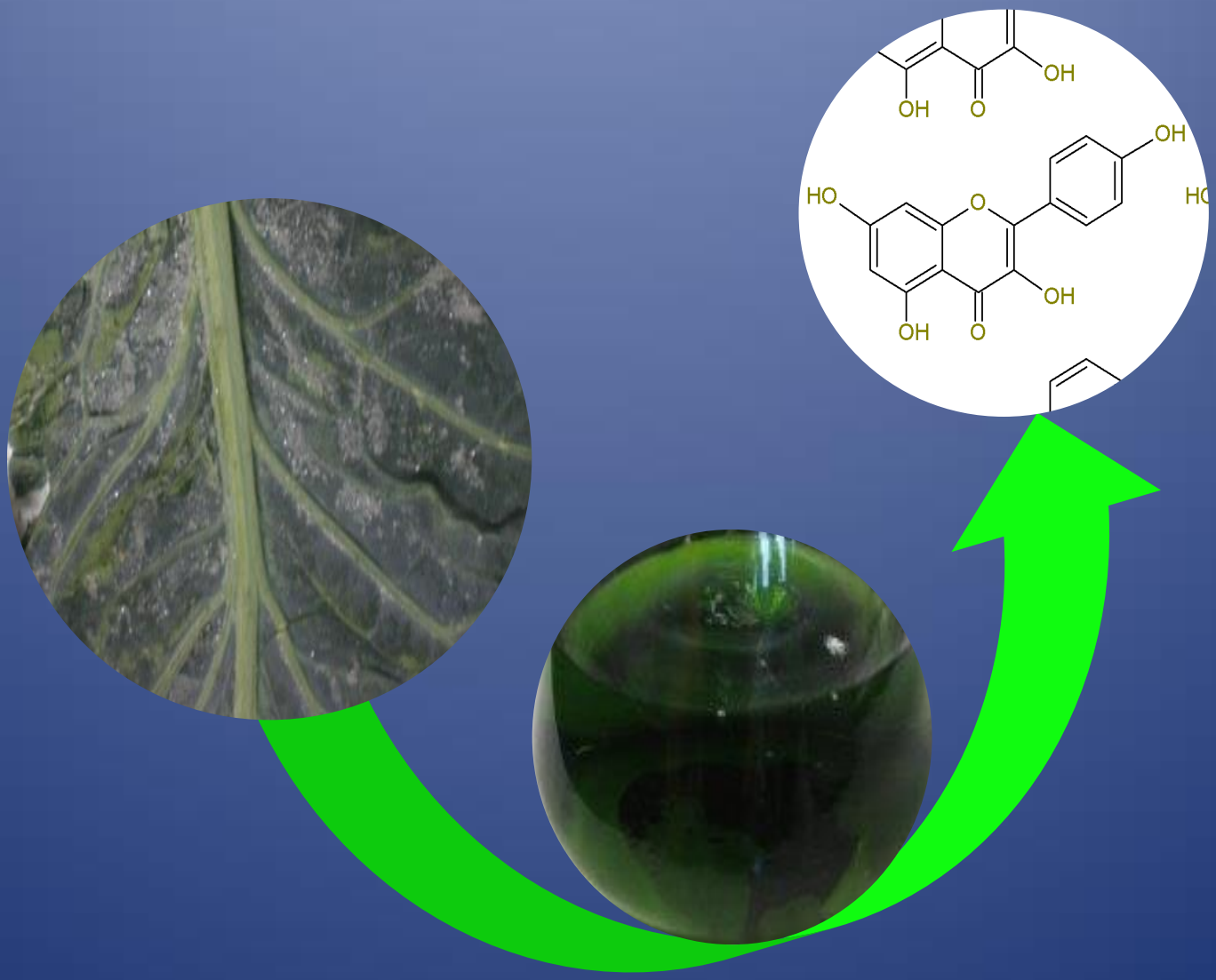
BIOLOGICAL TREATMENTS OF CAULIFLOWER (*BRASSICA OLERACEA* L. VAR. *BOTRYTIS*) OUTER LEAVES

IMPROVED EXTRACTION AND CONVERSION OF PHENOLIC COMPOUNDS



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Huynh Thai Nguyen

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BIOLOGICAL TREATMENTS OF CAULIFLOWER (*BRASSICA OLERACEA* L. VAR. *BOTRYTIS*) OUTER LEAVES: IMPROVED EXTRACTION AND CONVERSION OF PHENOLIC COMPOUNDS

Thesis submitted in fulfillment of the requirements
for the degree of Doctor (PhD) of Applied Biological Science

Dutch translation of the title:

Biologische voorbehandeling van bloemkool (*Brassica oleracea* L. Var. *Botrytis*) bladeren: impact op extractie en omzetting van fenolische componenten

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Table of contents

LIST OF ABBREVIATIONS

AE: Aqueous Extract

ANOVA: Analysis of Variance

CFU: Colony Forming Units

DAD: Diode Array Detection

E/S: Enzyme/Substrate

GAE: Gallic Acid Equivalent

HDMS-TOF: High Definition Mass Spectrometry - Time of Flight

H: hour

LAB: Lactic Acid Bacteria

LC-MS: Liquid Chromatography - Mass Spectrometry

L: liter

min: minute

MA: Methanol Extract

PCA: Principal Component Analysis

RE: Rutin Equivalent

SmF: Submerged Fermentation

SPE: Solid Phase Extraction

SSF: Solid State Fermentation

TPC: Total Phenolic Content

TP: Total Polyphenol Content

UPLC: Ultra Performance Liquid Chromatography

UV-vis: Ultraviolet–visible

Introduction and Objectives

Introduction and Objectives

Cauliflower and broccoli are the most common vegetables used in the world. In the last two decades, the production has steadily increased to meet the rising demand of consumers, up from globally 10 million tons in 1993 to around 22.2 million tons in 2013 (Food and Agriculture Organization, FAO) (FAOstat, 2015). Cabbage and Brassica are worldwide important vegetables, of which one third is compromised by cauliflower and broccoli. This increased worldwide production of cabbages simultaneously resulted in a huge amount of by-products generated. The main wastes, which might account for over 70 % of crop, are the non-edible parts of cauliflower and broccoli including leaves, stems, florets, and stalks produced during postharvest handling. Traditionally, most of these residues are discarded and left on the land causing additional environmental problems (e.g. imbalance in mineral composition of the soil, methane production, groundwater pollution), while a small proportion is utilized as animal feeds (Campas-Baypoli et al., 2009; Iñiguez-Covarrubias et al., 2001). Only a few studies recognized these by-products as a source of glucosinolates (Ares et al., 2014; Cabello-Hurtado et al., 2012; Dominguez-Perles et al., 2011), fiber (Nilnakara et al., 2009; Tanongkankit et al., 2012) and amino acids (Arnáiz et al., 2012). It has been reported that glucosinolates is present in a range of 2.1 to 2.5 mg/g dry weight, with broccoli leaves having the highest content. Crude fiber is present in outer leaves of cabbage (*B. oleracea* L. var. capitata) in concentrations around 20 % (Nilnakara et al., 2009), while amino acids are present in quantities around 10 mg/g dry weight in the leaves of *Nubia* (a kind of cultivar of broccoli)(Arnáiz et al., 2012).

During recent years, these by-products have also received a lot of attention due to their content of phenolic compounds, which have been documented as potential compounds for prevention and treatment of cardiovascular diseases (hypertension, obesity, arteriosclerosis, smoking, diabetes, and aging) and cancers (Nile et al., 2014; Ranilla et al., 2010; Rodriguez-Mateos et al., 2013). The majority of up-to-date technologies to obtain these bioactive compounds from their

plant matrices is based on conventional extraction in which organic solvents are frequently utilized. However, this method has been recognized to be more efficient in extraction of free phenolic compounds, while the bound phenolic compounds, which are linked to cell wall structure components like cellulose, hemicellulose, lignin and pectin, are normally ignored by this approach (Acosta-Estrada et al., 2014; Saura-Calixto, 2012). As such, a significant amount of phenolic compounds remains in the solid residues and are generally discarded. In addition, most phenolic compounds found in natural sources occur in highly glycosylated forms, whereby various sugar moieties are conjugated to the aromatic rings, in most cases resulting of a reduction in their biological activity (Fukumoto et al., 2000).

For these reasons, the main aim of this PhD-thesis was to investigate different methods for improving the release of phenolic compounds and their metabolites from cauliflower by-products using various biological approaches. Commercial carbohydrate-degrading enzymes, lactic acid bacteria, and filamentous fungi (all food-grade) were used for the performed studies in this PhD-thesis. The outline of the research is presented in different chapters (Figure 0. 1) as follows:

Chapter 1 gives a detailed overview of scientific literature covering the basic knowledge of phenolic compounds (structure, classification, localization and biological activity), the current status of agricultural and *Brassica* by-products and their phenolic content. The improved release of phenolic compounds and their metabolism through enzymatic and fermentation technology has been summarized and discussed. In **chapter 2**, two commercial carbohydrate-degrading enzymes (Visoczyme L and Rapidase) were investigated for their influence on the release of phenolic compounds extracted from cauliflower outer leaves. As potential alternatives for using commercial enzymes, **chapter 3, chapter 4 and chapter 5** were carried out to evaluate the improvement in extraction and bioconversion of phenolic compounds from cauliflower outer leaves through microbial fermentation.

Chapter 6 gives a general discussion related to the results obtained in the different research chapters. Finally, the perspectives for the future work are proposed in this part.

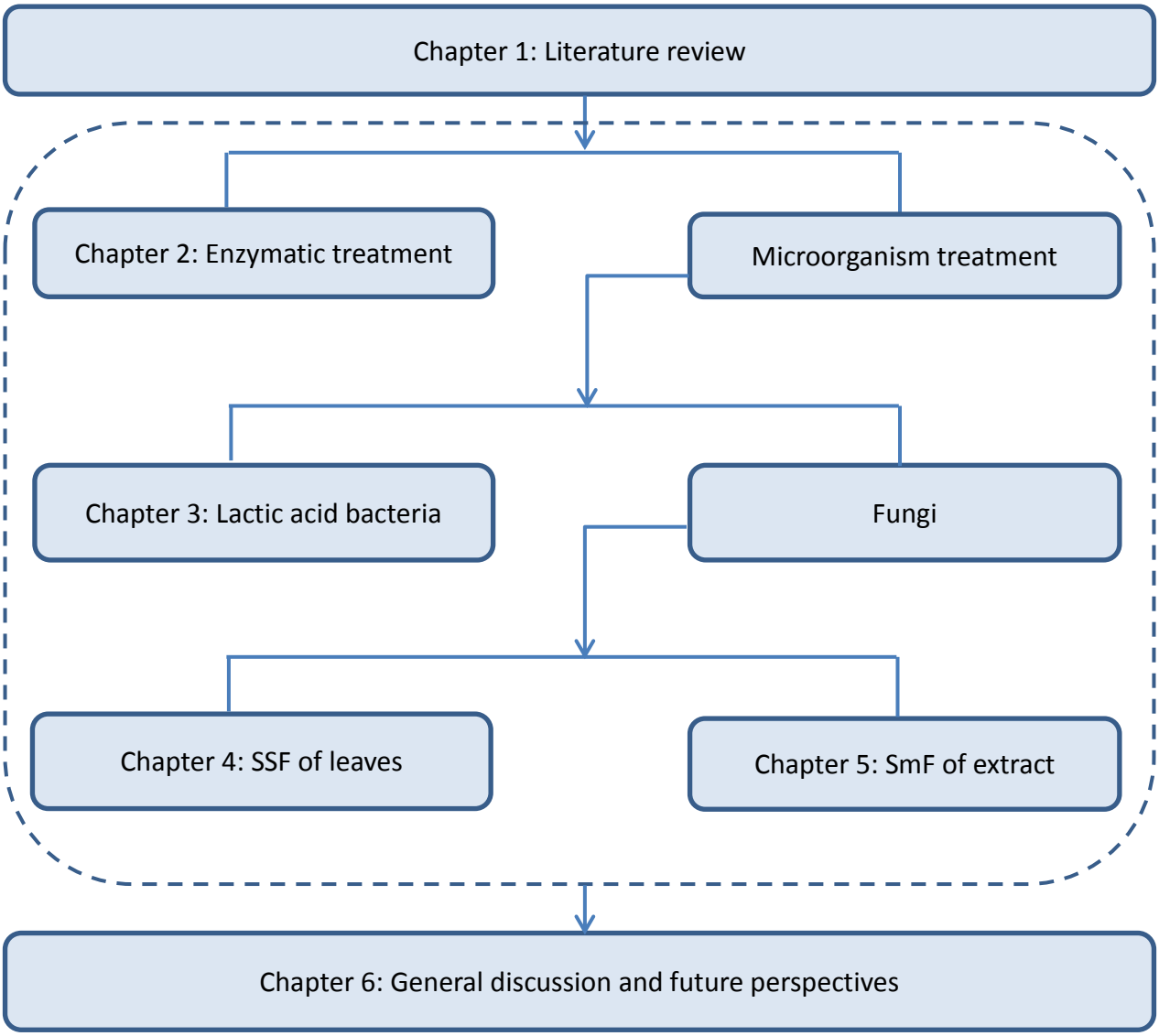


Figure 0.1. Structure and content of the PhD-dissertation; SSF: solid-state fermentation; SmF: submerged fermentation

Summary – Samenvatting

SUMMARY

This PhD thesis focuses on various biological approaches, i.e. using enzymes and microorganisms, for an enhanced extraction of phenolic compounds and their metabolites from cauliflower outer leaves as a model for by-products.

The first chapter of the thesis presents a review of literature currently available on this topic. The studies performed to date related to extraction and metabolism of phenolic compounds by enzymes or microorganisms were summarized and discussed. It became clear that many reports worked on extraction of bioactive compounds from various plant-based sources are available using different types of extraction techniques. However studies performed on cauliflower and broccoli by-products to produce extracts rich in phenolic compounds is scarce, especially with the help of enzymes and microorganisms. The research performed in this thesis tried to contribute to fill part of this research gap. Therefore several experimental studies were performed as described in chapter 2 to 5.

In chapter 2, the effect of enzymatic pretreatment on the release of phenolic compounds was evaluated. Cauliflower outer leaves were incubated with two carbohydrate-degrading enzymes (Viscozyme L and Rapidase) under various conditions. The optimal parameters determined for the treatment were pH 4.0, temperature 35 °C and enzyme/substrate ratio of 0.2 % (v/w) and 0.5 % (v/w) for Viscozyme L and Rapidase, respectively. Under optimal conditions, both these enzymes resulted in a more than 100% increase of total phenolic content in the extracts. As identified by UPLC-MS/MS, most of individual phenolic compounds in cauliflower outer leaves were present as different forms of kaempferol

glycosides and their combination with hydroxycinnamic acids. Although no aglycones were released, the amount of kaempferol-3-O-glycoside, one of the lowest glycosylated forms, found in samples incubated with Viscozyme L and Rapidase increased by 7.2 fold and 3.7 fold, respectively.

As an alternative for the use of commercial enzymes, lactic acid bacteria were selected for the study in chapter 3. Four strains including *Lactobacillus plantarum* (LMG6907), *Lactobacillus mali* (LMG6899), *Pediococcus pentosaceus* (LMG10740) and *Pediococcus damnosus* (LMG114884) were evaluated for their effect on the extraction of phenolic compounds through fermentation. Although a good growth on cauliflower outer leaves was observed, the level of total phenolic content in the samples remained stable during 6 days of fermentation. Similarly, there was no change in the profile of individual phenolic compounds in the extract, obtained after a fermentation with *Lactobacillus plantarum* during a period of 6 days. However during this incubation, the β-glucosidase, determined as intracellular, activity showed an increase of 1.37 (U*/mL) and 2.34 (U/mL) after 12 and 24 h of incubation respectively compared to the control (t=0). It could be concluded that these highly glycosylated flavonoids could not be taken up by the bacterial cells to come into contact with the intracellular β-glucosidase.

As lactic acid bacteria were not able to obtain extracts with a higher amount of phenolic compounds, food-graded fungi were screened. Therefore, Chapter 4 was performed with the aim at improved extraction of phenolic compounds and their unique metabolites through solid-state fermentation with various filamentous fungi: *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus sojae*, *Rhizopus oryzae*, *Rhizopus azygosporus* and *Phanerochaete chrysosporium*.

*One unit of enzyme activity is defined as the amount of enzyme that releases 1 mmol p-nitrophenol per minute under optimal condition (pH 4.6 and 50±1°C)

Among them*, Aspergillus sojae* exhibited the greatest capacity for an enhanced extraction, with an increase in the amount of phenolic compounds by a factor of three as compared with unfermented sample. The incubation with *Aspergillus sojae* also resulted in a considerable decrease in the concentration of high glycosylated compounds, while the massive increase in their corresponding metabolites, in which less or no sugar moieties attached at 3- and/or 7-position, was concomitantly observed.

Because no aglycones were observed through solid-state fermentation as described in previous work, in chapter 5, two fungal strains (*Rhizopus oryzae, Rhizopus azygosporus*) were evaluated for their influence on the bioconversion of kaempferol and quercetin metabolites under submerged fermentation condition. The fermentation with *Rhizopus oryzae* resulted in the accumulation of kaempferol aglycone in medium. Kaempferol aglycone was already detected after 1 day of incubation, and reached the highest level on 2nd day prior to reduction in the latter period. However this compound was not observed for the fermentations with *Rhizopus azygosporus*, except for day 4. The production of quercetin was observed for *Rhizopus oryzae* during 7 days of incubation, and the highest amount was recorded on the last day (day 7). In the incubations with *Rhizopus azygosporus*, another release pattern was observed i.e. the highest amount of quercetin was observed after 1 day, followed by a gradual decrease towards a complete disappearance at the last day (day 7).

In conclusion, this PhD dissertation mainly works on the techniques to obtain an improved extraction of kaempferol glycosides and their metabolites. With the results obtained, it can be concluded that fungi can be considered as a potential candidate for developing a biological extraction process of kaempferol derivatives from cauliflower by-products, as well

as of the production of their unique metabolites, which could be valuable substances for the medical, cosmetic or food demands.

SAMENVATTING

Dit doctoraatsonderzoek heeft zich toegespitst op verschillende biologische benaderingen, met name het gebruik van enzymen en micro-organismen, om een betere extractie te verkrijgen van fenolische componenten en hun metabolieten uit buitenste bladeren van de bloemkool, dit als modelmatrix voor nevenstromen.

In het eerste hoofdstuk van de thesis wordt een overzicht van de huidig beschikbare literatuur weergegeven. De studies welke extractie en metabolisme van fenolische componenten met behulp van enzymen en micro-organismen behandelden, werden besproken. Hieruit werd het duidelijk dat er reeds veel werk verricht is rond extractie van bioactieve componenten uit verschillende plantaardige matrices en dit met een grote variëteit aan extractietechnieken. Echter het aantal studies uitgevoerd op nevenstromen van bloemkool en broccoli is heel beperkt, zeker indien extracties uitgevoerd werden met behulp van enzymen of micro-organismen. Het onderzoek uitgevoerd gedurende deze doctoraatstudie hoopt bij te dragen aan deze gap in het onderzoek, dit via verschillende experimenten zoals beschreven in hoofdstuk 2 - 5.

In hoofdstuk 2 werd het effect van een enzymatische voorbehandeling op de vrijstelling van fenolische componenten onderzocht. Hiervoor werden bloemkoolbladeren geïncubeerd met 2 verschillende koolhydraatafbrekende enzymen nl. Viscozyme L en Rapidase, en dit bij verschillende omstandigheden. Optimale incubatieparameters waren pH = 4.0, temperatuur = 35 °C en enzym/substraat verhouding = 0.2 % (v/w) en 0.5 % (v/w) voor Viscozyme L en Rapidase, respectievelijk. Onder optimale omstandigheden werd met beide

enzympreparaten een meer dan 100% verhoogde vrijstelling aan fenolische componenten bekomen. Na identificatie met behulp van UPLC-MS/MS kon aangetoond worden dat de meeste fenolische componenten in bloomkoolbladeren verschillende vormen van kaempferol glycosiden en combinaties met hydroxykaneelzuren waren. Er konden geen aglyconen gedetecteerd worden na enzymatische behandeling, echter de hoeveelheid aan kaempferol-3-O-glucoside, als één van de minst geglycosyleerde vormen, was 7.2 en 3.7 keer hoger met respectievelijk Viscozyme L en Rapidase, in vergelijking met de controle.

In hoofdstuk 3 werd het gebruik van melkzuurbacteriën geëvalueerd als mogelijk alternatief voor het gebruik van commerciële enzymen. Vier verschillende stammen namelijk *Lactobacillus plantarum* (LMG6907), *Lactobacillus mali* (LMG6899), *Pediococcus pentosaceus* (LMG10740) and *Pediococcus damnosus* (LMG114884) werden gebruikt om hun effect op de extractie van fenolische componenten gedurende fermentatie na te gaan. Hoewel een goede groei waargenomen werd op de bloemkoolbladeren, was het gehalte aan fenolische componenten in de extracten ongewijzigd gedurende een incubatie van 6 dagen. Daarnaast werd er ook geen verschil in het profiel aan individuele componenten waargenomen in de extracten bekomen via fermentatie met *L. plantarum* gedurende 6 dagen. Echter tijdens deze fermentatie werd wel een toename in de intracellulaire β-glucosidase activiteit gemeten van respectievelijk 1.37 U/ml en 2.34 U/ml na 12 en 24h in vergelijking met de controle. Hieruit kan besloten worden dat deze hoog geglycosileerde verbindingen niet in staat zijn om door de bacteriële cel opgenomen te worden zodat er interactie kan zijn tussen het intracellulair enzym en de component.

*Eén unit enzym-activiteit wordt gedefinieerd als de hoeveelheid enzym dat 1 mmol p-nitrofenol per minuut vrijstelt onder optimale omstandigheden (pH 4.6 en 50±1°C)

Aangezien het met melkzuurbacteriën niet mogelijk was om extracten te bereiden met een hoger gehalte aan fenolische componenten, werd in een volgende fase gekozen om food-grade fungi te screenen. Zoals beschreven in Hoofdstuk 4, is het doel om extracten te bekomen met een verhoogd gehalte aan fenolische componenten en hun unieke metabolieten via solid-state fermentatie met verschillende filamenteuze schimmels: *Aspergillus niger, Aspergillus oryzae, Aspergillus sojae, Rhizopus oryzae, Rhizopus azygosporus* and *Phanerochaete chrysosporium*. Hieruit werd duidelijk dat *Aspergillus sojae* de grootste capaciteit bezat om na incubatie extracten te bekomen met een verhoogde hoeveelheid aan fenolische componenten, tot driemaal hoger in vergelijking met het niet-gefermenteerd monster. De incubatie met *Aspergillus sojae* leidde daarnaast ook tot een belangrijke daling in de hoeveelheid aan hoog geglyosyleerde componenten. Dit ging gepaard met een sterke toename in hun respectievelijke metabolieten, met weinig tot geen suikermoleculen op de 3- en/of 7-positie.

Met de voorgaand uitgevoerde experimenten was het niet mogelijk om aglyconen te vormen. Daarom werd in Hoofdstuk 5 nagegaan of 2 Rhizopus stammen (*Rhizopus oryzae, Rhizopus azygosporus*) onder submerged fermentatie in staat waren om bioconversie uit te voeren van kaempferol en quercetine metabolieten. De fermentatie met *Rhizopus oryzae* resulteerde in de accummulatie van kaempferol als aglycone. Het kaempferol aglycone werd reeds in het medium waargenomen na 1 dag incubatie, en bereikte een maximum concentratie na 2 dagen. Deze component werd daarentegen niet waargenomen in de fermentaties met *Rhizopus azygosporus*, behalve op dag 4. De productie van quercetine werd waargenomen tijdens de 7 dagen durende incubatie met *Rhizopus oryzae*, waarbij de

hoogste concentratie op dag 7 gemeten werd. In de incubaties met *Rhizopus azygosporus* werd een ander patroon waargenomen, namelijk de hoogste hoeveelheid quercetine werd gemeten na 1 dag incuberen, gevolgd door een continue afname tot het volledig verdwijnen van quercetine op dag 7.

Het werk uitgevoerd in het kader van deze doctoraatsthesis handelde hoofdzakelijk omtrent technieken om een verhoogde extractie te verkrijgen van kaempferol glycosiden en hun metabolieten. Uit de bekomen resultaten kan besloten worden dat fungi potentiele kandidaten zijn om een biologisch extractieproces te ontwikkelen om kaempferol derivaten te extraheren uit nevenstromen van bloemkolen. Ook kan dit aanleiding geven tot de vorming van nieuwe, unieke metabolieten, welke waardevolle componenten kunnen zijn voor medische, cosmetische en voedingstoepassingen.

Literature Review

Redrafted from: Huynh, N.T; Van Camp, J.; Smagghe, G.; Raes, K., Improved Release and Metabolism of Flavonoids by Steered Fermentation Processes: A Review, International Journal of Molecular Sciences. 15(2014) 19369-19388.

Chapter 1

Literature review

1.1. Phenolic compounds

1.1.1. Classification of phenolic compounds

Phenolic compounds are a vast variety of secondary metabolites that are naturally synthesized in plants by the shikimic acid pathway and malonic acid pathway (Rosa et al., 2009) (Figure 1.1). They play protectives roles in plants against pathogen and insect attack and ultraviolet radiation. They consist of a wide range of structures i.e. more than 8,000 compounds are identified, divided into a number of main groups including phenolic acids, flavonoids, lignans and stilbenes depending on the number of benzene rings as well as on their structure of the carbon skeleton (Celep et al., 2014) as listed in Table 1.1. Among these, flavonoids are the most widespread group of phenolic compounds, i.e., accounting for over half of those. The basic structure of these compounds is the flavan nucleus consisting of 15 carbon atoms, which are arranged in a C_6–C_3–C_6 skeleton labeled A, B and C (Figure 1.2) (Pietta, 2000). They are further divided into different subclasses i.e. chalcones, flavonols, flavones, flavanones, flavanols, isoflavones and anthocyanins, based on various substitution patterns on rings A and B as well as variations on ring C (Balasundram et al., 2006; Martins et al., 2011) such as hydrogenation, hydroxylation, methylation, malonylation, sulfation, and glycosylation (Cook et al., 1996). Flavonols are the most frequent flavonoids in fruits and vegetables, in which the flavonols kaempferol and quercetin are predominant compounds, occurring generally as glycosylated derivatives (Pietta, 2000; Rosa et al., 2009). While

flavones and flavanoves are not as common as flavonols and are considered as minor flavonoids in plants.

The second most popular group of phenolic compounds are phenolic acids, which can be divided into two categories, i.e. hydroxybenzoic acids and hydroxycinnamic acids (Figure 1.2). The most common hydroxybenzoic acids are gallic acid, salicylic acid, vanillic acid, protocatechuic acid and p-hydroxybenzoic acid, which commonly appear in the C_6–C_1 structure, while p-coumaric acid, caffeic acid, sinapic acid and ferulic acid are the best known hydroxycinnamic acids, arranged in a three-carbon framework (C_6–C_3) (Balasundram et al., 2006; Martins et al., 2011).

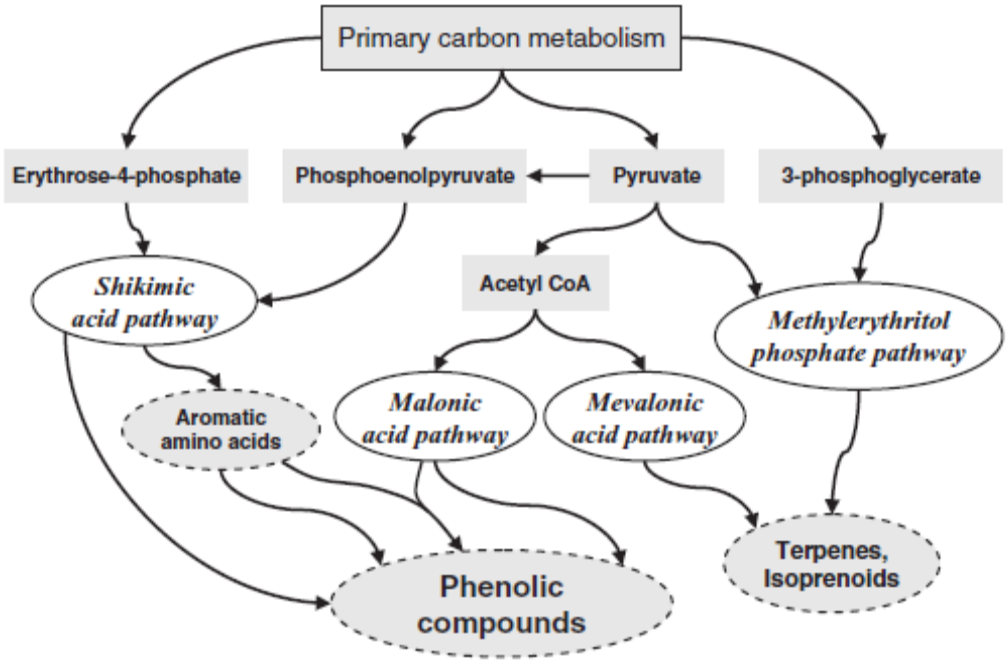
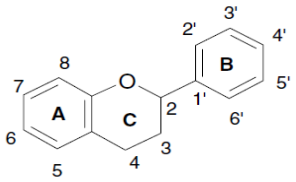


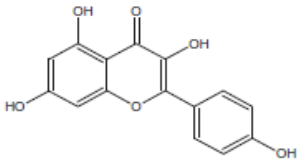
Figure 1.1. The overview of the biosynthetic pathways of phenolic compounds (Rosa et al., 2009)

FLAVONOIDS

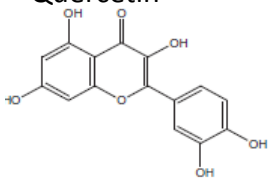


Flavonols

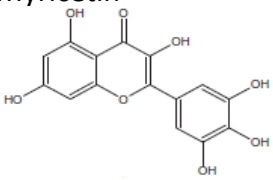
Kaempferol



Quercetin

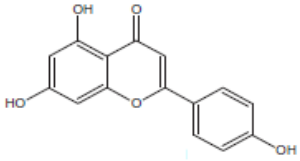


Myricetin

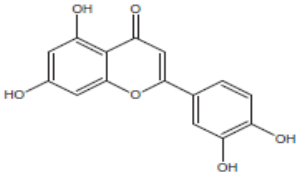


Flavones

Apigenin

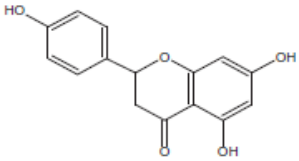


Luteolin

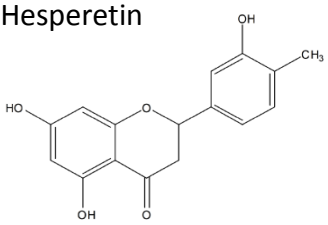


Flavanones

Naringenin

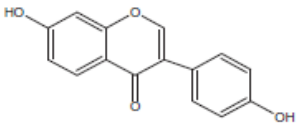


Hesperetin

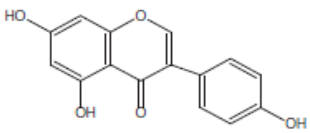


Isoflavones

Diadzein

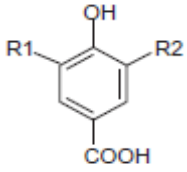


Genistein

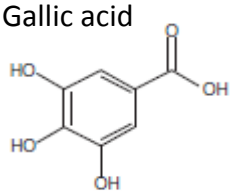


PHENOLIC ACIDS

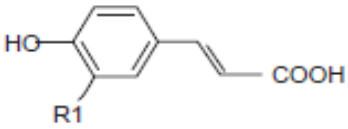
Hydroxybenzoic acids



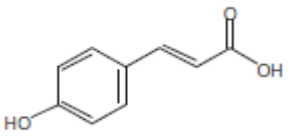
Gallic acid



Hydroxycinnamic acids



Coumaric acid



Ferulic acid

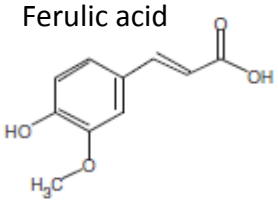


Figure 1.2. Basic structure of flavonoids, phenolic acids, and selected samples (Martins et al., 2011)

Table 1.1. Classification of phenolic compounds in plants, according to Balasundram et al. (2006)

Classes	Basic skeleton
Simple phenolics, benzoquinones	C_6
Hydroxybenzoic acids	C_6–C_1
Acethophenones, phenylacetic acids	C_6–C_2
Hydroxycinnamic acids, phenylpropanoids (coumarins, isocoumarins, chromones, chromenes)	C_6–C_3
Napthoquinones	C_6–C_4
Xanthones	C_6–C_1–C_6
Stilbenes, anthraquinones	C_6–C_2–C_6
Flavonoids, isoflavonoids	C_6–C_3–C_6
Lignans, neolignans	$(C_6–C_3)_2$
Biflavonoids	$(C_6–C_3–C_6)_2$
Lignins	$(C_6–C_3)_n$
Condensed tannins (proanthocyanidins or flavolans)	$(C_6–C_3–C_6)_n$

1.1.2. Localization of phenolic compounds in plant cells

Plant cell walls consist of a series of layers, i.e. the primary cell wall, secondary cell wall and the middle lamella, which are composed of two phases (microfibrils and wall matrix). The microfibrils are made of cellulose (β-1,4 glucan), while the wall matrix is formed from a

variety of polysaccharides (hemicelluloses and pectins), proteins, and phenolics (lignin and other phenolic compounds) (Brett et al., 1990).

Phenolic compounds often accumulate in the vacuole of plant cells, epidermal cells and subepidermal cells of leaves (Hutzler et al., 1998). They are also found in the cytoplasm, nucleus (not nucleolus) and plastid membranes (Hanson et al., 2014). In the vacuole, these compounds are generally present as free forms, while a significant proportion of them occurs as bound compounds mostly found in cell walls, plant barks, in which they are covalently linked to the plant matrix, e.g. cellulose, hemicellulose, lignin, and pectin, as graphically presented in Figure 1.3 (Acosta-Estrada et al., 2014).

In cell wall matrix, phenolic acids are bound to cell wall components through both ester- and ether-linked bounds. These bound compounds are covalently linked to carbohydrates and proteins through ester linkages, while they are either esterified or etherified to lignin through their hydroxyl group in the phenyl ring (Acosta-Estrada et al., 2014; Ozdal et al., 2013; Scalbert et al., 1985). Lam et al. (2001) found that most of hydroxycinnamic acids (80%), i.e. ferulic and p-coumaric acids, are etherified to the benzyl position of lignin (α-position). However, ester linkages between p-coumaric acid and lignin were also found in wheat straw (Sun et al., 1997). So there may exist both ether- and ester-bridges between hydroxycinnamic acids and lignin in plant cell walls (Iiyama et al., 1990).

