7

Soybean Oil and Meal as Substrates for Lipase Production by *Botryosphaeria ribis*, and Soybean Oil to Enhance the Production of Botryosphaeran by *Botryosphaeria rhodina*

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1. Introduction

Soybean oil, a common vegetable (plant seed) oil, has traditionally been used as a food in cooking and salad dressings, and more recently, as a feedstock for the production of biodiesel fuels (Bajaj et al., 2010; Ghaly et al., 2010). After extracting the oil from soybean seeds, the residue constitutes an important by-product called soybean meal, or oil-seed cake. This edible by-product, a rich source of proteins and amino acids, especially tryptophan, threonine and lysine (Ramachandran et al., 2007), has been used as a livestock feed, as well as a nutrient source and fermentable substrate for producing microbial lipases (Ul-Haq et al., 2002), and other enzymes (Singhania et al., 2009).

The fatty acid composition of soybean oil is typically: 53.8 % linoleic ($C_{18:2}$), 20.8 % oleic ($C_{18:1}$), 11.4 % palmitic ($C_{16:1}$), 9.3 % linolenic ($C_{18:3}$), 4.4 % stearic ($C_{18:0}$), and 0.3 % arachidic acids ($C_{20:0}$) (Ghaly et al., 2010). Besides the applications cited above, soybean oil was demonstrated an effective inducer for the production of fungal enzymes (lipases) that degrade plant seed oils (Messias et al., 2009). It has also been reported to enhance the synthesis of pleuromutilin by *Pleurotus mutilis*, an antibiotic effective against gram-positive bacterial pathogens (Hu et al., 2009), and promoted the production of laccases by *Botryosphaeria rhodina* MAMB-05 when added to nutrient medium (Dekker et al., 2007).

Lipases (EC 3.1.1.3; triacylglycerol acylhydrolases) are hydrolytic enzymes, which catalyse the hydrolysis of the ester linkages of long-chain acylglycerols to glycerol and free fatty acids. These enzymes also conduct interesterification, transesterification and ammonolysis reactions. Lipases are ubiquitous among microorganisms being produced by bacteria, actinomycetes, filamentous fungi and yeasts, and have found applications in various sectors of commerce (Li & Zong, 2010). The main industrial application of lipase is still restricted to their use in laundry detergents to remove fats and oil stains (Hasan et al., 2010). They are also used in various food and agro-chemical industries, e.g., processing foods, treatment of fatty effluents, synthesis of biosurfactants, removal of resins (pitch) in processing paper from wood cellulose pulps, and as

biocatalysts in biotransformation reactions in the semi-synthesis of drugs (Jaeger & Eggert, 2002; Gotor-Fernández et al., 2006). More recently, their applications have been extended to catalysis of transesterification of plant seed oils (triacylglycerols) for biodiesel production (Hasan et al., 2006; Bajaj et al., 2010; Ghaly et al., 2010).
Several genera of fungi, and mainly *Aspergillus, Colletotrichum, Penicillium*, and *Rhizopus*, have been studied for lipase production (Treichel et al., 2010), and more recently, the genus *Botryosphaeria* was described as a good producer of lipases by our research group. Among the nine isolates of *Botryosphaeria* studied, a strain of *Botryosphaeria ribis* EC-01 was found to produce high lipase titres (Messias et al., 2009).
The genus *Botryosphaeria* has been studied by our research group since 1995 (Barbosa et al., 1995) when an isolate of *Botryosphaeria* sp. MAMB-05 was found to be ligninolytic producing a polyphenol oxidase (laccase), and concomitantly secreted an exopolysaccharide (EPS) into the culture fluid during growth. The EPS was described as a β-glucan (Dekker & Barbosa, 2001), and characterized at the structure level as a β-(1→3),(1→6)-D-glucan, and named botryosphaeran (Barbosa et al., 2003). The production of botryosphaeran could be increased by the combined addition of soybean oil and the surfactant, Tween 80, to the nutrient media during submerged liquid fermentation (Silva et al., 2007).
In this chapter, we report on the comparison of different vegetable oils, including soybean oil, as substrates to produce lipases by *Botryosphaeria ribis* EC-01 by submerged fermentation, as well as the influence of nitrogen and phosphate sources on lipase production. A comparison of soybean meal with other oil-seed cakes (castor bean and corn kernel) as fermentative substrates for lipase production by *Botryosphaeria ribis* EC-01, as well as the effect of adding mineral nutrients to soybean meal to enhance lipase production by this fungal strain, is also discussed. The effect of adding soybean oil and Tween-80 to nutrient media to increase botryosphaeran production by *Botryosphaeria rhodina* MAMB-05 is also presented.

2. Experimental procedures

2.1 Materials

All plant seed oils (canola, coconut, corn, olive, soybean, and sunflower) were of food grade. Castor bean oil and meal were purchased from Remy Comércio e Beneficiamento de Mamona (Londrina-PR, Brazil); and hempseed oils (raw and refined) from Prairie Emerald (Hemp Oil, Canada). Corn steep liquor (Milhocina®) and corn meal were kindly donated by CornProducts Brazil (Mogi Guaçu-SP, Brazil), and soybean meal by IMCOPA (Cambé-PR, Brazil).

