JOSÉ VALDO MADEIRA JUNIOR

Tannase Production and Phenolic Compounds Obtainment by Biotransformation of *Paecilomyces variotii* From *Citrus* Residues

Produção de Tanase e Obtenção de Compostos Fenólicos através da Biotransformação por *Paecilomyces variotii* a partir de Resíduos de *Citrus*

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Orientadora: Gabriela Alves Macedo

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Abstract

Tannases, tannin acylhydrolases (EC 3.1.1.20), are an important group of biotechnologically relevant enzymes, which presents great potential in industrial applications, especially on antioxidant production. However, the practical use of this enzyme is at present limited due to insufficient knowledge about its properties, optimal expression and expensive production process. The tannase catalyzes the ester bonds present in hydrolyzed tannins, gallotannins and some glycosylated phenolic, such as hesperetin and naringin. Phenolic compounds have been the focus in research for its action in human metabolism, acting in the prevention of chronic diseases, and therefore, important antioxidants in the food industry. However, the processes of obtaintion are by chemical synthesis or extraction from plant sources, which acids, bases and organic solvents are used. An alternative to minimize these problems, environmentally friendly processes may be realized, such as by fermentation or enzymatic extraction. This project aimed to the production of tannase and phenolic compounds by the fungus *Paecilomyces variotii*, using as substrate *Citrus* residues (*Citrus latifolia*, *Citrus sinensis* Hamlin, Valencia, Pera riu e Pera Natal). Firstly, the tannase production was carried out under different conditions of the solid-state fermentation (SSF) process, such as particle substrate size, moisture of substrate, inductor compounds, nitrogen source and temperature of incubation. Therefore, the enzyme produced was semi-purified and biochemically characterized. The SSF process increased the tannase production at ten-fold, and biochemical characterization showed a stable and reliability enzyme. The second part, two biotransformation processes in *Citrus* residues for phenolic compounds obtainment were carried out, by fermentation and enzymatic reaction: for fermentation process, temperature of incubation, moisture of substrate and substrate particle size were evaluated; for enzymatic process, the conditions tested were the

enzymatic activity of the pectinase, cellulase and tannase (produced in this work). The two biotransformation processes increased, at least, ten-fold of bioactive phenolic concentration, such as hesperetin, naringenin and ellagic acid. The bioactive phenolic extracts obtained by *Citrus* residues biotransformation processes showed an increase of antioxidant and tumor anti-proliferation type colorectal potential. These results suggest that SSF and biotransformation processes may be viable alternatives for tannase and rich bioactive phenolic extract productions, respectively, using residues at low price values.

Resumo

As tanases, tanino acil hidrolases (EC 3.1.1.20), são um importante grupo de enzimas na área de biotecnologia, que apresenta grande potencial em aplicações industriais, especialmente na produção de antioxidantes. No entanto, o uso prático desta enzima está limitado, devido ao insuficiente conhecimento sobre suas propriedades, condições ótimas de ação e caro processo de produção. A tanase catalisa a hidrolise das ligações ésteres presentes em taninos hidrolisáveis, galotaninos e alguns fenólicos glicosilados, como hesperidina e naringina. Compostos fenólicos têm sido foco em pesquisas por sua ação no metabolismo humano, agindo na prevenção de doenças crônicas, sendo portanto, importantes antioxidantes na indústria de alimentos. Contudo, os processos de obtenção são por síntese química ou extração a partir de fontes vegetais, nos quais são usados ácidos, bases e solventes orgânicos. Uma alternativa para minimizar estes problemas são os processos ambientalmente corretos, que podem ser por fermentação ou extração enzimática. Este projeto teve como principal objetivo a produção da tanase e compostos fenólicos pelo fungo *Paecilomyces variotii*, utilizando como substrato resíduo de frutas cítricas (*Citrus latifolia*, *Citrus sinensis* Hamlin, Valencia, Pera riu e Pera Natal). Primeiramente foi realizada a produção da tanase em resíduo de frutas cítricas sob diferentes condições do processo de fermentação em estado sólido (FES), como granulometria do substrato, hidratação do substrato, adição de indutores, fonte de nitrogênio, temperatura de incubação, entre outros. Em seguida, esta enzima foi semi-purificada e caracterizada bioquimicamente. O processo de FES aumentou a produção de tanase em 10 vezes, e a caracterização bioquímica mostrou uma enzima estável para uso. Após esta etapa, foram realizados dois processos de obtenção de compostos fenólicos, via fermentativa e enzimática, a partir de resíduo de *Citrus*. Para o primeiro processo foram avaliadas as condições da temperatura de

incubação, hidratação do substrato e granulometria do resíduo; para o segundo, as condições testadas foram as atividades enzimáticas da pectinase, celulase e tanase (produzida neste trabalho). Os dois processos de biotransformação aumentaram, pelo menos, 10 vezes a concentração dos compostos fenólicos bioativos, como hesperetina, naringenina e ácido elágico. Os extratos de fenólicos bioativos obtidos pelos dois processos de biotransformação em resíduo de *Citrus* mostraram um aumento nos potenciais antioxidante e anti-proliferativo em células tumorais do colorretal. Estes resultados sugerem que os processos de FES e biotransformação podem ser alternativas viáveis para produção de tanase e compostos fenólicos bioativos, respectivamente, usando resíduos de baixo valor comercial.

SUMÁRIO

“With great power there must also come great responsibility”

Stan Lee (Spider-Man, 1962)

Dedicatória

À minha família: Mãe, Pai, Rafa, Kelly,

Michelle, Bruno, Gabs e Manuzita.

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Lista de Abreviaturas

SSF – Solid-State Fermentation

FES – Fermentação em Estado Sólido

UV – Ultraviolet Radiation

NRRL – Agricultural Research Service (ARS)

CBMAI – Confederação Brasileira de Micro-organismos de Ambiente e Indústria

PDA – Potato Dextrose Agar

CCD – Central Composite Design

ANOVA – Analysis of Variance

FAO – Food and Agriculture Organization

HPLC-DAD – High Pression Liquid Chromatographic – Diode Array Detector

AAPH – 2,2'-azobis(2-methylpropionamidine)

DPPH – 2,2-diphenyl-1-picrylhydrazyl

SRB – Sulforhodamine B sodium salt

ORAC – Oxygen Reaction Absorbance Capacity

HT29 – Human colorectal adenocarcinoma

Introdução Geral

A tanase, também conhecida como tanino acil hidrolase (EC 3.1.1.20), catalisa a hidrólise de ligações ésteres de taninos hidrolisáveis, liberando ácido gálico e glicose. Esta enzima tem grande potencial nas indústrias química, farmacêutica e alimentícia. Para a produção da tanase, a fermentação em estado sólido apresenta vantagens sob a submersa, como maior produção enzimática de forma extracelular, maior produtividade, maior estabilidade de pH e temperatura, e menor custo do produto. Este menor custo se dá, entre outros motivos, pelo uso de resíduos agroindustriais e menor quantidade de água no meio. O grupo de pesquisa do laboratório de Bioquímica de Alimentos (DCA – FEA – UNICAMP) iniciou um estudou da produção da tanase em resíduo de *Citrus*. Os resultados obtidos mostraram grande potencial da produção enzimática, e simultâneo aumento do potencial antioxidante no resíduo fermentado, no qual foi constatado que este potencial se dava pelo aumento da concentração de compostos fenólicos bioativos.

Compostos fenólicos são definidos como substâncias que possuem anel aromático ligado por uma ou mais hidroxilas. Eles são originados principalmente do metabolismo secundário de plantas a partir da fenilalanina (Treutter, 2001). Estes compostos são considerados importantes para os vegetais, tanto em estrutura quanto na sua fisiologia. Além disso, para as plantas, os fenólicos podem atrair insetos polinizadores, contribuir na pigmentação de flores e frutos, atuar na proteção oxidativa e proteger contra agentes da luz UV; desempenhando assim papel efetivo no crescimento e desenvolvimento vegetal (Martins et al., 2011; Soto et al., 2011).

Nos últimos anos, grande atenção tem se dado aos compostos fenólicos que apresentam atividade biológica. Estudos indicam que os mesmos podem reduzir a incidência de doenças neuro-degenerativas, cardiovasculares e diversos tipos de cânceres.

Estes compostos apresentam alta capacidade antioxidante, anti-mutagênica, anti-alergênica, anti-inflamatória e antimicrobiana (Ferk et al., 2011; Tsai et al., 2011; You et al., 2010). Devido a essa gama de características benéficas para o organismo, pesquisas têm sido intensificadas com o objetivo de encontrar frutas, vegetais e resíduos agroindustriais como fontes de compostos fenólicos. Cada espécie vegetal apresenta um perfil fenólico único, que vai de acordo com suas características fenotípicas, o que reforça o estudo destes compostos bioativos em diversos tipos de matrizes.

Estes compostos são obtidos através da extração sólido-líquido ou líquido-líquido, empregando solventes orgânicos em sistemas de fluxo de calor. Além destas, outras técnicas têm sido usadas, como processos de alta pressão, incluindo o uso de fluídos supercrítico, extração por micro-ondas ou ultrassom (Hayat et al., 2010; Setianto et al., 2009; Capote et al., 2007).

A obtenção de compostos bioativos por processos biológicos a partir de resíduos agroindustriais ricos em compostos fenólicos é também uma alternativa que merece atenção, já que minimiza o uso de compostos tóxicos na extração, como os solventes orgânicos. Neste processo, os fenólicos podem ser obtidos por duas vias: biotransformação microbiana, em que os fenólicos ficam disponibilizados através do metabolismo microbiano; ou por biotransformação enzimática, na qual se utilizam enzimas exógenas que liberam estes compostos a partir do substrato (Martins et al., 2011; Puri et al., 2012). Uma dessas enzimas com grande potencial é a tanase, que apresenta trabalhos na ação hidrolítica de polifenóis para aumento do potencial biológico (Battestin et al., 2008; Ferreira et al., 2013).

Assim, o primeiro objetivo do presente trabalho foi otimizar a produção da tanase em resíduo de *Citrus* pelo fungo *Paecilomyces variotii*, através da fermentação em estado

sólido. Em seguida, a enzima obtida foi semi-purificada e caracterizada bioquimicamente. A etapa seguinte foi a obtenção dos fenólicos bioativos em resíduo de *Citrus* por dois processos: pela biotransformação microbiana empregando o fungo *P. variotii*, e a biotransformação enzimática pelas enzimas celulase, pectinase e tanase produzida neste trabalho. Por fim, a partir dos produtos obtidos pelos processos de biotransformação, foram avaliados o potencial biológico, ou seja a capacidade antioxidante e anti-proliferativa de células tumorais.

O emprego do resíduo de *Citrus* nestes processos representa a busca por novas alternativas de produção de enzimas e compostos fenólicos, a fim de baixar o custo de produção, além de dar destino mais nobre aos resíduos abundantes em nosso país. Este trabalho poderá trazer uma significativa contribuição ao estudo de produção de compostos fenólicos bioativos e também das enzimas induzíveis, a partir de resíduos obtido da extração do suco de laranja. Muitos compostos fenólicos são de difícil obtenção e muitas vezes requerem tratamentos que são agressivos ao meio ambiente. Além disso, a tanase produzida e usada neste projeto, apresenta grande potencial na aplicação industrial, embora o custo e os poucos produtores no mundo dificultem sua inserção no mercado, adicionando assim mais um fator de importância no estudo dessa enzima.

O presente trabalho foi dividido em 4 capítulos, sendo que o capítulo 1 apresenta uma revisão bibliográfica sobre os bioprocessos de obtenção dos compostos fenólicos, publicado na revista Critical Reviews in Biotechnology.

No capítulo 2 foram abordados aspectos da produção da tanase em FES utilizando como substrato resíduo de *Citrus*, utilizando o fungo *P. variotii*. O estudo da produção da tanase foi realizado empregando a metodologia de superfície de resposta, avaliando os principais parâmetros de fermentação dessa produção. Em seguida, foram avaliadas as

características bioquímicas da enzima produzida, na forma semi-purificada, em relação a sua atividade catalítica e estabilidade em diferentes temperaturas e valores de pH.

O capítulo 3 descreve os dois processos de obtenção de compostos fenólicos: sendo a primeira parte através da fermentação em estado sólido e a segunda pela reação enzimática. Na primeira parte foram avaliadas as melhores condições de fermentação utilizando o *P. variotii* no resíduo de *Citrus* para maior liberação dos compostos fenólicos de interesse. Na segunda parte, a reação enzimática foi estudada nas melhores condições de atividade das enzimas, pectinase, celulase e tanase, também em resíduo de *Citrus* para maior liberação de compostos fenólicos. Este capítulo foi dividido em dois artigos científicos, sendo que a etapa da obtenção de fenólicos por FES foi aceita para publicação na edição especial "Green Processes and Eco-technologies" da revista Chemical Engineering Research and Design.

O último capítulo trata da avaliação biológica dos resíduos tratados tanto por fermentação em estado sólido quanto por reação enzimática. Foram avaliados a atividade antioxidante e potencial anti-proliferativo de células tumorais do cólon-retal dos resíduos tratados. Assim, com o objetivo de avaliar o potencial destes dois processos para obtenção de compostos fenólicos de alto valor biológico.

CAPÍTULO I. Biotransformation and bioconversion for phenolic compounds obtainment: an overview

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Abstract

Phenolic compounds have recently been recognized for their influence on human metabolism, acting in the prevention of some chronic diseases as well as proving to be important antioxidants in food. Nevertheless, the extraction and concentration processes are usually carried out by organic solvent extraction from natural sources and can generate some drawbacks like phenolic compound degradation, lengthy process times and low yields. As a solution, some eco-friendly technologies, including SSF or enzymatic-assisted reaction, have been proposed as alternative processes. This paper reviews the extraction of phenolic compounds from agro-industrial co-products by SSF, even as friendly enzyme-assisted extraction. It also discusses the characteristics of each bioprocess system and the variables that affect the product formation, as well as the range of substrates, microorganisms and enzymes that can be useful for the production of bioactive phenolic compounds.

Resumo

Os compostos fenólicos têm recebido constante atenção devido sua influência no metabolismo humano, agindo na prevenção de algumas doenças crônicas ou mesmo como antioxidantes em alimentos. De qualquer forma, os processos de extração e concentração

destes compostos são relativamente longos, de baixo rendimento e agressivos para o meio ambiente. Como alternativa, algumas tecnologias ambientalmente amigáveis podem ser utilizadas para obtenção dos compostos fenólicos, como a FES e as reações enzimáticas. Este trabalho trata de uma revisão da literatura sobre a obtenção de compostos fenólicos a partir de resíduos agroindustriais pela FES e também pela extração enzimática assistida. Este trabalho também discute as características de cada bioprocesso e das variáveis que afetam a formação do produto, como o tipo de substrato, micro-organismos e enzimas utilizadas, entre outros.

I.1. Introduction

In recent years, attention has turned to phenolic compounds with biological activity due to their ability to promote benefits for human health, such as reduction in the incidence of some neurodegenerative diseases, reduction in the occurrence of factors linked to cardiovascular disease and antioxidant, anti-mutagenic, anti-allergenic, anti-inflammatory and anti-microbial activities (Martins et al. 2011; Tripoli et al. 2007). Some phenolic compounds may have an important technological role in the prevention of lipid oxidation and other substances, thereby increasing their shelf life (Pokorný 2007). Research has been intensified in order to find plants and agricultural co-products that produce bioactive phenolic compounds. Agricultural co-products, as well as plant species, have a unique profile that will produce phenolics according to specific needs and the characteristics provided by the environment. Thus, each co-product has a concentration of a specific pronounced phenolic compound, enabling the production and extraction of a variety of bioactive compounds for use (Aliakbarian et al. 2011; Harrison et al. 2012; Martins et al. 2011; Pingret et al. 2012; Saad et al. 2012; Wu et al. 2012).

Phenolic compounds are derived mainly from the secondary metabolites of plants from phenylalanine (Treutter 2001). Numerous compounds of different chemical structure are grouped together, including: hydroxybenzoic acids, hydroxycinnamic acids, flavanols, anthocyanins and tannins (Martins et al. 2011). These compounds are considered important to the plants, both structurally and physiologically. In plants, they can attract pollinating insects, contribute to the pigmentation (flowers and fruits), act as antioxidant agents, protect against UV light, and finally, play a role in plant growth and development, preventing the action of pathogens and predators (Martins et al. 2011; Soto et al. 2011).

Currently, phenolic compounds are obtained by chemical synthesis or extraction. Phenolic compounds are recovered from natural sources by solid-liquid or liquid-liquid extraction employing organic solvents in systems of heat. However, other techniques have been used to obtain these compounds, including the use of supercritical fluids, high pressure processes, and extraction by microwave or ultrasound. Over the past five years, more than 1700 articles on extraction of phenolic compounds have been published, demonstrating that this type of process is already well studied. So far, most studies published discuss obtaining phenolic compounds using chemical and physical processes. As an example, commercial ellagic acid is obtained by chemical extraction of ellagitannin-rich materials, such as plants, using acidified methanol. Such a process results in high cost and high contamination of the product and is aggressive with a low income (Aguilera-Carbó et al. 2009; Lei et al. 2001; Wilson & Hagerman 1990).

Some of these processes are not feasible for the food and/or pharmaceutical industries, in both cases because of cost, use of organic solvents and low rate of productivity (Capote et al. 2007; Hayat et al. 2010; Setianto et al. 2009).

The biotransformation of bioactive compounds is also an interesting alternative that deserves attention, since it precludes the use of toxic compounds such as organic solvents in the extraction. In these processes, bioactive compounds are obtained from natural sources by microorganisms through their secondary metabolism or by exogenous enzymatic action (Martins et al. 2011; Puri et al. 2012).

The purpose of this paper is to provide an overview of the study of production and obtainment of phenolic compounds by biotransformation using agro-industrial co-products.

I.2. Obtaining Phenolic Compounds by Biotransformation

Biotransformation can be defined as chemical transformations that are catalyzed by biological systems through their effective enzyme activity or by microorganisms through SSF (Banerjee et al. 2012; Martins et al. 2011). In addition, this bioprocess has received great attention due to the potential conversion of inexpensive agro-industrial co-products, as well as plants, in a great variety of valuable compounds.

In this review, processes were shown using the technique of SSF and enzymatic reaction as alternative processes for obtaining different phenolic compounds.

I.2.1. Solid-State Fermentation in Phenolic Obtainment

SSF has many advantages, such as high biotechnological productivity, high concentration, product stability and growth of microorganisms in non-water soluble substrates. It also has some disadvantages, such as formation of a temperature gradient throughout the fermented substrate and difficulty in controlling the pH and the amount of water. These problems result in reduced mobility of nutrients derived from reduced movement of the water in the substrate. Changes in temperature and water content in the

substrate can be caused by heating resulting from fermentation, which makes it difficult to control the substrate under uniform conditions. However, there is great interest in the SSF process among researchers and industries, particularly due to the fact that the process is usually cheaper with higher productivity than submerged fermentation (Barrios-González 2012; Singhania et al. 2009).

SSF emerged as an attractive alternative for obtaining phenolic compounds. However, they are expensive compounds using current methods of extraction (Martins et al. 2011; Sepúlveda et al. 2011).

The first report of the production of phenolic compounds is from Betts et al. (1974), which describes a screening of over 40 microorganisms for the production of compounds with antitumor activity. According to the results, there was a 30% increase in the production of phenolic 9-hydroxyacronycine by microorganism *Cunninghamella echinulata* NRRL 3655 in a stirred fermentor.

There are patents reported for the production of polyphenol using microbial fermentation. Kanji (1991) reported the production of O-methylated phenolic compounds from the hydroxyl grouping by the microorganism *Aspergillus repens* in liquid medium. Another process for the production of gallic acid was created using a mixed culture of *Aspergillus foetidus* and *Rhizopus oryzae* residues, rich in tannins (Banerjee & Mukherjee 2003).

Table I-1 presents the process used for the production of phenolic compounds by SSF. Below, some works are cited regarding SSF used in the process of obtaining phenolic compounds.

The choice of residue interferes deeply in obtaining the phenolic compound of interest. Machado et al. (2012) studied the selection of fungal strains with potential growth

and release of phenolic compounds in two coffee residues, coffee silverskin (CS) and spent coffee ground (SCG), by SSF. The strains GH2 *Penicillium purpurogenum*, *Neurospora crassa* and *Mucor sp*. 3P showed higher growth conditions in the SCG and increased the release of phenolics by approximately 40%. The increasing availability of phenolic compounds varies according to the type of residue. Regarding the silverskin, the spent coffee ground has a higher content of phenolic compounds, such as catechin, epicatechin, chlorogenic acid, protocatechuic acid and ferulic acid, among others. Thus, the choice of residue as the substrate for fermentation is important for achieving different phenolic compounds.

Besides the residue, the microorganism interferes in the fermentation and obtainment of phenolic compounds. Cai et al. (2012) reported the effect of three different microorganisms in the fermentation of oat bran as a source of phenolic compounds. After the SSF, the phenolics, caffeic and ferulic acids, respectively, presented: 230 and 790% increase for *Aspergillus oryzae* var. effuses, 170 and 450% for *Aspergillus niger*, 37 and 0% increase for *Aspergillus oryzae*. According to the analyses, the phenolic compounds in larger quantities are caffeic and ferulic acids, which are in esterified form. With hydrolysis by microorganisms, these compounds increased solubility and facilitated their extraction, showing great advantage over other processes that use high temperatures and therefore generate high energy costs. This difference in clearance of phenolic compounds is due to the metabolic activity of each microorganism. This case is related to various types of microbial enzymes and their activities.

After selecting the microorganism, fermentation kinetics should be performed in order to evaluate the obtainment of the product during the process. Georgetti et al. (2009) evaluated the bioconversion of polyphenol glycosides from soybeans to form non-

glycosides through SSF by *Aspergillus awamori*. The conversion of the glycoside to the form of phenolic non-glycoside was accompanied by production of the enzyme β-glucosidase. The non-glycoside form presents a greater number of free hydroxyl groups in regard to glycoside, thus increasing their biological activity. According to the results, the concentration of genistein increased by 1880% in fermented soy. The production of β-glucosidase increased with fungal growth during 48 h of incubation at 30 °C, showing values of 1000 U.ml-1, leading to the conclusion that the enzyme was responsible for the release of phenolic compounds in the soybean during the fermentation. After 48 h, enzyme activity dropped approximately 20%. In this case, studies have indicated that high concentrations of genistein inhibited the activity of β-glucosidase, which in turn inhibits the hydrolysis of phenolic glycosides. Therefore, one should evaluate the kinetics of the release of phenolic compounds so that no interference inhibits its extraction.

Another important factor for obtaining phenolic compounds is the addition of water to the substrate for microbial growth. A strain of *Aspergillus* was subjected to fermentation in grape residue to increase phenolic antioxidants. Fermentation kinetic was performed to evaluate the time when gallic acid is present in higher concentration. The results showed that after 6 h of incubation, the concentration of gallic acid increased 100%. After 15 h of incubation, the concentration of gallic acid decreased abruptly, probably indicating that the phenolic compounds were present in the metabolism of the microorganisms. During the process, it was noted that in SSF, adding water to the substrate is extremely important, especially if the substrate has hemicellulose and pectin, which can absorb more water, potentially leading to an increase in microbial growth in the substrate. The most commonly used materials for SSF are those with high water absorption levels, since the moisture

content of these materials can be modified during the bioprocess (Martinez-Ávila et al. 2011).