Similar to phenolic acids, flavonoids are often located in the cell vacuoles, which may occupy 80-90% of the cell. Flavonoids are delivered into the vacuole from the endoplasmic reticulum (in which they are synthesized) through the action of membrane transporter systems, i.e.

multidrug and toxic compound extrusion (MATE) transporters, and ATP-binding cassette (ABC) proteins (Agati et al., 2012). Flavonoids present in the vacuole not only occur in free forms, but may also be linked to the protein matrix forming the vacuolar inclusions (Pinelo et al., 2006). The location and binding of vacuolar flavonoids may be influenced by the degree of the glycosidation and acylation of the flavonoid substances (Markham et al., 2000).

Flavonoids occurring in plants are not always present in the cell vacuoles. Some recent studies have demonstrated that these compounds are also found in the cell wall, in which they are bound to polysaccharides through hydroxyl groups (O-glycosides) or through carbon-carbon bridges (C-glycosides) (Acosta-Estrada et al., 2014). Strack et al. (1988) have found acylated kaempferol derivatives in the cell wall of leaf epidermal cells in Scots pine. Kaempferol and quercetin derivatives have also been detected in the cell wall of epidermal cells in lisianthus (*Eustoma grandiflorum*) flowers petals (Markham et al., 2000). Through transport mechanisms, i.e. vesicle-mediated and membrane-mediated transport, flavonoids are extruded from the cell to accumulate in the plasma membrane and the cell wall (Agati et al., 2012).

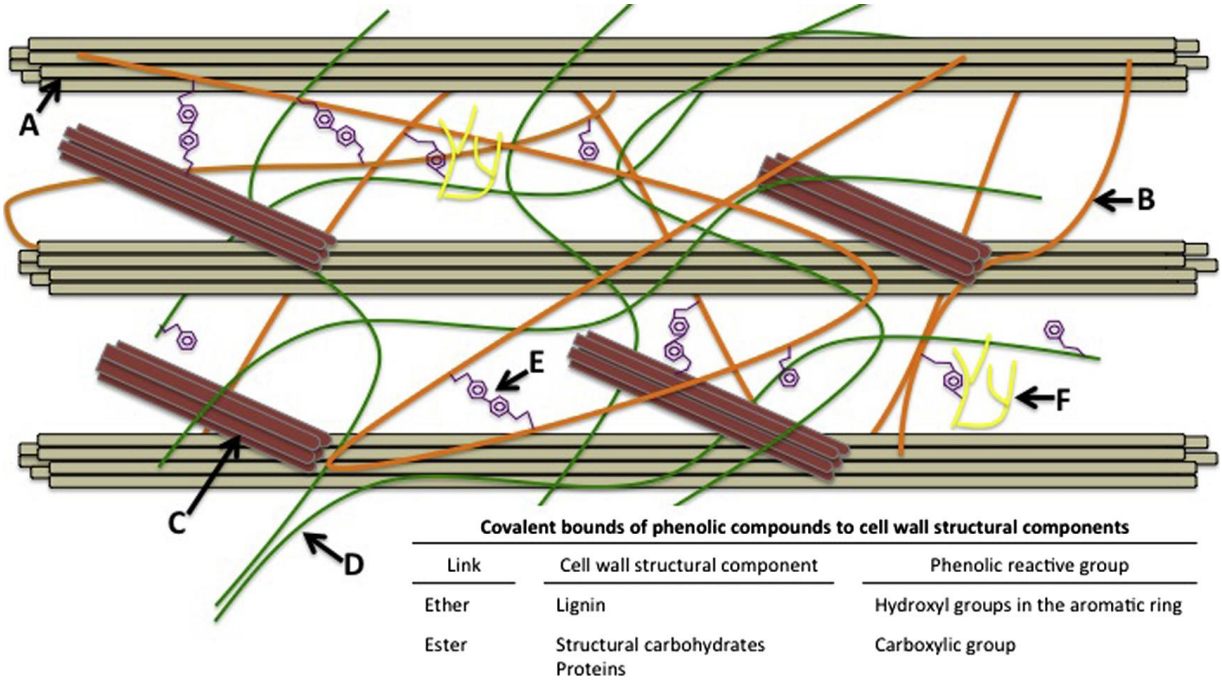


Figure 1.3. Primary cell wall structure of plant material and cross-linking between structural components and phenolic compounds. (A) Cellulose, (B) Hemicellulose, (C) Structural proteins, (D) Pectin, (E) Phenolic acids, (F) Lignin (Acosta-Estrada et al. (2014)).

1.1.3. Antioxidant activity and bioavailability of phenolic compounds

1.1.3.1. Biological activity

Phenolic compounds have been extensively studied in recent years due to their health-promoting effects. These compounds are found to display various beneficial properties, including anti-inflammatory, anticarcinogenic, antimutagenic, antimicrobial, and enzyme inhibition (Cartea et al., 2010; Jahangir et al., 2009). Many epidemiological studies have shown that phenolic compounds are protective factors against cardiovascular diseases (hypertension, obesity, arteriosclerosis, smoking, diabetes, and aging) and different types of cancers through their biological actions (Nile et al., 2014; Ranilla et al., 2010; Rodriguez-Mateos et al., 2013). Some examples are given below. Hertog et al. (1993) have indicated that a higher intake of flavonoids with about 30 mg/day resulted in an approximately 50%

decrease in mortality rate of coronary heart disease as compared to people who consumed the lower 19 mg/day of flavonoids. Similarly, a 50 % reduction in coronary mortality has observed for persons who have the highest consumption of apple as well as of onion which are both well known as an excellent source of dietary flavonoids (Knekt et al., 1996). Likewise, in another epidemiologic study, German et al. (2000) revealed that a significant reduction of cardiovascular disease was observed for individuals who daily consume a moderate amount of wine which was attributed to the inhibition of wine phenolic compounds on the oxidation of low-density lipoprotein (LDL) cholesterol and the increased antioxidant capacity of plasma. In terms of cancers, several studies on in vivo and in vitro investigation have demonstrated that flavonoids can interrupt various stages of certain cancers due to their antioxidant and anticarcinogenic properties (Chung S Yang et al., 2001; Hollman et al., 1996; Rice-Evans et al., 1996; Tham et al., 1998).

Also, a variety of phenolic compounds are known to possess antimicrobial properties. Puupponen-Pimiä et al. (2001) have indicated that phenolic acids (cinnamic acid, 3-coumaric acid, caffeic acid, ferulic acid and chlorogenic acid) at high concentration (500 µg/ well) exhibited their inhibitor activity on the growth of Gram-negative bacteria, while flavonoids such as myricetin and luteolin showed significant activity against Gram-positive bacteria. Not only on bacteria, phenolic compounds have also been assessed to be toxic to fungi (Duke, 2002) and viruses (Kaul et al., 1985).

The biological activity of phenolic compounds has been attributed to their antioxidant activity. These compounds possess the ability to inactivate ‘reactive oxygen species’ (ROS), which are generated in all living organisms during oxidation reactions as a result of normal

metabolic processes (Tripoli et al., 2005). Phenolic compounds can react with those radicals, resulting in scavenging the radical electrons and thus quenching the chain reaction, which is triggered by free radicals (Tripoli et al., 2005). Another antioxidant activity is related to their chelation ability of transition metals, i.e., Fe^{2+} and Cu^{+}, leading to a lower involvement of these metals in producing hydroxyl radicals (OH^{*-}) via the Fenton reaction (Tripoli et al., 2005) as seen in Figure 1.4.

As shown in recent studies, the degree of antioxidant activity of phenolic compounds, i.e., flavonoids, phenolic acids depends on the number as well as on the position of hydroxyl groups in their molecule (Cartea et al., 2010). Fukumoto et al. (2000) reported that a higher antioxidant activity could be attributed to an increase in hydroxyl groups. Regarding the localization of hydroxyl groups, Finotti et al. (2003) showed that the –OH group substituted on *ortho*- or *para*-position exhibits a higher antioxidant capacity with respect to the *meta*-radical form. A similar observation was reported by Choi et al. (2002), who found that the flavonoids with *ortho*-hydroxyl structures showed an increased scavenging activity. A double bond between C-2 and C-3 in ring C also enhances the radical scavenging capacity of flavonoids (Balasundram et al., 2006).

Moreover, glycosylated phenolic compounds resulted in a lower antioxidant activity. The more sugar moieties attached to the aglycone, the more their activity was reduced. This could be attributed to steric hindrance by the addition of sugar moieties (Fukumoto et al., 2000).

$Fe^{2+} + H_2O_2$ -------> $Fe^{3+} + OH^- + OH^{*-}$

$Fe^{3+} + H_2O_2$ -------> $Fe^{2+} + OOH^{*-} + H^+$

$Cu^+ + H_2O_2$ -------> $Cu^{2+} + OH^- + OH^{*-}$

Figure 1.4. Production of the hydroxyl radical via the Fenton reaction

1.1.3.2. Bioavailability

The health-promoting effects of phenolic compounds depend on their behavior during digestion, absorption and metabolism, which are influenced by their structure (size of molecule, conjugation or glycosylation with other components, location of glycosides) and their physical properties e.g. solubility (Balasundram et al., 2006). After consumption, in a first step phenolic compounds are released from their food matrix and then they are absorbed in the small intestine, accounting for 5-10% of the total intake of phenolic compounds (Balasundram et al., 2006). Generally, most of the aglycones can be absorbed efficiently through the gut wall, while the complexed phenolics (e.g., glycosidic forms), are too hydrophilic and too large to be absorbed as such. Therefore, they must be hydrolyzed by intestinal enzymes (β-glucosidases or lactase-phlorizin hydrolase) or by the colonic microflora to release their corresponding aglycones, which are better absorbed. However, the phenolic aglycones produced by hydrolysis can only be slowly absorbed due to their poor solubility. Thus, Hu (2007) suggested that a good balance between hydrophilicity and lipophilicity is very important for penetration of a phenolic compound into enterocytes. This could be the reason for the conclusion that quercetin-3-glucoside is 184 % better absorbed than its aglycone (Morand et al., 2000).

Absorbed phenolic compounds can undergo conjugation including O-methylation, O-sulfation and O-glucuronidation in the small intestines, liver or kidney (Balasundram et al., 2006). This is also known as a metabolic detoxification process producing more polar compounds, which facilitate their biliary and urinary elimination.

1.2. Brassica

1.2.1. Agricultural and Brassica by-products

In the last two decades, more fruits and vegetables have been produced to meet the demand for human consumption, up from globally around 980 million tons in 1993 to 1,800 million tons in 2013 (FAO, 2015) (Figure 1.5), with a major increased production in Asia. This change resulted in the generation of huge amounts of wastes and by-products, accounting for a substantial proportion, i.e. 10-60% of the raw materials depending on the plant. In 2011, the United states and China produced over 45 million tons of fruits and vegetables wastes (Wadhwa et al., 2013).

The major quantity of wastes is commonly generated during the harvest, i.e., agricultural residues (e.g. leaves, stems), and in the food processing industry (pomace, peel or seeds from apple, grape, and citrus fruits) (Madurwar et al., 2013). In the past, these materials were usually utilized as fertilizer or animal feed, and even as garbage disposals (Laufenberg et al., 2003). In recent years, however, several new methods to utilize these waste products have been developed to obtain higher added-value products (Laufenberg et al., 2003) because they have been reported to be a good source of bioactive compounds e.g. phenolic

compounds, glucosinolates, β-carotene, vitamin C, vitamin E, flavors, colorants and dietary fiber, as well as selenium and zinc (Shilpi et al., 2013).

On the side of *Brassica* vegetables, cauliflower and broccoli are commonly used vegetables all over the world. According to a report issued by Food and Agriculture Organization (FAO) in 2015, the world cauliflower and broccoli production in 2013 was around 22,2 million tons (Figure 1.6). Their edible parts include sprouts and flower head, while a larger quantity of non-edible proportion, over 70% of the crops such as leaves, stems, florets, and stalks, is generated during harvest handing. Most of these by-products from cauliflower and broccoli are more spread on the land, while a small amount is used for animal feeds and industrial fertilizer (Iñiguez-Covarrubias et al., 2001). However, in a few recent studies, it was recognized that they are a source of antioxidative compounds, e.g., phenolic compounds (Gonzales et al., 2014; Huynh et al., 2014; Llorach et al., 2003), glucosinolates (Ares et al., 2014; Cabello-Hurtado et al., 2012; Dominguez-Perles et al., 2011), fiber (Nilnakara et al., 2009; Tanongkankit et al., 2012) and amino acids (Arnáiz et al., 2012).

Like other fruits and vegetables, the consumption of cabbage and other brassica was increased steadily in the last two decades, ranging from around 45.3 million tons in 1993 up to approximately 71.4 million tonnes in 2013 (FAO, 2015) (Figure 1.6). Since these products are mostly consumed raw and fresh, the waste produced is less. The main cabbage wastes are leaves, which are generated from the packaging and storage processes, as well as by household cooking. These vegetables such as Chinese cabbage, white and red cabbage, Brussels sprouts, kale, have also been reported to be potential sources of bioactive compounds (Podsędek, 2007).

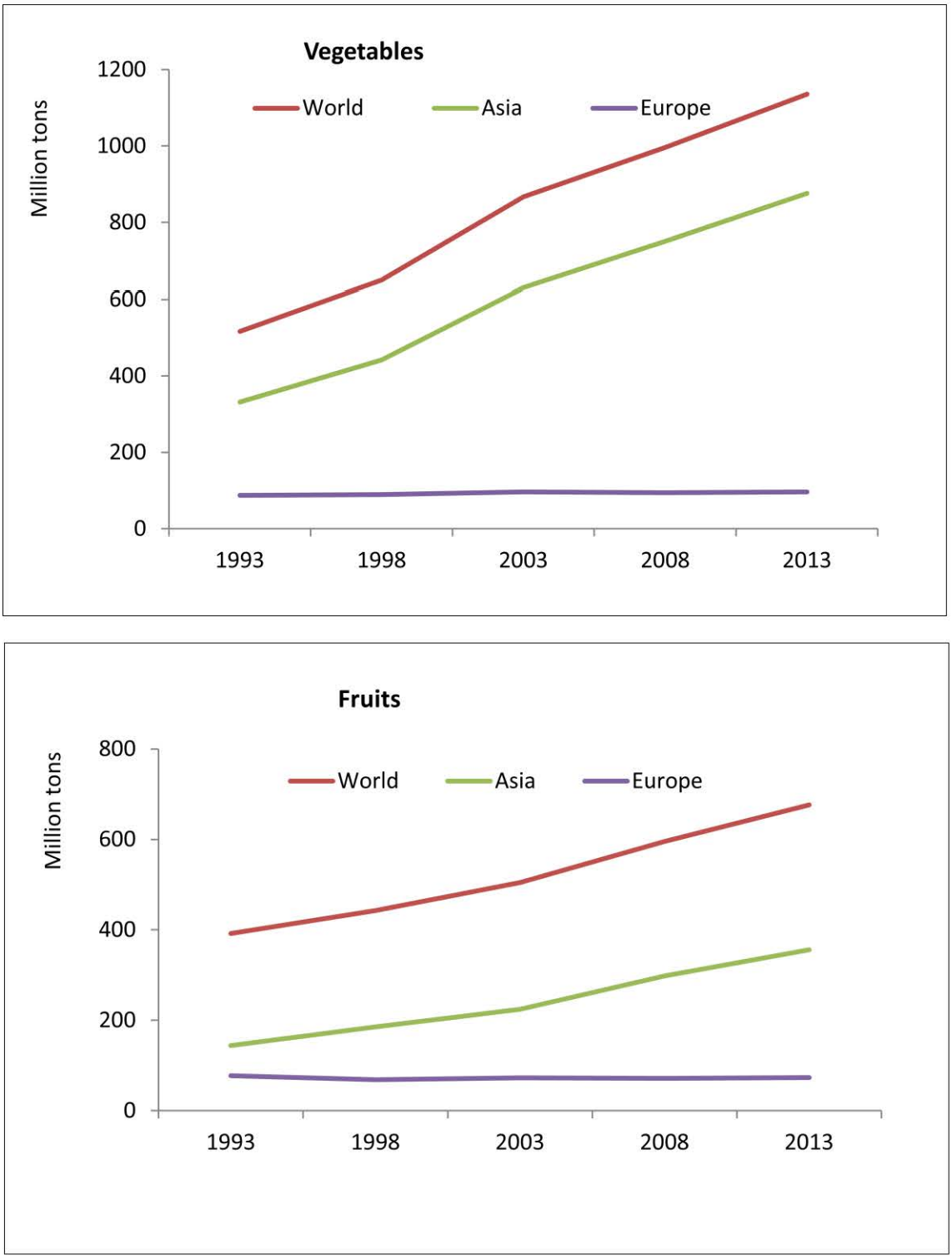
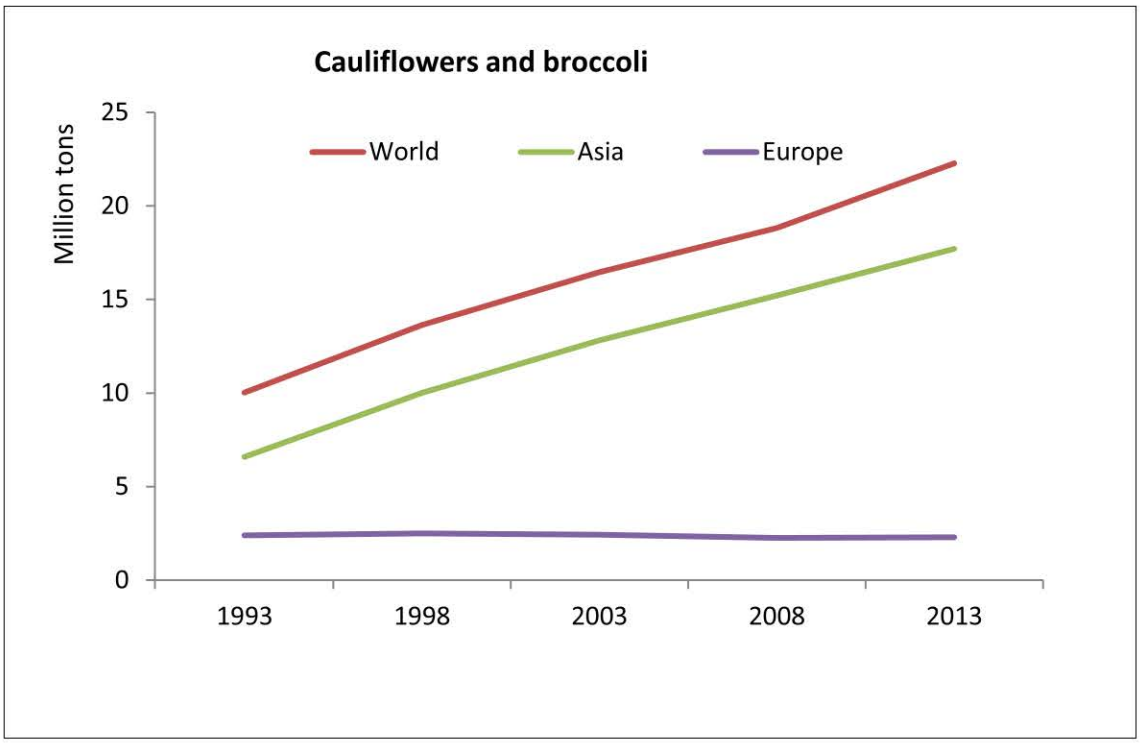


Figure 1.5. Production of vegetables and fruits in the last two decades (FAOstat, 2015)



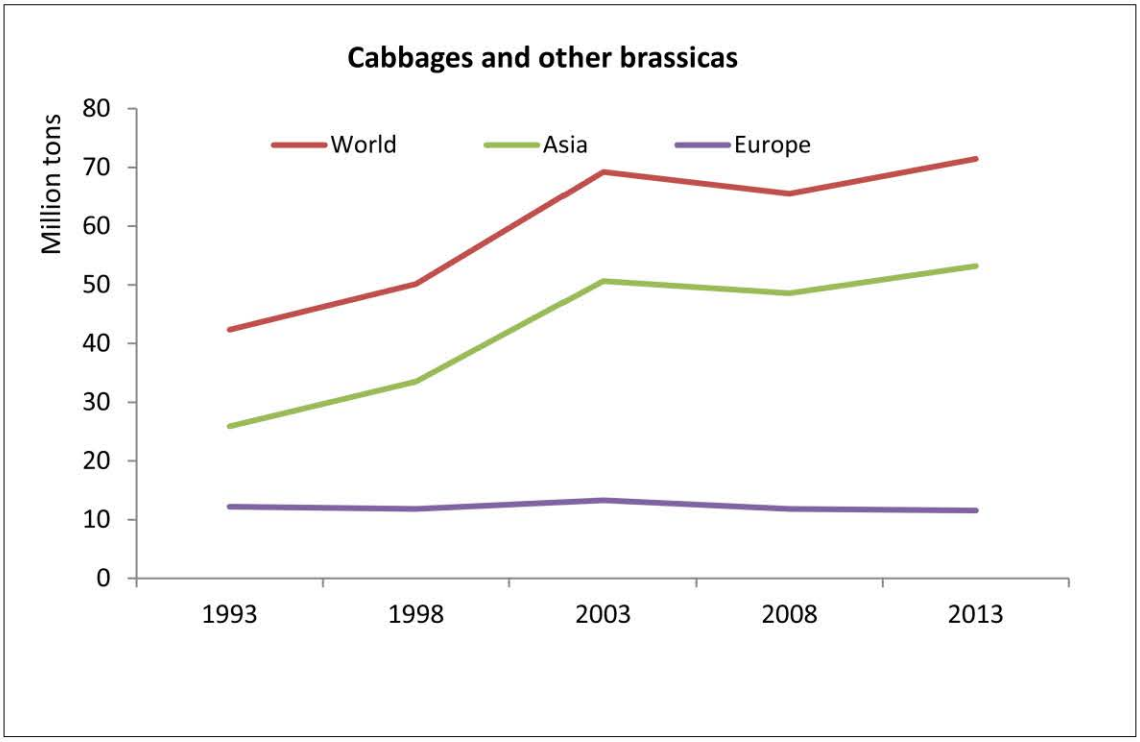


Figure 1.6. Production of cauliflower and broccoli, and other brassica vegetable in the last two decades (FAOstat, 2015)

1.2.2. Phenolic compounds in Brassica vegetables

Phenolic compounds are known to be the major antioxidant compounds of Brassica vegetables. The most abundant group found in these plant are flavonoids, which mainly exists as glycosides of kaempferol and quercetin as well as their derivatives in combination with hydroxycinnamic acids, e.g. caffeic, coumaric, ferulic and sinapic acid (Cartea et al., 2010; Lin et al., 2010). Lin et al. (2010) characterized the phenolic composition of 17 *Brassica* vegetables, finding fifty-one flavonoid glycosides that were all identified as glycosides of kaempferol, quercetin, and isorhamnetin, i.e. sixteen non-acylated flavonoid glycosides, and thirty-five acylated flavonoid glycosides. In another study on different Brassica species (Collard Greens, Kale, and Chinese Broccoli), these authors also reported that forty-five flavonoids and thirteen hydroxycinnamic acid derivatives were detected in these three vegetables (Lin et al., 2009). Among them, kaempferol glycosides were major compounds, while quercetin glycosides were identified as minor ones.

The phenolic profile can be quite different depending on the varieties or cultivars, species, and even the plant parts from the same species, climatic conditions, harvest and postharvest processes (Cartea et al., 2010; Vale et al., 2015). The concentration of phenolic compounds were evaluated in seven Italian Brassica varieties, i.e. Italian kale, broccoli, savoy and white cabbage, cauliflower, green cauliflower and Brussels sprouts by Heimler et al. (2006). They found that broccoli and kale edible varieties exhibit the highest content of both total phenolics and flavonoids. The phenolic content of the external and internal leaves of tronchuda cabbages (*Brassica oleracea* L. var. *costata* DC) was found to be different (Ferreres et al., 2006). These authors found seventeen phenolic compounds in the internal leaves,

while only two out of these compounds were detected in the external leaves. The concentration of individual phenolic compounds in internal leaves was about 10 times higher than their concentration in external leaves. The finding from Harbaum et al. (2008) who worked on variation in phenolic content in several pak choi cultivars indicated that the highest flavonoid content was obtained on cv. Ai Kang Qing (38 mg/g DW) while the lowest was reported on cv. Huang Xin Cai (15 mg/g DW).

In relation to seasonal effect on concentration of phenolic compounds, Koh et al. (2009) have investigated the change of content of kaempferol, quercetin in 80 commercial broccoli samples during a period of 2 years (2005-2007). During this two-year period, kaempferol was detected as the most abundant flavonoid, ranging between 0.24–13.20mg/100g, with an exception in August 2005 when quercetin was determined as the predominant compound.

Contrary to other Brassica vegetables, a few studies have been done to evaluate the phenolic profile of broccoli and cauliflower outer leaves. Llorach et al. (2003) found twenty-two phenolic compounds in this by-product, of these, twenty-one compounds were identified as derivatives of kaempferol and ferulic and sinapic acids. The most abundant compounds detected were kaempferol 3-diglucoside-7-glucoside and its combinations with different hydroxycinnamic acids. This finding is consistent with the results of Gonzales et al. (2014), who also found kaempferol-3-O-diglucoside-7-O-glucoside as one of the major compounds naturally occurring in cauliflower waste.

1.3. Improved release of phenolic compounds

Phenolic compounds present in plants can be classified into free phenolic compounds found in the vacuoles of plant cells, and bound phenolic compounds linked to cell wall structure components (cellulose, hemicellulose, lignin, pectin and protein) through several covalent bounds (Cerda et al., 2013; Pinelo et al., 2006; Yadav et al., 2013). Free phenolic compounds can be effectively extracted by conventional techniques, while several hydrolysis processes have been used to enhance the release of bound phenolics. Enzymatic treatment and fermentation has been considered as the alternative processes to obtain extracts with a high quality and a high activity, using economically and environmental friendly techniques (Martins et al., 2011).

1.3.1. Enzyme-assisted extraction

The phenolic compounds obtained from plant-based food based on the conventional extraction using different combinations of water and organic solvents generally constitute the extractable phenolic compounds (Pérez-Jiménez et al., 2011), which are typically lower molecular weight compounds, e.g. monomers and decamers (Saura-Calixto, 2012). However, it has been reported that these chemical approaches miss some of the phenolic compounds i.e. bound compounds, which are bound to cell-wall structure e.g. to cellulose, hemicellulose, lignin and pectin (Acosta-Estrada et al., 2014; Saura-Calixto, 2012). One of the more effective methods to enhance the release of these compounds is enzymatic pretreatments (Acosta-Estrada et al., 2014). The activity of carbohydrate-degrading enzymes such as cellulase, hemicellulase, and pectinase causes the degradation and disruption of the cell-wall matrix, resulting in facilitating the phenolic extraction and thus increasing the

amount of released bound phenolic compounds (Gómez-García et al., 2012). Besides, the enzymatic treatment also has an effect on the transformation of phenolic compounds resulting in a variation in their polarity and solubility, which could have an impact on the efficiency of the extraction treatment (Chen et al., 2011).

A number of studies related to the enzymatic extraction of phenolic compounds from plant materials have been reported in recent years (Table 1.2). The preparation of oat bran with various commercial enzymes, i.e. Viscozyme, Cellulast, α-amylase and amyloglucosidase led to an increase in total phenolic content (Alrahmany et al., 2012). Likewise, Alrahmany et al. (2013) showed that treatments with Viscozyme and cellulase resulted in a 4.4-fold and 3.7-fold increase in the release of vanillic and caffeic acid, respectively. In addition, an increase in of the ferulic acid content was observed with all enzyme treatments. The recovery of phenolic compounds from raspberry wastes by the enzyme Gryndamil increased by 18% as compared to the control (Laroze et al., 2010). Similarly, the content of p-coumaric acid, ferulic acid and caffeic acid increased by a factor of 8, 4, and 32, respectively, when unripe apples were treated with Viscozyme L (Zheng et al., 2009).

The parameters of treatments such as types and dosages of enzyme, time, temperature, and pH can influence the release and the recovery of phenolic compounds from plant matrices due to their effects on the enzyme activity. Zheng et al. (2009) found that the Viscozyme L treatment exhibited the highest concentration of phenolic compounds extracted from unripe apples, among all enzymes used, and its optimal condition was determined at 50 °C, pH 3.7 and 12 hours of incubation. The selection of enzymes also needs be considered during the extraction and depends upon the plant matrices used. In some cases, the combination of

multiple enzymes exert a higher effect in releasing phenolic compounds than only single enzymes due to synergistic actions between enzymes. For example, Chamorro et al. (2012) indicated that the treatment with a combination of both enzymes (Tannase and Pectozyme) resulted in the maximum amount of phenolic compounds released from grape pomace.

Table 1.2. Enzyme assisted extraction using different enzymes and matrices

Plants	Condition of enzymatic treatment	Results	Reference
Black currant juice	**Anthocyanidins treatment** **Enzymes**: Pectinex BE , Pectinex Ultra SP, Rohapect B5L, Vinozyme **Contact time:** 30 min **Temperature:** 60 min **E/S:** 0,18 **pH:** Pectinex Ultra: 3,5; Rohapect B5L: 3-4 **Total phenols treatment** **Contact time:** 210 min	**Enzyme** / **Antocyanidins (mg/kg)** / **Total phenols (mg/kg)** Pectinex BE: 1500-2190 / 31280-4460 Pectinex ultra SP: 1480-2170 / 3250-4690 Rohapect B5L: 1660-2130 / 3500-4470 Vinozyme: 900-2110 / 3050-4600 Control: 1300±70 / 3900±90	(Landbo et al., 2004))
Grape Pomace	**Enzymes:** Pektozyme®, Laminex ®, Tannase **Contact time:** 24hours **Temperature:**35°C **pH:** 5,6 **Enzyme concentration:** Pektozyme®: 6,75 U/g DM; Laminex®: 157,5 U/g DM; Tannase: 500U/ g DM **Results expressed as:** mg /100g DM	**Phen-Compounds** / **Control** / **Pektozyme** / **Laminex** / **Tannase** TP: 0.11 / 0.11 / 0.10 / 0.11 Gallic acid: 5.74 / 6.90 / 4.04 / 8.87 Gallocathechin: 0.028 / 0.036 / 0.028 / 0.026 Epigallocathechin: 0.075 / 0.098 / 0.086 / 0.076 Catechin: 2.96 / 2.73 / 2.77 / 2.44 Epicatechin: 2.03 / 2.14 / 2.08 / 1.82 Procyanidin B1: 4.63 / 4.93 / 4.56 / 4.22 Procyanidin B2: 2.62 / 2.87 / 2.63 / 2.89	(Chamorro et al., 2012)
Lentils	**Enzymes:** Phytase, α galactosidase,viscozyme, Tannase **Contact time:** Pythase-viscozyme 60 min α-galactosidase 90 min; tannase 120min **Temperature:** 37°C **Concentration(mg/g substrate)** Phytase(1,60) α galactosidase,(1,16) Viscozyme(1,50) Tannase(0,50) **Results expressed as:** µg/g dry matter	**Phenolic Compounds** / **Raw lentils** / **phytase** / **α-gal** / **Viscozyme** / **Tannase** Gallic acid: nda / nda / 0.80 / 0.66 / 0.83 phydrobenzoic acid: 3.25 / 2.48 / 1.57 / 1.49 / 2.00 trans-p- coumaric acd: 5.74 / 4.20 / 2.74 / 1.92 / 3.87 cis-p-coumaric acid: 0.73 / 0.35 / 0.46 / 0.66 / 0.45 Trans-ferulic acid: 0.74 / 0.62 / 0.91 / 0.34 / 0.84 Quercitin-3-O-rutinoside: 5.24 / 11.68 / 8.28 / 6.73 / 12.27 Trans-resveratrol: nda / 0.34 / nda / nda / 0.41	(Dueñas et al., 2007)
Lemon Balm	**Enzymes:** Cellulase, endo 1,4 β-xylanase, lafase® **Contact time:**2hours **Temperature:** 50°C **Concentration (%w/wdm):** 5% **pH:** 5 **Results expressed as:** mg GAE/g extract	**Enzyme** / **TPC** Cellulase: 71.58 Endo 1.4 β-xylanase: 73.79 Lafase®: 72.62 Mix(1:1:1 w/w/w): 78.55 Control: 65.39	(Miron et al., 2013)

Oat Bran	**Enzymes:** Cellulase, viscozyme, α-amylase amyloglucosidase **Contact time:** 2hours **Temperature:** 50°C **pH:** Cellulase(CELL) 5,5 viscozyme (VIS) 4,6 α-amylase (α-AMY)6,2 amyloglucosidase (AMYUGLU)5,0 **Concentration: :** Cellulase (4 EGU) viscozyme (60FBG) α-amylase (1500U/g) amyloglucosidase (500U/g) **Results expressed as:** µg/g of bran	**Phenolic Compounds** / **Control** / **VIS** / **α-AMY** / **CELL** / **AMYGLU** Vanillic acid 11.2 21.5 17.0 41.4 11.3 Caffeic acid 5.9 26.1 13.0 11.2 7.1 p-coumaric acid 6,1 17.7 13.9 5.2 7.5 Ferulic acid 29.5 203.2 290.8 214.4 35.5 Cinnamic cid 69.0 58.3 83.6 66.6 N.D	(Alrahmany et al., 2013)
Raspberry waste	**Enzymes:** Cellubrix, Olivex, Pectinex Ultra SPL, Ultrazym 100G, Viscozym ,Rohapect, Rohapect DA6L, Rohapect MAX, Rohavin L, Granozyme, Grindamyl CAL 50, Maxoliva **Contact time:** 18h **pH:** **Concentration:** 10g/100 gDM **Results expressed as:** mg GAE/g D.W.	**Enzyme Preparation** / **TPC** / **Enzyme Preparation** / **TPC** Control 11.35 Rohapect Max 12.17 Cellubrix 9.77 Rohavin L 11.06 Pectinex Ultra SPL 11.09 Granozyme 9.59 Ultrazym 100G 10.21 Gryndamil CA 150 15.79 Viscozyme 10.11 Maxoliva 15.40 Rohapect 10.48 Rohapect DA6L 10.70	(Laroze et al., 2010)
Apple skin	**Enzymes:** Pectinex Smash, Celluclast, Protease, Control **Contact time:** 0.5-3-5min **Temperature:** 50°C **pH** **Concentration (%, v/w):** **1.**PectinexSmash (0.1%), Celluclast (0.1%) Protease(0%) **2**.PectinexSmash(0%), Celluclast (0.1%) Protease(0.1%) **3.** Pectinex Smash (0.1%), Celluclast (0.1%) Protease (0.1%) **Results expressed as:** mg/L	**Enzyme Preparation** / **TPC** / **Rutin** / **Hydroxycinnamic acid** 1 98.45 0.19 0.1731 2 101.07 0.23 0.1795 3 104.94 0.28 0.2285 Control 90.82 0.15 0.1269	(Pinelo et al., 2008)
Pigeonpea leaves	**Enzymes:** Pectinase **Contact time:** 18hours **Temperature:** 30-35°C pH: 3,5-4 **Concentration (v/w):** 4% **Results expressed as:** mg/g	**Enzyme** / **Luteolin** / **Apigenin** Pectinase 0.268 0.132 Control 0.077 0.039	(Fu et al., 2008)

1.3.2. Fermentation process: the release of phenolic compounds

Several studies reported that fermentation influences the phenolic profile of extracts obtained from various plant sources or during the fermentation of plant sources. Table 1.3 summarizes the published papers in the last decade, including the phenolic profile changes, only measured by chromatographic techniques, and obtained by a steered fermentation process (no spontaneous or natural fermentations). Vattem et al. (Vattem et al., 2004; Vattem et al., 2003) have found that solid-state fermentation of cranberry pomace using a food-grade fungus *Lentinus edodes* resulted in a maximum of 49% increase in ellagic acid content after 5 days of incubation. Another study demonstrated that the phenolic acid profile in an ethanolic extract from oat fermented by three different filamentous fungi (*Aspergillus oryzae* var. *effuses, Aspergillus oryzae and Aspergillus niger*) at 25 °C for 3 days was remarkably improved in comparison with non-fermented oat (Cai et al., 2011). Indeed, the study showed that fermentation of oat using *Aspergillus oryzae* var. *effuses* or *Aspergillus oryzae* increased the content of caffeic acid and ferulic acid in oat (*Avena sativa* L.) up to about 2.7 to 3-fold and 5.5 to 9-fold, respectively, when compared to native oat. Fermentation with *Aspergillus oryzae* var. *effuses* also resulted in a more than 100% increase of chlorogenic and *p*-coumaric acids. In a recent study, Schmidt et al. (Schmidt et al., 2014) investigated the effect of solid-state fermentation by *Rhizopus oryzae* on the profile of phenolic acids derived from rice bran. The content of chlorogenic acid, *p*-hydroxybenzoic acid and vanillin significantly increased during fermentation. According to these authors, an incubation for 120 h at 30 °C with *Rhizopus oryzae* led to the most substantial increase in

gallic acid and ferulic acid content, ranging from 3 and 33 mg/g dried weight in native bran to 155 and 765 mg/g dried weight in fermented bran, respectively.

