PRODUCTION OF BIOACTIVE PHENOLIC COMPOUNDS FROM FRUIT RESIDUES BY SOLID-STATE FERMENTATION AND CARBOHYDRASE TREATMENT

PH.D. DISSERTATION

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LIST OF ABBREVIATIONS

anti-QS: anti-quorum sensing
ATCC: American Type Culture Collection, USA
CFU: colony forming unit
DA: oven-dried apple
DBG: oven-dried black grape
DM: dry matter
DMG: oven-dried mango
DN: oven-dried naranjilla
DP: oven-dried pitahaya
DPPH: 1,1-diphenyl-2-picrylhydrazyl
DWG: oven-dried white grape
FPase: filter paper degrading activity
FRAP: ferric reducing antioxidant power
GAE: gallic acid equivalent
HPLC: high-performance liquid chromatography
LA: lyophilized apple
LB: lysogeny broth
LBG: lyophilized black grape
LM: lyophilized mango
LN: lyophilized naranjilla
LP: lyophilized pitahaya
LWG: lyophilized white grape
MBC: minimum bactericidal concentration
ME: malt extract medium
MIC: minimum inhibitory concentration
NRRL: Agricultural Research Service Culture Collection, USA
QS: quorum sensing
ROS: reactive oxygen species
SmF: submerged fermentation
SSF: solid-state fermentation
SZMC: Szeged Microbiological Collection, Szeged, Hungary
TGE: tryptone glucose yeast extract
TPC: total phenolic content
TPTZ: 2,4,6-Tri(2-pyridyl)-s-triazine
TSB: tryptic soy broth

1. INTRODUCTION

Plants contain various phenolic compounds, e.g. hydroxybenzoates, hydroxycinnamates, flavonoids, stilbenes *etc*., that are involved in defense mechanisms, as well as in pigmentation and adaptation processes. Application of phenolics as additives in food, cosmetic and nutraceutical products has increased which is attributed to their bioactive properties beneficial to the human health. In addition, certain molecules can be used as natural food preservatives or sanitizers as well.

The agro- and food industrial waste of antioxidative plants is a good source of bioactive phenolics. To obtain the compounds, many approaches use different physical and chemical methods such as microwave/ultrasound assisted extraction and solvent treatment. However, most phenolics in plants are bounded in glycoside complexes that results in reduced bioavailability. The carbohydrases, e.g. cellulases and pectinases, can hydrolyze these glycosides releasing the phenolic aglycone molecule. Consequently, fermentation of the substrate using cellulolytic microorganisms, or a carbohydrase-aided extraction may facilitate the mobilization of the phenolic antioxidant.

Many filamentous fungi are used in the industry as valuable producers of carbohydrases. In this context, the enzyme production of zygomycetes fungi has also been continuously screened. In previous experiments performed by our group, the *Rhizomucor miehei* NRRL 5282 isolate proved to be an excellent cellulolytic fungus with high enzyme activity.

The main objective of our work was to enrich bioactive phenolic compounds from selected antioxidative fruit residues via two approaches: i) *in vivo* solid-state fermentation with the cellulolytic fungus *R. miehei* NRRL 5282 and ii) *in vitro* enzyme treatment of the substrate with *R. miehei* derived cellulase cocktail. The residues were planned to be subjected to oven-drying or lyophilization before treatments, and we wanted to supplement the enzyme-aided reactions with pectinases as well. Our goals included the analysis and comparison of the total and individual phenolic contents and the antioxidant activity before and after treatments. Evaluation of additional bioactive properties, i.e. antimicrobial, anti-quorum sensing and antibiofilm activities of extracts produced was also planned using food related bacteria and other model organisms.

2. LITERATURE REVIEW

2.1. Antioxidants

Antioxidants are substances that inhibit the oxidative processes. The antioxidant compounds could inhibit the formation of hydrogen peroxide, scavenge free radicals and reduce the activity of enzymes (e.g., lipoxygenase, NADH oxidase, cyclooxygenase) responsible for the development of reactive oxygen species (ROS) during aerobic cell metabolism. Source of antioxidants can be endogenous, i.e. produced by the organism, or exogenous, i.e. ingested through the intake (utilization of substances that contain them), natural (e.g., enzymes, vitamins, polyphenols, proteins) or synthetic (e.g., phenolic compounds such as butylated hydroxyanisole and butylated hydroxytoluene) (Anwar *et al.* 2018). The natural antioxidants are mainly phenolics that can be derived from various plant sources. Safety concerns towards the use of synthetic antioxidants led researchers to explore natural sources of antioxidants with economic viability, high antioxidant potential and improved bioavailability (Shahidi and Ambigaipalan 2015).

2.2. Plant phenolic compounds

2.2.1. General features

Phenolic phytochemicals are secondary metabolites participating in defense mechanisms in plants. They also have significant roles in other processes, such as pigmentation, adaptation to adverse environmental conditions and incorporation of attractive substances to accelerate pollination. Many fruits, vegetables and plant-based foods and beverages contain phenolic phytochemicals in high amount (Manach *et al.* 2004). The phenolic compounds can be classified into different groups depending on the number of the phenol rings present and the structural elements that link these rings together (Pandey and Rizvi 2009); there are both simple molecules and highly polymerized compounds (Figure 1).
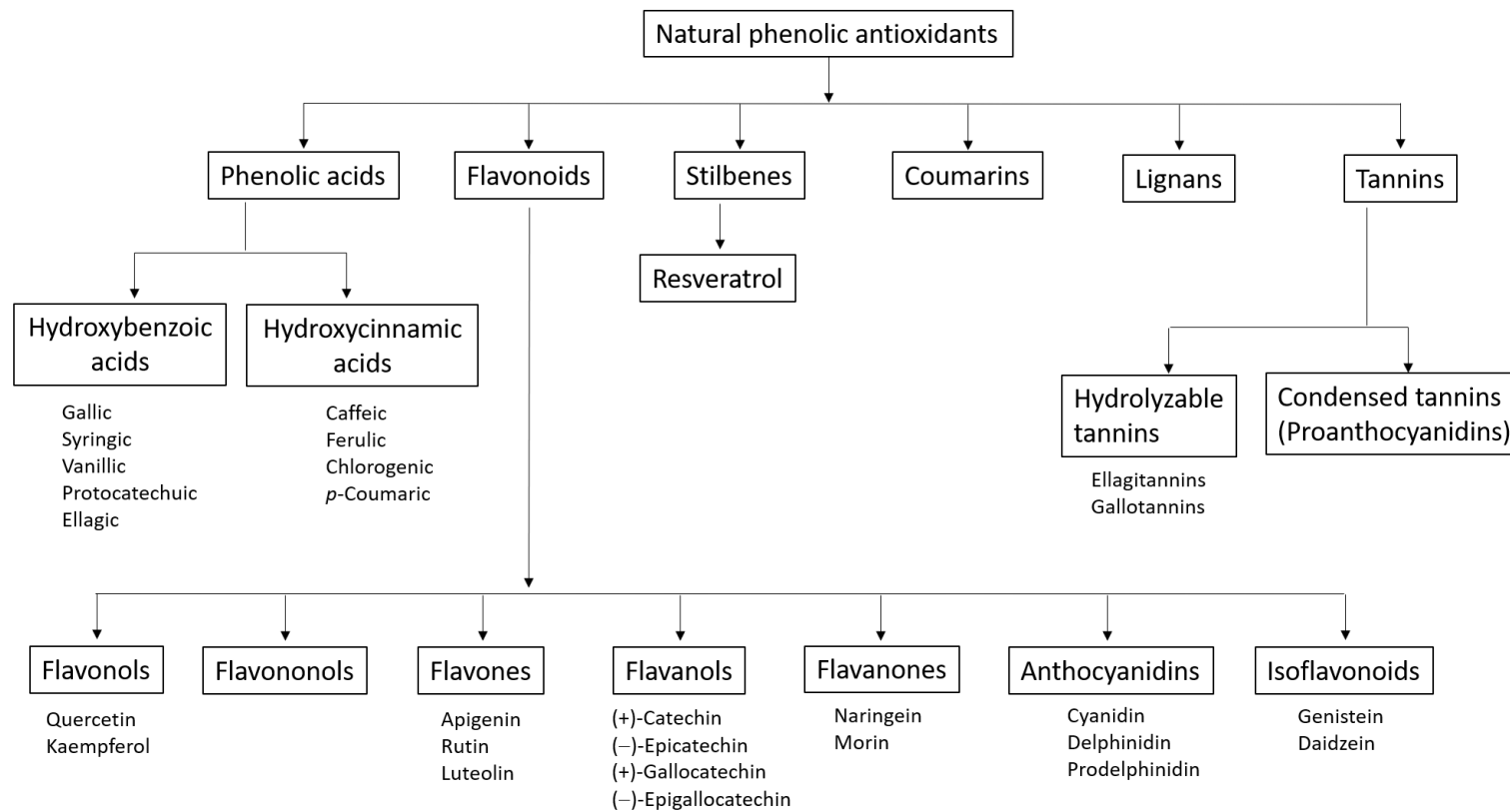
Figure 1. Classification of natural phenolic antioxidants (modified from Shahidi and Ambigaipalan 2015).

Polyphenols bear one or more hydroxyl substituents. Their number and position are responsible for the antioxidant properties of the phenolics (Balasundram *et al.* 2006). In plants, about one third of the phenolics is glycosylated by one or more sugars (Acosta-Estrada *et al.* 2014), mainly by glucose (Figure 2). Less frequent is their conjugated form containing carboxylic acid, organic acid, amine and lipid residues. The bioavailability of these bound phenolic compounds is limited due to their reduced absorption in the small intestine. Numerous studies deal with the antioxidant, anti-inflammatory, antibacterial, platelet antiaggregant, antiviral, antifungal, anti-mutagenic and free radical scavenging activities of phenolic substances (Pandey and Rizvi 2009, Bahadoran *et al.* 2013, Panche *et al.* 2016, Papuc *et al.* 2017). In addition, these molecules can modulate some important cellular signaling pathways, regulate cell cycle processes and inhibit certain digestive enzymes such as lipase, α-amylase and α-glucosidase (Han *et al.* 2007).
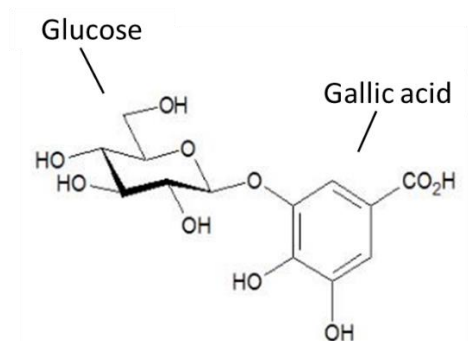
Figure 2. Structure of gallic acid 3-O-β-D glucopyranoside.

2.2.2. Main groups of plant phenolics

<u>Phenolic acids</u>

Phenolic acids are found in most foods of vegetable origin, generally in conjugated form. The phenolic acids are divided into two forms according to the acid component (Manach *et al.* 2004): (1) hydroxybenzoic acids, as derivatives of benzoic acid, can be found in red fruits, roots and bulbs (e.g., radish, onion) in high amount; (2) hydroxycinnamic acids, as derivatives of cinnamic acid, are the most abundant in foods. The most common hydroxybenzoic acids in plants are the gallic acid, *p*-hydroxybenzoic acid, vanillic acid and protocatechuic acid. Hydroxycinnamic acids are mostly found in bounded forms as glycosylated derivatives or esters of quinic acid, shikimic acid and

tartaric acid. The most common hydroxycinnamic acids in plants are *p*-coumaric, caffeic, ferulic and sinapic acids. These can be found in high concentration in several fruits such as kiwis, apples, cherries and blueberries. Caffeic acid is abundant mostly in fruits concentrated mainly in the peel. Its concentration increases with increasing maturity and represents about 75% of the total hydroxycinnamic acid content. Ferulic acid is the major phenolic acid in cereals and grains. It is concentrated mainly in the crust of the grain and represents about 90% of the total of polyphenol mass (Manach *et al.* 2004). As antioxidants, numbers of phenolic acids are outstanding in reducing power and free radical scavenging activities (Soobrattee *et al.* 2005, Pereira *et al.* 2009). Many caffeic acid derivatives can inhibit various enzyme activities such as lipoxygenase and protein kinase (Kang *et al.* 2008, Doiron *et al.* 2017).

<u>Flavonoids</u>

These compounds are widely distributed in plants. Their structure, composition and biological activities are reviewed by Chae *et al.* (2013) and Panche *et al.* (2016). Flavonoids generally represent two thirds of the consumed polyphenols (Scalbert and Williamson 2000). Most of them are of low molecular weight and soluble according to their polarity and degree of glycosylation, hydroxylation and acylation. More than 8,000 flavonoids have been identified that can be divided into several subclasses (Table 1). All share a common structure consisting of two aromatic rings that are joined by three carbon atoms that form an oxygenated heterocycle with various levels of hydroxylation and methoxylation (Bravo 1998). Antioxidant activity of flavonoids is based on its potential as reducing agents, hydrogen donors, oxygen radical and oxidative enzyme inhibitors, superoxide radical scavengers and metal chelators. In addition, these compounds are capable of activating antioxidant enzymes as well (Shahidi and Ambigaipalan 2015). Flavonols, e.g. quercetin, myricetin, kaempferol are ubiquitous flavonoids in foods. In nature, they are present in glycosylated and aglycone forms. Among foods and beverages, onions, broccoli, kale, blueberries, red wine and tea contain high amount of flavonols. Flavones are present as luteolin glycosides and apigenin in products such as parsley and celery, as well as in the skin of citrus fruits. Flavanones are found in tomatoes, aromatic plants such as mint, and in citrus fruits where their high concentration is responsible for the bitter taste. Isoflavonoids are found in legume plants, e.g. soybean, in high amount. Numerous forms, e.g. daidzein and genistein, are intensively studied as phytoestrogens (Bacciottini *et al.* 2007). Flavanols (catechins) are found mostly in nuts, red wine, green tea and chocolate (Shahidi and Ambigaipalan 2015). The (+)-catechin and (−)-epicatechin

are the main flavanols in fruits, while (+)-gallocatechin, (–)-epigallocatechin and (–)-epigallocatechin-3-gallate are found in legumes and tea (Kozłowska and Szostak-Wegierek 2014). Anthocyanins are glycosides of anthocyanidin aglycones. These are water soluble pigments that give red, purple or blue color to leaves, flowers and fruits. Main sources are berry fruits and vegetables such as aubergines, cabbages, beans and onions. Cyanidin is the most common anthocyanidin in foods (Clifford and Scalbert 2000).

Table 1. Types of flavonoids and their dietary sources (Data from Naczk and Shahidi 2004 and Kamboh *et al.* 2015).

Subclass	Commonly occurring compound	Dietary source
Flavones	Chrysin, Apigenin, Sibelin, Rutin, Luteolin	Peel of various fruits, parsley, celery, grape, apple
Flavanones	Naringin, Naringenin, Taxifolin, Eriodictyol, Hesperidin, Isosakuranetin	Citrus, grapefruit, lemon, orange, tomatoes
Flavonols	Kaempferol, Quercetin, Myricetin, Isorhamnetin	Leek, broccoli, endive, grapefruit, black tea, onion, lettuce, tomato, tea, berries, apples, olive oil, cranberry, buckwheat, citrus, red pepper
Isoflavonoids	Genistein, Daidzein, Biochanin A, Formononentin	Soybean and soy foods, fruits
Flavanols	Catechin, Gallocatechin, Epicatechin, Epigallocatechin, Epicatechin gallate, Epigallocatechin gallate	Tea, red grapes
Anthocyanins	Epigenidin, Cyanidin, Delphinidin, Pelargonidin, Malvidin	Stored fruits, cherry, raspberry, strawberry, grapes, dark fruits

Stilbenes

More than 400 natural stilbenes are known. These compounds are non-flavonoid polyphenols that are characterized structurally by the presence of a 1,2-diphenylethylene nucleus. Concentration of stilbenes in plants is depended on the presence of the stilbene synthase enzyme catalyzing the biosynthesis (Reinisalo *et al.* 2015). Main sources of stilbenes are grapes, wine, peanut and sorghum, but in low concentration, they can be found in pistachio, berries, spinach and cabbage. The most studied representatives are the trans-resveratrol and its glycosidic forms that have gained global attention due to their ability to prevent cardiovascular diseases and cancer (Jaganath and Crozier 2010,

Ravagnan *et al.* 2013). The study carried out by Gülçin (2010) showed that resveratrol is an effective antioxidant with reducing power, free radical scavenging capacity and hydrogen peroxide scavenging and metal chelating activities. It can also be used to minimize or prevent the oxidation of lipids in pharmaceutical products, maintaining its nutritional quality and prolonging its shelf life (Salehi *et al.* 2018).

Tannins

Tannins are highly hydroxylated molecules and can form insoluble complexes with carbohydrates and proteins. According to their chemical structure, they are divided into two groups: (1) hydrolysable tannins that are esterified derivatives of gallic acid and hexahydroxydiphenic acid; (2) condensed tannins known as proanthocyanidins, which are polymers of high molecular weight catechins formed by the oxidative condensation between the phenolic ring and the adjacent units or by enzymatic polymerization of flavan-3-ol and flavan-3,4-diol units (He *et al.* 2008). Both the condensed and hydrolysable tannins have high bioactive potential (Bele *et al.* 2010). The antimicrobial activity of hydrolysable tannins is summarized recently by Ekambaram *et al.* (2016). However, condensed tannins can inhibit the growth of many microorganisms as well (Abu Zarin *et al.* 2016, Tomiyama *et al.* 2016, Assefa *et al.* 2017).

2.2.3. Health promoting effect of phenolics

Effect on cardiovascular diseases

Polyphenols have a widely demonstrated vasodilator, anti-atherosclerotic, anti-thrombotic, anti-lipemic, anti-inflammatory and anti-apoptotic action (Quiñones *et al.* 2012). Compounds such as quercetin, epigallocatechin gallate, resveratrol, flavonols, flavanols, procyanidins and anthocyanins present in tea, wine and cocoa can be used in the prevention and/or treatment of cardiovascular diseases and pathologies associated with oxidative stress. These molecules inhibit the formation of ROS thanks to their antioxidant effect and capacity to chelate heavy metals as iron or copper (Orallo *et al.* 2002).

Anti-diabetes effect

Diabetes is a public health problem that decreases the quality of life and increases the mortality rate. Studies have shown that the consumption of foods with a high content of polyphenols such as catechins, epicatechins, chlorogenic acids, ferulic acids, caffeic and tannic acids, quercetin and naringenin slows the development of type 2 diabetes (Bahadoran *et al.* 2013). They reduce the intestinal absorption of carbohydrates (reduces

the postprandial glycemic level) (Johnston *et al.* 2005), regulate enzyme activity such as α-glucosidase and lipase, reduce inflammation, improve pancreatic function (Iwai 2008), and act synergistically with hypoglycemic medicine (Prabhakar *et al.* 2013). Therefore, they can be used as supplementary treatments for the control and management of the complications associated with this disease.

Anti-cancer effect

The main causes of cancer can be DNA damage caused by aging, exposure to chemicals, radiation and ultraviolet rays. The anticancer effect of flavonoids can be attributed to several activities such as antioxidative effect, inactivation of ROS, activation of protective enzymes such as glutathione S-transferase and glucuronosyl transferases, increase of apoptosis rate, and inhibition of cell proliferation, lipid peroxidation, angiogenesis and DNA oxidation (Chahar *et al.* 2011). Flavonoids such as genistein, quercetin, tangeretin, luteolin, daidzein, kaempferol, apigenin, resveratrol and epigallocatechin act in all three stages of cancer: initiation, promotion and progression (Chahar *et al.* 2011). Regular intake of phenolic compounds, such as green tea polyphenols, milk thistle silymarin and grape proanthocyanidins have been shown to protect the skin from the adverse effects of ultraviolet radiation, one of the causes of skin cancer (Afaq and Katiyar 2011).

Anti-aging effect

Aging is a natural process, a consequence of the accumulation of a great variety of molecular and cellular damages over time. At the same time, it decreases the effectiveness of the antioxidant and repair mechanisms, which produces a progressive decrease of motor and cognitive abilities, increases the risk of diseases and finally death (Harman 2006). Alzheimer's disease is a serious cognitive degenerative disease associated with age. In the study of Porquet *et al.* (2013), dietary resveratrol could prevent many Alzheimer's markers in mice model. For instance, it reduced cognitive impairment, decreased the amyloid burden and reduced tau hyperphosphorylation. Moreover, it can activate pro-survival routes as well. In another investigation, the epigallocatechin gallate was able to reduce the age-associated inflammation and oxidative stress in rat models (Niu *et al.* 2013).

2.2.4. Antimicrobial activity of phenolics

Phenolic compounds can suppress several microbial virulence factors. For instance, they can inhibit biofilm formation, reduce host ligand adhesion and neutralize bacterial

toxins (Li *et al.* 2014). Moreover, these substances can show synergy with antibiotics enhancing their effectiveness and reducing the dose of use. Besides antioxidant activity, the phenolic hydroxyl group plays important role in the antimicrobial activity as well. The hydroxyl group of phenolic compounds can destabilize the cell membrane structures and cause the leakage of cellular components (Xue *et al.* 2013). In addition, these groups can reduce the pH gradient across the cytoplasmic membrane (Ultee *et al.* 2002) and can form hydrogen bonds with active sites of enzymes (Farag *et al.* 1989).

Due to the above properties, the phenolic compounds can be good candidates for the development of efficient antimicrobials (Ayala-Zavala *et al.* 2011, Daglia 2012). In this regard, phenolic acids, flavonoids and tannins have gained attention over the last decades (Gyawali and Ibrahim 2014, Shahidi and Ambigaipalan 2015). Several laboratory pilot studies investigated the antimicrobial activity of phenolics. Some interesting recent assays are listed in Table 2.

Table 2. Examples about the antimicrobial activity of individual phenolic compounds.

Compounds	Target organism	Antimicrobial activity	Reference
Coumarin, quercetin	*Escherichia coli*, *Enterobacter aerogenes*, *Salmonella infantis*, *Salmonella* Typhimurium	Coumarin: MIC*, 0.625-5 mg/mL; MBC**, $\geq$ 5 mg/mL. Quercetin: no effect.	Nitiema *et al.* (2012)
Gallic acid, catechin	*Helicobacter pylori*	Inhibition haloes of 14 and 16 mm in the presence of 1 mg gallic acid and catechin, respectively.	Díaz-Gomez *et al.* (2013)
Ellagic acid, quercetin-3-galactoside, chlorogenic acid, quercetin	*Listeria monocytogenes*, *Salmonella* Enteritidis	Effective concentrations: chlorogenic acid, 500 µg/mL; quercetin and quercetin-3-galactoside, 200 µg/mL; ellagic acid, 44 µg/mL.	Shen *et al.* (2014)
Phloridzin, phloretin	*Staphylococcus aureus*, *E. coli*	MIC: *S. aureus* 0.50 and 0.10 mg/ml, *E. coli* 1.50 and 0.75 mg/ml.	Zhang *et al.* (2016)
25 phenolic compounds	*Streptococcus pyogenes*	1,2-Naphthoquinone and 5-hydroxy-1,4-naphthoquinone MIC: 0.39-6.25 mg/ml; MBC: 100 mg/ml.	Macé *et al.* (2017)

* MIC, minimum inhibitory concentration; ** MBC, minimum bactericidal concentration

In addition, García-Ruiz *et al.* (2011) analyzed the antimicrobial activity of 18 phenolic compounds, i.e. hydroxycinnamic acids, hydroxybenzoic acids, stilbenes, flavan-3-ols, flavonols and phenolic alcohols, against lactic acid bacteria isolated from wine.

Flavonols and stilbenes showed the strongest inhibitory effect, while the activity was negligible in case of the flavan-3-ols. In another study, the effect of 15 kinds of phenolic acids, stilbene and flavonoid compounds from grapes were investigated against the growth of spoilage yeasts and acetic acid bacteria. Pterostilbene, resveratrol and luteolin presented high inhibitory effect against all microorganisms tested. Myricetin, *p*-coumaric acid and ferulic acid showed selective antimicrobial activity depending on the species of yeasts and bacteria tested (Pastorkova *et al.* 2013). In the study of Tenore *et al.* (2012), the flavonoid and phenolic acid fractions of red pitahaya exhibited good inhibitory effect on the growth of various Gram-positive and Gram-negative bacteria, yeasts and molds.

2.2.5. Plant phenolics as anti-quorum sensing and anti-biofilm agents

Many bacteria can form biofilm, that is, communities enclosed in self-synthesized extracellular polymer matrices, attached to biotic or abiotic surfaces. One of the main communication processes regulating biofilm formation and development is the quorum sensing (QS) mechanism based on autoinducer signal molecules. Once colonization has begun, several cell divisions lead to the maturation of the biofilm and expression of certain biofilm related genes. Within the biofilm, the cells are metabolically less active than the planktonic form; therefore, they are significantly less sensitive to the action of antimicrobial agents, which is the main cause of chronic infections (Landini *et al.* 2010). Biofilms are a potential risk in the food industry, including brewing (Flemming and Ridgway 2009), seafood (Shikongo-Nambabi *et al.* 2011) and dairy processing (Chmielewski and Frank 2003). Therefore, elimination of biofilms and inhibition of their formation have great importance in today's preservative agent developments.

Certain plant phenolic substances can exhibit anti-biofilm and/or anti-quorum sensing (anti-QS) activities (Silva *et al.* 2016, Slobodníková *et al.* 2016, Asfour 2018). Therefore, an effective phenolics-based QS inhibitor could inhibit the biofilm formation of pathogenic bacteria as well (Kalia 2013). Many studies have addressed with this topic. For instance, catechin (Vandeputte *et al.* 2010), naringenin (Vandeputte *et al.* 2011) and quercetin (Ouyang *et al.* 2016) depicted strong anti-QS property against *Pseudomonas aeruginosa*. Additionally, kiwifruit pulp with high vanillic acid content showed anti-QS and anti-biofilm activities in *Serratia marcescens* (Sethupathy *et al.* 2017). Naringenin, quercetin, sinensetin, apigenin and grape seed extracts were effective QS antagonists and biofilm suppressors in pathogenic *Escherichia coli* strains (Vikram *et al.* 2010, Sheng *et al.*

2016). Studies were done to inhibit and eradicate the *Staphylococcus aureus* biofilms as well. For instance, the 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose from *Eustigma oblongifolium* could block the synthesis of intercellular adhesion compounds in *S. aureus*, preventing its adherence to solid surfaces (Lin *et al.* 2011). In addition, phenolic acids such as gallic acid (Luís *et al.* 2014), ginkgolic acid (Lee *et al.* 2014) and rosmarinic acid (Slobodníková *et al.* 2013), and several flavonoids (Cho *et al.* 2015) also modulated the *S. aureus* biofilm in subinhibitory concentrations. In a comprehensive study, which dealt with human bacterial pathogens, methanol extract from pomegranate, rich in ellagic acid, inhibited the biofilm formation of *S. aureus*, methicillin resistant *S. aureus* (MRSA) and *E. coli* (Bakkiyaraj *et al.* 2013). The phloretin, a flavonoid present in the apple inhibited the formation of *E. coli* O157:H7 biofilms without affecting the growth of planktonic cells (Lee *et al.* 2011). Regarding fungi, eugenol and ellagic acid proved to be effective inhibitors of *Candida albicans* biofilms (He *et al.* 2007, Bakkiyaraj *et al.* 2013).

Based on the above findings, phenolic compounds can be useful tools against various microorganisms, including many foodborne pathogens and spoilage bacteria. Application of phenolics as alternatives or supplements in antibiotic treatments could also be a cost-effective technology worth taken into consideration.

2.3. Antioxidative fruit substances relevant to this study

2.3.1. Grapes

Grapes are economically important plant species; wine, juice, jam and raisin can be produced from them. More than 77 million tons are produced annually from this fruit (http://www.fao.org/faostat). About 80% of the total amount is utilized for winemaking purposes (Ayala-Zavala *et al.* 2011). Pomace represents approximately 30% of the weight of grapes processed. Its composition varies considerably, depending on grape variety and technology of wine making. Major phenolic compounds in the grape pomace are phenolic acids (e.g., caftaric acid, coutaric acid and trans-fertaric acid), flavonols (e.g., glycosidic forms of quercetin and myricetin) (Souquet *et al.* 2000) and flavanonols (e.g., astilbin and engeletin) (Lu and Yeap Foo 1999). Stilbenes such as trans- and cis-resveratrol, piceids, astringins, resveratrolosides and pterostilbene can also be found in grapes (Naczk and Shahidi 2006). Grape seeds are a rich source of monomeric phenolic compounds such as (+)-catechin, (−)-epicatechin, (+)-gallocatechin, (−)-epigallocatechin, and their dimeric,

trimeric and tetrameric proanthocyanidins (Sun and Spranger 2005). Black grapes are one of the major sources of dietary anthocyanins. Majority of these compounds are accumulated in the berry skin. The phenolic compounds in grape pomace and other grape residue extracts exhibit antioxidant, anticancer, antidiabetic, anti-inflammation, antimicrobial and cardioprotective properties (Xia *et al.* 2010, Teixeira *et al.* 2014, Yadav *et al.* 2015). Majority of the flavonoids are found in the skin, whereas about 60-70% of total polyphenols are stored in grape seeds (Han *et al.* 2007). More than 70% of grape polyphenols remain in the pomace after juice processing (Ratnasooriya and Rupasinghe 2012). The grape seeds may be separated from the pomace and used either for production of grape seed oil or grape seed powder/extract as individual food, additive in cosmetics and pharmaceutical industry as well as biomass for biogas production (Cáceres *et al.* 2012, Dwyer *et al.* 2014).

