UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIA E TECNOLOGIA DE ALIMENTOS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA E TECNOLOGIA DE ALIMENTOS

RECUPERAÇÃO DE COMPOSTOS BIOATIVOS DE BAGAÇO DE UVA POR CULTIVOS FÚNGICOS EM ESTADO SÓLIDO COMPARADO AO MÉTODO DE EXTRAÇÃO SÓLIDO-LÍQUIDO

Natália Guilherme Graebin

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Natália Guilherme Graebin

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“Àqueles que me ensinam a viver cada dia
com muito respeito e muita dedicação.
Pai e mãe, amo vocês!”

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LISTA DE ABREVIATURAS

ABTS	2,2’-azino-bis(3-etilbenzotiazolina-6-ácido sulfônico)
BG	*β-glucosidase*
CPT	Conteúdo de polifenóis totais
DPPH	2,2-difenil-1-picrilhidrazila
GAE	*Gallic acid equivalent*
TEAC	*Trolox equivalent antioxidant capacity*
TPC	*Total phenolic content*

RESUMO

O bagaço de uva, fonte de antioxidantes naturais, é um resíduo agro-industrial muito abundante e inexplorado. A partir do uso de técnicas de extração adequadas, tais compostos de interesse podem ser recuperados. Assim, neste trabalho, o estudo de duas técnicas de extração dos compostos bioativos de bagaço de uva é apresentado. A extração sólido-líquido dos compostos antioxidantes do resíduo vinícola foi otimizada através de um planejamento experimental e a influência das variáveis temperatura, concentração de etanol e Tween 80 foi investigada. O conteúdo de polifenóis totais (CPT) e atividade antioxidante (AA) (metodologias com os radicais DPPH• e $ABTS^{\bullet+}$) das amostras foram medidos. As condições ótimas de extração foram: 75 ºC; etanol, 28,8 %; Tween 80, 5 %. O valor encontrado de CPT foi de 21,55 mg GAE/g_{dw}, enquanto que a AA obtida foi de 9,13 mmol TEAC/g_{dw} e 178,34 mmol TEAC/g_{dw}, para os ensaios com DPPH e ABTS, respectivamente. Todas as variáveis influenciaram significativamente ($p < 0.05$) o CPT, porém somente a temperatura e a concentração de etanol tiveram influência sobre a AA. Assim, os resultados sugerem que o etanol pode ser uma alternativa aos solventes orgânicos, de grau não-alimentício, ambientalmente incorretos. Além disso, observou-se que o uso do surfactante Tween 80 auxiliou a extração dos compostos bioativos. Outra técnica possível é o cultivo em estado sólido, uma vez que esse bioprocesso pode ser utilizado para recuperar os compostos bioativos de resíduos agroindustriais. As linhagens *Aspergillus niger* BLAn1, *Phanerochaete chrysosporium* BLPc1 e *Penicillium* sp. BLPen1 foram investigadas quanto à recuperação dos compostos bioativos e à produção de β-glicosidase (BG). As maiores atividades de BG foram, aproximadamente, 17,2, 8,3, e 13,5 U/g resíduo para as 3 linhagens, respectivamente. A atividade enzimática foi consideravelmente reduzida quando utilizada injeção de ar saturado no sistema. O CPT obtido foi de 21,15, 12,13, e 10,08 mg GAE/g resíduo, enquanto que a AA medida foi de 16,30, 11,35, e 10,57 mmol TEAC/g resíduo (método DPPH) e 158,61, 104,68, e 102,55 mmol TEAC/g resíduo (método ABTS) para BLAn1, BLPc1, e BLPen1, respectivamente. Tais resultados sugerem que a hidrólise enzimática dos fenólicos glicosilados permitiu maior concentração de fenólicos em sua forma aglicona, o que aumentou o seu poder sequestrante de radicais livres.

ABSTRACT

Grape pomace is an abundant agro-industrial residue that could be used as a source of natural antioxidants using appropriate techniques for their extraction. In this work, two different techniques are presented. The solid-liquid extraction of bioactive compounds from grape pomace was optimized by experimental design. Temperature, ethanol, and Tween 80 amounts were evaluated by measuring the total phenolic content and antioxidant activity of samples using DPPH and ABTS methods. Optimal conditions were: 75 ºC; ethanol amount, 28.8 %; Tween 80 amount, 5 %. The total phenolic content was 21.55 mg GAE/g_{dw}, whereas the antioxidant activity was 9.13 mmol TEAC/g_{dw} and 178.34 mmol TEAC/g_{dw}, respectively. The variables showed to significantly affect the total phenolic content ($p < 0.05$), but only temperature and ethanol amount influenced the antioxidant activity. Results suggest that ethanol could be used to replace non-food grade and environmentally problematic organic solvents, while the use of Tween 80 could improve the solid-liquid extraction. On the other hand, solid-state cultivation was also carried out, since is a bioprocess that represents an attractive alternative for the production of bioactive compounds from agro-industrial wastes used as substrates. The strains *Aspergillus niger* BLAn1, *Phanerochaete chrysosporium* BLPc1, and *Penicillium* sp. BLPen1 were evaluated in the recovery of antioxidant compounds and β-glucosidase (BG) production. The highest BG activities were approximately 17.2, 8.3, and 13.5 U/g pomace for the 3 strains, respectively. The enzyme activity was considerable reduced with moisture-saturated air injection in the system. The total phenolic content obtained were 21.15, 12.13, and 10.08 mg GAE/g pomace, while the highest antioxidant activity released were 16.30, 11.35, and 10.57 mmol TEAC/g pomace (by DPPH assay) or 158.61, 104.68, and 102.55 mmol TEAC/g pomace (by ABTS assay) for BLAn1, BLPc1, and BLPen1 respectively. These results suggest that the enzyme hydrolysis of phenolic glycosides leads to increased concentrations of free phenolics and enhanced radical-scavenging potential of pomace.

INTRODUÇÃO

A uva, fruta mundialmente cultivada, é matéria-prima no processamento de sucos e de vinhos. Tais setores são bem representativos no mundo e no Brasil, e por isso, vê-se a importância do correto aproveitamento dos resíduos dessa fruta, já que esse contém antioxidantes. Esses compostos bioativos obtidos de plantas, frutas e flores e seus resíduos têm sido relatados como protetores na peroxidação lipídica dos alimentos. Além disso, muitos estudos relatam os benefícios desses constituintes em doenças cardíacas, neurológicas, câncer, diabetes, uma vez que a partir da ingestão desses compostos, sintomas são amenizados.

Algumas técnicas de extração são utilizadas para a recuperação dos compostos bioativos. A extração sólido-líquido, operação unitária que envolve um fenômeno de transferência de massa, apresenta-se como uma técnica fácil e de baixo custo. Uma alternativa possível a ser empregada é o cultivo em estado sólido em substratos capazes de suprir o desenvolvimento de micro-organismos, como os resíduos agroindustriais, para a liberação dos compostos antioxidantes. A partir da produção de enzimas denominadas β-glicosidases pelos micro-organismos, as quais possibilitam a clivagem das formas glicosiladas dos compostos fenólicos, os antioxidantes são expostos ao meio de cultivo e assim, podem facilmente ser extraídos, além de adquirirem sua forma mais ativa.

Na literatura, parâmetros como granulometria, solvente, razão sólido/líquido, tempo de extração, temperatura têm apresentado forte influência na extração dos compostos fenólicos e da atividade antioxidante. Maiores temperaturas, solventes mais polares, menores granulometrias têm sido relatados como condições ótimas de processo. Ainda nesse âmbito, a utilização de solventes com grau alimentício na recuperação dos compostos bioativos apresentam grande relevância, visto que deste modo, esses podem ser utilizados em alimentos, cosméticos e fármacos.

Nesse sentido, o presente estudo apresenta pesquisas em relação ao aproveitamento do subproduto vinícola a um produto de alto valor agregado, os compostos antioxidantes. Da mesma forma, estão demonstrados estudos quanto ao crescimento de micro-organismos para a maior liberação dos compostos bioativos, bem como os parâmetros ótimos de extração sólido-líquido dos mesmos.

2 OBJETIVOS

2.1 Objetivo geral

Avaliar a recuperação dos compostos bioativos de bagaço de uva por cultivos fúngicos em estado sólido e por extração sólido-líquido, além de maximizar tal extração.

2.2 Objetivos específicos

- Caracterizar o bagaço de uva através da análise físico-química do resíduo;
- Determinar as condições ótimas de extração sólido-líquido dos antioxidantes;
- Avaliar o cultivo do bagaço de uva com três fungos filamentosos – *Aspergillus niger* BLAn1, *Phanerochaete chrysosporium* BLPc1 e *Penicillium* sp. BLPen1 – em relação aos compostos bioativos;
- Quantificar a atividade enzimática de β-glicosidase no cultivo em estado sólido;
- Avaliar o conteúdo de polifenóis totais e a atividade antioxidante dos compostos bioativos recuperados por ambos os métodos.

CAPÍTULO I

3 REVISÃO BIBLIOGRÁFICA

3.1 Compostos bioativos

As substâncias bioativas ou metabólitos secundários de origem vegetal, também são conhecidos como fitoquímicos ou fitonutrientes. Devido as suas importantes propriedades, efeitos biológicos e seus atributos sensoriais (KING e YOUNG, 1999; BELNSTEIN, 2001), são muito estudados e, especialmente em frutas e hortaliças, dá-se atenção às substâncias fenólicas.

Os compostos fenólicos constituem uma das principais classes de antioxidantes naturais. Eles são largamente distribuídos em frutos, legumes, grãos, sementes, folhas, raízes, cascas. Possuem composição quantitativa e qualitativa variada em cada alimento e, ainda, possuem ação antioxidante de acordo com a sua estrutura química e a sua concentração (MELO *et al.*, 2008). A biodisponibilidade desses fitoquímicos está relacionada à matriz alimentícia, tipo de planta, período de crescimento das plantas, estação, grau de luminosidade e maturação, preparação e processamentos dos alimentos (AHERNE e O'BRIEN, 2002).

De forma geral, os compostos fenólicos são constituídos por moléculas que contém grupamentos hidroxilas ligados aos anéis aromáticos. Particularmente, os ácidos fenólicos e os alcoóis fenólicos, possuem somente um radical fenol. Esses polifenóis são divididos em: flavonóides, ácidos fenólicos, taninos, estilbenos e lignanas. E os flavonóides, ainda, são classificados em flavonas, isoflavonas, flavonóis e antocianinas (TSAO e YANG, 2003).