2.2 Methods

2.2.1 Microorganisms

Botryosphaeria rhodina MAMB-05 (Barbosa et al., 1995; 1996) and *Botryosphaeria ribis* EC-01 (Silveira et al., 1996) were isolated from the stem of eucalypt trees.

2.2.2 Preparation of inoculum and growth conditions

Fungal isolates were maintained on potato-dextrose agar (PDA) slants at 4 °C, and sub-cultured at three-monthly intervals. From PDA the fungi were transferred to agar plates containing glucose (10 g/L), minimum salts medium (VMSM; Vogel, 1956), and agar (20 g/L), and left at 28 °C for 5 d. Following growth, four plugs (0.7 cm-diameter) were taken from the mycelial-colonized agar with the aid of a sterile cork borer and used to inoculate Erlenmeyer flasks containing nutrient medium.

2.2.3 Enzyme production by submerged fermentation

Fungal cultures were developed in submerged fermentation on nutrient medium (VMSM) containing plant seed oils (1 %, v/v) as described by Messias et al., (2009) for lipase production. To evaluate the effects of nitrogen (N) and phosphate (P) on lipase production, *Botryosphaeria ribis* EC-01 was cultured on VMSM containing soybean oil (1 %, v/v) as the carbon source, and each of the different N and P sources (see below) for 5 days at 28 °C. N (i.e., NH_4NO_3, 0.2 % (w/v) and P (i.e., KH_2PO_4, 0.5% (w/v) in the VMSM medium were replaced separately by each of the inorganic salts: $NaNO_3$ (0.4 % (w/v), NH_4Cl (0.4 % (w/v), $(NH_4)_2SO_4$, (0.2 % (w/v), and Na_2HPO_4, NaH_2PO_4, K_2HPO_4, $NH_4H_2PO_4$, $(NH_4)_2HPO_4$; each at 0.5 % (w/v). Organic N sources included: urea, peptone, yeast extract and corn steep liquor (each of 0.2 %, w/v, concentration). In experiments evaluating the effect of P source on lipase production when *Botryosphaeria ribis* EC-01 was cultured on soybean oil (1 %, v/v), three phosphates (KH_2PO_4, K_2HPO_4, Na_2HPO_4 each at 0.5 %, w/v) were added to VMSM, but the initial pH of the medium was not adjusted. In a separate experiment, the initial pH value of the nutrient media was adjusted from 3.5 to 9.5 with 1 M HCl or 1 M NaOH, and lipase production evaluated. In experiments examining the effect of nutrient supplementation of oil-seed meals on lipase production, water or VMSM were added to the oil-seed meals (castor bean, corn kernel and soybean at 1 %, w/v, final concentrations), and *Botryosphaeria ribis* EC-01 grown in submerged fermentation for 5 days at 28 °C. In separate experiments, the effect of soybean meal concentration was evaluated using final concentrations of 0.5, 1.0, 2.0, 4.0 and 6.0 % (w/v) made up in only distilled water. All experiments were conducted in replicates of three, and the results represent the means ± SD.

2.2.4 Enzyme assays

The extracellular fluids (ECF) arising from submerged fermentation were used as the source of lipases, and were obtained after removal of the fungal mycelium by centrifugation at 1,509 x *g* at room temperature for 15 min.

Lipase activity was assayed against *p*-nitrophenyl palmitate (*p*NPP, Sigma) as substrate according to Winkler & Stuckmann (1979). The reaction was carried out in 50 mM sodium phosphate buffer (pH 8.0) at 55 °C (Messias et al., 2009). Absorbance was measured spectrophotometrically (410 nm, molar extinction coefficient of *p*-nitrophenol (*p*NP) was 15,000 M^{-1} cm^{-1}). One unit of enzyme activity was defined as the release of 1 µmol of *p*NP per min under the assay conditions.

2.2.5 Harvesting and determination of mycelium biomass

Fungal cultures grown in submerged fermentation were harvested and mycelium removed by centrifugation (2,240 x *g*/15 min at 4 °C). The supernatant recovered was then filtered through glasswool, and collected for analysis. The fungal biomass (mycelium) was washed once with distilled water and measured gravimetrically after drying (70 °C) to constant weight in an oven. In experiments where fungal isolates were cultivated on soybean meal, fungal biomass was not quantified.

2.2.6 Analytical determinations

Extracellular protein was measured by a modified Lowry's method (1951) as described by Hartree (1972) when the fungus was grown on soybean oil, and by the Bradford method

(Bradford, 1976) where soybean meals were the fermentable substrate. Bovine serum albumin was used as the standard. Total sugars were determined by the phenol-sulfuric acid method (DuBois et al., 1956), and reducing sugars by the method of Nelson (1944) and Somogyi (1945); glucose was used as standard for both methods.

2.2.7 Determination of botryosphaeran and fungal biomass

Botryosphaeran production by *Botryosphaeria rhodina* MAMB-05 and its isolation from the ECF through precipitation with ethanol was developed as described by Steluti et al., (2004), and Silva et al., (2007).