2.3.2. Apple

The world apple production is estimated to nearly 89 million tons per year (http://www.fao.org/faostat). Antioxidant properties of apple materials highly dependent on the fruit variety, agricultural methods, weather, storage conditions and fruit processing. Apples are a good source of polyphenols, which are predominantly localized in the peels and are extracted into the juice to a minor extent. The major polyphenolic groups present in apple are hydroxycinnamic acids (chlorogenic and *p*-coumaroylquinic acids), cyanidin-3-galactoside, flavan-3-ols/procyanidins (mainly catechin, epicatechin and procyanidins B1 and B2), flavonols (quercetin glycosides), and dihydrochalcones (mainly phloridzin and phloretin-2′-xyloglucoside) (Tsao *et al.* 2005). Apple pomace is a solid waste generated by cider and apple juice making processes. This substance consists of peel, core, seed and stem residues, which constitute of about 25-35% mass of the fresh fruit. Apple pomace can be utilized to produce pectin (Schieber *et al.* 2003), enzymes (Favela-Torres *et al.* 2006), organic acids (Shojaosadati and Babaeipour 2002), protein, feed (Bhalla and Joshi 1994), ethanol (Parmar and Rupasinghe 2013), aroma compounds (Madrera *et al.* 2015) and natural antioxidants (Ajila *et al.* 2011, Zhang *et al.* 2016). In addition, the ethanolic extract from apple peel exhibited antimicrobial activity against *Bacillus cereus* and *E. coli* O157:H7 (Fratianni *et al.* 2011).

2.3.3. Pitahaya

Pitahaya (known as dragon fruit) is a tropical fruit produced mainly in Asian and Central and South American countries (Le Bellec and Vaillant 2011). There are three species of pitahaya: *Hylocereus polyrhizus* (red pitahaya), *Hylocereus undatus* (white pitahaya) and *Hylocereus megalanthus* (yellow pitahaya) (Lim *et al.* 2010). The difference is based on their shape, size and color of their flesh (Nurul and Asmah 2014). Bioactive potential of red and white pitahayas is intensively studied (Kim *et al.* 2011, Tenore *et al.* 2012), while less attention has been paid to the yellow variety in this regard. Pitahaya contains pectin, fiber, simple sugars (e.g., glucose, fructose and sucrose), vitamins and various minerals (e.g., phosphorus, calcium, potassium, magnesium, iron) (Le Bellec *et al.* 2006, Nurul and Asmah 2014). Pitahaya is a rich source of phenolic antioxidants; however, there are differences in the phenolic contents of the different varieties and fruit parts (Kim *et al.* 2011). In addition, red pitahaya contains betacyanins, responsible for the purple color of the peel and flesh (Tenore *et al.* 2012). After extraction, these compounds can be utilized as natural colorants in foods (Vaillant *et al.* 2005). The seed and peel fractions of pitahaya have higher antioxidative capacity compared to the flesh (Choo *et al.* 2016). Pitahaya consumption has many health benefits including hypocholesterolemic, prebiotic, anticancer, anti-inflammatory and antidiabetic effects (Choo *et al.* 2016, Song *et al.* 2016). The phenolic fractions of red and white flesh pitahaya fruit demonstrated antimicrobial activity against different pathogens (Nurmahani *et al.* 2012, Yong *et al.* 2018). However, there are only scarce data about yellow pitahaya (*H. megalanthus*) residue samples in this regard (Choo *et al.* 2016).

2.3.4. Mango

Mango (*Mangifera indica* L.) grows in many tropical countries in the world. From the available varieties, only a few are grown on commercial scale. The global production of mangoes was about 50 million tons in 2017 (http://www.fao.org/faostat). About 35-60% of the total weight of the fruit remained as byproduct after processing (O'Shea *et al.* 2012). Mango peel is an important source of dietary fiber, carotenoids, vitamins C and E, phenolic compounds with good antioxidant activity. The major phenolics in mango are gallic, protocatechuic and syringic acids, kaempferol and quercetin (Ajila and Prasada Rao 2013). The antimicrobial activity of mango seed extracts could be attributed to the presence of

flavonoids, terpenes, tannins and coumarins (Orijajogun *et al.* 2014). In this context, it has been reported that the kernel powder of an African mango variety displayed high antimicrobial activity against *S. aureus*, *P. aeruginosa*, *E. coli*, *Bacillus subtilis* and *C. albicans* (Ahmed *et al.* 2012). Moreover, the study of Gadallah and Abdel Fattah (2011) showed that mango seed extract was more effective against Gram-positive pathogens (*S. aureus* and *B. subtilis*) than Gram-negative bacteria (*E. coli* and *Salmonella sp.*) in minced beef during refrigerated storage. The mango fruit and byproducts displayed high antioxidant activity, radical-scavenging capacity, metal-chelating capacity and tyrosinase inhibitory activity, thus can be used in food, cosmetic, nutraceutical and pharmaceutical applications.

2.3.5. Naranjilla

Naranjilla (*Solanum quitoense*) is the fruit of a subtropical perennial plant that belongs to the Solanaceae family, native in the northwestern Andes. It is used to produce juice, ice creams, candies, jams, jellies, sauces and other cooked confections (Andrade-Cuvi *et al.* 2015). Two geographically separated varieties are recognized: *S.* var. *quitoense*, a spineless form, found in southern Colombia and Ecuador, and *S.* var. *septentrionale*, a form with spines that is found in central Colombia, Panama and Costa Rica (Acosta *et al.* 2009). During ripening, naranjilla turns from green to orange. Sometimes it is consumed and processed at earlier stages of maturity, but the late harvested fruit has sweeter pulp due to the high total soluble solid content. *S. quitoense* contains simple sugars (e.g., glucose, fructose, sucrose), organic acids, dietary fiber (15% dry matter, DM), proteins (7.4% DM), minerals (approx. 52 mg/100 g DM), carotenoids (e.g., lutein, zeaxanthin), vitamins (e.g., niacin, thiamine, riboflavin, vitamin A and C), caffeoylquinic acids and their derivatives, and different flavonol glycosides (e.g., quercetin and kaempferol derivatives) (Gancel *et al.* 2008). The chlorogenic acid and dihydrocaffeoyl spermidine compounds can be found in all parts of the fruit, while the flavonol glycosides are located mainly in the peel. The total phenolic content is superior in the peel than in the flesh. The antioxidant activity of naranjilla is close to those of red grape, peach and pear, but higher than those of kiwi fruit, banana, watermelon, pineapple and cantaloupe (Acosta *et al.* 2009).

2.4. Fruit and vegetable processing byproducts

The food and agricultural product processing industries generate a large volume of plant byproducts causing difficulties in their disposal and treatment (Socaci *et al.* 2017). Some possible utilization pathways for these substances are presented in Figure 3. Food processing residues are generally utilized as animal feed, natural fertilizers or as biomass for bioethanol production (Laufenberg *et al.* 2003, Kruczek *et al.* 2016). Peels, pulps and seeds from fruits and vegetables, however, could be rich in extractable phytochemicals including phenolic compounds, carotenoids and dietary fibers with relevant bioactivity (Moure *et al.* 2001). The content of bioactive compounds of the byproduct is often similar or higher than that of the main product (Kunradi Vieira *et al.* 2009, Ayala-Zavala *et al.* 2011). For instance, Kalogeropoulos *et al.* (2012) studied the bioactive composition of tomato whole raw material and its byproduct, wherein tomato peel and seeds had a higher concentration of carotenoids (lycopene and β-carotene) and phenolic compounds compared to the pulp fraction. Similar results were found in the investigation of brown-colored onion; concentration of flavonols in the outer skin was higher than in the inner parts (Benítez *et al.* 2011).
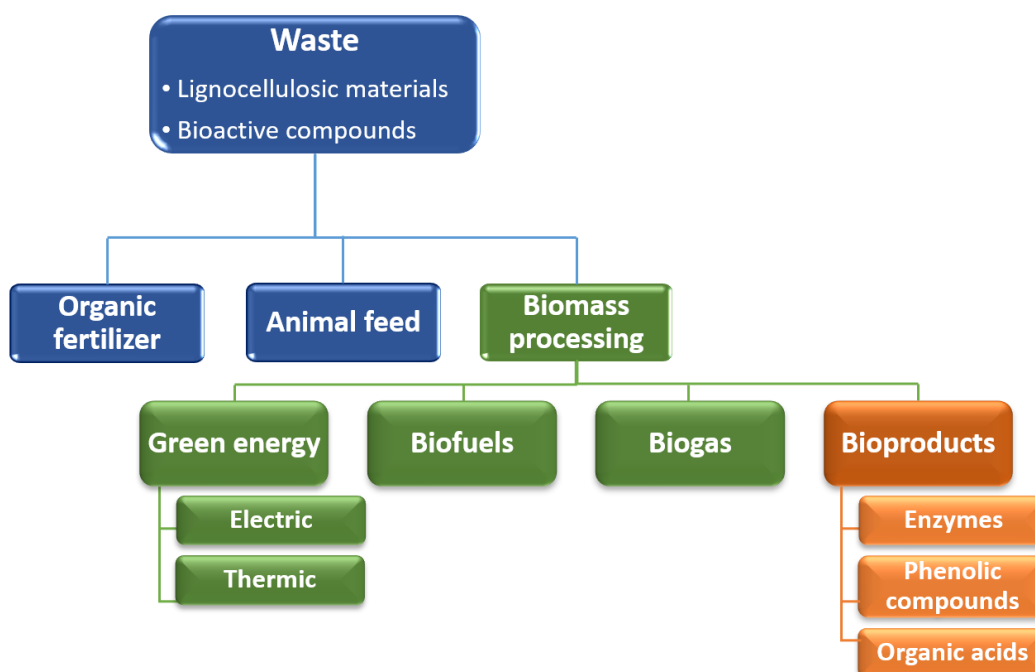
Figure 3. Bioconversion possibilities of agro-industrial residues into value-added products (edited by C. Zambrano).

Increasing interest can now be observed for the utilization of plant residues to produce natural additives and supplements rich in valuable compounds. The recycling and recovery of these byproducts could be economically attractive for industries to obtain health-promoting compounds as well as developing functional ingredients (Galanakis 2012, Kumar *et al.* 2017). Pomace is the solid part of the plant, which contains seeds, skin, pulp of the fruits or vegetables, and it can be defined as a byproduct remains after juice pressing. Approximately 25-35% mass of the raw material generally left after the pressing process (Kruczek *et al.* 2016). Fruit juices are the principal processed fruit products commercialized in the world.

According to the Food and Agriculture Organization of the United Nations (FAO), the cultivation of apples, grapes and mangoes corresponded to 39% of the world's fruit production in 2016 and about 45% of the produced fruits and vegetables are wasted throughout the entire production chain (http://www.fao.org/faostat). Also, pitahaya has growing interest in the exotic fruit market (Le Bellec and Vaillant 2011). In 2016, the waste materials coming from the industrial processing of these fruits were around 25 million tons and most of them were sent to the landfill or used to produce organic fertilizers and animal feed. In past decades, however, the research on the use of the biomass has gained interest for the extraction of biomolecules such as phenolic compounds, organic acids, enzymes, polysaccharides and other industrially important products (De Ancos *et al.* 2015).

2.5. Extraction of phenolics from plant materials

Extraction is the first step for the recovery of phenolic compounds from plant substrates. The yield and purity of the compounds obtained may vary if the phenolics are present in a simple or conjugated form (e.g., compounds linked with pectin, arabinoxylan or mono- or polysaccharide molecules), and other factors of the extraction process such as temperature, time, agitation, presence of inhibitors and sample particle size (Naczk and Shahidi 2004). Conjugated phenolic compounds can be separated by physical (e.g., ultrasound assisted) and chemical methods (e.g., acid, alkaline or enzymatic hydrolysis). The conventional solid-liquid extraction processes such as mechanical agitation and treatment with organic solvents alone or in combination generally achieve different extraction efficiencies (López-Miranda *et al.* 2016). In this context, the long extraction time at high temperatures demanded by certain solvents can induce thermal decomposition

leading to activity loss in the phenolic content (Wang and Weller 2006). Physical methods such as microwave and ultrasound-assisted extractions can be conducted with short conversion time and can improve the phenolic content and antioxidant activity of the extract compared to solvent extraction techniques (Nayak *et al.* 2015). However, these methods require high investment costs and are difficult to carry out on an industrial scale. To extract phenolics, many studies applied organic solvent/alcohol-water mixtures where the water can enlarge the permeability of the cell wall and the extraction of hydrolysable compounds by molecular diffusion (Jayaprakasha *et al.* 2001). For instance, the study of Alonso *et al.* (1991) suggests that the ethanol-water mixture improves the recovery of catechins and procyanidins from grape seeds. In another assay, the water-ethanol and water-acetone combinations resulted in higher phenolic yields from grapes compared to those obtained with acetone or ethanol alone (Kallithraka *et al.* 1995). However, the fermentation and enzyme-assisted extraction methods, alone or in combination with the abovementioned techniques, could provide an alternative way to obtain more phenolic yield with improved antioxidant capacity.

2.6. Solid-state fermentation

2.6.1. Application of solid-state fermentation on the production of biomolecules

Solid-state fermentation (SSF) is a well applicable method for bioactive compound production and enrichment from fruit and vegetable processing byproducts (Lizardi-Jiménez and Hernández-Martínez 2017). The fermentation process could be defined as the cultivation of microorganisms on the surface and inside of a solid substrate in the absence or in the presence of a very low amount of free water. Agro-industrial wastes generally have high sugar content that are easily assimilated by microorganisms; therefore, they are suitable raw materials as fermenting substrates to produce compounds relevant to industry (Rodríguez Couto 2008). Anyway, the SSF is considered as a technological way in the waste management, which is suitable for the ecofriendly utilization of many agro-industrial byproducts.

The selection of residues depends on its availability, composition and cost. Sugarcane and cassava bagasse, oil cakes, orange peel, apple and grape pomace, coffee husk and wheat bran are the most common fermentation materials used (Krishna 2005). There are important factors in terms of the efficient bioconversion, such as substrate

pretreatment, moisture level, water activity, particle size, growth medium supplementation, temperature, pH, agitation and aeration (Singh nee' Nigam and Pandey 2009). The advantages of SSF over submerged fermentation (SmF) are a consequence of the different physiology shown by fungi and other microorganisms on a solid substrate. In SSF, for instance, final yields of various extracellular enzymes of filamentous fungi are significantly higher than in SmF (Barrios-González 2012). The production yield of a heterologous chymosin by *Aspergillus oryzae* was 500-fold higher in wheat bran based SSF systems than in SmF conditions (Tsuchiya *et al.* 1994). In a comparative study, the economic yield of cellulase production in SSF was higher compared to SmF (Tengerdy 1998). Several studies reported that antibiotics, organic acids, polyunsaturated fatty acids, biopesticides and biofuels can be produced under SSF conditions in high yield. From biochemical engineering point of view, the temperature, oxygen limitation and moisture content are the main factors during the fermentation (Bhargav *et al.* 2008).

2.6.2. Microorganisms applied in solid-state fermentation

Microorganisms that grow naturally on solid substrates, e.g. pieces of wood, stems, roots, *etc*., are the most suitable for the bioconversion of agro-industrial wastes. Filamentous fungi are important microorganisms in the SSF processes owing to their physiological, enzymological and biochemical properties. The hyphal growth of filamentous fungi gives them a great advantage to penetrate and use the nutrients of solid substrates. The contact of hyphae with the substrate surface is enabled and the mycelium synthesizes hydrolytic exoenzymes in high yield (Raimbault 1998). Table 3 shows some examples of fungi used in SSF with the appropriate solid substrates to produce bioactive compounds and enzymes.

Table 3. Some SSF studies applying fungi as fermenting microorganism for bioactive compound production.

Filamentous fungi	Substrate	Product	References
Aspergillus niger	Cassava bagasse	Citric acid	Vandenberghe *et al.* (2000)
Aspergillus niger	Wheat bran, soybean meal, cottonseed meal, orange peel	Protease	de Castro *et al.* (2015)
Aspergillus niger	Sugarcane bagasse	Citric acid	Yadegary *et al.* (2013)
Aspergillus fumigatus	Wheat bran, rice straw	Cellulase	Das *et al.* (2013)
Aspergillus oryzae	Wheat bran+coconut oil cake	Neutral metalloprotease	Sumantha *et al.* (2006)
Fusarium oxysporium	Groundnut cake, soybean cake, and cottonseed cake	Protease	Apastambh and Baig (2017)
Monascus purpureus	Jack fruit seed	Pigment	Babitha *et al.* (2007)
Mortierella alpina	Rice bran	Polyunsaturated fatty acid	Jang and Yang (2008)
Mucor corticolus, *Rhizomucor miehei*, *Gilbertella persicaria*, *Rhizopus niveus*	Corn residues, wheat bran	Cellulase, lipase, protease	Takó *et al.* (2015)
Penicillium simplicissimum	Soybean cake	Lipase	Di Luccio *et al.* (2004)
Rhizopus arrhizus	Corn flour, wheat bran, rice bran, wheat flour, sunflower meal, sunflower cake, oat bran	Lipase	Dobrev *et al.* (2018)
Rhizopus oryzae	Coconut oil cake + sesame oil cake (1:1, w/w)	Phytase	Ramachandran *et al.* (2005)
Trichoderma reesei	Wheat bran	Cellulase	Singhania *et al.* (2007)
Trichoderma harzianum	Colloidal chitin	Chitobiase	Binod *et al.* (2007)

2.7. *Rhizomucor miehei*

The genus *Rhizomucor* has two well characterized species, *Rhizomucor miehei* and *Rhizomucor pusillus*. The saprotrophic *R. miehei* is a thermophilic homothallic fungus that produces numerous colored sporangiospores, therefore, its colony has dark gray color. The optimum growth temperature of *R. miehei* is between 35 and 45 °C, and the maximum is about 58 °C. It is an excellent producer of industrial enzymes, especially aspartic protease, lipase, β-1,3-glucanase, β-glucosidase and xylanase (Maheshwari *et al.* 2000, Ferreira *et al.* 2013). The aspartic proteases have been widely used as substitute for calf chymosin in the cheese production (Rao *et al.* 1998). Lipases of *R. miehei* are commercialized in soluble and immobilized forms; those have a very high activity and good stability (Rodrigues and Fernandez-Lafuente 2010, Takó *et al.* 2017). Other extracellular enzymes have also been

characterized from *R. miehei* including fibrinolytic enzymes and xylanases (Ali and Ibrahim 2008, Fawzi 2010).

2.8. Application of enzymes to produce plant bioactives

Enzymes are frequently used in industrial processes. These are mainly hydrolytic enzymes that are utilized in the textile-, pulp- and paper-, and food- and feed industries in large quantities (Li *et al.* 2012). In the last decades, the use of enzymes has gained relevance in the ecofriendly enrichment of polyphenols from plant cell-wall structures (Soto-Maldonado and Zúñiga-Hansen 2018). Namely, the carbohydrate cleaving enzymes can hydrolyze the phenolic glycosides releasing the aglycone phenols, thus, improving their bioavailability (Madeira Jr *et al.* 2015). Enzymes such as pectinase, cellulase, hemicellulase and glucanase are used in the extraction of complex polyphenols. Other enzymes can also be associated with the liberation of phenolic aglycone moieties. For instance, tannin acyl hydrolase (known as tannase) catalyzes the hydrolysis of the galloyl ester bond of hydrolysable tannins (mainly tannic acid). Tannins are present in a variety of plants processed in food industry (Sagar *et al.* 2018); therefore, their amount in byproducts is considerable. The released gallic acid then can be utilized as intermediate compounds in various organic synthesis processes and food industrial applications (Zhang *et al.* 2015).

Enzymatic extraction of phenolic compounds from food wastes has many advantages over the physical and solvent based methods, such as the high efficiency, large scale application and eco-friendly reaction conditions (Shahidi and Yeo 2016). Furthermore, as a non-denaturing condition, the enzyme treatment can preserve the antioxidant activity of the extracted phenolic compounds (Laroze *et al.* 2010). In addition, low-cost enzyme preparations with high carbohydrase activity can be obtained via fermentation of various agro-industrial wastes (Behera and Ray 2016), then, the produced cocktails can be used for efficient phenolic extraction. In most studies, however, commercially available enzyme preparations are applied as extraction biocatalysts (Soto-Maldonado and Zúñiga-Hansen 2018).

The operating condition of the enzyme treatment affects the release of phenolic compounds. The most important factors are composition, particle size and porosity of the raw material; type of enzyme and its activity (Hong *et al.* 2013); the method used for quantifying antioxidant capacity (Laroze *et al.* 2010); enzyme/substrate ratio and the reaction time; temperature of the treatment and the solid-liquid ratio in the reaction

environment (Azmir *et al.* 2013). For instance, reaction temperatures between 45 and 55 °C proved to be optimal to achieve the highest phenolic yield during the enzyme assisted extraction of pomegranate husk (Mushtaq *et al.* 2015). High temperatures could cause a decrease in phenolic yield because of the enzyme inactivation and phenolic degradation. Chandini *et al.* (2011) studied the recovery of antioxidants from tea extract by applying pectinase or tannase at different concentrations. Concerning pectinase, only the enzyme concentration of 20 U/g black tea caused significant increase in the free phenolic content. However, the higher the tannase concentration, the higher the phenolic yield was in the extract. In the study of Laroze *et al.* (2010), the highest Grindamyl CA 150 (pectinase) concentration used (10% enzyme/substrate ratio) caused a significant increase in the free phenolic yield and antioxidant activity of raspberry residues. Type of carbohydrases, enzyme-substrate ratio and temperature were the most relevant variables in the enzymatic extraction of polyphenols from grape residues (Kammerer *et al.* 2005).

2.9. Importance of pretreatment of fruit byproducts

Prior to fermentation and enzyme treatment, the byproduct residues are subjected to drying and subsequent grinding in most studies. Frequently used pretreatment methods are the heat treatment (vaporization), drying and grinding. First step could be wet grinding of the byproduct to decrease the particle size of wet residue for further actions (Galanakis 2012). To avoid the degradation of phytochemical compounds during storage, stabilization of the byproducts is needed. The reduction of free water in the substrate is essential to inactivate the enzymes responsible for the degradation of biologically active compounds and to decrease the microbial growth rate. Enzymatic browning could occur by the result of enzymatic oxidation of phenolic compounds in the presence of oxygen (Pirovani *et al.* 2015). For example, apple pomace without drying process could quickly suffer from brown discoloration thanks to polyphenol oxidase activities, and microbial spoilage (Kammerer *et al.* 2014). Due to the moisture content of the pomace, the risk that unwanted microorganisms populate fruit and vegetable pomace is high. However, if the pomace is dried immediately after harvesting, microbial contamination can be decreased, thereby, the shelf life of the pomace is expanded (Kruczek *et al.* 2016). Hot air drying (oven-drying) and freeze-drying (lyophilization) are frequently used drying procedures (Vashisth *et al.* 2011).

Heating has impact on activation or de-activation of some key enzymes (e.g., pectin methyl esterase or polyphenol oxidases), which determines the final amount and quality of bioactive compounds in the residues (Galanakis 2012). However, some bioactive phenolics are sensitive to heat. Temperatures higher than 50 °C often cause their chemical degradation, isomerization or polymerization affecting the antioxidant capacity of the byproduct (Sadilova *et al.* 2007, Yu and Ahmedna 2013). Drying procedures in oven use a temperature of about 60 °C for a few hours. This technique has low operating cost, but the risk of degradation of the phenolic compounds is relatively high. Alternatively, ohmic heating, also called as electro-conductive heating, could be applied for avoiding the thermal damage of heat sensitive substances, such as vitamins and pigments (Aggarwal and Jain 2019).

The freeze-drying procedure is based on sublimating the water from a previously frozen sample. As it needs vacuum, and the process could take several days to reduce the moisture content to the desired level, lyophilization is a costly and time-consuming technique compared to oven-drying (Galanakis 2012). Using lyophilization, however, high quality products could be obtained with minimal loss in their nutritional and organoleptic (e.g., content of volatile components) properties (Vashisth *et al.* 2011). This pretreatment method could preserve the physical and chemical structures of the byproduct thanks to the low temperature (Michalczyk *et al.* 2009). The study of Khanal *et al.* (2010) showed that the heating above 60 °C significantly decreased the concentrations of procyanidins and anthocyanins in blueberry and grape pomace. The authors concluded that drying at low temperature for three days may not be detrimental compared to heating at higher temperatures for more than eight hours. In another research, freeze-dried mango husk and seed retained phenolic compounds in higher percentage than the oven-dried samples, which could be a result of the lack of heat-related damage (Sogi *et al.* 2013). Lyophilization also proved to be a suitable pretreatment to stabilize the bioactive compounds in onion (De Ancos *et al.* 2015) and apple byproducts (Wolfe and Liu 2003).

For high yield recovery of phytochemicals from byproducts, grinding is needed before or/and after the drying process to get the appropriate particle size. Grinding supports the degradation of the lignin-cellulose network, thereby, can increase the surface contact area for enzyme treatment. Grinded particles could facilitate the accessibility of hydrolytic enzymes to degrade cellulose and improve the bio-digestibility and the porosity of the solid residues. When solvent extraction is applied, grinding provides higher diffusion of solvents into the byproduct matrix as well (Hendriks and Zeeman 2009, Galanakis 2012).

2.10. Foodborne pathogens and spoilage bacteria

Foodborne diseases frequently occur worldwide. The infection is caused by the consumption of food or water contaminated with a pathogen and/or microbial toxin. The pathogenesis varies according to the host's health condition, the type of food consumed, the type of microorganism and the number of microbes, to which the host is initially exposed. Foodborne pathogens can live in adverse environments such as cold, warm, acidic and high salt conditions and have the capacity to form biofilms on biotic or abiotic surfaces. Therefore, they could become tolerant against many food processing and conservation methods (Gyawali and Ibrahim 2014, Begley and Hill 2015). QS, as a mechanism of bacterial communication, plays an important role in the development, resistance and proliferation of bacterial biofilms, accordingly, its inhibition can contribute to the elimination of pathogenic and spoilage organisms (Alvarez *et al.* 2012). The risk of foodborne illnesses is enhanced by the contamination of foods before consumption and by the increased consumption of raw products, such as fruits and vegetables, packaged salads and ready-to-eat products (King *et al.* 2000). Another problem is the selective pressure generated by the use, misuse and overuse of antimicrobial drugs, which can lead to resistance of microorganisms against clinical antibiotic therapy (Ngwoke *et al.* 2011, Daglia 2012). Nevertheless, despite the high worldwide food demand and progresses in conservation techniques, the food losses due to microbial deterioration are large. Many synthetic antimicrobials are used successfully for shelf life extension; however, growing concern of consumers against such preservatives in foods encourages researchers to screen for natural alternatives.

A variety of natural antimicrobials is known that can be used against foodborne pathogens. These are obtained from animal, plant, bacterial, algal and fungal sources (Gyawali and Ibrahim 2014). Antimicrobial activity of plant extracts can be attributed to secondary metabolites (Ngwoke *et al.* 2011); of which polyphenolics is one of the largest groups (Daglia 2012). Besides the *in vitro* activity (see section 2.2.3), many studies have demonstrated the *in situ* efficacy of antimicrobial plant phenolic compounds as well (Stojković *et al.* 2013, Papuc *et al.* 2017).

The US Centers of Disease Control and Prevention has reported a list of selected multistate foodborne outbreak investigations from 2006 until date (www.cdc.gov/foodsafety/outbreaks/multistate-outbreaks/outbreaks-list.html) and found that the strains of *E. coli* and *Listeria monocytogenes* and various *Salmonella* serovars are

the main causes of foodborne disease outbreaks. In addition, other food-related bacteria such as *Shigella*, *Clostridium*, *Campylobacter*, *Staphylococcus* and *Bacillus* species may also be responsible for food and waterborne illnesses and/or toxic symptoms (Addis and Sisay 2015). In the present work, several representatives from the above genera were selected to test the antimicrobial capacity of phenolic extracts produced.

3. AIMS

There is a growing interest in the application of phenolic antioxidants as natural additives in functional foods. Fruit residues are excellent substrates to produce such phenolics, however, most of them occur in conjugated forms with sugar residues reducing their bioavailability. Carbohydrate-cleaving enzymes, i.e. cellulases and pectinases, can hydrolyze these glycosides releasing the phenolic aglycones. Foodborne pathogens and spoilage bacteria cause serious problems in the food industry despite preservation methods are applied. Because synthetic preservatives in foods provoke serious concern in consumers, there is a need to identify and produce novel preservatives. The abovementioned phenolic compounds can enhance the stability and shelf life of food products, increase their antioxidant capacity, and inhibit the growth of a range of bacteria and fungi.

In previous studies, the *Rhizomucor miehei* NRRL 5282 was identified as a high yield cellulase producer fungus (Takó *et al.* 2010, 2015). Therefore, the main objective of our work was to mobilize bioactive phenolic compounds from fruit byproducts via *in vivo* SSF with the *R. miehei* NRRL 5282 isolate and *in vitro* substrate treatment using *R. miehei* cellulolytic enzyme cocktail. The extraction efficiency of the phenolic compounds can be improved by pectinases, thus, addition of commercial *Aspergillus niger* pectinase to the *R. miehei* enzyme cocktail was also planned. We expect to show that the obtained phenolic rich extracts can be used as sources of natural antimicrobial agents.