Os flavonóides são moléculas de baixo peso molecular, constituídos por 15 átomos de carbono, arranjados em uma configuração C6-C3-C6. Basicamente, a estrutura consiste em dois anéis aromáticos, ligados por uma ligação no carbono 3, usualmente na forma de um anel heterocíclico (MERKEN e BEECHER, 2000). Esses compostos são importantes antioxidantes devido ao seu alto poder redox, o qual permite agirem como agentes redutores, doadores de hidrogênio e quelantes de oxigênio singlete, além de quelantes de metais, substâncias consideradas aceleradores das reações de oxidação (TSAO e YANG, 2003).

Dentre os flavonóides mais estudados, encontram-se as antocianinas. A estrutura básica das antocianinas são as antocianidinas. As antocianidinas são constituídas por um anel aromático [A] ligado a um anel heterocíclico [C] que contém oxigênio, o qual é ligado por ligação carbono-carbono a um terceiro anel aromático [B]. Quando essas estão em sua forma glicosilada (ligadas a uma molécula de açúcar), são denominadas antocianinas (KONCZAK e ZHANG, 2004).

As antocianinas são altamente instáveis e são muito suscetíveis à degradação (GIUSTI e WROLSTAD, 2003). Fatores como pH, temperatura de armazenamento, luminosidade, presença de enzimas, proteínas e íons metálicos influenciam a estabilidade desses compostos (REIN, 2005). Em alimentos, há destacada importância para os metabólitos pelargonidina, cianidina, delfinidina, peonidina, petunidina e malvidina devido ao seu alto poder antioxidante (FRANCIS, 2000).

Figura 1. Estrutura básica das antocianinas
Fonte: CASTAÑEDA-OVANDO *et al.* (2009)

3.2 Bagaço de uva

A uva é a fruta mais cultivada no mundo, com produção anual de mais 70 milhões de toneladas. Tal matéria-prima é utilizada no processamento de sucos e de vinhos. Tais setores são bem representativos no mundo e, aproximadamente, 80 % da produção da uva é utilizado para a produção de vinhos. Desse montante, cerca de 20 % em peso das uvas processadas é resultante como descarte. Sendo assim, calcula-se que mais de 10 milhões de toneladas desse resíduo é gerado pela indústria vinícola no mundo (KAMMERER *et al.*, 2005; FAO, 2011). O Brasil detém a décima posição no ranking de produção de uvas no mundo (FAO, 2011) e o Rio

Grande do Sul possui a maior produção de vinhos no país, com produção de, aproximadamente, 300 milhões de litros (vinhos de mesa e fino), gerando cerca de 55 milhões de quilos de resíduos (proporção de resíduo/vinho de 18 kg/100 L) (DE CAMPOS *et al*.,2008; IBRAVIN, 2011).

Esse bagaço consiste em cascas, sementes e talos, sendo que as cascas e as sementes correspondem à maior parte do mesmo (ZOCCA *et al.*, 2007). Esse resíduo da agroindústria tem sido utilizado como fertilizante, na produção de ácido tartárico e etanol, além de ser destinado ao consumo animal. Em relação a essas aplicações, relata-se problemas, uma vez que o alto conteúdo de fenólicos e a presença de polifenóis poliméricos do bagaço de uva inibem a germinação das sementes e reduzem a digestibilidade dos animais (KAMMERER *et al.*, 2005; SILVA *et al*., 2000). Tendo em vista que o bagaço de uva constitui uma fonte abundante e de baixo custo de compostos fenólicos, diversos estudiosos têm avaliado diferentes métodos de recuperação desses para posterior aplicação em medicamentos, alimentos e ingredientes funcionais (PINELO *et al.*, 2005; RUBERTO *et al.*, 2007; SPIGNO e DE FAVERI, 2007; GUERRERO *et al.*, 2008; VATAI *et al.*, 2009; MONRAD *et al.*, 2010). Mazza (1995) observou que cerca de 70 % dos compostos fenólicos presentes no bagaço de uva permanecem após o processamento vinícola, descoberta que agrega valor a esse subproduto.

Entre os principais constituintes do bagaço de uva estão os compostos fenólicos. Esses fitoquímicos possuem importância fisiológica e morfológica, já que provém proteção contra patógenos e predadores, e participam no desenvolvimento e reprodução das plantas (BRAVO, 1998). Para os seres humanos, tais componentes apresentam benefícios, visto que são considerados anti-alergênicos, anti-inflamatórios, anti-trombóticos, antioxidantes, cardioprotetores e vasodilatadores (BENAVENTE-GARCÍA *et al.*, 1997; SAMMAN *et al.*, 1998; MIDDLETON JR. *et al.*, 2000; PUUPPONEN-PIMIÄ *et al.*, 2001; MANACH *et al.*, 2005).

O bagaço de uva contém grande variedade de polifenóis, dentre esses, ácidos fenólicos (ácido gálico, ácido *trans*-caftárico, *cis* e *trans*-ácido cutárico), alcoóis fenólicos, flavan-3-óis (catequina, epicatequina e procianidina B1) e 8 flavonóides (quercetina, 3-glicosídeo e 3-glicoronídeo, campferol 3-glicosídeo e 3-galactosídeo, eucrifina, astilbina e engeletina. Lu e Yeap Foo (1999) além de identificarem esses compostos, relataram ter encontrado em tal resíduo da indústria vinícola, as formas 3-β-glicopiranosídeo e 4-β-glicopiranosídeo do ácido gálico, e,

ainda, o composto 2-hidroxi-5-(2-hidroxietil)fenil-β-glicopiranosídeo. Todos esses flavonóides, incluindo as procianidinas oligoméricas, representaram, aproximadamente, 4 % do peso seco do bagaço de uva estudado, sendo esse, ótima fonte de antioxidantes naturais.

O conteúdo dos compostos fenólicos está relacionado com fatores intrínsecos e extrínsecos. Fatores como cultivar, espécie, clima, solo, práticas de cultivo podem influenciar a quantidade bem como a variedade desses compostos bioativos nas matérias-primas (RAPISARDA *et al.*, 1999; TOMÁS-BARBERÁN e ESPÍN, 2001). Ainda, é possível citar que os parâmetros de extração desses compostos, como tipo de solvente e preparo de amostra, influenciam a quantidade e a variedade dos mesmos, como relatado por Louli *et al.* (2004), na obtenção de antioxidantes de bagaço de uva.

Pertencentes ao grupo mais representativo dos compostos fenólicos encontrado em uvas, as antocianinas, também possuem atividade antioxidante. Essa propriedade tem papel fundamental na prevenção de doenças cardiovasculares e neurológicas, diabetes (KONCZAK e ZHANG, 2004), e, ainda, agregam benefícios no tratamento do câncer (LULE e XIA, 2005; NICHENAMETLA *et al.*, 2006).

3.3 Extração de compostos bioativos

Dentre as inúmeras tecnologias envolvidas na extração de compostos bioativos de frutas, hortaliças e plantas, destacam-se a extração sólido-líquido, a extração com ondas eletromagnéticas – energia com micro-ondas e energia ultrassônica –, e a extração enzimática.

A extração sólido-líquido é uma operação unitária comumente utilizada na recuperação de compostos dos alimentos. Envolve um fenômeno de transporte de massa no qual os sólidos contidos na matriz do produto migram para o solvente que está em contato, o qual pode ser otimizado com alterações no gradiente de concentração e/ou coeficiente de difusão (CORRALES *et al.*, 2009). De forma geral, essa extração é utilizada em diversos nichos da indústria de alimentos, tais como na recuperação de açúcares da cana, lipídeos e proteínas de sementes oleaginosas, hidrocolóides funcionais de algas, e de compostos fenólicos de plantas, vegetais e frutas (IGNAT *et al.*, 2011).

Já as extrações por micro-ondas e por ultrassom são processos nos quais a energia é dissipada através das ondas eletromagnéticas. O princípio do mecanismo das micro-ondas é baseado no efeito direto das mesmas sobre as moléculas através da condução iônica e da rotação dos dipolos. Quando o campo magnético é aplicado, há uma migração dos íons e uma movimentação dos dipolos o que provoca calor (KINGSTON e JASSIE, 1988; THUÉRY, 1992; JASSIE *et al.*, 1997). Investigações quanto à extração com micro-ondas dos compostos fenólicos de tomates, resíduos de uva (cascas e sementes), sementes de uva e farelos de cereais revelam a eficiência dessa operação na recuperação dos antioxidantes (CASAZZA *et al.*, 2010; DAR e SHARMA, 2011; LI *et al.*, 2011; LI *et al.*, 2012).

Semelhante, o método de extração com ultrassom atua na superfície da matriz da amostra produzindo maior contato com o meio de extração. Esse procedimento tem sido utilizado para a extração de compostos orgânicos não-voláteis e pouco voláteis de sólidos provindos do solo e de resíduos (WANG *et al.*, 2008) e ainda, na extração de lipídeos, proteínas e compostos fenólicos das plantas, com sementes de uva, resíduos de maçã e de uva (ALONSO *et al.*, 2002; GHAFOOR *et al.*, 2009; AJILA *et al.*, 2011).

A liberação enzimática dos compostos fenólicos de frutas, hortaliças e plantas é outra técnica aplicada na extração dos polifenóis naturais. A eficácia de extração de tais constituintes bioativos está correlacionada com a lise da parede celular das plantas provocada pela ação de pectinases e celulases, por exemplo, o que viabiliza a exposição desses antioxidantes no meio de reação (MAILLARD e BERSET, 1995). Chamorro *et al.* (2012) e Meyer *et al.* (1998) estudaram a extração de antioxidantes de bagaço de uva, utilizando carboidrases com atividade celulolítica e pectinolítica. A exposição do resíduo aos complexos enzimáticos permitiu aumento da capacidade sequestrante de radicais livres, a partir da liberação de formas mais ativas dos compostos.

3.3.1 Extração sólido-líquido

A extração sólido-líquido apresenta-se como uma técnica fácil e de baixo custo. As condições do processo são responsáveis pela eficiência da extração dos compostos de interesse e por isso, é de extrema importância o estudo de

parâmetros como temperatura, razão sólido/líquido, tamanho de partícula e tempo de contato.

