anaerobic system; and
- increases the stability of the anaerobic treatment system.

If the two-stage fungal and anaerobic process resulted in an effluent that meets the legal requirements, it will be discharged into a suitable water source. If not, the effluent would be discharged onto land as a supplement to irrigation water, provided the effluent meets the requirements for land application.

6.3. Hazard Identification and Characterization:

As the treatment and downstream processes should be situated on-site, the hazards associated with the transport of industrial effluents are avoided. The following are the hazards that may be associated with the fungal treatment process:

Raw effluent:

The potential hazards that the raw effluent poses to humans and the environment are discussed in Section 3.3.

pH conditioning:

The hazards associated with the storage and handling of alkali chemicals used for pH adjustment are discussed in Section 4.3.

Downstream processing:

Again, this should be a closed system, with the reactor effluent flowing through a separation system to remove biomass, then a purification system to extract laccase. *T. pubescens* biomass is not known to be pathogenic, however if the biomass is to be sold as animal feed, extensive research is required concerning the toxicity of the biomass.

Furthermore, the laccase produced is also not known to be harmful to humans or the environment. The purification columns such as ion-exchange columns that could be used for laccase extraction should be re-usable in order for economical extraction. However, further research regarding the downstream process and its waste outputs are necessary in order for conclusions on the hazards of the extraction process to be drawn.

Treated effluent:

The impact of the treated effluent is dependent on its fate. If, as suggested, the effluent undergoes anaerobic digestion as the secondary treatment, the hazards associated with anaerobic digestion are applicable (Section 4.3). If the effluent is diluted and distributed onto land, the hazards associated with land application are applicable (Section 3.3).

6.4. Exposure and Risk Assessment:

Table 6.1 rates the hazards associated with the fungal process using the ratings table (Table 3.2). The risk value was calculated and the risk classified according to the classification table (Table 3.3).

Table 6.1: Classification of risk associated with the fungal treatment of wine distillery effluent

Process	Factor	Impact	Rating			Risk Value	Risk classification
			Severity	Probability	Period		
Raw effluent		Human health	3	2	2	12	Medium
		Environment	3	2	2	12	Medium
pH adjustment	Alkali storage	Human health	4	2	2	16	Medium
		Environment	3	2	2	16	Medium
Reactor contents		Human health	3	2	1	6	Low
		Environment	3	2	1	6	Low
Downstream processing	Biomass	Human health	ND	4	3	ND	ND
		Environment	ND	4	3	ND	ND
	Laccase purification	Human health	2	2	1	4	Low
		Environment	2	2	1	4	Low

Note: ND = not determined

Raw effluent:

As the process will be a closed system, it is unlikely that either humans or the environment will be in contact with the raw effluent or the reactor contents for an extended period of time unless mishaps such as a pipe burst occurred. As mentioned in Section 4.4, if such incidents occur, they should be repaired quickly, thereby reducing the period of exposure.

pH adjustment:

The impacts of alkali storage are discussed in Section 4.4.

Reactor contents:

The probability of the environment or humans being exposed to the reactor contents is low because it would be a closed system. If cleaning of the reactor is required, it would be done at the end of a treatment cycle. The reactor would then be allowed to empty into the downstream process before cleaning commences, reducing exposure to the reactor contents.

Downstream processing:

Humans and animals could be exposed to the dried biomass if the biomass is sold as animal feed or as a biosorbent (Section 6.8). Therefore the probability of exposure of humans, animals and the environment would be definite. The severity of exposure to humans and animals cannot be assessed as, although the fungus is not known to be pathogenic, toxicity and other hazards-related information is unavailable.

Laccase from *T. pubescens* is not known to have any severe effect on humans or the environment, thereby reducing the severity of the hazard. Furthermore, the purification system should be a closed system thus there is little probability of exposure. Exposure through a burst pipe or other occurrence is unlikely assuming regular maintenance and checks, and the period of exposure if it did occur would be low as the system should be repaired quickly.

Treated effluent:

As discussed in Section 4.4, the impacts of the treated effluent are dependent on the end fate of the treated effluent. If the treated effluent undergoes secondary treatment by anaerobic digestion, the hazards associated with anaerobic treatment are applicable (Section 4.3 and 4.4). The risk associated with anaerobic digestion may not decrease as similar pre-treated effluents tend to produce a larger volume of hazardous methane during the anaerobic stage (Hamdi & Garcia, 1993; Fenice *et al.*, 2003; Jimenez *et al.*, 2003; Jimenez *et al.*, 2004). If the treated effluent is diluted and applied to land, the hazards associated with land irrigation are applicable (Sections 3.3 and 3.4), although the risks associated with this process could be reduced from a high to a medium risk process due a reduction in the organic load of the effluent before irrigation.

6.5. Social Impacts:

One of the most significant social problems regarding the traditional disposal of wine distillery effluent in evaporation ponds is the release of foul odours. The fungal process itself is aerobic and does not generate foul smelling gases, therefore odours will no longer be problematic. However, as

the fungal process will more than likely be followed by an anaerobic "polishing" step, some odours may still be generated although due to the reduced strength of the feed to the anaerobic digester, the chances of digester failure and therefore odour production are diminished.

This process could have further positive social impacts in the form of job creation, which was assessed according to the method described in Section 3.5. It also allows for capacity development in the form of training technicians and operators. Table 6.2 shows the number of potential jobs created during the construction and operation of a fungal treatment process. The treatment process would require at least one trained technician to monitor the system and at least one trained operator would be required for the daily running of the system. A contracted mechanical engineer who is familiar with the system should also be available. Furthermore, at least one contracted consultant with detailed knowledge of the system is needed in order to help resolve any problems that may arise with the fungal treatment step or DSP.

