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Agro-Industrial Wastes: A Substrate for Multi-Enzymes Production by *Cryphonectria parasitica*

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Abstract: This study aims to produce a mix of enzymes through Solid State Fermentation (SSF) of raw materials. Four different, easily available, agro-industrial wastes were evaluated as SSF substrates for enzymes production by *Cryphonectria parasitica* (Murr.) Barr. environmental strains named CpA, CpB2, CpC4, and CpC7. Among the tested wastes, organic wheat bran for human use and wheat bran for animal feed better supports *C. parasitica* growth and protease production without any supplements. SDS-PAGE analyses highlighted the presence of three bands corresponding to an extracellular laccase (77 kDa), to the endothiapepsin (37 kDa), and to a carboxylesterase (60.6 kDa). Protease, laccase, and esterase activities by *C. parasitica* in SSF were evaluated for 15 days, showing the maximum protease activity at day 9 (3955.6 AU/gsf,). Conversely, the best laccase and esterase production was achieved after 15 days. The *C. parasitica* hypovirulent CpC4 strain showed the highest laccase and esterase activity (93.8 AU/gsf and 2.5 U/gsf, respectively). These results suggest the feasibility of a large-scale production of industrially relevant enzymes by *C. parasitica* strains in SSF process on low value materials.

Keywords: esterase; aspartic protease; laccase; solid state fermentation; wheat bran; by-products

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1. Introduction

Currently, a key theme in sustainable development is the requirement of appropriate waste management (UNEP, 2011; UNHSP, 2010), based on the notion that waste can be a resource [1,2]. Agriculture and food processing produce solid organic wastes in large quantities and their improper disposal may cause severe environmental, social, and economic impacts. Agro-food wastes and by-products have been receiving growing attention as they are abundant [3], and rich in proteins, sugars and minerals which can be converted and assimilated by microorganisms [4]. One attractive valorization of agro-food wastes is their application in bio-refinery processes as substrates for microbial growth to produce various commodity chemicals and industrial enzymes [5–7].

The industrial importance of enzymes as biological catalysts ranges from the food industry to paper, pulp, and detergent treatments. Currently, 60% of the global market is covered by proteases, with applications in detergents, leather processing, food and feed processing, pharmaceuticals, chemicals, and waste treatment [8]. Among the remaining enzymes, laccases are polyphenol oxidases involved in biopulping and bioleaching, denim washing, textile dye decolorization, and wastewater treatment [9,10] and esterases and lipases are used in many industrial applications, such as for detergents [11,12]. Enzymes are relatively expensive reagents: around 30–40% of their production cost is accounted for by the fermentation substrate that could be greatly reduced by using low-cost substrates such as agro-food residues [13].

Microorganisms, fungi in particular, have been regarded as a treasure source of useful enzymes for the positive environmental impact of their production processes. In fact, fungi can grow on low-cost materials while secreting high amounts of enzymes, which eases downstream processing [14]. In addition, fungal enzymatic production is not subjected to ethical evaluation and consequent regulatory issues, as long as its use is confined to specific fermentation facilities and proper disposal is implemented.

A relevant part of the world enzyme market derives from microorganisms under submerged fermentations (SmF) [15] due to high amounts of enzyme recovered, low-cost production, and, finally, stability of the polypeptides at various extreme conditions. However, over the last twenty years, the interest in solid state fermentation (SSF) processes have markedly increased, appearing in a large number of patents and publications [16]. SSF is a promising technology enabling microorganisms to produce biomolecules by growing on solid substrates used both as a physical support and as a source of nutrients in an environment with the absence or near absence of free-flowing liquid [13,17].

SSF has some advantages over SmF, since the provided environmental conditions resembles the natural habitat of the fungus [18], allowing adequate growth and high yield of target enzymes production. Furthermore, SSF has a lower energy requirement and demands simpler extraction processes to obtain the desired enzymes [4,19–21].

Therefore, there is an increasing interest in the bioprocessing of agro-industrial wastes through SSF, achieving their valorization to produce industrial enzymes [22–24]. In most cases, filamentous fungi are particularly interesting for SSF processes, since they have the unique capacities to colonize the interparticle spaces of solid matrices and to secrete mixtures of enzymes, in place of single enzymes, that allow to metabolize complex mixtures of organic compounds found in most residues, as required by several industries [25–27]. Unfortunately, few fungal strains meet the criteria for commercial production, so far. In fact, the production of low-cost and readily available enzymes possessing suitable operating characteristics is still challenging.

Cryphonectria parasitica (Murr.) Barr is a filamentous ascomycete well known for its ability to secrete a range of proteins with industrial applications that are being commercially produced through different fermentation procedures [28–31]. For example, milk-clotting enzymes were produced by *C. parasitica*, and they are considered as GRAS (generally recognized as safe) substances [32].

