ORANGE BAGASSE AS SUBSTRATE FOR THE PRODUCTION OF PECTINASE AND LACCASE BY *BOTRYOSPHAERIA RHODINA* MAMB-05 IN SUBMERGED AND SOLID STATE FERMENTATION

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Orange bagasse comprising pulp tissues, rind, and seeds, constitutes a major industrial food waste arising from processing oranges for juice, and represents a fermentation feedstock for the production of enzymes. *Botryosphaeria rhodina* MAMB-05 grown on essential oils-extracted orange bagasse in submerged (SmF) and solid-state fermentation (SSF) with and without added nutrients produced pectinase and laccase. Highest enzyme titres (pectinase, 32 U ml^{-1}; laccase, 46 U ml^{-1}) occurred in SSF without added nutrients, indicating nutrient sufficiency of orange bagasse at a solids concentration of 16 % (w v^{-1}) to sustain growth and high enzyme titres. Orange essential oil extract added to nutrient medium containing 1 % glucose in SmF strongly inhibited fungal growth with consequent lower laccase and pectinase activities. The results demonstrate the need to remove the essential oils fraction before citrus waste can be successfully used as a fermentation substrate for enzyme production.

Keywords: Botryosphaeria rhodina MAMB-05; Orange bagasse; Citrus waste; Orange essential oil; Laccase; Pectinase; SmF; SSF

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INTRODUCTION

Enormous quantities of agro-industrial waste residues are generated throughout the world from processing raw agricultural materials for foods. These, in turn, impose a high BOD burden on the environment when dumped. Thus, agro-industrial residues from the processing of sugarcane, orange, coffee, and rice present suitable feedstocks for bioconversion into chemicals, including enzymes by fermentation processes, thereby adding value to what normally constitutes a waste product.

Brazil is the world's largest producer of oranges (14.4 million tons during season 2005/2006), and exports frozen orange juice concentrates of >1 million tons per year (ABECitrus 2007). The processing of orange juice generates huge amounts of citrus wastes (approximately 50 % of the fruit weight), which offers potential applications in biotechnology. The solid material arising from processing oranges for juice consists of peel, seed, and pulp (collectively referred to as orange bagasse; OB), and primarily constitutes a waste product. Valuable by-products from the processing of citrus waste

residues are the essential oils such as d-limonene (Clark 2003). The solid waste residue following essential oils extraction is used as an energy source to generate process-steam at the factory. This waste has also been pelletized and incorporated into animal feeds because of its nutritive value (Ítavo et al. 2000). Orange bagasse contains large amounts of soluble carbohydrates, particularly fructose, glucose, sucrose, and pectins, as well as insoluble cellulose, and has been used as a fermentation feedstock for the production of fermentation products including enzymes (Rosales et al. 2002).

Pectinases are a heterogeneous group of enzymes catalyzing the degradation of the pectic substances, and are produced by various fungi including *Aspergillus* sp. (Dhillon et al. 2004; Freitas et al. 2006; Patil and Davanand 2006), *Penicillium* sp. (Silva et al. 2002), and *Thermoascus aurantiacus* (Martins et al. 2002) when cultivated on agro-industrial wastes. Pectinases have a wide range of technical applications that include clarification of fruit juices (including grape must for wine production) during processing of the fruit, to improve plant oil extraction, and to de-gum fibers (Couto and Sanromán 2006). They have also been used in simultaneous saccharification solid-state fermentation of citrus peel wastes into bioethanol (Wilkins et al. 2007). Likewise, other hydrolytic enzymes such as cellulases, as well as oxidative enzymes, can be obtained by fermentation processes from agro-industrial wastes. Laccases are particularly abundant in living organisms capable of degrading plant materials, albeit the lignified cell wall. Laccases catalyze the oxidation of a large variety of reducing phenolic and aromatic compounds, which make them useful for biotechnological purposes (Couto and Herra 2006). These polyphenol oxidases have been obtained from fermentation of agricultural wastes, e.g., sugarcane bagasse (Meza et al. 2007), and brans from barley (Gómez et al 2005), wheat, and soybean (Papinutti and Forchiassin 2007).

