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Enhancement of Rooibos (*Aspalathus linearis*) Aqueous Extract and Antioxidant Yield with Fungal Enzymes

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The leaves and stems of the Rooibos plant (*Aspalathus linearis*) are used for the production of an herbal tea known for its health promoting properties, which have been linked to its flavonoid content but which is substantially reduced by the traditional processing method employed. Selected food-grade fungi were screened for their potential to improve the yield of soluble matter extracted from rooibos plant material. Fungal cocktails of hydrolyzing enzymes enhanced either the yield of soluble solids (*Lentinula edodes* and *Rhizopus oryzae* cultured in yeast peptone-wheat straw medium) or the yield in antioxidants from fermented rooibos (*R. oryzae* cultured in potato dextrose or yeast peptone-wheat straw medium). When applied to green rooibos, *L. edodes* (cultured in yeast peptone-wheat straw medium) enhanced the release of soluble solids as well as color formation, leading to semifermented rooibos with a relatively high aspalathin content, compared to fermented rooibos.

KEYWORDS: Rooibos; *Aspalathus linearis*; *Lentinula edodes*; *Rhizopus oryzae*

INTRODUCTION

Rooibos (*Aspalathus linearis*) is an endemic South African fynbos plant, native to the Cedarberg Mountains of the Western Cape. The leaves and stems of the plant are used for the production of green and traditional ("fermented") rooibos tea, an herbal tea sold locally and on the international market. Anecdotal evidence suggests consumption of rooibos tea is linked to the relief of insomnia, nervous tension, mild depression, stomach cramps, constipation, and allergic symptoms (*1*). Polyphenols, or more specifically the flavonoids, present in rooibos tea have antioxidant and free radical scavenging activities (*2, 3*). However, "fermentation" of the plant material, an open-air oxidation process required for the development of the characteristic sweetish flavor and red-brown leaf and infusion color of traditional rooibos tea, results in a substantial decrease in aspalathin, a dihydrochalcone glycoside and the major monomeric antioxidant in rooibos (*4*). The low levels of aspalathin in the fermented rooibos aqueous extract, typically less than 0.5% (*1*), led to the development of green or unfermented rooibos (*5*) for the manufacturing of extracts with a high aspalathin content for inclusion in nutraceutical and cosmetic products, as well as for the herbal tea market. Green rooibos consumed as an herbal tea has a very grassy, hay-like flavor. Since color also plays an important role in the consumer's perception of quality, the yellowish-orange color of green rooibos infusions, instead of the red-brown color of traditional rooibos infusions, could be considered representative of a "weak" tea. There is thus a need to develop a rooibos tea with a substantial quantity of aspalathin, but with improved color.

Only 20% of traditional rooibos tea is hot water soluble in comparison to as much as 40% for black tea, produced from *Camellia sinensis*. Yields of soluble matter are further reduced by poor extraction of these compounds (50−60%) due to their chemical nature (*1*). Shortages of rooibos raw material due to increasing demand and persisting droughts necessitated the development of new technologies or processes to improve the extraction efficiency during the production of extracts from both green and traditional rooibos. Furthermore, local industries were obliged to use rooibos material with higher stalk content for the preparation of tea extracts. However, the extraction of soluble matter from the stalks is further impaired by the insoluble nature of the cellulosic backbone of the plant material. Higher yields from the plant material would also decrease production cost of extracts through lower material costs and higher production capacity.

It has been reported that the application of exogenous cellulolytic enzymes, e.g., cellulases, pectinases, and xylanases, which can degrade the cell walls of tea leaves, may enhance the maceration of black tea leaves (*6*). Crude enzyme extracts of *Aspergillus flavus*, *Aspergillus indicus*, and *Aspergillus niveus* improved a number of quality parameters in black tea, including

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theaflavin and thearubigen levels, total liquor color, dry matter, and total soluble solids (*7*). These crude fungal extracts contained a variety of enzymes, including cellulases, xylanases, pectinases, and laccases.

White-rot fungi are renowned for the production of polysaccharases and laccases and are of increasing biotechnological interest since they produce a wide range of extracellular enzymes that can convert insoluble lignocellulosic substrates to soluble substances (*8*). Although fungal enzymes are known for the efficient hydrolysis of various plant materials, they have not yet been evaluated for the bioprocessing of rooibos plant material. In principle, the complex polysaccharide structures of the rooibos plant material could be macerated by polysaccharases that target the cellulolysic material, which could assist in the release of polyphenols. This may improve the extraction of soluble solids as well as aromatic and antioxidant compounds. Polyphenols in turn may also act as substrates for laccases (benzenediol:oxygen oxidoreductases; EC 1.10.3.2) that play an important role in polymerization reactions through the oxidation of a variety of mono-, di-, and polyphenols, aminophenols, aromatic amines, etc., leading to browning of the plant material (*9*).

