PH.D. THESIS

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

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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. 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. 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. Furthermore, some plant phenolics can exhibit anti-biofilm and/or anti-quorum sensing activities as well. Hence, certain molecules can be used as natural food preservatives or sanitizers.

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. It has also been proven that the β-glucosidase of this fungus can release phenolic aglycones from their glycosidic bond.

AIMS

The present work aimed the mobilization of bioactive phenolic compounds from antioxidative fruit byproducts through solid-state fermentation (SSF) and direct enzyme treatment using the *R. miehei* NRRL 5282 strain and its cellulolytic enzyme 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 pectinase as well. Our goals included the analysis of the total and individual phenolic contents and the antioxidant and antimicrobial activities of the extracts produced.

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 *Aspergillus niger*.
4. Analysis and comparison of the total phenolic content (TPC) 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-quorum sensing 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.

METHODS

Solid-state fermentation

- Fermentation of residues moisturized with water and supplemented with soy flour as nitrogen source

Enzyme-assisted extraction

- Enzyme-free and cellulase, pectinase or cellulase-pectinase aided treatments

Cellulolytic activity assays

- Filter paper degrading activity
- Endoglucanase
- Cellobiohydrolase
- β-Glucosidase

Determination of total phenolics and antioxidant activity

- Folin-Ciocalteu's assay for TPC determination
- 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity
- Ferric reducing antioxidant power (FRAP) assay

Analysis of individual phenolics in extracts

- High-performance liquid chromatography (HPLC)

Antimicrobial activity tests

- Agar disk-diffusion method
- Minimum inhibitory concentration (MIC) by broth microdilution method
- Minimum bactericidal concentration (MBC) by plate count agar method

Anti-quorum sensing capacity assay

- Detection of quorum sensing inhibition in liquid cultures using the model organism *C. violaceum*

Biofilm formation assay

- Detection of biofilm formation inhibition by crystal violet staining method

RESULTS

Selection of food residue samples

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 experiments. 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.

Solid-state fermentation on fruit residues (Zambrano *et al.* 2018)

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 *R. miehei* NRRL 5282 isolate. Samples were collected at specified intervals, then, cellulase (β-glucosidase) activity, TPC, FRAP and 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 in excess, the enzymes and transporters necessary for the utilization of a carbon source different from glucose are repressed. 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.

Enzyme treatment of fruit residues (Zambrano *et al.* 2018)

Enzyme treatment of oven-dried and/or lyophilized grape, apple, pitahaya, mango and naranjilla 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 *A. 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.

Liquid chromatography analysis of individual phenolic compounds (Zambrano *et al.* 2018)

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.

Antimicrobial activity assays (Zambrano *et al.* 2019)

We investigated the antimicrobial activity of grape, apple, pitahaya, mango and naranjilla extracts before and after enzyme treatments against 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.

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.

Quorum sensing inhibition in *C. violaceum* (Zambrano *et al.* 2019)

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

The fruit residue extracts presented anti-quorum sensing 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-quorum sensing 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.

Biofilm formation in pathogen and spoilage bacteria (Zambrano *et al.* 2019)

It is known that the quorum sensing 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 quorum sensing 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.

SUMMARY

1. Phenolic rich extracts with improved antioxidant activity have been prepared by solid-state *R. miehei* fermentation and *R. miehei* cellulase and/or *A. niger* pectinase assisted extraction of grape, apple, pitahaya, mango and naranjilla residues. 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.
2. Concentration of 11 individual phenolics in the black grape, apple, pitahaya and mango extracts have been analyzed and compared before and after enzyme treatments. HPLC data also confirmed an increase in the phenolic content of extracts after cellulase treatments.
3. Antimicrobial activity of enzyme-free and enzyme-treated extracts and individual phenolics against food-related bacteria has been studied. Antioxidative samples with effective growth inhibitory potential have been identified. Our study is the first that determine MIC data of naranjilla samples against pathogenic bacteria.
4. Anti-quorum sensing activity of enzyme-free and enzyme-treated black grape, apple and pitahaya residue extracts, and phenolic compounds has been evaluated using the model organism *C. violaceum*. To our knowledge, the anti-quorum sensing capacity of pitahaya samples has not been studied so far.
5. Biofilm formation of foodborne pathogens and spoilage bacteria was studied in the presence of phenolic extracts and compounds. Our investigations revealed potential antibiofilm phenolics that can be obtained from black grape, apple and pitahaya extracts after cellulase and cellulase-pectinase combined treatments.

