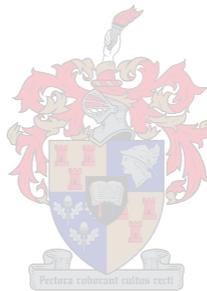


Evaluation and Optimisation of Fungal Enzymes for Microbial Bioprocessing of Rooibos Tea

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

Summary

Aspalathus linearis is a leguminous shrub native to the Cedarberg Mountains in the Western Cape, of which the leaves and stems are used for the preparation of rooibos tea. Over the past few decades, rooibos tea and other related products have gained popularity due to their health promoting properties. These beneficial properties can partly be ascribed to the phenolic constituents that are trapped within the cellulosytic plant material of the tea leaves as glycoconjugated aroma and phenolic compounds. Although many fungal species are known for their efficient hydrolysis of plant material, fungal enzymes have not been evaluated for the bioprocessing of rooibos tea to improve its commercial value. It was the objective of this study to identify a specific cocktail of microbial enzymes to enhance the maceration of the rooibos plant material, while retaining the antioxidant content.

During this study, 11 fungal species known for the production of hydrolytic enzymes, as well as 12 species isolated from rooibos tea products, were screened for their potential to improve aroma development and/or increased extraction of soluble matter and/or antioxidants from rooibos tea material.

After culturing in Potato Dextrose medium, the crude enzyme extracts of the 23 isolates were evaluated on spent rooibos tea for enhanced extraction of soluble solids (SS) and/or total polyphenols (TP). Nine strains increased the yield in SS (improvement varying from 3% to 42%), while 14 strains yielded higher levels of TP (increase varying from 1% to 36%). Little improvement in colour development from green (unfermented) rooibos tea was observed, but the enzyme extracts from *Pleurotus ostreatus* var. *florida*, *Lentinula edodes*, *Aspergillus oryzae*, *Aspergillus tubingensis*, *Paecilomyces variotti* and *Trichoderma reesei* improved the aroma development from green tea to some extent. Ten-fold concentrated enzyme extracts from four of these isolates were able to release at least an additional 10% in SS from the green tea.

The crude enzyme extracts prepared from three food-grade strains, i.e. *Aspergillus oryzae*, *Lentinula edodes* and *Pleurotus ostreatus* var. *florida*, contained relatively high levels of endoglucanase, xylanase and pectinase activities. Eight different culture media were evaluated for optimal hydrolase and laccase production by these food-grade fungi. MYPG proved to be the best growth medium, while 1% spent grain, 1% wheat straw and 1% pineapple peel gave the best induction of xylanase, cellulase, pectinase and laccase activities for *L. edodes*.

When cultured in the Yeast Peptone (YP) medium + 1% wheat straw, the *L. edodes* enzyme cocktail showed the best improvement in both the aroma and colour development of green tea and may be considered for shortening of the fermentation time required for green tea processing. Traditional open-air fermentation of rooibos tea can take up to 16 hours, which results in a significant loss in antioxidants and therefore also in its pharmaceutical and nutraceutical value. The *Rhizopus oryzae* cocktail prepared in YP + 1% wheat straw showed potential for the development of a quick-draw fermented tea made by infusion, where there is improved colour release and more than 20% improved extraction of soluble solids without a loss in the TP content.

When cultured in Potato Dextrose medium, the *L. edodes* cocktail can be used for aroma and colour development from green tea, while the *R. oryzae* cocktail can be used for increasing the antioxidant content in rooibos extracts from green or fermented tea. This was confirmed with small-scale industrial treatments of fermented tea where the *L. edodes* YP + wheat straw cocktail improved the release in SS by more than 10% and the *R. oryzae* YP + wheat straw cocktail increased the yield in SS by more than 30% and the TP by more than 20%.

Opsomming

Aspalathus linearis is 'n fynbosplant inheems aan die Sederberge in die Wes-Kaap, waarvan die blare en stingels vir die voorbereiding van rooibostee gebruik word. Die afgelope paar dekades het die gewildheid van rooibostee en verwante produkte aansienlik toegeneem weens die gesondheidsvoordele wat dit inhou. Hierdie voordelige eienskappe kan toegeskryf word aan die fenoliese komponente wat binne die sellulolitiese plantweefsel van die teeblare as gekonjugeerde geur- en fenoliese verbindings vasgevang is. Alhoewel verskeie swamspesies vir hul doeltreffende degradering van plantmateriaal bekend is, is fungale ensieme nog nie geëvalueer vir die prosessering van rooibostee om die kommersiële waarde daarvan te verbeter nie. Die doelwit van hierdie studie was om 'n spesifieke kombinasie van mikrobiële hidrolitiese ensieme te identifiseer wat die maserasie van rooibos plantmateriaal sal verhoog met behoud van die anti-oksidadant inhoud.

Tydens hierdie studie is 11 swamspesies wat bekend is vir die produksie van hidrolitiese ensieme, asook 12 swamspesies wat vanaf rooibostee produkte geïsoleer is, geëvalueer vir hul potensiaal om geurontwikkeling en/of ekstraksie van oplosbare stowwe en/of anti-oksidadante vanuit rooibostee materiaal te verbeter.

Die kru ensiemekstrakte van die 23 isolate, wat in Aartappel-Dextrose medium opgegroeï is, is op oorskot rooibostee geëvalueer vir verhoogde ekstraksie van oplosbare vastestowwe (SS) en/of totale polifenole (TP). Nege rasse het die opbrengs van oplosbare vastestowwe verhoog (verbetering tussen 3% en 42%), terwyl 14 rasse die totale polifenoliese vlakke laat toeneem het (tot so hoog as 36%). Baie min verbetering in kleurontwikkeling van groen (ongefermenteerde) rooibostee is waargeneem, maar ensiemekstrakte van *Pleurotus ostreatus* var. *florida*, *Lentinula edodes*, *Aspergillus oryzae*, *Aspergillus tubingensis*, *Paecilomyces variotti* en *Trichoderma reesei*, het wel die aroma ontwikkeling vanaf groen tee tot 'n mate verbeter. Tienvoudig gekonsentereerde ekstrakte van vier van hierdie isolate het 'n verbetering van meer as 10% in die ekstraksie van opgeloste vastestowwe uit groen tee tot gevolg gehad.

Die ensiemekstrakte van drie swamme bekend vir hul gebruik in die voedselindustrie, nl. *A. oryzae*, *L. edodes* and *P. ostreatus* var. *florida*, het relatief hoë vlakke van endoglukanase, xylanase en pektinase aktiwiteit getoon. Agt verskillende kultuur-media is vir die optimale produksie van hidrolitiese and lakkase ensieme vanaf hierdie voedsel-graad swamme geëvalueer. MYPG was die beste groeimedium vir *L. edodes*, -terwyl 1%

koringstrooi, 1% oorskot graan en 1% pynappelskil die beste induksie van xylanase, pektinase, endoglukanase en lakkase aktiwiteite vir hierdie organisme getoon het. *Lentinula edodes* opgegroeï in YP medium + 1% koringstrooi, het die beste verbetering in aroma en kleur getoon vanaf groen tee getoon. Hierdie ekstrak kan dus moontlik gebruik word vir die verkorting van die fermentasietyd wat vir groen tee benodig word. Ope-lug fermentasie van groen tee duur gewoonlik tot 16 uur en lei tot 'n aansienlike verlies in antioksidant-inhoud. Die *R. oryzae* ekstrak het die beste potensiaal vir die vervaardiging van 'n "quick-draw" tee getoon met 'n goeie kleurvrystelling sonder enige verlies in SS en TP opbrengs.

Wanneer die swamme in Aartappel-Dextrose medium opgegroeï word, kan die *L. edodes* ensiemekstrak vir aroma en kleurontwikkeling van groen tee aangewend word, terwyl die *R. oryzae* ensiemekstrak vir die verhoging van die antioksidant-inhoud in rooibos ekstrakte van groen tee of gefermenteerde tee gebruik kan word. Dit is bevestig met die kleinskaalse behandeling van gefermenteerde tee waar die *L. edodes* YP + 1% koringstrooi ensiemekstrak die vrystelling van SS met meer as 30% en die TP met meer as 20% verbeter het.

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Chapter 1

General Introduction and Project Aims

General Introduction and Project Aims

Rooibos tea is prepared from the fynbos plant *Aspalathus linearis* that is only cultivated in the Cedarberg mountains of the Western Cape of South Africa, where the rooibos plant grows in its natural state in an area of winter rainfall and coarse sandy soil (Morton, 1983). Rooibos tea, an infusion of the fermented leaves and stems, is a unique beverage that can be consumed hot or cold with a natural sweet taste and a clear red-brown colour (Joubert and Ferreira, 1996). This tea is becoming increasingly popular due to its health properties. It is claimed to help improve appetite and cure insomnia, allergies and nervous complaints and is also considered as an “anti-ageing” beverage by the Japanese (Joubert and Ferreira, 1996). Rooibos tea is rich in volatile components, polyphenols and minerals, contains no caffeine and has a low tannin content (Rabe et al., 1994). The antioxidant activities of Rooibos tea have long been recognised and can be attributed to the presence of flavonoids, which have been reported to inhibit lipid peroxidation, scavenge free radicals and active oxygen, chelate iron ions and inactivate lipoxygenase (Joubert and Ferreira, 1996; Yen et al., 1997).

The processing of rooibos tea includes the shredding and bruising of the plant material, followed by open-air ‘fermentation’ (oxidation) during which the distinctive colour, aroma and flavour of rooibos tea is released. After fermentation, the rooibos tea is spread out thinly and subjected to sun drying, with little control over processing conditions. Fermentation can take up to 16 hours (Morton, 1983), which results in a significant loss in antioxidants in the processed tea, and therefore also in its pharmaceutical and nutraceutical value (Von Gadow, 1997).

The composition of the rooibos plant results in the low solubility of rooibos tea components. It is likely that many of the flavour and medicinal compounds are trapped within the cellulolytic plant material of the tea leaves as glycoconjugated aroma and phenolic compounds. These compounds can then only be released upon cleavage of the chemical bonds by relevant hydrolases or esterases, or altered by oxidases. Polysaccharases could target the cellulolytic material, macerating the complex polysaccharide structures of the leaves, while other enzymes could target the glucosidic bonds and assist in the release of polyphenols. It has previously been demonstrated that the exogenous application of hydrolytic and oxidative enzymes, e.g. cellulase, xylanase, pectinase and laccase, increased quality parameters such as the concentrations of theaflavins, thearubigens and soluble solids, in black tea (Angayarkanni et al., 2002; Murugesan et al., 2002).

The objective of this study was to design a tailor-made enzyme cocktail that will enhance the maceration of the rooibos plant material to release more compounds of commercial value. The specific cocktail of microbial enzymes may achieve one or more of the following:

- (a) Improved solubility and thus yields of cold-water and hot-water soluble matter for rooibos extract preparation,
- (b) Improved antioxidant extraction for the production of nutraceutical products,
- (c) Enhanced colour and aroma development of green rooibos tea leaves (shorten fermentation time), and
- (d) Increase extraction of colour and soluble matter for quick-draw tea (infusion of tea leaves).

This dissertation represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore been unavoidable. A critical discussion of current literature on tea in general is given in Chapter 2, with the focus on the chemical composition of tea and the role of various chemicals during tea processing. The methodology and results of the evaluation of fungal enzymes for microbial bioprocessing of rooibos tea are described in Chapter 3. Chapter 4 comprises an overview of hydrolytic enzymes and their production by selected food-grade fungi used in this study. The optimisation of culture media for enzyme production by these food-grade fungi for application in rooibos tea processing is discussed in Chapter 5. This is followed by some concluding remarks in Chapter 6 and a combined reference list for Chapters 1 to 6.

Chapter 2

Literature Review:

General Introduction on Chemical Properties of Tea

2.1 Introduction

Tea, being the most widely used stimulant throughout the world (Murugesan et al., 2002), is one of the most widely consumed beverages, second only to water. The estimated average consumption is 1 L per person per day in the United Kingdom (Cabrera et al., 2003) and the per capita worldwide consumption is approximately 0,12 L per day (Graham, 1992). Tea is grown in about 30 countries worldwide and is cultivated on more than three million hectares in the world (Ravichandran and Parthiban, 1998a).

Tea originated from China and has gained the world's taste in the past 2000 years. Initially consumed only by Chinese monks, it was first introduced into continental Europe by the Dutch at the beginning of the 16th century and reached England and North America by the mid-1600s (Balentine et al., 1997). The consumption of tea is now part of people's daily routine as an everyday drink as well as a therapeutic aid in many illnesses (Cabrera et al., 2003).

It is well known that tea causes an increase in alertness, diminishes fatigue and improves physical and mental performance. These characteristics can mainly be attributed to the low caffeine content of tea. Four cups of tea with skimmed milk typically provide two thirds of the fluid humans need each day for optimum health, along with 5% of the selenium, 25% of the riboflavin, 6% of the pantothenate, 10% of the zinc, 10% of the folate, 9% of the potassium and 45% of the manganese humans are estimated to require, as well as smaller but significant quantities of trace nutrients (Marks et al., 1997), which play an important role in human metabolism (Cabrera et al., 2003). Tea is considered to have a wide range of physiological properties, for example being stimulants, anti-depressants, and anti-inflammatory, anti-hypersensitive, anti-atherosclerotic and hypercholesterolemia agents (Yen and Chen, 1995).

Tea is also a good dietary resource for natural antioxidants (Table 2.1) known as polyphenolic compounds (Wang et al., 1989; Joubert and Ferreira, 1996). Antioxidants decrease the adverse effects of reactive oxygen and/or reactive nitrogen species on normal physiological functions (Wang et al., 2000b) and appear to have a protective effect against lipid peroxidation in humans, which has been related to phenomena such as vascular disease, carcinogenesis, thrombosis and ageing, to mention a few (Duthie, 1991; Kinsella, 1993).

Flavonoids are phenolic compounds that occur naturally in plant foods and have demonstrated a wide range of biochemical and pharmacological effects, including anti-inflammatory and anti-allergenic effects (Middleton and Kandaswami, 1992). They have also

Table 2.1. The relative antioxidant potentials of vitamins, tea beverage, flavonoids, carotenes and xanthophylls typically found in black tea (Wiseman et al., 1997).

Antioxidant	Trolox equivalent antioxidant capacity (TEAC, mM) ^a
Vitamins	
Vitamin C	1.0 ± 0.02
Vitamin E	1.0 ± 0.03
Tea beverage	
Green tea (1000 ppm)	3.8 ± 0.03
Black tea (1000 ppm)	3.5 ± 0.05
Flavan 3-ols	
Epicatechin	2.5 ± 0.02
Epigallocatechin	3.8 ± 0.06
Epigallocatechin gallate	4.8 ± 0.06
Epicatechin gallate	4.9 ± 0.02
Theaflavins	
Theaflavin	2.9 ± 0.08
Theaflavin 3-monogallate	4.7 ± 0.16
Theaflavin 3'-monogallate	4.8 ± 0.19
Theaflavin 3-3'-digallate	6.2 ± 0.43
Flavonols	
Quercetin	4.7 ± 0.10
Kaempferol	1.3 ± 0.08
Rutin	2.4 ± 0.06
Flavones	
Apigenin	1.5 ± 0.08
Luteolin	2.1 ± 0.05
Carotenes	
Lycopene	2.9 ± 0.15
α -Carotene	1.3 ± 0.04
β -Carotene	1.9 ± 0.10
Xanthophylls	
β -Cryptoxanthin	2.0 ± 0.02
Zeaxanthin	1.4 ± 0.04
Lutein	1.5 ± 0.10

^aTEAC is the millimolar concentration of a Trolox Solution having the antioxidant capacity equivalent to a 1.0 mM of the substance under investigation.

been found to inhibit lipid peroxidation, scavenge free radicals and active oxygen, chelate iron ions and inactivate lipoxygenase (Yen et al., 1997). Several studies have also associated the consumption of tea with a lower risk of several types of cancer, including stomach, oral cavity, esophagus and lung cancers. Therefore, tea appears to be an effective chemopreventive agent for some toxic chemicals and carcinogens (Cabrera et al., 2003).

In general, tea is consumed directly either by brewing loose leaves or tea bags or in a ready-to-drink form. Tea extracts may be prepared in a variety of physical forms and range from liquids, such as strong infusions, through soft and dry extracts to purified catechins. A possible application of the natural antioxidant properties of catechins in green tea is the improvement of cereals, cakes and biscuits as well as traditional health food products and dietary supplements. The extracts can also be employed to give dairy products, instant noodles, confectionary, ice cream and fried snacks a healthier appeal to the consumer. The antibacterial and deodorising effect of catechins provides natural added value for toothpastes, mouthwashes, chewing gums and breath fresheners, with the potential for other body, skin and hair product applications. Many shampoos, moisturising creams, perfumes and sunscreens contain tea extracts as they are believed to have a soothing effect on the skin as well as acting as antioxidants to protect the skin from free radicals (Wang et al., 2000b).

2.2 *Camellia sinensis*

The tea plant, *Camellia sinensis* (L.) O. Kuntze, is a perennial evergreen shrub that belongs to the Theaceae family (Ravichandran, 2002). Tea bushes mature after seven years and can maintain a steady yield for up to 100 years. It thrives under temperatures within the range of 15-25°C and grows best in acidic soil with pH ranges from 3.5 to 5.6 (Wong et al., 2003). Tea is generally processed from the tender shoots of the tea plant as two leaves and a bud (Murugesan et al., 2002).

Depending on the leaf pose and growth habitat, two intra-specific forms of *C. sinensis* (L.) can be recognised, i.e. the China variety, *C. sinensis* var. *sinensis* (L.) and the Assam variety, *C. sinensis* var. *assamica* (Masters) Kitamura. The growth habitat for the China variety is dwarf, slow growing and shrub-like. Their leaves are small, erect, narrow, serrate and dark green whereas the Assam variety is a tall, quick growing tree with leaves that are large, horizontal, broad, mostly non-serrated and light green (Wong et al., 2003).

Based on distinct manufacturing processes, tea has been traditionally classified into six categories. These teas are green, black, dark green, oolong, white and yellow tea (Balentine et al., 1997). Black teas are fermented products where browning reactions are catalysed by polyphenol oxidase. Dark green teas are fermented products in which the browning reaction is non-enzymatic, while the oolong teas are semi-fermented products. White teas are unfermented products produced from the tender leaves or unopened leaf buds (Chen et al., 1996) and represent the least processed of teas since it goes through steaming and drying without an initial withering stage (Santana-Rios et al., 2001). Yellow teas are made similarly to green teas, except that they are slightly fermented and non-enzymatic browning reactions occur to a lesser extent (Chen et al., 1996).

Of the tea consumed worldwide, black tea is the most popular drink in Europe, North America and North Africa (except Morocco). Green tea is mainly consumed throughout Asia, whereas oolong tea is popular in China and Taiwan (Wu and Wei, 2002). Of the nearly 2,5 million tons of dried tea that are produced each year (Graham, 1992), 76-78% is black tea, 20-22% is green tea and less than 2% is oolong tea (Costa et al., 2002; Zuo et al., 2002).

The fermentation and heating of tea leaves can result in polymerisation of monophenolic compounds, leading to conformational changes (Costa et al., 2002), which are responsible for the variations in the flavour and quality of the various teas (Murugesan et al., 2002). During the fermentation process, these compounds are oxidised or condensed to other larger polyphenolic molecules such as theaflavins and thearubigens (Zuo et al., 2002; Wu and Wei, 2002).

2.2.1 Green tea

The term green tea refers to the product manufactured from fresh *C. sinensis* leaves in which significant oxidation of the major leaf polyphenols known as catechins is prevented (Ho et al., 1994). This is done by subjecting the fresh tea leaves to withering after which they are either panfried or steamed prior to rolling or shaping and drying (Santana-Rios et al., 2001). During the final drying step, many new aromatic compounds are formed that contribute to the characteristic flavour of green tea. Green tea beverage composition varies with the origin of the leaf and with manufacturing conditions and results in a light yellow-green infusion exhibiting almost no catechin oxidation (Graham, 1992).

Green tea is known to possess various beneficial pharmacological and physiological effects. These include antipyretic, diuretic, antioxidative and radioprotective activities (Wang

et al., 1989). As a cancer preventive, one of the most important advantages of green tea is its low-toxicity (Wang et al., 2000b). Green tea is very high in polyphenols, especially flavonoids, which have demonstrated greater antioxidant protection than vitamins C and E in experimental studies (Ho et al., 1992). One cup of green tea usually contains about 300-400 mg of polyphenols (Graham, 1992).

2.2.2 Oolong tea

Oolong tea is mainly produced in China (approx. 50 000 tons) and Taiwan (approx. 10 000). Catechins comprise 8-20% of the total dry matter of oolong tea extracts (Graham, 1992). The tea gets its unique floral, fruity and jasmine-like aroma from the particular manufacturing process (Wang et al., 2001a). Various processing methods are known for different kinds of oolong tea, but they generally involve tea leaf plucking, solar withering, indoor withering, parching, rolling and drying (Hara et al., 1995).

Solar withering comprises the freshly picked leaves being exposed to sunlight to lose 10-20% of their weight and grassy colour and give off a light fragrance. The leaves are then moved indoors for withering while they are being turned over at adequate intervals. This turnover treatment, which is a special operation unique to the processing of oolong tea, causes friction between leaves, disrupts the cellular organisation at the edge of the leaves, and brings about a limited degree of fermentation. Enzymatic reactions are activated during the solar withering and indoor withering stages, which directly affects the flavour of oolong tea (Wang et al, 2001a). It was also found that the flavour compounds improved substantially when potassium and magnesium were applied as soil fertilisers (Ruan et al., 1999).

2.2.3 Black tea

Black tea is the cheapest non-alcoholic stimulant taken throughout the world (Ravichandran and Parthiban, 1998b) and its flavour quality and taste have been shown to change with variations in geographical and climatic conditions (Takeo and Mahanta, 1983). India is the major producer, consumer and exporter of black tea (Borse et al., 2002). The quality of black tea depends mainly on the components and colour of the tea infusions (Liang et al., 2003). Previous studies have demonstrated that there were a quality reduction with the use of mechanical harvesters, although it improved plucker productivity. Hand plucked teas had higher theaflavins, caffeine, brightness, flavour index, Group II volatile flavour compounds

and sensory evaluations, and less of the undesirable Group I volatile flavour compounds (Owour and Odhiambo, 1993).

Black tea aroma is extremely complex. Over 600 volatile compounds have been identified and most of these are formed from lipids, amino acids, carotenoids and glucosides during the fermentation and firing steps (Graham, 1992). In terms of aroma, black tea can be classified either as flavoury or plain. Flavoury black teas are sold for their special aromas and flavours, while plain teas are bought mainly on the basis of strength, briskness, colour and quality (Owour et al., 1986).

During the processing of black tea, the enzyme polyphenoloxidase oxidises the polyphenols present in the tea leaves (Bhatia and Ullah, 1968). This enzymatic oxidation results in the formation of a series of quinones, such as theaflavins (TFs) and thearubigens (TRs), which are responsible for the colour, flavour and brightness of black tea (Graham, 1992; Liang et al., 2003). When potassium was applied as soil fertiliser to the plants, the contents of theaflavins and thearubigens increased considerably (Ruan et al., 1999). The total TF content of the tea leaves does not usually exceed 2% and can be as low as 0.3% (Balentine et al., 1997).

The processing of black tea comprises five basic steps: withering, rolling, fermentation, firing and sorting. The tea is allowed to dry or wither in open-air sheds, controlled lofts, tunnels or troughs for 18 hours in order to reduce the initial moisture content to approximately 60%. The next step is rolling (45 minutes to 3 hours) during which the cell structures are partially destroyed by crushing and maceration of the leaves (Wang et al., 2001b; Wong et al., 2003). During this important step, cytoplasmic flavonoids are progressively oxidised into quinones as a result of chloroplast polyphenol oxidase and cell wall peroxidase generating yellow to red-brown colours (Baruah and Mahanta, 2003). The primary reaction during tea fermentation is the oxidation and condensation of tea catechins, leading to the characteristic colour and flavour of black tea. The total time for fermentation is usually about 3 hours at 27°C. During the firing stage, the tea is passed on trays through hot air dryers (110°C for 20 minutes and then at 80°C for 30 minutes) to stop the enzymatic fermentation (Wang et al., 2001b; Wong et al., 2003). At the end of the firing step, the moisture content of the tea should be about 3% (Pintauro, 1977). After firing, the tea leaves are sorted into the appropriate grades (Wong et al., 2003).

Black tea can be divided into two principle categories, namely orthodox and CTC (crush-tear-curl) (Mahanta and Hazarika, 1985). Their production techniques differ considerably and have a pronounced impact on the formative and degradative patterns of

various pigments (Hazarika et al., 1984). In the conventional orthodox method, the leaves are twisted in a rolling machine to damage the cellular membranes, which releases the cell contents without significantly affecting the integrity of the plucked material. In the CTC machine, the leaf epidermis is stripped off, shredded from the stalk, enhancing the brown colour, due to exposure of the stalk (Pintauro, 1977). In some factories, dual manufacture is carried out where coarse material from the rolled leaf is separated out and subjected to CTC (Mahanta and Hazarika, 1985).

During the rolling and CTC stages of black tea manufacturing, ultrastructural changes in the cellular components take place, exposing the pigments to acids and degradative enzymes (Selvendran and King, 1976). At the same time, oxidative enzymes interact with catechins to form yellowish-red polymerised pigmented products (Millin and Swaine, 1981). The change of colour from coppery red to brown can be attributed to these various changes that gain momentum during fermentation. Further modification in shape, size, texture and colour of the fermented material takes place during water evaporation in the firing stage (Mahanta and Hazarika, 1985).

The golden yellow theaflavins and the reddish-brown thearubigens are mainly responsible for the liquor characteristics of black tea and are determined by several factors: the composition; distribution and tissue levels of the flavanol substrates in the fresh shoot; the activity of the oxidising enzymes in the green leaf; the degree of tissue damage and cellular disruption achieved during the processing phase; and the temperature and oxygen content of the fermenting leaf particles (Cloughley, 1980). Quality is also determined by physico-chemical parameters, such as total soluble solids (TSS), viscosity of the brew and bulk density (packed/loose) of the tea (Borse et al., 2002). During black tea manufacturing, however, catechins are reduced by up to 85% (Balentine et al., 1997). Black teas containing higher ratios of yellow theaflavin species to dark brown thearubigens, are always higher in demand in the auction market and by global consumers (Baruah and Mahanta, 2003). Black tea also contains no tannic acid (pentadigalloylglucose) (Graham, 1992).

2.2.4 Composition

Tea generally consists of important chemical constituents such as polyphenols, caffeine, sugars, organic acids, volatile flavour compounds and amino acids, which greatly influence the taste and flavour in tea brew (Table 2.2) (Borse et al., 2002). Some of the organic acids present in the tea leaf include free gallic and quinic acid, which enters into interesting

oxidation reactions during the manufacture of black tea. An unusual amino acid known as theanine is also found in tea leaves in addition to the normal complement of 19 amino acids. It is an N-methylated derivative of glutamine and constitutes about one half of the total amino acid content (Graham, 1992). The degradation of amino acids is involved with biogenesis of aroma (Balentine et al., 1997).

Tea owes part of its popularity to the presence of moderate amounts of caffeine (2.5 - 4.5%). Two isomeric dimethylanthines are also present, but in very small quantities, namely theobromine (0.1%) and theophylline (0.2%). Caffeine contributes to the characteristic taste and briskness of tea by forming a physico-chemical complex with polyphenols. When a tea

Table 2.2. Black tea leaf composition (Balentine et al., 1997).

Component	Percentage of dry weight
Flavanols	25.0
Flavanols and flavonol glycosides	3.0
Phenolic acids and depsides	5.0
Other polyphenols	3.0
Caffeine	3.0
Theobromine	0.2
Amino acids	4.0
Organic acids	0.5
Monosaccharides	4.0
Polysaccharides	13.0
Cellulose	7.0
Protein	15.0
Lignin	6.0
Lipids	3.0
Chlorophyll and other pigments	0.5
Ash	5.0
Volatiles	0.1

liquor is allowed to cool, thearubigens readily form insoluble complexes with caffeine and primarily contributes to the formation of the coloured insoluble precipitate known as “cream” (Graham, 1992). The caffeine content largely determines the degree of cream formation that is used by professional tea tasters as an indication of quality for the evaluation of a tea (Chandra and Pandey, 1998). Ruan et al. (1999) found that the contents of free amino acids and caffeine

in various tea types were increased by potassium and magnesium fertiliser application, with the maximum increase found after treatment with both nutrients. This may be explained by the role that both nutrients play in nitrogen metabolism, specifically on the nitrate reductase activity in tea plants, which is a prerequisite for the formation of these two compounds.

The mineral content of tea leaves are very similar to that of most plants. Depending on the soil conditions, tea has a tendency to accumulate aluminum and manganese, but is also relatively rich in potassium, calcium, magnesium and fluoride. Copper is also present and plays an essential role in polyphenol oxidase activity (Graham, 1992).

2.2.4.1 Polyphenols

The polyphenols of plants represent a diverse group of compounds of which the C₁₅-compounds constitutes the largest part. The term flavonoid is used to indicate all phenolic compounds that are based on the C₆-C₃-C₆ carbon structure (Joubert, 1984). Flavonoids are a broad class of low molecular weight, secondary plant metabolites that are widely distributed in the plant kingdom (Wang et al., 2000b). They occur predominantly in leaves, flowering tissues and pollens and are also abundant in woody parts such as stems and barks. The flavonoid concentration in plant cells often exceeds 1 mM with concentrations from 3 to 10 mM already been reported (Larson, 1988). They also form a common component of our diet. Important dietary sources of flavonoids are vegetables, fruit, wines, teas and cocoa (Heim et al., 2002), of which the beverages account for at least 25-30% of the total daily flavonoid intake. It was also estimated that the total intake of flavonoids in the United States was 1 g per day expressed as glycosides or 170 mg per day expressed as aglycones (Kühnau, 1976).

To date, over 4 000 flavonoids have been identified. They occur in foods primarily as *O*-glycosides and polymers that are degraded to variable extents in the digestive tract. Glucose is the most common glycosidic unit, but other examples include glucorhamnose, galactose, arabinose and rhamnose. Two β -endoglucosidases have been characterised which are capable of flavonoid glycoside hydrolysis (Heim et al., 2002).

Flavonoids are derivatives of benzo- γ -pyrone and consist of phenolic and pyrane rings as can be seen in Figure 2.1 (Heim et al., 2002). These rings are formed from condensation reactions of cinnamic acids and acetic acid (Balentine et al., 1997). Flavonoids are classified according to substitutions in these rings. The dietary flavonoids differ in the conjugation

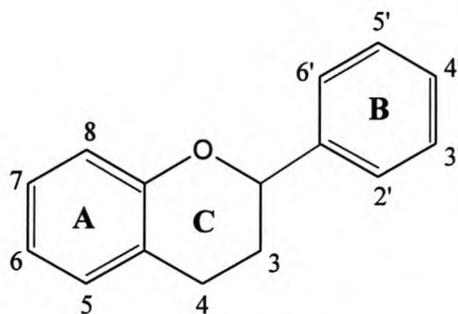


Figure 2.1 The basic structure of flavonoids in black tea (Wang et al., 2000b).

between the A- and B-rings, as well as in the arrangements of hydroxyl, methoxy and glycosidic side groups (Heim et al., 2002).

There can be distinguished between “normal” flavonoids, isoflavanoids (Wong, 1975) and neoflavonoids (Donnelly, 1975). They can further be subdivided into six classes based on the structure and conformation of the heterocyclic oxygen ring (C ring) of the basic molecule. These classes include flavones, flavanones, isoflavones, flavonols, flavanols and anthocyanins, of which flavanols and flavonols are the main classes found in tea (Wang et al., 2000b). The structures of the flavanols (Figure 2.2a) are analogous to those of the flavonols (Figure 2.2b), except that they are present in a different state of oxidation (Graham, 1992).

Quercetin, kaempferol and myricetin are the main flavonols in tea leaves and represent 2-3% of the water-soluble extractive in tea (Wang et al., 2000b). They are predominantly present as glycosides of glucose, rhamnose and possibly other sugars rather than as their non-glycosylated forms (aglycones) (Graham, 1992). Engelhardt et al. (1992) reported the presence of at least 14 glycosides of myricetin, quercetin and kaempferol in fresh tea shoots, green and black teas. Their aglycones are not found in significant quantities in tea beverages due to their poor solubility in water (Wang et al., 2000b).

Young tea leaves are used in the manufacturing process because they contain the most flavanols. As the leaves age, their total polyphenol content decrease (Wickremasinghe, 1978) and the relative amount of flavanols change (Bhatia and Ullah, 1968). Hertog et al. (1993) found that flavonoid levels in different types of black tea varied only slightly and that infusions of green tea contained a similar amount of flavonoids as black tea (30-40 mg/L).