3. Soybean oil as carbon source for lipase production by submerged fermentation

The endophytic, ascomyceteous fungus, *Botryosphaeria ribis* EC-01, is a constitutive and inductive lipase producer. A constitutive producer of lipase, because this enzyme was always expressed independent of the carbon source used in the culture medium including glucose (normally considered a catabolite repressor); and inductive, because lipase titres (U/mL) could be enhanced when carbon sources such as fatty acids (e.g., oleic acid), vegetable oils (soybean oil), surfactants (Tween-80), and emulsified lipids (stearic acid plus Triton X-100) were incorporated in nutrient medium (Messias et al., 2009). Several complex substrates have been reported to enhance lipase synthesis. Examples include wheat bran, rice bran, sugarcane bagasse, and oil-seed cakes derived from coconut, olive, sesame (Ul-Haq et al., 2002). However, vegetable oils, free fatty acids, hydrolysable esters, Tween surfactants, bile salts and glycerol appear to be essential supplements to enhance lipase yields (Gupta et al., 2004; Treichel et al., 2010).

Several vegetable oils were used as sole carbon source and compared to induce lipase production by *Botryosphaeria ribis* EC-01.

3.1 Effect of different vegetable oils as sole carbon source for the production of lipases by *Botryosphaeria ribis* EC-01

Among the vegetable oils tested as sole carbon source for *Botryosphaeria ribis* EC-01, raw hempseed oil and sunflower oil produced highest lipase titres, followed by canola oil, soybean oil, and olive oil (Table 1). Lowest activity was observed for coconut oil. In terms of specific lipase activity (U/mg), refined hempseed oil induced highest activities, followed by coconut oil and soybean oil, while olive oil was least. These results indicate that the composition of vegetable seed oils (nature of the fatty acids constituting the acylglycerols) affected *Botryosphaeria ribis* EC-01 metabolism (Table 1). Pogori et al., (2008) investigated lipase production by *Rhizopus chinensis* CCTCC M201021 and showed that soybean oil enhanced lipase production and was highest among other oils studied. Comparatively, *Botryosphaeria ribis* EC-01 was previously shown to produce highest lipase titres on soybean oil and glycerol, while eight isolates of *Botryosphaeria rhodina* produced significantly lower enzyme titres (Messias, 2008). Despite the source of the plant seed oils used as carbon source and the lipase yields produced by *Botryosphaeria ribis* EC-01, the fungal biomass observed did not have high variation (Table 1).

Plant seed oil	Lipase activity (U/mL)	Specific activity (U/mg*)	Fungal biomass (g/L)
Soybean	23.0 ± 0.82	138.4 ± 2.47	14.8 ± 0.42
Sunflower	27.7 ± 1.16	112.8 ± 1.79	12.0 ± 0.36
Olive	22.7 ± 2.41	40.1 ± 0.63	11.9 ± 0.20
Hempseed (refined)**	20.1 ± 1.35	227.6 ± 2.75	15.5 ± 0.24
Hempseed (raw)	28.0 ± 0.79	97.2 ± 3.52	14.8 ± 0.27
Canola	26.8 ± 2.45	44.7 ± 3.61	12.6 ± 0.58
Coconut	10.0 ± 0.50	170.0 ± 0.28	14.1 ± 0.35

* mg protein; ** degummed oil

Table 1. Comparison of lipase production by *Botryosphaeria ribis* EC-01 grown on different vegetable oils as sole carbon source for 5 days by submerged fermentation.

3.2 Effect of nitrogen source on lipase production by *Botryosphaeria ribis* EC-01 on soybean oil as sole carbon source

Nitrogen constitutes essential micronutrients for microbial growth, and can play an important role in enzyme production and their optimization. These nutritional requirements are present in defined nutrient media (synthetic medium), as well as complex components such as peptones, yeast extract, malt extract, and also agro-industrial residues containing all the components necessary for microorganism development (Treichel et al., 2010).

Both organic (urea, corn steep liquor, yeast extract, peptone) and inorganic (NH_4NO_3 or $(NH_4)_2SO_4$, NH_4Cl, $NaNO_3$) N sources were evaluated for the production of lipase by *Botryosphaeria ribis* EC-01. In all grown cultures, soybean oil and KH_2PO_4 were used as the carbon and P sources, respectively. The highest specific activities (32.2 U/mL and 89.3 U/mg) were observed in cultures containing NH_4NO_3 (Table 2). Microorganisms generally produce higher lipase levels on organic N sources (Sharma et al., 2001). *Aspergillus* sp., for instance, produced twice as much lipase in culture medium containing peptone than in the presence of NH_4NO_3 (Cihangir & Sarikaya, 2004), while the production of intracellular lipases by *Rhizopus oryzae* was higher on corn steep liquor (Essamri et al., 1998). For the genus *Botryosphaeria*, inorganic N sources, such as $(NH_4)_2SO_4$ and NH_4NO_3 were reported as having the best effect on production of laccases by *Botryosphaeria rhodina* MAMB-05 (Dekker et al., 2007), whereas for lipases, the highest specific activity produced by *Botryosphaeria ribis* EC-01 was observed for media containing NH_4NO_3 (Table 2).

The final pH values of the culture medium after 5 days growth ranged from 4.7 to 8.8, except that for NH_4Cl, where the final pH was 1.78. NH_4^+ is the most readily assimilated cation amongst inorganic N sources, as the N atom is at the same oxidation level (-3) as the N atom in biological molecules (amino acids, purines and pyrimidines). Considering that NH_4^+ is a weak acid, NH_4^+ can dissociate in fermentation medium to NH_3 and H^+. Ammonia enters cells by means of passive diffusion, whereas nitrate requires nitrate and nitrite reductase enzymes (both of which are NADPH-dependent) to be converted to NH_3. This may be a likely reason for the low final pH values observed with NH_4Cl, i.e., NH_3 was probably rapidly removed by the fungus from the culture medium leaving only H^+ and Cl^- ions, hence the acidity (Miranda et al., 1999; Galvagno & Forchiassin, 2004).