Table 6.2: The number of potential jobs created during the construction and operation of a fungal system treating wine distillery effluent (Low = <3, Medium = 3-10, High = >10)

Job category	Number of jobs	
	Construction	Operation
Skilled and permanent	Low	Medium
Skilled and temporary	Low	Low
Unskilled and permanent	Low	Low
Unskilled and temporary	Low	Low

Significant job opportunities could also arise as a result of downstream processing of the reactor effluent and by-products. Again, an operator and technician will be necessary for the running of the DSP system. As the DSP plant is likely to be on-site with the treatment process, only one engineer and contracted consultant would be needed for both processes.

The use of the fungal treatment process will also benefit society in the long run as the production and sale of laccase could lead to cleaner production systems in other industries. Furthermore, the development of the biomass for sale as animal feed could result in the availability of cheaper feed supplements as the biomass would be cultured on a cost-free waste product (vinasse). Therefore more farmers could afford to purchase the protein supplement in order to increase the quality of their herds. Further research regarding the quality of the biomass as a protein supplement, toxicity and the quantity of biomass that can be produced is required.

6.6. Performance Assessment:

The gaps in the information regarding the performance of the fungal process were addressed in Chapter 5. The fungal system was assessed on its ability to treat the effluent in terms of COD removal, and its ability to produce a valuable by-product, laccase.

The RSM model predicted that *P. sanguineus* would be the most efficient fungus at COD removal from wine distillery effluent at a dilution of 30% vinasse (Table 5.1). Although the fungal treatment was efficient at degrading diluted wine distillery effluent, this efficiency was not sufficient for the resultant effluent to be discharged into a water source as the COD concentration (1632mg/l) remained above the legal limit of 75mg/l. The resultant effluent from this method of treatment could, with pH adjustment, be used legally to irrigate land. Dilution of the effluent could possibly be accomplished by some recycling of the treated effluent. *P. sanguineus*, however produces little, if any, laccase under these conditions (Table 5.2). Under these conditions, although the fungal process could be considered an efficient treatment process, it was not as efficient as anaerobic digestion which can achieve 90 – 95% COD removal (Driessen *et al.*, 1994).

As discussed in Chapter 5, it was decided that the best option for fungal treatment of wine distillery effluent would be utilizing *T. pubescens* in undiluted effluent with the pH adjusted to 5.3, as these conditions allowed for 50% COD removal from undiluted effluent within 3 days (Figure 5.6). Furthermore, these conditions are additionally advantageous as the fungus produced large quantities of laccase, also within 3 days (Figure 5.6) which is of significant economic benefit. It must be noted that this was not an optimized process and higher COD removals and laccase production could possibly be achieved with effluent recycling, repeated cultures and/or optimal reactor design. However, the process under these conditions produced an effluent that still did not meet the legal standards for discharge into a water source or for disposal on land (Table 6.3). A two-stage aerobic-anaerobic process is suggested for post-fungal treatment in order to achieve sufficient degradation of the vinasse for the effluent to meet the legal limits for disposal.

6.7. Regulatory Status:

The effluent from the fungal treatment process will be subject to the legal limits for disposal into a water source (Section 4.7) unless land application is considered as a method of disposal, in which case the reactor effluent will be subject to the regulations stipulated in Section 3.7.

Table 6.3 compares the general limits applicable to the discharge of wastewater into a water resource and for the purpose of irrigation with <50m^3 effluent per day (specified by the General Authorizations in terms of Section 39 of the National Water Act) to the characteristics of undiluted wine distillery effluent (pH 5.3) treated with *T. pubescens*.

Although COD removals comparable to other studies with similar effluents (Jaouani *et al.*, 2003; Jimenez *et al.*, 2003) were recorded for fungal treatment in this study, it did not remove sufficient COD for the resultant effluent to be discharged into a water source or onto land without dilution (Table 6.3). pH adjustment of the resultant effluent would also be necessary, as the pH is slightly below the legal limits for discharge or irrigation (Table 6.3). Dilution or a post-treatment step is required before land irrigation or discharge into a water source is possible. Although *T. pubescens* sufficiently reduced the ammonium and orthophosphate content (<0.3mg/l and 2.5mg/l respectively) below the legal limit, it was unable to do the same for the chlorine content.

Table 6.3: Characteristics of treated wine distillery effluent compared to the discharge/disposal limits

Substance/Parameter	General limits for disposal into water source (mg/l)	General limits for land irrigation (<50m^3/day) (mg/l)	Undiluted effluent (pH 5.3) treated with *T. pubescens*
COD	75	5000mg/l	13 074 (52.5% removal)
pH	5.5 – 9.5	6.0-9.0	4.34
Ammonia-N (ionized and unionized)	3	-	<0.3
Nitrate/Nitrite-N	15	-	Inconclusive results
Chlorine	0.25 (free)	-	26.08 (total)
Total solids	-	-	8423
Orthophosphate (PO_4-P)	10	-	2.5

Note: All parameters aside from pH in mg/l
Note: ND = not determined

As discussed in Section 6.6, fungal treatment is insufficient and a two-stage aerobic-anaerobic process is suggested in order to sufficiently treat the effluent for disposal on land or into a water source.

6.8. Market Size and Information:

Effective treatment of the distillery effluent before disposal is vital if South African distilleries are to remain competitive as the negative views associated with irresponsible production and waste

management would impact negatively on the market for the products. The market for an efficient treatment process for wine distillery wastewater in South Africa is mostly centred in the Western Cape. However, as wine and distillation products are manufactured worldwide, the technology is relevant on a global scale.

According to current trends, the fungal treatment process should be well received and could possibly increase the market value of the wine products, as it is an efficient natural process using natural fungal isolates that have not undergone any genetic modification. Once the fungal treatment of wine distillery effluent is established, the process, with possibly small alterations regarding dilution, retention times, etc. could be adapted to treat similar effluents such as those from molasses, cane sugar, beet sugar, grain and rice wine distillation. Today, people are more willing to pay more for "environmentally friendly products" rather than buying cheaper products whose production processes are harmful to the environment.