For this purpose, our specific objectives were the followings:

1. Selection, preparation and pretreatment of residue substrates rich in polyphenolic compounds.
2. Completion of SSF studies using the *R. miehei* NRRL 5282 as the fermenting organism.
3. Direct enzyme treatment of the residues using a crude cellulolytic extract produced by *R. miehei* NRRL 5282 and/or a pectinase preparation from *A. niger*.
4. Analysis and comparison of the total phenolic content and the phenolic profile of the extracts before and after fermentation and enzyme treatment using spectrophotometric and chromatographic methods.
5. Evaluation of the bioactive properties of the extracts before and after fermentation and enzyme treatment by:

- antioxidant capacity studies,
- antimicrobial activity assays against foodborne pathogens and spoilage bacteria,
- investigation of the anti-QS capacity with the model organism *Chromobacterium violaceum*,
- study of the inhibitory effect of bioactive extracts on the biofilm formation of foodborne pathogens and spoilage bacteria.

4. MATERIALS AND METHODS

4.1. Substrates

Othello black grape (*Vitis vinifera x* (*Vitis labrusca x Vitis riparia*)), Saszla white grape (*Vitis vinifera*) and Jonagold apple (*Malus domestica* cv. Jonagold) were purchased at a local market in Szeged, Hungary. Yellow pitahaya (*Hylocereus megalanthus*), Tommy Atkins mango (*Mangifera indica* L.) and naranjilla (*Solanum quitoense*) were purchased at a local market in Guayaquil, Ecuador.

4.2. Media

The composition of the used media is presented in Table 4. Solid media were prepared by addition of 2% agar-agar.

Table 4. Composition of the media used during the experiments.

Malt extract medium (ME)	Tryptone glucose yeast extract (TGE)	Tryptic soy broth (TSB)	Lysogeny broth (LB)
20% (v/v) malt extract*, 50 mL/L; yeast extract, 5 g/L; glucose, 5 g/L	glucose, 1 g/L; yeast extract, 3 g/L; tryptone, 5 g/L	casein peptone, 17 g/L; K_2HPO_4, 2.5 g/L; glucose, 2.5 g/L; NaCl, 5 g/L; soya peptone, 3 g/L	tryptone, 10 g/L; yeast extract, 5 g/L; NaCl, 5 g/L

*All ingredients were purchased from VWR International (USA).

4.3. Microorganisms

The fungal and bacterial strains used for the experiments are listed in Table 5. The microorganisms were subcultured every four weeks on the corresponding solid medium and stored at 4 °C. Before use, fresh bacterial cultures were prepared by picking a colony from a 24-h old culture, suspended in 30 mL medium and incubated for 18 h at 30 or 37 °C depending on the requirements of the applied strain. At the end of the incubation period, the growth of each bacterium was in the stationary phase. Cell number was set by serial dilution in the respective growth medium followed by counting in a Bürker-chamber under a light microscope (Carl Zeiss Jena, LABOVAL 4, Germany).

Table 5. Fungal and bacterial strains used in the experiments.

Microorganism	Code	Growth medium	Growth temperature (°C)
Rhizomucor miehei NRRL 5282*	SZMC 11005	ME	37
Bacillus subtilis	SZMC 0209	TGE	30
Bacillus cereus	SZMC 0042	TGE	30
Listeria monocytogenes	SZMC 21307	TSB	37
Staphylococcus aureus	SZMC 0579	LB	37
Methicillin-resistant *Staphylococcus aureus*	SZMC 6270	LB	37
Escherichia coli	SZMC 0582	LB	37
Salmonella enterica subsp. *enterica* serovar Typhimurium ATCC 13311	SZMC 23289	LB	37
Pseudomonas putida	SZMC 6010	LB	30
Pseudomonas aeruginosa	SZMC 0568	LB	30
Chromobacterium violaceum 85WT	SZMC 6269	LB	30

*Abbreviations: NRRL, Agricultural Research Service Culture Collection; SZMC, Szeged Microbiological Collection; ATCC, American Type Culture Collection; ME, malt extract medium; TGE, tryptone glucose yeast extract; TSB, tryptic soy broth; LB, lysogeny broth.

4.4. Reagents, solvents and standards

Unless otherwise stated, all materials were purchased from VWR International (USA). All solutions were prepared freshly before use.

Solutions and reagents for the cellulase activity assays

- Dinitrosalicylic acid reagent: 1% dinitrosalicylic acid (Sigma-Aldrich, Germany), 1% NaOH, 0.2% phenol, 0.05% Na_2SO_3,
- 4 mM *p*-nitrophenyl-β-D-cellobioside (Sigma-Aldrich) solution in distilled water,
- 7 mM *p*-nitrophenyl-β-D-glucopyranoside (Sigma-Aldrich) solution in distilled water,
- Sodium acetate buffer (100 mM, pH 5.0) prepared by addition of 1.36 g sodium acetate x $3H_2O$ to 100 mL distilled water supplemented with 85 mg of 96% glacial acetic acid,
- 100 mM sodium carbonate buffer solution in distilled water,
- 8 mM glucose and 10 mM *p*-nitrophenol (Sigma-Aldrich) standard solutions in acetate buffer (100 mM, pH 5.0) for preparation of calibration curves.

Solutions for total phenolic concentration measurement
- 50% Folin-Ciocalteu's reagent (Sigma-Aldrich) solution in distilled water,
- 5% sodium carbonate solution in distilled water,
- gallic acid (Sigma-Aldrich) solutions of various concentrations prepared in 96% ethanol for calibration curve establishment.

Solutions for ferric reducing antioxidant power (FRAP) assay
- Sodium acetate buffer (300 mM, pH 3.6) prepared by addition of 4 g sodium acetate x $3H_2O$ to 100 mL distilled water supplemented with 3 mL of 96% glacial acetic acid,
- 40 mM HCl solution,
- 10 mM 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ; Sigma-Aldrich) solution prepared in 40 mM HCl by heating at 50 °C,
- 20 mM Iron (III) chloride solution in distilled water,
- FRAP reagent prepared by mixing 20 mL of 300 mM acetate buffer (pH 3.6), 4 mL of 10 mM TPTZ solution, 4 mL of 20 mM Iron (III) chloride solution and 2.2 mL distilled water under 37 °C condition,
- 1 mM Iron (II) sulfate solution for calibration curve preparation.

Solutions for free radical scavenging activity determination
- 100 µM 1,1-diphenyl-2-picrylhydrazyl (DPPH; Sigma-Aldrich) solution prepared in 96% ethanol. The solution was stored in the dark.

Solutions for high-performance liquid chromatography (HPLC) analysis
- Solvent A: 97 mL of distilled water, 3 mL 96% acetic acid
- Solvent B: 97 mL acetonitrile, 3 mL 96% acetic acid

Phenolic standards
- Gallic acid, vanillic acid, syringic acid, *p*-coumaric acid, 4-hydroxybenzoic acid, cinnamic acid, (+)-catechin, (−)-epicatechin, polydatin, quercetin and resveratrol were purchased from Sigma-Aldrich. Stock solutions (1 mg/mL) of pure phenolic compounds were prepared in 10% (v/v) ethanol.

Control solution in antimicrobial tests

- 100 µg/mL streptomycin (Sigma-Aldrich) solution prepared in distilled water. The solution was sterilized by filtration using 0.45 µm Millex-HV syringe filter (PVDF, Millipore).

4.5. Substrate preparation

The grapes and apple substrates were processed at the Institute of Food Engineering, University of Szeged, Hungary, while the pitahaya, mango and naranjilla residues at the Faculty of Food Engineering, Escuela Superior Politécnica del Litoral, Ecuador. In the case of grapes and naranjilla, the skin, stem, and seeds were obtained after juice pressing. The apple, pitahaya and mango fruits were peeled, and the flesh was separated from the other parts of the fruits. A mixture of peels, cores, peduncles and seeds was subjected to substrate pretreatment. These byproducts constitute the bulk of the pomace remained after wine, juice and jam production, therefore, the substrates used were close to those generated at industrial level. To pretreat the substrates, the residues were dried immediately in an oven or frozen at -20 °C and subsequently lyophilized. Time of drying processes varied depending on humidity, fruit sugar content and the used equipment (Table 6). Then, both types of samples were ground in a commercial mill into particles of 3 mm mean diameter. The ground samples were stored in dark at room temperature and used for subsequent experiments.

Table 6. Conditions of oven-drying and lyophilization processes.

Fruits	Weight of initial biomass (g)	Oven-drying			Lyophilization
		Temperature (°C)	Time (h)	Humidity (%)	Time (h)
Grapes	30	65	18	10.3	24
Apple	25	65	18	12.2	24
Pitahaya*	20	65	5	13.1	24-32
Mango*	25	65	4	10.8	24
Naranjilla*	30	65	4	11.1	24

*Electric dehydrator (KENGO- Guangdong, China)

4.6. Solid-state fermentation conditions

For SSF, 2 g of ground sample, 0.5 g of soy flour as a nitrogen source and 5 mL distilled water were mixed in 100-mL Erlenmeyer flasks and autoclaved at 121 °C for 25 min. The flasks were then inoculated with 5×10^6 spores of *R. miehei* NRRL 5282 and incubated at 37 °C for 18 days. Four parallel fermentation tests were carried out, two for distilled water extraction and two for 50% (v/v) ethanol extraction. During incubation, four flasks were taken on every second day for subsequent analytical measurements, and the remaining flasks were hydrated with 2 mL of sterile distilled water to ensure the humidity conditions until the end of fermentation.

4.7. Extraction processes after solid-state fermentation

The fungus-byproduct ferments were extracted with 30 mL of distilled water or a solution of 50% (v/v) ethanol macerating them with a spatula and incubated at 4 °C for 24 h. After the extraction, the samples were filtered through gauze and centrifuged at 16,200×*g* for 20 min at 4 °C. The supernatant was stored at -20 °C until analytical measurements. To avoid the inhibition effect of ethanol (Takó *et al.* 2010), aqueous extracts were used to measure the β-glucosidase activity in the samples, while crude extracts made with 50% (v/v) ethanol solution were utilized for total phenolic content (TPC), antioxidant activity and HPLC measurements.

4.8. Production of cellulolytic cocktail

To produce crude cellulolytic enzyme cocktail, 20 g of wheat bran was mixed with 20 mL of distilled water, then, the flask was sterilized at 121 °C for 25 min. After cooling to room temperature, the flask was inoculated with 800 μL of 10^6 spore suspension of *R. miehei* and incubated at 37 °C for 6 days (Takó *et al.* 2010). After incubation, the ferment was extracted with 120 mL of sodium acetate buffer (100 mM, pH 5.0) and incubated at 4 °C for 12 h. The extracted ferment was filtered through gauze and centrifuged at 5,040×*g* for 20 min at 4 °C. The supernatant was sterilized using 3.0 μm and 0.45 μm pore-size filters (PVDF; Millipore) and after cellulase activity measurements, the filtrate was stored at -20 °C until use.

4.9. Cellulolytic activity assays

To determine the cellulolytic property of the crude enzyme extract used for enzyme-assisted extraction tests, filter paper degrading (FPase), endoglucanase, cellobiohydrolase and β-glucosidase activity measurements were carried out. During SSF on fruit residue substrates, only the β-glucosidase activity was monitored. In case of the FPase and endoglucanase assays, one enzyme activity unit was defined as the amount of enzyme that released 1 μM of glucose from the treated substrate per minute under the assay conditions. One unit of cellobiohydrolase or β-glucosidase activity was defined as the activity of the enzyme that released 1 μM *p*-nitrophenol per minute under the conditions of the assay. Standard curves were established using stock solution of 8 mM glucose or 10 mM *p*-nitrophenol in sodium acetate buffer (100 mM, pH 5.0).

FPase activity

FPase activity was determined using Whatman No. 1 filter paper as a substrate. The reaction mixture consisted of 200 μL sodium acetate buffer (100 mM, pH 5.0), 50 μL crude enzyme extract and 2.5 mg filter paper. After incubation at 50 °C for 1 h, 250 μL of dinitrosalicylic acid was added and the mixture was incubated in hot water bath for 15 minutes. Then, absorbance was measured at 570 nm using a SPECTROstar Nano spectrophotometer (BMG Labtech, Germany).

Endoglucanase activity

To determine the endoglucanase activity, a reaction mixture was composed from 200 μL sodium acetate buffer (100 mM, pH 5.5), 50 μL crude enzyme extract and 1% carboxymethyl cellulose (Sigma-Aldrich). The mixture was incubated at 50 °C for 30 min, then, 250 μL of dinitrosalicylic acid was added and the mixture was subjected to boiling for 15 minutes. After cooling to room temperature, absorbance was recorded at 570 nm (SPECTROstar Nano spectrophotometer, BMG Labtech).

Cellobiohydrolase and β-glucosidase activities

The cellobiohydrolase and β-glucosidase activities were determined by using 4 mM *p*-nitrophenyl-β-D-cellobioside and 7 mM *p*-nitrophenyl-β-D-glucopyranoside stock solutions, respectively. The reaction mixtures consisted of 20 μL of appropriate substrate solution, 160 μL of 100 mM sodium acetate buffer (pH 5.0) and 20 μL of extract diluted to

10-fold by distilled water. After 30 min incubation at 50 °C, the reaction was stopped by addition of 50 µL of 100 mM sodium carbonate, and the *p*-nitrophenol release was measured at 405 nm using a SPECTROstar Nano spectrophotometer (BMG Labtech).

4.10. Enzyme treatment of plant residues

The *R. miehei* cellulolytic cocktail produced by wheat bran-based SSF and a commercial *A. niger* pectinase with 1.1 U/mg polygalacturonase activity (Sigma-Aldrich) were used for enzyme-assisted extraction of phenolics. For treatment 1 (designated as S1), 600 mg of oven-dried or lyophilized fruit residue was mixed with 6 mL of *R. miehei* cellulase cocktail. In the case of treatment 2 (designated as S2), this reaction mixture was supplemented with 0.6 mg of *A. niger* pectinase (1 mg/g DM). For treatment 3 (designated as C2) 600 mg of fruit residue was mixed with 6 mL of acetate buffer (50 mM, pH 5.0) and 0.6 mg *A. niger* pectinase. Enzyme-free acetate buffer (50 mM, pH 5.0)/fruit residue mixture was used as control (designated as C1). The reaction mixtures were then incubated at 50 °C for 5 h under constant stirring (200 rpm), and the reaction was stopped by boiling (100 °C) for 3 min. After centrifugation at 16,200×*g* for 10 min, the resulted clear supernatant was collected and used for TPC, antioxidant activity and HPLC measurements, and to prepare stock solutions for subsequent bioactivity tests. The reaction conditions (pH 5.0, 50 °C and 5 h incubation time) were selected according to the studies of Krisch *et al.* (2012) and Xu *et al.* (2014). All enzyme treatments were carried out in three independent experiments.

4.11. Extract stock solution preparation for microbiological tests

Clear supernatant of S1-2 and C1-2 treatments was placed to a 50-mL Falcon centrifuge tube and frozen overnight at -20 °C. Then, these samples were lyophilized until the water content was completely displaced. The remained DM of each sample was weighted and subsequently diluted in 10% (v/v) ethanol solution to reach the concentration of 200 mg/mL. After vortexing, this concentrated stock solution was sterilized by syringe filtration (Millex-HV, PVDF, pore size: 0.45 µm; Millipore).

4.12. Determination of total phenolics

TPC was measured using a reaction mixture containing 20 µL of ethanol (96%), 100 µL of distilled water, 10 µL of 50% Folin-Ciocalteu's phenol reagent and 20 µL of extract diluted 10-fold in ethanol (96%). The mixture was left at room temperature for 5 min, then, the reaction was initiated by the addition of 20 µL of sodium carbonate (5%), and incubated in dark condition for 60 min. After incubation, absorbance was measured at 725 nm (SPECTROstar Nano spectrophotometer, BMG Labtech). The standard curve was established using gallic acid in the concentration range of 0-100 µg/mL. The content of total phenolics was expressed as mg of gallic acid equivalent (GAE) in 100 g DM. The following equation was used to calculate the TPC:

$$TPC \ (mg \ GAE/100 \ g \ DM) = \frac{P \ (\mu g/mL) * D * ES \ (mL) * 1000}{amount \ of \ pomace \ (g)} * 100$$

where P is the phenol concentration determined from the calibration curve, D is the dilution factor, and ES is the volume of the extraction solvent.

4.13. Antioxidant activity assays

4.13.1. Determination of free radical scavenging activity

The free radical scavenging activity of the extracts and phenolic compounds was determined using DPPH solution in 100 µM concentration prepared freshly before the measurement. The mixture was shaken vigorously until complete dissolution and left in the dark at room temperature until use. The reaction mixture contained 150 µL of 100 µM DPPH and 25 µL of extract diluted in 50% (v/v) ethanol (10-fold dilution), or 25 µL of phenolic standard solution (1 mg/mL). The control sample contained 150 µL of 100 µM DPPH and 25 µL of 50% (v/v) ethanol. Absorption was measured at 517 nm (SPECTROstar Nano spectrophotometer, BMG Labtech), and the results were plotted as percent DPPH radical scavenging capacity against the control. The following equation was used to calculate the DPPH inhibition:

$$DPPH \ (\%) = \frac{control \ A - extract \ A}{control \ A} * 100$$

where A is the absorbance at 517 nm.

4.13.2. Ferric reducing antioxidant power assay

The reagent solution prepared for FRAP analysis contained 80 mL of 300 mM acetate buffer (pH 3.6), 8 mL of 10 mM TPTZ diluted in 40 mM HCl, 8 mL of 20 mM iron(III) chloride and 4.8 mL of distilled water. The power of phenolic extracts and compounds on ferric reducing was determined by mixing 200 µL FRAP reagent with 6 µL of extract diluted in 50% (v/v) ethanol (10-fold dilution), or 6 µL of phenolic standard solution (1 mg/mL). The reaction mixture was incubated at 37 °C for 30 min and then absorbance was measured at 593 nm (SPECTROstar Nano spectrophotometer, BMG Labtech). The standard curve was established using 1 mM iron(II) sulfate solution in the concentration range of 0.1-1.0 mM. The FRAP of the crude extracts and phenolics was expressed as mM Fe(II)/100 g DM. The following equation was used to calculate the reducing power:

$$\text{FRAP (mM Fe (II)/100 g DM)} = \frac{C\ (mM)*extraction\ solvent\ (mL)*dilution}{1000\ ml\ *amount\ of\ pomace\ (g)}$$

where C is the Fe(II) concentration determined from the calibration curve.

4.14. Antimicrobial activity tests

4.14.1. Agar disk-diffusion assay

Agar disk-diffusion method was used to screen the antimicrobial activity of the extracts and individual phenolic compounds. A volume of 1 mL of each bacterial suspension (10^6 colony forming unit (CFU)/mL) was uniformly spread on the surface of the corresponding solid medium prepared in Petri-dishes. After removing the excess suspension, plates were dried and six sterile paper disks (5 mm in diameter) were placed equidistantly on the surface of each agar plate. Then, stock solution of extracts was diluted to 100 mg/mL concentration using 10% (v/v) ethanol. A volume of 10 µL of fruit extract solution (1 mg per disk) or phenolic compound stock solution (10 µg per disk) was pipetted to the surface of the disks. Then, the cultures were incubated for 24 h under the condition appropriate for the bacteria tested. The antibacterial activity was estimated from the diameter of the growth inhibition zone (in mm) formed around the disks. Paper disks impregnated with 10% (v/v) ethanol and 100 µg/mL of streptomycin served as negative

and positive control, respectively. Three biological replicates were performed with each enzyme-treated sample and phenolic compound.

4.14.2. Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of the extracts and individual phenolics was determined by microplate assay. Using the corresponding stock solution, diluted samples were prepared in 10% (v/v) ethanol to the concentrations of 12.5, 25, 50 and 100 mg/mL for the fruit extracts and 250 and 500 µg/mL for the phenolic compounds. After 18 h incubation, the bacterial cells were collected (16,200×g, 10 min) and a 10^5 CFU/ml suspension was prepared using double concentrated growth medium appropriate for the specific bacterium. A volume of 100 µL from this bacterial suspension was added to each well of a sterile 96-well polystyrene microtiter plate (Sarstedt, Germany), followed by adding 100 µL of the stock or diluted fruit extract and phenolic compound samples, giving a final concentration from 6.25 to 100 mg/mL and 125 to 500 µg/mL, respectively. A volume of 100 µL of bacterial suspension supplemented with 100 µL of 10% (v/v) ethanol solution was considered as positive control, while the negative controls contained 100 µL of sterile double concentrated growth medium and 100 µL of diluted fruit extract or phenolic compound. Final volume of the reaction mixture was 200 µL. Subsequently, the optical density (OD) of the plates was measured at 600 nm (SPECTROstar Nano spectrophotometer, BMG Labtech) before and after the incubation (for 24 h under appropriate growth conditions). MIC was considered as the lowest concentration of fruit extract or phenolic compound where the absorbance was 10% or lower than the positive control, thus the growth inhibition was 90% or higher. Three biological replicates were performed with each enzyme-treated sample and phenolic compounds. The following equation was used to calculate the percentage of growth inhibition:

$$MIC = \frac{A - B}{C - D} * 100$$

where A and B are the absorbance of the sample after and before incubation, respectively, and C and D are the absorbance of the positive control after and before incubation, respectively.

4.14.3. Determination of minimum bactericidal concentration

A volume of 10 µL from the cultures where the MIC was recorded, and from the higher concentrations (up to 100 mg/mL for the fruit extracts or 500 µg/mL for the phenolic compounds), were inoculated equidistantly on solid medium appropriate for growth of the bacterium tested. Then, the plates were incubated for 24 h under different conditions depending on the bacterium used. The lowest concentration, at which no colonies were formed, was taken as the minimum bactericidal concentration (MBC). This value corresponds to at least 99.9% elimination of the cells from the inoculum.

4.15. Quorum sensing inhibition assay

Anti-QS activity of the extracts and individual phenolics was assessed through the inhibition of violacein synthesis in *C. violaceum* model organism, which uses N-acyl-L-homoserine lactones as signaling molecules like many other Gram-negative bacteria (Morohoshi *et al.* 2008). In this assay, the violacein pigment produced in liquid culture was extracted and detected spectrophotometrically, according to the modified method of Choo *et al.* (2006). Falcon tubes containing 9.4 mL of LB medium and 100 µL of fruit extract or phenolic compound stock solutions were inoculated with 500 µL of 18-h old bacterial culture with approximately 10^8 CFU/mL density. A single tube consisting 9.5 mL of LB medium and 500 µL of bacterial suspension (10^8 CFU/mL) was used as positive control, and tubes consisting 9.9 mL of growth medium and 100 µL of extract and phenolic compound stock solutions were the negative controls. Final concentrations of the fruit extracts and the phenolic compounds during the test were 2 mg/mL and 10 µg/mL, respectively. After the inoculation, culturing tubes were incubated at 30 °C for 24 h under continuous shaking at 150 rpm. The samples were then vortexed and 2-mL aliquots were transferred to sterile Eppendorf tubes and centrifuged at $16,200 \times g$ for 20 minutes to precipitate the insoluble violacein pigment and the bacterial cells. The supernatant was discarded, and the pellet was solubilized in 1 mL of dimethyl sulfoxide by vigorous vortexing for 20 min. Finally, cellular debris was removed by centrifugation ($16,200 \times g$, 20 min), and 200 µL of supernatant was transferred into the wells of a 96-well polystyrene microtiter plate. Absorption was measured at 585 nm via SPECTROstar Nano microplate reader (BMG Labtech), and the percentage inhibition of violacein production was calculated by using the following equation:

$$Inhibition\ of\ violacein\ production\ (\%) = \frac{a - b}{a} * 100$$

where "a" and "b" are the absorbance of the control and the sample, respectively.

The CFU was determined in each tube after the incubation, to make sure that the tested extracts and phenolic compounds did not inhibit the growth of *C. violaceum*, at the applied concentration. Three replicates were performed in case of each enzyme-treated sample and phenolic compound.

4.16. Biofilm formation assay

Effect of extracts and individual phenolics on the biofilm formation was determined by the crystal violet staining method. Following the procedure of Kerekes *et al.* (2013), 200 µL of 24-h old bacterial culture (approximately 10^8 CFU/ml) was pipetted into the wells of a sterile 96-well polystyrene microtiter plate and the cells were allowed to adhere for 4 h at temperatures appropriate for the bacteria tested. After removing the planktonic cells, the plate was rinsed with physiological saline solution and left to dry for 10 min under sterile conditions. A volume of 200 µL of medium containing fruit extracts or phenolic compounds in 50 mg/mL or 100 µg/mL concentrations, respectively, were then added to each treated well. The positive control was made by addition of 200 µL of growth medium to treated wells, while the negative controls contained fruit extracts or phenolic compounds in the growth medium. The plates were incubated for 24 h, then, the supernatant was removed, and the wells were rinsed with physiological saline. To fix the biofilm, 200 µL of methanol was added to each well and the plate was left to stand for 15 min at room temperature. The methanol was removed, and 200 µL of 0.1% crystal violet solution was added to each well. After incubation for 20 minutes at room temperature, the excess dye was removed by rinsing each well twice with distilled water. To release the bounded dye, 200 µL of 33% acetic acid solution was added to the wells followed by 10 min incubation at room temperature. Finally, the absorbance was measured at 590 nm (SPECTROstar Nano microplate reader, BMG Labtech), and the percentage of biofilm formation was calculated. Optical density of the positive control was considered as 100%. Three biological replicates were performed with each enzyme-treated sample and phenolic compounds. The percent biofilm formation was calculated according to the following equation:

$$Biofilm\ formation\ (\%) = \frac{b}{a} * 100$$

where "a" and "b" are the absorbance of the positive control and the sample, respectively.

4.17. HPLC analysis of phenolic compounds

Phenolic compounds were separated on Prodigy ODS 3 C18 (250 x 4.6 mm, 5 µ; Phenomenex, USA) column using the mixture of solvent A (distilled water:acetic acid, 97:3) and solvent B (acetonitrile:acetic acid, 97:3) as mobile phase at a flow rate of 1 mL/min; detection was carried out at 280 nm with an SPD-20AVP UV-VIS detector (Shimadzu, Germany). The following gradient program was used during the separation: 0 min 8% B, 20 min 25% B, 25 min 25% B, 40 min 60% B, 45 min 60% B, 46 min 8% B, and 60 min 8% B. The oven temperature and injection volume were 40 °C and 3 µL, respectively. The detector response was linear for all examined phenolic compounds (gallic, vanillic, syringic, p-coumaric, 4-hydroxybenzoic and cinnamic acids, and (+)-catechin, (−)-epicatechin, polydatin, resveratrol and quercetin) with correlation coefficients greater than 0.995. Recording of data and evaluation was carried out using the ClassVP v5.1 chromatography software.

4.18. Statistical analysis

Assays were performed in three independent experiments and data were expressed as an average of the replicates ± standard deviation. Two-way analysis of variance (ANOVA) was used for pairwise multiple comparisons. The independent variables considered for each study are given in the figure captions or table footnotes. One-way ANOVA was applied for the individual phenolic compounds in the antimicrobial activity tests. Significant differences between the means were further analyzed by Tukey's Multiple Comparison Test using the program GraphPad Prism version 6.00 (GraphPad Software Inc., San Diego, USA). A p value of < 0.05 was considered as statistically significant. Pearson's correlation coefficients were calculated using Microsoft Office Excel 2016 function.

5. RESULTS AND DISCUSSION

5.1. Selection of fruit residue samples

Pomace, peel, seed and/or stem residues of grape, apple, mango, pitahaya and naranjilla fruits contain substantial amounts of polyphenolic compounds; therefore, they could be a good basis to test the *in vivo* fermentative and *in vitro* enzyme-assisted production of bioactive phytochemicals using cellulolytic microorganisms and their carbohydrases. For our experiments, the antioxidative Othello black grape, (*Vitis vinifera x (Vitis labrusca x Vitis riparia)*), Saszla white grape (*Vitis vinifera*), Jonagold apple (*Malus domestica* cv. Jonagold), yellow pitahaya (*Hylocereus megalanthus*), Tommy Atkins mango (*Mangifera indica* L.) and naranjilla (*Solanum quitoense*) fruits have been selected. Pomace and/or different parts of the fruits were prepared according to the method described in subsection 4.5. Two pretreatment methods were used and compared to prepare the residues for fermentation and enzyme treatment assays: drying at 65 °C in a conventional oven (oven-dried sample) and freeze drying (lyophilized sample).

5.2. Solid-state fermentation on oven-dried and lyophilized fruit residues

A previous study pointed out the potential of *Rhizomucor miehei* NRRL 5282 β-glucosidase to enrich free, extractable and bioavailable phenolic compounds from fruit samples by liberating them from the glycosidic form (Krisch *et al.* 2012). Generally, plant wastes contain enough polysaccharides that can induce the fungal β-glucosidase production in solid cultures. The solid-state bioprocessing is a useful technique to test the fungus-mediated liberation of phenolics from plant wastes (Martins *et al.* 2011), by using the cellulolytic fungus *R. miehei* NRRL 5282 that grows well under such conditions (Takó *et al.* 2015).