The ascomyceteous fungus, *Botryosphaeria rhodina* MAMB-05, described as ligninolytic, produces enzymes degrading the lignified plant cell wall, including laccases (Barbosa et al. 1996; Dekker et al. 2007), pectinases (Cunha et al. 2003; Saldanha et al. 2007), cellulases, and xylanases (Dekker et al. 2001). Agro-industrial wastes have great potential for the production of enzymes, and as the applications of plant residue utilisation require large amounts of low-cost enzymes, it is essential to produce enzymes converting these wastes (e.g., through hydrolysis) at low cost, and solid state fermentation (SSF) is a means by which this is achievable.

The aim of the work presented here was to evaluate the potential of essential oil-extracted orange bagasse as a fermentation feedstock for the production of enzymes such as laccase and pectinase by *B. rhodina* MAMB-05 grown under different conditions of cultivation (submerged fermentation (SmF) and SSF), thereby utilizing an agro-industrial waste to produce value-added enzymes. We also report on the strong influence of orange essential oils on the growth of *B. rhodina* MAMB-05 when incorporated into basal medium, and its effect on the production of pectinase and laccase, with the view that if high enzyme titres are required, then it is necessary beforehand to remove the essential oils fraction from citrus waste materials.

EXPERIMENTAL

Materials

Orange (*Citrus sinensis* (L.) Osb) bagasse and essential oils extracted from orange waste were kindly provided by COROL/Rolândia, Paraná, Brazil. The bagasse consisted of pulp material including membrane tissues, seeds, and orange peel after extraction of the juice and essential oils. The material was sun-dried followed by pulverization in a ball-mill to a powder. Dried orange bagasse typically contains up to 12 % crude fibre (includes pectin and cellulose), 6.4 % protein, 19 % total sugar, and 9 % reducing sugars (Martins et al. 2002). The essential oil extract, the component of oil phase of orange liquor distillation, consists of a mixture of essential oils, predominantly d-limonene, and some aromatic compounds. The liquor was obtained from orange waste including peel, pulp, and seeds after juice extraction.

Microorganism and Cultivation

Botryosphaeria rhodina MAMB-05 isolate (Barbosa et al. 1995) was maintained through periodic transfer at 4 °C on potato-dextrose-agar. Inoculum was prepared by growing the fungus on glucose-agar plates [Vogel minimal salts medium (VMSM, Vogel 1956), which contains sodium citrate, citric acid, nutrients (NH_4NO_3, KH_2PO_4, $MgSO_4$, $CaCl_2$, and biotin) and trace elements (B, Cu, Fe, Mn, and Zn), agar (20 g l^{-1}), and glucose (10 g l^{-1})] at 28 °C for 5 days.

Two types of cultivations were performed, SmF and SSF. In the case of SmF, *B. rhodina* MAMB-05 was cultivated on 1 and 4 % (w v^{-1}) pulverized orange bagasse in 125 ml Erlenmeyer flasks containing 25 ml of either water, or supplemented with VMSM (1 ml of a 50-times concentrated VMSM solution (Vogel 1956) added per 24 ml water), and inoculated with three 7-mm diameter plugs taken from the periphery of freshly-grown mycelial-colonized agar plates. The cultures were maintained under agitation at 28 °C for 108 h and 180 rpm.

In the SSF experiments, 4 g of OB was placed in a 125 ml Erlenmeyer flask and the contents moistened with water to give a final solids concentration of 16 % (w v^{-1}), and were left stationary for 108 h at 28 ºC. In determining the growth profile for enzyme production by SSF, a set of 125 ml Erlenmeyer flasks each containing OB (4 g) to which 25 ml of water was added were maintained statically for times up to 144 h at 28 ºC. At 24-h intervals during the growth period, flasks were removed and the contents extracted as described below. All experiments were carried out in replicates of 4, and the results represent the mean value ± SD.