In addition to the presence of water in the substrate, the relative humidity also affects the product of interest. Bhanja et al. (2008) compared a new solid-state reactor to the conventional process, for the enrichment of the phenolic in rice by *Aspergillus oryzae* for 72 h at 30 °C. The results showed that the reactor increased the concentration of phenolics to 330 $mg.g^{-1}$ as compared to the conventional process, the increase of which was 270 $mg.g^{-1}$. This result is explained because in the first case, supplemental oxygen, heat transfer and water activity were monitored in the reactor, which increased the fungal growth and production of the phenolic. Probably, with circulation of humidified air, the heat produced by the fermentation was dissipated and also facilitated the oxygen permeability into the substrate. At the same time, this maintenance of humidified air maintains the water percentage in the substrate during fermentation, which increases fungal growth and, consequently, the release of aglycon phenolics.

Several other factors also influence the production of phenolic compounds, such as pH. The fermentation by fungus *Chaetomium globosum* was assessed in cottonseed and sugar cane bagasse, under alkaline conditions for the production of phenolic compounds. The alkaline stress is an important factor in the production and/or release of these compounds. According to the results, there was an increase of 620 and 500% of gallic acid in the sugar cane bagasse and cottonseed, respectively. The results showed a linear correlation between increasing pH and the amount of gallic acid. Probably at a pH level between 10 and 12, the enzymes β-endoglucanase, β-glucosidase and β-exoglucanase were produced, which have higher enzymatic activity and thus their respective substrates hydrolyzed, finally releasing the phenolic compounds (Ravindran et al. 2011).

Therefore, several works have been carried out in order to optimize the production of phenolic compounds, reducing the costs of the process, as well as evaluating the effects that influence the yield of the final product. The properties of the agro-industrial co-products used, such as particle size, biodegradability, water absorption and water activity, in addition to their chemical composition, should be evaluated for obtainment of high yield phenolic compounds (Martínez-Ávila et al. 2011). Most papers showed that the main factors affecting the fermentation are temperature, pH, aeration, water activity, microorganisms, moisture and substrate. According to studies, the latter three factors have further interference in the final product, in this case, the production of phenolic compounds (Cai et al. 2012; Georgetti et al. 2009; Machado et al. 2012; Martínez-Ávila et al. 2011; Martins et al. 2011; Ravindran et al. 2011).

I.2.2. Enzymatic Processing in Phenolic Obtainment

Enzyme production is an important field in biotechnology, having worldwide sales near five billion dollars annually, with a growth rate of approximately 6.5 to 10%, while the number of patents and research papers is listed in ascending publication (Panke et al. 2004). The use of enzymes, especially in biocatalysis in agro-industrial residues, has been introduced for the hydrolysis of plant cell walls, which is complexed with polyphenols. In this scenario, it should be concluded that enzymes can act on this substrate in plant cells.

Table I-2 presents some published procedures for the extraction of phenolic compounds by the action of microbial enzymes from agro-industrial waste. The main enzymes used in the process of obtaining phenolic compounds are reported below, as well as the work performed.

Cellulases, xylanases and ligninases are enzymes that are capable of breaking the structure of the hemicellulose, cellulose and lignin of a plant cell wall. They usually have three important complex enzymes, endo-1,4-β-glucanase, cellobiohydrolase and cellobiase. These enzymes work cooperatively to catalyze the hydrolysis of cellulose. The effect of these enzymes is the release of cellobiose and glucose. Cellulases have numerous applications and biotechnological potential for chemicals, fuel, beer, animal feed, textile, and pulp and paper (Gnana Soundari & Sashi 2009; Khandeparker & Numan 2008; Kirby 2005; Lin et al. 2011; Paës et al. 2012; Verma et al. 2011).

Min et al. (2006) studied the conditions of dry and humidified stems of sweet potatoes for release of ferulic acid using primarily commercial β-glucanase. The reaction was performed with enzyme mixtures, degrading plant cell walls, Ultraflo-L Viscozyme (Novozymes A/S) and the α-amylase (Sigma-Aldrich) in a buffer solution with pH 6.0 at 37 °C spinning at 12 rpm for 12 h. The results showed a concentration of 0.5% of Viscozyme-Ultraflo L, 50 mU of released α-amylase and 6 mg of ferulic acid / g of substrate humidified, which is 3 times higher compared to the dry substrate. The ferulic acid increase in the humidified substrate must have been due to the fact that the enzymes were easily adsorbed into the substrate, which facilitated and enhanced the catalytic action.

Moore et al. (2006) evaluated commercial enzymes and their actions regarding the release of phenolics in wheat bran. The reaction was conducted in the dark with 10% humidity at room temperature for 72 h. The results indicated that extracts of multi-enzyme complexes (e.g. Ultraflo-L - Novozymes A/S) had a greater effect than the addition of various purified enzymes. However, each enzyme complex responds differently to each type of substrate. As previously mentioned in this paper, the effects of moisture in the

reaction can influence the release of phenolics. However, this condition should be evaluated together with the catalytic activity of the enzyme.

The pectinases are a heterogeneous group of enzymes that degrade pectin. These enzymes are especially important in the industrial sector and are used in various segments, such as clarification of fruit juice and wine, and product manufacturing of pectin hydrolysate extract oil from seeds and pigments. Degradation of the pectin molecule occurs through a coordinated action of multiple synergistic and pectinolytic enzymes, including pectin, polygalacturonase, pectate-lyase and pectin-lyase. The pectin-lyase (EC 4.2.2.10) and polygalacturonase (EC 3.2.1.15) are of great relevance to the process of depolymerization of pectin, acting in the cleavage of glycosidic bonds α-1,4, polygalacturonic acid and pectin, respectively (Alimardani-Theuil et al. 2011; Kashyap et al. 2001; Pedrolli & Carmona 2009).

Oszmianski et al. (2011) studied the action of pectinase on apple pomace to increase the availability of phenolic compounds for the enrichment of apple juice. According to the results, pectinase increased the concentration of phenolic compounds by 245%, especially procyanidins, flavan-3-ols and flavonols. Apple peels always had tangibly higher concentrations of proanthocyanidins than whole apples. Procyanidins have been shown to bind readily to cell-wall polysaccharides through hydrogen-bonding and/or hydrophobic interactions.

Table I-1. Phenolic compounds production by microbial fermentation of agro-industrial residues.

substrate	micro-organism	incubation[a]	phenolic compounds	Increased (%)[b]	references
cranberry residue	*Lentinus edodes*	120h/28°C	ellagic acid	37.5	Vattem et al. (2004)
cheonggukjang	*Bacillus pumilus*	60h/37°C	gallic acid	233.3	Cho et al. (2009)
tar bush	*Aspergillus niger*	5d/30°C	pyrocatechol	150	Ventura et al. (2009)
creosote bush	*Aspergillus niger*	4d/30°C	gallic acid	1700	Ventura et al. (2009)
soybeans residue	*Lentinus edodes*	30d/25°C	catechin	83.3	McCue et al. (2004)
soybean seed	*Trichoderma harzianum*	7d/25°C	genistin	200	Singh et. al. (2010)
wheat bran	*S. cerevisiae*	48h/32°C	ferulic acid	75	Moore et. al. (2007)
Lupinus angustifolious seed	*Bacillus subtilis*	48h/30°C	catechin	489.5	Fernandez-Orozco et al. (2008)

[a] temperature and time of incubation.

[b] [(FC-IC)/IC]*100; IC: initial concentration, FC:final concentration.

The β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) catalyzes the hydrolysis of disaccharide glycosides and conjugates from the non-reducing end. The β-glucosidase enzyme has numerous applications in the food and pharmaceutical industries, working in the hydrolysis of cellobiose to glucose, the process of conversion of cellulose to glucose in combination with other cellulolytic enzymes, and the release of aroma compounds in fruit juices and wine. Also used in the hydrolysis of cyanogenic compounds present in plants for hormone replacement therapy (Puri et al. 2012; van den Brink & de Vries 2011).

Hamza et al. (2012) studied the action of the multienzymatic complex β-glucosidase (4600 U.ml-1), esterase (200 U.ml-1), α-amylase (92 U.ml-1), xylanase (5.4 U.ml-1) and carboxymethyl-cellulase (0.6 U.ml-1) in waste water from olives for the production of hydroxytyrosol. According to the results, the oleuropein could be hydrolyzed and obtain hydroxytyrosol. The reaction probably occurred during the breakdown of the glycosidic bond and resulted in the formation of hydroxytyrosol and elenolic acid (Figure I-1).

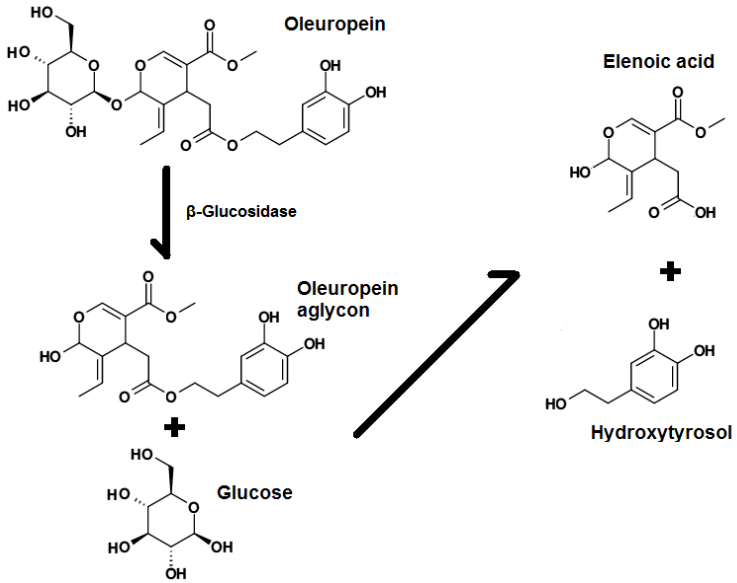

Figure I-1. Enzymatic reaction to Hydroxytyrosol from Oleuropein (Khoufi et al., 2011).

Table I-2. Phenolic compounds production by microbial enzyme of agro-industrial residues.

substrate	enzyme(s)	enzymatic activity	incubation[a]	phenolic compounds	Increased (%)[b]	references
corn cobs	esterase xylanase	0.367 nkat.g-1 1.483 nkat.g-1	45h/pH5.0/50°C	coumaric acid	1100	Topakas et al. (2004)
red dragon pomace	pectinase	10,292 PGU.ml-1	6h/30°C	gallic acid	87.5	Kunnika & Pranee (2011)
olive wastewater	β-glucosidase esterase	3,000 U.ml-1 100 U.ml-1	2h/pH4.8/50°C	hydroxytyrosol	610	Khoufi et al. (2011)
goldenberry pomace oil	cellulase pectinase	- 300 U.ml-1	2h/pH4.3/50°C	caffeic acid	102	Ramadan et al. (2008)
rice flour	α-amilase β-glucosidase	100 U.ml-1 6 U.ml-1	15min/50°C	free phenolics	139	Bhanja et al. (2008)

[a] time, pH and temperature of incubation.

[b] [(FC-IC)/IC]*100; IC: initial concentration, FC:final concentration.

According to the results, the highest concentrations of hydroxytyrosol were between pH 4-5 and coincided with the highest activity of β-glucosidase, leading to the conclusion that the release of phenolics is directly related to the aforesaid action of the enzyme. Also, it was observed that within 30 minutes of stirring, the hydroxytyrosol concentration tripled, and after that time these values decreased. On the other hand, the static extraction was doubled after 250 minutes, compared to the values before the enzymatic extraction. Thus, prolonged exposure of the phenolic compounds to O_2, changed their structures and bioactive functions.

Tannin acyl hydrolase, commonly referred to as tannase (EC 3.1.1.20) is an inducible enzyme produced by fungi, yeasts and bacteria. This enzyme is mostly characterized by its activity in the polyphenols complex and is capable of hydrolyzing ester bond (between gallic acid and glucose) and depside linkage (between two gallic acids) substrates, such as tannic acid, epicatechin-gallate, epigallocatechin-gallate and chlorogenic acid, among others. The enzyme has wide industrial applications, in juices, beer, cosmetics, pharmaceutics and chemicals. It is primarily used in the stabilization of the color of wine, in the leather treatment process, and for wastewater treatment and production of gallic acid and other phenolics (Banerjee et al. 2001; Battestin & Macedo 2007; Lekha & Lonsane 1997; Madeira et al. 2012).

Chamorro et al. (2012) studied the release of phenolic grape residue after its reaction to the carbohydrase enzyme (cellulase and pectinase) and tannase. The reaction medium was performed at pH 5.5 at 35 °C under agitation for 24 h, with the addition of pectinase (135 U.g-1), cellulase (3150 U.g-1) and tannase (200 U.mg-1). The results showed that both cellulase and pectinase used alone showed no change in the concentration of phenolics. However, with the action of tannase, the concentration of phenolic acids

increased, especially gallic acid. At the same time, the concentration of epigallocatechin-gallate, gallocatechin and epicatechin-gallate decreased, from which it can be concluded that the tannase hydrolyzed ester linkages of phenolic compounds have become an important factor in the release of phenolic compounds in grape residue.

Dueñas et al. (2007) evaluated the effect of different enzymes in the quantification of free phenolics in lentil flour. The residue was incubated in acetate buffer with pH 5.5 at 37 °C in four reactors, each of which received a different enzyme, α-galactosidase, Viscozyme, tannase and phytase. According to the results, both the phytase and the tannase released the greatest amount of gallic acid, 1-0.8 μg.g-1 lentil flour. Despite the considerable increase of gallic acid, phytase decreased the antioxidant activity of the matrix due to the release of phosphate groups of phytic acid chelates and other cations in them. Phytic acid is considered to be a potent iron chelator, which in turn prevents the formation of hydroxyl radicals. At the same time, there is an increase in oxidation cations released in the medium that is present. However, the use of tannase for the release of phenolic antioxidants has become interesting for various types of agro-industrial waste. This is because most of these residues can release the phenolic compounds without requiring a pre-treatment such as the action of the pectinase or cellulase, or variation in temperature or pH.

Briefly, the majority of published studies investigated the best conditions for enzyme activity added to the residue to release the phenolic compounds. The main variables are the enzyme concentration, reaction time, pH, and particularly the class of enzymes used, ranging in accordance with the type of residue (Chamorro et al. 2012; Dueñas et al. 2007; Hamza et al. 2012; Min et al. 2006; Moore et al. 2006). Thus, process modeling studies should be performed in order to increase the yield of a phenolic

compound of interest, wherein the class(es) of enzyme(s) and substrate are the major interferences in the final product.

I.3. Conclusion

The microbial and enzyme biotransformation of phenolic compounds seems to be a promising way to increase the concentration of free phenolics. These bioprocesses are clean technologies with great potential for obtainment of biologically active compounds from natural sources. In this case, the use of residues is of particular interest because of its availability, low cost and features that allow obtaining different bioactive phenolic compounds, as well as being an environmentally friendly alternative for their removal. For better obtainment of these compounds, factors such as type of substrate, microorganism, class of enzyme(s), moisture in the substrate, time and temperature of incubation can be assessed through bioprocesses. In most cases, it was noted that between the biological processes reviewed, the yield obtained with the enzymatic process was greater than that obtained with the microbial process. Therefore, modeling the bioprocess can increase the product yield and consequently lower the cost of the process.

I.References

Abbasi S, Vahabzadeh F, Mehranian M. (2007). Profiles of phenolics and antioxidant activity of pistachio hulls during solid-state fermentation by *Phanerochaete chrysosporium* – involvement of lignin peroxidase and manganese peroxidase. Sci Iran, 14, 373-378.

Aguilera-Carbó A, Hernández-Rivera JS, Augur C, Prado-Barragán LA Favela-Torres E, Aguilar CN. (2009). Ellagic acid production from biodegradation of creosote

bush ellagitannins by *Aspergillus niger* in solid state culture. Food Bioprocess Technol, 2, 208-212.

Aliakbarian B, Casazza AA, Perego P. (2011). Valorization of olive oil solid waste using high pressure-high temperature reactor. Food Chem, 128, 704-710.

Alimardani-Theuli P, Gainvors-Claisse A, Duchiron F. (2011). Yeasts: An attractive source of pectinases – From gene expression to potential applications: A review. Process Biochem, 46, 1525-1537.

Banerjee D, Mondal KC, Pati BR. (2001). Production and characterization of extracellular and intracellular tannase from newly isolated *Aspergillu aculeatus* DBF 9. J Basic Microbiol, 41, 313-318.

Banerjee R, Mukherjee G. (2003). Process for the preparation of gallic acid by co-culture. United State of American. US7118882 (B2).

Banerjee S, Singh S, Rahman LU. (2012). Biotransformation studies using hairy root cultures – A review. Biotechnol Adv, 30, 461-468.

Barrios-González J. (2012). Solid-state fermentation: Physiology of solid medium, its molecular basis and applications. Process Biochem, 47, 175-185.

Battestin V, Macedo GA. (2007). Tannase production by *Paecilomyces variotii*. Biores Technol, 98, 1832-1837.

Betts RE, Walters DE, Rosazza JP. (1974). Microbial transformations of antitumor compounds. 1. Conversion of acronycine to 9-hydroxyacronycine by *Cunninghamella echinulata*. J Med Chem, 17, 599-602.

Bhanja T, Rout S, Banerjee R, Bhattacharyya BC. (2008). Studies on the performance of a new bioreactor for improving antioxidant potential of rice. LWT – Food Sci Technol, 41, 1459-1465.

Cai S, Wang O, Wu W, Zhu S, Zhou FJB, Gao F, Zhang D, Liu J, Cheng Q. (2012). Comparative study of the effects of solid-state fermentation with three filamentous fungi on the total phenolics content (TPC), flavonoids, and antioxidants activities of subfractions from oats (*Avena sativa* L.). J Agric Food Chem, 60, 507-513.

Capote FP, Jiménez JR, De Castro MDL. (2007). Sequential (step-by-step) detection, identification and quantitation of extra virgin olive oil adulteration by chemometric treatment of chromatographic profiles. Anal Bioanal Chem, 388, 1859-1865.

Chamorro S, Viveros A, Alvarez I, Vega E, Brenes A. (2012). Changes in polyphenol and polysaccharide content of grape seed extract and grape pomace after enzymatic treatment. Food Chem, 133, 308-314.

Cho KM, Hong SY, Math RK, Lee JH, Kambiranda DM, Kim JM, Islam SMA, Yun MG, Cho JJ, Lim WJ, Yun HD. (2009). Biotransformation of phenolics (isoflavones, flavanols and phenolic acids) during the fermentation of cheonggukjang by *Bacillus pumilus* HY1. Food Chem, 114, 413-419.

Dueñas M, Hernández T, Estrella I. (2007). Changes in the content of bioactive polyphenolic compounds of lentils by the action of exogenous enzymes. Effect on their antioxidant activity. Food Chem, 101, 90-97.

Fernendez-Orozco R, Frias J, Muñoz R, Zielinski H, Piskula MK, Kozlowska H, Vidal-Valverde C. (2008). Effect of fermentation conditions on the antioxidant compounds and antioxidant capacity of *Lupinus angustifolius* cv. zapaton. Eur Food Res Technol, 227, 979-988.

Georgetti SR, Vicentini FTMC, Yokoyama CY, Borin MF, Spadaro ACC, Fonseca MJV. (2009). Enhanced in vitro and in vivo antioxidant activity and mobilization of free

phenolic compounds of soybean flour fermented with different β-glucosidase-producing fungi. J Appl Microbiol, 106, 459-466.

Gnana Soundari S, Sashi V. (2009). Bacterial xylanases. Asian J Microbiol Biotechnol Environ Sci, 11, 677-682.

Hamza M, Khoufi S, Sayadi S. (2012). Fungal enzymes as a powerful tool to release antioxidants from olive mill wastewater. Food Chem, 131, 1430-1436.

Harrison JE, Oomah BD, Diarra MS, Ibarra-Alvarado C. (2012). Bioactivities of pilot-scale extracted cranberry juice and pomace. J Food Process Preserv, DOI: 10.1111/j.1745-4549.2011.00655.x.

Hayat K, Zhang X, Farooq U, Abbas S, Xia S, Jia C, Zhong F, Zhang J. (2010). Effect of microwave treatment on phenolic content and antioxidant activity of *Citrus* mandarin pomace. Food Chem, 123, 423-429.

Juan M.-Y, Chou C-C. (2010). Enhancement of antioxidant activity, total phenolic and flavonoid content of black soybeans by solid state fermentation with *Bacillus subtilis* BCRC 14715. Food Microbiol, 27, 586-591.

Kanji D. (1991). O-Methylation of phenolic hydroxyl group by fermentation. Japan. JP3232495 (A).

Kashyap DR, Vohra PK, Chopra S, Tewari R. (2001). Applications of pectinases in the commercial sector: A review. Biores Technol, 77, 215-227.

Khandeparker R, Numan MTh. (2008). Bifunctional xylanases and their potential use in biotechnology. J Ind Microb Biotech, 35, 635-644.

Khoufi S, Hamza M, Sayadi S. (2011). Enzymatic hydrolysis of olive wastewater for hydroxytyrosol enrichment. Biores Technol, 102, 9050-9058.

Kirby R. (2005). Actinomycetes and lignin degradation. Adv Appl Microbiol, 58, 125-168.

Kunnika S, Pranee A. (2011). Influence of enzyme treatment on bioactive compounds and colour stability of betacyanin in flesh and peel of red dragon fruit *Hylocereus polyrhizus* (Weber) Britton and Rose. Int Food Res J, 18, 1437-1448.

Lei Z, Jervis J, Helm RF. (2001). Use of methanolysis for the determination of total ellagic and gallic acid contents of wood and food products. J Agric Food Chem, 49, 1165-1168.

Lekha PK, Lonsane BK. (1997). Production and application of tannin acyl hydrolase: State of the art. Adv Appl Microbiol, 44, 215-260.

Lin H, Li W, Guo C, Qu S, Ren N. (2011). Advances in the study of directed evolution for cellulases. Front Environ Sci Eng China, 5, 519-525.

Machado EMS, Rodriguez-Jasso RM, Teixeira JÁ, Mussatto SI. (2012). Growth of fungal strains on coffee industry residues with removal of polyphenolic compounds. Biochem Eng J, 60, 87-90.

Madeira Jr JV, Macedo JA, Macedo GA. (2012). A new process for simultaneous production of tannase and phytase by *Paecilomyces variotii* in solid-state fermentation of orange pomace. Bioprocess Biosyst Eng, 35, 477-482.

Maier T, Göppert A, Kammerer DR, Schieber A, Carle R. (2008). Optimization of a process for enzyme-assisted pigment extraction from grape (*Vitis vinifera* L.) pomace. Eur Food Res Technol, 227, 267-275.

