Beyond Enzyme Production: Solid State Fermentation (SSF) as an Alternative to Produce Antioxidant Polysaccharides

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Abstract:

Solid state fermentation (SSF) is considered more sustainable than traditional fermentation because it uses low amounts of water and transforms agro-industrial residues into value added products. Enzymes, biofuels, nanoparticles and bioactive compounds can be obtained from SSF. The key factor in SSF processes is the choice of microorganisms and their substrates. Many fungal species can be used and are mainly used due their lower requirements of water, O2 and light. Residues rich in soluble and insoluble fiber are utilized by lignocellulolytic fungi because they have the enzymes that break fiber hard structure (lignases, celullases or hemicelullases). During the hydrolysis of lignin, some phenolic compounds are released but fungi also synthetize compounds such as mycophenolic acid, dicerandrol C, phenylacetates, anthraquinones, benzo[5]furans and alkenyl phenols that have health beneficial effects such as antitumoral, antimicrobial, antioxidant and antiviral activities. Another important group of compounds synthetized by fungi during fermentation are polysaccharides that also have important health promoting properties. Fungal biofermentation has also proved to be a process which can release high contents of phenolics and it also increases the bioactivity of these compounds.

Keywords: solid state fermentation; phenolic compounds; enzymes; polysaccharides

1. Introduction

The innovation implied in the idea of turning food waste into valuable chemicals, which are used daily in human activities, is very attractive commercially and involves further research in areas like biotechnology, nanotechnology, food science and food technology [1]. There is an enormous demand for food and energy to fulfill the requirements of the increasing population and since food waste is growing too, the need of effective waste management strategies and procedures is urging as rapid urbanization continues in many countries [2]. The accumulation of food waste corresponds to 1/3 of the world’s total food production [3]. Currently, most countries are focused on the prevention of food waste and they tend to ignore procedures employed to dispose food and organic wastes and residues.

Solid residues from agricultural industries as well as vegetable waste are potential sources of important substances which could be employed in the chemical, food, pharmaceutical and cosmetic industry. Through recent studies of food supply chain...
residues and waste, it has been found that useful products like enzymes, biofuels, biodegradable plastics, nanoparticles, bioactive compounds, among others can be obtained [2]. Among the valuable compounds that can be obtained from organic waste, polyphenols are the most abundant as they are found in vegetables, cereals, beverages and fruits. These substances are the ones that give color and other important organoleptic features, but they also have many different beneficial bioactivities in health as well as on the prevention of chronic diseases [4]. Among the several technologies used to obtain valuable compounds from organic waste, solid state fermentation (SSF) is perhaps the most promising one because of its economic and sustainable characteristics: product yielding, high efficiency and productivity, low consumption of energy and water, and minimum concerns about solving disposal problems [1].

2. Solid State Fermentation (SSF)

Solid-state fermentation (SSF) is a process where microorganisms are able to grow in a complete, or almost complete, water-free environment [5]. This process is carried out using microorganisms growing on moist and solid substrates that has been used for thousands of years to produce food like bread and cheese. Although SSF is not a new technology, recently, it has become a very relevant process for the production of pharmaceutical, biochemical and food products, as well as for bioenergy generation. The products obtained enable higher enzymatic productivity for many enzymes because they are less susceptible to substrate inhibition [1]. Other applications of SSF include: pigments production, aroma production, phenolic compound production, composting, biobleaching, etc. [6]. Remnants of SSF (which have a different level of biodegradability) can be used for composting, anaerobic digestion or to produce biogas [1].

The most important aspect to consider while developing SSF process is the choice of microorganisms and substrates. Mainly fungi and mold are employed for SSF because they need less energy for substrate sterilization and are less susceptible to bacterial contamination. filamentous fungi are particularly appropriate for SSF because this method simulates their natural habitat. This kind of fungi is able to synthesize large amounts of enzymes and other metabolites under SSF conditions. Yeasts and some species of bacteria (e.g. Bacillus subtilis, Bacillus thuringiensis and Lactobacillus sp.) are considered as the second-best choice because of their ability to grow in environments with low water activity. Streptomyces sp. and other Actinomycetes can also be employed in SSF because of their resistance to extreme conditions and capacity to colonize solid residues abundantly [6].

The most common substrate utilized in SSF are agricultural and forestry residues because they are underutilized and abundant. All these materials are composed by cellulose, hemicellulose, starch, pectin and lignin and other fibers. Sugarcane bagasse, cassava bagasse, corn cobs, wheat bran and other cereal bran, fruit peels and pulps, coffee pulp and husks, straws and husks from different origins are the most frequent agro-residues used for SSF as substrates [6, 7].