No processo de extração dos compostos fenólicos de serragem de pinho e cascas de amêndoas, das frutas originárias da Tunísia *Quercus coccifera* L. e *Juniperus phoenicea*, e de resíduos de groselha, groselha preta e uva verificou-se que a utilização de solventes com diferentes polaridades, maiores razões sólido-líquido e maior tempo de contato entre amostra/solvente favorecem a recuperação dos compostos antioxidantes (PINELO *et al.*, 2004; LAPORNIK *et al.*, 2005; HAYOUNI *et al.*, 2007).

Na literatura, há um crescente interesse em estudar aspectos sobre os resíduos da indústria vinícola. Pinelo *et al.* (2005) reportaram que cada componente do bagaço de uva possui uma composição fenólica diferente. Além disso, tanto a natureza do composto (estrutura, grau de polimerização) quanto à concentração estão relacionadas com a fração considerada em estudo (casca, semente ou talo).

A diferença dos compostos bioativos encontrados em cada fração do bagaço de uva é relatada por Negro *et al.* (2003). Tais autores verificaram que as sementes possuem maior concentração de fenólicos totais. Os talos e as cascas foram responsáveis pela quantidade de antocianinas totais e livres. Os extratos desse subproduto demonstraram atividades antioxidantes de 85 % quando comparadas ao antioxidante sintético butil-hidroxitolueno (BHT).

Bucić-Kojić *et al.* (2009) investigaram a influência do solvente (água e etanol), bem como da temperatura na extração de polifenóis de sementes de uva. Os melhores resultados foram obtidos nas extrações com solvente 50 % etanol e temperatura de 80 ºC. Os autores ainda identificaram o composto em maior quantidade sendo esse a catequina, representando 45,11 % do total do conteúdo de polifenóis.

No estudo dos compostos fenólicos e da atividade antioxidante do bagaço de uva, Bonilla *et al.* (1999) avaliaram influência do solvente, tempo de extração e condição da partícula. Os resultados indicaram maiores extrações com bagaço de uva moído em 10 minutos utilizando como solvente, acetato de etila. Além disso, os autores identificaram extração seletiva de alguns compostos bioativos, à medida que monômeros flavan-3-óis, catequina e flavonóis foram mais extraídos na fase orgânica, enquanto que as procianidinas, na fase aquosa.

Louli *et al.* (2004) verificaram o efeito do tipo de solvente, da composição (casca, talo e semente) e do pré-tratamento na extração dos compostos bioativos e na atividade antioxidante do bagaço de uva. O solvente mais apropriado dentre os pesquisados foi o acetato de etila. Além de apresentar maior extração dos compostos de interesse, tal solvente apresenta baixo ponto de ebulição e não toxicidade, o que é desejável para a indústria de alimentos. A eficiência do processo de extração tornou-se maior, quando no bagaço de uva não continha talos, o que pode indicar que quando esse está presente, ocorre co-extração de compostos inativos.

Boonchu e Utama-Ang (2013) estudaram a otimização dos parâmetros de extração dos antioxidantes de resíduos de uvas tintas. Os resultados indicaram que o rendimento de extração, conteúdo de polifenóis totais, antocianinas e resveratrol foram maiores com o aumento da temperatura e do tempo de extração, enquanto que a quantidade de taninos foi reduzida. As condições ótimas foram 80 ± 1 ºC e 2 horas e 53 minutos.

Na investigação das variáveis do processo de extração de compostos bioativos de uvas Ghure, Shojaee-Aliabadi *et al.* (2013) relataram que a razão etanol/água do solvente foi o parâmetro mais significativo para o conteúdo de polifenóis totais e capacidade antioxidante e que, além disso, houve relação entre as duas respostas estudadas. As condições ótimas para temperatura, tempo e razão etanol/água foram 44,93 ºC, 19,34 horas, 70,08, respectivamente, liberando 388,79 mg GAE/100 g, com capacidade sequestrante de radicais livres de 91 %.

3.4 Cultivo em estado sólido

O cultivo em estado sólido consiste no crescimento de micro-organismos em partículas sólidas na ausência – ou quase ausência – de água livre. O substrato contém umidade suficiente para permitir que haja crescimento e metabolismo adequado do micro-organismo (PANDEY, 2003).

Tal processo biológico vem sendo utilizado para a extração de constituintes de interesse a partir de resíduos. Para que esse subproduto seja viável para o processo algumas características devem ser preenchidas: (1) o material deve ser poroso, biodegradável ou não, com grande área superficial por unidade de volume a fim de permitir o consumo por parte do micro-organismo; (2) a matriz do substrato

deve ser capaz de absorver grande quantidade de água, para que haja atividade de água relativamente alta no sistema capaz de permitir o crescimento microbiológico; (3) o resíduo não pode ser contaminado por quaisquer inibidores produzidos pelo micro-organismo e deve ser capaz de absorver ou conter componentes necessários à vida do micro-organismo, como carboidratos, fontes de nitrogênio e sais minerais. (PANDEY, 2003; ORZUA *et al.*, 2009).

Dentre as vantagens do cultivo em estado sólido, pode-se citar o baixo requerimento de custos e energia, facilidade de recuperação dos compostos de interesse pós-cultivo, utilização de substratos provindos de resíduos da agro-indústria, alta produtividade. Algumas desvantagens ainda são inerentes ao processo como difícil controle de parâmetros, heterogeneidade e problemas de escalonamento da operação (COUTO e SANROMÁN, 2006).

O cultivo em estado sólido vem sendo utilizado para inúmeros setores da indústria de alimentos. A partir dessa operação, tem-se a produção de metabólitos secundários como aromatizantes, enzimas (lipases, pectinases, amilases), ácidos orgânicos (ácido láctico e ácido cítrico), aminoácidos, goma xantana, além da produção de cogumelos comestíveis (PANDEY, 2003; COUTO e SANROMÁN, 2006).

Bactérias, leveduras e fungos são os micro-organismos utilizados no cultivo em estado sólido. Os fungos filamentosos são os mais comumente explorados devido a sua habilidade de crescer em substratos complexos em quase/ausência de água (NIGAM e SINGH, 1994). Além disso, os fungos são capazes de penetrar nesses substratos rígidos, pela pressão originada pelos seus micélios (RAMACHANDRAN *et al.*, 2004).

Dentre os diversos filos do reino Fungi, Phycomycetes (*Mucor* e *Rhizopus*), Ascomycetes (*Aspergillus* e *Penicillium*) e Basiodiomycetes são os mais citados nos estudos dos cultivos (PANDEY, 1992). Tais micro-organismos têm sido relatados como produtores de compostos bioativos (MAPARI *et al.*, 2005) e ainda de β-glicosidase (EC 3.2.1.21) (GEORGETTI *et al.*, 2009; DHILLON *et al.*, 2011), enzima capaz de hidrolisar as ligações dos compostos fenólicos glicosilados, permitindo maior atividade antioxidante dos mesmos (HSIEH e GRAHAM, 2001).

Recentemente, com o objetivo de recuperar os compostos bioativos de resíduos da indústria, diversos micro-organismos e técnicas de cultivo têm sido estudados. A soja foi utilizada como substrato no cultivo em estado sólido com

Trichoderma harzianum NBRI-1055 (SINGH *et al.*, 2010). Como objetivo, os autores verificaram a influência do processo biológico sobre a liberação das substâncias fenólicas e a atividade antioxidante dos compostos. O extrato cultivado apresentou significante conteúdo de flavonóides totais e atividade antioxidante com água como meio de extração. Além disso, esse extrato apresentou poder redutor, capacidade inibitória da oxidação lipídica, do DNA e de proteínas e atividade sequestrante de radicais hidroxilas e superóxidos.

Zheng e Shetty (2000) estudaram a ação da enzima β-glicosidase produzida pelo fungo GRAS ("*Generally Recognized as Safe*") *Lentinus edodes* durante o cultivo em resíduo de "*cranberry*". A importância dessa enzima no estudo foi correlacionada com a liberação dos fenólicos livres através da hidrólise dos fenólicos glicosídicos. Após 50 dias de cultivo, observou-se o máximo de fenólicos livres, à medida que a atividade de β-glicosidase foi de 9 unidades/g de resíduo. Ácido gálico, ácido clorogênico, ácido *p*-hidrobenzóico e ácido *p*-cumárico foram identificados por cromatografia líquida de alta eficiência como majoritários no resíduo de "*cranberry*".

Outro resíduo muito gerado pela indústria de alimentos é o subproduto da manufatura do café. A fim de não descartar no meio ambiente ou incinerar tal resíduo, os pesquisadores Machado *et al.* (2012) investigaram a liberação dos compostos fenólicos através do crescimento de 7 diferentes linhagens dos gêneros *Aspergillus*, *Mucor*, *Penicillium* e *Neurospora* em cultivo em estado sólido. Ao resíduo foram adicionados sais minerais e o cultivo foi conduzida por 6 dias. *Penicillium purpurogenum*, *Neurospora crasse* e *Mucor* foram os micro-organismos que demonstraram maior ação sobre o subproduto do café, com maiores conteúdos de fenólicos totais.

Com o intuito de atribuir valor ao resíduo de maçã, um estudo foi realizado para aumentar a liberação dos compostos fenólicos e da atividade antioxidante através do cultivo em estado sólido com *Phanerochaete chrysosporium*. O cultivo foi conduzido a 37 ºC por 14 dias. Ajila *et al.* (2011) descobriram que o conteúdo de fenólicos totais aumentou cerca de 4 vezes durante o processo biológico em relação ao extrato do resíduo com acetona.

Martínez-Ávila *et al.* (2011) avaliaram a recuperação compostos antioxidantes pelo cultivo em estado sólido de resíduo de uva por quatro espécies do gênero *Aspergillus*. A incubação do cultivo foi realizada a 30 ºC por 24 horas. Extratos

cultivados com *Aspergillus* GH1 foram aqueles com maior capacidade antioxidante contra os radicais DPPH e ABTS, os quais inibiram 91 % e 87 %, respectivamente. Extratos obtidos após 24 horas de cultivo foram capazes de inibir até 90 % da oxidação lipídica avaliada.