Martínez-Ávila GC, Aguilera-Carbó AF, Rodríguez-Herrera R, Aguilar CN. (2011). Fungal enhancement of the antioxidant properties of grape waste. Ann Microbiol, DOI: 10.1007/s13213-011-0329-z.

Martins S, Mussatto SI, Martínez-Avila G, Montañez-Saenz J, Aguilar CN, Teixeira JA. (2011). Bioactive phenolic compounds: Production and extraction by solid-state fermentation. A review. Biotechnol Adv, 29, 365-373.

McCue P, Horii A, Shetty K. (2004). Mobilization of phenolic antioxidants from defatted soybean powders by *Lentinus edodes* during solid-state bioprocessing is associated with enhanced production of laccase. Innov Food Sci Emerg, 5, 385-392.

Min J-Y, Kang S-M, Park D-J, Kim Y-D, Jung H-N, Yang J-K, Seo W-T, Kim S-W, Karigar CS, Choi M-S. (2006). Enzymatic release of ferulic acid from *Ipomoeba batatas* L. (sweet potato) stem. Biotechnol Bioprocess Eng, 11, 372-376.

Moore J, Cheng Z, Hao J, Guo G, Liu J-G, Lin C, Yu L. (2007). Effects of solid-state yeast treatment on the antioxidant properties and protein and fiber compositions of common hard wheat bran. J Agric Food Chem, 55, 10173-10182.

Moore J, Cheng Z, Su L, Yu L. (2006). Effects of solid-state enzymatic treatments on the antioxidant properties of wheat bran. J Agric Food Chem, 54, 9032-9045.

Oszmianski J, Wojdylo A, Kolniak J. (2011). Effect of pectinase treatment on extraction of antioxidant phenols from pomace, for the production of puree-enriched cloudy apple juices. Food Chem, 127, 623-631.

Paës G, Berrin J-G, Beaugrand J. (2012). GH11 xylanases: Structure/function/properties relationships and applications. Biotechnol Adv, 30, 564-592.

Panke S, Held M, Wubbolts M. (2004). Trends and innovations in industrial biocatalysis for the production of fine chemicals. Curr Opin Biotechnol, 15, 272-279.

Pedrolli DB, Carmona EC. (2009). Pectin lyase from *Aspergillus giganteus*: Comparative study of productivity of submerged fermentation on *Citrus* pectin and orange waste. Appl Biochem Microbiol, 45, 610-616.

Pingret D, Fabiano-Tixier AS, Le Bourvellec C, Catherine MGC, Chemat RF. (2012). Lab and pilot-scale ultrasound-assisted water extraction of polyphenols from apple pomace. J Food Eng, 111, 73-81.

Pokorný J. (2007). Are natural antioxidants better and safer than synthetic antioxidants? Eur J Lipid Sci Technol, 109, 629-642.

Puri M, Sharma D, Barrow CJ. (2012). Enzyme-assisted extraction of bioactives from plants. Trends Biotechnol, 30, 37-44.

Puupponen-Pimiä R, Nohynek L, Ammann S, Oksman-Caldentey K-M, Buchert J. (2008). Enzyme-assisted processing increases antimicrobial and antioxidant activity of bilberry. J Agric Food Chem, 56, 681-688.

Ramadan MF, Sitohy MZ. (2008). Solvent and enzyme-aided aqueous extraction of goldenberry (*Physalis peruviana* L.) pomace oil: impact of processing on composition and quality of oil and meal. Eur Food Res Technol, 226, 1445-1458.

Rashad MM, Mahmoud EA, Abdou MH, Nooman UM. (2011). Improvement of nutritional quality and antioxidant activities of yeast fermented soybean curd residue. Afr J Biotechnol, 10, 5750-5759.

Ravindran C, Varatharajan GR, Azagesan K. (2011). Role of alkaline-tolerant fungal cellulases in release of total antioxidants from agro-wastes under solid state fermentation. Biores, 6, 3142-3154.

Saad H, Bouhtoury FC, Pizzi A, Rode K, Charrier B, Ayed N. (2012). Characterization of pomegranate peels tannin extractives. Ind Crop Prod, 40, 239-246.

Salar RK, Certik M, Brezova V. (2012). Modulation of phenolic content and antioxidant activity of maize by solid state fermentation with *Thamnidium elegans* CCF 1456. Biotechnol Bioproc Eng, 17, 109-116.

Sepúlveda L, Ascacio A, Rodríguez-Herrera R, Aguilera-Carbó A, Aguilar CN. (2011). Ellagic acid: Biological properties and biotechnological development for production processes. Afr J Biotechnol, 10, 4518-4523.

Setianto WB, Yoshikawa S, Smith Jr RL, Inomata H, Florusse LJ, Peters CJ. (2009). Pressure profile separation of phenolic liquid compounds from cashew (*Anacardium occidentale*) shell with supercritical carbon dioxide and aspects of its phase equilibria. J Supercrit Fluids, 48, 203-210.

Singh HB, Singh BN, Singh SP, Nautiyal CS. (2010). Solid-state cultivation of *Trichoderma harzianum* NBRI-1055 for modulating natural antioxidants in soybean seed matrix. Biores Technol, 101, 6444-6453.

Singhania RR, Patel AK, Soccol CR, Pandey A. (2009). Recent advances in solid-state fermentation. Biochem Eng J, 44, 13-18.

Siriwardhana N, Kim K-N, Lee K-W, Kim S-H, Ha J-H, Song CB, Lee J-B, Jeon .-J. (2008). Optimisation of hydrophilic antioxidant extraction from Hizikiafusiformis by integrating treatments of enzymes, heat and pH control. Int J Food Sci Technol, 43, 587-596.

Soto ML, Moure A, Domínguez H, Parajó JC. (2011). Recovery, concentration and purification of phenolic compounds by adsorption: A review. J Food Eng, 105, 1-27.

Tapin S, Sigoillot J-C, Asther M, Petit-Conil M. (2006). Feruloyl esterase utilization for simultaneous processing of nonwood plants into phenolic compounds and pulp fibers. J Agric Food Chem, 54, 3697-3703.

Topakas E, Kalogeris E, Kekos D, Macris BJ, Christakopoulos P. (2004). Production of phenolic from corn cobs by coupling enzymic treatment and solid state fermentation. Eng Life Sci, 4, 283-286.

Treutter D. (2001). Biosynthesis of phenolic compounds and its regulation in apple. Plant Growth Regul, 34, 71-89.

Tripoli E, La Guardia M, Giammanco S, Di Majo D, Giammanco M. (2007). *Citrus* flavonoids: Molecular structure, biological activity and nutritional properties: A review. Food Chem, 104, 466-479.

Van Den Brink R, De Vries RP. (2011). Fungal enzyme sets for plant polysaccharide degradation. Appl Microbiol Biotechnol, 91, 1477-1492.

Vattem DA, Lin Y-T, Labbe RG, Shetty K. (2004). Phenolic antioxidant mobilization in cranberry pomace by solid-state bioprocessing using food grade fungus *Lentinus edodes* and effect on antimicrobial activity against select food borne pathogens. Innov Food Sci Emerg Technol, 5, 81-91.

Ventura J, Gutiérrez-Sanchez G, Rodríguez-Herrera R, Aguilar CN. (2009). Fungal cultures of tar bush and creosote bush for production of two phenolic antioxidants (pyrocatechol and gallic acid). Folia Microbiol, 54, 199-203.

Verma N, Bansal MC, Kumar V. (2011). Enzymatic deinking with cellulases: A review. J Solid Waste Technol Manage, 37, 297-306.

Wilson TC, Hagerman AE. (1990). Quantitative determination of ellagic acid. J Agric Food Chem, 38, 1678-1683.

Wu T, Yan J, Liu R, Marcone MF, Aisa HÁ, Tsao R. (2012). Optimization of microwave-assisted extraction of phenolics from potato and its downstream waste using orthogonal array design. Food Chem, 133, 1292-1298.

Zheng Z, Shetty K. (2000). Solid-state bioconversion of phenolics from cranberry pomace and role of *Lentinus edodes* β-glucosidase. J Agric Food Chem, 48, 895-900.

CAPÍTULO II. Efficient tannase production in Brazilian *Citrus* Residues and partial enzymatic biochemical characterization

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Abstract

Tannase is an interesting enzyme due to its capacity to hydrolyze galotannins and polyphenol compounds. Tannase from *P. variotii* has been produced from different residues, such as wheat, castor bean cake and pomegranate, and has shown great potential in Brazilian *Citrus* residue. The process of tannase production using SSF was optimized and activity increased ten-fold. The tannase produced showed optimum activity at pH 5.0, with 70 °C and 80% stability between pH 4.0-6.5 and 20-60 °C. Hence, the *P. variotii* produced a stable and reliability tannase, independent of the type of substrate. These properties can be further exploited in developing tannase for a wider range of applications in the pharmaceutical, food, feed industries and antioxidant compounds production. Therefore, this new optimized tannase production process would provide a new source for industrial applications.

Resumo

Tanase é uma interessante enzima devido a sua capacidade de hidrolisar galotaninos e compostos fenólicos. A tanase de *P. variotii* tem sido produzida em diferentes resíduos agroindustriais, como farelo de trigo, torta de mamona, casca de romã, e mostrou a partir de resíduos de *Citrus* grande potencial. A produção de tanase por fermentação em estado sólido foi otimizada e um aumento de 10 vezes da atividade enzimática foi obtida. A tanase

produzida apresentou atividade ótima em pH 5,0, temperatura de 70 °C e estabilidade enzimática de 80% nas temperaturas entre 20-60 °C e pH 4,0-6,5, com resultados similares aos obtidos anteriormente em farelo de trigo. Portanto, a *P. variotii* produziu uma tanase estável, independentemente do tipo de substrato. Estas propriedades podem ser exploradas no desenvolvimento da enzima para uma ampla gama de aplicações na indústria farmacêutica, de alimentos, indústrias de ração e produção de compostos antioxidantes. Portanto, estas condições otimizadas do processo de produção de tanase irá proporcionar uma nova fonte para aplicações industriais.

II.1. Introduction

The production of enzymes such as amylases, cellulases, pectinases and tannases by SSF has of late received increased attention since the product can be widely applied in the pharmaceutical and chemical industries due to its varied biological activities (antioxidant, anti-apoptotic, antibacterial, antiviral, analgesic etc.) (Dhillon et al., 2011; Madeira et al., 2013).

Tannin acyl hydrolase, commonly referred to as tannase (EC 3.1.1.20), is an enzyme that cleaves ester linkages in hydrolysable tannins to produce glucose and gallic acid (Battestin et al., 2004). Tannase is an extracellular, inducible enzyme produced in the presence of tannic acid by fungi, bacteria and yeasts (Belmares et al., 2004). There are several potential industrial applications for tannase. However, due to its high production cost and our limited knowledge of its catalytic actions, it is currently only used in limited circumstances. Using tannase in feed preparations increases the bioavaibility of nutrients by hydrolyzing phenolic (tannins) anti-nutritional factors (Belmares et al., 2004; Madeira Jr. et al., 2012).

The first step in the development of microbial enzyme production is lineage selection. Extracellular enzymes are ideal because they are easily extracted and do not require expensive extraction methods (Díaz et al., 2007). SSF provides several advantages over conventional enzyme production processes, and agro-industrial byproducts can be put to use and supplemented by tannic acid (Battestin et al., 2004; Madeira et al., 2012). Adapting the necessities of SSF to optimize the process of fermentation parameters includes adding physico-chemical parameters. Once the basic fermentation parameters like fermentation period, moisture content, inoculum size, substrate concentration, pH, and temperature are optimized, it is necessary to increase product yields. Since the agrobased residues are generally ill-defined substrates, supplementation of various carbon and nitrogen sources, other organic substances such as fatty acids, alcohols, acids, and vitamins have proved to be beneficial in achieving higher product yields (Belmares et al., 2004; Díaz et al., 2007).

In this study, *Paecilomyces variotii* was used to produce tannase. The enzyme was produced and biochemically characterizated. *Citrus* Brazilian residues were used as substrate for SSF.

II.2. Material and methods

II.2.1. Material

Hesperidin, hesperetin, naringin and naringenin were purchased from Sigma-Aldrich Corporation. *Citrus* residue from 5 different cultivars (*Citrus latifolia*, *C. sinensis* Hamlin, Valencia, Pera riu and Pera Natal) was kindly donated by CP Kelco industry headquarters (Limeira, SP, Brazil) from subsequent juice extraction, the origin of a low quality residue with a low commercial value.

II.2.2. Microorganism and inoculum preparation

The *P. variotii* strain was isolated and selected as the tannase producer. The fungus strain was deposited at the Brazilian Collection of Environmental and Industry Microorganisms (CBMAI) under the number 1157. The *P. variotii* was preserved in Potato Dextrose Agar (PDA) medium slants with vaseline and refrigerated at 4 °C. For sporulation, the fungal strain was inoculated on plates containing PDA medium and incubated at 30 ºC for 3 days. The spores were suspended in distilled water at a concentration of 9 x 10^6 spores/ml (Madeira et al., 2012).

II.2.3. Enzyme obtaining

Citrus residues were donated by CP Kelco industry headquarters (Limeira, SP, Brazil) in its dried state. The residue was ground in a knife mill (Philips, RI 1725) and separated in a sieve shaker (Mesh 10, particle size under 1.86 mm).

The initial fermentation medium was established in previous studies on tannase production, and consisted of: 10 g of the *Citrus* residue was added to 10 ml of distilled water in 250 ml Erlenmeyer flasks (Madeira Jr. et al., 2012). After sterilization, the flasks were inoculated with 1 ml of spore suspension (9 x 10^6) and incubated at 30 ºC at 90% relative humidity (Climate Chamber 420 CLD – Nova Etica, SP, Brazil) for up to 96 h.

After the incubation period, the tannase extraction was performed by adding 50 ml of acetate buffer (pH 5.5, 0.02 M) to 5 g of fermented substrate. The solution was shaken at 200 rpm for 1 h and then filtered and centrifuged at 10070 x *g* for 30 min at 4 ºC (Centrifuge Beckman J2-21, Beckman-Coulter, Inc., Fullerton, CA, USA). The supernatant was assayed for tannase activity.

II.2.4. Tannase assays

Tannase activity was evaluated according to Sharma et al. (2000) and adapted using tannic acid as a substrate. One unit of activity was defined as the amount of enzyme that released 1 μmol/min of gallic acid. Enzyme activity was expressed as total unit (U) per g of dry substrate (gds) of dry solid medium (based on the initial mass).

II.2.5. Optimization of fermentation parameters for tannase production

The fermentation parameters that had the greatest influence on tannase production were evaluated using two CCD (Central Composite Design) methodologies. The first design was run with the following physical parameters: particle size substrate (mm), water:substrate ratio (v:w) and temperature of incubation (°C). The water:substrate ratio was determined according to the maximum moisture absorption capacity of the orange pomace. The second design was carried out with the following nutritional parameters: ammonium sulphate concentration (w/w) and tannic acid concentration (w/w). The variables were coded, according to Equation 1:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad \text{(Equation 1)}$$

Where: x_i is the coded variable, X_i is the natural variable of the nutrient factor, X_o is the value of the natural variable at the center point, and ΔX_i is the step change value. The variables and levels are shown in Tables II-1 and II-2.

The first experimental design was defined as a full CCD methodology for 3 factors (2^3), consisting of 8 cubic points, 6 star points and 3 replicates at the center point (Table II-1). The second experimental design was defined as a full CCD methodology for 2 factors

(2^2), consisting of 4 cubic points, 4 star points and 3 replicates at the center point, which served to estimate the experimental error and investigate the suitability of the proposed model (Table II-2). The experimental results were fitted to a second-order polynomial function, and the Student t-test made it possible to check the statistical significance of the regression coefficients. Analysis of variance (ANOVA) was performed on the experimental data to evaluate the statistical significance of the model. The response model was expressed in terms of coded variables, ignoring the statistically non-significant terms.

II.2.6. Tannase Biochemical Characterization

After tannase production was optimized, the fermented material was added to 100 ml of 20 mM acetate buffer, at pH 5.0 and shaken at 200 rpm for 1 h. The solution was filtered and centrifuged at 9650 x *g* for 30 min at 4 °C (Beckman J2-21 centrifuge, Beckman-Coulter, Inc. Fullerton, CA, USA). The supernatant was then treated with solid ammonium sulphate (80% saturation) and left to stand overnight at 4 °C. The precipitate was collected by centrifugation (9650 x *g* – 30 min), resuspended in distilled water and dialysed against distilled water. The dialysed preparation was used as crude freeze-dried tannase.

Table II-1. Coded levels and decoded values (in parentheses) for the experimental design and results of CCD.

Trial	Substrate Particle Size (mm)	Water:Substrate Ratio (v/w - %)	Temperature of Incubation (°C)	Tannase (U/g)
1	-1 (1.20)	-1 (8.00)	-1 (30)	0.923
2	+1 (2.80)	-1 (8.00)	-1 (30)	0.301
3	-1 (1.20)	+1 (17.00)	-1 (30)	0.045
4	+1 (2.80)	+1 (17.00)	-1 (30)	0.537
5	-1 (1.20)	-1 (8.00)	+1 (34.5)	0.941
6	+1 (2.80)	-1 (8.00)	+1 (34.5)	0.515
7	-1 (1.20)	+1 (17.00)	+1 (34.5)	0.820
8	+1 (2.80)	+1 (17.00)	+1 (34.5)	0.846
9	-1.68 (0.70)	0 (12.50)	0 (32)	2.082
10	+1.68 (3.35)	0 (12.50)	0 (32)	0.598
11	0 (2.00)	-1.68 (5.00)	0 (32)	0.845
12	0 (2.00)	+1.68 (20.00)	0 (32)	0.450
13	0 (2.00)	0 (12.50)	-1.68 (28.5)	0.484
14	0 (2.00)	0 (12.50)	+1.68 (36)	0.161
15	0 (2.00)	0 (12.50)	0 (32)	1.386
16	0 (2.00)	0 (12.50)	0 (32)	1.354
17	0 (2.00)	0 (12.50)	0 (32)	1.398

Table II-2. Coded levels (in parentheses) and real values for the experimental design and results of the CCD.

Trial	Tannic Acid (w/v - %)	Ammonium Sulphate (w/v - %)	Tannase (U/g)
1	2.2 (-1)	1.5 (-1)	10.4
2	7.5 (+1)	1.5 (-1)	12.4
3	2.2 (-1)	8.5 (+1)	9.3
4	7.5 (+1)	8.5 (+1)	11.6
5	0 (-1.41)	5 (0)	5.3
6	15 (+1.41)	5 (0)	11.2
7	12.8 (0)	0 (-1.41)	6.9
8	12.8 (0)	10 (+1.41)	9.6
9	12.8 (0)	5 (0)	16.5
10	12.8 (0)	5 (0)	16.2
11	12.8 (0)	5 (0)	15.1

To assess the optimal temperature of tannase activity, the enzyme assay was carried out at different temperatures (25 – 90 °C). The optimal pH for tannase activity was determined by conducting assays at different pH levels by using appropriate buffers (0.2 M): acetate buffer (pH 3.5, 4.0, 4.5, 5.0, 5.5), phosphate buffer (pH 6.0, 6.5, 7.0), Tris-HCl (pH 8.0, 9.0) and borate–NaOH buffer (pH 9.5, 10.0). To determine the temperature and pH stability of tannase, the enzyme was pre-incubated at various temperatures (20 – 90 °C) and different pH values (3.5 – 10.0 at 0.02 M) for 60 min and assayed to calculate the residual

activity. The optimal and stable pH and temperature were determined through calculating the specific activity using the Bradford methodology for protein quantification.

II.2.7. Statistical Analysis

Statistica 7.0 software (Statsoft, Inc., Tulsa, USA) was used and all of the results reported in the characterization, application and production of tannase represent the mean value of three replicates and the standard deviation. T-tests showed significant differences ($p<0.05$) in fermentation times for phenolic production.

II.3. Results and Discussion

II.3.1. Tannase production

Paecilomyces variotii was utilized to produce extracellular tannase in *Citrus* residues using SSF. The maximum enzymatic activity occurred after 96 h of fermentation when experiments were performed using CCD.

The results obtained for the first experiment design are shown in Table II-1. Maximum tannase production, 2.08 U/g substrate was obtained at 0.70 mm of particle substrate size, 12.50 water:substrate ratio and a temperature of 32 °C (run 9). The regression analysis was performed to correspond with the response function (tannase activity) with the experimental data. The analysis of variance for the three variables (particle substrate size, water:substrate ratio and temperature) indicated that enzyme productivity could be described in detail by a polynomial model (Equation 2).

$$Y = 1.336 - 0.222x_1 - 0.273x_2^2 - 0.388x_3^2 + 0.196x_1x_2 \quad \text{(Equation 2)}$$

The ANOVA for tannase activity units/g (Y), indicating that the Fisher F-statistic ($F=9.347>F_{t0.1;4;12}=2.48$) was 3.77 times higher than the F_t, and the p-value of <0.01 demonstrated that this regression was statistically significant at a 90% confidence level (Table II-3). However, the lack of fit was also significant, although the ideal would be F-test value smaller than the F_t. However, as the means for the central points were similar and very low pure error, the model was considered valid for predictive purposes. The ANOVA indicated the R^2 value of 0.76 for response Y, which signified that 76% of data variability could be explained by the model.

The response surface plots for tannase production is shown in Figure II-1. The enzymatic activity yield varied significantly upon changing the particle substrate size, water:substrate ratio and temperature incubation. It indicated that the optimum values of each variable were identified based on the three-dimensional hump or the central point of the corresponding contour plot. The response surface indicates that the highest level of tannase activity was 1.6 U/gds at 0.8 mm of particle substrate size, 12.50 (v/w) water:substrate ratio and incubation at 32 °C.

Table II-3. Analysis of variance and regression analyses[a] for the response of the central composite design of tannase production.

Source of variation	Sum of squares	Degrees of freedom	Mean squares	F-test[b]	p-value
Regression	3.280507	4	0.82012675	9.347	0.01
Residual	1.052869	12	0.08773908		
Lack of fit	1.051858	10	0.10518580	208.083	
Pure error	0.001011	2	0.00050550		
Total	4.332365	16			

[a]Coefficient of determination: $R^2 = 0.76$

[b]$F_{t0.1;4;12} = 2.48$

The influence of substrate particle size was investigated in tannase production and increased enzyme production occurred with decreasing particle size. These effects may be due to the higher area of contact between the substrate's surface and the microorganism. Substrates with intermediate particle sizes provide a considerable contact area between the fungus and the substrate, favoring its growth. Very small particles are, however, more susceptible to compaction and the formation of agglomerates, resulting in decreased oxygen transfer, affecting respiration and fungal development. For some substrates, larger particles had a higher porosity than the smaller particles; however, particles with a larger diameter also presented a higher surface area. During the filamentous fungi growth on solid substrates, it is generally accepted that oxygen supply is limited to the cells that are in close contact with the substrate or that penetrate the substrate (Schmidt and Furlong, 2012; Membrillo et al., 2011).