Most agro-industrial residues (fruit byproducts, wheat straw, paddy straw and corn stove) can be transformed through the use of SSF to enrich their antioxidant properties for different uses, such as animal feed [6]. Lignino lytic exoenzymes which are produced during SSF break the chemical bonds of lignin by depolymerizing the complete structure into smaller ones. SSF can be used to obtain bioactive compounds since enzymes produced by microorganisms (like esterases, amylase, cellulases and xylanases) release bound phenolic compounds [7]. Among the positive aspects of SSF are: no need of organic solvents to extract
the released phenolic compounds; higher quality and higher activity of extracts; operating and capital costs are lower, etc. In the same way, SSF yields higher enzymatic productivity for many enzymes, it has less susceptibility to substrate inhibition and therefore it produces a higher final concentration of products [6]. SSF has a lot of potential to increase the extraction yield of bioactive compounds that may be recovered by emerging technologies such as ultrasound, pulsed electric fields or microwave that have been already tested in products such as potato peels [8].

3. Phenolic compounds in fungi

Phenolic compounds are secondary metabolites produced mostly by plants. The biosynthesis of phenolic acids begins with phenylalanine, which is the first substrate during the phenylpropanoid pathway [9]. Phenolics can enter the organs through the transporter protein penetrating the compartment membrane [10]. Phenolic acids content is largely influenced by factors like agronomic practices and environmental conditions, as well as different abiotic and biotic stimuli. Genetics influence phenolic acids content too because of the environmental interactions that cause a large variation among species and cultivars of the same species [9].

The phenolic compounds are synthesized in the intracellular organs (like the endoplasmic reticulum) then they are liberated and moved into the vacuole or cell wall matrix through the vesicle transfer system (which is a small lipid bilayer system). This system can contain phenolics and it also facilitates their migration into the cell wall matrix. It has been observed that the cytoplasmic and Golgi vesicles that contain phenolics move to the plasma membrane and secrete phenolics to the cell wall matrix. Additionally, the phenolic compounds can reach the cell wall matrix as they are moved to the plasma membrane via ABC transporters [10]. The phenolic compounds that were transferred are found now bound to macromolecules like structural proteins, cellulose and pectin by covalent bonds such as ether, ester and carbon-carbon bonds [11].

Phenolic compounds can be found inside the matrix of plants in three ways: free, soluble-bound (conjugated) or in insoluble-found form. Soluble conjugated phenolics are found esterified to compounds with low molecular mass (like carbohydrates, proteins and lipids) while insoluble are esterified or etherified to components of the cell wall structure [12]. Bound or insoluble phenolics are also called non-extractable phenolics and remain in the matrix of the residue after the extraction process of soluble phenolics using aqueous alcohol [13]. Insoluble-bound phenolics are found in cell wall matrix of the plant cells. They can only be released from the matrix through a process of acid, alkaline or enzyme hydrolysis [10].

Since phenolic compounds have a protecting role in plants, they also should be considered vital for the survival of endophytic fungus or at least for their mutually beneficial relationships. However, it is not clear yet if phenolic compounds have the same value for plants than for their associated microorganisms [14].

3.1 Biosynthesis of phenolic compounds in fungi

It has been reported that sometimes the phenolic compounds originally biosynthesized by plants may be found in the endophytes cultures. Among these compounds are: emodin,
capsaicin, luteolin, hypericin and chlorogenic acid [14]. In fungal endophytes, flavonoids, phenolics and saponins are very important bioactive constituents which can be considered as new possible antioxidant resources. For example, Pan et al. (2017) isolated fungal fermented filtrates from 53 different endophytes from the bulbs of *Fritillaria unibacteata* var. *wabuensis* (FUW), and all of these compounds showed antioxidant activities [15]. These researchers identified many natural antioxidant compounds from the fungal extracts, like phlorizin, rutin, gallic acid and 2,6-di-tert-butyl hydroquinone [15].

In fungi, the Shikimate pathway has been correlated with the presence of phenolic compounds in these microorganisms. Tyrosol, a well-known phenolic compound, has been isolated from extracts of endophytes from the fermentation of different plants and it is thought to be a signaling molecule in fungi. Besides this, several other phenolics have been reported in endophytic fungi and most of these compounds seem to have interesting bioactivities [14]. Bioactive properties of phenolic acids from fungi include antitumor, antimicrobial and antioxidant (Table 1). The interaction between the substrate and the fungi affects the production of bioactive metabolites [16]. For example, when *Stemphylium globuliferum* grew in solid white bean medium produced tetrahydroanthraquinones that were inactive against the L5178Y mouse lymphoma cell line but when it was grown in a rice culture, other anthraquinones that inhibit the growth of the lymphoma cells were produced [17].

*Ampelomyces* sp., is an endophytic fungus which can synthetize several phenolic secondary compounds through solid and liquid fermentation. Some of the phenolic compounds produced by this microorganism are: mycophenolic acid, dicerandrol C, cytosporone C, phomopsin A, phomopsin B, altersolanol A, desmethyldiaportinol, etc. These two last compounds showed promising cytotoxic activity *in vitro* against L5178Y mouse lymphoma cell lines; other phenolics produced have antimicrobial activity against *S. aureus*, *S. epidermidis* and *E. faecalis* with minimum inhibitory concentration values between 12.5-25 µg/mL. *Pestalotiopsis* sp. and *Phoma pinodella* can produce phenolics like phomodione, usnic acid, cercosporamide, pestalol D and E and several others, some of which have antiproliferative activity against a panel of human cancer cell lines (lung, prostate, ovary, colon, cervix, pancreas and malignant tumor of melanoma) [14].