Growth on Basal Medium Containing Orange Essential Oils

B. rhodina MAMB-05 was grown in SmF in 125 ml Erlenmeyer flasks containing 25 ml basal medium (VMSM and 10 g l^{-1} glucose) and different concentrations of orange oil extract (0, 0.5, 1.0, 1.5 and 2.0 %, v v^{-1}). The flasks were inoculated with three 7-mm diameter agar plugs, and the cultures maintained under agitation (180 rpm) at 28 °C for 108 h. Following growth, the mycelium was recovered by centrifugation, and the cell-free fluid (ECF) used to assay for enzyme activity as described below. Biomass was determined gravimetrically by drying mycelium recovered by centrifugation in an oven at

70 ºC to constant weight. All experiments were carried out in replicates of 4, and the results represent the mean value ± SD.

Sample Preparation following Fermentation

Upon completion of SmF, the ECF was assayed for enzyme activity after removal of the residual solid residue by centrifugation (30 min at 5000 x *g* and 4 ºC). Fermented samples from SSF (16 % (w v^{-1}) orange bagasse) had de-ionized water (30 ml) added to them, and were left for 30 min on a rotary shaker (180 rpm) at ambient temperature. The resulting solids suspension was centrifuged, and the supernatant broth used for the analytical measurements.

Enzyme Activity Determinations

Laccase was assayed against ABTS (2,2[1]-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid, Sigma) at pH 3.0 and 50 °C, and activity expressed in units as μmol product formed min^{-1} ml^{-1} of enzyme solution (Barbosa et al. 1996). Pectinase activity was determined by measuring the release of reducing sugars from citrus pectin by the cuproarsenate method. The reaction mixture containing 0.1 ml pectin (Citrus pectin, Sigma; 1 %, w v^{-1}) in 50 mM sodium acetate buffer (pH 5.0) and 0.4 ml ECF, was incubated at 50 ºC for 15 min. One unit of pectinase activity was defined as the amount of enzyme releasing 1 μmol of reducing sugars (as galacturonic acid) min^{-1} ml^{-1} of enzyme solution.

Analytical Techniques

Reducing sugars were determined by the cuproarsenate method of Somogyi (1945), using D-galacturonic acid as the standard sugar, and total sugars by the phenol-sulfuric acid method with D-glucose as the standard (Dubois et al. 1956).

RESULTS AND DISCUSSION

Enzyme Production by Submerged Fermentation

B. rhodina MAMB-05 produces laccases constitutively when grown on several mono- and di- saccharides, and polysaccharides including pectins; and constitutive pectinases on galacturonic acid, as sole carbon sources (Cunha et al. 2003). When cultured on pectin, this has resulted in the production of higher levels of pectinase (Cunha et al. 2003 ; Dekker et al. 2001).

B. rhodina MAMB-05 was capable (Table 1) of growing on orange bagasse as substrate in SmF, and produced laccases and pectinases, both in the absence and presence of supplemented nutrients, i.e., Vogel minimum salts medium. Supplementing 1 % orange bagasse with VMSM resulted in only a slight increase in laccase titres (0.7-fold), but that of pectinase increased significantly by 3.5-fold. The higher enzyme titres obtained in the presence of VMSM indicated poor nutrient level in orange bagasse at low substrate loadings, and that addition of VMSM to the substrate promoted enzyme titres presumably through increased fungal growth. A similar effect has also been reported when nutrients were added to nutritionally-poor de-seeded sunflower heads (Patil and

Davanand 2006) and pectin-rich apple pomace (Rodriguez et al. 2007). In both cases, microbial growth was supported by the added nutrients, and this greatly enhanced pectinase titres by the different strains of *A. niger* studied.

Table 1. Comparison of Laccase and Pectinase Production by *Botryosphaeria rhodina* MAMB-05 in Submerged Fermentation on Orange Bagasse Supplemented with (+) and without (only water) Nutrients.