The objective of this study was to screen selected food grade fungal species known for the production of hydrolytic and oxidative enzymes during wood degradation for their potential application to improve color development from green rooibos, while reducing loss of flavonoid antioxidants and/or increase the extraction of soluble matter from fermented rooibos products. The fungi investigated in this study included *Lentinula edodes* (Berk.) Pegler, the Asian shiitake mushroom, which produced high amounts of hydrolases and oxidases (including laccaces) during bioconversion of lignocellulosic wastes (*10–12*). *Rhizopus oryzae* is a valuable filamentous fungi used for the production of fermented foods, industrial enzymes, organic acids, and corticosteroids (*13*). *Pleurotus ostreatus* var. *florida* is a commercially important edible mushroom, commonly known as the oyster mushroom. It is industrially produced as human food, and together with *Pleurotus djamor*, it accounts for nearly a quarter of the world's production of cultivated fungi (*14, 15*). *Aspergillus niger* is probably the most biotechnologically important fungal species with GRAS status (*16*) and is one of the best known producers of citric acid (*17*). It is currently used in the food industry to produce numerous proteins and metabolites (*16*), including glucoamylase (*18*).

Fungal strains representing *A. niger*, *L. edodes*, *P. djamor*, *P. ostreatus* var. *florida*, and *R. oryzae* were characterized in terms of their cellulase (endoglucanase), xylanase, pectinase, and laccase activities. The supernatants from cultures containing the crude enzyme extracts were evaluated for their ability to enhance the quality of rooibos plant material, i.e., color development from green rooibos, or enhanced extraction of soluble solids, polyphenols, and/or antioxidants from green or fermented rooibos.

MATERIALS AND METHODS

Plant Material. Dried green (unfermented) rooibos tea was supplied by the Post-Harvest and Wine Technology Division of ARC Infruitec-Nietvoorbij (Stellenbosch, South Africa). Dried fermented rooibos with a high stalk content (sieved >2 mm) was supplied by Afriplex (Pty) Ltd. (Paarl, South Africa). The dry plant material was used as fresh plant material was not readily available for day-to-day experimental work. Due to large variations between different batches of rooibos material, a control treatment was included in every experiment, and the results are expressed as a percentage relative to the respective control.

Chemical Analysis. The most important chemical properties of the fermented rooibos material were determined by quantifying the alcohol-benzene and hot water soluble extractive contents, as well as lignin and cellulose content (all measurements were done in triplicate on a combined sample taken in a random fashion). The Seifert cellulose content was determined as described by Browning (*19*), whereas the TAPPI standard method number T222 om-88 (*20*) was used to determine the Klason lignin content. Alcohol-benzene extractive content was determined according to TAPPI standard method number T204 om-88, but the alcohol-benzene was substituted with a cyclohexane-ethanol mixture. This was followed by the determination of hot water soluble extractive content according to TAPPI standard method T264 om-88.

Polysaccharides were hydrolyzed by subjecting ~0.25 g of tea leaves to two-stage sulfuric acid hydrolysis (*21*). After neutralization with $CaCO_3$ to pH ~5.5, samples were amended with 20 mg of myo-inositol (internal standard) and then centrifuged (1500*g*, 15 min), and 10 mL of supernatant was lyophilized, resuspended in 1000 μL deionized water, and centrifuged at 12000*g* for 5 min. Supernatants were dried under an air stream and then subjected to reduction with Na-borodeuteride and acetylation with acetic anhydride, as described by Blakeney et al. (*22*). Gas−liquid chromatography of alditol acetates was performed using a Hewlett-Packard 6890 Plus GC fitted with a flame ionization detector and a Supelco SPB-225 capillary column (30 m × 0.25 mm, with 0.25 um film thickness). Samples (1 μL) were injected at a split ratio of 50:1 using Helium as carrier gas. The temperature program was as follows: 215 °C for 2 min, then increased at 4 °C per min for 3.75 min, then held at 230 °C for 11.25 min.

Strains and Culture Conditions. Fungal strains included in this study are *A. niger* ATCC 10864 (American type culture collection), *L. edodes* ABO 287, *P. djamor* ABO 283, *P. ostreatus* var. *florida* ABO 280 and *R. oryzae* MP1 (Department of Microbiology culture collection, Stellenbosch University). The strains were maintained by periodic transfer to malt extract agar plates (MEA, Sigma Chemical Co., St Louis, MO), incubated for 5 days at 30 °C (*A. niger* and *R. oryzae*) or at 25 °C (*Pleurotus* spp.), whereas *L. edodes* was grown for 21 days at 25 °C on MYPG plates (10 g/L malt extract, 2 g/L yeast extract, 2 g/L peptone, 10 g/L D-glucose, 2 g/L KH_2PO_4, 1 g/L $MgSO_4 \cdot 7H_2O$, 2.3% agar) (*23*).