<table>
<thead>
<tr>
<th>Phenolic compounds</th>
<th>Reported bioactivities</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td>Mycophenolic acid: (caproate)</td>
<td>Immunosuppression for kidney transplant recipients</td>
<td>[18]</td>
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<tr>
<td>Dicerandrol C (xanthone)</td>
<td>Antimicrobial actions against <em>S. aureus</em></td>
<td>[19, 20]</td>
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<tr>
<td>Cytosporone B and C (phenylacetates)</td>
<td>Inhibitory action against <em>C. albicans</em> and <em>Fusarium oxysporum</em></td>
<td>[20]</td>
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<tr>
<td>Phomopsin A and B (mycotoxins)</td>
<td>Antiviral, antibacterial and antifungal activities</td>
<td>[21]</td>
</tr>
<tr>
<td>Altersolanol A, B and N (anthraquinone)</td>
<td>Cytotoxic activity <em>in vitro</em> against L5178Y mouse lymphoma cell lines and</td>
<td>[22, 23]</td>
</tr>
<tr>
<td>Compound</td>
<td>Activity</td>
<td>Reference(s)</td>
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<tr>
<td>4-dehydroaltersolanol A</td>
<td>Antimicrobial activity against <em>S. aureus</em>, <em>S. epidermidis</em> and <em>E. faecalis.</em></td>
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<tr>
<td>(anthraquinone)</td>
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<tr>
<td>Cytotoxicity against L5178 mouse lymphoma cells.</td>
<td>[24, 25]</td>
<td></td>
</tr>
<tr>
<td>Dihydroaltersolanol C and acetylalterporriol E (anthraquinones)</td>
<td>Strong cytotoxicity against the murine lymphoma cell line L5178Y.</td>
<td>[26]</td>
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<tr>
<td>Alterporriol T</td>
<td>Inhibition of a-glucosidase</td>
<td>[26]</td>
</tr>
<tr>
<td>Nigbeauvin A and B (azaphilones)</td>
<td>Cytotoxic against tumor cells HL-60, A-549, SMMC-7721, SW480 and MCF-7.</td>
<td>[27]</td>
</tr>
<tr>
<td>Phomaether A and C (anthraquinones)</td>
<td>Strong antibacterial activity against <em>S. albus</em>, <em>S. aureus</em>, <em>E. coli</em> and <em>V. parahaemolyticus.</em></td>
<td>[28]</td>
</tr>
<tr>
<td>Desmethyldiaportinol (isocoumarine)</td>
<td>Cytotoxic activity <em>in vitro</em> against L5178Y mouse lymphoma cell lines.</td>
<td>[25]</td>
</tr>
<tr>
<td>Phomodione (benzofuran)</td>
<td>Activity against <em>S. aureus</em>, <em>Pythium ultimum</em>, <em>Sclerotinia sclerotiorum</em> and <em>Rhizoctonium solani</em></td>
<td>[25]</td>
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<tr>
<td>Usnic acid (benzofuran)</td>
<td>Antimicrobial and anti-inflammatory activity</td>
<td>[25]</td>
</tr>
<tr>
<td>Cercosporamide (benzofuran)</td>
<td>Mnk inhibitor through blockage of eIF4E phosphorylation. Anti-cancer activities in hepatic and lung cancer, leukemia and glioblastoma</td>
<td>[29, 30]</td>
</tr>
<tr>
<td>Pestalol D (alkenyl phenol)</td>
<td>Anticancer, antiviral</td>
<td>[31, 32]</td>
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3.2 Phenols release from vegetable cell walls using fungi

Cellulose and hemicellulose have limited accessibility to microorganisms in plant cell walls because of the interaction and/or chemical association with lignin and the relative ratio of its monomeric constituents. Fungi are the main microorganisms which are able to recycle cellulose in nature; they are well-adapted for the degradation of cellulosic biomass because they produce an elevated number of enzymes of wide diversity and with several supportive functions. Lignocellulosic materials include three main groups of polymers: cellulose (linear polymer of b 1-4 of glucose bonds), hemicellulose (polymers non-cellulosic which include glucans, mannans, arabinans, galactans and xylans) and lignin (complex polyphenol) [33].

Lignin is a network made by phenolic compounds and it is commonly found in secondary cell walls, especially in woody tissues. It provides structure and rigidity to cells and its proportion increases with maturation. It can be found embedded in cell walls between cellulose and hemicelluloses and its concentration depends upon cell type, stage of maturation and plant species. Lignin is synthesized in the secondary wall formation and it is found throughout the cell wall of plants [34]. Lignin contains about 40 different
oxygenated phenyl-propane units and it is chemically inert. Ferulic and coumaric acids are some of the most studied phenolic acids because of their abundance in plants. These compounds give rigidity to cell walls because they crosslink the sugar moieties and also lignin [12].