4 CAPÍTULO II: ARTIGO CIENTÍFICO

An eco-friendly design for bioactive compounds extraction from grape pomace

Os resultados referentes ao trabalho de otimização dos parâmetros – temperatura, solvente etanol/água, surfactante Tween 80 – na extração de compostos bioativos de bagaço de uva estão apresentados em forma de artigo submetido para publicação na revista *Food Chemistry*.

An eco-friendly design for bioactive compounds extraction from grape pomace

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Abstract

Grape pomace is an abundant agro-industrial residue that could be used as a source of natural antioxidants using appropriate techniques for their extraction. The solid-liquid extraction of bioactive compounds from grape pomace was optimized by experimental design. Temperature, ethanol, and Tween 80 amounts were evaluated by measuring the total phenolic content and antioxidant activity of samples using DPPH and ABTS methods. Optimal conditions were: 75 ºC; ethanol amount, 28.8 %; Tween 80 amount, 5 %. The total phenolic content was 21.55 mg GAE/g_{dw}, whereas the antioxidant activity was 9.13 mmol TEAC/g_{dw} (DPPH) and 178.34 mmol TEAC/g_{dw} (ABTS). The variables showed to significantly affect the total phenolic content ($p < 0.05$), but only temperature and ethanol amount influenced the antioxidant activity. Results suggest that ethanol could be used to replace non-food grade and environmentally problematic organic solvents, while the use of Tween 80 could improve the solid–liquid extraction.

Keywords: grape pomace; bioactive compounds; surfactant; solid-liquid extraction; central composite design.

1 Introduction

Grape (*Vitis vinifera*) is one of the most important fruit crops, with a global production of approximately 70 million tonnes, used to produce 28 million tonnes of wine (FAO, 2011). This industrial activity generates a large amount of residues, including grape pomace, skins, seeds, and stalks. The high content of organic matter and the seasonal production of grape residues can difficult their use and treatments, contributing to potential pollution problems, regarding the chemical and biological oxygen demand of groundwater, rivers, and soils (SPIGNO *et al.*, 2008). Therefore, several studies have been devised in order to aggregate value to these grape residues, mainly at recovering the bioactive compounds present on them, in particular grape pomace (MAKRIS *et al.*, 2007; SPIGNO and DE FAVERI, 2007; BUCIĆ-KOJIĆ *et al.*, 2009; ROCKENBACH *et al.*, 2011).

Waste solids from winemaking are heterogeneous, and the various materials present in them have different compositions. The major phenolic compounds in *Vitis vinifera* grape seeds are epicatechin (accounting for 60 % of the monomers) followed by catechin and gallic acid (SAITO *et al.*, 1998; PALMA *et al.*, 1999; YAMAGUCHI *et al.*, 1999), whereas the most abundant phenolics in grape peels are epicatechin, epigallocatechin, gallic acid, and catechin. The presence of phenolic acids such as catechin, epicatechin, astilbin, and engeletin as well as myricetin, kaempferol, and quercetin glucosides in stalks has also been reported (HURTADO *et al.*, 1997; SOUQUET *et al.*, 2000; PASTRANA-BONILLA *et al.*, 2003). These bioactive compounds have been reported to present health benefits, such as tumors inhibition and anti-inflammatory effects (GOD *et al.*, 2007), while reports on the properties of distilled grape pomace show it as having free-radical scavenger potential, with authors suggesting its therapeutic value against cardiovascular diseases (ÁLVAREZ *et al.*, 2012).

Solid-liquid extraction is a simple and inexpensive procedure to recovery bioactive compounds from biomasses. It consists in a process in that organic, acid or aqueous solvents enter in contact with solid substrates solubilizing several components from substrates such as grape seeds (JAYAPRAKASHA *et al.*, 2003), red grape marc (BONILLA *et al.*, 1999; NEGRO *et al.*, 2003), and winemaking solid wastes from red grapes after fermentation and distillation process (CRUZ *et al.*, 2004). There are many parameters that can influence the extraction of phenolic

compounds and their antioxidant activity, such as solvent type, temperature, surfactant amount, solid-liquid ratio, particle size, and extraction time.

Most relevant works concerning the use of solvent extraction of bioactive compounds from grape residues have been reported by Lapornik *et al.* (2005) who investigated the effects of solvent type and extraction time on the yield of antioxidants from grape and currant marcs; Larrauri *et al.* (1998), Kapasakalidis *et al.* (2006), Yilmaz and Toledo (2006) studied organic solvents, such as methanol and acetone as extractants on byproducts of winemaking and grape processing, black currant pomace and black currant press residues; and by Pinelo *et al.* (2005) the best conditions of flow-rate, sample and particle size in the continuous phenol extraction from *Vitis vinifera* wastes. Besides, ethanol has been used for polyphenol extraction from different plant sources instead of other organic solvents because of environmental and health concerns (MAKRIS *et al.*, 2008; BUCIĆ-KOJIĆ *et al.*, 2009; CASAZZA *et al.*, 2012).

The influence of temperature on phenolic extraction has been studied by Bucić-Kojić *et al.* (2009) that the best extractability of polyphenols from grape seeds was obtained at 80 ºC in comparison with 25 ºC, whereas Pinelo *et al.* (2005) reported that better temperature to extract antioxidants from grape was 50 ºC compared with 25 ºC and 37.5 ºC. However, Larrauri *et al.* (1998) studied the influence of high temperatures (80 ºC, 100 ºC, and 120 ºC) on bioactive compounds extraction from red and white grape pomace peels, found that higher temperatures were correlated with partial losses of the compounds and their free radical scavenging capacity above 100 ºC.

Surfactants can have an effect on solid-liquid extractions because of their reduction of surface tension, releasing the phenolic compounds entrapped in the complex solid matrix and increasing the contact between polyphenols and other components in the liquid phase (GOLDING and SEIN, 2004; AJILA *et al.*, 2011). Tween 80 is a polysorbate non-ionic surfactant, which is relatively stable, commercially inexpensive, and available as a food grade product. It is used in many domestic, scientific, and pharmacological applications.

In this context, the aims of this work were to optimize the solid-liquid extraction of bioactive compounds from red grape pomace derived from winemaking. A central composite design (CCD) and response surface methodology (RSM) analyses were used in order to verify the combined effects of temperature, surfactant and ethanol

amounts added to the system on the yields of extraction of total phenolic content (TPC) and antioxidant activity. The relationship between the phenolic compounds content and their antioxidant activities were also evaluated.

2 Materials and methods

2.1 Plant material

Red grape pomace (skins and seeds) used as the substrate for the solid-liquid extractions were kindly supplied by a winery of Bento Gonçalves (Rio Grande do Sul, Brazil; geographic coordinates: 29° 10' 26" S 51° 3 1' 7" O). This residue has the following *in natura* proximal chemical composition (%): moisture, 61; ash, 2.4; total fiber, 26; fat, 0.6; protein, 4; carbohydrate, 6. This material was immediately collected after the vinification process and stored at −20 ºC for its conservation. Once in the laboratory, the grape pomace was lyophilized in order to obtain a dry solid with around 5 % of moisture to avoid degradation of bioactive compounds before extraction (TSENG and ZHAO, 2012).

2.2 Chemicals

Folin-Ciocalteau reagent, 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma-Aldrich (St. Louis, USA). Tween 80 was purchased from Synth (Diadema, Brazil). Ethanol used in this work was of analytical grade (96 % mass fraction). All solvents used for extraction and chemical characterization of grape pomace were of analytical grade and purchased either from Dinâmica (Diadema, Brazil) or Vetec (Rio de Janeiro, Brazil).

2.3 Solid-liquid extraction

The red grape pomace was placed in a 50 mL conical flask containing 20 mL of solvent in a proportion of solvent-to-solid ratio of 20:1 (mass fraction). The

extractions were performed in a thermostatic orbital shaker at a constant stirring rate of 180 rpm for 6 h without light exposition to avoid photo-oxidation. Extraction temperature varied from 25 ºC to 75 ºC. The ethanol amount varied from 0 % (only distilled water) to 96 %, whereas the Tween 80 amount varied from 0.1 % to 5 %. The supernatant resulting after each extraction was stored at −20 ºC until used for the analyses.

2.4 Experimental design

A central composite design with three variables was carried out in order to obtain the optimal conditions for solid-liquid extraction. A number of 17 treatments of the 3 variables, each at 5 levels and their coded and uncoded values were performed. The design was constructed of 8 factorial points, 6 axial points and 3 replications at the central point. In each case, the yield of TPC, DPPH, and ABTS for solid-liquid extraction was determined. The second-order polynomial equation for the variables was as follows:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \quad (1)$$

Where Y is the response variable, b_0 the constant, b_i, b_{ii}, b_{ij} were the coefficients for the linear, quadratic, and for the interaction effects, respectively, and X_i and X_j the coded level of variables x_i and x_j. The above quadratic equation was used to plot surfaces for all variables.

2.5 Statistical analysis

The experimental design and analysis of results were carried out using Statistica 7.0 (Statsoft, USA). The statistical analysis of the model was performed as analysis of variance (ANOVA). The significance of the regression coefficients and the associated probabilities, *p(t)*, were determined by Student's t-test; the second order model equation significance was determined by Fisher's F-test. The variance explained by model is given by the multiple determination coefficients, R^2. For each variable, the quadratic models were represented as contour plots (2D).

2.6 Determination of TPC

TPC was determined following protocols described by Singleton and Esau (1969) and López *et al.* (2001) with the following modifications. A volume of 1 580 µL of distilled water and 100 µL of Folin-Ciocalteau reagent diluted were added to a tube followed by the addition of 20 µL of sample. The content was briefly mixed, and 300 µL of a 1.88 mM sodium carbonate solution was finally added to the reaction. The total solution was mixed and allowed to stand at room temperature in the dark for 2 h. Spectrophotometric analyses were carried out at 765 nm using a UV–Vis spectrophotometer, model Ultrospec 3100 Pro (GE Healthcare, USA) and the calibration curve was constructed using standard solutions of gallic acid with concentrations varying in the range 0.1 to 0.4 mg/mL. All analyses were performed in triplicate. TPC concentration was expressed as milligrams of gallic acid equivalent per gram of dried grape pomace (mg GAE/g_{dw}).