Orange bagasse (%, w v^{-1})	Enzymatic activity (U ml^{-1})		Final pH	Soluble residual sugars (g l^{-1})	
	Laccase	Pectinase		Reducing sugars	Total sugars
1 + water	0.93 ± 0.18	0.29 ± 0.09	7.13 ± 0.06	0.94 ± 0.09	1.99 ± 0.15
1 + VMSM	1.57 ± 0.56	1.31 ± 0.41	6.78 ± 0.05	0.58 ± 0.12	1.74 ± 0.18
4 + water	2.23 ± 0.19	1.99 ± 0.00	5.68 ± 0.00	2.46 ± 0.17	7.69 ± 1.54
4 + VMSM	2.37 ± 0.06	2.23 ± 0.20	6.67 ± 0.03	0.84 ± 0.02	3.72 ± 0.22

At a higher substrate loading (4 % OB), however, supplementation with VMSM resulted in no significant increases ($p>0.05$) in the titres of both laccase and pectinase compared to water. On the other hand, increasing the substrate loading of OB from 1 to 4 % (w v^{-1}) in the absence of VMSM increased enzyme titres; 1.4-fold for laccase, and 5.9-fold for pectinase. At the higher substrate loading in the presence of VMSM, however, there was only a slight corresponding increase in the titres of both enzymes (0.5-fold for laccase, and 0.7-fold for pectinase). In each case pectinase titres resulted in greater increases than laccase under SmF conditions. Other studies using orange waste materials have likewise reported that nutrient supplementation did not interfere with fungal growth, and effectively enhanced enzyme titres, for example, α-amylases (Djekarif-Dakhmouche et al. 2006).

The residual soluble total and reducing sugars content were higher in the cultures grown in the absence of VMSM, especially in the presence of higher amounts of orange bagasse (4 %). The residual total sugars were relatively high compared to the reducing sugar concentration, and this was most likely attributable to the production of the exopolysaccharide, botryosphaeran, by *B. rhodina* MAMB-05 during fungal growth (Steluti et al. 2004). The final pH increased in accordance with previous observations (Cunha et al. 2003).

Enzyme Production by SmF and SSF

As enzyme titres of laccase and pectinase could be increased in SmF by increasing the amount of OB from 1 to 4 % (w v^{-1}), it was of interest to examine the effect of increasing the solids loading of OB on enzyme titres. The solids loading was increased to 16 % w v^{-1}, and the experiment was run in the SSF mode with no nutrients added, as it was demonstrated above that adding VMSM to OB at 4 % w v^{-1} did not

promote enzyme production compared to water. The production of pectinase and laccase by *B. rhodina* MAMB-05 under the 2 different fermentation conditions (SmF on 4 % w v^{-1}; and SSF on 16 % w v^{-1} of orange bagasse) in the absence of added nutrients revealed that enzyme titres could be significantly increased. In SmF, the amount of laccase and pectinase produced was equivalent, but in SSF pectinase far exceeded laccase (Fig. 1). The difference can be explained by an increase of soluble nutrients, including residual pectin and free sugars in orange bagasse. This resulted in much higher levels of laccase (11.7 U ml^{-1}) and pectinase (24.1 U ml^{-1}) activity under the growth conditions. Orange bagasse has been used as a fermentable substrate for pectinase production by SSF (Silva et al. 2005), and recent studies comparing SSF with SmF using mandarin and apple peel wastes, indicated the type of enzyme produced (hydrolytic or oxidative) was highly dependent upon the method of cultivation of the basidiomyceteous fungi studied (Elisashvili et al. 2007).