Fungi degrade first lignin through the action of lignolytic enzymes (lignin peroxidase, manganese peroxidase and laccase) and then can access energy-rich polysaccharides for their metabolism and growth [5]. Enzymatic hydrolysis reactions on the phenolic conjugates generate free phenolics (for example acids like gallic, ferulic and ellagic acids) but also low molecular weight lignin molecules which have a higher antioxidant power [35]. In fruits and vegetables, phenolic compounds can be released from pectins and cellulose using enzymes like β-glucosidase [33].

3.2.1 Fungal enzymes for cellulose degradation

Three complementary enzymatic activities have been proposed to be responsible for cellulose degradation: β-glucosidases (GH), endoglucanases (EG) and cellobiohydrolases (CBH). These enzymes can hydrolyze the β-1,4 covalent bonds which connect the glucose units present in cellulose chains. β-glucosidases are non-processive enzymes because their substrate has to be liberated after each cleavage event to let the new glucose molecule to exit the pocket. Endo-1,4-β-D-glucanases, EC 3.2.1.1 randomly cleave β-1,4 bonds in cellulose amorphous areas; these enzymes generate new reducing and non-reducing ends with this activity. Endoglucanases, as well as β-glucosidases, are strongly inhibited by their reaction products: cellobiose and glucose respectively. Cellobiohydrolases (like cellulose 1,4-β-cellobiosidases and EC 3.2.191) are processive enzymes that release cellobiose (i.e., two units of glucose linked by a β-1,4 bond) from reducing or non-reducing ends from cellulose fragments produced by endoglucanases [36].

Different fungi have been used for the production of cellulases, including Aspergillus terreus M11, Trichoderma reesei RUT C30, Aspergillus niger MTCC 7956, Trichoderma asperellum, Pleurotus ostreatus, Aspergillus oryzae, among others. Fungi like Rhizomucor miehei and Aspergillus niger produce enzymes like cellulases and pectinases that break the cellulosic chains to obtain bioenergy from the residues [37] (Zambrano, et al., 2018). The extraction of these enzymes and in consequence the testing of their enzymatic activity may be affected by their absorption into the substrate lignocellulosic matrix. This gives an additional advantage to SSF since end products may be obtained without the need of using pure enzymes [38].

Aerobic fungi have multiple enzymes that are mostly active with cellulose: lytic polysaccharide monooxygenases (LPMOs) can carry out copper-mediated oxidative cleavage of cellulose and AA9 enzymes can act on xylan and xyloglucan. Additionally, expansins (non-catalytic proteins) are thought to have a role in cellulose degradation (reduction of substrate viscosity as well as disruption of cellulose fibers were observed sometimes) but their mechanism has not been totally clarified because no hydrolytic activity was reported [36].

3.2.2 Fungal enzymes to release phenolic compounds
Ligninases are a large group of complex enzymes (such as peroxidases and laccases) that work together to break down the phenolic compounds from lignin. These enzymes attack the carbon-carbon and carbon-oxygen bonds that hold in place the complex structure of 3D lignin. Some types of ligninases are a class of copper enzymes (called laccases), and other three types are peroxidases-lignin peroxidases, manganese peroxidases and versatile peroxidases. There is a strong synergy among all these types of enzymes and it is considered as one of the potential outstanding applications for lignin valorization [36].

Lignocellulolytic fungi are very common in the fungal kingdom. Simple and primitive fungal species like *Chytridiomycetes* up to more complex and advanced ones (like Basidiomycetes) degrade cellulose very efficiently as they use it as a carbon source. Fungi prefer to degrade lignin, cellulose and hemicellulose (e.g., white rot fungi) but also degrade polysaccharides and modify lignin (e.g., brown rot fungi); or may degrade lignin and polysaccharides at the same time (e.g., soft rot fungi). This kind of fungi produce enzymes that, either individually or as a consortium, degrade and metabolize recalcitrant lignocellulose [36].

Table 2 shows the chemical and bioactive effects of secondary metabolites obtained through enzymatic fermentation. In general, enzymes increased the extraction yield and had chemical effects on the secondary metabolites, affecting their bioactivity. The substrates utilized were mainly food and agricultural waste which commonly have no further use. By using enzymes, synthetized from microorganisms or bought from commercial brands, most of the metabolites display good bioactive effects. Additionally, the energy employed during the process is lower than in traditional extraction methods (like acid or heat extraction).

Table 2. Utilization of enzymes to obtain secondary metabolites from cellulosic materials.