The market for industrial enzymes is growing steadily due to improved production efficiency resulting in cheaper enzymes, new applications, new enzymes being screened or re-engineering to improve the properties of traditional enzymes. Furthermore, enzymes have become an indispensable tool in many industries as they are natural catalysts and boost chemical reactions several fold (Nand, 2001). Laccase is a very valuable enzyme that can be used for many purposes as is mentioned in the introduction (Section 1.11). For example, laccase can be a vital commodity chemical for the paper and pulp industry. It will increase the market value of the paper and pulp produced as use of laccase in the process will minimize the amount of toxic pollutants generated in the manufacture of pulp and paper and improve the existing technology in a cost-effective manner. This could be true of other industrial processes in which the use of laccase could replace the use of toxic, pollutant and recalcitrant chemicals. With a view to the immense number of uses of laccase (Section 1.11), the market for this enzyme is vast, ranging from industries that require a crude extract to those that require highly purified enzyme. The value of laccase increases with the enzyme purity (Table 6.4).

The South African market for feed protein is large. During the 1994/1995 season, for example, South Africa had to import 168 000 tons of fish meal and 256 000 tons of oil cake which cost over R600 million in order to meet the demand for feed protein (van der Westhuizen & Pretorius, 1998). A locally produced source of protein, grown in a cost-free medium for example a waste product such as wine distillery effluent, would be cheaper and therefore competitive in the market. Therefore the recovery of biomass from the fungal process for sale could be another option for a

return on costs. This would require a downstream separation step as indicated in Figure 6.1. Since the effluent being treated is organic one and the fungus is not known to be pathogenic, the use of the biomass produced as protein supplement in animal feed could feasible. The amount of biomass produced by *T. pubescens* treating undiluted effluent (pH 5.3) was 2.181g/l. Although not as high as, for example, the fungal biomass (3.7g/l) produced by the MyPro Water Treatment System treating industrial effluents and producing a saleable biomass (van der Westhuizen & Pretorius, 1998), the biomass production by this treatment process could be sufficient to make biomass sale feasible. However further research is required to determine the quantity of biomass that could be produced in an optimized system, which would confirm if biomass recovery could be commercially viable.

Additional research regarding the process and the fungus must be conducted if the biomass is to be marketed. The stability and toxicity of the fungus must be determined. Furthermore, although the treatment process should be fairly sterile, some contamination is always expected. The contaminant populations in the reactor at various times must be non-pathogenic and should be quantified as this is a prerequisite if the biomass is to be used as animal feed (van der Westhuizen & Pretorius, 1998). Information regarding the nutritional value of *T. pubescens* was not found, however this information is vital and should be investigated if the fungal biomass is to be used as feed. Atkinson & Mavituna (1991) stated that the nutritional value of the biomass should be determined in terms of:

- the amino acid profile (especially lysine and methionine), which must be acceptable as the biomass is a source of protein; and
- digestibility, as the test animal should digest the fungal material sufficiently in order for the uptake of amino acids to be possible.

If the research shows that the fungal biomass cannot be sold as feed, it can still be valuable as it can be sold for other treatment process. Kapoor & Viraraghavan (1998) and Zulfadhly *et al.* (2001) for example have shown that viable and dead fungal biomass can be used as an adsorbent in biosorption processes, especially for the removal of heavy metals from aqueous solutions.

Both the public and government are increasing the pressure on industries to reduce or manage their effluent streams to acceptable standards. However, although imperative, the installation of treatment systems is often met with resistance by industry as it is often thought that treatment plants require investment of capital without any return (van der Westhuizen & Pretorius, 1998). The fungal treatment plant however offers a potentially high return on costs from the sale of the high-value by-

products laccase and possibly even the fungal biomass, both of which have large markets. The fungal treatment process embraces the market trends of sustainability, cleaner production practices and value recovery treatment technologies.

6.9. Financial Considerations:

The costs involved in a treatment process are dependent on a number of factors. Normally, treatment of industrial process effluents involves the conversion of dissolved organic matter to solid biological material (van der Westhuizen & Pretorius, 1998). This sludge then requires appropriate disposal. A treatment process that can recover its costs (and even generate profits) by the production of a valuable by-product and/or recovery of organic material (in the form of biomass) that can be marketed is very attractive. The fungal treatment of wine distillery effluent offers both these benefits.

All the steps in the treatment system and downstream processing can be accomplished on-site, therefore transport costs are avoided. As it stands, the minimum retention time required is 3 days (for maximum COD removal and laccase production by *T. pubescens*), therefore the construction and size of holding tanks is dependent on the peak effluent flow rate of the distillery and the reactor volumes. A conditioning tank is necessary in order to adjust the pH of the vinasse. The cost of construction of holding and conditioning tanks can be reduced by modifying the evaporation lagoons already present at most distilleries. Yet another cost involved in the process at this step is the pH conditioning itself which would require the purchase of a suitable chemical. As the fungus tended to decrease the pH of the initial effluent from pH 5.3 to 4.3 (Figure 5.5), effluent recycling cannot be used to adjust the initial effluent pH.

One of the most significant capital costs involved would be the construction and installation of the aerobic reactor. Further research is required in order to determine the best reactor design for both optimal effluent treatment and optimal laccase production. However, these reactors could be constructed to be adaptable to alternative processes when they are not in use such as treatment of similar wastes, for example, in the off-season when the volumes of distillery effluent are much lower. Further research is required to determine if the reactors could be adaptable. Furthermore, the fungal process treats undiluted effluent, which implies that smaller reactors can be used. Another major cost involved in aerobic processes is the supply of oxygen to the microorganism. Careful reactor design would help ensure sufficient oxygen supply at the lowest cost.