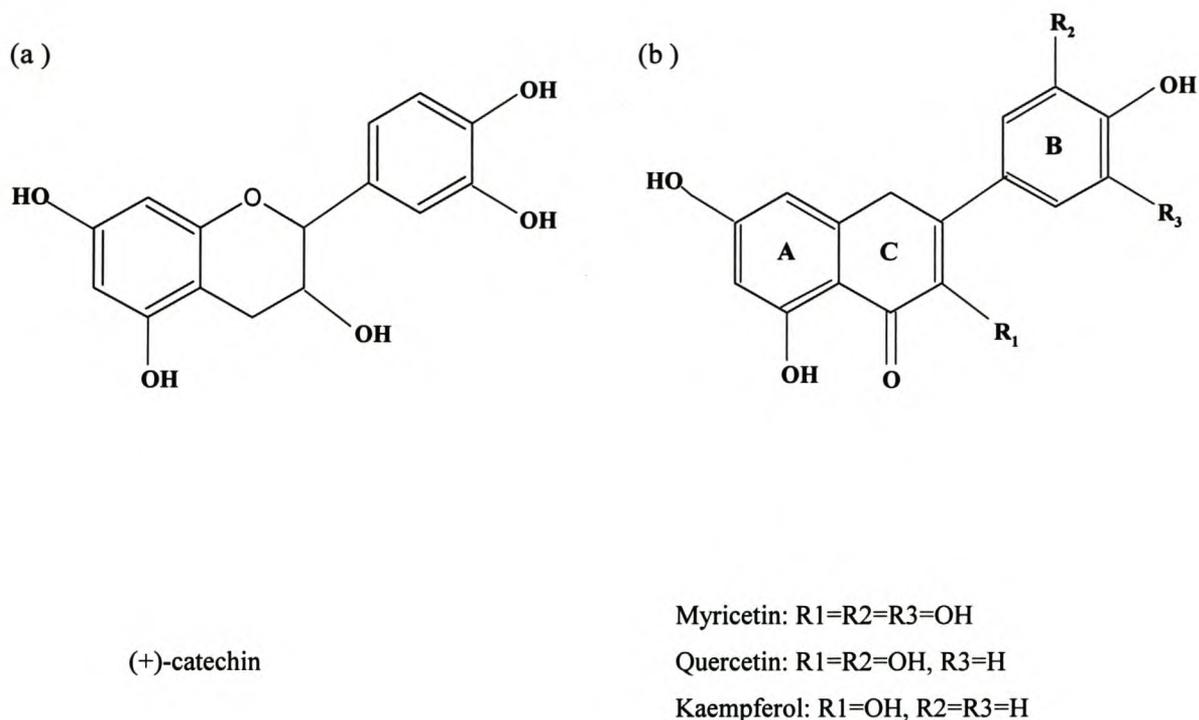


Figure 2.2 The basic structure of (a) flavanols and (b) flavonols in black tea (Joubert en Ferreira, 1996; Wang et al., 2000b).

During fermentation, the enzymatic oxidation of green tea leaves to black tea results in polymerisation of flavonols to tannins and other complex compounds (Figure 2.3). The condensed tannins are also known as proanthocyanidins. They consist of flavonol units and may reach high molecular weights consisting of up to 17 flavonol units. Of all these compounds, the proanthocyanidins are the most relevant to the human diet and consist of (+)-catechin and (-)-epicatechin monomers. The chelating and radical scavenging properties of these compounds in green tea can be attributed to the galloyl fraction of the tannins and the monomeric catechins (Heim et al., 2002).

Flavonoids have also been shown to demonstrate a wide range of biochemical and pharmacological effects, including anti-inflammatory and anti-allergenic effects (Middleton and Kandaswami, 1992), which can be attributed to their antioxidant and chelating abilities (Heim et al., 2002).

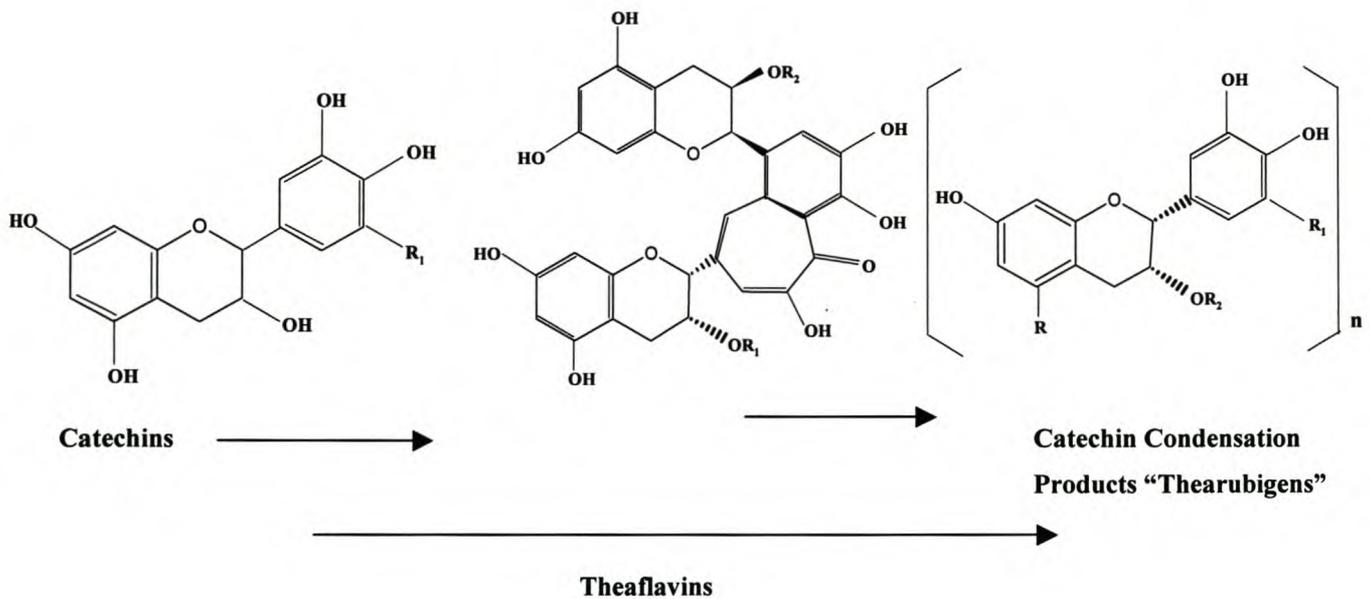
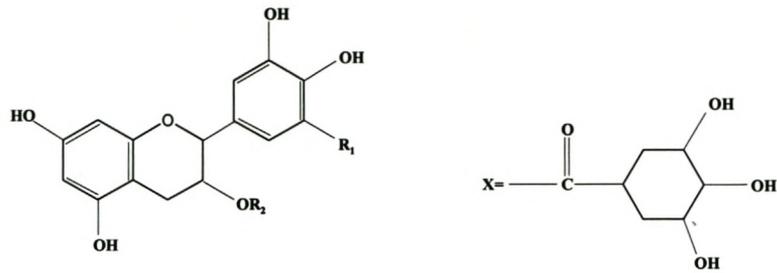


Figure 2.3 Chemical conversion of flavonoids during the manufacturing of black tea (Wiseman et al., 1997).

2.2.4.2 Tea catechins

Tea catechins are structurally and primarily flavanols and represent 20-30% of the dry weight of green tea (Balentine et al., 1997). They play the most important role in the series of oxidations and condensations that occur during the production of black tea. Catechins can be present in two possible configurations, but most of the catechin mass is in the so-called "epi-" form. They are also known as gallocatechins if there are three hydroxy groups present on the "B" ring of the substances (Graham, 1992). The six major catechins in fresh tea leaves are (+)-catechin (C), (+)-gallocatechin (GC), (-)-epicatechin (EC), (-)-epigallocatechin gallate (EGCG), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin (EGC) (Wright et al., 2002), of which the latter three may comprise over 90% of the total flavanol fraction (Bhatia and Ullah, 1968). Figure 2.4 illustrates the chemical structures of the four main tea catechins. It was also found that the gallates were always associated with chlorophyll-bearing organs. They are colourless, water-soluble compounds that contribute to the bitterness and astringency of green tea. Although their oxidation potentials vary, they are readily oxidisable. The concentration of the catechins is highly dependent on leaf age with the leaf bud and first leaf being the richest in epigallocatechin gallate. Levels of catechin also vary greatly between different tea varieties (Graham, 1992).



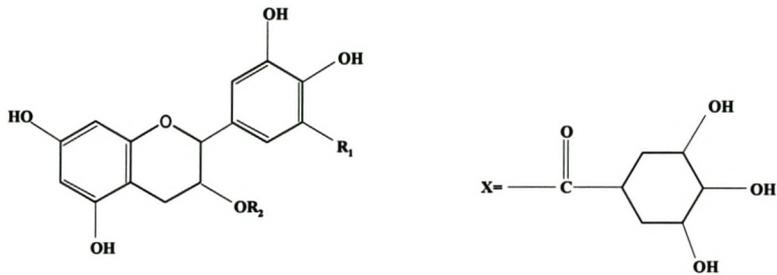
(-)-Epi-form (2R, 3R)

(-)-Epicatechin: R1=R2=H

(-)-Epigallocatechin: R1=OH, R2=H

(-)-Epicatechin gallate: R1=H, R2=X

(-)-Epigallocatechin gallate: R1=OH, R2=X



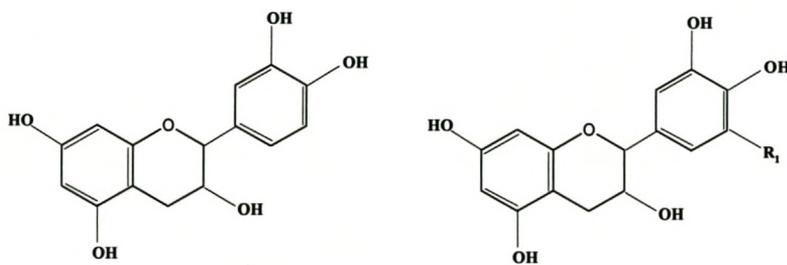
(-)-form (2R, 3R)

(-)-Catechin: R1=R2=H

(-)-Gallocatechin: R1=OH, R2=H

(-)-Catechin gallate: R1=H, R2=X

(-)-Gallocatechin gallate: R1=OH, R2=X



(+)-Epi-form (2S, 3S)

(+)-form (2R, 3S)

(+)-Epicatechin

(+)-Gallocatechin: R1=OH

(+)-Catechin: R1=H

Figure 2.4 The chemical structures of black tea catechins and their epimers (Wang et al., 2000b).

Almost all of the characteristics of manufactured tea are associated directly or indirectly with modifications to the catechins, including its taste, colour and aroma (Wang et al., 2000b). Liang and Xu (2001) determined that EGCC was the most important catechin in unfermented green tea with a concentration of 29.8 mg/g tea, while GC was the most important catechin to be found in black tea.

2.2.4.3 Theaflavins and thearubigens

During black tea manufacturing, catechins can be oxidised to form the typical colour and flavour of black tea (Wang et al., 2000b). The two principle classes of pigments present in tea beverage are the orange coloured theaflavins (TFs) and the brownish thearubigens (TRs) (Cloughley and Ellis, 1980).

The quinone derived from a catechin or from its gallate may react with a quinone derived from a gallo catechin or its gallate to form seven-membered ring compounds known as theaflavins, which contain the benzotropolone group (Figure 2.5a) (Graham, 1992).

In black tea, there are four main TFs namely theaflavin, theaflavin 3-gallate, theaflavin 3'-gallate and theaflavin 3,3'-digallate (Wang et al., 2000b) and they account for 2-6% of the dry weight in brewed black tea (Murugesan et al., 2002). As the fermentation period is prolonged, they will diminish in quantity (Graham, 1992).

The four major theaflavins are formed from specific pairs of substrates, but if neither of the flavan-3-ol substrates (EC and EGC) is gallated, free theaflavin (TF-f) will form. The flavan-3-ol composition of the green tea leaf should thus determine the theaflavin profile of the black tea. Other types of theaflavins and dimers are also formed but on a smaller scale and include theasinensis (bisflavanols), theaflavates, oolongtheanin and theacitrins. These compounds can contribute to the colour of the tea and might also be utilised in thearubigen formation (Wright et al., 2002). Bisflavanols are a group of colourless substances formed by the paired condensation of two gallo catechins and are thought to rearrange to form undefined black tea flavonoids (Balentine et al., 1997).

The TRs (Figure 2.5b) are a heterogenous group of phenolic pigments with a molecular weight distribution of 700 – 40,000 Da and accounts for 10-20% of the dry matter of black tea (Wang et al., 2000b) and 30-60% of the soluble solids (Wright et al., 2002). This wide range may be the result of rapid molecular condensation during holding in aqueous solution (Graham, 1992). They can be divided into three groups: (i) SI, soluble in ethyl

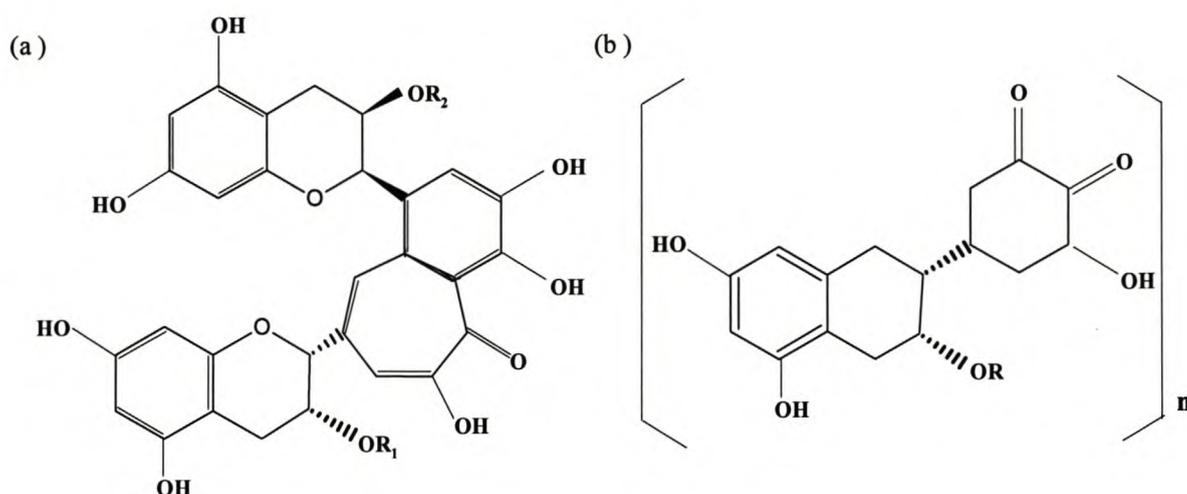


Figure 2.5 The chemical structure of (a) theaflavins and (b) thearubigens in black tea (Wang et al., 2000b).

acetate; (ii) SIa, soluble in water and diethyl ether, and (iii) SII, soluble in water (Davies et al., 1999).

Directly after plucking, TRs start forming and continue until drying of the tea particles. The amount of TR increases linearly during fermentation and attain their maximum level in over-fermented leaves (Murugesan et al., 2002).

2.2.4.4 Polyphenoloxidase (PPO) and Peroxidase (POD)

The enzymes polyphenoloxidase (PPO) and peroxidase (POD) play an important role in the oxidation of flavanols to black tea pigments such as theaflavins (TFs) and thearubigens (TRs). They oxidise *o*-diphenols to their corresponding orthoquinones (Figure 2.6a) (Finger, 1994). Subsequently, the orthoquinones condense to bis-flavanols by a process known as dimerisation. Bis-flavanols (theasinensis) are colourless compounds that are only present in very small quantities (Graham, 1992). These bis-flavanols then also rapidly condense into theaflavins. Further oxidation results in the transformation of theaflavins in thearubigens via an enzyme-dependent process. An ideal fermentation will result in a proper balance of theaflavins and thearubigens (Murugesan et al., 2002).

There is also some evidence that tea leaf peroxidase (POD) may contribute to the fermentation process. The specific POD activity is more than five times higher than that of PPO in fresh leaves and was found to increase during black tea processing. POD can also catalyse the oxidation of *o*-diphenols to their quinones but requires a peroxide, e.g. hydrogen peroxide, as an oxidising agent (Figure 2.6b). Hydrogen peroxide is formed during the action of PPO on certain flavonols and can then be used as an oxidising agent by POD. The maximal enzyme activities for POD were observed at pH 5.0, while for PPO it was at pH 5.5. A decline of PPO and POD activities can be observed with increasing fermentation time and temperature, with PPO declining more rapidly than POD (Finger, 1994).

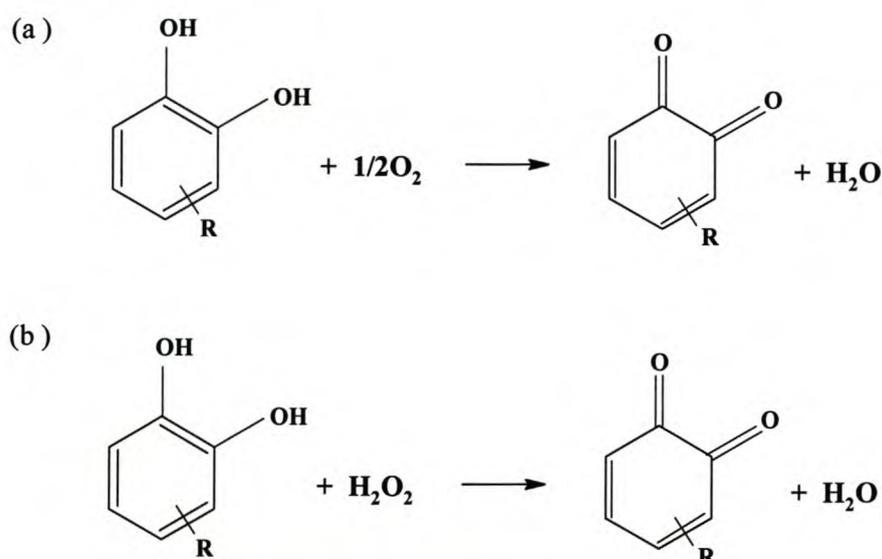


Figure 2.6 The oxidation of *o*-diphenols to their corresponding orthoquinones as catalysed by (a) polyphenoloxidase (PPO) and (b) peroxidase in black tea (Finger, 1994).

2.2.4.5 Aroma components

Tea aroma is an important quality parameter in determining the price of made tea (Ravichandran and Parthiban, 1998a). Fresh tea leaves are virtually odourless or slightly smell of green note (Mizutani et al., 2002). More than 60 volatile compounds have been identified in fresh tea leaves, including alcohols, carbonyls, esters, acids and cyclic compounds, of which the alcoholic compounds that contribute to the floral fruity aroma, are known to be important aroma components of both oolong and black tea (Graham, 1992; Wang et al., 2001a). It is well known that these alcoholic tea aroma compounds are mainly present as

glycosides in fresh tea leaves and are released by endogenous glycosidases during the manufacturing process. Analysis of these glycosides during the black tea manufacturing process, showed that they were all substantially hydrolysed during black tea manufacturing and contributed greatly to the formation of the black tea aroma (Wang et al., 2001a). These glycosides (Figure 2.7) have been isolated and identified as β -D-glucopyranoside (Glc), 6-*o*- β -D-xylopyranosyl- β -D-glucopyranoside (primeveroside, Prim), 6-*o*- α -L-arabino-pyranosyl- β -D-glucopyranoside (vicianoside, Vic) and 6-*o*- β -D-apiofuranosyl- β -D-glucopyranoside (acuminoside, Acu) (Wang et al., 2000a). Guo et al. (1993) isolated a new geranyl glycoside, geranyl 5-O- β -D-xylopyranosyl- β -D-glucopyranoside, as an aroma precursor from tea leaves.

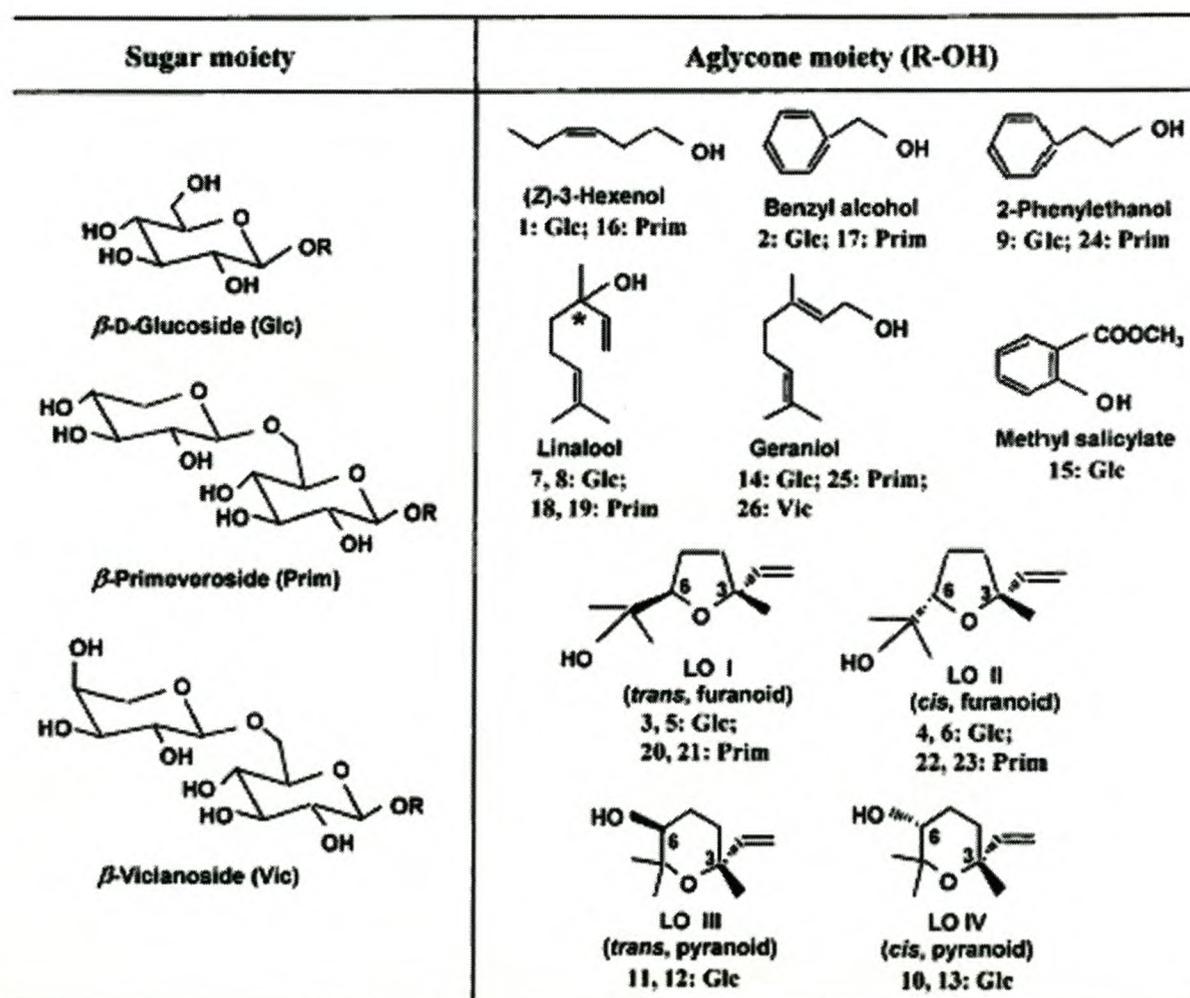


Figure 2.7 Structures of the glycosides synthesised as aroma precursors in black tea (taken from Wong et al., 2000a).

The quantitative analysis of glycoside aroma precursors in tea leaves showed that disaccharide glycosides, especially β -primeverosides, were about three times more abundant than other glucosides (Wang et al., 2000a). From these results it can be concluded that disaccharide glycosides, especially β -primeverosides, are the main precursors for the tea aroma formation (Mizutani et al., 2002). According to Ma et al. (2001), β -primeverosidase shows very narrow substrate specificity with respect to the glycon moiety and prominent specificity for the β -primeverosyl (6-O- β -D-xylopyranosyl β -D-glucopyranosyl) moiety. These volatile flavour compounds (VFC) can be divided into two main groups (Table 2.3).

Table 2.3 A total of 31 principle aroma compounds identified in dried fresh black tea leaves that constitute ~35% of the oolong tea aroma compounds (Wang et al., 2001a).

Group I	Group II
(Z)-3-hexenol	2-butanone
LO I*	hexanal
LO II*	1-penten-3-ol
linalool	pentanol
LO III*	(Z)-2-pentenol
LO IV*	(E,E)-2,4-heptadienal
methyl salicylate	(E,E)-3,5-octadien-2-one
geraniol	2,6,6-trimethyl-2-hydroxycyclohexanone
benzyl alcohol	hotrienol
2-phenylethanol	hexanoic acid
	3,7-dimethyl-1,5-octadiene-3,7-diol
	2-acetylpyrrole
	5,6-epoxy-, -ionone
	furaneol
	<i>trans</i> -nerolidol trace
	jasmine lactone
	4-hydroxymaltol
	3-ethyl-4-methyl-1 <i>H</i> -pyrrole-2,5-dione
	dihydroactinidiolide
	2,3-dihydrobenzofuran
	indole

* Four isomers of linalool oxide

The Group I compounds are products of lipid breakdown, mainly of the unsaturated fatty acids (Ravichandran and Parthiban, 1998a). During ageing and mechanical injury (Saikia and Mahanta, 2002), lipoxygenase attack the lipoprotein membrane storage lipids to release fatty acids, which undergo further degradation. The volatiles derived from these biochemical changes are major undesirable components of tea flavour. South Indian tea industries experience the “Pacha Taint” problem that causes off-flavours in tea on storage and this can be ascribed to poor lipid metabolism (Ravichandran and Parthiban, 2000). Guo et al. (1994) isolated three glycosides of the aroma constituents, linalool, 2-phenylethanol and benzyl alcohol, as aroma precursors of which the linalyl glycoside is the first example of naturally occurring (S)-linalyl β -primeveroside.

Group II compounds are mainly derived from glycosides of terpenoid glycosides and impart a sweet flowery aroma to black tea. They mainly comprise of terpenoids, aromatics and other non-terpenoids and are formed by enzyme-initiated oxidative breakdown of carotenoids and hydrolysis of terpenoid glycosides (Ravichandran and Parthiban, 1998a). The two most important group II compounds in oolong tea are jasmine lactone, which possesses a jasmine-like floral and fruity odour (31.55 mg/100 g tea) and indole (12.52 mg/100 g tea). Together, they constitute ~35% of the oolong tea aroma compounds (Wang et al., 2001a).

2.2.4.6 Carotenoids

Carotenoids are yellow pigments present in the fresh leaf (Hazarika and Mahanta, 1983). They occur in amounts comparable to those in carrot and spinach and are important precursors in green leaves for the manufacturing of high quality black tea (Ravichandran, 2002). Of the 14 carotenoids identified, β -carotene, lutein, violaxanthine and neoxanthine are thought to be the major components (Hazarika and Mahanta, 1983). The carotenoid content mostly depends on the type of leaf and ranges from 36 to 73 mg/100 g dry weight (Ravichandran, 2002).

During black tea manufacturing, the carotenoids are oxidatively degraded to several volatile and non-volatile products (Hazarika and Mahanta, 1983). Ravichandran (2002) found that carotenoid degradation was greater in the CTC (Crush, Tear, Curl) process than the orthodox process and in the order β -carotene > zeaxanthin > lutein.

2.2.4.7 Chlorophylls

During tea manufacturing, chlorophyll is degraded to pheophytin and pheophorbide. It was also found that the quantitative distribution of pheophytin, the darker pigment of the two, is not only dependant on the content of chlorophyll in the raw material, but also on the surrounding physico-chemical environment in the type of tea processing. Chlorophyll and concomitant pigments are insoluble in water and do not contribute to the liquor characteristics of tea. It is presumed that it is the pheophytin, pheophorbide and residual chlorophyll that influence the blackness in orthodox teas and thus the ratio of pheophytin to thearubigen, which will determine the shade of colour in the finished product (Mahanta and Hazarika, 1985).

2.2.5 Anti-microbial activity

The anti-bacterial effects of green, oolong and black teas have been studied intensively and it was found that the extracts of these teas showed a strong anti-microbial activity against Gram-positive and Gram-negative bacteria. These bacteria include members of the species *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium*, *Clostridium perfringens* and *Pseudomonas fluorescens*. However, the extracts were not found to be effective against yeasts and moulds (Ahn et al., 1991; Oh et al., 1999; Toda et al., 1989; Yeo et al., 1995).

Tea polyphenols in green tea can prevent teeth from decaying by inhibiting the biological activities of the cariogenic streptococci, *Streptococcus mutans* and *Streptococcus sobrinus*, which are responsible for dental caries, the most common of all microbial related diseases. It is believed that tea exerts its anti-caries effect by reducing the acid on the tooth enamel for the whole time that starch is trapped in the teeth (Wang et al., 2000b). Suzuki (1983) found that green tea polyphenols, especially EGCG, have stronger deodorising activity than sodium copper chlorophyllin and so should improve breath freshness.

Tea catechins have also been found to exhibit inhibitory effects against phytopathogenic bacteria such as *Erwinia*, *Pseudomonas*, *Clavibacter*, *Xanthomonas* and *Agrobacterium* and it was found that EGC and EGCG are more effective than EC and ECG (Fukai et al., 1991). Diker and Hascelik (1994) found that tea catechins at a minimal inhibitory concentration (MIC) of 32 µg/ml are able to inhibit growth of *Helicobacter pylori*, which causes chronic gastritis and gastric cancer. It has been shown that tannins inhibit the

growth of many filamentous fungi (Scalbert, 1991), while yeasts appear to be more resistant. Tannic acid and propyl gallate are toxic to foodborne bacteria such as *E. coli*, *S. aureus*, *Streptococcus faecalis* and *Yersinia enterocolitica* (Chung et al., 1998).

Volatile flavour compounds have also shown anti-bacterial activity, but are only effective in combination with each other. The green tea flavour compounds also show additional anti-fungal activity, which may contribute to the fact that these compounds can be used as anti-microbial agents for cosmetic and food products (Kubo et al., 1992).

Tea extracts have been used for therapy in cholera patients in epidemic areas, as well as the prevention of influenza virus infections. A 50% inhibition of the AIDS virus has been reported using ECG and EGCG at concentrations of 0.01-0.02 µg/ml. It has also been demonstrated that a component of tea extracts is able to reverse methicillin resistance in methicillin-resistant *S. aureus* (MRSA), the so-called “superbug” (Wang et al., 2000b).

2.3 *Aspalathus linearis*

Two indigenous South African herbal teas, honeybush tea (*Cyclopia intermedia*) and rooibos tea (*Aspalathus linearis*) have both been shown to contain a complex mixture of polyphenolic compounds, of which a dihydrochalcone, aspalathin, is unique to rooibos tea while luteolin is one of the most important flavones in honeybush tea. These phenolic compounds are thought to contribute towards the scavenging ability of these herbal teas, which also exhibit antimutagenic properties. Steps in the processing of these teas, include fermentation of leaves and stems, followed by sun-drying and sieving and in the case of rooibos tea, steam pasteurisation of the dried product before packaging (Marnewick et al., 2000; 2003)

Honeybush tea is prepared from the leaves, fine stems and flowers of mainly two species, *Cyclopia intermedia* E. Mey. and *Cyclopia subternata* Vogel (Kamara et al., 2003). Both *Cyclopia* species belong to the genus, *Cyclopia* Vent. (Tribe Podalyrieae), which consists of ± 24 species of woody legumes endemic to the fynbos region of South Africa (De Nysschen et al., 1996). Research on honeybush tea has only started in the 1990's and initial studies on the phenolic content of *C. intermedia* E. Mey, have revealed the presence of phenolic metabolites thought to have significant pharmacological properties (Kamara et al., 2003). Honeybush tea has a very low tannin content, contains no caffeine and comprises various antioxidants. It is therefore especially valuable for children and patients with digestive and heart problems where stimulants and tannins should be avoided. It is also used for

restorative properties such as soothing coughs and alleviating bronchial complaints, including tuberculosis, pneumonia and catarrh (Kamara et al., 2003).

2.3.1 Introduction

Rooibos tea is prepared from the leaves and stems of the rooibos tea plant, *Aspalathus linearis*, a unique South African, leguminous shrub native to the mountainous areas of the northwestern Cape Province and one of the major crops cultivated in the Clanwilliam region. The coarse sandy soil and winter rainfall of this region are the ideal conditions for the cultivation of rooibos (Morton, 1983). It is one of the relatively few economically important plants that have made the transition from a local wild resource to an important cultivated crop in the 20th century (Bramati et al., 2003).

It was members of the indigenous Khoikhoi group who first discovered in 1772 that the fine, needle-like leaves of the *A. linearis* plant made a tasty, aromatic tea. It was only in 1904 that a Russian immigrant and pioneer in the area, Benjamin Ginsberg, became interested in rooibos and realised its marketing potential (Morton, 1983). Cultivation of the tea started rather slowly, but during and after World War II it was favoured due to the high price of the Ceylonese tea (Dahlgren, 1968). In South Africa, an average of six cups of rooibos tea is consumed per person per day (Joubert and Ferreira, 1996) and it is becoming more popular worldwide with a 10% increase in sales from 1995 to 1997. Currently, rooibos tea is exported to many countries, including Britain, Germany, Japan, the Far East and the United States of America. In 1998, about 7 500 tons of tea was yielded from a harvest of about 23 090 hectares of rooibos plantations (Schepers, 2001).

The *Aspalathus* genus consists of more than 200 species and is endemic to South Africa (Dahlgren, 1968). The *A. linearis* species is exceptionally polymorphic and distinct geographical forms can be distinguished that differs in habitat, fire-survival strategy, vegetative and reproductive morphology, enzyme patterns and flavonoids. Most of the variation within populations is quantitative and therefore it can be expected that the relative quantities of phenolic compounds will show large qualitative and quantitative differences between the wild types (Van Heerden et al., 2003). In general, *A. linearis* is a bushy plant, with long slender branches and thin, needle-like leaves, varying from 2-6 cm in length (Habu et al., 1985).

Although the plant requires a production area with winter rainfall, its active growth only starts in spring, increasing towards summer after which growth declines. Rooibos seed is

by nature very hard-shelled and is scrubbed by means of mechanical scourers to increase the germination potential from approximately 25-30% to 85-95%. After further treatment with fungicides and insecticides, the seed is ready to be sown. Sowing takes place from February to March after which the plants are tended carefully for 18 months when they are ready for the first harvesting (Morton, 1983). The life span of the tea shrublets is about 6-18 years (Dahlgren, 1968).

