Evaluation of Commercial Enzymes for the 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.

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Date

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Summary

The Rooibos tea plant (*Aspalathus linearis*) is indigenous to South Africa and occurs only in the Western Cape's Cedarberg region. Rooibos tea is produced from the leaves and fine stems of the plant. The tea is normally prepared by brewing the leaves and consuming the liquor. However, the Rooibos plant is not only used to prepare tea; the plant extracts are also used in various neutraceutical and pharmaceutical products, including health drinks, iced tea, soaps and moisturising creams.

Although the tea plant contains native enzymes responsible for the colour and aroma development of Rooibos tea, the disruption and maceration of the plant material during processing is insufficient to allow these enzymes proper access to the substrates responsible for Rooibos tea's characteristics. The current processing of Rooibos tea is also time consuming and is done under uncontrolled conditions, leading to unnecessary loss in aroma and antioxidant content. The addition of enzymes could improve the maceration of the plant material, shorten the processing time and improve the extraction of aroma, colour and antioxidant components.

During this study, 16 commercially available microbial enzymes were evaluated on three different Rooibos substrates for the improvement of aroma and colour development, as well as the extraction of soluble solids (SS) and total polyphenols (TP). Thirteen enzymes were evaluated on spent tea for the enhanced extraction of soluble solids and to determine the best candidates for further evaluation on fermented and green Rooibos tea. Seven of the enzymes improved the yield in SS from spent tea. Up to 232% improvement was obtained, depending on the type of enzyme and dosage applied.

The best six enzyme preparations were further evaluated on fermented Rooibos tea. For Depol™ 670L at 20 µl/g tea, the laboratory treatment increased the yield in SS by 44%, while small-scale industrial simulations increased the SS by 26%. However, an increase in the yield in SS was usually accompanied by a decrease in the %TP/SS ratio, indicating that mainly inactive compounds were extracted. Based on the results with the commercial enzymes, twelve "synthetic" enzyme cocktails, consisting of

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different combinations of commercial enzymes were designed, of which three cocktails released increased amounts of SS without decreasing the %TP/SS ratio significantly.

Thirteen enzymes were evaluated on dried and freshly cut green Rooibos tea, with three enzymes (Depol™ 670L, Pectinex Ultra SP-L and Depol™ 692L) increasing the yield in SS between 21% and 66%, and the TP content between 11% and 47%. Laccase was the best candidate in improving colour development from green tea, with the improvement being slightly better at 50°C than at 40°C.

All the "synthetic" cocktails containing laccase improved the colour extract of all three substrates evaluated, but also significantly decreased the TP and antioxidant content. However, lower dosages of laccase resulted in colour development with little loss in the antioxidant content. Due to the promising results obtained with the treatments of Rooibos tea with laccases, it was decided to clone and express the laccase gene (*lacA*) of *Pleurotus ostreatus* into *Aspergillus niger*. The gene was successfully transformed into *A. niger*, but the expression of the recombinant gene was not effective.

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Opsomming

Die Rooibostee plant (*Aspalathus linearis*) is inheems tot Suid-Afrika en kom slegs in die Sederberg-omgewing in die Wes-Kaap voor. Rooibostee word van die blare en fyn stingels van die plant geproduseer. Die tee word normaalweg voorberei deur die blare in kookwater te laat trek en dan die aftreksel te drink. Die Rooibos plant word nie net gebruik om tee te maak nie; die tee ekstrak word ook gebruik vir verskeie neutraseutiese en farmaseutiese produkte, insluitende gesondheidsdrankies, ystee, seep en bevogtigingsrome.

Ten spyte daarvan dat die teeplant sy eie ensieme vir die kleur en aroma ontwikkeling van Rooibostee bevat, is die verbreking en maserasie van die plantmateriaal tydens prosessering onvoldoende om die ensieme genoeg toegang tot die substrate verantwoordelik vir die kenmerkende eienskappe van Rooibostee te gee. Die huidige prosessering van Rooibostee is ook tydrowend en geskied onder onbeheerde toestande, wat tot 'n onnodige verlies in aroma en antioksidante lei. Die toevoeging van ensieme kan die afbraak van die plantmateriaal verbeter, die behandelingsproses verkort en die aroma, kleur en antioksidant inhoud van ekstrakte verbeter.

Tydens hierdie studie is 16 kommersieël-beskikbare mikrobiese ensieme op drie verskillende Rooibos substrate vir die verbetering van aroma, kleur en ekstraksie van oplosbare vastestowwe (SS) en totale polifenole (TP) getoets. Dertien ensieme is op oorskot tee vir die verbeterde ekstraksie van oplosbare vastestowwe geëvalueer, waarna die beste kandidate vir evaluering op gefermenteerde en ongefermenteeede Rooibostee gekies is. Sewe ensieme het die SS vanaf oorskot tee verhoog. Tot 232% verhoging is waargeneem, afhangende van die tipe ensiem en die dosis wat gebruik is.

Die beste ensiem preparate is verder op gefermenteerde Rooibostee geëvalueer. Labarotorium behandelings met Depol™ 670L teen 20 µl/g tee het die SS inhoud met 44% verhoog, terwyl die kleinskaalse industriële simulasie die SS inhoud met 26% verhoog het. 'n Verhoging in SS het egter gewoonlik met 'n afname in die %TP/SS verhouding gepaard gegaan, wat aandui dat hoofsaaklik onaktiewe stowwe vrygestel is. Na aanleiding van die resultate met die kommersiële ensieme, is twaalf

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"sintetiese" ensiemmengsels met verskillende ensiemkombinasies getoets, waarvan drie mengsels ook meer SS vrygestel het met byna geen verlaging in die %TP/SS verhouding nie.

Dertien ensieme was op gedroogde en vars gekerfde groen Rooibostee getoets met drie ensieme (Depol™ 670L, Pectinex Ultra SP-L en Depol™ 692L) wat die SS met tussen 21% en 66%, en die TP inhoud met tussen 11% en 47% verhoog het. Lakkase was die beste kandidaat vir die verbetering van kleur ontwikkeling by groen Rooibostee met die verbetering effens beter by 50°C as by 40°C.

Al die "sintetiese" ensiem mengsels wat lakkase bevat het, het die kleur by al die verskillende substrate verbeter, maar het ook die TP en antioksidant inhoud aansienlik verlaag. Laer lakkase dosisse het goeie kleurontwikkeling tot gevolg gehad met minimale verlies in die antioksidant inhoud. Vanweë die goeie resultate wat met die lakkase behandelings verkry is, is daar besluit om die lakkase geen (*lacA*) van *Pleurotus ostreatus* te kloneer en in *Aspergillus niger* uit te druk. Die geen is suksesvol in *A. niger* getransformeer, maar die uitdrukking daarvan was nie effektief nie.

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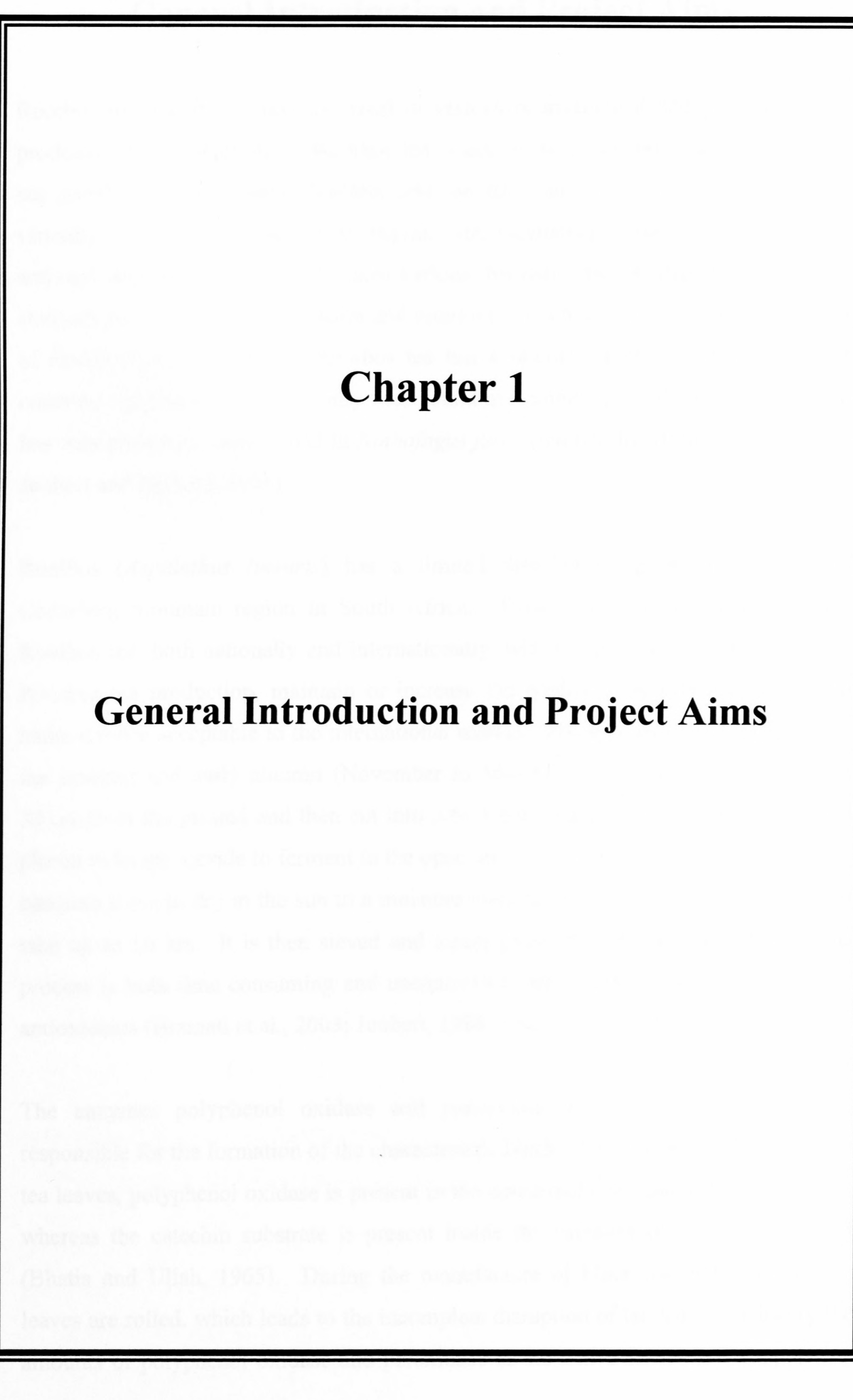

Chapter 1

General Introduction and Project Aims

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General Introduction and Project Aims

Rooibos tea and its extracts are used in various neutraceutical and pharmaceutical products. This popularity of Rooibos tea is due to its medicinal characteristics that are ascribed to its flavonoid content and the fact that it contains no caffeine and virtually no tannins. It has anti-mutagenic, anti-carcinogenic, anti-inflammatory and antiviral activities and also alleviates various disorders such as digestive disorders, stomach problems, nervous tension and allergies. It is also used as topical treatment of dermatological diseases. Rooibos tea has a unique flavonoid composition. It contains aspalathin, which has only been found in Rooibos tea, and nothofagin, which has only previously been found in *Nothofagus fusca* (red beech) (Bramati et al., 2003; Joubert and Ferreira, 1996)

Rooibos (*Aspalathus linearis*) has a limited distribution, growing only in the Cedarberg mountain region in South Africa. There is an increasing demand for Rooibos tea, both nationally and internationally, which require strategies to increase Rooibos tea production, maintain or increase the quality of Rooibos products and make it more acceptable to the international market. Rooibos tea is harvested during the summer and early autumn (November to March). The plants are cut to about 30 cm from the ground and then cut into 3 to 4 mm lengths, moistened, bruised and placed in heaps outside to ferment in the open air. The tea is then spread out on large concrete areas to dry in the sun to a moisture content of 8 to 10%, a process that can take up to 16 hrs. It is then sieved and steam pasteurised before packaging. This process is both time consuming and uncontrolled and results in considerable loss in antioxidants (Bramati et al., 2003; Joubert, 1988; Von Gadow et al., 1997).

The enzymes polyphenol oxidase and peroxidase in *Camellia* tea leaves are responsible for the formation of the characteristic black tea components. In the native tea leaves, polyphenol oxidase is present in the epidermal cells and vascular bundles, whereas the catechin substrate is present inside the vacuoles of the palisade cells (Bhatia and Ullah, 1965). During the manufacture of black tea, the withered tea leaves are rolled, which leads to the incomplete disruption of tea leaves, reducing the amounts of polyphenol oxidase and peroxidase in the native leaves and suppressing

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the formation of black tea components. The cell wall polysaccharides also act as an additional barrier for the interaction between enzyme and substrate. The application of exogenous enzymes responsible for cell wall degradation could therefore lead to maceration of the tea leaf and release more tea phenolic compounds that could improve the therapeutic value of tea. The application of cellulase, xylanase, pectinase and laccase, as well as the crude enzyme extracts from various microbial organisms, improved black tea quality with the crude extracts (combinations of enzymes) being superior (Angayarkanni et al., 2002; Murugesan et al., 2002).

The main objective of this study was to apply commercially available microbial enzymes or combinations of enzymes to Rooibos substrates to:

a) reduce the fermentation period required for the development of the characteristic colour and flavour from green Rooibos tea material and allow the fermentation to be conducted inside a factory under controlled conditions,

b) enhance the flavour and colour release during infusion of the fermented tea leaves, resulting in a "quick-draw" tea that is favoured by the fast-food industry,

c) improve the release of antioxidants from Rooibos substrates, thereby improving the nutraceutical or health-promoting value of Rooibos tea,

d) improve the release of soluble solids (plant material rendered soluble due to processing) from the plant material. This will increase the yield of tea extracts per kg raw material. The Rooibos tea extract can be used for the manufacturing of beverages (e.g. iced tea), nutraceutical supplements, cosmetic and health products or for the pharmaceutical industry, and

e) treat spent Rooibos tea for the extraction of additional soluble solids from the left-over plant material following conventional physio-chemical extraction processes.

Of particular interest was the effect that laccases had on the colour development in green tea. To this end, it was decided to express the laccase gene from *P. ostreatus* in *A. niger* to obtain a laccase that may be of commercial importance for the bioprocessing of Rooibos tea.

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Literature Review:

General Introduction on the Manufacture and Properties of Tea

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2.1 Introduction

The tea plant, *Camellia sinensis*, was domesticated in Szechuan about 2000 years ago. During the first millennium AD it spread through much of China and reached Korea and Japan in the eight century AD. The production of tea was largely confined to these countries and only spread outside their confines in the 1840's (Grigg, 2002). The traditional tea varieties are all made from the tea plant *Camellia sinensis* (L.) O. Kuntze of which two major varieties exist. These varieties are *var. sinensis* and *var. assamica* which differ largely in leaf size, with the former being small-leaved (5 – 12 cm) and the latter with leaves up to 20 cm in length. Tea is not only derived from *Camellia*, but also from other plants - these teas are referred to as herbal or organic teas (Graham, 1992). Rooibos tea, which is the focus of this study, is one of these herbal teas and will be discussed in more detail in later sections.

Tea is consumed worldwide at a per capita consumption of 0,12 liters per year. The highest consumption of tea per capita is in southwest and south Asia, North Africa, Russia, British Isles, South Africa, Australia and New Zealand. The consumption of tea is much lower in the Americas, tropical Africa and Europe (Graham, 1992; Grigg, 2002) (Figure 2.1). In 2003, about 3,2 million metric tons of tea was produced in over 30 countries, of which China and India were the largest contributors (http://faostat.fao.org) (Table 2.1). There are three main types of tea manufactured, namely black tea, green tea and oolong tea. Of all the tea manufactured worldwide, 76 – 78% is black tea, 20 – 22% is green tea and <2% is oolong tea (Cabrera et al., 2003). Green tea is primarily produced in Asia while oolong tea is only produced in China and Taiwan. These three types of tea differ in the way they are manufactured and consequently, in the type of polyphenols present in the tea.

The two most well known types of herbal tea in South Africa are Rooibos tea and Honeybush tea. Rooibos tea is derived from the plant *Aspalathus linearis* (Burm. f.) Dahlgren of the family *Fabaceae*. Rooibos is a shrubby legume that is indigenous to the mountains of South Africa's Cedarberg region and this genus contains more than 200 species native to this country. It is a polymorphic species of which various wild forms exist, each with its own distinct morphology and geographical distribution. These types also have distinct profiles in their polyphenolic constituents.

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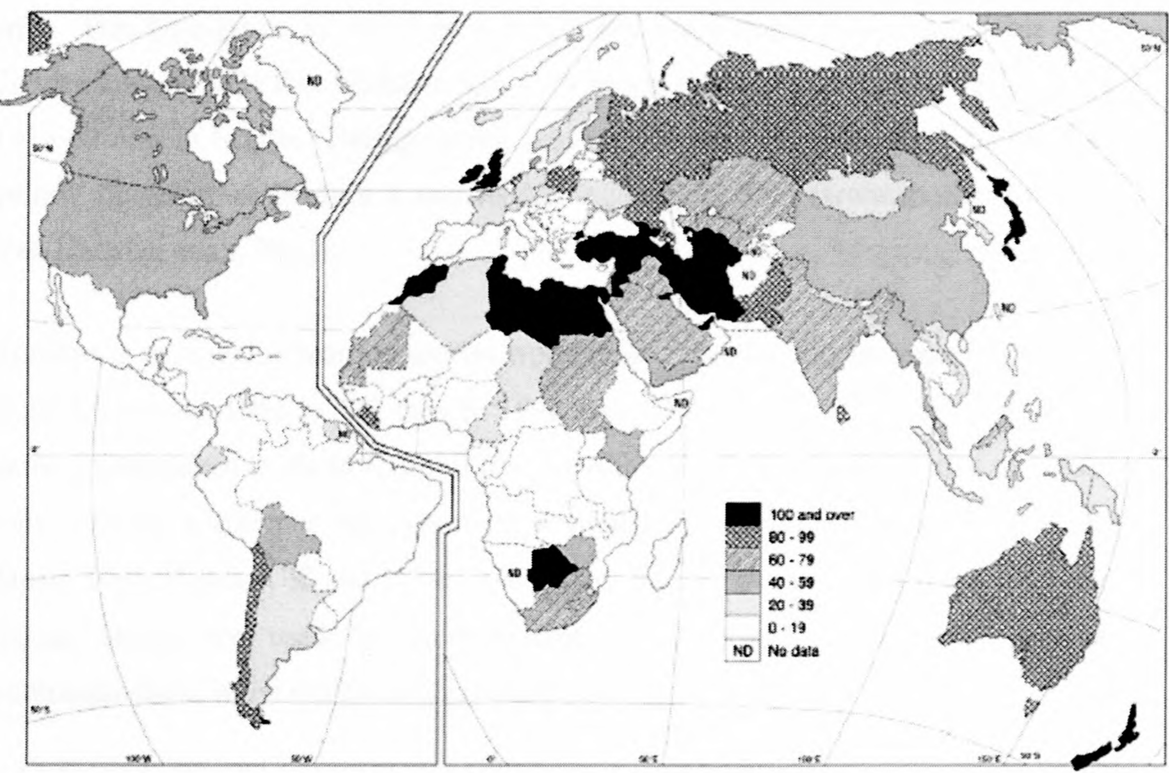

Figure 2.1 Tea consumption, liters per capita per year (Grigg, 2002).

Table 2.1 Tea production in metric tons for 2003 (http://faostat.fao.org).

Country	Tea production (Mt)
Argentina	63 500
Bangladesh	60 000
China	800 345
India	885 000
Indonesia	158 843
Islamic Republic of Iran	51 500
Japan	92 000
Kenya	290 000
Sri Lanka	303 230
Turkey	131 000
Other countries (combined)	371 649
World	3 207 067

There are several types of wild rooibos that are used to make tea. They are sometimes referred to as red, black, grey, yellow and Wupperthal types. The type generally used commercially for the production of tea is the red type, also known as the Rocklands

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type. This type grows up to 1,5 m in height and has a single basal stem. This stem divides into multiple thin branches that carry bright green, needle-like leaves of about 10 – 40 mm in length. During spring into early summer, the plant produces small yellow flowers that produce a one-seeded leguminous fruit (www.rooibosltd.co.za; Van Heerden et al., 2003).

Rooibos is exported to many countries worldwide. The amounts increased drastically from 8.6 tons in 1966 to 432 tons in 1990 and 1800 tons in 1999 when the foreign earnings amounted to about R20 million. In 2003, Rooibos sales amounted to 10 600 tons of which 6 400 tons was exported. Germany was the largest buyer, followed by Japan, Netherlands, UK and USA. Currently, the sales exceed the production and rescue stores are used to accommodate the increase (E. Joubert, personal communication; www.wesgro.org.za; www.ppecb.com).

Honeybush tea is made from the leaves of the *Cyclopia* Vent. (tribe Podalyrieae) plant of which approximately 24 species exist. This woody leguminous shrub is endemic to the coastal areas of the Western and Eastern Cape Provinces. The plant can grow up to a meter in height with green and golden yellow stems. The size and shape of the leaves differ between species but are mostly thin, needle-like to elongated and broadish. Honeybush tea was initially made from *C. genistoides*, but the main production is now from the two species *C. intermedia* and *C. subternata*. In 2003, approximately 230 tons of Honeybush tea was sold, mainly *C. intermedia* with smaller amounts contributed by *C. subternata*, *C. genistoides* and *C. sessiliflora*. About 80% was exported with Germany being the biggest buyer (De Nysschen et al., 1996; www.ppecb.com).

Tea is available for drinking either by brewing loose leaves or tea bags, or in a ready to drink form. However, these are not the only applications for tea: green tea extracts can be used to improve cereals, cakes and biscuits, traditional health food products and dietary supplements. These extracts can also be used to improve the health aspects of dairy products, instant noodles, confectionery, ice cream and fried snacks. Many antioxidant-rich health drinks containing green and black tea extracts are now available. Tea contains catechins that have an antibacterial and deodorising effect, slows tooth decay and improves breath freshness. These compounds naturally adds

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value to toothpastes, mouthwashes, chewing gums and breath fresheners. Tea extracts are also added to shampoos, moisturising creams, perfumes and sunscreens since they are reputed to have a soothing effect on the skin and acting as antioxidants to protect the skin from free radicals (Wang et al., 2000a). Tea is truly a global product and any means of improving the quality and production thereof will be of great importance in increasing the value and meeting the increasing demands of the consumer.

2.2 Tea manufacturing

2.2.1 *Camellia sinensis*

The chemical composition and quality of tea can be influenced even before the manufacturing process has started. Tea composition varies with climate, season, horticultural practices, variety and the age of the leaf. The age of the leaf greatly influences catechin concentration with the leaf bud and the first leaf being richest in epigallocatechin gallate. Varietal differences also influences the catechin levels: the tea leaf used for green tea usually has somewhat lower catechin levels than those used for black tea (Graham, 1992). The levels of two enzymes in black tea (polyphenol oxidase and phenylalanine ammonia lyase) have been shown to be influenced by seasonal variations and shoot maturity. The activities declined with the mature shoots while the tender shoots showed higher enzyme activity. The seasons also had an effect with the activity decreasing during dry seasons (January – March) (Ravichandran and Parthiban, 1998a). Ruan et al. (1999) showed that the application of fertiliser can also greatly influence tea composition and quality. The application of K and Mg fertiliser increased the levels of free amino acids in black, green and oolong tea and markedly increased the flavour compounds in brewed oolong tea. The theaflavin and thearubigin content in black tea increased with the application of K.

The harvesting methods and intervals of harvesting also influence tea quality. There are two methods of harvesting. The tea is either plucked by hand or harvested by mechanical harvesters. Hand plucked teas showed higher levels of theaflavins and caffeine, both important in black tea quality. Mechanical plucked teas showed lower levels of these compounds, which may be due to mechanical injury and non-selective

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plucking by the mechanical harvesters. Plucking intervals also influenced the theaflavin and caffeine levels with shorter plucking intervals being superior (Owuor and Odhiambo, 1993; Ravichandran and Parthiban, 1998b).

The lipid content of tea has a negative effect on tea flavour. The lipid content in the plant is high during stress conditions like drought and monsoon. The flavour of the tea improved outside these seasonal conditions due to low lipid content. It was also shown that shoot maturity and longer plucking intervals increased lipid content, whereas increasing the pruning intervals decreased the lipid content (Ravichandran and Parthiban, 2000).

2.2.1.1 Black tea

In black tea manufacturing the tea is allowed to dry or wither in open-air sheds, controlled lofts, tunnels or troughs after being plucked from the plant. The tea leaf is allowed to wither for 18 hours in which time 45 kg of fresh tea leaf may lose 11 to 22 kg of moisture. After the tea leaf has lost about 45 to 70% of its moisture, it goes on to the rolling phase where a rolling process damages the cell membranes to release the enzymes in the leaves for the fermentation reaction (Pintauro, 1977).

There are two diverging rolling methods, i.e. the orthodox method where the leaves are twisted in a rolling machine that damages the cellular membranes without unduly affecting the integrity of the plant material. The other method is called C.T.C. (curling, tearing, cutting) where machines cause considerable more damage to the material (Mahanta and Hazarika, 1985). During this process, the leaf heats up mainly due to friction, but also because fermentation starts immediately after the leaf is damaged and the enzymes released. The term "fermentation" is historical, since the process is actually one of oxidation rather than one of anaerobic or bacterial fermentation.

After rolling, the leaves are spread on trays or cement floors. Heat is generated during fermentation, therefore the thickness of the layers are important since thicker layers retain more heat. This increased heat may lead to undesirable flavours. The humidity should be controlled and the temperature not allowed to rise above 27°C

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inside the fermenting room. The tea is allowed to ferment for 3 hours and the reaction is stopped by firing or treating the tea through a dryer with the hot-air inlet at 82°C to 93°C. The moisture content of the tea should be about 3% after firing and the enzymes responsible for oxidation are destroyed through this process. The teas are sifted to obtain different commercial grades and dust and fiber are removed from the tea through winnowing (air-stream) (Pintauro, 1977).

2.2.1.2 Green tea

Whereas the catechins in black tea manufacturing are oxidised to quinones during the fermentation process, this reaction is inhibited in green tea manufacturing. The process for the production of green tea is similar to that of black tea, but in the green tea process, the leaves are either steamed or pan-fried after plucking to inactivate the enzymes and to exclude the fermentation (oxidation) step. This is then followed by rolling and high temperature air drying. In Japan, enzyme inactivation is mostly carried out by steaming in large rotating cylinders for 20 – 50 sec after which a series of twisting and drying steps are done to improve appearance and reduce moisture gradually to about 3%. Tea produced in this way is called sencha (Joy, 1986). Most Chinese green tea is produced by rapid pan firing or roasting followed by twisting and drying. In India, a Chinese-style green tea is manufactured by using long, rotating heated cylinders to inactivate the enzymes. This step may last from 7 – 10 min (Forster, 1990).

2.2.1.3 Oolong tea

Oolong teas are referred to as being partially fermented since a short period of oxidation takes place. Oolong tea manufacturing is carried out in several different ways and results in variation in the degree of catechin oxidation in the end product. The general steps for oolong tea manufacture are tea leaf plucking, solar withering, indoor withering, parching, rolling and drying. Solar withering involves exposing the freshly picked leaves to sunlight until 10 – 20% of the weight is lost. The tea leaves are then moved indoors for further withering while being turned over at regular intervals. The turn-over process causes friction between leaves which disrupts the cellular organization at the edge of the leaves. This allows a limited degree of

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fermentation and this process is unique to oolong tea manufacture. Compared to black tea, pouching tea is about a third fermented and oolong tea about half fermented. Therefore, oolong tea composition would be expected to be intermediate between green and black teas (Wang et al., 2001a; Graham, 1992).

2.2.2 South African Herbal teas

2.2.2.1 Rooibos tea

Rooibos tea is harvested during the South African summer and early autumn (November to March). The plants are cut to about 30 cm from the ground and then cut into 3 to 4 mm lengths, moistened, bruised and placed in heaps outside to ferment in the open air. Fermentation time is about 8 hours, depending on the ambient temperature and degree of bruising. The tea is then spread out on large concrete areas to dry in the sun to a moisture content of 8 to 10%. The tea used for commercial purposes is screened to remove dust and coarse material and to select tea material that is 0.4 to 1.68 mm in size. Before packing, the tea is steam pasteurised to remove bacteria and other impurities (Joubert, 1988; www.rooibosltd.co.za).

To improve the quality of rooibos tea, the fermentation process would have to be done under controlled conditions as opposed to the current open-air method. Different fermentation and drying temperatures were therefore tested, as well as controlled drying as opposed to sun-drying. The quality of rooibos tea improved with increased fermentation temperature and decreased with increased drying temperature. There was no significant difference between the two drying methods, suggesting that sunlight is not necessary for processing of rooibos tea as heat energy without ultraviolet light supports the same process (Joubert and De Villiers, 1997; Marais et al., 2000).

2.2.2.2 Honeybush tea

Harvesting occurs during the flowering period which may be either May or September, depending on the species. Two methods for the fermentation of honeybush tea exists: the harvested material is cut and placed into fermentation heaps to ferment, or is immersed in water and then fermented in a preheated "baking oven".