Nitrogen Source	Lipase activity (U/mL)	Specific activity (U/mg)	Final pH of culture medium	Fungal biomass (g/L)
None	1.29 ± 0.05	3.59 ± 0.46	6.48 ± 0.04	8.67 ± 1.09
Urea	18.26 ± 0.87	12.75 ± 0.71	8.80 ± 0.07	7.84 ± 0.28
Corn Steep Liquor	8.93 ± 0.35	25.51 ± 2.27	6.41 ± 0.03	10.37 ± 0.36
Yeast extract	1.89 ± 0.45	5.71 ± 1.40	6.88 ± 0.10	11.51 ± 0.66
Peptone	15.41 ± 2.20	32.46 ± 5.90	7.36 ± 0.02	13.08 ± 0.85
$(NH_4)_2SO_4$	7.00 ± 1.69	27.46 ± 4.37	4.73 ± 0.30	10.00 ± 0.85
NH_4Cl	25.24 ± 4.51	37.40 ± 5.71	1.78 ± 0.13	11.19 ± 0.50
NH_4NO_3 *	32.19 ± 1.97	89.34 ± 2.56	6.87 ± 0.12	12.27 ± 0.14
$NaNO_3$	4.52 ± 0.73	15.69 ± 2.99	7.17 ± 0.05	12.19 ± 0.27

* N source normally used in minimum salts medium (VMSM)

Table 2. Effect of nitrogen sources on lipase production by *Botryosphaeria ribis* EC-01 grown on soybean oil for 5 days by submerged fermentation.

Low lipase yields were observed in the presence of yeast extract, as well as the inorganic salt, $NaNO_3$. Despite the low lipase yields, *Botryosphaeria ribis* EC-01 growth was generally higher when the nutrient media was supplemented with N rather than without added N (Table 2).

After 5 days growth on nutrient medium containing soybean oil and supplemented with corn steep liquor, yeast extract or peptone as N sources, the total sugars (represented by polysaccharides and glyco-conjugates in these N sources) consumed by the fungus were low (2, 18 and 32 %, respectively). The sugar content (measured as reducing sugars), however, presented by these N sources increased 10-fold at the end of fermentation. The results indicated that *Botryosphaeria ribis* EC-01 preferentially used soybean oil as the primary carbon source for growth and lipase production, as evidenced by the sugar content remaining at the end of fermentation.

3.3 Effect of phosphate source and initial pH on the production of lipase by *Botryosphaeria ribis* EC-01 using soybean oil as sole carbon source

K_2HPO_4, KH_2PO_4 or Na_2HPO_4 are typical phosphates used in nutrient media for lipase production by fungi (Macris et al., 1996; Gulati et al., 1999; Fadiloglu & Erkmen, 1999; Lima et al., 2003; Lin & Ko, 2005; Wang et al., 2008). Several P sources were compared for lipase production by *Botryosphaeria ribis* EC-01, and are shown in Table 3. The highest specific activities of lipase produced were observed in media containing K_2HPO_4. Fungal biomass was highest on KH_2PO_4, followed by K_2HPO_4.

The final pH values in fungal cultures grown on media containing Na_2HPO_4 and K_2HPO_4 were alkaline (8.0 and 8.2, respectively), and acidic (pH 3.8) for $NH_4H_2PO_4$, probably due to the rapid uptake of NH_4^+ by the fungus as explained above.

A comparison of the effect of three different P sources (K_2HPO_4, KH_2PO_4, Na_2HPO_4) on lipase production by *Botryosphaeria ribis* EC-01 grown on soybean oil and NH_4NO_3 as N source is presented in Figure 1. The profiles of the specific lipase activities differed for each of the P sources evaluated, with highest activity (285 U/mg) being produced on K_2HPO_4

Sources of phosphate	**Lipase activity** (U/mL)	**Specific activity** (U/mg)	**Final pH of culture medium**	**Fungal biomass** (g/L)
Na_2HPO_4	40.83 ± 1.95	104.23 ± 3.20	8.05 ± 0.06	9.40 ± 1.00
NaH_2PO_4	29.07 ± 1.10	94.50 ± 9.19	6.45 ± 0.06	7.25 ± 0.30
KH_2PO_4 *	32.19 ± 1.98	89.34 ± 2.56	6.87 ± 0.12	12.27 ± 0.14
K_2HPO_4	38.23 ± 3.71	201.91 ± 2.51	8.15 ± 0.09	11.76 ± 0.41
$NH_4H_2PO_4$	39.93 ± 6.47	68.50 ± 4.62	3.81 ± 0.57	10.92 ± 0.33
$(NH_4)_2HPO_4$	10.43 ± 2.09	30.89 ± 5.80	5.48 ± 0.18	10.40 ± 0.25

* P source normally used in the minimum salts medium (VMSM)

Table 3. Effect of phosphate sources on the growth and production of lipases by *Botryosphaeria ribis* EC-01 on soybean oil for 5 days by submerged fermentation.