Precultures were grown in 200 mL of liquid peptone-dextrose medium (PD, Sigma Chemical Co., St Louis, MO) with 5% citrate buffer (pH 5) in 1 L cotton-plugged Erlenmeyer flasks. Spores were harvested from *A. niger* and *R. oryzae* as previously described (*24, 25*). Flasks with 3 L of PD medium or yeast peptone medium plus wheat straw (YP-wheat straw, containing 2 g/L yeast extract, 2 g/L tryptone peptone, and 10 g/L wheat straw sieved at 2 mm) were inoculated at an initial concentration of 2×10^7 spores/mL for *A. niger* and *R. oryzae* or with five mycelial covered agar blocks (6 mm diameter) for *P. ostreatus* var. *florida*, *P. djamor*, and *L. edodes*. The flasks were incubated on a rotary shaker at 125 rpm at 30 °C for 4 to 5 days (25 °C for *Pleurotus* spp.).

Liquid Enzyme Assays. After incubation, fungal biomass was collected via centrifugation (12000*g* for 20 min), and the supernatant (enzyme source) was concentrated 10-fold with the Pellicon Casette and Filter cross-flow ultra filtration device (Millipore, Billerica, MA) with a 5 kDa cutoff membrane. The endoglucanase, xylanase, and pectinase activities in the supernatant were measured using 1% birchwood xylan (Sigma Chemical Co., St Louis, MO), 1% carboxymethylcellulose (Sigma Chemical Co.), and 0.1% polygalacturonic acid (Fluka BioChemika AG, Buchs, Switzerland) as respective substrates (*26*). One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of xylose or glucose equivalent per milliliter per minute under the assay conditions. Reducing sugars were determined with the dinitrosalicyclic acid (DNS) method allowing a reaction time of 5 min (*27*). Laccase activity was determined according to the method of Jönsson et al. (*28*), where one unit of laccase activity was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol ABTS in a 100 μL reaction mixture for 1 min at 30 °C.

Values given for each experiment are the means of at least three treatment replications. Enzyme and substrate controls were routinely included, and all enzyme preparations were appropriately diluted to determine activity in the presence of a negligible background.

Laboratory-Scale Evaluation of Fungal Cocktails. Duplicate batches of 100 g of green rooibos were treated with 150 mL of the five fungal enzyme extracts for 6 h at 40 °C (controls received no enzymes). The tea was dried in an air tunnel for 3 h at 40 °C. On the basis of the results obtained with green rooibos, duplicate batches of 100 g of fermented rooibos (stalky material) were treated with 150 mL of the *R. oryzae* and *L. edodes* extracts for 2 h at 40 °C and then dried in an air tunnel for 3 h at 40 °C.

Analysis of Quality Parameters. Tea extracts were prepared in duplicate by adding 150 mL of freshly boiled purified and deionized water to 2 g of tea and stirring for 2 min on a magnetic stirrer, whereafter the extract was filtered through a Whatman #4 filter paper. The soluble solid content, expressed as g SS/100 mL extract (%SS), was determined gravimetrically in duplicate after 20 mL of the extract, pipetted in duplicate into nickel moisture dishes, was evaporated on a steam bath and dried for 1 h at 100 °C in a laboratory oven. Aliqouts of the extracts were stored frozen at −20 °C until further analyses.

The polyphenol (TP) content of the extract, expressed as mg gallic acid equivalents (GAE)/g tea, was determined according to the Folin-Ciocalteu assay of Singleton and Rossi (*29*) with gallic acid as standard reference. Each extract was analyzed in triplicate. The total antioxidant activity of extracts, expressed as μmol Trolox/g SS, was determined in duplicate using the ABTS cation radical scavenging method with Trolox as standard (*30*). The industry benchmarks of 20%TP/SS and 1200 μmol Trolox/g SS were used throughout the study.

Quantification of the major flavonoids in the extracts was carried out in duplicate with UV detection at 288 and 255 nm, depending on the chemical structure, using authentic standards. Separation of extracts (4) was done by reversed-phase HPLC on a Lichrophere 100 RP-18 (5 μm particle size, 250 × 4 mm, ID) column with a LiChrospher 100 RP-18 (5 μm particle size) guard column at 30 °C, using a Waters LC Module I equipped with a 2996 PDA detector using Millenium^{32} version 4.0 software (Waters, Milford, MA). Objective color measurements of extracts (L_h, a_h, and b_h) was done with a Colorgard 2000 system (BYK-Gardner, Geretsried, Germany) with a TM-M transmission attachment (5 mm path length quartz cell) to the 05 sensor (*31*). Triplicate readings of each extract were done according to the three-dimensional Hunter-scale where L_h indicates black (−) to white (+) tones; a_h indicates green (−) to red (+) tones, and b_h indicates blue (−) to yellow (+) tones.

Small-Scale Simulated Industrial Treatments of Fermented Tea with Fungal Cocktails. Duplicate batches of 50 g of fermented material were treated with concentrated *L. edodes* and *R. oryzae* supernatants (cultured in 3 L of YP-wheat straw medium) for 2 h at 40 °C followed by drying in an air tunnel for 4 h at 40 °C. Duplicate batches of 40 g of dried tea were extracted with 400 mL of deionized water at 90−93 °C for 30 min, whereafter it was decanted and filtered hot through Whatman #4 filter paper. The filtrate was cooled to room temperature and cleaned by filtration through a 0.8 μm pore size glass fiber prefilter (Millipore AP15). Samples were collected after each filtration stage for further analysis.