Downstream processing would add to both the capital and utility costs involved in the process. The downstream process will have to include a filtration or centrifugation system for the removal of biomass. The biomass recovered has two possible uses: as a protein product for animal feed supplementation and as an adsorbent in biosorption processes. Extensive research regarding the biomass stability, toxicity and nutritional value must be conducted if biomass is to be sold as animal feed as it will have to meet the requirements set out in the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act (Act 36 of 1947). The use of viable and dead fungal biomass for removal of heavy metals from aqueous solutions has also proved successful (Kapoor & Viraraghavan, 1998; Zulfadhly *et al.*, 2001).

Once the biomass has been separated from the effluent stream, laccase can then be recovered. This step would require at least one purification column, such as a suitable ion-exchange or affinity column. Although purification systems can be costly (Internet Reference 16), laccase is valuable and the capital costs can be expected to be recovered and profits generated. Although the costs increase with the complexity of the purification process, the market value of the enzyme increases with purity. The price of the enzyme is also dependent on the fungus from which it is produced. The following (Table 6.4) provides an indication of the price of laccase:

Table 6.4: Price of laccase from different sources and different activities

Fungus	Laccase activity (U/mg)	Quantity	Price (Euros)	Company
Agaricus bisporus	≥ 4	1g	31.80	Fluka Chemicals
Coriolis (Trametes) versicolor	≥ 0.8	1g	44.20	Fluka Chemicals and Sigma-Aldrich
Coriolis (Trametes) versicolor	≥ 20	1g	57.00	Fluka Chemicals
Rhus vernificera	50U/mg (crude acetone extraction) (1U produces a ΔA_{530} of 0.001/min, pH 6.5, 30°C, 3ml reaction volume, syringaldazine as substrate)	10 000 units	124.40	Sigma-Aldrich

The financial considerations discussed regarding anaerobic digestion (Section 4.9) or land irrigation (Section 3.9) may also be relevant, depending on the fate of the treated effluent.

6.10. Energy Impacts:

Aerobic treatment is an energy-demanding process as the energy required for aeration is high although dependent on the reactor configuration. However, the construction of small, intensive

treatment plants should decrease the amount of energy required. Unlike the anaerobic process, aerobic digestion using white-rot fungi does not produce biogas or any other form of recoverable energy. Furthermore, energy will also be required for downstream processes of biomass and laccase recovery. Since the biomass to be sold will have to be dewatered and dried, it is thought that the drying process will require the most energy input. If a two-stage aerobic-anaerobic process is constructed as suggested earlier, the biogas produced during the anaerobic digestion step could possibly be used to offset at least some of the energetic requirements of the aerobic process and/or downstream processes. If the resultant effluent is used for irrigation purposes, the energy impacts discussed in Section 3.10 would be relevant.

6.11. Resource Use/Conservation:

Aside from the energy input, the fungal treatment technology does not require the input of other resources as dilution water is unnecessary because *T. pubescens* is able to treat undiluted wine distillery effluent. Further research regarding DSP is required in order to determine the resources used/conserved by the operation. Water could be necessary for cleaning of the enzyme extraction equipment. Again, the fate of the treated effluent would determine any further resource use/conservation.

6.12. Conclusions:

Treatment with *T. pubescens* using undiluted pH-adjusted effluent was the most feasible process. Although COD removal was slightly lower than achieved with *P. sanguineus* under the same conditions, the laccase production was significantly higher and the necessary retention time shorter. It must be noted that this was not an optimized process. Optimization of the process would usually lead to greater process efficiency in terms of effluent degradation and laccase production. Overall, although the treated effluent did not meet the legal limits for disposal, the fungal treatment process was very attractive in that it has the potential to remove more than 50% of the COD content from undiluted effluent and produce almost 800U/l laccase within 3 days. A secondary treatment process such as anaerobic digestion is recommended in order to further reduce the COD content of the effluent prior to discharge.

Furthermore, the biomass produced as a by-product of the fungal system may prove valuable as a feed supplement which is an added economic benefit. Further research is required to determine the

potential quality, quantity, toxicity and potential hazards of the biomass that could be produced by the system. The biomass could also be used for other treatment processes as an adsorbent.

The final step of the ETSA was to complete the final information module which compares the novel process with the established systems and draw conclusions as to whether the novel process should be developed further.

CHAPTER VII: COMPARISONS, CONCLUSIONS AND FUTURE WORK

7.1. Introduction:

The current study had two main objectives, the first of which was to determine whether an ETSA approach is useful in terms of assessing the future viability of an emerging technology and based on this, to compare the potential viability of a novel fungal technology for treatment to two existing technologies, specifically irrigation and anaerobic digestion.

In this chapter, the last module of the ETSA called the relative technology evaluation is completed. The two most popular methods of disposal/treatment of wine distillery effluent, land application and anaerobic digestion, are compared to the emerging technology, fungal treatment. If the novel alternative proves feasible, sustainable and competitive, its development into pilot-scale should be considered.

Any novel technology basically goes through two stages of development before implementation and development of a full-scale process. These are the laboratory scale and pilot-scale development. An emerging technology should ideally go through two separate ETSAs, at different stages in its development in order to assess the process potential at each stage. On completion of the pilot-scale studies, a new ETSA should be conducted as scale-up poses its own problems and the process may not show the same potential on a larger scale. If the process still demonstrates feasibility and competitiveness, a full-scale system can be established.

7.2.Relative technology evaluation:

A qualitative relative evaluation method was used to compare the three alternative processes for treatment of wine distillery effluent. The previous information modules regarding potential risks to human and the environment, social impacts, process performance, regulatory status, market and financial considerations, energy impacts and resource uses from the ETSA of each process was rated on a scale of 1 to 5, with 1 being undesirable and 5 being highly desirable. Therefore, according to this rating system, the higher the total scores of a process, the higher it desirability and therefore its competitiveness. The results of this process are recorded in Table 7.1. It must be noted that the fungal technology was assessed as a single-stage operation, without considering final disposal via anaerobic digestion or land application.