PUBLICATIONS UNDERLYING THE PH.D. THESIS

Journal articles:

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. ***IF: 3.714***

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. ***IF: 3.714***

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.

Zambrano, C., Kerekes, E.B., Kotogán, A., Bencsik, O., Szekeres, A., Vágvölgyi, Cs., Papp, T., Krisch, J., Takó, M. (2018) Production of bioactive phenolic compounds from mango residues. In: Monostori, T. (ed.) 16th Wellmann International Scientific Conference: Book of Abstracts. pp. 102-103.

Zambrano, C., Kovács, F., Kotogán, A., Papp, T., Mondal, K.C., Hargitai, F., Vágvölgyi, Cs., Krisch, J., Takó, M. (2017) Phenolic antioxidant mobilization in black grape, apple and dragon fruit residues by enzymatic treatment using a cellulolytic cocktail from *Rhizomucor miehei*. In: 7th Congress of European Microbiologists (FEMS 2017) p. 1327.

Zambrano, C., Kerekes, E.B., Kotogán, A., Bencsik, O., Mondal, K.C., Vágvölgyi, Cs., Papp, T., Takó, M., Krisch, J. (2017) Production of bioactive phenolic compounds from fruit residues by carbohydrase enzymes. Mikológiai Közlemények-Clusiana 56:1 pp. 16-18.

Zambrano, C., Kotogán, A., Kerekes, E., Vágvölgyi, Cs., Krisch, J., Takó, M. (2017) Effect of carbohydrate hydrolyzing enzyme treatment on the antioxidant and antimicrobial activity of fruit pomace extracts. In: 19th Danube-Kris-Mures-Tisa (DKMT) Euroregional Conference on Environment and Health: Program and abstracts. p. 34.

Takó, M., **Zambrano, C.**, Kotogán, A., Krisch, J., Papp, T., Mondal, K.C., Vágvölgyi, Cs. (2016) Solid-state fermentation of dragon fruit residues to produce phenolic antioxidants using *Rhizomucor miehei*. In: Škrbić, B. (ed.) 18th Danube-Kris-Mures-Tisa (DKMT) Euroregional Conference on Environment and Health: Book of abstracts. pp. 86-87.

Zambrano, C., Kotogán, A., Komáromi, L., Takó, M., Krisch, J., Vágvölgyi, Cs. (2016) Production of phenolic antioxidants from apple residues. In: Keszthelyi-Szabó, G., Hodúr, C., Krisch, J. (eds.) International Conference on Science and Technique Based on Applied and Fundamental Research (ICoSTAF'16): Book of Abstracts. p. 48.

OTHER PUBLICATIONS

Journal article:

Kotogán, A., **Zambrano, C.**, Kecskeméti, A., Varga, M., Szekeres, A., Papp, T., Vágvölgyi, Cs., Takó, M. (2018) An organic solvent-tolerant lipase with both hydrolytic and synthetic activities from the oleaginous fungus *Mortierella echinosphaera*. *International Journal of Molecular Sciences* 19(4), 1129. ***IF: 4.183***

Abstract:

Takó, M., **Zambrano, C.**, Kotogán, A., Kerekes, E.B., Krisch, J., Papp, T., Vágvölgyi, Cs. (2016) Antimicrobial effect of antioxidative extracts obtained after solid-state bioprocessing of oilseed residues using the zygomycete *Rhizomucor miehei*. In: Mrša, V; Teparić, R; Kifer, D (szerk.) Power of Microbes in Industry and Environment 2016: Programme and abstracts. p. 66.

Cumulative impact factor: 11.611