<table>
<thead>
<tr>
<th>FOOD/ SUBSTRATE</th>
<th>ENZYME</th>
<th>CHEMICAL EFFECTS</th>
<th>BIOACTIVE EFFECTS</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sweet potato (<em>Ipomoea batatas</em> (L.))</td>
<td>Feruloyl esterases from <em>L. acidophilus</em></td>
<td>The pH values of sweet potato fell from 6.2 to 3.45. Higher contents of free ferulic acid and p-coumaric acid.</td>
<td>Higher inhibitory effects on pheochromocytoma-cancer-cell proliferation.</td>
<td>[39]</td>
</tr>
<tr>
<td>Spent espresso grounds (SEGs)</td>
<td>Cellulase and hemicellulase</td>
<td>A maximum reducing sugar yield. Flavonoids and polyphenols increased by 24.0% and 33.9%, respectively</td>
<td>DPPH free-radical scavenging activity increased by 59.9%</td>
<td>[40]</td>
</tr>
<tr>
<td>Fermented oat (Avena sativa L.) in solid state using mold Monascus anka</td>
<td>( \alpha )-amylase and xylanase</td>
<td>The phenolic content increased significantly, especially the ferulic acid in the insoluble fraction and the vanillic acid in the soluble fraction.</td>
<td>[41]</td>
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<tr>
<td>Rice bran</td>
<td>Carbohydrases (Viscozyme, Termamyl, Celluclast, AMG, Ultraflo and Pentopan)</td>
<td>Increased the amount of extractable phenolic acids by 2.5-3.0 times.</td>
<td>Significant increase in ferric reducing power (1.5-3.3 times).</td>
<td>[42]</td>
</tr>
<tr>
<td>Steamed rice bran fermented with lactic acid bacteria and hydrolyzed with complex enzymes</td>
<td>( \alpha )-amylase, complex enzymes</td>
<td>Enhanced the total phenolics and flavonoids of aqueous solutions from rice bran pretreated with ( \alpha )-amylase.</td>
<td>The antioxidant activity of aqueous solutions also increased after the process.</td>
<td>[43]</td>
</tr>
<tr>
<td>Lyophilized and oven-dried black grape (Vitis vinifera x (Vitis labrusca x Vitis riparia)) pomace, and apple (Malus domestica cv. Jonagold) and yellow pitahaya (Hylocereus megalanthus) peel, core, peduncle and seed mixture.</td>
<td>Cellulase and pectinase cocktails from R. miehei NRRL 5282 and Aspergillus niger, respectively</td>
<td>Release of free phenolic compounds.</td>
<td>Improve the antioxidant properties of the phenolics as determined by 1,1-diphenyl-2-picrylhydrazyl radical inhibition or ferric reducing antioxidant power analyses.</td>
<td>[37]</td>
</tr>
<tr>
<td>Underutilized watermelon (Citrullus lanatus Thunb) rind (WMR)</td>
<td>Enzyme cocktail “Kemzyme” Kemine, Germany composed of pectinase, endo-1,3 (4)-β-glucanase, α-amylase, endo-1, 4-β-xylanase and bacillolysine (protease)</td>
<td>Release of antioxidant phenolics up to 3 folds on fresh weight basis compared to conventional solvent extraction with substantial level of total phenolics</td>
<td>WMR extracts retained most of their antioxidant properties.</td>
<td>[44]</td>
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<tr>
<td>Pigmented and non-pigmented rice bran</td>
<td>Cellulase and/or xylanase.</td>
<td>Increase in the content of soluble oryzanol.</td>
<td>Increase in the free radical scavenging activity and total antioxidant activity.</td>
<td>[45]</td>
</tr>
<tr>
<td>Guava (Psidium guajava) leaves</td>
<td>Xylanase, cellulase and β-glucosidase.</td>
<td>Improved the soluble phenolics content and flavonoids content by 103.2% and 81.6%, respectively.</td>
<td>Higher antioxidant activity and preventative effects against supercoiled DNA damage.</td>
<td>[46]</td>
</tr>
<tr>
<td>Ulmus pumila barks (UPB)</td>
<td>Cellulase, pectinase and β-glucosidase.</td>
<td>Higher extraction yield of TP.</td>
<td>Higher in vitro antioxidant activity was observed using the FRAP and DPPH methods.</td>
<td>[47]</td>
</tr>
<tr>
<td>Black tea leftover (BTLO)</td>
<td>Kemzyme, alcalase, acid cellulase, Pectinex, viscozyme and using supercritical carbon dioxide</td>
<td>The hydrolysis of BTLO with 2.9% (w/w) kemzyme at 45°C and pH 5.4 for 98 improved the release of non-extractable</td>
<td></td>
<td>[48]</td>
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</table>
and ethanol as co-solvent (SC-\(\text{CO}_2\) + EtOH) and a conventional solvent extraction (CSE) with ethanol and water (80:20, v/v) was used to compare results. Polyphenols (NEPPs). The polyphenols extracts obtained by SC-\(\text{CO}_2\) + EtOH were cleaner and richer in polyphenols compared to those obtained by CSE.