Interestingly, *C. parasitica* is also the agent of the plant disease chestnut blight, causing relevant economic losses worldwide [33]. *C. parasitica* is also a model study for mycovirus-fungi interactions, since it naturally harbors a number of viruses, among which is Cryphonectria hypovirus-1 (CHV1), a mycovirus that alters the host virulence and is used for natural biocontrol [34]. CHV1 is also known to down- and up-regulate the expression of several genes, with a corresponding reduction/increase of the relative products including enzymes [35,36].

The present study aims at the definition of an eco-friendly multiple enzymes production protocol through SSF. More precisely, the feasibility of different food wastes as sole substrates, with no supplements, has been evaluated for the growth and the production of aspartic endopeptidase (EC 3.4.23), carboxylesterase, and laccase (EC 1.10.3.2) by environmental *C. parasitica* strains under SSF.

2. Materials and Methods

2.1. Microorganisms and Their Molecular Characterization

Four *Cryphonectria parasitica* strains, named CpA, CpB2, CpC4, and CpC7 (Table 1), newly isolated from cankers on a *Castanea sativa* Mill in two Italian regions, were morphologically identified and stored in the collection of the Agri-food and Environmental Microbiology Platform (PiMiAA), University of Brescia, Italy. The strains were maintained as agar plugs of mycelium in sterile distilled water at 4 °C. The cultures were grown on Potato Dextrose Agar (PDA, Merck, Milano, Italy), and incubated for 6 days at 26 °C under the illumination of 16 h light/8 h dark cycles, using daylight tubes 24 W/m^2, 9000 lx.

Fungal inocula were prepared as mycelial slurry by grinding a fully-grown culture plate (diameter 9 cm) in a Waring blender, with the addition of 26 mL of sterile deionized water.

Table 1. Environmental strains of *Cryphonectria parasitica* used in this study. The presence of the hypovirus CHV1 is indicated by +.

Strain	Origin	Canker Type	CHV1
CpA	Tuscany	evolutive	–
CpB2	Tuscany	evolutive	–
CpC4	Lombardy	superficial	+
CpC7	Lombardy	evolutive	–

To assess the presence of Cryphonectria hypovirus-1 (CHV1), the *C. parasitica* strains were grown on PDA covered with a sterile cellophane disc, inoculated with mycelial plugs, and incubated at 26 °C for four days [37]. Mycelium was harvested by scraping with a sterile scalpel, lyophilized, and the RNA was extracted using the PureLink RNA purification kit (Ambion, Thermo Scientific, Milano, Italy). Synthesis of cDNA from RNA was performed using a cDNA first strand synthesis kit (Thermo Fisher, Milano, Italy) with hexamer primer, according to the manufacturer's instruction.

CHV1 was detected by qRT-PCR using cDNA, prepared as described above, as a template. Reactions were performed using the Real-time PCR PowerUp SYBR Green Master Mix kit (Applied Biosystem, Milano, Italy) in the ViiA7 Real-time PCR system (Applied Biosystems, Milano, Italy) and by adopting the specific primers (5337) ACCTGGTTCGCCGAAGAAC Rev (5405) GCAACCTCTAAGGCAACCA [38].

2.2. Agro-Food Waste Based Substrates and Solid State Fermentation

2.2.1. Effect of the Carbon Source on the Fungal Growth and Protease Activity

Wheat bran for human (organic wheat bran, OW) or for animal consumption (zootechnical wheat bran, ZW), and rice husk (RH), all bought at local stores, and spent espresso coffee grounds (CG) collected from bars after espresso preparation in Brescia, Italy, were the by-products used as substrates for fungal growth and enzymes production (Table 2). SSF was carried out in 340 mL micropropagation boxes equipped with a 0.45 µm filter (Micropoli, Cesano Boscone, Italy) containing 11 g of each waste. The dry substrates in boxes were autoclaved twice at 121 °C for 20 min.

Table 2. Approximate chemical composition (g/100 g dry material) retrieved from scientific literature of feed stocks used in this study.

Chemical Components	Substrate for SSF		
	Wheat Bran (ZW *)	Rice Husk (RH)	Spent Coffee Grounds (CG)
Cellulose	32	33.4	12.5
Hemicellulose	21	21	39
Lignin	22	18.3	24
Carbohydrates	26.6	1.1	30
Protein	14.1	2	17.5
Lipids	5.5	0.8	2.3
Ash	0.5	15.5	1.3

* Similar to OZ.

2.2.2. Effect of Water Content on the Fungal Growth and Protease Activity

Different moisture contents were tested depending on the substrate composition. The optimum water content of the dried substrates was studied over a range of 1:1 (wet basis) (OWA, ZW, RH, and CGA); 1.5:1 (OWB) and 2:1 (CGB). Different moisture contents were adjusted with deionized water.

2.2.3. Effect of Substrate Density on the Fungal Growth and Protease Activity

To analyze the effect of the substrate density, the activity was tested at different porosity using rice husk. RH substrate was separated into two series, one was milled with a Waring blender (RHM), and the other series was unmilled (RHU).

2.3. SSF Protocol Set Up

Fungal inocula were prepared as mycelial slurry by grinding a fully-grown culture plate (diameter 9 cm) in a Waring blender, with the addition of 26 mL of sterile deionized water.