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There is no withering stage in the manufacture of honeybush since both the leaves and stems are used, which leads to a relatively low moisture content (<50%). The tea is then spread out in the sun and allowed to dry for 24 – 48 hours (Du Toit and Joubert, 1998).

Du Toit and Joubert (1998) found that certain pretreatments could improve the quality of honeybush tea and increase the fermentation rate. They found that treatment with water (hot and cold) resulted in a faster fermentation rate and a better quality tea. Hot water treatment inactivated the polyphenol oxidase and peroxidase, but did not have a detrimental effect on fermentation. The inactivation of the enzymes, combined with high temperature fermentation (>60°C) would suggest a chemical oxidation rather than an enzymatic oxidation process.

2.3 Antioxidants and Tea

Flavonoids are a broad class of low molecular weight, plant phenolics characterized by the flavan nucleus (Figure 2.2). At present, more than 4 000 flavonoids have been identified and are commonly found in leaves, seeds, barks and flowers of plants. The function of these compounds in plants include protection against ultraviolet radiation, pathogens and herbivores.

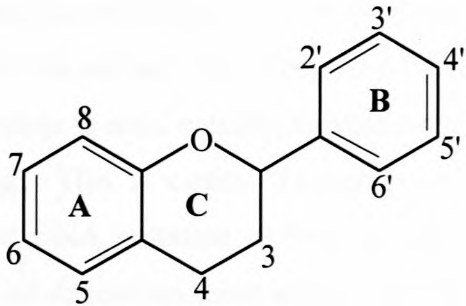

Figure 2.2 The flavan nucleus (Wang et al., 2000a).

Vegetables, wines, tea and berries form a large part of the human diet and are an important source of flavonoids. The estimated daily intake of flavonoids through plant foods is 1g, with black tea contributing 48% of the flavonoid intake. There is, however, no recommended daily allowance for antioxidants. The human antioxidant

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intake depends on factors such as fat intake, life-style, age and smoking. About 31% (wt/wt) of black tea beverage are flavonoids of which 9% is catechins, 4% theaflavins, 3% flavonols and 15% undefined catechin condensation products. In green tea beverage, 33% (wt/wt) are flavonoids that consist of 3% flavonols and 30% catechins. The characteristic colours of certain foods are obtained from anthocyanin copigments, which are also responsible for attracting pollinating insects to flowers (Heim et al., 2002; Joubert and Ferreira, 1996; Kühnau, 1976; Wiseman et al., 1997).

Flavonoids can be subdivided into six classes: flavones, flavanones, isoflavones, flavonols, flavanols and anthocyanins (Table 2.2). These classes are based on the structure and conformation of the heterocyclic oxygen ring (C – ring) of the basic molecule (Wang et al., 2000a). Flavonoids are benzo-γ-pyrone derivatives consisting of phenolic and pyrane rings and are classified according to substitutions. The differences in these dietary flavonoids are the arrangements of hydroxyl, methoxy and glycosidic side groups which can be added, methylated, sulfated or glucuronidated during metabolism. There are also differences in the conjugation between the A- and B-rings. In food, flavonoids exist primarily as 3-*O*-glycosides and polymers. The two main classes of flavonoids in tea are the flavanols and the flavonols (Hammerstone et al., 2000; Wang et al., 2000a).

Most of the beneficial health effects of flavonoids are due to their antioxidant and chelating abilities. Biological oxidation is a normal process in the human body and a source of energy for survival and activity. The powerful reactivity of oxygen species can cause functional damage to man, causing mutagenesis, carcinogenesis, circulatory disturbances and ageing. This is caused through lipid peroxidation (Figure 2.3), protein denaturation and DNA mutation as free radicals attack these substances in living cells. Ironically, we depend on these oxygen species for metabolism of foreign matter, prostaglandin biosynthesis and antibacterial cell activities. However, free radicals can also be generated in cells after exposure to chemical agents in the environment such as NO_2 and tobacco smoke (Joubert and Ferreira, 1996; Namiki, 1990; Thomas, 1995) .

There are enzymatic and non-enzymatic defenses to protect the body against oxidative damage, but they are not 100% effective. Enzymatic defenses include enzymes such

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as superoxide dismutase (SOD), catalase and glutathione peroxidase that destroy the active oxygen species. The non-enzymatic system is food-derived antioxidants such as tocopherol (vitamin E), ascorbic acid (vitamin C) and various flavonoids (Halliwell et al., 1995; Namiki, 1990; Thomas, 1995).

Table 2.2 The six classes of flavonoids (adapted from Heim et al., 2002).

Class	General Structure	Flavonoid	Substitution Pattern	Dietary Sources
Flavanol	3', 4', 7, A, B, C, 5, OH	(+)-catechin	3,5,7,3',4'-OH	Tea (camellia sinensis)
		(-)-epicatechin	3,5,7,3',4'-OH	Tea
		epigallocatechin gallate	3,5,7,3',4',5'-OH,3-gallate	Tea
Flavone		chrysin	5,7-OH	Fruit skins
		apigenin	5,7,4'-OH	Parsley, celery
		rutin	5,7,3',4'-OH,3-rutinose	Red wine, buckwheat
				Citrus, tomato skin
		luteolin	5,7,3',4'-OH	Red pepper
		luteolin glucosides	5,7,3'-OH,4'-glucose	
			5,4'-OH,4',7-glucose	
Flavonol	OH	kaempferol	3,5,7,4'-OH	Leek, broccoli, endives
				Grapefruit, black tea
		quercetin	3,5,7,3',4'-OH	Onion, lettuce, broccoli
				Tomato, red wine, tea
				Berries, olive oil, appleskin
		myricetin	3,5,7,3',4',5'-OH	Cranberry, grapes, red wine
		tamarixetin	3,5,7,3'-OH,4'-OMe	
Flavanone (dihydroflavon)		naringin	5,4'-OH,7-rhamnoglucose	Citrus, grape fruit
		naringenin	5,7,4'-OH	Citrus fruits
		taxifolin	3,5,7,3',4'-OH	Citrus fruits
		eriodictyol	5,7,3',4'-OH	Lemons
		hesperidin	3,5,3'-OH,4'-OMe,7-rutinose	Oranges
Isoflavone		genistin	5,4'-OH,7-glucose	Soybean
		genistein	5,7,4'-OH	Soybean
		daidzin	4'-OH,7-glucose	Soybean
		daidzein	7,4'-OH	Soybean
Anthocyanidin		apigenidin	5,7,4'-OH	Coloured fruits
		cyanidin	3,5,7,4'-OH,3,5-OMe	Cherry, raspberry,strawberry

Antioxidants can be classified as free radical terminators, chelators of metal ions capable of catalysing lipid oxidation, or as oxygen scavengers that react with oxygen in a closed system. Primary antioxidants react with oxygen high-energy lipid radicals to convert them to more thermodynamically stable products. Secondary antioxidants,

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also known as preventive antioxidants, function by retarding the rate of chain initiation by breaking down hydroperoxides. Flavonoids and phenolic antioxidants are considered as primary antioxidants, but may also be considered as secondary antioxidants due to their metal chelating ability (Joubert and Ferreira, 1996; Shahidi et al., 1992; Torel et al., 1986).

Antioxidants in tea has very strong free radical scavenging activities that are even superior to that of vitamins E and C in protecting cells against free radical damage. Numerous studies have shown effects on coronary heart disease and inhibition of carcinogenesis in experimental animals by tea or tea catechins. These inhibitions were apparent at all three levels of cancer progression, namely initiation, promotion and transformation. It has been shown that Japanese male smokers are at lower risk of lung cancer than their US counterparts even though they smoke more cigarettes per day. This may be due to the diet in Japan which is higher in flavonoids of green tea and vegetables and lower in fat and cholesterol (Hertog et al., 1993; Higdon and Frei, 2003; Wang et al., 2000a; Weisburger and Chung, 2002).

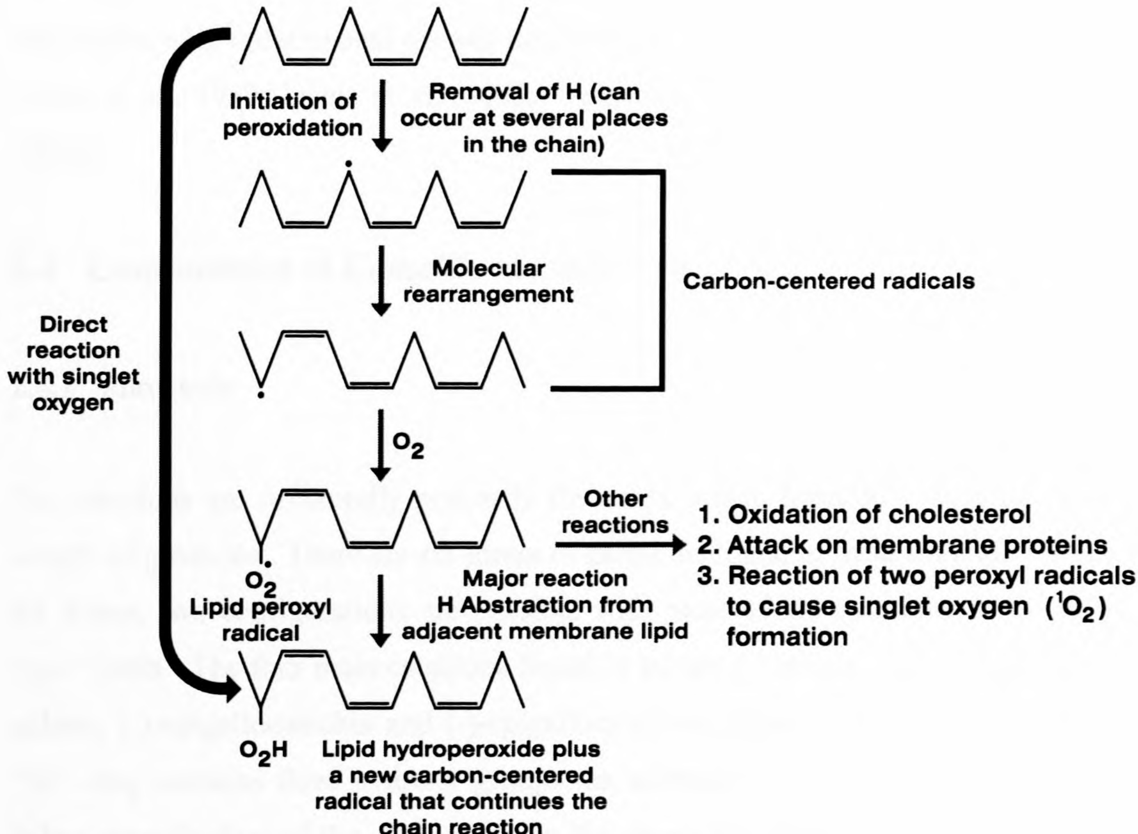

Figure 2.3 Diagram of lipid peroxidation (Halliwell et al., 1995).

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Tea also has numerous antimicrobial activities. Green tea polyphenols can inhibit the biological activities of the cariogenic streptococci, *Streptococcus mutans* and *Streptococcus sobrinus* and therefore prevent tooth decay. Tea extracts has also been used for therapy in cholera patients and for the prevention of influenza virus infections. It has also been found that the AIDS virus can be inhibited by up to 50% by using epicatechin gallate and epigallocatechin gallate at concentrations of 0.01 – 0.02 µg/ml. Furthermore, it was demonstrated that a component of tea extracts reverse the methicillin resistance in methicillin-resistant *Staphylococcus aureus*. Gastric cancer, caused by a combination of salted foods and *Helicobacter pylori*, was found to be lower in tea drinkers. Tea polyphenols are bacteriostatic and bacteriocidal and therefore lowers the titers of *H. pylori* and the risk of gastric cancer. Tea and tea polyphenols lead to a reduction in *Enterobacteriaceae* in the intestine that produce ammonia, skatole and other amines that are responsible for the unpleasant odour of stools. Tea increases the level of Lactobacilli and Bifidobacteria that have beneficial odour-free metabolites. In another study, it was shown that *Bacillus subtilus*, *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas fluorescens*, *Salmonella* sp. and *S. aureus* were all inhibited to various degrees by tea extracts. The level of inhibition was higher with unfermented tea and decreased as the level of fermentation increased (Chou et al., 1999; Gupta et al., 2002; Weisburger and Chung, 2002; Wang et al., 2000a).

2.4 Composition of *Camellia sinensis* Tea

2.4.1 Flavanols

Tea catechins are structurally primarily flavanols, which form 20 – 30% of the dry weight of green tea. There are six forms of catechins found in fresh tea leaf. Of these six forms, two configurations are possible with most of the catechins found in the "epi-" form. The four main catechins found in tea are (-)-epicatechin, (-)-epicatechin gallate, (-)-epigallocatechin and (-)-epigallocatechin gallate (Figure 2.4). When the "B"– ring contains three hydroxy groups the substance is known as gallocatechins. When esterification of the -OH group on the pyran ring with gallic acid occurs, the substance is called catechin gallate. Catechins are the predominant group of

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substances in green tea. They are colourless, water-soluble compounds, which imparts bitterness and astringency to green tea (Graham, 1992; Wang et al., 2000a).

(-)-Epicatechin: R1=R2=H

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

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

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

Figure 2.4 The major catechins in fresh tea leaves (Wang et al., 2000a).

2.4.2 Flavonols

The other main component in tea is the flavonols with the main flavonols being quercetin, kaempferol and myricetin (Figure 2.5). They make up 2 – 3% of the water-soluble extracts in tea (Balentine et al., 1997). In tea, flavonols are found in their glycosidic form rather than in their non-glycosylated forms (aglycones). Of the main flavonols myricetin, quercetin and kaempferol, at least 14 glycosides have been found in fresh tea shoots, green and black tea. The sugar moieties consist of glucose, rhamnose, galactose, arabinose and fructose with mono-, di- and tri-glycosides also present. Due to their poor solubility in water, aglycones are not found in considerable amounts in tea. Polyphenol oxidase does not affect glycosides to the same extent as it does catechins and therefore their content in black tea is nearly the same as in green leaves (Finger and Engelhardt, 1991; Wang et al., 2000a).

Alcoholic tea aroma compounds are mainly present as glycosides in fresh tea leaves. These aroma compounds are released by endogenous glycosidases during the manufacturing process of particularly oolong tea and black tea. These glycosidases

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Myricetin: R1=R2=R3=OH

Quercetin: R1=R2=OH, R3=H

Kaempferol: R1=OH, R2=R3=H

Figure 2.5 Structures of flavonols in tea (Wang et al., 2000a).

can act on non-volatile mono- or oligoglycoside constituents to produce volatile aglycones that add to the characteristic flavour or aroma of black tea (Halder and Bhaduri, 1997). Various glycosides have been isolated and identified from fresh tea leaf as aroma precursors. These glycosides are β-D-glucopyranoside (Glc), 6-*O*-β-D-xylopyranosyl-β-D-glucopyranoside (primeveroside, Prim), 6-*O*-α-L-arabinopyranosyl-β-D-glucopyranoside (vicianoside, Vic) and 6-*O*-β-D-apiofuranosyl-β-D-glucopyranoside (acuminoside, Acu) with monoterpene alcohol aglycons of geraniol, linalool and linalool oxides (LO), with aromatic alcohol aglycons of benzyl alcohol, 2-phenyl-ethanol, with phenol aglycone of methyl salicylate, and with the aliphatic alcohol aglycone of (*Z*)-3-hexenol. Recently, the glucosides of LO I, LO II, LO III and LO IV, 2-phenylethanol, geraniol and methyl salicylate were also detected in tea leaves (Guo et al., 1993; Wang et al., 2000b).

Fresh leaves contain about three times more disaccharides than glucosides due to the high primeveroside content. Both these glycoside levels remained effectively unchanged during withering, but during the rolling stage, the primeveroside decreased greatly while ~70% of the glucosides still remain. This led to the conclusion that primeveroside is the main black tea aroma precursor (Mizutani et al., 2002; Wang et al., 2001b). In the manufacture of oolong tea, the glycoside content increased during

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the solar withering stage, reaching its highest level at the end of oolong tea production. In contrast to black tea there was no decrease in any of the glycosides (Wang et al., 2001a).

2.4.3 Theaflavins and Thearubigins

During black tea manufacture, the enzymatic oxidation of catechins results in the formation of quinones that can then react to form other complex substances. The quinone derived from a catechin or its gallate can react with a quinone derived from a gallocatechin or its gallate to form a compound known as theaflavins. Theaflavins are only present at levels of 1.5 – 2.5% in the dry leaf. They are orange-red astringent compounds that are responsible for the briskness, brightness and quality of tea liquor. There are four main theaflavins, i.e. theaflavin, theaflavin 3-gallate, theaflavin 3'-gallate and theaflavin 3,3'-digallate (Figure 2.6) and they account for 2 – 6% of the dry weight in brewed black tea (Angayarkanni et al., 2002; Balentine et al., 1997; Graham, 1992; Murugesan et al., 2002; Wang et al., 2000a; Wright et al., 2002).

OH
OR2
OH
HO
O
OH
HO
O
O
OR1
OH
OH

Theaflavin: R1=R2=H

Theaflavin 3-gallate: R1=gallate, R2=H

Theaflavin 3'-gallate: R1=H, R2=gallate

Theaflavin 3,3'-digallate: R1=R2=gallate

Figure 2.6 Structures of theaflavins (Wang et al., 2000a).

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During black tea manufacture the catechins are reduced by ~85% with the residual unoxidised catechins of black tea amounting to about 5 – 10% of the original levels. Combined with all the other mentioned constituents of catechins, this still accounts for less than 20% of the fresh leaf catechins. The rest of the catechins form a heterogenous, undefined, water-soluble group of phenolic pigments with relative molecular masses of between 700 – 40 000 Da. These compounds are called thearubigins and they are responsible for colour, body and taste (Figure 2.7). Therefore, optimal quality tea requires the proper balance between theaflavins and thearubigins. One subgroup of these thearubigins is classified as proanthocyandin polymers. They are also found in fresh tea leaves and may be condensation products of catechins linked by C-C bonds between the "A"- ring and pyran ring. After acid hydrolysis, this polymer forms cyanidin and delphinidin, which are the flavone equivalents of epicatechin and epigallocatechin, respectively (Angayarkanni et al., 2002; Balentine et al., 1997; Graham, 1992; Wang et al., 2000a).

Catechins → Theaflavins → Catechin Condensation Products "Thearubigens"

Figure 2.7 The formation of theaflavins and thearubigins from catechins (Wiseman et al., 1997).

Black teas are classified as either flavoury or plain. Special aromas and flavours are important for flavoury black teas while plain teas are sought after for their strength, briskness, colour and quality. However, the factors determining the quality and therefore the valuation of black teas are subjective. Many studies aimed to find measurable chemical groups to determine the valuations of black teas. Some results have shown that theaflavins and thearubigins are related to colour and strength and theaflavins are also responsible for briskness and quality (Owuor et al., 1986). Certain manufacturing parameters influence theaflavins and thearubigins and thus black tea quality. Cloughley and Ellis (1980) found that reducing the fermentation pH

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from physiological pH (pH 5.5) to the range of 4.5 – 4.8, resulted in an increase in theaflavin content and a reduction in thearubigin content. This led to an increase in the evaluation of the black tea, probably due to the activities of two important fermentation enzymes present in the tea leaf, namely polyphenol oxidase (PPO) and peroxidase (POD).

PPO is responsible for the oxidation of catechins to reactive *o*-quinone intermediates that are spontaneously converted into the black tea pigments, theaflavins and thearubigins. PPO produces H_2O_2 while oxidising catechins. At the physiological pH of 5.5 in the tea leaf (PPO's optimum pH) a greater amount of H_2O_2 is produced than at the lower pH. POD contributes to the rapid decomposition of theaflavins and also oxidises theaflavins in the presence of H_2O_2, leading to the formation of thearubigins. Therefore, at a higher pH, PPO is more active and produces more H_2O_2, which POD utilises to oxidise theaflavins to thearubigins. At the lower pH, PPO is less active, producing less H_2O_2 for POD to use and that leads to a higher theaflavin content and lower thearubigin levels (Finger, 1994; Subramanian et al., 1999). Cloughley (1980a) also showed that the activities of PPO and POD declined at longer fermentation times with PPO showing the greater decrease. High temperatures also affected PPO with little effect on POD. A change in the ratio of these enzymes may alter the ratio of their end products and consequently have an effect on tea quality.

Temperature and fermentation time also has an effect on black tea quality. Cloughley (1980b) found that fermenting tea at 15°C led to higher theaflavin levels than at 35°C, but at 35°C more thearubigins were formed and the tea had a more intense colour and lower theaflavin levels. Furthermore, theaflavin levels increase to a maximum and then decline as the fermentation time increased. As the fermentation time increased to 3 hrs the thearubigin content and colour also increased. Although a higher quality tea is produced at lower temperatures, a longer fermentation time will be required. However, the cost for keeping the fermentation temperature at low levels for extended periods of time will be too great for the improvement in quality (Owuor and Obanda, 2001).

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2.4.4 Theasinensins

Bisflavonols are formed when gallocatechin quinones, in either their free or gallated forms, react to form these substances. Although these compounds are found in fresh green leaves, they are synthesised additionally during the manufacture of black tea. The bisflavonols, also known as theasinensins, are colourless compounds which are not found in large amounts in black tea (Figure 2.8). They may undergo further changes as fermentation proceeds, but their influence on black tea quality is minimal (Balentine et al., 1997; Graham, 1992)

OH OGallate OH OH HO O OH HO O OH OH OGallate OH OH

OH OGallate OH OH HO O OH HO O OH OGallate OH OH

Figure 2.8 The chemical structure of Theasinensin A and Theasinensin F (Balentine et al., 1997).

2.4.5 Theaflavic acid

Although gallic acid is not a substrate for polyphenol oxidase, its quinone can be generated by reaction with the quinones derived from some of the other catechins. This reaction leads to the formation of a benztropolone molecule known as theaflavic acid, which is similar in structure to theaflavins. Another compound formed with gallic acid is theaflagallins, which are formed by gallocatechins and gallic acid (Figure 2.9). The mechanism for the formation of theaflagallins is not clear but they may be formed by the condensation of two quinones derived from trihydroxy molecules (Balentine et al., 1997; Berkowitz et al., 1971; Graham, 1992).

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Figure 2.9 The chemical structures of epitheaflavic acid and epitheaflagallin (Balentine et al., 1997).

2.4.6 Amino acids

Tea also consists of a nitrogenous fraction of ~17% wt/wt. This fraction consists of ~6% protein and ~1% amino and nucleic acids. Theanine (γ-*n*-ethyl glutamine), a N-methylated derivative of glutamine, is one of the 19 amino acids in green and black tea and is unique to tea. It constitutes almost half of the total amino acid content in tea (Balentine et al., 1997). Amino acids have been implicated in contributing to the aroma in tea leaf and the destinctive flavour of Japanese green tea is in part due to amino acids (Balentine et al., 1997; Sanderson and Graham, 1973). During fermentation, the α-amino acids in tea leaf will degrade to corresponding aldehydes in the presence of oxidising tea flavanols. However, not all α-amino acids will give rise to volatile aldehydes (Sanderson and Graham, 1973).

2.4.7 Methylxanthines

Tea contains 2.5 – 4.0% caffeine. Other methylxanthines have also been reported but at considerably smaller quantities. Theobromine and theophylline have been reported at 0.1 and 0.02%, respectively, the presence of theophylline has recently been questioned (Balentine et al., 1997). Caffeine forms a physiochemical complex with theaflavin gallates and thearubigins, which contributes to the characteristic taste of and briskness of tea infusions. When tea is allowed to cool, this complex forms an insoluble precipitate known as "cream". The creaming phenomenon is largely dependent on the caffeine content and the presence of high molecular weight

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thearubigens and theaflavins. The cream also contains small amounts of other flavonoids, non-caffeine compounds, chlorophyll and inorganic material. Creaming is used by professional tea tasters as an indication of tea quality and therefore price. Creaming down can also be a problem in production of cold water-soluble iced tea, because it makes the iced tea become a turbid liquor that is detrimental to the product (Boadi and Neufeld, 2001; Chandra and Pandey, 1998; Graham, 1992; Hazarika et al., 1984; Liang et al., 2003; Liang and Xu, 2001).

2.4.8 Carotenoids

Carotenoids are present in tea leaves in amounts ranging from 36 – 73 mg/100 g dry weight. Fourteen of these carotenoids have been identified in tea with β-carotene, lutein, violaxanthine, neoxanthine and zeaxanthin being the major components. Carotenoids are yellow components in fresh leaves and their degradation during manufacturing leads to the formation of terpenoid flavour compounds in black tea. The degradation of these compounds were found to be greater in CTC (crush, tear, curl) teas than in orthodox teas and also greater in withered than in unwithered teas. The degradation of carotenoids also differed during the different manufacturing stages. The degradation was much less during withering and showed a minor increase during fermentation. The greatest loss was during the firing stage. The mode of carotenoid breakdown is by oxidative enzymatic reaction during withering and fermentation, and a pyrolytic reaction during firing. Despite the greater carotenoid degradation in CTC teas, the orthodox teas with lesser degradation turned out to be more flavoursome (Hazarika and Mahanta, 1983; Ravichandran, 2002; Sanderson and Graham, 1973).

2.4.9 Lipids

The lipid content of tea leaf is only 3 – 7%, but plays an important role in the biogenesis of flavour in black teas (Mahanta et al., 1993). Almost 50% of the total lipids are glycolipids with linolenic acid being the main constituent. Phospholipids were present at 15% and consisted mainly of oleic, linoleic and palmitic acids. Neutral lipids were present at 35% and had a high content of lauric, myristic, palmitic,

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stearic, oleic and linoleic acids (Bhuyan et al., 1991; Ravichandran and Parthiban, 2000). The volatiles derived from these fatty acids contribute to the Group I volatile flavour compounds, which exhibits a greenish, grassy, undesirable odour. The Group I compounds are derived from lipid breakdown, primarily of unsaturated fatty acids. (Ravichandran and Parthiban, 1998d; Selvendran et al., 1978; Wright and Fishwick, 1979). Group II is responsible for the sweet aroma and are derived from glycosides of terpenoid related compounds. These consist of terpenoids, aromatics and non-terpenoids and they are derived from the enzyme-initiated oxidative breakdown of carotenoids and the hydrolysis of terpenoid glycosides. The ratio of the sum of group II VFC's (volatile flavour compounds) to the sum of group I VFC's constitutes the VFC index (Ravichandran and Parthiban, 1998c).

During the withering stage of black tea manufacture, lipids/fatty acids decreased significantly and even more during the firing process. The group II compounds increase during the firing process, which leads to an increase in the flavour index. During the rolling and fermentation stages, only slight changes occurred in lipid/fatty acid content. The orthodox teas show better flavour than the CTC teas and also better storage quality. The reason for this is the additional lipid degradation during the manufacture of orthodox teas, mainly during the rolling stage. The higher withering, slow rolling over an extended period and longer fermentation time also contributes to the lipid degradation (Ravichandran and Parthiban, 2000).

In some parts of southern India, off-flavours develop only after storage. These flavours are described as grassy, fishy or painty and is called "Pacha taint". This could be due to residual lypoxygenase activity found in manufactured tea. Lypoxygenase activity increase with the degree of withering and rolling, but decrease steadily during fermentation and drying process. However, the enzyme activity cannot be completely inactivated and this may be the reason for "Pacha taint" (Ravichandran and Parthiban, 1998d).

2.4.10 Chlorophyll

In black teas, chlorophyll is degraded into pheophytin and pheophorbide. Pheophytin is a darker pigment than pheophorbide. The quantitave distribution of these pigments

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in black tea does not only depend on the content of chlorophyll in the raw material, but also on the neighboring physicochemical environment and the type of black tea processing. The degradation of chlorophyll to pheophorbide is higher in orthodox teas than in CTC teas (Mahanta and Hazarika, 1985).

The blackness of orthodox teas is influenced by pheophytin, pheophorbide and remaining chlorophyll. Carotenoids, theaflavin and thearubigin contribute towards the brown appearance of the leaf. Thearubigins formed are the most important reason for the brownness of black tea due to their high concentration and intense colour. Therefore, the ratio of pheophytin to thearubigin may be the reason for the shade of colour in orthodox teas (Mahanta and Hazarika, 1985).

2.5 Composition of South African Herbal Teas

2.5.1 Honeybush tea

Honeybush tea (*C. intermedia*) contains primarily xanthones, flavanones, flavones, coumestans with no caffeine and virtually no tannins. De Nysschen et al. (1996) were one of the first to analyse dry honeybush tea leaves for phenolic compounds and found that the three major constituents were xanthone (mangiferin) and glycosides of the flavanones, hesperitin and isosakuranetin. They also found that the combination of these three compounds is a unique characteristic of the *Cyclopia* genera. Later studies identified the flavone, luteolin and other flavanones, hesperidin, naringenin and eriodictyol. However, processing of honeybush teas results in a significant decrease in mangiferin, isomangiferin and hesperidin (Marnewick et al., 2000; 2003; 2004).