2.7 Determination of antioxidant activity by the DPPH method

The antioxidant activity of the grape pomace extracts was measured in terms of hydrogen-donating or radical-scavenging ability by means of the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•), according to a modified version of the method described by Kim *et al*. (2002). A volume of 1 425 µL of DPPH solution ($6.1 \cdot 10^{-5}$ M) was added to a tube and briefly mixed with 75 µL of sample. The reaction mixture was incubated for 30 min in the dark, at 25 ºC. The absorbance was read at 515 nm. Calibration curve was made with standard solution of trolox varying in the range 10 to 80 µmol/mL. All analyses were performed in triplicate. Total antioxidant activity of dried grape pomace was expressed in mmol TEAC (Trolox equivalent antioxidant capacity)/g_{dw}.

2.8 Determination of antioxidant activity by the ABTS method

The measurement of antioxidant activity of grape pomace extracts using radical 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) was determined spectrophotometrically as described by Re *et al.* (1999) with some modifications. A mixture of 5 000 µL of $7 \cdot 10^{-3}$ M ABTS and 88 µL of 140 mM potassium persulphate

was prepared. This concentrated ABTS reagent was stored in the dark for 16 h. Then, a volume of 3 000 µL of this ABTS radical solution was placed into a tube and the reaction was started by the addition of 30 µL of sample. This mixture was incubated for 6 min in the dark at 25 ºC. The colorimetric analysis was performed at 734 nm. Calibration curve was made using a standard solution of trolox varying in the range 100 to 2 000 µmol/mL. All analyses were performed in triplicate. Total antioxidant activity of dried grape pomace was also expressed in mmol TEAC/g_{dw}.

3 Results and discussion

3.1 Experimental results of CCD

Table 1 shows the experimental results and the matrix of the independent variables according to the CCD. The highest value of TPC, 32.50 mg GAE/g_{dw}, was obtained in treatment 10 (75 ºC; ethanol amount, 48 %; Tween 80, 2.5 %), and the lowest, 6.05 mg GAE/g_{dw} was obtained in treatment 3 (35 ºC; ethanol amount, 76.6 %; Tween 80, 1.1 %), whereas the best results for the antioxidant activities, DPPH and ABTS, 13.66 mmol TEAC/g_{dw} and 344.07 mmol TEAC/g_{dw}, respectively, were both found in treatment 6 (65 ºC; ethanol amount, 19.4 %; Tween 80, 4 %) and the lowest results, 2.77 mmol TEAC/g_{dw} and 39.70 mmol TEAC/g_{dw}, in treatment 9 (25 ºC; ethanol amount, 48 %; Tween 80, 2.5 %).

Table 1. Extraction conditions of experimental design (uncoded and coded levels) and their responses: TPC, DDPH and ABTS values

Run	(X_1) T (ºC)	(X_2) Ethanol (% v/v)	(X_3) Tween 80 (% v/v)	TPC[a]	DPPH[b]	ABTS[b]
1	(-1) 35	(-1) 19.4	(-1) 1.1	10.09	3.98	91.05
2	(-1) 35	(-1) 19.4	(1) 4	14.16	4.16	100.77
3	(-1) 35	(1) 76.6	(-1) 1.1	6.05	3.09	74.42
4	(-1) 35	(1) 76.6	(1) 4	9.86	3.92	89.16
5	(1) 65	(-1) 19.4	(-1) 1.1	11.72	9.50	265.55
6	(1) 65	(-1) 19.4	(1) 4	28.11	13.66	344.07

7	(1) 65	(1) 76.6	(-1) 1.1	16.24	13.00	311.05
8	(1) 65	(1) 76.6	(1) 4	11.69	9.08	123.26
9	(-1.68) 25	(0) 48	(0) 2.5	9.47	2.77	39.70
10	(1.68) 75	(0) 48	(0) 2.5	32.50	12.05	234.42
11	(0) 50	(-1.68) 0	(0) 2.5	17.85	4.53	89.09
12	(0) 50	(1.68) 96	(0) 2.5	9.52	4.11	61.70
13	(0) 50	(0) 48	(-1.68) 0.1	10.13	8.03	116.98
14	(0) 50	(0) 48	(1.68) 5	21.18	9.00	147.32
15	(0) 50	(0) 48	(0) 2.5	17.79	7.40	126.25
16	(0) 50	(0) 48	(0) 2.5	15.00	7.28	131.88
17	(0) 50	(0) 48	(0) 2.5	16.27	8.38	143.34

[a]Total phenolic content expressed as milligrams of gallic acid equivalent per gram of dried grape pomace (mg GAE/g_{dw});

[b]Antioxidant activity expressed in concentration of trolox per gram dried grape pomace (mmol TEAC/g_{dw});

Despite different best treatments for TPC and antioxidant activities analyses, it is possible to observe that an increase in temperature up to 65 ºC led to an increase of both studied responses. As reported by Bucić-Kojić *et al.* (2009), the temperature of 80 ºC compared with 25 ºC was more efficient to release bioactive compounds from grape seeds. Spigno and De Faveri (2007) reported that the yields percent of polyphenols duplicated when higher temperature were tested, 28 ºC to 60 ºC, in 5 h of extraction.

This increased recovery may be explained by temperature influence over polyphenols linked to the cellulosic matrix of grape pomace. These bonds could be more susceptible to cleavage allowing the migration of bioactive compounds to the reaction medium. However, as the results suggest, at least for grape pomace under the experimental conditions of this work, above 65 ºC the extraction of TPC is also followed by partial losses in the bioactive compounds and its free radical scavenging capacity, probably caused by denaturation of these molecules as also noticed by Larrauri *et al.*, (1998) and Moure *et al.* (2001).

Similar profile was observed concerning the use of surfactant amount. Reporting the recovery of phenols from olive mill wastewater (OMW) and red-flesh

orange juice (RFOJ), Katsoyannos *et al.* (2012) tested the effect of surfactants Span 20, PEG 400, Tween 80 and 20. They found that the most appropriate Tween 80 concentration was 5 % in the case of OMW and 7 % for RFOJ recovering 94.4 % of the total phenol from OMW and 79.8 % of the total carotenoids from RFOJ in a double step extraction keeping higher antiradical activity. Ajila *et al.* (2011) reported that concentrations of 0.10 % up to 5 % of Tween 20 improved the extraction of total phenolic of fermented apple pomace comparing to apple pomace *in natura* samples in both ultrasonic assisted and microwave-assisted extraction tested. Otherwise, polyphenol extracts (fermented or not) presented the highest inhibitory activity against DPPH• radical by both microwave and ultrasonic assisted extraction with only 1 % Tween 20 (volume fraction). The reports on the use of surfactants, especially Tween, as an extractant suggest that the contact between these chemicals and food surface, lowering the surface tension, eases the mass transport phenomena, enhancing the release of polyphenols entrapped in the cell wall by exposing the bioactive compounds to solvent phase.

Shojaee-Aliabadi *et al.* (2013) studied the optimization of extraction of antioxidant compounds from grape pomace. Their optimal condition for the ratio of ethanol to water was 70.08, using 45 ºC and 19 h. The authors obtained 389 mg GAE/100 g of TPC, which is almost 10 times lower than our results. In this work, we reached a higher value of TPC in a system of extraction using 30 % less ethanol amount and surfactant addition. These results suggest that the amount of surfactant in the extraction reaction could be combined with lower ethanol amounts, which is important to characterize a less aggressive process to the environment and cheaper.

Finally, our results for ethanol concentration showed that a decrease in alcohol amount allowed a better extraction of phenolic content, at the same time allowing the high antioxidant activity. Similar results were reported by Bucić-Kojić *et al.* (2009) who showed that the highest content of individual polyphenolic compounds in grape seed extracts were obtained with the lower amount of ethanol used in their experiments. However, Yilmaz and Toledo (2006) found out that ethanol was ineffective as a solvent for the extraction of phenolic compounds from Muscadine seed powder.

In our work, different ethanol amounts lead to different profiles of extractions. Since the highest TPC was found with 48 % ethanol content, those compounds with more free scavenging capacity of both radicals evaluated – DPPH and ABTS – were

extracted with approximately 20 %. It suggests that there is an optimal alcoholic amount, in which the antioxidant compounds are more soluble due to polar characters, not only affecting the quantity of total phenolics that were extracted, but also the composition and activity of these compounds. Similarly, it could be explained by a selective extraction that enhances free scavenging capacity. It has been reported this solvent dependency of antioxidant capacity may be attributed to structural differences of extracted bioactive molecules (YU *et al.*, 2005; KAPASAKALIDIS *et al.*, 2006; KARACABEY and MAZZA, 2010). This solvent reduction in the extraction system allows a green process with several applications on pharmaceutical, cosmetic and feed and food industries without complex steps of purification.

3.2 Statistical analysis of TPC

The quadratic model calculated for TPC after neglecting the statistically calculated insignificant terms ($p > 0.05$) was:

$$Y_1 = 4.92X_1 - 2.45X_2 - 1.97X_2^2 + 2.87X_3 - 1.27X_3^2 - 2.76X_2X_3 \qquad (2)$$

Where Y_1 is the TPC response and X_1, X_2 and X_3 are temperature, ethanol amount, and Tween 80 amount, respectively.

Fisher's statistical test for analysis of variance (ANOVA) showed a computed F-value of 7.64, which is significant ($p = 0.05$). The determination coefficient ($R^2 = 0.82$) implies that the variation of 82 % for total phenolic compounds recovery is attributed to the independent variables, and can be explained by the model (eq. 2). Observing the contour plots (Fig. 1a) it can be seen that simultaneously increasing the temperature and surfactant concentration, while reducing the ethanol amount, the recovery of phenolic content was enhanced, which was confirmed by the linear effects estimated for the extraction parameters, all statistically significant ($p < 0.05$): the positive effects of the temperature (9.84) and Tween 80 concentration (5.74), and negative effect of ethanol content (−4.89).

Rockenbach *et al.* (2011) studied the maximization of the extraction of antioxidant compounds from grape pomace using acidified methanol at 4 ºC.

According to grape variety, the authors found TPC values of 32.62 ± 0.68 mg GAE/g_{dw} like our results (Table 1). However, in our work, using a food grade and environmentally friend solvent, it was reached a 35-fold higher free scavenging capacity with this similar phenolic content. Casazza *et al.* (2012) evaluated the extraction of bioactive compounds from Pinot noir skins. In their study, the extraction process, only with ethanol at 25 ºC, was inefficient able to release the TPC since their values varied 1.63 to 2.98 mg GAE/g_{dw}. This demonstrates that our optimization including the surfactant addition is suitable once it is possible to obtain higher recovery of TPC with antioxidant activity.