In addition to phenolic acids, the enhancement of the flavonoid content has also been observed in recent studies. Soybeans incubated with *Aspergillus oryzae* at 30 °C for 48 h resulted in a 23-fold increase in genistein aglycones when compared to the content found in unfermented soybean flour (da Silva et al., 2011). The amount of these aglycones was also found to be higher in solid-state fermentations of soybean with *Rhizopus* sp. (Cheng et al., 2013) and *Monascus purpureus* (Handa et al., 2014) compared to unfermented soybeans.

Similar to filamentous fungus, different food-graded lactic acid bacteria (LAB) and *Bacillus* spp. have been evaluated for their potential to release phenolic acids as well as flavonoids from plant sources such as soybean (Cho et al., 2009; Chung et al., 2011), apple (Ankolekar et al., 2012) and cereals (Hole et al., 2012). The fermentation with *Lactobacillus johnsonii, Lactobacillus reuteri* and *Lactobacillus acidophilus* showed a 20-fold increase in the content of total free phenolic acids in both barley and oat flour, compared to the unfermented sample, with the largest increase observed for free ferulic acid up to 39-56 μg/g dried weight depending on the strains used, while the amount of this compound found in unfermented samples was around 1 μg/g dried weight (Hole et al., 2012). This study also found that fermentation with *Lactobacillus johnsonii* had a much higher effect on the release of free phenolic acids than the other strains. A similar effect on the release of bound phenolic compounds was observed. Fermentation of grain barley with three LAB strains resulted in a significant increase of ferulic acid and *p*-coumaric acid which contributed to an increase in

total content of bound phenolic acids by around 23%, compared to native grain barley. Also, enhancing the release of phenolic acids and flavanols was reported in a recent study (Cho et al., 2009), showing 2.8-fold, 7.6-fold and 4.5-fold increases in gallic acid, catechin and epicatechin, respectively after 60 h of fermentation with *Bacillus pumilus.* Soybean seeds fermented with *Bacillus subtilis* for 3 days yielded an increase in chlorogenic acid and naringin (Chung et al., 2011). More so, spontaneously fermentation have been found as a reason for increase in phenolic compounds (e.g. ferulic acid, hydroferulic acid, kaempferol-3-glucoside) content in leek (Bernaert et al., 2013).

Table 1.3. The effect of microbial fermentation on the increase in phenolic compounds from various plant-based foods

Microorganism	Source	Compounds increased by fermentation	Reference
Bacteria			
Bacillus pumilus	Soybean	Gallic acid, catechin, epicatechin	(Cho et al., 2009)
Bacillus subtilis	Soybean	Chlorogenic acid, naringin	(Chung et al., 2011)
Bacillus subtilis	Cheonggukjang (soybean paste)	Daidzein, genistein,	(Cho et al., 2011; Shin et al., 2014)
Lactobacillus acidophilus	Apple juice	Gallic acid	(Ankolekar et al., 2012)
Lactobacillus johnsonii, Lactobacillus reuteri *Lactobacillus acidophilus*	Whole grain barley, oat groat	Sinapic acid, caffeic acid, *p*-coumaric acid, ferulic acid	(Hole et al., 2012)
Lactobacillus plantarum	Cowpeas	Quercetin	(Dueñas et al., 2005)
Lactobacillus plantarum, Lactobacillus delbrueckii supsp. *lactis*	Soybean	Daidzein, genistein	(Pyo et al., 2005)
Yeast *Saccharomyces cerevisiae*	Wheat bran	Syringic acid, *p*-coumaric acid, ferulic acid	(Moore et al., 2007)
Fungi			
Aspergillus oryzae, Monascus purpureus	Soybean	Daidzein, genistein	(da Silva et al., 2011; Handa et al., 2014; Hwan Nam et al., 2011)
Aspergillus oryzae var. *effuses* *Aspergillus oryzae*, *Aspergillus niger*	Oat (*Avena sativa* L.)	Chlorogenic acid, ferulic acid, *p*-coumaric acid, caffeic acid	(Cai et al., 2011)
Aspergillus oryzae	Green tea	Gallic acid, gallocatechin, epigallocatechin, epicatechin, 3-*p*-coumaroylquinic acid, keampferol-ruinoside	(Kim et al., 2013)
Lentinus edodes	Cranberry pomace (*Vaccinium acrocarpon*)	Ellagic acid	(Vattem et al., 2004; Vattem et al., 2003)
Rhizopus oryzae	Rice bran	Gallic acid, ferulic acid, *p*-hydroxybenzoic acid, caffeic acid, chlorogenic acid, vanillin	(Schmidt et al., 2014)
Rhizopus oligosporus, Rhizopus oryzae	Black soybean	Daidzein, genistein	(Cheng et al., 2013)

Not only fungi, LAB strains and *Bacillus* spp. have been used, but also yeast were screened for their improvement of the free phenolic profile. Moore et al. (2007) reported that solid-state fermentation of wheat bran with *Saccharomyces cerevisiae* yielded a maximum increase of 48 %, 51 % and 333 % in the content of soluble free *p*-coumaric, ferulic and syringic acid, respectively, compared to unfermented samples.

However, a fermentation process does not exclusively increase all phenolic compounds. Also a decrease in some components is observed as they are e.g. deglycosylated, metabolized in other, less toxic components. Indeed, phenolic compounds are known for their antimicrobial activity and their ability to retard microbial growth. Also type of microorganism, conditions of the fermentation process and fermentation time play a role herein (Hwan Nam et al., 2011; Kim et al., 2013).

The change in the profile of phenolic compounds by the fermentation process is due to the action of cellulolytic, ligninolytic and pectinolytic enzymes, mainly produced during the growth of the microorganisms, as described in Figure 1.7. An overview of possible enzymes involved in the release of phenolic compounds, by breaking down the cell wall matrix and produced by the fermentation microorganisms is given in Table 1.4. These enzymes are known to be capable of completely breaking down the chemical components of plant cell walls, resulting in the hydrolysis of the ester bonds, which link phenolic compounds to the cell wall matrix, and in the oxidative degradation of lignin. As a consequence, the free phenolic compounds as well as bound forms are released more efficiently from the plant matrix. Among these enzymes, β-glucosidase has been widely reported as an enzyme

responsible for catalyzing the hydrolysis of glycosidic linkages in alkyl and aryl-β-D-glucosides to release phenolic aglycone moieties. Vattem et al. (2003) demonstrated that the increased release of the aglycone form of ellagic acid from cranberry pomace could be attributed to crude β-glucosidase produced, during solid-state fermentation by the food-grade fungi *Lentinus edodes*. Similarly, previous studies pointed out that esterases produced by filamentous fungi through solid-state fermentation of cereal sources such as oat (Cai et al., 2011), rice bran (Schmidt et al., 2014) caused an increase in the content of phenolic acids such as ferulic acid, caffeic acid and *p*-coumaric acid.

The changes in phenolic profile were observed as a result of microbial fermentation of plant-based matrices. This confirms that the fermentation of plant substrates, both edible parts as well as agricultural by-products and food waste, with different microorganisms, including filamentous fungi, lactic acid bacteria, yeast, could be considered as a potential process to increase the release of phenolic compounds contributing to the production of extracts and food products with an added value.

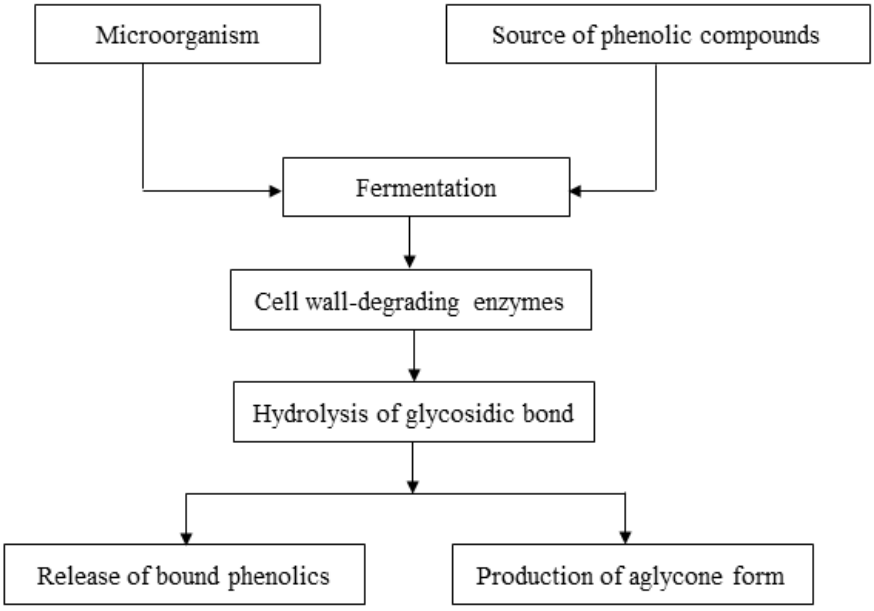


Figure 1.7. A schematic diagram of the release and bioconversion of phenolic compounds

Table 1.4. Enzymes system produced by different microorganism strains to degrade the cell wall matrix

Microorganisms	Species	Enzymes	References
Bacteria	*Lactobacillus lactis*	Esterase, decarboxylase	(Hur et al., 2014)
	Lactobacillus plantarum	β-Glucosidase, decarboxylase	(Hur et al., 2014)
	Lactobacillus rhamnosus	Cellulase, esterase, β-glucosidase	(Hur et al., 2014)
	Bacillus cereus	Cellulase, tannase	(Hur et al., 2014)
	Bacillus subtilis	Cellulase, β-glucanase	(Hur et al., 2014)
	Bacillus thuringiensis	Cellulase, tannase	(Hur et al., 2014)
Fungi	*Aspergillus awamori nakazawa*	Xylanase, α-L-arabinofuranosidase, feruloyl esterase	(Bhanja et al., 2009)
	Aspergillus niger	Cellulase, esterase, β-glucosidase, xylanase	(Bhanja et al., 2009)
	Aspergillus oryzae	Cellulase, β-glucosidase, xylanase, pectinase	(Wu et al., 2011)
	Aspergillus sojae	Cellulase, xylanase, β-glucosidase	(Sardjono et al., 1998) (Kimura et al., 1995) (Kimura et al., 1999)
	Lentinus edodes	Cellulase, β-glucosidase, xylanase, manganese peroxidase, laccase	(Zheng et al., 2000)
	Penicillium brasilianum	Feruoylesterase	(Panagiotou et al., 2007)
	Pleurotus ostreatus	Laccase, α-/β-glucosidase	(Żuchowski et al., 2013)
	Rhizopus oligosporus	β-glucosidase, β-glucuronidase, xylanase	(Correia et al., 2004)
	Phanerochaete chrysosporium	β-Glucosidase, lignin peroxidases, manganese peroxidase, laccase	(Ajila et al., 2011)
	Rhizopus oryzae	β-glucosidase, tannase, pectinase	(Yadav et al., 2013)
	Rhizopus azygosporus	β-glucosidase	(Lee et al., 2006)
Yeast	*Crytococcus flavus*	β-glucosidase, β-glucanase, esterase, xylanase	(Hur et al., 2014)
	Rhodotorula glutimis	β-Glucosidase	(Hur et al., 2014)
	Sacharomyces cerevisiae	β-Glucosidase, feruoylesterase	(Hur et al., 2014)
	Wickerhamomyces anomalus	β-Glucosidase, esterase	(Restuccia et al., 2011)

1.4. Metabolism of flavonoids during fermentation

Various metabolic pathways of phenolic compounds by microbial fermentation are summarized in Figure 1.8. The studies dealing with the bioconversion of flavonoids into their metabolites by a controlled microbial fermentation are summarized in Table 1.5. However, it should be mentioned that not all of the studied microorganisms are food-graded ones. Besides, only wild-type microorganisms are included, possessing the characteristics to modify phenolic compounds. Although some recent studies are investigating genetically modified organisms to obtain higher conversion yields by expressing certain enzymes, these are not included. A more detailed discussion of the different processes is given below.

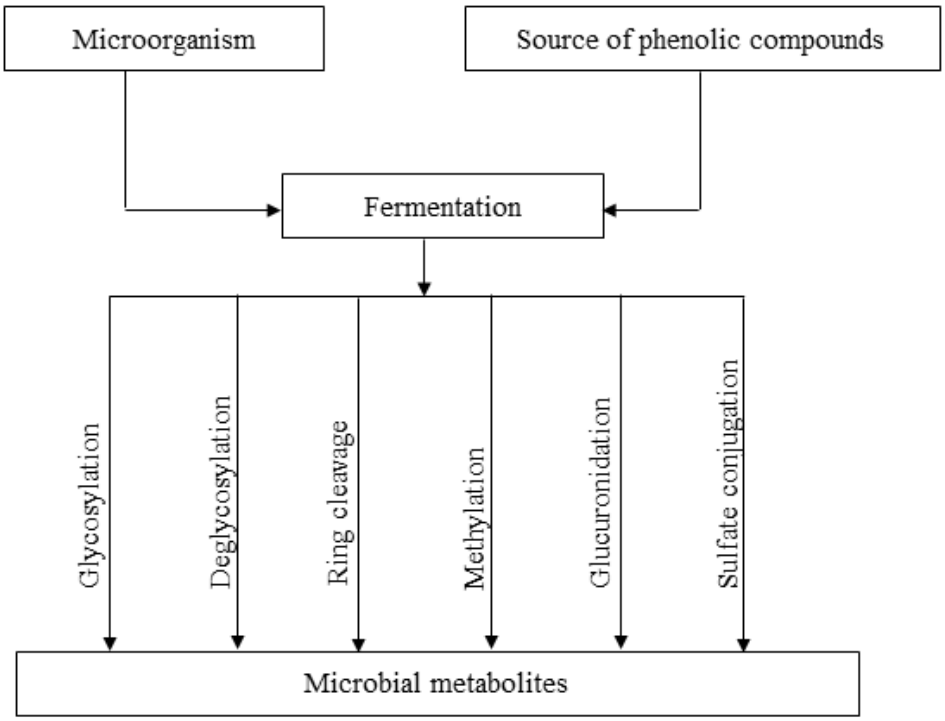


Figure 1.8. A schematic diagram of microbial conversion of phenolic compounds

1.4.1. Glycosylation and deglycosylation of flavonoids

1.4.1.1. Glycosylation of flavonoids

Glycosylation is the reaction in the biosynthesis of phenolic compounds whereby an activated glycosyl donor is attached to a phenolic aglycone through linkage to hydroxyl groups. This reaction could be performed by glycosyltransferases, resulting in a higher hydrophilic solubility of these, mainly lipophilic compounds. The glycosylation process could be applied to enhance the stabilization, detoxification and solubilization of the substrates (Hyung Ko et al., 2006). Some microorganisms such as *Bacillus cereus, Streptomyces rimosus, Cunninghamella elegans* and *Cunninghamella echinulata* are known to be capable of glycosylating phenolic compounds (Hyung Ko et al., 2006; Ma et al., 2013; Miyakoshi et al., 2010; Rao et al., 1981; Zi et al., 2011). Quercetin can be converted into isoquercetin (quercetin-3-glucoside) with a 20 % bioconversion yield using a fermentation process with *Bacillus cereus* at 30 °C (Rao et al., 1981). As reported by Zi et al. (2011), incubation of quercetin with *Cunninghamella elegans* ATCC9245 yielded quercetin-3-O-β-D-glucopyranoside. Similar to quercetin, kaempferol was glycosylated by the filamentous fungus *Cunninghamella echinulata* (Miyakoshi et al., 2010). According to Slana et al. (2011), under conditions whereby flavonoids are toxic, e.g. high concentrations, microorganisms such as fungi could produce glycosylating enzymes (glycosyltransferase) transforming the phenolic compounds into less toxic metabolites. Not only glycosyltransferases but also other enzymes can be involved in the glycosylation of phenolic compounds, as it was shown e.g. for glucansucrase of *Leuconostoc mesenteroides* acting on luteolin, quercetin and myricetin (Bertrand et al., 2006), cellulase of *Penicillium decumbrens* on quercetin (Chen et al., 2011),

Aspergillus niger on catechin (Gao et al., 2000), or α-amylase of *Bacillus* sp. on catechin (Gao et al., 2000).

1.4.1.2. Deglycosylation of flavonoids

In contrast to glycosylation, many studies indicated that deglycosylation of phenolic compounds could be performed through microbial fermentation due to glycosyl hydrolase activities, such as β-glucosidase (Vattem et al., 2004; Zheng et al., 2000), naringinase (Liu et al., 2013), α-rhamnosidase and hesperidinase (Lin et al., 2014). Park et al. (2006) reported that glucose moieties attached to flavonoids at the C3 and C7 positions can be a substrate for β-glucosidase [EC 3.2.1.21]. This enzyme is well known for its deglycosylation capability by hydrolyzing the β-1,4 glycosidic bonds in aryl and alkyl β-D-glucosides as well as glycosides containing disaccharides and oligosaccharides (Turner et al., 2006; Zheng et al., 2000). Recently, several studies for increasing the concentration of isoflavone aglycones in soy products have been performed (Cho et al., 2009; Di Gioia et al.; Lee et al., 2013). Cho et al. (Cho et al., 2009; Cho et al., 2011) reported that fermented soybeans with *Bacillus pumilus* HY1 or *Bacillus subtilis* CS90 for 48h incubation resulted in the highest concentration of isoflavone aglycones (daidzein) and thus in a decrease in isoflavone glucosides. This finding was also reported by Lee et al. (2013), who found that soybeans fermentation by *Aspergillus oryzae* KACC 40247 seemed to cause a significant increase in the amount of isoflavone aglycones including daidzein, glycitein and genistein. In general, the biotransformation of glycosidic flavonoids occurring in soybeans into their corresponding aglycones during fermentation was attributed to microbial β-glucosidase activity (Cho et al., 2009; Cho et al., 2011; Lee et al., 2013). This enzyme could be considered as a possible reason for the

deglycosylation of kaempferol-3-O-glucoside into kaempferol through fermentation with *Bifidobacterium pseudocatenulatum* B7003 (Di Gioia et al.) or *Aspergillus awamori* (Lin et al., 2014) and quercetin-3-glucoside into quercetin by *Aspergillus awamori* (Lin et al., 2014).

Another enzyme also produced by fungal fermentation is α-L-rhamnosidase [E. C. 3.2.1.40] which cleaves terminal α-L-rhamnose present in many natural glycosides such as naringin, rutin, quercitrin, hesperidin, diosgene, terpenyl glycosides (Yadav et al., 2010). For example, fermentation of rutin with food-grade *Aspergillus niger* for 4 h (You et al., 2010) or *Aspergillus awamori* for 4 days (Lin et al., 2014) resulted in transformation of rutin into (isoquercetin) quercetin-3-glucoside which could be attributed to α-L-rhamnosidase activity well known for removing one rhamnose moiety. Also some α-rhamnosidase activity was measured in different lactic acid bacteria, with the most positive strains belonging to *Lactobacillus plantarum* and able to release rhamnose units from hesperidin and rutin rhamnose metabolites (Avila et al., 2009).

1.4.2. Ring cleavage of flavonoids

Many flavonoids undergo a ring-opening reaction in which their C-ring is split and chalcones along with hydroxylations at different C-positions are produced. As shown by Udupa et al. (1969), a number of hydroxylated chalcone metabolites (2'-hydroxychalcone; 2',4-dihydroxydihydrochalcone; 2',4-dihydroxychalcone) are produced when flavanone was incubated with the fungal strain, *Gibberella fujikuroi*. The ring fission of the heterocylic C-ring of flavanone is known to occur among several fungal species such as *Aspergillus, Penicillium, Rhizopus, Monacus* (Das et al., 2006). Incubation of unsubstituted flavanone with *Aspergillus*

niger x172 yielded the chalcone products 2'-hydroxydibenzoylmethane and 2',3'',4''-trihydroxydihydrochalcone (Das et al., 2006). Another strain, *Penicillium chrysogenum* cleaves the C-ring of flavanone into 2'-hydroxydihydrochalcone (Das et al., 2006).

Similarly, biotransformation of quercetin to 2-protocatechuoylphloroglucinol carboxylic acid was observed by Das et al. (2006), who indicated that the C-ring of quercetin was oxidized and cleaved by the enzyme quercetinase produced by *Aspergillus flavus*. The cleavage of quercetin to 2-protocatechuoylphloroglucinol carboxylic acid was also performed by flavonol 2,4-dioxygenase, an enzyme produce by *Aspergillus niger* DSM 821 (Hund et al., 1999).

1.4.3. Methylation of flavonoids

O-methylated flavonoids, known as xenobiotic transformation metabolites, is a common hepatic metabolite obtained by phase II reaction occurring in mammalians by O-methyl transferases (Celep et al., 2014). However, some fungal species have been evaluated for their capability of methyl conjugation (Araújo et al., 2013; Edyta et al., 2012; Eula Maria de et al., 2008; Zi et al., 2011). According to Eula Maria de et al. (2008), some of *Beauveria* strains used in their study exhibited the ability to produce 3'-O-methylquercetin by incubation of quercetin at 29 °C for 72 hours. Another flavonoid, rutin was also methylated into methylrutin by *Cunninghamella echinulata* (Araújo et al., 2013). The fermentation of 7-hydroxyflavanone with *Penicillum chermesinum* 113 at 25 °C for 6 days resulted in two methylated products: 7-methoxyflavanone and 3', 4'-dihydroxy-7-methoxyflavanone (Edyta et al., 2012). O-Methylation was also found in the transformation pathway of quercetin into

isorhamnetin 3-O-β-D-glucopyranoside as reported by Zi et al. (2011) using *Cunninghamella elegans* ATCC 9245 at 28 °C for 72 hours.

1.4.4. Glucuronidation of flavonoids

Bioconversion of bioactive compounds with microorganisms to produce specific mammalian metabolites have been investigated in the last decade. A few microbial strains are known for their ability to produce flavonoid metabolites by glucuronidation such as *Beauveria bassiana (Eula Maria de et al., 2008), Cunninghamella echinulata* (Araújo et al., 2013) and *Streptomyces* sp. (Marvalin et al., 2011). Araújo et al. (2013) reported that fermentation of quercetin and rutin with *Beauveria bassiana* and *Cunninghamella echinulata* respectively resulted in their corresponding glucuronide. Recently, a study has demonstrated that a fermentation process of several phenolic compounds (e.g. naringenin, rutin, quercetin) using a *Streptomyces* sp. can produce glucuronidated products (Marvalin et al., 2011). According to these authors, quercetin incubated with a culture of *Streptomyces* M52104 at 28 °C for 65 h resulted in several glucuronidated compounds including quercetin-4'-O-β-D-glucuronide, quercetin-3-O-β-D-glucuronide and quercetin-7-O-β-D-glucuronide. Similarly, both naringenin and naringenin-7-O-glucoside were also glucuronidated into naringenin-7-O-β-D-glucuronide and naringenin-4'-O-β-D-glucuronide by fermentation with *Streptomyces* M52104 at 28 °C for 65 h (Marvalin et al., 2011). The microbial production of glucuronidates could be attributed to the detoxification pathways in which bioactive compounds are conjugated with glucuronic acid leading to an increased solubility and a higher molecular weight (Marvalin et al., 2011).

1.4.5. Sulfate conjugation of flavonoids

Sulfate conjugation is a major pathway for the phase II metabolism of phenolic compounds in humans via the bile using arylsulphotransferase, originating from human colonic bacteria (Celep et al., 2014). However, recent studies have shown that bioconversion of phenolic compounds into their sulfated conjugated form could also be performed by a few fungal strains including *Cunninghamella echinulata* (Araújo et al., 2013), *Cunninghamella blakesleeana* (Ibrahim et al., 2008), *Streptomyces fulvissimus* (Das et al., 2006), and *Mucor ramannianus* (Herath et al., 2011). Rutin incubated with *Cunninghamella echinulata* induced rutin sulfate (Araújo et al., 2013). 5-hydroxyflavone was converted into 5,4'-dihydroxyflavone-4'-sulfate by *Streptomyces fulvissimus* (Das et al., 2006). Ibrahim et al. (2008) reported that incubation of kaempferol with *Cunninghamella blakesleeana* (ATCC 8688A) led to the production of kaempferol-4'-sulfate. A similar result was observed by Herath et al. (2011) indicating that *Mucor ramannianus* (ATCC 2628) was able to convert hesperitin into hesperetin-7-sulfate.

Table 1.5. Microbial metabolism of flavonoids through fermentation process

Substrate	Production	Microoganism	Reference
Glycosylation			
Quercetin	Isoquercetin (quercetin-3-glucoside)	*Bacillus cereus*	(Rao et al., 1981)
Catechin	Catechin 7-α-D-glucopyranoside	*Bacillus stearothermophilus*	(Gao et al., 2000)
	Catechin 5-α-D-glucopyranoside	*Aspergillus niger*	(Gao et al., 2000)
Luteolin	Luteolin-3'-O-α-D-glucopyranoside	*Leuconostoc mesenteroides*	(Bertrand et al., 2006)
	Luteolin-4'-O-α-D-glucopyranoside		
Kaempferol	Kaempferol 3-β-O-glucopyranoside	*Cunninghamella blakesleeana*	(Ibrahim et al., 2008)
	Kaempferol 4'-O-α-L-rhamnopyranoside		
Kaempferol	Kaempferol 3-β-O-glucopyranoside	*Cunninghamella echinulata*	(Miyakoshi et al., 2010)
Flavonol	Flavonol 3-β-O-glucopyranoside	*Cunninghamella echinulata*	(Miyakoshi et al., 2010)
Quercetin	Quercetin 3-O-β-D-glucopyranoside	*Cunninghamella elegans*	(Zi et al., 2011)
Quercetin	Quercetin glycoside	*Penicillium decumbens*	(Chen et al., 2011)
Kaempferol	Kaempferol glycoside	*Penicillium decumbens*	(Chen et al., 2011)
Isorhamnetin	Isorhamnetin glycoside	*Penicillium decumbens*	(Chen et al., 2011)
Deglycosylation			
Daidzin	Daidzein	*Bacillus pumilus*	(Cho et al., 2009)
Daidzin	Daidzein	*Bacillus subtilis*	(Cho et al., 2011)
Kaempferol-3-O-glucoside	Kaempferol	*Bifidobacterium*	(Di Gioia et al.)
Narigin	Prunin	*pseudocatenulatum*	(Kaur et al., 2010)
Quercetin-glucoside	Quercetin	*Clostridium stercorarium*	(Landete et al., 2014)
Ploridzin	Phloretin	*Lactobacillus plantarum*	(Landete et al., 2014)
Kaempferol-3-rutinoside	Kaempferol, kaempferol-3-glucoside	*Lactobacillus plantarum*	(Lin et al., 2014)
Rutin	Quercetin, quercetin-3-O-glucoside	*Aspergillus awamori*	(Lin et al., 2014)
Rutin	Quercetin, quercetin-3-O-glucoside	*Aspergillus awamori*	(You et al., 2010)
Daidzin, glycitin, genistin	Daidzein, glycitein, genistein	*Aspergillus niger*	(Lee et al., 2013)
Narigin	Naringenin	*Aspergillus oryzae*	(Liu et al., 2013)
Ring cleavage			
Quercetin	2-protocatechuoylphloroglucinol carboxylic acid	*Curvularia lunata*	(Das et al., 2006)
Flavanone	2'-hydroxydibenzoylmethane	*Aspergillus flavus*	(Das et al., 2006)

Quercetin	2-protocatechuoylphloroglucinol carboxylic acid	*Aspergillus niger*	(Hund et al., 1999)
Flavanone	2'-hydroxychalcone; 2',4-hydroxydihydrochalcone 2,4-dihydroxychalcone	*Asperillus niger*	(Udupa et al., 1969)
Flavanone	2',3'',4''-trihydroxydihydrochalcone	*Gibberella fujikuroi*	(Das et al., 2006)
	2'-hydroxydihydrochalcone	*Penicillium chrysogenum*	
Methylation			
Quercetin	3'-O-methylquercetin	*Beauveria sp.*	(Eula Maria de et al., 2008)
Quercetin	Methylquercetin	*Beauveria bassiana*	(Araújo et al., 2013)
Rutin	Methylrutin	*Cunninghamella echinulata*	(Araújo et al., 2013)
Quercetin 3-O-β-D-glucopyranoside	Isorhamnetin 3-O-β-D-glucopyranoside	*Cunninghamella elegans*	(Zi et al., 2011)
7-hydroxyflavanone	7-methoxyflavanone	*Penicillium chermesinum*	(Edyta et al., 2012)
	3', 4'-dihydroxy-7methoxyflavanone		
Glucuronidation			
Quercetin	Quercetin glucuronide	*Beauveria bassiana*	(Araújo et al., 2013)
Rutin	Rutin glucuronide	*Cunninghamella echinulata*	(Araújo et al., 2013)
Quercetin	Quercetin-4'-O-β-D-glucuronide	*Streptomyces sp.*	(Marvalin et al., 2011)
	Quercetin-3'-O-β-D-glucuronide		
	Quercetin-3-O-β-D-glucuronide		
	Quercetin-7-O-β-D-glucuronide		
Rutin	Quercetin-4'-O-β-D-glucuronide	*Streptomyces sp.*	(Marvalin et al., 2011)
	Quercetin-3-O-β-D-glucuronide		
Naringenin	Quercetin-7-O-β-D-glucuronide		
	Naringenin-7-O-β-D-glucuronide	*Streptomyces sp.*	(Marvalin et al., 2011)
	Naringenin-4'-O-β-D-glucuronide		
Sulfate conjugation			
Kaempferol	Kaempferol-4'-sulfate	*Cunninghamella blakesleeana*	(Ibrahim et al., 2008)
Rutin	Rutin sulfate	*Cunninghamella echinulata*	(Araújo et al., 2013)
Hesperitin	Hesperetin-7-sulfate	*Mucor ramannianus*	(Herath et al., 2011)
5-hydroxyflavone	5,4'-dihydroxyflavone-4'-sulfate	*Streptomyces fulvissimus*	(Das et al., 2006)

Enzyme-assisted extraction enhancing the phenolic release from cauliflower (Brassica oleracea L. var. botrytis) outer leaves

Redrafted from: Huynh, N. T.; Smagghe, G.; Gonzales, G. B.; Van Camp, J.; Raes, K., Enzyme-Assisted Extraction Enhancing the Phenolic Release from Cauliflower (*Brassica oleracea L. var. botrytis*) Outer Leaves, J. Agric. Food Chem. 62(2014) 7468-7476.

Chapter 2

Enzyme-assisted extraction enhancing the phenolic release from cauliflower (*Brassica oleracea* L. var. *botrytis*) outer leaves

ABSTRACT

Phenolic compounds are highly present in by-products from the cauliflower (*Brassica oleracea* L. var. *botrytis*) harvest and are thus a valuable source for valorization towards phenolic rich extracts. In this study, we aimed to optimize and characterize the release of individual phenolic compounds from outer leaves of cauliflower, using two commercially available polysaccharide-degrading enzymes, Viscozyme L and Rapidase.

As major results, the optimal conditions for the enzyme treatment were: Enzyme/Substrate ratio of 0.2 % for Viscozyme L and 0.5 % for Rapidase, temperature 35 °C, and pH 4.0. Using a UPLC-HD-TOF-MS setup, the main phenolic compounds in the extracts were identified as kaempferol glycosides and their combinations with different hydroxycinnamic acids. The most abundant components were kaempferol-3-feruloyldiglucoside and kaempferol-3-glucoside (respectively 37.8 and 58.4 mg rutin equivalents/100 g dry weight). Incubation of the cauliflower outer leaves with the enzyme mixtures resulted in a significantly higher extraction yield of kaempferol-glucosides compared to the control treatment.