The young, green shoots are harvested during summer and early autumn (November to March) (Joubert, 1988). Workers cut 30-45 cm branches from the plants with scythes or sickles after which the shoots are immediately processed by passing them through a carving machine, where they are cut into 3 to 4 mm lengths to prevent the loss of moisture (Cheney and Scholtz, 1963; Dahlgren, 1968). The harvested tea is then moistened and bruised between rollers, triggering the fermentation process during which the distinctive colour, aroma and flavour of rooibos tea is released (Morton, 1983). The bruising is done preferably in the late afternoon to allow for the fermentation of the tea during the night and the subsequent drying of the tea the next day. The tea is then thrown into heaps that are turned two to three times during the night to ensure uniform oxidation of the wet tea. The recommended temperature for fermentation is about 38-42°C (Cheney and Scholtz, 1963). The fermentation time depends on the climate, the composition of the plant material, the degree of bruising and the amount of water added to the fermentation heaps, but on average it lasts about 12 to 18 hours (Schepers, 2001). Joubert (1990) found that extraction of the different phenolic groups from tea leaves significantly increased with increasing temperature. This also led to an increase in the phenolic content of extract soluble solids.

The rooibos tea is then spread out in thin layers of about 15-20 mm on concrete floors in the hot sunlight to dry which reduces the moisture content to 8-10% (Morton, 1983). If drying takes up to 24 hours or longer, it will result in a tea that lacks flavour. When the tea is not properly dried and remains moist, over-fermentation can occur that causes the tea to smell sour (Cheney and Scholtz, 1963). After the tea has dried it is screened to remove dust and coarse material and only material from 0.4 to 1.68 mm is used for commercial purposes (Joubert, 1988). The rooibos tea material undergoes a special process of pasteurisation and is dried once more over an airbed drier. The rooibos tea is then finally weighed and packed in tea bags or in loose leaf form (Standley, 1999).

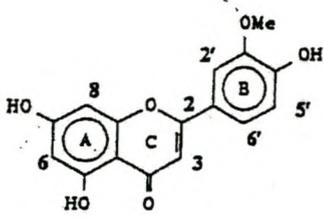
2.3.2 Chemical composition

According to Koeppen (1970), the organoleptic properties of rooibos tea can be attributed to polyphenols and therefore they are important in the preparation of soluble rooibos products. Processing, or more specifically, fermentation, greatly influences the phenolic composition of rooibos tea (Von Gadow et al., 1997). Studies showed that fermented rooibos tea contained approximately 7% of the dihydrochalcones originally present in the green tea. This decrease can be ascribed to the enzymatic and chemical oxidation of polyphenols that were initiated with comminution of the tea at which time exposure to oxygen and cell damage occurred. The ratio of dihydrochalcones to total ethyl acetate soluble polyphenols also decreased with processing which can be attributed to its conversion to other ethyl acetate soluble polyphenols (Joubert, 1996).

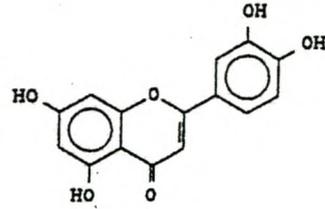
The polyphenols can be classified into two groups, namely flavonoids and phenolic acids (Koeppen, 1970). The flavonoids (Figure 2.8) can further be divided into three groups, namely flavones, flavonols and the flavanones (Schepers, 2001). Polyphenols characteristic to rooibos tea include the dihydrochalcones aspalathin (49.92 mg/g) and nothofagin, the flavones *iso*-orientin (3.57 mg/g), orientin (2.336 mg/g) and their 4'-deoxy analogues *iso*-vitexin, vitexin, the aglycons luteolin and chrysoeryol as well as the flavonol quercetin and its 3-*O*- β -D-glucopyranoside derivative *iso*-quercitrin. These compounds were more abundant in unfermented rooibos than in fermented, with the levels of aspalathin almost 50 times higher (Bramati et al. 2002; 2003). Values obtained were from single studies only and therefore substantial variation might occur between different tea samples tested.

The dihydrochalcone, aspalathin (2',3,4,4',6'-pentahydroxy-3-*C*- β -D-glucopyranosyldihydrochalcone), is unique to rooibos tea and contributes significantly to the characteristic red-brown colour of processed tea (Joubert, 1996). Aspalathin constitutes approximately 0.55% of the soluble solids of processed tea (Joubert and Ferreira, 1996). During fermentation, aspalathin undergoes extensive degradation and is enzymatically oxidised to the flavanones dihydro-2,3-orientin and dihydro-3,4-*iso*-orientin and unknown polymeric substances (Bramati et al., 2003; Joubert and Ferreira, 1996; Von Gadow et al., 1997), while the *C*-glycosyl-flavones *iso*-orientin, orientin, vitexin and *iso*-vitexin are degraded at a lower extent (Bramati et al., 2003).

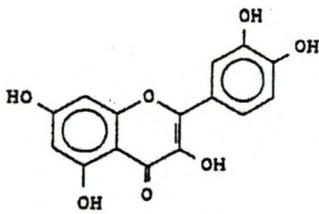
Nothofagin is another rare β -hydroxydihydrochalcone present in relatively large amounts in unprocessed rooibos tea and constitute about 0.19% of the soluble solids of the



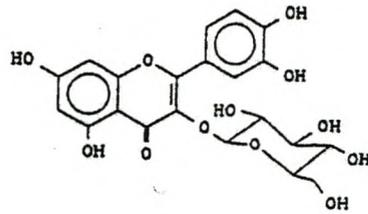
Chrysoeriol



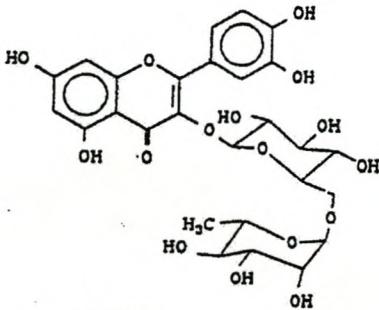
Luteolin



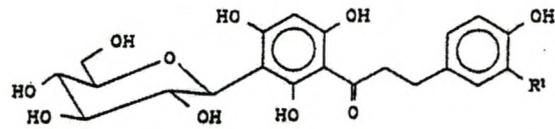
Quercetin



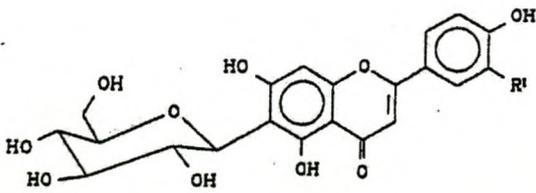
Isoquercitrin



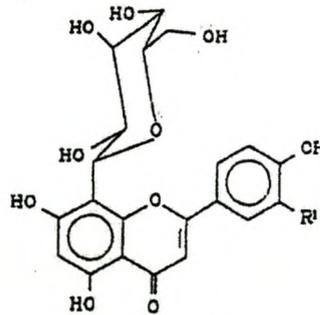
Rutin



Aspalathin: R¹=OH
Nothofagin: R¹=H



Orientin: R¹=OH
Vitexin: R¹=H



Iso-orientin: R¹=OH
Isovitexin: R¹=H

Figure 2.8 The major flavonoids found in rooibos tea (taken from Joubert and Ferreira et al., 1996).

processed tea (Joubert and Ferreira, 1996). Structurally it is similar to aspalathin except for the hydroxylation pattern of the B-ring (Joubert, 1996). It has previously only been isolated from *Nothofagus fusca* (red beech) (Hills and Inoue, 1967). Rutin, the main flavonol-glycoside, is partly converted to the aglycone quercetin as can be observed by its increased level in fermented rooibos (Bramati et al., 2003). The average amount of quercetin per cup was found to be 1.5 mg (Joubert, 1990). It has been postulated that aspalathin and nothofagin might contribute to the naturally sweet taste of rooibos tea (Jaganyi and Wheeler, 2003). Joubert (1996) also found that the aspalathin and nothofagin contents decreased with processing and depended on the degree of oxidation of the tea.

Rooibos tea contains only very small quantities of (+)-catechin, procyanidin B3 and the profisetinidin triflavanoid, bis-fisetinidol-(4 β ,b:4 β B,8)-catechin (Schepers et al., 2001). The low contents of these three compounds results in rooibos tea having a low tannin content (3.2%) (Cheney and Scholtz, 1963). Rooibos tea is also caffeine-free, which contributes to its growing popularity as a health beverage (Bramati et al., 2002). Caffeine is associated with a number of physiological and pharmacological effects that can mainly be attributed to its effect of blocking adenosine receptors. It also acts as a mild central nervous system stimulant and has broncho-dilatory properties. Researchers are also looking at the potential relationship between caffeine and cardiovascular disease (Bimms, 1995).

Phenolic acids isolated from rooibos tea include 4-coumaric, protocatechuic, vanillic, syringic, caffeic, ferulic, cinnamic and 4-hydroxybenzoic acid (Rabe et al., 1994). Rooibos tea also contains non-phenolic metabolites, which include the inositol (+)-pinitol, the nucleoside uridine, and a phenylpyruvic acid-*O*- β -D-glucopyranosyl derivative (Schepers, 2001). Other substances isolated from rooibos tea extracts are minerals and volatile components that contribute to the characteristic sweet flavour of rooibos tea (Kawakami et al., 1993; Rabe et al., 1994). Some of the major volatile components include guaiacol (24.0%), damascenone (5.0%), geranylacetone (4.2%) and β -phenylethyl alcohol (4.1%) (Habu et al., 1985).

2.3.3 Health promoting benefits

Rooibos tea is becoming increasingly more popular due to its unique taste, versatility and for its reputation as a health drink (Jaganyi and Wheeler, 2003). It gained further popularity when Annatjie Theron discovered in 1968 that a rooibos tea infusion, when administered to her baby, cured the infant of chronic restlessness, vomiting and stomach cramps. Rooibos tea is

claimed to help improve appetite and cure insomnia, allergies and nervous complaints (Morton, 1983). It is also considered as an “anti-ageing” beverage by the Japanese and used as an ingredient in a natural medicinal product believed to be effective in relieving a variety of inflammatory diseases as well as reducing serum lipid peroxide levels (Niwa et al., 1988). Studies also revealed that rooibos tea has antioxidative activities by superoxide dismutase mimetic substances and has effects on dermatological diseases such as Bechet’s disease, Sweet disease and photosensitive dermatitis (Kawakami et al., 1993).

The low tannin content of rooibos tea presents little or no interference with the digestibility of protein and has an overall beneficial effect on the human diet (Morton, 1983), with no significant effect on iron absorption (Hesseling et al., 1979). It has also been reported that small amounts of quercetin, kaempferol and myricetin have anti-microbial activity against Gram-positive bacteria and phytopathogenic fungi and it was determined that quercetin had a minimal inhibitory concentration (MIC) value of 37 µg/ml against *S. aureus* (Hamilton-Miller, 1995). Table 2.4 summarises the contribution of chemical compounds isolated from *A. linearis* on human health.

2.3.4 Antioxidant activity

Interest in antioxidants has increased in recent years since an imbalance in oxidative levels may be an important factor in causing diseases, such as arteriosclerosis, arthritis, heart disease, Alzheimer’s disease and cancer (Joubert and Ferreira, 1996). Oxygen is essential for life, but for more than 100 years, it has been recognised as the principal agent responsible for the deterioration of organic materials exposed to air (Larson, 1988). The normal metabolism of oxygen results in formation of the free radical superoxide ($O_2^{\bullet-}$) and hydrogen peroxide that can be detrimental to health (Thomas, 1995). In the presence of suitable transition metals (e.g. iron), superoxide and H_2O_2 can generate the highly active OH^{\bullet} radical *in vivo* (Halliwell et al., 1992). OH^{\bullet} randomly attacks lipids, proteins and DNA and free radical damage is demonstrated in lipid peroxidation, protein denaturation and DNA mutation as the radicals attack different substances in living cells and tissue (Halliwell and Gutteridge, 1984). Under certain circumstances, e.g. high levels of air pollutants, tobacco smoke, pesticides, certain drugs, transition metals (iron) and alcohol, more free radicals are formed (Joubert and Ferreira, 1996). Fortunately, biological systems have antioxidant enzymes and antioxidant nutrients, such as vitamin A, C and E, at their disposal to protect them against the harmful effects of these oxidative substances (Halliwell et al., 1995). The radical defense

Table 2.4. Some of the chemical compounds found in rooibos tea and their contribution to human health.

Chemical compound	Health promoting benefit	References
4-Coumaric acid	Antimicrobial activity	(Eklund, 1985)
Protocatechuic acid	Antimicrobial and antioxidant activity	(Larson, 1988)
Vanillic acid	Antimicrobial activity	(Eklund, 1985)
Syringic acid	Antimicrobial and antioxidant activity	(Larson, 1988)
Caffeic acid	Antimicrobial and antioxidant activity	(Larson, 1988)
Ferulic acid	Antimicrobial and antioxidant activity	(Larson, 1988)
Cinnamic acid	Antimicrobial activity	(Eklund, 1985)
4-Hydroxybenzoic acid	Antimicrobial activity	(Eklund, 1985)
(+)-Pinitol	Role in cellular communication and has anti-viral properties	(Schepers, 2001)
Phenylpyruvic acid- <i>O</i> - β -D-glucopyranosyl derivative	Used for prevention of dermatological diseases and plays important role in biosynthesis of C ₆ -C ₃ -C ₆ backbone of flavonoids	(Schepers, 2001)
Luteolin	Anti-spasmodic properties	(Schepers, 2001)
Quercetin	Anti-spasmodic properties. Anti-atherosclerotic activity. Causes the prevention of the oxidation of low-density lipoproteins (LDL)	(Schepers, 2001)

mechanisms can be weakened as a result of ageing and inadequate nutrition, resulting in oxidative stress that can cause cell damage and death in severe cases (Joubert and Ferreira, 1996). There is no recommended daily intake for antioxidants, but our antioxidant requirements are determined by factors such as fat intake, life-style, age, smoking, alcohol intake, infections, occupation, etc. that influence our oxidative stress levels (Block, 1992; Thurnham, 1992).

The term antioxidant has been defined as a substance capable of delaying, retarding or preventing the oxidation process (Schuler, 1990). Generally, antioxidants can be classified into two major groups. The first group is the primary or chain-breaking antioxidants that interfere with lipid peroxidation by reacting with lipid radicals to form more stable products. They also have the ability to bind active oxygen species (Torel et al., 1986). The second group is the secondary antioxidants, which slow down the rate of lipid oxidation through mechanisms such as chelation of metals, scavenging of oxygen, quenching of singlet oxygen and reduction of hydroperoxides to non-radical products (Gordon, 1990).

Nature provides us with an abundance of antioxidants that originate from compounds that occur naturally in foodstuff or from substances formed during its processing. There is an increasing consumer resistance to the use of synthetic antioxidants and therefore the use of natural antioxidants in foods for stabilisation against oxidative changes is gaining more acceptance. In the past, research has focused mainly on the antioxidant properties of β -carotene (provitamin A) and vitamins C and E, but scientists began to realise the potential of other dietary substances such as flavonoids in cancer prevention and reduction of the frequency of coronary heart disease (Joubert and Ferreira, 1996).

Rooibos tea infusions have been shown to exhibit good antioxidant activity which can be attributed to the presence of flavonoids and phenolic acids. These compounds play an active role in reducing the formation of reactive oxygen species (ROS) since they affect enzymes that catalyse redox reactions. Flavonoids display secondary antioxidant activity by quenching singlet oxygen and in addition, they also have the ability to chelate metal ions, such as Cu^{2+} and Fe^{2+} (Bramati et al., 2002). Several of the flavonoids found in rooibos tea have shown antioxidant activity, for example quercetin, luteolin, rutin, isoquercitrin and isovitexin. All the flavonoids of the 3', 4'-dihydroxy configuration also have antioxidant activity (Pratt and Hudson, 1990). It is thought that the ene-diol functionality in the electron-rich aromatic B-ring system could supply the electrons that are required for the reduction of the active oxygen species, rendering them harmless (Joubert and Ferreira, 1996).

Quercetin is oxidised by superoxide to phenolic carboxylic acids and cannot be regenerated, while protocatechuic acid, one of the breakdown products of quercetin, is an effective antioxidant (Onyeneho and Hettiarachy, 1992). It was also found that dihydrochalcones are more effective antioxidants than their corresponding flavonones and therefore it can be expected that aspalathin would be a more effective antioxidant than its corresponding flavonones, i.e. dihydro-orientin and dihydro-*iso*-orientin (Joubert and Ferreira, 1996).

When the antioxidant activity of fermented and unfermented rooibos tea was compared, the results indicated that unfermented tea has an inhibition of 86.6%, while fermented tea has an inhibition of 83.4% based on the scavenging of the DPPH (α,α -diphenyl- β -picrylhydrazyl) radical by compounds in the tea. The higher inhibition can be attributed to the fact that aspalathin is an active scavenger of DPPH and the major flavonoid present in unfermented rooibos tea (Von Gadow et al., 1997). Tabel 2.5 shows the antioxidant activity of different teas as a measure of their DPPH radical scavenging ability.

Table 2.5. Antioxidant activity of different teas as obtained with the DPPH radical scavenging methods (Von Gadow et al., 1997).

Type of tea	Inhibition ^a (%)	AAC ^b
Green	90.8	695
Oolong	71.2	597
Black	81.7	650
Unfermented rooibos	86.6	557
Semi-fermented rooibos	81.9	522
Fermented rooibos	83.4	605

^aDetermined by DPPH radical scavenging method; mass ratio of tea solids to DPPH = 0.52.

^bAAC, antioxidant activity coefficient as determined by β -carotene bleaching method; concentration of stock solution = 500 mg soluble solids per 100 ml solution.

It is clear from the literature that not much is known about the composition of the rooibos tea leaf. It was therefore essential to search for novel enzymes or enzyme combinations that would be able to macerate the rooibos tea leaves. In the next chapter, the methodology and results for the evaluation of a number of fungal strains for their production of hydrolytic enzymes are described. The same hydrolytic enzymes used for the degradation of the leaves of *C. sinensis*, were used as starting point.

Chapter 3

Research Results:

Evaluation of Fungal Enzymes for Microbial Bioprocessing of Rooibos Tea

Written in a style suitable for submission to a scientific journal

Evaluation of Fungal Enzymes for Microbial Bioprocessing of Rooibos Tea

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Abstract

The leaves of the rooibos tea plant, *Aspalathus linearis*, require a long fermentation period (14-16 hours) during which the characteristic organoleptic qualities of rooibos tea develop. This results in a significant loss in antioxidants in the processed tea, and therefore also in its pharmaceutical and nutraceutical value. Many of the aroma and medicinal compounds in rooibos tea are trapped within the cellulolytic plant material of the tea leaves as glycoconjugated aroma and phenolic compounds. Since the plant material and phenolic composition of *A. linearis* is totally different from that of *Camellia sinensis*, used for manufacturing of black tea, it was essential to search for novel enzymes or enzyme combinations that would specifically macerate the rooibos plant material. Various fungal species known for the production of hydrolytic and oxidative enzymes were screened for their potential to improve aroma development as well as the extraction of soluble matter from rooibos tea. The results suggest that different fungal strains can be used for different applications, i.e. extraction of additional soluble solids and/or polyphenols for rooibos extracts, or enhanced colour and aroma development from green rooibos tea. Fungal species that showed good potential for bioprocessing of rooibos tea were further characterised in terms of the enzymes produced and their corresponding activities.

Introduction

Rooibos tea (*Aspalathus linearis*), an indigenous South African herbal tea, is a unique beverage that is rich in volatile components, minerals and ascorbic acid. It is also caffeine-free and has a low tannin content (Rabe et al., 1994). These aspects, together with its unique taste and versatility, contribute to the increasing popularity of rooibos tea as a health beverage (Bramati et al., 2002; Janganyi and Wheeler, 2003). Rooibos tea infusions are known to have good antioxidant activity that can be attributed to the presence of polyphenols, more specifically flavonoids (Bramati et al., 2003; Joubert and Ferreira, 1996). Aspalathin, one of

the flavonoids unique to rooibos tea, contributes significantly to the characteristic red-brown colour of processed rooibos tea (Joubert, 1996). Other flavonoids like quercetin and nothofagin have also been isolated from rooibos tea (Joubert, 1990). During rooibos tea processing, the enzyme polyphenol(II)oxidase oxidises the polyphenols present in the tea leaves, resulting in the characteristic organoleptic qualities of rooibos tea (Murugesan et al., 2002). This processing includes shredding and bruising of the plant material, followed by exposure of the moistened tea material to sunlight at 30-35°C for 14-16 hours, also referred to as “fermentation” (Marais et al., 2000).

Enzymes present in the *Camellia sinensis* black tea plant cells play an important role in tea processing. They are responsible for the large number of chemical and biochemical changes occurring during fermentation, resulting in the development of aroma and black tea characteristics (Jain and Takeo, 1984). The enzyme polyphenol oxidase (PPO) plays an important role in the oxidation of flavanols to black tea components such as theaflavins and thearubigens. It catalyses the oxidation of *o*-diphenols to their corresponding quinones, which are then spontaneously transformed to more complex fermentation products. Tea leaf peroxidase (POD) can also catalyse the oxidation of *o*-diphenols to their quinones, but requires a peroxide, e.g. hydrogen peroxide, as an oxidising agent (Finger, 1994). During the CTC (crush, tear and curl) process in the conventional method of black tea manufacturing, the partial disruption of tea leaves reduces the levels of peroxidase and polyphenol oxidase in the native leaves, thereby suppressing the formation of black tea components. Maceration of the tea leaves by shredding and bruising during the rolling process causes only partial rupture of the cells, resulting in incomplete oxidation of oxidisable matters (Angayarkanni et al., 2002). The cell wall polysaccharides can also act as a barrier for the interaction of the enzymes (PPO and POD) with the tea leaves (Murugesan et al., 2002).

It has been reported that the application of external cellulolytic enzymes, e.g. cellulases, pectinases and xylanases, that can degrade the cell walls of tea leaves may result in complete maceration of the tea leaves (Murugesan et al., 2002). Angayarkanni et al. (2002) evaluated the addition of external crude enzyme extracts of *Aspergillus flavus*, *Aspergillus indicus* and *Aspergillus niveus* to black tea for a number of quality parameters, including theaflavin, thearubigen, highly polymerised substances, total liquor colour, dry matter and total soluble solids. Thearubigens are responsible for colour, body and taste, while theaflavins contribute to the briskness, brightness and quality of the liquor (Murugesan et al., 2002). Although the purified pectinase from the tea fungus (a symbiont of two yeasts, *Pichia* sp. NRRL Y-4810 and *Zygosaccharomyces* sp. NRRL Y-4882 and a bacterium, *Acetobacter* sp.

NRRL B-2357) improved some of these parameters, the crude enzyme extracts from the three *Aspergillus* fungal extracts showed better results. A possible reason for this is that the crude extracts contained a variety of enzymes, e.g. cellulases, xylanases, pectinases and laccases (Angayarkanni et al., 2002). Furthermore, it has also been shown that the combination of purified cellulase (also from the tea fungus) and a *Trametes versicolor* laccase in a ratio of 3:2 (v/v) improved the tea quality above that of the purified cellulase (Murugesan et al., 2002).

Although fungal species are known for the efficient hydrolysis of various plant materials, fungal enzymes have not been evaluated for the bioprocessing of rooibos plant material. Many of the aroma compounds in rooibos tea are linked to the hemicellulotic backbone of plant material by way of β -glycosidic bonds. The complex polysaccharide structures of the tea leaves could therefore be macerated by polysaccharases that target the cellulolytic material, while enzymes that target the glucosidic bonds could assist in the release of the polyphenols. This combined effect may improve the extraction of soluble solids, as well as aromatic and antioxidant compounds.

The objective of this study was to screen various food grade fungal species known for the production of hydrolytic and oxidative enzymes, for their potential application to improve aroma and colour development from green rooibos tea, and/or increase the extraction of soluble matter from rooibos tea. The quality parameters included aroma and colour development, total soluble solids, total polyphenols and antioxidant content. Two different substrates were treated, i.e. spent tea (i.e. after extraction with physical-chemical methods) and green rooibos tea leaves (dried unfermented tea). The fungal strains identified as effective for improved aroma development from green tea or the extraction of soluble solids from spent tea, were further characterised with respect to enzymes that could be included in an enzyme cocktail that can enhance the maceration of the rooibos plant material without unnecessary loss in aroma development or antioxidant content.

Materials and methods

Plant material and chemicals

Dried spent rooibos tea (i.e. after extraction with conventional physical-chemical methods) was provided by Benedict (Pty) Ltd (Stellenbosch) and dried green unfermented rooibos tea

leaves were supplied by the Post-Harvest & Wine Technology Division of ARC Infruitec-Nietvoorbij (Stellenbosch, South Africa).

All chemicals used were standard or analytical grade and obtained from Sigma Chemical Co. (St Louis, USA); Merck (Darmstadt, Germany); Fluka AG (Buchs, Switzerland); Riedel-de Haën AG (Seelze, Hannover) and BDH Chemicals Ltd (Poole, England).

Organisms and culture conditions

Fungal strains used in this study (Table 3.1) represent a phylogenetically diverse group of fungi including ascomycetous and basidiomycetous groups from the culture collection of the Department of Microbiology at Stellenbosch University, South Africa, or the American Type Culture Collection, Rockville, Maryland, USA. All of these strains are food-grade organisms and known for the production of hydrolytic enzymes. They were maintained by periodic transfer to malt extract agar (MEA) (Sigma) plates, incubated for 5 days at 30°C (or 25°C for the *Pleurotus* spp.) and stored at 4°C. However, *Lentinula edodes* Abo 287 was maintained on 2.3 % agar plates with 1 x MYPG medium containing 1% malt extract (Sigma), 0.2% yeast extract (Merck), 0.2% tryptone peptone (Merck), 1% glucose (Merck), 0.2% KH₂PO₄ (Sigma), 0.1% MgSO₄·7H₂O (Sigma), 0.1% L (+)-asparagine (Merck) and 0.1% thiamine (Merck), and incubated at 25°C for 21 days.

The other fungal strains listed in Table 3.1 were isolated from either spent rooibos tea or different brands of commercial rooibos tea bags. Approximately 1 g of rooibos tea was wet with sterile dH₂O in a petri dish and incubated at 30°C for 3 to 4 days. Individual colonies were transferred to malt extract agar (MEA) plates and incubated for 3 to 4 days at 30°C. This procedure was repeated two to three times to isolate pure cultures that were further identified using a combination of morphological and molecular techniques.

Identification of fungal strains isolated from rooibos tea products

The fungal isolates were identified based on the DNA sequence of their ITS regions. Genomic DNA was extracted based on the protocol of Raeder and Broda (1985). Using acid washed sand, frozen mycelia were ground to a fine powder with a mortar and pestle. Approximately 0.5 ml of the powdered mycelia was transferred to a 1.5 ml micro-centrifuge tube containing 500 µl ice-cold extraction buffer (200 mM Tris-HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). The sample was vortexed briefly, extracted with 350 µl phenol and 150 µl chloroform:isoamylalcohol (24:1), vortexed and centrifuged (13 000 rpm, 60 min, 4°C).

Table 3.1. Source and identity of fungal strains used in this study

Isolate number	Organisms	Source
Dsm 2344	<i>Aspergillus aculeatus</i>	German Collection of Microorganisms and Cell Cultures
NRRL 3135 / ATCC 66876	<i>Aspergillus ficuum</i>	ARS Culture Collection, National Center for Agricultural Utilisation Research, U.S Department of Agriculture, Rockville, Maryland, USA, American Type Culture Collection
Abo 511	<i>Aspergillus oryzae</i> *	Department of Microbiology, Stellenbosch University
ATCC 10864	<i>Aspergillus niger</i>	American Type Culture Collection
ATCC 52430	<i>Aspergillus terreus</i>	American Type Culture Collection
Abo 287	<i>Lentinula edodes</i>	Department Microbiology, Stellenbosch University
SAM 3	<i>Penicillium candidum</i>	Rhoda Foods, France
Abo 286	<i>Pleurotus citrinopileatus</i>	Department Microbiology, Stellenbosch University
Abo 283	<i>Pleurotus djamor</i>	Department Microbiology, Stellenbosch University
Abo 280	<i>Pleurotus florida</i>	Department Microbiology, Stellenbosch University
RUT C30	<i>Trichoderma reesei</i>	Department Microbiology, Stellenbosch University
MP1	<i>Rhizopus oryzae</i>	Isolated from spent tea
MP2	<i>Aspergillus fumigatus</i>	Isolated from green tea
MP3	<i>Rhizomucor pusillus</i>	Isolated from green tea
MP4	<i>Aspergillus fumigatus</i>	Isolated from spent tea
MP5	<i>Rhizopus</i> sp.	Isolated from spent tea
MP6	<i>Neurospora sitophila</i>	Isolated from spent tea
MP7	<i>Paecilomyces variotti</i>	Isolated from commercial tea bags
MP8	<i>Aspergillus tubingensis</i>	Isolated from green tea
MP9	<i>Paecilomyces variotti</i>	Isolated from commercial tea bags
MP10	<i>Aspergillus fumigatus</i>	Isolated from commercial tea bags
MP11	<i>Paecilomyces variotti</i>	Isolated from commercial tea bags
MP12	Unidentified	Isolated from commercial tea bags

*Referred to as *Aspergillus oryzae* throughout the study but subsequently molecularly identified as *Aspergillus japonicus*.

The aqueous phase was treated with 25 µl RNase A (20 mg/ml) and incubated at 37°C for 10 min. The DNA was extracted with 1 volume chloroform:isoamylalcohol (24:1), precipitated with 0.54 volumes isopropanol and dissolved in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

Polymerase chain reactions (PCR) were carried out with Expand™ High Fidelity DNA Polymerase (Roche, Germany) in a Perkin Elmer 2 400 Thermal Cycler. Primers ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3') and ITS 5 (5'-GGA AGT AAA AGT CGT AAC AAG-3'), obtained from Roche (Germany) (White et al., 1990), or universal primers F63 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and LR3 (5'-GGT CCG TGT TTC AAG ACG-3') were used to amplify the conserved ITS region (Fell et al., 2000). The PCR products were purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences) and sequenced using a Perkin Elmer ABI PRISM™ genetic sequencer, Model 3100 (Version 3.7). The fungal isolates were identified by comparing the sequencing results with known sequences using the BLAST software (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov/blast).

Morphological criteria were employed to confirm the identity of the isolates as well as for the strains from the culture collection. For this purpose, the descriptions of Domsch et al. (1980), Shipper (1973, 1975, 1976, 1978, 1984) and Klich (2002) were used.

Preparation of fungal enzyme extracts

The fungal strains were cultivated in 200 ml liquid potato dextrose medium (containing infusions of potatoes and glucose) (Sigma) with 5 % citrate buffer (pH 5) in 1 L cotton-plugged Erlenmeyer flasks. Spores were harvested in 100 ml 0.85% sterile sodium chloride, using either 0.1% or 0.01% (v/v) Tween 80 (Merck) or 0.1% or 0.01% Triton X-100 (BDH Chemicals Ltd), and inoculated into 200 ml medium at an initial concentration of 2×10^7 spores/ml for the spore-forming fungi, or with five mycelial covered agar blocks (6 mm diameter) for non-spore forming fungi. The flasks were incubated on a shaker at 125 rpm at 30°C for 4 to 5 days or at 25°C for 21 days in the case of *L. edodes*. After incubation, the biomass was collected via centrifugation (12, 000 x g for 20 min) and the supernatant (enzyme source) used as is or concentrated ten-fold through the Minitan cross-flow ultrafiltration device (Millipore Corporation, Bedford, Massachusetts, USA).

Enzyme assays

The supernatant from the fungal cultures was used to quantify cellulase, endoxylanase and pectinase activities in the broth according to the method of Bailey et al. (1992). β -1,4-xylanase activity was measured using 1% birchwood xylan (Sigma) as substrate prepared in 0.05 M citrate buffer (pH 5.0) with 0.01 M D-xylose (Sigma) as standard. For endoglucanase and pectinase activity, 1% carboxymethylcellulose (Sigma) and 0.1% polygalacturonic acid (Fluka, BioChemika), both prepared in 0.05 M citrate buffer (pH 5.0), were used as substrates with 0.01 M D-glucose (Merck) as standard. A reaction time of 5 min were allowed before DNS solution were added. One unit of enzyme activity was defined as the amount of enzyme that released 1 μ mol xylose or glucose equivalent per ml per min under the assay conditions. Reducing sugars were determined with the dinitrosalicylic acid (DNS) method done according to Miller (1959). The values given for each experiment are the means of at least three treatment replications. Enzyme and substrate controls were routinely included and all samples were appropriately diluted for the determination of enzyme activity in the presence of a negligible background reducing sugar concentration.

β -xylosidase activity was determined by using a combination of the methods of Kersters-Hilderson (1982) and Poutanen and Puls (1988), which measures the amount of *p*-nitrophenol released from *p*-nitrophenyl- β -D-xylopyranoside (pNPX) (Sigma). Esterase and glucosidase activities were measured similarly using *p*-nitrophenyl β -D-Glucopyranoside (pNPG) (Sigma) and *p*-nitrophenyl acetate (pNPA) (Sigma) as substrates. One unit of enzymatic activity was defined as the amount of enzyme that released 1 μ mol of *p*-nitrophenol per minute under the assay conditions.

Treatment conditions for fungal cocktails

Batches of 100 g dried spent or green tea were treated in duplicate with 150 ml of the crude supernatant from the fungal cultures, either as is or ten-fold concentrated. The spent tea was treated for 6 hours at 40°C, followed by drying in an air tunnel for 2 days at 50°C. The green tea was also treated at 40°C for 6 hours, evaluated for aroma development and then dried in an air tunnel for 3 hours at 50°C.