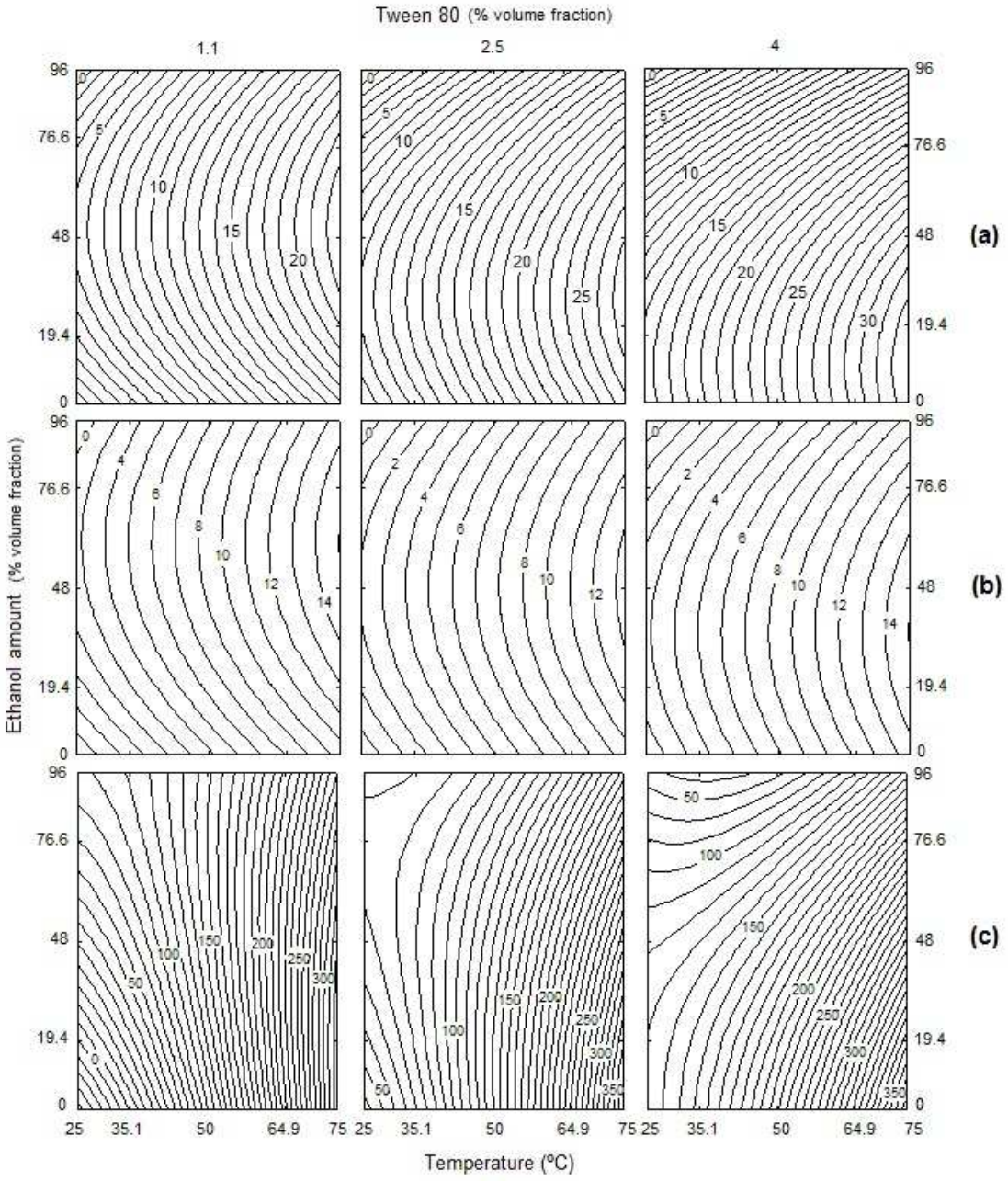

Figure 1. Contour plots of TPC (a), DPPH (b) and ABTS (c) values. The numbers inside the contour plots indicate TPC (expressed as milligrams of gallic acid equivalent per gram of dried grape pomace (mg GAE/g_{dw})) and antioxidant activity of grape pomace by DPPH and ABTS methods (expressed in concentration of trolox per gram dried grape pomace (mmol TEAC/g_{dw})) – at given reactions conditions.

3.3 Statistic analysis of antioxidant activity (DPPH and ABTS)

According to the experimental design, to best describe the antioxidant activity of grape pomace measured by DPPH method, the second-order is represented by the equation:

$$Y_2 = 3.34X_1 - 1.03X_2^2 + 0.46X_3^2 - 0.93X_2X_3 \quad (3)$$

Where Y_2 is the DPPH response and X_1, X_2, and X_3 are temperature, ethanol amount, and Tween 80 concentration, respectively. The insignificant terms ($p > 0.05$) were not considered to describe the equation (3).

Statistical testing of the model was done by the Fisher's statistical test for ANOVA. The computed F-value (30.64) was highly significant ($p = 0.05$). The determination coefficient ($R^2 = 0.91$) implies that the sample variation of 91 % for antioxidant activity measured by DPPH method is attributed to the independent variables and can be explained by the model while the correlation coefficient (R = 0.88) suggests a highly satisfactory representation of the process model and a good correlation between the experimental results and the theoretical values predicted by the model equation 3, which was adjusted with experimental data.

The quadratic model adjusted to antioxidant activity measured by ABTS methodology within studied levels, after neglecting the statistically calculated insignificant terms ($p > 0.05$) was:

$$Y_3 = 74.44X_1 + 14.81X_1^2 - 18.29X_2 - 7.04X_2^2 + 13.07X_3^2 - 18.38X_1X_2 - 16.72X_1X_3 - 32.66X_2X_3 \quad (4)$$

Where Y_3 is the ABTS response and X_1, X_2, and X_3 are temperature, ethanol amount and Tween 80 amount, respectively.

The computed F-value (3.78) and the determination coefficient (R^2 = 79 %) were significant at 95 % confidence level. The correlation coefficient (R = 0.58) suggests a good representation of the process model and correlation between the experimental results and the theoretical values predicted by the model equation 4, which was adjusted with experimental data.

Temperature was the most significant extraction factor affecting the antioxidant activity of grape pomace as determined by the DPPH and ABTS methods, with positive effects 6.70 and 148.87, respectively. As observed for TPC, increasing the temperature and reducing the ethanol amount, the antioxidant activity reached at highest values (Fig 1b and 1c). Although not statistically significant, increasing the amount of Tween 80 from 1.1 % to 4 %, improved the recovery of grape pomace antioxidant activity. More important, the addition of surfactant can be correlated with the amount of ethanol. In particular, when using the ABTS method, increased amounts of Tween 80 produced higher free scavenging radical with only 5 % ethanol in the system. These results show that the cost of the extraction could be reduced with less solvent being used.

Karacabey and Mazza (2010) reported that temperature and ethanol content were important for bioactive compounds extraction from grape cane extracts. These authors determined the antioxidant activity by TEAC assay and the oxygen radical absorbance capacity using fluorescein (ORACLF) method, reporting similar profiles between the two methods. Optimizing the solid-liquid extraction of antioxidants from apple pomace, Wijngaard and Brunton (2010), found that the increase of ethanol concentration had a higher influence over TPC extraction than it did on the antioxidant activity. Kapasakalidis *et al.* (2006) evaluated black currant residues as a source of antioxidants, reporting that the radical scavenging capacity varied widely from 0.04 to 0.45 mmol of TEAC/g of material, approximately 800-fold lower than our findings for grape pomace residue. These authors performed a two-steps extraction procedure, using formic acid in methanol and methanol/water/acetic acid besides an acid hydrolysis.

3.4 Optimal reaction conditions, model validation and kinetics of bioactive compounds

The optimal conditions for bioactive compounds extraction were determined by the response desirability profile calculated using the Statistica 7.0 software. The optimal values of each variable were obtained for the three desired responses, in this case the equilibrium between of total phenolic content and antioxidant activity after 6 h of solid-liquid extraction. The optimal conditions were found to be as: temperature, 75 ºC; ethanol concentration, 28.8 % (volume fraction); Tween 80 concentration, 5 %. Under these conditions, the theoretical values for the TPC, DPPH, ABTS, predicted by the model, were 20.33 mg GAE/g_{dw}, 7.49 mmol TEAC/g_{dw}, and 198.71 mmol TEAC/g_{dw}, respectively. The fact that the calculated values of the response desirability profile are lower than the CCD responses shows that it is necessary that the phenolic compounds must keep their antioxidant activities using less stringent extraction conditions, therefore, being considered as bioactive compounds to be used in the pharmaceutical, cosmetic, food and feed industries.

For the validation of the proposed model, an experiment was conducted observing the optimal conditions where the three responses were maximized and the 6 hours kinetics of the studied responses is presented in Figure 2.