Table 7.1: Rating and comparison of land application, anaerobic digestion and fungal technology for the treatment of wine distillery effluent (with 1 being undesirable and 5 being most desirable)

Information module	Land application	Anaerobic digestion	Fungal technology
Exposure and Risk Assessment	1	3	4
Social Impacts	1	3	4
Performance Assessment	1	3	3
Regulatory Status	1	3	3
Market size and information	1	3	4
Financial considerations	3	3	4
Energy impacts	3	5	2
Resource Use/Conservation	3	3	3
Total	***14***	***26***	***27***

Exposure and Risk Assessment:

Fungal technology has the least associated probability and severity of hazards to humans and the environment, as it does not produce any toxic by-products such as H_2S, nor is the probability of exposure of gases and effluent to humans and the environment as high as those for land application and anaerobic digestion.

Social Impacts:

The most positive social impacts are offered by the fungal technology in the form of future benefits for farmers in South Africa and training and permanent employment opportunities both during the treatment process and downstream processing. Anaerobic treatment offers permanent employment only to highly skilled people, with detailed knowledge of the often unstable anaerobic process. Employment offered by the irrigation process would mostly be seasonal, during the planting and harvesting periods.

Performance Assessment:

Land application does not effectively treat the effluent. Furthermore, the effects on vegetation are crop-specific, although treatment before disposal does increase the fertilizer quality of the effluent. Long-term damage to the soil is a concern due to the high salt content of the effluent. Anaerobic digestion can be very effective at degrading wine distillery effluent, however the process parameters must be optimal and changes such as shock loads or seasonal variations in effluent composition could result in reactor failure. Furthermore, large effluent dilutions are necessary for efficient

treatment. Although the fungal technology is not as effective at COD removal from wine distillery effluent as anaerobic systems, it is likely to be a more stable process and dilution is unnecessary, therefore the reactors can be smaller.

Regulatory Status:

The distribution of raw effluent on land via irrigation is prohibited by law as the untreated effluent does not meet the legal standards. Effluent applied to land has to undergo primary treatment and high dilution before land application. The treated effluents from anaerobic digestion and fungal technology require less dilution before application to land or disposal to water sources.

Market Size and Information:

Although the application of untreated effluent to land is common, it is now considered irresponsible waste management. Some effective pre-treatment is needed before land application can be legally acceptable and the future market of this technology is likely to be limited. Anaerobic digestion is a well-established treatment process; therefore it has a large share of the treatment technology market. It also produces biogas which can be collected and used to provide the energy for at least part of the process, or sold. Fungal technology however targets three high value markets, the treatment technology, the production of a valuable enzyme that has a multitude of uses and the possible production of biomass for animal feed and/or biosorbent. However, markets are often reluctant to accept new technologies and will more than likely require proof of performance from pilot-scale systems.

Financial considerations:

Land irrigation is currently the cheapest disposal option, but users may face future liability regarding remediation of contaminated water and soil. Anaerobic digesters are very expensive to construct and maintain and the quality of the biogas produced dictates its energy potential and thus its price. As anaerobic reactors can be unstable, the biogas quality, and thus its value, also fluctuates. This instability and fluctuation decreases the score for financial considerations for this technology. The fungal process produces large quantities of laccase. The enzyme is very valuable (Table 6.4) as it can be used in various processes and its price is dependent on the level of purity achieved. The biomass could also be used as a protein supplement for animal feed or as a biosorbent in other treatment processes.

Energy Impacts:

The fungal technology is the most energy intensive of the three processes. Anaerobic technology on the other hand, requires little energy input and the process produces energy in the form of combustible biogas.

Resource Use/Conservation:

Land application of raw effluent requires high dilution rates with fresh water although it can lead to distilleries establishing plantations of trees and other plants. Anaerobic digestion requires little energy input and therefore has reduced requirement of fossil fuels. Excess energy may also be used for other applications such as pumping the effluent from the distillery to the digester. The process does, however require some dilution of the effluent for effective treatment. The fungal technology is energy intensive and does not produce any energy; however no dilution water is required. In the final process design, the fungal process is likely to be coupled with anaerobic digestion, which would reduce the external energy requirements.

From the comparison above (Table 7.1) one can conclude that the practice of land application of raw distillery effluent can no longer continue unchecked as it had the lowest score (14) according to the rating system used.

Anaerobic digestion with a score of 26 (Table 7.1) is a very attractive alternative, however the process instability and production of potentially toxic offgas (such as hydrogen sulphide) lower its desirability. This desirability is further lowered by the high cost of the technology. Although the process generates some form of financial recovery in the production of biogas, the production of high quality, valuable biogas can fluctuate due to the instability of reactors treating high-strength waste streams which have a variable flow rate.

The fungal treatment process compares admirably to anaerobic digestion and receives the highest score of 27 (Table 7.1). As the COD removal efficiency by the fungal technology is not satisfactory, i.e. the reactor effluent does not meet the regulations for discharge, a further post-fungal treatment step such as anaerobic digestion is suggested. The effluent from the fungal process has a lower organic load, which would be advantageous for the anaerobic system. The fungal process out-competes the anaerobic process economically, in that it produces the laccase enzyme as a by-product. The enzyme is very valuable and has a large number of uses (Section 1.11). Furthermore, if the biomass can be sold as animal feed, this will be another valuable by-product of the system.

Based on the information available, the fungal treatment process is the most feasible, especially from an economic standpoint.

It can therefore be concluded that the fungal technology is a feasible, sustainable and very competitive process and the research and development of this system should proceed.