2.1. Introduction

Cauliflower (*Brassica oleracea* L. var. *botrytis*) is one of the cruciferous vegetables belonging to the *Brassicaceae* family that are widely consumed all over the world. These products contain considerable amounts of health beneficial compounds, such as phenolic compounds, glucosinolates and vitamins (Cabello-Hurtado et al., 2012; Hodges et al., 2006; Picchi et al., 2012; Podsędek, 2007). These vegetables are also characterized by their high amount of non-edible parts, such as outer leaves, stems, pods. These non-edible parts are now valorized only as raw materials for industrial fertilizer, animal feed (Iñiguez-Covarrubias et al., 2001), and fiber production (Nilnakara et al., 2009; Tanongkankit et al., 2012), or they are left on the fields. However, as they contain high amounts of bioactive compounds, their valorization potential can be much higher.

In the past, a number of techniques has been applied to obtain phenolic compounds from plant materials, such as cold pressing, supercritical fluid extraction and organic solvent extraction (Puri et al., 2012; Wijngaard et al., 2012). Nevertheless, the drawback of these methods is the low extraction yield as the phenolic compounds are bound to plant cell wall material. In cauliflower leaves, just as in other vegetables, phenolic compounds may be classified as bound phenolics found in cell walls in which they are linked to polysaccharides by ester bonds, hydrophobic interactions and hydrogen bonds, and as free phenolic compounds found in the vacuoles of plant cells (Cerda et al., 2013; Pinelo et al., 2006; Yadav et al., 2013). As a consequence, the use of preprocessing techniques prior to extraction may be used to reduce the loss of bioactive components and to improve the yields of the extraction process. Degradation and disruption of the cell-wall matrix have been considered

as an appropriate step to improve the release of phenolic compounds, keeping their stability and antioxidant activity (Gómez-García et al., 2012; Li et al., 2006). The mechanism for this treatment is based on the use of cell-wall degrading enzymes to depolymerize cell-wall polysaccharides (Kim et al., 2005), and to hydrolyze the glycosidic linkages between phenolic compounds and cell-wall polymers (Yadav et al., 2013). In addition, enzyme systems originating from microorganisms can transglycosylate the target compounds (Chen et al., 2011; Hansson et al., 2001). As a result, not only the structure of cell walls can be weakened and broken down, whereby intracellular materials are more exposed for extraction (Gómez-García et al., 2012; Li et al., 2006), but also the solubility of the target compounds in the extractant can be improved (Chen et al., 2011).

The successful application of carbohydrate-cleaving enzymes for the extraction of phenolic compounds has been reported in several studies, mainly focusing on other plant sources, such as apple peel, citrus peel, grape pomace, *Thymus vulgaris*, *Ginkgo biloba* leaves, berries and oat bran (Alrahmany et al., 2012; Cerda et al., 2013; Chen et al., 2011; Gómez-García et al., 2012; Kim et al., 2005; Li et al., 2006; Maier et al., 2008; Pimpão et al., 2013; Saulnier et al., 2001). However, the investigation field was restricted to the factors influencing enzyme-assisted extraction of phenolic components (Li et al., 2006; Zheng et al., 2009), and information on the impact of enzymatic treatment on the release of individual components from plant waste material is lacking. The aim of this study was to investigate the potential of using enzyme-assisted extraction and to evaluate its effect on the yield and the profile of extracted phenolic compounds from *Brassica* cauliflower outer leaves.

2.2. Materials and Methods

2.2.1. Materials

Plant material

The outer fresh leaves, a by-product from the cauliflower harvest, were collected from a local farm in West-Flanders, Belgium, during the harvesting of cauliflowers from one field in July 2012. The leaves were kept frozen at -18 °C until further analysis.

Chemicals and Reagents

Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3) were purchased from VWR International (Leuven, Belgium), and gallic acid, hesperetin, rutin from Sigma-Aldrich (Bornem, Belgium). Viscozyme L and Rapidase vegetable juice were purchased from Novozyme (Bagsvaerd, Denmark) and DSM Food Specialties (Delft, the Netherlands), respectively.

Viscozyme L, produced by *Aspergillus aculeatus*, contains different polysaccharide-degrading enzymes, including beta-glucanase, cellulase, hemicellulase and xylanase. The specific enzyme activity of Viscozyme L, as given by the supplier, is 120 Fungal Beta-Glucanase units (FBG)/mL, in which 1 FBG is the amount of enzyme required under standard conditions (30 ^{o}C, pH 5.0 and 30 min reaction time) to degrade barley α-glucan to reducing carbohydrates with a reducing power corresponding to 1 µmol glucose/min.

Rapidase vegetable juice, is an enzyme mixture produced by both *Aspergillus niger* and *Trichoderma longibrachiatum*. As stated by the supplier, it contains pectinase and hemicellulase (optimal pH 4.0-5.0, optimal temperature 10-55 °C).

2.2.2. Experimental procedure

Enzyme-assisted pretreatment

The frozen outer leaves of cauliflower were cut into pieces (2×5 cm), followed by 3 min mixing using a kitchen homogenizer. Enzymes were first dissolved in water taking into account the appropriate concentrations to obtain a correct E/S (cauliflower outer leaves) ratio. Then, the cabbage mixture (60 g) was placed in a 500 mL glass vial and 90 mL of aqueous enzyme solution was added. In a first series of experiments, we searched for the optimal conditions for the two enzyme pretreatments. A first set of incubations was done at 40 °C, pH 4.0 with different enzyme concentrations (0–5 % E/S ratio). In the second set with the optimal enzyme concentrations (E/S ratio of 0.2 % for Viscozyme L and 0.5 % for Rapidase), the incubation temperature varied from 30 °C to 50 °C, always at pH 4.0. In a last step, the optimal enzyme concentrations, and optimal temperature (35 °C) was used while the pH value varied from 3.0 to 6.0. All incubations were performed in a temperature-controlled water bath with agitation. Samples of the treated cabbage were taken at 0, 6 and 24 h for methanol extract, and a sample of the supernatans was taken at 24 h, representing the aqueous extract. All samples were stored at -18 °C for further analysis of total phenolic content. All incubations were done in triplicate.

Using the optimal conditions for both enzymes obtained in the first experiment, a second series of experiments was performed to investigate the enzymatic kinetics of the two enzymes. All the experimental parameters were the same as in the first series of experiments, except that samples were taken at 2 h intervals during the first 12 h and then at

30 h. All incubations were done in triplicate. Samples were stored at -18 °C for analysis of total phenolic content, and profile of individual phenolic compounds.

Extraction of phenolic compounds

Samples were divided into two parts. Supernatant (= liquid phase), after 24 h of incubation, was obtained by centrifugation (13,000xg, 10 min, 4 °C) and filtered through filter paper (VWR, grade 413) (aqueous extract, AE). The solid samples at 0, 6, 12 and 24 h of incubation were extracted by the method according to Olsen et al. (2009). In brief, 15 mL of methanol was added to 5 g of sample, the suspension was homogenized at 9500 rpm using an ultraturrax for 40 s, followed by incubation in ice water for 15 min. Supernatant was collected after centrifugation (13,000xg, 10 min and 4 °C). The pellet obtained was then re-extracted by the same procedure, using 10 mL methanol/water (80/20; v/v). Both extracts were pooled and filtered through filter paper (VWR, grade 413) before adding methanol to a final volume of 25 mL (methanolic extract, ME).

2.2.3. Analytical Methods

Determination of total phenolic content

Total phenolic content was determined according to Folin-Ciocalteu[23], with slight modifications. In brief, 1.8 mL deionized water was added to 0.2 mL of sample. After thorough mixing, Folin-Ciocalteu reagent (0.5 mL) was added, and the tube was shaken vigorously. After 6 min, 1.5 mL of sodium carbonate solution (20 %) was added, followed by adding deionized water to a final volume of 5 mL and mixing well again. The solution was then allowed to stand in the dark for 2 h at ambient temperature, and the absorbance was read at 760 nm. Total phenolic content (TPC) was expressed as milligrams of gallic acid

equivalents per 100 gram of dry weight (mg GAE/100 g DW) by using a standard curve of gallic acid in the range of 10-100 mg/L. The blank was prepared with 0.2 mL of methanol instead of sample solution.

Sample purification using SPE

In brief, 1 mL of sample was diluted in water (0.1 % formic acid) up to 20 mL of total volume, followed by adding 100 µL of 100 µg/mL hesperetin as internal standard. The mixture was slowly loaded onto a solid phase extraction (SPE) C18 column (50 mg, 4 mL; Davison Discover Science, Deerfield, IL, USA) which was previously preconditioned with methanol (0.1 % formic acid). Then, the column was washed with 5 mL of water (0.1 % formic acid) prior to loading 3 mL of methanol (0.1 % formic acid). The eluent obtained was evaporated under nitrogen flux. After drying, the residue of the sample was re-dissolved in 100 µL of methanol (0.1 % formic acid) prior to adding 900 µL of water (0.1 % formic acid). The sample obtained was kept at -18 °C until UPLC-DAD-HDMS-TOF-MS analysis.

Quantification and identification of phenolic compounds by UPLC-DAD-HDMS-TOF-MS

UPLC-DAD analysis was performed on a bride ethylene hybrid (BEH) C18 column (150 x 2.1 mm, 1.7 µm particle size) by using an Ultimate U(H)PLC (Dionex, Breda, The Netherlands). The column and autosampler temperature were kept at 40 °C and 4 °C, respectively. The mobile phase was formed by two solvents, solvent A (water containing 0.1 % formic acid; v/v) and solvent B (methanol containing 0.1 % formic acid; v/v). A stable flow rate of 250 µL/min was applied at 0 min, 90 % A; followed by linear and isocratic gradient of eluting solvents as: 0–6 min, 20 % B linear; 6-12 min, 20 % B isocratic; 12-13 min, 30 % B linear; 13-23 min, 50 % B linear, 23-30 min, 90 % B linear; 30-35 min, 90 % isocratic; 35-40 min, 10 % B linear; and 40-

45 min 10 % B isocratic. Quantification was achieved by comparing the peak areas to a calibration curve made using rutin. Quantities of the compounds were expressed as rutin equivalents (RE).

Identification was achieved using an ACQUITY UPLC™ system (Waters, Milford, MA) system coupled to a Synapt HDMS-TOF mass spectrometer (Micromass, Manchester, UK), equipped with an electrospray ionization source. The working conditions of MS analysis were: capillary voltage, 2 kV; sampling cone voltage, 40 V; extraction cone voltage, 4 V; source temperature 150 °C; desolvation temperature, 350 °C; cone gas flow rate, 50 L/h; desolvation gas flow rate, 550 L/h. Collision energies for the trap and transfer were set at 6 and 4 V for low energy while a ramp of 15-45 V was applied for the high energy collision-induced dissociation. The instrument was operated in negative and positive continuum mode and data was acquired using Waters MassLynx software. Detailed information on the MS analysis and peak identification is described by Gonzales et al. (2014).

2.2.4. Statistical analysis

The data are given as mean ± standard deviation (SD) for the optimization of the enzyme treatment and as mean with the standard error of mean (SEM) for the individual phenolic compounds. One-way and two-way ANOVA were used to determine significant differences between individual means using Tukey post-hoc test for P values < 0.05. Principal component analysis (PCA) was applied to visualize differences in effects between treatments as well as incubation times based on the contents of individual phenolic compounds. The SPSS software v.22 (IBM, Chicago, IL, USA)) was used for all statistical analyses.

2.3. Results

2.3.1. Optimization of enzyme-assisted extraction of phenolic compounds

Independent of the treatment (type of enzyme, concentration of enzyme, temperature and pH), the TPC at 0h of incubation in the ME was 336 ± 30 mg GAE/100g DW. During 24h of incubation, it was observed that enzyme dosages, temperature, and pH, as well as their two-way interaction term with time significantly affected the total phenolic release (P-value < 0.001), with an exception for Rapidase concentration × time.

The effect of enzyme concentration. The total phenolic contents of AE (Figure 2.1 A) and of ME (Figure 2.1 B) after 24h enzyme-assisted extraction at 40 °C and pH 4.0 of the cauliflower outer leaves were measured in response to the different concentrations of enzymes (Viscozyme L and Rapidase) used. The level of phenolic compounds in both extracts was significantly increased after 24 h of incubation compared to the control incubation, when 0.1 % Viscozyme L and 0.5 % Rapidase were added. Viscozyme L resulted in a higher concentration of phenolic compounds in AE, ranging from 429 ± 28 mg GAE/100g DW at 0 % E/S (control) to 614 ± 23 mg GAE/100 g DW at 0.5 % E/S, with no further significant increase in TPC at higher enzyme concentrations (Figure 2.1 A). Similar results were found for the ME, whereby the TPC in ME significantly increased to 650 ± 18 mg GAE/100 g DW at 0.2 % E/S, without further significant improvement between 0.2 and 5 % E/S (Figure 2.1 B). The Rapidase treatment with 0.5 % E/S resulted in a greater release of phenolic compounds, mainly in the ME and to a lesser extent in the AE, as compared to the control sample, whereas an increased Rapidase concentration from 0.5 to 5 % did not enhance the TPC in AE and ME, except for AE from sample treated with 5 % Rapidase (Figure 2.1 B). Based on the

results of the different enzyme concentrations, it was chosen to work further with 0.2 % Viscozyme L and 0.5 % Rapidase.

Temperature. The influence of incubation temperature on the extraction of phenolic compounds from cauliflower outer leaves using 0.2 % Viscozyme L or 0.5 % Rapidase at a pH of 4 is given in Figure 2.1 C and Figure 2.1 D. The treatments with Viscozyme L and Rapidase resulted in a significant improvement of TPC at all temperature points examined, ranging from 30 °C to 50 °C, compared to the control (no enzyme) incubation, except for ME at 30 °C, where no difference between enzymatic treatment and control sample was found (Figure 2.1 D). Although a trend towards a higher release of phenolic compounds with higher temperatures was observed in the ME, this was not observed in AE. The TPC in ME was significantly higher at 35 °C than at 30 °C for Viscozyme L and Rapidase treatment, whereas no further increase in total phenolic release between 35 °C to 50 °C was observed for both ME and AE. Even the TPC at 50 °C was lower than at 40 °C with Viscozyme L treatment. Therefore, 35 °C was chosen as optimal incubation temperature for both enzymes.

The effect of pH. During enzymatic treatment, the pH values were varied from 3 to 6 to investigate its effect on extraction yields of total phenolic compounds (Figure 2.1E and Figure 2.1 F). The results obtained showed that the TPC did not significantly change in response to the difference in pH values during extraction for both AE and ME, within one enzymatic treatment.

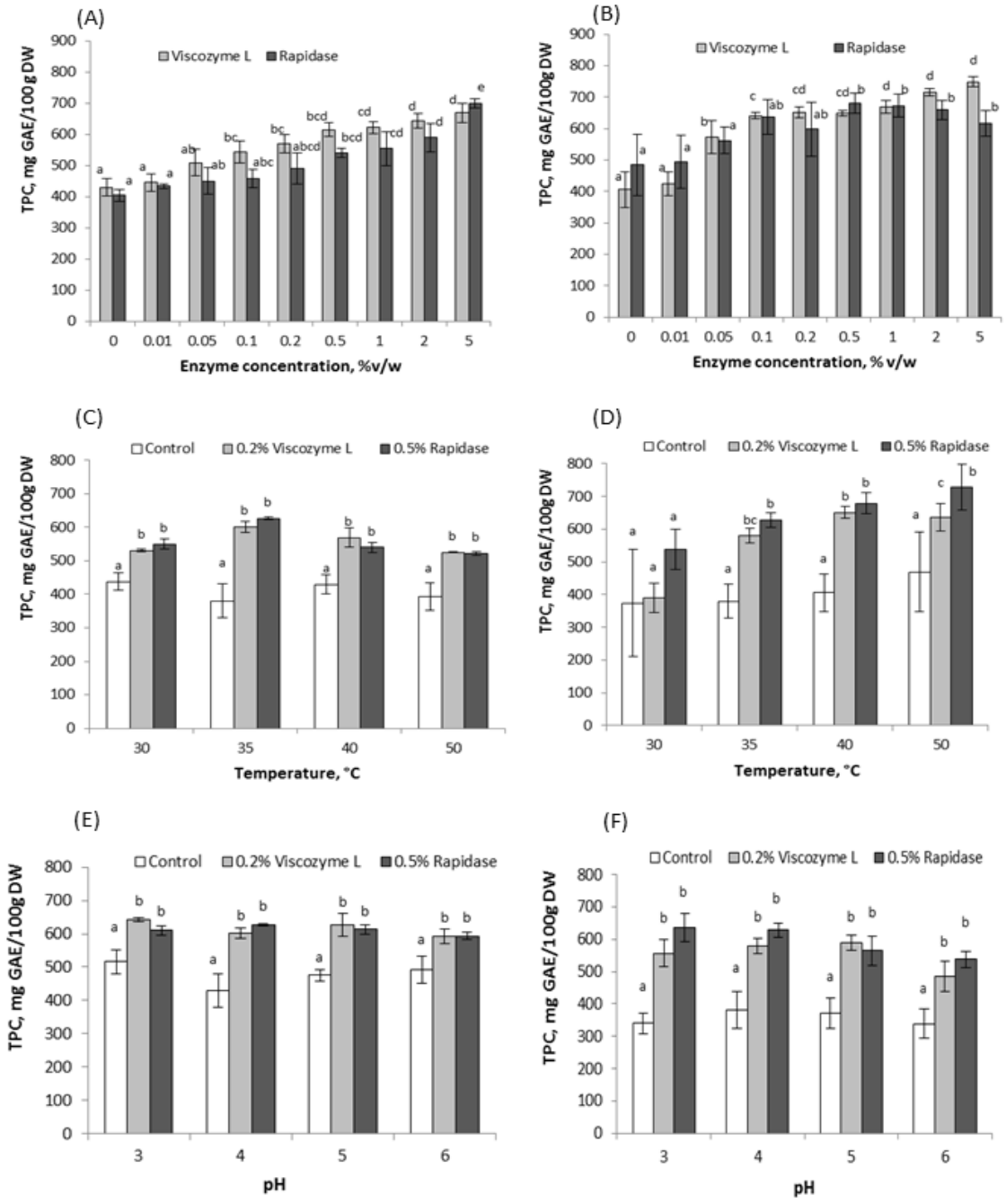


Figure 2.1. The effect of enzyme concentration (A, B), temperature (C, D), and pH (E, F) on the extraction of phenolic compounds from outer leaves of cauliflower after 24 h of incubation. (A), (C), (E): total phenolic content in AE assisted by enzymes. (B), (D), (F): total phenolic content in ME after enzymatic pretreatment. Different letters (a, b, c, d, e) indicate significant difference (P value < 0.05) within the same color column. The total phenolics of methanolic extract from cabbage solid residue at 0 h of incubation (Control sample): 336 ± 30 mg GAE/100g DW (n = 3)

2.3.2. Identification and quantification of phenolic compounds

Using the optimal conditions for the enzyme treatment, the individual phenolic compounds released from the cauliflower outer leaves were identified and quantified. The shift in the phenolic profile during time of incubation and type of enzyme was studied.

Kinetics of the release of TPC at optimal conditions. Selecting the optimal conditions obtained for enzyme treatment (0.2 % Viscozyme L or 0.5 % Rapidase, temperature 35 °C and pH 4), we evaluated the effects of incubation time on the phenolic extraction from outer leaves of cauliflower (Figure 2.2). The TPC in ME of the control incubation remained stable. A significant increase in the TPC in the ME after enzyme treatment was observed, with a more than 100% increase after 12 h of incubation for the enzyme-treated incubations compared to the control ones. The TPC of ME increased as a function of incubation time. No difference in TPC in the ME extract was observed between the two enzyme treatments. After 12 h of incubation, the TPC in the ME remained constant, and this trend was observed for both enzymes. The enzymatic treatments also caused significant effects on released phenolic compounds in AE after 30 h of incubation, increasing to 581 ± 16 mg GAE/100 g DW (with Viscozyme L) and to 604 ± 9 mg GAE/100 g DW (with Rapidase) compared to 457 ± 23 mg GAE/100 g DW for the control without enzyme treatment.

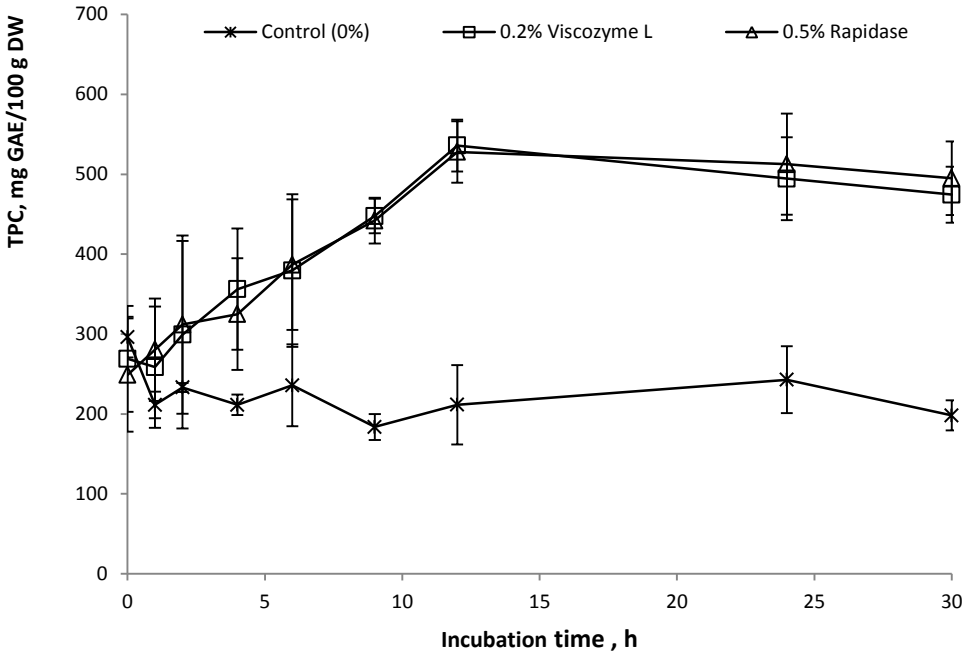


Figure 2.2. Changes in total phenolic content in ME from solid residue incubated with Viscozyme L and Rapidase at 35 °C, pH 4.0 during 30 h (n = 3)

Identification and quantification of individual compounds by UPLC-DAD-HDMS-TOF-MS

The phenolic compounds in cauliflower outer leaves extracts were quantified by UPLC-DAD and identified by the UPLC-MS/MS method (Table 2.1). As there was no interaction effect between treatment and time of incubation on the content of the individual phenolic compounds, results are presented for the main factors separately, i.e. treatment and time of incubation in Table 2.2 and Table 2.3, respectively.

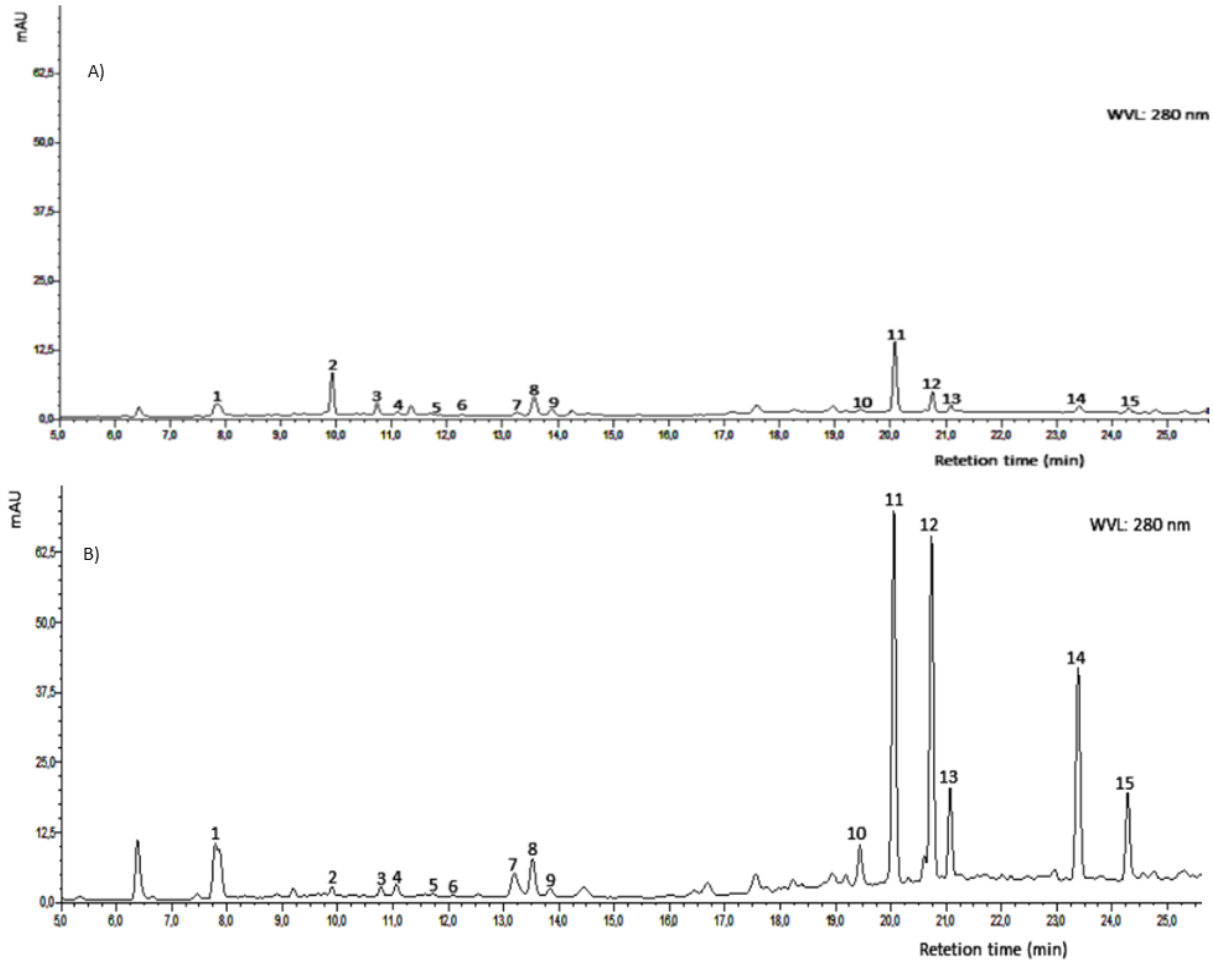


Figure 2.3. U(H)PLC profile of phenolic compounds from cauliflower byproduct extracts obtained by an incubation 0 h (A) and 12 h (B) with Rapidase, recorded at 280 nm (Table 2.1 for identification of peaks)

Table 2.1. Identification of flavonoid glycosides from Cauliflower by-product extract

Peak	Rt (min)	m/z Values		λ max (nm)	Identity
		Molecular	Fragments		
1	7.8	359.07	359.07	213, 307	Unknown 1
2	9.9	950.23	771, 609, 285	nd	Kaempferol-C-glucoside-3-O-glucose units
3	10.8	963.25	963, 609, 771, 801, 284	nd	Kaempferol-3-O-glucoside-7-O-hydroxyferuloyldiglucoside
4	11.11	933.21	933, 609, 285	265, 345	Kaempferol-3-O-diglucoside-7-O-diglucoside
5	11.79	785	nd	nd	Unknown 2
6	12.16	nd	nd	269, 318	Unknown 3
7	13.26	977.35	977, 815, 609, 284, 446	268, 338	Kaempferol-3-O-sinapoyldiglucoside-7-O-glucoside
8	13.59	1109.30	1109, 947, 771, 609 , 447	268, 331	Kaempferol-3-O-feruloyltriglucoside-7-O-glucoside
9	13.92	947.24	947, 785, 609, 284	269, 322	Kaempferol-3-O-feruloylglucoside-7-O-diglucoside
10	19.46	787.18	787, 625, 462, 300	254, 348	Kaempferol-3-O-diglucoside-7-O-glucoside
11	20.07	609.12	609, 284	265	Kaempferol-3-O-diglucoside
12	20.75	785.18	785, 609, 284	329	Kaempferol-3-O-feruloyldiglucoside
13	21.08	755.18	755, 609, 284	320	Kaempferol-3-O-coumaroyldiglucoside
14	23.39	447.07	447, 284	nd	Kaempferol-3-O-glucoside
15	24.28		623, 447, 284	nd	Kaempferol-C-feruloylglucoside/Kaempferol-3-O-feruloylglucoside

Rt: retention time; nd: not detected

Table 2.2. The effect of different treatments on the profile of phenolic release in ME from cauliflower outer leaves (n=3)

Methanol extraction							
Peak	Individual compound (mg RE/100 g DW)	Rt (min)	Treatment			SEM	P value
			Control	Viscozyme	Rapidase		
1	Unknown 1	7.8	10.1^{a}	16.0^{b}	12.2^{ab}	1.1	0.003
2	Kaempferol-C-glucoside-3-O-glucose units	9.9	10.0	19.4	15.0	3.9	0.250
3	Kaempferol-3-O-glucoside-7-O-hydroxyferuloyldiglucoside	10.8	6.1^{a}	10.8^{b}	8.1^{ab}	1.2	0.034
4	Kaempferol-3-O-diglucoside-7-O-diglucoside	11.11	5.7^{a}	8.1^{b}	6.0^{a}	0.5	0.007
5	Unknown 2	11.79	5.0^{a}	6.8^{b}	5.1^{ab}	0.5	0.030
6	Unknown 3	12.16	5.0	6.4	4.8	0.6	0.125
7	Kaempferol-3-O-sinapoyldiglucoside-7-O-glucoside	13.26	6.5^{a}	11.5^{b}	8.2^{ab}	1.2	0.026
8	Kaempferol-3- O-feruloyltriglucoside-7-O-glucoside	13.59	9.0^{a}	21.0^{b}	14.8^{ab}	3.2	0.046
9	Kaempferol-3-O-feruloylglucoside-7-O-diglucoside	13.92	5.8^{a}	10.0^{b}	7.3^{ab}	0.8	0.007
10	Kaempferol-3-O- diglucoside-7-O-glucoside	19.46	7.1^{a}	12.5^{b}	11.8^{ab}	1.5	0.033
11	Kaempferol-3-O-diglucoside	20.07	48.0	52.8	43.6	4.5	0.369
12	Kaempferol-3-O-feruloyldiglucoside	20.75	17.2^{a}	37.8^{b}	28.7^{ab}	3.5	0.002
13	Kaempferol-3-O-coumaroyldiglucoside	21.08	10.0^{a}	16.7^{b}	13.1^{ab}	1.3	0.004
14	Kaempferol-3-O-glucoside	23.39	8.1^{a}	58.4^{b}	29.9^{c}	5.4	< 0.001
15	Kaempferol-C-feruloylglucoside/Kaempferol-3-O-feruloylglucoside	24.28	7.1^{a}	12.2^{b}	10.7^{a}	1.1	0.012
	Total compounds		160.3^{a}	300.0^{b}	219.2^{a}	19.8	< 0.001

Different letters (a, b, c) indicate significant difference (P value < 0.05) within a row. SEM, standard error of mean

Table 2.3. The effect of incubation time on the profile of phenolic release in ME from cauliflower outer leaves (n=3)

Methanol extraction								
Peak	Individual compound (mg RE/100 g DW)	Rt (min)	Incubation time (h)				SEM	P value
			0	6	12	24		
1	Unknown 1	7.8	13.0	12.7	13.7	11.6	1.3	0.732
2	Kaempferol-C-glucoside-3-O-glucose units	9.9	32.8^{b}	10.9^{a}	8.6^{a}	6.9^{a}	4.5	0.001
3	Kaempferol-3-O-glucoside-7-O-hydroxyferuloyldiglucoside	10.8	12.3^{b}	8.8^{ab}	6.6^{a}	5.7^{a}	1.4	0.011
4	Kaempferol-3-O-diglucoside-7-O-diglucoside	11.11	6.1	7.7	7.0	6.1	0.6	0.441
5	Unknown 2	11.79	5.0	6.2	6.2	5.2	0.6	0.364
6	Unknown 3	12.16	4.4	5.9	6.1	5.1	0.7	0.276
7	Kaempferol-3-O-sinapoyldiglucoside-7-O-glucoside	13.26	9.2	9.7	8.1	7.8	1.4	0.757
8	Kaempferol-3-O- feruloyltriglucoside-7-O-glucoside	13.59	23.2^{b}	15.7^{ab}	12.6^{ab}	8.1^{a}	3.7	0.051
9	Kaempferol-3-O-feruloylglucoside-7-O-diglucoside	13.92	9.6^{b}	8.5^{ab}	7.1^{ab}	5.7^{a}	1.0	0.047
10	Kaempferol-3- O-diglucoside-7-O-glucoside	19.46	8.0	12.8	9.4	11.6	1.7	0.230
11	Kaempferol-3-O-diglucoside	20.07	31.6^{b}	61.2^{a}	59.6^{a}	39.9^{b}	5.2	0.001
12	Kaempferol-3-O-feruloyldiglucoside	20.75	12.0^{b}	26.2^{ab}	33.7^{a}	39.7^{a}	4.1	< 0.001
13	Kaempferol-3-O-coumaroyldiglucoside	21.08	6.6^{b}	15.3^{a}	16^{a}	15^{a}	1.5	< 0.001
14	Kaempferol-3-O-glucoside	23.39	6.1^{c}	26.3^{bc}	37.2^{ab}	59.0^{a}	6.3	< 0.001
15	Kaempferol-C-feruloylglucoside/Kaempferol-3-feruloylglucoside	24.28	5.7^{b}	10.7^{a}	11.6^{a}	11.6^{a}	1.3	0.008
	Total compounds		185.6	237.9	243.6	239.1	22.9	0.258

Different letters (a, b, c) indicate significant difference (P value < 0.05) within a row. SEM, standard error of mean

As shown in Figure 2.3 and Table 2.1, fifteen individual compounds were identified, of which three components in the extracts could not be identified by our protocol (unknown 1, 2 and 3). All individual phenolic compounds present in the extracts and identified by our analysis

belonged to different derivatives of kaempferol. The results also indicated that the treatment with Rapidase did not enhance the amount of all identified compounds released as compared to the control with an exception for kaempferol-3-glucoside. In contrast, there was a clear increase in identified compounds by Viscozyme L treatment. The release of kaempferol-3-feruloyltriglucoside-7-glucoside (compound 8; 21.0 mg RE/100 g DW), kaempferol-3-feruloyldiglucoside (compound 12; 37.8 mg RE/100 g DW) and kaempferol-3-glucoside (compound 14; 58.4 mg RE/100 g DW) was sharply improved by Viscozyme L treatment, compared to the control sample (9.0; 17.2; and 8.1 mg RE/100 g DW, respectively) (Table 2.2). As shown in Table 2.3, in general the release of the total phenolic compounds increased during time of incubation, although the increase was not significant. However, the amounts of individual components including kaempferol-3-feruloyldiglucoside (compound 12), kaempferol-3-coumaroyldiglucoside (compound 13), kaempferol-3-glucoside (compound 14), kaempferol-C-feruloylglucoside/ kaempferol-3-feruloylglucoside (compound 15) were significantly increased after 12 or 24 h of incubation in comparison with non-treatment samples (0 h).