5.2.1. Black grape pomaces

Weak negative associations were found between β-glucosidase activity and FRAP for both oven-dried (DBG) ($r = -0.477$) and lyophilized black grape (LBG) ($r = -0.111$) pomaces. β-Glucosidase activity showed a slow increase from the 7th day of the fermentation and reached maximal yields at the 15th day (Figure 4). The slow increase in

the activity may be due to the high glucose content of the medium. Namely, glucose in the growth medium represses the cellulase production at the beginning of the fermentation (Adnan *et al.* 2018). Another explanation could be that sugar compounds, for example cellobiose and glucose, can bind to the active site of the β-glucosidase inhibiting its activity if they are present in excess (Srivastava *et al.* 2019). Additionally, interaction of polyphenols and free aglycones with glucosidases may also reduce the enzyme activity (Cairns and Esen 2010, Ximenes *et al.* 2010, Yu and Ahmedna 2013).

The high phenolic content observed at the beginning of the fermentation (Figure 4) is related to the flavanol, stilbene and proanthocyanin content of the crude pomace (Ali *et al.* 2010). Polyphenol concentration in DBG samples decreased from 1236 ± 74 to 875 ± 50 mg GAE/100 g DM, while it reached 1956 ± 31 mg GAE/100 g DM TPC at the 7th day on the LBG residue. The decrease in TPC may be due to degradation and/or enzymatic polymerization of the released phenolics by the fermenting fungus. Phenolic compound degradation and polymerization have been documented for other filamentous fungi as well (Correia *et al.* 2004, Mendonça *et al.* 2004, Santos and Linardi 2004).

In black grape pomaces, the main phenolic compounds are gallic acid, catechin and epicatechin (Makris *et al.* 2007). HPLC analysis detected an increase in the concentration of these components when LBG pomace was used as a fermenting medium. The highest gallic acid, (+)-catechin and (–)-epicatechin yields, i.e. 0.79 ± 0.12, 3.49 ± 0.52 and 1.37 ± 0.08 mg/100 g DM, respectively, were determined at the 7th day of fermentation. In case of the DBG samples, only the gallic acid content improved reaching 1.27 ± 0.03 mg/100 g DM at the 7th day of incubation.
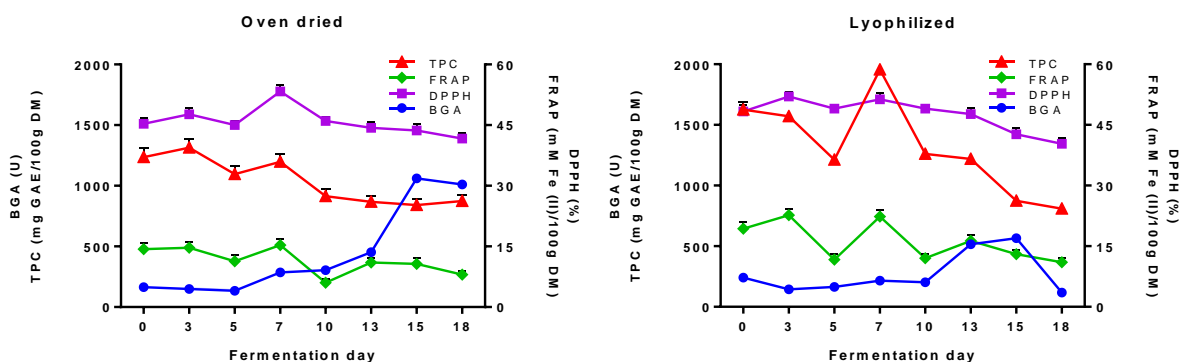
Figure 4. β-Glucosidase activity (BGA), total phenolic content (TPC), antioxidant power (FRAP) and free radical scavenging activity (DPPH) of oven-dried and lyophilized black grape byproducts during solid-state fermentation by *R. miehei* NRRL 5282. Error bars represent SD values of replicates.

Antioxidant potential in the fermented samples varied depending on the incubation time. FRAP for the DBG and LBG samples was the highest at the 3rd day (14.6 ± 0.9 and 22.5 ± 1.1 mM Fe(II)/100 g DM, respectively) and 7th day (15.0 ± 1.0 and 22.4 ± 1.1 mM Fe(II)/100 g DM, respectively). The ability of polyphenolics to inhibit DPPH was 53 ± 1 and $51 \pm 1\%$ for DBG and LBG, respectively, in the 7th day extracts (Figure 4). Fermentation on LBG resulted in higher free phenolic yield compared to DBG ($p < 0.05$), which is directly related to the increased FRAP.

5.2.2. White grape pomaces

The β-glucosidase activity was low during fermentation on both oven-dried (DWG) and lyophilized (LWG) white grape residues (Figure 5). There was no association between the total phenolic content and the antioxidant potential on DWG residues, whereas LWG displayed a sharp positive correlation ($r = 0.7$). The initial β-glucosidase activity obtained on DWG and LWG was 108 ± 1 and 90 ± 1 U, respectively, and remained constant around these values until the end of the fermentation process. The TPC displayed an increase on the 3rd, 10th and 13rd days (DWG), and on the 5th and 7th days (LWG) of fermentation compared to initial data. The maximum TPC yields were 1118 ± 11 (DWG) and 1102 ± 10 mg GAE/100 g DM (LWG) at the 13th and 7th day, respectively. The FRAP was maximum at the 5th fermentation day reaching 183 ± 2 and 163 ± 2 μM Fe(II)/g DM for DWG and LWG samples, respectively, while the highest scavenging capacity was 45 ± 0.3 and $50 \pm 0.3\%$ detected at the 10th and 13th day in DWG and LWG pomaces, respectively (Figure 5).

Figure 5. β-Glucosidase activity (BGA), total phenolic content (TPC), antioxidant power (FRAP) and free radical scavenging activity (DPPH) of oven-dried and lyophilized white grape byproducts during solid-state fermentation by *R. miehei* NRRL 5282. Error bars represent SD values of replicates.

Overall, fermentation on DWG substrate resulted in slightly higher free phenolic yield and FRAP than LWG. Although the white grape and black grape have different phenolic composition (Rodríguez Montealegre *et al.* 2006), results of the current study didn't show a big difference in antioxidant activity between the two types of grape variety tested.

5.2.3. Apple residues

β-Glucosidase activity of the oven-dried (DA) and the lyophilized (LA) apple substrates showed a considerable increase from the 7th to the 10th day of bioconversion (Figure 6). TPC of DA continuously decreased during the fermentation, while TPC of LA increased for 5 days reaching a maximum value of 477 ± 37 mg GAE/100 g DM (Figure 6). Similarly, Ajila *et al.* (2012) described an increase in the free phenolic content after bioconversion of apple samples by *Phanerochaete chrysosporium*. β-Glucosidase activity reached its maximum by the 6th to 8th day, which coincided with the liberation of phenolic compounds. In the current study on LA, however, TPC increased slowly and reached a maximum on the 5th fermentation day before the β-glucosidase activity increased.

Hydroxybenzoic acids are one of the major polyphenolic compounds of apple residues (Watkins and Liu 2011). Interestingly, among hydroxybenzoic acids analyzed by HPLC, only the gallic acid content of LA samples demonstrated an increase during the fermentation. The highest gallic acid concentration was 0.79 ± 0.06 mg/100 g DM at the 5th sampling day. However, free gallic acid may be formed not only from its glucosidic derivatives but by hydrolysis of the gallic acid esters of flavan-3-ol (Rentzsch *et al.* 2009).
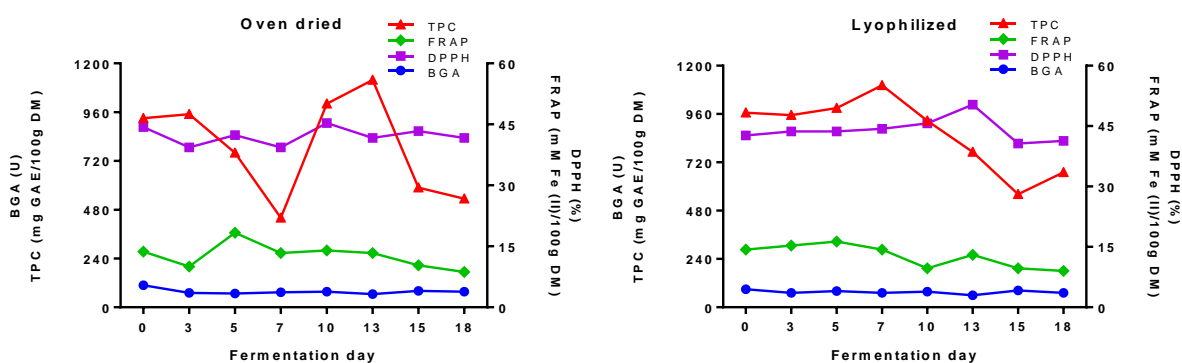

Figure 6. β-Glucosidase activity (BGA), total phenolic content (TPC), antioxidant power (FRAP) and free radical scavenging activity (DPPH) of oven-dried and lyophilized apple byproducts during solid-state fermentation by *R. miehei* NRRL 5282. Error bars represent SD values of replicates.

FRAP was the highest at the 13th and 10th days for DA and LA, respectively, and subsequently reduced during later course of fermentation (Figure 6). In this context, the phenolic compound stability and antioxidant activity might be affected by a number of factors including enzymatic degradation (Santos and Linardi 2004), oxidation of phenolics and storage conditions of the extracts (Heras-Ramírez *et al.* 2012, Shah *et al.* 2015). It is remarkable that the FRAP increased in parallel to the β-glucosidase activity in both fermentation systems. Furthermore, the DPPH radical scavenging activity showed a slight increase up to the 5th day of fermentation (Figure 6). In the case of LA-based fermentation, this may be related to the TPC increase during the first phase of fermentation.

5.2.4. Yellow pitahaya residues

Despite the large antioxidant compound content of the fruit, fermentative production of bioactive phenolics from pitahaya residues has not been studied so far. In case of the lyophilized pitahaya (LP) sample, β-glucosidase activity found to be correlating with TPC ($r = 0.866$) and FRAP ($r = 0.41$) suggesting that the *R. miehei* β-glucosidase has an important role in the liberation of phenolics with reducing power (Figure 7). A similar trend has been reported during the bioconversion of cranberry and pineapple pomaces by *Lentinus edodes* and *Rhizopus oligosporus*, respectively (Zheng and Shetty 2000, Correia *et al.* 2004). β-Glucosidase activity on LP was maximal at the 10th day, while it was the highest at the 13th day in the case of oven-dried pitahaya (DP) residue. TPC of DP substrate exhibited a decrease up to the 13th fermentation day, then, a moderate increase was observed, potentially due to the β-glucosidase action (Figure 7). In contrast, TPC of LP did not decrease during the fermentation, moreover, it increased in parallel to the β-glucosidase activity. Concerning antioxidant properties, FRAP was 7.8 ± 0.3 mM Fe(II)/100 g DM at the 10th fermentation day when LP was used as substrate, while it was 5.8 ± 0.2 µM Fe(II)/100 g at the 3rd day on DP. The DPPH radical scavenging activity of DP residues showed an increase in parallel to the β-glucosidase activity (Figure 7).
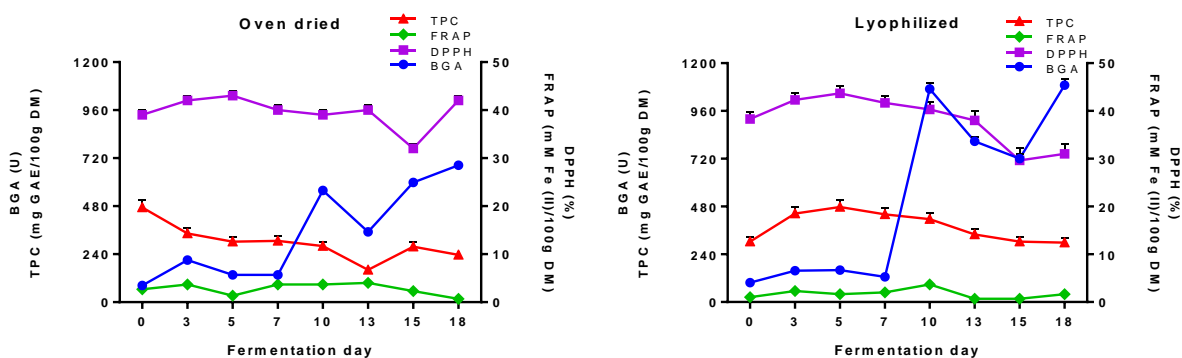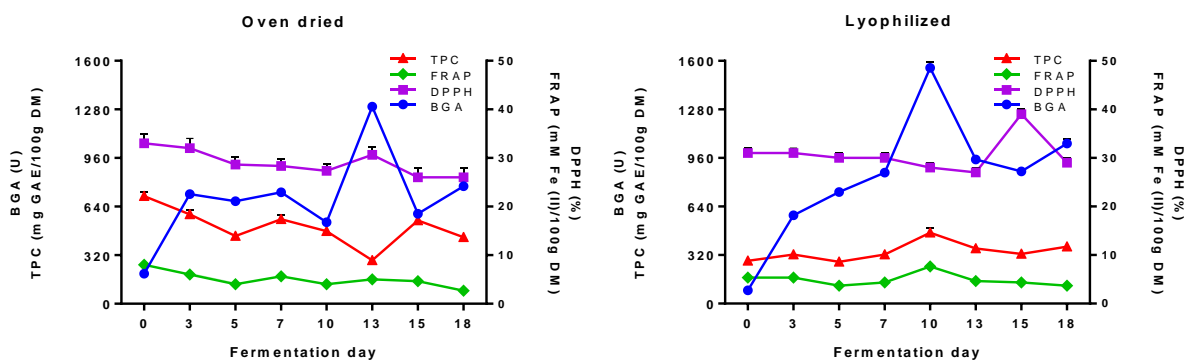
Figure 7. β-Glucosidase activity (BGA), total phenolic content (TPC), antioxidant power (FRAP) and free radical scavenging activity (DPPH) of oven-dried and lyophilized pitahaya byproducts during solid-state fermentation by *R. miehei* NRRL 5282. Error bars represent SD values of replicates.

Gallic acid, 4-hydroxybenzoic acid, (+)-catechin and (−)-epicatechin content of DP samples increased during the fermentation (Table 7). Concentration of vanillic acid, syringic acid, cinnamic acid, quercetin, polydatin and resveratrol compounds were below the detection limit. The quantity of gallic acid and (−)-epicatechin increased considerably and hydroxycinnamate *p*-coumaric acid was also detected after 15 days (Table 7). Hydroxycinnamates were previously identified in large quantities in red and white pitahaya varieties (Mahattanatawee *et al.* 2006). The initial concentration of gallic acid in LP samples was 2.46 ± 0.11 mg/100 g DM. After fermenting for 15 days, it increased to 10.38 ± 0.34 mg/100 g DM. The content of 4-hydroxybenzoic acid, vanillic acid, syringic acid, (+)-catechin, quercetin and polydatin decreased, while the content of *p*-coumaric acid, cinnamic acid, (−)-epicatechin and resveratrol did not change after fermentation.

Table 7. Liberation of phenolic substances (mg/100 g DM) from oven-dried pitahaya residues fermented with *R. miehei* NRRL 5282. Data were obtained from 50% (v/v) ethanol solution.

Fermentation time (day)	Gallic acid	4-Hydroxybenzoic acid	*p*-Coumaric acid	(+)-Catechin	(−)-Epicatechin
0	<DL*	<DL	<DL	<DL	<DL
3	1.24 ± 0.09	<DL	<DL	<DL	1.88 ± 0.30
7	7.88 ± 1.00	2.06 ± 0.38	<DL	4.49 ± 0.19	14.32 ± 0.49
15	37.93 ± 2.19	3.33 ± 0.31	0.77 ± 0.01	6.77 ± 0.29	25.49 ± 1.21

* <DL, below detection limit; detection limit values were 1.95 and 0.98 mg/L for (+)-catechin and quercetin, respectively, and 0.49 mg/L for the other phenolics tested. Values are averages from measurements performed in triplicates ± standard deviation.

There is little information in the literature regarding to the phenolic content and antioxidant activity of pitahaya samples. The study of Wu *et al.* (2006) detected 42.4 and

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39.7 mg GAE/100 g TPC of red pitahaya flesh and peel, respectively. They also concluded that this variety was rich in polyphenols and was a good source of antioxidants, for instance gallic acid, which acted as a strong inhibitor of the growth of B16F10 melanoma cancer cells. In addition, the antioxidant activity of the peel extracts was significantly higher than in fruit flesh (Wu *et al.* 2006) which may be attributed to the considerable amount of betalain and betacyanin in the peel (Harivaindaran *et al.* 2008). In another studies, 52.3 and 17.25 mg GAE/100 g TPC have been reported for white pitahaya flesh samples (Mahattanatawee *et al.* 2006, Chen *et al.* 2014).

5.2.5. Mango residues

Concerning mango, surprisingly, *R. miehei* did not grow on the lyophilized residue (LM), while it grew on oven-dried mango (DMG) sample. In DMG, a moderate negative correlation (r = -0.5) was identified between the β-glucosidase activity and the antioxidant potential. The β-glucosidase activity significantly increased (p < 0.05) during the fermentation process while the antioxidant parameters decreased (Figure 8). β-Glucosidase activity was maximal at the 18th day reaching 1734 ± 14 U activity. The highest total phenolic content, antioxidant power and scavenging capacity decreased during the course of the fermentation. Ajila *et al.* (2007) reported 54.7 - 109.7 mg GAE/g DM TPC for Indian Raspuri and Badami (ripe and raw) mango peel extracts. In another study, it was found that bound polyphenolic acids (gallic, protocatechuic and syringic) and flavonoid contents (kaempferol and quercetin) ranged from 8.1 to 29.5 and 0.101 to 0.392 mg/g, respectively, in the mango peel (Ajila *et al.* 2013). In our experiments, the maximum TPC determined was about 10 times lower than those measured by Ajila et al (2007), which can be attributed to the different mango variety, maturity grade, sugar content, substrate pretreatment method and/or extraction procedure. Dorta *et al.* (2012) studied the effects of different drying methods on the polyphenol and antioxidant activity of mango peels and seeds. In their assays, a decrease in antioxidant capacity was detected after oven-drying of the samples, while this was not observed for the freeze-dried residues. The freeze-drying method could improve the antiradical and scavenge capacity of the mango peel against ABTS⁄+ and free radicals, respectively, and inhibit the lipid peroxidation in the seed (Dorta *et al.* 2012).
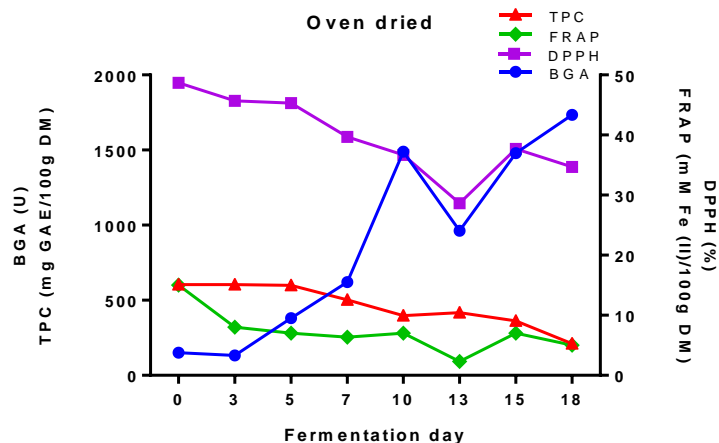
Figure 8. β-Glucosidase activity (BGA), total phenolic content (TPC), antioxidant power (FRAP) and free radical scavenging activity (DPPH) of oven-dried mango byproducts during solid-state fermentation by *R. miehei* NRRL 5282. Error bars represent SD values of replicates.

5.2.6. Naranjilla residues

There was no fungal growth on the oven-dried naranjilla (DN) samples probably due to the low pH condition generated by the substrate (pH = 3.0 ± 0.5). On lyophilized naranjilla (LN), the β-glucosidase activity and TPC significantly increased at the beginning of the fermentation (Figure 9) reaching the maximum activity at the 5th and 3rd day, respectively. Then, a significant drop in their yields was observed. The enzyme activity increased again at the 10th and 15th days followed by the increase of TPC at the 13th and 18th days. Therefore, it seems that the *R. miehei* β-glucosidase plays an important role in the free phenolic liberation with antioxidant capacity from LN residues. In this experiment, the maximal β-glucosidase activity, TPC, antioxidant power and DPPH inhibition values were 1976 ± 54 U, 2118 ± 100 mg GAE/100 g DM, 7.1 ± 1 mM Fe(II)/100 g DM and 34 ± 2%, respectively (Figure 9). In previous studies, the TPC and antioxidant properties of naranjilla samples from Ecuador (Gancel *et al.* 2008), Costa Rica (Acosta *et al.* 2009) and Colombia (Contreras-Calderón *et al.* 2011) were determined, and it was found that the TPC varied between 48 ± 3 and 505 ± 63 mg GAE/100 g fresh fruit depending on the sample origin. In a comprehensive assay, the DPPH radical inhibition activity of the Ecuadorian naranjilla was measured about 23% (Vasco *et al.* 2008), which is agreed with the overall values determined here during the fermentation process (Figure 9).
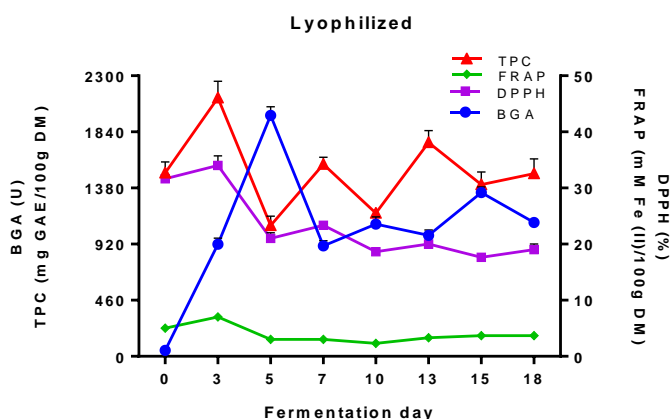
Figure 9. β-Glucosidase activity (BGA), total phenolic content (TPC), antioxidant power (FRAP) and free radical scavenging activity (DPPH) of lyophilized naranjilla byproducts during solid-state fermentation by *R. miehei* NRRL 5282. Error bars represent SD values of replicates.

5.2.7. Effect of substrate pretreatment on free phenolics enrichment

Proper selection of the sample preparation technique is important to achieve high phenolic yield during the fermentation. It influences not only the growth of fermenting fungus but affects the stability of the fruit byproducts and phenolics (Tseng and Zhao 2012). The β-glucosidase activities were higher when lyophilized substrates were used except for both grape residues (Figure 10A). However, the fermentation on DP sample resulted higher maximum TPC compared to the yield obtained on the lyophilized residue (Figure 10B). In the case of apple, pitahaya and white grape samples, there were no significant differences in the FRAP between the two pretreatments (Figure 10C). The DPPH scavenging activity obtained was not remarkable different between pretreatments except for the pitahaya and white grape samples that showed the highest activity on lyophilized substrate (Figure 10D).

In general, some bioactive compounds in fruit byproducts were sensitive to heat and may undergo chemical degradation, isomerization or polymerization when subjected to elevated temperature (Yu and Ahmedna 2013). Freeze-drying at low temperature and vacuum conditions is a more gently process preventing the bioactive compounds, especially the heat sensitive polyphenolics, from thermal degradation (Michalczyk *et al.* 2009).
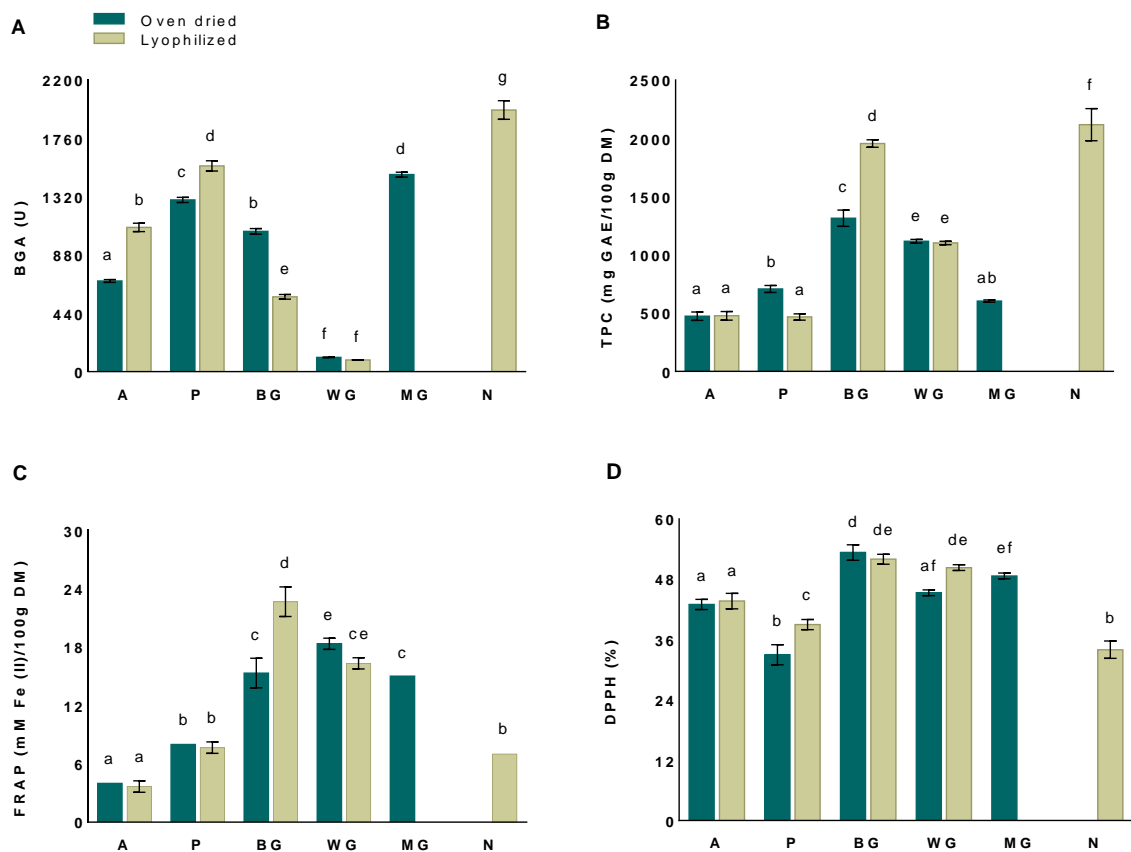
Figure 10. Comparison between pretreatment methods and fruit residue types according to the best β-glucosidase activity (A), total phenolic content (B) and antioxidant activity (C and D) data achieved on oven-dried and lyophilized residues during solid-state *R. miehei* NRRL 5282 bioconversion. A: apple, P: pitahaya, BG: black grape, WG: white grape, MG: mango, N: naranjilla. Different letters on the bars show significant differences according to two-way ANOVA followed by Tukey's Multiple Comparison Test (p < 0.05; independent variables: pretreatment and the fruit residue).

The optimal fermentation day to achieve the highest β-glucosidase and antioxidant activities and TPC yield depended on the type of fruit and was found to be different for the β-glucosidase activity and the antioxidant potential. Highest β-glucosidase activity yields were achieved at 5th day for naranjilla, 10th day for pitahaya, 15th day for white and black grapes and 18th day for apple and mango. On the other hand, the FRAP exposed the maximum values at the 3rd day for black grape, mango and naranjilla, 5th day for white grape, 10th day for pitahaya and 13th day for apple. The highest TPC for DA, and the highest TPC, FRAP and DPPH radical scavenging activity for DP and DMG substrates presented on Figure 10 were obtained at the initial phase of fermentation. Then, a decrease was observed in these values. This may be caused by the action of phenolics-degrading and/or stress induced polymerizing enzymes of the fermenting fungus, similar to that observed for black grape substrates (see 5.2.1.), and reported in other studies (Vattem *et al.*

2004, Dulf *et al.* 2016). However, the recovery of some small phenolics of DP sample was increased during the fermentation (see Table 7), which may be explained by the hydrolysis of total phenolic compounds.

5.3. Enzyme treatment of oven-dried and lyophilized fruit residues

Carbohydrate cleaving enzymes can rapidly and totally hydrolyze plant tissue polymers, and some studies pointed out that these enzymes can mobilize free phenolics during the hydrolysis (see 2.8.). Therefore, in the following experiments, both oven-dried and lyophilized fruit residues were treated with a cellulolytic enzyme cocktail produced by *R. miehei* NRRL 5282 on wheat bran substrate (S1 samples). The cellulase cocktail exhibited 75, 203, 35 and 1727 U/g DM of FPase, endoglucanase, cellobiohydrolase and β-glucosidase activities, respectively. To loosen the cell wall matrix in the fruit residues, the reaction mixture was supplemented by *Aspergillus niger* produced pectinase (S2 samples). The TPC and antioxidant activities (FRAP and DPPH radical scavenging) were determined after 5-h treatments, and data were compared to those measured in enzyme-free samples (C1 samples) and samples treated with pectinase exclusively (C2 samples).