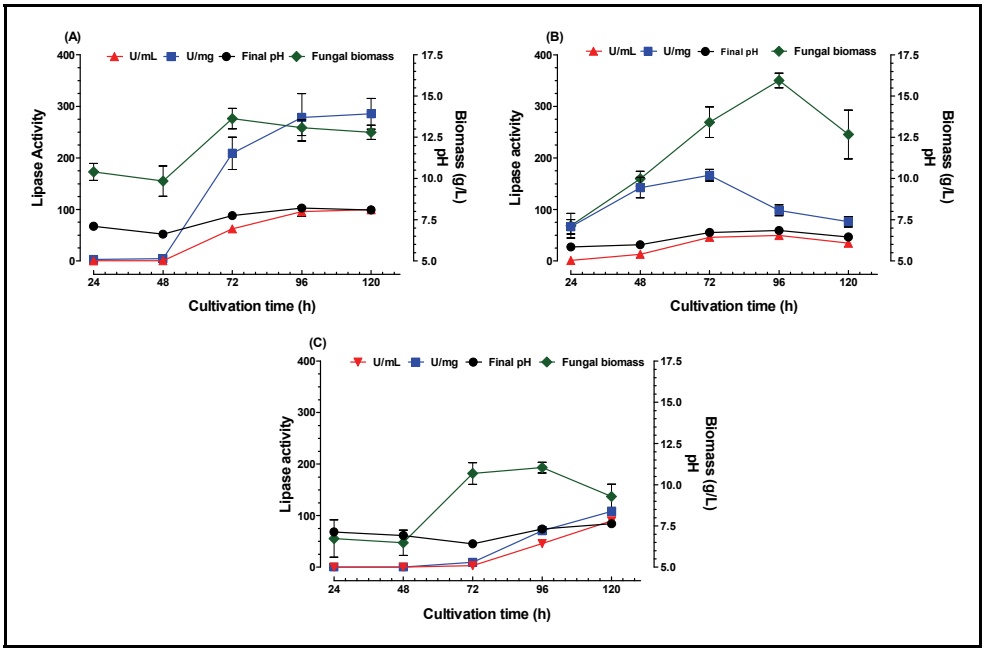

Fig. 1. Growth profiles comparing lipase production by *Botryosphaeria ribis* EC-01 on soybean oil in media containing NH_4NO_3 as N source, and three different phosphate sources: (**A**) K_2HPO_4, (**B**) KH_2PO_4 and (**C**) Na_2HPO_4 in submerged fermentation. The initial pH of the culture medium was not adjusted.

and the least on Na_2HPO_4 (108 U/mg). In the case of the di-cation phosphate salts, lipase production occurred during late log phase of growth (after 72 h), whereas for KH_2PO_4, lipase commenced after 24 h and decreased after 72 h.

According to Jaeger and coworkers (1994), the initial pH of the nutrient medium may be related to an increase of lipase production, and values ranging from 7.0 to 8.0 had a positive

effect in lipase synthesis. The same observation was described for a strain of *Aspergillus oryzae* which produced highest lipase titres at initial pH 6.0, whereas the peak of lipase production was observed when the final pH reached 8.0 (Ohnishi et al., 1994).
The influence of initial pH within the range 3.5 to 9.5 on lipase production by *Botryosphaeria ribis* EC-01 grown on soybean oil in media containing the 3 different P sources (KH_2PO_4, K_2HPO_4 and Na_2HPO_4) is presented in Figure 2.

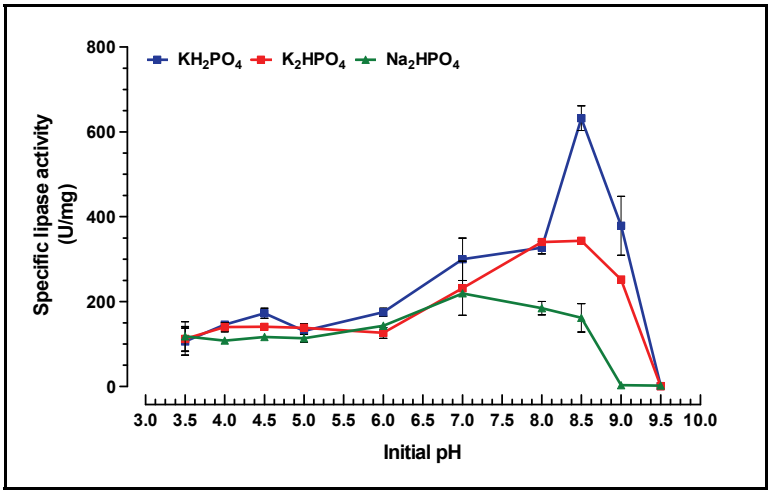

Fig. 2. Influence of initial pH on the production of lipases by *Botryosphaeria ribis* EC-01 cultured for 5 days on soybean oil, and media containing NH_4NO_3 as N source and either KH_2PO_4, K_2HPO_4 or Na_2HPO_4 as phosphate sources.