The two major species of *Cyclopia* used for honeybush tea production were further analysed. The tea leaves and stems of *C. intermedia* yielded tyrosol, five glycosylated flavonols, two isoflavones, four flavanones and two flavones. *Cyclopia subternata* contained pinitol, shikimic acid, *p*-coumaric acid, 4-glucosyltyrosol, epigallocatechin gallate, the isoflavone orobol, the flavanones hesperidin, narirutin and eriocitrin, a glycosylated flavan, the flavones luteolin, 5-deoxyluteolin, and scolymoside, the

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xanthone mangiferin, and the flavonol C-6-glucosylkaempferol (Kamara et al., 2003; 2004).

As is the case with *C. sinensis*, the phenolic compounds of *Cyclopia* have various health properties. Hesperidin has Vitamin C-like activity as well as anti-inflamatory, antimicrobial and antiviral properties. Hesperidin produces an analgesic and exertes mild antipyresis. It also reduces aggregation of blood cells and abnormal capillary permeability and fragility. Luteolin has antispasmodic and antioxidant properties. It has also been shown to stimulate antihypertensive activity even in excess of the reference drug, papaverine. Mangiferin has potential as an antioxidant and an antiviral agent and has been reported to be a powerful scavenger of free radicals. It has been used for melancholia and could be a potential cure for diabetes mellitus (Kamara et al., 2004).

2.5.2 Rooibos tea

Rooibos tea is rich in volatile compounds, minerals and ascorbic acid, is caffeine-free and is claimed to have a low tannin content (as gallic acid). Habu et al. (1985) showed that a volatile oil extraction consisted of 99 components of which there were 26 ketones, 19 aldehydes, 16 alcohols, 12 esters, nine hydrocarbons, seven phenols, four acids, three ethers and three miscellaneous components. Analysis of the dry leaves yielded 218 compounds: 47 alcohols, 41 ketones, 39 aldehydes, 27 hydrocarbons, 24 esters, 13 ethers, seven phenols, six acids and 14 miscellaneous components.

The flavonoid fraction of rooibos tea contains the flavonol quercetin and aspalathin, which is unique to rooibos tea. Nothofagin, a β-hydroxydihydrochalcone, has only been found in rooibos tea and *Nothofagus fusca* (red beech). Aspalathin and nothofagin comprise about 0.55% and 0.19%, respectively, of the total soluble solids in rooibos tea. It has been thought that aspalathin may contribute to the naturally sweet taste of rooibos tea. Other components contributing to the flavonoids found in rooibos tea are the flavones, orientin, iso-orientin, vitexin, iso-vitexin, chrysoeriol, 5,7,4'-trihydroxy-3-methoxyflavone, luteolin and the flavonols, iso-quercitrin, rutin

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and their aglycone, quercetin (Figure 2.10). More flavonoids are derived from aspalathin during processing. These are the flavanones dihydro-2,3-orientin and dihydro-3,4-iso-orientin. Two more flavanones were derived from aspalathin under conditions that mimicked the fermentation process, namely (*S*)- and (*R*)-eriodictyol-6-*C*-*β*-D-glucopyranoside. Antioxidant activity has been displayed by quercetin, luteolin, rutin, isoquercitrin and iso-vitexin (Jaganyi and Wheeler, 2003; Joubert and Ferreira, 1996; Marais et al., 2000).

Catechin, procyanidin B3 and a profistinidin triflavonoid have also been reported, but they are found at extremely low quantities and therefore support the claim that Rooibos tea has a low tannin content. The phenolic acids found in rooibos tea are caffeic acid, ferulic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, vanillic acid, protocatechuic acid and syringic acid. Hydroxybenzoic acid is well known for its antimicrobial properties and may serve as a natural preservative in rooibos tea (Bramati et al., 2002; Rabe et al., 1994).

As was mentioned above, some of the phenolic compounds are derived from aspalathin during processing. The oxidation of aspalathin starts even before fermentation due to cell damage and exposure to oxygen when the plant material is cut into smaller pieces. The oxidation of aspalathin is both chemical and enzymatic in nature. All the above mentioned flavonoids were more abundant in the unfermented rooibos tea than the fermented type, with the levels of aspalathin almost 50 times higher in unfermented tea. The flavones iso-orientin, orientin, vitexin and iso-vitexin are degraded to a lower degree, while rutin is partly transformed to the aglycone quercetin (Bramati et al., 2003; Joubert, 1996).

Standley et al. (2001) reported that fermented rooibos tea showed a lower antimutagenic and antioxidant potential than the unfermented tea. This may be due to the reduction in polyphenolic content during processing. The most significant reduction of these properties were found during fermentation. The sun-drying, sieving and steam pasteurisation also reduced these properties, but to a lesser extent. Joubert (1990) showed that the extraction of different phenolic groups from rooibos tea leaves increased markedly with increasing temperature, probably due to the

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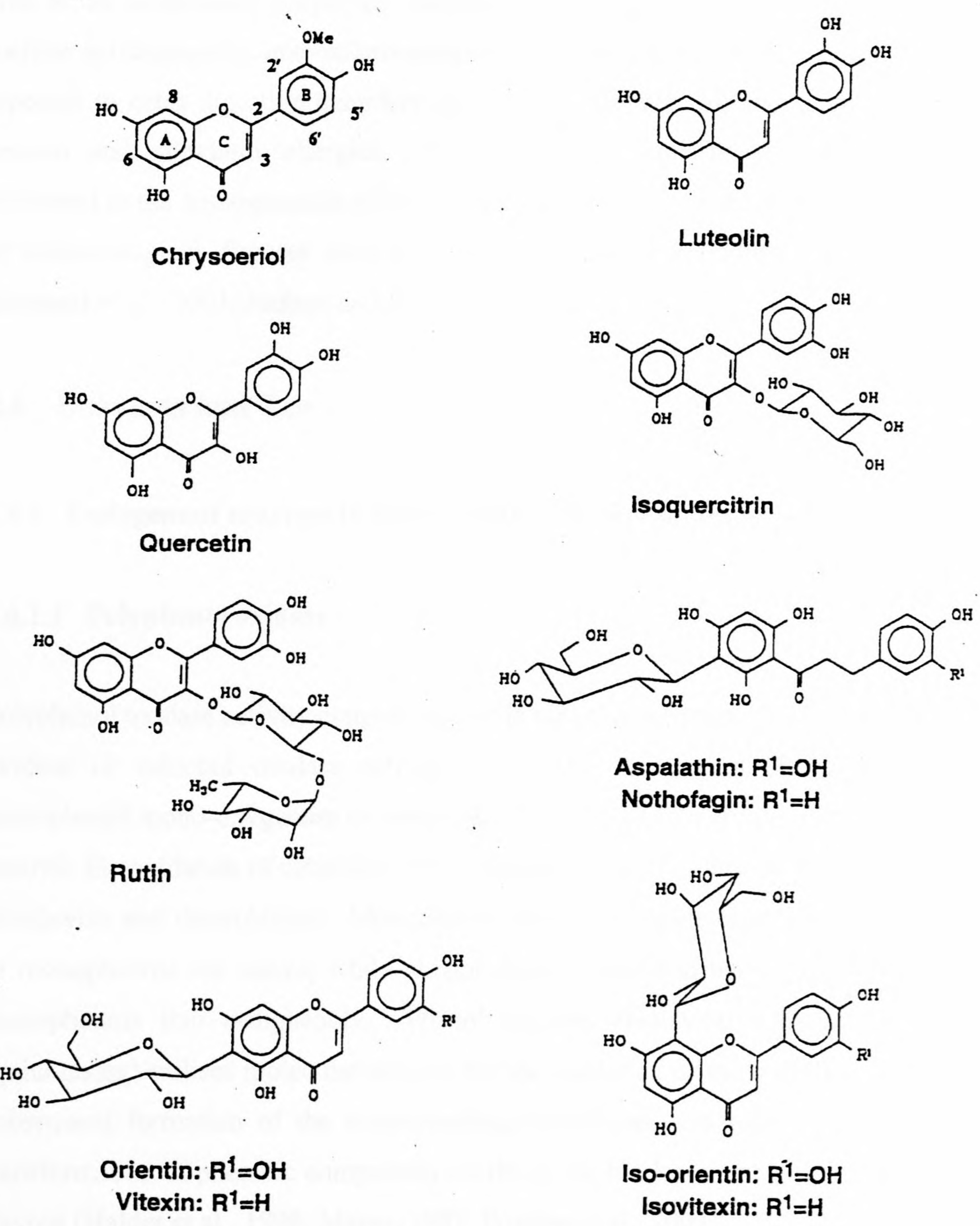

Figure 2.10 The flavonoids in Rooibos tea (Joubert and Ferreira, 1996).

extraction of flavonoids. Von Gadow et al. (1997) compared the antioxidant activity of unfermented and fermented rooibos tea with that of green, oolong and black tea. They found that the antioxidant activity decreased in the following order: green > unfermented rooibos > fermented rooibos > semifermented rooibos > black > oolong.

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Due to its antioxidant properties, rooibos tea has numerous health benefits. This include antimutagenic, anti-inflammatory and antiviral activity while it has also been reported to calm digestive disorders and various stomach problems, reduce nervous tension and alleviates allergies. The anti-allergic property of rooibos tea was attributed to the antispasmodic effect of quercetin. It is also used for topical treatment of dermatological diseases such as Behcet's disease and photosensitive dermatitis (Bramati et al., 2003; Joubert and Ferreira, 1996).

2.6 Enzymes and Tea

2.6.1 Endogenous enzymes in tea and their influence on tea

2.6.1.1 Polyphenol oxidase

Polyphenol oxidase activity consists of a wide range of activities that include diphenol oxidase or catechol oxidase activity (EC 1.10.3.1), laccase (EC 1.10.3.2) and monophenol mono-oxygenase or tyrosinase (EC 1.14.18.1) activities. These enzymes catalyse the oxidation of catechins into *o*-quinones, which in turn are polymerised into theaflavins and thearubigins. Monophenol mono-oxygenase catalyses hydroxylation of monophenols tea leaves, while phenol oxidase found in tea leaves, hydroxylises monophenols into *o*-diphenols. Phenol oxidase displaying dehydrating activity (quinonising) utilises molecular oxygen for the oxidation of the *o*-diphenols with the subsequent formation of the corresponding *o*-quinones and water. The oxidative transformation of phenolic compounds results in the black tea taste, liqour, aroma and flavour (Halder et al., 1998; Mayer, 1987; Pruidze et al., 2003).

Polyphenol oxidases in tea are the most important enzymes in determining the rate and direction of these fermentative processes. The enzyme is found in the stems with lower levels of activity in the leaves, but the activity differs between cultivars. The enzymes were found to be associated with the chloroplasts (Jain and Takeo, 1984). Halder et al. (1998) isolated a chloroplastic polyphenol oxidase from Indian tea leaf (*Camellia sinensis*) that was unable to utilise monophenols and *p*-diphenols as substrate. It could, however, very effectively utilise *o*-diphenols, including the tea

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flavonols, and was therefore regarded as a *o*-diphenol oxidase or catechol oxidase. Pruidze et al. (2003) isolated five forms of chloroplastic phenol oxidases from Kolkhida tea leaves (*Camellia sinensis* L.) with molecular weights (MW) of 28 000, 41 000, 58 000, 118 000 and 250 000. The largest three enzymes were all catechol oxidases, while the other two enzymes exhibited high monophenol mono-oxygenase activity. The latter catalyses the hydroxylation of *p*-coumaric acid into caffeic acid, which serves as a substrate for catechol oxidase that oxidises the cafeic acid into *o*-quinones. The higher MW forms oxidised catechins, mainly (-)-epigallocatechin and (-)-epigallocatechin gallate. The theaflavins and thearubigins subsequently produced at high concentrations completely inhibited these enzymes.

2.6.1.2 Peroxidase

Peroxidase (EC 1.11.1.7) is a hemoprotein that catalyses the oxidation of substrates in the presence of H_2O_2. It is found in all parts of the plant, but the activity is higher in the roots than in the aerial organs. It was also reported that 74% and 70% of the total amount of enzyme in the root and the aerial parts, respectively, was insoluble. The enzyme was found to be localised in the mitochondria and chloroplasts of the leaves with the higher activity found in the mitochondria (Jain and Takeo, 1984).

Peroxidase (POD) plays an oxidative role in the tea process and in conjunction with polyphenol oxidase (PPO), contributes to the colour and flavour of black tea. POD could also be responsible for the removal of H_2O_2 if formed during the fermentation of tea. Finger (1994) found that although PPO produced high levels of theaflavins and resolved thearubigin compounds, the application of POD led to the formation of even more unresolved and higher MW thearubigins. He also concluded that POD transforms the flavanols into oxidised products more rapidly than PPO and that POD also contributes to the rapid decomposition of theaflavins. However, when POD is the only enzyme present, a certain amount of theaflavins remains. It has been reported that, even in the presence of catechins, theaflavins and thearubigins inhibited the enzyme activity. Therefore, the reactions leading to the formation of pigments and flavour during fermentation is in part by the relative action of PPO and POD. PPO generates H_2O_2 during catechin oxidation which POD utilises for further

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oxidation of the products of PPO-catalysed reactions (Jain and Takeo, 1984; Subramanian et al., 1999).

2.6.1.3 Lypoxygenase

Lypoxygenase (EC 1.13.11.12) in tea catalyses the peroxidation of 1,4-diene unsaturated fatty acids, chlorophyll and carotene to produce carbonyl compounds responsible for the characteristic odour of fresh leaves. Lypoxygenase activity is significantly affected by seasonal and clonal variations. The lypoxygenase activity increased with leaf maturity and decreased with age from pruning. Increasing plucking intervals increased the enzyme activity. During CTC manufacture the enzyme showed an increase in activity with the degree of withering and upon rolling, but a decline was observed with fermentation and drying. The enzyme was found in the lamellae fraction of chloroplasts from tea. Lypoxygenase oxidises polyunsaturated fatty acids to peroxides and these compounds act as substrates for hydroperoxide lyase to form C6-aldehydes (Jain and Takeo, 1984; Ravichandran and Parthiban, 1998d).

2.6.1.4 Alcohol dehydrogenase

Alcohol dehydrogenase (EC 1.1.1.1) reversibly catalyses the reduction of aldehydes to their matching alcohols. These aldehydes may include those produced by hydroperoxide lyase. These volatile compounds are major black tea aroma constituents and have been found in the seeds and leaves of the tea plant. The over-oxidation of polyunsaturated acids during food processing, may lead to an off-flavour due to the production of aldehydes at increased concentrations. Alcohol dehydrogenase may decrease the concentration of these aldehydes by converting them to alcohols (Figure 2.11). However, this role has yet to be evaluated in tea (Jain and Takeo, 1984).

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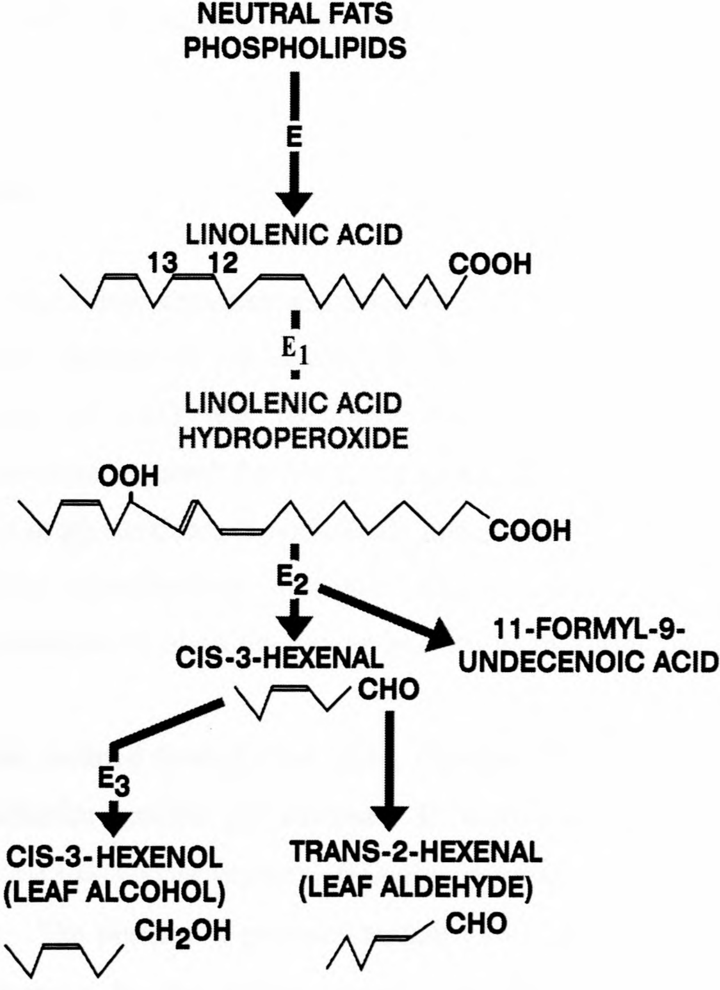

Figure 2.11 Biosynthetic pathway of leaf alcohol and leaf aldehyde. E_1 is lipoxygenase; E_2 is hydroperoxide lyase; E_3 is alcohol dehydrogenase (Jain and Takeo, 1984).

2.6.1.5 L-phenylalanine Ammonia Lyase

L-phenylalanine amonia lyase (PAL; EC 4.3.1.5) was found in tea seedlings with the activity of the enzyme in the plant organs distributed as follows: leaves > roots > seeds > cotyledons. PAL activity was high in the shoots containing two leaves and a bud, but the highest activity was found in the apical bud. The activity decreased with maturity with the lowest levels in the stems. The activities of PAL may be significantly influenced by different clones as well as seasonal variations. Plucking rounds and pruning had a marked effect on enzyme activity while the activity during different manufacturing stages differed widely. PAL plays an important role in the biosynthesis of flavanols, the prime substrate for PPO. PAL activity was also found

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to be associated with chloroplasts (Jain and Takeo, 1984; Ravichandran and Parthiban, 1998a).

2.6.1.6 Glycosidases

A broad range of glycosidase activities was detected by Halder and Bhaduri (1997) in the acetone powder extract of tea leaves of the Assam variety (*C. sinensis*). β-galactosidase (EC 3.2.1.23) was the most active of these enzymes at the fermentation temperature required for black tea processing and at acidic pH. The exogenous addition of glycosidases to nonvolatile material from tea leaves produced a mixture of volatile monoterpene alcoholic aroma constituents. Therefore, glycosidases can contribute to black tea flavour and aroma by acting on glycosides.

Another glycosidase isolated from the tea plant, β-primeverosidase (E.C. 3.2.1.149), is a unique disaccharide-specific glycosidase. It hydrolyses aroma precursors of β-primeverosides (6-*O*-β-D-xylopyranosyl-β-D-glucopyranosides) to release various aroma compounds. The purified β-primeverosidase exhibited very narrow substrate specificity with respect to the glycon moiety, specifically the β-primeverosyl (6-*O*-β-D-xylopyranosyl-β-D-glucopyranosyl) moiety. The activity and quantity of β-primeverosidase was high in younger leaves and decreased with leaf age. β-primeverosidase was also found in considerable amounts in the stems. The physiological function of β-primeverosidase in the tea plant may be one of defense, where the enzyme may hydrolyse β-primeverosides to release aglycons in response to fungal infection and herbivore feeding (Ma et al., 2001; Mizutani et al., 2002).

2.6.2 Exogenous application of enzymes to improve tea quality

Although the above mentioned enzymes are present in the tea plant, their efficacy is influenced by the tea manufacturing process. As was previously described, the enzymes PPO and POD in tea leaves are responsible for the formation of black tea components. In the native tea leaves, PPO is present in the epidermal cells and vascular bundles, whereas the catechin substrate is present inside the vacuoles of the palisade cells (Bhatia and Ullah, 1965). During the manufacture of black tea, the

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withered tea leaves are rolled. This process leads to the incomplete disruption of tea leaves, which reduces the amounts of POD and PPO in the native leaves and suppresses the formation of black tea components. The cell wall polysaccharides also act as an additional barrier for the interaction between enzyme and substrate. However, the application of exogenous enzymes responsible for cell wall degradation would lead to complete maceration of the tea leaf and may also release more tea phenolic compounds which could improve the therapeutic value of tea (Angayarkanni et al., 2002; Murugesan et al., 2002).

Ravichandran and Parthiban (1998a) supplemented dhool with cellulase and pectinase before fermentation. The tea showed a marked improvement in liquor colour and water soluble solids. This was reflected in the tasters' scores and price valuations that were higher than that of the control. In two further studies the application of exogenous enzymes were tested using various parameters to assess the tea quality, namely theaflavin (TF), thearubigin (TR), highly polymerised substances (HPS), total liquor colour (TLC), dry matter content (DMC) and total soluble solids (TSS). Angayarkanni et al. (2002) purified pectinase enzyme from crude enzyme preparations of *Aspergillus indicus*, *Aspergillus flavus* and *Aspergillus niveus*, and applied the crude enzyme extract, the purified pectinase or a commercial pectinase to the cut dhool before fermentation. They found that the separate crude enzyme extracts from all three fungi was more effective at improving the tea quality than the purified pectinase or the commercial enzyme. This was because the crude enzyme extracts from the fungi include cellulase, hemicellulase (xylanase), proteinase, pectinase, etc., that acted on the tea leaf that consists of cellulose, hemicellulose and pectin. Murugesan et al. (2002) isolated cellulases, pectinases and xylanases from a 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). The crude cellulase, pectinase and xylanase extracts, as well as the purified form of these enzymes, were applied to cut dhool before fermentation. They found that all the enzymes improved the quality parameters, but the purified cellulase treatment was the most effective. The purified cellulase was then combined with a laccase from *Trametes versicolor* in a 3:2 (v/v) ratio, which improved the tested parameters above that of the purified cellulase.

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About 25% of the water soluble components in tea leaves are tannins. These tannins, along with caffeine, are responsible for the development of turbidity of the tea extract upon cooling. This phenomenon is known as creaming and is an undesireble feature in cold-soluble instant tea. Various methods have been used for the decreaming of soluble tea, including maintaining the temperature above 65°C or cooling and precipitation of the soluble tea. Both these methods adversely affect tea flavour or colour. Soluble tannase has been shown to be very effective at removing cream: the cream is hydrolysed to lower molecular weight components which reduces the turbidity and increases the solubility of the tea extracts. However, the enzyme must be deactivated by heating to over 90°C, which results in a loss of tea flavour. Tannase immobilised to various substrates and encapsulated tannase made recovery easier, circumvents the need to heat the tea and improved the flavour and processing costs (Boadi and Neufeld, 2001).

Fungal pectinases are being used in the manufacture of tea where the enzyme treatment accelerates tea fermentation and improves the foam forming property of instant tea powders by destroying tea pectins (Hoondal et al., 2002; Kashyap et al., 2001). These studies on the addition of exogenous enzymes to tea and its products showed that they are effective at improving the quality of tea and tea products.

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Chapter 3

Research Results:

Evaluation of Commercial Enzymes for the Bioprocessing of Rooibos Tea

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Evaluation of Commercial Enzymes for the Bioprocessing of Rooibos Tea

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Abstract

Rooibos tea is well known for its neutraceutical and pharmaceutical value. The current processing of Rooibos tea takes from 14 –16 hrs and occurs under uncontrolled open-air conditions. During this extended fermentation period, a considerable amount of antioxidants are lost that reduces the value of the final product. The bruising of the tea leaves is also not sufficient in disrupting the plant cell walls to allow the naturally occurring plant enzymes access to the substrates inside the plant cells to catalyse the reactions that contribute to the organoleptic properties of Rooibos tea. In this study, the application of 16 commercially available microbial enzymes were evaluated on Rooibos tea for the improved release of soluble solids, total polyphenols, colour and aroma. Several enzymes showed improvement in the yield of either soluble solids or total polyphenols, but combinations of enzymes are required to improve both parameters. The enzymes evaluated are typical of those required for the breakdown of plant material i.e. cellulases, xylanases, pectinases and laccases. Laccases proved to be the best enzyme for improving colour and aroma development from green Rooibos tea, with treatment times reduced to only 2 hrs.

Introduction

The Rooibos tea plant (*Aspalathus linearis* (Burm. Fil.) R. Dalhgr. spp. *linearis*) is indigenous to South Africa with a very limited distribution in the Western Cape, occurring particularly in the Cedarberg mountains. The plant is a leguminous shrub about 1.35 – 2 m tall with red-brown branches and linear, needle-like leaves of 2 – 6 cm long. The genus contains more than 200 species, all native to South Africa (Morton, 1983; Van Heerden et al., 2003).

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Rooibos tea is produced from the leaves and fine stems of the plant. The tea is normally prepared by brewing the leaves and consuming the liquor. However, the Rooibos plant extracts are also used in various neutraceutical and pharmaceutical products, including iced tea, health drinks, soaps and moisturising creams (Jaganyi and Wheeler, 2003; www.rooibosltd.co.za).

Rooibos tea is well known as a health drink due to its polyphenolic content and the fact that it contains no caffeine and virtually no tannins. Its benefits include antimutagenic, anti-inflammatory and antiviral activities. It has been reported to calm digestive disorders and various stomach problems, reduce nervous tension and alleviates allergies. It is also used for topical treatment of dermatological diseases such as Behcet's disease and photosensitive dermatitis. The flavonoid fraction of Rooibos tea includes aspalathin, nothofagin, quercetin, luteolin, rutin, isoquercitrin and iso-vitexin, which have all been shown to have antioxidant activity. Aspalathin is unique to Rooibos and has been implicated in contributing to the natural sweet taste of Rooibos tea. Nothofagin has only previously been found in *Nothofagus fusca* (red beech). Aspalathin and nothofagin make up 0.55% and 0.19%, respectively, of the soluble solids of the processed Rooibos tea (Bramati et al., 2002; 2003; Jaganyi and Wheeler, 2003; Joubert and Ferreira, 1996; Standley et al., 2001).

Rooibos tea is harvested during the summer and early autumn (November to March). The plants are cut to about 30 cm from the ground and then cut into 3 to 4 mm lengths, moistened, bruised and placed in heaps outdoors to ferment in the open air. Fermentation time is about 8 hrs, depending on the ambient temperature and degree of bruising. The tea is then spread out on large concrete areas to dry in the sun to a moisture content of 8 to 10%. It is then sieved and steam pasteurised before packaging (Joubert, 1988; www.rooibosltd.co.za).

The current manufacturing process has a significant effect on the flavonoid content and consequently the antimutagenic and antioxidant properties of Rooibos tea. Bramati et al. (2003) reported that all the main polyphenols found in Rooibos were more abundant in the unfermented Rooibos than in the fermented. The level of aspalathin was almost 50 times higher in the unfermented Rooibos while it is extensively degraded to dihydro-*iso*-orientin during the fermentation process. Von

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Gadow et al. (1997) showed that aqueous extracts prepared from fermented Rooibos were less effective as antioxidants than extracts from unfermented Rooibos. The most significant reduction in the antimutagenic and antioxidant potential of the fermented tea occurs during the fermentation phase, while sun-drying, sieving and steam pasteurisation contribute to a lesser degree. The antimutagenic and antioxidant activities were linked to tea polyphenols since processing led to a significant reduction in the polyphenol content and subsequently to a reduction in these activities (Standley et al., 2001).

The demand of the local and international market for high quality Rooibos tea and extracts is continuously increasing. However, high quality Rooibos tea can only be produced under controlled conditions as opposed to the current uncontrolled open-air method of floor fermentation and sun-drying. It has already been shown that an increase in fermentation temperature and a decrease in drying temperature improved tea quality, whilst no significant difference in quality was observed between sun-drying and controlled drying in a tray drier (Joubert and De Villiers, 1997).

Angayarkanni et al. (2002) purified pectinase enzymes from crude enzyme preperations of *Aspergillus indicus*, *Aspergillus flavus* and *Aspergillus niveus*, and applied the crude enzyme extracts, the purified pectinase or a commercial pectinase to the cut dhool (tea leaves cut into small particles and allowed to ferment) of black tea before fermentation. They found that the individual crude enzyme extracts from all three fungi were more effective at improving the tea quality than the purified pectinase or the commercial enzyme. This was ascribed to the crude enzyme extracts that included cellulase, hemicellulase (xylanase), proteinase, pectinase, as well as other enzyme activities that acted synergistically on the tea leaf that consists of mostly cellulose, hemicellulose and pectin. Murugesan et al. (2002) isolated cellulases, pectinases and xylanases from a 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). When the crude extracts or the purified enzymes were applied to cut dhool of black tea before fermentation, all the enzymes improved the quality parameters, but the purified cellulase treatment was the most effective. When the purified cellulase was combined with a laccase from *Trametes versicolor* in

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a 3:2 (v/v) ratio, the enzyme cocktail was even more effective than the purified cellulase.

In this study, 16 commercially available microbial enzymes were studied for their ability to improve Rooibos tea quality as measured by the increase in total phenols (TP) and soluble solids (SS) or improved colour and aroma development. The enzymes were applied to four different Rooibos tea substrates, i.e. spent tea (after extraction with physical-chemical methods), dried fermented tea, dried green Rooibos tea and freshly cut green Rooibos tea leaves.