Statistical Analyses. All results were statistically analyzed using one-way ANOVA and the Bonferroni posthoc tests and, where applicable, were expressed as a percentage relative to the values obtained with the respective controls.

RESULTS

Chemical Analysis of Plant Material. The dried fermented tea material contained 12.85 ± 1.32% water-soluble and 3.74 ± 0.75% solvent-extractable matter, as well as 41.96 ± 0.32% cellulose and 26.88 ± 0.57% lignin. This suggested that especially cellulases could be effective to hydrolyze the plant material to release additional soluble matter. The high lignin content possibly reflects the total polyphenol content, i.e., lignin as well as antioxidants in the plant material.

Table 1. Neutral Sugar Analysis of Polysaccharides in Fermented Rooibos Tea

monosaccharide	mole fraction[a]
glucose	0.668
xylose	0.206
arabinose	0.042
galactose	0.035
mannose	0.026
rhamnose	0.020
fucose	0.003

[a] Fraction each monosaccharide represented of the total measurable neutral sugars.

Table 2. Quantitative and Qualitative Analysis of Selected Enzymes in 10-Fold Concentrated Fungal Extracts

	activity (IU/mL)[a]			
cocktail	endoglucanase	pectinase	xylanase	laccase
R. oryzae-YP-wheat straw	**8.1**[b]	1.2	**344.5**	583.7
A. niger-YP-wheat straw	**7.3**	1.7	**183.1**	570.3
P. djamor-YP-wheat straw	4.6	**2.2**	9.6	**594.6**
P. ostreatus var. *florida*-YP-wheat straw	3.9	1.2	14.0	568.9
L. edodes-YP-wheat straw	1.2	**3.3**	4.1	176.1

[a] Values given show the means of duplicate or triplicate measurements. [b] The best two values for each type of enzyme activity are indicated in bold.

Neutral sugar analysis of the polysaccharides in fermented rooibos plant material (**Table 1**) indicated that glucose and xylose represent the majority of sugar moieties in the plant material (67% and 21%, respectively). Glucose could be released via the action of a combination of enzymes, including endoglucanases, whereas xylose indicated that xylanases could play an important auxiliary role in the maceration of the plant material.

Quantitative Analyses of Enzymes in Fungal Extracts. It was imperative to develop an enzyme cocktail with high levels of those enzymes identified as important for value-addition to rooibos (**Table 1**), i.e., endoglucanase, pectinase, and xylanase (improved solubility of plant material) and laccase (improved color and flavor). Previous results (data not shown) suggested that YP-wheat straw medium supported the production of especially laccases, endoglucanase, and xylanase by *L. edodes*. The five fungal strains were therefore evaluated in YP-wheat straw medium for their ability to produce endoglucanase, pectinase, xylanase, and laccase. The best results were obtained with the concentrated *R. oryzae* cocktail that displayed the highest level of endoglucanase and xylanase activities (8.1 and 344.5 IU/mL, respectively), as well as a very high level of laccase activity (583.7 IU/mL, **Table 2**). Relatively high levels of laccase activity were also shown by the *A. niger*, *P. djamor*, and *P. ostreatus* var. *florida* strains, all exceeding 500 IU/mL.

Laboratory-Scale Treatment of Green and Fermented Rooibos with Fungal Cocktails. The presence of high laccase activities in the supernatants of *R. oryzae*, *A. niger*, *P. djamor*, and *P. ostreatus* var. *florida* suggested that these fungal cocktails could have the potential to improve the color and flavor development from green rooibos tea. However, when cultured in YP-wheat straw, only the *L. edodes* extract improved the perceived aroma and color development from green tea as

Table 3. Improvement in Soluble Solids (%SS), Total Polyphenols (TP) and Color Determinants from Green Rooibos Material after Treatment with Fungal Cocktails in Laboratory-Scale Treatments[a]