Quantitative measurement of quality parameters

Soluble solids from the dried tea were extracted in duplicate by adding 150 ml boiling purified and deionised water to 2 g tea and stirring for 2 min on a magnetic stirrer, whereafter the extract was filtered through filter paper (Whatman nr. 4 or equivalent). The **soluble solid**

content, expressed as g SS/100 ml extract (%SS), was determined gravimetrically after 20 ml of the extract was evaporated on a steam bath and dried for 1 hour at 100°C. The values given for each sample are the means of four replications. **Total polyphenol content** of the extract, expressed as mg GAE/g tea (TP), was determined according to the Folin-Ciocalteu assay of Singleton and Rossi (1965) with gallic acid as standard. The values given for each sample are the means of six replications. **Objective colour measurement** of the extract (L^* , a^* and b^*) was done with a Colorgard 2000 system with a TM-M transmission attachment (5 mm path length quartz cell) to the 05 sensor (Joubert, 1995). Readings were done according to the three-dimensional Hunter-scale where L indicates black (-) to white (+) tones; a indicates green (-) to red (+) tones, and b indicates blue (-) to yellow (+) tones. The values given for each sample are means of six replications.

Total antioxidant activity of extracts expressed as $\mu\text{mol TROLOX/gSS}$, was determined according to the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) cation radical scavenging method using TROLOX as standard, according to the method of Re et al. (1999). The values given for each sample are the means of duplicate replications. Quantification of specific antioxidants was carried out by reversed-phase HPLC with UV detection at 288 and 255 nm, depending on the chemical structure, using authentic standards. The values given for each sample are the means of four replications. Treatments were compared using one way ANOVA and the Bonferroni post-hoc tests.

Results

Identification of fungal isolates

The 12 strains isolated from spent, green, or commercial rooibos tea products were identified based on morphological and molecular characteristics. Strains identified include *Paecilomyces variotti*, *Neurospora sitophila*, *Aspergillus tubingensis*, *Rhizomucor pusillus* and *Rhizopus oryzae*. Three of the strains were found to be associated with *Aspergillus fumigatus*, a ubiquitous pathogen. Isolate MP12 was not subjected to molecular identification since it did not produce good results in general.

Evaluation of fungal cocktails on rooibos tea

The best results obtained with aroma development on green tea are summarised in Table 3.2. In most of the treated samples, the strong green note from unfermented green rooibos tea was

absent with a slight development of a sweet aroma, although not characteristic of the sweet aroma of rooibos tea. Treatment with ten-fold concentrated enzyme extracts prepared from six strains that appeared slightly better in aroma development, showed that the treatment with the *P. ostreatus* var. *florida* enzyme extract slightly improved the fermented character of green tea, but not significant enough to pursue further evaluation.

Eleven of the 23 known or newly isolated fungal strains improved the yields of soluble solids and/or total polyphenols from spent tea (Table 3.3) or green tea (Table 3.4). On spent tea, the yield in soluble solids (g SS/100 g tea, or %SS) increased by more than 20% when treated with crude enzyme extracts from *A. oryzae*, *P. ostreatus* var. *florida* and *P. djamor* (Table 3.3). More than 20% increase in total polyphenols (mg GAE/g tea) was obtained with *L. edodes*, *A. ficuum*, *P. candidum*, *P. citrinopileatus*, *P. ostreatus* var. *florida*, *P. djamor*, MP1, MP2, MP3 and MP4. For *L. edodes*, the values obtained for the control were much higher than that of the previous controls due to the use of a new batch of tea.

On green tea, the yield in soluble solids increased by more than 15% when treated with ten-fold concentrated enzyme extracts from *P. ostreatus* var. *florida*, *A. oryzae* and *L. edodes* (Table 3.4). Only the ten-fold concentrated enzyme extracts of *A. oryzae* yielded an increase of more than 10% in total polyphenols.

Table 3.2. Aroma and colour development from green tea after treatment with fungal cocktails

Treatment	Aroma and colour development
MP1 – <i>R. oryzae</i>	Weak, atypical fruity aroma
MP8 – <i>A. tubingensis</i>	Fruity aroma with soft green note
MP9 – <i>P. variotti</i>	Aroma development. Slightly fruity and sweet.
<i>A. oryzae</i>	Sharp green note more absent
<i>A. oryzae</i> (10x concentrated)	Underfermented note absent, but not sweet
<i>A. aculeatus</i>	Sharp green note more absent. Slightly better than controls
<i>P. djamor</i>	Sharp green note more absent. Slight development of sweet aroma.
<i>P. candidum</i>	Sharp green note more absent. Colour very good.
<i>P. ostreatus</i> var. <i>florida</i>	Vague sweet aroma development.
<i>P. ostreatus</i> var. <i>florida</i> (10x concentrated)	More fermented than controls.
<i>L. edodes</i>	Unusual aroma present.
<i>L. edodes</i> (10x concentrated)	Unusual, non-characteristic aroma present
<i>T. reesei</i>	Strong underfermented character absent.

Table 3.3. Improvement in soluble solids (%SS), total polyphenols (TP) and colour determinants from spent rooibos tea leaves after treatment with fungal cocktails. Values given show the means of duplicate or triplicate measurements on treatments, expressed as % relative to the control. Improvements of more than 10% are indicated in bold.

Treatment of spent tea	%SS (g SS/100 g dry leaves)	TP (mg GAE/g tea)	%TP/SS (mg GAE/100 g SS)	Colour - Rooibos extract		
				L*	a*	b*
Control - Potato Dextrose	5.32 ± 0.19	1.13 ± 0.12	21.31 ± 2.95	92.30 ± 0.31	-1.78 ± 0.10	15.72 ± 0.15
<i>A. oryzae</i>	#7.07 ± 0.17	1.09 ± 0.05	15.38 ± 1.08	#91.00 ± 0.25	-1.91 ± 0.13	#19.41 ± 0.15
%	133	96	73	99	108	124
<i>A. aculeatus</i>	5.98 ± 0.17	1.13 ± 0.08	18.85 ± 1.86	#91.20 ± 0.32	-1.80 ± 0.11	18.70 ± 0.10
%	113	100	89	99	102	119
<i>A. niger</i>	5.83 ± 0.12	1.07 ± 0.14	18.30 ± 2.72	92.44 ± 0.57	-1.85 ± 0.18	15.05 ± 0.56
%	110	95	86	100	104	96
MP1 - <i>R. oryzae</i>	4.18 ± 0.70	1.07 ± 0.05	26.05 ± 5.51	92.32 ± 0.72	-2.00 ± 0.11	18.60 ± 0.85
%	74	136	169	100	105	123
MP2 - <i>A. fumigatus</i>	4.88 ± 0.16	1.07 ± 0.01	22.04 ± 0.43	92.09 ± 0.17	-1.88 ± 0.10	17.94 ± 0.19
%	86	137	143	99	98	119
MP3 - <i>R. pusillus</i>	4.99 ± 0.07	1.04 ± 0.00	20.77 ± 0.33	91.96 ± 0.24	-1.79 ± 0.14	16.79 ± 0.14
%	88	132	135	100	94	111
MP4 - <i>A. fumigatus</i>	5.20 ± 0.83	0.96 ± 0.02	18.57 ± 2.49	92.02 ± 0.21	-1.90 ± 0.07	16.65 ± 0.27
%	92	122	121	100	99	110

Indicates significant differences in means on a 5% (p<0.05) significance level

Table 3.3. (continued)

Treatment of spent tea	%SS (g SS/100 g dry leaves)	TP (mg GAE/g tea)	%TP/SS (mg GAE/ 100 g SS)	Colour - Rooibos extract		
				L*	a*	b*
<i>A. ficuum</i>	5.51 ± 0.02	0.96 ± 0.04	17.38 ± 0.61	92.21 ± 0.20	-1.80 ± 0.06	16.33 ± 0.27
%	97	122	113	100	95	108
Control - Potato Dextrose	5.69 ± 0.05	0.88 ± 0.13	15.43 ± 2.38	92.61 ± 0.09	-1.91 ± 0.06	15.12 ± 0.12
MP5 - <i>Rhizopus</i> sp.	4.36 ± 0.17	1.00 ± 0.06	23.10 ± 0.43	#90.47 ± 0.08	-1.90 ± 0.06	#21.16 ± 0.14
%	77	114	150	98	100	140
MP6 - <i>N. sitophila</i>	4.88 ± 0.12	1.00 ± 0.09	20.38 ± 2.34	92.10 ± 0.04	-1.90 ± 0.06	17.07 ± 0.08
%	86	114	132	99	100	113
MP7 - <i>P. variotti</i>	5.00 ± 0.21	1.00 ± 0.11	22.00 ± 3.14	91.00 ± 0.10	-2.00 ± 0.05	16.00 ± 0.35
%	88	114	143	98	105	106
MP8 - <i>A. tubingensis</i>	5.87 ± 0.53	0.93 ± 0.06	15.81 ± 0.37	91.91 ± 0.18	-1.81 ± 0.05	14.98 ± 0.22
%	103	106	102	99	95	99
MP9 - <i>P. variotti</i>	6.41 ± 0.91	1.00 ± 0.01	15.84 ± 2.46	91.59 ± 0.15	-1.76 ± 0.05	15.93 ± 0.48
%	113	114	100	99	92	105
MP10 - <i>A. fumigatus</i>	6.44 ± 0.18	1.02 ± 0.04	15.90 ± 1.15	#90.61 ± 0.26	-1.65 ± 0.05	17.15 ± 0.11
%	113	116	103	98	86	113
Control - Potato Dextrose	6.00 ± 0.61	1.10 ± 0.02	19.76 ± 2.52	90.94 ± 0.06	-1.74 ± 0.05	17.45 ± 0.06
MP11 - <i>P. variotti</i>	5.46 ± 0.01	#1.11 ± 0.01	20.28 ± 0.12	#93.57 ± 0.06	-1.85 ± 0.08	16.20 ± 0.12
%	91	101	103	103	106	93

Indicates significant differences in means on a 5% (p<0.05) significance level

Table 3.3. (continued)

Treatment of spent tea	%SS (g SS/100 g dry leaves)	TP (mg GAE/g tea)	%TP/SS (mg GAE/100 g SS)	Colour - Rooibos extract		
				L*	a*	b*
MP12 - unidentified	5.00 ± 0.07	#1.12 ± 0.00	22.33 ± 0.35	#93.28 ± 0.25	-2.19 ± 0.10	18.75 ± 0.19
%	83	102	113	103	126	108
<i>A. terreus</i>	#6.37 ± 0.04	#1.16 ± 0.04	18.27 ± 0.54	90.80 ± 0.16	-1.74 ± 0.12	18.75 ± 0.14
%	106	105	92	100	100	107
Control – Potato Dextrose	4.92 ± 0.60	0.91 ± 0.04	18.70 ± 3.18	90.76 ± 0.16	-2.05 ± 0.08	15.02 ± 0.25
<i>P. candidum</i>	5.03 ± 0.35	#1.10 ± 0.03	21.97 ± 0.99	#93.33 ± 0.15	-2.03 ± 0.12	#18.96 ± 0.16
%	102	121	117	103	99	126
<i>P. citrinopileatus</i>	5.56 ± 0.38	#1.10 ± 0.02	19.80 ± 1.65	#93.12 ± 0.13	-1.94 ± 0.15	18.75 ± 0.27
%	113	120	106	102	95	125
<i>P. ostreatus</i> var. <i>florida</i>	#6.83 ± 0.32	#1.13 ± 0.00	16.47 ± 0.84	91.71 ± 0.07	-1.81 ± 0.14	17.71 ± 0.09
%	139	124	88	101	88	118
<i>P. djamor</i>	#6.98 ± 0.02	#1.16 ± 0.03	16.67 ± 0.38	90.93 ± 0.10	-1.80 ± 0.09	#19.51 ± 0.13
%	142	127	89	100	88	130
Control - Potato Dextrose	17.28 ± 0.05	2.67 ± 0.01	15.47 ± 0.24	90.94 ± 0.06	-1.74 ± 0.05	17.45 ± 0.06
<i>L. edodes</i>	17.30 ± 0.70	3.24 ± 0.18	18.75 ± 0.11	91.52 ± 0.14	-1.84 ± 0.11	16.35 ± 0.09
%	100	121	121	101	106	94

Indicates significant differences in means on a 5% (p<0.05) significance level

Table 3.4. Improvement in soluble solids (%SS), total polyphenols (TP) and colour determinants from green rooibos tea leaves after treatment with fungal cocktails. Values given show the means of duplicate or triplicate measurements on treatments, expressed as % relative to control. Improvements of more than 10% are indicated in bold.

Treatment of green tea	%SS (g SS/100 g dry leaves)	TP (mg GAE/g tea)	%TP/SS (mg GAE/ 100 g SS)	Colour - Rooibos extract			Colour - Rooibos leaves		
				L*	a*	b*	L*	a*	b*
Control - Potato Dextrose	17.17 ± 0.79	2.90 ± 0.02	16.89 ± 0.24	85.03 ± 0.27	-2.18 ± 0.38	72.42 ± 1.94	31.88 ± 0.30	12.06 ± 0.16	22.87 ± 0.25
MP1 - <i>R. oryzae</i>	#15.49 ± 0.84	2.72 ± 0.12	#17.55 ± 1.34	84.85 ± 0.47	-1.77 ± 0.26	74.12 ± 1.12	31.96 ± 0.50	12.19 ± 0.17	22.71 ± 0.69
%	90	94	104	100	82	102	100	101	99
MP2 - <i>A. fumigatus</i>	#16.72 ± 0.88	2.90 ± 0.34	#17.33 ± 1.01	84.67 ± 0.68	-2.10 ± 0.16	71.77 ± 0.26	31.08 ± 0.63	11.91 ± 0.32	22.05 ± 0.55
%	97	100	103	100	96	100	98	99	96
Control - Potato Dextrose	18.00 ± 0.68	2.82 ± 0.03	16.51 ± 1.06	82.72 ± 4.43	-0.86 ± 0.59	78.64 ± 1.80	30.63 ± 0.23	11.82 ± 0.15	21.45 ± 0.23
MP9 - <i>P. variotti</i>	15.51 ± 1.10	2.87 ± 0.01	18.50 ± 1.12	84.99 ± 0.57	-2.09 ± 0.12	74.07 ± 0.37	31.72 ± 0.41	12.12 ± 0.29	22.70 ± 0.39
%	86	101	112	100	96	102	104	103	106
MP10 - <i>A. fumigatus</i>	15.71 ± 0.70	2.92 ± 0.03	18.60 ± 0.29	85.95 ± 0.59	-2.39 ± 0.34	72.18 ± 1.21	31.15 ± 0.23	11.67 ± 0.09	21.61 ± 0.37
%	88	103	113	104	277	92	102	99	101
<i>P. ostreatus</i> var. <i>florida</i>	16.16 ± 0.50	3.1 ± 0.59	19.19 ± 3.58	85.96 ± 0.49	-2.33 ± 0.23	72.57 ± 0.92	32.15 ± 0.34	12.21 ± 0.27	#22.64 ± 0.63
%	90	110	116	104	270	93	105	103	106
Control - Potato Dextrose	17.87 ± 0.09	3.66 ± 0.14	20.49 ± 1.28	85.04 ± 0.87	-0.75 ± 1.20	77.41 ± 4.80	30.72 ± 0.33	11.71 ± 0.43	20.99 ± 1.04
<i>A. aculeatus</i>	#16.98 ± 0.28	3.55 ± 0.30	#20.95 ± 2.12	84.19 ± 0.17	-1.15 ± 0.19	78.79 ± 0.31	31.01 ± 0.35	12.23 ± 0.31	21.90 ± 0.81
%	95	97	103	99	153	102	101	105	104
<i>P. djamor</i>	#16.90 ± 0.61	3.44 ± 0.20	#20.35 ± 1.04	84.08 ± 0.42	-1.27 ± 0.40	75.34 ± 1.73	30.46 ± 0.68	12.08 ± 0.12	21.45 ± 0.97
%	95	94	100	99	170	97	99	103	102

Indicates significant differences in means on a 5% (p<0.05) significance level

Table 3.4. (continued)

Treatment of green tea	%SS (g SS/100 g dry leaves)	TP (mg GAE/ g tea)	%TP/SS (mg GAE/ 100 g SS)	Colour - Rooibos extract			Colour - Rooibos leaves		
				L*	a*	b*	L*	a*	b*
Control - Potato Dextrose	17.57 ± 0.22	3.54 ± 0.08	20.16 ± 0.81	85.26 ± 0.10	-1.49 ± 0.12	78.29 ± 0.35	30.34 ± 0.57	12.18 ± 0.16	21.45 ± 0.42
<i>P. citrinopileatus</i>	#17.90 ± 0.11	#3.34 ± 0.61	#18.65 ± 3.30	85.02 ± 0.35	-1.12 ± 0.17	78.24 ± 0.46	30.03 ± 1.05	12.04 ± 0.35	21.12 ± 0.45
%	102	94	93	100	75	100	99	99	98
<i>P. candidum</i>	#17.75 ± 0.30	#3.67 ± 1.20	#20.66 ± 0.88	84.78 ± 0.27	-1.18 ± 0.31	77.27 ± 0.84	30.02 ± 0.39	11.79 ± 0.33	20.63 ± 0.57
%	101	104	103	100	80	99	99	97	96
<i>L. edodes</i>	#18.12 ± 0.57	#3.50 ± 0.26	#19.89 ± 1.51	84.15 ± 0.86	-1.35 ± 0.15	75.80 ± 1.11	30.54 ± 0.57	11.89 ± 0.15	20.90 ± 0.41
%	103	99	96	99	91	97	100	98	97
Control - Potato dextrose	18.65 ± 0.55	4.39 ± 0.12	23.53 ± 0.78	87.69 ± 1.77	-4.19 ± 1.18	60.20 ± 5.87	33.87 ± 1.12	10.04 ± 0.95	25.30 ± 1.67
MP8 - <i>A. tubingensis</i>	19.45 ± 0.10	4.57 ± 0.56	23.52 ± 0.79	86.82 ± 0.21	-3.45 ± 0.04	67.39 ± 0.63	34.32 ± 0.08	10.65 ± 0.08	25.13 ± 0.07
%	104	104	100	99	82	112	101	106	99
<i>T. reesei</i>	#18.83 ± 1.04	#4.19 ± 0.74	#22.25 ± 1.21	#84.79 ± 0.40	#-0.95 ± 0.18	³ 82.74 ± 1.95	29.82 ± 0.05	12.33 ± 0.05	21.51 ± 0.14
%	101	95	95	97	23	137	88	123	85
<i>P. ostreatus</i> var. <i>florida</i>	#20.09 ± 0.29	#4.09 ± 0.38	#20.37 ± 0.66	#85.44 ± 0.70	#-1.74 ± 0.33	#78.67 ± 0.93	30.79 ± 0.15	12.47 ± 0.09	19.55 ± 0.22
%	108	93	87	97	42	131	91	124	77
<i>A. oryzae</i>	20.52 ± 0.08	4.04 ± 0.42	19.67 ± 0.45	86.44 ± 0.17	-3.40 ± 0.78	66.86 ± 1.67	31.30 ± 0.02	11.25 ± 0.03	23.01 ± 0.10
%	110	92	84	99	81	111	92	112	91

Indicate significant differences in means on a 5% (p<0.05) significance level

Table 3.4. (continued)

Treatment of green tea	%SS (g SS/100 g dry leaves)	TP (mg GAE/ g tea)	%TP/SS (mg GAE/100 g SS)	Colour - Rooibos extract			Colour - Rooibos leaves		
				L*	a*	b*	L*	a*	b*
Control - Potato dextrose	17.30 ± 0.72	4.22 ± 0.04	24.41 ± 0.63	85.19 ± 0.21	-2.52 ± 0.47	68.50 ± 2.28	31.83 ± 0.12	11.77 ± 0.04	22.27 ± 0.07
MP8 – <i>A. tubingensis</i> (10x concentrated)	17.19 ± 0.63	4.36 ± 0.67	25.34 ± 0.47	85.86 ± 0.10	-3.27 ± 0.16	74.12 ± 0.62	31.42 ± 0.03	12.10 ± 0.12	22.39 ± 0.36
%	103	103	104	101	138	108	99	103	101
MP9 – <i>P. variotti</i> (10x concentrated)	19.18 ± 0.23	4.60 ± 0.29	23.97 ± 0.02	83.83 ± 0.12	-0.51 ± 0.00	85.1 ± 0.64	28.75 ± 0.19	12.42 ± 0.10	19.9 ± 0.16
%	111	109	98	98	20	124	90	105	89
<i>T. reesei</i> (10x concentrated)	18.22 ± 0.95	4.57 ± 0.31	25.07 ± 0.05	85.05 ± 0.04	-2.03 ± 0.33	79.90 ± 0.07	28.34 ± 0.50	11.8 ± 0.19	20.38 ± 0.55
%	105	108	103	100	91	117	89	100	91
<i>P. ostreatus</i> var. <i>florida</i> (10x concentrated)	20.48 ± 1.07	4.58 ± 0.30	22.36 ± 0.45	85.20 ± 0.14	-2.55 ± 0.04	74.91 ± 0.06	28.30 ± 0.07	12.37 ± 0.05	20.44 ± 0.20
%	118	108	92	100	101	109	89	105	92
<i>A. oryzae</i> (10x concentrated)	[#] 22.37 ± 0.76	4.79 ± 0.15	21.40 ± 0.42	84.86 ± 0.10	-1.92 ± 0.01	79.32 ± 0.48	29.67 ± 0.08	12.48 ± 0.07	21.88 ± 0.11
%	129	113	88	100	76	116	93	106	98
<i>L. edodes</i> (10x concentrated)	20.07 ± 0.00	3.99 ± 0.00	19.87 ± 0.09	84.25 ± 0.53	-1.93 ± 0.02	79.31 ± 0.49	27.63 ± 0.11	12.42 ± 0.07	19.81 ± 0.13
%	116	94	81	99	77	116	87	105	89

Indicate significant differences in means on a 5% (p<0.05) significance level

Quantitative analyses of antioxidants released from spent and green rooibos tea

An increase in total polyphenol content usually suggests that there may also be an increase in the antioxidant content. Treatments that increased the TP content significantly, were further subjected to quantitative analyses of ten antioxidants extracted from the spent or dried green tea after treatment with the fungal enzyme extracts (Table 3.5). On spent tea, the most significant effect was obtained with the enzyme extract from MP1 (identified as *R. oryzae*), which improved the extraction of aspalathin, nothofagin and most of the minor compounds, resulting in a 60% increase in the flavonoid content of the soluble solids (% Total Flavonoids/SS). On green tea, the two food-grade strains, *P. ostreatus* var. *florida* and *L. edodes*, increased the levels of aspalathin and nothofagin from green tea by 57% and 43% for *P. ostreatus* var. *florida*, and 32% and 26% for *L. edodes*. Although there were reduced levels for some of the minor antioxidants, there was a 29% and 17% increase in the total polyphenol content (%Total Flavonoids/SS) for *P. ostreatus* var. *florida* and *L. edodes* respectively.

Screening for the production of hydrolytic enzymes by fungal strains

The endoglucanase, xylanase and pectinase activity levels for all 23 of the fungal strains were determined (Table 3.6). The *A. oryzae*, *L. edodes* and *P. ostreatus* var. *florida* strains had relatively high levels of xylanase, endoglucanase, and pectinase activity. Although very low levels of activity were in general observed for β -glucosidase, xylosidase and esterase in these three strains, *A. oryzae* did show much higher levels of glucosidase and especially xylosidase activity.

Table 3.5. HPLC analysis of the effect of fungal enzyme treatment on the release of ten antioxidants from spent and green rooibos tea leaves. Results are given as % deviation from the respective controls and show the means of duplicate or triplicate measurements on treatments. Improvements of more than 10% are indicated in bold.**Spent tea**

Treatment	*Asp	Orient	Isoorien	Vitex	Noth	Isovitex	Isoquer/ Rutin	Quer	Luteol	Chrys	% Tot Flav/SS	% Tot Flav/TP
Control - Potato Dextrose	0.213	1.126	1.411	0.196	0.091	0.254	0.090	0.012	0.004	0.018	3.34 ± 0.19	21.96 ± 3.20
MP1 - <i>R. oryzae</i>	0.263	#1.742	#2.237	#0.315	#0.128	#0.382	0.206	0.100	0.031	0.030	#5.36 ± 1.10	20.46 ± 1.82
%	123	155	159	161	141	150	228	833	775	167	160	93
MP2 - <i>A. fumigatus</i>	0.226	1.332	1.744	0.203	0.111	#0.320	0.068	0.027	0.015	0.007	#3.98 ± 0.18	18.03 ± 0.37
%	106	118	124	104	122	126	76	225	375	39	119	82
MP3 - <i>R. pusillus</i>	0.108	0.912	1.200	0.119	0.052	0.213	0.048	0.018	0.005	0.005	2.59 ± 0.02	12.33 ± 0.37
%	51	81	85	61	57	84	53	150	125	28	78	56
Control - Potato Dextrose	0.238	1.315	1.635	0.219	0.094	0.293	0.139	0.013	0.004	0.032	3.91 ± 0.33	21.02 ± 1.23
MP10 - <i>A. fumigatus</i>	0.222	0.943	1.237	0.145	0.089	0.239	0.048	0.019	0.006	0.007	2.90 ± 0.24	18.43 ± 2.97
%	93	72	76	66	95	82	35	146	150	22	74	88
Control - Potato Dextrose	0.154	0.960	0.878	0.179	-	0.216	0.074	0.018	0.010	0.009	2.29 ± 0.66	13.05 ± 2.39
<i>A. terreus</i>	0.114	1.013	1.227	0.216	-	0.220	0.105	0.014	0.009	0.005	2.09 ± 0.09	14.85 ± 0.25
%	74	106	140	121	-	102	142	77	90	56	91	114

*Abbreviations for antioxidants: Asp, aspalathin; Orient, orientin; Isoorien, isoorientin; Vitex, Vitexin, Noth, nothofagin, Isovitex, isovitexin; Isoquer/Rutin, isoquercitrin/Rutin; Quer, quercetin; Luteol, luteolin; Chrys, chrysoeryol

Indicate significant differences in means on a 5% (p<0.05) significance level

Table 3.5. (continued)**Green tea**

Treatment	Asp	Orient	Iso orien	Vitex	Noth	Isovitex	Isoquer/ Rutin	Quer	Luteol	Chrys	%Tot Flav/SS	% Tot Flav/TP
Control - Potato Dextrose	2.000	0.640	0.775	0.204	0.124	0.196	0.286	0.007	0.008	0.003	4.24 ± 0.34	27.43 ± 2.13
<i>P. ostreatus</i> var. <i>florida</i>	3.149	0.668	0.825	0.200	0.177	0.216	0.239	0.007	0.003	0.003	5.49 ± 0.11	30.21 ± 0.90
%	157	104	106	98	143	110	84	100	38	100	129	110
<i>P. djamor</i>	2.025	0.546	0.675	0.175	0.125	0.172	0.252	0.005	0.002	0.003	3.98 ± 0.82	22.24 ± 6.85
%	101	85	87	86	108	88	88	74	25	100	92	82
<i>L. edodes</i>	2.631	0.693	0.845	0.217	0.156	0.213	0.179	0.005	0.003	0.003	4.94 ± 0.04	26.34 ± 0.47
%	132	108	109	107	126	109	63	71	33	106	117	96
MP9 - <i>P. variotti</i>	2.120	0.635	0.780	0.198	0.130	0.197	0.291	0.007	0.004	0.003	4.36 ± 0.46	24.51 ± 1.88
%	106	99	101	93	105	101	102	100	50	100	103	89
Control - Potato dextrose	3.783	0.689	1.000	0.480	0.175	0.172	0.465	0.003	0.004	0.002	6.44 ± 0.12	26.37 ± 1.18
MP9 - <i>P. variotti</i>	3.931	0.759	0.984	0.137	0.179	0.192	0.527	0.035	0.021	0.001	6.74 ± 0.29	27.12 ± 0.76
%	104	110	98	29	102	112	113	1167	525	50	105	103
<i>A. oryzae</i>	4.245	0.643	0.835	0.115	0.208	0.156	0.438	0.021	0.015	0.002	6.67 ± 0.94	31.15 ± 4.24
%	112	93	84	24	119	91	94	700	375	100	104	118

*Abbreviations for antioxidants: Asp, aspalathin; Orient, orientin; Isoorien, isoorientin; Vitex, Vitexin, Noth, nothofagin, Isovitex, isovitexin; Isoquer/Rutin, isoquercitrin/Rutin; Quer, quercetin; Luteol, luteolin; Chrys, chrysoeryol

Indicate significant differences in means on a 5% (p<0.05) significance level

Table 3.6. Enzyme activities in the supernatant from fungal strains. The three strains yielding the best results are highlighted in bold.

Fungal strain	Activity (IU/ml)		
	Xylanase	Endoglucanase	Pectinase
<i>A. aculeatus</i>	2.9	2.6	2.7
<i>A. ficuum</i>	2.3	3.0	3.4
<i>A. niger</i>	2.4	2.7	3.9
<i>A. oryzae</i>	20.5	22.4	22.3
<i>A. terreus</i>	1.3	22.0	18.9
<i>L. edodes</i>	21.6	18.0	29.0
<i>P. candidum</i>	21.0	18.4	17.3
<i>P. citrinopileatus</i>	15.6	15.3	26.5
<i>P. djamor</i>	16.9	15.6	13.3
<i>P. ostreatus</i> var. <i>florida</i>	20.7	29.6	26.7
MP1 – <i>R. oryzae</i>	1.0	3.2	1.7
MP2 – <i>A. fumigatus</i>	1.5	4.1	2.0
MP3 – <i>R. pusillus</i>	1.9	3.1	3.7
MP4 – <i>A. fumigatus</i>	1.6	2.2	2.6
MP5 – <i>Rhizopus</i> sp.	1.2	2.1	3.7
MP6 – <i>N. sitophila</i>	1.4	2.8	7.4
MP7 – <i>P. variotti</i>	2.0	3.1	2.8
MP8 – <i>A. tubingensis</i>	1.6	3.9	2.0
MP9 – <i>P. variotti</i>	2.4	2.4	3.5
MP10 – <i>A. fumigatus</i>	1.5	3.2	5.3
MP11 – <i>P. variotti</i>	1.9	3.5	2.7
MP12 - unidentified	9.5	22.4	19.4
	Activity (mU/ml)		
	Esterase	Glucosidase	Xylosidase
<i>A. oryzae</i>	0.05	4.14	21.30
<i>L. edodes</i>	0.12	0.00	0.06
<i>P. ostreatus</i> var. <i>florida</i>	0.72	0.06	0.18

Discussion

Microorganisms that grow naturally on a substrate are often most suitable for the hydrolysis of that specific substrate, although the specific strains may not be suitable for commercial application in the food and beverage industry. In this study, 12 fungal strains isolated from rooibos tea products were evaluated for the bioprocessing of rooibos plant material, i.e. spent tea or green tea. However, identification of these isolates revealed that three of the isolates are associated with *A. fumigatus*, a ubiquitous pathogen. All further investigations with isolates MP2, MP4 and MP10 were therefore suspended. Other strains identified include *P. variotti*, a common agent in the deterioration of foodstuff and responsible for soft rot of timber (Domsch et al., 1980), while *N.sitophila* have powerful cellulolytic capability and is one of the faster growing fungi (Shojaosadati et al., 1999). *Aspergillus tubingensis* is one of the most commonly reported fungi from foods (Klich, 2002), while *R. pusillus* can mainly be found on composting and fermenting substrates, such as municipal wastes (Domsch et al., 1980). Since rooibos tea products are generally marketed as tea or health promoting products, it was decided to focus on the food-grade organism represented by the isolate MP1 (*R. oryzae*).

An additional 11 food-grade fungal strains proved to be of particular importance for the bioprocessing of rooibos tea to improve the extraction of soluble solids from spent tea for the manufacturing of rooibos tea extracts, or to enhance the release of soluble solids and/or antioxidants from green tea to improve the quality of tea made by infusion of green rooibos tea.

From the total of 23 strains evaluated on spent tea, the crude enzyme extracts of nine fungal strains increased the release of soluble solids (improvement in %SS varying from 3% to 42%), while 14 strains yielded higher levels of total polyphenols (increase varying from 1% to 37%). The 12 strains that gave the best results on spent rooibos tea leaves were also evaluated for enhanced colour and aroma development from green rooibos tea. However, improvements in the colour and aroma were difficult to determine due to the relative low enzyme concentration and the strong background from spontaneous tea fermentation. Six of the strains, namely *P. ostreatus* var. *florida*, *A. oryzae*, *L. edodes*, *T. reesei*, *A. tubingensis* and *P. variotti*, were selected for the preparation of more concentrated enzyme extracts for further evaluations. Four of the concentrated enzyme extracts, i.e. MP9, *A. oryzae*, *L. edodes* and *P. ostreatus* var. *florida*, released more than 10% additional soluble solids and the *A. oryzae* extract also increased the release of TP by more than 10%.

Cellulases, pectinases, xylanases and laccases have previously shown to be important for the release of black tea components (Murugesan et al., 2002; Angayarkanni et al., 2002). Of the 23 fungal enzyme profiles determined for these four enzyme activities, the three most promising profiles were those of *A. oryzae*, *L. edodes* and *P. ostreatus* var. *florida*, which all showed relatively high levels of xylanase, cellulase and pectinase activities. Attempted verification of the identity of the *A. oryzae* strain with molecular and morphological analyses, indicated that it was actually a strain of *Aspergillus japonicus*, a fungus not widely known for its application in the food industry and a known phytopathogenic fungus (Sanyal et al., 1988). This fungus has also been isolated from cases of otitis (Wadhvani and Srivastava, 1984) and produces sterigmatocystin, a mycotoxin known to cause diseases in man and animals (Begum and Samajpati, 2000). Any further work with this strain was suspended.