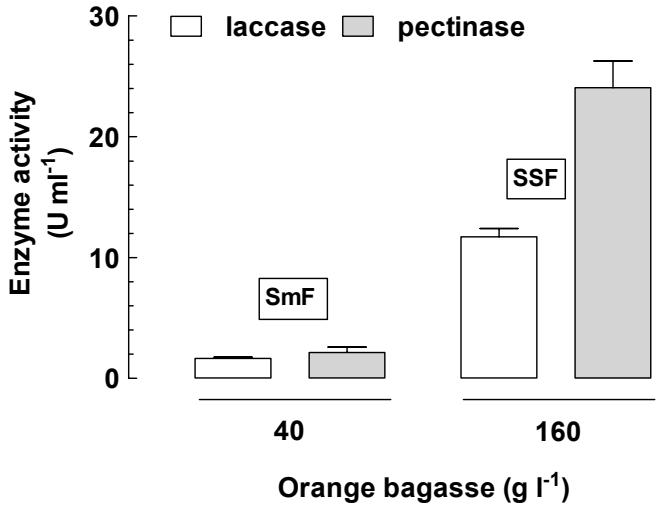

Fig 1. Production of pectinase and laccase by *Botryosphaeria rhodina* MAMB-05 cultivated on orange bagasse by submerged (SmF) and solid state fermentation (SSF).

Enzyme Production by SSF

Growth on orange bagasse by SSF greatly increased the production of pectinase and laccase by *B. rhodina* MAMB-05. To evaluate the course of fungal growth and enzyme production, *B. rhodina* MAMB-05 was cultured on orange bagasse by SSF over a period of 5 days (Fig. 2). During the first 2 days, a slight decline in the reducing sugars content was observed, and after 72 h the reducing sugars content decreased significantly. The pH decreased during the first 24 h of fermentation, possibly due to microbial production of organic acids, and a shift in pH was observed from 3.6 at 24 h to 5.7 at 96 h. As shown in Figure 2, pectinase activity increased from the beginning of SSF and attained a peak after 72 h (32 U ml^{-1}) of growth, while the activity of laccase reached a maximum at 96 h (46 U ml^{-1}). The value of pectinase was similar to that reported by Patil and Dayanand (2006) (30.3 U ml^{-1}) for *A. niger*. The value obtained for laccase (46 U ml^{-1}) was among the highest of values reported in the literature for fungal laccases by SSF grown on food waste residues.

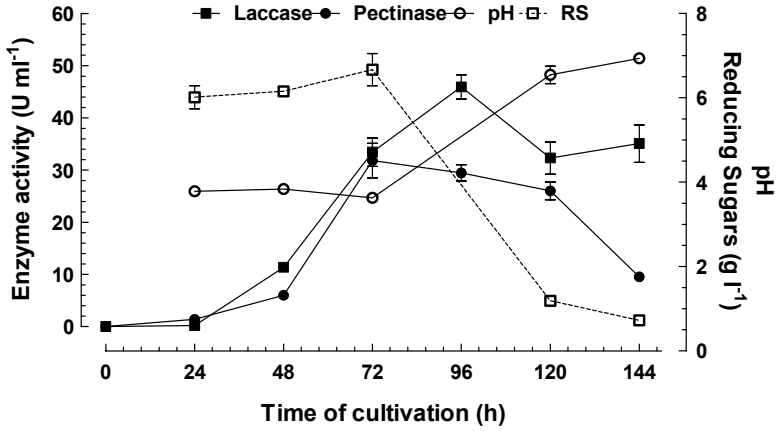

Fig 2. Growth profile showing the production of laccase and pectinase by *Botryosphaeria rhodina* MAMB-05 cultured on 16 % (w v^{-1}) orange bagasse by solid-state fermentation.

High laccase titres have been reported for basidiomycetes (e.g., *Trametes* sp.) grown on various fruit processing waste materials by SmF that included banana skin (15.7 U ml^{-1}) (Osma et al. 2007), and orange peel (31.8 U ml^{-1} with 5 mM added copper as inducer) (Rosales et al. 2002). Whereas different strains of *Lentinus edodes* and *Pleurotus* species were also shown to produce high levels of laccase in SmF using mandarin, apple, and banana peel, and with mandarin peel at a concentration of 50 g l^{-1} resulted in an enzyme titre of 7.7 U ml^{-1} (Silva et al. 2005; Elisashvili et al. 2006). The group of Songulashvili et al. (2007), on further screening of basidiomycetes for laccase production by SmF of food wastes including mandarin peels, were successful in promoting the yields of laccase to 36 U ml^{-1} (40 g l^{-1} substrate concentration and nutrient supplemented) by *Ganoderma lucidum* 447. However, these laccase yields were still much lower than that achieved in this work (46 U ml^{-1}) by *B. rhodina* MAMB-05 in SSF on orange bagasse without nutrients supplementation.