Materials and Methods

Plant material, chemicals and commercial enzymes

The various tea substrates were obtained from Rooibos (Pty) Ltd., Clanwilliam, South Africa, the Post-Harvest and Wine Technology Division of ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa and Cape Natural Tea Products (Pty) Ltd., Brackenfell, South Africa. Fermented tea (stalky material not suitable for commercial tea bags) was supplied by Afriplex (Pty) Ltd. (Paarl, South Africa). All chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany). Commercial enzymes (Table 3.1) were supplied by Novozymes (Bagsvaerd, Denmark) and Biocatalysts Ltd. (Wales, UK).

Neutral sugar analysis of polysaccharides in green tea

Polysaccharides were hydrolysed by subjecting ~0.25 g of the tea sample to two-stage sulphuric acid hydrolysis (Moore and Johnson, 1967). After neutralisation with $CaCO_3$ to pH ~5.5, samples were amended with 20 mg of myo-inositol (internal standard), then centrifuged (1 500 x g, 15 min), and 10 ml of supernatant was lyophilised. The lyophilised samples were resuspended in 1 000 µl deionised water, then centrifuged at 12 000 x g for 5 min. Supernatants were dried under an air stream and subjected to reduction with Na-borodeuteride and acetylation with acetic anhydride, as described by Blakeney et al. (1983).

Gas-liquid chromatography of alditol acetates was performed using a Hewlett-Packard 6890 Plus GC fitted with flame ionisation detector and a Supelco SPB-225 capillary

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column (30 m x 0.25 mm, with 0.25 μm film thickness). Samples of 1 μl were injected at a split ratio 50 using helium as carrier gas. The temperature program was set at 215°C for 2 min, then increased at 4°C per min for 3.75 min, and held at 230°C for 11.25 min.

Table 3.1 Commercial enzymes evaluated in this study.

Commercial Enzyme	Activities*	Source
Cellulase 13L	Cellulase 1 500 U/g	Biocatalysts
Depol™ 112L	β-Glucanase 7 000 U/g Xylanase 4 000 U/g	Biocatalysts
Depol™ 670L	Cellulase 1 200 U/g Pectinase 800 U/g Ferulic acid esterase 7 U/g	Biocatalysts
Depol™ 740L	Ferulic acid esterase 36 U/g	Biocatalysts
Fructozyme L	Exo-inulinase 2 000 ING/g	Novozymes
Glucanase 5XL	β-Glucanase 12 500 U/g	Biocatalysts
Laccase L603P	Laccase 112 000 POU/g	Biocatalysts
Lactase L107P	Lactase 65 000 U/g	Biocatalysts
Pectinex® Ultra SP-L	Pectinase 26 000 PG/ml	Novozymes
PpL Laccase	Laccase 2 500 U/g	Novozymes
Suberase	Phenol oxidase 10 500 PCU/ml	Novozymes
Tannase C	Tannase 90 U/ml	Novozymes
Tannase P	Tannase 200 U/g	Novozymes
Depol™ 692L	Cellulase 800 U/g Ferulic acid esterase 3 U/g	Biocatalysts
Ultraflo® L	β-Glucanase 45 FBG/g	Novozymes
Ultrazym P	Proteolytic enzyme 200 000 U/g	Novozymes

*Activities as defined by manufacturers

Evaluation of commercial enzymes on spent tea

Batches of 50 g dried spent tea were treated in duplicate with enzymes (Table 3.2) diluted in 100 ml citrate buffer (pH 3.5 to pH 6.5). The tea was treated for 1 or 6 hrs at 40°C followed by drying in an oven for 2 days at 50°C. Enzyme dosages were calculated as U/g tea or where the commercial enzyme product consisted of more than one enzyme, as μl/g tea.

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Evaluation of commercial enzymes on fermented tea

Batches of 150 g dried fermented tea were treated in duplicate with Depol™ 670L (10 µl/g tea), Ultraflo® L (1 U/g tea), PpL Laccase (1 U/g tea) or Pectinex Ultra SP-L (260 U/g tea) diluted in Citrate buffer (pH 5.5) to a final volume of 225 ml. The tea was treated at 50°C for different time intervals, i.e. 30 min, 1 hr, 2 hrs, 4 hrs or 6 hrs. The tea was dried in an air tunnel for 3 hrs at 40°C.

Table 3.2 Parameters for treatment of spent tea.

Enzyme	Dosage*	Treatment time (hrs)	pH
Cellulase 13L	15; 30	6	5.5
Depol™ 112L	10; 20 (µl/g tea)	6	5.5
Depol™ 670L	2; 20; 200 (µl/g tea)	1	4.5
Depol™ 740L	0.1; 1; 10	1	4.5
Glucanase 5XL	1; 10	6	5.5
Laccase L603P	100; 1000	6	5.5
Lactase L107P	1; 10	6	5.5
Pectinex® Ultra SP-L	260	1	3.5
PpL Laccase	1	1	4.5
Suberase	2	1	4.5
Depol™ 692L	5; 10 (µl/g tea)	6	5.5
Ultraflo® L	0.009; 1	1	6.5

*Dosage is defined as U/g tea unless stated otherwise

Batches of 150 g dried fermented tea were treated in duplicate with Depol™ 670L (2 µl and 20 µl/g tea), Pectinex Ultra SP-L (26U and 260U/g tea), Depol™ 692L (1 µl and 10 µl/g tea), Ultraflo® L (0.01U and 0.1U/g tea), Depol™ 740L (0.1 µl and 1 µl/g tea), Depol™ 112L (1 µl and 10 µl/g tea) or two "synthetically" designed enzyme cocktails (Cocktail #1 and Cocktail #2 in Table 3.3) diluted in Citrate Buffer (pH 5.5) to a final volume of 225 ml. The tea was treated at 40°C for 2 hrs and dried in an air tunnel for 3 hrs at 40°C.

Duplicate batches of 150 g fermented tea (Afriplex waste) were treated with 150 ml Citrate buffer (pH 5.5) containing Cocktail #5 to #12 (Table 3.3) at 40°C for 2 hrs and then dried in an air tunnel for 3 hrs at 40°C.

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Table 3.3 Composition of synthetic cocktails used in this study.

Enzyme cocktail	Dosage	Treatment time (hrs)	pH
Cocktail #1	1.5 µl/g tea Depol™ 112L 1.62 µl/g tea Depol™ 670L 3.9 U/g tea Pectinex Ultra SP-L 1002 U/g tea Laccase L603P	2	5.5
Cocktail #2	1.5 µl/g tea Depol™ 112L 2.44 µl/g tea Depol™ 692L 3.9 U/g tea Pectinex Ultra SP-L 1002 U/g tea Laccase L603P	2	5.5
Cocktail #3	1.5 µl/g tea Depol™ 112L 2.44 µl/g tea Depol™ 692L 3.9 U/g tea Pectinex Ultra SP-L 250 U/g tea Laccase L603P	2; 3; 4	5.5
Cocktail #4	1.5 µl/g tea Depol™ 112L 2.44 µl/g tea Depol™ 692L 3.9 U/g tea Pectinex Ultra SP-L 500 U/g tea Laccase L603P	2; 3; 4	5.5
Cocktail #5	1.5 µl/g tea Depol™ 112L 1.62 µl/g tea Depol™ 670L 3.9 U/g tea Pectinex Ultra SP-L	2	5.5
Cocktail #6	1.5 µl/g tea Depol™ 112L 2.44 µl/g tea Depol™ 692L 3.9 U/g tea Pectinex Ultra SP-L	2	5.5
Cocktail #7	2 µl/g tea Depol™ 670L 0.1 U/g tea Depol™ 740L	2	5.5
Cocktail #8	2 µl/g tea Depol™ 670L 1 µl/g tea Depol™ 112L	2	5.5
Cocktail #9	26 U/g tea Pectinex Ultra SP-L 0.1 U/g tea Depol™ 740L	2	5.5
Cocktail #10	26 U/g tea Pectinex Ultra SP-L 1 µl/g tea Depol™ 112L	2	5.5
Cocktail #11	1 µl/g tea Depol™ 692L 0.1 U/g tea Depol™ 740L	2	5.5
Cocktail #12	1 µl/g tea Depol™ 692L 1 µl/g tea Depol™ 112L	2	5.5

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Small-scale simulated industrial treatments of fermented tea

Duplicate batches of 150 g fermented tea (Afriplex waste) were treated with 150 ml dH_2O containing Pectinex Ultra SP-L (26U or 260U/g tea), Depol™ 670L (2 µl or 20 µl/g tea), Depol™ 692L (1 µl or 10 µl/g tea), Depol™ 112L (1 µl/g tea) or Depol™ 740L (0.1U/g tea) at 40°C for 2 hrs and dried in an air tunnel for 3.5 hrs at 40°C. The batches were pooled and samples of 100 g dried tea were extracted with 1000 ml of deionised water at 90 - 93°C for 30 min, where after it was decanted and filtered through Whatman #4 filter paper. The filtrate was cooled to room temperature and cleaned up by filtration through a 0.8 µm pore size filter (AP15).

Evaluation of commercial enzymes on green tea

Duplicate batches of 100 g dried green tea were treated with different enzymes (Table 3.4) diluted in Citrate buffer (pH 3.5 or 5.5) to a final volume of 150 ml. The tea was treated for 6 hrs at either 40°C or 50°C and dried in an air tunnel for 3 hrs at 40°C. It was then evaluated for %SS, TP and colour.

Table 3.4 Parameters for treatment of dried green tea.

Enzyme	Dosage*	Temperature (°C)	pH
Cellulase	5	40	5.5
Cellulase 13L	1; 10	40	5.5
Depol™ 112L	0.25; 2.5 (µl/g tea)	40	5.5
Depol™ 670L	20 (µl/g tea)	40	5.5
Depol™ 740L	0.1; 1	40; 50	5.5
Fructozyme	1; 10	40	5.5
Glucanase 5XL	1; 10	40; 50	5.5
Laccase L603P	1; 10	40	5.5
Lactase L107P	1; 10	40	5.5
Pectinex® Ultra SP-L	1; 260	40	3.5
PpL Laccase	1	40; 50	5.5
Suberase	2	40	5.5
Depol™ 692L	10; 100 (µl/g tea)	40	5.5
Ultraflo® L	0.009; 0.09; 1	40; 50	5.5

*Dosage is defined as U/g tea unless stated otherwise

Duplicate batches of 230 g freshly cut green plant material were treated with different enzymes or Cocktails #3 and #4 (Table 3.5) diluted in Citrate buffer (pH 5.5) to a

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final volume of 100 ml. The batches treated with the enzymes were treated for 1.5 – 6 hrs at 35°C, 40°C, 50°C or 60°C and evaluated for aroma and colour development. The batches treated with the Cocktails (see Table 3.3 for detail) were treated for 2, 3 or 4 hrs at 40°C and dried in an air tunnel for 3 hrs at 40°C and evaluated for SS, TP, antioxidants and extract colour. Enzyme dosages allowed for the moisture content of the freshly cut plant material and were comparable to those used for 150 g dried tea. Aroma and colour was evaluated orally and visually by an experienced tea taster of Rooibos tea. Enzyme dosages were calculated as U/g tea or where the commercial enzyme product consisted of more than one enzyme, as µl/g tea.

Table 3.5 Parameters for treatment of freshly cut Rooibos.

Enzyme	Dosage*	Temperature (°C)	Treatment time (hrs)
Cocktail #3	see Table 3.3	40	2; 3; 4
Cocktail #4	see Table 3.3	40	2; 3; 4
Laccase L603P	1; 10; 50; 100; 500; 1000; 2000	40; 60	4
PpL Laccase	1; 10	35; 40; 50	1.5; 3; 4; 6
Suberase	10	40	4
Tannase C	0.09; 1; 5	40	4
Tannase P	0.2; 1; 5	40	4
Depol™ 692L	10 (µl/g tea)	50	3; 6
Ultraflo® L	1; 2	40; 50	3; 6
Ultrazym P	1; 10	40	4

*Dosage is defined as U/g tea unless stated otherwise

Analysis of enzyme-treated tea

The dried tea was extracted by adding 150 ml boiling purified and deionised water to 2 g of tea and stirring for 2 min on a magnetic stirrer after which the extract was filtered through filter paper (Whatman Nr 4 or equivalent). The **soluble solid** content of the extract was determined gravimetrically with 20 ml of the extract being evaporated on a steam bath and dried for 1 hr at 100°C. The values given for each sample are the means of four replications. **Total phenol** content of the extract 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 was described according to the CIELAB scale (L*, a*, b*) and determined with a Colorgard 2000/05 system

with a TM-M transmission attachment (5 mm path length quartz cell) to the 05 sensor (Joubert, 1995). Colour improvement is defined as a lower L*, higher a* and higher b* values. The values given for each sample are the mean of six replications. Increases of 10% indicate statistically significant differences (at 5% level).

Quantative analysis of antioxidants released from green tea

Quantification of specific **antioxidants** was carried out by reversed-phase HPLC with UV detection at 288 and 255 nm, depending on the chemical structure. Authentic standards were used for quantification. The values given for each sample are the means of four replications. **Total antioxidant activity (AOX)** of extracts was determined according to the ABTS cation radical scavenging method with TROLOX as standard, according to the method of Re et al. (1999). The values given for each sample are the means of duplicate replications.

Results

Neutral sugar analysis of polysaccharides in green tea

The major fractions of the polysaccharides released by chemical hydrolysis from green tea (Table 3.6) is glucose, followed by xylose. Glucose and xylose usually form the backbones of cellulose and xylan, a major hemicellulose. The concentrations of the other sugars are considerably less and are often side groups associated with these backbones. Mannose and galactose can also be constituents of mannans and galactans (or galactomannan).

Table 3.6 Neutral sugar composition of duplicate green tea samples.

Sample	Rhamnose	Fucose	Arabinose	Xylose	Mannose	Galactose	Glucose	Totals
1	0.0222	0.0017	0.0428	0.197	0.0312	0.0361	0.6689	**1.000**
2	0.0186	0.0033	0.0404	0.2143	0.0215	0.0341	0.6678	**1.000**

	Other sugars	% Arabinoxylan	% Galactomannan	% Cellulose	
1	**2.39**	**23.98**	**6.73**	**66.89**	**99.99**
2	**2.19**	**25.47**	**5.56**	**66.78**	**100**

Evaluation of commercial enzymes on spent tea

Thirteen commercial enzymes were evaluated on spent tea (Table 3.7). The Depol™ 740L, Depol™ 670L, Ultraflo® L, Pectinex® Ultra SP-L, Depol™ 692L, Depol™ 112L and Cellulase 13L all released additional SS from spent tea. At increased

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Table 3.7 Change in yields of %SS and TP from spent tea treated with commercial enzymes. Deviations of more than 10% from the respective controls are indicated in bold.

Treatment	Dosage (U/g tea or µl/g tea)	%SS (g/100 g dry leaves)	TP (mg GAE/g tea)	%TP/SS
Control – pH 4.5		8.50	1.84	21.68
Depol 670L	200 µl	28.27	1.83	6.50
		332 ± 1.84%	99 ± 4.10%	**30%**
Depol 740L	10 U	21.35	1.59	7.46
		251 ± 3.80%	**86 ± 5.20%**	**34%**
PpL Laccase	1 U	4.81	0.34	7.35
		57 ± 2.96%	**18 ± 5.38%**	**34%**
Suberase	2 U	8.30	1.61	19.36
		98 ± 4.99%	**88 ± 1.55%**	**89%**
Control – pH 4.5		8.72	1.75	20.06
Depol 670L	2 µl	14.56	1.81	12.45
		167 ± 5.06%	104 ± 3.75%	**62%**
Depol 670L	20 µl	18.75	1.80	9.63
		215 ± 3.42%	103 ± 1.53%	**48%**
Depol 740L	0.1 U	8.78	1.91	21.92
		101 ± 7.31%	109 ± 3.68%	109%
Depol 740L	1 U	10.27	1.67	16.24
		118 ± 4.24%	95 ± 2.52%	**81%**
Control - pH 3.5		7.77	1.86	23.95
Control - pH 6.5		9.94	2.25	22.67
Pectinex (pH 3.5)	260 U	16.56	1.49	9.03
		213 ± 2.05%	**80 ± 5.65%**	**38%**
Ultraflo (pH 6.5)	0.009 U	11.09	2.31	20.81
		112 ± 4.38%	103 ± 5.38%	92%
Ultraflo (pH 6.5)	1 U	11.95	2.32	19.38
		120 ± 2.32%	103 ± 2.12%	**85%**
Control - pH 5.5		8.79	1.93	22.0
Depol 692L	5 µl	17.74	1.86	10.53
		202 ± 5.86%	97 ± 2.53%	**48%**
Depol 692L	10 µl	18.38	1.95	10.64
		209 ± 3.39%	101 ± 1.08%	**48%**

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Table 3.7 (continued)

Treatment	Dosage (U/g tea or µl/g tea)	%SS (g/100 g dry leaves)	TP (mg GAE/g tea)	%TP/SS
Glucanase 5XL	1 U	9.36 107 ± 5.36%	1.99 103 ± 2.57%	21.25 97%
Glucanase 5XL	10 U	9.57 109 ± 3.00%	2.04 106 ± 3.91%	21.36 97%
Lactase	1 U	8.12 92 ± 6.04%	1.93 100 ± 1.24%	23.77 108%
Lactase	10 U	9.17 104 ± 6.87%	2.14 **111 ± 1.96%**	23.33 106%
Control – pH 5.5		8.98	1.87	20.8
Cellulase 13L	15 U	11.88 **132 ± 3.64%**	1.96 105 ± 3.98%	16.49 **79%**
Cellulase 13L	30 U	13.09 **146 ± 1.30%**	1.98 106 ± 2.22%	15.16 **73%**
Depol 112L	10 µl	12.17 **135 ± 1.82%**	1.78 95 ± 2.16%	14.63 **70%**
Depol 112L	20 µl	12.32 **137 ± 5.98%**	1.76 94 ± 3.20%	14.28 **69%**
Laccase L603P	100 U	4.40 **49 ± 1.43%**	0.30 **16 ± 2.13%**	6.72 **32%**
Laccase L603P	1000 U	4.37 **49 ± 4.08%**	0.18 **10 ± 5.47%**	4.22 **20%**

dosages, Depol™ 740L and Depol™ 670L showed significant increases in the release of SS (more than two-fold) while Ultraflo® L, Depol™ 692L, Depol™ 112L and Cellulase 13L obtained only slight improvements at the higher dosages. All the laccases decreased the %SS and TP content significantly. For the other enzyme treatments, the TP remained relatively unchanged compared to the controls except for Suberase, Depol™ 740L (10U/g tea) and Pectinex® Ultra SP-L where the TP content was reduced by more than 10%. Wherever there was a strong increase in the SS released from the spent tea, the TP/%SS ratio was reduced dramatically, suggesting the extraction of "inactive" compounds, i.e. non-phenolic components with little antioxidant activity.

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Evaluation of commercial enzymes on fermented tea

The effect of treatment time was evaluated for Depol™ 670L, Ultraflo® L, PpL Laccase and Pectinex® Ultra SP-L. The results indicated that Depol™ 670L and Pectinex® Ultra SP-L improved the release of SS from fermented tea, especially when the treatment time was 1 hr or longer (data not shown), while PpL Laccase improved the extract colour after only 30 min of treatment. However, large variability between the samples was observed due to the stalky substrate material and the results can therefore only be taken as trends.

In the laboratory-scale treatment of fermented tea (Table 3.8), Depol™ 670L only increased the yield in SS significantly at the higher dosage while Pectinex® Ultra SP-L showed increases at both dosages, with the higher dosage being more effective. Depol™ 692L also increased the yield in SS, but with no difference between the two dosages. Treatment with Depol™ 670L and Pectinex® Ultra SP-L both decreased the antioxidant content of the extract by more than 10%, suggesting that the increase in SS was due to the extraction of mostly inactive compounds. However, Depol™ 740L and Depol™ 112L increased the antioxidant content of the extract with no significant increase in the yield in SS, suggesting the extraction of active compounds. Of particular interest is that the lower dosages of both enzymes increased the antioxidant content more than at the higher dosages.

Similar trends were observed in the industrial simulations (Table 3.9) where Depol™ 670L, Pectinex® Ultra SP-L and Depol™ 692L increased the yield in SS with the higher dosages being more effective (25%, 26% and 23% increase, respectively). The treatments with Depol™ 112L and Depol™ 740L were also more effective than the laboratory-scale treatments with an increase of 10% in the TP content for both treatments. The higher efficacy of the enzymes can be ascribed to the extraction method used for the industrial simulations of Rooibos extract preperation that differ from that used for the laboratory evaluations, which were based on the tea made by infusion.

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Table 3.8 Change in yields of %SS, TP and antioxidants from fermented tea treated with commercial enzymes. Deviations of more than 10% from the respective controls are indicated in bold.

Treatment	Dosage (U/g tea or µl/g tea)	%SS (g/100 g dry leaves)	TP (mg GAE/g tea)	%TP/SS	AOX (µmol Trolox/g SS)
Control – pH 5.5		11.50	3.13	25.69	1730.50
Depol 670L	2 µl	12.57	3.32	23.54	1540.08
		109 ± 2.84%	106 ± 5.08%	92%	**89 ± 3.29%**
Depol 670L	20 µl	16.62	3.11	22.05	1476.14
		144 ± 3.01%	99 ± 3.94%	**86%**	**85 ± 3.26%**
Depol 692L	1 µl	12.82	3.19	26.36	1728.29
		111 ± 3.76%	102 ± 3.53%	103%	100 ± 3.57%
Depol 692L	10 µl	12.91	3.31	24.90	1558.62
		112 ± 1.15%	106 ± 2.38%	97%	**90 ± 4.95%**
Pectinex	26 U	13.91	3.29	22.72	1511.74
		121 ± 2.35%	105 ± 1.02%	**88%**	**87 ± 5.37%**
Pectinex	260 U	16.23	3.37	22.39	1405.30
		141 ± 2.55%	108 ± 4.18%	**87%**	**81 ± 2.53%**
Control – pH 5.5		12.60	2.99	24.83	1343.50
Depol 112L	1 µl	11.52	3.05	26.27	1708.61
		91 ± 5.80%	102 ± 3.98%	106%	**127 ± 2.95%**
Depol 112L	10 µl	11.47	3.05	26.07	1598.34
		91 ± 5.02%	102 ± 2.04%	105%	**119 ± 5.75%**
Depol 740L	0.1 U	11.78	3.18	25.58	1860.88
		94 ± 4.00%	106 ± 5.19%	103%	**139 ± 5.20%**
Depol 740L	1 U	13.08	3.12	23.67	1530.47
		104 ± 3.33%	104 ± 3.74%	95%	**114 ± 4.57%**
Ultraflo	0.01 U	11.48	3.08	26.89	1371.14
		91 ± 1.79%	103 ± 1.02%	108%	102 ± 5.34%
Ultraflo	0.1 U	11.99	3.09	25.38	1389.53
		95 ± 6.54%	103 ± 2.74%	102%	103 ± 5.92%

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Table 3.9 Change in yields of %SS and TP from fermented tea in industrial simulations with commercial enzymes. Deviations of more than 10% from the respective controls are indicated in bold.

Treatment	Dosage (U/g tea or µl/g tea)	%SS (g/100 g dry leaves)	TP (mg GAE/g tea)	%TP/SS
Control - dH_2O (AP15 filtrate)		12.46	3.82	30.71
Depol 670L	2 µl/g	14.54	3.88	26.67
		117 ± 1.00%	102 ± 2.73%	**87%**
Depol 670L	20 µl/g	15.65	3.71	23.69
		126 ± 0.4%	97 ± 2.29%	**77%**
Depol 692L	1 µl/g	13.94	4.05	29.03
		112 ± 0.52%	106 ± 1.25%	95%
Depol 692L	10 µl/g	15.39	3.98	25.89
		123 ± 1.94%	104 ± 3.42%	**84%**
Pectinex	26 U	14.00	3.92	27.98
		112 ± 0.74%	103 ± 1.14%	91%
Pectinex	260 U	15.53	3.64	23.46
		125 ± 1.65%	95 ± 1.23%	**76%**
Control - dH_2O (AP15 filtrate)		13.92	3.89	27.97
Depol 112L	1 µl/g	14.52	4.29	29.56
		104 ± 0.54%	**110 ± 3.08%**	106%
Depol 740L	0.1 U	14.30	4.26	29.78
		103 ± 0.17%	**110 ± 2.66%**	106%

As shown in Table 3.10, the enzyme Cocktails #1 and #2 decreased the SS, TP and antioxidant content of the extract, probably due to the high laccase concentrations (Table 3.3). Cocktails #6, #7 and #8 all increased the yield in SS from fermented tea while Cocktail #10 increased both the SS and TP content. It was shown in Table 3.6 that Rooibos has a high cellulose and xylose content, indicating that cellulase and xylanase will be needed for the breakdown of the plant cell walls. This explains the apparent lack of improvement with Cocktail #9, which contains no cellulase or xylanase activities. Cocktail #6 contains xylanase, cellulase, pectinase, β-glucanase and some ferulic acid esterase (FAE) activities. These are all enzymes involved in the breakdown of plant material, which is reflected in the increase in %SS, but mostly inactive compounds are extracted as is reflected in the decrease in the %TP/SS ratio. Cocktail #7 contains cellulase, pectinase and FAE activities, cocktail #8 has xylanase activity substituted for the FAE activity of cocktail #7, and cocktail #10 contains

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xylanase, pectinase and β-glucanase as main activities. Cocktails #11 and #12 have similar composition to Cocktails #7 and #8 (Table 3.3), but with different sources and concentrations of cellulase (2.4 U from Depol™ 670L/g tea for #7 and #8; 0.8 U from Depol™ 692L/g tea for #11 and #12). Therefore, it is not surprising that cocktails #7 and #8 show an increase in %SS while cocktails #11 and #12 do not.

Table 3.10 Change in yields of %SS and TP from fermented tea treated with synthetic enzyme cocktails. Deviations of more than 10% from the respective controls are indicated in bold.

Treatment	%SS (g/100 g dry leaves)	TP (mg GAE/g tea)	%TP/SS
Control - pH 5.5	11.36	3.01	26.42
Cocktail #1	9.99	1.13	11.32
	88 ± 2.07%	**38 ± 0.60%**	**43%**
Cocktail #2	9.53	1.22	12.83
	84 ± 2.11%	**41 ± 0.45%**	**49%**
Control - pH 5.5	10.36	3.04	26.44
Cocktail #5	11.31	2.98	26.18
	109 ± 5.88%	98 ± 0.63%	99%
Cocktail #6	11.77	2.97	23.12
	114 ± 3.52%	98 ± 0.82%	**87%**
Cocktail #7	11.58	3.05	25.82
	112 ± 3.82%	100 ± 1.50%	98%
Cocktail #8	11.70	3.16	26.91
	113 ± 4.40%	104 ± 4.50%	102%
Control - pH 5.5	11.51	3.41	29.59
Cocktail #9	11.38	3.12	27.43
	99 ± 4.63%	92 ± 3.77%	93%
Cocktail #10	12.76	3.88	30.38
	111 ± 2.60%	**114 ± 1.74%**	103%
Cocktail #11	11.80	3.20	27.17
	103 ± 2.68%	94 ± 5.52%	92%
Cocktail #12	11.71	3.30	28.21
	102 ± 3.63%	97 ± 0.29%	95%

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Evaluation of commercial enzymes on green tea

Ten commercial enzymes were evaluated on freshly cut Rooibos plant material (Table 3.11) at different treatment times, dosages and temperatures for aroma and colour development. PpL Laccase (isolated from *Polyporus pinsitus*) consistently improved the colour and aroma development at 10 U/g tea, especially at 40°C, whereas Laccase L603P (isolated from *Trametes* sp.) showed some potential only at 1000 U/g tea.

Thirteen commercial enzymes were evaluated on dried green tea (Table 3.12), with Depol™ 670L, Pectinex® Ultra SP-L and Depol™ 692L increasing both the SS and TP content by more than 10%. For Pectinex® Ultra SP-L and Depol™ 670L, the %TP/SS ratio was more than 10% lower than for the control treatment, suggesting the additional SS extracted had a low phenol content. PpL Laccase decreased the TP content, but improved the extract colour at a dosage of 1U/g tea.

Table 3.11 Colour and aroma development of green tea treated with commercial enzymes.