Further evaluations of fungal strains for rooibos processing focused on the other two food-grade fungi, i.e. *P. ostreatus* var. *florida* and *L. edodes*, as well as the isolate MP1, identified as *R. oryzae*. This study showed that crude enzyme extracts from *P. ostreatus* var. *florida* increased the release of TP and %SS with more than 20% from spent tea. When ten-fold concentrated, the extract also increased the release of %SS by more than 10% from green tea. The *L. edodes* extract increased the release of TP by more than 20% from spent tea and the release of %SS by more than 10% from green tea. It also increased the levels of aspalathin and nothofagin from green tea by 32% and 26%, respectively. When evaluated on spent tea, the *R. oryzae* extract increased the release of TP by more than 30% and improved the extraction of aspalathin, nothofagin and most of the minor compounds, resulting in a 60% increase in the total flavonoid content of the soluble solids.

Our results therefore confirmed that microbial enzymes can be applied for the improved release of soluble solids, polyphenols and/or improved fermentation characteristics of rooibos products. Furthermore, it became clear that different strains (and therefore different enzyme combinations) release different components from rooibos products.

Chapter 4

Literature Review:

Hydrolytic Enzymes and Expression thereof by Filamentous Fungi

4.1 Hydrolytic Enzymes

Enzymes are referred to as the catalytic cornerstone of metabolism and are therefore the focus of international research by biologists, process designers/engineers, chemical engineers and scientists working in other related fields to improve industrial processes. For hundreds of years, enzymes have played a central role in many manufacturing processes, e.g. in the production of wine, cheese, bread and the modification of starch to name a few (Beg et al., 2001). Since their discovery in the latter half of the 19th century, enzymes have been extensively used in several industrial processes. They are extremely efficient and highly specific biocatalysts. Enzymes have found their way into many new industrial processes with the advancement in biotechnology over the last three decades, especially in the fields of genetics and protein engineering. During the past century, there has been a tremendous increase in awareness of the effects of pollution and therefore an increasing demand to replace some traditional chemical processes with biotechnological processes involving microorganisms and enzymes such as pectinases, xylanases, cellulases, mannanase, α -galactosidase, laccases and ligninases. These enzymes not only provide an economically viable alternative, but are also more environmentally friendly (Hoondal et al., 2002).

The most abundant organic compounds found in nature are the plant cell wall polysaccharides which make up almost 90% of the plant cell wall (De Vries and Visser, 2001). Enzymes degrading these polysaccharides have many industrial applications. For example, xylanolytic enzymes used for biobleaching in the pulp and paper industry, improvement of bread volume and dough quality in the baking industry, and increasing the feed conversion efficiency of animal feeds. Pectinolytic enzymes are used to improve pressing and clarification of concentrated fruit juices, for the production of carrot puree and for enzymatic debarking in the pulp and paper industry (De Vries, 2003).

The plant cell wall polysaccharides consist of cellulose, hemicellulose (xyloglucans, xylan, galactomannan) and pectin. The hemicellulose and pectin polysaccharides, as well as the aromatic polymer lignin, interact with the cellulose microfibrils by forming hydrogen bonds. This results in the formation of a rigid structure that strengthens the plant cell walls. Diferulic acid bridges between different hemicellulose chains and between hemicellulose and lignin, lead to the formation of covalent cross-links. Ester linkages between glucuronic acid present in xylan and lignin have also been reported. These cross-links strengthen the plant cell

wall, but they may also limit cell growth and reduce cell wall biodegradability (De Vries, 2003).

4.1.1 Biodegradation of cellulose

Cellulose is a polymer consisting of β -D-glucopyranose units linked together by the β -1,4-glucosidic bonds (Eriksson et al., 1990). The resulting linear polymeric chains can vary from 15 to more than 10 000 glucose residues and are insoluble. The individual chains do not exist in isolation but adhere to each other in a parallel way to form crystalline microfibrils. The crystallinity is not uniform along the microfibrils and contains both crystalline and 'amorphous' regions that can adopt various shapes. Most of the 'amorphous' regions correspond to chains that are located at the microfibril surface, whereas the crystalline components occupy the core (Bayer et al., 1998; Schülein, 2000). Cellulose molecules are linear and can easily form intra- and intermolecular hydrogen bonds. The cellulose molecule's size is usually given in terms of the degree of polymerisation, which is the number of glucose moieties in one cellulose molecule. Cellobiose is the major product from hydrolysis of cellulose and thus forms the basic structural unit of cellulose. Cellulose exists in several crystalline forms (cellulose I-IV) of which only cellulose I is found in significant amounts in nature (Eriksson et al., 1990).

Cellulose usually exists in a complex of cellulose, hemicellulose and lignin, which is collectively known as lignocellulose. In industrial processes, the cellulose and hemicellulose fractions are converted to sugars through acid and/or enzymatic hydrolysis which are further fermented to produce ethanol or other valuable products. The co-existence of lignin with cellulose in lignocellulose makes enzymatic hydrolysis difficult and without pre-treatment, the final sugar yield from enzymatic hydrolysis of lignocellulosic materials is generally less than 20% of the theoretical maximum. A number of pre-treatment options have been explored, for example pre-treatment with supercritical carbon dioxide (SC-CO₂), an extraction solvent, which resulted in a significant increase in the final sugar yields (Kim and Hong, 2001).

Cellulose constitutes almost half of the biomass synthesised by photosynthetic fixation of carbon dioxide. In 1883 it was first reported that cellulose is decomposed in nature mainly through the action of microorganisms (Eriksson et al., 1990). However, only a limited number of species have been found suitable for the production of cellulolytic enzymes and more specifically extracellular enzymes are required for commercial production of cellulases (Busto

et al., 1995). In a typical cellulose-degrading ecosystem, a variety of cellulolytic bacteria and fungi work in concert with related microorganisms to convert insoluble cellulosic substrates to soluble sugars. These sugars are primarily cellobiose and glucose that can be assimilated by the cell. To catalyse this process, the cellulolytic microbes produce a range of different enzymes, collectively known as cellulases (Bayer et al., 1998). However, due to its low solubility, cellulose is resistant to hydrolysis, and enzymes that degrade crystalline materials must also disrupt the hydrogen bonding of a single glucan chain forcing it away from the crystal and maintaining it whilst performing numerous catalytic events (Schülein, 2000). The hydrolysis of cellulose is accompanied by either the inversion or retention of the configuration of the anomeric carbon and in both cases, are catalysed primarily by two carboxyl groups located in the active site of the enzyme. Inverting enzymes (endoglucanases) make use of a single-step concerted mechanism, while retaining enzymes (cellobiohydrolases) function via a double-displacement mechanism during which a transient covalent enzyme-substrate intermediate is formed (Bayer et al., 1998).

Cellulases are the main enzymes that hydrolyse the β -(1 \rightarrow 4) glucosidic bonds in cellulose (Cao and Tan, 2002). They form part of the glycoside hydrolase families of enzymes (Schülein, 2000), which have been classified into over 97 families to date, on the basis of amino acid sequence similarities (<http://afmb.cnrs-mrs.fr/CAZY/GH.html>). They are also known to hydrolyse oligosaccharides and/or some polysaccharides (Bayer et al., 1998). Cellulases are produced as a multi-component enzyme system that consists of three classes of enzymes that act synergistically in the hydrolysis of cellulose: 1,4- β -D-glucan cellobiohydrolases (CBH, EC 3.2.1.91) cleave cellobiosyl units from the ends of cellulose chains, endo-1,4- β -D-glucanases (EG, EC 3.2.1.4) cleave internal glucosidic bonds, and 1,4- β -D-glucosidase (EC 3.2.1.21) cleaves glucose units from cello-oligosaccharides (Cao and Tan, 2002; Jørgensen et al., 2003). The first two enzymes act directly on cellulose, with cellobiose and glucose being the main reaction products yielded.

Endoglucanases and cellobiohydrolases are responsible for the degradation of soluble cellodextrins and amorphous cellulose, although it is cellobiohydrolases that degrade crystalline cellulose most efficiently (Cao and Tan, 2002). β -glucosidases can be divided into three groups based on their substrate specificity. Some β -glucosidases exhibit high specificity towards aryl β -D-glucosides, while others preferentially hydrolyse cellobiose and cello-oligosaccharides and are therefore called cellobiases. Cellobiase thus hydrolyses the cellobiose to glucose. Most β -glucosidases are reported to be broad-specificity-glucosidases, cleaving both types of substrates to the same extent (Decker et al., 2001). Beside volatile

aroma compounds, grape berries and several fruits contain different aroma precursors of which glycosidic precursors represent an important aroma source. A main proportion of potent flavourants, monoterpenes, shikimate-derived compounds and C₁₃-norisoprenoids in grapes are linked to the sugars consisting of β -D-glucosides or diglycosides. As a consequence, there is growing interest in the exploitation of this hidden aromatic potential by the use of exogenous enzymes (Belancic et al., 2003).

A broad range of organisms including fungi, bacteria, plants and insects are known to produce cellulases. Since fungi produce large amounts of cellulase, it has been extensively studied and effectively used for industrial purposes. So far, fungal cellulases have been isolated mainly from the members of the subdivision *Deuteromycotina* (Moriya et al., 2003). The extensive intermolecular bonding pattern of cellulose generates a fascinating crystalline substrate that is particularly resistant to microbial degradation and thus requires multiple enzyme systems to efficiently degrade cellulase. Such systems constitute either a collection of free cellulases and/or multi-component complexes known as cellulosomes. The cellulosome was first observed in *Clostridium thermocellum*, where the cellulases are organised into a high molecular weight cellulolytic complex (Bayer et al., 1998).

In addition to these hydrolytic enzymes, oxidative enzymes are also involved in aerobic fungal cellulose degradation. These enzymes include quinone oxidoreductase (cellobiose dehydrogenase), which reduces quinones and phenoxy radicals in the presence of cellobiose which is oxidised to cellobiono- δ -lactone, and cellobiose oxidase which oxidises cellobiose and higher cellodextrins to their responding -onic acids using molecular oxygen. Some aerobic and anaerobic bacteria are known to produce another type of enzymes involved in cellulose degradation, namely phosphorylases (Eriksson et al., 1990).

In recent years the industrial potential of microbial cellulases has been recognised for their application in the saccharification of cellulosic material, in the formulation of washing powders and in the textile and pulp and paper industries. The growing concerns about shortage of fossil fuels, the emission of greenhouse gases and air pollution by incomplete combustion of fossil fuel has also led to an increased focus on production of bioethanol from lignocellulosics (Jørgensen et al., 2003). For economically feasible bioethanol production, the utilisation of both the cellulose and hemicellulose fraction is required. For cellulose to be enzymatically hydrolysed to glucose, physical and chemical pre-treatment is required to break down the lignin and overcome the resistance of cellulose to hydrolytic cleavage due to its partly crystalline structure. Although cellulose-degrading enzymes are commercially

available, they are still too expensive for commercial production of fuel ethanol (Thygesen et al., 2003).

4.1.2 Biodegradation of Hemicelluloses

Hemicelluloses are complex polysaccharides and the second most abundant organic structure in the plant cell wall after cellulose (De Vries and Visser, 2001). It was first thought that hemicelluloses were precursors of cellulose, but it was found that they represent a distinct and separate group of plant polysaccharides, which have no part in cellulose biosynthesis (Eriksson et al., 1990). Hemicellulose consists of both linear and branched heteropolymers, i.e. xylan, mannan, galactan and arabinan of which D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose and D-glucuronic acid form the principle monomers. Most hemicelluloses contain two to six of these sugars that can either be acetylated or methylated (Beg et al., 2001; Eriksson et al., 1990). Most of the hemicelluloses are based on a 1,4- β -linkage of their backbone sugars, except for the galactose-based hemicelluloses with 1,3- β -linkages (Eriksson et al., 1990).

Xylan is one of the most complex polysaccharides present in the plant cell wall, representing up to 30-35% of the total dry weight (Beg et al., 2001). Its backbone consists of β -1,4-linked D-xylopyranoses, but can be substituted with a large number of different carbohydrate residues, depending on the origin. In cereals, xylans are highly substituted with α -1,2- or α -1,3-linked L-arabinofuranose residues and are therefore commonly referred to as arabinoxylans (De Vries, 2003). In hardwood hemicellulose, an *O*-acetyl-4-*O*-methylglucurono- β -D-xylan, often called glucuronoxylan, forms the major component (Eriksson et al., 1990). Every tenth xylose residue carries a 4-*O*-methylglucuronic acid that is attached to the 2 position of xylose. Hardwood xylans are highly acetylated and these acetyl groups are responsible for the partial solubility of xylan in water. Xylans from softwood are composed of arabino-4-*O*-methylglucuroxylans and have a higher 4-*O*-methylglucuronic acid content than the hardwood xylans. The softwood xylans are not acetylated and have α -L-arabinofuranose units linked by α -1,3-glycosidic bonds at the C-3 position of the xylose instead of an acetyl group (Beg et al., 2001).

Another hemicellulose found in hardwoods is glucomannan that comprises 2-5% of the wood. This polysaccharide is composed of β -D-glucopyranose and β -D-mannopyranose units linked by 1,4-bonds (Eriksson et al., 1990). Galactomannans and galactoglucomannans are the main hemicelluloses found in softwoods with their backbone being a linear chain

consisting of 1,4-linked β -D-glucopyranose and β -D-mannopyranose units (Eriksson et al., 1990). These residues can also be substituted with α -1,6-linked D-galactopyranose residues. Two different types of galacto(gluco)mannans are found, one with a low galactose content and one with a high galactose content, depending on the origin of the galacto(gluco)mannan (De Vries, 2003). β -mannosidase (EC 3.2.1.25), together with α -galactosidase (EC 3.2.1.22), are responsible for the complete conversion of galactomannan into D-galactose and D-mannose by catalysing the cleavage of terminal α -1,6-linked D-galactosyl and β -1,4-linked D-mannopyranosyl residues, respectively (Ademark et al., 1998).

4.1.2.1 Xylan degradation

D-xylose, a five-carbon sugar that can be converted to single cell protein and chemical fuels, forms the main component of xylan. Due to the heterogeneity and complex chemical nature of plant xylan, the complete enzymatic degradation of hemicelluloses involves several specific activities (Figure 4.1) (Beg et al., 2001). The hemicellulolytic system involves several different hydrolytic enzymes, i.e. endo-1,4- β -D-xylanases (EC 3.2.1.8), which cleaves internal bonds in the xylan chain; exo-1,4- β -D-xylosidases (EC 3.2.1.37), which cleaves xylo-oligosaccharides to produce xylose; endo-1,4- β -D-mannanases (EC 3.2.1.78), which cleaves manno-oligosaccharides to mannose as well as α -glucuronidase (EC 3.2.1). A number of enzymes are also involved in the removal of the side chains including α -glucuronidases (EC 3.2.1.139) and acetyl xylan esterases (EC 3.1.1.6) (De Vries, 2003). Hemicelluloses can also be degraded by xylanosomes, which are discrete, multifunctional, multi-enzyme complexes found on the surface of several microorganisms (Beg et al., 2001).

Two families of xylanases are found, namely families 10 (formerly known as fam F) and 11 (formerly known as fam G). Family 10 xylanases consist of a carbohydrate binding module and a catalytic domain, which is connected by a flexible linker region. Family 11 comprises highly specific low molecular weight endoxylanases from eukaryotic and bacterial species, in which the sequence identity varies from 40-90% (Törrönen and Rouvinen, 1997).

Xylanases are usually inducible enzymes secreted in media containing pure xylan or xylan-rich residues that contain little or no free glucose or metabolisable sugars. Constitutive production of xylanase has also been reported (Beg et al., 2001). Since xylan is unable to enter the microbial cell, low levels of certain hydrolases are constitutively formed and excreted. These enzymes release small soluble fragments from the xylan, which then can penetrate the cell membrane. Following uptake by the organism, these compounds signal the

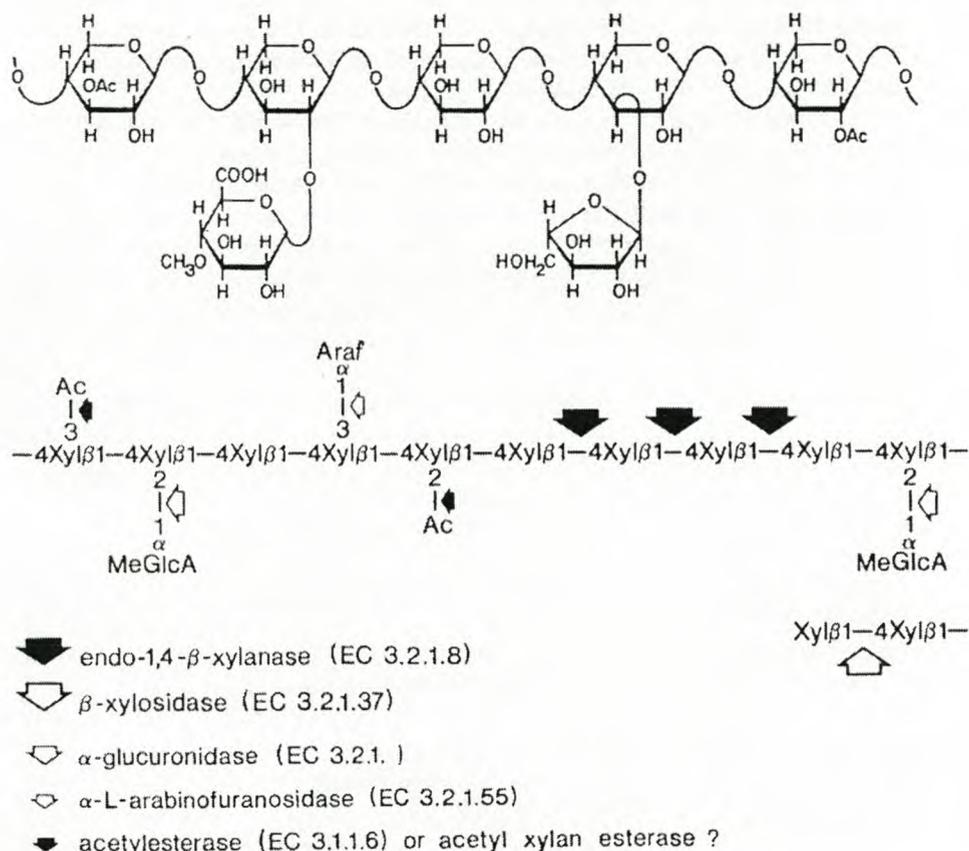


Figure 4.1. A hypothetical scheme of a plant xylan structure showing the different substituent groups with sites of attack by microbial xylanolytic enzymes. The fragment comprising five D-xylose units is presented in the upper part of the figure. *Ac*, Acetyl Group; *Araf*, L-arabinofuranose; *MeGlcA*, 4-O-methyl D-glucuronic acid; *Xyl*, D-xylose (taken from Eriksson et al., 1990).

presence of the polymeric substrate and stimulate the formation of larger quantities of the enzymes necessary for its breakdown. Catabolites derived from xylan are frequently found to induce the synthesis of xylanase in both filamentous fungi and yeasts. Xylobiose was found to be the most prominent signal molecule and an effective inducer in a range of microorganisms (Haltrich et al., 1996). Xylanase can also be induced by several other compounds, such as L-sorbose, various xylo-oligosaccharides, xylose and lignocellulosic residues, while readily metabolisable sugars, such as glucose and/or xylose, can be suppressors of xylanase synthesis (Beg et al., 2001).

In the last decade, microbial xylanolytic enzymes have attracted a great deal of attention, particularly because of their biotechnological potential in various industrial processes. From an industrial point of view, filamentous fungi are particularly interesting producers of xylanases due to the fact that they excrete xylan-degrading enzymes into the

medium, thus eliminating the need for cell disruption. Xylanase levels from filamentous fungal cultures are also much higher than those from yeasts or bacteria. Filamentous fungi also produce several auxiliary enzymes that are necessary for debranching of the substituted xylans (Haltrich et al., 1996).

Currently, the most promising application of xylanases is in the prebleaching of kraft pulps where the application of the enzyme improves pulp fibrillation and water retention, reduction of beating times in virgin pulps, restoration of bonding and increased freeness in recycled fibres, and selective removal of xylanases from dissolving pulps. Xylanases are also incorporated into a rye-based diet of broiler chicks to reduce the intestinal viscosity and thus improving both the weight gain of chicks and their feed conversion efficiency (Beg et al., 2001).

Xylanases can also improve the quality of bread by increasing the specific bread volume. Wastes from agricultural and food industries contain large amounts of xylan and xylanases are used for the conversion of xylan into xylose in wastewater. Glycosylation and fatty acetylation of phytosterols can be induced in plant cells treated with xylanases. Together with cellulase and pectinase, xylanase are used for clarifying must and juices and for liquefying fruits and vegetables. It is also used in the pretreatment of forage crops to improve the digestibility of ruminant feeds and to facilitate composting (Beg et al., 2001).

Another potential application of the xylanolytic enzyme system in conjunction with the pectinolytic enzyme system, is in the degumming of base fibres such as flax, hemp, jute and ramie. A xylanase-pectinase combination is already being used in the debarking process, which is the first step in wood processing. Biological fuels, such as ethanol, can be generated from lignocellulosic biomass when xylanase are used in synergism with other enzymes, such as mannanase, ligninase, xylosidase, glucanase, glucosidase, etc. (Beg et al., 2001).

The use of α -L-arabinofuranosidase and β -D-glucopyranosidase for aromatising musts, wines and fruit juices, have also been employed in food processing. The maceration of cell walls for the production of plant protoplasts can also be improved by some xylanases. A more recent application is that of a truncated bacterial xylanase gene from *C. thermocellum* involved in rhizosecretion in transgenic tobacco plants. Some of the species used for the commercial production of xylanase include *Trichoderma reesei*, *Thermomyces lanuginosus*, *Aureobasidium pullulans*, *Bacillus subtilis* and *Streptomyces lividans* (Beg et al., 2001).

4.1.3 Biodegradation of Lignin

4.1.3.1 Lignin structure

Being the most abundant aromatic compound on earth, lignin is the second biggest contributor to living terrestrial biomass. Lignin is a phenylpropanoid structural polymer and its biological functions include providing vascular plants the rigidity they need to stand upright and to protect their structural polysaccharides (cellulose and hemicellulose) from attack by other organisms (Hammel, 1997). It also decreases water permeation across cell walls of xylem tissue and makes the wood more resistant to attack by microorganisms. Lignin biodegradation has diverse effects on soil quality because it can ultimately be degraded to humus, water and carbon dioxide following the death of the plant tissue (Eriksson et al., 1990). Since decomposing (as opposed to already decomposed) lignocellulose supports high populations of microorganisms that may produce phytotoxic metabolites, undegraded lignocellulose, e.g. in the form of straw, can have a deleterious effect on soil fertility (Hammel, 1997).

Lignin is the most important renewable material next to cellulose, but it also constitutes a barrier that must be breached before most cellulose is accessible to enzymes. It is also a hindrance for efficient utilisation of the cellulose in the production of ethanol, sugars and protein, which explains the importance of research into lignin degradation (Eriksson et al., 1990). Aspergilli are versatile ascomycetes that are able to degrade a wider spectrum of lignin-related aromatic compounds at a more rapid rate than many white-rot fungi which are known to degrade phenolic and polysaccharide components from lignocellulosic material (Duarte and Costa-Ferreira, 1994).

4.1.3.2 Lignin degrading enzymes

One of the first groups of ligninolytic enzymes to be discovered were the lignin peroxidases (LiP). Although they occur in some frequently studied fungi, e.g. *Phanerochaete chrysosporium* and *Trametes versicolor*, they are also evidently absent in others, e.g. *Pleurotus ostreatus* and *Dichomitus squalens*. LiP is oxidised by H₂O₂ to a two-electron deficient intermediate. This intermediate then returns to its resting state by performing two one-electron oxidations of donor substrates. As LiPs are more powerful oxidants than typical peroxidases are, they oxidise the usual peroxidase substrates such as phenols and anilines, as well as a variety of non-phenolic lignin structures and other aromatic ethers that resemble the

basic structural unit of lignin. Another lignin degrading enzyme found mostly in white-rot fungi, is manganese peroxidases (MnPs). They are similar to conventional peroxidases, except that Mn(II) is the obligatory electron donor for reduction of the one-electron deficient enzyme to its resting state, producing Mn(III) as a result. These LiP and MnP-catalysed reactions cannot provide the only means of cleaving polymeric lignin, and the production of diffusable oxyradicals by MnP may contribute to a combined MnP/lipid peroxidation system that oxidises and cleaves non-phenolic lignin compounds. White-rot fungi require sources of extracellular H₂O₂ and this is met by extracellular oxidases (e.g. glyoxal oxidase and aryl alcohol oxidases) that reduce molecular oxygen to H₂O₂, with the concomitant oxidation of a cosubstrate (Hammel, 1997).

In the absence of lignin peroxidase and manganese peroxidase, laccases can also degrade lignin. Laccases can roughly be divided into two major groups, i.e. those from higher plants and those from fungi, although the presence of laccase-like enzymes has been reported in bacteria and insects. Laccases have been shown to be glycoproteins and in some higher plants, they may be bound to the cell walls (Mayer and Staples, 2002). In fungi, laccases are believed to be involved in degradation of lignin and/or in the removal of potentially toxic phenols during degradation. They are also thought to take part in the synthesis of dihydroxynaphthalene melanins, in fungal morphogenesis, and in plant pathogenesis and fungal virulence (Galhaup et al., 2002).

Laccases (EC 1.10.3.2, *p*-diphenol:dioxygen oxidoreductases) form part of a larger group of enzymes known as the multicopper enzymes belonging to the blue oxidases and are known to catalyse the one-electron oxidation of a wide variety of organic and inorganic substrates, including mono-, di- and polyphenols, aminophenols, methoxyphenols, aromatic amines and ascorbate, with the concomitant four-electron reduction of oxygen to water (Figure 4.2) (Minussi et al., 2002). It was first described from the sap of the Japanese lacquer tree *Rhus vernicifera* by Yoshida (1883), which makes it one of the longest known enzymes (Galhaup et al., 2002; Mayer and Staples, 2002). Laccases often occur as isoenzymes that oligomerise to form multimeric complexes. The laccase molecule is a monomeric, dimeric or tetrameric (glyco)protein in its active holoenzyme form (Valderrama et al., 2003) of which the molecular mass of the monomers can range from about 50 to 100 kDa. An important feature is a covalently linked carbohydrate moiety (10-45%). This may contribute to the high stability of these enzymes (Claus, 2004). For catalytic activity, the holoenzyme usually contains four copper atoms per monomer bound to three redox sites (Valderrama et al., 2003).

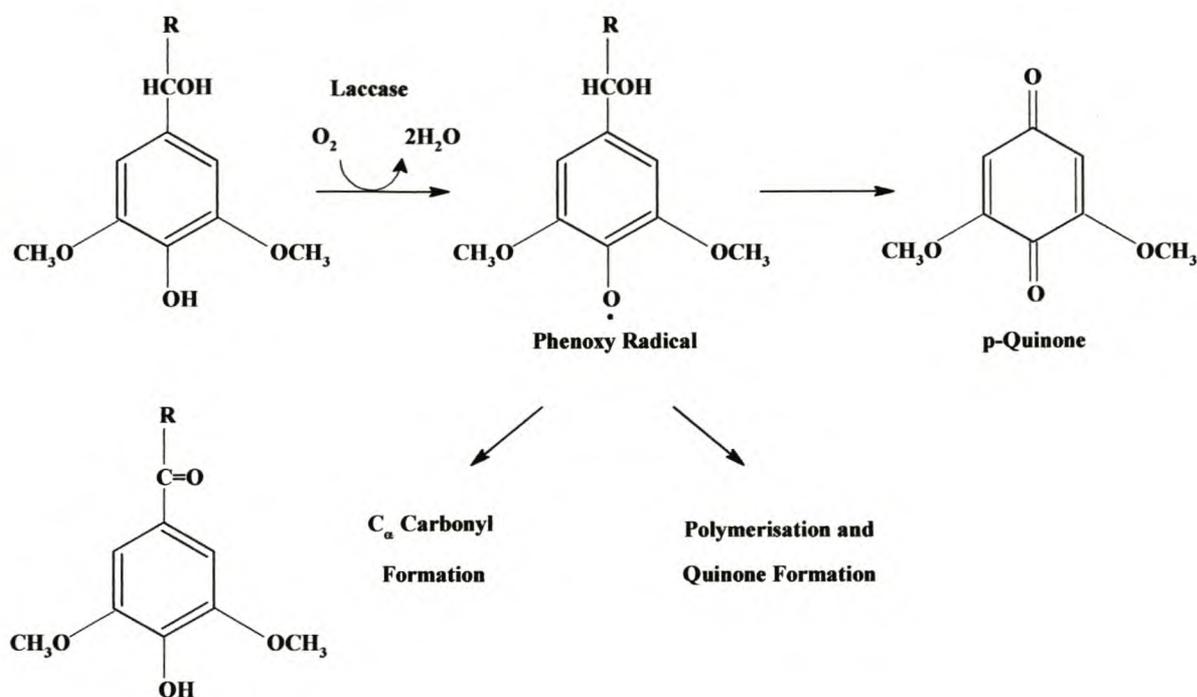


Figure 4.2. A typical reaction of laccase with the oxidation of phenol (Minussi et al., 2002).

More recently, it was shown that white-rot fungi cultivated on natural solid lignin-containing substrates produce another form of laccases, the so-called “yellow laccases”. These laccases do not show the characteristic absorption spectrum at about 600 nm (blue colour of blue laccases) and are capable of oxidising non-phenolic compounds in the absence of mediators. It is assumed that these yellow laccases form as a result of binding of aromatic products of lignin degradation with the blue laccases and that they might contain endogenous mediators derived from lignin, which carry out the role of exogenous mediators in the reaction of oxidation of non-phenolic compounds (Pozdnyakova et al., 2004).

Laccases may be constitutive or inducible enzymes and several compounds have been shown to induce and improve laccase formation. These compounds mainly include phenolic compounds, strictly related to lignin or lignin derivatives (Valderrama et al., 2003), such as ferulic acid, 2,5-xylidine, *p*-anisidine or veratryl alcohol (Galhaup et al., 2002). However, nonlignin compounds and extracts from different origins were also found to be effective inducers of laccase production (Valderrama et al., 2003) in the presence of aromatic electron-transfer or radical-forming mediators such as 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 1-hydroxybenzo-triazole (HBT) and hydroxyanthranilic acid (Pozdnyakova et al., 2004). Laccase production can also be

influenced by the nitrogen concentration in the culture medium and higher nitrogen levels are often required to increase laccase formation (Galhaup et al., 2002). The reactive radicals that are generated lead to the cleavage of covalent bonds as well as the release of monomers. Laccase catalysed ring-cleavage of aromatic compounds have also been reported in several cases (Claus, 2004).

4.1.3.3 Industrial application of laccases

Laccases have become important, industrially relevant enzymes because of a number of diverse applications, especially in biotechnology (Galhaup et al., 2002). They play an important role in removing aromatic compounds, such as phenols and aromatic amines, which constitute one of the major classes of pollutants in drinking and irrigation water, as well as in beer-factory and distillery wastewater. One of the main applications of laccases in the food industry is the stabilisation of wine. Wine and musts contains phenolic compounds, which include cinnamic acid derivatives and catechins, which are mainly responsible for the colour and taste of the different types of wine. Removal of these compounds in must should be very selective to avoid an undesirable alteration in the wine's organoleptic characteristics. Laccases oxidise and polymerise these polyphenolic substances and then remove them by clarification. *Botrytis cinerea* secretes laccases that may cause various forms of rot in grapes and the presence of laccase may serve as an indicator of *B. cinerea* infection in must (Minussi et al., 2002).

Laccases are also among the various enzymatic treatments proposed for the stabilisation of fruit juices where the excessive oxidation of phenolics is considered detrimental to the organoleptic quality of the product. A persistent problem in the brewing industry, is the tendency for hazes to develop in some beers during storage as a result of protein precipitation that is usually stimulated by small quantities of naturally occurring proanthocyanidins. The addition of laccase may remove any excess oxygen whereby storage life of beer is enhanced, and at the same time, the laccase will remove some of the polyphenols that may still remain in the beer (Minussi et al., 2002).

The flavour quality of oils could be improved by eliminating dissolved oxygen in oils or products comprising an oil, e.g. salad dressings prepared with vegetable oils, by adding an effective amount of laccase. When laccase is added to dough used for baked products, it may exert an oxidising effect on the dough constituents and thereby serve to improve the strength of gluten structures in dough and/or baked products (Minussi et al., 2002).

Laccases have also been shown to be an important virulence factor in many diseases caused by fungi. They protect the fungal pathogen from the toxic phytoalexins and tannins in the host environment. Laccase and its product melanin are an important virulence factors of *Cryptococcus neoformans*, an encapsulated fungus that has emerged as a life-threatening infection in immunocompromised patients, especially those infected with the human immunodeficiency virus. Since the mammalian host does not produce laccase, it can be used as a potential drug target. The expression of laccase is sometimes regulated by the presence of mycoviruses that infect the fungal pathogen, which can both up-regulate and down-regulate fungal virulence (Mayer and Staples, 2002).