Highest specific lipase activity (632.6 U/mg) was achieved on KH_2PO_4 and an initial pH of 8.5. The enzyme profile for K_2HPO_4 was similar, but the specific activity was lower (350 U/mg), while in the case of Na_2HPO_4, the best initial pH was 7.0 producing a specific activity of 200 U/mg. It is clear from these results that not only the initial pH value of the nutrient media affected lipase activity, but also the type of P source was important in enhancing lipase specific activities. Similar observations of the effect of initial pH on lipase production have been reported for *Fusarium globulosum* (Gulati et al., 2005), and *Aspergillus terreus* (Gulati et al., 1999) when grown on maize oil, and reported the optimal initial pH being 7.0 and 9.0, respectively. In another example, *Cryptococcus* sp. S-2, produced highest lipase activity when grown on triolein and yeast extract at an initial pH of 5.6 (Kamini et al., 2000). From these observations, it is concluded that initial pH has a significant effect on the production of fungal lipase. The composition of the culture medium such as carbon, N and P sources, inoculum size, as well as culture conditions (shaking rate, temperature, air/medium ratio) will also have interaction effects on microbial lipase production (Lin et al., 2006). Response surface methodology was employed to optimize medium nutrients to produce lipase by *Geotrichum* sp, in order to study the effect of carbon sources (soybean oil, olive oil and glucose) and concentrations of N sources (corn steep liquor and NH_4NO_3). The optimized condition was obtained using NH_4NO_3 (2.1 - 2.5 %), corn steep liquor (13 - 15 %) and soybean oil (0.6 %), and resulted in lipase titres of 20 U/mL (Burket et al., 2004).

4. Soybean meal as substrate for lipase production by submerged fermentation

As is common with most commercial enzymes, lipase is associated with high production costs, generally because the enzymes are produced by submerged fermentation (Castilho et al., 2000). *Botryosphaeria ribis* EC-01 is able to produce lipases both in submerged and solid-state fermentations (Messias et al., 2009; Costa et al., 2009).

Oil-seed cakes are rich in protein and are recognized as being good food supplements, and some have been used for feed applications in poultry, fish and pig production. They also add value to various biotechnological processes such as the production of enzymes, antibiotics and mushrooms by fermentation (Ramachandran et al., 2007). Oil-seed cakes, being rich in protein, can serve as a source of nitrogen for enzyme production including lipases.

Three oil-seed meals (soybean, castor bean, corn kernel) were compared as substrates for their effect on lipase production by *Botryosphaeria ribis* EC-01 when cultivated at a concentration of 1 % (w/v) in the absence (distilled water only) and presence of minimum salts medium (VMSM). The results are presented in Table 4 and showed that the addition of VMSM to the nutrient medium containing soybean and castor meals strongly decreased lipase production by *Botryosphaeria ribis* EC-01. However, the addition of VMSM increased lipase production when corn kernel meal was the substrate. These results indicate that soybean and castor bean meals contain sufficient nutrients to support the growth of *Botryosphaeria ribis* EC-01 and subsequent production of lipases. In this case, the use of these meals could lower the costs of lipase production.

The results presented in Table 5 shows that soybean and castor bean meals have higher protein concentrations than corn kernel meal, which may also explain the capacity by *Botryosphaeria ribis* EC-01 to produce higher lipase activity.

Minimal salts medium (VMSM)	Oil-seed meal	Lipase activity		
		(U/mL)	(U/mg*)	(U/g ds**)
None ***	Soybean	13.4 ± 0.63	142.5 ± 5.31	47.5 ± 2.26
	Castor bean	12.3 ± 0.38	88.0 ± 1.30	43.8 ± 1.37
	Corn kernel	0.8 ± 0.20	17.3 ± 3.26	3.0 ± 0.75
Presence	Soybean	0.1 ± 0.03	1.7 ± 0.66	1.1 ± 0.67
	Castor bean	0.8 ± 0.07	7.2 ± 0.41	2.8 ± 0.23
	Corn kernel	4.5 ± 0.09	28.9 ± 0.49	16.7 ± 0.33

* mg protein; ** dry substrate; *** replaced by distilled water

Table 4. Comparison of lipase production by *Botryosphaeria ribis* EC-01 grown on three different oil-seed meals in the absence and presence of minimal salts medium for 5 days by submerged fermentation.

Different soybean meal concentrations (from 0.5 to 6 %, w/v) were evaluated on lipase production by *Botryosphaeria ribis* EC-01 in submerged fermentation in order to enhance enzyme activities. The results are presented in Table 6, which shows a comparison of lipase production [titres (U/mL), specific activity (U/mg of protein), and yield (U/g of dry meal substrate, ds)].

Oil-seed meal	Carbohydrate	Lipid	Protein	Ash	Moisture
Soybean	30.7	0.7	49.4	6.2	13.0
Castor bean	47.2	1.4	31.8	7.1	12.5
Corn kernel	66.9	1.2	23.1	1.8	7.1

Table 5. The chemical composition of oil-seed meals (g/100 g; Costa, 2008).

One percent soybean meal was the concentration that promoted highest lipase activity when expressed as specific activity (182.5 U/mg) and enzyme yield (67.6 U/ds). Considering that the oil-seed meals are rich in proteins, highest lipase titres (79.4 U/mL) were obtained on 6 % soybean meal (Table 6), and do not really correspond as an expression of high lipase activity compared to the specific activity (182.5 U/mg). Hence, when using oil-seed meals as fermentable substrates for lipase production, one should consider all 3 measures of enzyme activity (U/mL, U/mg, and U/g ds).