cocktail	%SS (g SS/100 g dry leaves)	%TP (g GAE/100 g tea)[b]	%TP/SS (g GAE/100 g SS)	extract color		
				L_h	a_h	b_h
control-PD	17.3 ± 0.7	4.2 ± 0.0	24.4 ± 0.6	81.5 ± 0.3	−2.3 ± 0.03	44.3 ± 0.4
P. ostreatus var. *florida*-PD	20.5 ± 1.0	4.6 ± 0.3	22.4 ± 0.4	81.5 ± 0.	−2.3 ± 0.0	46.4 ± 0.2
	118%[c]	108%	92%	100%	100%	105%
control-YP-wheat straw	13.3 ± 0.5	3.9 ± 0.2	29.1 ± 0.2	83.9 ± 0.5	−1.1 ± 0.0	25.9 ± 0.6
L. edodes-YP-wheat straw	15.6 ± 0.8	3.0 ± 0.1[c]	19.4 ± 1.5[c]	74.9 ± 1.1[c]	4.7 ± 0.8[c]	44.5 ± 0.0[c]
	117%	78%	67%	**89%**	**−417%**	**172%**
R. oryzae-YP-wheat straw	13.9 ± 0.7	4.0 ± 0.2	29.0 ± 0.0	83.2 ± 1.9	−0.5 ± 0.2	27.7 ± 0.6
	105%	105%	100%	99%	**42%**	107%
control-PD	14.1 ± 0.4	3.8 ± 0.1	26.9 ± 0.2	87.3 ± 1.3	−1.0 ± 0.1	15.8 ± 0.6
L. edodes-PD	14.1 ± 1.1	3.8 ± 0.2	26.8 ± 0.5	82.6 ± 1.3	0.5 ± 0.7	41.1 ± 0.6
	100%	99%	100%	95%	**−47%**	**261%**
R. oryzae-PD	13.6 ± 1.0	4.2 ± 0.4	30.6 ± 0.3[c]	87.4 ± 1.3	−1.1 ± 0.1	24.5 ± 1.2
	96%	109%	**113%**	100%	110%	**155%**

[a] Values given are the means of duplicate or triplicate measurements on duplicate treatments. Improvements of more than 10% are indicated in bold. [b] Benchmark quality parameters: 20 g GAE/100 g SS (%TP/SS), 1200 μmol Trolox/g SS. [c] Values expressed as a percentage relative to control.

determined by expert visual and sensory analyses (data not shown). The color development was also reflected in the color of the extract (**Table 3**, lower L_h with higher a_h and b_h values). This was of particular interest given that the *L. edodes*-YP-wheat straw cocktail had the lowest laccase activity (176 IU/mL) of the five strains that were evaluated.

The efficacy of the YP-wheat straw cocktails were subsequently compared to cultures prepared in PD medium that yielded a lower laccase activity for *R. oryzae* (74 IU/mL). Please note that only results that significantly influenced the %SS, TP/SS, or extract color are shown in **Table 3**. The concentrated *P. ostreatus* var. *florida*-PD and the *L. edodes*-YP-wheat straw cocktails increased the yield in soluble solids from green rooibos by 17−18% (**Table 3**). However, the TP content (%TP/SS ratio) of the soluble solids prepared with the *L. edodes*-YP-wheat straw cocktail was reduced by 33%, suggesting extraction of mostly nonphenolic compounds. This was confirmed by the HPLC analyses of the flavonoids (**Table 4**) that showed a 66% decrease in the total flavonoid content (% total flavonoids/SS ratio), as well as a 90% reduction in the level of aspalathin, the most important antioxidant in rooibos tea. In contrast, the *L. edodes*-PD cocktail reduced the aspalathin content of the extract to a lesser degree, while the content of all the other flavonoids increased (**Table 4**). The *R. oryzae*-YP-wheat straw cocktail had little effect on the TP (**Table 3**) and total flavonoid (**Table 4**) content of the soluble solids, nor was the treatment detrimental to the aspalathin content (**Table 4**). On the other hand, the *R. oryzae*-PD cocktail increased the %TP/SS ratio of the extract by 13% (**Table 3**), and HPLC analyses confirmed a 39% increase in the total flavonoid content (**Table 4**). This coincided with an increase in the levels of the major antioxidants in rooibos, such as aspalathin (46%), orientin (29%), and isoorientin (29%). The *P. ostreatus* var. *florida*-PD cocktail also had little effect on the TP content of the soluble solids (**Table 3**), but it showed a 29% increase in the total flavonoid content (**Table 4**), which coincided with an increase in the levels of aspalathin (57%) and nothofagin (43%).

For comparative purposes, the extracts from *R. oryzae* and *L. edodes* were also evaluated on fermented tea. On this substrate, the *R. oryzae*-YP-wheat straw cocktail improved the yield in %SS by 47% (**Table 5**) with 99% and 95% of the TP content and antioxidant activity retained, respectively. When treated with the *R. oryzae*-PD cocktail, there was no increase in soluble solids, but the TP content and antioxidant activity were increased by 14% and 13%, respectively. The improvement in antioxidant activity coincided with a 28% increase in the total flavonoid content of the soluble solids, as well as increases of at least 20% in the levels of seven of the major flavonoids found in rooibos (**Table 6**). The levels of aspalathin and nothofagin in these experiments compare well with the levels generally found in fermented tea produced with conventional methods (*32*): the extract treated with the *R. oryzae*-PD cocktail had an aspalathin content of 0.69 g/100 g SS compared to the average of 0.35 g aspalathin/100 g substrate reported for fermented tea extract powder produced by aqueous extraction of stalky material (*32*).

Small-Scale Simulated Industrial Treatment of Fermented Tea with Fungal Cocktails. Concentrated extracts from *L. edodes* and *R. oryzae* prepared in YP-wheat straw were used for simulated industrial extraction of soluble solids from stalky fermented rooibos material, which usually has less soluble solids than material with a high leaf content. The *L. edodes* and *R. oryzae* cocktails improved the yield in %SS by 19% and 30% in the 0.8 μm AP15 filtrates, respectively, whereas the *R. oryzae* cocktail also improved the release of TP by 31% (**Table 7**). This confirmed that the *R. oryzae* extract could assist in the release of additional 'active' soluble solids, i.e., solids with a high antioxidant value.