Enzyme	Time (hr)	Dosage*	Temp. (°C)	Result
PpL Laccase	3	1	40	Good colour, a bit less green aroma
PpL Laccase	6	1	40	Good colour, similar to 3 hr
PpL Laccase	1.5	10	40	Underfermented, colour very dark
PpL Laccase	1.5	10	50	Less green than 40°C, colour slightly lighter
PpL Laccase	3	10	35	Sweet aroma with hint of green. Dark brown colour
PpL Laccase	3	10	40	More fermented than 35°C
PpL Laccase	3	10	40	More fermented than 50°C, aroma better, colour darker
PpL Laccase	3	10	50	Sweet aroma developing, not as dark as 40°C
Laccase L603P	4	1000	40	Best, but still underfermented. Colour good
Suberase	4	10	40	Strange aroma
Tannase C	4	1	40	Best of all tannases
Tannase P	4	5	40	Still green, other sweet aroma present, not characteristic
Depol 692L	3	10 (µl /g tea)	40	Sour aroma
Ultraflo	3	2	40	Similar to control
Ultraflo	3	2	50	Less underfermented, but not characteristic Rooibos aroma
Ultrazym P	4	10	40	Green

*Dosage is defined as U/g tea unless stated otherwise

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Table 3.12 Change in yield of %SS, TP and colour of green tea treated with commercial enzymes. Deviations of more than 10% from the respective controls are indicated in bold.

Treatment	Dosage (U/g tea or µl/g tea)	%SS (g/100 g dry leaves)	TP (mg GAE/ g tea)	%TP/ SS	Colour - Rooibos extract			Colour - Rooibos leaves		
					L*	a*	b*	L*	a*	b*
Control - Buffer pH 3.5		16.45	4.76	28.95	88.35	-4.74	47.40	33.31	9.49	22.60
Control - Buffer pH 5.5		17.29	4.32	24.96	84.53	-1.15	73.65	31.70	12.70	24.35
Control - Buffer pH 6.5		18.00	4.13	22.95	82.86	1.57	79.88	31.96	13.80	24.76
Cellulase 13L	1 U	17.45	4.29	24.59	84.88	-0.26	81.14	29.66	12.82	22.00
		105 ± 3.19%	104 ± 4.28%	99%	99%	**29%**	101%	102%	102%	**145%**
Cellulase 13L	10 U	17.80	4.04	22.66	83.48	-0.61	78.74	29.97	14.07	21.71
		104 ± 4.73%	100 ± 1.77%	96%	99%	**56%**	101%	99%	**111%**	97%
Depol 112L	0.25 µl	16.57	4.01	24.19	84.31	-0.65	80.47	28.99	12.23	20.44
		98 ± 1.27%	98 ± 0.95%	100%	100%	95%	99%	98%	97%	95%
Depol 112L	2.5 µl	18.46	3.89	21.37	84.94	-1.46	77.64	29.78	12.31	21.11
		108 ± 2.64%	99 ± 3.11%	92%	100%	**122%**	96%	99%	97%	95%
Depol 670L	20 µl	21.83	4.68	21.45	85.27	-2.72	64.89	32.92	11.31	25.15
		133 ± 2.02%	**117 ± 4.85%**	**88%**	100%	**124%**	94%	100%	91%	101%
Depol 740L	0.1 U	16.89	4.15	24.55	84.87	-1.67	70.03	31.08	12.37	23.36
		98 ± 0.88%	96 ± 1.14%	98%	100%	**145%**	95%	98%	97%	96%
Depol 740L	1 U	17.81	3.97	22.29	84.02	-0.74	80.76	30.50	12.85	22.28
		104 ± 3.49%	101 ± 1.97%	97%	100%	**139%**	98%	103%	101%	102%

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Table 3.12 (continued)

Treatment	Dosage (U/g tea or µl/g tea)	%SS (g/100 g dry leaves)	TP (mg GAE/ g tea)	%TP/ SS	Colour - Rooibos extract			Colour - Rooibos leaves		
					L*	a*	b*	L*	a*	b*
Fructozyme	1 U	17.09	4.32	25.30	85.21	-0.45	80.56	30.64	12.52	22.53
		103 ± 3.18%	105 ± 2.61%	102%	100%	**51%**	100%	105%	100%	**149%**
Fructozyme	10 U	16.35	3.88	23.76	84.90	-1.55	76.21	30.66	12.57	22.26
		96 ± 4.78%	96 ± 2.58%	100%	101%	**143%**	98%	102%	99%	99%
Glucanase 5XL	1 U	17.14	4.11	23.96	85.70	-0.84	77.51	29.23	12.49	21.19
		103 ± 2.62%	99 ± 1.43%	96%	100%	95%	97%	100%	100%	**140%**
Glucanase 5XL	10 U	17.14	4.08	23.79	84.09	-1.06	78.81	30.11	12.28	21.66
		103 ± 3.48%	104 ± 4.61%	101%	101%	98%	101%	102%	97%	102%
Laccase L603P	1 U	17.06	3.94	23.13	84.47	-0.88	78.66	30.35	12.35	22.09
		101 ± 2.27%	97 ± 3.82%	96%	100%	**128%**	97%	102%	98%	103%
Laccase L603P	10 U	16.85	4.07	24.16	83.12	0.43	83.34	29.38	12.87	21.02
		101 ± 3.51%	104 ± 2.78%	103%	99%	**-39%**	107%	100%	102%	99%
Lactase	1 U	17.04	4.00	23.52	84.41	-0.74	79.18	28.77	12.32	20.84
		101 ± 4.24%	98 ± 1.78%	97%	100%	108%	98%	97%	97%	97%
Lactase	10 U	16.99	4.23	25.03	84.62	-1.00	79.46	29.69	12.80	21.71
		102 ± 1.98%	108 ± 1.00%	106%	101%	92%	102%	101%	101%	103%
Pectinex (pH 3.5)	1 U	16.99	4.75	27.95	88.72	-4.70	46.60	34.85	9.61	24.40
		103 ± 2.38%	100 ± 4.13%	97%	100%	99%	98%	105%	101%	108%
Pectinex (pH 3.5)	260 U	21.49	5.30	24.66	85.16	-3.75	37.47	34.26	8.75	23.36
		131 ± 2.23%	**111 ± 1.09%**	**85%**	96%	**79%**	**79%**	103%	92%	103%

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Table 3.12 (continued)

Treatment	Dosage (U/g tea or µl/g tea)	%SS (g/100 g dry leaves)	TP (mg GAE/ g tea)	%TP/ SS	Colour - Rooibos extract			Colour - Rooibos leaves		
					L*	a*	b*	L*	a*	b*
PpL laccase	1 U	16.54	3.46	20.87	76.78	7.66	88.27	27.18	12.65	18.64
		96 ± 4.95%	**80 ± 4.40%**	**84%**	91%	**-665%**	**120%**	**86%**	100%	**77%**
PpL laccase	1 U	16.89	2.37	14.04	78.04	6.93	89.69	27.60	12.47	19.07
		95 ± 3.46%	**78 ± 1.51%**	**82%**	93%	**-547%**	**124%**	**87%**	105%	**81%**
Suberase	2 U	16.69	4.33	25.93	84.18	-0.70	75.29	31.15	12.58	23.16
		97 ± 1.65%	100 ± 1.90%	104%	100%	**61%**	102%	98%	99%	95%
Depol 692L	10 µl	20.63	4.90	23.74	84.13	-1.07	77.98	29.98	12.58	22.13
		121 ± 3.25%	**121 ± 2.82%**	100%	100%	98%	100%	99%	99%	98%
Depol 692L	100 µl	28.27	5.80	20.49	81.49	-0.40	74.32	30.41	11.74	21.78
		166 ± 3.38%	**147 ± 3.13%**	**89%**	96%	**34%**	92%	101%	92%	98%
Ultraflo (pH 6.5)	0.009 U	16.96	3.33	19.64	82.72	1.20	79.59	29.63	12.70	20.91
		94 ± 2.61%	109 ± 2.09%	**116%**	100%	**84%**	99%	100%	103%	97%
Ultraflo (pH 6.5)	1 U	19.62	4.27	21.80	82.03	1.84	79.65	30.59	13.27	22.60
		109 ± 3.84%	103 ± 2.54%	95%	99%	**117%**	100%	96%	96%	91%
Ultraflo (pH 5.5)	0.01 U	14.74	3.82	25.95	86.40	-0.28	32.05	35.02	10.00	22.41
		93 ± 3.56%	93 ± 5.08%	100%	100%	101%	99%	101%	**86%**	99%
Ultraflo (pH 5.5)	0.1 U	14.52	3.86	26.61	86.32	-0.29	30.73	36.17	9.98	23.07
		91 ± 4.87%	94 ± 1.88%	103%	100%	104%	95%	104%	**86%**	102%
Ultraflo (pH 5.5)	1 U	15.65	3.68	23.53	86.29	-0.27	31.52	35.89	10.13	23.36
		98 ± 6.28%	**89 ± 2.73%**	91%	100%	96%	98%	103%	**87%**	103%

(1) All % are relative to respective controls (data only shown for one example of controls).
(2) All treatments were done at pH 5.5 unless stated otherwise.

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Depol^{TM} 670L substantially increased the two major antioxidants, aspalathin and nothofagin (increase by 38% and 90%, respectively), but the levels of some of the minor compounds decreased markedly (Table 3.13), contributing to a lower than expected %TP/SS ratio. Nevertheless, the flavonoid content of the TP was increased by 21%. Given the fact that this treatment increased the yield in SS by 33% (Table 3.12), it indicates the extraction of both active and inactive compounds. Pectinex Ultra SP-L was not as effective, but still increased the %Total Flavonoids/TP ratio by 18%.

The commercial enzymes that showed potential for extraction of additional SS and TP from green tea were further evaluated in different combinations on freshly cut Rooibos plant material (Table 3.14). Enzyme cocktails #3 and #4 with varying laccase dosages (250 and 500 U/g tea, respectively) had no effect on the yield in SS. However, the TP and antioxidant content decreased significantly with cocktail #4 (higher laccase concentration) and longer treatment time having a greater effect. Both the cocktails had a significant impact on the extract colour with a more profound effect at increasing treatment times (indicated by a decline in the L* value, more black components and an increase in the a* and b* values ,more red and yellow components, respectively). However, the cocktails also had a marked effect on the leaf colour suggesting that the laccase treatment resulted in the polymerisation of phenols to such an extent that the products became insoluble. The effect of laccase on the colour development of green tea was clearly visible when the samples treated with or without laccase were compared (Figure 3.1).

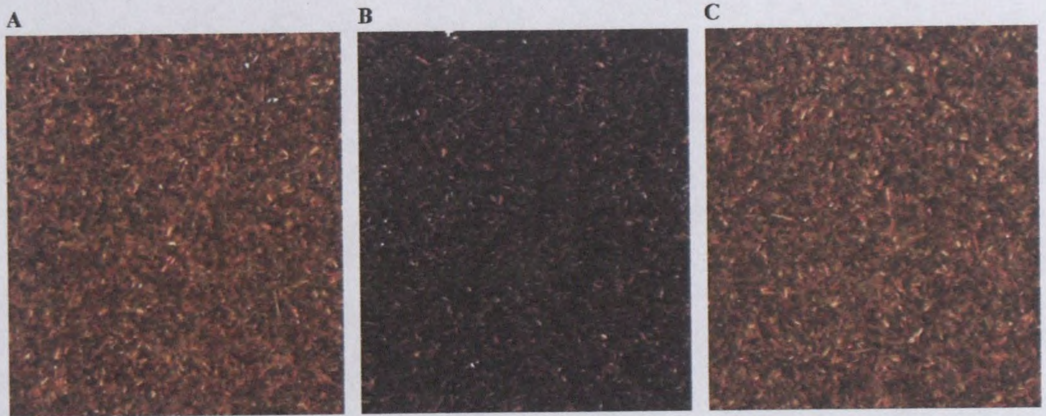

Figure 3.1 Examples of dried green tea treated with **(A)** Control – citrate buffer pH 5.5, **(B)** Cocktail #2 (includes 1002 U Laccase L603P per gram tea) and **(C)** Cocktail #6 (no laccase) after treatment for 2 hrs at 40°C.

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Table 3.13 Effect of enzyme treatment on the release of ten different antioxidants from green Rooibos tea. Results are given as % deviation from the respective controls. Values in bold indicate statistically significant differences (at 5% level).

	% Deviation from control														
Enzyme	**Asp**	**Orient**	**Isoorien**	**Vitex**	**Noth**	**Isovitex**	**Isoquer/ Rutin**	**Quer**	**Luteol**	**Chrys**	**%Tot Flav/SS**	**% Tot Flav/TP**	**%TP/SS**	**μmol Trolox/g SS**	**μmolTrolox /g TP**
Control - pH 5.5	2.686	0.757	0.924	0.165	0.083	0.148	0.435	0.009	0.015	0.002	5.210	21.385	24.367	1408.478	5780.785
Depol 670L (20μl/ g tea)	3.696	0.573	0.638	0.133	0.158	0.130	0.129	0.011	0.011	0.002	5.469	25.781	21.438	1243.904	5854.035
	138%	**76%**	**69%**	**81%**	**190%**	**87%**	**30%**	**119%**	**73%**	103%	105%	**121%**	**88%**	**88%**	101%
Control - pH 3.5	8.115	0.919	1.156	0.163	0.387	0.169	0.172	0.006	0.003	0.003	11.091	38.360	28.971	1570.012	5425.278
Pectinex (260U/g tea)	8.732	0.721	0.908	0.103	0.473	0.142	0.096	0.007	0.003	0.002	11.185	45.438	24.633	1300.495	5303.722
	108%	**78%**	**79%**	**63%**	**122%**	**84%**	**56%**	**127%**	**83%**	**86%**	101%	**118%**	**85%**	**83%**	98%

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

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Table 3.14 Change in yields of %SS, TP, antioxidants and colour of green tea treated with enzyme cocktails.

Treatment	%SS (g/100 g dry leaves)	TP (mg GAE/ g tea)	%TP/ SS	AOX (μmol Trolox/g SS)	Colour - Rooibos extract			Colour - Rooibos leaves		
					L*	a*	b*	L*	a*	b*
Control - pH 5.5 (2hr)	18.30	3.68	20.13	1349.77	87.55	-0.34	30.53	37.66	8.61	24.65
Control - pH 5.5 (3hr)	18.24	3.78	20.75	1412.60	87.13	-0.26	30.84	36.11	9.33	24.01
Control - pH 5.5 (4hr)	19.53	3.93	20.11	1385.82	86.95	-0.18	32.00	36.45	9.60	24.36
Cocktail #3 (2hr)	19.51	3.24	16.60	947.97	79.13	5.55	76.55	29.69	13.58	22.55
	107 ± 3.71%	**88 ± 3.82%**	**82%**	**70 ± 1.39%**	**90%**	**-1633%**	**251%**	**79%**	**158%**	91%
Cocktail #3 (3hr)	19.85	3.16	15.91	853.06	77.93	6.82	79.14	28.04	13.66	21.35
	109 ± 2.82%	**84 ± 2.37%**	**77%**	**60 ± 1.46%**	**89%**	**-2621%**	**257%**	**78%**	**146%**	**89%**
Cocktail #3 (4hr)	20.77	3.36	16.17	832.92	77.26	8.13	81.88	28.34	14.22	21.33
	106 ± 1.00%	**85 ± 1.99%**	**80%**	**60 ± 1.40%**	**89%**	**-4515%**	**256%**	**78%**	**148%**	**88%**
Cocktail #4 (2hr)	19.14	2.87	14.98	665.26	74.67	9.09	80.07	25.30	12.98	19.60
	105 ± 0.90%	**78 ± 1.35%**	**74%**	**49 ± 1.47%**	**85%**	**-2675%**	**262%**	**67%**	**151%**	**80%**
Cocktail #4 (3hr)	18.74	2.85	15.23	805.16	75.59	7.25	78.87	26.45	12.78	19.95
	103 ± 0.06%	**75 ± 1.12%**	**73%**	**57 ± 2.13%**	**87%**	**-2789%**	**256%**	**73%**	**137%**	**83%**
Cocktail #4 (4hr)	19.80	2.63	13.29	668.98	75.12	8.33	78.98	24.07	13.33	19.43
	101 ± 3.17%	**67 ± 2.10%**	**66%**	**48 ± 1.16%**	**86%**	**-4628%**	**247%**	**66%**	**139%**	**80%**

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Discussion

Cellulase is the major cell wall polysaccharide and consists of a linear polymer of β-1,4-linked D-glucose residues (De Vries and Visser, 2001). The second most abundant organic structure in the plant cell wall polysaccharides are the heterogeneous polysaccharides called hemicelluloses. Xylan is the major hemicellulose polymer in cereals and hardwood. Xylan consists of a β-1,4-linked D-xylose backbone and can be substituted by various side groups such as L-arabinose, D-galactose, acetyl, feruloyl, *p*-coumaroyl and glucoronic acid residues. Cereal xylans contain large amounts of L-arabinose and are therefore referred to as arabinoxylans. Other hemicellulolytic structures in cell walls are galactomannans and galactoglucomannans, which are the major hemicellulose fraction of gymnosperms, representing 12 to 15% of the cell wall biomass. Galactomannans are most frequently found in the family *Leguminosae*, in which it represents 1 – 38% of seed dry weight. This correlates well with the polysaccharide fractions found in green Rooibos tea. The major fraction is cellulose (~66%) with the arabinoxylan fraction (arabinose and xylose) representing 24% of the dry weight. Since the Rooibos plant is a legume, the mannose and galactose fractions could be considered as galactomannan (representing ~6% of dry weight), with rhamnose and fucose being minor sugars. It is therefore not surprising that enzyme cocktails containing cellulases and xylanases are the ones that consistently increased the yield in SS on all three Rooibos substrates, i.e. spent, fermented and green tea.

The various commercial enzymes were evaluated on spent tea to determine which enzymes could release additional SS and/or TP from tea that has already gone through an extraction process and would therefore be more accessible to enzymes. Depol™ 740L, Depol™ 670L, Ultraflo® L, Pectinex® Ultra SP-L, Depol™ 692L, Depol™ 112L and Cellulase 13L all increased the yield in SS considerably. However, it was only Depol™ 740L and Depol™ 670L that showed a dosage-dependent effect, suggesting that the reaction was already saturated at the lower dosages for the other enzymes.

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The enzymes that increased the yield in SS from spent tea had the same effect on fermented tea, except for Depol^{TM} 670L that was dosage-dependent and Pectinex® Ultra SP-L that increased in the yield in SS at a lower dosage than for spent tea. It is clear that the spent tea material that received a "pre-treatment" during conventional hot water extraction was more accessible to the hydrolases and more SS were released by the enzymes, relative to the treatments of fermented tea. However, most of the commercial enzymes that significantly improved the release of SS on fermented tea decreased the antioxidant content of the extract due to the extraction of inactive compounds. This problem may be overcome if lower enzyme dosages are used that will still improve the SS yield, but with no or minor loss in antioxidant content. For example, Depol^{TM} 740L and Depol^{TM} 112L both increased the antioxidants extracted from fermented tea at the lower dosage to a greater extent.

When the most effective enzymes were evaluated on "waste" fermented tea in industrial simulations, it increased the yield in SS without greatly influencing the TP content. However, the increase in the yield in SS was lower than for the corresponding laboratory treatments since dH_2O instead of citrate buffer was used, and the waste fermented Rooibos tea had a higher stem content. Although similar trends were observed for the extraction of SS, proper industrial evaluations on larger batches need to be done to determine the cost-effectiveness of the bioprocessing.

On green tea, Depol^{TM} 670L, Pectinex® Ultra SP-L and Depol^{TM} 692L increased both the yield in SS and TP with the higher dosages of Pectinex® Ultra SP-L and Depol^{TM} 692L showing a stronger effect. However, these enzymes showed no significant improvement in the colour of the extract. Significant changes in the extract colour was only evident when laccases were present, with PpL Laccase resulting in a marked improvement in extract colour, but also considerably decreases the TP content. Laccase L603P only improved the extract colour when freshly cut Rooibos plant material was treated at a dosage of 1002 U/g tea (Table 3.11). This also suggests that treatments with laccases may reduce the fermentation time of green tea to 2 hrs, a considerable saving in view of the current processing time of up to 16 hrs.

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All the laccases evaluated on spent tea markedly decreased the yield in SS and TP content of the extract. This was also the case with fermented tea where cocktail #1 and #2 decreased the antioxidant content, as well as the yield in SS and TP. This may indicate that excessive dosages of laccase may result in the polymerisation of components that render them insoluble, while polymerisation of phenols may lead to a decrease in antioxidants.

When green tea was treated with cocktail #3 and #4 (both containing Laccase L603P at dosages of 250 U/g tea and 500 U/g tea, respectively), there was a significant decrease in TP and antioxidant content, as well as a marked increase in both extract and leaf colour. However, the colour development was much darker than what is desired for the normal red-brown colour of Rooibos tea (Figure 3.1). An interesting observation is that this darker colour was achieved at a fraction (250 and 500U/g tea) of the Laccase L603P dosage required for colour development. This may be due to the synergistic effect of all the enzymes contained in cocktails #3 and #4. The desired colour and a smaller or no decrease in TP content and antioxidant content may be achieved at even lower laccase dosages in the cocktails.

Two previous studies on the effect of fungal enzymes on black tea evaluated the parameters theaflavin (TF), thearubigin (TR), highly polymerized substances (HPS), total liquor colour (TLC), dry matter content (DMC) and total soluble solids (TSS). The tea was treated according to conventional methods, i.e. withered, CTC, sprayed with enzyme and then fermented and dried. When crude pectinase preparations from three *Aspergillus* sp. were applied at 2.5 IU/750g tea leaf, increases of 0.91% and 6.21% were found for TSS, while the purified enzyme (25 IU/750g tea leaf) showed increases of 1.31%, 1.04% and 4.02% (Angayarkanni et al., 2002). Murugesan et al. (2002) applied crude and purified cellulase, pectinase and xylanase preparations (20 IU/750g tea leaf, 24 IU/750g tea leaf and 20.75 IU/750g tea leaf, respectively) and found an increase of 1.31% in TSS for the purified cellulase treatment, while the crude extracts of pectinase and xylanase increased the TSS by only 0.003% and 0.001%, respectively. However, the purified cellulase treatment was the best and increased the TF (52.4%), TR (8.89%), HPS (24.8%) and TLC (20.2%).

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In conclusion, it has been shown that laccases consistently improves colour and aroma development of Rooibos tea substrates. This effect is greater at 50°C than at 40°C, but the difference may not be worth the additional cost required to ferment the tea at the higher temperature. Depol™ 670L (cellulase, pectinase and ferulic acid esterase), Pectinex® Ultra SP-L (pectinase) and Depol™ 692L (cellulase, ferulic acid esterase) consistently improved the release of SS while retaining most of the antioxidants when applied at lower dosages. It is also apparent that a combination of enzymes are more effective since no one particular enzyme improved all the parameters. For example, cocktail #10 containing β-glucanase, xylanase and pectinase improved both the yield in SS and TP. This is also shown in the fact that less laccase is required in the cocktails to achieve the same effect as the higher dosage of laccase alone.

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Chapter 4

Literature Review:

Laccases: Distribution, Properties and Industrial Applications

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4.1 Introduction

Laccases proved to be the best enzyme, individually or in combination with other enzymes, in improving the colour and aroma development from green Rooibos tea. However, during the course of this study Novozymes suspended their development of PpL laccase and further evaluations with this enzyme would have been redundant. Laccase L603P required higher dosages to obtain the same effect as PpL laccase, making it a very expensive treatment. However, the successful expression of a food-grade laccase for possible commercial production in South Africa could reduce the cost of treatment for Rooibos tea. In this chapter, the distribution, properties, reaction mechanisms and current and future industrial applications of laccase will be discussed, with the cloning and expression of the *Pleurotus ostreatus lacA* gene in *Aspergillus niger* discussed in the following chapter.

4.2 Laccase

4.2.1 Distribution

The blue copper oxidases include ascorbate oxidase, laccase and ceruloplasmin. These multi-copper enzymes catalyse the four-electron reduction of molecular oxygen to water with simultaneous one-electron oxidation of the substrate. The blue copper oxidases are a subgroup of the copper proteins and their metal ions were classified consequent to their distinct spectroscopic properties. Ascorbate oxidase is found in higher plants with cucumber (*Cucumis sativus*) and green zucchini squash (*Cucurbita pepo medullosa*) being the most common sources. The biological role of ascorbate oxidase is still unclear. It is thought to be involved in plant growth due to its abundance in the cell wall fraction and a role in plant respiration has also been suggested. Ascorbate oxidase is highly specific for reducing ascorbate and other substrates containing a lactone ring with an enediol group next to a carbonyl group (Messerschmidt and Huber, 1990).

All mammals and birds studied contain ceruloplasmin in their sera. Various functions have been attributed to ceruloplasmin, including mobilisation, transport and

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homeostasis of copper, ferroxidase, amino oxidase and perhaps superoxide dismutase activity. This enzyme is also an acute-phase protein in the inflammatory response. Ceruloplasmin is lacking in Wilson's disease, an autosomal recessive trait with a deficiency in copper metabolism. Ceruloplasmin catalyses the oxidation of a wide variety of organic and inorganic compounds, including amines like *p*-phenylenediamine, dopamines and serotonin, and the catechol derivatives, aminophenols and Fe(II) (Messerschmidt and Huber, 1990).

Laccase (EC 1.10.3.2, *p*-diphenol: dioxygen oxidoreductases) uses molecular oxygen to oxidise various aromatic and non-aromatic substances by a radical-catalysed reaction mechanism. Laccases have been found in eukaryotes (fungi, plants and insects), but recently strong evidence has been presented for their extensive distribution among prokaryotes (Alexandre and Zhulin, 2000; Mayer and Staples, 2002).

Laccase was first described by Yoshida in 1883 in the latex of *Rhus vernicifera* (lacquer tree) (Benfield et al., 1964), a member of the family *Anacardiaceae*. This entire family appears to contain laccase in the resin ducts and in the secreted resin of the plant. Two further examples from this family having been shown to contain laccases are *Schinus molle* and *Melanorrhea* (Bar-Nun et al., 1981; Mayer and Harel, 1979). The prevalence of laccases in higher plants seem to be far more limited than in fungi and their characterisation less convincing. A *p*-diphenol oxidase, which may be a laccase, has been shown to be present in many gymnosperms, particularly in species of the *Podocarpaceae*. Further evidence of laccases in plants was found in *Acer pseudoplatanus*, *Pinus tida* (eight laccases expressed in the xylem tissue), in the leaves of *Aesculus parviflora* and the xylem tissue of *Populus euramericana* have five distinct laccases (Mayer and Harel, 1979; Mayer and Staples, 2002).

Evidence for the presence of proteins with typical characteristics of the multi-copper oxidase family in prokaryotes has been mounting. They have been reported in Gram-negative and Gram-positive bacteria. The first reports of laccases in actinomycetes were based on rather non-specific substrate reactions, but have now been confirmed for *Streptomyces griseus*. A strain of *Pseudomonas maltophila* has a nucleoside oxidase that exhibits laccase-like activity in the presence of nucleosides. The first

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credible data for a prokaryotic laccase activity were presented for *Azospirillum lipoferum*. Table 4.1 shows some prokaryotes with laccase-like enzymes and their possible functions (Claus, 2003; 2004; Diamantidis et al., 2000).

Table 4.1 Occurence of some laccase-like proteins in prokaryotes (Adapted from Claus, 2003).

Species (gene)	Possible function
Aquifex aeolicus (sufI)	Cell division protein
Azospirillum lipoferum	Pigmentation
	Oxidation of phenolic compounds
	Electron transport
Bacillus sp. *(mnxG)*	Sporulation, Mn oxidation
Bacillus sphaericus	Sporulation, pigmentation
Bacillus subtilis (cotA)	Pigmentation of spores
	UV and H_2O_2 resistance
Escherichia coli (yacK)	Cu efflux
	Oxidation of phenolate-siderophores
	Ferrooxidase activity
Marinomonas mediterranea (ppoA)	Pigmentation
Oceanobacillus iheyensis (cotA)	Sporulation
Pseudomonas maltophila	Nucleoside oxidase activity
Pseudomonas putida (cumA)	Mn oxidation
Pseudomonas sp. *(CumA)*	Mn oxidation
Pseudomonas syringae (copA)	Cu resistance
Pyrobaculum aerophilum (pae1888)	Unknown
Streptomyces antibioticus	Phenoxazinone synthesis
Streptomyces griseus (epoA)	Pigmentation, morphogenesis
Xanthomonas campestris (copA)	Cu resistance

Laccases are widespread among fungi and have been found in various classes. This includes the ascomycetes *A. nidulans*, *Neurospora crassa* and *Podospora anserine*, the deuteromycete *Botrytis cinerea*, and several basidiomycetes. Schanel (1967) reported that of 130 wood-rotting basidiomycetes tested, 87 gave a positive polyphenol oxidase reaction. The basidiomycetes such as *Collybia velutipes*, *Fomes annosus*, *Fomes fomentarius*, *Lentinula edodes*, *Phanerochaete chrysosporium*, *Pholiota mutabilis*, *Pleurotus ostreatus*, *Poria subacida*, *Sporotrichum pulverulentum*, *Trametes sanguinea* and *T. versicolor* are all known as

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lignin degraders. Laccase has also been shown to be present in the ectomycorrhizal fungi *Thelephora terrestris* (Bollag and Leonowicz, 1984; Burke and Cairney, 2002). Table 4.2 shows some laccases that have been isolated and characterised from fungi.