4.1.4 Biodegradation of Pectin

Pectic constituents are ubiquitous in the plant kingdom and form the major components of middle lamella, a thin layer of adhesive extracellular material found between the primary cell walls of adjacent young plant cells (Hoondal et al., 2002). Pectic substances are classified into protopectin, pectic acid, pectinic acid and pectin based on the type of modification of the backbone chain. Protopectin is the parent pectic substance and yields pectin or pectinic acid upon restricted hydrolysis. Pectic acids are the galacturonans that contain negligible amounts of methoxyl groups, whereas pectinic acids contain various amounts of methoxyl groups (Kashyap et al., 2001). Pectins is the generic name given for the mixture of widely differing compositions containing pectinic acid, which is the demethylated form of pectin, as the major component. These pectic substances are commonly amorphous with a degree of polymerisation of about 200-400 and account for about 0.5-4% of the weight of fresh plant material (Hoondal et al., 2002). When the tissue is ground, an increase in viscosity and the pulp particles are observed. This is caused by the pectin found in the liquid phase (soluble pectin), whereas other pectin molecules remain bound to cellulose fibrils by means of side chains of hemicellulose and thus facilitate water retention (Kashyap et al., 2001). Primarily, pectic compounds are found in fruits and vegetables and occur in low concentrations (less than 1%) in forestry or agricultural residues. They also constitute a large part of some algal biomass (up to 30%) (Hoondal et al., 2002).

Pectins are complex colloidal acid polysaccharides that contain two defined regions with highly different characteristics. The “smooth” regions consist of a backbone of α -1,4-linked D-galacturonic acid residues and may contain acetyl residues at O-2 or O-3 or methyl residues at O-6. The “hairy” regions comprise of two different structures, a xylogalacturonan

consisting of a homogalacturonan backbone substituted with β -1,3-linked D-xylose residues and rhamnogalacturonan I, consisting of a α -1,4-linked D-galacturonic acid backbone that is interrupted by α -1,2-linked L-rhamnose residues. Long arabinan and galactan chains can be attached to O-4 of the rhamnose residues, giving this region its name (hairy). The arabinan chain consists of a main chain of α -1,5-linked L-arabinose residues that can be substituted with α -1,5-linked L-arabinose and by feruloyl residues attached terminally to O-2 of the arabinose residues (De Vries, 2003; De Vries and Visser, 2001). Two types of arabinogalactan side chains have been identified. Type I comprises of a chain of β -1,4 linked D-galactopyranose linkages, while Type II consists of a backbone of β -1,3 linked D-galactopyranose residues that can be substituted with β -1,6-linked D-galactopyranose residues. In the pectic hairy regions, ferulic acid residues have been identified attached to O-2 of arabinose and O-6 of galactose (De Vries, 2003).

These pectic substances are hydrolysed by enzymes broadly known as pectinases, which include polygalacturonases, pectin esterases, pectin lyases and pectate lyases. They are produced by a wide variety of microbial sources such as bacteria, yeast, fungi and actinomycetes (Hoondal et al., 2002). Pectinases are classified under three headings according to a number of criteria: (1) whether pectin, pectic acid or oligo-D-galacturonate is the preferred substrate, (2) whether pectinases act by trans-elimination or hydrolysis, and (3) whether the cleavage is random (endo-, liquefying or depolymerising enzymes) or endwise (exo- or saccharifying enzymes). The pectin esterases, also known as pectin methylhydrolase, catalyse the de-esterification of the methoxyl group of pectin forming pectic acid. The second group comprises the depolymerising enzymes involved in the hydrolysis of glycosidic linkages, including the polymethylgalacturonases (PMG) and the polygalacturonases (PG). The PMGs catalyse the hydrolytic cleavage of α -1,4-glycosidic bonds while the PGs catalyse the hydrolysis of α -1,4-glycosidic linkages in pectic acid. Another group includes the polymethylgalacturonate lyases (PMGL), which catalyse the breakdown of pectin by trans-eliminative cleavage, and the polygalacturonate lyases (PGL) which catalyse the cleavage of α -1,4-glycosidic linkages in pectic acid by trans-elimination. The third group of enzymes consists of protopectinase which solubilises protopectin while forming highly polymerised soluble pectin in doing so (Kashyap et al., 2001).

Enzymes acting on the backbone include pectin lyase (EC 4.2.2.10), pectate lyases (EC 4.2.2.2), rhamnogalacturonan lyases, endopolygalacturonases (EC 3.2.1.15), exopolygalacturonases (EC 3.2.1.67), rhamnogalacturonan hydrolases, rhamnogalacturonan galacturonohydrolases, rhamnogalacturonan rhamnohydrolases, α -rhamnosidases (EC

3.2.1.40), pectin methyl esterases (EC 3.1.1.11), pectin acetyl esterases and rhamnogalacturonan acetyl esterases. Other enzymes that act on the side chains of the hairy regions include α -L-arabinofuranosidases (EC 3.2.1.55), endoarabinanases (EC 3.2.1.99), exoarabinanases, β -1,4-endogalactanases (EC 3.2.1.89), β -1,6-endogalactanases, β -galactosidases (EC 3.2.1.23), β -1,3-exogalactanases, β -1,4-exogalactanases, β -1,6-exogalactanases and ferulyl esterases (EC 3.1.1.73) (De Vries, 2003).

Pectolysis is one of the most essential processes for plants, as it plays a role in cell elongation and growth, as well as in fruit ripening. Microbial pectolysis is also important in plant pathogenesis, symbiosis and decomposition of plant deposits. The pectolytic enzymes play a very important role in nature by breaking down pectin polymers for nutritional purposes and by doing so, contribute to the natural carbon cycle (Hoondal et al., 2002). Pectinases are either acidic or alkaline, based on their applications. Acidic pectinases are mainly used in the fruit juice industries as well as wine making and often come from fungal sources, especially *Aspergillus niger*. The alkaline pectinases are mainly used in the degumming and retting of fibre crops and in the pretreatment of pectic wastewater from fruit juice industries. In coffee fermentation, pectinolytic enzymes are used to remove the mucilage coat from the coffee beans with the help of some cellulases and hemicellulases present in the enzyme preparation. Pectinolytic enzyme treatment can also accelerate and reduce the fermentation stage of coffee processing from the usual 40-80 hours to about 20 hours. Fungal pectinases are also applied in the manufacturing of tea. Tea fermentation is accelerated with pectinase treatment, although the enzyme dosage must be adjusted carefully to avoid damage to the tea leaf. It also improves the foam-forming property of instant tea powders by destroying tea pectins (Kashyap et al., 2001). Pectinases are also used in the extraction of oils, the preparation of cellulose fibres for linen, jute and paper manufacture (Dinu, 2001), liquefaction and saccharification of biomass and isolation of protoplasts used for genetic recombination experiments (Kashyap et al., 2001).

4.1.5 Biodegradation of Tannins

Tannin acyl hydrolase (tannase EC 3.1.1.20) is an industrially important enzyme and is known to be produced by a number of fungi. It hydrolyses the ester and depside bonds of gallotannins and gallic acid esters. Tannase is widely used in the manufacturing of instant tea, acorn wine and gallic acid. Gallic acid is an important substrate used for the synthesis of propyl gallate in the food industry and trimethoprim in the pharmaceutical industry. It also

has potential applications in the clarification of beer and fruit juices, manufacturing of coffee flavoured soft drinks, improvement of grape wine and as an analytical probe for determining the structure of naturally occurring gallic acid esters (Seth and Chand, 2000).

Tannins account for 25% of the water-soluble ingredients in tea leaves. Interaction with caffeine results in the development of turbidity upon cooling of the tea extract. This precipitate formation is known as creaming and under certain conditions the precipitate may flocculate. The main constituents in this cream are theaflavins, thearubigens and caffeine, plus smaller amounts of other flavonoids, non-caffeine compounds, chlorophyll and inorganic material. During manufacturing of soluble tea, the processing of tea cream can be done by either maintaining the temperature above 65°C or through removal by cooling and precipitation or centrifugation. However, this cooling and precipitation causes a decrease in yield and quality due to a loss in flavour. Hydrolysis of the tannin with tannase at lower temperatures and under gentle conditions can reduce the loss of flavour. The cream is hydrolysed to lower molecular weight components, leading to reduced turbidity and an increase in cold-water solubility. Immobilised tannase facilitates recovery, avoids the need for the tea to be heated, improves flavour and leads to a reduction in processing costs (Boadi and Neufeld, 2001).

4.2 Expression of Hydrolytic and Oxidative Enzymes by Filamentous Fungi

Many microorganisms are known to produce extracellular enzymes that are able to degrade complex plant polysaccharides into monomers. These organisms include filamentous fungi and bacteria that often act synergistically with each other. One of the most efficient cellulolytic organisms is the soft rot fungus, *T. reesei* (Margolles-Clark et al., 1997). Cellulases produced by this filamentous fungus have a high industrial interest and are widely used in the food and feed industries (Domingues et al., 2000).

The white-rot fungus, *P. chrysosporium*, is the most commonly used fungus in studies on enzymes involved in cellulose degradation. During the degradation of cellulose to glucose, these fungi utilise an assortment of extracellular hydrolytic enzymes, including endo-1,4- β -D-glucanase (EC 3.2.1.4), exo-1,4- β -D-glucanase (EC 3.2.1.91) and β -glucoside-glucohydrolases (β -glucosidases, EC 3.2.1.21) (Eriksson et al., 1990; Hamada et al., 1999; Morais et al., 2001). β -glucosidase affects the hydrolysis of cellobiose, sophorose and other β -linked glucose oligomers. This enzyme is produced constitutively at low levels, but it can also be

induced to produce much higher quantities by methyl- β -glycoside and other substrates (Busto et al., 1995).

Among all known microorganisms, white-rot fungi are the best lignin degraders (Eriksson et al., 1990) and are therefore of increasing biotechnological interest. These fungi produce a wide variety of extracellular enzymes that enable them to degrade insoluble lignocellulosic substrates into soluble substances. These compounds then again serve as nutrition for the fungi. White-rot fungi typically secrete one or more of the three principle ligninolytic enzymes, i.e. lignin peroxidase (LiP, EC 1.11.1.14), Mn-dependent peroxidase (MnP, EC 1.11.1.13) and phenol oxidase (laccase) (LAC, EC 1.10.3.2) (Novotný et al., 2004). They are classified into five main groups (Table 4.1) based on their ability to produce these oxidative enzymes (Tuor et al., 1995).

Table 4.1. The five main groups of white-rot fungi and the oxidative enzymes they produce (Tuor et al., 1995).

GROUP	ENZYMES PRODUCED	EXAMPLE
I	LiP, MnP and laccase	<i>Phanerochaete chrysosporium</i>
II	Simultaneous production of MnP and laccase, but not LiP	<i>Lentinula edodes</i>
III	LiP and one of the phenoloxidases	<i>Coriolus pruinorum</i>
IV	LiP without phenoloxidases	Hardwood degraders
V	None	<i>Trametes cingulata</i>

Other enzymes that are produced in parallel with above-mentioned enzymes, include other peroxidases, enzymes producing H₂O₂ required by peroxidases (e.g. glyoxal oxidase and superoxide dismutase) and enzymes linked to lignocellulose degradation pathways (e.g. glucose oxidase and aryl alcohol oxidase) (Novotný et al., 2004).

According to Dartora et al. (2002), the filamentous fungi *A. niger* and *Aspergillus oryzae* are the best producers of endo-polygalacturonase. This pectinolytic enzyme hydrolyses the internal α (1 \rightarrow 4) linkage between D-galacturonic acid units of pectin. Of these two

species, *A. niger* is mostly used because it is classified as ‘generally regarded as safe’ (GRAS) by the United States Food and Drugs Administration (USFDA).

White-rot fungi, mutants thereof and isolated ligninolytic enzymes have potential industrial application in several areas. These include delignification, bleaching and modification of pulp fibres in the biobleaching and biopulping industry, as well as treatment of waste bleach liquors to reduce colour, toxicity and mutagenicity. They are also involved in the delignification of wood, straw and bagasse to increase digestibility by ruminants (Eriksson et al., 1990). Soil is the principal environment for litter-decomposing white-rot fungi and under natural conditions, they compete with different microorganisms for resources and space. When these fungi and indigenous soil microorganisms are cocultured, enhanced degradation of xenobiotics occur. Xenobiotics, such as fuel additives, organochloride pesticides, polychlorinated biphenyls, polycyclic aromatic hydrocarbons (PAH) and pentachlorophenol (Hatvani and Mécs, 2003) are commonly found in contaminated soil and only a few species of the white-rot fungi are potentially useful in the removal of these contaminants (Baldrian, 2004). White-rot fungi are also used in the treatment of wastewater to remove environmental pollutants like PCB, DDT and dioxins (Eriksson et al., 1990).

Microbial cells are potentially an excellent source of protein and over the past decades, considerable research has been done on the possibility of using single-cell protein (SCP) to help prevent famine in the less developed countries. Fungi provide an ideal source of protein because of their rapid growth and high protein content. By using cheap waste products as nutrient source, fungal protein can be produced in large amounts in a relatively small area. Fungi can be grown on almost any waste product that contains carbohydrates, e.g. confectionary and distillery waste, vegetable waste and wood processing effluents. Fungal cells also contain carbohydrate, lipids and nucleic acids, as well as a favourable balance of lysine, methionine and tryptophane, amino acids that plant proteins often lack. However, filamentous fungi tend to have a relatively low vitamin content (Wainwright, 1992).

During the past century, knowledge of the production of traditional fermented foods such as tempeh and miso, has led to the identification of various fungal strains that can process certain plant substrates. A biotechnological strategy that has been identified as an efficient means of enriching plant food substrates for beneficial bioactive phytochemicals, is the solid-state bioprocessing (SSB) of edible plant substrates by dietary fungi. Phenolic phytochemicals have received a lot of interest due to their potential use as functional foods and supplements in complementary and alternative therapies (McCue and Shetty, 2004).

Traditional technologies that employ fungi include the production and flavouring of foods (Table 4.2), as well as the production of biochemicals such as citric acid. For thousands of years, yeasts have been used to leaven bread and to produce alcoholic drinks, which include beers, wines and spirits. A number of filamentous fungi have also traditionally been used to improve the flavour of cheese, while others are used in Asian cultures to produce food such as sufu, tempeh and miso. Yeast wastes from the brewing industry are also formulated into products such as Marmite and Vegemite, which are used as flavourings and spreads and serves as a source of B group vitamins. One of the more recent applications of fungi in the food industry include the production of flavourants and colouring agents. This is possible due to the fact that fungi produce flavour compounds such as terpenes, menthol and lactones. Fungi are also particularly useful in removing bitter tastes and are used to debitter citrus fruits. They are also implemented as a protein supplement used to mimic meats. Quorn is perhaps the best-known example of fungal protein. This is a high-technology product that is

Table 4.2. Direct food uses of fungi (Wainwright, 1992).

Application	Species
Edible macrofungi	
Common edible mushroom	<i>Agaricus bisporus</i>
Shiitake	<i>Lentinula edodes</i>
Chinese or straw mushroom	<i>Volvariella volvacea</i>
Winter mushroom	<i>Flammulina velutipes</i>
Oyster mushroom	<i>Pleurotus</i> sp.
Truffle	<i>Tuber melanosporum</i>
Cheeses	
Roquefort, Stilton, 'blue'	<i>Penicillium roquefortii</i>
Camembert, Brie, soft ripened cheeses	<i>Penicillium camemberti</i>
Oriental food fermentations	
Ang-kak	<i>Monascus purpurea</i>
Hamanatto	<i>Aspergillus oryzae</i>
Miso	<i>Aspergillus oryzae/Aspergillus sojae</i>
Ontjom	<i>Neurospora intermedia</i>
Shoyu (soy sauce)	<i>Aspergillus oryzae/Aspergillus sojae</i>
Tempeh	<i>Rhizopus oligosporus</i>

used to simulate meat or is sold to consumers as a high-value, low fat, low-energy health food (Wainwright, 1992).

The first important use of fungi was probably the large edible mushrooms produced by higher fungi. Since this discovery more than 300 years ago, much development and refinement of the highly efficient methods, now typically used to convert composted horse or poultry manure and straw into the commonly cultivated white mushroom (*Agaricus bisporus*), took place (Leatham, 1992). From more than 30 genera, about 2 000 species are regarded as prime edible mushrooms of which only 80 of them are experimentally grown, 40 economically cultivated, around 20 commercially cultivated and only five to six have reached an industrial scale in many countries (Chang, 1991).

Mushrooms have long been used as food or as food flavouring materials because of their unique and subtle flavour (Zawirska-Wojtasiak, 2004) and methods are now being developed to cultivate efficiently new desirable mushrooms with bold memorable flavours and large market potential (Leatham, 1992). Mushrooms were known and consumed as far back as prehistoric times. The Greeks considered mushrooms as providing strength for warriors in battle, the Romans regarded them as the “food of gods” and the Pharaohs prized mushrooms as delicacies (Chang, 1991).

Edible mushrooms are considered to be health food because their mineral content is higher than that of meat or fish and almost twice that of vegetables, while the protein content of fresh mushrooms is about twice that of vegetables and four times that of oranges. Mushrooms also contain all nine amino acids essential to the diet of humans and are especially rich in lysine and leucine, which are lacking in most cereal foods. Various mushrooms also produce a number of unique non-protein amino acids, some of which are biologically active. For example, ibotenic acid, from the mushrooms *Amanita muscaria* and *Tricholoma muscarium*, binds the glutamate receptor as an agonist and exhibits excitotoxicity (Watanabe et al., 2003). They also contain many vitamins, are devoid of starch and low in carbohydrates which make it an ideal food for diabetics (Chang, 1991).

The main mushroom odourants are eight carbon atom (C8) compounds of which 1-octen-3-ol is the most important. Studies indicated that (R)-(-)-octen-3-ol had a fruity mushroom-like characteristic, while (S)-(+)-1-octen-3-ol had a mouldy, grassy note. The character impact flavour compound of mushrooms has been described as the levorotatory antipode, formed by the enzymatic oxidative breakdown of linoleic acid (Zawirska-Wojtasiak, 2004). Mushrooms also tend to accumulate a variety of secondary metabolites, which include phenolic compounds, polyketides, terpenes and steroids. It has been found that these phenolic

compounds are excellent antioxidants and synergists that are not mutagenic. This proves to be a valuable characteristic since carcinogenic properties have been reported for some of the synthetic antioxidants used in the stabilisation of foods (Cheung et al., 2003).

Two of the most commercially important edible mushrooms cultivated in the world are *Lentinula edodes* (Berkeley) Pegler, commonly known by its Japanese name, shiitake (Chen, 2001), and *Pleurotus ostreatus*, commonly known as the oyster mushroom (Peñas et al., 2002).

4.2.1 *Lentinula edodes* (Shiitake mushroom)

Lentinula edodes (Figure 4.2) is the second most popular edible mushroom worldwide thanks to its delicious flavour, great nutritional value and immunity-enhancing components (Hatvani and Mécs, 2002). In 1997, worldwide production of shiitake reached 1 564 400 ton that constituted about 25.4% of the total mushroom supply (Ohga and Royse, 2001). This mushroom is primarily of temperate climate and indigenous to the Far East, but sightings of wild shiitake have been reported in the states of Washington and California. Although cultivation of shiitake on natural logs began in China almost a thousand years ago, today, shiitake cultivation using synthetic-log cultivation, is widely practiced in southeast Asia



Figure 4.2 *Lentinula edodes*, the widely cultivated shiitake mushroom.

(China, Taiwan, Japan, Korea, Singapore, the Philippines, Sri Lanka and Thailand) as well as in North America, Europe, Australia and New Zealand (Chen, 2001). The use of sawdust-based cultivation to replace natural logs has contributed to expanding the production and consumption of *L. edodes*. The ability of *L. edodes* strains to grow depends mainly on the temperature, their tolerance of moisture and the lignocellulosic substrates used (Silva et al., 2005).

4.2.1.1 Composition

Several important bioactive compounds have been isolated from this fungus. These include protein (23%), lipids (72-77% oleic and linoleic acid), carbohydrates, fibre, minerals, vitamins (A, B₁, B₂, B₁₂, C, D and niacin) and ergosterol (Sugui et al., 2003; Vatter and Shetty, 2003). Shiitake also contains eight essential amino acids (methionine being the limiting amino acid) (Longvah and Deosthale, 1998) in better proportions than soybeans, meat, milk or eggs (Vatter and Shetty, 2003). Watanabe et al. (2003) found free D-alanine in a cell extract of the fruit-body of *L. edodes*, as well as amino acid racemase activity that catalyses the interconversion between D- and L-amino acids.

The flavour of *L. edodes* can be attributed to the presence of C8 compounds such as 3-octanone, 3-octanol and 1-octen-3-ol as well as dimethyl disulphide and dimethyl trisulphide. The formation of these compounds is affected by the pH, while the characteristic aroma of shiitake can be ascribed to lenthionine, a cyclic sulfurous compound (Zawirska-Wjtasiak, 2004). When grown on cranberry pomace during solid-state growth, *L. edodes* produced high amounts of β -glucosidase, which released the phenolic aglycones from the cranberry pomace and, therefore, increased the antioxidant capacity (Vatter and Shetty, 2003). Cheung and Cheung (2005) found that a water extract of *L. edodes* showed high antioxidant activity against lipid peroxidation of rat brain homogenate with IC₅₀ values of 0.109 and 1.05 mg/ml. These antioxidant activities were found to correlate with the phenolic content in different subfractions of the extract (Cheung and Cheung, 2005). A lectin with a molecular weight of 43 kDa showing specificity for *N*-acetylgalactosamine and *N*-acetylglucosamine, have also been isolated from fruiting bodies of *L. edodes*. This lectin is unique because it only shows weak interaction with sugars in contrast with other glycoproteins that bind specific to sugars (Wang et al., 1999).

4.2.1.2 Potential health benefits

Studies of *L. edodes* have demonstrated that the use of its extracts results in improved liver function, reduction of viremia in patients with chronic hepatitis B, an inhibition of human immunodeficiency virus infection *in vitro*, lowering of cholesterol and blood pressure levels and also shows anti-viral, anti-fungal and anti-tumor activities (Hirasawa et al., 1999; Wojtas et al., 2004). Three kinds of antibacterial substances have been extracted from *L. edodes*, possessing efficient antibacterial activities against *Streptococcus* spp., *Actinomyces* spp., *Lactobacillus* spp., *Prevotella* spp. and *Porphyromonas* spp. of oral origin. Other general bacteria, such as *Enterococcus* spp., *Staphylococcus* spp., *Escherichia* spp., *Bacillus* spp. and *Candida* spp. are relatively resistant to these substances (Hirasawa et al., 1999).

Lentianan, a β -glucan, was the first anti-tumor polysaccharide isolated from *L. edodes* (Sugui et al., 2003) and one of several mushroom-based anti-cancer drugs approved by the FDA in Japan in 1985 (Wojtas et al., 2004). The structure of lentianan was reported as a branched molecule having a backbone of (1 \rightarrow 3)- β -D-glucan and side chains of both β -D-(1 \rightarrow 3)- and β -D-(1 \rightarrow 6)-linked D-glucose residues with a molecular weight of about one million Da (Sasaki and Takasuka, 1976). Lentianan was found to reduce the size of malignant tumors, including sarcoma-180, methylcholanthrene (MCA)-induced sarcoma and mammary carcinomas in mice. It was also found to modify the process of endotoxin shock in rabbits (Tamura et al., 1997). Although its mechanism of action is not completely clear, it mainly involves lentianan inhibiting tumorigenesis by activating the immune system and inducing gene expression of immunomodulatory cytokines and their receptors. Lentianan also displayed protective effects on DNA damage induced by antineoplastic agents *in vivo* (Sugui et al., 2003).

Lenthionine, a sulphur-containing compound, has antibacterial and antifungal activity, while bis[(methylsulfonyl)-methyl]disulphide, a derivative of lenthionine, has strong inhibitory effects against *S. aureus*, *B. subtilis* and *E. coli* (Hatvani, 2001). Some sulfated polysaccharide derivatives were found to exhibit antiviral action, e.g. sulfates of the β -(1 \rightarrow 3)-D-glucan from *L. edodes* showed considerable anti-HIV activity as well as low anti-coagulant activities (Zhang et al., 2002).

4.2.1.3 Cultivation

The cultivation of *L. edodes* is regarded as the largest bioconversion process utilising wood (Makkar et al., 2001). Efficient colonisation and utilisation of wood logs and artificial sawdust logs currently used as growth substrates, depends upon the capacity of the fungus to produce an array of oxidative and hydrolytic enzymes required to degrade the major components of lignocellulose, i.e. lignin, cellulose and hemicellulose (Buswell et al., 1995) to low-molecular weight compounds that can be absorbed and assimilated for nutrition (Zhao and Kwan, 1999). One group of enzymes that are responsible for the degradation of lignin includes two phenol-oxidising enzymes, namely laccase (EC 1.10.3.2 benzenediol:oxygen oxidoreductase) and manganese (II)-dependent peroxidase (MnPs, EC 1.11.1.7). *Lentinula edodes* produces high amounts of laccase during primary growth, whereas MnP expression occurs during the secondary metabolism (idiophase) in the presence of nitrogen starvation conditions (Makkar et al., 2001; Vinciguerra et al., 1997). Lignin peroxidase does not play an important part in this enzyme system. The production of these extracellular ligninolytic enzymes are strongly affected by the nature and amount of the nutrients, especially nitrogen (N) and microelements, in the growth substrate (Hatvani and Mécs, 2002).

The main problem occurring during the cultivation of *L. edodes* is the contamination of the logs by competing fungi. Species belonging in the genus *Trichoderma* are the group of competing fungi causing the greatest losses. Some of the mechanisms that may be involved in the antagonism between *L. edodes* and *Trichoderma* spp., include competition for space and nutrients and chemical interference by the production of antibiotics and extracellular enzymes. The levels of the extracellular enzymes of both organisms are altered during the antagonism between these two organisms (Hatvani et al., 2002).

The laccases produced by *L. edodes* have been implicated in pigmentation, fruiting-body formation and pathogenicity. The highest laccase activity was found in the pigmented rind of the pileus and in the stipe. Nagai et al. (2003) isolated a laccase, Lcc 2, from the fully browned gills of *L. edodes*. The production of this monomeric protein of 58 kDa, was associated with the biosynthesis of melanin, which causes a brown surface discolouration as a result of a stress response (Nagai et al., 2003). An increase in laccase activity was found to be associated with rapid growth of nonpigmented aerial mycelium and formation of pigmented primordial and fruiting bodies. DNA sequence analysis demonstrated that the *lac1* gene encodes a putative polypeptide of 526 amino acids, while competitive PCR showed that expression of *lac1* and *lac2* genes was different under various conditions (Zhao and Kwan,

1999). Ohga and Royse (2001) found that laccase activity was high during colonisation and then declined rapidly during fruit body development, while laccase activity decreased, cellulase activity peaked at the veil break stage of fruit body development.

Extracellular ligninolytic enzymes have been attracting wide spread attention due to their ability to degrade environmentally persistent xenobiotics such as pentachlorophenol (PCP) and dioxins. MnP is considered to be the key enzyme for degradation of these pollutants (Makkar et al., 2001). Several dyes containing one or more aromatic rings have similar structures to those of xenobiotics, but are water-soluble and exhibit low toxicity. *Lentinula edodes* mycelium was shown to decolourise the dyes Poly R-478, remazol Brilliant Blue R (RBBR) and Orange II (Hatvani and Mécs, 2002). The capability of dye decolourisation may be ascribed to the activities of lignin peroxidase or laccase (Boer et al., 2004).

In addition to laccase, *L. edodes* also produces other hydrolytic enzymes, such as cellulases and xylanases. Lee et al. (2001) cloned and characterised two cellulase genes from *L. edodes* that may work synergistically to digest cellulose. The first gene, *cel7A*, is a 1551-bp gene that encodes a 516-amino acid protein, has a predicted molecular mass of 53.5 kDa and belongs to the glycosyl hydrolase family 7. The other gene, *cel6B*, encodes a 444-amino acid protein with a predicted molecular mass of 46.4 kDa and belongs to the glycosyl hydrolase family 6. The production of these cellulases is strongly regulated during fruit body development (Silva et al., 2005).

Zheng and Shetty (2000) found that *L. edodes* produced a high level of polygalacturonase activity during solid-state fermentation on strawberry pomace. Starch is a polymer of glucose and is perhaps, next to cellulose, the most widely available polymeric glucoside made by plants and therefore, available to fungi growing on plants or plant residues. Digestion of starch requires a complex of enzymes, e.g. glucoamylases (1,4- α -D-glucan glucohydrolases, EC 3.2.1.3). *Lentinula edodes* was found to be a promising strain for amylase production. The glucoamylase gene *glal* from *L. edodes* codes for a putative polypeptide of 571 amino acids. Its expression was induced by starch and increased during fruit body formation (Zhao et al., 2000).

4.2.2 *Pleurotus ostreatus* (oyster mushroom)

This white-rot fungus is an edible basidiomycete, industrially produced as human food and accounts for nearly a quarter of the world mushroom production (Peñas et al., 2002). The cap of this fungus resembles an oyster in shape (Christensen, 1981) and can grow up to 150 mm across (Watling, 1973). It grows in clumps weighing a pound or more, each including five to ten fruit bodies (Figure 4.3). The upper side is white or ivory-coloured and quite smooth, while the gills are white and the white flesh is soft and spongy (Christensen, 1981). This mushroom produces long, hyaline spores with a size of 10-11 x 4 µm. They are commonly found clustered in tiers on stumps, trunks, posts, etc. and form white sheets of mycelium immediately under the bark of fallen trees. It may be found throughout the year although they are more frequent in autumn (Watling, 1973).



Figure 4.3 *Pleurotus ostreatus* var. *florida* found growing on the barks of trees (Christensen, 1981).

Besides its importance for food production, *P. ostreatus* is also used as a source of enzymes and secondary metabolites for industrial and pharmaceutical applications, as an agent for bioremediation and as organic fertiliser (Peñas et al., 2002). *P. ostreatus* is also of increasing biotechnological interest due to its ability to degrade both wood and chemicals related to lignin degradation products and can be implemented in the bioconversion of agricultural and industrial wastes (Larraya et al., 1999; Peñas et al., 2002).

Quite a number of different *P. ostreatus* varieties are industrially produced and the commercial varieties *florida* and *ostreatus* were found to differ in size, colour and temperature tolerance (Peñas et al., 2002). Despite its economic importance, only a limited number of studies of *P. ostreatus* have been conducted. This is due to the difficulty in performing directed crosses between strains, contradictory data about the size and organisation of its genetic material as well as a lack of a genetic linkage map for it. The first linkage map for *P. ostreatus* identified 11 linkage groups corresponding to the chromosomes of *P. ostreatus* and has a total length of 1,000 centimorgans (cM) (Larraya et al., 2000).

The laccase isoenzymes produced by *P. ostreatus* have been extensively studied. POXC (phenol oxidase) is the most abundantly produced under all growth conditions. Three other isoenzymes that have also been purified and characterised, is POXA1a, POXA1b and POXA2. Studies involving the laccase coding genes in *P. ostreatus* have led to the identification of three different genes and of the corresponding cDNAs, *poxc* (previously named *pox2*), *pox1* (which codes for a laccase isoenzyme that has not been identified yet) and *poxa1b*. Palmieri et al. (2003) purified and characterised two strictly related laccase isoenzymes (POXA3a and POXA3b) produced by *P. ostreatus*. It has also been demonstrated that the amount of all laccase isoenzymes increases substantially in copper-supplemented cultures and in these conditions, laccase expression is regulated at transcriptional level. Recently, it has been shown that *P. ostreatus* extracellular protease can play a regulatory role in laccase activity.

The high degradative potential and high capacity to compete well with an indigenous soil microflora, makes *P. ostreatus* a promising candidate for use in soil bioremediation (Martens et al., 1999). Polycyclic aromatic hydrocarbons (PAH) are high molecular weight pollutants exhibiting recalcitrant properties and are typically found at wood preservation plants and gas work sites (Eggen, 1999). Bezalel et al. (1996) demonstrated that *P. ostreatus* metabolises several PAHs similarly to nonligninolytic fungi and suggested that cytochrome P-450 monooxygenase might be responsible for the initial attack. It has also been reported that *Pleurotus* species are able to secrete, in both liquid and solid-state fermentation cultures, versatile peroxidase (VP), laccase and aryl-alcohol oxidase (AAO), which are all involved in the degradation of lignin. VP is a recently discovered type of peroxidase oxidising phenolic and non-phenolic aromatic compounds, while AAO oxidises aromatic and aliphatic polyunsaturated primary alcohols (Rodríguez et al., 2004).

4.2.3 *Rhizopus oryzae*

Rhizopus is a valuable filamentous fungus (Figure 4.4) with a history of use in the production of fermented foods, industrial enzymes (e.g. glucoamylase and lipase), organic acids (e.g. lactate and fumerate) and corticosteroids. It also has the unfortunate reputation of being a food spoilage organism, a plant pathogen and an opportunistic human pathogen (Skory, 2002). It is often used for industrial production of L (+)-lactic acid, which currently has an estimated global market in excess of 100,000 tons per year. Some advantages of using *Rhizopus* species as an alternative to lactic acid bacteria for production of lactic acid, include production of optically pure lactic acid, which is preferred for many applications, and the ability of the fungus to grow in a chemically defined minimal medium without the need for complex components such as yeast extract. However, the shortcoming of using *Rhizopus*, is that the production efficiency is still generally considered low compared to bacterial fermentations (Skory, 2003).



Figure 4.4 *R. oryzae* viewed under a microscope.

Rhizopus oryzae is able to assimilate mannose, xylose, glucose and galactose in spent sulfite liquor (SSL) from the paper pulp industry. The presence of these sugars contributes to the high biological oxygen demand (BOD) of SSL, which is an environmental concern. This fungus can also produce ethanol under anaerobic or oxygen limiting conditions and has at least two alcohol dehydrogenases, ADHI and ADHII. The mycelium biomass is also of great

interest, since it can be further processed to produce the cell wall skeleton and other products, which have several potential applications (Taherzadeh et al., 2003).