5.3.1. Total phenolics and antioxidant activity after enzyme hydrolysis

Black grape pomace

TPC and FRAP of LBG pomace showed significant ($p < 0.05$) increase after cellulase and cellulase-pectinase treatments (Figures 11A and B). For DBG samples, cellulase treatment also improved the TPC and FRAP compared to the enzyme-free control, however, interestingly, this was not observed when the reaction mixture was supplemented with pectinase. The highest TPCs, 5221 mg and 5393 mg GAE/100 g substrate, were obtained on LBG sample after cellulase and cellulase-pectinase treatments, respectively. In parallel, the FRAP of the lyophilized sample displayed about 1.5-fold and 1.3-fold increment, respectively. Considering enzyme-free samples, the DPPH radical scavenging activity was slightly higher in DBG than in LBG (Figure 11C). However, the pectinase treatment of DBG and the cellulase-pectinase treatment of LBG caused an increase in the DPPH radical scavenging activity compared to the ezyme-free control. Overall, the *R. miehei* cellulase-pectinase combined treatment was effective to liberate free phenolics and increase the antioxidant activities of the LBG samples. In a previous study

testing tannase treatment of grape pomace, the TPC increased by six-fold, and it was observed that tannase combined with pectinase could release gallic acid from galloylated catechins and thus, increased the antioxidant activity of the extracts (Chamorro *et al.* 2012).
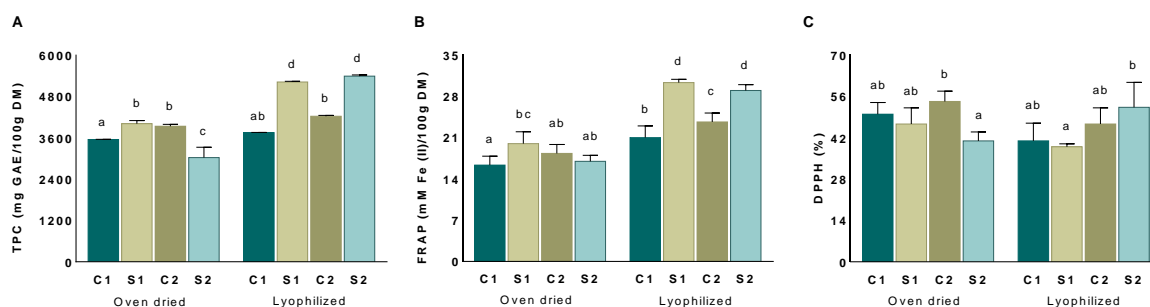
Figure 11. Effect of *R. miehei* NRRL 5282 cellulolytic cocktail (S1), *A. niger* pectinase (C2) and *R. miehei* cellulase-*A. niger* pectinase (S2) treatments on the TPC (A) and FRAP (B) and DPPH scavenging activity (C) of oven-dried and lyophilized black grape pomaces. Enzyme-free (C1) mixture was used as control. Different letters above the columns indicate significant differences according to two-way ANOVA followed by Tukey's Multiple Comparison Test (p < 0.05; independent variables: pretreatment and the type of the enzyme treatment).

White grape pomace

The LWG residue displayed significantly higher (p < 0.05) TPC and FRAP compared to DWG sample (Figures 12A and B). All three types of enzyme treatments increased (p < 0.05) the TPC of LWG sample, while none of the enzymes used affected the TPC in DWG residue.
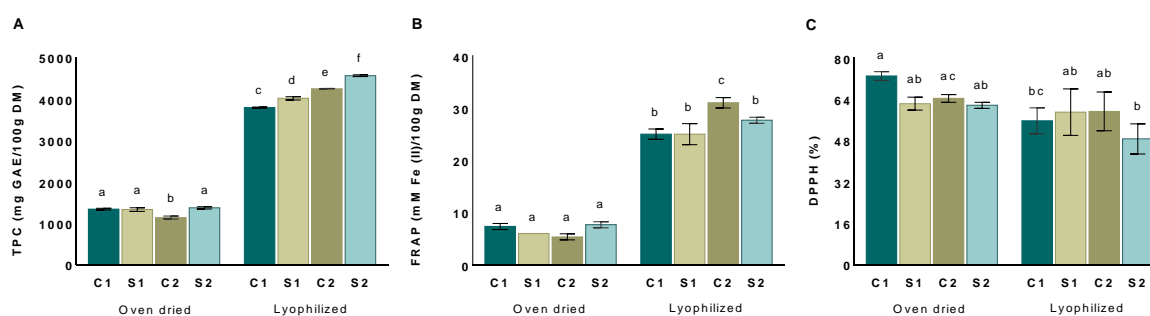
Figure 12. Effect of R. miehei NRRL 5282 cellulolytic cocktail (S1), A. niger pectinase (C2) and R. miehei cellulase-A. niger pectinase (S2) treatments on the TPC (A) and FRAP (B) and DPPH scavenging activity (C) of oven-dried and lyophilized white grape pomaces. Enzyme-free (C1) mixture was used as control. Different letters above the columns indicate significant differences according to two-way ANOVA followed by Tukey's Multiple Comparison Test (p < 0.05; independent variables: pretreatment and the type of the enzyme treatment).

The a maximum TPC yield was 4564 ± 14.3 mg GAE/100 g DM obtained after the cellulase-pectinase combined treatment of LWG sample. Compared to enzyme-free sample, only the pectinase treatment caused a significant ($p < 0.05$) increase in FRAP of LWG residue achieving a 30.8 mM Fe (II)/100 g DM maximum yield. However, the DPPH radical scavenging activity of enzyme treated samples did not change significantly compared to the corresponding enzyme-free control (Figure 12C).

Apple residues

For apple residues, both cellulase and cellulase-pectinase treatments positively affected the TPC yield. After 5 h incubation, the overall TPC showed 1.7 and 1.3 times increase ($p < 0.05$) in cellulase treated DA and LA samples, respectively, compared to that of untreated samples (Figure 13A). Furthermore, statistically significant ($p < 0.05$) increase in the phenolics yield was also observed for the samples treated with pectinase supplemented cellulolytic enzymes. Oven-dried and lyophilized samples subjected to combined cellulase-pectinase treatment resulted in the maximum phenolic contents (1521 ± 90 and 1732 ± 17 mg GAE/100 g, respectively). This could be attributed to the cell wall depolymerization by pectinases, which is responsible for releasing soluble compounds, including phenolic substances, from the residues (Will *et al.* 2000).

Figure 13. Effect of *R. miehei* NRRL 5282 cellulolytic cocktail (S1), *A. niger* pectinase (C2) and *R. miehei* cellulase-*A. niger* pectinase (S2) treatments on the TPC (A) and FRAP (B) and DPPH scavenging activity (C) of oven-dried and lyophilized apple residues. Enzyme-free (C1) mixture was used as control. Different letters above the columns indicate significant differences according to two-way ANOVA followed by Tukey's Multiple Comparison Test ($p < 0.05$; independent variables: pretreatment and the type of the enzyme treatment).

In most studies, the optimal pectinase concentration for liberation of phenolics is different, which variation is due to the variations of enzyme spectrum and -activities between the commercially distibuted enzyme products. The Pectinex AFP L-4, Pectinex Yield Mash and Pectinex XXL, for example, proved to be strong biocatalysts for the

liberation of phenolics from apple residues, while the Pectinex Ultra SPL has no effect on the free phenolics yield (Oszmiański *et al.* 2011). The TPC of LA substrate was higher in enzyme treated and untreated systems compared to those detected in the corresponding DA samples. The antioxidant activity followed this trend (Figures 13B and C); however, DPPH radical scavenging activity detected in cellulase-pectinase treated DA residues was slightly superior compared to the other samples. The DPPH radical scavenging activity of enzyme treated LA samples did not differ from that of untreated control.

Pitahaya residues

TPC and FRAP of the DP and the LP samples exhibited an increase after a 5-h incubation with the cellulase and cellulase-pectinase cocktails (Figures 14A and B). Since the pectin content of the pitahaya residues is relatively low (0.12-0.27 g/100 g fruit) compared to those of grapes (0.7-0.8 g/100 g) and apple (0.25-0.63 g/100 g) (Mahattanatawee *et al.* 2006), the pectinase treatment was expected to have insignificant effect on the extractable phenolic content. Remarkably, the added pectinase resulted in about 1.4- and 1.2-fold increase in the TPC yield on DP and LP, respectively, compared to the non-treated sample. The cellulase-pectinase cocktail further enhanced the TPC by 1.1- and 2.2-fold reaching maximum yields of 2207 ± 43 and 1314 ± 19 mg GAE/100 g for DP and LP, respectively. Furthermore, the cellulase-pectinase combined treatment affected positively the DPPH radical scavenging activity of the DP substrate (Figure 14C). To our knowledge, TPC and antioxidant activity of cellulolytic and pectinolytic enzyme treated pitahaya residues have not been reported so far.
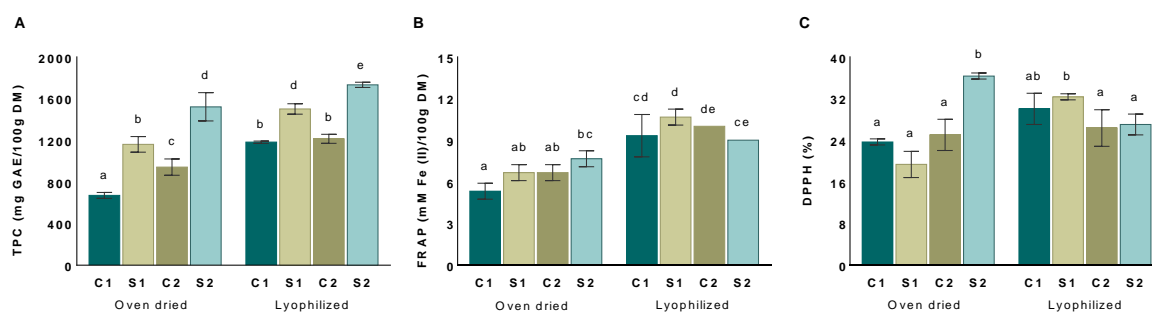

Figure 14. Effect of *R. miehei* NRRL 5282 cellulolytic cocktail (S1), *A. niger* pectinase (C2) and *R. miehei* cellulase-*A. niger* pectinase (S2) treatments on the TPC (A) and FRAP (B) and DPPH scavenging activity (C) of oven-dried and lyophilized pitahaya residues. Enzyme-free (C1) mixture was used as control. Different letters above the columns indicate significant differences according to two-way ANOVA followed by Tukey's Multiple Comparison Test ($p < 0.05$; independent variables: pretreatment and the type of the enzyme treatment).

Mango residues

Concerning mango, only the oven-dried residue (DMG) was subjected to enzyme treatment. All three types of enzyme treatments increased the TPC yields significantly (p < 0.05) (Figure 15A), while only the pectinase and the cellulase-pectinase treatments improved the FRAP values (Figure 15B) The highest TPC yield (2350 ± 38 mg GAE/100 g DM) and DPPH scavenging activity (47 ± 6%) were in the extract obtained by cellulase-pectinase treatment (Figures 15A and C). FRAP was maximum in cellulase-pectinase treated samples (20.1 ± 0.8 mM Fe(II)/100 g DM).
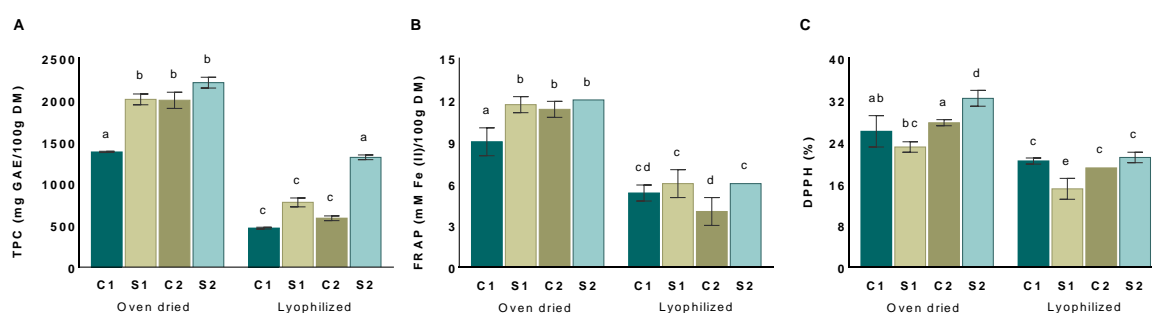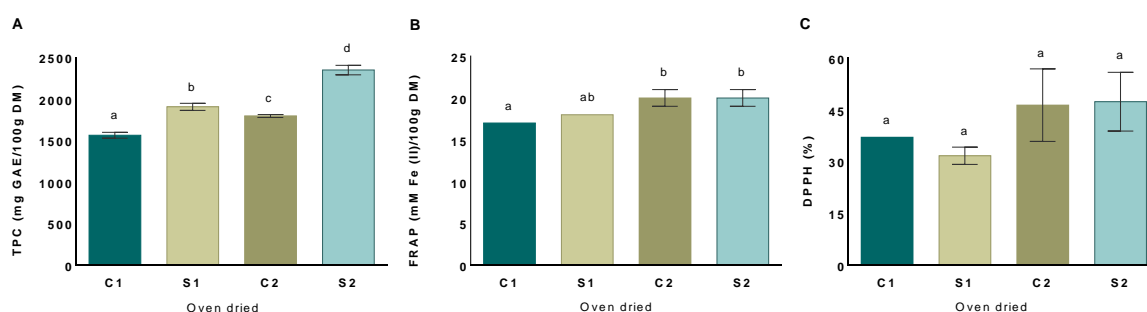

Figure 15. Effect of *R. miehei* NRRL 5282 cellulolytic cocktail (S1), *A. niger* pectinase (C2) and *R. miehei* cellulase-*A. niger* pectinase (S2) treatments on the TPC (A) and FRAP (B) and DPPH scavenging activity (C) of oven-dried mango residues. Enzyme-free (C1) mixture was used as control. Different letters above the columns indicate significant differences according to two-way ANOVA followed by Tukey's Multiple Comparison Test (p < 0.05; independent variables: pretreatment and the type of the enzyme treatment).

Naranjilla residues

The TPC and the FRAP of the LN showed a significant increase (p < 0.05) in the cellulase treated sample compared to the untreated one. The DPPH radical scavenging activity improved only for the oven-dried residue (Figure 16). Among all naranjilla extracts analyzed, the highest TPC and FRAP yields were in the oven-dried enzyme-free extract, however, the values decreased after the cellulase treatment. In contrast, LN sample exhibited improved phenolics yield and reducing power after the cellulase-assisted extraction: the TPC and FRAP values increased to 1.5 and 1.3 times, respectively, compared to enzyme-free sample. The maximum TPC and FRAP in cellulase treated LN sample were 1654 ± 69 mg GAE/100 g DM and 10 ± 0.5 mM Fe(II)/100 g DM, respectively.
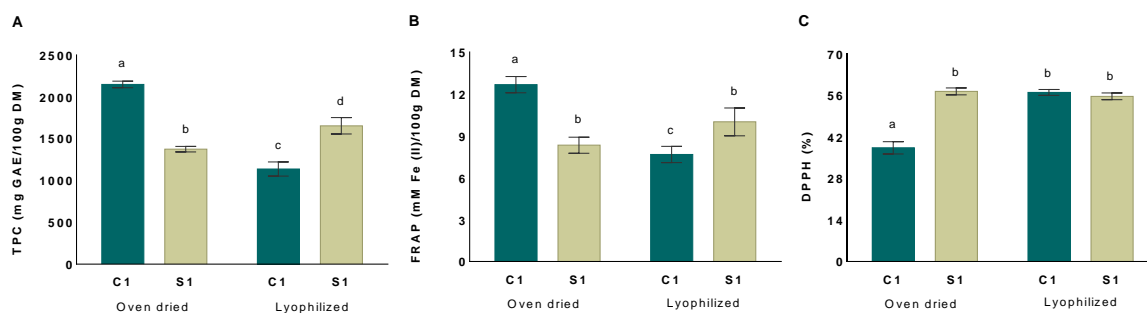
Figure 16. Effect of *R. miehei* NRRL 5282 cellulolytic cocktail (S1) treatment on the TPC (A) and FRAP (B) and DPPH scavenging activity (C) of oven-dried and lyophilized naranjilla residues. Enzyme-free (C1) mixtures were used as controls. Different letters above the columns indicate significant differences according to two-way ANOVA followed by Tukey's Multiple Comparison Test ($p < 0.05$; independent variables: pretreatment and the enzyme treatment).

5.3.2. Analysis of individual phenolic compounds after enzyme treatments

Tables 8, 9 and 10 summarize the concentrations of distinct phenolic compounds belonging to the groups of hydroxybenzoates, hydroxycinnamates, flavan-3-ols, flavonols and stilbenoids in black grape, apple, pitahaya and mango samples before and after enzyme treatments. Analytical HPLC data indicate that total yield of the tested individual phenolics increased after enzyme treatments, which agrees with the TPC results. However, it is worth to note that the Folin-Ciocalteu's phenol reagent used for the TPC assay reacts not only with phenolic compounds but also with amino acids, sugars and other reducing substances present in the extracts (Rapisarda *et al.* 1999). Therefore, differences between the degree of phenolic content determined by Folin-Ciocalteu's phenol reagent and analytical HPLC method are expected.

With regards to the cellulase-pectinase treatments, chromatographic measurements showed a decrease in the amount of some individual phenolics. This was most obvious for the data of the DBG and DMG extracts (Tables 8 and 10). A probable reason for this phenomenon can be the transformation of the phenolics into volatile compounds during incubation (Sandri *et al.* 2011, Shah *et al.* 2015) and the release of additional phenolic acid derivatives from their ester linkages by the action of the enzyme.

Table 8. Concentration of phenolic compounds (mg/100 g DM) detected in oven-dried black grape, apple and yellow pitahaya extracts pre- and post-enzyme treatment. Data were obtained from the supernatant of the reaction mixture.

Compounds	Fruit residues								
	Black grape			Apple			Pitahaya		
	Control*	*R. miehei* cellulase	*R. miehei* cellulase + *A. niger* pectinase	Control	*R. miehei* cellulase	*R. miehei* cellulase + *A. niger* pectinase	Control	*R. miehei* cellulase	*R. miehei* cellulase + *A. niger* pectinase
Gallic acid	2.28 ± 0.32 a	6.21 ± 0.46 b	3.61 ± 0.26 ab	2.17 ± 0.24 a	<DL	0.58 ± 0.06 a	19.68 ± 1.31 c	27.53 ± 2.02 d	34.16 ± 2.95 e
4-Hydroxybenzoic acid	<DL**	6.31 ± 0.52 a	2.88 ± 0.38 b	<DL	1.99 ± 0.17 b	1.78 ± 0.28 b	5.44 ± 0.52 a	5.04 ± 0.64 a	5.93 ± 0.84 a
Vanillic acid	2.11 ± 0.21 a	12.61 ± 0.93 b	11.27 ± 0.39 b	<DL	3.56 ± 0.34 a	3.18 ± 0.31 a	5.16 ± 0.35 a	11.99 ± 1.13 b	15.86 ± 1.35 c
Syringic acid	2.27 ± 0.31 a	7.97 ± 0.80 b	14.58 ± 0.81 c	<DL	2.09 ± 0.21 a	2.29 ± 0.33 a	5.94 ± 0.66 b	6.19 ± 0.93 b	7.69 ± 0.77 b
p-Coumaric acid	<DL	2.56 ± 0.16 a	10.35 ± 0.35 b	<DL	<DL	1.78 ± 0.37 a	2.61 ± 0.28 a	3.21 ± 0.60 ac	4.41 ± 0.84 c
Cinnamic acid	<DL	<DL	<DL	<DL	<DL	<DL	1.59 ± 0.18 a	1.66 ± 0.37 a	1.73 ± 0.35 a
(+)-Catechin	3.59 ± 0.42 a	22.14 ± 1.42 b	7.67 ± 0.52 c	<DL	8.93 ± 0.55 c	6.22 ± 0.57 c	22.86 ± 2.08 b	18.03 ± 0.78 d	3.74 ± 0.51 a
(−)-Epicatechin	3.44 ± 0.38 a	21.73 ± 0.91 b	7.82 ± 0.42 c	<DL	2.59 ± 0.26 a	3.35 ± 0.41 a	14.02 ± 0.93 d	6.95 ± 0.84 c	10.87 ± 1.21 e
Quercetin	<DL	6.11 ± 0.32	<DL	<DL	<DL	<DL	<DL	<DL	<DL
Polydatin	<DL	3.97 ± 0.34 a	<DL	<DL	<DL	<DL	22.56 ± 1.17 b	28.56 ± 1.11 c	36.56 ± 3.15 d
Resveratrol	<DL	<DL	<DL	<DL	1.99 ± 0.24 a	<DL	2.46 ± 0.44 a	2.82 ± 0.62 a	<DL
Total	13.69	89.61	58.18	2.17	21.15	19.18	102.32	111.98	120.95

* without enzyme treatment; ** <DL, below detection limit; detection limit values were 1.95 and 0.98 mg/L for (+)-catechin and quercetin, respectively, and 0.49 mg/L for the other phenolics tested. Values are the average of measurements performed in triplicates ± standard deviation; mean values within a row with different letters are significantly differ according to two-way ANOVA followed by Tukey's Multiple Comparison Test (p < 0.05; independent variables: fruit residue and type of the enzyme treatment).

Table 9. Concentration of phenolic compounds (mg/100 g DM) detected in lyophilized black grape, apple and yellow pitahaya extracts pre- and post-enzyme treatment. Data were obtained from the supernatant of the reaction mixture.

Compounds	Fruit residues								
	Black grape			Apple			Pitahaya		
	Control*	*R. miehei* cellulase	*R. miehei* cellulase + *A. niger* pectinase	Control	*R. miehei* cellulase	*R. miehei* cellulase + *A. niger* pectinase	Control	*R. miehei* cellulase	*R. miehei* cellulase + *A. niger* pectinase
Gallic acid	3.05 ± 0.59 a	43.77 ± 4.10 b	37.23 ± 3.10 b	<DL	5.11 ± 0.74 a	3.91 ± 0.86 a	2.24 ± 0.35 a	2.61 ± 0.34 a	2.56 ± 0.24 a
4-Hydroxybenzoic acid	4.21 ± 0.66 a	68.23 ± 4.27 b	67.45 ± 6.11 b	2.35 ± 0.41 a	8.32 ± 0.98 c	7.30 ± 0.92 c	1.53 ± 0.21 a	1.67 ± 0.38 a	1.61 ± 0.25 a
Vanillic acid	2.75 ± 0.37 a	60.26 ± 6.11 b	60.27 ± 6.64 b	1.52 ± 0.35 a	14.64 ± 1.42 c	12.49 ± 0.57 c	<DL	2.87 ± 0.34 a	2.84 ± 0.36 a
Syringic acid	2.45 ± 0.48 a	33.06 ± 2.68 b	35.46 ± 3.47 b	0.76 ± 0.15 a	3.23 ± 0.44 a	3.92 ± 0.49 a	2.08 ± 0.18 a	2.29 ± 0.24 a	2.22 ± 0.16 a
p-Coumaric acid	1.83 ± 0.36 a	10.35 ± 1.16 b	15.36 ± 2.11 c	0.89 ± 0.09 a	0.95 ± 0.11 a	2.97 ± 0.67 a	<DL	1.80 ± 0.19 a	1.81 ± 0.18 a
Cinnamic acid	<DL**	1.51 ± 0.36 a	1.49 ± 0.35 a	<DL	<DL	<DL	<DL	<DL	<DL
(+)-Catechin	7.48 ± 0.89 a	209.83 ± 22.31 b	200.51 ± 20.33 b	<DL	33.23 ± 3.62 c	2.81 ± 0.55 a	<DL	<DL	<DL
(−)-Epicatechin	8.75 ± 0.82 a	215.81 ± 17.17 b	188.13 ± 19.93 b	6.71 ± 0.84 a	10.05 ± 1.22 a	99.92 ± 9.68 c	2.79 ± 0.34 a	2.83 ± 0.28 a	2.77 ± 0.34 a
Quercetin	<DL	8.54 ± 0.81 a	7.61 ± 0.71 a	<DL	<DL	6.49 ± 0.75 a	<DL	<DL	<DL
Polydatin	2.63 ± 0.43 a	<DL	<DL	<DL	<DL	<DL	3.35 ± 0.41 a	3.51 ± 0.37 a	3.49 ± 0.36 a
Resveratrol	<DL	4.28 ± 0.61 a	4.67 ± 0.53 a	<DL	0.51 ± 0.02 b	<DL	<DL	2.17 ± 0.19 c	2.12 ± 0.17 c
Total	33.15	655.64	618.18	12.23	76.04	139.81	11.99	19.75	19.42

* without enzyme treatment; ** <DL, below detection limit; detection limit values were 1.95 and 0.98 mg/L for (+)-catechin and quercetin, respectively, and 0.49 mg/L for the other phenolics tested. Values are the average of measurements performed in triplicates ± standard deviation; mean values within a row with different letters are significantly differ according to two-way ANOVA followed by Tukey's Multiple Comparison Test ($p < 0.05$; independent variables: fruit residue and type of the enzyme treatment).

Table 10. Concentration of phenolic compounds (mg/100 g DM) detected in oven-dried mango extract pre- and post-enzyme treatment. Data were obtained from the supernatant of the reaction mixture.

Compounds	Control*	*R. miehei* cellulase	*R. miehei* cellulase + *A. niger* pectinase
Gallic acid	3.29 ± 0.48 a	80.25 ± 5.10 b	6.98 ± 0.10 a
4-Hydroxybenzoic acid	<DL**	33.39 ± 2.77 a	3.24 ± 0.11 b
Vanillic acid	<DL	21.61 ± 2.11 a	2.94 ± 0.64 b
Syringic acid	<DL	10.48 ± 1.68 a	2.2 ± 0.47 b
p-Coumaric acid	<DL	3.84 ± 0.16 a	1.8 ± 0.11 b
Cinnamic acid	<DL	<DL	<DL
(+)-Catechin	<DL	2.67 ± 0.31 a	2.98 ± 0.33 a
(−)-Epicatechin	6.29 ± 0.89 a	163.75 ± 12.17 b	13.41 ± 2.93 a
Quercetin	<DL	10.35 ± 0.83 a	5.93 ± 0.61 b
Polydatin	<DL	<DL	2.27 ± 0.21 a
Resveratrol	<DL	<DL	<DL
Total	9.58	326.34	41.75

* without enzyme treatment; ** <DL, below detection limit; detection limit values were 1.95 and 0.98 mg/L for (+)-catechin and quercetin, respectively, and 0.49 mg/L for the other phenolics tested. Values are the average of measurements performed in triplicates ± standard deviation; mean values within a row with different letters are significantly differ according to one-way ANOVA followed by Tukey's Multiple Comparison Test ($p < 0.05$).

After the cellulase treatment, (+)-catechin was found in the largest concentration in DBG and LBG pomaces (Tables 8 and 9), while (−)-epicatechin content was the highest in LBG and DMG samples (Tables 9 and 10). Compared to the control treatment, the quantities of (+)-catechin and (−)-epicatechin in the DBG and LBG residues had increased about 6 and 26 times, respectively, while the amount of (−)-epicatechin enhanced by 27-fold in DMG samples ($p < 0.05$). The enzyme treatments increased the (+)-catechin content in DMG residues as well, although it remained in low concentration (Table 10). The contents of these flavan-3-ols also increased when apple residues were incubated with the cellulase cocktail. Furthermore, chromatography detected an about 10 times increase ($p < 0.05$) in the (−)-epicatechin content of LA after addition of pectinase (Table 9). This could ascribe to the decomposition of apple pectin fiber, supporting the liberation of epicatechin from its insoluble, fiber- and pectin bounded form. The bioavailability of the entrapped apple epicatechin is limited (Hollands *et al.* 2013), which could be enhanced by the *R. miehei* cellulase-*A. niger* pectinase combined treatment. However, a decrease in the flavan-3-ol content was detected in some samples after pectinase addition, which may be due to both oxidation and several polymerization reactions (Ye *et al.* 2014).

As a result of enzyme treatments, the content of hydroxybenzoates (gallic, 4-hydroxybenzoic, vanillic and syringic acids) and hydroxycinnamates (*p*-coumaric and cinnamic acids) increased by different degrees in both oven-dried and lyophilized pomaces. From DMG samples, for instance, the cellulase treatment markedly supported the mobilization of most of these molecules (Table 10). The LBG residues contained the highest level of the hydroxybenzoates and hydroxycinnamates analyzed, with concentrations from 1.49 to 68.19 mg/L, respectively. Except for cinnamic acid, the content of these above phenolics increased by about 10 to 20 times ($p < 0.05$) after the enzyme treatments (Table 9). The same trend was reported during the treatment of grape pomace from red wine using Novoferm 106 pectinolytic and Cellubrix ®L cellulolytic enzyme mixtures (Maier *et al.* 2008). The DP samples showed the highest initial gallic acid concentration among the fruit residues studied. Moreover, its content increased to 1.7 times ($p < 0.05$) after cellulase-pectinase treatment (Table 8). The gallic acid is the major contributor to hydroxybenzoates and has attracted considerable interests because of its antioxidant properties (Strlič *et al.* 2002).

The detected stilbenoids during enzyme treatment were the resveratrol and its glycosidic form, the polydatin. These compounds are the major stilbenoids found in foods of plant origin and display a broad variety of beneficial effects on human health (Ravagnan *et al.* 2013). Except for DP samples, the initial content of resveratrol in residues was below the detection limit (Tables 8, 9 and 10). This low level may be due to the poor extractability of resveratrol from the residues in the applied aqueous environment. However, the resveratrol yield increased in all lyophilized and the DA samples after cellulase treatment. Polydatin, was found in the largest concentration in the DP samples. Compared to the initial content, it showed an about 1.6 times increase ($p < 0.05$) during enzyme treatments (Table 8). The amount of polydatin in DP residues was higher than that of the resveratrol, which finding is consistent with the results of studies on grape samples (Romero-Pérez *et al.* 1999).