The Effect of Orange Essential Oils on Enzyme Production in SmF

The essential oils from orange peel and orange seed-press are rich in terpenes, especially d-limonene (4-isopropenyl-1-methylcyclo hexene), and aromatic compounds Corazzi et al. 2001). Terpenoids can be active against fungi and bacteria as antimicrobial agents (Cowan 1999). It is known that aromatic compounds such as guaiacol, catechol, and veratryl alcohol, for example, can increase the production of laccase by *B. rhodina* MAMB-05 at enzyme titres higher than constitutive levels Dekker et al. (2002). It was therefore of interest to determine whether *B. rhodina* MAMB-05 when cultured on basal medium supplemented with orange oil extract at different concentrations could likewise enhance laccase production. Table 2 shows that fungal growth was strongly inhibited by orange oil extract at concentrations greater than 1.0 % (v v^{-1}), and laccase titres were more greatly reduced compared to pectinase. Contrary to expectations, the aromatic compounds and monoterpenes in the orange oil extract could not serve as an inducer of laccase activity. On the other hand, pectinase production continued up to 2.0 % (v v^{-1}), but at titres considerably lower than the control, and was dependent upon the concentration of oil used.

Table 2. Production of Pectinase and Laccase by *Botryosphaeria rhodina* MAMB-05 Cultivated on Basal Medium (VMSM and glucose, 10 g l^{-1}) Supplemented with Different Amounts of Orange Oil Extract.

Orange oil extract (%. v v^{-1})	Biomass production		Laccase activity (U ml^{-1})	Pectinase activity (U ml^{-1})
	Amount (g l^{-1})	Degree of inhibition (%)		
0	6.649 ± 0.67	0	0.275 ± 0.009	0.565 ± 0.068
0.5	4.680 ± 0.19	30	0.093 ± 0.008	0.474 ± 0.017
1.0	2.880 ± 0.65	57	0.010 ± 0.000	0.385 ± 0.022
1.5	1.748 ± 0.20	74	0.023 ± 0.001	0.262 ± 0.063
2.0	1.024 ± 0.12	85	0.011 ± 0.001	0.320 ± 0.042

CONCLUSION

Conclusions can be drawn from this study that *B. rhodina* MAMB-05 produced higher enzyme titres of laccase and pectinase when grown on the agro-industrial waste, orange bagasse, as nutrient source than on refined carbohydrates (e.g., glucose, Cunha et al 2003; Saldanha et al. 2007) as fermentable substrates. In this case, orange bagasse was used in which the essential oils fraction had first been extracted, and the enzyme titres observed under un-optimised conditions in SSF for laccase were much higher than those reported for other industrial food processing waste residues as fermentation feedstocks for enzyme production.

We also showed that *B. rhodina* MAMB-05, grown on basal medium containing orange oil extract in SmF, was strongly inhibited, producing significantly lower enzyme titres that were considerably lesser for laccase than pectinase, compared to a control with no citrus oil added. It has recently been reported by Wilkins et al. (2007), that d-limonene strongly interfered with the growth of the yeast *Saccharomyces cerevisiae* during simultaneous saccharification fermentation of citrus peel wastes to which d-limonene was added. It is therefore important when using citrus wastes such as orange bagasse as a fermentable substrate for bioconversion (enzyme or bioethanol production), that the essential oils fraction be removed prior to bioconversion. Our findings are interesting and demonstrate the potential of orange bagasse as a low-cost fermentation feedstock for enzyme production. This is advantageous, as orange bagasse constitutes a common industrial food waste that is generated in enormous quantities on both continents of the Americas.

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