Although this is an organism with industrial importance, the techniques for its genetic manipulation are still in an early stage of development, compared to those used for many other fungi (Skory, 2004). Recently, a transformation system was developed with uracil auxotrophs of *R. oryzae* (Skory, 2002). It was found that *R. oryzae* has two genes, *ldhA* and *ldhB*, which code for the two iso-enzymes of NAD⁺-dependent lactate dehydrogenase, LdhA and LdhB, respectively. LdhA is probably active during the conversion of pyruvate to lactate, whereas LdhB is responsible for the reaction in the opposite direction (Taherzadeh et al., 2003).

Bakir et al. (2001) isolated an endo- β -1,4-xylanase, with a molecular weight of about 22 kDa, produced by *R. oryzae* from low-cost lignocellulosic byproducts. Two major endoglucanases, RCE1 and RCE2, were also isolated from *R. oryzae*. They were shown to possess similar hydrolytic properties (Moriya et al., 2003). Recently, an endopolygalacturonase with a molecular weight of 29.7 kDa was isolated from the solid-state culture of *R. oryzae* (Saito et al., 2004).

From the discussions above, it is clear that various fungal species are known for the efficient production of hydrolytic and or oxidase enzymes. This is also reflected in the results obtained for bioprocessing of rooibos tea (Chapter 3), where five fungal strains induced xylanase, cellulase, pectinase and laccase activity when evaluated on laboratory-scale. For industrial-scale evaluations, larger amounts of enzyme extract are required that is optimised specifically for the hydrolysis of rooibos substrate. Chapter 5 describes the methodology and results obtained to develop a growth medium that will ensure a high level of expression for the above-mentioned enzymes.

Chapter 5

Research Results:

Optimisation of Enzyme Production by Food-grade Fungi for Rooibos Tea Processing

Written in a style suitable for submission to a scientific journal

Optimisation of Enzyme Production by Food-grade Fungi for Rooibos Tea Processing

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Abstract

The leaves of *Aspalathus linearis* are processed to develop the characteristic organoleptic qualities of rooibos tea. It was previously demonstrated that the external application of fungal supernatants, containing a cocktail of hydrolysing enzymes, improved the extraction of aroma, colour, soluble tea components and/or antioxidants from rooibos tea. Different types of media were evaluated for the increased production of hydrolytic enzymes and laccase by the food-grade fungus, *Lentinula edodes*. Yeast peptone medium supplemented with 1% wheat straw consistently produced increased levels of xylanase, endoglucanase and laccase activities. Crude enzyme extracts from four other fungal strains, i.e. *Rhizopus oryzae*, *Aspergillus niger*, *Pleurotus djamor* and *Pleurotus ostreatus* var. *florida*, were also evaluated for rooibos tea treatment after cultivation of the strains in yeast peptone supplemented with 1% wheat straw. The *L. edodes* enzyme cocktail improved both the aroma and colour development of green rooibos tea leaves and the release of soluble solids from fermented tea. When cultured in potato dextrose medium, *R. oryzae* improved the antioxidant content of rooibos extracts from fermented tea with more than 10%, although no increase in soluble solids was observed.

Introduction

Rooibos (*Aspalathus linearis*) is a unique South African fynbos plant, native to the Cedarberg mountains of the Western Cape in South Africa (Joubert, 1996). The leaves and fine stems of the plant are used for the production of rooibos tea (Bramati et al., 2003), which is associated with a number of health promoting properties, including the relief of insomnia, nervous tension, mild depression, stomach cramps, constipation and allergic symptoms. The polyphenols, or more specifically the flavonoids, present in rooibos tea also have very strong antioxidant and free radical scavenging activities that contribute to their potential as anti-carcinogenic and anti-arteriosclerotic agents (Joubert and Ferreira, 1996).

Fermentation of the rooibos tea leaves can take up to 16 hours, which results in a significant loss in antioxidants in the processed tea, and therefore also in its pharmaceutical and nutraceutical value. This fermentation is routinely done outdoors with little control over the processing conditions. In future, increasing resistance from foreign buyers to this kind of processing might be expected due to the unhygienic nature of this process and the absence of GMP (Good Manufacturing Practices) and HACCP (Hazard Analysis Critical Control Point). The ineffective processing of rooibos also results in the low solubility of rooibos tea components. Only 20% of traditional rooibos tea is soluble in hot water in comparison to as much as 40% for black tea. Due to their chemical structure, these compounds are not easily extracted (50-60%) which causes a further reduction in yields of soluble matter.

As discussed in Chapter 3, the external application of fungal supernatant containing a cocktail of hydrolytic enzymes, resulted in enhanced extraction of soluble solids (SS) and/or total polyphenols (TP), and/or an improvement in the colour and aroma of the tea leaves. Hydrolytic enzymes identified in this cocktail included endoglucanases, xylanases, pectinases as well as laccases. Although all the above-mentioned enzymes are known for their ability to degrade plant material, the laccases are of particular importance due to their role in polymerisation reactions that involve polyphenolic substrates.

Laccases (benzenediol:oxygen oxidoreductases; EC 1.10.3.2) oxidise a wide variety of organic and inorganic substrates, including mono-, di- and polyphenols, aminophenols, methoxyphenols, aromatic amines as well as ascorbate (Galhaup et al., 2002). During a typical laccase reaction, a phenol undergoes a one electron oxidation to form a free radical that can be transferred into a quinone in a second oxidation step. This free quinone can then undergo polymerisation (Minussi et al., 2002).

Over the past few decades, laccases have become increasingly important because of a number of diverse industrial applications, e.g. bioremediation (waste detoxification and textile dye transformation), beverage (wine, fruit juices and beer) processing, as biosensors, and for analytical applications. Laccases have been found in the majority of white-rot fungi but are also produced by insects and some bacteria (Galhaup et al., 2002; Minussi et al., 2002). White-rot fungi are of increasing biotechnological interest since they produce a wide range of extracellular enzymes that enable them to convert insoluble lignocellulosic substrates to soluble substances (Morais et al., 2001). In these fungi, laccases are believed to be involved in the degradation of lignin and/or in the removal of potentially toxic phenols produced during wood degradation (Galhaup et al., 2002).

Based on the results presented in Chapter 3, *L. edodes*, *R. oryzae*, *A. niger*, *P. djamor* and *P. ostreatus* var. *florida* were chosen for further evaluation due to their ability to produce sufficient levels of hydrolytic enzymes, as well as their GRAS (Generally Regarded As Safe) status, which adds to the attractiveness of their enzymes in the food industry (Bakir et al., 2001).

Lentinula edodes (Berk.) Pegler, the Asian shiitake mushroom, is the second most popular edible mushroom on the global market. Its importance can be attributed to its nutritional value and the possibility of its medical application (Hatvani and Mécs, 2003). The consumption of these mushrooms has been shown to lead to significantly lower blood cholesterol levels and to lower high blood pressure in laboratory animals (Vattem and Shetty, 2003). It has been shown that *L. edodes* produces high amounts of hydrolases and oxidases for bioconversion of lignocellulosic wastes (Silva et al., 2005). Preparations of this fungus have also exhibited strong laccase activity, with the highest activity found in the pigmented rind of the pileus and in the stipe (Nagai et al., 2003; Zhao and Kwan, 1999). *Rhizopus oryzae* is a valuable filamentous fungi used for the production of fermented foods, industrial enzymes (e.g. glucoamylase, lipase), organic acids (e.g. lactate, fumarate), and corticosteroids (Skory, 2004). *Pleurotus ostreatus* var. *florida* is a commercially important edible mushroom, commonly known as the oyster mushroom. It is industrially produced as human food and together with *P. djamor*, accounts for nearly a quarter of the world's production of cultivated fungi (James et al., 2004; Peñas et al., 2002). *Aspergillus niger* is probably the most biotechnologically important fungal species with GRAS status (Wallis et al., 2001) and is one of the best known producers of citric acid (Ul-Haq et al., 2005). It is currently used in the food industry to produce numerous proteins and metabolites (Wallis et al., 2001), including glucoamylase (Silva et al., 2005).

The objective of the present study was to improve the cultivation media for optimal hydrolase and laccase production by these fungal strains. The efficiency of the respective supernatants was evaluated on different rooibos substrates for their ability to improve aroma and colour development, increased extraction of soluble solids, total polyphenols and antioxidants. Best results were obtained with *R. oryzae*, which was further evaluated in a fermentation process for optimal expression of the relevant enzymes.

Materials and methods

Plant material and chemicals

Dried spent rooibos tea (i.e. after extraction with conventional physical-chemical methods) was provided by Benedict (Pty) Ltd (Stellenbosch, South Africa) and dried green (unfermented) rooibos tea leaves were supplied by the Post-Harvest & Wine Technology Division of ARC Infruitec-Nietvoorbij (Stellenbosch, South Africa). Dried fermented Rooibos leaves (stalky tea material, not suitable for commercial tea bags, sieved 2 mm) were supplied by Afriplex (Paarl, South Africa).

For the growth media, spent grain was obtained from South African Breweries (Newlands, South Africa); pineapple cores and peels from Collondale Cannery, East London, South Africa; corn steep liquor from the Council for Science and Industrial Research, Pretoria, South Africa; wheat straw (sieved 2 mm) from the Department of Animal Science, Stellenbosch University, South Africa; Eucalyptus saw dust from Cape Sawmills (Pty) Ltd, Stellenbosch, South Africa and molasses from Hulett Sugar, Epping Industrial, South Africa.

All chemicals used were standard or analytical grade and obtained from Sigma Chemical Co. (St Louis, USA); Merck (Darmstadt, Germany); Fluka AG (Buchs, Switzerland); Riedel-de Haën AG (Seelze, Hannover) and BDH Chemicals Ltd (Poole, England).

Strains and culture conditions

Lentinula edodes Abo 287, *A. niger* ATCC 10864, *P. ostreatus* var. *florida* Abo 280 and *P. djamor* Abo283 were obtained from the fungal culture collection of the Department of Microbiology at Stellenbosch University. The *R. oryzae* strain was isolated from spent tea as described in Chapter 3. All the strains were maintained by periodic transfer to malt extract agar (MEA) (Sigma) plates and incubated at 30°C (*R. oryzae* and *A. niger*) or 25°C (*L. edodes*, *P. ostreatus* var. *florida* and *P. djamor*).

For inoculum development, 500 ml Erlenmeyer flasks containing 100 ml of the specific medium, were inoculated with three mycelial covered agar plugs (6 mm in diameter) of *L. edodes*. The *L. edodes* strain was grown in seven different media, i.e. malt extract (ME) (Sigma), potato dextrose (PD) (Sigma), MYPG medium (10 g/L malt extract, 2 g/L yeast extract, 2 g/L peptone, 10 g/L D-glucose, 2 g/L KH₂PO₄, 1 g/L MgSO₄·7H₂O), MYPG + Amino Acids (1g/L L(+)-asparagine and 1 g/L thiamine chloride), yeast peptone dextrose

(YPD) (Merck), yeast malt extract (YM) (Merck) and GYP (2 g/L D-glucose, 5 g/L yeast extract and 10 g/L peptone).

More cost-effective nitrogen and/or carbon sources, i.e. combinations of D-glucose (Merck) and soybean flour (Sigma), D-glucose (Merck) and corn steep liquor, molasses with yeast extract and peptone (Merck), molasses and soybean flour, molasses and corn steep liquor, as well as 3% molasses, were evaluated as an alternative to MYPG. These nitrogen and/or carbon sources, 125 ml Erlenmeyer flasks containing 25 ml of the specific combination of media, were inoculated with three mycelial covered agar plugs (6 mm in diameter) of *L. edodes*. The cotton-plugged flasks were incubated on a shaker at 125 rpm for 21 days at 25°C.

Possible inducers of laccase and/or hydrolase enzyme activity (i.e. spent grain, pineapple core, pineapple peel, saw dust, wheat straw, carboxymethylcellulose (CMC), xylan and pectin) were added at 1% to 50 ml MYPG or MYPG without glucose in 250 ml Erlenmeyer flasks. Each flask was inoculated with *L. edodes* as described above. Plate assays were performed to qualitatively determine endoglucanase, xylanase, pectinase and laccase activities.

The best inducers (1% spent grain, 1% pineapple peel and 1% wheat straw) were then tested in combination with 1 x MYPG, 0.1 x MYPG, 1 x YP (2 g/L yeast extract, 2 g/L tryptone peptone), 0.1 x YP, 1 x ME (33.6 g/L malt extract) or 0.1 ME. Erlenmeyer flasks containing 50 ml of the specific medium were inoculated with 2 ml of a 12 day-old *L. edodes* culture, incubated as described above and sampled weekly. Enzyme activity was qualitatively determined for the eight cultures that gave the best results with plate assays.

Plate Assays

Plate assay were performed using 1% (CMC) (Sigma) for cellulase, 1% xylan (Sigma) plus 0.2% Remazol Brilliant Blue (Sigma) for xylanase, 1% glucose (Merck) plus 0.03% 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Sigma) for laccase, and 0.4% polygalacturonic acid (Fluka, BioChemika) for pectinase activity. Each substrate was dissolved in 200 ml 0.05 M citrate buffer (pH 6) and 2% Difco-BactoYeast Nitrogen Base was added to each solution. Small wells in the agar (6 mm in diameter) were filled with 60 µl of the supernatant from the different cultures and checked for zones of clearing after overnight incubation at 30°C. The CMC plates were flooded with 0.1% Congo Red dye (Merck) and the polygalacturonic acid plates with 1 M CaCl₂ (Sigma) to visualise zones.

Liquid Enzyme Assays

After incubation, the biomass was collected via centrifugation (12, 000 x g for 20 min) and the supernatant (enzyme source) used as is or concentrated ten-fold. The supernatant from each culture was used to quantify endoglucanase, xylanase and pectinase activities in the broth according to the method of Bailey et al. (1992). The xylanase activity was measured using 1% birchwood xylan (Sigma) prepared in citrate buffer (0.05 M, pH 6.0) as substrate with 0.01 M D-xylose (Sigma) as standard. For endoglucanase and pectinase activity, 1% carboxymethylcellulose (Sigma) and 0.1% polygalacturonic acid (Fluka, BioChemika), both prepared in 0.05 M citrate buffer (pH 5.0), were used as substrates with 0.01 M D-glucose (Merck) as standard. A reaction time of 5 min were allowed before DNS solution were added. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol xylose or glucose equivalent per ml per min under the assay conditions. Reducing sugars were determined with the dinitrosalicylic acid (DNS) method done according to Miller (1959). The values given for each experiment are the means of at least three treatment replications. Enzyme and substrate controls were routinely included, and all enzyme preparations were appropriately diluted for the determination of activity in the presence of a negligible background reducing sugar concentration.

Laccase activity was determined according to the method of Jönsson et al. (1997), using ABTS as substrate. The formation of the cation radical was detected by measuring the increase in absorbance at 420 nm ($\epsilon_{420}=36\ 000\ \text{M}^{-1}\text{cm}^{-1}$). One unit of laccase activity was defined as the amount of enzyme that catalysed the oxidation of 1 μmol ABTS in a 100 μl reaction mixture for 1 min at 30°C.

Gel filtration chromatography

The molecular mass of the hydrolytic enzymes present in the supernatant of *A. niger*, *R. oryzae*, *L. edodes*, *P. djamor* and *P. ostreatus* var. *florida* were determined using Fast Performance Liquid Chromatography (FPLC)-gel filtration chromatography with the ÄKTA purifier (Amersham Pharmacia Biotech). Samples were loaded on a Superdex 75 HR 10/30 (300 x 100 cm) column that had been calibrated with molecular mass standards (BSA, 66 kDa; ovalbumin, 46 kDa; lysine, 46 kDa; carbonic anhydrase, 29 kDa; cytochrome c, 12.4 kDa; aprotin, 6.5 kDa). As elution buffer 0.05 M citrate buffer (pH 5) were used. The flow rate was set at 0.5 ml/min and 0.5 ml fractions were collected. Samples from the peak fractions were then assayed for enzyme activity and further analysed by SDS-PAGE (Laemmli, 1970) and staining with a Zinc Stain Kit (BioRad).

Laboratory-scale evaluation of fungal cocktails on green and fermented rooibos tea

The fungal strains were cultivated in Erlenmeyer flasks containing either 3L YP medium plus 1% wheat straw or potato dextrose broth buffered with 0.05 M citrate buffer (pH 5). Spores were harvested in 100 ml 0.85% sterile sodium chloride, using either 0.1% or 0.01% (v/v) Tween 80 (Merck) or 0.1% or 0.01% Triton X-100 (BDH Chemicals Ltd), and inoculated into 200 ml media at an initial concentration of 2×10^7 spores/ml for the spore-forming fungi, or with five mycelial covered agar blocks (6 mm diameter) for non-spore forming fungi. The flasks were incubated on a shaker at 125 rpm at 30°C for 4 to 5 days or at 25°C for 21 days in the case of *L. edodes*. After incubation, the supernatant from 3 litre cultures was collected via centrifugation ($12,000 \times g$ for 20 min) and concentrated ten-fold with the Pellicon Cassette and Filter cross-flow ultrafiltration device with a 5 kDa cut-off membrane. DNS liquid enzyme assays were performed on the concentrated and unconcentrated fungal cocktails to quantify the levels of enzyme activity as described above for liquid assays.

Duplicate batches of 100 g dried green tea were treated with 150 ml of the concentrated and unconcentrated extracts for 6 hours at 40°C. The tea was evaluated for aroma development and dried in an air tunnel for 3 hours at 40°C, after which the tea was further evaluated for the yield in soluble solids (%SS), antioxidants and total polyphenols (TP). Duplicate batches of 100 g dried fermented rooibos tea (Afriflex waste) were treated with 150 ml of the unconcentrated and concentrated extracts of *R. oryzae* and *L. edodes*. The tea was treated at 40°C for 2 hours, dried in an air tunnel for 3 hours at 40°C and further analysed for total %SS, antioxidants and TP.

Analysis of quality determinants in dried tea

The dried tea was extracted by adding 150 ml boiling purified and deionised water to 2 g tea and stirring for 2 min on a magnetic stirrer, whereafter the extract was filtered through filter paper (Whatman Nr 4 or equivalent). The **soluble solid content**, expressed as g SS/100 ml extract (%SS), was determined gravimetrically after 20 ml of the extract was evaporated on a steam bath and dried for 1 hour at 100°C. The values given for each sample are the means of four replications. **Total polyphenol content** of the extract, expressed as mg GAE/g tea (TP), was determined according to the Folin-Ciocalteu assay of Singleton and Rossi (1965) with gallic acid as standard. The values given for each sample are the means of six replications.

Total antioxidant activity of extracts, expressed as $\mu\text{mol TROLOX/g SS}$, was determined according to the ABTS cation radical scavenging method using TROLOX as standard, according to the method of Re et al. (1999). The values given for each sample are

the means of duplicate replications. Quantification of ten **antioxidants** was carried out by reversed-phase HPLC with UV detection at 288 and 255 nm, depending on the chemical structure. For quantification, authentic standards were used. The values given are the means of four replications. **Objective colour measurement** of extracts (L^* , a^* and b^*) was done with a Colorgard 2000 system with a TM-M transmission attachment (5 mm path length quartz cell) to the 05 sensor (Joubert, 1995). Readings were done according to the three-dimensional Hunter-scale where L indicates black (-) to white (+) tones; a indicates green (-) to red (+) tones, and b indicates blue (-) to yellow (+) tones. The values given for each sample are the means of six replications. Treatments were compared using one way ANOVA and the Bonferroni post-hoc tests.

Small-scale simulated industrial treatments of fermented tea with fungal cocktails

Concentrated supernatant from *L. edodes* and *R. oryzae* cultured in 3 L YP medium plus 1% wheat straw was used to treat duplicate batches of 50 g “waste fermented tea” (coarse material with high stalk content routinely used by Afriplex for production of rooibos extracts) at 40°C for 2 hours followed by drying in an air tunnel for 4 hours at 40°C.

Batches of 40 g dried tea were extracted with 400 ml of deionised water at 90-93°C for 30 min, whereafter it was decanted and filtered hot through Whatman #4 filter paper. The filtrate was cooled to room temperature and cleaned by filtration through a 0.8 µm pore size filter and 120 ml of this was subjected to ultrafiltration at 400-500 kPa, using a NMWL (nominal molecular weight limits) membrane of 30 kDa (50 ml permeate collected). Samples were collected after each filtration stage for further analysis.

Upscaled production of *R. oryzae* via fermentation technology

Potato dextrose broth inoculated with *R. oryzae* spores at a concentration of 2×10^7 spores/ml was prepared for fermentation in a 14 L BioFlo 110 non-jacketed vessel Fermentor (New Brunswick Scientific). The fermentation was monitored on-line for pH, dissolved oxygen, carbon dioxide (out) and oxygen (out). The temperature was maintained at 30°C with the speed of agitation at about 150 rpm. Anti-foam A (Sigma) was used to control foaming. Samples were taken every 24 hours to determine the levels of enzyme activity in the broth as described for liquid assays. Fermentation broth was used as is or ten-fold concentrated for treatment of green and fermented tea as previously described.

Results

Selection and optimisation of media for inoculum development and expression of selected *L. edodes* enzymes

In terms of biomass production by *L. edodes*, good results were obtained with MYPG and MYPG medium with added amino acids (Figure 5.1). For the different combinations of nitrogen and carbon sources, glucose plus corn steep liquor gave the best results, followed by molasses plus soybean flour (Figure 5.2). Based on plate assay results, MYPG in combination with 1 % spent grain, 1 % wheat straw and 1 % pineapple peel proved to be the best inducers for the production of xylanase, endoglucanase, pectinase and laccase (data not shown). Enzyme liquid assays done on the supernatant of the cultures grown in the different media combinations showed that 1 x YP medium supplemented with 1% wheat straw or 1% spent grain, consistently gave the highest levels of xylanase, endoglucanase and laccase, as well as good induction for pectinase (highlighted in Table 5.1). In general, high levels of laccase were observed for all 12 substrates evaluated.

Quantitative and qualitative analyses of enzymes in fungal extracts

Results of enzyme assays performed on the ten-fold concentrated and unconcentrated supernatant of the fungal cocktails prepared in YP + 1% wheat straw medium are given in Table 5.2. In general, an increase in enzyme activity could be observed in the ten-fold concentrated enzyme cocktail of each fungal strain, although not ten-fold higher as expected. A possible explanation for this could be that the enzymes are degraded to a certain extent during the concentration of the extract. The highest level of cellulase and xylanase activities were obtained with both the unconcentrated and concentrated enzyme extracts of *A. niger*. Although high levels of laccase activity was also observed in the concentrated *A. niger* extract, the concentrated *R. oryzae* cocktail had the highest laccase activity.

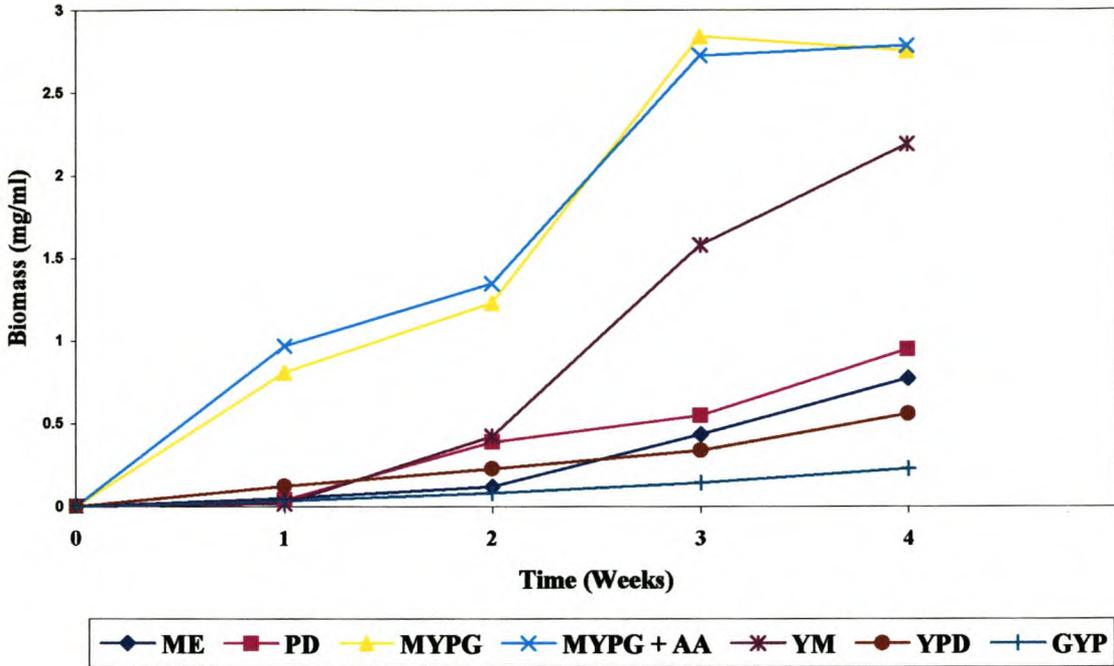


Figure 5.1. Growth curve of *L. edodes* showing biomass production in different media over a period of 4 weeks. Values given show the means of duplicate samples. ME: Malt Extract; PD: Potato Dextrose; MYPG+AA: Malt Extract, Yeast Extract, Peptone, Glucose, KH_2PO_4 , MgSO_4 , Asparagine, Thiamine; YM: Yeast Malt Extract; YPD: Yeast Peptone Dextrose; GYP: Glucose, Yeast Extract, Peptone.

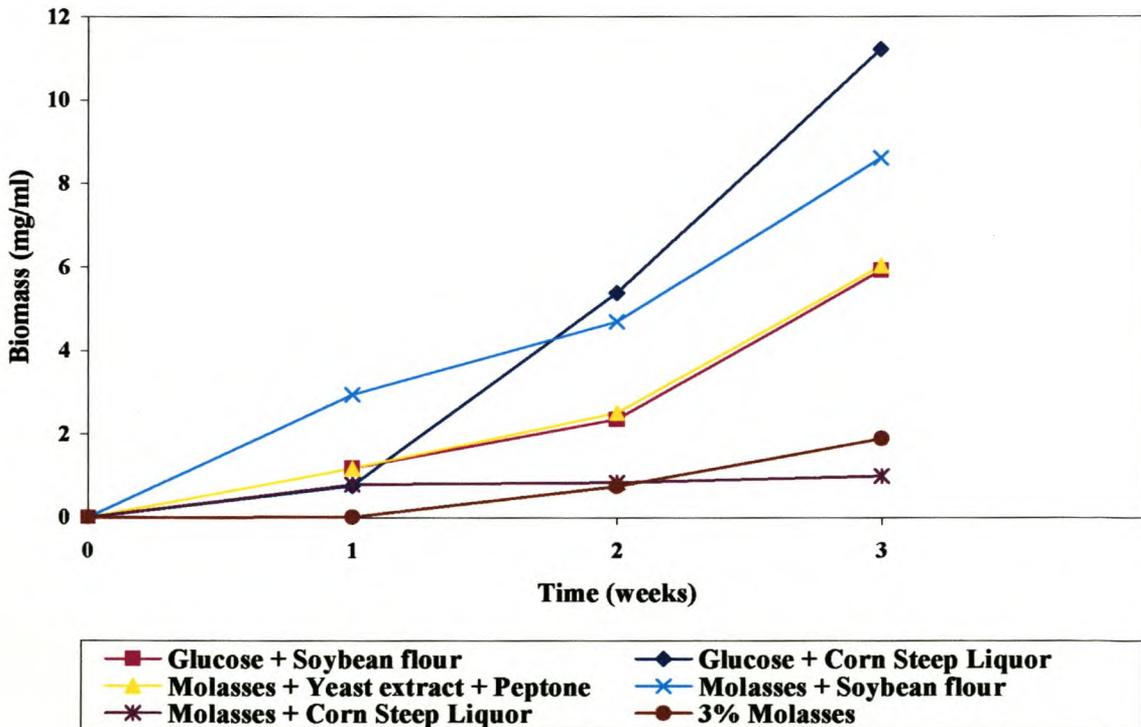


Figure 5.2. Growth curve of *L. edodes* showing biomass production in the presence of different carbon and nitrogen sources over a period of 3 weeks. Values given show the means of duplicate samples.

Table 5.1. Enzyme activities in *L. edodes* cultures grown in different media combinations for 2 or 3 weeks. Values given show the means of duplicate or triplicate measurements.

Substrate	Inducer at 1% (w/v)	Activity (IU/ml)							
		Endoglucanase		Pectinase		Xylanase		Laccase	
		2 w	3 w	2 w	3 w	2 w	3 w	2 w	3 w
0.1 x MYPG	Pineapple peel	0.3	0.9	0.3	0.2	0.2	0.1	36.6	96.1
0.1 x MYPG	Spent grain	0.4	0.2	0.8	0.1	0.3	1.5	27.7	10.3
0.1 x MYPG	Wheat straw	0.1	0.4	0.0	0.3	0.8	2.7	29.7	95
1 x YP	Pineapple peel	0.4	0.6	1.3	0.9	1.2	3.8	49.5	47.3
1 x YP	Spent grain	1.3	1.0	1.7	0.3	1.4	6.5	66.6	52.7
1 x YP	Wheat straw	2.0	1.1	0.8	0.9	4.3	7.1	75.1	108
0.1 x ME	Pineapple peel	0.1	0.2	1.1	0.2	0.1	0.0	28.4	21.5
0.1 x ME	Spent grain	0.4	0.6	0.9	0.2	1.9	2.5	49.5	18.0
0.1 x ME	Wheat straw	0.1	0.5	0.5	0.4	1.6	1.1	65	12.3
1 x ME	Pineapple peel	0.7	0.2	1.1	0.4	0.1	0.3	58.7	35.0
1 x ME	Spent grain	0.8	0.3	0.5	0.6	0.3	1.0	33.3	36.9
1 x ME	Wheat straw	0.3	0.6	0.8	0.5	1.0	1.1	28.4	49.9

*Best two values for each enzyme at 2 or 3 weeks are indicated in bold

Table 5.2. Quantitative and qualitative analysis of enzymes in fungal extracts (as is or ten-fold concentrated). Values given show the means of duplicate or triplicate measurements on treatments.

Cocktail	Activity (IU/ml)			
	Endoglucanase	Pectinase	Xylanase	Laccase
<i>A. niger</i>	6.1	1.0	25.4	75.6
<i>A. niger</i> (10x)	7.3	1.7	183.1	570.3
<i>R. oryzae</i>	0.2	0.6	1.0	129.0
<i>R. oryzae</i> (10x)	1.3	2.6	4.0	667.8
<i>L. edodes</i>	1.3	0.9	2.7	102.8
<i>L. edodes</i> (10x)	1.2	3.3	4.1	176.1
<i>P. ostreatus</i> var. <i>florida</i>	0.7	1.0	8.8	153.7
<i>P. ostreatus</i> var. <i>florida</i> (10x)	3.9	1.2	14.0	568.9
<i>P. djamor</i>	1.2	1.4	1.3	180.1
<i>P. djamor</i> (10x)	4.6	2.2	9.6	594.6

*Best two values for each enzyme are indicated in bold

Quantification and enzymatic profiles of fungal cocktails

The supernatant of the five fungal cocktails were separated on Superdex 75 into several peaks (only data for *L. edodes* are shown in Figure 5.3), after which enzyme assays were performed only on the peak fractions. In general, very low levels of endoglucanase, pectinase and xylanase activity, but higher levels of laccase activity were observed for fractions from all ten fungal cocktails evaluated (Table 5.3). Similar to results presented in Table 5.2, high levels of laccase activity were detected for both the unconcentrated and concentrated *R. oryzae* cocktails. A proper enzymatic profile will therefore require quantification and identification of all the enzymes present in the various gel-filtration fractions. Enzyme activities were only quantified for endoglucanase, pectinase, xylanase and laccase, although it is clear from the peaks shown in Figure 5.3 that there are several other enzymes also present in the cocktails. However, SDS-PAGE analyses yielded protein species that were faint or barely visible due to the low enzyme concentrations in the various fractions (Figure 5.4).

Laboratory-scale treatment of green and fermented tea with fungal cocktails

For the treatment of green rooibos tea, the best results were obtained with *L. edodes* cultured in 1 x YP medium supplemented with 1 % wheat straw, where both the unconcentrated and ten-fold concentrated extracts improved the aroma and colour development from green tea (Table 5.4). The colour development was also reflected in the colour of the rooibos extract as well as the leaves (Table 5.5, lower L*, higher a* and b* values). Only the concentrated *L. edodes* YP + 1 % wheat straw cocktail increased the yield in soluble solids from green tea, but this coincided with a decrease in the antioxidant content, suggesting the extraction of mostly inactive compounds. The unconcentrated and concentrated *R. oryzae* potato dextrose cocktail improved the antioxidant content of the rooibos extract by more than 10 % while retaining most of the soluble solids, but the aroma and colour development was unsatisfactory. HPLC analyses of the antioxidants released by the *R. oryzae* potato dextrose cocktails showed a significant increase in the flavonoid content of the rooibos extract after treatment with either the concentrated or unconcentrated *R. oryzae* potato dextrose cocktail (Table 5.6). This coincided with an increase in the levels of the major antioxidants in rooibos, such as aspalathin, orientien and iso-orientien.