Most of the reports on laccases in fungi refer to it as an extracellular enzyme, but several suggest the existence of intracellular laccases. It is generally true that laccase is secreted by the hyphae of fungi such as *Botrytis* and *Polyporus* but in *Podospora* the enzyme is clearly intracellular. It has also been suggested that *T. versicolor* and the chestnut blight fungus *Cryphonectria parasitica* may contain intracellular laccase. In *Neurospora* and *Glomerella* there is both an intracellular and extracellular laccase. In ascomycetes, the intracellular enzyme may be related to colour formation during fruiting. The intracellular laccase have been distinguished from their extracellular equivalent on the basis of pH optimum, isoelectric point and/or molecular mass, but all seem to have similar substrate ranges. It is yet unclear whether the intracellular activities signify actual intracellular laccases, extracellular laccases in the process of being exported or a combination of the two. Fungal laccases have not yet been reported to be other than cytoplasmic, similar to higher plant laccases (Burke and Cairney, 2002; Mayer and Harel, 1979).

4.2.2 Regulation of laccase expression in fungi

The regulation of laccase production in fungi is complex and may vary between different taxa. Zhao and Kwan (1999) reported the production of laccase during various developmental stages, with laccase activity in the mushroom cap being the highest, followed by the primordia and then the stalk. Tyrosinase, manganese peroxidase and lignin peroxidase activities were not present at various fruiting stages. It has been reported that increased nitrogen availability led to increasing levels of laccase in *Trametes pubescens* and *T. versicolor*. This substantiates the importance of the nitrogen source and high nitrogen levels for the formation of laccase. However, some reports dispute this since it has been found that laccase activity increased under nitrogen limiting conditions since it has been reported that an increase in the C:N ratio increased laccase activity in *Pycnoporus cinnabarinus* (Burke and Cairney, 2002; Collins and Dobson, 1997; Galhaup et al., 2002a)

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Table 4.2 Characteristics of some laccases isolated from fungi.

Organism	Enzyme	Carbohydrate %	Molecular mass (kDa)	pI	pH (substrate)	Temperature	References
Pleurotus ostreatus	POXA1	3	61	6.7		45 – 65	Palmieri et al., 1997
	POXA2	9	67	4.0		25 -35	
Pleurotus eryngii	Laccase I	7	65	4.1	4.0	65	Munoz et al., 1997
	Laccase II	1	61	4.2	3.5	55	
Chaetomium thermophilium			77	5.1	6 - 8	50 - 60	Chefetz et al., 1998
Panaeolus sphinctrinus			60	<3.5	3.0 (ABTS) 7.0 (2,6-DMP)		Heinzkill et al., 1998
Panaeolus papilionaceus			60	≤3.6	3.0 (ABTS) 7.0 (2,6-DMP)		Heinzkill et al., 1998
Coprinus friesii			60	3.5	5.0 (ABTS) 8.0 (2,6-DMP)		Heinzkill et al., 1998
Polyporus pinsitus			66	≤3.5	3.0 (ABTS) 5.0 (2,6-DMP)		Heinzkill et al., 1998
Coriolopsis rigida		9	66	3.9	2.5 (ABTS) 3.0 (2,6-DMP)		Saparrat et al., 2002
Rhizoctonia solani	r-lcc1		50 – 80	5.0	≤2.7 (ABTS) 6.0 (syringaldazine)		Wahleithner et al., 1996
	r-lcc4		70 – 85	7.5	≤2.7 (ABTS) 7.0 (syringaldazine)		
	wt-lcc4		66	7.5	≤2.7 (ABTS) 7.0 (syringaldazine)		
Trametes sp. strain AH28-2	Laccase A	11 - 12	62	4.2	4.5	50	Xiao et al., 2003
Trametes sanguinea M85-2		9.1	62	3.5	5.0	60	Nishizawa et al., 1995

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Srinivasan et al. (1995) reported that glucose repressed the laccase production in *P. chrysosporium* irrespective of the amount of nitrogen present. However, in the presence of the more recalcitrant microcrystalline cellulose, increasing nitrogen availability increased the laccase activity. Glucose repression was also shown to be the case in *T. pubescens* where the laccase formation only started after glucose was depleted from the medium. Glucose is often found to inhibit genes that are used in the metabolism of different carbon sources. This phenomenon (catabolite repression) is widely known in fungi and yeasts and is thought to be an energy saving response.

It has also been shown that the addition of various aromatic compounds have an effect on laccase production and activity. Collins and Dobson (1997) showed that the addition of 2,5-xylidine (XYL) and 1-hydroxybenzotriazole (HBT) induced the transcription of the laccase gene in *T. versicolor*, while ferulic acid and veratric acid had no effect. The addition of XYL and HBT also led to an increase in laccase activity for *Pleurotus sajor-caju*, which was also induced by ferulic acid and veratric acid. At very high concentrations, all of these compounds inhibited fungal growth. Of the four isozyme genes (Psc *lac1, 2, 3, 4*) isolated from *Pl. sajor-caju*, the *lac3* transcription appears to be constitutively expressed while transcription levels for the other three seemed to be differentially regulated by the above compounds (Soden and Dobson, 2001).

Of the seven aromatic compounds tested on *T. pubescens* by Galhaup et al. (2002a), only gallic acid and catechol slightly increased laccase activity, while *p*-anisidine and vanillic acid reduced laccase activity. Two sites matching the xenobiotic response elements (XRE) consensus sequence, TNGCGTG, are present in the region upstream from the *lac4* promoter in *Pl. sajor-caju*. The presence of these putative XRE elements suggests that the transcription of laccase genes may be activated by aromatic compounds. The absence of these putative XRE elements in the promoter regions of laccase genes in other fungal species may indicate that these genes are not induced by aromatic compounds, or that other unknown aromatic response element(s) could exist (Soden and Dobson, 2001).

Soden and Dobson (2001) showed that the addition of high copper and manganese concentrations increased laccase transcript levels and therefore laccase activity in

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Pl. sajor-caju. Galhaup et al. (2002b) also reported an increase in laccase activity in *T. pubescens* after addition of copper, but also saw an increase with the addition of 5μM Cd^{2+} and Hg^{2+}. However, at higher concentrations these heavy metals appear to be toxic for the fungus as seen from reduced fungal growth. Copper also increases the laccase activity in *N. crassa* and increased the laccase gene transcription in *T. versicolor* and *Pl. ostreatus*. A response element has been found in the promoter region of the *lac1* gene of the basidiomycete PM1(CECT 2971). This response element shows some similarity to the binding site for the ACE1 transcription factor in the *Saccharomyces cerevisiae SOD1* gene. This gene encodes for a Cu-Zn superoxide dismutase, which is regulated by copper and zinc. Given that laccases contain binding sites for copper ions that are essential for enzyme activity, free copper ions in the growth media could result in the induction of laccase in a analogous manner to how yeast respond to copper ion toxicity through the production of the copper-chelator Cu-metallothionein (Collins and Dobson, 1997; Huber and Lerch, 1987; Palmieri et al., 2000; Soden and Dobson, 2001).

Manganese is present in lignin which is primarily degraded by white rot fungi. The induction of manganese peroxidase (MnP) transcription has been reported. A number of putative metal response elements (MRE's) have been recognized in the promoter regions of the MnP and laccase of *Pl. ostreatus* (Faraco et al., 2003; Giardina et al., 1999). These putative MRE's conform precisely to the consensus sequence found in the promoters of metallothionein genes in higher eukaryotes. The expression of these genes are induced by a variety of heavy metals, with regulation via a metal-regulatory protein that functions as both a metal receptor and a *trans*-acting transcription factor. However, Chen et al. (2004) showed that despite the addition of copper or various aromatic compounds to defined medium, no transcripts were detected from the *lac4* gene of *Volvariella volvacea*.

4.2.3 Structure and properties

Laccases oxidise a phenolic substrate with the resultant reduction of oxygen to H_2O. This reaction is characteristic of laccase and is the primary requirement by which the enzyme is classified (Figure 4.1). Laccase can attack a very broad range of substrates

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and will oxidise any substrate with characteristics similar to a *p*-diphenol and all known laccases oxidise *o*-diphenols of the catechol type. Some fungal laccases can also oxidise monophenols such as cresol, and some are able to oxidise ascorbic acid, *m*-diphenols and *p*-phenylenediamine, but not tyrosine. However, these classifications are not always that clear (Mayer and Harel, 1979; Mayer and Staples, 2002). The melanogenic marine bacterium, *Marinomonas mediterranea*, expresses both an SDS-activated tyrosinase and a laccase. The laccase, which was heterologously expressed in *E. coli*, exhibited the typical copper-binding domains of laccases and two extra potential copper binding sites near the N-terminus. These sites may be responsible for the tyrosinase activity of the enzyme. Two more examples are *Filobasidiella neoformans* and *A. nidulans*, which may not have true laccases in that they do not belong to the same evolutive branch, regardless of their ability to utilise typical substrates (Sanchez-Amat and Solano, 1997; Sanchez-Amat et al., 2001; Valderrama et al., 2003).

R
HCOH
Laccase
O_2 $2H_2O$
CH_3O OCH_3
OH
R
HCOH
CH_3O OCH_3
O
Phenoxy Radical
O
CH_3O OCH_3
O
p-Quinone
R
C=O
CH_3O OCH_3
OH
C_α Carbonyl Formation
Polymerisation and Quinone Formation

Figure 4.1 Oxidation of phenols by laccase (adapted from Minussi et al., 2002).

Various techniques have been used to study the metal centers of fungal laccases, that seem to be relatively stable compared to the secondary structure of the protein.

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Bonomo et al. (2001) showed that under varying conditions (pH, temperature, ionic strength), 'heavy' changes often occurred with respect to native conformation, while the metal site geometric arrangements remained fairly unaltered. It is this secondary protein structure that can undergo changes thereby altering the substrate specificity. A multitude of substrates have been used to study laccase activity. It is proposed that the only conclusive proof of laccase activity is that it is able to oxidise quinol as determined by oxygen uptake, regardless what other substances are attacked. The use of syringaldazine and 2,2-azobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) should be done with caution since both are also oxidised by peroxidases in the presence of H_2O_2. Laccases show extreme diversity with regard to molecular weight, pH optima, substrate specificity etc., but it is unclear to what extent this diversity may be as a result of isolation and purification procedures (Mayer and Staples, 2002).

Fungal laccases often occur as multimeric complexes consisting of oligomerised isoenzymes. The molecular mass of these monomers is in the range of 50 – 100 kDa. The enzyme is a glycoprotein, which has a covalently linked carbohydrate moiety (10 – 45%), which may contribute to the high stability of the enzymes. A sugar content of 32 – 45% was reported for *Rhus*, 11% for *Neurospora*, 14% for *Polyporus* and 23% for *Podospora* laccases. The carbohydrate moiety consists of hexosamine, glucose, mannose and galactose in *Podospora* and *Polyporus*, while that of *Rhus* laccase includes hexosamine, frucose, mannose, galactose, glucose and arabinose (Claus, 2004; Mayer and Harel, 1979). For the catalytic activity the enzyme requires a minimum of four copper atoms per active protein unit and the copper atoms differ in their light absorbance and electron-paramagnetic behaviour (Claus, 2004). However, Palmieri et al. (1997) reported a "white laccase" in *P. ostreatus* that did not have the typical absorption around 600 nm and contained one copper, two zinc and one iron atom/each protein molecule.

Three types of copper exist in laccase, namely, type I is a paramagnetic "blue" copper with an absorbance at 610 nm (oxidised), type II is a paramagnetic "non-blue" copper and type III is a diamagnetic spin-coupled copper-copper pair with an absorbance at 330 nm (oxidised). Type I copper has a trigonal coordination, with two histidines and a cysteine as conserved equatorial ligands and one changeable position. This axial

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ligand is methionine in the bacterial and leucine or phenylalanine in fungal laccases (Figure 4.2). The blue colour of the type I copper is as a result of the strong electronic

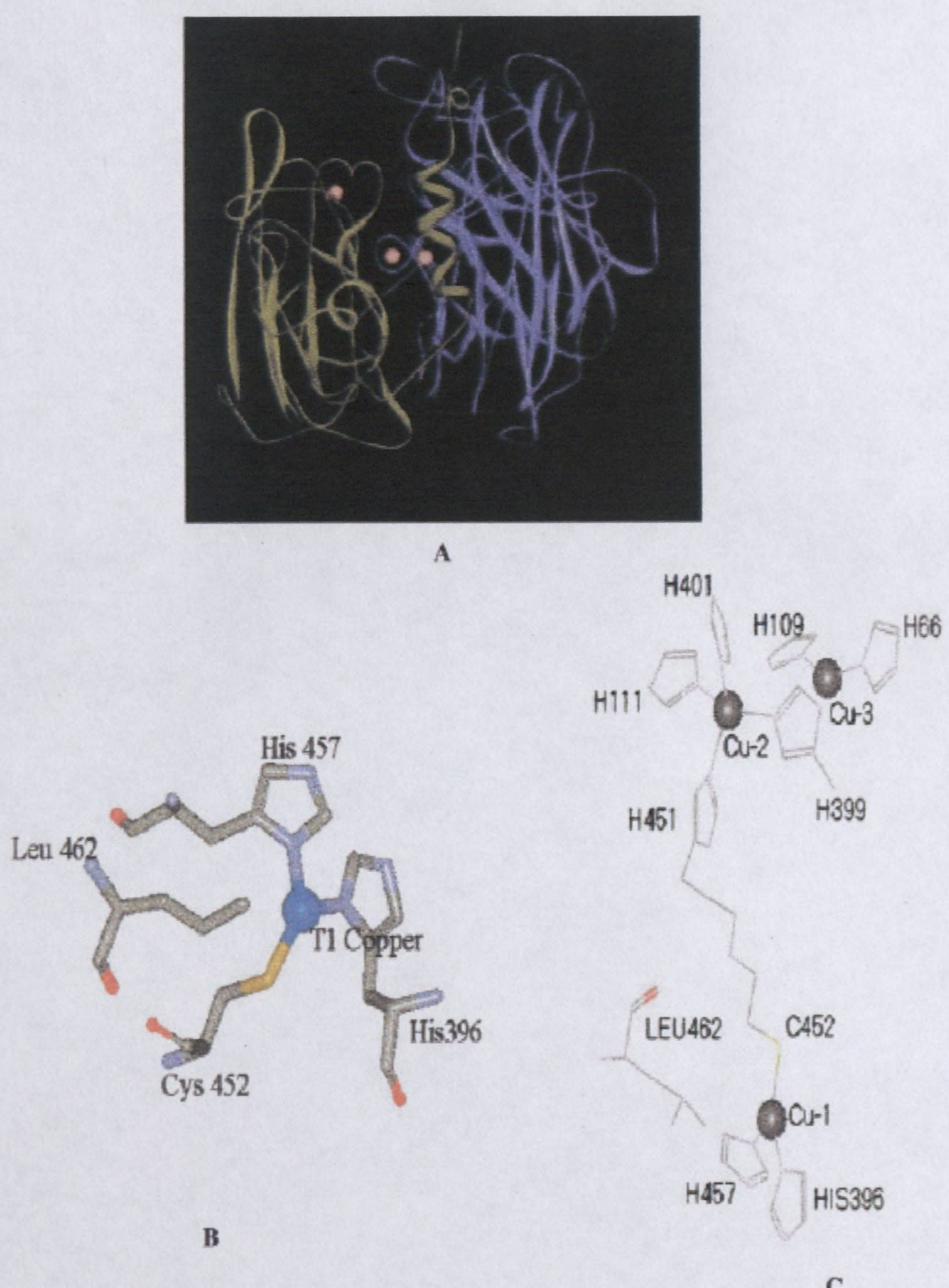

Figure 4.2 View of the ligands at T1 copper centers in *Coprinus cinereus.* (A) Three dimensional structure of backbone of entire domain of *C. cinereus* showing all the copper ions at T1 and T3 copper centers. (B and C) Amino acid ligands in the vicinity of the T1 copper center and the trinuclear copper center with T2 and T3 copper. For T1, copper His396, His457 and Cys452 are equatorial ligands while Leu462 is an axial ligand. The His451-Cys452 electron bridge is highlighted (Kumar et al., 2003).

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absorption of the covalent copper-cysteine bond, which is the site where substrate oxidation takes place. The type II copper is well positioned close to the type III copper. The type III has no EPR signal due to the anti-ferromagnetic coupling of the copper pair. The type II and type III copper form a trinuclear cluster and this is where the reduction of molecular oxygen and the release of water takes place (Claus, 2004).

Multiple sequence alignments of more than 100 laccases led to the identification of four ungapped sequence regions, L1 – L4, as the overall signature of laccases, discerning them within the broader class of multi-copper oxidases. The 12 amino acid residues in the enzymes serving as the copper ligands, are situated within these four identified regions of which L1 and L3 are distinctive of laccases. The amino acid ligands of the trinuclear cluster are the eight histidines. These occur in a highly conserved pattern of four HXH motifs. In one of these motifs, X is the cysteine bound to the type I copper, while each of the histidines is bound to one of the two type III coppers. Intraprotein homologies between signatures L1 and L3 and between L2 and L4 imply the incidence of duplication events (Kumar et al., 2003).

4.2.4 Reaction mechanism

The different copper centers of laccases drive electrons from a reducing substrate to molecular oxygen without liberating toxic peroxide intermediates. The enzyme oxidises the substrate molecules with type I copper by four step-wise transfers of one electron. Full reduction of the laccase requires four electrons. Through the type III copper pair, four electrons are transferred in two electron steps to oxygen. This is probably achieved via a peroxide intermediate. Reduction of oxygen and the resultant release of water occur at the type III copper (figure 4.3). The oxidation of substrates produces reactive radicals, which can undergo the following non-enzymatic reactions:

Cross-linking of monomers: The enzymatic oxidation of phenolic compounds and anilines by laccases generate radicals. These radicals can react with each other to form dimers, oligomers or polymers covalently coupled by C-C, C-O and C-N bonds. In the case of substituted compounds, the reaction can be accompanied by incomplete demethylations and dehalogenations.

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Degradation of polymers: Complex natural polymers (lignin, humic acids) are in part degraded by laccase. The reactive radicals created results in the cleavage of covalent bonds and the release of monomers.

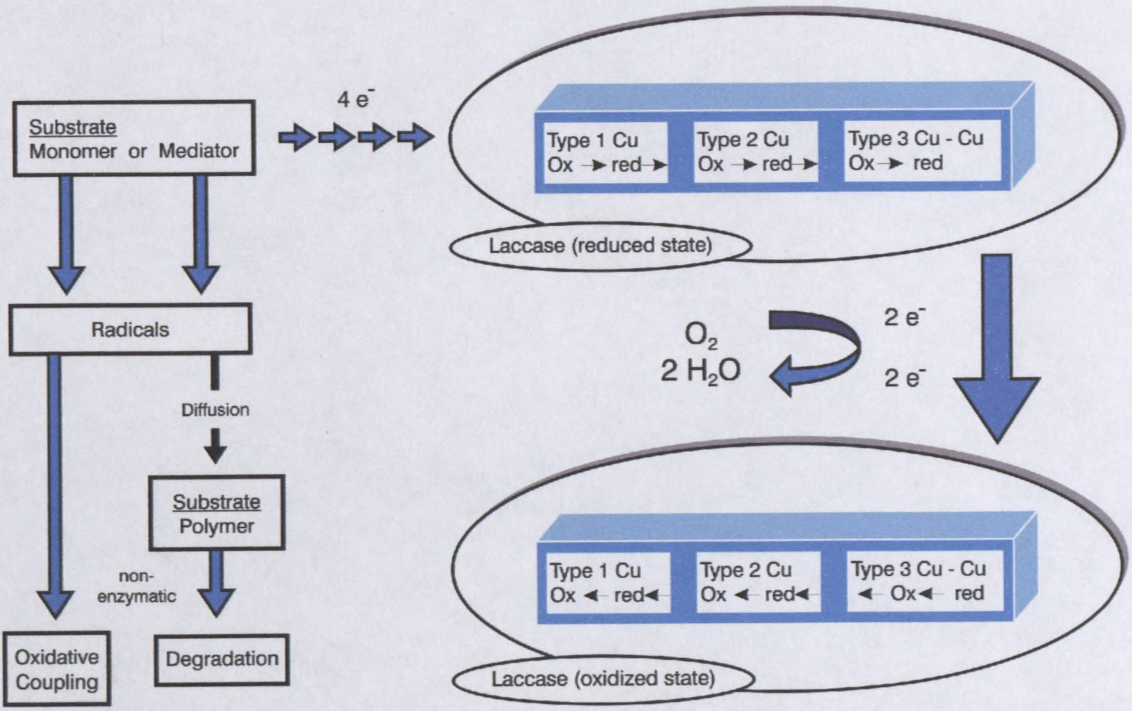

Figure 4.3 A schematic representation of the step-wise oxidation mechanism of laccase (Claus, 2003).

Ring cleavage of aromatics: There have been several reports of laccase-catalysed ring cleavage of aromatic compounds. This reaction can be applied in biotechnology for the degradation of xenobiotics and synthetic dyes (Claus, 2003; 2004; Mayer and Harel, 1979).

4.2.5 Physiological functions

4.2.5.1 Lignin degradation

Lignin is the second most abundant polymer in nature and a integral part of all higher plants. It is an important part of the biospheric carbon cycle and is degraded to various degrees by different microorganisms. Lignocellulosic materials are produced through photosynthesis and consists of 15 – 36% lignin. It is an amorphous phenylpropanoid polymer with arbitrary distribution of stable C-C and other linkages

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between the phenylpropane units. Due to lignin's structural complexity, it is somewhat resistant to microbial attack. Brown-rot fungi, which are unable to degrade lignin, do not secrete laccase extracellularly. White-rot fungi are well known for their ability to degrade lignin. Analyses of lignin residues after fungal attack have indicated that the extracellular degradation of lignin is not only oxidative, but also non-specific and non-stereoselective. Laccase and peroxidase utilise aromatic compounds as substrates to produce aromatic radicals or reactive oxygen species able to attack lignin non-specifically (Youn et al., 1995).

Due to the oxidising characteristics of laccase and the fact that it is produced during lignin degradation by some white-rot fungi, it has been proposed to play a part in lignin degradation by oxidising free phenolic groups to phenoxy cation radicals. When *Pleurotus* species were cultivated on lignin media, the tempo of lignin degradation correlates with the production of laccase, whereas *Pleurotus* mutants deficient of laccase degraded lignin poorly. This was also the case for a laccase-deficient mutant of *Sporotrichum pulverulentum* that was unable to degrade kraft lignin, whereas the laccase-positive revertant regained this ability. However, there are some examples that contradict the function of laccases in lignin degradation. When the laccase of *C. versicolor* was inhibited it was still able to degrade milled wood lignin. Despite being generally described as a white-rot fungus lacking a laccase activity, *P. chrysosporium* still has the ability to degrade lignin. It was therefore concluded that laccase is not necessary for lignin degradation (Burke and Cairney, 2002; Call and Mücke, 1997; Youn et al., 1995).

It has been reported that laccase can degrade non-phenolic lignin model compounds, in systems including naturally occurring or synthetic redox mediators. Redox mediators are defined as oxidisable, low molecular weight, laccase substrates that generate radicals that can diffuse away from the reactive site and act as oxidants for other compounds. Certain mediators can oxidise lignin, which would not be oxidisable by laccase alone. For example, *P. cinnabarinus* completely degrades lignin without producing other lignin-degrading enzymes. It produces a metabolite (3-hydroxyanthranilate) that acts as a redox mediator for the depolymerisation of non-phenolic lignin by laccase (Bourbonnais and Paice, 1990; Burke and Cairney, 2002; Call and Mücke, 1997). In the presence of primary substrates, the laccase from

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C. versicolor can generate Mn(III) chelates from Mn(II). The small size of Mn(III) chelates (malonate, oxalate, pyrophosphate) could allow these oxidants to penetrate regions of wood inaccessible to wood decaying enzymes (Youn et al., 1995).

4.2.5.2 Lignification

Lignin is formed through the oxidative polymerisation of monolignols within the plant cell wall matrix. Peroxidases are widespread in virtually all cell walls and have been held to be the major catalyst for this reaction. However, it has been reported that laccase secreted into the secondary walls of vascular tissue are equally capable of polymerising monolignols in the presence of O_2. One study showed that laccase from *Acer pseudoplatanus* was able to polymerise monolignols in the total absence of peroxidases, concluding that laccase was involved in the early stages of lignification, followed later by peroxidases (Mayer and Staples, 2002).

The laccase activity of *Pinus taeda* and *Zinnia elegans* were correlated with lignification. The laccase in *P. taeda* was found in the xylem, associated with the cell wall, present in lignifying cells and able to oxidise monolignols. The laccase of *Z. elegans* was active in the stem tissue and a laccase-like enzyme was found in the xylem of lignifying tobacco. Although, laccases have been found in plant tissue that undergoes lignification (Mayer and Staples, 2002), the role of laccase in lignification has been re-evaluated. Wallace and Fry (1999) studied the relative rates by which purified laccases and peroxidases were able to oxidise various phenolic substrates. They reported higher specific activities towards the tested phenols by peroxidases than laccases. It is not clear if specific activity alone is an adequate indicator for the role of the enzymes in lignification. Due to the differences between plant and fungal laccases, it is not expected that the fungal laccases would be involved in lignification (Mayer and Staples, 2002).

4.2.5.3 Wounding and herbivory

Laccase activity may be a factor in cell-wall reconstruction in regenerating protoplasts of higher plants. The laccase activity was measured in regenerating and non-regenerating protoplasts isolated from tobacco leaves (*Nicotiana tabacum*). During a

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six day period, the activity increased steadily in regenerating protoplasts while it was undetectable in non-regenerating protoplasts. Wounding of the leaf stimulated an instantaneous increase of laccase activity while peroxidase activity increased very slowly. Therefore, it could be concluded that laccase was the only effective polymerising enzyme during the first day of protoplast culture and could partake in the initial steps of healing wounded leaves, substituting for peroxidase activity in cell wall reconstitution before H_2O_2 became available (De Marco and Roubelakis-Angelakis, 1997). Constabel et al. (2000) cloned and characterised a PPO expressed in a wound induced hybrid poplar. They proposed that the enzyme played an important role in the defense of hybrid poplar against folivore insects. They also suggested that it may function in reducing additional herbivore damage rather than in wound repair.

4.2.5.4 Growth and development

Laccase activity has been linked with various growth and developmental stages of fungi, insects and bacteria. Laccase activity was only associated with rhizomorph growth in *Armillaria* sp. when the rhizomorphs were induced, and it was localised mainly in the rhizomorphs rather than in the mycelium. The appearance of rhizomorphs was preceded by that of laccase, and laccase activity increased and declined with rhizomorph growth rate. When laccase inhibitors were introduced, laccase activity as well as rhizomorph growth decreased while mycelial growth was uninhibited (Worrall et al., 1986). In *A. nidulans*, the laccase enzyme has been shown to be important for the production of the green spore pigment that gives a greater resistance to UV light (Clutterbuck, 1972). In a later study, two isozymes from *A. nidulans* were shown to be expressed during different sexual phases. Laccase I is expressed during asexual development and is needed for formation of a green pigment from a yellow polyketide derived precursor. The laccase II is exclusively expressed during cleistothecial development and may be involved in hyphal aggregation or pigment conversion (Burke and Cairney, 2002).

In *P. cinnabarinus*, laccase has been shown to interact with cellobiose dehydrogenase (CBH) in pigment formation. In the presence of glucose as carbon source, *P. cinnabarinus* produces a red pigment, cinnabarinic acid (CA), which is formed by

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laccase catalysed oxidation of 3-hydroxyanthranilic acid (3-HAA). Laccase oxidation products of the metabolite 3-HAA were identified as *in vivo* electron acceptors for *P. cinnabarinus* CDH. CA is the typical phenoxazinone pigment in *P. cinnabarinus* which is formed from the precursor 3-HAA by a laccase-mediated oxidation. In the absence of cellulose, laccase oxidises 3-HAA to CA while in the presence of cellulose or cellodextrins, CDH is created which catalyses the reduction of 3-HAA oxidation intermediates. As long as a appropriate electron donor, preferably cellobiose, is present for the oxidative half-reaction, this cycle is sustained indefinitely, which prevents the build up of CA. This seems to be consistent with the fact that wood colonised by *P. cinnabarinus* has the typical white rot appearance. CA and other related phenoxazinones do not amass until fruiting bodies are formed (Temp and Eggert, 1999).

The transcript for the laccase gene from *Volvariella volvacea* was detected only at a late stage of sporophore formation, which may indicate that gene expression is important for fruit body development. Low levels of gene expression were first detected during the closing phase of substrate colonisation. The amount of transcripts increased quickly at the start of primordia formation and reached maximum levels at the pinhead stage. The levels of gene expression declined during the button stage and varied between 21% and 42% of peak values during the subsequent stages of fruiting. This pattern strongly implies that the enzyme may play an important role in the development of the *V. volvacea* sporophore. Other possible roles, such as lignin degradation and detoxification mechanisms, are not likely since the gene is not expressed in the earlier stages of colonization when it is expected for these functions to be most apparent. Furthermore, *V. volvacea* is not well known for its ability to degrade lignin (Chen et al., 2004).