The changes in profiles of phenolic compounds affected by different treatments were visualized by using PCA (Figure 2.4). The PC 1 (86.69 %) described the distinct difference between the samples without adding enzymes (control-AE) and the samples treated by enzymes, which are mostly localized on the positive axis of PC1, except for control-ME. All of phenolic components released were positively related to the Viscozyme L and Rapidase used. The PC2 (13.20 %) can be expressed as a factor associated with the profile of individual compounds in ME, compared to AE. The observation from the graph indicated that the high

value of compounds 7, 8, 12, 13, 14, and 15 are associated with AE (cluster of Viscozyme-AE, Rapidase-AE), while the phenolic profile in ME exhibited higher content of compounds 2, 4, 5, 6, 9 and 11.

Figure 2.5 shows the effect of incubation time on the profile of phenolic release. Two PCs are responsible for 94.63 % of the total variance of data. An incubation of 6 h with Viscozyme L characterized for the high amount of compounds 2, 3, 4, 5 and 6, whilst the longer period of Viscozyme L and Rapidase treatments (12 h, 24 h) positively contributed to the high content of compounds 1, 12, 13, 14 and 15.

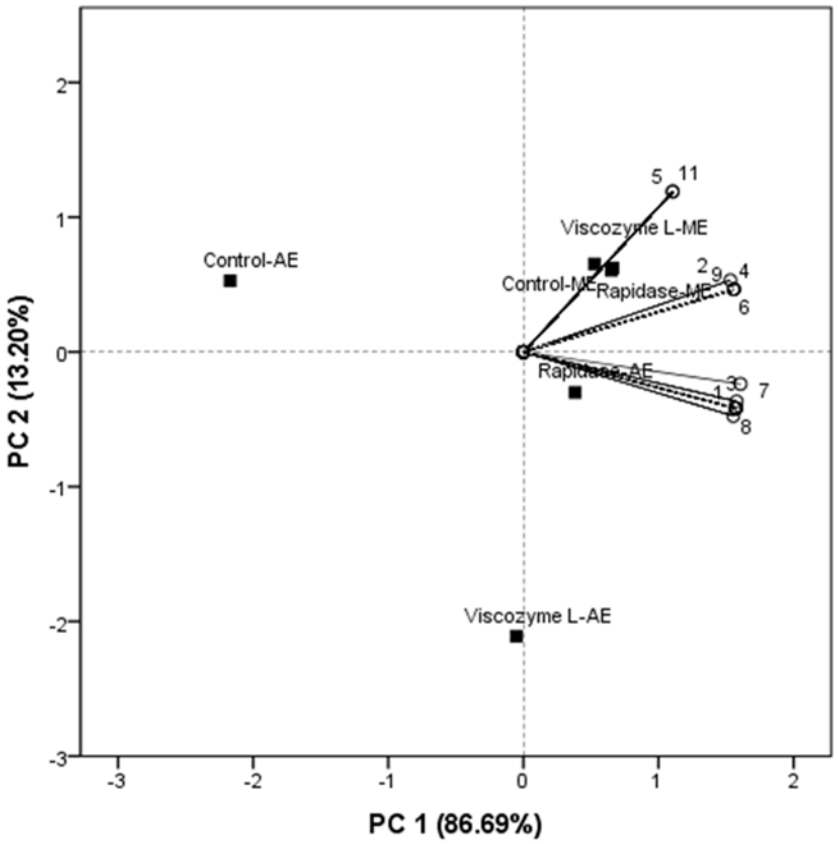


Figure 2.4. Principal component analysis of the individual phenolic compounds in AE and ME by different treatments (n = 3). The numbers in the PCA plot represents the different phenolic compounds as described in Table 1. Control-AE, aqueous extract obtained by an incubation of 30 h without enzyme; Viscozyme L-AE, aqueous extract obtained by an incubation of 30 h with Viscozyme L; Rapidase-AE, aqueous extract obtained by an incubation of 30 h with Rapidase; Control-ME, methanolic extract obtained by an incubation of 12 h without enzyme; Viscozyme L-ME, methanolic extract obtained by an incubation of 12 h with Viscozyme L; Rapidase-MA, methanolic extract obtained by an incubation of 12 h with Rapidase.

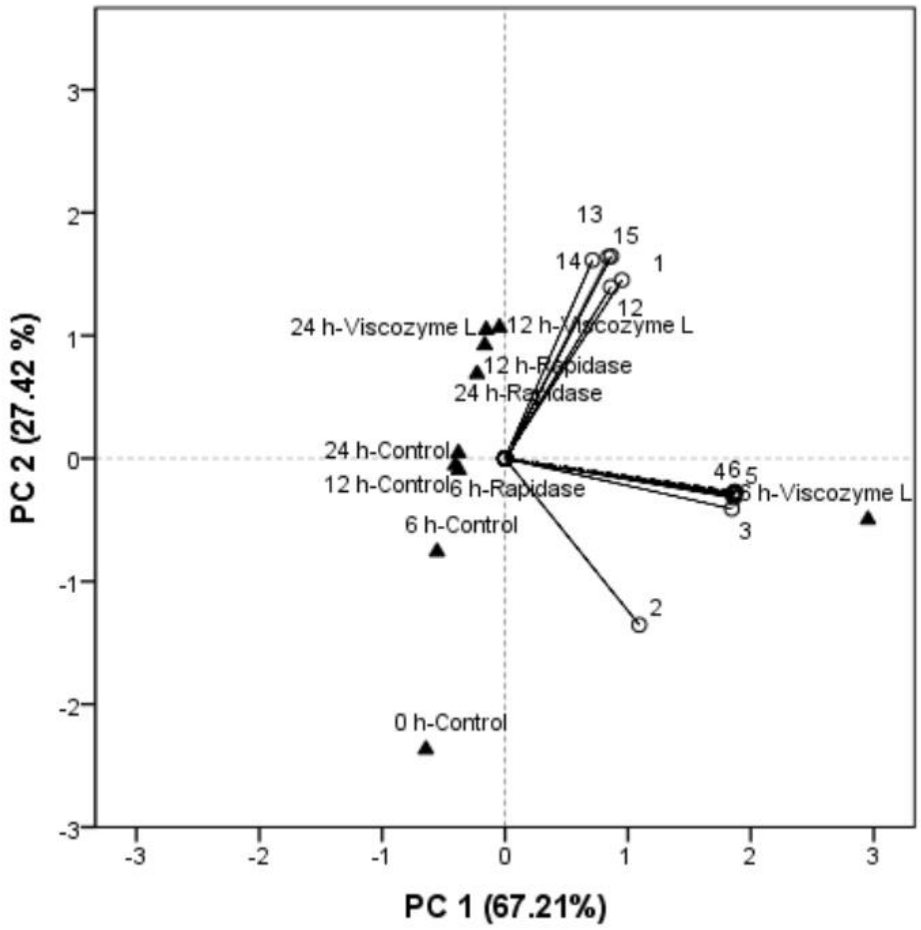


Figure 2.5. Principal component analysis of the individual phenolic compounds in ME from different time of treatments (n = 3). The numbers in the PCA plot represent the different phenolic compounds as described in Table 1. 0 h-Control, 6 h-Control, 12 h-Control, 24 h-Control: methanolic extracts obtained by an incubation of 0 h, 6 h, 12 h and 24 h without enzyme, respectively; 0 h-Viscozyme L, 6 h-Viscozyme L, 12 h-Viscozyme L, 24 h-Viscozyme L: methanolic extracts obtained by an incubation of 0 h, 6 h, 12 h and 24 h with Viscozyme L, respectively; 0 h-Rapidase, 6 h- Rapidase, 12 h- Rapidase, 24 h- Rapidase: methanolic extracts obtained by an incubation of 0 h, 6 h, 12 h and 24 h with Rapidase, respectively.

2.4. Discussion

The enzyme-assisted extraction yield of phenolic compounds depends on multiple variables including type of enzyme and concentration, incubation temperature, pH and incubation time. In this experiment, the optimization of Viscozyme L and Rapidase-assisted enzymatic pretreatment was studied on cauliflower outer leaves, with the focus on the identification of the released phenolic compounds. The significantly improved release of total phenolic compounds in both AE and ME for Viscozyme L (0.2 %) and Rapidase (0.5 %) treatment compared to control, clearly indicated that the hydrolysis of cell-wall components of cabbage improves the extraction yields of phenolic compounds. This observation could be explained by an increased degradation of the cell-wall structure as a result of the hydrolysis of cell-wall components, especially glycosidic bonds/linkages between phenolic compounds and cell-wall polysaccharides (Bagger-Jørgensen et al., 2004; Yadav et al., 2013). In addition, it is known that the permeability and porosity of plant cells increase by the breakdown of cell-wall polymers, improving the solubility of cell internal components, and in turn this results in an increase in the concentration of phenolic compounds present in the extracts (Cerda et al., 2013; Chen et al., 2011). The treatments with higher enzyme doses did not increase the release of total phenolic compounds ($p > 0.05$), which could be due to competitive adsorption to the cell-wall polysaccharide when a higher enzyme concentration is applied. This leads to steric hindrance of binding positions of enzymes to the substrate which negatively influences the breakdown of cell-wall components (Kapasakalidis et al., 2009; Norsker et al., 1999). The lack of improvement at higher enzyme concentrations is in

accordance with previous studies e.g. on black currant pomace (Kapasakalidis et al., 2009), or on unripened apples (Zheng et al., 2009).

The increased phenolic concentration in both AE and ME at all temperature incubations in comparison with the control sample demonstrates the importance of enzymatic catalysis to release phenolic compounds from cauliflower leaves. The significant enhancement of TPC in ME by treatments with 0.2 % Viscozyme L and 0.5 % Rapidase at higher temperature (35, 40 and 50 °C) could be caused by an increase of the hydrolysis activity and overall porosity of cell-wall structure, improving hydrolysis of the linkages between bound-phenolics and cell-wall compositions (Landbo et al., 2004; Yadav et al., 2013). However, at 50 °C for Viscozyme L, there was a significant decrease in TPC in ME, compared to 40 °C. This finding might be explained by a decreased activity of one of the enzymes in Viscozyme L, which is lower at 50 °C. Also, a possible loss of phenolic compounds has been observed due to thermal degradation (Bagger-Jørgensen et al., 2004), which is mainly attributed to an increased oxidation (Patras et al., 2010). This observation is consistent with results of Kapasakalidis et al. (2009), who reported a reduction of phenolic concentration by cellulase-assisted extraction at 50 °C compared to 40 °C. As the different pH values tested did not affect the level of TPC in both AE and ME, it can be concluded that the commercial enzyme mixture has a broad pH activity range.

The kinetic study of phenolic release at optimal conditions (Figure 2) indicates that the TPC in ME greatly increased during the first 12 h of enzymatic treatment with the maximum level reached at 12 h of incubation. This finding can be attributed to the catalytic action of carbohydrate-cleaving enzymes (Jamal et al., 2011) as pre-treatment process. It can also be

concluded that an incubation of 12 h is sufficient for Viscozyme L and Rapidase to hydrolyze the substrate. The stable level of total phenolic content in ME between 12 h and 30 h incubation can be attributed to deactivation, even inactivation of cellulase, hemicellulase and pectinase by bioactive inhibitors, such as phenolic compounds and lignin, released from the substrate. Indeed, Berlin et al. (2006) and Tejirian et al. (2011)reported that phenolics, flavonoids and polymeric lignin could strongly adsorb onto cellulase and pectinase, limiting the enzyme activity and/or precipitation of enzyme. Our results confirm the possibility of enzymes as an effective method to improve the yield of phenolic compounds released from waste material. However it is also important to know if the enzyme treatment resulted in the release of specific phenolic compounds or in a change in their structure, compared to the non-enzymatic treated outer leaves. As far as we know, the effect of enzymatic treatment on the release of individual phenolic compounds from vegetable waste material has not been reported before.

The LC-MS analysis revealed that the main components found in ME and AE from controls (no-enzyme treatment) were kaempferol glycosides. This indicates that most phenolic compounds, naturally produced by cauliflower plants, are present in conjugated form, as was also reported on cauliflower by-products by Llorach et al. (2003) and Gonzales et al. (2014). Similar results were also observed after the enzymatic treatment with Viscozyme L and Rapidase, meaning that no aglycones were formed. This is probably due to the insufficiency or absence of β-glycosidase, which is known for its capacity of deglycosylation by hydrolyzing the β-1,4 glycosidic bonds in aryl and alkyl β-D-glucosides as well as glycosides containing disaccharides and oligosaccharides (Turner et al., 2006; Zheng et al., 2000). The presence of

the β-glycosidase was not specified by the supplier for the enzyme mixtures used. Viscozyme L was more efficient in hydrolyzing the substrate to obtain higher amounts of kaempferol-3-feruloyldiglucoside (compound 12), kaempferol-3-glucoside (compound 14). The large increase in kaempferol-3-feruloyldiglucoside (compound 12) can be the result of the deglycosylation of two glucose molecules from kaempferol-3-feruloyltriglucoside-7-glucoside (compound 8). The large increase of kaempferol-3-glucoside (compound 14) by both enzymatic treatments is probably the result of removing one glucose molecule from kaempferol-3-diglucoside (compound 11). These cleavages may be caused by the presence of cellobiase activity or other unknown enzymatic actions in the commercial enzymes Viscozyme L and Rapidase. However, a full characterization of these commercial enzyme mixtures and their activity is not available. This behavior has also been confirmed by the results on time of incubation in the profile of phenolic release. The longer the samples were treated, the lower the concentration of compounds 2, 8, and the higher the amount of compounds 11, 12 and 14. However, no difference in the individual components of AE was observed as a function of the treatment, although a substantial increase in TPC was measured by Folin-Ciocalteu assay for the enzymatic treatments compared to the control. This apparent difference can be attributed to the reaction of Folin-Ciocalteau reagent with other interfering components such as protein, enzymes, sugars, as well as ascorbic acid present in AE (Kapasakalidis et al., 2009; Roura et al., 2006; Singleton et al., 1999).

2.5. Conclusions

Although no phenolic aglycones were found in comparison with extract from native cauliflower by-product, the study showed that the use of Viscozyme L or Rapidase enzyme

mixture results in a higher yield of kaempferol-glucosides extracted from the cauliflower outer leaves. Further treatment with β-glycosidase, either or not with Viscozyme L or Rapidase, could result in the release of kaempferol as aglycone form, a flavonoid which is characterized by several bio-activities (Ferreres et al., 2009; Spencer et al., 2004).

The impact of lactic acid fermentation on the release of phenolic compounds from cauliflower outer leaves

Chapter 3

The impact of lactic acid fermentation on the release of phenolic compounds from cauliflower outer leaves

ABSTRACT

A study was carried out to understand the release and bioconversion of phenolic compounds in cauliflower outer leaves as well as of their extract by fermentation with various lactic acid bacteria. Total phenolic content in unfermented sample was approximately 47-54 mg GAE/100 g fresh weight, however this level remained stable during lactic acid fermentation of cauliflower outer leaves. Similarly, no considerable change in phenolic profile of twelve different identified kaempferol glycosides was observed during fermentation of a phenolic extract obtained from cauliflower outer leaves with *L. plantarum*. These findings were the result of the inaccessibility of glycosylated phenolic compounds to interact with β-glucosidases, which were only found intracellularly in the lactic acid bacterial cells. Due to the highly complex structure and lipophilicity of the phenolic compounds present in the cauliflower outer leaves, they were not able to penetrate into the bacterial cells.

Keywords: lactic acid fermentation, localization of β-glucosidase, phenolic compounds, cauliflower wastes

3.1. Introduction

Cauliflower and broccoli are members of the *Brassica* family with a global production in 2013 of about 22 million tons, of which approximately 80% of these products are produced in Asia (Food and Agriculture Organization, FAO, 2015). During the postharvest handling, a large amount of the crops remain, i.e., leaves and stems, florets and stalks, accounting for about 45-50% of the total production. Traditionally, most of these residues were left on the fields, resulting in environmental problems. However, they have recently been valorized as valuable materials such as animal feeds (Martínez-Sánchez et al., 2006), as a source of glucosinolates (Ares et al., 2014; Cabello-Hurtado et al., 2012; Dominguez-Perles et al., 2011), as a source of fiber (Campas-Baypoli et al., 2009; Nilnakara et al., 2009; Tanongkankit et al., 2012) or amino acids (Arnáiz et al., 2012).

Most of the studies on Brassica vegetables were focused on the edible parts, while there is limited information available on their corresponding byproducts. In a recent study, it has been reported that phenolic compounds, i.e. chlorogenic acid and sinapic acid derivatives, and flavonoids were found in broccoli wastes (Domínguez-Perles et al., 2010). Llorach et al. (2003) have also shown that cauliflower outer leaves are a good source of phenolic compounds, in which most of the compounds were identified as flavonoid derivatives. Similarly, recent work from our group also found fifteen phenolic compounds, occurring mainly as different combinations of kaempferol with glycoside moieties and hydroxycinnamic acids (Martins et al., 2011).

Fermentation by lactic acid bacteria has been traditionally used for maintaining and improving nutritional, sensory and shelf-life properties of vegetables. However, this process has been recently applied to the extraction and bioconversion of phenolic compounds from various plant-based foods such as cabbage (Sun et al., 2009), Chinese *Brassica* vegetable (Harbaum et al., 2008), onion (Bisakowski et al., 2007), apple juice (Ankolekar et al., 2012), soy beans (Cho et al., 2009; Chung et al., 2011), cowpeas (Dueñas et al., 2005) and cereals (Hole et al., 2012). The mechanism that seems to be responsible for the change in the phenolic concentration as well as in the distribution of phenolic compounds is the activity of lactic acid bacteria. This activity is mediated by enzymes catalyzing different chemical reactions during fermentation resulting in the release and/or metabolism of phenolic compounds. In fact, it has been shown that β-glucosidase can be produced by different strains/species such as *Lactobacillus delbrueckii*, *Bifidobacterium breve* and *Bifidobacterium thermophilum* (Pyo et al., 2005), *Lactobacillus plantarum* (Hur et al., 2014; Pyo et al., 2005), *Lactobacillus rhamnosus* (Hur et al., 2014). Moreover, other enzymes, i.e., amylase, esterase, decarboxylase are also possessed by several LAB strains (Hur et al., 2014). Hence, the fermentation with lactic acid bacteria could be considered as not only a biological pretreatment, but also as a biotransformation prior to extraction of phenolic compounds.

As shown in our previous study (Huynh et al., 2014), the incubation with carbohydrate-cleaving enzymes resulted in a substantial enhancement of extraction of flavonoids from cauliflower leaves. Nevertheless, the price of such commercial enzymes is rather high, especially towards the valorization of such by-products. The aim of this study was thus to evaluate the impact of fermentation with several lactic acid bacteria on the extraction of

phenolic compounds from cauliflower outer leaves as an alternative for the use of commercial enzyme preparations.

3.2. Materials and Methods

3.2.1. Materials

Plant material

The outer leaves of cauliflower were collected from a local farm in West-Flanders, Belgium. They were transported to the laboratory and stored frozen (at-20°C) until further use.

Chemicals and reagents

Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), HPLC-grade methanol, formic acid were purchased from VWR International (Leuven, Belgium), and gallic acid, hesperetin, rutin from Sigma-Aldrich (Bornem, Belgium), while MRS broth and bacteriological agar were obtained from Oxoid (Erembodegem, Belgium).

Microorganisms and inoculum preparation

Lactobacillus plantarum (LMG6907), *Lactobacillus mali* (LMG6899), *Pediococcus pentosaceus* (LMG10740) and *Pediococcus damnosus* (LMG114884) were obtained from the BCCM/LMG bacterial collection of Ghent University. To prepare the inoculum, stock cultures stored in glycerol/MRS (80/20, v/v) were propagated twice in MRS broth at 30 °C for 24 h. The starter inoculum was prepared by transferring 1 mL of mother culture into MRS and incubated at 30 °C until the viable cell was 10^8 CFU/mL.

Fermentation of cauliflower outer leaves

The frozen leaves were cut into 2x5 cm pieces and homogenized in a stirrer for 3 minutes. NaCl was added to reach a final concentration of 2% (w/v), followed by 1 minute mixing. The fermentation was performed in a sterilized 500 mL-glass bottle (Duran, Schott). The amount of mixed material (120 g) and 20 mL of sterilized water were added to each bottle. After adding the inoculum (1%), the prepared bottles were incubated at 30 °C for 6 days. Also one treatment consisted of non-inoculated samples, and is further denoted as control. At different time points, 2 g of samples were taken to measure pH and viable cell count directly, also 5 g of samples were taken for the extraction of phenolic compounds by the method described by Huynh et al. (2014).

Fermentation of an extract obtained from cauliflower outer leaves

The methanol extract from cauliflower outer leaves was prepared by the method (Huynh et al., 2014). Methanol was then removed by using a rotary vacuum evaporator to obtain the concentrated extract which was used in this experiment.

A 0.1 mL of the prepared inoculum for *L. plantarum* was added to an Erlenmeyer flask containing 10ml of MRS broth. The amount of phenolic extract was then added to obtain a concentration of 0.5 mg GAE/mL. Flasks were then incubated for 1, 3, and 6 days at 30°C with shaking. At the different time points, a flask was taken for the analysis of pH, viable cell count, and phenolic profile. All incubations were done in triplicate.

3.2.2. Analytical methods

pH and viable cell count

2 g of samples were transferred to a stomacher bag in a sterilized way, followed by a 10 times dilution with physiological water (0.85%). This slurry was then homogenized for 1 minute. The liquid obtained was used for the measurement for pH and cell counts.

The pH of fermented cabbage was measured using a pH meter and viable cell counts (CFU/mL) were done by standard plate method on MRS agar. The plates were incubated at 30 °C for 48h for viable cell count.

Total phenolic content

Determination of the total phenolics content (TPC) was done by the modified Folin-Ciocalteu procedure (Singleton et al., 1999). Therefore, 250 µL of methanolic extracts were diluted with 250 µL of destilled water and mixed with 500 µL of Folin-Ciocalteu reagent. The samples were incubated for 6 minutes at room temperature before sodium carbonate 20 % (w/v) was added. The mixture was incubated for 2h at room temperature. Then the absorbance was measured at 765 nm using an UV-vis spectrophotometer. Total phenol content was determined against a calibration standard curve of gallic acid (10-100 mg/L) and the results were expressed in mg GAE/100g FW.

The profile of phenolic compounds

Sample purification using solid phase extraction (SPE), quantification and identification of phenolic compounds by LC-MS/MS were based on the methods published in our previous works (Gonzales et al., 2014; Huynh et al., 2014).

Preparation of crude enzyme solution and assay of β-glucosidase activity

The supernatants obtained using centrifugation from 200 mL of the culture of *L. plantarum* grown in MRS broth at 30 °C for 12 h and 24 h were measured for their extracellular activity of β-glucosidase, while the pellets (the cells) were collected for a preparation of the crude enzyme extract following the method of Mesas et al. (2012). The cells were washed twice with physiological water (0.85% NaCl) and then resuspended in 10 mL of disruption buffer (sodium acetate buffer, pH 4.6). The cell slurry was maintained in an ice water batch prior to sonication at 60 W for 30 s. The cell debris was removed by centrifugation, while the supernatant was tested for its activity of intracellular β-glucosidase.

The enzyme activity was measured by a modified procedure based on the methods of Gunata et al. (1990) and Hang and Woodams (1994). A standard reaction mixture contained 0.1 ml of 9 mM p-nitrophenol β-D-glucopyranoside, 0.8 ml of 200 mM sodium acetate buffer (pH 4.6), and 0.1 ml of enzyme solution. After 30 min incubation at 50±1°C, the reaction was stopped by addition of 1 ml of 0.1 M sodium carbonate and the released p-nitrophenol was measured at 400 nm. The standard curve was established using pure p-nitrophenol (Fisher Scientific Co., Fair Lawn, NJ). One unit of enzyme was defined as the amount of enzyme that releases 1mmol p-nitrophenol per minute at pH 4.6 at 50±1°C under the assay conditions.

3.2.3. Statistical analysis

The data were described as a mean of three independent experiments. One-way and two-way ANOVA were used to test for the significant differences between two factors (time and microorganism) using Tukey post hoc test at a significance of $p < 0.05$.

3.3. Results

Changes in pH, cell growth and total phenolic content during fermentation of cauliflower outer leaves

The pH values were measured during the fermentation as presented in Figure 3.1. The initial pH value (pH = 6.1 ± 0.2) decreased during the first two days of incubation and reached the lowest value (pH = 4.3 ± 0.1) of the sample fermented with *L. mali* on the second day (p-value < 0.05). Further incubation resulted in an increase in pH values for all treatments with the highest value of 7.9 ± 0.1 and 8.0 ± 0.1 for the non-inoculated (control) and fermented leaves with *P. damnosus* at the end of fermentation (day 6), respectively.

The growth of lactic acid bacteria was also determined during the fermentation period through counting the viable cells (Figure 3.2). In general, the increase in bacterial population was observed for all strains along with time of incubation. Similarly, a considerable population of lactic acid bacteria were also found in the control treatment. The maximum viable cell counts were recorded for the control treatment on day 2 with 9.2 Log CFU/mL, and for fermented sample with *L. mali* on day 1 with 9.0 Log CFU/mL.

The total phenolic content found in unfermented leaves (control treatment, day 0) varied between 48-54 mg GAE/100 g fresh weight, and was not influenced by the day of incubation ($p > 0.05$) (Figure 3.3). Nevertheless, none of the fermentations with the tested strains resulted in the enhancement of phenolic compounds extracted ($p > 0.05$).

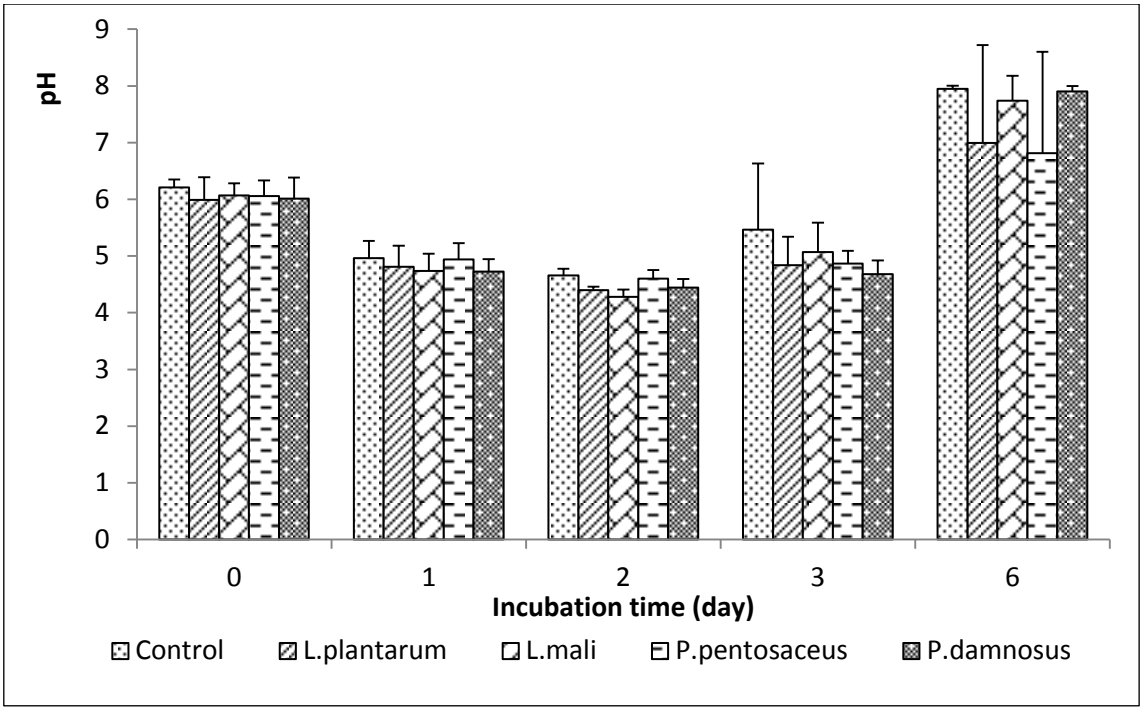


Figure 3.1. Change in pH during fermentation of cauliflower outer leaves using lactic acid bacteria at 30 °C (n = 3)

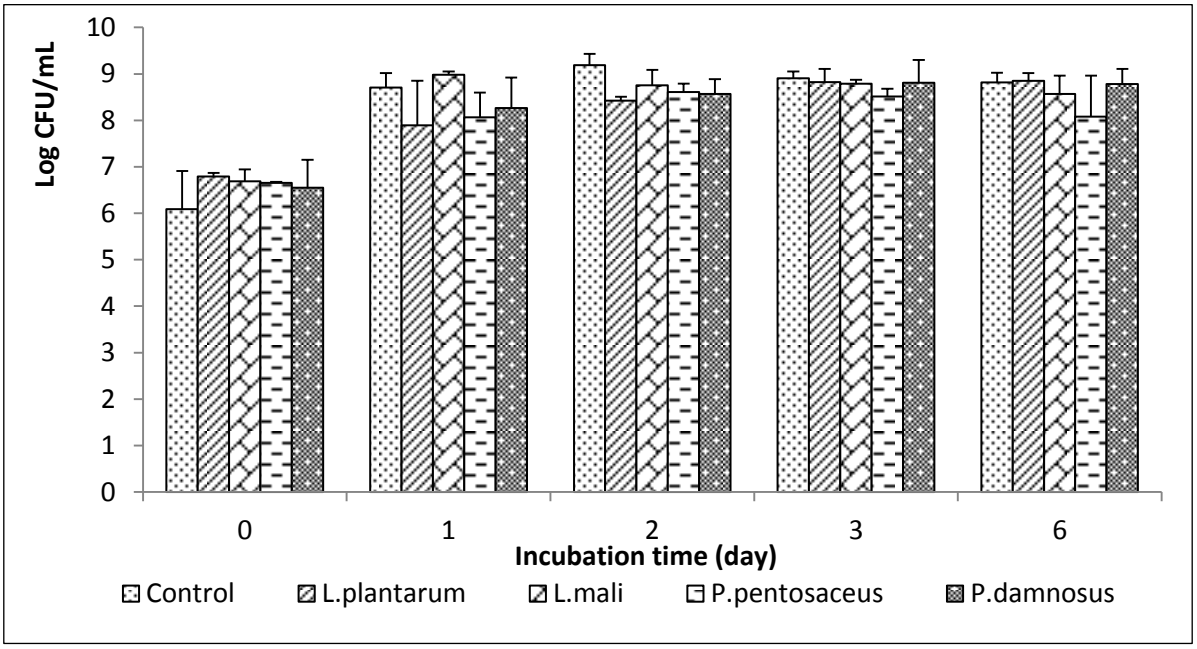


Figure 3.2. Microbial growth during fermentation of cauliflower outer leaves with lactic acid bacteria at 30 °C (n = 3)

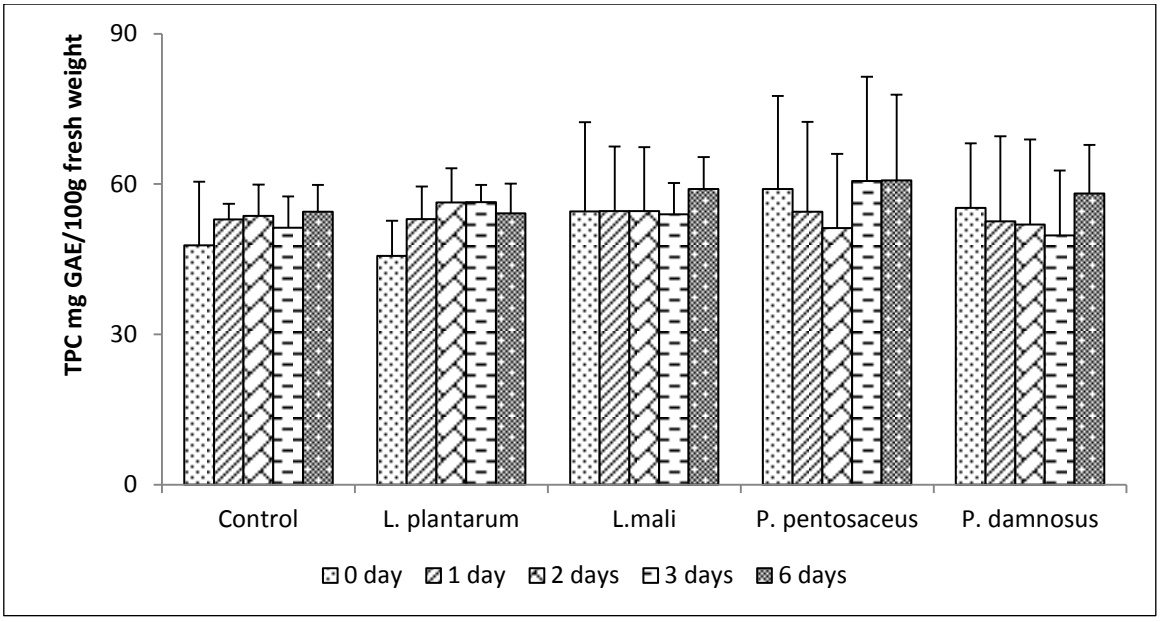


Figure 3.3. Total phenolic content (TPC) in cauliflower outer leaves fermented with lactic acid bacteria at 30 °C (n = 3)

Fermentation profile (cell growth, pH) and phenolic profile during fermentation of extract from cauliflower outer leaves

In this experiment, an extract from cauliflower outer leaves was used as the substrate for fermentation, using *L. plantarum* as starter culture. Viable cell count (log CFU/mL), pH and the profile of phenolic compounds were determined during the fermentation period. As shown in Figure 3.4, there was an increase of approximately 2.5 log CFU/mL of *L. plantarum* population after 1 day of fermentation. Further incubation did not result in a further increase of the CFU/ml. Concerning the pH measurements, after 1 day of fermentation, the pH decreased from an initial value of 6.1 to 3.7, and remained then constant during further incubation.

To gain more insight into the changes in the profile of phenolic compounds in cauliflower extract and the possible bioconversion during fermentation, the individual phenolic compounds in the samples were identified and quantified by using LC-MS/MS. As presented in Figure 3.5, twelve different flavonoids were identified in both unfermented (control, day 0) and fermented samples, and listed in Table 3. 1. Nevertheless, no changes in their profile, nor in the peak area, as an indication of content, were observed during 3 days of fermentation.