When cultured in YP + 1% wheat straw, the *R. oryzae* cocktail improved the yield in SS from fermented tea (Table 5.7) without a loss in antioxidant content (% TP/SS),

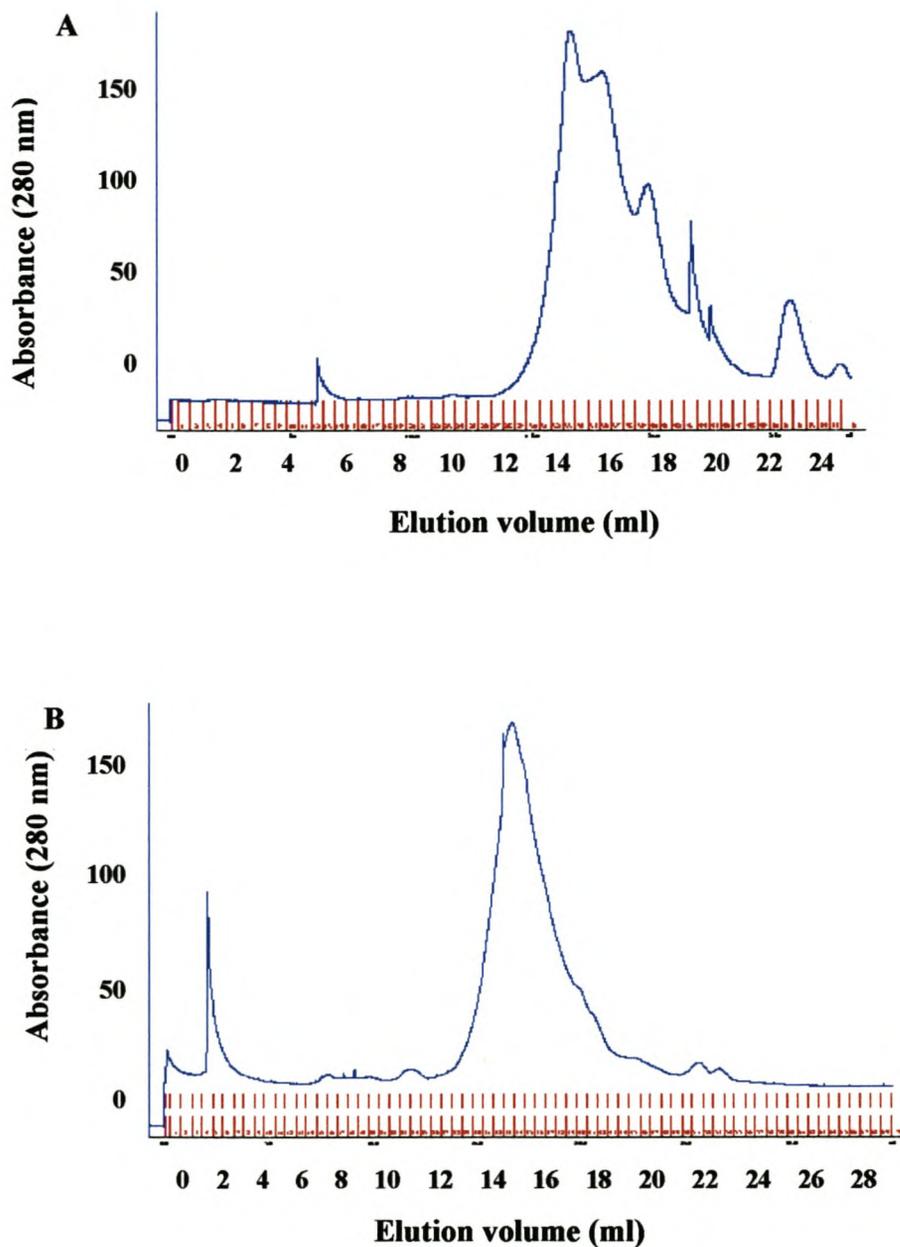


Figure 5.3. Gel-filtration by fast protein liquid chromatography on Superdex 75. A: *L. edodes* (unconcentrated) and B: *L. edodes* (10x concentrated).

suggesting that additional active compounds with antioxidant capabilities were extracted. This was confirmed with the strong increase in antioxidant content in the extract after treatment with the treated concentrated extract. When treated with the potato dextrose cocktail, there was no increase in soluble solids, but more than 10% improvement in the antioxidant content.

Table 5.3. Enzyme activities of the eluted fractions of the fungal cocktails.

Cocktail	Fraction number	Activity (IU/ml)			
		Endoglucanase	Pectinase	Xylanase	Laccase
<i>A. niger</i>	17	0.02	0.20	0.20	19.00
	24	0.04	0.20	0.10	201.00
<i>A. niger</i> (10x concentrated)	17	0.00	0.10	0.10	9.00
	24	0.20	0.10	0.00	9.00
<i>P. djamor</i>	16	0.02	0.20	0.20	25.00
	17	0.01	0.10	0.04	22.00
	34	0.01	0.20	0.02	26.00
<i>P. djamor</i> (10x concentrated)	16	0.02	0.10	0.10	22.00
	17	0.02	0.05	0.10	20.00
	34	0.02	0.20	0.30	23.00
<i>P. ostreatus</i> var. <i>florida</i>	15	0.02	0.10	0.10	11.00
	16	0.02	0.10	0.10	10.00
	34	0.02	0.20	0.10	123.00
<i>P. ostreatus</i> var. <i>florida</i> (10x concentrated)	16	0.05	0.10	0.04	206.00
	17	0.04	0.20	0.04	18.00
	34	0.04	0.20	3.00	18.00
<i>R. oryzae</i>	16	0.01	0.10	0.10	287.00
	23	0.01	0.10	0.10	284.00
	34	0.01	0.10	0.10	62.00
<i>R. oryzae</i> (10x concentrated)	16	0.03	0.30	0.10	57.00
	22	0.10	0.30	0.04	193.00
	34	0.03	0.30	0.10	228.00
<i>L. edodes</i>	33	0.00	0.10	0.10	38.00
	36	0.00	0.30	0.10	39.00
	40	0.04	0.30	3.50	20.00
	51	0.02	0.10	0.20	12.00
	55	0.02	0.20	0.10	12.00
<i>L. edodes</i> (10x concentrated)	16	0.02	0.20	0.10	9.00
	24	0.02	0.20	0.10	23.00
	33	0.02	0.04	0.20	24.00
	51	0.03	0.10	0.20	9.00
	53	0.03	0.20	0.30	13.00

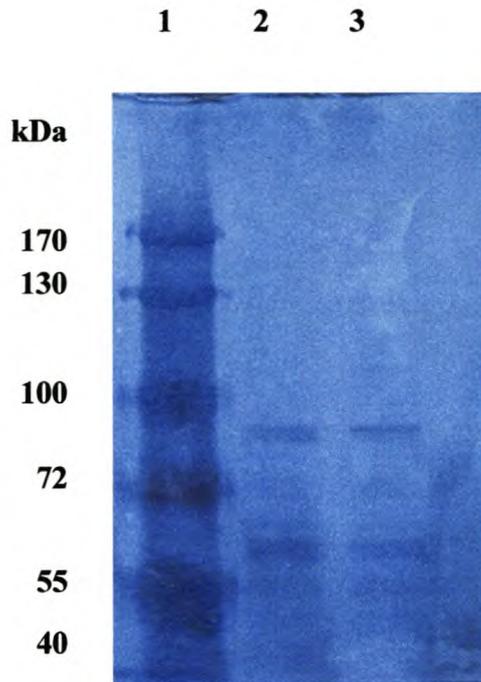


Figure 5.4. An example of results obtained with SDS-PAGE. Lane 1, molecular mass marker; Lane 2 and 3, fractions 16 and 17 of *P. ostreatus* var. *florida* (10x concentrated).

The lower efficacy of the potato dextrose cocktail in the extraction of soluble solids from fermented rooibos tea could be due to the lower levels of enzyme activity, particularly that of endoglucanase and xylanase, than in the YP plus 1% wheat straw medium (Table 5.8). The improvement in antioxidant content was confirmed with HPLC analysis (Table 5.9) where both the unconcentrated and concentrated *R. oryzae* cocktail increased the % total flavonoids/SS, as well as the levels of seven of the major antioxidants found in rooibos tea, by more than 20%.

Small-scale simulated industrial treatment of fermented tea with fungal cocktails

Concentrated extracts from *L. edodes* and *R. oryzae*, prepared in YP + 1% wheat staw, were used for simulated industrial extraction of SS from “waste fermented tea”, a stalky substrate, not suitable for commercial sale. Both cocktails improved the release of soluble solids and that of *R. oryzae* also improved the release of TP (Table 5.10), which suggests an improvement in the antioxidant content. The TP/SS ratio (mg GAE/100 g SS) for most of the treatments were above the critical benchmark of 20% (E. Joubert, personal communication), except for the ultrafiltration (UF) 30 kDa filtrates. However, the 30 kDa cut-off filter is more tight than the 0.25 μm filter

Table 5.4. Effect of fungal cocktails on aroma and colour development from green tea.

Isolate	Aroma and Colour development
Control – YP + wheat straw	Underfermented
<i>A. niger</i>	Musty, dusty aroma with unidentified, uncharacteristic note
<i>A. niger</i> (10x conc.)	Musty, dusty aroma with unidentified, uncharacteristic note
<i>R. oryzae</i>	Similar to control
<i>R. oryzae</i> (10x conc.)	Similar to control
<i>L. edodes</i>	Strange, nutty aroma. Well fermented character. Colour very good
<i>L. edodes</i> (10x conc.)	Nutty aroma. Well fermented character. Colour very good
<i>P. ostreatus</i> var. <i>florida</i>	Strange aroma with underfermented character
<i>P. ostreatus</i> var. <i>florida</i> (10x conc.)	Not much different than unconcentrated sample
<i>P. djamor</i>	Vague sweet aroma development. Better than concentrated sample
<i>P. djamor</i> (10x conc.)	Vague sweet aroma development.

membrane routinely used by the industry, which will have a substantial effect on the final recovery of soluble solids and thus yields in soluble solids and total polyphenols.

Fermentation studies to increase enzyme production in *L. edodes*

To obtain sufficient amounts of enzyme cocktail for further evaluations, *R. oryzae* was cultured in potato dextrose in a 14L bench-top fermentor. To simulate the conditions previously used in shake flask experiments, the pH and dissolved oxygen were not controlled during the first fermentation. The results showed that a decrease in dissolved oxygen after Day 2 resulted in a drop in the pH (data not shown), subsequently leading to a decrease in enzyme activity due to proteolytic activity (Table 5.11). Based on these results, a second and third fermentation were done in which the pH was maintained at 4.5 units and dissolved oxygen at 20%, with agitation increased to 200 rpm. In both fermentation experiments a similar trend was observed, i.e. a decrease in enzyme activity at Day 2 due to proteolysis. It was therefore decided to harvest the supernatant at Day 2 and concentrate it ten-fold by ultrafiltration with a 5 kDa cut-off membrane. The concentrated *R. oryzae* cocktail did not increase the yield in %SS or TP from fermented tea, although an improvement in the colour of the leaves and abstract was obtained (Table 5.12). The aroma and colour development from the green tea was not significant, but the enzyme cocktail improved the TP content of the extract by more than 10%.

Table 5.5. Laboratory scale treatment of green tea with fungal cocktails. Values given show the means of duplicate or triplicate measurements on treatments, expressed as % relative to the control. Improvements of more than 10% are indicated in bold.

Treatment	%SS (g SS/100 g dry leaves)	TP (mg GAE/ 100g SS)	%TP/SS (*mg GAE/100 g SS)	Colour – Rooibos extract			Colour – Rooibos leaves		
				L*	a*	b*	L*	a*	b*
Control – YP + wheat straw	13.28 ± 0.48	3.87 ± 0.17	29.12 ± 0.21	83.96 ± 0.49	-1.13 ± 0.00	25.88 ± 0.61	30.38 ± 0.58	7.41 ± 0.07	12.56 ± 0.31
<i>L. edodes</i> (unconcentrated)	14.42 ± 0.25	3.83 ± 0.10	26.55 ± 0.24	#78.36 ± 1.14	-0.61 ± 1.25	#42.36 ± 0.03	26.50 ± 0.36	10.16 ± 0.59	11.48 ± 0.37
%	109	99	91	93	54	164	87	137	91
<i>L. edodes</i> (10x concentrated)	15.57 ± 0.79	#3.01 ± 0.09	#19.40 ± 1.54	#74.87 ± 1.07	#4.70 ± 0.83	#44.45 ± 0.00	23.53 ± 0.33	9.94 ± 0.04	10.17 ± 0.59
%	117	78	67	89	-417	172	77	134	81
<i>R. oryzae</i> (unconcentrated)	13.33 ± 0.07	3.89 ± 0.17	29.21 ± 1.40	84.43 ± 0.67	-1.00 ± 0.35	26.34 ± 1.62	28.84 ± 0.11	7.48 ± 0.37	11.73 ± 0.19
%	100	101	100	101	88	102	95	101	93
<i>R. oryzae</i> (10x concentrated)	13.92 ± 0.72	4.04 ± 0.21	29.03 ± 0.04	83.16 ± 1.87	-0.47 ± 0.24	27.69 ± 0.63	29.24 ± 0.54	7.40 ± 0.43	11.97 ± 0.68
%	105	105	100	99	42	107	96	100	95
Control – Potato Dextrose	14.11 ± 0.41	3.80 ± 0.08	26.94 ± 0.23	87.32 ± 1.26	-1.02 ± 0.05	15.76 ± 0.58	28.97 ± 0.02	7.41 ± 0.41	12.46 ± 0.38
<i>L. edodes</i> (unconcentrated)	13.91 ± 0.12	3.96 ± 0.09	28.45 ± 0.44	86.61 ± 0.26	-0.97 ± 0.26	29.28 ± 0.30	28.60 ± 0.29	7.85 ± 0.11	12.44 ± 0.39
%	99	104	106	99	95	186	99	106	100
<i>L. edodes</i> (10x concentrated)	14.06 ± 1.10	3.77 ± 0.22	26.82 ± 0.51	82.62 ± 1.34	0.48 ± 0.71	41.09 ± 0.62	25.69 ± 0.22	9.70 ± 0.21	11.58 ± 0.29
%	100	99	100	95	-47	261	89	131	93
<i>R. oryzae</i> (unconcentrated)	12.59 ± 0.17	3.85 ± 0.10	#30.58 ± 0.41	88.67 ± 0.06	-1.30 ± 0.18	16.44 ± 0.73	30.01 ± 0.70	7.13 ± 0.62	12.87 ± 0.09
%	89	101	114	102	127	104	104	96	103
<i>R. oryzae</i> (10x concentrated)	13.61 ± 1.04	4.16 ± 0.36	#30.55 ± 0.29	87.39 ± 1.30	-1.13 ± 0.12	24.50 ± 1.24	31.72 ± 0.20	7.13 ± 0.62	13.39 ± 0.43
%	96	109	113	100	110	155	110	96	107

*Quality parameters: 20 mg GAE/100 g SS (%TP/SS), 1 200 µmol Trolox/g SS

Indicate significant differences in means on a 5% (p<0.05) significance level

Table 5.6. HPLC analysis of antioxidants extracted from green rooibos tea leaves with fungal cocktails. Values given show the means of duplicate treatments, expressed as % relative to the control. Improvements of more than 10% are indicated in bold.

Cocktail	Asp	Orient	Iso-orient	Vitex	Noth	Isovitex	Iso-quer/Rutin	Quer	Luteol	%Total Flavonoids/SS	% Total Flavonoids/TP
Control – YP + wheat straw	3.246	0.648	0.749	0.175	0.325	0.208	0.869	0.013	0.012	6.245 ± 1.05	21.433 ± 0.49
<i>L. edodes</i> (unconcentrated)	1.767	0.682	0.778	0.190	0.357	0.220	0.921	0.017	0.012	4.946 ± 0.02	18.628 ± 0.05
%	54	105	104	108	110	106	106	129	102	79	87
<i>L. edodes</i> (10x concentrated)	#0.338	0.556	0.611	0.178	0.195	0.188	0.684	0.013	0.015	#2.777 ± 0.03	14.371 ± 1.08
%	10	86	82	102	60	90	79	99	120	44	67
<i>R. oryzae</i> (unconcentrated)	3.590	0.711	0.825	0.2	0.365	0.223	0.952	0.019	0.013	6.897 ± 0.09	23.627 ± 0.63
%	111	110	110	114	112	107	110	144	104	110	110
<i>R. oryzae</i> (10x concentrated)	3.350	0.647	0.755	0.199	0.336	0.2	0.831	#0.029	0.014	6.361 ± 0.07	21.911 ± 0.28
%	103	100	101	113	103	96	96	217	116	102	102
Control – Potato Dextrose	3.063	0.549	0.639	0.147	0.303	0.178	0.743	0.016	0.008	5.646 ± 0.24	20.957 ± 0.73
<i>L. edodes</i> (unconcentrated)	3.253	0.617	0.709	0.162	0.355	0.197	0.824	0.018	0.012	6.146 ± 0.65	21.628 ± 0.56
%	106	112	111	110	117	110	111	110	151	109	103
<i>L. edodes</i> (10x concentrated)	2.006	0.651	0.730	0.174	0.350	0.207	0.856	0.020	0.013	5.007 ± 0.04	18.670 ± 0.45
%	66	118	114	119	115	116	116	120	163	89	89
<i>R. oryzae</i> (unconcentrated)	4.004	0.659	0.768	0.172	0.393	0.208	0.897	0.021	0.006	7.128 ± 0.33	23.302 ± 0.81
%	131	120	120	117	130	117	121	126	82	126	111
<i>R. oryzae</i> (10x concentrated)	#4.463	0.711	0.825	0.186	#0.441	0.224	0.969	0.017	0.013	#7.849 ± 0.24	25.694 ± 0.60
%	146	129	129	127	145	126	131	104	163	139	123

*Abbreviations for antioxidants: Asp, aspalathin; Orient, orientin; Isoorient, isoorientin; Vitex, Vitexin, Noth, nothofagin, Isovitex, isovitexin; Isoquer/Rutin, isoquercitrin/Rutin; Quer, quercetin; Luteol, luteolin; Chrys, chrysoeryol

Indicate significant differences in means on a 5% (p<0.05) significance level

Table 5.7. Laboratory scale treatment of fermented tea with *R. oryzae* cocktails. Values given show the means of duplicate treatments, expressed as % relative to the control. Improvements of more than 10% are indicated in bold.

Cocktail	%SS (g SS/100g dry leaves)	%TP/SS (*mg GAE/100 g SS)	Antioxidant (* μ mol Trolox/g SS)	Trolox/ g TP
Control – YP + wheat straw	7.72	29.54	2024	6860
<i>R. oryzae</i>	9.48	30.06	1942	6460
%	123	102	96	94
<i>R. oryzae</i> (10x concentrated)	#11.33	#29.18	1914	8937
%	147	99	95	130
Control - Potato Dextrose	12.53	25.45	1547	9271
<i>R. oryzae</i>	11.58	28.54	1732	8579
%	92	112	112	93
<i>R. oryzae</i> (10x concentrated)	11.45	28.92	1752	8415
%	91	114	113	91

*Quality parameters: 20 mg GAE/100 g SS (%TP/SS), 1 200 μ mol Trolox/g SS

Indicate significant differences in means on a 5% (p<0.05) significance level

Table 5.8. Enzyme activities in *R. oryzae* cocktails. Values given show the means of duplicate treatments.

Cocktail	Activity (IU/ml)			
	Endoglucanase	Pectinase	Xylanase	Laccase
YP + wheat straw (Day 5)				
<i>R. oryzae</i>	3.2	0.9	47.0	158.1
<i>R. oryzae</i> (10x concentrated)	8.1	1.2	344.5	583.7
Potato Dextrose (Day 5)				
<i>R. oryzae</i>	2.1	1.2	0.7	3.2
<i>R. oryzae</i> (10x concentrated)	2.3	3.4	0.6	73.8

Table 5.9. HPLC analysis of antioxidants extracted from fermented tea with fungal cocktails. Values given show the means of duplicate treatments, expressed as % relative to the control. Improvements of more than 10% are indicated in bold.

Cocktail	Asp	Orient	Iso-orien	Vitex	Noth	Isovitex	Iso-quer/ Rutin	%Total Flavonoids/SS	% Total Flavonoids/TP
Control – YP + wheat straw	0.708	0.123	0.083	0.242	0.069	0.106	0.672	2.003 ± 0.28	6.791 ± 0.14
<i>R. oryzae</i> (unconcentrated)	0.639	0.116	0.079	#0.218	#0.061	0.100	#0.641	1.855 ± 0.50	6.173 ± 0.03
%	90	95	95	90	88	94	95	93	91
<i>R. oryzae</i> (10x concentrated)	0.731	0.116	0.079	#0.218	#0.052	0.111	#0.584	1.891 ± 0.03	6.485 ± 0.21
%	103	94	95	90	75	105	87	94	95
Control – Potato Dextrose	0.515	0.091	0.062	0.163	0.048	0.073	0.520	1.474 ± 0.08	5.795 ± 0.40
<i>R. oryzae</i> (unconcentrated)	#0.683	#0.111	#0.074	#0.193	#0.064	0.086	0.639	#1.849 ± 0.06	6.479 ± 0.08
%	133	121	119	118	130	117	123	126	112
<i>R. oryzae</i> (10x concentrated)	#0.691	#0.116	#0.076	#0.201	#0.064	#0.100	0.641	#1.890 ± 0.02	6.542 ± 0.18
%	134	127	122	123	130	137	123	128	113

*Abbreviations for antioxidants: Asp, aspalathin; Orient, orientin; Isoorien, isoorientin; Vitex, Vitexin, Noth, nothofagin, Isovitex, isovitexin; Isoquer/Rutin, isoquercitrin/Rutin; Quer, quercetin; Luteol, luteolin; Chrys, chrysoeryol.

Indicate significant differences in means on a 5% (p<0.05) significance level

Table 5.10. Small-scale simulated treatment of fermented tea with fungal cocktails. Values given show the means of duplicate treatments, expressed as % relative to the control. Improvements of more than 10% are indicated in bold.

Cocktail	Filtrate	%SS (g SS/100 ml extract)	TP (mg GAE/100 ml extract)	%TP/SS (mg GAE/100 g SS)
Control - dH₂O	W 4	0.838	280.90	33.52
	Ap 15	0.77	347.78	45.17
	UF 30K	0.19	45.67	24.04
Control – YP + wheat straw	W 4	0.93	302.88	32.57
	Ap 15	0.91	345.87	38.01
	UF 30K	0.28	60.96	21.77
<i>L. edodes</i> (10x concentrated)	W 4	1.093	311.48	28.50
	%	118	103	88
	Ap 15	1.085	302.88	27.91
%	119	88	73	
%	UF 30K	0.513	58.66	11.44
%	183	96	53	
<i>R. oryzae</i> (10x concentrated)	W 4	1.235	389.82	31.56
	%	133	129	97
	Ap 15	1.185	480.58	40.55
%	130	139	107	
%	UF 30K	0.455	64.78	14.24
%	163	106	65	

W4: Whatman #4; Ap15: Millipore glass fibre prefilter (pore size ~ 1.0 µm); UF 30K: Millipore/Amicon Ultrafiltration membrane

Table 5.11. Enzyme activities in *R. oryzae* cocktails obtained with fermentation broth. Values given show the means of triplicate measurements.

	Activity (IU/ml)			
	Endoglucanase	Pectinase	Xylanase	Laccase
Day 1	3.1	14.7	2.6	345.5
Day 2	5.7	9.1	2.8	365.7
Day 3	1.9	8.0	2.2	72.1
Day 4	1.0	4.9	1.3	72.0
Day 5	0.2	0.4	1.1	65.7

Table 5.12. Effect of *R. oryzae* cocktails (fermentation broth) on yields in %SS, TP and colour development from rooibos tea material. Values given show the means of duplicate treatments, expressed as % relative to the control. Improvements of more than 10% are indicated in bold.

Treatment	%SS (g SS/100 g dry leaves)	TP (mg GAE/ g tea)	%TP/SS (mg GAE/ 100 g SS)	Colour - Rooibos extract			Colour - Rooibos leaves		
				L*	a*	b*	L*	a*	b*
Fermented tea									
Control - Potato Dextrose	14.52 ± 0.70	52.52 ± 0.02	24.95 ± 0.34	83.06 ± 0.51	-0.6 ± 0.32	58.38 ± 0.70	27.60 ± 0.24	14.52 ± 0.44	24.38 ± 0.44
<i>R. oryzae</i>	14.10 ± 0.75	51.04 ± 2.19	25.83 ± 0.17	84.35 ± 0.56	-1.57 ± 0.37	54.01 ± 1.56	27.71 ± 0.82	14.31 ± 0.41	24.81 ± 0.74
%	97	97	104	102	262	93	100	99	102
<i>R. oryzae</i> (10x concentrated)	14.20 ± 0.22	51.98 ± 2.61	26.47 ± 0.18	83.98 ± 0.33	-1.09 ± 0.23	56.88 ± 1.28	27.93 ± 0.07	14.36 ± 0.04	24.82 ± 0.12
%	98	99	106	101	182	97	101	99	102
GREEN tea									
Control - Potato Dextrose	14.51 ± 2.03	42.74 ± 4.28	24.08 ± 0.43	85.86 ± 0.20	-2.38 ± 0.14	55.29 ± 0.17	30.35 ± 0.46	13.85 ± 0.11	28.13 ± 0.62
<i>R. oryzae</i>	15.92 ± 0.84	43.88 ± 2.27	22.29 ± 0.74	85.73 ± 0.70	-2.25 ± 0.77	70.13 ± 0.52	30.55 ± 0.30	14.48 ± 0.41	27.51 ± 0.14
%	111	103	93	100	95	127	101	105	98
<i>R. oryzae</i> (10x concentrated)	17.29 ± 0.85	45.41 ± 1.77	21.53 ± 0.22	86.08 ± 0.27	-2.65 ± 0.31	68.07 ± 0.22	30.48 ± 0.44	13.99 ± 0.38	26.84 ± 0.17
%	119	106	89	100	111	123	100	101	95

Discussion

Based on their food-grade status, *L. edodes*, *A. niger*, *R. oryzae*, *P. ostreatus* var. *florida* and *P. djamor* were selected for evaluations on rooibos tea substrates for aroma and colour development from green tea, or for the release of soluble solids and/or antioxidants from fermented tea. However, as these evaluations require large amounts of enzyme extract that is optimised specifically for the hydrolysis of rooibos substrate, it was imperative to develop a growth medium that will ensure a high level of expression for those enzymes identified to be important for improved processing of rooibos, i.e. endoglucanase, pectinase, xylanase and laccase (Chapter 3).

The *L. edodes* enzyme cocktail was in particular effective for processing of fermented tea (Chapter 3) and was selected for the evaluation of different types of media to determine whether the growth media can be optimised for the production of endoglucanase, pectinase, xylanase and laccase. From the 12 substrates evaluated, 1 x MYPG proved to be the best growth medium while 1% spent grain or 1% wheat straw gave the best induction for xylanase, cellulase and laccase activities. DNS liquid assays (Table 5.1) confirmed that 1 x YP medium supplemented with 1% wheat straw consistently gave the highest levels of xylanase and endoglucanase activities, as well as good induction of pectinase and laccase levels. YP medium supplemented with 1% wheat straw was therefore used for cultivation of *L. edodes* and the other four strains on larger scale.

Quantitative enzyme profiles for the four targeted enzymes, i.e. endoglucanase, xylanase, pectinase and laccase, were obtained (Table 5.3), but qualitative analyses of all the enzymes in the different cocktails are required to compile a complete enzyme profile.

When cultured in YP + 1% wheat straw medium, the *L. edodes* cocktail showed the best improvement in both the aroma and colour development on green tea and may be considered for reducing the fermentation time required for green tea (Table 5.4 and 5.5). This may allow for a factory-based process opposed to the current outdoor fermentation process. In general, the *R. oryzae* cocktail seems to be the best candidate for application in a quick-draw fermented tea made by infusion where there is an improved colour release and extraction of soluble solids (more than 20%) (Table 5.7), without loss in the TP and antioxidant content. The YP + 1% wheat straw enzyme cocktails of *L. edodes* and *R. oryzae* (Table 5.8), showed relatively high levels of laccase activity, which could assist in the release of polyphenols and therefore increase the antioxidant content, as well as contribute to colour development.

Previous results (see Chapter 3) indicated that *R. oryzae* cultured in potato dextrose medium could increase the release of antioxidants from green tea. This was, however, not observed when the fungus was cultured in YP + 1% wheat straw medium. The potato dextrose cocktails from *L. edodes* and *R. oryzae* were therefore re-evaluated on green tea (Table 5.5) to confirm previous observations. On green tea, the *L. edodes* cocktail proved to be the most effective for aroma and colour development, and that of *R. oryzae* for increasing the antioxidant levels in rooibos extracts. For fermented tea, the culture conditions and therefore the enzyme concentrations in the fungal extract, determined the efficacy of *R. oryzae* (Table 5.7) for either the extraction of more than 20% additional soluble solids (YP + 1 % wheat straw) or more than 10% additional antioxidants (potato dextrose).

Optimisation of expression of the four enzymes via fermentation of *R. oryzae* was done with only potato dextrose medium since the YP + 1 % wheat straw medium yielded extremely low, if any, levels of enzyme activity. The enzyme levels in the potato dextrose fermentation broth seemed to be high enough after Day 2 (Table 5.11) to be used for small-scale industrial simulations. However, the fermentation broth was not as effective as the concentrated cocktails previously isolated from shake flasks. It is possible that proteolysis already started at Day 2, resulting in a further loss in enzyme activity during the treatment of rooibos substrates. Further fermentation optimisation may be needed to yield high antioxidant extraction, as observed for shake flask cultures.

It can be concluded that the use of optimised YP + 1% wheat straw medium induced the production of several hydrolytic enzymes in both *L. edodes* and *R. oryzae*. This led to an improvement of both the colour and aroma development of green tea with the *L. edodes* cocktail. However, when cultured in potato dextrose, the *L. edodes* cocktail can be used for aroma and colour development from green tea, while *R. oryzae* can be used for increasing the antioxidant levels in rooibos extracts from green or fermented tea. This was confirmed with small-scale industrial treatments of fermented tea where the *L. edodes* YP + wheat straw cocktail improved the release in SS by more than 10% and the *R. oryzae* YP + wheat straw cocktail increased the yield in SS by more than 30% and the TP by more than 20%.

Chapter 6

Concluding Remarks

Concluding Remarks

Since many of the flavour and medicinal compounds in rooibos tea are trapped within the cellulolytic plant material of the tea leaves as glycoconjugated aroma and phenolic compounds, we believed that these compounds could be released upon cleavage of the chemical bonds by applying external microbial hydrolytic enzymes. Eleven white-rot fungal strains from the Departmental collection (all food-grade organisms), as well as 12 newly isolated strains from commercial rooibos tea products, were selected for their potential to improve various quality parameters such as aroma and colour development, extraction of soluble matter and antioxidants from rooibos tea. The enzyme profiles of cellulase, pectinase, xylanase and laccase for all 23 fungal strains were determined with the most promising profiles presented by *L. edodes*, *A. niger*, *P. djamor*, *P. ostreatus* var. *florida* and isolate MP1, later identified as *R. oryzae*. The crude enzyme extracts of nine fungal strains increased the release of soluble solids with an improvement in the yield in soluble solids of up to 42% (*P. djamor*), while 14 strains yielded higher levels of total polyphenols with increases up to 36% (MP1 – *R. oryzae*).

Processing of rooibos tea includes the shredding and bruising of green plant material, followed by fermentation during which the characteristic organoleptic qualities of rooibos tea develop. However, the long fermentation period of 14-16 hours results in a significant loss in antioxidants in the processed tea and therefore also in its pharmaceutical and nutraceutical value. Twelve of the strains that gave the best results on spent tea were therefore evaluated for enhanced colour and aroma development from green rooibos tea, but improvements were difficult to determine due to relative low enzyme concentrations.

It was imperative to develop a growth medium that will ensure a high level of expression for cellulase, xylanase, pectinase and laccase to evaluate the suitability of the enzyme extract for rooibos processing. From the 12 substrates evaluated, 1 x YP medium supplemented with 1% wheat straw gave the best induction for all four hydrolytic enzymes and was therefore selected for large-scale cultivation of the fungal strains for further evaluations on rooibos tea. Of the five strains evaluated, the *L. edodes* cocktail showed the best improvement in both aroma and colour development of green tea. This extract may therefore be considered for shortening of the fermentation time required for green tea to allow a factory-based process under controlled conditions as opposed to the current out-door fermentation process. For application in a quick-draw fermented tea made by infusion, the *R. oryzae* cocktail proved to be the best candidate, showing an improved colour release and

extraction of soluble solids (more than 20%) from rooibos tea, without a loss in total polyphenol content.

When cultured in Potato Dextrose medium, it was found that the *L. edodes* cocktail was the most effective for aroma and colour development from green tea, whereas the *R. oryzae* was able to increase the antioxidant levels in rooibos extracts from green and fermented tea. Fermentation studies with *R. oryzae* in Potato Dextrose medium yielded high levels of enzyme activity after two days. Although similar trends were observed as for the laboratory-scale evaluations, the presence of proteases reduced the efficiency of the fermentation broth.

It can be concluded that different microbial enzymes can be used for the improved release of soluble solids, polyphenols, antioxidants and/or improved fermentation characteristics of rooibos plant material. Depending on the strain and culture conditions, some enzyme extracts can enhance the extraction of soluble solids from green and/or spent tea, whereas others may be more suitable for releasing additional antioxidants from green or fermented tea, or to reduce the fermentation time of green tea through improved colour and aroma development. These findings will provide the rooibos industry with the opportunity to exploit value-added products from rooibos for the local and international functional food/nutraceutical, pharmaceutical and cosmetic markets.

Further research is necessary to determine the exact polysaccharide and polyphenol composition of the rooibos tea leaf, which will assist in the choice of microbial hydrolytic enzymes for optimal bioprocessing. This will lead to optimal release of the colour and aroma components trapped in the cellulolytic plant material, and extraction of additional soluble solids from rooibos products.

Chapter 7

Literature Cited

Literature Cited

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