Each substrate was inoculated with 2 mL of the mycelium slurry, and incubated for 15 days at 24 °C, with a photoperiod of 16 h, and 50% relative humidity in a climatic chamber (Binder Model 720, Milano, Italy). Samples (approximately 1–2 g) were aseptically withdrawn at various time intervals (6, 9, 12, and 15 days) for the determination of carboxylesterase, aspartic endopeptidase, and laccase activity, or frozen at −20 °C until analyses. Five replicates for each substrate were prepared, and the experiment was repeated twice. The fungal growth was visually quantified by assigning a score based on the timing of appearance and the abundance of both substrate colonization and pycnidia production, with a scale from very poor (−/+) to abundant (++) growth.

2.4. Extraction of Crude Enzyme from the Fermented Media

Liquid-solid enzymes extraction was performed from solid fermented substrates with distilled water (1:10, *w*/*v*) by shaking at 170 rpm for 2 h at room temperature, followed by centrifugation of the whole content at 5000× *g* for 15 min at 4 °C. The supernatants were used as crude enzyme source.

2.5. Protease Activity Assay

The proteolytic activity was measured by a modification of the hemoglobin method [39]. In short, 20 µL of diluted enzyme solution were added to 500 µL of pre-warmed bovine serum hemoglobin (Merck, Milano, Italy) solution, mixed, and incubated at 37 °C for 15 min. The reaction was stopped by adding trichloroacetic acid (TCA), and the mixture was cooled on ice and centrifuged at 20,000 g for 10 min at 4 °C to remove the unhydrolyzed protein. The absorbance of the clear supernatant solution was measured at Abs 280 nm; the blanks were prepared by using the same amount of assay solution, and reversing the order of addition of TCA and hemoglobin solutions. The protease activity is expressed as Abs 280 nm in arbitrary units (AU). One AU is defined as the enzyme amount that produces an increase of Abs 280 nm of 0.01 under the assay conditions [40,41], measured as the quantity of TCA-soluble products [42]. The observed AU values are normalized by gram of fermented substrate (AU/g_{sf}).

2.6. TBA Assay

As a preliminary screening for lipolytic activity of crude extracts, the modified TriButyrin Agar Plate Assay [43] was carried out on TBA medium containing 1% tributyrin (*v*/*v*) and 1.5% agar (*w*/*v*) in 10 cm diameter plates. Aliquots (20 µL) of the crude enzyme extractions were placed in wells in the agar. All plates were incubated at 25 °C overnight, and lipolytic hydrolysis was verified as presence of clear zone around the wells.

2.7. Esterase Activity Assay

The esterase activity was determined spectrophotometrically by measuring the hydrolysis of 4-nitrophenyl butyrate (pNPB) (Sigma, Milano, Italy) as a substrate [44]. The pNPB dissolved in acetonitrile (50 mM) was added to 200 µL Na phosphate buffer (100 mM, pH 7.5) with 0.5% Triton X-100 (*v*/*v*) (Sigma, Milano, Italy), to 0.5 mM pNPB as a final concentration; 10 µL crude enzyme extraction were used as enzyme source. The enzymatic reaction was carried out at 25 °C for 15 min. The release of 4-nitrophenol (pNP) at 405 nm was measured by using a UV-VIS micro-plate reader (EnSight multimode reader, Perkin-

Elmer Waltham, MA, USA). Enzyme activity calculated using the extinction coefficient of pNP corresponding to 18.5 $mM^{-1} \cdot cm^{-1}$, One U (µmol/min) is defined as the amount of the enzyme that catalyzes the conversion of one micromole of substrate per minute under the specified conditions of the assay method, and normalized by gram of fermented substrate (U/g_{sf}).

2.8. Laccase Activity Assay

The laccase activity was determined with 2,6-dimethyoxyphenol (DMP) (Carlo Erba, Milano, Italy) as a substrate [45]. The oxidation of DMP was spectrophotometrically determined by continuously recording the increase in absorbance at 468 nm at 25 °C for 15 min in a micro-plate reader (EnSight multimode reader, Perkin-Elmer Waltham, MA, USA). One arbitrary unit (AU) is defined as an increase Abs 468 nm of 1.0 per min at 25 °C [46].

2.9. SDS-PAGE

SDS–PAGE of the crude extracts was performed using a 12% (*w*/*v*) running gel, (Laemmli, 1970). Protein bands were visualized by Coomassie Brilliant Blue staining, and their size estimated with the aid of the Protein Marker VI (molecular weight range from 10 to 245 kDa).

2.10. Protein Determination

Protein concentrations were determined by Coomassie Protein Assay Reagent (Sigma, Milano, Italy), according to the method of Bradford [47], using bovine serum albumin (Sigma, Milano, Italy) as the standard.