<p>| Cherry (\textit{Cerasus pseudocerasus} \textit{G. Don}) seeds | An ultrasonic-microwave assisted aqueous enzymatic extraction (UMAAEE) was employed. The enzyme cocktail included cellulase, hemicellulase, and pectinase | The fatty acid composition of cherry seeds oil was not changed significantly by UMAAEE. More bioactive components (like a-tocopherol, b-carotene, phospholipids and phytosterols) were obtained by UMAAEE. | [49] |
|---------------------------------------------------------------|----------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------|
| Pomegranate (\textit{Punica granatum}) peel extracts         | Pectinase and cellulase                                                          | Enzymatic extraction did not improve the extraction yields. Selective antimicrobial activity against \textit{S. aureus}, \textit{Methycillin-resistant Staphylococcus aureus} and \textit{Listeria monocytogenes} |
| Seeds of \textit{Cuscuta chinensis Lam.}                     | Cellulase and proteases                                                          | The combination of enzymes The antioxidant properties of the flavonoids were                                             |
|                                                              |                                                                                   | [51]                                                                                                                      |</p>
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<tr>
<th><strong>Sargassum muticum:</strong> whole algae (Sm) and residual algae from alginate production (AESm).</th>
<th>Enzyme assisted extraction coupled with ultrasound extraction. Alcalase, alcalase + Protamex, amylase, Protamex, cellulase, Rapidase Press, Rapidase TF, Rapidase UF L and Viscozyme L.</th>
<th>Aqueous enzyme extraction coupled with ultrasound treatment lead to the highest yield rates.</th>
<th>One gram of extract was equivalent to 30 mg of ascorbic acid and 200 mg Trolox. The treatments did not influence the cytotoxicity of <em>Sargassum muticum</em> aqueous extracts on melanoma and liposarcoma cells.</th>
</tr>
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<tr>
<td><strong>Crude and waste seeds of guarana (<em>Paullinia cupana</em>).</strong></td>
<td>Aqueous enzymatic maceration (AEM) using cellulase and pectinase.</td>
<td>AEM enhanced the global yield in the extract but did not efficiently extracted bioactive molecules from guarana seeds.</td>
<td></td>
</tr>
<tr>
<td><strong>Longan (<em>Dimocarpus longan</em> Lour.) pulp.</strong></td>
<td>Superfine grinding-assisted enzymatic treatments</td>
<td>Yield, sugar content, solubility, arabinose and mannose percentage</td>
<td>Strong stimulation on the proliferation of <em>Lactobacillus plantarum</em>, <em>L. bulgaricus</em>, <em>L.</em></td>
</tr>
<tr>
<td>Source Material</td>
<td>Process/Enzymes</td>
<td>Characteristics</td>
<td>Antioxidant Potential</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
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<tr>
<td>Soy pulp by-product (okara)</td>
<td>Endoproteases</td>
<td>Half of the original insoluble proteins were turned into soluble peptides. The solubilization of isoflavones trapped in the insoluble protein matrix was detected too.</td>
<td>Higher antioxidant potency than the original source.</td>
</tr>
<tr>
<td>Sesame (Sesamum indicum L.) bran</td>
<td>Viscozyme L., alcalase, ultrasound and ultrasound-assisted enzymatic extractions.</td>
<td>Alcalase had higher protein and total phenolic compounds recovery than viscozyme L.</td>
<td>The highest antioxidant capacities (determined with the DPPH method and the ABTS method) were found in the ultrasound-assisted enzymatic extraction.</td>
</tr>
<tr>
<td>Grape pomace and wheat bran</td>
<td>Two enzymatic cocktails obtained from the solid fermentation of grape pomace and wheat bran and only</td>
<td>Total phenolic content increased with the increase of time of enzyme production in both mediums. The activities of polygalacturonase and tannase</td>
<td>Higher antioxidant potential.</td>
</tr>
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<td><strong>wheat bran,</strong> by the mutant strain <em>Aspergillus niger</em> 3T55B8 were used.</td>
<td>showed a linear relationship with released of total phenolic compounds and proanthocyanidins, respectively.</td>
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<tr>
<td><strong>Leaves of <em>Viscum coloratum</em> (Kom) Nakai (VCP)</strong></td>
<td>Cellulase.</td>
<td>The VCP extract inhibited the replication of HBV-DNA and the secretion of HBV antigens and showed a better antioxidant capacity. [58]</td>
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<td><strong>Watermelon (<em>Citrullus lanatus</em>)</strong></td>
<td>A papain digestion process was employed</td>
<td>The monosaccharide composition included: galactose (38.26%), arabinose (26.12%), rhamnose (17.86%), mannose (9.94%), xylose (5.10%) and glucose (2.70%). PWR showed cytotoxicity ability to human laryngeal carcinoma Hep-2 cell in a dose and time dependant manner. [59]</td>
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<tr>
<td><strong>Alfalfa (<em>Medicago sativa</em> L.)</strong></td>
<td>Four different methods were used to extract polysaccharides from alfalfa: hot-water extraction (HWE), ultrasonic-assisted extraction (UAE),</td>
<td>The results showed that the monosaccharide composition of the four extracts were the same (glucuronic acid, glucose, rhamnose, galactose and xylose) but the UEAE had the largest extraction yield, the highest uronic acid content and the best antioxidant activities. [60]</td>
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enzyme-assisted extraction (EAE) and ultrasonic-enzyme-assisted extraction (UEAE). The enzyme complex used consisted of cellulose, papain and pectinase. Proportions were different.