Tan et al., (2004) compared soybean bean meal, defatted soybean meal and soybean protein (all at 4 %) as substrates for lipase production by *Penicillium camembertii* Thom PG-3. The lipase activities obtained were 75.2, 128.4 and 78.8 U/mL, respectively. Also present in the nutrient medium were cyclodextrin (0.5 %), olive oil (0.75 %), K_2HPO_4 (0.5 %) and $(NH_4)_2SO_4$ (0.1 %), and these supplements would surely affect lipase production.

Soybean meal (%, w/v)	Lipase activity		
	(U/mL)	(U/mg*)	(U/g ds**)
0.5	5.6 ± 0.50	95.1 ± 7.73	39.7 ± 2.73
1.0	19.0 ± 1.37	182.5 ± 2.64	67.6 ± 5.14
2.0	34.0 ± 0.03	129.7 ± 0.11	59.1 ± 0.29
4.0	35.9 ± 0.92	38.5 ± 0.90	32.3 ± 2.94
6.0	79.4 ± 6.56	76.3 ± 6.58	46.0 ± 3.82

* mg protein; ** dried substrate

Table 6. Comparison of lipase production by *Botryosphaeria ribis* EC-01 grown on increasing concentrations of soybean meal under submerged fermentation for 5 days.

Some comparisons on lipase production by submerged and solid-state fermentations have been discussed in terms of U/mL and U/g ds for several microorganisms cultivated on different substrates, and no conclusion could be achieved in terms of highest enzyme titres because the processes are dependable on the microorganism, the type of substrate fermented, and the conditions used for cultivation (Treichel et al., 2010). *Botryosphaeria ribis* EC-01, for example, produced lower yields of lipase activity when grown by solid-state fermentation (Costa, 2008) than compared to the results obtained herein by submerged fermentation.

Phosphate was also added to the oil-seed meal in order to supplement and enhance lipase production. A comparison of adding KH_2PO_4 and K_2HPO_4 is shown in Figure 3. The addition of KH_2PO_4 significantly enhanced lipase production in terms of specific activity.

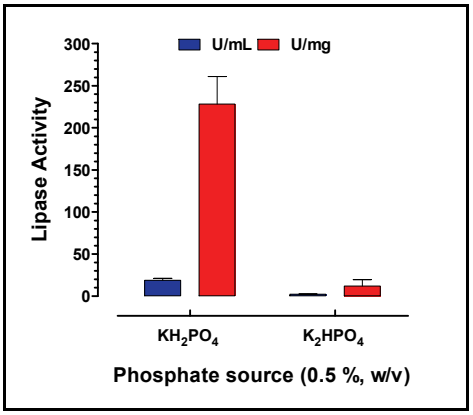

Fig. 3. Comparison of phosphate source on the production of lipases by *Botryosphaeria ribis* EC-01 grown on soybean meal (1% w/v) for 5 days by submerged fermentation.

5. Soybean oil emulsification to enhance the production of the exopolysaccharide botryosphaeran produced by *Botryosphaeria rhodina* MAMB-05

Exopolysaccharides (EPS) are biopolymers secreted extracellularly by several microorganisms including fungi. These carbohydrate macromolecules possess important industrial applications and interesting biological activities. Because of their physical properties, they have been used in foods as emulsifying, stabilizing and thickening agents, as well as in pharmaceutical formulations, and as drug delivery agents (Sutherland, 1998).

Botryosphaeria rhodina isolate MAMB-05 produces an EPS named botryosphaeran, a β-1,3;1,6-D-glucan comprising 22 % ramification. The side branches consist of glucosyl and gentiobiosyl residues linked to the β-1,3-D-glucan backbone chain by β-1,6-bonds (Barbosa et al., 2003). When grown on different carbohydrate substrates, *Botryosphaeria rhodina* MAMB-05 produced a family of botryosphaerans that differed only in the degree of branching (Steluti et al., 2004). *Botryosphaeria rhodina* MAMB-05 grown on fructose presented a higher degree of branching (31 %) compared to that when grown on sucrose and glucose (21-22 %) as carbon sources. In each case, the degree of branching affected the physical properties (viz., rheology) of the botryosphaerans produced (Corradi da Silva et al., 2005). Botryosphaeran exists in solution in a triple helical conformation (Giese et al., 2008); an important property manifesting biological response modifying activity.

Vegetable seed oils, fatty acids and surfactants such as Tween-80 (polyoxyethylene sorbitan mono-oleate) when added to nutrient media, are known to enhance the production of fungal β-glucans (West & Reed-Hamer, 1995). The enhanced production is possibly due to the oils providing an additional energy source to the fungus, thus shunting glucose for synthesis into β-glucan.

As some β-1,3-glucans have important industrial and pharmaceutical applications, and botryosphaeran is no exception, their inclusion as new materials in commercial applications is dependent upon their scale of production. Effective strategies to increase the yields of exopolysaccharides in fermentation processes are therefore important. Vegetable seed oils are readily available commercially and are considered low-cost. Their use in nutrient media

to enhance fungal β-glucan production can therefore be a promising means of increasing their yields for commercial purposes.

Botryosphaeria rhodina MAMB-05 when grown on basal media (glucose (10 g/L) plus VMSM) in which soybean oil (1 %, v/v) was incorporated demonstrated that botryosphaeran production could be enhanced during the course of submerged fermentation (Figure 4).