4.2.5.5 Pathogenicity

Fungi causes many diseases with laccase as an important virulence factor. The aggressiveness of the root pathogen *Heterobasidion annosum* is associated with the presence of laccase. *Cryptococcus neoformans* is an encapsulated fungus that has emerged as a life-threatening infection in immunocompromised patients, particularly those infected with HIV. Laccase is present in this yeast as a tightly associated cell

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wall enzyme that is easily accessible for interactions with host immune cells. The melanin product of laccase is an important virulence factor in *C. neoformans*. It was proposed that in human patients, melanin may protect *C. neoformans* by acting as an antioxidant or by interacting with the cell wall surface. In this way it may protect the cell against a multitude of effectors of cellular immunity (Burke and Cairney, 2002; Mayer and Staples, 2002). It has been shown that in infected mouse brain, which does not contain melanin, the iron oxidase activity of laccase may protect *C. neoformans* from alveolar macrophages. Fe(III) is reduced to Fe(II) in the phagosome by reductants, which may consist of nitric oxide (NO) in murine macrophages, microglia cells, human astrocytes and macrophages with cryptococcal granulomas or other reductants, such as ascorbate or reduced glutathione in other human macrophages. Fe(II) is converted back to Fe(III) when it converts H_2O_2 to hydroxyl radicals. The laccase activity from *C. neoformans* oxidises phagosomal iron to Fe(III) with a resultant decrease in hydroxyl radical formation (Liu et al., 1999).

Plants have two chemical mechanisms of protecting themselves against microbial infection, namely phytoanticipins and phytoalexins. Antibiotic compounds that are in the plant tissue before microbial infection and those that are created from preformed constituents during infection, are known as phytoanticipins. These compounds provide a protective barrier against microbial infection. It was proposed that laccase secreted by *B. cinerea* operates as a detoxifying enzyme to protect the fungus from toxic metabolites and to reduce lignification activities by the host. Cucumber fruits (*Cucumis prophetarium*) and the common weed (*Ecballium elaterium*) contain a family of cyclic triterpenoids (cucurbitacins) that confers protection against fungal attack by *B. cinerea*. Resistance to *B. cinerea* is linked with the ability of extracts from the fruit to inhibit laccase secretion. When laccase formation in *B. cinerea* is repressed by cucurbitacins, the fungus turns out to be a weak or disabled parasite. This verifies the significance of laccase in pathogen virulence (Mayer and Staples, 2002).

Plants synthesise phytoalexins in response to microbial infection. Certain fungal pathogens can endure phytoalexin produced by the host. However, the capacity to metabolise a phytoalexin does not always impart tolerance to the fungus. The laccases from the necrotrophic fungal plant pathogen, *B. cinerea*, is involved in

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phytoalexin destruction. The fungus is resistant to the toxic affects of plant defense compounds with differing structures such as stilbenes, isoflavonoids, coumarins and sesquiterpenes. It has been reported that in grapevine, the phytoalexin resveratrol is transformed into fungitoxic compounds, the viniferins, around a *B. cinerea* infection site. The synthesis of resveratrol can also be induced to high levels by UV irradiation or infection with the fungus *Plasmopara viticola*. However, no build up of viniferins could be detected under these conditions. It was further showed that *B. cinerea* carries a laccase gene that is induced by resveratrol and is responsible for changing non-toxic resveratrol into fungitoxic substances, causing self-intoxication. This agrees with the observations that viniferins is only detected in grapevine tissue infected with *B. cinerea* (Mayer and Staples, 2002; Schouten et al., 2002).

4.2.6 Potential industrial applications of laccases

4.2.6.1 Bioremediation

Laccase has been studied for their use in bioremediation, for example industrial dyes that are released into the environment as effluents from synthesis plants and from dye using industries. Rodríguez et al. (1999) showed that laccase was the main enzyme involved in dye decolorisation due to the correlation of laccase activity with decolorisation. The two purified laccases from *Trametes hispida* was capable of decolorising several synthetic dyes *in vitro*. Balan and Monteiro (2001) showed a link with the decolorisation of dyes and the production of laccase. The purified laccase from *Trametes hirsuta* and the organism itself was able to degrade triarylmethane, indigoid, azo and anthroquinonic dyes. However, there was no correlation between decolorisation and detoxification, since some dye degradation products were still toxic after enzyme treatment (Abadulla et al., 2000).

Laccase has also been shown to be capable of degrading wastewaters from beer factories, distilleries and olive mills. Wastewaters from beer factories contain high levels of polyphenols and have a dark-brown colour. *Coriolopsis gallica*, a white-rot laccase producer, degraded this high tannin containing wastewater and resulted in the decrease of polyphenols in the wastewater (Minussi et al., 2002). Distillery effluent produced from ethanol production from fermentation of vinasses has a significant impact on the environment due to its high soluble organic matter content and dark-

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brown colour. A *Trametes* sp. decolorised the effluent and reduced the COD. During this process a 35 fold increase in laccase production was observed (Minussi et al., 2002). Greco et al. (1999) showed that a PPO naturally immobilised in olive husk and a purified laccase from *T. versicolor* could polymerise phenols in olive mill wastewater. Sigoillot et al. (2004) tested three *P. cinnabarinus* laccases, one in natural form and two recombinant forms expressed in either *A. oryzae* or *A. niger*. Approximately 75% delignification of wheat straw Kraft pulp was obtained with laccases from *P. cinnabarinus* or *A. niger* in the presence of HBT as redox mediator, but the laccase from *A. oryzae* was unable to delignify pulp.

Other toxic compounds been have shown to be degraded by laccase. Amitai et al. (1998) reported the complete and rapid oxidative degradation of the insecticide analog *O,O*-diethyl *S*-[*N,N*-diisopropylaminoethyl]phosphorothiolate and the nerve agents *O*-ethyl *S*-[*N,N*-diisopropylaminoethyl]methylphosphonothiolate (VX) and *O*-isobutyl *S*-[*N,N*-diethylaminoethyl]methylphosphonothiolate (RVX) in the presence of ABTS. *Panus tigrinus* and *C. versicolor* were both tested for their ability to transform trichlorophenols. Both the intact fungi and purified lignolytic enzymes were able to transform the toxic isomers of 2,4,6-trichlorophenols to 2,6-dichloro-1,4-hydroquinol and 2,6-dichloro-1,4-benzoquinone. However, the initial attack on 2,4,6-TCP by *P. tigrinus* was catalysed by Mn-peroxidase, while it was laccase in *C. versicolor* (Mayer and Staples, 2002). The herbicide, isoxaflutole, is activated in soils and plants to its active diketonitrile derivative, which undergoes cleavage to the inactive benzoic acid analogue. Laccases from *P. chrysosporium* and *T. versicolor* are able to convert the diketonitrile to the acid (Mougin et al., 2000).

4.2.6.2 Food industries

One of the primary applications of laccase in the food industry is the stabilisation of wine. Musts and wines are complex assortments of various chemical compounds such as ethanol, organic acids, salts and phenolic compounds. The wine aroma is dependent on the organic acids and alcohol, while the colour and the taste rely predominantly on the phenolic compounds present in the different types of wine. The sensorial properties of wine should remain constant until consumption. The polyphenols in wine (derived from coumaric acid derivatives, flavans and anthocyanidins) play a significant role in oxidative reactions in musts and wines

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catalysed by iron, copper and enzymes, which involve aldehydes, amino acids and proteins leading to turbidity, colour intensification, and aroma and flavour modification. A mutant laccase from *Polyporus versicolor* removed up 70% catechin and 90% of anthocyanidins in a model solution in a 3 hour treatment. The laccase also removed 50% of the total polyphenols from black grape juice. The use of the laccase in must produced a stable wine with good flavour and the method was better in action specificity and stabilisation than the physico-chemical treatment. It was suggested that the laccase could be used in conjunction with other treatments and the oxidised products and enzyme protein could be removed afterwards (Minussi et al., 2002).

B. cinerea could cause several forms of rot in grapes due the secretion of laccase. Musts and wines made from grapes infected with this fungus have laccase present, while the wines and musts made from healthy grapes do not have the enzyme present (Macheix et al., 1991). Although, the presence of laccase from *B. cinerea* does indicate the contamination of the grapes, there is disagreement about as to whether the laccase activity correspond to the degree of contamination of the must since the rise in laccase activity is not always proportional to the increase in *Botrytis* infected berries (Minussi et al., 2002).

There are various causes for the formation of hazes and sediments in beer, wine and fruit juices, but the most common is protein-polyphenol interactions. The stabilisation of these beverages result in a delay of protein-polyphenol haze formation. The use of laccase in the stabilisation of fruit juices has been studied, but the results were contradictory. One study showed that the laccase treatment of apple juice increased the propensity of browning during storage and that the conventionally treated apple juice was more stable than the laccase treated one. Another study showed that the treatment of laccase in conjunction with other processes (cross-flow-filtration) without the addition of fining agents, resulted in stable and clear apple juice (Minussi et al., 2002).

The haze forming property of beer during long-term storage, even in haze free products at the time of packaging, is a constant problem for the brewing industry. The storage life of beer relies on many factors including temperature, haze forming

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potential and oxygen content. Protein precipitation is the cause of haze formation in beer and this is stimulated by small quantities of proanthocyanidins polyphenols present in beer. It was suggested that a laccase could be added to the wort instead of performing the traditional treatment. When added at the end of the process, the laccase may remove the excess unwanted oxygen and may simultaneously remove a quantity of the polyphenols that are still present in the beer. The resultant polyphenol complexes could then be removed by filtration (Minussi et al., 2002).

4.2.7 Recombinant expression

The development of molecular biology techniques offer new ways to employ yeasts and moulds as microbial cell factories for the production of homologous and heterologous (particularly mammalian) proteins, antibiotics, pigments and fatty acids. The selection of a fungal strain used in the food industry depends on the basis of production yields and regulatory issues. The host strains are generally selected from those that has attained GRAS (Generally Regarded As Safe) status by the U.S. Food and Drug Administration (FDA). Numerous species of fungi have GRAS status and are utilised for large-scale production of recombinant proteins and metabolites (Punt et al., 2002).

Saccharomyces cerevisiae is a food organism and is regarded as a safe host for the production of pharmaceutical proteins such as human interferon, human epidermal growth factor, human hemoglobin and hepatitus B surface antigens. The advantages of *S. cerevisiae* as a host, is its ability to be grown rapidly and to a high cell density, the secretion of heterologous proteins into the extracellular broth and advanced knowledge of its genetics. However, it is not always an optimal host for large-scale production of mammalian proteins due to hyperglycosylation, the presence of α-1,3-linked mannose residues that could cause antigenic responses in patients, as well as the lack of strong and tightly regulated promoters (Adrio and Demain, 2003).

Another host, *Pichia pastoris*, has become one of the most widely used expression systems due to these deficiencies in *S. cerevisiae*. The advantages of *P. pastoris* include an efficient and tightly regulated methanol promoter (AOX1), less extensive glycosylation, integration of multiple copies of foreign DNA into the chromosomal

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DNA yielding stable transformants, the ability to secrete high levels of foreign proteins, the high density of growth and the simple scale-up. However, its main disadvantage is its non-GRAS status. *Hansenula polymorpha* heterologous gene expression is comparable to that of *P. pastoris* with a highly expressed and tightly regulated MOX promoter. MOX is drastically derepressed in the absence of glucose or during glucose limitation, therefore, tight regulation of the promoter is lost under high glucose conditions typically used for high-biomass fermentations (Adrio and Demain, 2003).

Numerous black aspergilli are regularly used for industrial applications, such as the production of metabolites (organic acids) and extracellular proteins (plant-cell-wall-degrading enzymes). The advantages of fungi are their high secretory potential and their ability to perform all the posttranslational modifications that are required for the correct production of proteins from higher eukaryotes. However, the production of heterologous proteins is lower than that of homologous proteins. This may be due to high level of secreted protease activity that degrade heterologous proteins and the acidification of the growth medium by the production of organic acids. This acidification of the medium stimulates the production of protease and may also reduce the stability of heterologous proteins. A promising *Aspergillus* sp. (*Aspergillus vadensis*) has been identified with low extracellular protease activity and does not acidify the medium when nitrate is the nitrogen source (De Vries et al., 2004). Table 4.3 shows some laccases expressed in different hosts and their yields.

Table 4.3 Recombinant expression of laccase.

Source	Host	Yield (mg/l)	Reference
Pycnoporus cinnabarinus I-937	*Pichia pastoris*	8	Otterbein et al., 2000
Myceliophthora thermophila	*Saccharomyces cerevisiae*	18	Bulter et al., 2003
Pleurotus sajor-caju	*Pichia pastoris*	4.85	Soden et al., 2002
Trametes versicolor	*Pichia pastoris*	11	Gelo-Pujic et al., 1999
Coriolus hirsutus	*Saccharomyces cerevisiae*	5	Yasuchi et al., 1990
Melanocarpus albomyces	*Trichoderma reesei*	230 (shake-flask) 950 (fed-batch)	Kiiskinen et al., 2004
Pycnoporus cinnabarinus	*Aspergillus niger*	70	Record et al., 2002
Myceliophthora thermophila	*Aspergillus oryzae*	11 - 19	Berka et al., 1997
Coprinus cinereus	*Aspergillus oryzae*	8 - 135	Yaver et al., 1999

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Chapter 5

Research Results:

Heterologous Expression of the *Pleurotus ostreatus* Laccase Gene in *Aspergillus niger*

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Heterologous Expression of the *Pleurotus ostreatus* Laccase Gene in *Aspergillus niger*

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Abstract

Pleurotus ostreatus is an edible, lignin-degrading basidiomycete that is industrially produced for human consumption, while its enzymes and secondary metabolites are used for industrial and pharmaceutical applications. The *Pleurotus ostreatus lacA* gene, encoding a *p*-diphenol: dioxygen oxidoreductase, was previously cloned and expressed intracellularly in *Saccharomyces cerevisiae* at 6 U/l. In this study, the cDNA of *P. ostreatus lacA,* including its native secretion signal, was subcloned under the control of the *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase promoter sequence and transformed into *Aspergillus niger* D15. Integration of the *lacA* gene into the host genome was confirmed, but the transformants showed no extracellular laccase activity. Minimal intracellular laccase activity was observed (0.7 – 0.9 U/g total protein), suggesting that the gene was not effectively expressed and/or processed.

Introduction

Laccases (EC 1.10.3.2, *p*-diphenol: dioxygen oxidoreductases) use molecular oxygen to oxidise various aromatic and non-aromatic substances by a radical-catalysed reaction mechanism. Laccases can attack a broad range of substrates and will oxidise any substrate with characteristics similar to a *p*-diphenol, and all known laccases oxidise *o*-diphenols of the catechol type. Some fungal laccases can also oxidise monophenols such as cresol, whereas some are able to oxidise ascorbic acid, *m*-diphenols and *p*-phenylenediamine, but not tyrosine (Mayer and Harel, 1979; Mayer and Staples, 2002).

Fungal laccases often occur as multimeric complexes consisting of oligomerised isoenzymes with the molecular mass of the monomers ranging between 50 and 100 kDa. The enzyme is a glycoprotein, which has a covalently linked carbohydrate

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moiety (10 – 45%) that may contribute to the high stability of the enzyme. For catalytic activity, the enzyme requires a minimum of four copper atoms per active protein unit; the copper atoms differ in their light absorbance and electron-paramagnetic behaviour (Claus, 2004). However, Palmieri et al. (1997) reported a "white laccase" from *P. ostreatus* that contained one copper, two zinc and one iron atom per each protein molecule.

Laccases have been reported in various eukaryotes (fungi, plants and insects), but recently strong evidence have also been presented for their extensive distribution among prokaryotes (Alexandre and Zhulin, 2000; Mayer and Staples, 2002). The laccase enzyme was first described by Yoshida in 1883 in the latex of *Rhus vernicifera* (lacquer tree) (Benfield et al., 1964), a member of the family *Anacardiaceae*. This entire family appears to contain laccases in the resin ducts and in the secreted resin of the plant (Bar-Nun et al., 1981; Mayer and Harel, 1979). Evidence for the presence of proteins with typical characteristics of the multi-copper oxidase family in both Gram-negative and Gram-positive bacteria has been mounting. The first credible data for a prokaryotic laccase activity were presented for *Azospirillum lipoferum* (Diamantidis et al., 2000). The first reports of laccases in actinomycetes were based on rather non-specific substrate reactions, but have now been confirmed for *Streptomyces griseus* (Claus, 2003; 2004).

Laccases are widespread among fungi and have been found in various classes, including the ascomycetes *Aspergillus nidulans*, *Neurospora crassa* and *Podospora anserine*, the deuteromycete *Botrytis cinerea*, and several basidiomycetes. Schanel (1967) reported that of 130 wood-rotting basidiomycetes tested, 87 gave a positive polyphenol oxidase reaction. The basidiomycetes such as *Collybia velutipes*, *Fomes annosus*, *Fomes fomentarius*, *Lentinula edodes*, *Phanerochaete chrysosporium*, *Pholiota mutabilis*, *Pleurotus ostreatus*, *Poria subacida*, *Sporotrichum pulverulentum*, *Trametes sanguinea* and *Trametes versicolor* are all known as lignin degraders. A laccase has also been identified in the ectomycorrhizal fungi *Thelephora terrestris* (Bollag and Leonowicz, 1984; Burke and Cairney, 2002).

The industrial importance of laccases have been recognised in a number of industries, such as the food and beverage industry where it has been used for the stabilisation of

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wine musts, preventing haze formation in beer and as an indicator for *B. cinerea* infection in musts and wines (Macheix et al., 1991; Minussi et al., 2002). It has also been used for bioremediation such as the degradation, decolourisation and detoxification of industrial dyes (Abadulla et al., 2000; Balan and Monteiro, 2001; Rodríguez et al., 1999), the degradation of wastewaters from beer factories, distilleries and olive mills (Greco et al., 1999; Minussi et al., 2002), as well as herbicide degradation (Mougin et al., 2000).

A number of recombinant laccases have been expressed in either yeast or fungal hosts. The laccases from *Pycnoporus cinnabarinus I-937*, *Pleurotus sajor-caju* and *T. versicolor* were expressed in *Pichia pastoris* (Gelo-Pujic et al., 1999; Otterbein et al., 2000; Soden et al., 2002), while the laccases from *P. cinnabarinus*, *Myceliophthora thermophila* and *Coprinus cinereus* were expressed in *Aspergillus* sp. (Berka et al., 1997; Record et al., 2002; Yaver et al., 1999). Of all the laccases expressed heterologously, those using fungi as hosts produced higher yields of the enzyme opposed to expression in yeast hosts (Otterbein et al., 2000; Record et al., 2002; Soden et al., 2002; Yaver et al., 1999).

This study focussed on the heterologous expression of a laccase from *P. ostreatus,* an edible mushroom that is produced as food for human consumption and whose enzymes are used for industrial and pharmaceutical applications (Peñas et al., 2002). Recombinant expression of the laccase from this food-grade organism should therefore be acceptable for use in the food and beverage industry. Several laccase isoenzymes have been characterised in *P. ostreatus*. The *pox1* gene consists of 2 592 bp with the sequence being interrupted by 19 introns (Giardina et al., 1995). Two other enzymes isolated from *P. ostreatus*, POXA1 and POXA2, have molecular masses of 61 and 67 kDa, respectively (Palmieri et al, 1997). POXA2 contains the usual four copper atoms per mol of enzyme, while POXA1 contains one copper, two zinc and one iron atom per mol enzyme. Giardina et al. (1999) isolated an isoenzyme (POXA1b) that showed similarities to the *pox1* gene structure, but these two genes belong to different subfamilies. POXA1b also contains four copper atoms per molecule and the gene coding sequence consists of 1 599 bp interrupted by 15 introns. More recently, Palmieri et al. (2003) isolated two isoenzymes (POXA3a and

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POXA3b) from *P. ostreatus*. The complete determined nucleotide sequence (5 700 bp) of the *poxa3* gene contained 21 introns.

The cDNA of a laccase from *P. ostreatus* was previously cloned and expressed in *S. cerevisiae* under control of the glucose-repressible alcohol dehydrogenase II promoter and the MFα1 secretion signal (Roth, 1997). The cloned gene, *lacA*, had a 98% sequence identity with *pox1*. However, the recombinant strain expressed the *lacA* gene at very low levels intracellularly, i.e. 6 U/l. The enzyme presumably accumalated intracellularly within the periplasmic space and was probably degraded within the cell. In this study, we attempted to express the *P. ostreatus lacA* gene under regulation of the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase promoter sequence in *Aspergillus niger* D15, a strain previously used for effective heterologous expression of the *Trichoderma reesei* β-1,4-xylanase (*xyn2*) and β-1,4-endoglucanase genes (*egI*) (Rose and Van Zyl, 2002).

Materials and Methods

Chemicals

All chemicals used were of analytical grade and were obtained from Merck (Darmstadt, Germany), Sigma Chemical Co. (St. Louis, MO, USA), Difco Laboratories (Detroit, MI, USA), Becton, Dickenson and Company (Sparks, MD, USA) and Oxoid Ltd. (Basingstoke, Hampshire, UK).

Sequencing

Cycle sequencing reactions were performed using the BigDye 3 V1 Terminator kit (Applied Biosystems) and a GeneAmp PCR system 9700 (Applied Biosystems). Unincorporated terminators were removed and electrophoresis was performed on an ABI PRISM (R) 3100 Genetic Analyser (Applied Biosystems). Results were analyzed by using the VAX-based Genetics Computer Group Inc. sequence analysis package (version 7.1), its associated programs and the PC-based DNAMAN software (version 4.1) from Lynnon BioSoft. Comparison searches were performed using the nucleotide BLAST program of NCBI (http://www.ncbi.nih.gov/).

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Strains, plasmids and media

Recombinant plasmids were constructed and amplified in *Escherichia coli* DH5α using standard molecular biology protocols. Bacterial cells were cultivated at 37°C in Luria-Bertani liquid media or on Luria-Bertani agar with ampicillin added to a final concentration of 100 μg/ml for selection and propagation of transformed bacterial cells (Ausubel et al., 1998; Hanahan, 1983). The strains and plasmids used in this study are summarised in Table 5.1. The recipient strain for transformation, *A. niger* D15, is a uridine auxotrophic (*pyrG*), protease-deficient (*prtT*), non-acidifying (*phmA*) mutant (Wiebe et al., 2001). The *A. niger* D15 strain was inoculated to a concentration of $1x10^6$ spores per ml and cultivated in 250 ml *Aspergillus* minimal growth media containing 0.5% (w/v) yeast extract, 0.2% casamino acids, 1% glucose, 0.6% $NaNO_3$, trace elements and 0.01 M uridine in 1 L Erlenmeyer flasks at 30°C on a rotary shaker at 150 rpm (Punt and van den Hondel, 1992). Transformants were selected on *Aspergillus* minimal medium without uridine, containing 1.2 M sorbitol as osmotic stabilizer and 1.5% agar (Oxoid). For quantification of enzyme activities, transformants were cultivated without uridine on the selective minimal media and double strength (2xMM) minimal media containing 10% glucose.

Table 5.1 Genotypes and sources of strains and plasmids used in this study.

	Genotype	Source/reference
Strains:		
E. coli DH5α	*supE44' ΔlacU169 (ø80lacZΔM15) hsdR17 rec A1 endA1 gyrA96 thi-1 relA1*	Hanahan, 1983
A. niger D15	*pyrG prtT phmA*	Wiebe et al., 2001
A. niger D15 (*lacA*)	*$pyrG^+$ prtT phmA $amdS^+$ $gpdA_p$-lacA-$trpC_T$*	This study
Plasmids:		
pGEM®-T Easy	*bla*	Promega Corporation
pGEM®-T Easy (*lacA*)	*bla lacA*	This study
pBS-LacA	*bla*	Roth, 1997
pANN6	*bla pyrG amdS $gpdA_p$-$trpC_T$*	This study
pGC1	*bla pyrG amdS $gpdA_p$-lacA-$trpC_T$*	This study

DNA manipulations and transformation of *A. niger*

Plasmids were isolated from *E. coli* DH5α with High Pure Plasmid Isolation Kit (Roche Diagnostics Corporation, Indianapolis, IN, USA) as per manufacturers

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instructions. Standard protocols were followed for all other DNA manipulations and *E. coli* transformations (Ausubel et al., 1998). Restriction endonucleases and T_4 DNA ligases were obtained from Roche and DNA fragments was isolated from agarose gels with the QIAquick® Gel Extraction Kit (QIAGEN Corp., Valencia, CA, USA).

Transformation of *A. niger* spheroplasts was done as described by Punt and Van den Hondel (1992). Mycelia were harvested from fungal strains grown in minimal media for 72 hrs and ground to a powder in a mortar under liquid nitrogen for the isolation of genomic DNA (La Grange et al., 1996).

Plasmid construction and analysis

The cDNA copy of the *lacA* (*P. ostreatus pox1*) gene was amplified from plasmid pBS-LacA (Roth, 1997) via overlapping PCR, using primers LacCL and Rep1 (Table 5.2). The resulting 434 bp fragment (Frag1) was used as the 5' primer for the next round of PCR with Rep2 as the 3' primer. The 1.1 kb fragment from this reaction (Frag2) was used as the 5' primer for the subsequent round of PCR with LacCR as the 3' primer. The PCR reactions were performed with Expand High Fidelity PCR System (Roche Diagnostics Corporation, Indianapolis, IN, USA) using a thermocycler (PCR Sprint Temperature Cycling System, Hybaid Ltd., Ashford, Middlesex, UK) with initial denaturation at 94°C for 2 min, followed by 31 cycles of annealing at 55°C for 30 sec, elongation at 72°C for 30 sec and denaturation at 94°C for 30 sec and final annealing at 55°C for 30 sec and elongation at 72°C for 10 min.

Table 5.2 DNA sequences of the primers used in this study. Restriction sites are indicated in bold.

Primer name	Sequence	Restriction enzyme sites
LacCL	5' GACT**GCGGCCGC**ATGTTTCCAGGCGCACGGATTCTCGCTACGCTTACA 3'	*NotI*
LacCR	5' GACT**ATTTAAAT**CTAAGCTATGCCACCTTTGT 3'	*SwaI*
Rep1	5' GGTCCTCTAAGACCATCGCAATACTGTGTGGAAAGATG 3'	
Rep2	5' ACAGGAGCAGTCGGCGCTTTGAAGGGGGAACCATTG 3'	
LacSL	5' ATTCCTTTGAACCTTTCAGT 3'	
LacSR	5' ACACCAATTTGTCTCAACTCC 3'	

Similar conditions were used for the second and third rounds of PCR, except for elongation of 70 sec for the second round and 90 sec for the third round. The

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resulting 1.6 kb fragment was ligated into the pGEM®-T Easy Vector System (Promega Corporation, Madison, WI, USA) (Figure 5.1A) resulting in pGEM-LacA. Plasmid pGEM-LacA was digested with *SwaI* and *NotI* and the 1.6-kb fragment subcloned into the *NotI, SwaI* sites of plasmid pANN6, resulting in plasmid pGC1 (Figure 5.1B) that was transformed into competent *A. niger* D15 protoplasts.

The directional cloning of *lacA* into pGC1 was verified by restriction digest with combinations of *NotI*, *SwaI* and *PvuI,* as well as by PCR with primer sets LacSL (based on the sequence of $gpdA_P$) and LacCR, and LacCL and LacSR (based on the sequence of $trpC_T$). Integration of plasmid pGC1 into the *A. niger* D15 genome was also verified by PCR amplification of the *lacA* gene with primers LacCL and LacCR, using TaKaRa *Ex taq*™ (TAKARA BIO INC., Otsu, Shiga, Japan) and elongation at 72°C for 90 sec.

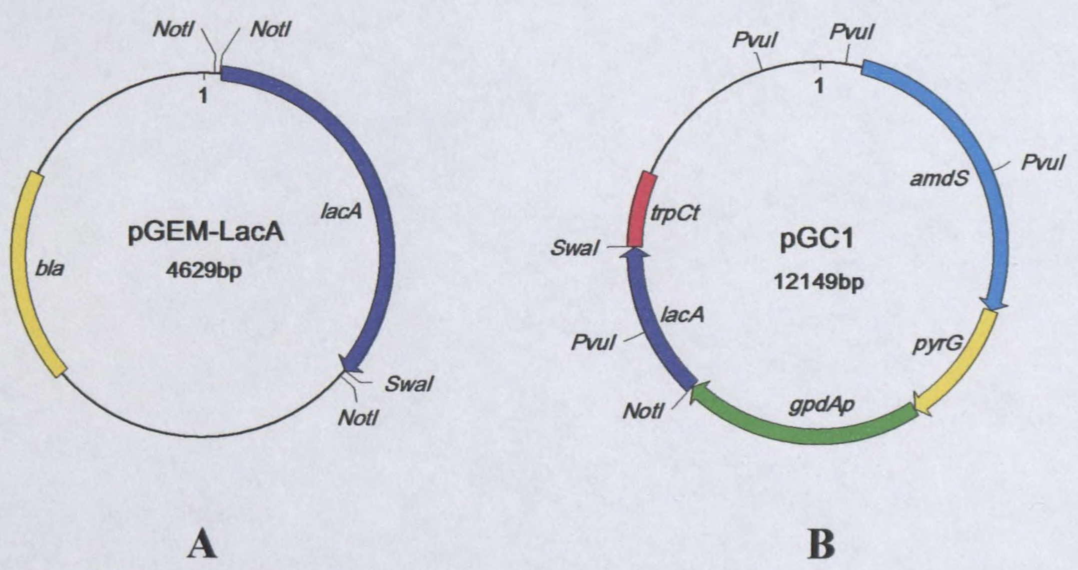

Figure 5.1 Schematic representation of **(A)** pGEM-LacA vector containing the *lacA* gene and *E. coli bla* gene (β-lacatamase) as selectable marker. **(B)** The *Aspergillus* expression vector containing the *lacA* gene used in this study. *pyrG, A. niger* orotidine-5'-decarboxylase gene used as a selectable marker; $GpdA_P$, the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase gene promoter; $trpC_T$, the *A. nidulans* gene terminator; and *amdS, A. nidulans* acetamidase gene used as selectable marker.