2.11. Statistical Analysis

For all the experiments described in the manuscript, at least three biological replicates were performed, and the mean enzymes activities were statistically analyzed by analysis two-way analysis of variance (ANOVA) followed by Tukey's means grouping tests using GraphPad Prism software 6 for Windows (GraphPad Software, San Diego, CA, USA). Differences were considered significant at p-values < 0.05.

3. Results and Discussion

Cryphonectria (formerly *Endothia*) *parasitica* is a well-known producer of a rennin-like protease and endothiapepsin [48], mainly used in the food industry, thanks to the GRAS status of the fungus derived additives [32]. In addition, *C. parasitica* has also been shown to produce and secrete a number of hydrolytic enzymes, including laccase [31], cellulase [28], polygalacturonase [49], cutinase [29], and tannase [30]. All of these enzymes are normally produced in liquid cultures, in fact, to the best of our knowledge; *C. parasitica* has never been cultivated in SSF regime so far. Consequently, the feasibility of using agro-food wastes as sole substrate for the growth and multiple enzymes production by *C. parasitica* in SSF needed to be explored.

Agro-food wastes are defined as lignocellulosic biomass, with lignin and celluloses as the main constituents. It is well known that the different materials used as substrate can modify the fungal gene expression and, consequently, the type and quantity of the produced enzymes, thus affecting their application in the biotechnological industry in many ways [50,51]. In the literature, the raw materials used as SSF substrates in this study are reported as characterized by the compositions shown in Table 2 [52–56]; as such they could support the growth of fungi and their enzymes production under SSF.

3.1. SSF Protocol Set Up

3.1.1. Fungal Growth on Different Raw Material

The growth of the strain CpC7 on OW, ZW, RH and CG as solid substrate during a period of 15 days is summarized in Table 3.

Table 3. Growth of the CpC7 strain on the different substrates used in this study. (a) "−" unmilled; "+" milled; (b) "−/+" very poor growth; "+" normal growth; "++" abundant growth.

Agro-Food Waste	Abbreviation	Ratio Water/Waste	Milled [a]	*C. parasitica* Growth [b]
Organic wheat bran	OWA	1:1	−	++
	OWB	1.5:1	−	++
Zootechnical wheat bran	ZW	1:1	−	++
Rice husk	RHM	1:1	+	+
	RHU	1:1	−	−/+
Spent coffee grounds	CGA	2:1	−	−/+
	CGB	1:1	−	−/+

The best growth was achieved on all the wheat bran substrates, OWA, OWB and ZW; representative samples on OWA at different times of incubation are shown in Figure 1.

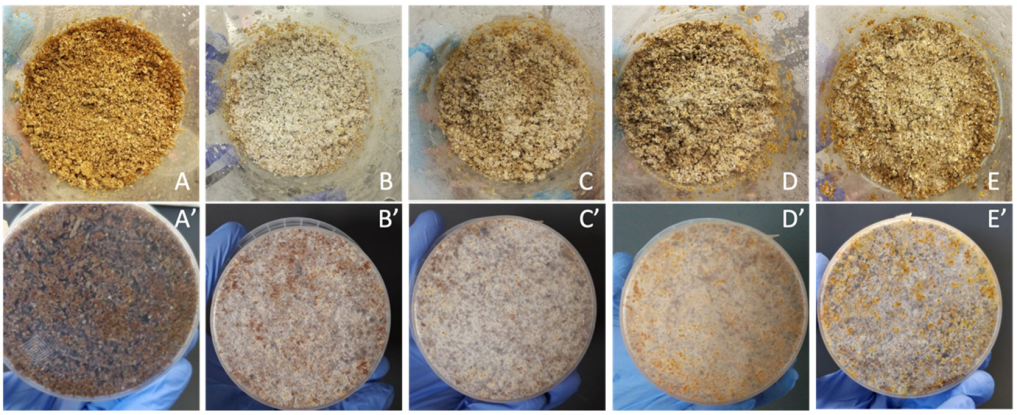

Figure 1. Growth of CpC7 strain under SSF using organic wheat bran as solid substrate, at (**B**) 6, (**C**) 9, (**D**) 12 and (**E**) 15 days of fermentation. The top (**A**–**E**) and bottom (**A′**–**E′**) surfaces of the OWA colonized substrates are shown. (**A**,**A′**), not inoculated substrate.

In detail, fungal white mycelium was visible at day 4 after inoculation on the upper surface. It completely colonized the biomass at day 6 of incubation, with abundant orange pycnidia production visible at day 12 (Figure 1D′). As far as the effect of the water content is concerned, the fungal growth appeared earlier, and fructification was more abundant on OWB than on OWA, indicating that both water conditions were conducive to growth, but the higher water content of OWB was more supportive (data not shown).

Conversely, RH and CG resulted less conducive of fungal growth. In detail, the substrate density represented another decisive factor for the rate and extent of the biomass colonization and should be cautiously altered, as it resulted strictly associated to aeration of the biomass and eventually to its colonization. A very high density of the matrix elements occurred in CGA and CGB, where the small particles appeared to pack together tightly, and corresponded to a very poor fungal growth, limited only to the biomass surface (Figure S1). This behavior could be determined by the scarce oxygenation of the media that limited an extensive fungal growth [57].