7.3. Critical evaluation of the ETSA approach:

The main aim of the ETSA is to prevent the use of resources on developing a technology from the conceptual phase that would not be competitive. In the current study, the ETSA was effective in determining that a fungal process for the treatment of wine distillery effluent could compete with the current popular treatment options. The ETSA has a holistic approach and compares the technologies on more than one level. For example, although the COD removal by the fungal technology is lower than achieved by anaerobic digestion, the fungal technology still scores higher on the overall rating due to the increased positive social, environmental and financial impacts, and the appeal to more than a single market. The ETSA is therefore considered an effective tool to determine the feasibility and competitiveness of an emerging technology.

The ETSA itself should not be a resource intensive process as this would defeat the purpose of the tool. It is therefore important that a preliminary ETSA is conducted to identify the information that is required and the sources from which they may be acquired. Most of the information, especially for the established processes may be found in literature or obtained from experts. Some experimental analysis may be required, especially regarding the emerging technology. Tools such as experimental design software can be used to minimize the resources spent on experimental data collection and to provide the specific information required for completion of the ETSA information modules.

The comparisons and conclusions drawn are reliable as all the technologies are assessed according to the same criteria and evaluated on the same rating system. Overall, the ETSA can be a very valuable and effective tool in assessing the viability and competitiveness of an emerging technology, with the minimum use of resources.

7.4. Future work:

As the ETSA determined that the fungal process has the potential to be competitive and further development is recommended, the future research on this process should include:

- Optimization of the fungal process and research regarding process stability.
- Bioreactor design (batch or continuous) and downstream process design.
- Pilot-scale studies including recycling ratios and retention times to improve effluent qualities and laccase production.
- Research concerning the quantity, stability, nutritional value, toxicity and potential hazards of the biomass produced.
- The possibility of a two-stage aerobic-anaerobic system to improve effluent quality if it is not sufficiently improved by optimization of the fungal system.
- An ETSA on completion of the pilot-scale studies to ensure that the fungal technology process will be feasible, sustainable and competitive in full-scale operation.

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APPENDICES

Appendix 1: Design matrix for the Box-Behnken experiment created with Design-Expert version 6.0.10 (Stat-Ease Inc., Minneapolis, USA):

Run	Block	Factor 1: Glucose	Factor 2: Phosphate	Factor 3: Inorganic nitrogen	Factor 4: Organic nitrogen	Factor 5: Polyphenol extraction	Factor 6: Fungal strain
1	Block 1	0	0	-1	-1	no extraction.	*P. sanguineus*
2	Block 1	0	0	-1	-1	extraction	*C. subvermispora*
3	Block 1	0	0	-1	-1	extraction	*T. pubescens*
4	Block 1	0	0	1	1	extraction	*T. pubescens*
5	Block 1	-1	-1	0	0	no extraction.	*P. sanguineus*
6	Block 1	0	0	-1	-1	no extraction.	*T. pubescens*
7	Block 1	0	0	1	-1	no extraction.	*C. subvermispora*
8	Block 1	-1	1	0	0	no extraction.	*C. subvermispora*
9	Block 1	0	0	1	-1	extraction	*P. sanguineus*
10	Block 1	1	1	0	0	extraction	*C. subvermispora*
11	Block 1	0	0	0	0	no extraction.	*C. subvermispora*
12	Block 1	1	-1	0	0	extraction	*T. pubescens*
13	Block 1	-1	-1	0	0	extraction	*T. pubescens*
14	Block 1	-1	1	0	0	no extraction.	*P. sanguineus*
15	Block 1	0	0	-1	-1	no extraction.	*C. subvermispora*
16	Block 1	0	0	0	0	no extraction.	*C. subvermispora*
17	Block 1	0	0	0	0	extraction	*T. pubescens*
18	Block 1	1	-1	0	0	extraction	*C. subvermispora*

Run	Block	Factor 1: Glucose	Factor 2: Phosphate	Factor 3: Inorganic nitrogen	Factor 4: Organic nitrogen	Factor 5: Polyphenol extraction	Factor 6: Fungal strain
19	Block 1	0	0	0	0	extraction	*C. subvermispora*
20	Block 1	-1	-1	0	0	extraction	*C. subvermispora*
21	Block 1	0	0	-1	1	no extraction.	*P. sanguineus*
22	Block 1	0	0	1	1	no extraction.	*T. pubescens*
23	Block 1	-1	1	0	0	extraction	*T. pubescens*
24	Block 1	1	-1	0	0	no extraction.	*C. subvermispora*
25	Block 1	0	0	1	-1	extraction	*C. subvermispora*
26	Block 1	0	0	-1	1	no extraction.	*T. pubescens*
27	Block 1	-1	-1	0	0	no extraction.	*C. subvermispora*
28	Block 1	0	0	1	-1	no extraction.	*T. pubescens*
29	Block 1	1	-1	0	0	no extraction.	*P. sanguineus*
30	Block 1	0	0	-1	-1	extraction	*P. sanguineus*
31	Block 1	0	0	0	0	no extraction.	*T. pubescens*
32	Block 1	1	1	0	0	no extraction.	*T. pubescens*
33	Block 1	-1	1	0	0	extraction	*P. sanguineus*
34	Block 1	0	0	0	0	extraction	*P. sanguineus*
35	Block 1	1	1	0	0	extraction	*P. sanguineus*
36	Block 1	-1	-1	0	0	extraction	*P. sanguineus*
37	Block 1	0	0	0	0	extraction	*P. sanguineus*
38	Block 1	1	1	0	0	no extraction.	*C. subvermispora*
39	Block 1	0	0	-1	1	extraction	*P. sanguineus*
40	Block 1	0	0	1	1	extraction	*P. sanguineus*
41	Block 1	1	1	0	0	extraction	*T. pubescens*
42	Block 1	0	0	1	1	no extraction.	*C. subvermispora*
43	Block 1	0	0	-1	1	no extraction.	*C. subvermispora*