Quercetin was observed after cellulase treatment in DBG (6.11 mg/100 g DM), LBG (8.54 mg/100 g DM) and DMG (10.35 mg/100 g DM) pomace extracts (Tables 8, 9 and 10), and after cellulase-pectinase treatment of LBG (7.61 mg/100 g DM), LA (6.49 mg/100 g DM) and DBG (5.93 mg/100 g DM) samples (Tables 9 and 10). In line with our previous study testing sour cherry pomace (Krisch *et al.* 2012), it could be a result of the hydrolysis of the quercetin aglycone from quercetin glycosides by the *R. miehei* cellulase. No quercetin was detected in the pitahaya samples.

After HPLC analysis of phenolic compounds of the fruit extracts, their antioxidant activity was also determined by FRAP and DPPH methods. In this assay, the syringic and gallic acids and the flavonoid (+)-catechin exhibited the highest antioxidant capacity among the tested phenolic compounds ($p < 0.05$) (Figure 17). After evaluation of results, correlation could be observed between the antioxidant activity and concentration of some individual phenolics after enzyme treatments. The above phenolics could be responsible for the increase of antioxidant potential in the samples after enzyme treatment.
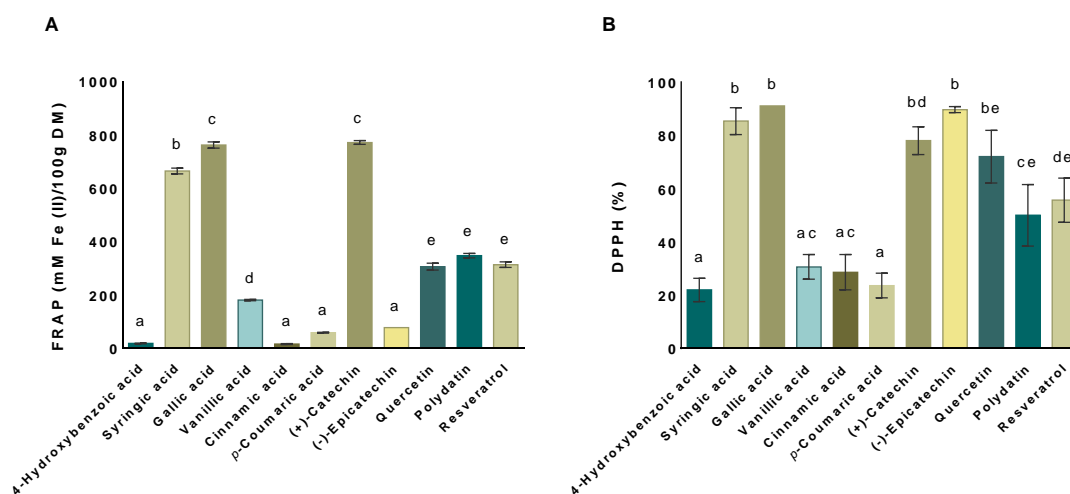
Figure 17. FRAP (A) and DPPH scavenging (B) activity of phenolic compounds. Different letters above the columns indicate significant differences according to one-way ANOVA followed by Tukey's Multiple Comparison Test ($p < 0.05$).

5.4. Antimicrobial activity of selected phenolic extracts

In these studies, additional bioactive properties, i.e. the antimicrobial, anti-QS and anti-biofilm forming activities of selected enzyme-free and enzyme-treated fruit extracts were screened and compared. In addition, the above examined 4-hydroxybenzoic acid, syringic acid, gallic acid, vanillic acid, cinnamic acid, *p*-coumaric acid, (+)-catechin, (−)-epicatechin, quercetin, polydatin and resveratrol compounds were also subjected to bioactivity analyses in order to study the possible correlation between the activity changes in the extracts and the increase in the phenolic compound concentrations after the enzyme treatments. The foodborne pathogen and/or spoilage *Bacillus subtilis*, *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium, *Pseudomonas putida* and *Pseudomonas aeruginosa* bacteria were involved

in the antimicrobial and anti-biofilm forming activity tests. The model organism *Chromobacterium violaceum* was used in the anti-QS activity assays.

5.4.1. Screening of antimicrobial capacity on solid medium

In this experiment, antimicrobial activity of enzyme-free and treated fruit extracts and individual phenolics was screened and compared by using a disk-diffusion method. Majority of the fruit residue extracts inhibited the growth of the bacteria studied (Table 11). Overall, *B. subtilis*, *S. aureus* and *S. enterica* were the most sensitive bacteria to all extracts investigated. The lowest growth inhibition effect was detected against *L. monocytogenes* and the *Pseudomonas* strains. By comparing the fruit samples, in general, extracts made from enzyme treated and untreated grape pomaces presented slightly stronger antimicrobial effect against the tested bacteria than those observed for apple, pitahaya, mango and naranjilla residues (Table 11). Most grape samples resulted in inhibition zones between 4 and 8 mm, whereas this was mainly between 1 to 5 mm for the other fruits tested. Grapes were effective growth inhibitors of *S. aureus*, *S. enterica* and both *Bacillus* strains; for instance, the cellulase treated DBG and DWG samples presented 8- and 9-mm inhibition zone, respectively, against the *S. enterica*. The white grape extracts exhibited stronger inhibition against the growth of *B. subtilis* and *B. cereus* than the black grape ones. Apple extracts were the most effective against *B. subtilis* and *S. enterica*, while the *B. subtilis*, *B. cereus*, *S. aureus* and *S. enterica* were the most sensitive bacteria to pitahaya extracts. In the case of many residues, the cellulase and/or the cellulase-pectinase combined treatments enhanced the antimicrobial activity. For instance, the cellulase and cellulase-pectinase treatments enhanced the inhibitory effect of the DP extracts against *B. cereus* and *S. aureus*, respectively. All three enzyme treatments supported the inhibition potential of DP residue against *P. aeruginosa*. Both the pectinase treated DP and LP samples considerably suppressed the growth of *S. enterica*, and the pectinase increased the activity of LP samples against *B. cereus* and *S. aureus* as well. The cellulase treatment remarkably increased the growth inhibitory effect of DMG samples against *L. monocytogenes* and *S. aureus* as compared to enzyme-free extract (Table 11). And the cellulase-pectinase treatment positively affected the inhibitory potential of both the DA and LA samples against *S. aureus*, MRSA and *E. coli* (Table 11).

Table 11. Antimicrobial activity of fruit residue extracts against pathogenic and spoilage bacteria before and after treatment with *R. miehei* cellulase and/or *A. niger* pectinase. Results were calculated from the growth inhibition zone (mm) measured around the paper disk.

Fruit residues		Growth inhibition*								
		B. subtilis	*B. cereus*	*L. monocytogenes*	*S. aureus*	MRSA	*E. coli*	*S. enterica*	*P. putida*	*P. aeruginosa*
Antibiotic control										
Streptomycin (100 μg/mL)		++++	++++	++++	++++	++++	++++	++++	++++	++++
Black grape										
Oven-dried	Enzyme-free	++	+	+	++	+	+	++	NI	+
	Cellulase	++	+	++	++	+	++	+++	+	+
	Pectinase	+	+	+	++	+	+	+	NI	+
	Cellulase + Pectinase	++	++	NI	+++	++	+	+	+	+
Lyophilized	Enzyme-free	++	+	+	+	+	+	++	+	+
	Cellulase	++	+++	+	++	+	+	+	+	NI
	Pectinase	+	++	+	++	+	+	++	+	+
	Cellulase + Pectinase	++	+	+	++	++	++	+++	+	+
White grape										
Oven-dried	Enzyme-free	+++	+	++	++	+	++	+	+	NI
	Cellulase	++	NI	++	++	++	+++	++++	+	+
	Pectinase	+++	+++	++	+	+	+	NI	NI	NI
	Cellulase + Pectinase	++	+++	+	++	+	+	+	+	+
Lyophilized	Enzyme-free	+++	+	++	+	+	+	+	+	NI
	Cellulase	++	++	+	++	++	++	++	+	+
	Pectinase	+++	+++	+	+	+	+	+	+	NI
	Cellulase + Pectinase	+++	++	++	++	++	++	++	+	+
Apple										
Oven-dried	Enzyme-free	++	+	NI	+	+	+	++	NI	NI
	Cellulase	++	++	NI	++	+	+	+++	+	+
	Pectinase	++	+	NI	+	+	+	++	NI	NI
	Cellulase + Pectinase	NI	++	+	++	++	++	++	+	+
Lyophilized	Enzyme-free	+	+	+	+	+	+	+	NI	NI
	Cellulase	+++	+	+	+	+	+	++	+	+
	Pectinase	+	+	+	+	+	+	++	+	NI
	Cellulase + Pectinase	++	+	+	++	+++	++	+	+	+

Table 11. Continued.

Fruit residues			Growth inhibition*								
			B. subtilis	*B. cereus*	*L. monocytogenes*	*S. aureus*	MRSA	*E. coli*	*S. enterica*	*P. putida*	*P. aeruginosa*
Pitahaya											
Oven-dried	Enzyme-free		+++	+	+	++	+	+	++	+	NI
	Cellulase		++	+++	+	++	+	+	++	+	+
	Pectinase		+++	+	NI	++	+	+	+++	+	+
	Cellulase + Pectinase		+	+	+	+++	+	+	+	+	+
Lyophilized	Enzyme-free		+++	+	+	+	+	+	+	NI	+
	Cellulase		+++	+	+	+	+	+	+	NI	NI
	Pectinase		+	++	+	++	+	+	+++	NI	+
	Cellulase + Pectinase		+++	+	+	+	+	+	+	NI	+
Mango											
Oven-dried	Enzyme-free		+	+	+	+	+	+	NI	NI	NI
	Cellulase		++	+	+++	+++	+	+	+	+	+
	Pectinase		++	+	+	+	+	++	NI	NI	NI
	Cellulase + Pectinase		++	+	++	++	+	+	+	+	NI
Naranjilla											
Oven-dried	Enzyme-free		+	+	NI	+	NI	NI	NI	NI	NI
	Cellulase		++	+	NI	+	NI	NI	NI	NI	NI
Lyophilized	Enzyme-free		++	++	NI	NI	+	+	+	NI	NI
	Cellulase		++	+	NI	NI	+	+	NI	NI	NI

* Slight antimicrobial activity (+), inhibition zone: 1-3 mm; moderate antimicrobial activity (++), inhibition zone: 4-5 mm; high antimicrobial activity (+++), inhibition zone: 6-8; strong antimicrobial activity (++++), inhibition zone > 8 mm; NI: No inhibition. There was no bacterial growth inhibition around the disks contained the negative control (10% (v/v) ethanol).

Although there were significant differences between the oven-dried and lyophilized samples in terms of their TPC and antioxidant capacity (see 5.3.1.), antimicrobial activity of both types of pretreatment was generally comparable. However, a strong positive correlation ($r > 0.900$) was observed between the TPC (see 5.3.1.) and the antimicrobial activities (Table 11) of the extracts against certain pathogens (Appendix 1), except for the LP residue. These findings suggested that the higher the TPC in those samples, the better their antimicrobial activity against the corresponding bacteria.

There are some reports in which the antimicrobial capacity of various grape, apple and pitahaya residue extracts were screened against microorganisms in agar diffusion systems (Özkan *et al.* 2004, Fratianni *et al.* 2011, Cheng *et al.* 2012, Nurmahani *et al.* 2012, Oliveira *et al.* 2013, Jahurul *et al.* 2015, Xu *et al.* 2016, Zhang *et al.* 2016). However, comparison of the results is quite difficult because the inhibitory potential of each residue sample strongly depends on the mode of extraction, the microorganism tested, and the fruit cultivar involved in the study.

There was no detectable growth inhibition by the individual phenolic compounds with the disk-diffusion method applied, at a phenolic quantity of 10 µg per disk. Therefore, antimicrobial activity of the compounds was further analyzed at higher concentrations in broth microdilution antimicrobial susceptibility tests.

5.4.2. Minimum inhibitory and minimum bactericidal concentrations

Complementing the results on the inhibitory potential of the enzyme-free and treated fruit residue extracts, MICs against the pathogenic and spoilage bacteria were determined via broth microdilution assay. As it can be seen in Table 12, majority of the samples had MIC value within the tested concentration range against all bacteria studied, with the exemption of DWG that did not exhibit MIC against both *Bacillus* isolates.

Table 12. Minimum inhibitory concentration (MIC) of fruit residue extracts against pathogenic and spoilage bacteria before and after treatment with *R. miehei* cellulase and/or *A. niger* pectinase.

Fruit residues		MIC (mg/mL)								
		B. subtilis	*B. cereus*	*L. monocytogenes*	*S. aureus*	MRSA	*E. coli*	*S. enterica*	*P. putida*	*P. aeruginosa*
Black grape										
Oven-dried	Enzyme-free	25	25	100	50	100	100	100	50	50
	Cellulase	12.5	12.5	100	50	50	50	50	25	50
	Pectinase	12.5	>100*	100	50	50	100	>100	50	25
	Cellulase + Pectinase	25	25	50	50	50	100	50	25	25
Lyophilized	Enzyme-free	12.5	25	50	50	50	50	100	25	25
	Cellulase	12.5	12.5	>100	25	50	50	50	50	25
	Pectinase	12.5	12.5	50	50	50	50	50	25	25
	Cellulase + Pectinase	50	25	100	25	50	100	50	25	25
White grape										
Oven-dried	Enzyme-free	>100	>100	50	25	100	50	50	25	25
	Cellulase	>100	>100	>100	50	>100	50	50	25	25
	Pectinase	>100	>100	>100	>100	>100	>100	>100	>100	>100
	Cellulase + Pectinase	>100	>100	>100	>100	>100	>100	>100	>100	>100
Lyophilized	Enzyme-free	100	100	50	50	>100	>100	50	>100	100
	Cellulase	>100	>100	50	50	>100	>100	>100	100	100
	Pectinase	>100	>100	50	25	50	50	25	50	25
	Cellulase + Pectinase	>100	>100	>100	>100	>100	>100	>100	>100	100
Apple										
Oven-dried	Enzyme-free	50	50	>100	100	>100	>100	>100	100	100
	Cellulase	50	25	>100	50	100	100	>100	50	50
	Pectinase	100	50	25	100	>100	100	100	50	50
	Cellulase + Pectinase	25	25	>100	50	100	100	>100	25	25
Lyophilized	Enzyme-free	100	100	50	>100	>100	>100	>100	50	100
	Cellulase	25	25	>100	50	100	>100	100	12.5	25
	Pectinase	100	100	100	100	>100	>100	100	100	50
	Cellulase + Pectinase	25	25	50	50	100	50	50	50	25

Table 12. Continued.

Fruit residues			MIC (mg/mL)								
			B. subtilis	*B. cereus*	*L. monocytogenes*	*S. aureus*	MRSA	*E. coli*	*S. enterica*	*P. putida*	*P. aeruginosa*
Pitahaya											
Oven-dried	Enzyme-free		100	50	100	100	>100	100	100	50	100
	Cellulase		50	50	>100	100	100	>100	100	12.5	50
	Pectinase		50	50	100	100	>100	>100	100	12.5	50
	Cellulase + Pectinase		50	50	50	50	>100	100	100	25	50
Lyophilized	Enzyme-free		50	100	100	100	100	100	100	12.5	50
	Cellulase		50	25	100	>100	100	>100	100	25	50
	Pectinase		50	50	100	100	100	100	100	12.5	50
	Cellulase + Pectinase		50	25	50	100	50	100	50	12.5	25
Mango											
	Enzyme-free		50	50	>100	100	>100	>100	100	>100	>100
Oven-dried	Cellulase		12.5	12.5	100	>100	>100	>100	50	>100	50
	Pectinase		12.5	12.5	100	50	50	25	25	25	25
	Cellulase + Pectinase		12.5	12.5	100	50	>100	>100	50	50	50
Naranjilla											
Oven-dried	Enzyme-free		>100	100	>100	>100	>100	>100	>100	>100	100
	Cellulase		12.5	>100	>100	12.5	25	12.5	25	25	25
Lyophilized	Enzyme-free		>100	>100	>100	>100	>100	100	>100	>100	>100
	Cellulase		12.5	25	25	12.5	12.5	12.5	12.5	12.5	25

* MIC is higher than 100 mg/mL.

The most sensitive bacteria were the *Bacillus* and *Pseudomonas* strains, while sensitivity of the other strains varied depending on the type of the fruit residue involved. Amongst the enzyme-free extracts, grape samples showed higher inhibitory effect against most of the bacteria to that observed for apple, pitahaya, mango and naranjilla samples, which correlated with the results of the disk diffusion test. Irrespectively of the residue pretreatment and enzyme treatments, MICs of black grape extracts started from 12.5 mg/mL against the *Bacillus* and from 25 mg/mL against the *Pseudomonas* and *S. aureus* strains. MICs against MRSA, *L. monocytogenes*, *E. coli* and *S. enterica* were found in a range of 50 to 100 mg/mL. The white grape extracts did not display inhibitory activity against the *Bacillus* and MRSA bacteria. On the other hand, the oven-dried enzyme-free and cellulase treated white grape extracts showed clear antibacterial effect against *S. aureus*, *E. coli*, *S. enterica*, *P. putida* and *P. aeruginosa* (the MIC ranged from 25 to 100 mg/mL). In addition, the pectinase treated LWG extract exhibited a remarkable inhibition against most of the tested bacteria (Table 12).

Literature describes various MICs to different grape extracts depending on the variety of grape, the mode of extraction and the waste fraction and microorganism applied for the test (Cheng *et al.* 2012). For instance, Dias *et al.* (2015) reported MICs from 66.7 to 133.3 mg/mL for stem phenolic extracts derived from different grape varieties against digestive pathogens. Their results are comparable to those reported here as the grape pomace subjected to enzyme treatments contained stem residues in high amount as well. Furthermore, in a recent study, MIC ranges from 4.69 to 18.8 and 40.6 to 250 mg/mL have been documented against *L. monocytogenes* and *S. aureus*, respectively, depending on the grape pomace extracts tested (Xu *et al.* 2016). The apple and pitahaya extracts were most effective against *P. putida* (MICs from 12.5 mg/mL) followed by *P. aeruginosa* and both *Bacillus* strains (Table 12). Growth inhibition capacity of the yellow pitahaya extracts studied here were close to those reported by Yong *et al.* (2017) for red pitahaya pulp samples after methanol extraction. However, red and white pitahaya peel extracts, with slightly lower MIC ranges found in this study, also showed inhibitory effect against foodborne pathogenic bacteria (Nurmahani *et al.* 2012).

The carbohydrase treatment could decrease the initial MIC of some of the extracts (Table 12). Depending on the type of bacteria tested, the MIC values detected in many enzyme-free extracts could be reduced by using *R. miehei* cellulase and/or *A. niger* pectinase mixtures. This was most significant for the apple and mango samples reducing some MICs about 2 to 4 times; a decrease was also observed for many extracts where

initial MIC was not found up to 100 mg/mL. Furthermore, a considerable decrease in the inhibitory potential was also identified for the LP and DP extracts against *B. cereus* and *P. putida*, respectively (Table 12). All three enzyme treatments supported the inhibition potential of DMG extract against *B. subtilis*, *B. cereus*, *L. monocytogenes*, *S. enterica* and *P. aeruginosa*; the highest effect was found in case of the *Bacillus* isolates (MIC = 12.5 mg/mL) (Table 12). The study of Gupta *et al.* (2010) reported that the MIC of ethanolic extract of dried unripe pulp of *Mangifera indica* was 250 mg/mL against *B. subtilis*, *B. cereus*, *L. monocytogenes* and *E. coli*, and 125 mg/mL for *S. aureus* and *P. aeruginosa*. The cellulase treated LN extract displayed strong inhibitory effect against all the bacteria tested. Studies showing the antimicrobial activity of enzyme-treated fruit extracts are scarce. In the work of Mandalari *et al.* (2007), the pectinase treatment efficiently increased the antimicrobial activity of flavonoids extracted from bergamot peel. Other papers reported effective carbohydrase-assisted extraction of phenolic antimicrobials from bilberry (pectinases; Puupponen-Pimiä *et al.* 2008), pumpkin and flaxseed (α-amylase, β-glucosidase and β-glucanase; Ratz-Łyko and Arct 2015) and *Sargassum* (Viscozyme® carbohydrase mix; Puspita *et al.* 2017) samples.

Majority of the samples had no MBC against the pathogens within the concentration range studied (Appendix 2). Where MBC was identified, it was the same or at least double of the MIC determined for the corresponding sample. Interestingly, despite their high sensitivity exhibited during the MIC assays, the *Bacillus* strains were not killed by extract concentrations less than 100 mg/mL. Similar trend was observed in the inhibitory effect of red and white flesh pitahaya extracts against a *B. cereus* isolate (Nurmahani *et al.* 2012).

MIC results obtained by individual phenolic compounds, i.e. 4-hydroxybenzoic acid, syringic acid, gallic acid, vanillic acid, cinnamic acid, *p*-coumaric acid, (+)-catechin, (−)-epicatechin, quercetin, polydatin and resveratrol quantified in the extracts are shown in Table 13. In this experiment, although it is lower than the MICs commonly found for most phenolic compounds against other pathogenic microorganisms (Mandalari *et al.* 2007, Cheng *et al.* 2012, Alves *et al.* 2013, Sabel *et al.* 2017), 500 µg/mL was applied as the maximum concentration of each phenolics because their yield in all residue extracts (see 5.3.2.) remained less than this value. For many phenolics, no inhibitory effect was recorded below 500 µg/mL concentration against most of the bacteria tested (Table 13). Hence, an elevated amount of these components after the enzyme treatments could not be responsible for the antimicrobial activity of the corresponding extract. However, it is worth pointing

out that our investigations do not exclude the occurrence of other phenolic compounds in the extract, and/or a synergic work between some of the phenolics present in the mixture (Dias *et al.* 2015), which could contribute to the growth inhibitory effect against the tested pathogens as well. Nonetheless, the two *Bacillus* strains examined were quite sensitive to cinnamic acid and resveratrol (MIC = 125 µg/mL), *p*-coumaric acid (MIC = 250 µg/mL) and quercetin (MIC = 500 µg/mL) (Table 13). Resveratrol inhibited the *Pseudomonas* strains as well (250 and 500 µg/mL MICs for *P. aeruginosa* and *P. putida*, respectively). The yield of some of the above phenolics increased after the enzyme treatments (see 5.3.2.), which could explain the high sensitivity of the *Bacillus* and *Pseudomonas* isolates towards many extracts. No MBC was found for the individual phenolics against any of the pathogens tested, at the concentration range used in this experiment (from 125 to 500 µg/mL).

Table 13. Minimum inhibitory concentration (MIC) of phenolic acids and other phenolic compounds against foodborne pathogenic and spoilage bacteria.

Compounds	MIC (µg/mL)								
	B. subtilis	*B. cereus*	*L. monocyt.*	*S. aureus*	MRSA	*E. coli*	*S. enterica*	*P. putida*	*P. aeruginosa*
4-Hydroxybenzoic acid	250	>500	>500	>500	>500	>500	>500	>500	>500
Syringic acid	250	>500	>500	>500	>500	>500	>500	>500	>500
Gallic acid	>500*	>500	>500	>500	>500	>500	>500	>500	>500
Vanillic acid	500	>500	>500	>500	>500	>500	>500	>500	>500
Cinnamic acid	125	125	>500	>500	>500	>500	>500	>500	>500
p-Coumaric acid	250	250	>500	>500	>500	>500	>500	>500	>500
(+)-Catechin	>500	>500	>500	>500	>500	>500	>500	>500	>500
(−)-Epicatechin	>500	>500	>500	>500	>500	>500	>500	>500	>500
Quercetin	500	500	>500	>500	>500	>500	>500	>500	>500
Polydatin	>500	>500	>500	>500	>500	>500	>500	>500	>500
Resveratrol	125	125	>500	>500	>500	>500	>500	500	250

* MIC is higher than 500 µg/mL.

5.4.3. Quorum sensing inhibition

It has been documented that certain phenol-rich plant extracts and phenolic compounds could inhibit the QS system of bacterial communities (Asfour 2018), which is responsible for many food deterioration phenotypes (Papuc *et al.* 2017). Therefore, we considered important to screen this potential of the enzyme treated and untreated extracts and the individual phenolics. From the extracts prepared, we selected the samples of black

grape, apple and pitahaya residues for the analysis that was conducted by using the violacein pigment producer *C. violaceum* 85WT model strain. The synthesis of violacein in *C. violaceum* is under QS regulation, which property makes this bacterium suitable for screening of compounds with anti-QS capacity (Kerekes *et al.* 2013). The extracts and phenolic compounds at the applied concentrations did not inhibit the growth of *C. violaceum* according to the CFUs determined.

Based on the liquid-culture assays performed, all fruit extracts exhibited anti-QS activity at the studied concentration (2 mg/mL), irrespectively from the enzyme treatments (Figure 18). Percent inhibition of violacein production ranged from 9 to 36%, 15 to 24% and 6 to 23% for black grape, apple and pitahaya extracts, respectively. Except for apple samples, there was no significant difference in the inhibitory effect of samples subjected to different substrate pretreatment methods (see the C1 column in Figure 18).
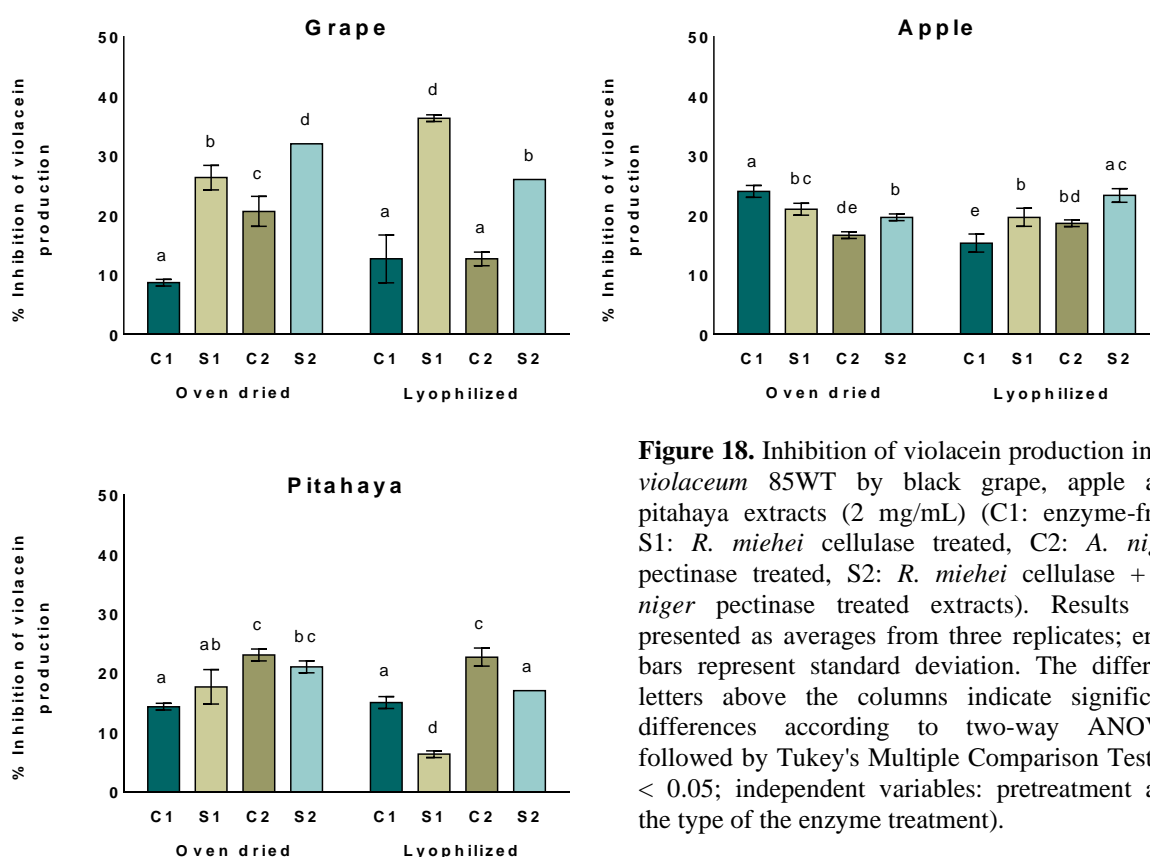
Figure 18. Inhibition of violacein production in *C. violaceum* 85WT by black grape, apple and pitahaya extracts (2 mg/mL) (C1: enzyme-free, S1: *R. miehei* cellulase treated, C2: *A. niger* pectinase treated, S2: *R. miehei* cellulase + *A. niger* pectinase treated extracts). Results are presented as averages from three replicates; error bars represent standard deviation. The different letters above the columns indicate significant differences according to two-way ANOVA followed by Tukey's Multiple Comparison Test (p < 0.05; independent variables: pretreatment and the type of the enzyme treatment).