DISCUSSION

The low extraction efficiency usually associated with rooibos plant material suggests that many of the flavor and valuable compounds in rooibos may be trapped within the cellulolysic plant material of the leaves. Chemical analyses of fermented rooibos plant material indicated a 41.96 ± 0.32% cellulose and 26.88 ± 0.57% lignin content. We therefore assumed that some of these compounds could be released upon maceration of the plant material through treatment with microbial hydrolytic enzymes produced by fungi known for the production of complex polysaccharase systems (including cellulases, hemicellulases, and accessory enzymes responsible for the hydrolysis of β-glycosidic bonds and esters). Neutral sugar analysis of the polysaccharides indicated that glucose and xylose represent more than 86% of the subunits that could potentially be released via enzyme hydrolysis (**Table 1**).

On the basis of their food-grade status, *L. edodes*, *A. niger*, *R. oryzae*, *P. ostreatus* var. *florida*, and *P. djamor* were eval-

Table 4. HPLC Analysis of Flavonoids Extracted from Green Rooibos Material with Fungal Cocktails[a]

cocktail	asp[b]	orient	iso-orien	vitex	noth	isovitex	iso-quer/ rutin	quer	luteol	% total flavonoids/SS	% total flavonoids/TP
control-PD	2.00 ± 0.23	0.64 ± 0.04	0.78 ± 0.04	0.20 ± 0.01	0.12 ± 0.01	0.20 ± 0.01	0.29 ± 0.01	0.01 ± 0.00	0.01 ± 0.01	4.24 ± 0.34	27.43 ± 2.13
P. ostreatus var. *florida*-PD	3.15 ± 0.01[c]	0.67 ± 0.00	0.83 ± 0.00	0.200 ± 0.00	0.18 ± 0.00[c]	0.22 ± 0.00	0.24 ± 0.09	0.01 ± 0.00	0.00 ± 0.00	5.49[d] ± 0.11[c]	30.21 ± 0.90
	157%[d]	104%	106%	98%	**143%**	110%	83%	86%	40%	**129%**	110%
control-YP-wheat straw	3.33 ± 0.60	0.67 ± 0.11	0.77 ± 0.11	0.18 ± 0.03	0.33 ± 0.6	0.21 ± 0.03	0.89 ± 0.14	0.01 ± 0.00	0.01 ± 0.00	6.25 ± 1.05	21.43 ± 0.49
L. edodes-YP-wheat straw	0.35 ± 0.00[c]	0.57 ± 0.01	0.63 ± 0.01	0.18 ± 0.00	0.20 ± 0.00	0.19 ± 0.00	0.70 ± 0.01	0.01 ± 0.00	0.02 ± 0.00	2.78 ± 0.03[c]	14.37 ± 1.08
	10%	86%	82%	102%	60%	90%	79%	99%	120%	44%	67%
R. oryzae-YP-wheat straw	3.44 ± 0.01	0.66 ± 0.03	0.78 ± 0.02	0.20 ± 0.00	0.34 ± 0.01	0.21 ± 0.00	0.85 ± 0.00	0.03 ± 0.00[c]	0.01 ± 0.00	6.36 ± 0.07	21.91 ± 0.28
	103%	100%	101%	**113%**	103%	96%	96%	**217%**	**116%**	102%	102%
control-PD	3.41 ± 0.13	0.56 ± 0.02	0.66 ± 0.03	0.15 ± 0.01	0.31 ± 0.01	0.18 ± 0.01	0.76 ± 0.04	0.02 ± 0.00	0.01 ± 0.01	5.65 ± 0.24	20.96 ± 0.73
L. edodes-PD	2.06 ± 0.04	0.67 ± 0.00	0.75 ± 0.00	0.18 ± 0.00	0.36 ± 0.01	0.21 ± 0.00	0.88 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	5.01 ± 0.04	18.67 ± 0.45
	66%	**118%**	**114%**	**119%**	**115%**	**116%**	**116%**	**120%**	**163%**	89%	89%
R. oryzae-PD	4.58 ± 0.12[c]	0.73 ± 0.03	0.85 ± 0.03	0.19 ± 0.01	0.45 ± 0.01[c]	0.23 ± 0.01	0.99 ± 0.04	0.02 ± 0.00	0.01 ± 0.00	7.85 ± 0.24[c]	25.69 ± 0.60
	146%	**129%**	**129%**	**127%**	**145%**	**126%**	**131%**	104%	**163%**	**139%**	**123%**

[a] Values given are the means of triplicate measurements on duplicate treatments. Values expressed as g/100 g SS. Improvements of more than 10% are indicated in bold. [b] Abbreviations for flavonoids: asp, aspalathin; orient, orientin; isoorien, iso-orientin; vitex, vitexin, noth, nothofagin, isovitex, isovitexin; isoquer/rutin, isoquercitrin/rutin; quer, quercetin; luteol, luteolin. [c] Indicate significant differences in means on a 5% ($p < 0.05$) significance level. [d] Values expressed as a percentage relative to control.

uated on rooibos substrates for color development from green rooibos (most likely via the action of laccases), while retaining higher levels of aspalathin than traditional fermented rooibos, or for the release of soluble solids and/or antioxidants from fermented tea (via the action of a combination of cellulolytic enzymes).