| Japanese grape (Hovenia dulcis) | A treatment with cellulase produced by *Bacillus amyloliquefaciens* DL-3 was used to extract reducing sugars from Japanese grape. | The enzymatic process increased the release of sugars and decreased the extraction temperature and time. | [61] |
| Grape (Vitis vinifera L.) pomace of Syrah, Cabernet Sauvignon, Malbec Pinot-Noir and Marselan varieties. | The following enzymes were employed: pectinase and cellulase from *Aspergillus niger*; and tannase from *A. oryzae* | The enzymatic treatment augmented the extraction yield of phenolics by up to 66%. Tannase released gallic acid and cellulase *p-*coumaric acid and malvidin-3-O-glucoside. | The enzymatic treatment increased the antioxidant capacity by up to 80%. | [62] |

Direct treatments with enzymes (carbohydrate-cleaving enzymes mostly) are also used in preparations to release the phenolic aglycones [37]. Dey and Kuhad (2014) employed fungi species like *Rhyzopus oryzae*, *Rhyzopus oligosporus* and *Aspergillus oryzae* in order to perform
solid state fermentation in several cereals [7]. These authors found that the scavenging properties of phenolic compounds released increased after the fermentation in all the microorganisms and cereals studied. They concluded that, although antioxidant properties of cereal grains can be increased through solid-state fermentation, these properties vary among species of fungi and grains. It is also possible that some other metabolic pathways could be involved in solid-state fermentation besides the enzymatic release of phenolic compounds [5]. Shin et al. (2019) employed Aspergillus awamori and Aspergillus oryzae in order to ferment during 5 days black rice bran (BRB) [63]. They found that, although a moisturizing and autoclaving pre-treatment before fermentation decreased total phenolic content (TPC), fermentation increased TPC specially protocatechuic acid and ferulic acid, which displayed the most significant increases [63]. Wang et al. [64] fermented guava leaves (GL) utilizing Monascus anka and Bacillus sp. and found that the fermenting process affect the composition and contents of biochemical compounds. Specifically, quercetin, kaempferol, gallic acid and quercetin-3-O-α-L-arabinopyranoside were the marker components responsible for the changes in the bioactivities of GL during fermentation; total flavonoids and phenolics content, kaempferol and quercetin correlated with their bioactivities very well [64].

During fermentation, the glycosylation degree of phenolic compounds changes and in consequence their bioactivity. For example, Wang et al. [64] used complex enzyme-assisted extraction (CEAE) in guava leaves (GL) to enhance the bioavailability of insoluble-bound phenolics. By using this enzymatic treatment these authors found that the soluble phenolics content, the flavonoids content, as well as the ABTS, DPPH and FRAP improved greatly by 103.2%, 81.6%, 104.4%, 126.5% and 90.3%, respectively. Total soluble phenolics extracts from GL after CEAE displayed the highest antioxidant activity and protective effects against supercoiled DNA damage. In the same way, quercetin and kaempferol contents augmented their bioactivity by 3.5 and 2.2 folds, respectively. They also found that after the CEAE, most of the phenolic compounds were in their soluble form and scarcely in their insoluble-bound form [64].

3.3 Accumulation of phenolic acids in fungi cell walls

In general, fungal endophytes coexists asymptotically with their hosts and they represent an underused group of microorganisms for the discovery of new valuable compounds because they can produce diverse metabolites and have the ability to synthesize substances that are only produced and isolated from higher plants [65]. In the same manner, the antioxidant activity of fungal endophytes from medicinal plants has gained recognition in natural product research during the last decades. The antioxidant compound synthetized by fungal endophytes probably help host plants to neutralize free radicals. Fungal endophytes can provide effective tolerance to free radicals under abiotic stress conditions and they also can promote growth through biosynthesis of plant hormones and the acquisition of nutrients [15].

Edible fungi contain bioactive glycolipids, aromatic phenols, fatty acid derivatives, polyacetylamine, poliketides, shikimic acid derivatives, sesquiterpenoids and many others [66]. Yeasts are enclosed by a rigid cell wall which is about 25-30% of the cell’s dry weight.
Polysaccharides extracted from yeasts’ cell walls have showed important properties for human health beyond their function as structural organ, like antioxidant, antiproliferative and immunomodulatory. For example, Galinari et al. have studied the pharmacological properties of the α-mannan-rich fraction of the cell wall of the yeast *Kluyveromyces marxianus* CCT7735 in order to evaluate its chelating and cytotoxic activities [67].

Phenolic acids are commonly found in their free form in fungi and there is evidence that they are absorbed by the human body in their free form as well. After being ingested, they are absorbed and conjugated (glucuronated mainly) in the same pathways as flavonoids and other polyphenols [16]. Although some authors have found flavonoids in *Pleurotus* spp, this is unlikely because edible fungi lack the main enzymes which are involved in the flavonoids metabolic pathway; besides this, fruiting bodies from fungi are not able to store flavonoids present in growth substrates [68,69].