The cellulase and cellulase-pectinase treatments significantly stimulated (p < 0.05) the anti-QS activity of both the LBG and the DBG grape samples compared to enzyme-free control (Figure 18). A moderate decrease in the violacein production could be detected for

the LA and DP samples after carbohydrase treatments. In case of the LP sample, only the *A. niger* pectinase treated extract reduced the pigment production significantly compared to enzyme-free sample ($p < 0.05$) (Figure 18). In fact, no results have been published in the literature that the carbohydrase treatment could increase the anti-QS property of a fruit residue sample. Additionally, although many studies have been conducted so far with plant sources as potential QS inhibitors (Viswanathan *et al.* 2015), relatively few reports are available about grape and apple extracts in this regard (Vattem *et al.* 2007, Fratianni *et al.* 2011, Sheng *et al.* 2016), and no studies were found on the capacity of pitahaya samples.

Concerning the individual phenolics tested, the syringic acid, vanillic acid, (+)-catechin and resveratrol compounds exhibited high inhibition against the violacein production compared to the control ($p < 0.05$), at 10 µg/mL concentration used in this assay (Figure 19). More detailed analysis of the data, comparing with the individual phenolic concentrations (see 5.3.2.), revealed a correlation between the inhibition of violacein production by certain extracts and the yield of some of these phenolics after the enzyme treatments. This correlation was strong positive ($r > 0.900$) for syringic acid in DBG, LA and DP, for vanillic acid in DBG, LBG and DP and for (+)-catechin in LBG extracts (Appendix 3). Negative associations were found between the violacein inhibition values and the concentration of syringic and vanillic acids in DA and LP samples, (+)-catechin in DA and DP samples and resveratrol in DA and both pitahaya extracts (Appendix 3). However, after enzyme treatments, the (−)-epicatechin, 4-hydroxybenzoic acid and gallic acid yields were higher in the LBG extract (from 37 to 215 µg/mL) (Table 9) than the individual phenolic concentration used in this experiment (10 µg/mL), suggesting that these components can also contribute to the inhibition of violacein production. The positive correlation ($r = 0.820$) between the violacein production inhibition values (Figure 19) and (−)-epicatechin content of the untreated and treated LA extracts (Table 9) could also support this suggestion. In this context, Borges *et al.* (2014) reported noticeable activity against the violacein production of *C. violaceum* for gallic acid and (−)-epicatechin at 1 mg/mL concentration. Also, in different concentrations, catechin, resveratrol and vanillic acid demonstrated anti-QS activity in *P. aeruginosa* (Vandeputte *et al.* 2010), *C. violaceum* (Alvarez *et al.* 2012, Erdönmez *et al.* 2018) and *Serratia marcescens* (Sethupathy *et al.* 2017), respectively.
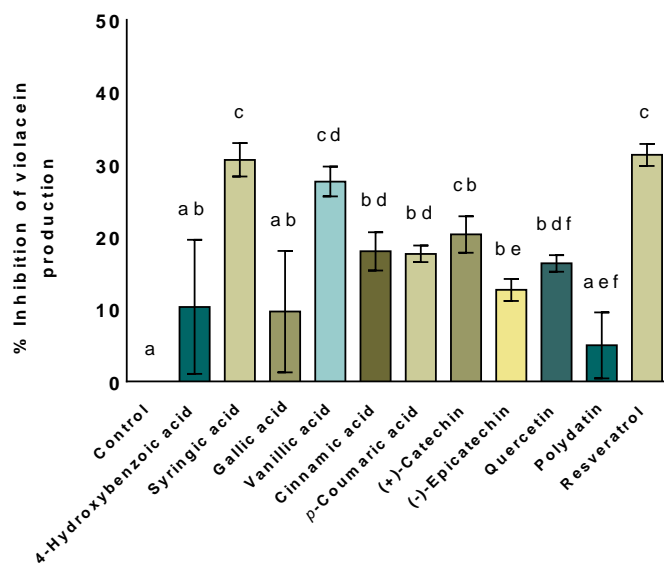
Figure 19. Percentage inhibition of violacein production in *C. violaceum* 85WT by phenolic acids and other phenolic compounds. The violacein inhibition measured in the absence of a given compound was taken as the control. Results are presented as averages from three replicates; error bars represent standard deviation. The different letters above the columns indicate significant differences according to one-way ANOVA followed by Tukey's Multiple Comparison Test (p < 0.05).

5.4.4. Effects on biofilm formation of food-related bacteria

QS inhibitory potential of the enzyme treated/untreated extracts and phenolics led us to investigate their influence on the biofilm formation. In these studies, the biofilm formation of seven pathogens involved in the antimicrobial tests in the presence or absence of extracts and individual phenolics was investigated. As can be seen on Table 14, majority of the extracts inhibited the biofilm formation *in vitro*, however, the effect varied depending on the types of substrate pretreatment and enzyme treatment, and the sensitivity of the bacterium analyzed. Overall, the most sensitive bacteria were the *P. putida*, *P. aeruginosa* and *S. aureus*. In this context, earlier studies verified that phenolic extracts and certain phenolic compounds, especially flavonoids, can efficiently prevent the biofilm formation in *P. aeruginosa* and *S. aureus* (Vandeputte *et al.* 2010, Rasamiravaka *et al.* 2015, Pratiwi *et al.* 2015, Lopes *et al.* 2017). All the studied extracts except LP contained considerable amounts of flavonoids, mainly (+)-catechin and (−)-epicatechin (see 5.3.2.).

Table 14. Effect of the black grape, apple and yellow pitahaya extracts (50 mg/mL) on biofilm formation against foodborne pathogenic and spoilage bacteria before and after treatment with *R. miehei* cellulase and/or *A. niger* pectinase. Biofilm formation occurred in the absence of a given extract was taken as the positive control.

Fruit residues		Biofilm formation (%)						
		L. monocytogenes	*S. aureus*	MRSA	*E. coli*	*S. enterica*	*P. putida*	*P. aeruginosa*
Black grape								
	Positive control	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 ab	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a
Oven-dried	Enzyme-free	44 ± 3.3 b	48 ± 6.0 b	53 ± 8.0 bc	87 ± 7.7 a	75 ± 4.8 b	48 ± 6.0 bc	52 ± 3.5 bc
	Cellulase	70 ± 11.7 c	35 ± 0.4 b	60 ± 7.3 b	128 ± 16.4 b	66 ± 8.6 b	33 ± 7.3 b	28 ± 3.7 d
	Pectinase	49 ± 5.1 bc	44 ± 8.4 b	33 ± 4.4 d	88 ± 13.1 a	81 ± 5.5 ab	51 ± 6.4 cde	55 ± 2.0 bc
	Cellulase + Pectinase	66 ± 10.0 bc	37 ± 1.3 b	41 ± 5.3 cd	107 ± 12.2 ab	133 ± 7.1 c	44 ± 4.2 bd	49 ± 2.6 be
Lyophilized	Enzyme-free	56 ± 6.2 bc	55 ± 2.0 b	105 ± 4.6 a	105 ± 10.0 ab	200 ± 0.4 d	62 ± 1.7 c	59 ± 4.4 ce
	Cellulase	115 ± 6.8 ad	101 ± 6.6 a	105 ± 2.4 a	43 ± 3.5 c	193 ± 2.4 d	35 ± 1.7 be	43 ± 4.2 b
	Pectinase	54 ± 1.7 bc	53 ± 6.0 b	106 ± 3.3 a	85 ± 10.4 a	188 ± 6.8 d	52 ± 2.4 dc	47 ± 4.8 be
	Cellulase + Pectinase	138 ± 4.6 d	88 ± 9.3 a	105 ± 2.2 a	44 ± 5.1 c	196 ± 5.7 d	40 ± 2.0 bd	62 ± 2.0 c
Apple								
	Positive control	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a
Oven-dried	Enzyme-free	40 ± 5.3 b	38 ± 2.6 b	74 ± 4.2 b	53 ± 4.6 b	193 ± 4.8 b	57 ± 7.5 bc	36 ± 7.1 bc
	Cellulase	138 ± 0.4 c	104 ± 3.5 a	71 ± 5.3 b	140 ± 11.1 c	176 ± 14.4 bc	71 ± 7.5 b	58 ± 4.4 d
	Pectinase	26 ± 4.2 b	5 ± 0.8 c	67 ± 4.2 b	60 ± 5.5 b	143 ± 12.0 cd	32 ± 3.1 de	34 ± 4.0 bc
	Cellulase + Pectinase	121 ± 4.6 d	104 ± 2.0 a	54 ± 7.3 bc	49 ±10.0 b	139 ± 17.3 de	55 ± 4.2 bc	48 ± 4.2 bd
Lyophilized	Enzyme-free	61 ± 5.1 e	38 ± 5.1 b	42 ± 4.0 c	56 ± 11.7 b	190 ± 12.6 b	53 ± 8.2 bc	33 ± 5.5 bc
	Cellulase	80 ± 4.8 f	64 ± 7.5 d	36 ± 4.6 c	38 ± 5.3 b	79 ± 1.1 a	22 ± 3.1 e	22 ± 5.1 c
	Pectinase	34 ± 6.0 b	40 ± 3.3 b	44 ± 7.3 c	62 ± 5.7 b	90 ± 3.3 a	44 ± 4.4 dc	40 ± 6.0 be
	Cellulase + Pectinase	93 ± 3.7 af	46 ± 8.4 b	74 ± 6.8 b	68 ± 12.0 b	105 ± 2.6 ae	28 ± 2.6 de	25 ± 1.7 ce
Pitahaya								
	Positive control	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 ab	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a
Oven-dried	Enzyme-free	76 ± 2.6 b	37 ± 3.1 bc	108 ± 0.4 a	154 ± 14.6 b	143 ±12.6 b	86 ± 7.1 ab	72 ± 0.8 b
	Cellulase	129 ± 7.1 c	74 ± 4.2 d	104 ± 1.5 a	26 ± 5.3 c	195 ± 8.0 c	100 ± 1.3 a	89 ± 4.4 c
	Pectinase	30 ± 1.7 d	18 ± 4.8 e	102 ± 4.0 ab	36 ± 1.5 c	50 ± 2.6 d	38 ± 0.4 c	17 ± 1.5 d
	Cellulase + Pectinase	127 ± 5.3 c	99 ± 1.7 a	106 ± 3.1 a	198 ± 6.0 d	189 ± 6.2 c	28 ± 3.7 c	45 ± 3.5 e
Lyophilized	Enzyme-free	66 ± 5.1 b	33 ± 5.7 be	105 ± 2.6 a	44 ± 7.5 c	54 ± 5.7 d	43 ± 5.5 c	22 ± 2.0 d
	Cellulase	79 ± 3.1 b	42 ± 3.7 b	95 ± 4.0 b	169 ± 16.2 bd	183 ± 9.7 c	65 ± 9.7 d	51 ± 3.1 ef
	Pectinase	33 ± 4.2 d	23 ± 4.6 ce	77 ± 2.4 c	43 ± 3.5 c	34 ± 6.4 d	31 ± 2.0 c	21 ± 1.7 d
	Cellulase + Pectinase	82 ± 3.5 b	92 ± 5.7 a	73 ± 1.5 c	33 ± 3.1 c	180 ± 7.1 c	67 ± 7.7 bd	61 ± 3.1 f

Average values from three tests ± standard deviation. Different letters represent significant changes ($p < 0.05$) compared to respective positive controls (two-way ANOVA followed by Tukey's Multiple Comparison Test; independent variables: pretreatment and type of the enzyme treatment).

Concerning the type of pretreatment of fruit residues, biofilm formation decreasing effect of the DBG extracts was superior against MRSA and *S. enterica* than that detected for the lyophilized ones (p < 0.05), irrespectively from the enzyme treatments (Table 14). However, in case of the other fruit residues, no general conclusion could be deduced regarding to the influence of pretreatment on the biofilm formation, since the effect of the extracts was different depending on the enzyme treatment. For example, a slight decrease (p > 0.05) in biofilm formation was noted in the *S. aureus* biofilms when cellulase treated DBG extract was added, while biofilm formation similar to positive control was detected in the presence of cellulase treated LBG extract, compared to the corresponding enzyme-free sample (Table 14). If samples presenting the highest inhibitory potential were considered, oven-drying pretreatment was effective for black grape and pitahaya residues to inhibit the formation of most of the bacterial biofilms analyzed. In contrast, except for *L. monocytogenes* and *S. aureus*, apple residues subjected to lyophilization pretreatment resulted the maximal reduction in biofilm formation.

Surprisingly, in some cases, addition of the extract affected positively the formation of the biofilms (Table 14). This was particularly found for *S. enterica*; for instance, the DA and LBG extracts, irrespectively from the enzyme treatments, caused 1.5- and 2-fold increase in the biofilm formation (p < 0.05), respectively. Also, some extracts had stimulating effect on the *L. monocytogenes* and *E. coli* biofilms. Another important observation is that despite the increased QS inhibition activity of some of the extracts after enzyme treatments (see Figure 18), the cellulase and/or cellulase-pectinase combined treatments decreased the antibiofilm activity of many enzyme-free extracts; however, it depended on the type of the bacterium tested. *L. monocytogenes* biofilm, for example, was significantly enhanced by the cellulase treated DA and DP extracts, and the cellulase-pectinase treated LBG and DP samples compared to the positive control (p < 0.05) (Table 14). Interestingly, all pectinase treated extracts facilitated the biofilm formation reduction in *L. monocytogenes*, and pectinase treated samples presented the highest inhibition against MRSA (DBG sample), *S. aureus* (DA sample), *S. enterica* (LP sample) and *S. aureus* and *P. aeruginosa* (DP sample) biofilms (Table 14).

The reason for the enhanced biofilm formation caused by some of the extracts could ascribe to the influences of various factors, such as the different sensitivity of the bacteria towards the tested extract, the amount of individual phenolic compounds in the extract, and the polysaccharides derived from the fruit residues. Namely, the cellulose fibers, oligomers and other polysaccharides, e.g., pectin and xylan, present in the residue

extracts can induce more structural stability and protection to the cells in the biofilm layer (Beauregard *et al.* 2013, Limoli *et al.* 2015). In this sense, cellulase treatment could facilitate the formation of such oligomers from the residue causing enhanced biofilm formation in some of the bacteria tested. Concerning plant phenolics, several studies have shown their excellent antibiofilm properties (Slobodníková *et al.* 2016), and numbers of them have been tested as potential antibiofilm agent in the food industry (Papuc *et al.* 2017). However, some results exist on that certain fruit extracts (Priha *et al.* 2014) and phenolic compounds (Vikram *et al.* 2010, Plyuta *et al.* 2013) can trigger biofilm formation by the bacterium with a concentration dependent interaction. As an example, Plyuta *et al.* (2013) found that the 4-hydroxybenzoic, gallic and cinnamic acids in subinhibitory concentrations up to 100 µg/mL, and the vanillic acid and (–)-epicatechin up to 750 µg/mL can stimulate the biofilm formation of *P. aeruginosa*. Presence of these compounds in the extracts studied here, at concentrations up to 215 µg/mL (see Tables 8 and 9), may also explain the observed biofilm-stimulating effects. In this context, correlations ($r > 0.636$) between the amounts of vanillic acid, syringic acid, (+)-catechin, (–)-epicatechin and resveratrol (Tables 8 and 9) and the biofilm-modulating capacity of LBG and DA extracts toward *L. monocytogenes* (Table 14) were observed. Also, vanillic and syringic acid concentrations in the pitahaya extracts found to be correlated with their influence on *L. monocytogenes* biofilm ($0.582 < r < 0.983$).

In further assays, the antibiofilm capacity of phenolic compounds identified in the extracts was examined. Here, a sub-MIC concentration (100 µg/mL) of the chemicals was applied and results showed an inhibitory effect by them against the biofilm formation of the bacteria tested (Table 15). Based on these experiments, it appears that a more detailed characterization of the polyphenolic profile in the extracts, followed by bioactivity analysis, is needed to prove the function of phenolics in the biofilm formation enhancement. Nevertheless, our investigations revealed potential antibiofilm phenolics that can be obtained from black grape, apple and pitahaya extracts after cellulase and cellulase-pectinase combined treatments. Vanillic and syringic acid content of LA extracts (Table 9), and (+)-catechin and (–)-epicatechin yields of DBG samples (Table 8), for instance, correlated well with their antibiofilm activity against *P. putida* and *P. aeruginosa* (Table 15), respectively ($0.905 < r < 0.998$).

Table 15. Effect of phenolic acids and other phenolic compounds (100 μg/mL) on biofilm formation against foodborne pathogenic and spoilage bacteria. Biofilm formation occurred in the absence of a given compound was taken as the positive control.

	Biofilm formation (%)						
	L. monocytogenes	*S. aureus*	MRSA	*E. coli*	*S. enterica*	*P. putida*	*P. aeruginosa*
Positive control	100 ± 0 a	100 ± 0 a	100 ± 0 a	100 ± 0 a	100 ± 0 a	100 ± 0 a	100 ± 0 a
Compounds							
4-Hydroxybenzoic acid	64 ± 7 bc	40 ± 2 b	54 ± 3 bc	50 ± 7 b	57 ± 6 b	77 ± 5 b	48 ± 7 b
Syringic acid	48 ± 6 b	49 ± 6 b	59 ± 11 cd	25 ± 6 c	38 ± 4 c	40 ± 4 cd	33 ± 8 bc
Gallic acid	89 ± 3 ac	45 ± 6 b	59 ± 2 cd	39 ± 10 bc	52 ± 5 bc	58 ± 2 ce	45 ± 4 b
Vanillic acid	40 ± 3 b	40 ± 1 b	49 ± 10 bc	29 ± 6 bc	49 ± 8 bc	57 ± 4 ce	39 ± 6 bd
Cinnamic acid	47 ± 4 b	40 ± 2 b	63 ± 6 cd	31 ± 3 bc	51 ± 3 bc	72 ± 6 be	38 ± 6 bd
p-Coumaric acid	41 ± 11 b	38 ± 3 b	68 ± 5 c	33 ± 8 bc	43 ± 3 bc	60 ± 6 be	50 ± 4 b
(+)-Catechin	47 ± 11 b	15 ± 1 c	43 ± 7 bc	19 ± 1 c	16 ± 2 d	18 ± 3 f	15 ± 0 c
(−)-Epicatechin	61 ± 10 bc	18 ± 4 c	41 ± 0 bd	38 ± 6 bc	20 ± 3 d	24 ± 5 df	25 ± 2 cd
Quercetin	40 ± 5 b	14 ± 0 c	33 ± 5 b	23 ± 6 c	17 ± 2 d	19 ± 2 f	20 ± 2 cd
Polydatin	49 ± 9 b	37 ± 5 b	40 ± 4 bd	16 ± 2 c	17 ± 3 d	19 ± 4 f	19 ± 1 cd
Resveratrol	60 ± 10 bc	17 ± 3 c	41 ± 7 bd	17 ± 3 c	18 ± 3 d	19 ± 1 f	20 ± 2 cd

Average values from three tests ± standard deviation. Values within a column with different letters are significantly different according to the one-way ANOVA followed by Tukey's Multiple Comparison Test ($p < 0.05$).

6. SUMMARY

Nowadays, the phenolic antioxidants have become frequently applied as natural additives in functional foods. These compounds are capable to enhance the stability of food and beverage products, increase their antioxidative capacity, and inhibit the growth of a range of foodborne pathogenic and spoilage bacteria and fungi. Many fruit residues contain such phytochemicals. However, most phenolic antioxidants in plants are bounded in glycosides forming a sugar linked compound with reduced bioavailability. Through hydrolytic action, the carbohydrate-cleaving enzymes can mobilize the phenolic aglycone molecules from their conjugated forms. For enrichment purposes, substrate treatment with carbohydrases and/or fermentation with the producing organisms can be a well-applicable and ecofriendly approach. Thus, extracts rich in bioactive and bioavailable natural phenolics can be generated from antioxidative plant byproducts.

There are good carbohydrase producers among filamentous fungi, and the obtained enzyme activities can be utilized in many food industrial applications. For instance, organoleptic properties and/or shelf life of certain food products can effectively be improved by these activities. In a preliminary research performed by our group, the zygomycete fungus *Rhizomucor miehei* NRRL 5282 was identified as an excellent cellulase producer. Hence, the present work aimed the mobilization of bioactive phenolic compounds from different fruit byproducts through solid-state fermentation (SSF) and direct enzyme treatment using the *R. miehei* NRRL 5282 strain and its cellulolytic enzyme cocktail. The antioxidative Othello black grape (*Vitis vinifera x* (*Vitis labrusca x Vitis riparia*)), Saszla white grape (*Vitis vinifera*), Jonagold apple (*Malus domestica* cv. Jonagold), yellow pitahaya (*Hylocereus megalanthus*), Tommy Atkins mango (*Mangifera indica* L.) and naranjilla (*Solanum quitoense*) fruits have been selected for the studies. From these substances, byproducts usually remaining after wine, juice and/or jam production were prepared, dried in an oven or lyophilized, and used for subsequent fermentation and enzyme treatments tests. Results achieved during our researches are the follows:

1. Phenolic rich extracts with improved antioxidant activity have been prepared by SSF of residues.

In this assay, mobilization of antioxidative phenolics from oven-dried and lyophilized black and white grape, apple, pitahaya, mango and naranjilla residues was

studied after SSF using the high yield cellulase producer *R. miehei* NRRL 5282 isolate. Samples were collected at specified intervals, then, cellulase (β-glucosidase) activity, total phenolic content (TPC), ferric reducing antioxidant power (FRAP) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity measurements were conducted. Results showed positive associations between the β-glucosidase activity and/or the TPC and FRAP on lyophilized apple (LA), pitahaya (LP), white grape (LWG) and naranjilla (LN) substrates. The β-glucosidase activity increased during the fermentation process on most fruit residues. In some experiments, the β-glucosidase activity increased slowly, most probably due to the high sugar content of the substrate. These compounds, mainly the cellobiose and the glucose, can bind to the active site of the β-glucosidase inhibiting its activity if they are present in excess. Furthermore, the catabolite repression can also explain the phenomenon. When glucose is present, the enzymes and transporters necessary for the utilization of a carbon source different from glucose are repressed. Additionally, the interaction of polyphenols and free aglycones with glucosidases may also reduce the enzyme activity. A positive correlation between the TPC, the FRAP and DPPH radical scavenging activity was detected for LP and oven-dried pitahaya (DP), oven-dried and lyophilized black grape (DBG and LBG), oven-dried mango (DMG) and LWG, LA and LN substrates. The phenolic content increased during the fungal growth when LWG, LA, LP and LN substrates were used as substrate. Depending on the fruit residue, the type of pretreatment (oven-drying or lyophilization) also influenced the TPC, β-glucosidase activity and/or antioxidant potential of the samples. In addition, the fermentation time required to achieve maximal enzyme activity and antioxidant effect also depended on the type of fruit residue. The highest β-glucosidase activity was achieved at different days during the bioconversions: 5th day for naranjilla, 10th day for pitahaya, 15th day for white and black grapes and 18th day for apple and mango. The FRAP was highest mostly during the first phase of fermentations, reaching maximal yields on the 3rd day for black grape and naranjilla, 5th day for white grape, 10th day for pitahaya and mango and 13th day for apple.

2. Enzyme-assisted extraction of antioxidative phenolics from fruit residues has been performed.

Enzyme treatment of oven-dried and/or lyophilized residues using a cellulolytic cocktail produced from *R. miehei* NRRL 5282 on wheat bran was carried out in these experiments. Except for naranjilla residues, commercial *Aspergillus niger* pectinase was

also added to the reaction mixtures to improve the extraction of the phenolic compounds and decrease the tissue rigidity in the residues. TPC, FRAP and DPPH radical scavenging activity were analyzed in the obtained phenolic-rich extracts.

Overall, enzyme treatments enhanced the TPC and antioxidant potential in many fruit residues tested. The FRAP was superior for DP and LBG after each enzyme treatment, and for DBG and LN after cellulase, for LWG after pectinase, for DMG after pectinase and cellulase-pectinase and for oven-dried apple (DA) after cellulase-pectinase treatments than that measured in the corresponding control. Level of TPC and FRAP exposed similar pattern for most of the fruit extracts examined; moreover, in some samples, positive association was found between the TPC and the FRAP/DPPH radical scavenging activity. It seems that the substrate pretreatment was an important factor in the enzyme aided liberation tests as well. In the case of grape, apple and naranjilla fruit residues, for instance, lyophilized samples showed higher TPC after enzyme treatment than the oven-dried ones. However, irrespectively from enzyme treatment, extracts obtained on DP exhibited more TPC than the lyophilized forms. The antioxidant effect depended on both the type of fruit residues and the pretreatment.

3. Individual phenolics in the extracts have been analyzed and compared.

We studied the concentration of 11 individual phenolic compounds, namely the 4-hydroxybenzoic acid, syringic acid, gallic acid, vanillic acid, cinnamic acid, *p*-coumaric acid, (+)-catechin, (−)-epicatechin, quercetin, polydatin and resveratrol, in black grape, apple, pitahaya and mango samples before and after enzyme treatments. In these assays, the analytical HPLC data also confirmed a phenolic content increase after cellulase treatments. The concentration of phenolic compounds changed by different degrees after the enzyme treatments depending on the substrate and the pretreatment: (+)-catechin and (−)-epicatechin were the major small compounds in the black grape and apple residue extracts, while the content of gallic and vanillic acids increased significantly in case of the DP samples. Except for stilbenoids and cinnamic acid, concentrations of all phenols tested were enhanced in DMG sample after the cellulase treatment. Anyway, HPLC analysis also confirmed an increase in the phenolic content after enzyme treatment of residues.

Antioxidant activity of the individual phenolic compounds investigated in the extracts was also determined. The syringic acid, gallic acid and (+)-catechin exhibited the highest antioxidant activity among the tested phenolic compounds. For some phenolic compounds, results showed correlation between the antioxidant activity and the

concentration found in the extracts, indicating a possible role of the studied phenolics in the antioxidant potential increase of samples.

4. Antimicrobial activity of extracts and individual phenolics against food-related bacteria has been studied. Antioxidative samples with effective growth inhibitory potential have been identified.

We investigated the antimicrobial activity of grape, apple, pitahaya, mango and naranjilla extracts before and after enzyme treatments against selected food spoilage and foodborne pathogen bacteria. Results were compared with the antimicrobial properties of individual phenolics analyzed to investigate the association between the concentration of the compounds in the extracts and the growth inhibitory effect displayed. *Bacillus subtilis*, *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), *Escherichia coli*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium, *Pseudomonas putida* and *Pseudomonas aeruginosa* isolates were included in the assay.

In disk-diffusion tests, grape samples performed the highest growth inhibition potential followed by apple and pitahaya residues. Moreover, the antimicrobial activity of most fruit samples was improved after enzyme treatments. In general, the cellulase and cellulase-pectinase treated samples were more effective against most of the pathogens tested. No significant differences were found in the inhibitory effects between the oven-dried and the lyophilized samples. The *B. subtilis*, *S. aureus* and *S. enterica* were the most sensitive to all extracts, while the lowest inhibitory effect was identified in case of the *L. monocytogenes* and the both *Pseudomonas* strains.

Minimum inhibitory and minimum bactericidal concentrations (MIC and MBC) of the extracts were also studied. In these assays, majority of samples had MIC value within the tested concentration ranging from 12.5 to 100 mg/mL. The *Pseudomonas* and *Bacillus* strains were quite sensitive to the extracts, while growth inhibition was the lowest towards *L. monocytogenes*, *E. coli* and *S. enterica*. Among the samples tested, black grape and enzyme treated mango and naranjilla displayed the highest bacteriostatic effect. In many extracts, carbohydrase treatments reduced the initial MIC depending on the bacteria tested. Moreover, the MBC of black grape extract towards *S. aureus*, MRSA, *E. coli* and *S. enterica* was improved after the cellulase treatment, while the pectinase treatment caused an increase in bactericidal effect of apple samples against *Pseudomonas*. All three enzyme

treatments affected positively the bactericidal effect of pitahaya residues against *P. aeruginosa* and *P. putida*.

Concerning individual phenolic compounds, no inhibitory effect has been recorded at the tested quantity in disk-diffusion tests (10 µg per disk). In the concentration range (from 125 to 500 µg/mL) studied, MIC was determined only for cinnamic acid, *p*-coumaric acid and quercetin against the two *Bacillus* strains, and resveratrol against the *Bacillus* and *Pseudomonas* isolates. This suggests that most phenolic compounds analyzed would have a minor role in the antimicrobial activity of the samples. No MBC was identified for the compounds within the used concentration range.

5. Anti-quorum sensing (anti-QS) activity of residue extracts, and phenolic compounds has been evaluated using the model organism *Chromobacterium violaceum*.

Because the cell-to-cell communication with chemical molecules, namely the quorum sensing (QS), is responsible for the development of many food deterioration phenotypes, we have screened the enzyme-free and enzyme-treated black grape, apple and pitahaya extracts and the phenolic compounds as possible inhibitors of QS by monitoring the QS regulated pigment (violacein) production of *C. violaceum* in liquid cultures.

The fruit residue extracts presented anti-QS effect in an inhibition range of 6 to 36%, at 2 mg/mL concentration used. Except for apple samples, inhibitory effect of the enzyme-free sample was not influenced by the nature of pretreatment. More importantly, the enzyme treatments enhanced the anti-QS activity of the DBG, LBG, DP, LP and LA extracts. The individual phenolics tested in 10 µg/mL concentration showed inhibition within the range of 5 to 31%. Results revealed that syringic acid, vanillic acid, (+)-catechin, (−)-epicatechin, 4-hydroxybenzoic acid, gallic acid and resveratrol yields obtained after enzyme treatments might have contributed to the violacein production inhibitory effect of some extracts.