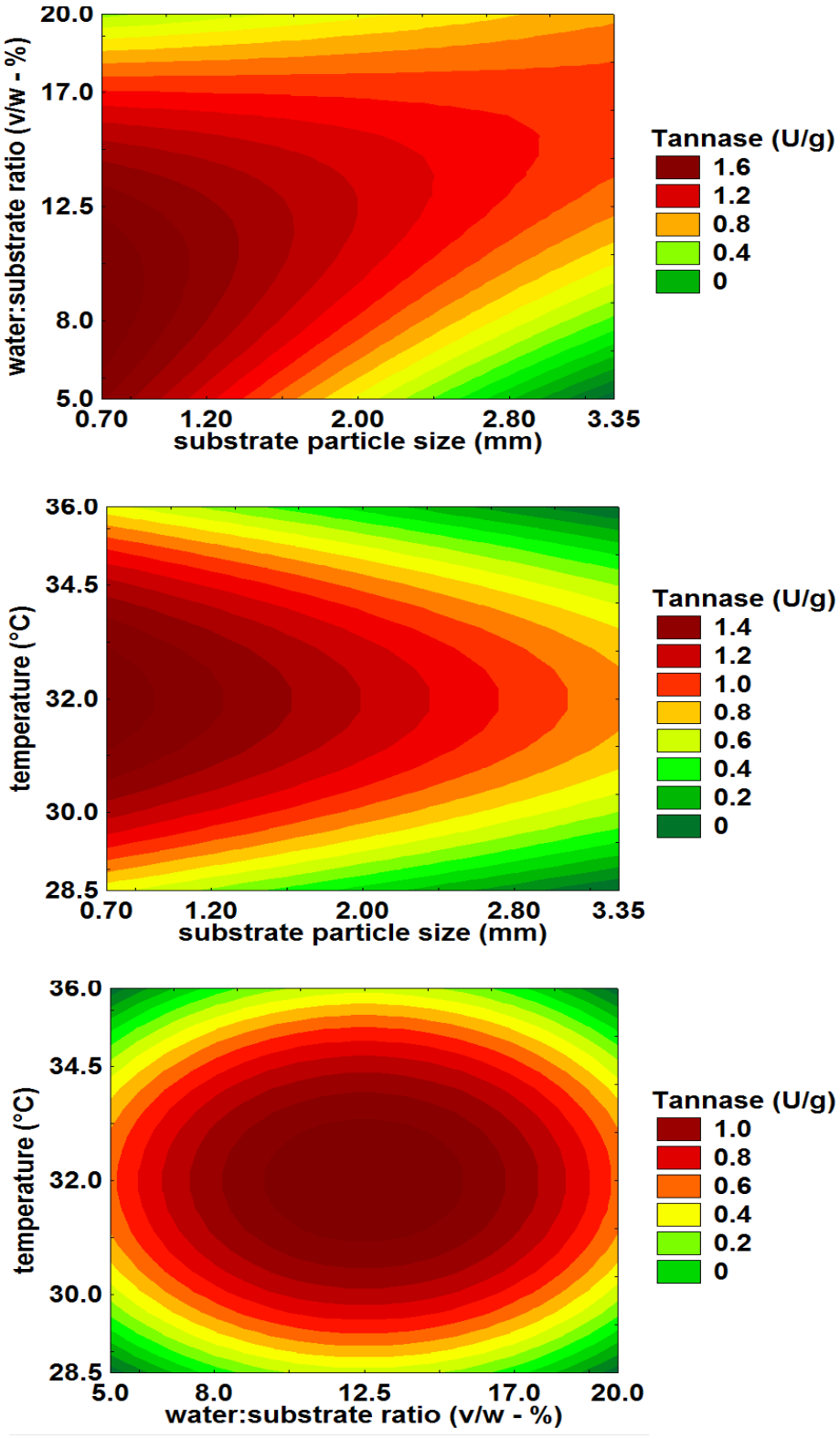

Figure II-1. Contour curve and response surface for tannase activity as a function of water:substrate ratio versus substrate particle size versus temperature according to the CCD.

Tannase production decreased as the water:substrate ratio was increased. This effect may be due to the poor oxygen supply that occurs with increased moisture levels, which results in decreased amounts of enzymes (Yadav et al., 2008; Madeira et al., 2012). The lower production of tannase at a lower water:substrate ratio might also be due to reduced water availability for biomass growth or reduced mobility of the substrate during the SSF process (Yadav et al., 2008; Ito et al., 2011).

Higher temperatures, such as 36 °C, caused a significant decrease in the release of tannase. The higher enzymatic activity arsenal most likely occurred at its optimum temperature (32 °C) (Ferreira et al., 2013; Holopainen-Mantila et al., 2013; Pistarino et al., 2013).

After optimization of the physical fermentation conditions: substrate particle size, water:substrate ratio and temperature of incubation, a second experimental design was performed. The conditions for the concentration of ammonium sulphate and tannic acid were selected and results are shown in Table II-2.

Maximum tannase production was obtained at run number 9 (16.50 U/g substrate), the parameters included were 12.8% of tannic acid and 5.0% of ammonium sulphate. The regression analysis was performed to correspond with the response function (tannase activity) with the experimental data. The analysis of variance for the two variables (tannic acid and ammonium sulphate concentration) indicated that enzyme productivity could be clearly described by a polynomial model (Equation 3).

$$Y = 15.93 + 1.58x_1 - 3.17x_1^2 - 3.17x_2^2 \quad \text{(Equation 3)}$$

Table II-4 shows ANOVA for tannase activity units/g (Y) indicating that the Fisher F-statistic ($F=11.43>F_{t0.1;3;7}=3.07$) was 3.72 times higher than the F_t, and the p-value of <0.01 demonstrated that this regression was statistically significant at a 90% confidence level. ANOVA indicated the R^2 value of 0.83 for response Y, which indicated that 83% of data variability could be explained by the model.

The response surface plots of tannase production is shown in Figure II-2. The enzymatic activity yield varied significantly upon changing the ammonium sulphate and tannic acid concentration. It indicated that the optimum values of each variable were identified based on the three-dimensional hump or from the central point of the corresponding contour plot. The response surface indicates that the highest level of tannase activity was 16.0 U/g at 7.5% (w/v) of tannic acid and 5.0% (w/v) of ammonium sulphate concentration.

Table II-4. Analysis of variance and regression analyses[a] for the response of the central composite design of tannase production.

Source of variation	Sum of squares	Degrees of freedom	Mean squares	F-test[b]	p-value
Regression	15989522	3	5329841	11.64	0.004
Residual	3204567	7	457795		
Lack of fit	2893964	5	578793	3.73	
Pure error	310603	2	155302		
Total	19194089	10			

[a]Coefficient of determination: $R^2=0.83$

[b]$F_{t0.1;3;7}=3.07$

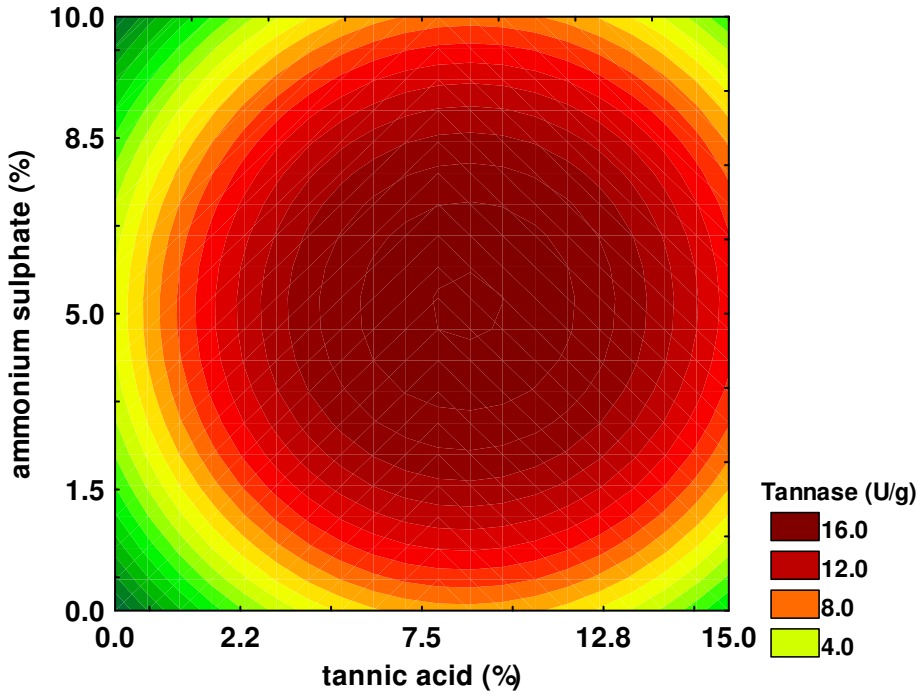

Figure II-2. Contour curve and response surface for tannase activity as a function of ammonium sulphate versus tannic acid concentration according to the CCD.

Higher concentrations of tannic acid led to a reduction in tannase activity. High concentrations of inducer molecules can have toxic effects on *P. variotii* (Battestin and Macedo, 2004), which may explain why lower concentrations of tannic acid were more effective at inducing tannase production. The concentration of tannic acid as an inducer molecule in the present study was reduced by approximately 5% (w/w) compared to the concentration of tannic acid required for fermentation reported by Battestin and Macedo (2004). Battestin and Macedo (2004) also used *P. variotii* for tannase production, but with wheat bran and coffee husk residues. Our results could represent the possibility of a significant reduction in tannase production costs.

Supplementation of the fermentation medium with the nitrogen source resulted in an increase in enzyme production. This may be due to the fact that the substrate does not have

enough nitrogen to supply the nutrients with the required quantity for fungal growth and tannase production (Kumar et al. 2007).

The optimal conditions realized from the optimization experiment were verified experimentally and compared with the calculated data from the model (0.70 mm substrate particle size, 12.50 water:substrate ratio, incubation at 32 °C, 7.5% of tannic acid and 5.0% of ammonium sulphate concentration). The estimated tannase activity was 16.0 U/g (Table II-5), the same obtained for the predicted value from the polynomial model (16.01 U/g). Which revealed the model's high degree of accuracy, at over 99%, and was proven by the validation model under the investigated conditions.

Table II-5. Validation of optimized tannase production by SSF in *Citrus* residues by *P. variotii*.

	Tannase Activity (U/g)		**Accuracy**
	Predicted[a]	**Experimental[b]**	
Optimized process	16.00	16.01 ± 2.19	99.94%

[a] value of the tannase activity predicted by second order polynomial equation of 2 CCD.

[b] data are means of 10 experimental tests at the predicted optimal values of independent variables.

The present study showed advantages over other recently published results from our group, Battestin and Macedo (2004) studied tannase production by SSF by the fungus *P. variotii* in wheat bran with 10% of tannic acid at 30 °C after 120h incubation. The process utilized *Citrus* residue, a less commercial value residue, and a lower concentration of tannic acid to enzyme induction, thus making the final cost of the process lower.

II.3.2. Tannase Biochemical Characterization

The use of tannase in biotechnological processes is growing and the characterization of new enzymes is required. The results of biochemical characterization of the tannase obtained in this work (Figure II-3) are presented below.

Tannase almost completely retained its activity level at a pH ranging from 4.0 to 6.5, when incubated for 24 h at 30 °C. At pH 7.0 the enzyme retained about 50% of its activity; a decrease in pH resulted in a fast decay in enzyme activity. At pH 3.5 the enzyme retained 68% of its activity. The activity of the enzyme decreased gradually at neutrality pH; retaining 10% of its activity at pH 7.5.

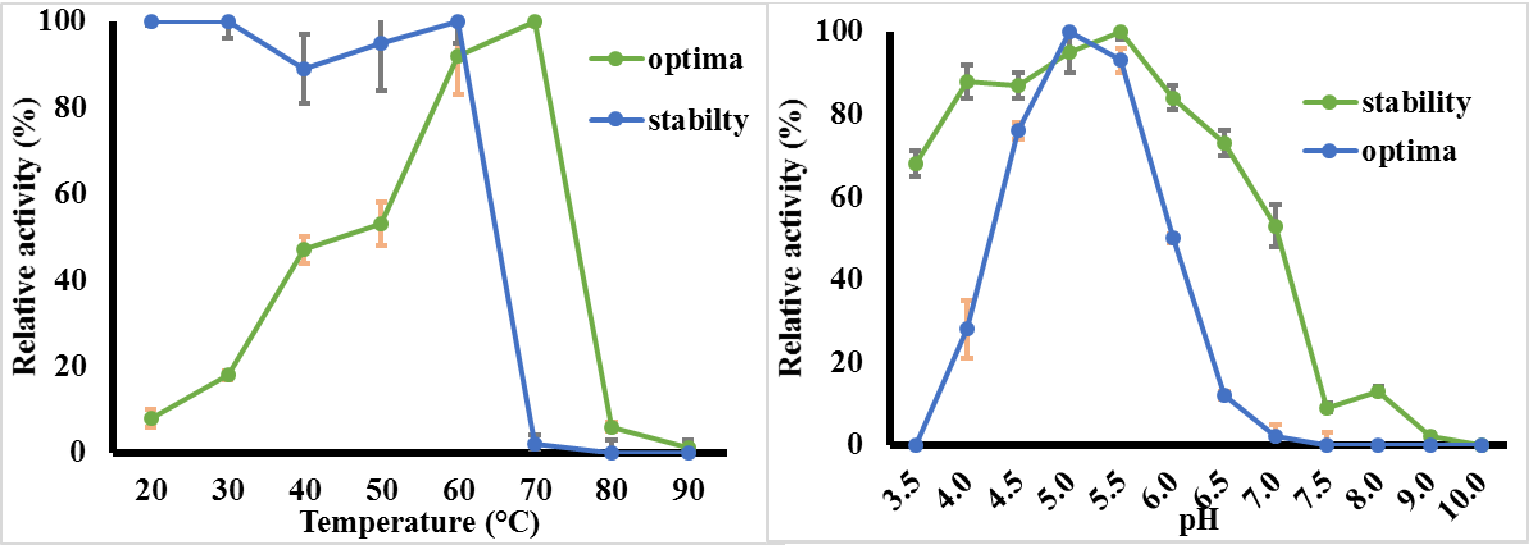

Figure II-3. Effects of temperature and pH on enzyme activity and stability of tannase.

The tannase almost completely retained its activity level at the temperature range of 20 °C to 60 °C when incubated for 1 h. At 70 °C the enzyme retained 4% of its activity, decreasing slowly with increasing temperature. At 90 °C the enzyme retained 1% of its activity.

The semi-purified tannase produced by *P. variotii* showed optimum activity at pH 5.0 (Figure II-3). This result is in agreement with earlier reports by Battestin et al. (2007) and Mahendran et al. (2006). The enzyme was active at acidic pH levels and the activity decreased as the pH approached the alkaline range. Any change in pH affects the protein structure and a decline in enzyme activity beyond the optimum pH could be due to enzyme inactivation or instability. The effect of pH on the enzyme activity is determined by the nature of the amino acids, which undergo protonation and deprotonation, at the active site, and by the conformational changes induced by the ionization of the amino acids. Enzymes are very sensitive to changes in pH and they function best over a very limited range, with a definite pH optimum (Sabu et al. 2005).

The temperature for optimum activity of *P. variotii* was 70 ºC for semi-purified tannase thermostable. Enzymes with an optimum high temperature are preferred for industrial applications. Thus, tannase activity did not increase continuously with a rise in temperature. When the temperature increases, the kinetic energy of the substrate and enzyme molecules also increase which affects the reaction rate. With a rise in temperature, the number of collisions per unit of time of tannase and its substrate, tannic acid, result in a higher activity. When the temperature was increased above the optimum level, the energy of the molecules increased further. When the chemical potential energy increases enough, however, some of the weak bonds determining the three-dimensional shape of the active proteins break. This leads to thermal denaturation of the tannase protein causing it to become inactive. Thus, either an increase in temperature beyond the optimum level caused a decrease in the catalytic rate of tannase, or the substrate became denatured and inactive. Temperatures above the optimum level also affect the protein ionization state, and the

solubility of species in solution, which resulted in a reduction in enzyme activity (Mukherjee and Banerjee, 2006).

Previous work conducted in our laboratory with tannase from *P. variotii* produced by SSF using wheat bran as substrate presented similar results (Battestin et al., 2007). With these results, we can conclude that the microorganism is able to produce the same enzyme, showing the stability and reliability of the process, independent of the type of substrate. The production of *Citrus* residues in Brazil is increasing every year. The availability and price of this waste is therefore of great importance for use as a substrate in SSF for the production of tannase (FAO, 2011). As a result, we can produce the enzyme substrates at a lower commercial value, as in the case of *Citrus* residues.

II.4. Conclusions

The tannase production by *P. variotii* showed attractive potential for industrial exploitation, especially for reducing the amount of tannic acid needed and use of Brazilian *Citrus* residues as substrate to reduce the costs of production. Additionally, the tannase produced was functional at a wide range of temperature and pH values. Hence, the *P. variotii* produced a stable and reliability tannase, independent of the type of substrate. These properties can be further exploited in developing tannase for a wider range of applications in the pharmaceutical, food, feed industries and antioxidant compounds production. Therefore, this new optimized tannase production process would provide a new source for industrial applications.

II.References

Battestin V, Macedo GA. (2004). Tannase production by *Paecilomyces variotii*. Bioresour Technol, 98, 1832-1837.

Battestin V, Macedo GA. (2007). Effect of temperature, pH and additives on the activity of tannase produced by *Paecilomyces variotii*. Eletron J Biotechnol, DOI: 10.2225/vol10-issue2-fulltext-9.

Belmares R, Contreras-Esquivel JC, Rodríguez-Herrera R, Coronel AR, Aguilar, CN. (2004). Microbial production of tannase: an enzyme with potential use in food industry. LWT – Food Sci Technol, 37, 857-864.

Chamorro S, Viveros A, Alvarez I, Vega E, Brenes A. (2006). Changes in polyphenol and polysaccharides content of grape seed extract and grape pomace after enzymatic treatment. Food Chem, 133, 308-314.

Dhillon GS, Oberoi HS, Kaur S, Bansal S, Brar SK. (2011). Value-addition of agricultural wastes for augmented cellulose and xylanase production through solid-state tray fermentation employing mixed-culture of fungi. Ind Crops and Prod, DOI:10.1016/j.indcrop.2011.04.001.

Díaz AB, Caro I, Ory I, Blandino A. (2007). Evaluation of the conditions for the extraction of hydrolytic enzymes obtained by solid-state fermentation from grape pomace. Enz Microb Technol, 41, 302-306.

FAO (Food and Agriculture Organization of the United Nations), 2011. http://faostat.fao.org/site/339/default.aspx (accessed December 2013).

Ferreira LR, Macedo JA, Ribeiro ML, Macedo GA. (2013). Improving the chemopreventive potential of orange juice by enzymatic biotransformation. Food Res Int, 51, 526-535.

Holopainen-Mantila U, Marjamaa K, Merali Z, Käsper A, de Bot P, Jääskeläinen A-S, Waldron K, Kruus K, Tamminen T. (2013). Impact of hydrothermal pre-treatment to chemical composition, enzymatic digestibility and spatial distribution of cell wall polymers. Bioresour Technol, 138, 156-162.

Ito K, Kawase T, Sammoto H, Gomi K, Kariyama M, Miyake T. (2011). Uniform culture in solid-state fermentation with fungi and its efficient enzyme production. J Biosci Bioeng, 111, 300-305.

Kumar R, Sharma J, Singh R. (2007). Production of tannase from *Aspergillus ruber* under solid-state fermentation using jamun (*Syzygium cumini*) leaves. Microbiol Res, 162, 384-390.

Madeira Jr JV, Macedo JA, Macedo GA. (2011). Detoxification of castor bean residues and the simultaneous production of tanase and phytase by solid-state fermentation using *Paecilomyces variotii*. Bioresour Technol, DOI:10.1016/j.biortech.2011.04.099.

Madeira Jr JV, Macedo JA, Macedo GA. (2012). A new process for a simultaneous production of tannase and phytase by *Paecilomyces variotii* in solid-state fermentation of orange pomace. Bioprocess Biosyst Eng, 35, 477-482.

Madeira Jr JV, Teixeira CB, Macedo GA. (2013). Biotransformation and bioconversion of phenolic compounds obtainment: an overview. Crit Rev Biotechnol, DOI: 10.3109/07388551.2013.803020.

Mahapatra K, Nanda RK, Bag SS, Banerjee R, Pandey A, Szakacs G. (2006). Purification, characterization and some studies on secondary structure of tannase from *Aspergillus awamori* nakazawa. Proc Biochem, 40, 10, 3251-3254.

Mahendran B, Raman N, Klim DJ. (2006). Purification and characterization of tannase from *Paecilomyces variotii*: hydrolysis of tannic acid using immobilized tannase. Appl Microbiol Biotechnol, 70, 444-450.

Membrillo I, Sánchez C, Meneses M, Favela E, Loera O. (2011). Particle geometry affects differentially substrate composition and enzyme profiles by *Pleurotus ostreatus* growing on sugar cane bagasse. Bioresour Technol, 102, 1581–1586.

Mukherjee G, Banerjee R. (2006). Effects of temperature, pH and additives on the activity of tannase produced by a co-culture of *Rhizopus oryzae* and *Aspergillus foetidus*. World J Microbiol Biotechnol, 22, 207-212.

Pistarino E, Aliakbarian B, Casazza AA, Paini M, Cosulich ME, Perego P. (2013). Combined effect of starter culture and temperature on phenolic compounds during fermentation of Taggiasca black olives. Food Chem, 138, 2043-2049.

Sabu A, Kiran GS, Pandey A. (2005). Purification and characterization of tannin acyl hydrolase from *Aspergillus niger* ATCC 16620. Food Technol Biotechnol, 43, 133-138.

Sharma KP, John PJ. (2011). Purification and characterization of tannase and tannase gene from *Enterobacter sp*. Process Biochem, 46, 240-244.

Shmidt CS, Furlong EB. (2012). Effect of particle size and ammonium sulfate concentration on rice bran fermentation with the fungus *Rhizopus oryzae*. Bioresour Technol, 123, 36-41.

Yadav A, Aggarwal NK, Kumar K, Kumar A. (2008). Tannase production by *Aspergillus fumigatus* MA under solid-state fermentation. World J Microbiol Biotechnol, 24, 3023-3030.

Yao J, Liu YH. (2013). Production, characterization and applications of tannase. J Mol Catal B-Enzym, DOI: 10.1016/j.molcatb.2013.11.018.