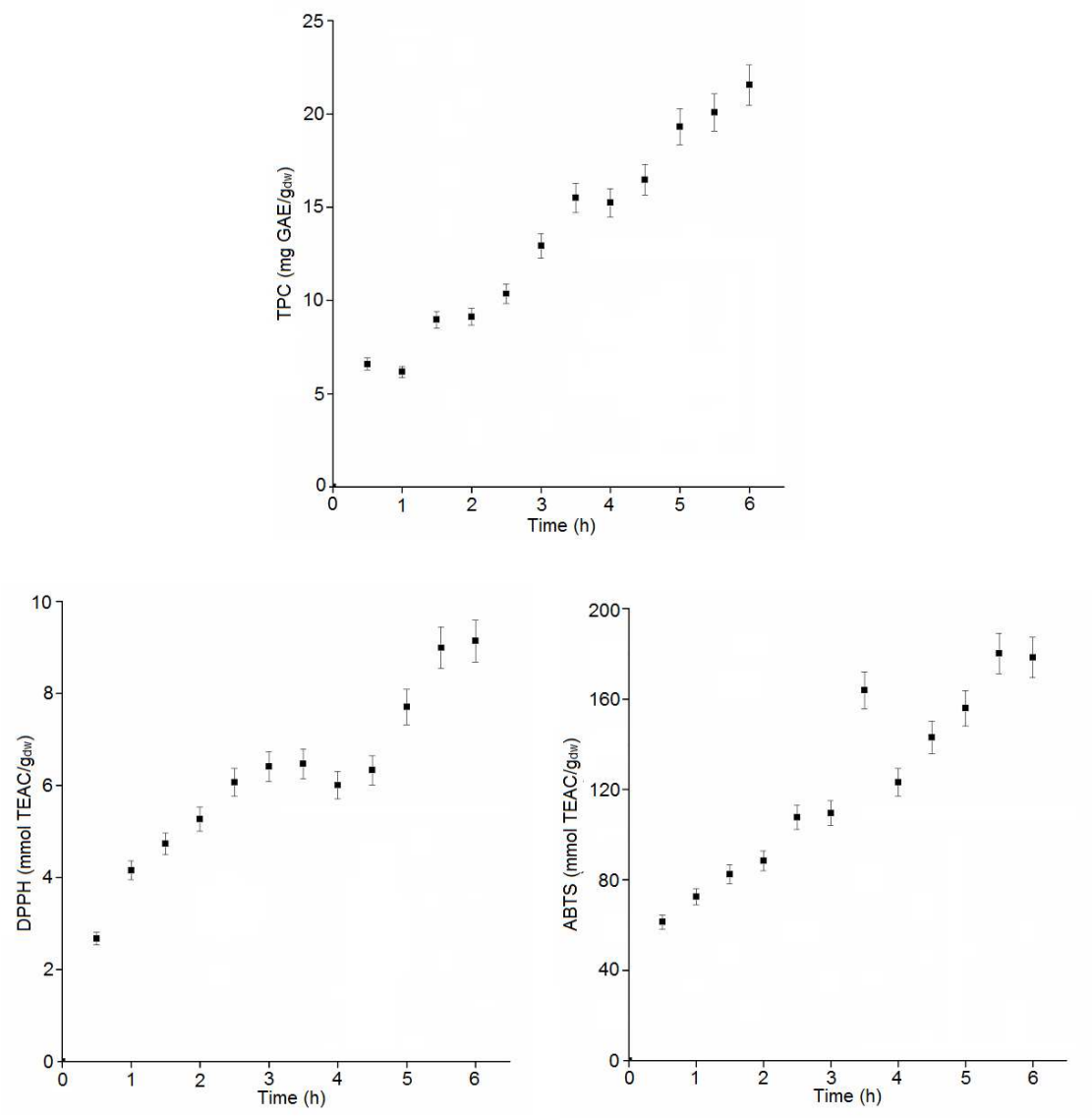

Figure 2. Kinetics of TPC, DPPH and ABTS values at optimal point. TPC yield was expressed in mg GAE/g_{dw} and antioxidant activity of grape pomace by DPPH and ABTS methods were expressed in mmol TEAC/g_{dw}.

Casazza *et al.* (2012) found that 19 h of extraction was a too long time for the recovery of bioactive compounds from skins of Pinot Noir cultivar, with reported loss of antioxidants and their radical-scavenging ability. In the same way, Pekić *et al.* (1998) reported that the kinetics of proanthocyanidins yield from grape seeds using ethyl acetate with different contents of water as extractant were of parabolic shape with the initial part being linear (up to 8 h), whereas the second part showed a slower increase. Therefore, in our work, an intermediate extraction time was considered optimal in order to avoid the oxidative phenomena and to improve the antioxidant

activity since a slight stabilization can be observed after 5 hours of extraction by both methods of free scavenging radicals.

In order to consider the feasibility of the extraction process in large scale applications, it would be important to obtain high yields of extractable phenolic compounds with high antioxidant activities, in the shortest times and using the lowest possible amounts of ethanol, which is compatible with the results obtained in our research.

3.5 Correlation between TPC and antioxidant activity

Because phenolic compounds do not show all the same antiradical activity, the correlation between TPC of grape pomace and their antioxidant capacity is an important topic to be studied.

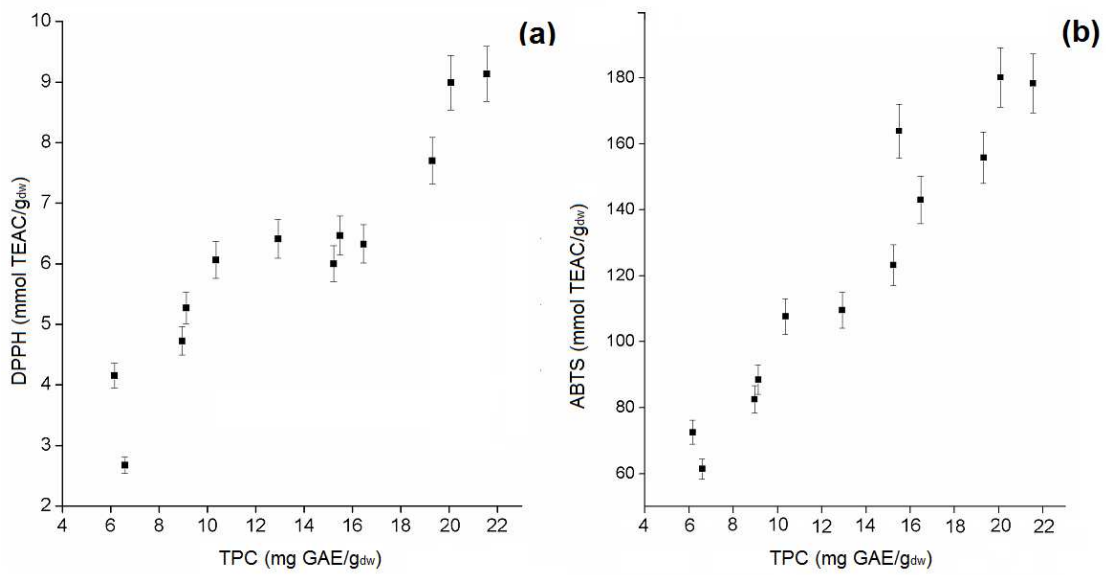

Figure 3. Correlation between total phenolic content and DPPH and ABTS free radical scavenging methods at 95 % confidence level.

Figure 3 reveals that there were positive correlations between the activities as measured by DPPH (86.97 %, Fig. 3a) and ABTS (92.97 %, Fig. 3b) assays with the values of TPC. These results suggest that the phenolic compounds, such as anthocyanin, phenolic acids, tannic acid, and proanthocyanidin, might contribute to the antioxidant activity in grape pomace. Shojaee-Aliabadi *et al.* (2013) reported a strong and positive correlation between inhibition percentage of DPPH and TPC of

Ghure grape marc extract (98 %), similar to findings of Yildirim *et al.* (2005), who reported positive correlations between total phenols and their radical scavenging capacity of organic grape, pomace, juice, must and wine. The high correlation between antioxidant activity and TPC of grape cane measured by two methods, TEAC and ORACFL, 95 % and 89 %, respectively, were also reported by Karacabey and Mazza (2010).

4 Conclusions

In this work, a central composite design was used to optimize the effects of ethanol and Tween 80 amounts, and temperature on the extraction of bioactive compounds present in grape pomace. The results obtained in this study suggest that grape pomace could be a good low-cost source for the productions of antioxidants, using a simple and inexpensive extraction procedure. Moreover, the surfactant Tween 80 allowed the use of lower amounts of ethanol, which leaves the process more environmentally friendly.

Acknowledgments

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5 CAPÍTULO III: ARTIGO CIENTÍFICO

Enhanced antioxidant activity of free phenolic compounds of grape pomace cultivated with three different β-glucosidase fungi producers

Os resultados referentes ao estudo do cultivo em estado sólido do bagaço de uva com três fungos filamentosos – *Aspergillus niger* BLAn1, *Phanerochaete chrysosporium* BLPc1 e *Penicillium* sp. BLPen1 – em relação à produção enzimática de β-glicosidase e à recuperação dos compostos bioativos e seu poder antioxidante estão apresentados em forma de artigo a ser submetido para publicação na revista *Bioresource Technology*.

Enhanced antioxidant activity of free phenolic compounds of grape pomace cultivated with three different β-glucosidase fungi producers

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Abstract

Solid-state cultivation (SSC) was carried out using grape pomace as substrate in cultivations of *Aspergillus niger* BLAn1, *Phanerochaete chrysosporium* BLPc1, and *Penicillium* sp. BLPen1 aiming at the recovery of antioxidant compounds and measuring the cultures β-glucosidase (BG) activities. The highest BG activities were approximately 17.2, 8.3, and 13.5 U · g^{-1} pomace for the 3 strains, respectively. The enzyme activity was considerable reduced with moisture-saturated air injection in the system. The total phenolic content obtained were 21.15, 12.13, and 10.08 mg GAE · g^{-1} pomace, while the highest antioxidant activity released were 16.30, 11.35, and 10.57 mmol TEAC · g^{-1} pomace (by DPPH assay) or 158.61, 104.68, and 102.55 mmol TEAC · g^{-1} pomace (by ABTS assay) for BLAn1, BLPc1, and BLPen1 respectively. These results suggest that the enzyme hydrolysis of phenolic glycosides leads to increased concentrations of free phenolics and enhanced radical-scavenging potential of pomace.

Keywords: grape pomace, solid-state cultivation, bioactive compounds, β-glucosidase activity

1 Introduction

Grape is the world largest fruit crop with an annual production of more than 69 million tonnes, of which approximately 80 % being used for winemaking (FAO, 2011). Around 20 % of the weight of grapes processed for wine remains as pomace (grape pomace, GP), generating more than 10 million tonnes annually of this by-product from wineries (KAMMERER *et al.*, 2005; FAO, 2011).

Pomace contains a large amount of bioactive compounds in skins, pulp, and seeds, which are partially transferred to wine during winemaking. These antioxidants can be divided into two groups: phenolic acids and flavonoids. The most common phenolic acids in grapes include cinnamic acids (coumaric acid, caffeic acid, ferulic acid, chlorogenic acid) and benzoic acids (*p*-hydroxybenzoic acid, protocatechuic acid, vanillic acid, and gallic acid). Flavonoids include colorless flavan-3-ols, such as catechin, epicatechin, and their polymers and ester forms, whereas colored flavanones include red and blue anthocyanins (SHI *et al.*, 2003).