6. Biofilm formation of foodborne pathogens and spoilage bacteria was studied in the presence of phenolic extracts and compounds.

It is known that the QS mechanism has important role in the biofilm formation of many microorganisms. Because most extracts of black grape, apple and pitahaya residues before and after enzyme treatments, as well as many phenolic compounds inhibited the QS communication system, we considered analyzing their effects against biofilm forming ability of food-related bacteria to be important. The above studied *L. monocytogenes*, *S.*

aureus, MRSA, *E. coli*, *S. enterica*, *P. putida* and *P. aeruginosa* bacteria were included in this assay. Most extracts exhibited biofilm formation inhibitory effect, and *P. putida*, *P. aeruginosa* and *S. aureus* showed the highest sensitivity to them. However, the degree of inhibition depended on the substrate pretreatment and enzyme treatment procedures as well as on the sensitivity of bacterium subjected to analysis. For instance, the DBG and DP residues provided more inhibition against the biofilms compared to LBG and LP samples, respectively. For apple extracts, however, most bacterial biofilms were inhibited by samples obtained from lyophilized materials. In addition, it was interesting to observe that some extracts, irrespectively from enzyme treatments, supported the formation of certain biofilms. This was particularly found for the *S. enterica*, *L. monocytogenes* and *E. coli* biofilms. The different sensitivity of the bacteria to extracts and/or the concentration of phenolic compounds and polysaccharides in the samples might be responsible for the biofilm stimulating effect. The phenolics could be stressors forcing the cells to biofilm formation while saccharides can stabilize the biofilm matrix during reaction. Biofilm inhibitory effect of phenolic compounds was the highest in the case of *S. aureus*, *E. coli*, *S. enterica* and the two *Pseudomonas* bacteria. Flavonoids, i.e. (+)-catechin, (−)-epicatechin and quercetin, and stilbenoids, i.e. polydatin and resveratrol, showed greater activity against most bacterial biofilms than the phenolic acids.

In conclusion, our study provides useful data on the application of *R. miehei* and its cellulase enzymes to generate free phenolic compounds from grape, apple, pitahaya, mango and naranjilla wastes. The obtained phenolic extracts can be used as a source of natural compounds having antioxidative, antimicrobial, anti-biofilm and anti-QS potential. We expect that the phenolic compounds and extracts may substitute or support the activity of synthetic additive products, which, by the way, can provoke serious concern in consumers. In line with this, the produced extracts could be potential sources of food preservatives and/or sanitizers as well. In addition, the ecofriendly utilization of food processing residues can also be achieved during the treatment processes. Moreover, as we know, this study is the first that has applied fermentation and enzyme aided techniques for production of natural bioactives from pitahaya and naranjilla residues.

7. ÖSSZEFOGLALÁS

A fenolos antioxidánsok természetes eredetű kiegészítőként alkalmazhatók funkcionális élelmiszerekben. Jelenlétükkel növelhetik az élelmiszerek stabilitását és antioxidáns képességét, valamint gátolhatják az élelmiszer eredetű patogén és romlást okozó mikroorganizmusok növekedését. Bioaktív fenolokat sokféle gyümölcs feldolgozás utáni maradványa tartalmaz. Többségük biológiailag nehezen hozzáférhető, cukormolekulákkal képzett glikozidokban lekötve található a növényben. A szénhidrátbontó enzimek azonban hidrolitikus aktivitásukkal mobilizálni képesek a fenolos aglikon molekulákat. Ezáltal a növényi anyag szubsztrát szénhidrázokkal történő kezelése és/vagy fermentációja enzimtermelő mikroorganizmusokkal egy jól alkalmazható megközelítés lehet a szabad fenolos összetevők környezetbarát kivonására. A módszerrel bioaktív és biológiailag hozzáférhető, természetes fenolokban gazdag kivonatok állíthatók elő antioxidáns növényi melléktermékekből.

A fonalas gombák között vannak jó hozamú szénhidráz-termelő törzsek. A kinyert enzimek számos élelmiszeripari célra hasznosíthatók; alkalmazásukkal például hatékonyan javítható egyes élelmiszerek eltarthatósági és/vagy érzékszervi tulajdonsága. Előzetes kutatások a *Rhizomucor miehei* NRRL 5282 járomspórás gombát kitűnő celluláz termelőnek azonosították. Ezt a gombatörzset és az általa termelt celluláz enzimet felhasználva célul tűztük ki bioaktív szabad fenolok szilárd-fázisú fermentációval és közvetlen enzimatikus kezeléssel történő kivonását különféle gyümölcsök feldolgozás utáni maradványaiból. A kutatási munkához az antioxidatív Otelló fekete szőlő (*Vitis vinifera x* (*Vitis labrusca x Vitis riparia*)), Saszla fehér szőlő (*Vitis vinifera*), Jonagold alma (*Malus domestica* cv. Jonagold), sárga sárkánygyümölcs (*Hylocereus megalanthus*), Tommy Atkins mangó (*Mangifera indica* L.) és naranjilla (*Solanum quitoense*) gyümölcsöket választottuk ki. Az alapanyagokból bor-, gyümölcslé- és/vagy lekvárkészítés után rendszerint visszamaradó melléktermékeket állítottunk elő, melyeket ezután szárítószekrényben szárítottunk vagy liofilizáltunk. Aprítást követően fermentációs és enzimatikus kezeléseknek vetettük alá a mintákat. A következő eredményeket értük el:

1. Megnövelt antioxidáns aktivitású, fenolokban dúsított kivonatokat állítottunk elő a maradványok szilárd-fázisú fermentációjával.

Ezekben a vizsgálatokban az antioxidáns hatás és a szabad fenol hozam változását tanulmányoztuk szárítószekrényben szárított és liofilizált szőlő, alma, sárkánygyümölcs,

mangó és naranjilla maradványok *R. miehei* gombával történő szilárd-fázisú fermentációját követően. A kijelölt időközönként levett mintákban meghatároztuk az összfenol-tartalmat és a celluláz (β-glükozidáz) aktivitást, majd az antioxidáns aktivitást a vasredukáló képesség (FRAP módszer) és szabadgyök-fogó kapacitás (DPPH módszer) monitorozásával követtük. A liofilizált alma (LA), sárkánygyümölcs (LP), fehér szőlő (LWG) és naranjilla (LN) szubsztrátok esetén pozitív összefüggést tapasztaltunk a β-glükozidáz aktivitás és/vagy az összfenol-tartalom és a vasredukáló képesség között. A β-glükozidáz aktivitás a legtöbb minta esetén növekedést mutatott a fermentáció alatt. Néhány mintánál az enzimaktivitás lassú emelkedését tapasztaltuk, mely valószínűleg a szubsztrát nagy cukortartalmának tudható be. Nagy koncentrációban való jelenlét esetén a cellobióz és a glükóz kötődhet a β-glükozidáz aktív centrumához gátolva annak működését. A jelenséget a katabolit represszió szintén magyarázhatja, illetve a glükozidáz aktivitás polifenolokkal és szabad aglikonokkal való interakció okán is csökkenhet. Vizsgálatainkban pozitív korrelációt mutattunk ki az összfenol-tartalom, a vasredukáló képesség és a szabadgyök-fogó aktivitás között szárítószekrényben szárított sárkánygyümölcs (DP), fekete szőlő (DBG) és mangó (DMG), illetve liofilizált fekete szőlő (LBG), LWG, LP, LA és LN mintákon. A fenoltartalom folyamatos növekedését azonosítottuk LWG, LA, LP és LN szubsztrátokon a fermentáció alatt. Gyümölcsmaradványtól függően azonban az előkezelés típusa (szárítószekrényben szárítás vagy liofilizálás) is befolyásolta a minták összfenol-tartalmát, β-glükozidáz aktivitását és/vagy antioxidáns potenciálját. Emellett, a maradvány típusától függően változott a legnagyobb enzimaktivitás és antioxidáns hatás eléréséhez szükséges fermentációs idő is. A legnagyobb β-glükozidáz aktivitást például naranjilla mintánál az 5., sárkánygyümölcsnél a 10., szőlőnél a 15., míg alma és mangó mintáknál a 18. napokon kaptuk. A vasredukáló képesség ezzel szemben a fermentációk kezdeti fázisában volt a legnagyobb; fekete szőlő és naranjilla esetén a 3., fehér szőlőn az 5., sárkánygyümölcs és mangó mintákon a 10., míg almán a 13. napokon mértünk maximális értékeket.

2. Antioxidatív fenolokat vontunk ki gyümölcsmaradvány mintákból enzimatikus kezelésekkel.

Szárítószekrényben szárított és/vagy liofilizált gyümölcsmaradvány minták enzimatikus kezelését végeztük búzakorpa alapú fermentációban előállított *R. miehei* celluláz koktéllal. A fenolkivonás hatékonyságának növelése érdekében *Aspergillus niger* pektinázt is adtunk a legtöbb minta reakcióelegyéhez. A maradványokat így háromféle

kezelésnek vetettük alá: celluláz, pektináz és celluláz-pektináz kombinált kezelés. A kivonatokban ezután meghatároztuk az összfenol-tartalmat és az antioxidáns aktivitást.

A legtöbb tesztelt gyümölcsmaradvány-kivonatban fokozódott az összfenol-tartalom és az antioxidáns potenciál az enzimatikus kezeléseket követően. A vasredukáló képesség a DP és LBG mintáknál mindegyik enzimes kezelés után, míg DBG és LN esetén celluláz, LWG mintánál pektináz, DMG szubsztrátnál pektináz és celluláz-pektináz, illetve szárítószekrényben szárított alma (DA) minta esetén celluláz-pektináz kombinált kezelést követően jelentősen nagyobb volt, mint az enzimmentes kontrollban. A legtöbb kivonatnál az összfenol-tartalom és vasredukáló képesség hasonló mintázatban változott az enzimes kezeléseket követően. Néhány minta esetében pozitív asszociációt tapasztaltunk az összfenol-tartalom és a vasredukáló képesség/gyökfogó kapacitás között. A szubsztrát előkezelése ezekben a kísérletekben is jelentős hatással volt a fenol felszabadulásra. Liofilizált szőlő, alma és naranjilla maradványok kivonataiban például nagyobb összfenol-tartalmat mértünk az enzimatikus kezeléseket követően, mint szárítószekrényben szárított mintáknál. Sárkánygyümölcsnél a szárítószekrényben szárított minták kivonataiban volt nagyobb összfenol-tartalom, enzimatikus kezeléstől függetlenül. Az antioxidáns aktivitás mértéke is függést mutatott a maradványok típusától és az előkezelések jellegétől.

3. Vizsgáltuk fenolos vegyületek jelenlétét és koncentrációját a kivonatokban.

A fekete szőlő, alma, sárkánygyümölcs és mangó minták enzimatikus kezelés előtti és utáni kivonatait HPLC analízisnek is alávetettük, melyben egyedi fenolos vegyületek (4-hidroxi-benzoesav, galluszsav, vanillinsav, sziringsav, *p*-kumársav, fahéjsav, kvercetin, polidatin, rezveratrol, (+)-katekin és (−)-epikatekin) mennyiségi meghatározását végeztük el. Számos vegyület esetén koncentrációnövekedést tapasztaltunk a celluláz kezelést követően. Az egyes fenolok mennyisége azonban az enzimatikus kezelésektől, a szubsztrát típusától és az előkezeléstől függően különböző módon változott: fekete szőlő és alma kivonatokban a (+)-katekin és az (−)-epikatekin volt a meghatározó vegyület, míg DP esetén a galluszsav és vanillinsav tartalom mutatott jelentős növekedést. A stilbenoid és a fahéjsav vegyületeken kívül növekedést tapasztaltunk a vizsgált fenolok koncentrációjában a DMG minták celluláz kezelését követően.

A kivonatokban vizsgált fenolos összetevők antioxidáns aktivitását is meghatároztuk. A legnagyobb aktivitást sziringsav, galluszsav és (+)-katekin vegyületeknél tapasztaltuk. Emellett, néhány fenol esetén korrelációt azonosítottunk az

antioxidáns hatás és a kivonatban mért koncentráció között, ami az adott vegyület lehetséges szerepére utal a minta antioxidáns aktivitásának emelkedésében.

4. Felmértük az enzimatikus kezeléssel előállított kivonatok antimikrobiális hatását élelmiszerekkel kapcsolatos baktériumokon. Azonosítottunk növekedést gátló tulajdonsággal rendelkező mintákat.

Élelmiszer-eredetű patogén és romlást okozó baktériumokon vizsgáltuk enzimes kezelés előtti és utáni gyümölcsmaradvány-kivonatok növekedést gátló aktivitását. A kísérletekbe bevontuk az előzetesen azonosított egyedi fenolvegyületeket is, majd vizsgáltuk az összefüggést a kivonatokban található vegyületek koncentrációja és a növekedést gátló hatás között. A kísérletekbe *Bacillus subtilis*, *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, meticillin-rezisztens *S. aureus* (MRSA), *Escherichia coli*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium, *Pseudomonas putida* és *Pseudomonas aeruginosa* baktériumokat vontunk be.

Korongdiffúziós tesztekben a szőlő, alma és sárkánygyümölcs kivonatai mutatták a legnagyobb növekedésgátló hatást. Az enzimatikus kezelés megnövelte a legtöbb kivonat gátló hatását: a celluláz és a celluláz-pektináz kezelt kivonatok számos patogén ellen nagyobb aktivitást mutattak az enzimmentes kivonattal összehasonlítva. A különböző módon előkezelt (szárítószekrényben szárított vagy liofilizált) minták kivonatai között viszont nem azonosítottunk szignifikáns eltéréseket az aktivitásban. A *B. subtilis*, *S. aureus* és *S. enterica* baktériumok érzékenyek voltak minden tesztelt kivonatra, míg a legkisebb gátló hatást *L. monocytogenes* és a két *Pseudomonas* izolátum esetén tapasztaltuk.

A kivonatok minimális gátló és minimális baktericid koncentrációját (MIC és MBC) is meghatároztuk. A 12,5-100 mg/mL vizsgált koncentráción belül a legtöbb mintánál azonosítottunk MIC értéket. Ezekben a tesztekben a *Pseudomonas* és a *Bacillus* baktériumok érzékenyek voltak a kivonatokra. A növekedést gátló hatás *L. monocytogenes*, *E. coli* és *S. enterica* esetén volt a legkisebb. A tesztelt minták közül a fekete szőlő és az enzimkezelt mangó és naranjilla kivonatok mutatták a legnagyobb bakteriosztatikus hatást. Baktériumtól függően ugyan, de számos kivonat kezdeti MIC értéke csökkent a szénhidráz kezeléseket követően. Sőt, a *S. aureus*, MRSA, *E. coli* és *S. enterica* baktériumokra azonosított MBC is csökkent fekete szőlő maradvány celluláz kezelését követően. Alma mintáknál pektináz kezelés után tapasztaltunk hasonlót *Pseudomonas* baktériumokon. Mindhárom enzimatikus kezelés pozitívan befolyásolta a sárkánygyümölcs minták *P. aeruginosa* és *P. putida* ellen mutatott baktericid hatását.

A fenolos vegyületek a vizsgált koncentrációban (10 µg per korong) nem mutattak gátló hatást a korongdiffúziós tesztek alatt. MIC értéket a fahéjsav, *p*-kumársav és kvercetin vegyületeknél *Bacillus* baktériumok ellen, és rezveratrol alkalmazásakor *Bacillus* és *Pseudomonas* izolátumoknál azonosítottunk a vizsgált 125-500 µg/mL koncentráción belül. Eszerint a vizsgált fenolos vegyületek többsége kismértékben járul hozzá a kivonatok antimikrobiális aktivitásához. A tesztelt koncentrációtartományon belül MBC adatot nem kaptunk a fenolos vegyületeknél.

5. Vizsgáltuk a kivonatok és a fenolos vegyületek quorum sensing folyamatot gátló képességét.

A kémiai molekulákkal történő sejt-sejt kommunikáció, azaz a quorum sensing (QS), számos élelmiszerromlást és fertőzést okozó fenotípusért felelős, ezért fontosnak tartottuk megvizsgálni a kivonatok és fenolos komponenseik esetleges QS folyamatot gátló hatását is. A kísérletsorozatba a fekete szőlő, alma és sárkánygyümölcs maradványok enzimes kezelés előtti és utáni kivonatait vontuk be. A hatást *Chromobacterium violaceum* modellorganizmus QS szabályzás alatt álló pigmenttermelésének (violacein) monitorozásán keresztül követtük, folyadék tenyészetben.

A kivonatok az egységesen alkalmazott 2 mg/mL koncentrációban 6-36% közötti mértékű QS gátló aktivitást mutattak. Szőlő és sárkánygyümölcs esetén az enzimmentes minta gátló hatását az előkezelés jellege nem befolyásolta. Ugyanakkor az enzimatikus kezelés képes volt megnövelni a DBG, LBG, DP, LP és LA kivonatok által kifejtett QS gátló hatást. A fenolos vegyületeket 10 µg/mL koncentrációban vittük be a tesztekbe, és 5-31% között azonosítottunk QS gátló hatást. Eredményeink alapján az enzimkezelések után kapott sziringsav, vanillinsav, (+)-katekin, (−)-epikatekin, 4-hidroxi-benzoesav, galluszsav és rezveratrol hozamok hozzájárulhattak egyes kivonatok QS gátló aktivitásához.

6. Tanulmányoztuk élelmiszer eredetű patogén és romlást okozó baktériumok biofilmképzését fenolos kivonatok és vegyületek jelenlétében.

A QS mechanizmus fontos szerepet játszik mikroorganizmusok biofilmképzésében. Mivel a legtöbb enzimmentes és enzimkezelt fekete szőlő, alma és pitahaya kivonat, valamint számos fenolos vegyület jól gátolta a QS folyamatot, fontosnak tartottuk megvizsgálni mintáink élelmiszerekkel kapcsolatos baktériumok biofilmképző képességére gyakorolt hatását is. Az előző tesztjeinkben alkalmazott *L. monocytogenes*, *S. aureus*, MRSA, *E. coli*, *S. enterica*, *P. putida* és *P. aeruginosa* baktériumokkal dolgoztunk. A legtöbb kivonatnál tapasztaltunk biofilmképzést gátló hatást; a *P. putida*, *P. aeruginosa* és

S. aureus biofilmeknél mutattunk ki nagy érzékenységet a kivonatokra. A gátló hatás a szubsztrát előkezelés módszerétől, az enzimkezelési eljárástól, valamint az vizsgált baktérium érzékenységétől függően változatos mértéket mutatott. A szárítószekrényben szárított fekete szőlő és sárkánygyümölcs maradványok kivonatai például nagyobb gátló hatást mutattak, mint a liofilizált szubsztrátokon készített minták. Alma kivonatok esetén ezzel szemben a liofilizált maradványokból származó minták hatékonyabban gátolták a legtöbb baktérium biofilmképzését. Néhány kivonat azonban, enzimkezeléstől függetlenül, támogató hatással volt bizonyos biofilmek képződésére. A jelenség a *S. enterica*, *L. monocytogenes* és *E. coli* biofilmeknél volt leginkább jellemző. A biofilmképzést fokozó hatásért a baktériumok kivonatokkal szembeni eltérő érzékenysége és/vagy a minták fenolos vegyület és poliszacharid koncentrációja lehet felelős. A fenolok stresszorként biofilm képzésre kényszeríthetik a sejteket, míg a szacharidok a képződés folyamata során stabilizálhatják a biofilm mátrixot. A fenolos vegyületek a *S. aureus*, *E. coli*, *S. enterica* és a *Pseudomonas* biofilmeket gátolták a legnagyobb mértékben. A flavonoidok, azaz a (+)-katekin, (−)-epikatekin és kvercetin, valamint a stibenoidok, azaz a polidatin és a rezveratrol, nagyobb gátló aktivitást mutattak a legtöbb általunk vizsgált baktérium biofilmképzésére, mint a fenolsavak.

Eredményeink alapján a *R. miehei* és az általa termelt cellulázok alkalmazhatók szabad fenolokat tartalmazó antioxidatív kivonatok előállítására szőlő, alma, sárkánygyümölcs, mangó és naranjilla maradványokból. A kapott fenolos kivonatok felhasználhatók antioxidáns, antimikrobiális, anti-biofilm és anti-quorum sensing tulajdonságokkal rendelkező természetes vegyületek forrásául. Reményeink szerint a fenolvegyületek és kivonatok helyettesíthetik vagy támogathatják szintetikus élelmiszer-adalékok aktivitását, mely utóbbi szerek napjainkban komoly fogyasztói ellenérzéssel bírnak. Ezzel összhangban az előállított kivonatok tartósítószerek és/vagy fertőtlenítőszerek potenciális forrásai is lehetnek. Emellett a kutatási programban alkalmazott kezelési módszerekkel élelmiszerek feldolgozás utáni maradványainak környezetbarát hasznosítása is elérhető. Tudomásunk szerint elsőként alkalmaztunk fermentációs és enzimatikus kezelés technikákat bioaktív természetes vegyületek előállítására sárkánygyümölcs és naranjilla mintákból.

8. REFERENCES

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9. ACKNOWLEDGEMENTS

10. APPENDICES

Appendix 1. Correlation coefficients (Pearson *r*) between the antimicrobial activity against different pathogens and total phenolic content of oven-dried and lyophilized black grape, apple and pitahaya extracts after enzyme treatments.

Fruit residues	*B. subtilis*	*B. cereus*	*L. monocytogenes*	*S. aureus*	MRSA	*E. coli*	*S. enterica*	*P. putida*	*P. aeruginosa*
Black grape									
Oven-dried	-0.291	-0.369	0.974	-0.180	-0.180	0.571	0.998	0.453	-0.291
Lyophilized	0.922	0.597	-0.796	0.992	0.778	0.960	-0.239	0.387	-0.387
White grape									
Oven-dried	0.291	0.978	-0.974	0.010	-0.179	-0.897	-0.703	0.984	0.626
Lyophilized	-0.284	0.972	0.234	0.006	0.284	0.006	0.460	-0.284	0.972
Apple									
Oven-dried	-0.720	0.906	0.819	0.906	0.888	0.962	0.087	0.639	0.906
Lyophilized	0.687	-0.584	0.996	0.746	0.961	0.746	0.276	0.907	0.411
Pitahaya									
Oven-dried	-0.999	0.584	-0.411	0.861	0.584	0.887	-0.231	-0.231	0.985
Lyophilized	0.385	0.607	0.385	0.385	-0.607	0.385	0.061	-0.607	0.385
Mango									
Oven-dried	0.982	0.827	0.312	0.329	NT*	0.827	0.827	0.827	-0.073
Naranjilla									
Oven-dried	-1	-1	NT	1	NT	NT	NT	NT	NI
Lyophilized	1	-1	NT	NT	1	-1	-1	NT	NI

* not tested. Neither the control nor the treated sample showed antimicrobial activity.

Appendix 2. Minimum bactericidal concentration (MBC) of fruit residue extracts against foodborne pathogenic and spoilage bacteria before and after treatment with *R. miehei* cellulase and/or *A. niger* pectinase.

Fruit residues		MBC (mg/mL)								
		B. subtilis	*B. cereus*	*L. monocytogenes*	*S. aureus*	MRSA	*E. coli*	*S. enterica*	*P. putida*	*P. aeruginosa*
Black grape										
Oven-dried	Enzyme-free	>100	>100	>100	>100	>100	>100	>100	100	100
	Cellulase	>100	>100	100	100	100	100	100	100	100
	Pectinase	>100	NT*	>100	100	100	100	NT	100	100
	Cellulase + Pectinase	>100	>100	50	100	100	100	>100	100	>100
Lyophilized	Enzyme-free	>100	>100	>100	100	>100	>100	>100	100	100
	Cellulase	>100	>100	NT	50	100	100	100	>100	100
	Pectinase	>100	>100	>100	100	>100	>100	100	100	100
	Cellulase + Pectinase	>100	>100	>100	100	100	100	100	100	>100
White grape										
Oven-dried	Enzyme-free	>100	>100	100	100	>100	100	100	100	100
	Cellulase	>100	>100	>100	100	100	100	100	100	100
	Pectinase	>100	>100	>100	>100	>100	>100	>100	>100	>100
	Cellulase + Pectinase	>100	>100	>100	>100	>100	>100	>100	>100	>100
Lyophilized	Enzyme-free	>100	>100	100	100	>100	>100	100	>100	>100
	Cellulase	>100	>100	100	100	>100	>100	>100	>100	>100
	Pectinase	>100	>100	100	100	100	100	100	100	100
	Cellulase + Pectinase	>100	>100	>100	>100	>100	>100	>100	>100	>100
Apple										
Oven-dried	Enzyme-free	>100	>100	NT	>100	NT	NT	NT	>100	>100
	Cellulase	>100	>100	NT	>100	>100	>100	NT	100	>100
	Pectinase	>100	>100	>100	>100	NT	>100	>100	100	100
	Cellulase + Pectinase	>100	>100	NT	>100	>100	>100	NT	>100	>100
Lyophilized	Enzyme-free	>100	>100	>100	NT	NT	NT	NT	>100	>100
	Cellulase	>100	>100	NT	100	>100	NT	>100	>100	>100
	Pectinase	>100	>100	>100	>100	NT	NT	>100	100	100
	Cellulase + Pectinase	>100	>100	>100	100	>100	>100	100	>100	>100

Appendix 2. Continued.

Fruit residues			MBC (mg/mL)								
			B. subtilis	*B. cereus*	*L. monocytogenes*	*S. aureus*	MRSA	*E. coli*	*S. enterica*	*P. putida*	*P. aeruginosa*
Pitahaya											
Oven-dried	Enzyme-free		>100	>100	>100	>100	NT	>100	>100	100	>100
	Cellulase		>100	>100	NT	>100	>100	NT	>100	>100	>100
	Pectinase		>100	>100	>100	>100	NT	NT	>100	100	100
	Cellulase + Pectinase		>100	>100	>100	100	NT	100	>100	100	100
Lyophilized	Enzyme-free		>100	>100	>100	>100	>100	>100	>100	>100	>100
	Cellulase		>100	>100	>100	NT	>100	NT	100	100	100
	Pectinase		>100	>100	>100	>100	>100	>100	>100	100	100
	Cellulase + Pectinase		>100	>100	>100	>100	>100	100	100	100	100
Mango											
	Enzyme-free		100	100	>100	>100	>100	>100	>100	>100	>100
Oven-dried	Cellulase		100	100	>100	>100	>100	>100	100	>100	100
	Pectinase		100	100	>100	100	100	100	100	100	100
	Cellulase + Pectinase		100	100	>100	100	>100	>100	100	100	100
Naranjilla											
Oven-dried	Enzyme-free		NT	>100	NT	NT	NT	NT	NT	NT	>100
	Cellulase		>100	NT	NT	>100	>100	>100	>100	>100	>100
Lyophilized	Enzyme-free		NT	NT	NT	NT	NT	>100	NT	NT	NT
	Cellulase		>100	>100	>100	>100	>100	>100	>100	>100	>100

* not tested.

Appendix 3. Correlation coefficients (Pearson *r*) between violacein production inhibitory activity and syringic acid, vanillic acid, (+)-catechin and resveratrol content of oven-dried and lyophilized black grape, apple and pitahaya extracts after enzyme treatments.

Fruit residues	Syringic acid	Vanillic acid	(+)-Catechin	Resveratrol
Black grape				
Oven-dried	0.951	0.933	0.463	n.d.*
Lyophilized	0.878	0.906	0.923	0.874
Apple				
Oven-dried	-0.974	-0.919	-0.820	-0.215
Lyophilized	0.981	0.848	0.193	0.117
Pitahaya				
Oven-dried	0.923	0.991	-0.958	-0.793
Lyophilized	-0.574	-0.337	n.d.	-0.352

* no data

11. PUBLICATIONS UNDERLYING THE THESIS

Full papers

Zambrano, C., Kerekes, E.B., Kotogán, A., Papp, T., Vágvölgyi, Cs., Krisch, J., Takó, M. (2019) Antimicrobial activity of grape, apple and pitahaya residue extracts after carbohydrase treatment against food-related bacteria. *LWT-Food Science and Technology* 100, 416-425.

Zambrano, C., Kotogán, A., Bencsik, O., Papp, T., Vágvölgyi, Cs., Mondal, K.C., Krisch, J., Takó, M. (2018) Mobilization of phenolic antioxidants from grape, apple and pitahaya residues via solid state fungal fermentation and carbohydrase treatment. *LWT-Food Science and Technology* 89, 457-465.

Book chapter

Takó, M., **Zambrano, C.**, Kotogán, A., Kerekes, E.B., Papp, T., Krisch, J., Vágvölgyi, Cs. (2019) Fermentative and enzyme-assisted production of phenolic antioxidants from plant residues. In: Thatoi, H.N., Das Mohapatra, P.K., Mondal, K.C., Mohapatra, S. (eds.) Microbial Fermentation and Enzyme Technology. Boca Raton (FL), USA: CRC Press - Taylor and Francis Group, Paper: Chapter 12 (*accepted, in press*)

Abstracts

Takó, M., **Zambrano, C.**, Kotogán, A., Kerekes, E.B., Bencsik, O., Szekeres, A., Papp, T., Vágvölgyi, Cs., Krisch, J. (2018) Carbohydrase-assisted extraction of bioactive phenolic compounds from fruit residues. In: A Magyar Mikrobiológiai Társaság 2018. évi Nagygyűlése és a XIII. Fermentációs Kollokvium: Absztraktfüzet. pp. 63-64.

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12. FINANCIAL SUPPORT