Run	Block	Factor 1: Glucose	Factor 2: Phosphate	Factor 3: Inorganic nitrogen	Factor 4: Organic nitrogen	Factor 5: Polyphenol extraction	Factor 6: Fungal strain
44	Block 1	0	0	-1	1	extraction	*T. pubescens*
45	Block 1	0	0	0	0	no extraction.	*P. sanguineus*
46	Block 1	0	0	-1	1	extraction	*C. subvermispora*
47	Block 1	0	0	1	1	no extraction.	*P. sanguineus*
48	Block 1	0	0	1	-1	no extraction.	*P. sanguineus*
49	Block 1	0	0	0	0	no extraction.	*T. pubescens*
50	Block 1	1	-1	0	0	no extraction.	*T. pubescens*
51	Block 1	0	0	1	-1	extraction	*T. pubescens*
52	Block 1	0	0	0	0	extraction	*T. pubescens*
53	Block 1	-1	1	0	0	no extraction.	*T. pubescens*
54	Block 1	-1	1	0	0	extraction	*C. subvermispora*
55	Block 1	-1	-1	0	0	no extraction.	*T. pubescens*
56	Block 1	1	1	0	0	no extraction.	*P. sanguineus*
57	Block 1	0	0	0	0	no extraction.	*P. sanguineus*
58	Block 1	0	0	0	0	extraction	*C. subvermispora*
59	Block 1	1	-1	0	0	extraction	*P. sanguineus*
60	Block 1	0	0	1	1	extraction	*C. subvermispora*
61	Block 2	0	1	-1	0	no extraction.	*T. pubescens*
62	Block 2	0	0	0	0	no extraction.	*T. pubescens*
63	Block 2	-1	0	0	1	no extraction.	*C. subvermispora*
64	Block 2	-1	0	0	1	extraction	*C. subvermispora*
65	Block 2	0	-1	1	0	extraction	*P. sanguineus*
66	Block 2	0	0	0	0	extraction	*P. sanguineus*
67	Block 2	0	1	1	0	no extraction.	*P. sanguineus*
68	Block 2	1	0	0	-1	no extraction.	*C. subvermispora*

Run	Block	Factor 1: Glucose	Factor 2: Phosphate	Factor 3: Inorganic nitrogen	Factor 4: Organic nitrogen	Factor 5: Polyphenol extraction	Factor 6: Fungal strain
69	Block 2	0	1	1	0	no extraction.	*C. subvermispora*
70	Block 2	-1	0	0	1	extraction	*T. pubescens*
71	Block 2	0	0	0	0	extraction	*P. sanguineus*
72	Block 2	1	0	0	-1	no extraction.	*P. sanguineus*
73	Block 2	0	0	0	0	no extraction.	*P. sanguineus*
74	Block 2	0	-1	1	0	extraction	*T. pubescens*
75	Block 2	0	1	-1	0	no extraction.	*C. subvermispora*
76	Block 2	0	-1	1	0	extraction	*C. subvermispora*
77	Block 2	1	0	0	-1	extraction	*C. subvermispora*
78	Block 2	0	0	0	0	extraction	*T. pubescens*
79	Block 2	-1	0	0	1	extraction	*P. sanguineus*
80	Block 2	1	0	0	-1	extraction	*P. sanguineus*
81	Block 2	1	0	0	1	extraction	*T. pubescens*
82	Block 2	0	1	1	0	no extraction.	*T. pubescens*
83	Block 2	0	-1	-1	0	extraction	*P. sanguineus*
84	Block 2	-1	0	0	-1	extraction	*C. subvermispora*
85	Block 2	0	-1	-1	0	extraction	*C. subvermispora*
86	Block 2	1	0	0	1	no extraction.	*P. sanguineus*
87	Block 2	1	0	0	1	extraction	*P. sanguineus*
88	Block 2	0	0	0	0	no extraction.	*P. sanguineus*
89	Block 2	0	-1	1	0	no extraction.	*T. pubescens*
90	Block 2	1	0	0	1	no extraction.	*T. pubescens*
91	Block 2	0	1	-1	0	extraction	*T. pubescens*
92	Block 2	0	-1	1	0	no extraction.	*C. subvermispora*
93	Block 2	-1	0	0	-1	no extraction.	*C. subvermispora*

Run	Block	Factor 1: Glucose	Factor 2: Phosphate	Factor 3: Inorganic nitrogen	Factor 4: Organic nitrogen	Factor 5: Polyphenol extraction	Factor 6: Fungal strain
94	Block 2	-1	0	0	-1	no extraction.	*T. pubescens*
95	Block 2	0	-1	-1	0	no extraction.	*C. subvermispora*
96	Block 2	0	-1	-1	0	no extraction.	*T. pubescens*
97	Block 2	0	0	0	0	no extraction.	*T. pubescens*
98	Block 2	0	1	1	0	extraction	*T. pubescens*
99	Block 2	1	0	0	1	extraction	*C. subvermispora*
100	Block 2	-1	0	0	-1	extraction	*P. sanguineus*
101	Block 2	0	-1	-1	0	extraction	*T. pubescens*
102	Block 2	1	0	0	1	no extraction.	*C. subvermispora*
103	Block 2	0	0	0	0	extraction	*C. subvermispora*
104	Block 2	0	0	0	0	no extraction.	*C. subvermispora*
105	Block 2	0	1	-1	0	extraction	*C. subvermispora*
106	Block 2	-1	0	0	-1	no extraction.	*P. sanguineus*
107	Block 2	0	0	0	0	extraction	*C. subvermispora*
108	Block 2	0	0	0	0	extraction	*T. pubescens*
109	Block 2	0	1	-1	0	extraction	*P. sanguineus*
110	Block 2	0	0	0	0	no extraction.	*C. subvermispora*
111	Block 2	1	0	0	-1	extraction	*T. pubescens*
112	Block 2	1	0	0	-1	no extraction.	*T. pubescens*
113	Block 2	-1	0	0	1	no extraction.	*T. pubescens*
114	Block 2	0	1	1	0	extraction	*C. subvermispora*
115	Block 2	-1	0	0	1	no extraction.	*P. sanguineus*
116	Block 2	-1	0	0	-1	extraction	*T. pubescens*
117	Block 2	0	-1	-1	0	no extraction.	*P. sanguineus*
118	Block 2	0	1	-1	0	no extraction.	*P. sanguineus*