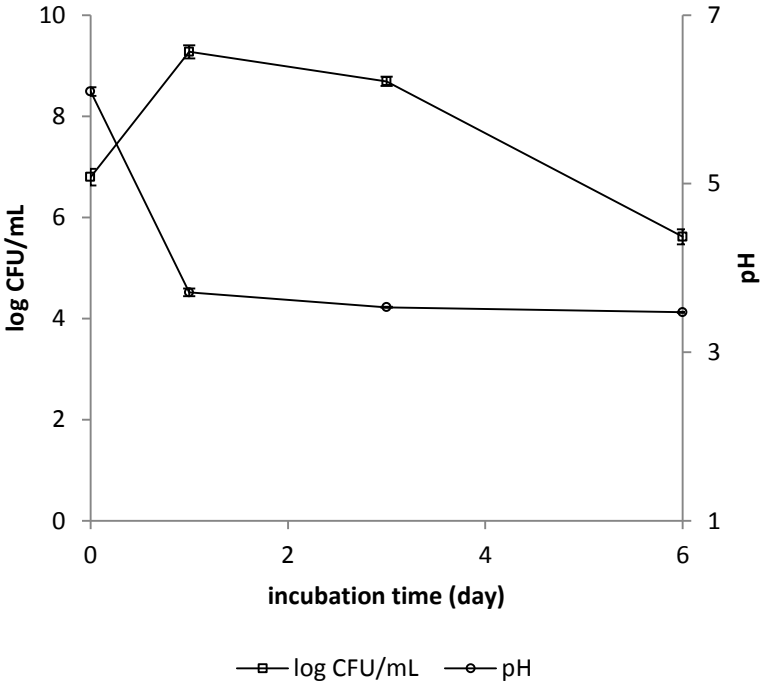


Figure 3.4. Changes in pH and bacterial counts during fermentation of a phenolic extract from cauliflower outer leaves at 30 °C by *L. plantarum* (n = 3)

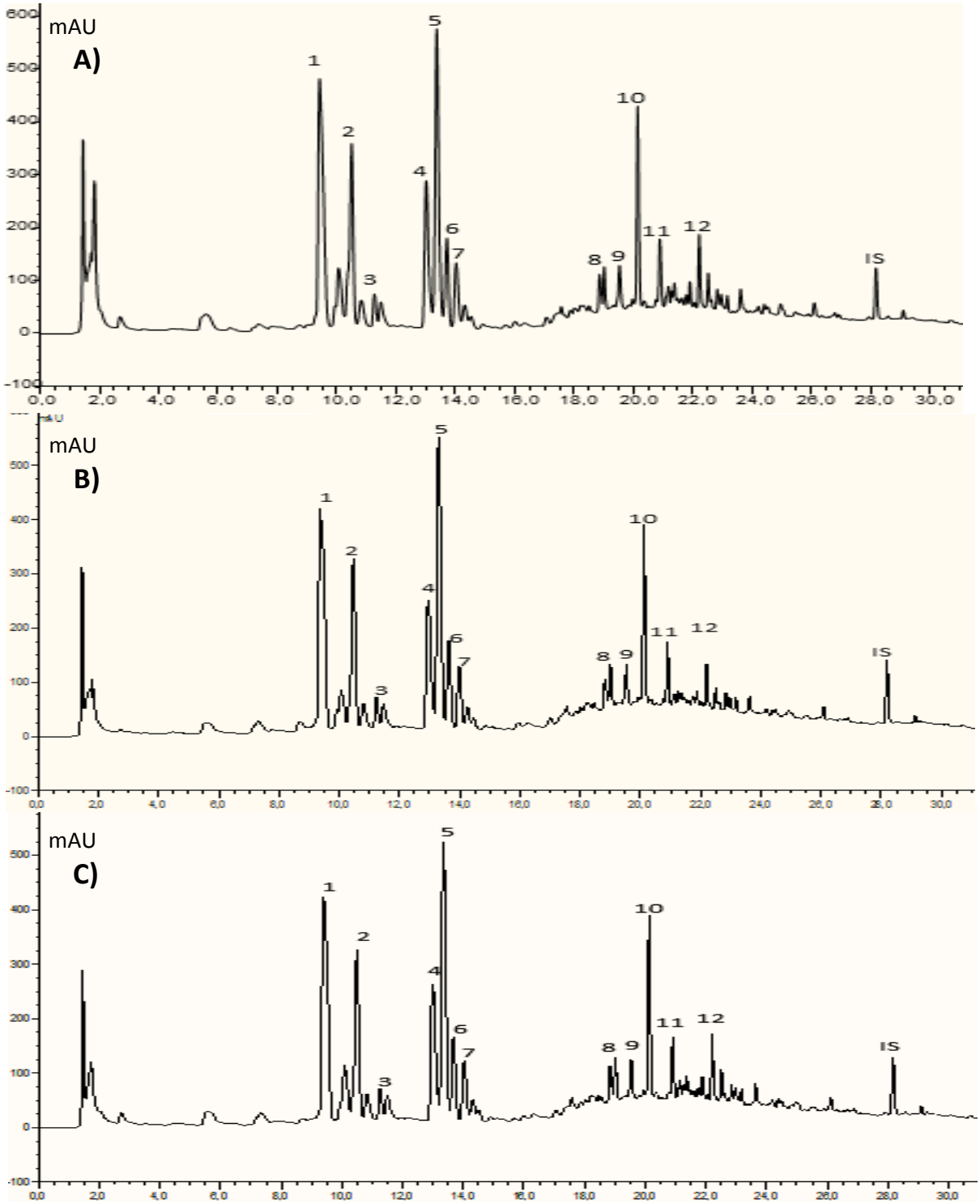


Figure 3.5. The profile of phenolic compounds during fermentation of an extract of cauliflower outer leaves at 30 °C by *L. plantarum*. A, unfermented sample; B, fermented sample for 1 day; C, fermented sample for 3 days. Peaks no. see Table 3. 1

Table 3. 1. Identification of flavonoid glycosides from cauliflower outer leaves

Peaks	Rt (min)	m/z value		Identity
		Exact mass	Fragments	
1	9.82	771.2195	771, 609, 447, 284	Kaempferol-3-O-diglucoside-7-O-glucoside
2	10.63	933.2191	933, 609, 285	Kaempferol-3-O-diglucoside-7-O-diglucoside
3	10.99	1095.343	1095, 771, 609, 284	Kaempferol-3-O-triglucoside-7-O-diglucoside
4	13.09	977.3577	977, 815, 609, 284, 446	Kaempferol-3-O-sinapoyldiglucoside-7-O-glucoside
5	13.39	947.2400	947, 785, 609, 284	Kaempferol-3-O-feruloyldiglucoside-7-O-glucoside
6	13.69	1109.302	1109, 755, 947, 284	Kaempferol-3-O-coumaroyldiglucoside-7-O-hydroxyferuloylglucoside
7	13.99	917.2275	917, 755, 284, 609	Kaempferol-3-O-coumaroyldiglucoside-7-O-glucoside
8	18.82	625.1302	625, 301	Quercetin-7-O-diglucoside
9	19.44	771.1885	771.284	Kaempferol-3-O-triglucoside
10	19.95	609.1251	609.284	Kaempferol-3-O-diglucoside
11	20.38	947.2400	947, 609, 771, 785, 284	Kaempferol-3-O-diglucoside-7-O-feruloylglucoside
12	20.65	785.1868	785,609,284	Kaempferol-3-O-feruloyldiglucoside

Screening of extra- and intracellular β-glucosidase activity of *Lactobacillus plantarum*

Among the various strains of LAB used in our experiment, *L. plantarum* was selected to examine its capacity of β-glucosidase production. To understand the localization of β-glucosidase, the cell-free supernatant from the culture medium was analysed for extracellular β-glucosidase activity, while the crude solution extracted from the cells of *L. plantarum* was determined for intracellular activity. As seen in Figure 3.6, an increased activity of β-glucosidase compared to 0h was detected in the crude extract from the cell of *L. plantarum*, which was 1.37 ± 0.17 U/mL and 2.34 ± 0.24 U/mL after 12 and 24 hours

respectively. However no β-glucosidase activity was found in the supernatants of the growth medium.

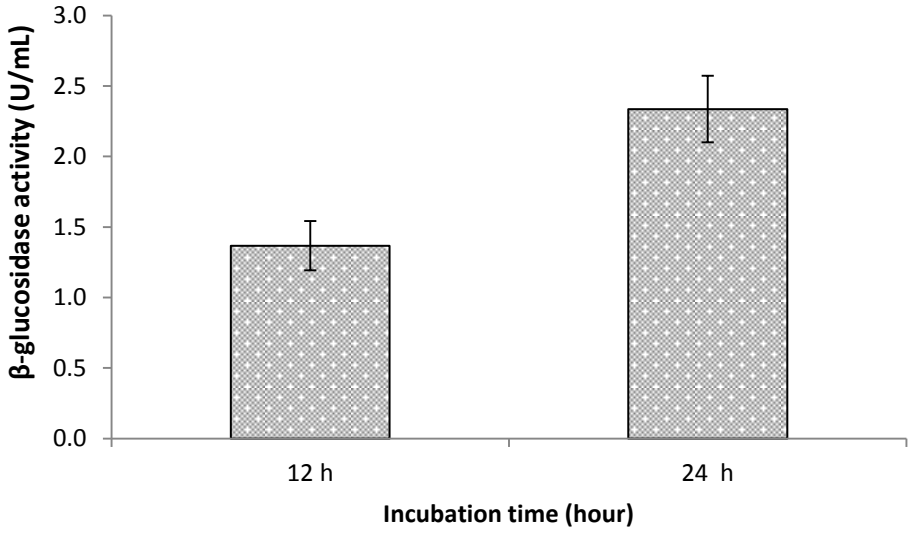


Figure 3.6. Increase in the intracellular β-glucosidase activity of *L. plantarum* compared to 0h incubation (n= 3)

3.4. Discussion

The higher acidity observed for all strains during the first two days of fermentation could be attributed to acidic compounds produced during bacterial activity. This is in agreement with the change in pH seen in many previous reports. Bratu et al. (2012) found a decrease in the pH from 6.0 to 4.0 after 24h fermentation of cucumber or cabbage juices with *L. acidophilus*. A similar result was obtained by Pistarino et al. (2013) who found a decrease in pH of 29.25% in the olive samples inoculated with *L. plantarum* after 24 hours of fermentation. The growth of molds was visually observed during the last days of fermentation, which could explain the observed increase in pH values.

LAB fermentation has been reported as a potential approach to release phenolic compounds from food-based plants in recent studies (Dueñas et al., 2005; Hole et al., 2012; Kusznierewicz et al., 2008; Pyo et al., 2005). However in this study, no variation in total phenolic content (TPC) was observed during 6 days of fermentation. Therefore, it was hypothesized that this finding could be due to the fact that no degradation of cauliflower leaves during fermentation took place. In other words, the cell-wall degrading enzymes e.g. xylanase, hemicellulase, cellulase, esterase were not produced by LAB growth on cauliflower leaves. Indeed, no cell wall degrading enzymes, e.g. cellulase have been reported in lactic acid bacteria (Moraïs et al., 2013). The results obtained in this study are not in accordance with the recent studies indicating an improved release of phenolic compounds by fermentation combined with a combination of fermentation with brining treatment (Kusznierewicz et al., 2008; Sun et al., 2009), or with heating treatment (Jaiswal et al., 2012; Kusznierewicz et al., 2008); or dealt with other phenolic compounds i.e. phenolic acids extracted from different matrices (Hole et al., 2012).

For this reason, another experiment was performed using an extract from cauliflower outer leaves, which was fermented with *L. plantarum*. As an extract was made before, the interaction of the phenolic compounds and the cell wall matrix was not present anymore. In that way, the lack of cell wall degrading enzymes of LAB could not be the critical point anymore in this experiment. To follow up the conversion of phenolic compounds, the phenolic profile was determined using LC-MS/MS during time of incubation. Twelve flavonoid compounds were found in the unfermented extract, all present as glycosidic forms of kaempferol. Nevertheless, there was almost no change in their concentration during

fermentation. Also no conversion to lower glycosidic compounds was observed as well. This observation might be explained by the inaccessibility of β-glucosidase to the substrate (glycosylated phenolic compounds). On other hand, these highly glycosylated compounds could not penetrate into the bacterial cells. Therefore, an experiment was conducted aiming to know if the β-glucosidase was produced and to achieve a better understanding of the localization of β-glucosidase in *L. plantarum*. No β-glycosidase activity was found in fermented medium, meaning that this strain was unable to produce β-glucosidase extracellularly. The enzyme activity was detected only in the crude solution extracted from the cells indicating that only an intracellular β-glycosidase was synthesized or this enzyme may be linked to cell walls. This result was in accordance with findings of previous authors who have also found intracellular β-glucosidase produced by *L. plantarum* (Giraud et al., 1993; Lei et al., 1999), or *Oenococcus oeni* (Mesas et al., 2012) or cell-wall associated enzymes in *L. rhamnosus* (Pham et al., 2000) and in *L. brevis* (De Cort et al., 1994). As only intracellular β-glucosidase was present, and as no conversion of the highly glycosylated molecules into lower glycosylated ones was observed, this could be the consequence of the inaccessibility of the enzyme, i.e. intracellular β-glucosidase to its substrates i.e. kaempferol glycosides present in the medium.

However, recent studies have proven that the bioconversion of glycosidic isoflavones i.e. genistin and daidzin into their aglycones i.e. genistein and daidzein was performed through the fermentation with lactic acid bacteria (Choi et al., 2002; Pyo et al., 2005). This finding reveal that these LAB allowed genistin and daidzin to diffuse into their cells in which their aglycones were produced and turned back into medium. It is recognized that there is a

variation in penetration behavior between these isoflavones and kaempferol glycosides. In fact, glucose moieties attached to kaempferol found in cauliflower leaves resulted in the formation of larger compounds with a higher polarity and higher molecular weight, while the plasma membrane is considered as the main diffusion barrier for penetration of hydrophilic and complex compounds from the medium and the escape of many hydrophilic metabolites from the cells (Kepes, 1985; Russ et al., 2000). On the other hand, *L. plantarum* is known as a gram-positive bacterium that has no outer membrane which plays an important role for effective transport of hydrophilic compounds via protein transporter i.e. porins (Benz et al., 1988). For example, the water solubility of both daidzin and genistin is determined to be 24.71 µM (Li et al., 2004), 23 µM (Li et al., 2005), respectively, meaning approximately 0.010 mg/mL for both, whereas that of kaempferol-3-glucoside is calculated to be 262-fold higher (2.61 mg/mL, ALOGPS source). In addition, Li et al. (2005) have proven that the degree of glycoslylation of phenolic compounds is proportional to their water solubility, e.g. the solubility of daidzein 7-O-triglucoside is 7.5×10^4 times that of daidzin (Li et al., 2004), glucosyl-α(1−4)-genistin is 3.75×10^3 times that of genistin (Li et al., 2005).

Therefore, it could be hypothesized that no bioconversion observed during fermentation of cauliflower outer leaves as well as of the extract. This was most likely the result of the inaccessibility of high glycosylated kaempferol in the extract to the intracellular β-glycosidase. This was due to the impenetrability of high polar and complex compounds into LAB cells.

3.5. Conclusions

Although LAB have shown their high rate of growth on cauliflower outer leaves as well as in phenolic-rich media i.e. MRS broth containing 0.5 mg GAE/mL of phenolic extract, the fermentation with LAB seem to be an ineffective process for an increase in phenolic compounds released from cauliflower outer leaves. From the results obtained it can be concluded that no bioconversion of kaempferol glycosides occurred during fermentation. Together with the fact that only intracellular β-glucosidase activity was found, it is hypothesized that phenolic compounds with high degree of glycosylation present in cauliflower outer leaves are unable to diffuse into *L. plantarum* cells in which their metabolism could take place. Further research on the effect of molecular weight and polarity of phenolic compounds on their penetration into LAB cells should be required. The characterization, localization and purification of β-glucosidase from LAB, as well as the incubation of this enzyme with pure highly glycosylated phenolic compounds requires further investigation.

Extraction and bioconversion of kaempferol metabolites from cauliflower outer leaves through fungal fermentation

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Chapter 4

Extraction and bioconversion of kaempferol metabolites from cauliflower outer leaves through fungal fermentation

ABSTRACT

Cauliflower outer leaves contain bioactive compounds, therefore fermentation could be a strategy to release phenolic compounds and their metabolites and thus increase their valorization potential. This study aimed to evaluate the release and metabolism of phenolic compounds by solid-state fermentation of cauliflower outer leaves with six different filamentous fungi. The fermentation with *Aspergillus sojae* was found to extract the highest level of total phenolic compounds 321mg rutin equivalents (RE)/100g fresh weight (FW)) after 1 day, which was 3 times higher compared to the unfermented sample (113mg RE/100g FW). The most dominant kaempferol metabolites were kaempferol-3-O-diglucoside in all fermented samples (38–126mg RE/100g FW) and kaempferol-3-O-diglucoside-7-O-glucoside in the unfermented sample (34.8mg RE/100g FW). Furthermore, in all fungal treated samples, the phenolic profile shifted to a profile with less or no carbohydrate moieties at the 3- or 7-carbon position. These results indicate the potential of solid-state fermentation to obtain different phenolic-rich extracts, with a unique profile in phenolic compounds, depending on the fungal strain used.

4.1. Introduction

The non-edible parts of cauliflower (*Brassica oleracea* L. var. *botrytis*), consisting of outer leaves, stems and pods, are important by-products from the cauliflower harvest. These residues still contain high amounts of bioactives e.g. phenolic compounds, vitamins, that are known for their bioactivities, such as the potential prevention of health risks as cardiovascular diseases, obesity, diabetes and cancer (Nile et al., 2014; Ranilla et al., 2010; Rodriguez-Mateos et al., 2013). Therefore, the recovery of bioactive compounds from the cauliflower waste streams could contribute to a high-value valorization process, instead of its current use in fiber production (Nilnakara et al., 2009), animal feed (Iñiguez-Covarrubias et al., 2001) or left on the fields.

The majority of phenolic compounds produced by plants are present in a bound form to cell walls in which they are conjugated with polysaccharides, organic acids and proteins (Ajila et al., 2012; Cerda et al., 2013; Pinelo et al., 2006; Yadav et al., 2013). Thus, the application of a hydrolysis process prior to conventional solvent extraction could be promising to maximize the extraction yield. Often chemical pretreatments such as acidic or alkaline hydrolysis, are used. However, these techniques cause several drawbacks, e.g. thermal degradation of phenolic compounds and safety hazards of final products (Puri et al., 2012; Robbins, 2003). Another technique to depolymerize cell-wall polysaccharides is based on the use of cell-wall degrading enzymes to break down the cell wall matrix, resulting in the release of bound phenolic compounds (Alrahmany et al., 2012; Cerda et al., 2013; Gómez-García et al., 2012; Puri et al., 2012; Yadav et al., 2013; Yang et al., 2009). However, the main limitation for the application of enzymes has been their high cost (Laroze et al., 2010).

Currently, microbial fermentation has been shown to be an alternative process to improve the release, stability, as well as bioavailability of phenolic compounds. The mechanism is based on the degradation of the cell-wall matrix and the bioconversion of released compounds by the system of carbohydrate-cleaving enzymes produced by fungi or bacteria during fermentation (Ajila et al., 2012; Bhanja et al., 2009; Martins et al., 2011). Moreover, through fermentation several filamentous fungi have been found to be capable of producing these carbohydrate-cleaving enzymes such as β-glucosidase (Ajila et al., 2012) which can be effective in catalyzing the hydrolysis of glycosidic linkages of aryl- or alkyl-β-glucoside and cellobiose (Bhatia et al., 2002). As a result of this, phenolic glycosides can be converted to aglycones having a higher antioxidant activity (Fukumoto et al., 2000) as well as bioavailability (Silberberg et al., 2006). Besides β-glucosidases, fungi are well-known for their production in cell-wall degrading enzymes, such as cellulases, hemicellulases and lignin-degrading enzymes (Corrêa et al., 2014).

Extracting the bioactive compounds from plant products using a microbial fermentation process has been reported before, e.g. with apple pomace (Ajila et al., 2012), black soybeans (Cheng et al., 2013) and *Larrea tridentate* leaves (Martins et al., 2013). In addition, bioconversion of the phenolic glycosides into their aglycones has also been shown in recent studies, mainly focusing on fermentation with filamentous fungi such as bioconversion of isoflavones from soybean by *Aspergillus oryzae* (da Silva et al., 2011), *Rhizopus* spp. (Cheng et al., 2013) or *Monascus* sp. (Hong et al., 2012), and quercetin and kaempferol from litchi pericarp by *Aspergillus awamori* (Lin et al., 2014).

Despite the fact that cauliflower outer leaves are one of the best substrates for solid-state fermentation as they contain 16% cellulose, 8% hemicellulose and 16% protein (Wadhwa et al., 2006), as well as they are a potential source of flavonoids (Gonzales et al., 2014; Llorach et al., 2003), there is no report dealing with the release and bioconversion of individual phenolic compounds from *Brassica* outer leaves during fungal solid-state fermentation. The objective of this study therefore was to determine the effect of a solid-state fermentation with six different filamentous food-graded fungi (*Aspergillus niger, Aspergillus oryzae, Aspergillus sojae, Rhizopus oryzae, Rhizopus azygosporus* and *Phanerochaete chrysoporium*) on the changes in profile and quantity of individual phenolic compounds released from cauliflower outer leaves. Focus is put on the release and conversions of flavonoid glycosides during the fermentation.

4.2. Materials and Methods

4.2.1. Materials

Plant material

After the cauliflower harvest (July 2012), the outer fresh leaves were collected from a local farm in West-Flanders, Belgium. The leaves were kept frozen at -18 °C until further analysis.

Chemicals and reagents

Hesperetin and rutin were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA), while malt extract, soy peptone, bacteriological agar, casein tryptone were purchased from Oxoid (Erembodegem, Belgium).

Fungal strains and preparation of the inoculum

The cultures of different food-grade fungi, including *A. niger* (MUCL 29039), *A. oryzae* (MUCL 3310), *A. sojae* (CBS 126.59), *Rhizopus oryzae* (CBS 1125.86), *R. azygosporus* (CBS 357.93) and *P. chrysoporium* (MUCL 19343) were used. The cultures were maintained at 4 °C on malt extract agar (MEA) slant. Each fungal strain was first cultured on 2 MEA slants and incubated at 30 °C for 7 days to obtain subcultures. A volume of 2 mL of Tween 80 (0.05%) was added into the subculture slant to make a suspension of the inoculum. Subsequently, the suspension was transferred into a sterile Erlenmeyer flask containing 5 g of sterile glass beads and shaken during 1 min prior to transfer to tubes for centrifuging at minimum 2000g during 20 min to remove mycelia. The supernatant was removed, while the pellet was suspended again with physiological water and centrifuged 2 times (2000g, 20 min). The pellet obtained was used to prepare the spore suspension as initial inoculum (10^6 spores/mL) for the fermentation process.

4.2.2. Experimental procedure

Fermentation process

The frozen outer leaves of cauliflowers were cut into pieces (2×5 cm), followed by 3 minutes mixing using a kitchen homogenizer. The mixture (120 g) was placed in a 500 mL-glass bottle (Duran, Schott). Then, 2 mL of the initial inoculum and 20 mL of sterilized water were pooled prior to adding to the bottle. A sample without inoculum was also included as natural fermentation (NF). The fermentation bottles were incubated on a shaker at 30 °C during 7 days. At different time points (0, 1 and 7 days of incubation) a fermentation bottle was taken and stored at -18°C for further analysis. Three independent replicates were performed.

Extraction of phenolic compounds

The fermented samples were extracted by the method of Olsen et al. (2009). In brief, 15 mL of methanol was added to 5 g of sample, stirred for 40 s using an ultraturrax, followed by incubation in ice water for 15 min. Supernatant was collected after centrifugation (13000g, 10 min and 4 °C). The pellet obtained was then re-extracted by the same procedure, using 10 mL methanol/water (80/20; v/v). Both extracts were pooled and filtered through filter paper (VWR, grade 413) before adding methanol to a final volume of 25 mL.

4.2.3. Analytical methods

Sample purification using solid phase extraction (SPE)

Methanol extract (1 mL) was diluted by water (0.1 % formic acid) up to 20 mL of total volume, followed by adding 100 µL of 100 µg/mL hesperitin as internal standard. The mixture was slowly loaded onto a SPE C18 column (50 mg, 4 mL), preconditioned with MeOH (0.1 % formic acid). The column was then washed with 5 mL of water (0.1 % formic acid), followed by loading 3 mL of MeOH (0.1 % formic acid). The eluent obtained was placed under nitrogen gas for evaporating the solvents. After drying, the residue was re-dissolved in 100 µL MeOH (0.1 % formic acid), followed by adding 900 µL water (0.1 % formic acid). The sample obtained was kept at -18 °C for UPLC-DAD-HDMS-TOF-MS analysis.

Quantification and identification of phenolic compounds by UPLC-DAD-HDMS-TOF-MS

The extracts obtained after SPE purification were analyzed through UPLC-DAD (Liquid Chromatography-Diode Array Detector) with a Waters Acquity UPLC system (Waters Corp., Milford, MA, USA). DAD detection was carried out between 280 nm and 370 nm. A bride ethylene hybrid (BEH) C18 column (150 x 2.1 mm, 1.7 µm particle size) was used for the separation of phenolic compounds. The column temperature was held at 40 °C, whilst the

auto-sampler was kept at 4 °C. The mobile phase consisted of solvent A (0.1 % formic acid in water) and solvent B (0.1 % formic acid in methanol). The elution gradient established was as follows: 0 min, 10 %B; 0-6 min, 20% B linear; 6-12 min, 20% B isocratic; 12-13 min, 30% B linear; 13-23 min, 50% B linear, 23-30 min, 90% B linear; 30-35 min, 90% isocratic; 35-40 min, 10% B linear; and 40-45 min 10% isocratic. The flow-rate was maintained at 250 µL/min.

A Synapt HDMS-TOF mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ionization (ESI) source was connected to an ACQUITY UPLC system to detect phenolic compounds. The setting for MS were as follows: capillary voltage, 2kV; sampling cone voltage, 40V; extraction cone voltage, 4V; source temperature 150 °C; desolvation temperature, 350°C; cone gas flow rate, 50L/h; desolvation gas flow rate, 550 L/h. Collision energies were set at 45 V and 6 V for high and low energy, respectively. The spectra was recorded between 100 and 1500 Da using MassLynx software (Waters, Corp.). The identification of structural phenolic compounds was based on the method described by Gonzales et al. (2014). The identified compounds were quantified based on an external calibration curve of rutin and are expressed as mg rutin equivalents (RE)/100 g fresh weight (FW).

4.2.4. Statistical analysis

The data are given as mean ± standard deviation (SD). One-way and two-way ANOVA were used to determine significant differences and then means were separated with a post-hoc Tukey's test with p-value < 0.05 in SPSS version 22. Principle component analysis (PCA) was applied to interpret differences between treatments and incubations based on the contents of individual phenolic compounds.

4.3. Results

The profile of phenolic compounds extracted from cauliflower outer leaves was quantified by using UPLC-DAD and identified by UPLC-MS/MS. Since there was a significant interaction ($p < 0.05$) between fungal treatments and time of fermentation for more than half of the identified components, the results are described for the two main factors (fungal treatment and time of fermentation) separately.

The retention time, exact mass and fragments of fifteen flavonoid compounds in the methanol extracts identified by our method (Gonzales et al., 2014) are listed in Table 4.1. The major compounds present in both unfermented and fermented cauliflower outer leaves were kaempferol glycosides (compounds 1, 2, 3, 9, 10 and 14) and their combinations with coumaric acid (compounds 6, 7, and 13), ferulic acid (compounds 5, 6, 11, 12 and 15) and sinapic acid (compound 4), whereas only one compound was detected as a derivative of quercetin (compound 8).

A representative chromatogram of the phenolic profile of the control sample, i.e. before fermentation, is given in Figure 4.1 . The major compounds in the control samples are compound 1 (34.8 mg RE/100g FW) and compound 10 (24.8 mg RE/100g FW), accounting for almost 50% of the total amount of phenolic compounds identified (113 mg RE/100g FW) (Table 4.2). After 7 days of fermentation, only a very low amount of total phenolic compounds was observed, independent of the fungal strain used (representative chromatogram in Figure 1C). On average, a total phenolic content of 25.3 ± 13.1 mg RE/100g FW was measured at day 7, which is about 4-5 times lower compared to the control, unfermented samples. All compounds were highly degraded, to even undetectable levels for compound 7 and 11. No

differences between different fungi, nor in the different phenolic compounds were observed for the degradation pattern.

Table 4.1. Identification of flavonoid glycosides from cauliflower outer leaves

Peaks	Rt (min)	m/z value		Identity
		Exact mass	Fragments	
1	9.82	771.2195	771, 609, 447, 284	Kaempferol-3-O-diglucoside-7-O-glucoside
2	10.63	933.2191	933, 609, 285	Kaempferol-3-O-diglucoside-7-O-diglucoside
3	10.99	1095.343	1095, 771, 609, 284	Kaempferol-3-O-triglucoside-7-O-diglucoside
4	13.09	977.3577	977, 815, 609, 284, 446	Kaempferol-3-O-sinapoyldiglucoside-7-O-glucoside
5	13.39	947.2400	947, 785, 609, 284	Kaempferol-3-O-feruloyldiglucoside-7-O-glucoside
6	13.69	1109.302	1109, 755, 947, 284	Kaempferol-3-O-coumaroyldiglucoside-7-O-hydroxyferuloylglucoside
7	13.99	917.2275	917, 755, 284, 609	Kaempferol-3-O-coumaroyldiglucoside-7-O-glucoside
8	18.82	625.1302	625, 301	Quercetin-7-O-diglucoside
9	19.44	771.1885	771.284	Kaempferol-3-O-triglucoside
10	19.95	609.1251	609.284	Kaempferol-3-O-diglucoside
11	20.38	947.2400	947, 609, 771, 785, 284	Kaempferol-3-O-diglucoside-7-O-feruloylglucoside
12	20.65	785.1868	785,609,284	Kaempferol-3-O-feruloyldiglucoside
13	20.99	755.1899	755,609,284	Kaempferol-3-O-coumaroyldiglucoside
14	23.60	447.0789	447, 284	Kaempferol-3-O-glucoside
15	24.28	623.1889	623, 447, 284	Kaempferol-C-feruloylglucoside/ Kaempferol-3-feruloylglucoside

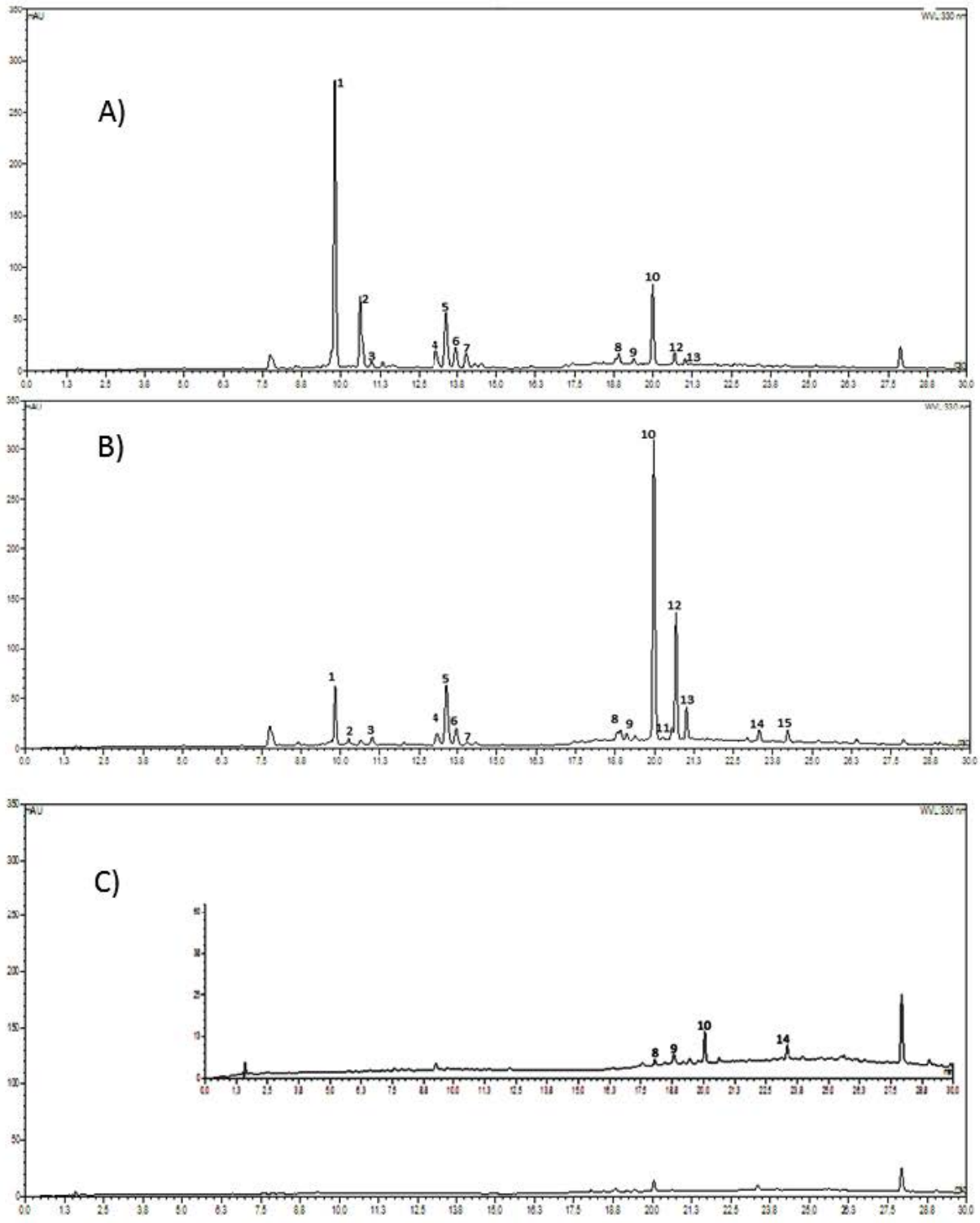

Figure 4.1. UPLC profile of phenolic compounds from cauliflower outer leaves unfermented (A), or fermented with *Aspergillus sojae* for 1 day (B) and 7 days (C), recorded at 330 nm (see Table 4.1 for identification of peaks).