Inversely, when the very coarse matrix RHU was compared to the more regular RHM (Figure S1), the latter allowed a better fungal growth with a faster colonization than RHU. In fact, the mycelium covered the substrate at day 7 with a good production of pycnidia, but nonetheless with a lower performance than on wheat bran. The coarse RHU turned out as the less appropriate substrate for *C. parasitica* growth.

No growth was observed on not inoculated substrates.

3.1.2. Protease Production under SSF

The fungal enzymatic production was analyzed in the crude extract obtained from SSF at different time points. No aspartic protease activity was detected in CGA, CGB and RHB. Higher aspartic protease activity was found when CpC7 grew on OW and ZW than on the other substrates, possibly due to the better fungal growth achieved on these wastes. On wheat bran substrates, aspartic protease activity was measured starting from day 4 of fermentation, with values above 2000 AU/g_{sf}. Protease production was not significantly influenced by wheat bran substrates composition. The maximum enzymatic activity was detected on day 8, with values of 3980.6 ± 646 AU/g_{sf}, 4123.5 ± 1259.9 AU/g_{sf}, and 3703.2 ± 350.7 AU/g_{sf}, for OWA, OWB, and ZW, respectively (Figure 2). Inversely, the protease activity was 133.6 ± 32,7 AU/g_{sf} after 7 days of growth on RHA that was significantly lower (95.0% of confidence interval, $p < 0.05$). After this peak, protease activity decreased to 20% of the correspond peak values.

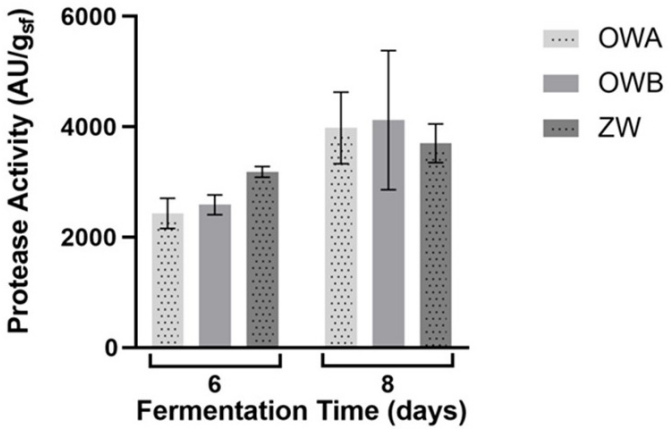

Figure 2. Effect of different wheat bran substrates (OWA, OWB and ZW) on protease production by CpC7 strains under SSF conditions, after eight days of fermentation. Data represent the average ± standard deviation of biological replicate cultures (n = 3).

In summary, wheat bran resulted to be conducive to fungal growth and to aspartic protease production without further supplementation. Moreover, wheat bran is a local waste for Italy, readily available in many regions, and hence a low-cost substrate.

As far as the water content of the substrate is concerned, the ratio water/waste 1:1 proved to be sufficient to ensure a very good growth and enzymatic production, making the process more affordable and sustainable, being far less water consuming in comparison to other reported methods for enzymes production either in SSF and SmF [40,57–61]. The potential presence of pesticide residues in the ZW, below the commercial threshold, but still able to interfere with the fungal growth, did not occur. Thus, even if the organic wheat bran OW gave a faster colonization, ZW was the economical choice as the sole substrate for further experiments of multiple enzymes production through SSF.

3.1.3. Enzymes Putatively Identification

Electrophoretic analysis of the crude enzymatic extracted at different time points of the CpC7 culture showed three main bands (Figure 3). These potentially corresponded to an extracellular laccase that migrates as a polypeptide with a molecular mass of approximately 77 kDa [46] to the endothiapepsin of 37 kDa (Brown et al., 1990), and to an unidentified protein of 27 kDa (Figure 3). Moreover, a band potentially corresponding to a carboxylesterase of 60.6 kDa [63] was present. This result suggests a good enzyme production and a purity of the crude extracts that might not require further purification steps.

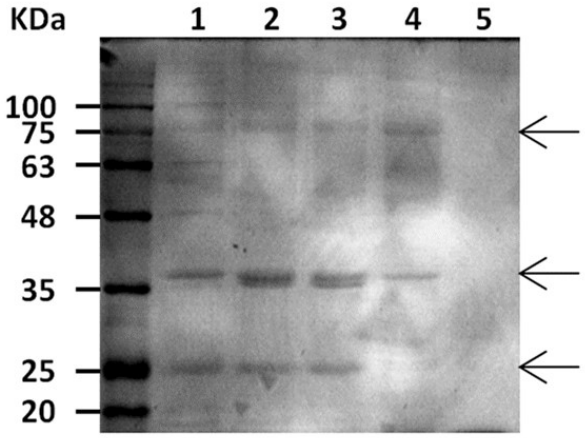

Figure 3. SDS-PAGE separation of enzyme crude extract produced at 4 (lane 1), 6 (lane 2), 8 (line 3), and 12 (line 4) SSF days by CpC7 on ZW; negative control (line 5). Arrows point to three main bands, respectively at 77, 37 and 27 KDa, putatively corresponding to extracellular laccase, endothiapepsin, and to an uncharacterized peptide.