Quite surprisingly, given the low laccase activity in the extract, the *L. edodes*-YP-wheat straw cocktail showed the best improvement in color development of green rooibos, although it greatly reduced the aspalathin content. It was also noted that the aroma reminiscent of the traditional rooibos developed when treated with the *L. edodes*-YP-wheat straw cocktail. This suggested that this cocktail could be further developed for the treatment of green rooibos material in order to reduce the fermentation time required for the development of the characteristic rooibos color and flavor. If such a process can be established in industry, it may also allow for a factory-based process as opposed to the current outdoor "fermentation" that exposes the material to microbial contamination and is dependent on favorable weather conditions, or it may shorten the fermentation period, thereby increasing the capacity of the traditional processing facility.

The *L. edodes*-PD cocktail showed promise in improving the extract color of green tea, without losing most of the aspalathin content. Analysis of a large number or green rooibos samples ($n = 340$) showed that ca. 25% of these samples contains between 1 and 1.99% aspalathin (*33*), indicating that the aspalathin content of the treated rooibos was comparable to that of a large percentage of green rooibos.

In general, the *R. oryzae*-YP-wheat straw cocktail seems to be the best candidate for the preparation of extracts from fermented rooibos, with an increased yield of 47% in soluble solids and no significant loss in the TP and flavonoid content and antioxidant activity. In contrast, the *R. oryzae*-PD cocktail was most effective in increasing the flavonoid levels of extracts from both green and fermented rooibos (13−14% increase), with increased levels of aspalathin, nothofagin, and most of the minor compounds by more than 22%. This translates into a 39% and 28% increase in the total flavonoid content of the soluble solids extracted from green and fermented rooibos, respectively. The higher efficacy of the *R. oryzae*-YP-wheat straw cocktail (as apposed to the *R. oryzae*-PD cocktail) in the extraction of soluble solids from fermented rooibos could be ascribed to the higher levels of endoglucanase and xylanase activities in the YP-wheat straw broth (8.1 and 344.5 IU/mL, respectively) than in the PD broth (2.3 and 0.6 IU/mL, respectively). As discussed earlier, glucose and xylose constitute more than 86% of the sugar monomers in the plant material and could therefore contribute to the soluble solids content following enzyme hydrolysis. Although there are many other enzyme activities in the crude enzyme extracts that were not quantified in this study, the most important finding is that the culture conditions, and therefore the enzyme concentrations in the fungal extract, determined the efficacy of *R. oryzae* for either the extraction of additional soluble solids (YP-wheat straw) or additional TP and antioxidants (PD) from fermented tea.

The laboratory observations were confirmed with an industrial extraction process of fermented rooibos material simulated on a laboratory scale with concentrated enzyme cocktails prepared in YP-wheat straw. The *L. edodes* and *R. oryzae* cocktails improved the %SS of the AP15-filtrates by 19% and 30%, respectively, whereas the *R. oryzae* cocktail improved the TP content by 39% when compared to the control treatment. For both treatments, the %TP/SS ratios (28% and 41% for *L. edodes*

Table 5. Laboratory Scale Treatment of Fermented Tea with *R. oryzae* Cocktails[a]

cocktail	%SS (g SS/100 g dry leaves)	%TP/SS (g GAE/100 g SS)[b]	antioxidant activity (μmol Trolox/g SS)[b]
control-YP-wheat straw	7.7 ± 0.6	29.5 ± 1.7	2024 ± 23
R. oryzae-YP-wheat straw	11.3 ± 0.9[c]	29.2 ± 1.0[c]	1914 ± 63
	147%[d]	99%	95%
control-PD	12.5 ± 0.7	25.5 ± 0.6	1547 ± 61
R. oryzae-PD	11.5 ± 0.7	28.9 ± 1.1	1752 ± 102
	91%	**114%**	**113%**

[a] Values given are the means of triplicate measurements on duplicate treatments. Improvements of more than 10% are indicated in bold. [b] Benchmark quality parameters: 20 g GAE/100 g SS (%TP/SS), 1200 μmol Trolox/g SS. [c] Indicate significant differences in means on a 5% ($p < 0.05$) significance level. [d] Values expressed as a percentage relative to the control.