CAPÍTULO III. Rich bioactive phenolic extract production by microbial and enzymatic biotransformations from Brazilian *Citrus* Residues

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Abstract

Flavanones in *Citrus* are molecules that play an important role as antioxidant in nutraceutical products. Recent studies point to reduction the incidence of some cancers, diabetes, and neuro-degenerative diseases, as well as use as antioxidant in foods. Recent studies indicate that molecules of the simplest classes of phenolics have higher biological activity and absorption capacity. Currently, phenolic compounds are obtained by chemical synthesis or extraction. An interesting environmentally friendly alternative that deserves attention regarding phenolic compound production is the biotransformation of these molecules. The aim of this study was to develop two processes of biotransformation of phenolics from Brazilian *Citrus* residues: by SSF with the fungus *Paecilomyces variotii*; and by enzymatic reaction with cellulase, pectinase and tannase. The development of these processes has generated, simultaneously, an increase of 900, 1400 and 1330% of hesperetin, naringenin and ellagic acid concentration through microbial biotransformation, respectively, and an increase of 96, 64 and 64% of hesperetin, naringenin and ellagic acid concentration in enzymatic biotransformation, respectively. These results suggest that the two bioprocesses obtained innovative responses: flavanones from agro-industrial residues, without the use of organic solvents, therefore a green technology.

Resumo

Flavanonas de *Citrus* são moléculas que desempenham importante papel na atividade antioxidante em produtos nutracêuticos. Estudos recentes apontam que estes compostos atuam na redução da incidência de alguns tipos de câncer, diabetes, e doenças neuro-degenerativas, assim na utilização como antioxidante em alimentos. Estudos indicam que as moléculas das classes mais simples de compostos fenólicos possuem maior atividade biológica e capacidade de absorção no intestino. Atualmente, os compostos fenólicos são obtidos por síntese química ou extração a partir de plantas. Uma alternativa ambientalmente interessante, que merece atenção quanto à produção de compostos fenólicos, é a biotransformação destas moléculas. O objetivo deste estudo foi desenvolver dois processos de biotransformação de compostos fenólicos a partir de resíduos de *Citrus*: por fermentação em estado sólido com o micro-organismo *Paecilomyces variotii*; e por reação enzimática com a celulase, pectinase e tanase. O desenvolvimento desses processos gerou, simultaneamente, um aumento de 900, 1.400 e 1.330% de hesperetina, naringenina e ácido elágico, respectivamente, na biotransformação microbiana; e um aumento de 96, 64 e 64% de hesperetina, naringenina e ácido elágico, respectivamente, na biotransformação enzimática. Estes resultados sugerem que os dois bioprocessos propiciaram obter respostas inovadoras: obtenção de flavanonas de resíduos agroindustriais, sem o uso de solventes orgânicos, sendo portanto uma tecnologia ambientalmente amigável.

III.1. Introduction

In recent years, *Citrus* flavonoids have gained much interest due to their chemoprotective effects. *Citrus* flavonoids exhibit antioxidant, antimicrobial, anticarcinogenic, antiviral, anti-allergic and anti-inflammatory activities. Through these

benefits, there is interest in replacing synthetic food antioxidant substances with natural ones, which has fostered research on vegetable sources and the screening of waste materials aimed at identifying new and/or better antioxidant sources (Ferreres et al., 2012; Lin et al., 2012; Sergent et al., 2012; Tripoli et al., 2007).

There are many classes of flavonoids, flavanones being the most abundant group in *Citrus* fruits (Barros et al., 2012; Ferreira et al., 2013). Flavanones are highly present in plant species from the genus *Citrus*, abundant in its by-products, mostly in peels and pectinolytic material, accounting for 4–12% of the dry weight (Marín et al., 2007). The most prevalent flavanones in tissues and peels of *Citrus* fruits are naringin and hesperidin. Naringin exhibits many health benefits, including the ability to prevent cancer by suppression of carcinogenesis and inducing cell apoptosis (Meiyanto et al., 2012). Hesperidin has also been reported to reduce the proliferation of many cancer cells and also possesses an anti-inflammatory effect (Ferreira et al., 2013; Nazari et al., 2011; Park et al., 2008). The glycoside and aglycon forms of flavanones possess several different biological functions. Several studies have revealed that aglycons are superior to glycosides in various bioactivities, due to their effective absorption (Murakami et al., 2008). Only free flavonoids without a sugar molecule were thought to be able to pass through the stomach wall. Hydrolysis only occurs in the colon by microorganisms, which at the same time degrade flavonoids (Hollman & Katan, 1997). According to Nielsen et al. (2006) and Ohguchi et al. (2006), when free phenolics (hesperetin and naringenin) in *Citrus* residues are released from their glycosides (hesperidin and naringin), the photoprotective functionality of these phytochemicals can be improved.

Other important compounds found in *Citrus* fruits are hydroxybenzoic acids, however in lower concentrations than flavanones. Ellagic acid is a very important

compound from this category and has a variety of benefits for anti-mutagenic, antimicrobial and antioxidant properties, as well as being an inhibitor of human immunodeficiency viruses (Nutan et al., 2013; Martins et al., 2011, Sepúlveda et al., 2011). The presence of ellagic acid in various functional commercial products is observed. Majority of published articles describe the few plant species that have ellagitannins such as oak tree and pomegranate (Sepulveda et al., 2011). Improving ellagic acid content in *Citrus* residues could provide an interesting source of this compound for the industry. Microbial degradation of tannins is highly documented, and most works report that the selective hydrolysis of galloyl groups from ellagitannins was catalyzed by tannase (Prasad et al., 2012).

Currently, phenolic compounds are obtained by chemical synthesis or extraction. An interesting environmentally friendly alternative that deserves attention regarding phenolic compound production is the biotransformation of these molecules. Biotransformation can be defined as chemical transformations that are catalyzed by biological systems through their effective enzyme structures or cells (Madeira Jr et al., 2013; Banerjee et al., 2012; Martins et al., 2011). In addition, this bioprocess has received great attention due to the potential conversion of inexpensive agro-industrial residues, as well as plants, to a great variety of valuable compounds. Orange residue is the waste product of the large orange juice industry in Brazil, considered to be an abundant and low cost substrate (FAO, 2004). The material used in this project was residue of two industrial subsequent processes: orange juice extraction and pectin extraction, giving origin to a very low cost co-product.

The current SSF has many advantages, such as high concentration, product stability and growth of microorganisms in non-water soluble substrates, the process is usually

cheaper with higher productivity than submerged fermentation (Barrios-González 2012). The SSF on *Citrus* residue using the *P. variotii* strain was initially developed by this research group for the production of tannase enzyme. The potential of this enzyme to produce more bioactive forms of the polyphenol molecule extract from vegetables was studied in previous works (Madeira et al., 2012). Georgetti et al. (2009) evaluated the biotransformation of polyphenol glycosides from soybeans to form non-glycosides through SSF by *Aspergillus awamori*. The conversion of the glycoside to the form of phenolic non-glycoside was accompanied by production of the enzyme β-glucosidase. The non-glycoside form presents a greater number of free hydroxyl groups in regard to glycoside, thus increasing their biological activity. The microbial biotransformation of phenolic compounds seems to be a promising way to increase the concentration of phenolics with high biological potential.

Enzyme production is an important field in biotechnology, having worldwide sales near five billion dollars annually, with a growth rate of approximately 6.5 to 10% while the number of patents and research papers is listed (Panke et al., 2004). The use of enzymes, especially in biocatalysis in agro-industrial residues, has been introduced for the hydrolysis of plant cell walls, which is complexed with polyphenols. In this scenario, it should be concluded that enzyme can act on this substrate in plant cells (Madeira Jr. et al., 2013).

The present work aimed to optimize some important parameters of these two biotechnological processes in order to produce phenolic compounds with high bioactivity, such as naringenin, hesperetin and ellagic acid, from *Citrus* residue. These have no viable source of extraction so far, being present in very low concentration in vegetables; however, literature shows their functional potential is increasing more and more every day.

III.2. Material and Methods

III.2.1. Material

Hesperidin, hesperetin, naringin, naringenin, ellagic acid and Potato Dextrose Agar (PDA) were purchased from Sigma-Aldrich Co. The enzymes cellulase (Powercell – 180 U/g) and pectinase (10,620 U/ml) were purchased from Prozyn (Sao Paulo – Brazil). *Citrus* residue (from 5 different cultivars: *C. latifolia*, *C. sinensis* Hamlin, Valencia, Pera riu and Pera Natal) was kindly donated by CP Kelco industry headquarters (Limeira, SP, Brazil) from juice and pectin extraction, giving origin to a residue of low quality and commercial value.

III.2.2. Microbial Process

III.2.2.1. Microorganism and inoculum preparation

Paecilomyces variotii strain was isolated and selected to tannase production and grew on different agro-industrial residues such as castor bean cake, wheat bran and *Citrus* residue (Battestin and Macedo, 2004; Madeira et al., 2011; Madeira et al., 2012). The fungus strain was deposited at the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI) under the number 1157. The *P. variotii* was preserved in PDA medium slants and refrigerated at 4 °C with vaseline. For sporulation, the fungal strain was inoculated on plates containing PDA medium and incubated at 30 ºC for 3 days. The spores were suspended in distilled water at a concentration of 9×10^6 spores/ml (Madeira Jr. et al., 2011).

III.2.2.2. Phenolic compound production

Citrus residue was donated by CP Kelco industry headquarters (Limeira, SP, Brazil) as a dry residue. The residue was ground in a knife mill (Philips, RI 1725) and separated in a sieve shaker (Mesh 10, particle size under 1.86 mm).

The initial fermentation medium established in a previous work (Madeira Jr. et al., 2012) for tannase production consisted of 10 g of the *Citrus* residue added to 10 ml of distilled water in 250 ml Erlenmeyer flasks. After sterilization, the flasks were inoculated with 1 ml of spore suspension (9×10^6) and incubated at 30 ºC at 90% relative humidity (Climate Chamber 420 CLD – Nova Etica, SP, Brazil) for up to 120 h.

After the incubation period, the tannase extraction was performed by adding 50 ml of acetate buffer (pH 5.5, 0.02 M) to 5 g of fermented substrate. The solution was shaken at 200 rpm for 1 h, and subsequently filtered and centrifuged at 10070 x *g* for 30 min at 4 ºC (Centrifuge Beckman J2-21, Beckman-Coulter, Inc., Fullerton, CA, USA). The supernatant was assayed for tannase activity. The extraction of the phenolic compounds was performed by adding 25 ml of 70% methanol to 1 g of fermented residue. The solution was submitted to sonication in ultrasonic (Unique UltraSonic Cleaner model USC-1800A) at 40 kHz for 30 min, after being shaken at 200 rpm for 30 min and then passed through a 0.45 µm filter. The filtered extract was assayed for identification and quantification of phenolic compounds by HPLC-DAD (High Pressure Liquid Chromatography-Diode Array Detector).

III.2.2.3. Tannase activity assay

Tannase activity was evaluated according to Sharma et al. (2000), adapted using tannic acid as substrate. One unit of activity was defined as the amount of enzyme that

released 1 μmol/min of gallic acid. Enzyme activity was expressed as total units (U) per g of dry substrate (gds) of dry solid medium (based on initial mass).

III.2.2.4. Identification and quantification of phenolic compounds by HPLC-DAD analysis

HPLC phenolic analysis of the extracts was performed on a Dionex – Ultimate 3000 equipped with a 150 × 4.6 mm i.d. reversed phase C18 column (Waters, Massachusetts); detection was carried out at 260, 280 and 330 nm using a diode array detector. The solvents were A, H_2O (0.1% Formic acid); and B, Methanol (0.1% Formic acid). The gradient elution was as follows: 90% A (0-5 min), 20% A (5-80 min), 90% A (80-85 min), and 90% A at a flow rate of 0.6 ml/min. All HPLC analyses were performed at 30 °C. The Chromeleon software (version 6.8) was used for the data processing. Compounds were identified according to retention time and UV–Vis spectra (260 nm of wavelength). A series of standards (hesperidin, hesperetin, naringin, naringenin, ellagic and gallic acid) solutions were used for the calibration curves and quantification of these phenolics on samples, the quantification was carried out at 280 nm using a diode array detector (Ferreira et al., 2013).

III.2.2.5. Fermentation parameter optimization for target phenolic production

Phenolic compounds and tannase production by *P. variotii* were observed for 120 h. The time of peak products was determined for the experimental design study.

The fermentation parameters that had the greatest influence on phenolic production were evaluated using the CCD (Central Composite Design) methodology. The independent variables were: particle size substrate (mm), water:substrate ratio (v:w) and temperature of

incubation (°C). The water:substrate ratio was determined according to the maximum moisture absorption capacity of the *Citrus* residue. The variables were coded, according to Equation 1:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad \text{(Equation 1)}$$

Here, x_i is the coded variable, X_i is the natural variable of the nutrient factor, X_o is the value of the natural variable at the center point, and ΔX_i is the step change value. The variables and levels are shown in Table III-1.

The experimental design was defined as a full CCD methodology for 3 factors (2^3), consisting of 8 cubic points, 6 star points and 3 replicates at the center point, which served to estimate experimental error and to investigate the suitability of the proposed model, the details of which are presented in Table III-1. The experimental results were fitted to a second-order polynomial function, and the Student t-test allowed for checking of the statistical significance of the regression coefficients. Analysis of variance (ANOVA) was performed on the experimental data to evaluate the statistical significance of the model. The response model was expressed in terms of coded variables, ignoring the statistically non-significant terms. Therefore, the optimum conditions of fermentation process were performed to evaluate the productivity of phenolic compounds obtained (hesperetin, naringenin and ellagic acid) in Equation 2.

$$Y = \left[\frac{(PCf - PCi)}{PCi}\right] * 100\% \quad \text{(Equation 2)}$$

Here, *Y* is the yield of final product, *PCf* is the final phenolic concentration, *PCi* is the initial phenolic concentration (the data were obtained from Table III-5).

Table III-1. Coded levels and real values (in parentheses) for the experimental design and results of the CCD.

Run	Independent Variables[a]			Response (Δ µg/g substrate)[b]		
	Substrate particle size (mm)	water:substrate ratio (v:w)	Temperature (°C)	Hesperetin	Naringenin	Ellagic Acid
1	-1 (1.2)	-1 (0.8:1)	-1 (30.0)	21.0	42.0	2,320
2	1 (2.8)	-1 (0.8:1)	-1 (30.0)	25.0	42.0	7,160
3	-1 (1.2)	1 (1.7:1)	-1(30.0)	50.0	57.0	8,950
4	1 (2.8)	1 (1.7:1)	-1 (30.0)	33.0	46.0	13,230
5	-1 (1.2)	-1 (0.8:1)	1 (34.0)	23.0	44.0	2,910
6	1 (2.8)	-1 (0.8:1)	1 (34.0)	26.0	47.0	4,510
7	-1 (1.2)	1 (1.7:1)	1 (34.0)	38.0	57.0	6,710
8	1 (2.8)	1 (1.7:1)	1 (34.0)	35.0	6.0	9,000
9	-α (0.7)	0 (1.3:1)	0 (32.0)	68.0	72.0	6,030
10	α (3.4)	0 (1.3:1)	0 (32.0)	33.0	31.0	14,540
11	0 (2.0)	-α (0.5:1)	0 (32.0)	9.0	33.0	2,120
12	0 (2.0)	α (2.0:1)	0 (32.0)	54.0	70.0	16,570
13	0 (2.0)	0 (1.3:1)	-α (28.5)	30.0	41.0	8,220
14	0 (2.0)	0 (1.3:1)	α (35.5)	32.0	36.0	6,280
15	0 (2.0)	0 (1.3:1)	0 (32.0)	37.0	54.0	10,610
16	0 (2.0)	0 (1.3:1)	0 (32.0)	44.0	60.0	11,800
17	0 (2.0)	0 (1.3:1)	0 (32.0)	45.0	58.0	11,270

[a] – independents variables: substrate particle size (mm); water:substrate ratio (v:w); temperature (°C);

[b] – response of phenolic compounds production between 0 and 48 h of incubation (µg phenolic/g substrate).

III.2.3. Enzymatic Process

III.2.3.1. Tannase obtaining

The tannase from *P. variotii* was obtained according to chapter II (tannase production). After fermentation, 100 ml of 20 mM acetate buffer, pH 5.0 were added and shaken at 200 rpm for 1 h. The solution was filtered and centrifuged at 9650 x g for 30 min at 4 °C (Beckman J2-21 centrifuge, Beckman-Coulter, Inc. Fullerton, CA, USA). The supernatant was then treated with solid ammonium sulphate (80% saturation) and stood overnight at 4 °C. The precipitate was collected by centrifugation (9650g – 30 min), re-suspended in distilled water and dialyzed against distilled water. The dialyzed preparation was used as crude freeze-dried tannase.

III.2.3.2. Enzymatic reaction for the obtainment, assessment and biotransformation of phenolic compounds

Citrus residue was ground in a knife mill (Philips, RI 1725) and separated in a sieve shaker (particle size under 0.80 mm).

The initial enzymatic reaction medium consisted of: 25 ml Erlenmeyer flasks, in which 2 g of the *Citrus* residue was added to 25 ml of 20 mM acetate buffer with tannase, pectinase and cellulase (all in 1.0 U/ml), incubated in a shaker at 40 ºC at 130 rpm for up to 30 h.

After the incubation period, the phenolic compound extraction was performed by adding 1.40 ml of methanol, to 0.60 ml of enzymatic reaction medium. The solution was treated in an ultrasonic bath (Unique UltraSonic Cleaner model USC-1800A) at 40 kHz for 30 min and then passed through a 0.45 µm filter. The filtered extract was assayed for identification and quantification of phenolic compounds by HPLC-DAD (Item III.2.2.4).

III.2.3.3. Enzymatic processing parameter optimization for target phenolic production

Phenolic compounds were observed for 30 h. The time of peak products were determined for the experimental design study.

The enzymatic parameters that had the greatest influence on phenolic production were evaluated using CCD (Central Composite Design) methodology. The independent variables were: tannase, pectinase and cellulase concentration (U/ml). The variables were coded, according to Equation 1:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad \text{(Equation 1)}$$

Where, x_i is the coded variable, X_i is the natural variable of the nutrient factor, X_o is the value of the natural variable at the center point, and ΔX_i is the step change value. The variables and levels are shown in Table III-2.

The experimental design was defined as a full CCD methodology for 3 factors (2^3), consisting of 8 cubic points, 6 star points and 3 replicates at the center point, which served to estimate experimental error and to investigate the suitability of the proposed model, the details of which are presented in Table III-2. The experimental results were fitted to a second-order polynomial function, and the Student t-test permitted checking the statistical significance of the regression coefficients. Analysis of variance (ANOVA) was performed on the experimental data to evaluate the statistical significance of the model. The response model was expressed in terms of coded variables, ignoring the statistically non-significant terms. Therefore, the optimum conditions of enzymatic process were performed to evaluate the productivity of phenolic compounds obtained (hesperetin, naringenin and ellagic acid) in Equation 2.

$$Y = \left[\frac{(PCf - PCi)}{PCi}\right] * 100\% \quad \text{(Equation 2)}$$

Where, *Y* is the yield of final product, *PCf* is the final phenolic concentration, *PCi* is the initial phenolic concentration (the data were obtained from Table III-5).

III.2.4. Statistical analysis

A Statistica 7.0 software (Statsoft, Inc., Tulsa, USA) package was used, and all values reported in the biotransformation of *Citrus* residue represent the mean from three replicates and standard deviation. Significant differences ($p<0.05$) in times of fermentation for phenolic production were determined by t-tests.

III.3. Results and Discussion

III.3.1. Microbial Biotransformation

The wild *P. variotii* strain was able to grow in Brazilian *Citrus* residue by SSF, which resulted in the production of the extracellular enzyme tannase and phenolic compounds such as hesperetin, naringenin and ellagic acid (Figure III-1). The maximum concentrations of these phenolic compounds occurred after 48 h of fermentation; and the maximum enzymatic activity of tannase was obtained after 96 h of fermentation.

According to the results obtained, the total amount of hesperidin and naringin in the *Citrus* residue extract decreased 50 and 100% after 120 h of incubation, respectively. On the other hand, the initial concentration of hesperetin, naringenin and ellagic acid in the *Citrus* residue were below the detection limit of the method, a practical zero. The three reached their highest value of 100% within 48 h and after that, decreased to 20, 0 and 0% at 120 h of incubation, respectively.

Table III-2. Coded levels and real values (in parentheses) for the experimental design and results of the CCD.

Run	Enzymes Concentrations[a]			Response (Δ µg/ml extract)[b]		
	Tannase (U/ml)	Pectinase (U/ml)	Cellulase (U/ml)	Hesperetin	Naringenin	Ellagic Acid
1	-1 (2)	-1 (2)	-1 (2)	4.5	3.1	734.2
2	1 (8)	-1 (2)	-1 (2)	8.1	4.4	840.8
3	-1 (2)	1 (8)	-1(2)	4.7	2.9	739.1
4	1 (8)	1 (8)	-1 (2)	7.8	4.5	868.0
5	-1 (2)	-1 (2)	1 (8)	5.1	3.9	751.6
6	1 (8)	-1 (2)	1 (8)	8.9	4.7	889.5
7	-1 (2)	1 (8)	1 (8)	5.2	4.1	745.3
8	1 (8)	1 (8)	1 (8)	8.7	4.9	855.4
9	-α (0)	0 (5)	0 (5)	0.6	2.1	725.7
10	α (10)	0 (5)	0 (5)	11.2	5.3	919.8
11	0 (5)	-α (0)	0 (5)	10.1	4.2	790.2
12	0 (5)	α (10)	0 (5)	10.4	4.1	791.3
13	0 (5)	0 (5)	-α (0)	4.7	3.5	754.1
14	0 (5)	0 (5)	α (10)	8.1	5.9	839.6
15	0 (5)	0 (5)	0 (5)	11.2	5.7	795.2
16	0 (5)	0 (5)	0 (5)	10.9	5.5	792.1
17	0 (5)	0 (5)	0 (5)	11.0	5.6	791.7

[a] – independents variables: tannase, pectinase, cellulose (U/ml);

[b] – response of phenolic compounds production between 0 and 24 h of reaction (µg phenolic/ml extract).