4. **Bioavailability and digestibility of bound phenolics**

Bioavailability of phenolic compounds is essential for their biological properties. Plants, grains and vegetables in general tend to have high concentrations of phenolic acids but they are mainly found biologically unavailable and in an insoluble-bound structure. In maize, for example, most of the phenolic acids are insoluble-bound hydroxycinnamic acids. Through the effect of different processing technologies (like nixtamalization, extrusion/cooking and steaming/autoclaving) many of these insoluble-bound phenolics are converted to their bioavailable, soluble state [70].

Phenolic acids are metabolized and move through the organism in their sulphated, methylated and glucuronated forms and thus they can show either higher or lower bioactivities. Polyphenols in general suffer this process, which is a very important one because it involves not only detoxification, but also because the hydrophilicity is increased and phenolics can be eliminated easily via urine and billis. For example, in plasma from patients who had ingested a specific quantity of coffee, several methylated, sulphated and glucuronated metabolites of phenolic acids were found [16]. Additionally, polyphenols found in plasma are conjugated derivatives bound to albumin. In general, polyphenols are excreted through the biliary pathway into the duodenum where they are exposed to bacterial enzymes, specially β-glucuronidase. After the action of enzymes on them, polyphenols can be absorbed again which can cause a longer presence of these compounds inside the body [16].

In humans, the β-glucosidases and esterases do not degrade dietary fiber that is water-insoluble and highly cross-linked. To improve bioavailability, it is convenient to convert insoluble cereal phenolics into soluble form; this process increases the health benefits obtained from cereal phenolics consequently [7]. Free phenolic acids are rapidly absorbed by the small intestine and later conjugated [16]. Conjugated phenolics have more potent antioxidant activity than free phenolic compounds [41].

5. **Release of soluble conjugated phenolics during fermentation and role of microbiota**

Gastro-intestinal microbiota is present all along from the mouth to the anus and it is composed by bacteria, fungi, viruses and archaea; bacteria being the majority group overwhelmingly. Microbiota is an ecosystem which is partially stable and bacteria here can
resist important changes that happen in their dynamic environment [34]. These changes in the composition of microbiota in humans can be understood in terms of diet, the influence of the immune system, chemical exposures or founder effects of initial colonizers [71].

Commonly, phenolics which are present in food are in the form of glycosides, esters or polymers that can’t pass through enterocytes and thus, they have to be hydrolyzed by enzymes in the colon or they also can be catabolized by microbiota before their absorption [72]. For example, it has been shown that existence of a highly complex xylan-degrading system inside the large intestinal microbiota that can recognize different types of complex carbohydrates and can respond accordingly [73]. Polymerized polyphenols like ellagitannins and proanthocyanins (commonly present in walnuts and pistachios) can be digested by microbiota present in the colon and subsequently, providing a large range of low molecular weight phenolic metabolites (like alkylphenols, tyrosols and phenolic acids) which seem to alter the microbial ecosystem, including its profile, which causes prebiotic effects too [74]. All these processes carried out by gastro-intestinal microbiota also leads to the biosynthesis of short-chain fatty acids and the release of bound phenolics (or non-extractable-polyphenols) [75].

The chemical structures of phenolic compounds have influence over the conjugation reactions and also over the quantity of metabolites formed by the microflora in the colon. An example of this is chlorogenic acid, because the bound ester can modify its biological properties; the only local reaction in the human body where chlorogenic metabolism is involved takes place in the colon and is performed by bacteria because there are no esterases in the human body which can release caffeic acid from chlorogenic acid. In the same way, ferulic and other hydroxycinnamic acids bound to cell walls can’t be released by human enzymes; to release these compounds, microbial enzymes from the colonic microflora are needed, such as esterases and xylanases. But when these molecules are hydrolyzed by microbial enzymes, the efficiency absorption decreases because they can degrade aglycones and simple aromatic acids are released. Because of this, the absorption efficiency of phenolic acids is notably reduced when they are in their esterified form rather than in their free forms [16].

Bioavailability of anthocyanins is generally low but through gut fermentation, microbiota can increase this bioavailability as well their antioxidant and antidiabetic properties in high-anthocyanin content fruits like blackberries [76]. Gut metabolites of blackberry anthocyanins improve the glucose consumption and glycogen content significantly in HepG2 cells and show important antioxidant properties [77]. In gluten-free flours, microbiota also plays a key role in intestinal fermentation of polyphenols, like anthocyanins and isoflavones, which are biotransformed into lower-molecular weight phenolics (such as protocatechuic aldehyde and 4-hydroxybenzoic acid, while isoflavones can be transformed into equol- or O-desmethylandangolensin derivatives) [78].

CONCLUSIONS
SSF processes using lignocellulolytic fungi not only has proved to be more sustainable than traditional fermentations, but it also yields higher amounts of valuables compounds as well as higher bioactivities of these substances. Additionally, there is a lot of potential to identify new metabolites besides those that the fungi release from the substrate but new compounds
with enhanced bioactivity in addition to the polysaccharide characterization as prebiotic substrate to treat or prevent diseases.

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