Table 6. HPLC Analysis of Antioxidants Extracted from Fermented Tea with *R. oryzae* Cocktails[a]

cocktail	asp[b]	orient	isoorien	vitex	noth	isovitex	iso-quer/ rutin	% total flavonoids/SS	% total flavonoids/TP
control-YP-wheat straw	0.71 ± 0.01	0.12 ± 0.00	0.08 ± 0.00	0.24 ± 0.00	0.07 ± 0.00	0.11 ± 0.00	0.67 ± 0.001	2.00 ± 0.28	6.79 ± 0.14
R. oryzae-YP-wheat straw	0.73 ± 0.02	0.12 ± 0.04	0.08 ± 0.00	0.22 ± 0.00[d]	0.05 ± 0.00[d]	0.11 ± 0.00	0.58 ± 0.01[d]	1.89 ± 0.03	6.49 ± 0.21
	103%[c]	94%	95%	90%	75%	105%	87%	94%	95%
control-PD	0.52 ± 0.04	0.09 ± 0.00	0.06 ± 0.00	0.16 ± 0.01	0.05 ± 0.00	0.07 ± 0.00	0.52 ± 0.04	1.47 ± 0.08	5.80 ± 0.40
R. oryzae-PD	0.69 ± 0.01[d]	0.12 ± 0.00[d]	0.08 ± 0.00[d]	0.20 ± 0.00[d]	0.06 ± 0.00[d]	0.10 ± 0.00[d]	0.64 ± 0.01	1.89 ± 0.02[d]	6.54 ± 0.18
	134%	**127%**	**122%**	**123%**	**130%**	**137%**	**123%**	**128%**	**113%**
extract powder prepared with conventional processes[e]	0.35 ± 0.13	0.16 ± 0.02	0.05 ± 0.01	0.24 ± 0.01	0.11 ± 0.02	0.09 ± 0.03	0.32 ± 0.09		

[a] Values given are the means of duplicate treatments, expressed as g/ 100 g SS. Improvements of more than 10% are indicated in bold. [b] Abbreviations for flavonoids: asp, aspalathin; orient, orientin; isoorien, isoorientin; vitex, vitexin, noth, nothofagin, isovitex, isovitexin; isoquer/rutin, isoquercitrin/rutin. [c] Values expressed as percentage relative to the control. [d] Indicate significant differences in means on a 5% ($p < 0.05$) significance level. [e] Rooibos extract powder prepared by aqueous extraction of stalky fermented tea (*32*).

Table 7. Industrial Extraction Process of Stalky Fermented Tea Simulated on Laboratory Scale[a]

cocktail	filtrate	%SS (g SS/100 mL extract)	TP (g GAE/100 mL extract)	%TP/SS[b] (g GAE/100 g SS)
control-YP-wheat straw	Whatman #4	0.93	303 ± 2	32.6
	Ap15	0.91	345 ± 3	38.0
L. edodes-YP-wheat straw	Whatman #4	1.09	311 ± 2	28.5
		118%[c]	103%	88%
	Ap15	1.09	303 ± 3	27.9
		119%	88%	73%
R oryzae-YP-wheat straw	Whatman #4	1.24	387 ± 3	31.6
		133%	**128%**	97%
	Ap15	1.19	451 ± 3	40.6
		130%	**131%**	107%

[a] Values given are the means of duplicate treatments. Improvements of more than 10% are indicated in bold. [b] Benchmark quality parameter: 20 g GAE/100 g SS (%TP/SS). [c] Values expressed as a percentage relative to the control.

and *R. oryzae*, respectively) exceeded the industry benchmark of 20%TP/SS by a large margin. These results were very significant given that plant material with a high stalky content was used for the simulated industrial extraction; this material is usually considered 'low value' material that is less suitable for the production of extracts. It was purposefully selected to determine the applicability of the enzyme cocktails on lower value rooibos material that is not suitable for tea made by infusion (high quality tea leaves).

Our results therefore confirmed that exogenous microbial enzymes can be applied for the improved release of soluble solids and polyphenols from rooibos plant material. In addition, color development of green rooibos could be improved, but further studies on aroma development are required before the manufacturing process of traditional rooibos can be replaced with an enzyme-enhanced process. Furthermore, it became clear that different strains and the corresponding culture conditions (and therefore different enzyme combinations) determine the efficacy of the respective cocktail on different rooibos materials, i.e., green or fermented tea. The *R. oryzae*-PD cocktail was more suitable for the extraction of TP and flavonoids from fermented tea, whereas the *R. oryzae*-YP-wheat straw cocktail was more effective for the extraction of soluble solids, most likely due to the relatively high levels of endoglucanase and xylanase in the crude enzyme extract. It was therefore concluded that a mixture of cellulase and xylanase activities are required for hydrolysis of the rooibos plant material to release additional soluble solids.

PATENT INFORMATION

The findings presented here have been described in the following two patents. (1) Patent application PCT/IB2005/052490: A process of producing rooibos tea extract. The University of Stellenbosch, Agricultural Research Council and Cape Natural Tea Products (Pty) Ltd. (2) Patent ZA2005/06040: A process of treating green rooibos tea. The University of Stellenbosch, Agricultural Research Council and Cape Natural Tea Products (Pty) Ltd.

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