The total amount of phenolic compounds extracted after 1 day incubation with *A. sojae* was approximately 3 times higher compared to the control sample, while for the other fungi less difference in total phenolic content was observed compared to the control sample. Detailed information of the changes in the phenolic profile after 1 day incubation for the different fungal treatments is given in Table 4.2. Significant differences between fungi are observed for compound 1, 3, 6 and 7 ($p < 0.05$). The concentration of compound 1 (kaempferol-3-O-diglucoside-7-O-glucoside) in cauliflower outer leaves fermented with *R. oryzae* and *R. azygosporus* decreased by approximately 94% and 90%, respectively, after 1 day of incubation, from an initial 34.8 mg RE/100 g FW in the control sample to 2.1 mg RE/100 g FW and 3.6 mg RE/100 g FW respectively. Although a 50% decrease was observed for this compound in the incubation of *A. sojae* compared to the control, this value was the highest among all fungal treatments after 1 day of incubation. Furthermore, a complete disappearance of compound 7 (kaempferol-3-O-coumaroyldiglucoside-7-O-glucoside) was observed when samples were incubated for 1 day with *A. sojae*, *R. azygosporus* and *P. chrysoporium*.

In contrast to the decrease observed for compounds 1, 2 and 7, the concentration of all other compounds massively increased after 1 day of fermentation of cauliflower outer leaves by *A. sojae* in comparison with the control samples. The highest content in compound 3 (8.3 mg RE/100g FW) and compound 6 (7.1 mg RE/100g FW) was observed for *A. sojae,* while the latter was almost completely degraded by *R.* oryzae. Interestingly, in comparison with the control samples, the fermented cauliflower outer leaves using *A. sojae* for 1 day resulted in a 6-fold and 18-fold increase in concentration of compound 13 and compound 14, respectively.

In addition, almost a 5-fold and 8-fold increase was observed for compounds 10 and 12, respectively. A similar behavior was found after 1 day of fermentation with *R. oryzae*, which showed an enhancement in the content of compounds 13 (14.8 mg/100 g FW) and 15 (6.1 mg/100 g FW), compared to their concentration present in the control sample (4 mg/100 g FW and 1.2 mg/100 g FW, respectively).

In order to visualize the change in phenolic profile affected by different fungal incubations, principal component analysis (PCA) was carried out (Figure 4.2). The first component (PC 1) accounting for 75.2% of the variation, described the distinct difference between the samples fermented with *A. sojae* and other treatments. While the second component (PC 2) responsible for 24.8 % of the variation can be expressed as a factor for the profile of individual compounds in unfermented samples (control) in comparison with fermented samples. The observation from Figure 4.2 indicates that the higher amount of compounds 8, 9, 10, 11, 12, 13, 14 and 15 is associated with fermentation by *A. sojae*, while the control sample exhibited a higher level of compounds 1, 2 and 7.

Table 4.2. Effect of fungi on the profile of individual phenolic compounds extracted from outer leaves of cauliflower for the control samples (day 0) and after 1 day of incubation (n=3)

Individual No. compound	Incubation time (1 day)									
(mg RE/100g FW)	Control	NF	*A. niger*	*A. oryzae*	*A. sojae*	*R. oryzae*	*R. azygosporus*	*P. chrysosporium*	SEM	P value
1	34.8 ± 19.5	14.2^{ab}	11.2^{ab}	9.3^{ab}	16.1^{a}	2.1^{b}	3.6^{ab}	9.0^{ab}	1.4	0.024
2	9.1 ± 5.4	4.3	3.5	2.0	4.9	1.9	0.74	3.6	0.42	0.121
3	1.2 ± 0.5	3.5^{ab}	0.13^{a}	3.7^{ab}	8.3^{b}	3.6^{ab}	3.1^{ab}	0.11^{ab}	0.81	0.048
4	2.0 ± 1.3	4.1	1.4	4.1	8.9	4.5	4.1	1.5	0.84	0.216
5	12.5 ± 7.0	7.9	11.1	9.2	14.0	1.2	7.7	7.7	1.54	0.452
6	3.9 ± 2.1	3.9	3.4	3.9	7.1	0.62	2.7	2.7	0.58	0.050
7	3.2 ± 1.9	3.0^{a}	1.1^{b}	0.54^{b}	nd	0.3^{b}	nd	nd	0.25	<0.001
8	5.4 ± 2.0	5.9	10.3	7.8	15.2	4.9	6.1	7.9	1.11	0.121
9	5.4 ± 2.5	4.3	6.9	5.9	12.2	5.0	5.3	5.4	0.97	0.335
10	24.8 ± 12.9	51.4	38.4	61.8	126	63.9	65.0	38.2	10.5	0.273
11	0.07 ± 0.06	0.2^{a}	nd	4.1^{ab}	17.4^{b}	2.7^{ab}	nd	2.7^{ab}	1.87	0.042
12	4.9 ± 2.0	11.8	17.3	19.9	38.3	21.5	20.6	13.5	3.74	0.591
13	4.0 ± 1.7	9.4	8.1	13.1	26.1	14.8	13.8	6.0	2.24	0.211
14	1.0 ± 1.4	3.8	6.9	5.1	18.0	9.3	9.5	5.7	1.68	0.315
15	1.2 ± 0.1	3.2	3.8	4.3	9.2	6.1	5.1	2.3	0.80	0.298
Total	113 ± 60	131	124	155	321	142	147	106	24.9	0.228

nd, not detected; NF, natural fermentation. Individual No compounds corresponds with the peak numbers as presented in Table 1. Different letters (a, b) indicate significant difference after 1 day of fermentation (P-value < 0.05) within a row.

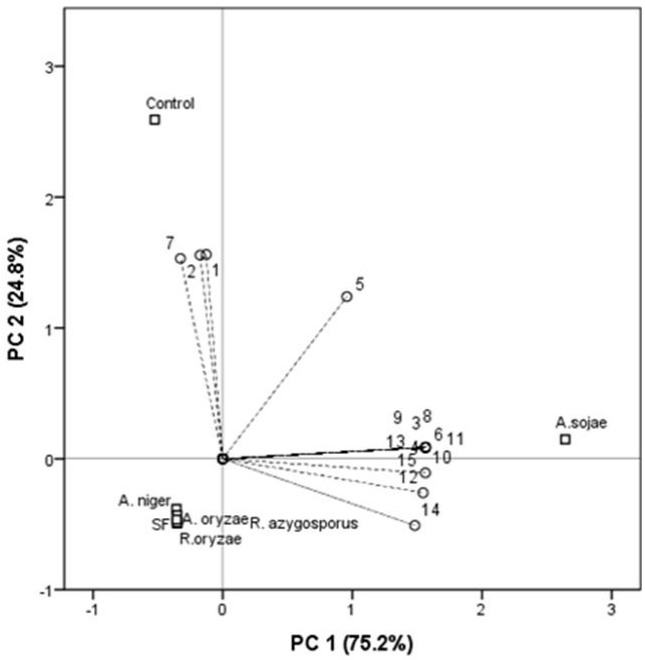

Figure 4.2. Principal component analysis of the individual phenolic compounds by 1 day of fungal fermentation (n=3). The numbers in the PCA plot represent the different phenolic compounds as described in Table 4.1.

4.4. Discussion

Filamentous fungi are widely used in fermentation of agricultural by-products. Nevertheless, the release and bioconversion of phenolic compounds during fermentation of cauliflower outer leaves by these organisms has not been characterized yet. This study aimed at reporting the effect of fungi and incubation time on the profile of individual phenolic compounds during 7 days of fermentation of this matrix.

Based on the identification by LC-MS, there is no phenolic aglycone detected in the unfermented sample (control), implicating that the phenolic compounds present in cauliflower outer leaves occur mainly in glycosidic forms. This finding is in agreement with

previous reports by Llorach et al. (2003) and Huynh et al. (2014) who also showed that phenolic compounds found in cabbage outer leaves are mainly kaempferol glycosides and their combinations with different hydroxycinnamic acids.

As compared to the control sample, the decreased concentration of compounds 1, 2, 5 and 7 in fermented cauliflower outer leaves after 1 day incubation may be related to the action of cell-wall degrading enzymes produced by the fungi during solid-state fermentation, thus providing a bioconversion of these compounds into their corresponding metabolites. In agreement with previous reports, the starter fungi used in this study were known to produce these type of enzymes during fermentation, such as β-glycosidase by *A. niger* (Hur et al., 2014), *A. oryzae* (Bhanja et al., 2009), *A. sojae* (Kimura et al., 1999), *R. oryzae* (Yadav et al., 2013) , *R. azygosporus* (Vattem et al., 2002) and *P. chrysoporium* (Ajila et al., 2011). This enzyme is able to hydrolyse the β-1,4 glycosidic bonds in aryl and alkyl β-D glucosides, as well as glycosides containing disaccharides and oligosaccharides (Zheng et al., 2000). It was thereby proposed that β-glycosidase produced by the fungus catalyses the bioconversion of compounds 1 and 2 to their possible corresponding metabolite (compound 10) through the cleavage of 7-O-glycosidic bond of kaempferol to glucose molecules, and then metabolised further to compound 14 as described in pathway 1 (Figure 4.3). This enzyme can also be responsible for the metabolism of compound 3 to compound 9 (pathway 2) by the removal of two glucose moieties at the 7-substitution. A similar behavior was observed with compound 5 and compound 7, where the metabolisation could also be attributed to a deglycosylation of these compounds through glycosyl hydrolase activity of β-glycosidase, converting compound 5 to compound 12 (pathway 3), and compound 7 to compound 13

(pathway 4). These metabolic pathways resulted in the loss of compounds 1, 2, 5 and 7, while their corresponding less glycosylated metabolites (compounds 10, 12, 13, and 14) increased after 1 day of fermentation. In general, these pathways of bioconversion stated above also give rise to an increased release of more simple compounds without any carbohydrate moiety at the 7-carbon position on the A ring (compounds 9, 10, 12, 13, and 14). These less glycosylated compounds have a higher human bioavailability and biological activity (Heim et al., 2002; Mora et al., 1990), and deglycosylation may thus improve the functional properties of this by-products.

Among the microorganisms examined, *A. sojae* has been reported as a good producer of xylanase (Kimura et al., 1995) which cleaves internal xylosidic linkages in xylans (Wong et al., 1988), and thus breaks down hemicellulose. In addition, other pectinolytic enzymes (exo-polygalacturonase) which are well known for their ability to catalyse the hydrolysis of 1,4-α-D-glycosidic bonds in pectic polysaccharides (Verlent et al., 2007), were also produced by this strain through solid-state fermentation (Ustok et al., 2007). It seems that the action of these enzymes produced by *A. sojae* during fermentation could result in the degradation and disruption of the cell-wall matrix of cauliflower outer leaves consisting of cellulose (16%), hemicellulose (8%) (Stojceska et al., 2008) and pectin, and thus enhancing the release of individual phenolic compounds into the methanolic extract. This could be the reason for the higher amount of the total phenolic compounds found in the incubations with *A. sojae*.

A vast reduction in phenolic compounds caused by 7 days of fermentation could be associated with the biotransformation of these glycosylated compounds into other

metabolites. One possibility is the metabolism to less toxic metabolites for the fungi, possible by mechanisms such as ring cleavage (Ajila et al., 2011). The occurrence of a ring cleavage mechanism on phenolic compounds by the fungi used in this study has not been reported before. Also the breakdown of these phenolic compounds to be used as a carbon source by the fungi is a possibility.

4.5. Conclusions

The findings obtained from our study indicated that the improved release of kaempferol glucosides from cauliflower outer leaves can be performed by solid-state fermentation with filamentous fungi, especially *Aspergillus sojae*. However the period of incubation is very important to obtain a maximum recovery of released phenolics. Furthermore, although none of flavonoid aglycones has been found, the fungal metabolism of flavonoid compounds by these treatments resulted into their corresponding derivatives with less or no carbohydrate moieties at the 3- or 7- carbon position on ring C and A. Therefore, the metabolic pathways proposed in this work could contribute to the development of biorefinery concepts for the valorization of agricultural (by-)products.

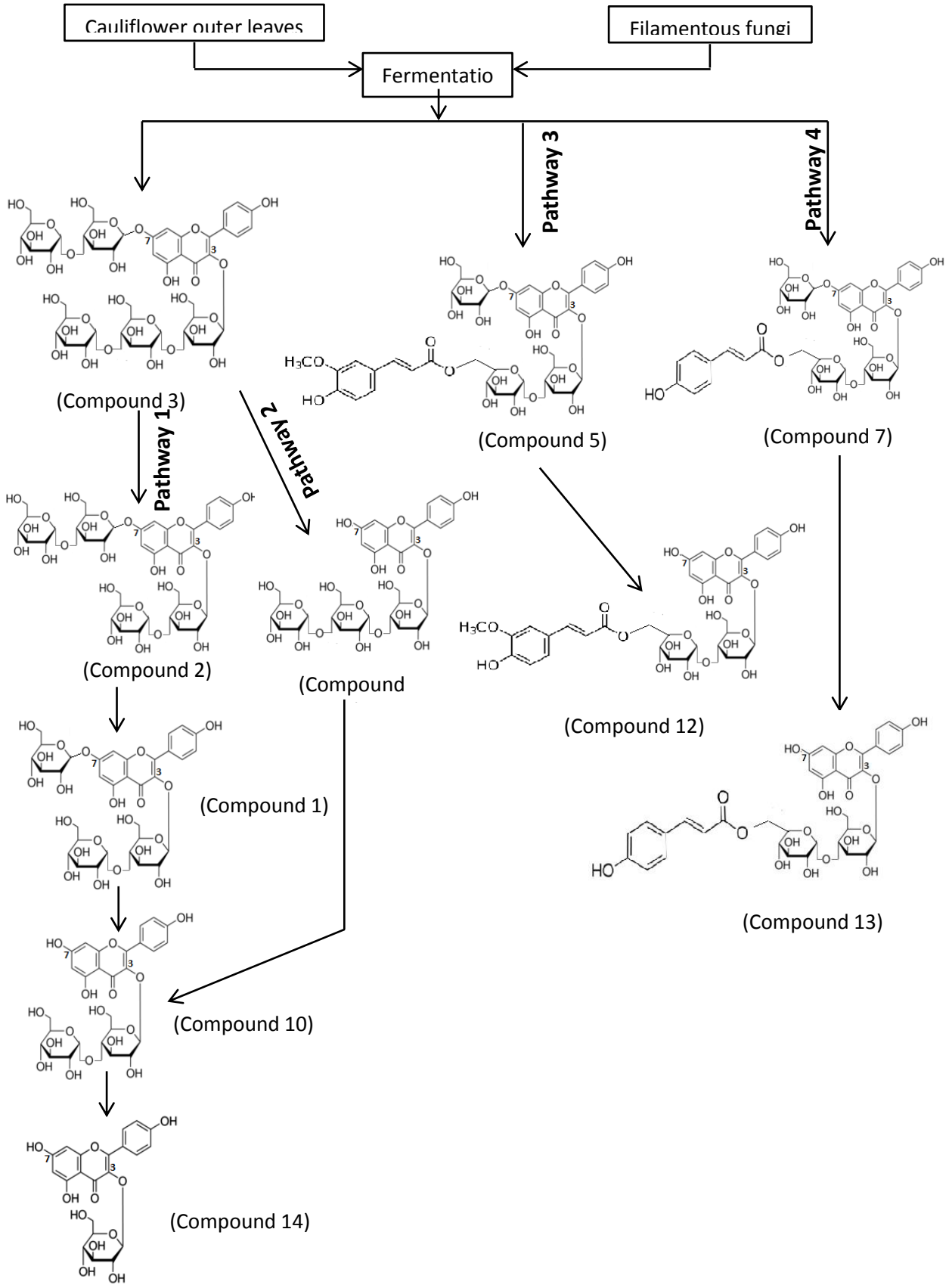

Figure 4.3. Proposed metabolic pathways of phenolic compounds from cauliflower outer leaves by solid-state fermentation with filamentous fungi

Release of kaempferol and quercetin from cauliflower outer leaves and onion powder extracts by Rhizopus fermentation

Chapter 5

Release of kaempferol and quercetin from cauliflower outer leaves and onion powder extracts by Rhizopus fermentation

ABSTRACT

Fermentation with filamentous fungi is known for their ability to bioconvert bioactive compounds. The aim of this study was to investigate the metabolism of glycosidic derivatives of kaempferol and quercetin during fungal fermentation of extracts from cauliflower outer leaves and onion by the two strains *R. oryzae* and *R. azygosporus*. The highest concentration of kaempferol and quercetin was observed after 2 days and 1 day of fermentation with *R. oryzae*, respectively. It was proposed that glycosidic compounds were initially deglycosylated to form kaempferol-3-glycoside and quercetin-3-glucoside and then further metabolized into their aglycones, i.e., kaempferol and quercetin. Hence, the fermentation with *R. oryzae* and *R. azygosporus* could be considered as a way to produce kaempferol and quercetin aglycone from their glycosidic derivatives.

5.1. Introduction

Brassica vegetables are one of the most produced and consumed vegetables worldwide. Their consumption increased steadily in the last two decades, up from globally around 45 million tons in 1993 to approximately 71 million tons in 2013 (FAO, 2015). This change resulted in the generation of huge amounts of wastes and by-products, i.e., non-edible fractions of the vegetable (outer leaves, stems, florets). In a few recent studies, these residues were recognized to be a source of antioxidant compounds, i.e. flavonoids (Gonzales et al., 2014; Huynh et al., 2014; Llorach et al., 2003), which are known as a protective factor against cardiovascular diseases (hypertension, obesity, arteriosclerosis, smoking, diabetes, and aging) (Nile et al., 2014; Ranilla et al., 2010; Rodriguez-Mateos et al., 2013).

Kaempferol and quercetin are the dominant flavonoids found generally in vegetables and fruits. While kaempferol derivatives are commonly found in *Brassica* vegetables (Cartea et al., 2010), onions have been reported as one of the major sources of quercetin (Hollman et al., 1999; Pérez-Gregorio et al., 2010). These authors found that quercetin in the form of glycosides are dominantly present in both red and white onion. The main structural difference between quercetin and kaempferol is a hydroxyl group added to 3'-position on ring B which results in differences in their microbial metabolism (Lin et al., 2003; Oka et al., 1972; Serra et al., 2012).

Fukumoto et al. (2000) stated that glycosylated phenolic compounds resulted in a lower antioxidant activity compared to their aglycones. The more sugar moieties attached to the aglycon, the more their antioxidant activity is reduced. Moreover, the glycosidic forms

cannot be well absorbed in the intestine, leading to low bioavailability, due to their large structure and their solubility (Cartea et al., 2010). Thus, preprocessing techniques prior to consumption could be used as an effective means to obtain smaller structures of phenolic compounds, as well as aglycones, which might result in the improvement in their activity as well as bioavailability.

In our previous study (Huynh et al., 2015), the lower glycosylated forms of kaempferol were found in the matrix (cauliflower outer leaves) fermented with fungi, but the formation of aglycone was not observed during this incubation time. The objective of this study was to evaluate whether the cell wall matrix is a barrier for the formation of aglycon from their parent compounds, and whether the structure of flavonoids are able to have an effect on the extent of deglycosylation. For this purpose, the fermentation of glycosidic kaempferol and quercetin derivatives extracted from their matrices, i.e. cauliflower outer leaves and onion, with two different filamentous fungi, i.e, *Rhizopus oryzae*, *Rhizopus azygosporus*, was performed under submerged conditions.

5.2. Materials and Methods

5.2.1. Materials

Plant material

After cauliflower harvest (July, 2012), the outer fresh leaves were collected from a local farm in West-Flanders, Belgium. The leaves were kept frozen at -18 °C until further analysis. Onion powder was kindly provided by Rapps (Belgium).

Chemicals and reagents

HPLC-grade methanol, formic acid were purchased from VWR International (Leuven, Belgium), and gallic acid, hesperetin, rutin from Sigma-Aldrich (Bornem, Belgium). Dox Czapek, malt extract, soy peptone, bacteriological agar, casein tryptone were purchased from Oxoid (Erembodegem, Belgium).

Fungal strains and preparation of the inoculum

The cultures of two food-grade fungi, i.e., *Rhizopus oryzae* (CBS 1125.86), *Rhizopus azygosporus* (CBS 357.93) were used. The cultures were maintained at 4°C on malt extract agar (MEA) slant. Each fungal strain was first cultured on 2 MEA slants and incubated at 30 °C for 7 days to obtain subcultures. A volume of 2 mL of Tween 80 (0.05%) was added into the subculture slant to make a suspension of the inoculum. The suspension was subsequently transferred into a sterile Erlenmeyer flask containing 5 g of sterile glass beads shaken during 1 min prior to transfer to centrifugal tubes for centrifuging at minimum 2000g during 20 min to remove mycelia. The supernatant was removed, while the pellet was suspended again with physiological water and centrifuged 2 times (2000g, 20min). The pellet obtained was used to prepare the spore suspension as initial inoculum (10^6 spores/ml) for the fermentation process.

5.2.2. Experimental procedure

Preparation of extract

The samples (cabbage outer leaves, onion powder) were extracted by the method of Olsen et al. (2009). In brief, 15 ml of methanol was added to 5 g of sample, stirred for 40 seconds using an ultraturrax, followed by incubation in ice water for 15 min. Supernatant was

collected after centrifugation (13,000xg, 10 minutes and 4 °C). The pellet obtained was then re-extracted by the same procedure, using 10 ml methanol/water (80/20; v/v). Both extracts were pooled and filtered through filter paper (VWR, grade 413) before adding methanol to a final volume of 25 ml. Finally, methanol present in the extract was evaporated using a rotary evaporator to obtain a concentrated extract which was used in this experiment.

Fermentation process

The inoculum for the different fungi prepared as described in the procedure was added to an Erlenmeyer flask containing 10ml of Dox Czapek medium, the flasks were then incubated for 3 days at 30 ^{0}C with shaking. After the third day, 1 mL of phenolic extracts (obtained from cabbage outer leave or onion powder) were added to the Erlenmeyer flasks. The extracts were fermented with *Rhizopus oryzae, Rhizopus azygosporus* during 7 days, and samples were taken on day 0, 1, 2, and day 4 and 7 days.

5.2.3. Analytical methods

Sample purification using solid phase extraction (SPE), quantification and identification of phenolic compounds by LC-MS were based on the methods published in our previous work as described by Huynh et al. (2014). The purification of samples was done using SPE C18 column (50 mg, 4 mL). LC-MS performed on a Waters Acquity UPLC system (Waters, Milford, MA) linked to a Synapt HDMS TOF mass spectrometer (Micromass, Manchester, UK) was applied to identify the phenolic compounds present in samples. Hesperidin was used to as an internal standard for LC-MS analysis.

5.2.4. Statistical analysis

The experiments were performed in triplicates. Data were subjected to Analysis of variance (ANOVA), and the Tukey's procedure was used to test the significant differences of peak areas of metabolite, with the level of significance at $p<0.05$, using SPSS v.22.

5.3. Results

The profile of phenolic compounds of the extracts from cauliflower outer leaves and onion powder and their metabolites was analyzed at different time points of fungal incubations (Figure 5. 1 and Figure 5. 2, respectively). The identification of phenolic compounds (kaempferol, kaempferol-3-O-glucoside, kaempferol-3-O-diglucoside; quercetin, quercetin-3-O-glucoside, quercetin-3-O-glucoside-7-O-glucoside) was carried out through LC-MS/MS based on their retention time, exact mass and fragments which is summarized in Table 5. 1. The change in the main constituents during the fermentation was determined based on their peak area (mAU).

Table 5. 1. Identification of flavonoid compounds selected from cauliflower outer leaves and onion extract

Extract	Rt (min)	m/z values		Identity
		Exact mass	Fragments	
Cauliflower outer leaves	18.15	609	609, 284	Kaempferol-3-O-diglucoside
	21.58	447	447, 284	Kaempferol-3-O-glucoside
	26.91	285	285	Kaempferol
Onion	16.57	625	625, 463, 300	Quercetin-3-O-glucoside-7-O-glucoside
	19.42	463	463, 300	Quercetin-3-O-glucoside
	24.29	301	301	Quercetin

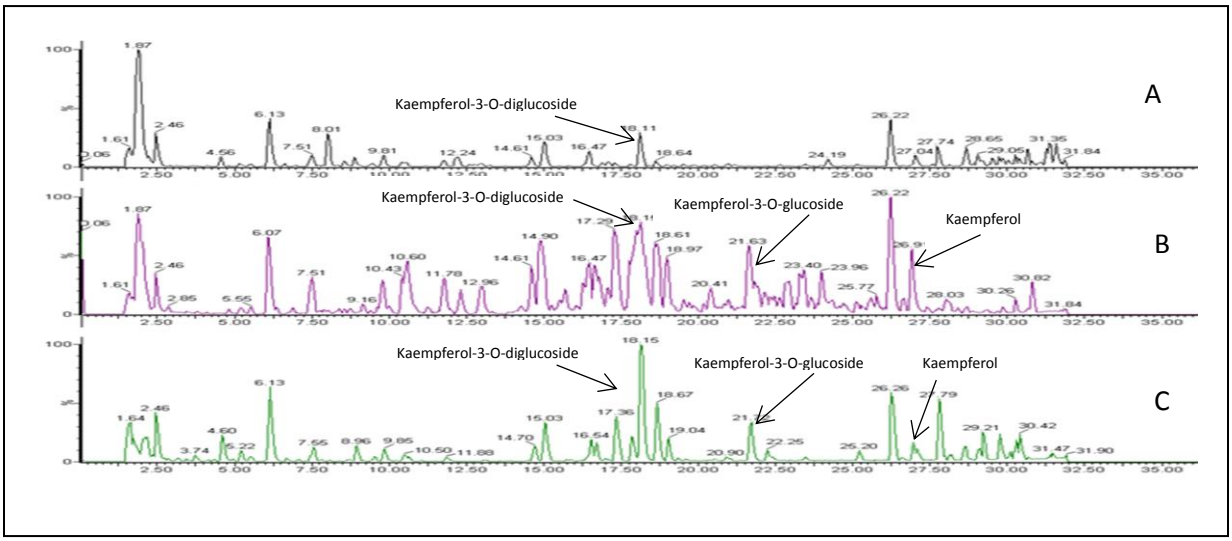

Figure 5. 1. UPLC chromatograms of cabbage extract of cauliflower outer leaves, A, control; B, fermented with *Rhizopus oryzae* for 2 days; C, fermented with *Rhizopus azygosporus* for 4 days.

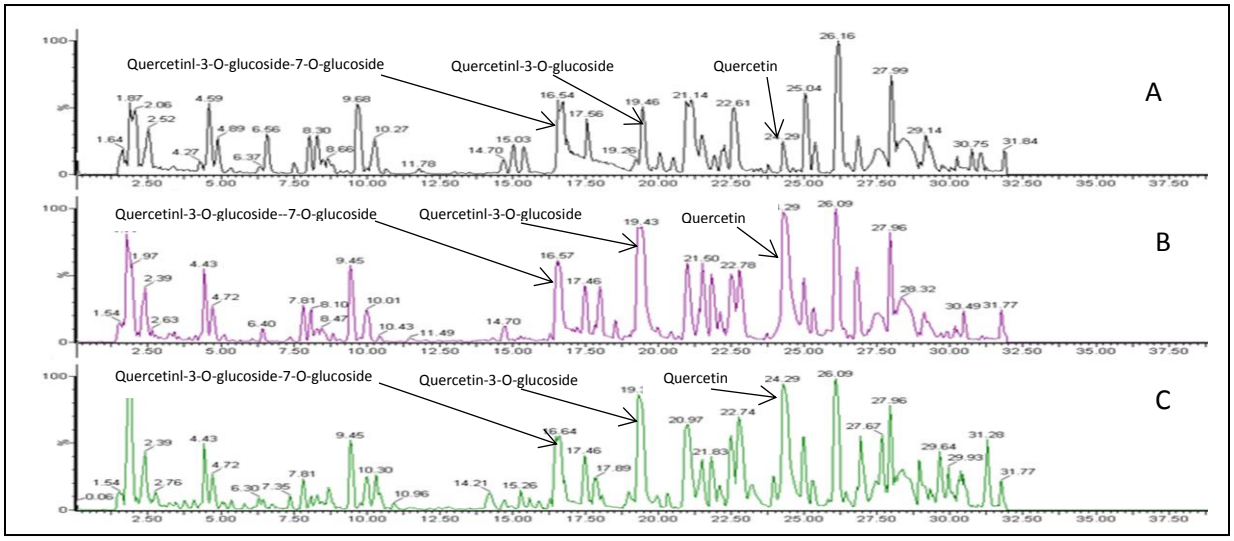

Figure 5. 2. Figure 2. UPLC chromatograms of onion extract, A, control; B, fermented with *Rhizopus oryzae* for 1 days; C, fermented with *Rhizopus azygosporus* for 1 days

As shown in Figure 5.3, both fermentation treatments with *R. oryzae* and *R. azygosporus* increased the concentration of kaempferol-3-O-glucoside, with the highest level obtained on the 2nd day of incubation for *R. oryzae* and on 4th day for *R. azygosporus*, i.e. a 4.8 fold and 8.6 fold increase compared to its content in the control sample, respectively. While the increase in concentration of kaempferol-3-O-diglucoside was only observed on the 1st day of incubation by *R. oryzae* and *R. azygosporus*, with an increase by 50% and 12%, respectively compared to the control samples (t = 0h).

Regarding the production of kaempferol aglycone from the extract of cauliflower outer leaves, kaempferol was found at all 4 days of fermentation with *R. oryzae*, reaching its highest concentration at day 2 and decreased on 4th day of incubation, while it was only detected in the sample fermented for 4 days with *R. azygosporus* at very low amount prior to disappearing on day 7.

To obtain more information on the possible metabolism of different glycosidic flavonoids, extracts from onion powder, which has been reported to contain mainly quercetin glycosides, was also incubated during 4 days with *R. oryzae* and *R. azygosporus*. The change in concentration of three main flavonoid compounds identified, i.e., quercetin, quercetin-3-O-glucoside, quercetin-3-O-glucoside-7-O-glucoside during the incubation period is presented in Figure 5.4. Both treatments with *R. oryzae* as well as with *R. azygosporus* after 1 day of incubation resulted in a more than 10–fold higher concentration of quercetin, ranging from peak areas of 62.6 mAU in the control (t=0h) to 747.9 mAU and 719.5 mAU in the *R. oryzae* and *R. azygosporus* respectively. However, a longer fermentation up to 4 days led to a reduction in quercetin concentration, especially clear for the incubations with *R.*

azygosporus. A similar result was observed with quercetin-3-O-glucoside. This compound increased significantly after 1 day of fermentation with *R. oryzae* and *R. azygosporus*, to 190% and 50% of its concentration in the control sample, respectively, and decreased gradually with time of incubation.

In contrast to the changes observed for the concentration of quercetin and quercetin-3-O-glucoside, the concentration of quercetin-3-O-glucoside-7-O-glucoside decreased continuously during the fermentation by both *R. oryzae* and *R. azygosporus*, with a 5 fold lower content after 4 days incubation compared to the control.

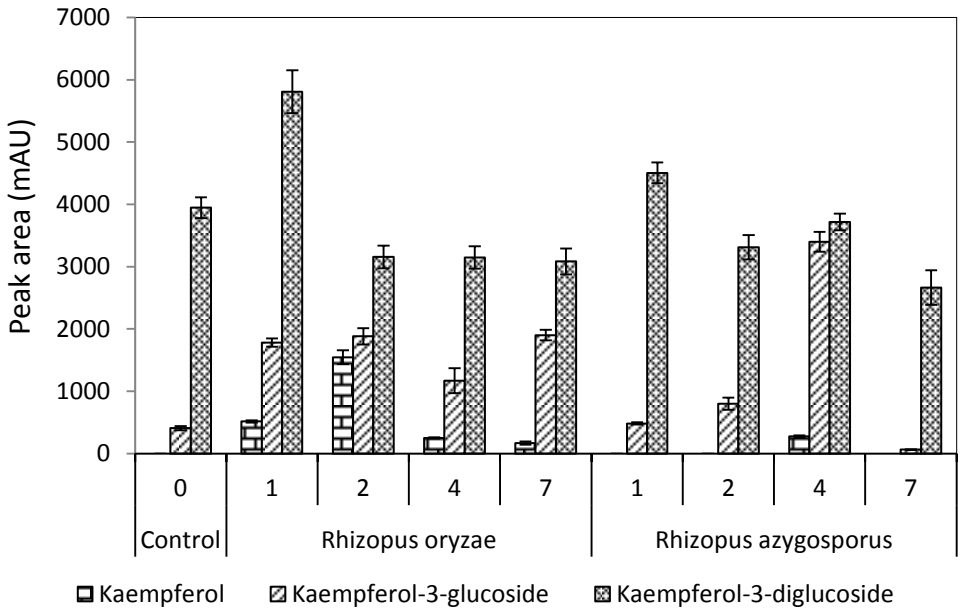

Figure 5.3. Change of kaempferol, kaempferol-3-O-glucoside, kaempferol-3-diglucoside during 7 days of fermentation

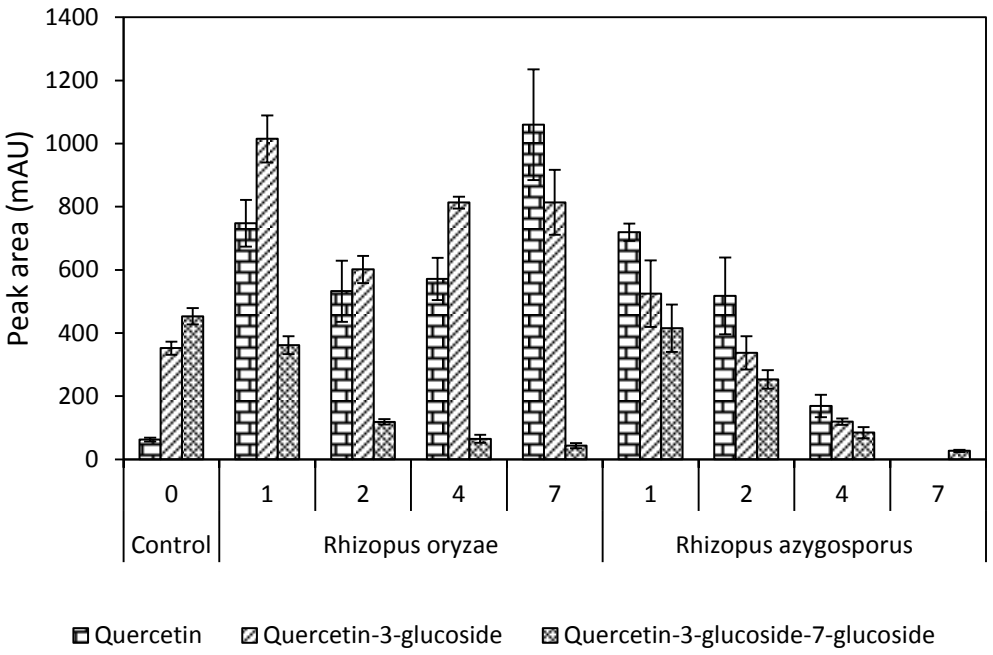

Figure 5.4. Change of quercetin, and quercetin-3-O-glucoside, and quercetin-3-O-glucoside-7-O-glucoside during 7 days of fermentation

5.4. Discussion

The use of filamentous fungi in solid-state fermentations of agricultural by-products and industrial waste from food processing is widely performed, mostly focusing on enzyme production and release of bioactive compounds. Since agricultural by-products are rich in phenolic compounds, fungal solid-state fermentation can be a possible way to improve its valorization potential. However, the possible metabolism of phenolic compounds by these fungi is poorly characterized and should be considered when using this valorization strategy. In this study, two Rhizopus strains, i.e. *R. oryzae* and *R. azygosporus* were selected, based on the results obtained in a former study using cauliflower outer leaves (Irimat, 2014). In general, the free form of the aglycone (kaempferol and quercetin) detected in fermented

medium indicated the presence of β-glucosidase in medium. The β-glucosidase secreted from fungi was able to remove the glucose molecule both on the 3-O and 7-O position.