3.2. Multi-Enzymes Production under SSF

Successively, the SSF protocol previously established was applied to grow four environmental strains of *C. parasitica* for the simultaneous multiple production of enzymes.

3.2.1. Strains Characterization

C. parasitica is a well-known fungus among plant pathologist as the agent of a severe disease of chestnut trees, where it normally produces perennial necrotic lesions, the so-called cankers. Some strains, however, are associated with superficial or healing cankers on susceptible host trees; these hypovirulent strains usually host a mycovirus, the RNA hypovirus CHV1, that decreases their ability to produce the disease on chestnut trees [64,65] by affecting the expression of several genes.

Therefore, the environmental *C. parasitica* strains used in this work, isolated from cankers on chestnut trees, were characterized for the presence of CHV1, since it could influence their enzymatic production [36,51,66]. The RT-qPCR revealed the presence of CHV1 only in the CpC4 strain (Table 1).

3.2.2. Multiple Enzymes Activities

To the best of our knowledge, the concomitant production of protease, esterase, and laccase, by environmental *C. parasitica* strains under SSF on ZW was here reported for the first time.

The highest protease activity was registered at day 9 of fermentation, with comparable values among the 4 strains, ranging from 3955.6 $\pm$ 279.1 AU/g_{sf} for CpA, to 3785.8 $\pm$ 263.3 AU/g_{sf} for CpB2 (Figure 4A) in spite of the similar total protein content of the different crude extracts of the isolates (Table S1).

The time course of the protease production was quite similar among all strains. In detail, the protease production increased from the sixth day up to the ninth, when it reached its maximum; a subsequent decrease of activity was observed with a plateau of production from day 12 in CpB2 and CpC7, or a more or less sharp decline in CpC4 and CpA, respectively (Figure 4A). In general, this growth-associated protease production profile was already observed for other fungi [59,67], and it is related to the degradation of extracellular enzymes and/or the depletion of available nutrients.

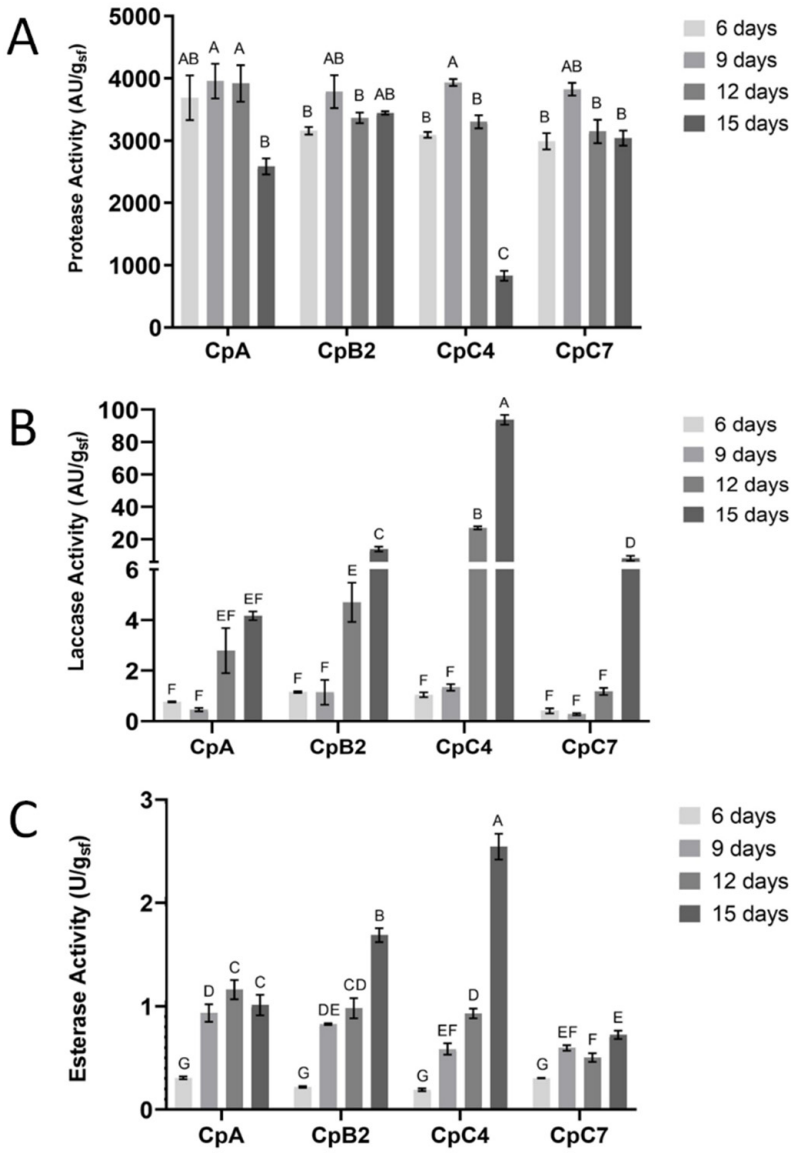

Figure 4. Time courses of protease (**A**), laccase (**B**) and esterase activities (**C**) produced by four *C. parasitica* environmental strains (CpA, CpB2, CpC4, CpC7), grown on ZW for 15 days. Different letters indicate significant differences ($p < 0.05$); means sharing a letter are not significantly different ($p > 0.05$). Data represent the average ± standard deviation of biological replicate cultures ($n = 3$).