Run	Block	Factor 1: Glucose	Factor 2: Phosphate	Factor 3: Inorganic nitrogen	Factor 4: Organic nitrogen	Factor 5: Polyphenol extraction	Factor 6: Fungal strain
119	Block 2	0	-1	1	0	no extraction.	*P. sanguineus*
120	Block 2	0	1	1	0	extraction	*P. sanguineus*
121	Block 3	1	0	-1	0	no extraction.	*C. subvermispora*
122	Block 3	0	0	0	0	extraction	*P. sanguineus*
123	Block 3	0	1	0	-1	no extraction.	*T. pubescens*
124	Block 3	0	1	0	1	extraction	*C. subvermispora*
125	Block 3	0	-1	0	-1	no extraction.	*T. pubescens*
126	Block 3	0	0	0	0	extraction	*P. sanguineus*
127	Block 3	-1	0	1	0	no extraction.	*P. sanguineus*
128	Block 3	-1	0	-1	0	extraction	*C. subvermispora*
129	Block 3	0	0	0	0	no extraction.	*C. subvermispora*
130	Block 3	0	-1	0	1	extraction	*C. subvermispora*
131	Block 3	-1	0	-1	0	extraction	*P. sanguineus*
132	Block 3	-1	0	-1	0	extraction	*T. pubescens*
133	Block 3	0	0	0	0	no extraction.	*P. sanguineus*
134	Block 3	0	1	0	1	no extraction.	*C. subvermispora*
135	Block 3	0	1	0	-1	no extraction.	*C. subvermispora*
136	Block 3	0	-1	0	-1	extraction	*P. sanguineus*
137	Block 3	1	0	1	0	extraction	*C. subvermispora*
138	Block 3	0	1	0	1	no extraction.	*P. sanguineus*
139	Block 3	0	-1	0	1	no extraction.	*C. subvermispora*
140	Block 3	1	0	1	0	extraction	*P. sanguineus*
141	Block 3	-1	0	-1	0	no extraction.	*T. pubescens*
142	Block 3	0	1	0	-1	extraction	*C. subvermispora*
143	Block 3	1	0	-1	0	extraction	*P. sanguineus*

Run	Block	Factor 1: Glucose	Factor 2: Phosphate	Factor 3: Inorganic nitrogen	Factor 4: Organic nitrogen	Factor 5: Polyphenol extraction	Factor 6: Fungal strain
144	Block 3	1	0	-1	0	extraction	*T. pubescens*
145	Block 3	0	0	0	0	no extraction.	*T. pubescens*
146	Block 3	1	0	-1	0	no extraction.	*T. pubescens*
147	Block 3	0	1	0	-1	no extraction.	*P. sanguineus*
148	Block 3	0	1	0	1	extraction	*T. pubescens*
149	Block 3	0	-1	0	-1	no extraction.	*C. subvermispora*
150	Block 3	0	-1	0	1	extraction	*T. pubescens*
151	Block 3	-1	0	1	0	extraction	*C. subvermispora*
152	Block 3	1	0	1	0	extraction	*T. pubescens*
153	Block 3	-1	0	-1	0	no extraction.	*P. sanguineus*
154	Block 3	0	0	0	0	extraction	*C. subvermispora*
155	Block 3	0	0	0	0	extraction	*C. subvermispora*
156	Block 3	0	1	0	-1	extraction	*T. pubescens*
157	Block 3	0	1	0	-1	extraction	*P. sanguineus*
158	Block 3	0	-1	0	1	no extraction.	*P. sanguineus*
159	Block 3	-1	0	-1	0	no extraction.	*C. subvermispora*
160	Block 3	0	0	0	0	extraction	*T. pubescens*
161	Block 3	0	1	0	1	no extraction.	*T. pubescens*
162	Block 3	0	0	0	0	no extraction.	*T. pubescens*
163	Block 3	1	0	-1	0	extraction	*C. subvermispora*
164	Block 3	0	-1	0	1	no extraction.	*T. pubescens*
165	Block 3	0	-1	0	-1	no extraction.	*P. sanguineus*
166	Block 3	1	0	1	0	no extraction.	*T. pubescens*
167	Block 3	-1	0	1	0	no extraction.	*T. pubescens*
168	Block 3	0	-1	0	-1	extraction	*T. pubescens*

Run	Block	Factor 1: Glucose	Factor 2: Phosphate	Factor 3: Inorganic nitrogen	Factor 4: Organic nitrogen	Factor 5: Polyphenol extraction	Factor 6: Fungal strain
169	Block 3	0	0	0	0	no extraction.	*C. subvermispora*
170	Block 3	0	0	0	0	extraction	*T. pubescens*
171	Block 3	0	-1	0	-1	extraction	*C. subvermispora*
172	Block 3	1	0	-1	0	no extraction.	*P. sanguineus*
173	Block 3	1	0	1	0	no extraction.	*C. subvermispora*
174	Block 3	-1	0	1	0	no extraction.	*C. subvermispora*
175	Block 3	1	0	1	0	no extraction.	*P. sanguineus*
176	Block 3	-1	0	1	0	extraction	*T. pubescens*
177	Block 3	0	1	0	1	extraction	*P. sanguineus*
178	Block 3	0	-1	0	1	extraction	*P. sanguineus*
179	Block 3	0	0	0	0	no extraction.	*P. sanguineus*
180	Block 3	-1	0	1	0	extraction	*P. sanguineus*