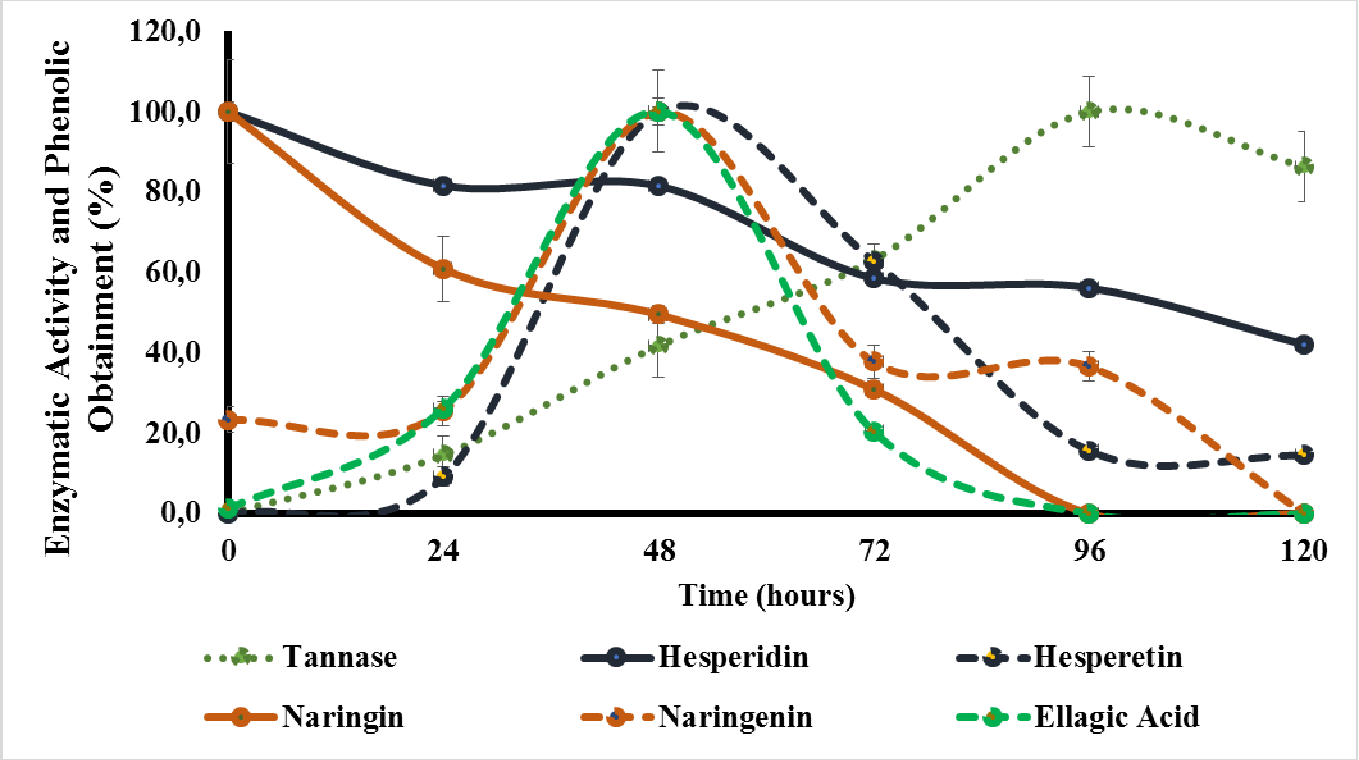


Figure III-1. Kinetics of fermentation of the *Citrus* residue by *P. variotii*: evaluating the tannase production and concentration of hesperidin, hesperetin, naringin, naringenin and ellagic acid.

The fungus probably used hesperidin, naringin and ellagitannins as sources of carbon and energy during the fermentation. In the initial phase of this bioprocess, a partial cleavage in the phenolic glycosides took place in association with the microbial enzymatic breakdown of the plant cell wall, and hence the aglycones were released (Starzynska-Janiszewska et al., 2008; Vattem and Shetty, 2003). The tannase from *P. variotii* was proven to have the ability to catalyze the deglycosylation of flavanones, such as hesperidin (Ferreira et al., 2013). In this manner, tannase is likely an important enzyme synthesized by *P. variotii*, with the ability of hydrolyzing polyphenolic compounds into their aglycone forms as a first step for the microbial consumption of this compound as an energy source. Although the maximum tannase activity occurs after 96 h, from the beginning of the fermentation process it is possible to observe tannase activity, justifying the early

hydrolysis of the hesperidin, naringin and ellagitannins, generating hesperetin, naringenin and ellagic acid, respectively.

Numerous studies have been conducted on biodegradation of tannins and on the degradation mechanism of some simple tannins, such as gallotannin. There is less knowledge about the pathways and enzymes involved in breaking down complex tannins, especially regarding the accumulation mechanism of the microorganism for some intermediates. However, some studies have indicated that tannase participates in ellagic acid obtainment from ellagitannins (Prasad et al., 2012). Shi et al. (2005) reported the ellagic acid accumulation by fungal fermentation of valonea (*Quercus aegilops*) tannins. The results showed simultaneous increase in tannase activity, accumulation of ellagic acid and decrease in ellagitannins during fermentation.

Consequently, as observed in Figure III-1, the concentration of hesperetin, naringenin and ellagic acid increased during fermentation. After 48 h of incubation, the concentration of these phenolics achieved its maximum and started to decrease. This decrease happened even in the presence of a significantly high concentration of the antecedent hesperidin, naringin and tannase activity. This fact led to the belief that the biotransformation from hesperidin and naringin to hesperetin and naringenin was still happening. However, the microorganism, at this point, probably had its metabolic arsenal ready to consume the hesperetin and naringenin molecules.

Justesen et al. (2000) also evaluated the microbial fermentation of hesperidin and hesperetin, and the results observed seem to corroborate our hypothesis. The results showed similar effects, in which degradation of hesperidin occurred during 72 h of incubation, with high hydrolysis values at 24 h. However, the hesperetin was also used as a substrate by the microorganisms after 24 h, being stored for 24 h and then degraded within

72 h. The hesperetin degradation showed some possible products after 48 h, and this showed that the C-ring of the compound was hydrolyzed, releasing compounds such as 4-hydroxyphenyl-propionic acid and 3-hydroxy-4-methoxyphenyl-acetic acid.

After the time-course assay, a CCD was designed to determine the optimal particle substrate size (mm), water:substrate ratio (v:w) and temperature of incubation (°C) for SSF, and the results are shown in Table III-1. According to previous analyses, in which innumerable conditions were tested in fermentation, these three variables were the most significant; therefore, they were used in the CCD.

The quadratic models used to calculate the hesperetin (Y_a), naringenin (Y_b) and ellagic acid (Y_c) after eliminating the statistically insignificant terms (x_1: particle size; x_2: moisture; x_3: temperature of incubation) are as follows (Equations 3, 4 and 5):

$$Y_a = 44.0 - 5.0x_1 + 10.0x_2 - 6.0x_2^2 - 6.0x_3^2 \qquad \text{Equation 3}$$

$$Y_b = 54.0 - 5.0x_1 + 8.0x_2 - 5.0x_3^2 \qquad \text{Equation 4}$$

$$Y_c = 10{,}300 + 2{,}000x_1 + 3{,}300x_2 - 900x_2^2 - 1{,}600x_3^2 \qquad \text{Equation 5}$$

The analyses of variance were reproduced and are shown in Table III-3. The Fisher F-statistic for hesperetin, naringenin and ellagic acid concentrations were higher than the Ft and p-value of <0.01, demonstrating that this regression model was statistically significant at a 90% confidence level. Additionally, the R^2 values obtained for the models were 0.77, 0.70 and 0.83, respectively.

Table III-3. Analysis of variance and regression analyses for the response of the central composite design of phenolic compounds obtainment.

Response	Source of variation	Sum of squares	Degrees of freedom	Mean squares	F test/Ftab	p-value/R^2
Hesperetin	Regression	2.373	4	0.59	9.8/2.5	0.001/0.77
	Residual	0.730	12	0.06		
Naringenin	Regression	1.623	3	0.54	9.0/2.6	0.003/0.69
	Residual	0.746	13	0.06		
Ellagic acid	Regression	240,393	4	60,098	14.8/2.5	0.0001/0.83
	Residual	48,877	12	4,073		

The highest hesperetin, naringenin and ellagic acid concentrations obtained in the experimental design tests were 50.0, 60.0 and 10,000 µg/g substrate, under the conditions: 2:1 water:substrate, 32 °C incubation temperature and 1.20 mm particle size substrate (Figure III-2).

Higher temperatures significantly decreased the release of phenolic compounds. The higher amount of phenolic compounds found in the *Citrus* residue fermented by *P. variotii* can be attributed to the ability of this species to hydrolyze structural carbohydrates pectin, cellulose, hemicelluloses and lignin (polyphenolic macromolecule closely bound to cellulose and hemicellulose in cell walls of plants) by way of various glycoside hydrolases (cellulases, hemicellulases). Also, polyphenol compounds such as hesperidin, naringin and ellagitannins can be hydrolyzed by active esterases (β-glucosidase, tannase) present in its metabolism. The higher enzymatic activity arsenal probably occurred at its optimum

temperature (32 °C) (Ferreira et al., 2013; Holopainen-Mantila et al., 2013; Pistarino et al., 2013).

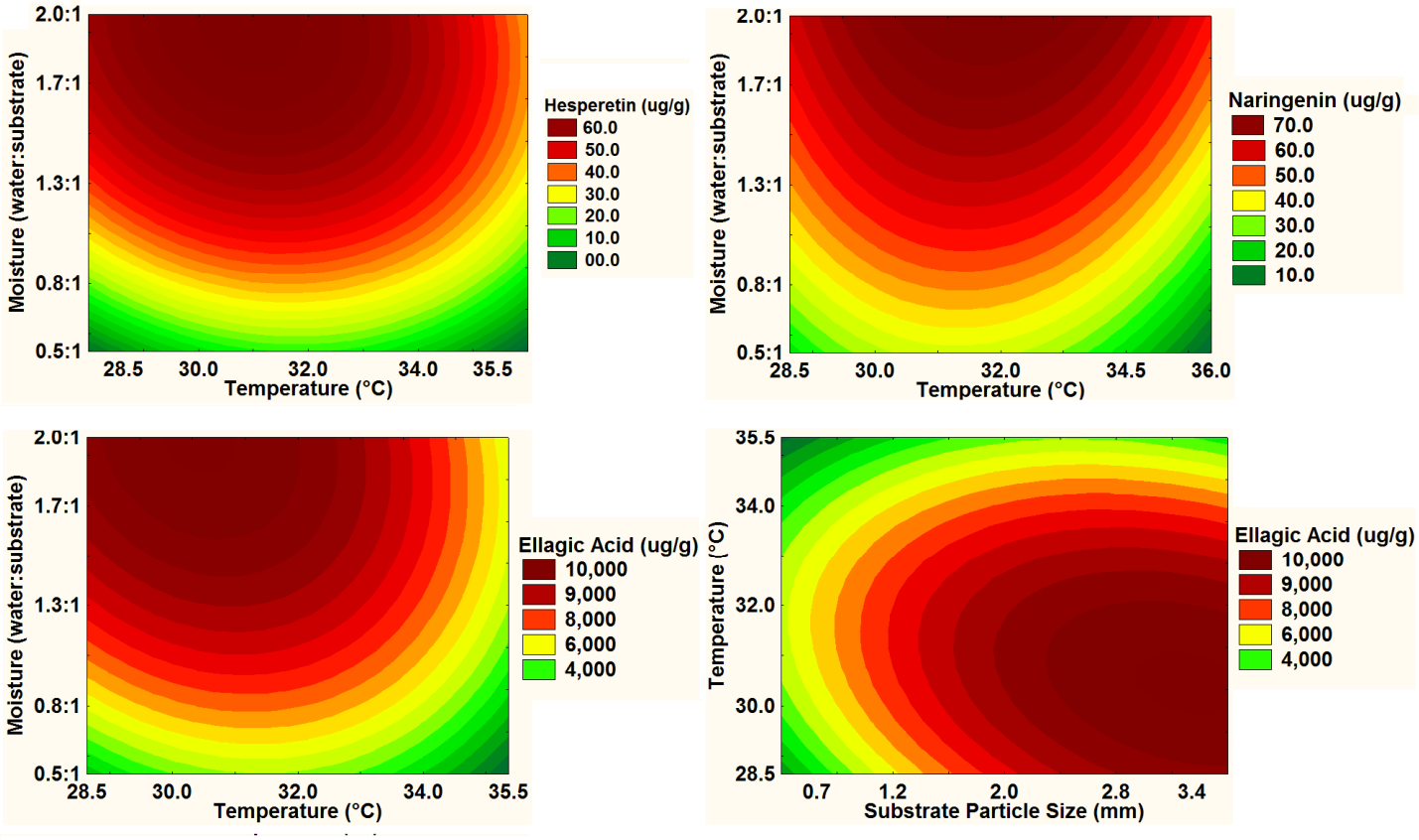


Figure III-2. A response surface representative of hesperetin, naringenin and ellagic acid production as a function of temperature vs. water:substrate ratio vs. particle substrate size, according to the CCD.

The substrate particle size was studied in phenolic compounds obtainment. For ellagic acid, the highest production was obtained with increasing particle size. Increased production for hesperetin and naringenin occurred with decreasing particle size. However, this variable showed less interference in obtaining the latter two phenolics. These effects might be due to the higher superficial area of contact between the substrate and the microorganism. Substrates with intermediate particle size provide a considerable contact area between the fungus and the substrate, favoring its growth; whereas very small particles

are more susceptible to compaction and the formation of agglomerates, resulting in decreased oxygen transfer, affecting respiration and fungal development. For some substrates, larger particles had higher porosity than smaller particles; however, particles with larger diameter also presented higher surface area. Despite the larger surface area and pore volume, smaller particles had pores that did not exceed 7 nm. Since the diameter of the fungal hyphae is usually greater than 10 nm, the microorganism would likely grow on the outside of the particles. The more porous particles benefited from aeration, dissipation of gases and heat produced during microbial growth. During the filamentous fungi growth on solid substrates, it is generally accepted that there is a limitation in oxygen supply to the cells that are in close contact with the substrate or that penetrate the substrate (Schmidt and Furlong, 2012; Membrillo et al., 2011).

Higher phenolic compound production was obtained at a maximal water:substrate ratio. Lower production of phenolic compounds at a lower water:substrate ratio might also be due to reduced water availability for biomass growth or reduced mobility of the substrate during SSF. During the bioprocess, the water content available to the substrate is extremely important, especially if the substrate has hemicellulose and pectin, which can absorb more water, potentially leading to an increase in microbial growth in the substrate and, consequently, the release of bioactive aglycon phenolic compounds (Madeira et al., 2013).

III.3.2. Enzymatic Biotransformation

Enzymatic kinetics in *Citrus* residue by biotransformation was realized, which resulted in the production of phenolic compounds such as hesperetin, naringenin and ellagic acid (Figure III-3).

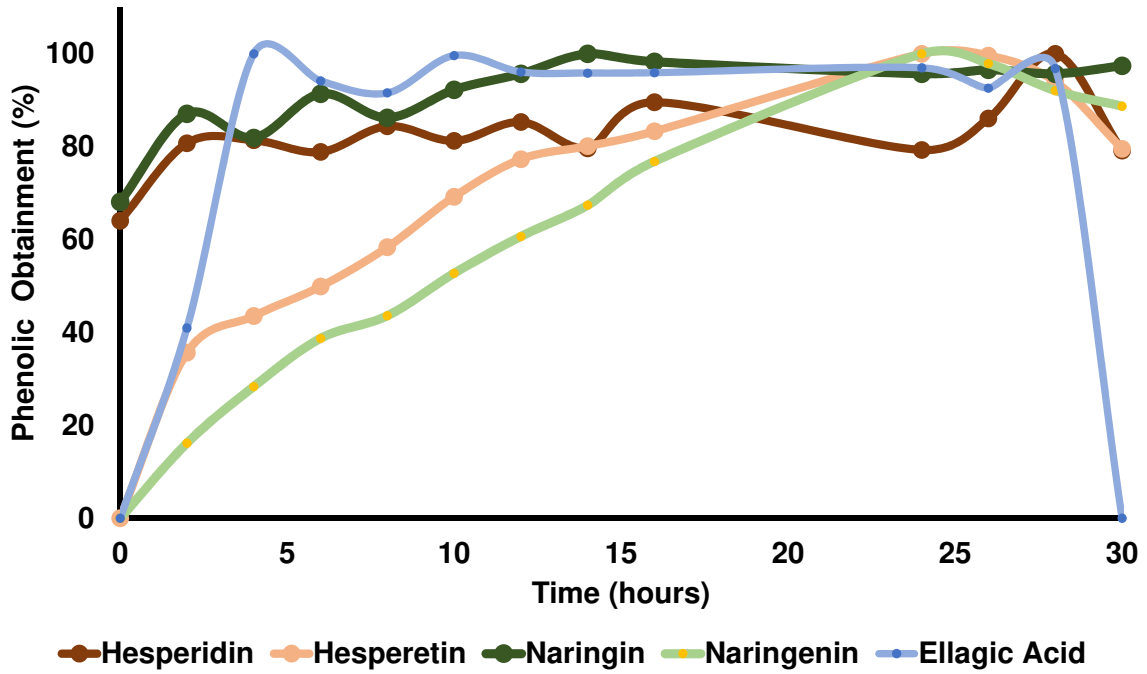


Figure III-3. Kinetics of enzymatic reaction of the *Citrus* residue by biotransformation: evaluating the concentration of hesperidin, hesperetin, naringin, naringenin, ellagic and gallic acid.

According to the results obtained, the initial concentrations of ellagic acid, hesperetin and naringenin in the *Citrus* residue were below the detection limit of the method, at practically zero. The last two reached their highest value within 24 h and then decreased to 80 and 90% at 30 h of incubation, respectively. The ellagic acid concentration was increased to 100% after 5 h of incubation. However, ellagic acid stayed at almost 90% during the next 20 h of incubation. Meanwhile, the concentration of hesperidin and naringin significantly increased. These compounds were most likely released from the complex matrix during the enzymatic reaction.

All phenolic compounds mentioned above decreased in concentration after 25 h of reaction, and it was not possible to transform all of the glycosylated forms into aglycon phenolics. These results may have been caused by two factors: the degradation of the final product and/or inhibition of the enzymes used in the reaction. The degradation of phenolic

compounds may occur from sustained exposure of the reaction medium with the atmospheric air. The hydroxyls present in phenolic compounds may be oxidized by exposure to the air of the environment (Hamza et al., 2012).

In addition, when vegetal cells are disrupted, the materials released from the cells may also include proteins, which are known to complex with phenolic compounds (Smith and Hossain et al., 2006). Enzymatic inhibition may occur, in particular, on the final product, as in the case of cellobiose, glucose and phenolic compounds for cellulase, pectinase, metal ions and phenolic compounds for the tannase (Battestin et al., 2007; Alvira et al., 2010; Andric et al., 2010; Ximenes et al., 2010). According to these results, the experimental design was carried out at 24 h of biotransformation reaction time.

The CCD experiment was designed to determine the optimal cellulase, pectinase and tannase activity (U/ml) for enzymatic biotransformation, and the results are shown in Table III-2.

The quadratic model used to calculate the hesperetin (Y_{HE}), naringenin (Y_{NE}) and ellagic acid (Y_{EA}) concentration (Equation 6, 7 and 8), after eliminating the statistically insignificant terms ($p>0.1$), is as follows (x_1: tannase; x_2: pectinase; x_3: cellulase):

$$Y_{HE} = 0.0105 + 0.0023x_1 - 0.0018x_1^2 - 0.0006x_3 - 0.0017x_3^2 \quad \text{Equation 6}$$

$$Y_{NE} = 0.0056 + 0.0007x_1 - 0.0007x_1^2 - 0.0005x_2 + 0.0005x_3 - 0.0003x_3^2 \quad \text{Equation 7}$$

$$Y_{EA} = 0.7932 + 0.0593x_1 + 0.0103x_1^2 + 0.0149x_3 - 0.0091x_2x_3 \quad \text{Equation 8}$$

The analysis of the variance was reproduced and is shown in Table III-4. The Fisher F-statistics for hesperetin, naringenin and ellagic acid were 10, 117 and 31 times higher than the F_t and p-value of <0.01, respectively, demonstrating that these regression models were statistically significant at the 90% confidence level. Additionally, the R^2 values obtained were 0.92, 0.93 and 0.97, respectively.

Table III-4. Analysis of variance and regression analyses for the response of the central composite design of phenolic compounds obtainment.

Response	Source of variation	Sum of squares	Degrees of freedom	Mean squares	F test/Ftab	R^2
Hesperetin	Regression	0.141	4	0.035	35/3.3	0.92
	Residual	0.012	12	0.001		
Naringenin	Regression	0.017	5	0.0034	377/3.2	0.93
	Residual	0.001	11	0.000009		
Ellagic acid	Regression	53.093	4	13.2733	108/3.4	0.97
	Residual	1.487	12	0.123		

The highest hesperetin, naringenin and ellagic acid production in the experiment (final concentration – initial concentration) were 9.5, 6.3 and 890 µg/ml, respectively. The response surface in Figure III-4 indicates that optimum hesperetin and naringenin concentration may have been found at 5.0 and 7.0 U/ml of cellulase and tannase, respectively. A different concentration of tannase and cellulase decreased naringenin and hesperetin concentration. In terms of ellagic acid, the highest enzymatic activities of cellulase and tannase (10.0 U/ml of cellulase and tannase) obtained the highest concentrations of phenolic compounds. The results showed that pectinase did not affect the ability to obtain phenolic compounds. According to the study of the quadratic model to obtain phenolic compounds, the tannase showed a higher impact in all responses (hesperetin, naringenin and ellagic acid).

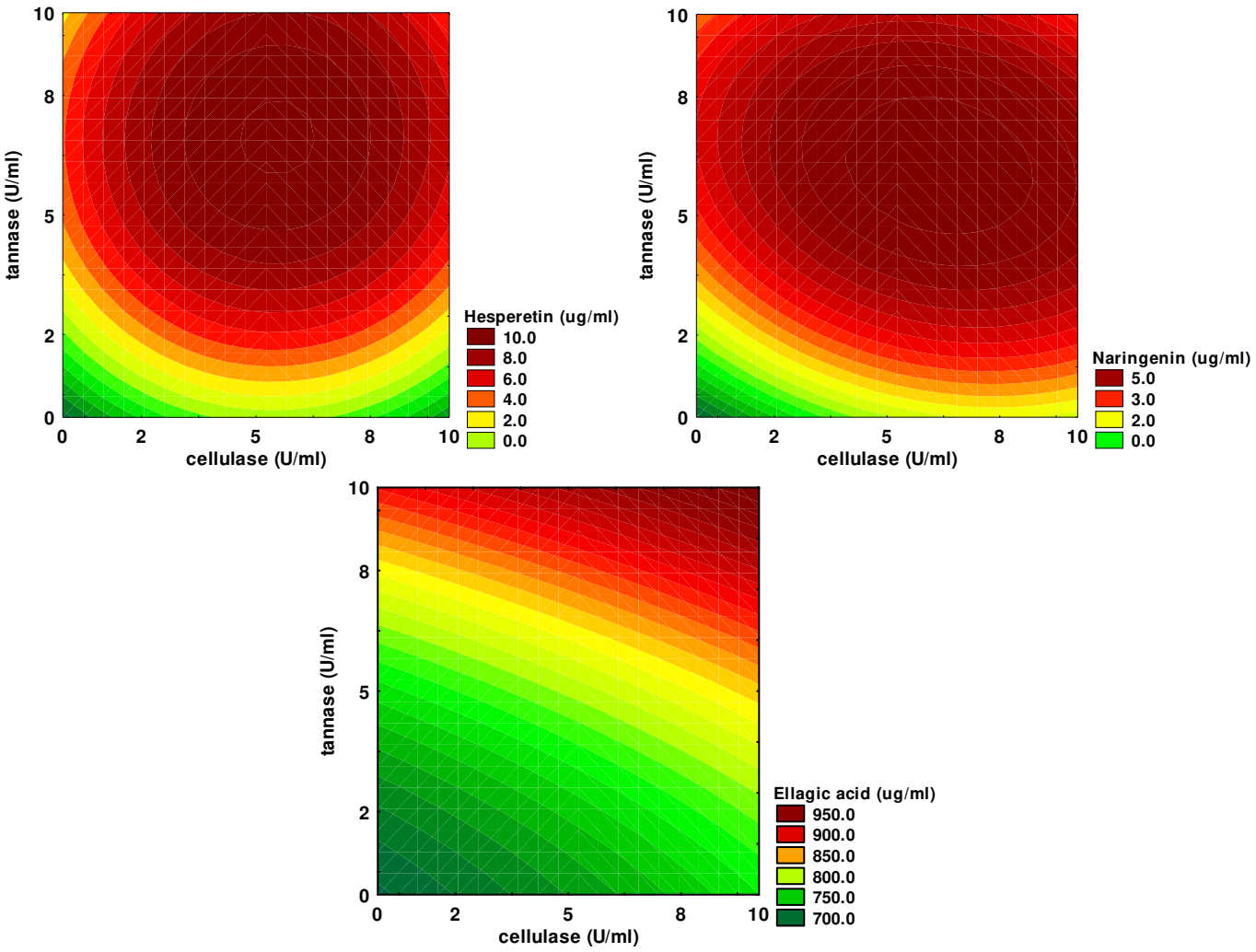


Figure III-4. A response surface representative of hesperetin, naringenin and ellagic acid production as a function of cellulase vs. tannase activity, according to the CCD.