Laccase enzyme assays

For plate assays, spores of transformants were spotted onto the center of SC plates containing trace elements and 0.3 g/l 2,2'-azino-bis(3-ethylbenzothiazoline-6-

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sulphonic acid) (ABTS) incubated for 5 days and monitored for the formation of green zones. For liquid assays, transformants were cultivated in 25 ml 2xMM in 125 ml Erlenmeyer flasks for 72 hrs. The mycelia were seperated from the growth media through Miracloth (Calbiochem) and ground to a powder in a mortar under liquid nitrogen. It was resuspended in 10 ml of 50 mM NaOAc buffer (pH 5.2) and centrifuged at 13 000 rpm for 20 min at 4°C. The supernatant was transferred to clean tubes and laccase assays were performed on both the liquid growth media and the supernatant as described by Jönsson et al. (1997). Total protein concentrations were determined with D_C Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) as per manufacturers instructions, and the laccase activity expressed as units/g total protein.

Results

Plasmid construction

Sequencing of the *P. ostreatus lacA* gene in plasmid pBS-LacA revealed that PCR amplification introduced point mutations that resulted in four amino acid substitutions (Figure 5.2). The mutations were a His22 → Asn, Cys149 → Trp, Lys377 → to Gln, and Leu385 → Phe. Two of these point mutations were rectified with overlap PCR, i.e. Trp149 → Cys and Gln377 → Lys, and the 1.6 kb fragment was subcloned into the pGEM®-T Easy Vector System to yield pGEM-LacA. Due to the presence of a *NotI* restriction site on both sides of the gene with one near the *SwaI* site, the plasmid was first digested with *SwaI* and then with *NotI* to allow subcloning in the *NotI*, *SwaI* sites of plasmid pANN6. The resulting recombinant plasmid containing the *lacA* gene under control of the constitutive *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase promoter ($gpdA_P$), was designated pGC1.

Mapping of plasmid pGC1 was done with *NotI, SwaI* and *PvuI* to verify the directional cloning of the gene (Figure 5.3). A single band of 12 kb was expected for digestion with either *NotI* or *SwaI*, but two bands were visible with *NotI* digestion (10.5 kb and 1.6 kb), and none with *SwaI*. It was concluded that the *lacA* gene was ligated as a *NotI-NotI* fragment and not as a *NotI-SwaI* fragment, possibly due to complete degradation of pGEM-LacA with *SwaI*. The directional cloning of the

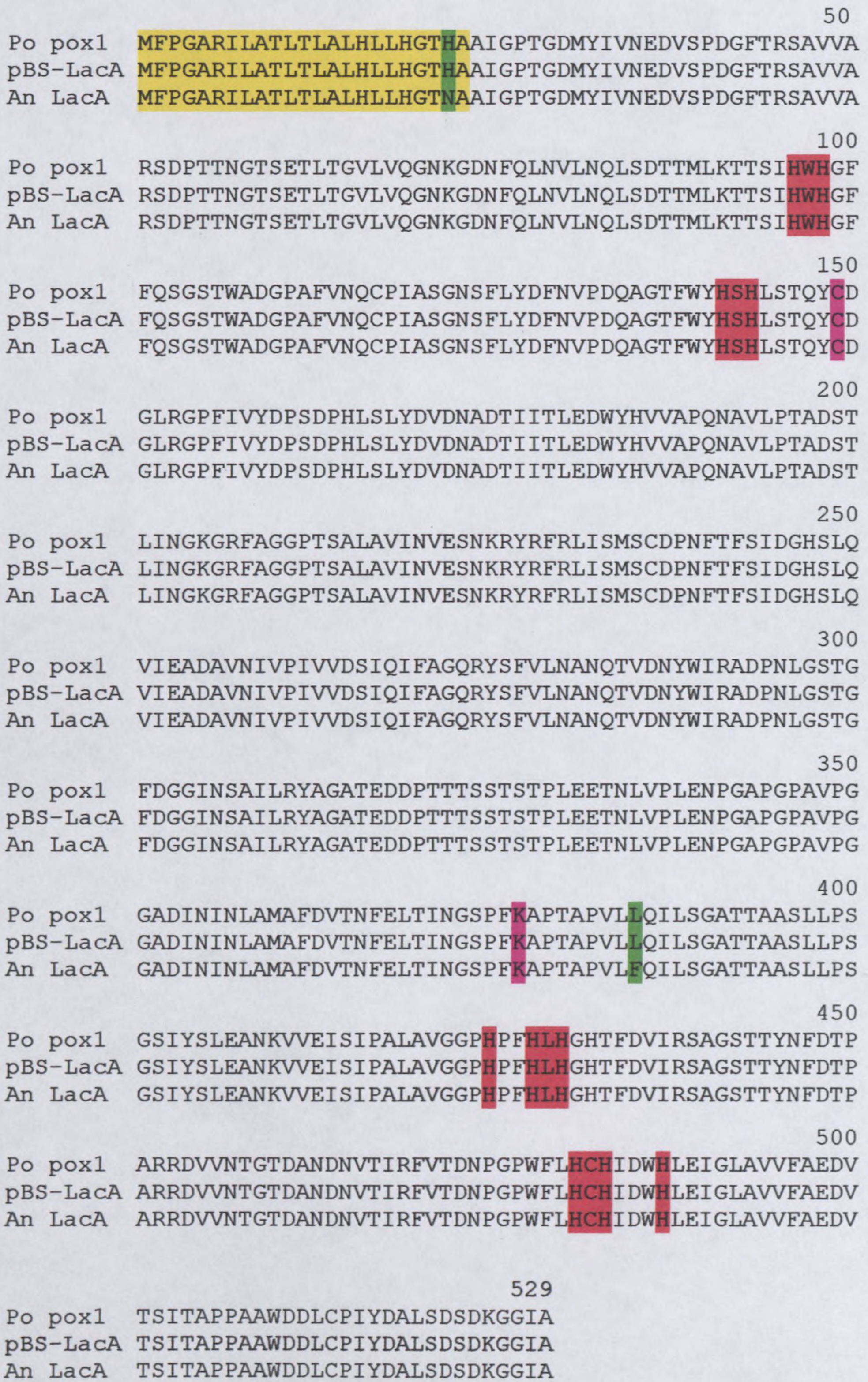


Figure 5.2 Alignment of the amino acid sequences of the *P. ostreatus pox1* cDNA as reported by Giardina et al., 1995 (Po pox1), Roth, 1997 (pBS-LacA) and those found in the *A. niger* D15 transformants (An LacA). The signal peptide is indicated in yellow. The amino acids corrected via overlap PCR in An LacA are indicated in purple and the remaining mismatches are indicated in green. The four copper binding sites are indicated in red.

NotI-NotI fragment under control of the *gpdA*-promoter was confirmed with restriction analysis with combinations of *PvuI* and *NotI* (Figure 5.3), as well as by PCR with primer set LacCL (anneals to 5' end of *lacA*) and LacSR (anneals to $trpC_T$), and primer set LacSL (anneals to $gpdA_P$) and LacCR (anneals to 3' end of *lacA*) which both yielded a 1.6 kb fragment (Figure 5.4).

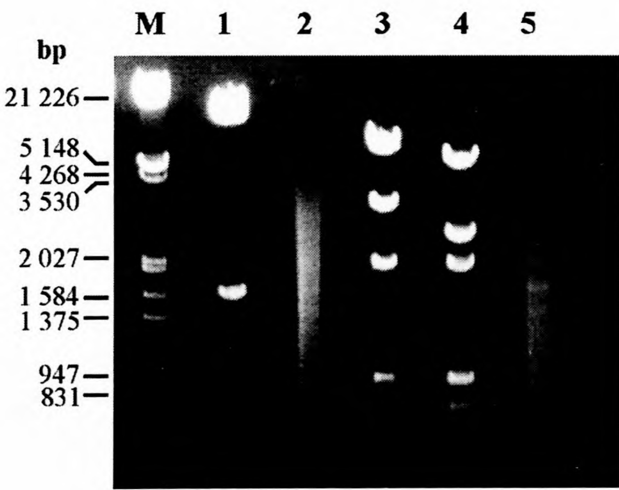


Figure 5.3 Plasmid pGC1 digested with **(1)** *NotI*, **(2)** *SwaI*, **(3)** *PvuI*, **(4)** *NotI* + *PvuI* and **(5)** *NotI* + ***SwaI*.** **M,** molecular weight marker III (Roche Diagnostics).

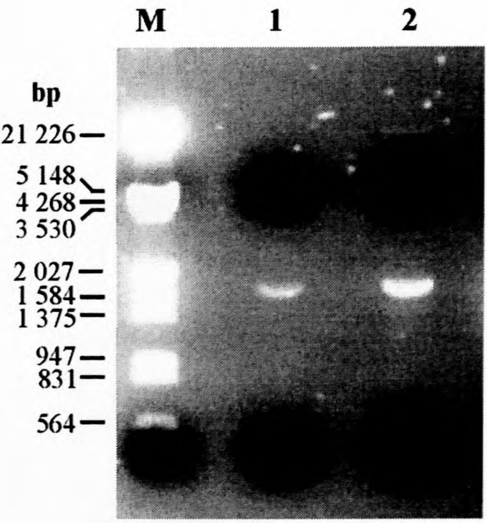


Figure 5.4 PCR analysis on two pGC1 clones to confirm directional cloning of the *lacA* gene. **M,** molecular weight marker III (Roche Diagnostics).

Plasmid pGC1 was transformed into *A. niger* D15 and twelve uridine-prototrophic transformants were obtained. Transformants were transferred three times to selective

media and integration of the plasmid into the genome was determined by PCR for three transformants (Figure 5.5). Two of the transformants produced the expected 1.6 kb fragments, confirming the integration of the *lacA* gene into the host genome. The two recombinant *A. niger* strains were designated ANL-1 and ANL-2.

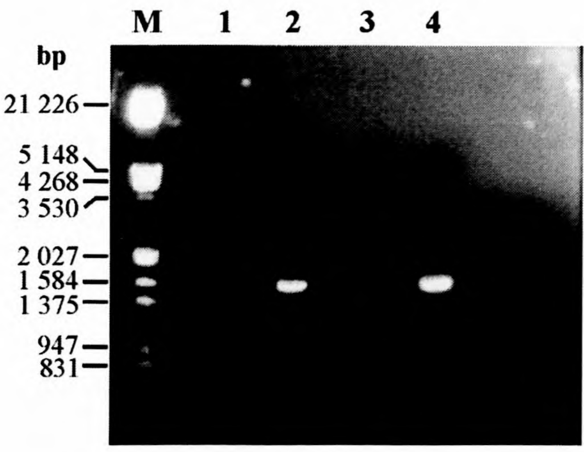


Figure 5.5 PCR analysis to confirm integration of pGC1 into the genome of three *A. niger* D15 transformants. **M**, molecular weight marker III (Roche Diagnostics Corporation); **1,** *A. niger* transformed with expression vector pANN6 without *lacA*; **Lanes 2-4,** PCR products from three *A. niger* transformants.

Laccase activity in *A. niger* transformants

Spores of the ANL-1 and ANL-2 strains were inoculated on SC plates containing ABTS and incubated for 5 days at 30°C. No definitive zones were formed, and although a green colour appeared beneath the mycelia after 5 days (data not shown), colour formation was also visible on the negative control plates. When cultivated in 2xMM liquid media, there was no detectable laccase activity in the supernatant from either the host strain or the two transformants. However, some activity was detected within the cell extracts, i.e. 0.9 U per g total protein and 0.7 U per g total protein for ANL-1 and ANL-2, respectively. Although the control strain also showed some activity in the cell extracts (0.5 U per g total protein), it was almost two-fold less than for ANL-1.

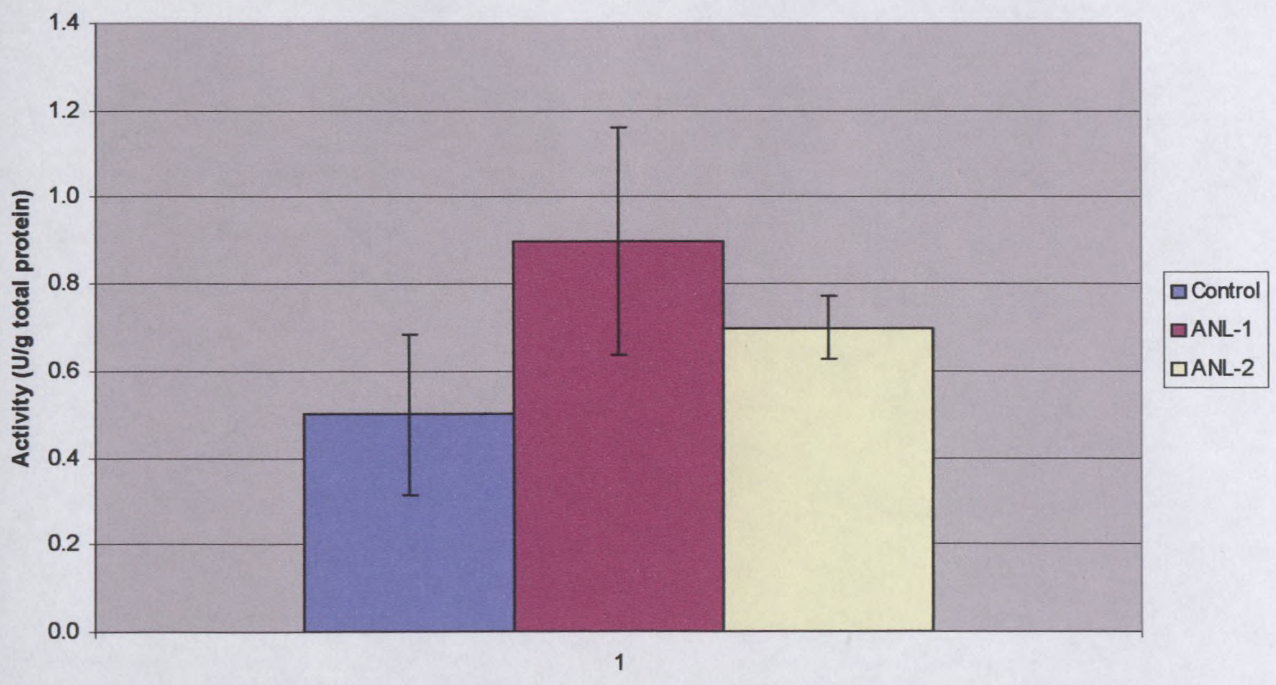


Figure 5.6 Enzyme activities in cellular extracts from ANL-1 and ANL-2.

Discussion

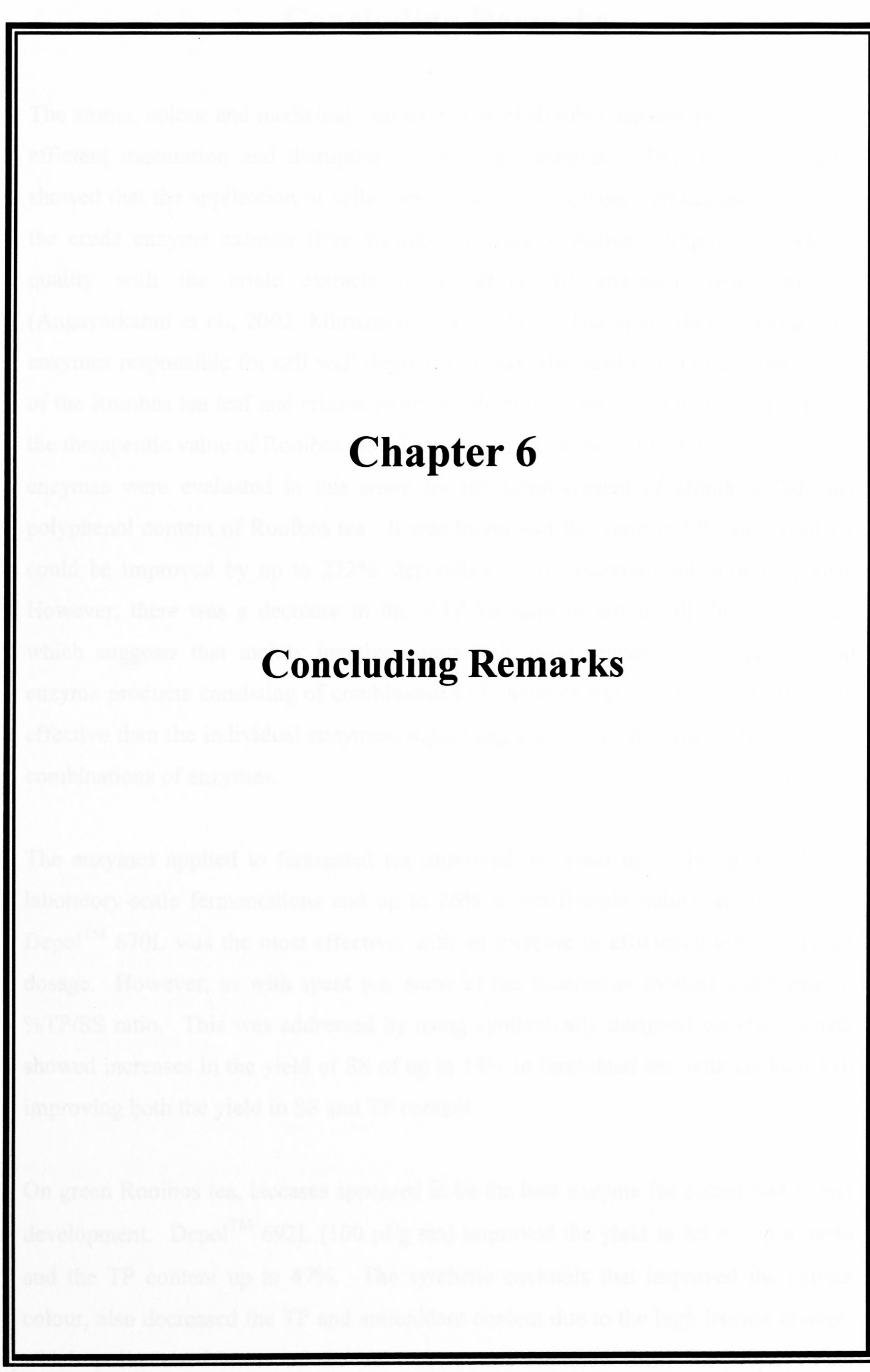
Laccases from various organisms have been expressed in recombinant hosts, such as the laccase from *M. thermophila* expressed in *A. oryzae* that yielded 11 – 19 mg/l laccase with a specific activity of 45 SOU/mg of enzyme extract (Berka et al., 1997). Record et al. (2002) expressed the *P. cinnabarinus* laccase gene in two different expression vectors: transformants containing the native laccase signal peptide yielded an enzyme activity of 90 IU/l, while those containing the glucoamylase prepro-sequence of *A. niger* yielded an activity of 7000 IU/l. The latter was also more successful in enzyme production, i.e. 70 mg/l as opposed to less than 1 mg/l. The laccase gene from *P. sajor-caju* was expressed in *P. pastoris* with an enzyme concentration of 10.2 U/ml and a specific activity of 92.7 U/mg crude cell-free extract (Soden et al., 2002).

The yields obtained with the heterologous expression of the *P. ostreatus lacA* gene in this study do not compare well with those previously reported. Low levels of laccase activity was observed intracellularly, with no evidence for secretion of the enzyme into the media as evaluated by liquid and plate assays. Although the intracellular

levels in the two transformants were higher than for the control strain, the the data suggest that these differences are not significant.

The ineffective expression and secretion of the recombinant *lacA* gene can be ascribed to a number of possible reasons. The integration of the *lacA* gene and its correct orientation in the genome of the *A. niger* host was confirmed. However, there are still two amino acid substitutions in the *lacA* sequence with one located within the signal peptide, which may inactivate the enzyme. The signal sequence may not be recognized or the gene may be incoreectly processed. If the enzyme is not effectively secreted, the intracellular accumalation of the enzyme will be avoided by the cell to avoid intracellular damage. Enzyme activity could possibly be improved if the gene encoding the mature protein was fused to the glucoamylase preprosequence of *A. niger* as was done by Record et al. (2002).

The *A. nidulans gpdA* promoter has been used successfully for the expression of the *Trichoderma reesei* β-1,4-xylanase (*xyn2*) and the β-1,4-endoglucanase (*egI*) genes in *A. niger* (Rose and Van Zyl, 2002). However, further investigations are required to confirm whether the *lacA* gene is transcribed and translated correctly and if so, if it is functional. Transcription of the gene can be confirmed through either northern blot analysis or RT-PCR. If not, the other two point mutations in the *lacA* gene would have to be also corrected with overlap-PCR. Translation and secretion of the gene can then be evaluated by laccase activity assays on intracellular and extracellular extracts from the recombinant host.



Chapter 6

Concluding Remarks

Concluding Remarks

The aroma, colour and medicinal characteristics of Rooibos tea depend largely on the efficient maceration and disruption of the plant material. Two previous studies showed that the application of cellulases, xylanase, pectinases and laccase, as well as the crude enzyme extracts from various microbial organisms, improved black tea quality with the crude extracts (combinations of enzymes) being superior (Angayarkanni et al., 2002; Murugesan et al., 2002). The application of exogenous enzymes responsible for cell wall degradation may also lead to complete maceration of the Rooibos tea leaf and release more tea phenolic compounds that could improve the therapeutic value of Rooibos tea. Therefore, 16 commercially available microbial enzymes were evaluated in this study for the improvement of aroma, colour and polyphenol content of Rooibos tea. It was found that the yield in SS from spent tea could be improved by up to 232%, depending on the enzyme and dosage applied. However, there was a decrease in the %TP/SS ratio in almost all these instances, which suggests that mainly inactive compounds were released. The commercial enzyme products consisting of combinations of enzymes were also found to be more effective than the individual enzymes, suggesting a need for the synergistic action of combinations of enzymes.

The enzymes applied to fermented tea improved the yield in SS by up to 44% in laboratory-scale fermentations and up to 26% in small-scale industrial simulations. Depol^{TM} 670L was the most effective, with an increase in efficiency with increased dosage. However, as with spent tea, some of the treatments showed a decrease in %TP/SS ratio. This was addressed by using synthetically designed cocktails which showed increases in the yield of SS of up to 14% in fermented tea, with cocktail #10 improving both the yield in SS and TP content.

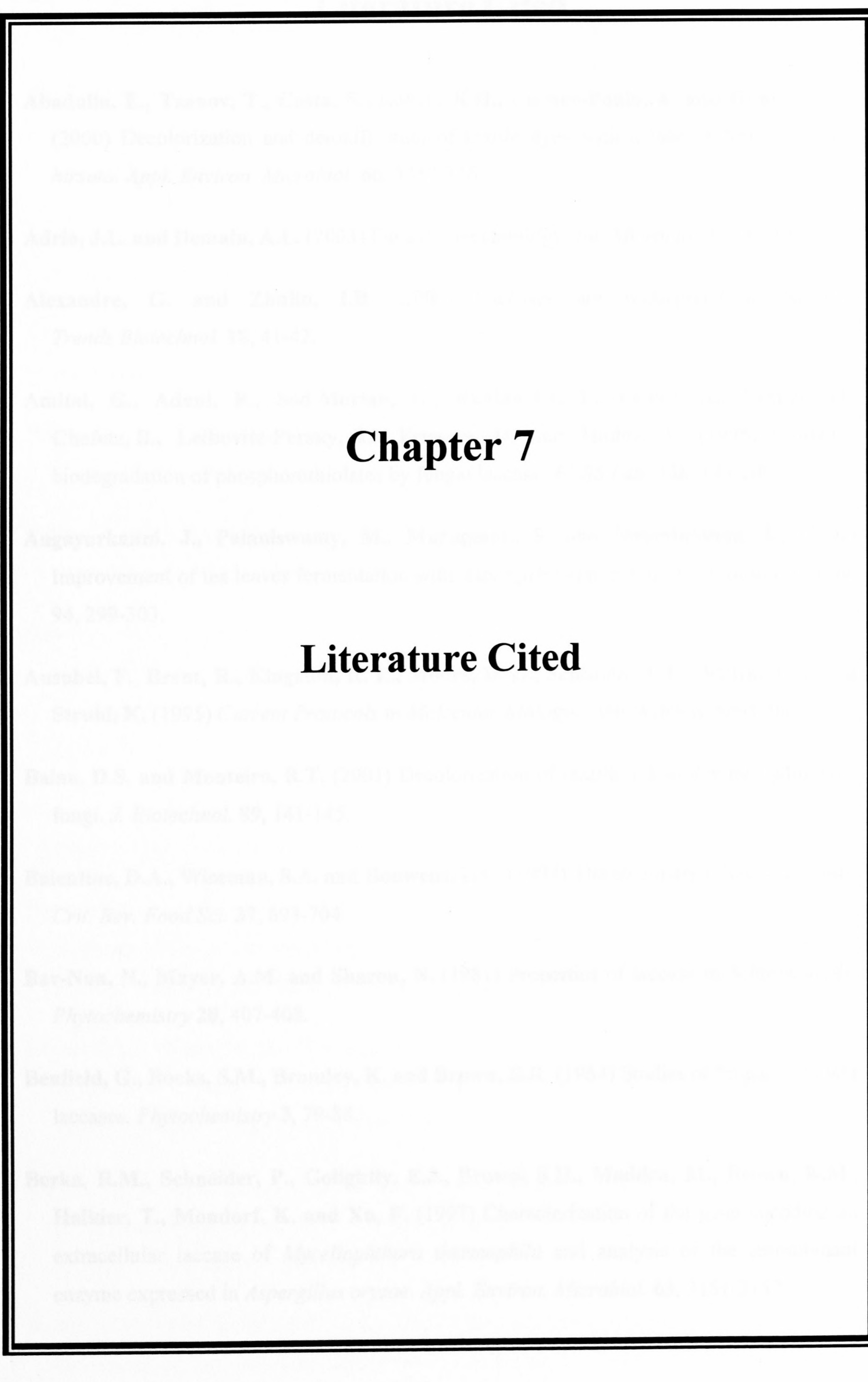
On green Rooibos tea, laccases appeared to be the best enzyme for colour and aroma development. Depol^{TM} 692L (100 μl/g tea) improved the yield in SS by up to 66% and the TP content up to 47%. The synthetic cocktails that improved the extract colour, also decreased the TP and antioxidant content due to the high laccase dosage, which polimerised most of the substances and rendered them insoluble. This

phenomenon was evident in all the substrates evaluated with laccases, either individually or in combinations with other enzymes. Lower laccase dosages were less efficient for colour development, but also reduced the loss in SS and TP.

Due to the encouraging results obtained from treatments of Rooibos tea with laccases, it was decided to clone and express the laccase gene, *lacA,* of *Pleurotus ostreatus* in *Aspergillus niger*. The gene was successfully transformed into *A. niger,* but very low levels of activity were obtained in the two transformants evaluated (ANL-1 and ANL-2). Further experiments will be required to determine whether the gene is efficiently transcribed and translated.

The results from this study showed that the exogenous application of microbial enzymes can improve the release of SS and TP (and thus antioxidants) from different Rooibos tea products, thereby improving the overall quality and commercial value of the product. The enzymes used in combinations appear to be more efficient than when used individually, since lower dosages of enzyme are required for a similar effect. The fermentation time of green tea was also reduced to 2 hrs, which would make it possible to process tea inside a factory under more controlled conditions. This will not only improve the quality of Rooibos tea, but also make the processing procedures more acceptable to the international market.

Further research on enzyme combinations and the respective ratios are required to improve the development of the characteristic Rooibos tea aroma from green tea. To this end, the exact composition of Rooibos plant cell wall structure would help immensely in choosing the correct enzymes for the treatment of the plant material. The inner workings of what occurs during Rooibos tea fermentation and which substances are responsible for colour and aroma formation, are still largely unknown. This knowledge could also further our understanding as to what enzymes are necessary for a high quality Rooibos tea product.



Chapter 7

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Literature Cited

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