The same amount of protease was obtained by the virulent strains, as well as by the hypovirulent CpC4. As reported previously for the *C. parasitica* virus-free strain EP155 and its hypovirus infected isogenic strain, no significant changes upon hypovirus infection were detected in the production of extracellular enzymes taking part in nutrient utilization [63], such as the endothiapepsin precursor.

As far as laccase is concerned, its production occurred later in the fungal growth cycle, with a significant increase of the activity starting at day 12 in all the strains (Figure 4B). In detail, CpC7 was characterized by a sharp increase reaching the maximum production at day 15 of growth. CpC4 strain proved to be the best producer, with a laccase yield of 93.8 ± 2.9 AU/g_{sf} at day 15 of growth, significantly higher than the other strains, corresponding to 6.5-fold increase. No activity was observed on non-inoculated samples.

While both protease and laccase production by *C. parasitica* in various types of liquid culture has already been reported, esterase and lipase activities have been scarcely studied in this fungus.

Preliminary evaluation of lipolytic activity by TBA assay evidenced a transparent halo around the disks with fungal crude extracts. These halos increased with the age of the cultures; notably, they appeared in the samples corresponding to day 9 and remained constant up to day 15. (Figure S2).

Here, for the first time, esterase activity was quantified in the crude extracts produced by environmental strains of *C. parasitica.* Esterase activity was significantly affected by the fermentation time following two different trends. In CpB2 and CpC4, the maximum esterase activity was reached after 15-day incubation with the yield of 1.69 ± 0.07 and 2.55 ± 0.12 U/g_{sf}, respectively. In detail, the enzyme activity in CpC4, the best esterase producer, was 2.73-fold higher at 15 days than at 12 days of incubation (0.93 ± 0.48 U/g_{sf}). Inversely, CpA and CpC7 esterase activity slightly increased to their maximum value of 1.16 ± 0.09 after 12 days, and 0.72 ± 0.040 U/g_{sf} after 15 days of fermentation, respectively (Figure 4C). No activity was observed on the non-inoculated substrate. Esterase and laccase production in SSF process seems to be associated with fungal growth phase, likely due to the very metabolic development of the microorganisms as previously reported [68–70].

Laccase, extracellular lipase and carboxylesterase are reported to be downregulated upon hypovirus infection [62]. In detail, the three laccase isoforms produced by *C. parasitica* [65] are modulated by the presence of hypovirus, and their activity is differentially suppressed by different CHV1 isolates, and also influenced by the culture conditions [66]. Surprisingly, in our study, CpC4 is the best laccase and esterase producer after 15 days of fermentation. The hypovirulent strain showed a level of activities 10-fold and 2-fold higher, respectively, than the other strains, in spite of being the only strain hosting CHV1. This apparently discordant behavior could be explained by the different genetic background of the pair Ep155 and its isogenic hypovirus infected strain, and the pair CpC4 and its virus free isogenic strain, producing quite different enzyme yields. In this regard, it would be interesting to cure CpC4 from the hypovirus to verify the CHV1 effect on the laccase production of the virus-free strain. Moreover, different hypoviruses and different CHV1 variants could cause a differential suppression of extracellular laccase, with some allowing a laccase activity to nearly virus-free levels [71]. Lastly, the culture conditions [71,72], such as the composition of the culture medium [72], could affect the enzymatic productions. Effectively, the different production of laccase and esterase found in the present study could be associated to specific features of the applied substrate and to the different timing used to assess the enzymes production compared to those applied in Rigling and Van Alfen [46], who found a 5-fold higher level of extracellular laccase expression by the dsRNA-free strain, grown in complete liquid medium for 5 days than the hypovirus infected isogenic strain.

The current finding of a hypovirulent strain characterized by an excellent enzymatic production makes the SSF method here presented a sustainable and completely circular technology. It offers the simultaneous production of multiple enzymes, and the possibility to use the SSF substrates entangled with the hypovirulent strain as fungal biomass to be applied in biological control treatment of chestnut cankers. The appreciable production of a cocktail of 3 enzymes (protease, lipase/esterase, and laccase) by single strains could be applied in textile industry [73].