As shown in previous studies, the strains used in this work have the ability to produce cell-wall degrading enzymes through fermentation, such as β-glucosidase produced by *R. oryzae* (Takii et al., 2005; Yadav et al., 2013), as well as by *R. azygosporus* (Lee et al., 2006; Lee et al., 2008). The action of this enzyme results in the bioconversion of these compounds into their corresponding metabolites and could have been involved in the increase in concentration of kaempferol-3-O-glucoside. It is hence proposed that the production of kaempferol-3-O-glucoside could originate from various kaempferol glycosides present in the extract such as kaempferol-3-O-diglucoside-7-O-glucoside, kaempferol-3-O-diglucoside-7-O-diglucoside, kaempferol-3-O-triglucoside-7-O-diglucoside, kaempferol-3-O-triglucoside and kaempferol-3-O-diglucoside, as determined in earlier research (Huynh et al., 2015). Similarly, quercetin glycosides from onion extract, e.g., quercetin-diglycoside, rutin (Marotti et al., 2002; Rodríguez Galdón et al., 2008) may be converted into quercetin-3-O-glycoside under fermentation conditions, and thus leading to the increase in its concentration during incubation with *R. oryzae* or on the first day with *R. azygosporus*. The concentration of quercetin-3-O-glycoside increased as a possible explanation for the reduction of quercetin-3-O-glycoside-7-O-glucoside during fermentation.

The cleavage of the 3-O-glycosidic bond of the sugar moiety to the aglycone molecule is in agreement with the previous findings that the aglycone could come from their corresponding glycosidic form by the activity of β-glycosidase, such as the bioconversion of rutin into quercetin by *Aspergillus awamori* (Lin et al., 2014) or *Aspergillus niger* (You et al., 2010);

daidzin, glycitin, genistin into daidzein, glycitein, genistein by *Aspergillus oryzae* (Lee et al., 2013) or *Rhizopus azygosporus*, *Rhizopus* sp. (Lee et al., 2006).

Among two fungal strains employed in this work, *R. oryzae* may exhibit a higher ability to produce β-glycosidase or to produce a more active enzyme than *R. azygosporus*. Indeed, Lee et al. (2006) reported low β-glycosidase activity derived from *R. azygosporus*, while *R. oryzae* has been considered as a good producer of this enzyme (Battaglia et al., 2011; Takii et al., 2005). This could cause the higher concentration of kaempferol aglycone found in medium prepared with *R. oryzae* than that incubated with *R. azygosporus*. Another possible explanation for our results is that *R. azygosporus* probably produces phenolic-degrading enzymes with a higher activity, which immediately degrade the formed kaempferol produced into other (none-) phenolic metabolites during fermentation(Gesell et al., 2004; Schlueter et al., 2013; Serra et al., 2012). This thus results in the lack of or in the presence of a modest amount of kaempferol in fermented medium with *R. azygosporus*.

Although the biotransformation of glycosylated forms into their aglycones (kaempferol and quercetin) undergo the same pathway for the removal of sugar molecules attached at the 3- and/or 7- position, the formation of kaempferol was limited for *R. azygosporus* during fermentation except on the 4th day where a modest amount was detected. On the contrary, a higher accumulation of quercetin was found in the fermented medium, which may be attributed to the existence of an additional –OH group at the 3'-position on ring B of quercetin compared to the kaempferol structure. Oka et al. (1972) and Lin et al. (2003) indicated that a hydroxyl group on the aromatic ring A and B of both flavonoids resulted in a considerable decrease in maximum velocity and Michaelis contants of the phenolic-

degrading enzymes. Therefore, quercetin, which possesses one extra –OH group at the 3'-position, was degraded at a lower rate compared to kaempferol during fungal degradation. This could also be a reason for the faster degradation of kaempferol by *R. oryzae* after 2 days when its accumulation in fermented medium seems to be sufficient for fungal detoxification as mediated by the fungal enzymes (Slana et al., 2011).

5.5. Conclusions

This study evaluated the bioconversion of phenolic compounds supported by food-degrading microorganisms. The fermentation with filamentous fungi used in this study (*R. oryzae* and *R. azygosporus*) revealed that glycoside derivatives of kaempferol and quercetin extracted from cauliflower outer leaves and onion can be deglycosylated to form their precursor products (kaemperol-3-O-glycoside and quercetin-3-O-glycoside), which were further metabolised into their corresponding aglycones (kaempferol and quercetin). The results obtained in this study also indicate that *R. oryzae* has a higher ability to produce aglycone compared to *R. azygosporus*. Further researches need to be performed on fungal metabolism of various flavonoids with different substitutions in hydroxyl groups.

General discussion, and future perspectives

Chapter 6

General discussion, and future perspectives

6.1. General discussion

6.1.1. The profile of phenolic compounds and their amount in cauliflower outer leaves

Total phenolic content (TPC), as measured by Folin-Ciocalteu reagent, in the raw material (cauliflower outer leaves) was in a range of 47-54 mg GAE/100 g fresh weight. This indicates that an interesting amount of phenolic compounds is present in this by-product. In comparison with other sources, this level is similar to those of edible parts of cabbage, carrot (Chu et al., 2002), artichoke by-products (Llorach et al., 2002) and higher than in lemon peels (Li et al., 2006) and grape pomace (Chamorro et al., 2012); but lower than in grape seeds (Chamorro et al., 2012). However, these values will vary depending on the origin of the raw material (variety, growth conditions, storage of the plant material) as well as on the extraction parameters such as solvents, time, temperature, sample-solvent ratio (Ignat et al., 2011; Stalikas, 2007).

Although the Folin-Ciocalteu assay is seen as a rapid, simplified and inexpensive measurement of phenolic compounds, the main disadvantage of this colorimetric method is its low specificity due to the color reaction that could be obtained from any oxidizable phenolic hydroxyl group (Hrncirik et al., 2004). Also it is affected by several interfering non-phenolic compounds such as sulfur dioxide, ascorbic acid, sugar, organic acids, aromatic

amines, proteins and enzymes (Roura et al., 2006; Singleton et al., 1999). For this reason, more and more interest is paid to the use of state-of-the-art instrumental analytical methods for the evaluation of the phenolic constituents. In our research work, also the quantification and identification of the individual phenolic compounds in cauliflower outer leaves were evaluated based on the use of SPE-LC-MS/MS.

As previously shown in chapter 2, chapter 3 and chapter 4, the most abundant phenolic compounds found in cauliflower outer leaves were identified as forms of kaempferol glycosides i.e. different combinations of kaempferol with sugar molecules and/or different hydroxycinnamic acids (coumaric acid, ferulic acid and synapic acid). The total phenolic compounds quantified using SPE-UPLC was 113 mg RE/100 g FW, of which flavonoids with a high degree of glycosylation such as kaempferol-3-O-diglucoside-7-O-glucoside, kaempferol-3-O-diglucoside-7-O-diglucoside, and kaempferol-3-O-feruloyldiglucoside-7-O-glucoside accounted for approximately 50 % of all identified kaempferol glucosides. Most of the compounds reported here have been previously described by Llorach et al. (2003). Also in other *Brassica* vegetables such as tronchuda cabbage (*Brassica oleracea L. var. costata*), white cabbage (*Brassica oleracea L. var. capitata*), pak choi (*Brassica rapa*) and rape leaves (*Brassica napus*), the majority of the phenolic compounds was identified as different kaempferol derivatives (Cartea et al., 2010). It is therefore suggested that cauliflower outer leaves in particular, or vegetables of the family *Brassicaceae* in general are potentially an excellent source of kaempferol glycosides, while onion (Pérez-Gregorio et al., 2010; Rodríguez Galdón et al., 2008), and apple (Schieber et al., 2001; Tsao et al., 2003) has been previously evaluated as a good source of quercetin derivatives, the other major flavonoid.

6.1.2. The release of phenolic compounds from cauliflower outer leaves and their metabolites by biological treatments

Phenolic compounds present in plants occur in both free and bound forms. The free ones generally accumulate in the vacuoles of plant cells, while the bound compounds are mostly found in the cell walls, where they are covalently linked to cell wall components such as cellulose, hemicellulose, lignin, or pectin (Acosta-Estrada et al., 2014). The free phenolic compounds are usually obtained by conventional solvent extraction, while their bound forms are normally missed by such methods, and thus a high amount still remains in the extraction residues (Pérez-Jiménez et al., 2011). For this reason, several novel extraction methods including chemical treatments (alkaline, acidic hydrolysis) and physical techniques (microwave, sonication) have been developed for the improved release of phenolic compounds from their matrices (Acosta-Estrada et al., 2014; Azmir et al., 2013; Wang et al., 2006). However, the phenolic extraction under chemical hydrolysis conditions may cause unwanted effects such as thermal degradation of phenolic compounds, product safety, and environmental problems (e.g. changes in pH value of soil, surface water which could result in the death of microorganisms, plants, aquatic animals). Also, the physical treatments could result in the undesirable degradation of unstable bioactive compounds (Joana Gil-Chávez et al., 2013; Zhao et al., 2006). Therefore, it was suggested to focus on the application of more environmentally friendly treatments to release and metabolize phenolic compounds from the outer leaves of cauliflower. Various biological approaches including use of enzymes (**chapter 2**), lactic acid bacteria (**chapter 3**) and fungi (**chapter 4** and **chapter 5**) were investigated in this research work.

Enzyme-assisted extraction

Based on the characterization of plant cell walls which mainly consist of polysaccharide components, i.e. cellulose, hemicellulose, pectin (Brett et al., 1990), two commercial products containing carbohydrate-cleaving enzymes (Viscozyme L and Rapidase) were selected for this study. Both are multi-enzyme complexes. Viscozyme L contains a range of carbohydrases (beta-glucanase, cellulase, hemicellulase, and xylanase), while Rapidase exhibits hemicellulase and pectinase activity. The activity of these enzymes on cauliflower outer leaves might lead to a weakening and degradation of the cell-wall structure of the cauliflower outer leaves. Consequently, the intracellular substrate e.g. phenolic compounds can be more efficiently released.

As presented in chapter 2, treatments with 0.2 % Viscozyme L (v/w) or 0.5 % Rapidase (v/w) during 12h resulted in a more than 100 % increase of the total phenolic content (as determined by the Folin-Ciocaltau method). This was an impressive observation in comparison with other enzymatic treatments which have been previously reported e.g. an increase by an enzyme-assisted extraction from apple peels by pectinase (50 %) (Kim et al., 2005), from lemon peels by cellulase (30 %) (Li et al., 2006), from black current pomace by pectinase Macer 8 FJ (approximately 75 %) or by pectinase Grindamyl (approximately 60 %) (Landbo et al., 2001). However, our extraction yield is lower than those reported by Kapasakalidis et al. (2009) (120 % increase in total phenolic content of black current pomace by cellulase), or by Zheng et al. (2009) (around 130 % phenolic improvement by Viscozyme L treatment on unripened apple). Efforts to release bound phenolics from plant-based foods through enzymatic treatment have been widely reported. Most of those papers mainly

focused on fruit processing by-products, while studies dealing with vegetables and their by-products remained scarce.

In the same way, the treatment with Viscozyme L also resulted in approximately 88 % increase in the amount of individual phenolic compounds, as determined by UPLC. Although no aglycone was observed, the amount of kaempferol-3-O-glycoside found in the treated sample was about 7.2-fold higher compared to the control sample (no enzyme treatment). These levels of release are much higher than those reported from pigeon leaves by pectinase (improved release of luteolin (248 %) and apigenin (239 %)) (Fu et al., 2008), but lower than the release of ferulic acid (9.8 fold) from oat bran by alpha-amylase (Alrahmany et al., 2013) or p-coumaric acid (around 1400 %) from grape pomace by a combined preparation of Novoferm 106 and Cellubrix L (Kammerer et al., 2005). These differences in the release of individual phenolic compounds observed between matrices could possibly be related to the higher proportion of bound phenolic compounds in bran or pomace compared to the other matrices studies as e.g. vegetables (Acosta-Estrada et al., 2014; Chu et al., 2002).

From our results obtained, it is clear that the enzymes used in this work were efficient for the release of flavonoids leading to a phenolic-rich extract which can contribute to add value to the cauliflower outer leaves. However, some drawbacks may occur while using these enzymatic treatments which may be summarized as follows:

i. The enzymes used in this process are commercialized at a high price e.g Viscozyme L-50 mL (90 EUR, Sigma); 100 mL 15 £, Novozymes), while this by-product (cauliflower outer leaves) is treated as a valorization substrate. It is therefore suggested to search

for a cheaper alternative which might contribute to reducing the cost of producing the extract or specific kaempferol derivatives.

ii. The costs of electric energy consumption used for maintenance of optimal temperature (35°C) for these enzymatic treatments.

iii. The risk of enzyme inactivation due to the fact that phenolic compounds may be considered as enzyme inhibitors.

Fermentation

Cauliflower outer leaves, consisting of cellulose (16 %), hemicellulose (8 %), and protein (16.1 %) (Wadhwa et al., 2006), offer an extraordinary carbon and energy source that can be utilized by various microorganisms i.e. fungi, yeasts and bacteria, of which several strains/species have been characterized for their ability to produce a copious variety of cellulolytic, lignolytic and pectinolytic set of enzymes under fermentation conditions. These enzymes are well known for their potential to catalyze the breakdown of these polysaccharide components in the plant cell wall resulting in a release of phenolic compounds. Furthermore, the phenolic compounds released can be converted during fermentation into their corresponding metabolites, which are estimated to exhibit a higher biological activity. In this sense, microbial fermentation may be considered as a cheaper alternative for enzymatic treatment.

Among the microorganisms previously studied, some lactic acid bacteria (LAB) have been shown to synthesize relevant enzymes such as esterase, laccase, β-glucosidase (Aguilar et al., 2000; Hur et al., 2014; McCue et al., 2005). Interestingly, β-glucosidase activity, responsible

for cleaving the linkage between the flavonoid skeleton and the sugar moiety, as well as glycosidic bonds in disaccharides and oligosaccharides, has been observed for several LAB strains/species i.e. *Lactobacillus plantarum, Lactobacillus rhamnosus, Lactobacillus lactis, Lactobacillus acidophilus* (Hur et al., 2014; Rekha et al., 2011), *Streptococcus thermophilus* (Chien et al., 2006).

With the aim to liberate and metabolize kaempferol glucosides from cauliflower outer leaves, four different strains, i.e. *Lactobacillus plantarum* (LMG6907), *Lactobacillus mali* (LMG6899), *Pediococcus pentosaceus* (LMG10740) and *Pediococcus damnosus* (LMG114884) were selected for our study, as presented in **chapter 3**. Unfortunately, there was no difference in the total phenolic content (TPC) as well as the phenolic profile during incubation (6 days). This confirms the lack of ability to produce cell wall degrading enzymes by the studied LAB. Also no change in phenolic profile was observed on a previous prepared phenolic rich extract from cauliflower outer leaves incubated with *L. plantarum*. However β-glucosidase activity has been found in the cells of *L. plantarum* as intracellular activity. This finding is in accordance with previous observations, e.g. the intracellular β-glucosidase has been characterized for other *L. plantarum* spp. (Giraud et al., 1993; Lei et al., 1999), *L. delbrueckii* subsp. *delbrueckii* (Choi et al., 2002) or *Oenococcus oeni* (Mesas et al., 2012), whereas a very low β-glucosidase activity was found in a cell-free extract of *L. casei* subsp. *rhamnosus* (Matsuda et al., 1994).

However from the reported data of soybean fermentations with similar LAB as described above, it is observed that the sugar moiety conjugated at position 7 of the isoflavonons (genistin and daidzin) can be removed through fermentation, hereby forming their

corresponding aglycones (genistein and daidzein). This metabolism indicates that genistin and daidzin were allowed to diffuse into the cells of LAB in which they can be hydrolyzed to produce genistein and daidzein, respectively, by the intracellular β-glucosidase activity. In contrast to these compounds, the similarity of the phenolic profile obtained from fermented samples of cauliflower outer leaves is hypothesized to result from the inaccessibility of the intracellular enzyme, i.e. β-glucosidase, to its substrate (kaempferol glycosides) resolved in the medium. The difference in permeability between these phenolic compounds may arise from their variation in solubility, complex structure and molecular weight. Indeed, most phenolic compounds found in cauliflower outer leaves are kaempferol derivatives with a high degree of glycosylation which result in larger, complex and more hydrophilic forms which cannot penetrate the membrane of Gram positive-bacteria such as LAB (Kepes, 1985; Russ et al., 2000).

The failure in the application of LAB for the improved release and metabolism of phenolic compounds from cauliflower outer leaves has been assessed as a result of their enzyme-producing ability, whereas various fungi have been reported for their capacity to produce a variety of carbohydrate-degrading enzymes. Plenty of fungi naturally secrete the full array of carbohydrate-degrading enzymes (cellulases, hemicellulases, pectinases, α-amylase, xylanases *etc*.). Moreover, various food-grade filamentous fungi such as *Aspergillus niger* (Bhanja et al., 2009), *Aspergillus oryzae* (Wu et al., 2011), *Lentinus edodes* (Zheng et al., 2000), *Rhizopus oryzae* (Yadav et al., 2013), and *Rhizopus oligosporus* (Correia et al., 2004) have also been reported to produce large amounts of β-glucosidase needed to remove sugar

moieties attached to the benzene rings of the phenolic compounds. These observations encouraged us to do another experiment involving fungi.

Based on these promising results in literature, four strains/species of fungi were selected for their potential effect on the release and metabolism of individual phenolic compounds through fermentation (**chapter 4**). As shown, *Aspergillus sojae* was the best one among the fungi used in this work. An interesting 2.8-fold increase (180%) in total amount of individual phenolic compounds was observed for the incubation of cauliflower outer leaves with this fungal strain compared to the non-inoculated sample. In comparison with previous work, this level is much higher than that for *Larrea tridentata* leaves fermented with *Phanerochaete chrysosporium* (33%) (Martins et al., 2013), but lower than that for fermented cranberry pomace with *Lentinus edodes* (around 400 %) (Zheng et al., 2000), or wheat incubated with *R. oryzae oligosporus* (494 %) (Bhanja Dey et al., 2014). Compared to vegetables, the higher amount of bound phenolics in cranberry pomace and wheat could be considered as one of the factors which contribute to these different observations. Indeed, the bound phenolic fraction found in cranberry pomace and wheat account for approximately 75-76 % (Acosta-Estrada et al., 2014) of the total phenolics, while vegetables have 10-32 % bound forms as observed by Chu et al. (2002).

As presented in Figure 5, compared to our own enzymatic treatments, the incubation with *Aspergillus sojae* also exhibited the highest level of phenolic compounds released. More importantly, this treatment resulted in a reduction in the concentration of highly glycosylated compounds, while a huge increase in their corresponding metabolites with lower sugar conjugation was concomitantly observed. For example, two major compounds, i.e.

kaempferol-3-O-glucoside and kaempferol-3-O-diglucoside showed an18-fold and 5-fold increase compared to the unfermented sample, which accounted for around 45 % of the total phenolic content in the extract from fermented samples with *Aspergillus sojae*. While their possible parent compounds, i.e. kaempferol-3-O-diglucoside-7-O-diglucoside and kaempferol-3-O-diglucoside-7-O-glucoside, accounting for about 40 % of the total phenolic content in raw (untreated) sample, decreased remarkably, and thus remained only in a modest proportion (of around 8 % of total phenolic content) in the fermented extract. It can be stated that these shifts in phenolic profile in the fermented extract can contribute to the improvement in their potent biological activity. In fact, Fukumoto et al. (2000) have indicated that the more sugar moieties attached to the aglycone, the more their activity was reduced. In another study, Heijnen et al. (2001) have also reported that the presence of an -OH group at 7-position in ring A enhances the radical scavenging activity of flavonoid compounds.

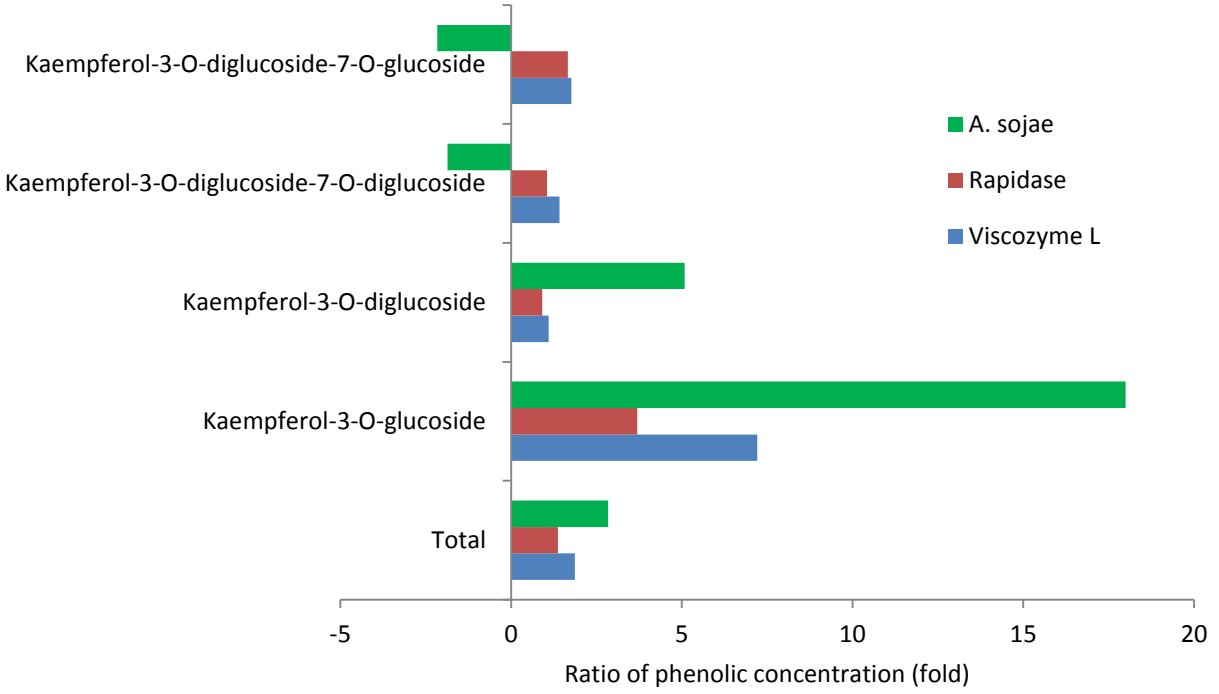

Figure 5. The ratio of average concentration of major and total flavonoid compounds in treated cauliflower outer leaves by Viscozyme L, Rapidase and *A. sojae* relative to untreated one.

It is therefore suggested that the application of fungal fermentation can be considered as the process needed to generate value-added compounds and products for the valorization of cauliflower by-products as a source of phenolic compounds, and this because of the following outcomes obtained: a large increase of phenolic compounds released from the substrate, the metabolism of parent compounds into simpler compounds improving their potential for biological activity, the low price and high rate of fungal growth. However, one of our pursuing aims was the formation of a kaempferol aglycone from its glycosylated compounds which we did not observe during the solid-state fermentation with *A. sojae*. There are some questions that remain to be understood. For instance, it is not known whether the limited accessibility of fungi-producing β-glycosidase to phenolic compounds

could be caused by the solid-state fermentation (SSF) process; or whether the structure of the flavonoid as well as the position of the conjugated sugar (3- or 7-) are able to have an effect on the extent of deglycosylation. Different fungi used may result in a different profile of enzymes produced, or whether there is an inhibition of enzymes or fungi by phenolic compounds released. These hypotheses led to perform our investigation as presented **in chapter 5**.

Therefore, the extract from cauliflower outer leaves was used as a source of kaempferol glycosides, while the source of quercetin derivatives was extracted from onion powder. These extracts were incubated with *R. oryzae, R. azygosporus*, that appeared to exhibit the desirable effect on phenolic metabolism (Irimat, 2014), under submerged fermentation conditions (SmF). As described in chapter 5, the fermentation resulted in the accumulation of kaempferol aglycone in the medium, already detected after 1 day of fermentation. The same is true for quercetin aglycone, with a concentration that increased largely after 1 day of incubation. The amount of these aglycones was also found to be different between the fungi examined and the time of incubation. The deglycosylation of flavonoids to produce their aglycones through submerged culture conditions has also been published recently (Di Gioia et al., 2014; Lin et al., 2014; Liu et al., 2013). It seems to be that in SSF the plant cell wall may be the barrier for the accessibility of phenolic compounds to β-glycosidase secreted from fungi, while this resistance could be removed by SmF and thus improving the accessibility of them to the enzymes i.e. β-glycosidase.

However, some disadvantages could also be seen from our studies using a fungal fermentation to study the extent of release and metabolism of phenolic compounds:

i. The 3-O-glycosidic linkage of aglycone to sugar moieties has not been hydrolyzed by fungi examined under SSF. It is only cleaved under SmF, in which the barrier of plant cell wall for the release of phenolic compounds is removed, and thus the accessibility of substrate and metabolic enzymes are improved.

ii. The differences in period of fermentation result in a shift in phenolic profile in cauliflower outer leaves. The forceful degradation of phenolic compounds occurs under long incubation times.

A proposed pathway for the release and metabolism of phenolic compounds

Based on the results obtained from our investigations (chapter 2, chapter 3, chapter 4 and chapter 5), a possible process for the effective valorization of cauliflower by-products is shown in Figure 6.1. The treatment with commercial enzymes results in the degradation of the cell wall matrix which assists in the extraction of phenolic compounds. Solid-state fermentation with fungi is evaluated as an efficient and low cost alternative for the improved release and metabolism of phenolic compounds from their matrix, thus achieving high value-added phenolic products. Furthermore, the formation of flavonoid aglycones, i.e. kaempferol, and quercetin from their parent glycosidic compounds under SmF conditions with fungi offers a promising tool to develop a biorefinery concept for producing specific aglycones which may meet their demands for laboratory, medical and cosmetic purposes.

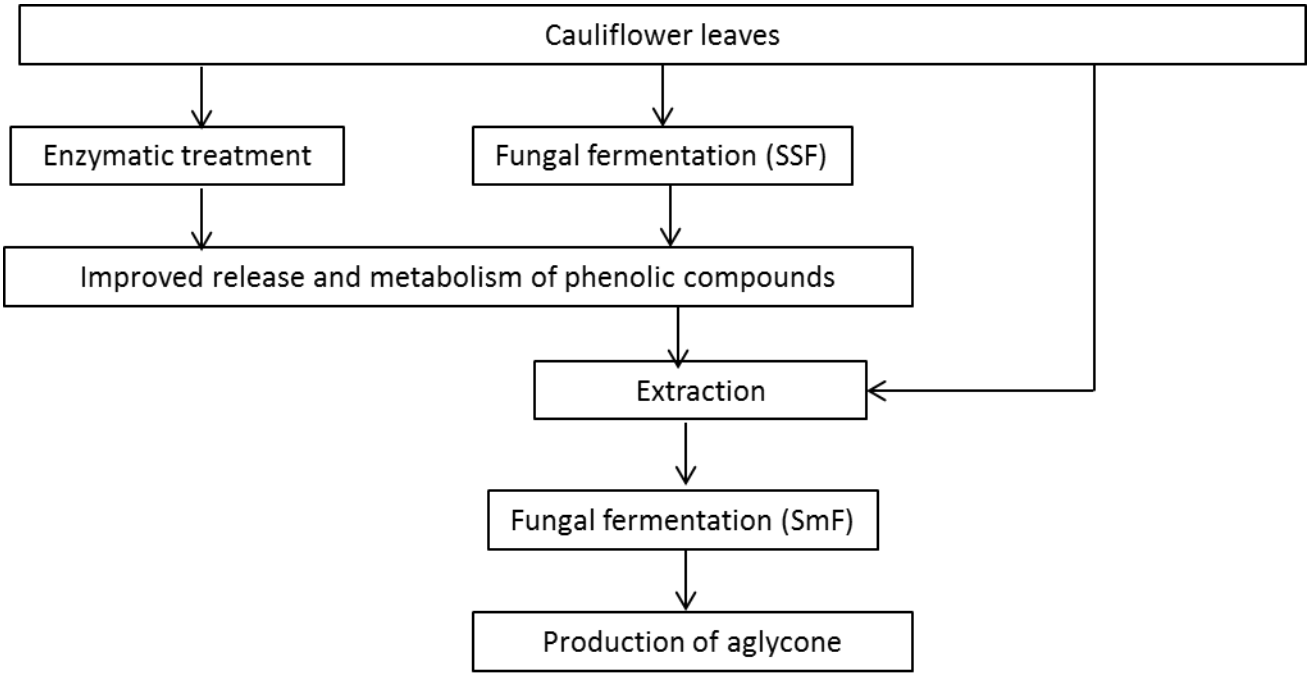

Figure 6.1. A schematic diagram for the release and bioconversion of phenolic glycosides into their metabolites and aglycones

6.2. Further perspectives

This study has mainly been focused on different biological approaches for an improved release and metabolism of phenolic compounds by biological treatments (enzymes and microorganisms). Some further studies need to be performed including:

i. *Optimization of the process parameters in combination with scale-up units*: During the thesis, the phenolic compounds were extracted from samples at fixed, standardized conditions in terms of solvent uses, incubation conditions (pH, temperature, …). It is, however, recognized that the amount and profile of phenolic compounds in the extract might vary as a function of extraction conditions used (Liyana-Pathirana et al., 2005; Silva et al., 2007). Many factors such as solvent composition, ratio of solvent to material,

extraction time and temperature could be further determined to obtain the optimum conditions for the extraction of phenolic compounds. Also, in chapter 4 the samples were taken on the fixed time points during fungal incubation i.e. for all the studied fungi the same time points for sampling. However it should be considered to optimize the sampling times depending on the used fungi, so that the optimum time can be determined for the maximum release of the phenolic compounds in the fermentation broth. Furthermore, the scaling up of the process for biological treatment as well as the extraction conditions needs to be developed from the laboratory unit to pilot scale as a long-term ambition of this study.

ii. *Studying the possibility to use "green solvent", i.e. water for the extraction step to obtain similar results*: As mentioned above, methanol was used during this study as the organic solvent for extraction. With the stricter regulations on the use of organic solvents for extraction of natural products, due to their negative environmental impact and potential hazard to the human health (Mustafa et al., 2011), more environmentally friendly techniques are preferred to date. In this sense, the "green" extraction approach using water as a solvent can be further taken into account, instead of using methanol as described in this study.

iii. *Development of the biorefinery concept including a series of extraction, separation, and purification methods to produce purified extract or specific compounds*: The extract obtained from cauliflower outer leaves is considered as an extract of dilute solvent due to the methanol and water that remained in the final product. Besides, various non-phenolic components (e.g. toxic components and contaminants) from the matrix can also be

present. In order to obtain purified extracts/individual (target) phenolic components as final products, the raw extract need to be processed to a food-grade or pharmaceutical-grade state using a series of steps including concentration, separation and purification which need to be further considered.

iv. *Testing the biological activity of the extract, or fungal metabolites obtained in both in vitro and in vivo analysis*: The analysis of the biological activity of the extract/metabolites has not been conducted in this study. Therefore, the determination of their activity (i.e. antioxidant capacity, antimicrobial property, bioavailability, angiotensin I-converting enzyme inhibitory properties, …) using both *in vitro* and *in vivo* tests is needed

v. *Indicate the specific enzymes playing a key role for the release as well as biotransformation of phenolic compounds*. *Based on this finding, the specific microorganism responsible for producing these extracellular enzymes can be further studied:* This study was done to draw attention to the biological approaches suitable to enhance the release and metabolism of phenolic compounds from its matrix. There is still a lack of comprehension of the specific enzymes responsible for these catalysis, and their producer microorganisms, which can be recommenced for further research.

vi. *The inhibitory and/or deactivator effect of phenolic compounds obtained in this study on common cell wall-degrading enzymes, i.e. cellulase, pectinase, and β-glucosidase*: Many phenolic compounds have been documented as inhibitors of cell wall-degrading enzymes (Kim et al., 2011; Tejirian et al., 2011; Ximenes et al., 2010), but literatures available involving phenolic compounds obtained in this study still remain scarce. This could be taken into account as future work.

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A1 Publications in international scientific journals with peer-review

Huynh, N.T., Smagghe, G., Gonzales, G.B., Van Camp, J. & Raes, K. (2015). Extraction and bioconversion of kaempferol metabolites from cauliflower outer leaves through fungal fermentation. Biochemical Engineering Journal, http://dx.doi.org/10.1016/j.bej.2015.12.005.

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Presentations

Huynh, N.T., Smagghe, G., Gonzales, G.B., Van Camp, J. & Raes, K. (2015). Improved release and metabolism of flavonoids from brassica waste by biological treatments. Symposium 'Belgo-Vietnamese Rector's Mission 2015. Gent, Belgium. October 2015 (poster presentation).

Huynh, N.T., Smagghe, G., Gonzales, G.B., Van Camp, J. & Raes, K. (2014). Enhancement and bioconversion of phenolic compounds from Brassica using solid-state fermentation by filamentous fungi. 24th International ICFMH conference-FoodMicro 2014. Nantes, France, September 2014 (poster presentation).

Huynh, N.T., Smagghe, G., Gonzales, G.B., Van Camp, J. & Raes, K. (2014). Enzyme-assisted extraction to enhance the release of phenolic compounds from cauliflower leaves. 8th World congress of Polyphenols Application. Lisbon, Portugal, June 2014 (poster presentation).

Participation in School Specialist Courses

EUBIS training school (2014). Food waste processing in the frame of the biorefinery concept. Lisbon, Portugal, July 2014.

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