Cellulase plays the role of a hydrolyzed vegetable cellular wall, facilitating the access of other enzymes in the cell. The tannase, therefore, operates in particular on phenolic compounds, hydrolyzing the ester bonds between the phenolic and saccharides, resulting in aglycon compounds (Alvira et al., 2010; Andric et al., 2010; Madeira et al., 2013).

The main reason that recovery increases with the use of enzymes is that the enzymes disrupt the integrity of the cell walls. However, in high enzyme concentrations,

when cells are disrupted, the materials released from the cells may also include proteins, which are known to complex with phenolic compounds. This may be due to the depletion of the substrates and/or product inhibition of enzymes. There are studies showing that long periods of incubation at high temperatures could decrease the phenolic compounds, suggesting the thermal degradation of phenolic over long periods of time (Kapasakalidis et al., 2009). Enzyme concentration also had a significant impact on levels of phenolic compounds in the extracts, with the higher enzyme dosage enhancing extraction of phenolics. The increase in extractability of phenolic depended on hydrolysis time and enzyme dosage; however there was no interaction between enzyme/substrate ratio and temperature (Madeira et al., 2013).

III.3.3. Productivity of Enzymatic and Microbial Biotransformations

The concentrations of phenolics before and after the processes, as well as their increase are shown in Table III-5.

Table III-5. Phenolic Compounds Productivity by Microbial and Enzymatic Biotransformation in *Citrus* Residues.

Samples	Hesperetin	Naringenin	Ellagic Acid
***Citrus* Residues (µg/g)**	1.25	1.25	172.5
Enzymatic Biotransformation (µg/g)	120.0	80.0	11,250.0
Increase of the Product (%)	9,500	6,300	6,422
***Citrus* Residues (µg/g)**	5.0	4.0	700.0
Microbial Biotransformation (µg/g)	50.0	60.0	10,000.0
Increase of the Product (%)	900	1,400	1,330

According to the results, the phenolic compounds yield obtained by enzymatic biotransformation was higher than that of microbial. The hesperetin, naringenin and ellagic acid yields in the enzymatic biotransformation were 10, 4.5 and 4.8 times higher than those of the microbial, respectively. Cai et al. (2012) reported the SSF on oats (*Avena sativa* L.) with three different filamentous fungi. The results showed that fermentation increased the amount of simplest phenolic compounds, such as ferulic and caffeic acid. Also, the species *Aspergillus oryzae* var. *effuse* was the microorganism that obtained the highest yield of phenolic compounds. In conclusion, biotransformation of phenolic compounds from fermentation is highly dependent upon the species employed. Madeira et al., (2013) published a review of published works, analyzing the two types of processes and concluded

that the enzymatic biotransformation showed better results in most papers published. One possible argument for this is that the action of enzymes are higher than microorganism for obtaintion products of interest. The *P. variotii* shows great adaptation to its environment, however its growth was 48 h to obtain higher concentration of phenolic, and the enzymatic process was only 24 h. (Battestin et al., 2008; Madeira et al., 2011; Madeira et al., 2012; Serva et al., 2012).

III.4. Conclusion

The microbial and enzymatic biotransformations are clean and viable biotechnologies with great potential for application in the obtainment of phenolic resources. The utilization of agro-industrial *Citrus* residues is particularly interesting because of its availability, low cost and features that allow obtaining different bioactive phenolic compounds. The processes studied were low cost and used an abundant source of hesperidin and naringin. It provided an interesting commercial source of hesperetin, naringenin and ellagic acid. These molecules have no commercial source to be extracted from and have been demonstrating much more important bioactivity potential than their glycosylate forms.

III.References

Aguilar CN, Aguilera-Carbo A, Robledo A, Ventura J, Belmares R, Martinez D, Rodríguez-Herrera R, Contreras J. (2008). Production of antioxidant nutraceuticals by solid-state cultures of pomegranate (*Punica granatum*) peel and creosote bush (*Larrea tridentate*) leaves. Food Technol Biotechnol, 46, 218-222.

Alvira P, Tómas-Pejó E, Ballesteros M, Negro MJ. (2010). Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. Biores Technol, 101, 4851-4861.

Andric P, Meyer AS, Jensen PA, Dam-Johansen K. (2010). Reactor design for minimizing product inhibition during enzymatic lignocellulose hydrolysis: I. Significance and mechanism of cellobiose and glucose inhibition on cellulolytic enzymes. Biotechnol Adv, 28, 308-324.

Banerjee S, Singh S, Rahman LU. (2012). Biotransformation studies using hairy root cultures – A review. Biotechnol Adv, 30, 461-468.

Barrios-González J. (2012). Solid-state fermentation: Physiology of solid medium, its molecular basis and applications. Process Biochem, 47, 175-185.

Barros HRM, Ferreira TAPC, Genovese MI. (2012). Antioxidant capacity and mineral content of pulp and peel from commercial cultivars from Brazil. Food Chem, 134, 1892-1898.

Battestin V, Macedo GA. (2004). Tannase production by *Paecilomyces variotii*. Biores Technol, 98, 1832-1837.

Chamorro S, Viveros A, Alvarez I, Vega E, Brenes A. (2012). Changes in polyphenol and polysaccharide content of grape seed extract and grape pomace after enzymatic treatment. Food Chem, 133, 308-314.

FAO (Food and Agriculture Organization of the United Nations), 2011. http://faostat.fao.org/site/339/default.aspx.

Ferreira LR, Macedo JA, Ribeiro ML, Macedo GA. (2013). Improving the chemopreventive potential of orange juice by enzymatic biotransformation. Food Res Int, 51, 526-535.

Ferreres F, Gil-Izquierdo A, Vinholes J, Silva ST, Valentão P, Andrade PB. (2012). *Bauhinia forficata* Link authenticity using flavonoids profile: Relation with their biological properties. Food Chem, 134, 894-904.

Georgetti SR, Vicentini FTMC, Yokoyama CY, Borin MF, Spadaro ACC, Fonseca MJV. (2009). Enhanced in vitro and in vivo antioxidant activity and mobilization of free phenolic compounds of soybean flour fermented with different β-glucosidase-producing fungi. J Appl Microbiol, 106, 459-466.

Hamza M, Khoufi S, Sayadi S. (2012). Fungal enzymes as a powerful tool to release antioxidants from olive mill wastewater. Food Chem, 131, 1430-1436.

Hollman PCH, Katan MB. (1997). Absorption, metabolism and health effects of dietary flavonoids in man. Biom Pharm, 51, 305-310.

Holopainen-Mantila U, Marjamaa K, Merali Z, Käsper A, de Bot P, Jääskeläinen A-S, Waldron K, Kruus K, Tamminen T. (2013). Impact of hydrothermal pre-treatment to chemical composition, enzymatic digestibility and spatial distribution of cell wall polymers. Bior Technol, 138, 156-162.

Justesen U, Arrigoni E, Larsen BR, Amado R. (2000). Degradation of flavonoid glycosides and aglycones during in vitro fermentation with human faecel flora. Lebensm Wiss Technol, 33, 424-430.

Kapasakalidis PG, Rastall RA, Gordon MH. (2009). Effect of a cellulase treatment on extraction of antioxidant phenols from black currant (*Ribes nigrum* L.) pomace. J Agric Food Chem, 2009, 57, 4342-4351.

Li BB, Smith B, Hossain MDM. (2006). Extraction of phenolics from *Citrus* peels: solvent extraction method. Sep Pur Technol, 48, 189-196.

Lin J-T, Chen Y-C, Lee Y-C, Rolis Hou C-W, Chen F-L, Yang D-J. (2012). Antioxidant, anti-proliferative and cyclooxygenase-2 inhibitory activities of ethanolic extracts from lemon balm (*Melissa officinalis* L.) leaves. LWT – Food Sci Technol, 49, 1-7.

Macedo JA, Battestin V, Ribeiro ML, Macedo GA. (2011). Increasing the antioxidant power of tea extracts by biotransformation of polyphenols. Food Chem, 126, 491-497.

Madeira Jr JV, Macedo JA, Macedo GA. (2011). Detoxification of castor bean residues and the simultaneous production of tannase and phytase by solid-state fermentation using *Paecilomyces variotii*. Bioresour Technol, 102, 7343-7348.

Madeira Jr JV, Macedo JA, Macedo GA. (2012). A new process for simultaneous production of tannase and phytase by *Paecilomyces variotii* in solid-state fermentation of orange pomace. Bioprocess Biosyst Eng, 35, 477-482.

Madeira Jr JV, Teixeira CB, Macedo GA. (2013). Biotransformation and bioconversion of phenolic compound: an overview. Crit Rev Biotechnol, DOI:10.3109/07388551.2013.803020.

Marín FR, Soler-Rivas C, Benavente-García O, Castillo J, Pérez-Álvarez JA. (2007). By-products from different *citrus* processes as a source of customized functional fibres. Food Chem, 100, 734-741.

Martins S, Mussatto SI, Martínez-Avila G, Montañez-Saenz J, Aguilar CN, Teixeira JA. (2011). Bioactive phenolic compounds: Production and extraction by solid-state fermentation. A review. Biotechnol Adv, 29, 365-373.

Meiyanto E, Hermawan A, Anindyajati. (2012). Natural products for cancer-targeted therapy: *Citrus* flavonoinds as potent chemoprotective agents. Asian Pac J Cancer Prev, 13, 427–436.

Membrillo I, Sánchez C, Meneses M, Favela E, Loera O. (2011). Particle geometry affects differentially substrate composition and enzyme profiles by *Pleurotus ostreatus* growing on sugar cane bagasse. Bioresour Technol, 102, 1581–1586.

Murakami A, Ashida H, Terao J. (2008). Multitarged cancer prevention by quercetin. Cancer Letters, 269, 315-325.

Nazari M, Ghorbani A, Hekmat-Doost A, Jeddi-Tehrani M, Zand H. (2011). Inactivation of nuclear factor-κB by *Citrus* flavanone hesperidin contributes to apoptosis and chemo-sensitizing effect in Romos cells. Eur J Phamacol, 650, 526–533.

Nielsen IL, Chee WS, Poulsen L, Offord-Calvin E, Rasmussen SE, Frederiksen H, Enslen M, Barron D, Horcajada M-N, Williamson G. (2006). Bioavailability is improved by enzymatic modification of the *Citrus* flavonoid hesperidin in humans: a randomized, double-blind, crossover trial. J Nutr, 136, 404-408.

Nutan, Modi M, Goel T, Das T, Malik S, Suri S, Rawat AKS, Srivastava S.K., Kumar, S, Tuli R, Malhotra S, Gupta SK. (2013). Ellagic acid & gallic acid from *Lagerstroemia speciosa* L. inhibit HIV-1 infection through inhibition of HIV-1 protease & reverse transcriptase activity. Ind J Med Res, 137, 540-548.

Ohguchi K, Akao Y, Nozawa Y. (2006). Stimulation of melanogenesis by the *Citrus* flavonoid naringenin in mouse B16 melanoma cells. Biosci Biotechnol Biochem, 70, 1499-1501.

Panke S, Held M, Wubbolts M., 2004. Trends and innovations in industrial biocatalysis for the production of fine chemicals. Curr. Opin. Biotechnol. 15, 272-279.

Park HJ, Kim M-J, Ha Chung J-H. (2008). Apoptosis effect of hesperidin through caspase 3 activation in human colon cancer cells, SNU-C4. Phytomedicine, 15, 147–151.

Pistarino E, Aliakbarian B, Casazza AA, Paini M, Cosulich ME, Perego P. (2013). Combined effect of starter culture and temperature on phenolic compounds during fermentation of Taggiasca black olives. Food Chem, 138, 2043-2049.

Prasad D, Gupta RK, Mathangi G, Kamini NR, Gowthaman MK. (2012). Advances in production and characteristic features of microbial tannases: an overview. Current Trends Biotech Pharm, 6, 145-165.

Schmidt CS, Furlong EB. (2012). Effect of particle size and ammonium sulfate concentration on rice bran fermentation with the fungus *Rhizopus oryzae*. Bior Technol, 123, 36-41.

Sepúlveda L, Ascacio A, Rodríguez-Herrera R, Aguilera-Carbó A, Aguilar CN. (2011). Ellagic acid: Biological properties and biotechnological development for production processes. Afr J Biotechnol, 10, 4518-4523.

Sergent T, Vanderstraeten J, Winand J, Beguin P, Schneider Y-J. (2012). Phenolic compounds and plant extracts as potential natural anti-obesity substances. Food Chem, 135, 68-73.

Sharma S, Bhat TK, Dawra RK. (2000). A spectrophotometric method for assay of tannase using rhodanine. Anal Biochem, 279, 85-89.

Shi B, He Q, Yao K, Huang W, Li Q. (2005). Production of ellagic acid from degradation of valonea tannins by *Aspergillus niger* and *Candida utilis*. J Chem Technol Biotechnol, 80, 1154-1159.

Starzynska-Janiszewska A, Stodolak B, Jamroz M. (2008). Antioxidant properties of extracts from fermented and cooked seeds of Polish cultivars of *Lathyrus sativus*. Food Chem, 109, 285–292.

Tripoli E, La Guardia M, Giammanco S, Di Majo D, Giammanco M. (2007). *Citrus* flavonoids: Molecular structure, biological activity and nutritional properties: A review. Food Chem, 104, 466-479.

Vattem DA, Shetty K. (2003). Ellagic acid production and phenolic antioxidant activity in cranberry residue (*Vaccinium macrocarpon*) mediated by *Lentinus edodes* using a solid-state system. Process Biochem, 39, 367–379.

Ximenes E, Kim Y, Mosier N, Dien B, Ladisch M. (2010). Inhibition of cellulases by phenols. Enz Microb Technol, 46, 170-176.

CAPÍTULO IV - Chemopreventive potential of biotransformed *Citrus* residue extracts on colon cancer cells

Submetido para *Food Research International*

Abstract

Biotransformation is deemed economically/ecologically competitive in the search for phenolic compounds. Nowadays, attention has turned to phenolic compounds with biological activity. Colorectal cancer constitutes one of the most frequent malignancies worldwide and is one of the prevalent causes of cancer-related mortality in the western world. The objective of this study was to evaluate the biological potential of the enzymatic and microbial biotransformation process in *Citrus* residues. Concerning the bioactivity potential, the enzymatic and microbial biotransformation increased 65 and 118% of the antioxidant capacity, respectively. The antiproliferative activity achieved 58 and 42% of tumoral cellular inhibition by enzymatic and microbial biotransformation, respectively. These results suggest that biotransformation process may be a viable alternative for rich bioactive phenolic extract production using residues at low price values.

Resumo

A biotransformação é considerada econômica e ecologicamente competitiva na busca de compostos fenólicos. Atualmente, a atenção tem sido focada nos compostos fenólicos com alta atividade biológica. O câncer colorretal constitui uma das neoplasias mais frequentes em todo o mundo, e é uma das causas prevalentes de mortalidade relacionada ao câncer no mundo ocidental. O objetivo deste estudo foi avaliar o potencial

biológico dos processos de biotransformação enzimática e microbiana em resíduo de *Citrus*. No que se refere ao potencial biológico, as biotransformações enzimática e microbiológica aumentaram 65 e 118% da capacidade antioxidante, respectivamente. A atividade anti-proliferativa obtida foi de 58 e 42% de inibição celular tumoral pelas biotransformações enzimática e microbiana, respectivamente. Estes resultados sugerem que o processo de biotransformação pode ser uma alternativa viável para a produção de extratos fenólicos bioativos, usando resíduos com baixo valor comercial.

IV.1. Introduction

Colorectal cancer constituts one of the most frequent malignancies worldwide and is one of the prevalent causes of cancer-related mortality in the western world (Cooper et al., 2010). Therefore, further development of therapeutic and preventive approaches to control this disease is clearly needed. The use of bioactive plant compounds for the treatment of disease has become an interesting subject for scientific research.

Phenolic compounds are derived mainly from the secondary metabolites of plants from phenylalanine (Treutter, 2001). Numerous compounds of different chemical structure are grouped together (Martins et al., 2011). Among them, flavanones constitute the main subgroup, and it has been extensively reported that they have high antioxidant activity. The most prevalent flavanones in tissues and peels of *Citrus* fruits are naringin and hesperidin. The Brazilian share of the world market of concentrated orange juice is around 53%, and production was about 19.8 million tons of oranges in 2011, an increase of 7% from the previous year (FAO, 2011). Approximately half of *Citrus* fruit is discarded during the manufacture of juice, thus generating large amounts of residue. The residue generated was

approximately 8% and consisted of phenolic compounds, including flavanones (Marín et al., 2007).

Recent studies indicate that molecules of the simplest classes of phenolics have higher biological activity and absorption capacity, such as naringenin and hesperetin (Hollman & Katan, 1997; Shigeshiro et al., 2013). Numerous studies have evaluated that *Citrus* phenolic compounds promote cancer cell growth inhibition and apoptosis, as well as reducing invasion, angiogenesis and metastasis (Erlund, 2004; Chen et al., 2003; Huang et al., 2012; Meiyanto et al., 2012). A large amount of molecular mechanisms of phenolics has been suggested, including anti-oxidant and pro-oxidant effects, inhibition of protein kinases, or modulation of growth factor receptor tyrosine kinases (Ferreres et al., 2012; Kim et al., 2003; Lin et al., 2012).

Currently, phenolic compounds are obtained by chemical synthesis or extraction. An interesting environmentally friendly alternative that deserves attention regarding phenolic compound production is the biotransformation of these molecules. A biotransformation process can be applied to a specific modification of a defined compound for a defined product with structural similarity and performed by cells or isolated enzymes. The number and diversity of applications of biotransformation are still modest considering the great availability of useful microorganisms and the broad scope of reactions that they can produce. Furthermore, biotransformation reaction technology is deemed economically and ecologically competitive in the search for new compounds. Enzyme availability, substrate scope, and operational stability are some of the limitations of biotransformation that could be overcome by scientific research (Banerjee et al., 2012; Madeira et al., 2013).

The objective of this study was to evaluate the antioxidant and antiproliferative potential increased by the enzymatic and microbial biotransformation of a phenolic rich extract from *Citrus* residues.

IV.2. Material and methods

IV.2.1. Material

Hesperetin, naringenin and ellagic acid 2,2′-azobis(2-methylpropionamidine) (97%) (AAPH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), sulforhodamine B sodium salt (SRB), trichloroacetic acid and T1503 Trizma® base were purchased from Sigma-Aldrich Co. Fluorescein was purchased from ECIBRA, and Trolox® (97%) was purchased from ACROS Organics. Cell culture reagents were purchased from Invitrogen®. *Citrus* residue was kindly donated by CP Kelco industry headquarters (Limeira, SP, Brazil), from juice and pectin extraction, origin to a residue of low quality and commercial value.

IV.2.2. Microbial Biotransformation

The bioactive phenolic compounds from *Citrus* residue were obtained according to the following procedure (chapter III): A 250 ml conical flask containing the following constituents was used for the fermentation process: 10 g of *Citrus* residue (2.00 mm particle size of substrate) and 20.0 ml of distilled water. The culture medium was sterilized at 120 °C for 20 min. After sterilization, the flasks were inoculated with 1.0 ml (9.0×10^6 cells/ml) of the pre-inoculum suspension and incubated at 32 °C at 90% relative humidity (Climate Chamber 420 CLD, Nova Etica, SP, Brazil) for 48 h. The control treatment was carried out in the same conditions without the microorganism. The phenolic compound extraction was performed by adding 250 ml of methanol, 70%.

IV.2.3. Enzymatic Biotransformation

The bioactive phenolic compounds from *Citrus* residue were obtained according to the following procedure (chapter III): A 125 ml conical flask was used, containing the following constituents: 2 g of *Citrus* residue (0.80 mm particle size of substrate) and 25.0 ml of 20 mM acetate buffer with 8.0 U/ml of tannase and 5.0 U/ml of cellulase, incubated in a shaker at 40 ºC and 130 rpm for 24 h. The control treatment was carried out in the same conditions without the enzymes. After the incubation period, the phenolic compound extraction was performed by adding 58 ml of methanol, with a final methanol concentration of 70%.

IV.2.4. *Citrus* Residue Biotransformed Extraction

An aliquot 25 ml of *Citrus* residue biotransformed and non-biotransformed methanolic extracts were evaporated on a rota-evaporator at 40 °C under reduced pressure, from which were obtained 5 ml of extracts of methanol-free solution. The final solution was treated by a lyophilizer for evaluation of non-biotransformed (NBE), enzymatic biotransformation (EBE) and microbial biotransformation extract (MBE) and measurement of dry weight.

IV.2.5. Bioactivity Potential

IV.2.5.1. Antioxidant Capacity

IV.2.5.1.1. DPPH

According to Macedo et al. (2011), the antioxidant capacity of standard and biotransformed hesperidin was assessed on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. The reaction mixtures, consisting of 50 µl of test samples and 150 µl of 0.2 mM DPPH in methanol, were carried out on a NovoStar Microplate reader (BMG LABTECH, Germany) with absorbance filters for a wavelength of 520 nm. The decolorizing process was recorded after 90 min of reaction and compared with a blank control, instead of DPPH. The DPPH solution and reaction medium were freshly prepared and stored in the dark. The measurement was performed in triplicate. Antiradical activity was calculated from the equation determined from the linear regression after plotting known solutions of Trolox with various concentrations. Antiradical activity was expressed as µmol of Trolox equivalent/mg of standards or ml of extracts (NBE, EBE and MBE).

IV.2.5.1.2. ORAC

ORAC assays were performed using fluorescein (FL) as the fluorescent probe, as described by Macedo et al., (2011). The automated ORAC assay was carried out on a NovoStar Microplate reader (BMG LABTECH, Germany) with fluorescence filters for an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The measurements were made in a COSTAR 96 plate. The reaction was performed at 37 °C, having been started by thermal decomposition of AAPH in a 75 mM phosphate buffer (pH

7.4) due to the sensitivity of FL to pH. The measurements were performed in triplicate. ORAC values were defined as the difference between the area under the FL decay curve and the blank (net AUC). Regression equations between net AUC and antioxidant concentration were calculated for all of the samples. ORAC-FL values were expressed as μmol of Trolox/mg of the standards or ml of extracts (NBE, EBE and MBE).