As evidenced in this study, *C. parasitica* shows a high potential for its further study as an industrial enzyme-producing fungus. Several industries require cocktail of enzymes in place of a single enzyme, as the latter will not function as efficiently for many applications e.g., for biomass hydrolysis or detergents developments [74]. Development of a cost-effective cocktail is still one of the major challenges. Hence single microorganisms able to produce multiple enzymes are needed as well as protocols for a concurrent production of multiple enzymes are of great value.

A summary of the protease, laccase and esterase activities from fungi is reported in Table 4. Although a direct comparison between data from the literature and our data is not possible due to the differences in the enzymatic assays used, protease and laccase production by *C. parasitica* registered higher yields than several values mentioned in the literature and, notably, without the need of any nutritional supplements.

Table 4. Comparison of enzyme activity of protease, laccase and esterase/lipase from C. parasitica strains of current study with other reports.

Enzyme	Microorganism	SSF Substrate	Fermentation Period	Enzyme Substate	Unit Definition	Enzyme Activity	Reference
Protease	*A. oryzae MTCC 5341*	Wheat bran with supplements	120 h	Hemoglobin	1 U = 0.001 increase in $A_{280\ nm}$/min	864,000 U/g_{ds} [a]	Vishwanatha et al., 2010
	C. parasitica CpA	Wheat bran	9 days	Hemoglobin	1 AU = 0.01 increase in $A_{280\ nm}$/min	3955.6 U/g_{sf} [b]	Current study
	A. oryzae (Ozykat-1)	Wheat bran and rice bran	4 days	Casein	1 U = release 1 µg tyrosine/min	1200 U/g_{ds}	Chutmanop et al., 2008
Laccase	*P. acaciicola AGST3*	Wheat bran with supplements	12 days	ABTS *	1 U = oxidation 1 µmol ABTS/min	535,000 U/g_{ds}	Thakur and Gupte 2015
	T. versicolor JSRK13	*Parthenium* sp. with supplements	17 days	Guaiacol	1 U = 0.01 increase $A_{470\ nm}$/min	185 U/g_{sf}	Singh et al., 2019
	C. parasitica CpC4	Wheat bran	15 days	2,6-Dimethoxyphenol	1 AU = 1.0 increase $A_{468\ nm}$/min	93.8 U/g_{sf}	Current study
Esterase /Lipase	*R. microsporus CPQBA 312-07 DRM*	Wheat bran and sugarcane bagasse with supplements	18 h	Olive oil	1 U = release 1 µmol fatty acid/min	265 U/g_{sf}	Pitol et al., 2017
	A. ibericus MUM 03.49	Olive pomace and wheat bran with supplements	7 days	pNP-butyrate **	1 U = release 1 µmol di pNP/min	223 U/g_{ds}	Oliveira et al., 2017
	C. parasitica CpC4	Wheat bran	15 days	pNP-butyrate	1 U = release 1 µmol di pNP/min	2.55 U/g_{sf}	Current study

Bold: *Cryphonectria* stain isolated in this work; [a] $_{ds}$: dry substrate, [b] $_{sf}$: substrate fermented; * ABTS: 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); ** pNP-butyrate: p-Nitrophenyl butyrate.

According to the obtained results, further studies will be carried out to optimize the SSF protocol here presented. Parameters such as variation in C/N ratio besides several other physicochemical factors are important in the development of fermentation process and their interactions should be evaluated in order to increase the expression of each enzyme of the cocktail to levels comparable to the best single producers.

4. Conclusions

The ability of the filamentous ascomycete *C. parasitica* to colonize agro-industrial wastes without any pre-treatment and supplements as a source of value-added bio-products by means of SSF was verified in this study.

To the best of our knowledge, this is the first report on multiple enzymes production by *C. parasitica* on agro-industrial wastes. All newly isolated *C. parasitica* strains were able to (i) grow, metabolize the substrate, and (ii) produce multiple enzymes on ZW without any other supplement. Interestingly, the crude extract obtained from the hypovirulent strain CpC4 showed the highest enzymatic activities, resulting an ideal candidate for further studies on the feasibility of large-scale production of industrially relevant enzymes.

In the circular economy view, the here presented approach could lead to develop a biorefinery without waste production. The fermentation substrates could be addressed for the production of microorganism-based fertilizer.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/fermentation7040279/s1, Table S1. Total protein content of crude extract of four *C. parasitica* environmental strains (CpA, CpB2, CpC4, CpC7), at different time points of fermentation, grown on ZW for 15 days. Data represent the average ± standard deviation of biological replicate cultures (n = 3). Figure S1. Growth of CpC7 strain under SSF using as solid substrate spent coffee grounds (CGA and CGB) and rice husk (RHM and RHU). Cnt, not inoculated substrate (negative control). Figure S2. Preliminary evaluation of lipolytic activity by TBA assay. It showed that the zone of hydrolysis evidenced a transparent halo around the disks with fungal crude extracts starting at day

6 (B), and increased with the age of the cultures day 9, 12, 15 (respectively, C, D, E); no transparent halo around in the samples corresponding to day 2 (A) and negative control not inoculated substrate (F).

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