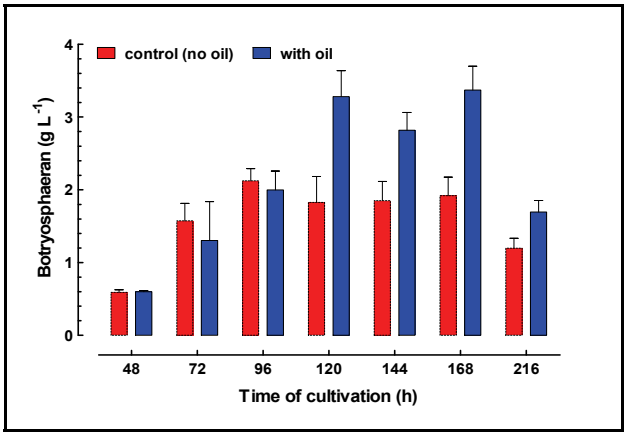

Fig. 4. Profile for the production of botryosphaeran by *Botryosphaeria rhodina* MAMB-05 grown in the presence and absence of soybean oil (Silva, 2007).

The addition of the surfactant Tween 80 to basal medium was also effective in promoting botryosphaeran production, and the combined presence of soybean oil and Tween-80 could further increase botryosphaeran yields (Figure 5). Structural characterization of the derived products revealed no structural abnormalities compared to botryosphaeran produced in the absence of oil and Tween-80 (Silva et al., 2007).

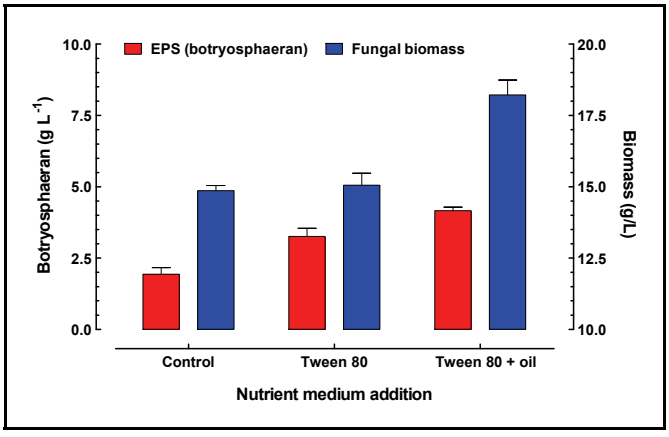

Fig. 5. Comparison of botryosphaeran and biomass production by *Botryosphaeria rhodina* MAMB-05 grown on glucose in the absence (control) and combined presence of Tween-80 and soybean oil, when added to the basal medium (Silva, 2007).

There was no significant difference in botryosphaeran production when using different commercial brands of soybean oil (Figure 6). The presence of *tert*-butylhydroquinone (preservative for unsaturated vegetable oils) in one commercial soybean oil brand did not affect botryosphaeran production by *Botryosphaeria rhodina* MAMB-05.

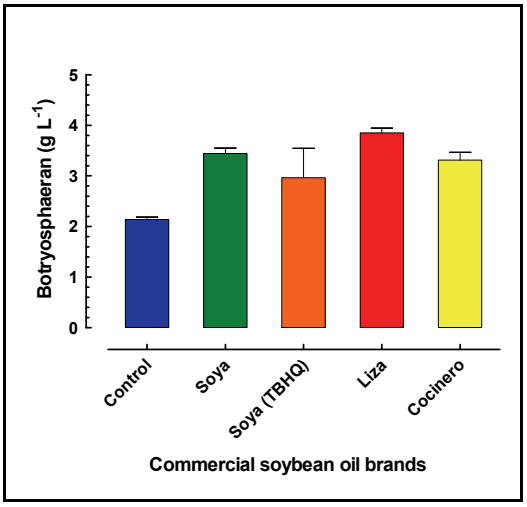

Fig. 6. Influence of different Brazilian commercial soybean oil brands on the production of botryosphaeran by *Botryosphaeria rhodina* MAMB-05 (n = 3; $p < 0{,}001$); TBHQ, *tert*-butylhydroquinone (Silva, 2007).

6. Conclusions

Soybean oil was an effective carbon source for *Botryosphaeria ribis* EC-01 to produce lipases by submerged fermentation, and NH_4NO_3 was the best nitrogen source. The source of phosphate also influenced lipase production, and the initial pH of the nutrient medium had a significant effect on promoting lipase activity.

Soybean meal was also an excellent substrate for lipase production by *Botryosphaeria ribis* EC-01, and did not require supplementation with nutrients to increase lipase activity when grown by submerged fermentation. KH_2PO_4 was an exception, and when added to soybean meal significantly increased the specific lipase activity.

Soybean oil in the presence of Tween 80 enhanced the production of botryosphaeran for *Botryosphaeria rhodina* MAMB-05, and there was no significant difference using various commercial brands of soybean oil.

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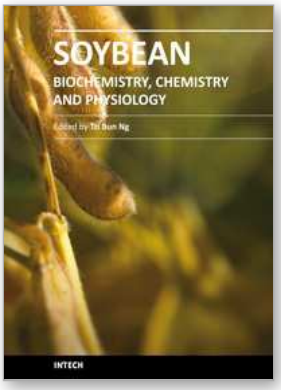

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Soybean is an agricultural crop of tremendous economic importance. Soybean and food items derived from it form dietary components of numerous people, especially those living in the Orient. The health benefits of soybean have attracted the attention of nutritionists as well as common people.

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