IV.2.5.2. Antiproliferative Potential

IV.2.5.2.1. Cell culture and maintenance

The human colon adenocarcinoma grade II cell line, HT29 was donated from the collection of the Pharmacology and Toxicology Division from CPQBA-UNICAMP and established at the Bioactive Compounds Laboratory at FEA-UNICAMP. The cells were cultured in DMEM supplemented with 5% fetal bovine serum (Invitrogen©). The cells were maintained at 37 °C and 5% CO2–95% humidified air.

IV.2.5.2.2. Cell proliferation assay

For chemosensitivity testing, the SRB assay, which is based on the staining of cellular proteins, has many advantages over other in vitro tests. The SRB assay shows a wide linear range with cell number, higher sensitivity and a stable end-point that does not require time-sensitive measuring. Ninety-six-well microplates were inoculated with HT29 cells at a density of 5×10^4 cells per well. Following incubation for 24 h, the adherent cells were washed once with a PBS (phosphate-buffered solution). Cells were then incubated in DMEM containing 50–250 μg/ml commercial standards (naringin, naringenin, hesperidin, hesperetin and ellagic acid), enzymatic biotransformed (EBE), microbial biotransformed (MBE) and non-biotransformed (NBE) *Citrus* residue extracts. Positive and negative

controls were also performed. After incubation at 37 °C in an atmosphere of 5% CO_2 and 100% relative humidity for 48 h, the cultures were assayed to detect the effects of the tested compounds on cellular proliferation.

Cellular proliferation was measured using the sulforhodamine B (SRB) assay, which has been described in detail by Monks et al., (1991). Briefly, adherent cell cultures were fixed *in situ* by adding 50 μL of cold 50% (w/vol) trichloroacetic acid (TCA) (final concentration, 10% TCA) and incubating the samples for 60 min at 4 °C. The supernatant was then discarded, and the plates were washed five times with deionized water and dried. One hundred microliters of SRB solution (0.4% w/vol in 1% acetic acid) was added to each microliter well, and the cultures were incubated for 10 min at room temperature. Unbound SRB was removed by washing the samples five times with 1% acetic acid. The plates were then air-dried. Bound stain was solubilized with a Tris buffer, and the optical densities were read at a single wavelength of 515 nm using an automated spectrophotometric plate reader. The results are expressed as percentages of the cell growth of the positive control (a cell line cultivated under the same conditions, without contact with the tested sample) (Macedo et al., 2012).

IV.2.6. Statistical Analyses

The Statistica 7.0 software (Statsoft, Inc., Tulsa, USA) package was used, and all values reported in the biotransformation of *Citrus* residue represent the mean from three replicates and standard deviation. Significant differences ($p<0.05$) in data biological tests were determined by t-tests.

IV.3. Results and Discussion

IV.3.1. Antioxidant Capacity

The non-biotransformed (NBE), microbial (MBE) and enzymatic biotransformation extracts (EBE) were produced according to the experimental conditions mentioned in item IV.2.5.1. The antioxidant capacity before and after the processes, as well as their yields, are shown in Table IV-1.

Table IV-1. Antioxidant Potential by Microbial and Enzymatic Biotransformation in *Citrus* Residues.

Sample	ORAC					DPPH
	Trolox equivalents (µmol/ml extract)[b]	**Sample concentration range (ml extract)**	**Slope**	**Intercept**	**r2**	**Trolox equivalents (µmol/ml extract)[b]**
NBE[a]	6538 ± 853[c]	0.031 - 0.25	1378	7.4	0.99	260 ± 6[a]
EBE[a]	10767 ± 890[b]	0.02 – 0.13	10.8	82.5	0.98	259 ± 13[a]
MBE[a]	14287 ± 869[a]	0.016 - 0.063	2935	4.4	0.99	266 ± 9[a]

[a] – NBE: non-biotransformed extract; EBE: enzymatic biotransformed extract; MBE: microbial biotransformed extract.

[b] - Results are presented as the mean (n = 3) ± SD, and those with different letters are significantly different, with $p < 0.05$.

According to the results, there was an increase of over 70% in the antioxidant capacity, by the ORAC method, of the residue against the free radical APPH after the two biotransformation processes. Among the results of the two processes of biotransformation there was statistical difference in antioxidant potential. MBE showed greater protection

against the oxidative APPH radical. One of the possible hypotheses for these increased activities is that the biotransformation process transformed the phenolics presented on the substrate into aglycone phenolic molecules; it is known that the aglycons have higher antioxidant capacity.

To verify this hypothesis, the antioxidant activity of phenolic commercial standards representing the major phenolics presented in the original extract (hesperidin and naringin) and in the biotransformed one (hesperetin, naringenin and ellagic acid) was measured. Table IV-2 shows the antioxidant capacity of commercial standard phenolic compounds. It also shows an increase in antioxidant capacity (ORAC and DPPH) of phenolic compounds with low molecular weight, compared with those with higher molecular weight.

Table IV-2. Trolox equivalents and linearity ranges for the ORAC (net AUC vs. concentration) and DPPH assay performed on the phenolic standards.

Sample	ORAC					DPPH
	Trolox equivalents (µmol/mg standard)	Sample concentration range (mg/ml)	Slope	Intercept	r2	Trolox equivalents (µmol/g standard)
Hesperidin	2333 ± 202[d]	0.04 – 0.08	68.5	1.3	0.99	55 ± 3[e]
Hesperetin	6552 ± 1250[c]	0.04 – 0.08	110.9	8.1	0.96	865 ± 91[a]
Naringin	7958 ± 610[b]	0.04 – 0.08	247.5	3.6	0.95	67 ± 2[e]
Naringenin	9955 ± 887[b]	0.04 – 0.08	293.4	5.4	0.99	428 ± 10[c]
Ellagic acid	1246 ± 226[d]	0.05 – 0.50	1170.6	0.9	0.99	192 ± 27[d]

[a,b,c,d,e] Results are presented as the mean (n = 3) ± SD, and those with different letters are significantly different, with $p < 0.05$.

The hesperetin and naringenin antioxidant capacities were 2.81 and 1.25 times greater than hesperidin and naringin, respectively. In addition, the ellagic acid presents lower antioxidant activity than other compounds. Besides its low activity, the ellagic acid showed high concentration in the two biotransformation process extracts. Therefore, this compound presents relative importance in obtainment of bioactive phenolic compounds by biotransformation, and may be important for other biological activities.

Beyond ellagic acid, two of the main compounds responsible for the increase of antioxidant activity were hesperetin and naringenin production in enzymatic and microbial biotransformation processes. The two phenolic compounds were produced in high concentration compared with other compounds, and simultaneously the hesperetin and naringenin presented high antioxidant capacity.

Therefore, it can be assumed that part of the increased antioxidant activity of the fermented extract was associated with the higher content of phenolic compounds of lower molecular weight, such as hesperetin, naringenin and ellagic acid.

Among the many possible forms of action of natural compounds, the antioxidant activity has an important effect on the increase of the tumor. Oxidative damage to DNA is considered a critical step in cancer development. Numerous experimental and epidemiological studies have shown that antioxidant compounds are able to prevent or slow down oxidative stress-induced damage leading to carcinogenesis by upsetting the molecular events in the initiation, promotion or progression conditions.

Yu et al. (2014) showed the influence of flavonoids from *Citrus* on antioxidant activity. *Citrus* peel presents a high amount of naringin and hesperidin, and these compounds have direct impact on antioxidant capacity in related residue.

Ferreira et al., (2013) studied the accumulation of antioxidant phenolic compounds by tannase reaction in orange juice, and an increase in aglycon phenolics and antioxidant capacity was observed. Madeira et al., (2012) described the fermentation of *Citrus* residue by *Paecilomyces variotii* for tannase production. After 120 h of incubation, the fermented substrate showed antioxidant activity 10 times greater than the unfermented substrate by the TEAC method. These results strongly indicate that the *Citrus* residue fermented by *Paecilomyces variotii* was improved in antioxidant compounds.

IV.3.2. Antiproliferative Potential

There are many tests on human tumoral cells, considering that the same compound does not affect different tissues in the same manner. For this reason, human colon adenocarcinoma cells (HT29) were chosen for initial antiproliferative modulation evaluation of the newly generated extracts. The results of the biotransformation processes were summarized in Table IV-3 and expressed as the concentration of extract tested and the related cell viability (%).

Table IV-3. Antiproliferative potential in cell line HT29 by Microbial and Enzymatic Biotransformation in *Citrus* Residues[1].

Cell viability (%)					
Sample	**Sample Concentration (µg/ml)[3]**				
	0.025	**0.25**	**2.5**	**25.0**	**250.0**
NBE[2]	97[a]	108[a]	111[a]	89[ab]	37[d]
EBE[2]	58[c]	77[b]	84[ab]	69[b]	72[b]
MBE[2]	75[b]	73[b]	77[b]	70[b]	42[d]

[1] - The results are expressed in percentage of growth (%);

[2] - NBE: non-biotransformed extract; EBE: enzymatic biotransformed extract; MBE: microbial biotransformed extract;

[3] - Results are presented as the mean (n = 3) ± SD, and those with different letters are significantly different, with $p < 0.05$.

According to the results, the biotransformed extracts (EBE and MBE) obtained moderate cell growth inhibition compared to the non-biotransformed extract (NBE), in lower extract concentration (0.0025 to 2.5 µg/ml). In addition, the highest concentrations of non-biotransformed extracts (25.0 and 250.0 µg/ml) inhibited 11 and 63% of cell growth, respectively, therefore obtaining a pronounced cytostatic effect. Probably in these concentrations, the high amount of bioactive compounds, including the glycosylated flavanones, prevented tumor cell growth. Furthermore, the two highest concentrations showed an initial dose-dependent response of cell growth inhibition.

The enzymatic and microbial biotransformation extracts obtained about 30% of cell growth inhibition between 0.25 and 250.0 µg/ml, and 0.025 and 25.0 µg/ml, respectively.

This bioactivity could be related to the antioxidant capacity of the two processes, which means it is related to the phenolic compounds obtained during the bioprocesses.

Most works showed no relation between antiproliferative potential and amount of phenolic compounds in extracts. However, there are specific phenolic compounds with high biological capacity. Table IV-4 shows important phenolic compounds obtained by enzymatic and microbial biotransformation in *Citrus* residues. According to the results, the aglycon phenolic forms presented higher antiproliferative potential than the glycosylated forms. The hesperetin and naringenin in 250 µg/ml presented a cytocidal effect, with 4 and -6% of tumor cell growth, respectively. Probably these two aglycon flavanones were responsible for the antiproliferative effect of the two biotransformation processes. This cytocidal effect observed on the commercial aglycone molecules happened in concentrations similar to the concentration of the chemotherapical drug tested as control (Doxorubicin). These concentrations were much higher than those present in the biotransformed extracts. Even so, the diluted compounds of the extracts produced achieved around 30-40% of tumoral growth inhibition. Higher concentrations of the extracts produced may have stronger effects.

Table IV-4. Antiproliferative potential of naringin, naringenin, hesperidin, hesperetin and ellagic acid, in cell line HT29[1].

Cell viability (%)					
Sample	**Sample Concentration (µg/ml)[3]**				
	0.025	**0.25**	**2.5**	**25.0**	**250.0**
Hesperidin	100[a]	100[a]	100[a]	97[a]	58[c]
Hesperetin	100[a]	99[a]	92[ab]	71[b]	4[e]
Naringin	102[a]	104[a]	106[a]	111[a]	83[b]
Naringenin	99[a]	99[a]	104[a]	89[ab]	-6[e]
Ellagic Acid	74[b]	79[b]	89[b]	99[a]	102[a]
Doxorubicin[2]	100[a]	79[b]	39[d]	0[e]	-5[e]

[1] – The results are expressed in percentage of growth (%);

[2] – Chemotherapy drug as control;

[3] - Results are presented as the mean (n = 3) ± SD, and those with different letters are significantly different, with $p < 0.05$.

Miranda et al. (1999) showed different flavonoids (chalcone and flavanone purified extract) by antiproliferative effects in human cancer cell lines. The flavonoids inhibited 100% cancer cell growth on HT29 with 100 µM after 2 days of incubation. One of the main possibilities is the inhibition of DNA synthesis, which stopped the tumor cell from growing.

Ferreira et al., (2013) studied the antiproliferative potential of orange juice. According to the results, the highest value of antiproliferative capacity in orange juice was 72% of cell viability in 10 µg/ml of sample. This result is similar to the antiproliferative

activity of enzymatic biotransformed extract (EBE) and higher than that of microbial biotransformed extract (MBE). In general, peels or residues show higher contents of phenolic compounds than pulps. Therefore, the use of residue for production of antiproliferative activity phenolic compounds seems to provide a major advantage over pulp extraction.

IV.3. Conclusion

The microbial and enzymatic biotransformation of phenolic compounds from *Citrus* residues seems to be a promising way to increase the concentration of free smaller and simpler phenolics. These bioprocesses are clean technologies with great potential for obtainment of biologically active compounds from natural sources. In this case, the re-use of residues is of particular interest because of its availability, low cost and features that allow obtaining different bioactive phenolic compounds, as well as being an environmentally interesting alternative for their removal. These molecules have no commercial source to be extracted from and have demonstrated much more important bioactivity potential than their original glycosylate forms. Also demonstrated was the usefulness of biotechnology on natural food molecules to improve their nutraceutical potential and provide a commercial source of these compounds.

IV.Reference

Banerjee S, Singh S, Rahman LU. (2012). Biotransformation studies using hairy root cultures – A review. Biotechnol Adv, 30, 461-468.

Battestin V, Macedo GA, de Freitas VAP. (2008). Hydrolysis of epigallocatechin gallate using a tannase from *Paecilomyces variotii*. Food Chem, 108, 228-233.

Cai S, Wang O, Wu W, Zhu S, Zhou FJB, Gao F, Zhang D, Liu J, Cheng Q. (2012). Comparative study of the effects of solid-state fermentation with three filamentous fungi on the total phenolics content (TPC), flavonoids, and antioxidants activities of subfractions from oats (Avena sativa L.). J Agric Food Chem, 60, 507-513.

Chen Y-C, Shen S-C, Lin H-Y. (2003). Rutinoside at C7 attenuates the apoptosis-inducing activity of flavonoids. Biochem Pharmacol, 66, 1139-1150.

Cooper K, Squires H, Carroll C, Papaioannou D, Booth A, Logan RF, Maguire C, Hind D, Tappenden P. (2010). Chemoprevention of colorectal cancer systematic review and economic evaluation. Health Technol Assessment, 14, DOI: 10.3310/htal4320.

Erlund I. (2004). Review of the flavonoids quercetin, hesperetin and naringenin. Dietary sources, bioactivities, bioavailability, and epidemiology. Nutrit Res, 24, 851-874.

FAO (Food and Agriculture Organization of the United Nations) (2011). http://faostat.fao.org/site/339/default.aspx.

Ferreira LR, Macedo JA, Ribeiro ML, Macedo GA. (2013). Improving the chemopreventive potential of orange juice by enzymatic biotransformation. Food Res Internat, 51, 526-535.

Ferreres F, Gil-Izquierdo A, Vinholes J, Silva ST, Valentão P, Andrade PB. (2012). Bauhinia forficata Link authenticity using flavonoids profile: Relation with their biological properties. Food Chem, 134, 894-904.

Hollman PCH, Katan MB. (1997). Absorption, metabolism and health effects of dietary flavonoids in man. Biomedic Pharmacother, 51, 305-310.

Huang Y-C, Liu K-C, Chiou Y-L. (2012). Melanogenesis of murine melanoma cells induced by hesperetin, a *Citrus* hydrolysate-derived flavonoid. Food Chem Toxicol, 50, 653-659.

Kim HK, Jeong TS, Lee MK, Park YB, Choi MS. (2003). Lipid-lowering efficacy of hesperetin metabolites in high-cholesterol fed rats. Clin Chim Acta, 327, 129-137.

Lin J-T, Chen Y-C, Lee Y-C, Rolis Hou C-W, Chen F-L, Yang D-J. (2012). Antioxidant, anti-proliferative and cyclooxygenase-2 inhibitory activities of ethanolic extracts from lemon balm (*Melissa officinalis* L.) leaves. LWT – Food Sci Technol, 49, 1-7.

Macedo JA, Battestin V, Ribeiro ML, Macedo GA. (2011). Increasing the antioxidant power of tea extracts by biotransformation of polyphenols. Food Chem, 126, 491-497.

Macedo JA, Ferreira LR, Camara LE, Santos JC, Gambero A, Macedo GA, Ribeiro ML. (2012). Chemopreventive potential of the tannase-mediated biotransformation of green tea. Food Chem, 133, 358-365.

Madeira Jr JV, Macedo JA, Macedo GA. (2011). Detoxification of castor bean residues and the simultaneous production of tannase and phytase by solid-state fermentation using *Paecilomyces variotii*. Biores Technol, 102, 7343-7348.

Madeira Jr JV, Macedo JA, Macedo GA. (2012). A new process for simultaneous production of tannase and phytase by *Paecilomyces variotii* in solid-state fermentation of orange pomace. Biop Biosyst Eng, 35, 477-482.

Madeira Jr JV, Teixeira CB, Macedo GA. (2013). Biotransformation and bioconversion of phenolic compound: an overview. Crit Rev Biotechnol, DOI:10.3109/07388551.2013.803020.

Marín FR, Soler-Rivas C, Benavente-García O, Castillo J, Pérez-Álvarez JA. (2007). By-products from different *Citrus* processes as a source of customized functional fibres. Food Chem, 100, 734-741.

Martins S, Mussatto SI, Martínez-Avila G, Montañez-Saenz J, Aguilar CN, Teixeira JA. (2011). Bioactive phenolic compounds: Production and extraction by solid-state fermentation. A review. Biotechnol Adv, 29, 365-373.

Meiyanto E, Hermawan A, Anindyajati. (2012). Natural products for cancer-targeted therapy: *Citrus* flavonoinds as potent chemoprotective agents. Asian Pac J Cancer Prev, 13, 427–436.

Miranda CL, Stevens JF, Helmrich A, Henderson MC, Rodriguez RJ, Yang Y-H, Deinzer ML, Barnes DW, Buhler DR. (1999). Antiproliferative and cytotoxic effects of prenylated flavonoids from Hops (*Humulus lupulus*) in human cancer cell lines. Food Chem Toxicol, 37, 271-285.

Monks A, Scudiero D, Skehan P, Shoemaker R, Paull K, Vistica D, Hose C, Langley J, Cronise P, Vaigro-Wolff A. (1991). Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. J Nat Cancer Inst, 83, 757-766.

Serva AP, Stathopoulou PM, Katsifas EA, Karagouni AD, Hatzinikolaou DG. (2012). The filamentous fungus *Paecilomyces variotii* as a potential candidate for bioethanol production via consolidated bioprocessing of lignocellulosics. New Biotechnology, 29, S5.

Shigeshiro M, Tanabe S, Suzuki T. (2013). Dietary polyphenols modulates intestinal barrier defects and inflammation in a murine model of colitis. J Funct Foods, 5, 949-955.

Treutter D. (2001). Biosynthesis of phenolic compounds and its regulation in apple. Plant Growth Regul, 34, 71-89.

Yu EA, Kim GS, Jeong SW, Park S, Lee SJ, Kim JH, Lee WS, Bark KM, Jin JS, Shin SC. (2014). Flavonoid profile and biological activity of Korean *Citrus* varieties (II): Pyunkyul (*Citrus tangerine* Hort. Ex Tanaka) and overall contribution of its flavonoids to antioxidant effect. J Funct Foods, 6, 637-642.

Conclusão Final

Foi estudado um processo para obtenção da enzima tanase através da fermentação em estado sólido, utilizando resíduo de citrus. Alguns parâmetros deste processo foram otimizados e foi possível minimizar a quantidade de ácido tânico adicionada ao meio, o que reduz os custos do processo.

Ao estudar este novo processo da produção da tanase, foi obtida uma enzima com características bioquímicas semelhantes à produzida em outros substratos, concluindo que o fungo *P. variotii* produziu uma enzima estável e confiável, independentemente do substrato, tornando mais vantajoso o uso de resíduos agroindustriais como substrato na fermentação.

A obtenção de compostos fenólicos utilizando resíduo de citrus através da fermentação em estado sólido e/ou por reação enzimática assistida, apresentaram grande potencial na concentração e extração dos compostos de alto valor biológico. Ambos os processos foram realizados de forma ambientalmente amigável, diminuindo o uso de solventes tóxicos para extração.

O resíduo da indústria do suco de laranja apresenta grande potencial biotecnológico para obtenção de produtos de interesse comercial como enzimas microbianas e compostos fenólicos, adicionando portanto outras finalidades deste resíduo na indústria do suco.

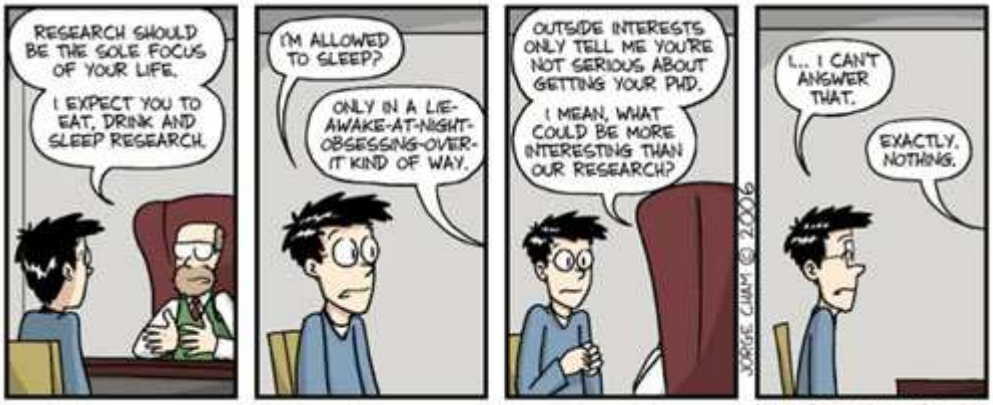
Os compostos fenólicos obtidos apresentaram grande potencial biológico, mostrando que os processos biotecnológicos estudados não só extraíram os fenólicos, mas obtiveram os mesmos de maior atividade biológica.

Trabalhos Futuros

Produção da tanase em escala piloto utilizando resíduo de *Citrus* como substrato e o fungo *Paecilomyces variotii*.

Avaliar outras características biológicas do resíduo de *Citrus* biotransformado, como antimicrobiano, anti-viral, anti-inflamatório, entre outros.

Estudar um processo de concentração ou purificação dos fenólicos obtidos no resíduo de *Citrus*, como por coluna por adsorção, carvão ativado.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I'M ALLOWED TO SLEEP?
ONLY IN A LIE-AWAKE-AT-NIGHT-OBSESSING-OVER-IT KIND OF WAY.
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