These polyphenols have known health-promoting effects and other important properties in different biological and food systems. Many researchers have suggested that these compounds may play an important role in preventing obesity, heart disease, cancer, and diabetes (GOD *et al.*, 2007; HOGAN *et al.*, 2010). They act as antioxidants by donating hydrogen to highly reactive radicals, thereby preventing further radical formation and as potential metal ion chelators (DUGAS JR. *et al.*, 2000).

Increased demand for sustainable process and for the use of natural antioxidants has led to efforts to minimize the environmental impact of industrial residues, in this case to recycle the GP, recovering its phytochemicals compounds that could be used in pharmaceutical, cosmetics, and food industries. Conventional extraction methodologies of GP phytochemicals such as heating, boiling, or refluxing, are commonly used for this purpose. However, they produce loss of polyphenols caused by ionization, hydrolysis, and oxidation during the process, as well as requiring long extraction times (LI *et al.*, 2005). Another simple procedure is the extraction by solvent (liquid-liquid or solid-liquid) depending on the biomass quality. Although usually cheap, these processes require the use of organic solvents that are toxic, acidified and non-food grade (KAPASAKALIDIS *et al.*, 2006). Robust methods of extraction such as the use of supercritical fluid, high hydrostatic pressure,

ultrasound-assisted, microwave-assisted, and enzymatic release of phenolic compounds, can be used (MAIER *et al.*, 2008; AJILA *et al.*, 2011; DAL PRÁ *et al.*, 2013). These techniques are generally environmental-friendly and represent good alternatives to the solvent-based extraction techniques. On the other hand, they need sophisticated equipment, require high-energy inputs, and are not fully developed and optimized for industrial scales (WANG and WELLER, 2006).

Solid-state cultivation (SSC) is a bioprocess that represents an attractive alternative for the production of bioactive compounds from agro-industrial wastes used as substrates. This technology requires low-input energy and low-cost equipment, also with simplified downstream processing (COUTO and SANROMÁM, 2006). In SSC a broad range of organisms including fungi, bacteria and plants have shown to produce β-glucosidase (EC 3.2.1.21), the enzyme that hydrolyzes the β-glucosidic bonds of several phenolic compounds, consequently increasing the concentration of free polyphenols from several biomasses (HSIEH and GRAHAM, 2001). The capacity of many fungi species cultivated in different biomasses such as pineapple waste (CORREIA *et al.*, 2004), apple residue (AJILA *et al.*, 2012) and cranberry pomace (VATTEM and SHETTY, 2003) were investigated for the bioconversion of bioactive compounds.

In this context, the aim of this work was to investigate the recovery of bioactive compounds present in grape pomace by means of SSC using three different strains of fungi belonging to genera *Aspergillus*, *Phanerochaete,* and *Penicillium*. In addition, it was also investigated the β-glucosidase (BG) activities of cultures and their correlation with the liberated antioxidant activities of phenolic compounds.

2 Materials and methods

2.1 Substrate and microorganisms

Red grape pomace (skins and seeds) was used as the substrate for the solid-liquid extractions and it was kindly supplied by a winery of Bento Gonçalves (Rio Grande do Sul, Brazil; geographic coordinates: 29° 10' 26" S 51° 31' 7" O). This residue has the following *in natura* proximal chemical composition (%): moisture, 61; ash, 2.4; total fiber, 26; fat, 0.6; protein, 4; carbohydrate, 6. This material was

immediately collected after the vinification process and stored at −20 ºC for its conservation until the cultivation.

A screening was performed according to radial growth to select the most promising fungal (data not shown). The fungal strains used in this study were *Aspergillus niger* (designated BLAn1), *Phanerochaete chrysosporium* (designated BLPc1), and *Penicillium* sp. (designated BLPen1). All fungal strains were isolated from soil samples of the Amazon rain forest (geographic coordinates: 03° 06' 26" S 60° 01' 34" O) and were kindly provided by the Sta te University of Amazonas, Brazil. Certified stocks of these strains are kept and available at Microbiology Culture Collection of BiotecLab (UFRGS, Porto Alegre, Brazil). Working stocks were stored in a cryo-protector medium containing glycerol 50 % at −80 ºC.

2.2 Chemicals

Folin-Ciocalteau reagent, 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma-Aldrich (St. Louis, USA). Tween 80 was purchased from Synth (Diadema, Brazil). All solvents used for bioactive compounds and enzyme extractions of grape pomace were of analytical grade and purchased either from Dinâmica (Diadema, Brazil) or Vetec (Rio de Janeiro, Brazil).

2.3 Solid-state cultivation (SSC)

The columns reactors (5.5 mm of diameter x 170 mm of height) used in this work were designed and constructed in our laboratory. An amount of 100 g of fresh grape pomace (containing 60 % water (mass fraction, wet basis) was used for solid-state cultivation without mineral medium added. The media contained in columns were autoclaved at 121 ºC for 15 min. For the inoculum preparation, spores from 7 days-sporulated cultures (plates with grape pomace and agar 4 %) were collected in sterilized water and counted in a Neubauer chamber in order to prepare a suspension containing around 10^5 spores $\cdot$ g^{-1} substrate. The bioreactors were incubated at 28 ºC. Sterilized moisture-saturated air was forced into bioreactors at 0.65 vvm rate in order to maintain the substrate moisture. The treatments were

monitored throughout growth for 288 h. In order to investigate the influence of the moisture-saturated air injection in the system, 125 mL conical flask containing 10 g of grape pomace was inoculated with the same spore suspension and it was maintained at 28 ºC without air injection. At the end of the cultivation, samples were collected in order to recover the cultivated media for the estimation of β-glucosidase activity, total phenolic content and its antioxidant activity.

2.4 Crude enzyme extraction

A volume of 5 mL of sodium citrate buffer (50 mM, pH 6.0) was added into cultivated-pomace-containing flasks, and the culture was vigorously shaken for 2 min and then centrifuged at 1 000 g at 4 ºC for 30 min. The supernatant was filtered and stored at −20 ºC until used for the analyses.

2.5 Bioactive compounds extraction

The fermented grape pomace was placed in a 50 mL conical flask containing 10 mL of solvent mixture in a proportion of solvent-to-solid ratio of 20:1 (mass fraction). The solvent mixture was 28.8 % absolute ethanol and 5 % Tween 80, and 66.2 % water. The extractions were performed in a thermostatic orbital shaker at 75 ºC and at a constant stirring rate of 180 rpm for 6 h in the dark to avoid photo-oxidation. At the end of the extraction procedure, the resulting supernatants were stored at −20 ºC until used for the analyses.

2.6 Total phenolic content

The total phenolic content (TPC) in the cultivated media was determined using the Folin-Ciocalteu reagent according to the colorimetric method described by Singleton and Esau (1969).

2.7 Antioxidant activity by the DPPH method

The antioxidant activity of the grape pomace extracts was measured in terms of hydrogen-donating or radical-scavenging ability by means of the radical 2,2-

diphenyl-1-picrylhydrazyl (DPPH•), according to a modified version of the method described by Kim *et al*. (2002). A volume of 1 425 µL of DPPH solution ($6.1 \cdot 10^{-5}$ M) was added to a tube and briefly mixed with 75 µL of sample. The reaction mixture was incubated for 30 min in the dark, at 25 ºC. The absorbance was read at 515 nm. Total antioxidant activity of cultivated grape pomace extracts was expressed in mmol TEAC (Trolox equivalent antioxidant capacity) $\cdot$ g^{-1}.

2.8 Antioxidant activity by the ABTS method

The measurement of antioxidant activity of grape pomace extracts using radical 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) was determined spectrophotometrically as described by Re *et al.* (1999) with some modifications. A mixture of 5 000 µL of $7 \cdot 10^{-3}$ M ABTS and 88 µL of 140 mM potassium persulphate was prepared. This concentrated ABTS reagent was stored in the dark for 16 h. Then, a volume of 3 000 µL of this ABTS radical solution was placed into a tube and the reaction was started by the addition of 30 µL of sample. This mixture was incubated for 6 min in the dark at 25 ºC. The colorimetric analysis was performed at 734 nm. Total antioxidant activity of fermented grape pomace extracts was expressed in mmol TEAC $\cdot$ g^{-1}.

2.9 β-glucosidase assay

The β-glucosidase (BG) activity was measured following the method described by Zheng and Shetty (2000) with some modifications. The β-glucosidase activity was assayed in a reaction mixture containing 100 µL sodium citrate buffer (50 mM, pH 4.8), 100 µL of *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG; 9 mM), and 100 µL of the culture supernatant. After the incubation at 50 ºC during 30 min, the reaction was stopped by adding 1.5 mL of sodium carbonate (500 mM). The activity was estimated spectrophotometrically by reading the absorbance of the liberated *p*-nitrophenol at 400 nm. One unit of BG was defined as the amount of enzyme required for the hydrolysis of 1 µmol of substrate (*p*NPG) per minute, under the assay conditions.

3 Results and discussion

3.1 β-glucosidase activity

In Figure 1 are presented the results of the variation of BG activity comparing the non-cultivated grape pomace control (time 0) in the culture of the 3 fungal strains studied. BG activities were low at early stages of culture, but increased 2 to 8-fold at 144 h of cultivation. *A. niger* BLAn1 exhibited the highest enzymatic activity (17.26 ± 0.47 U · g^{-1} pomace) at 144 h of cultivation in the absence of moisture-saturated air injection. *A. niger* strains is well known for higher production of β-glucosidase in cellulosic substrates (GEORGETTI *et al.*, 2009; DHILLON *et al.*, 2011). *P. chrysosporium* BLPc1 and *Penicillium* sp. BLPen1 had their maximum BG activities (8.29 ± 0.07 U · g^{-1} pomace and 13.54 ± 2.82 U · g^{-1} pomace) at 144 h and 240 h, respectively. Although the white-rot fungus *P. chrysosporium*, is a widely studied basidiomycete generally regarded as a producer of extracellular hydrolytic enzymes, strain BLPc1 showed lower BG productions compared to the other strains. Its genome has been largely sequenced, providing information on genes and proteins that play important roles in lignocellulose degradation (MARTINEZ *et al.*, 2004; KERSTEN and CULLEN, 2007). *Penicillium* strains were also investigated for the production of cellulases on many low-cost substrates, such as sugar cane bagasse and wheat bran (CAMASSOLA and DILLON, 2010). On grape pomace, Martínez-Ávila *et al.* (2011) verified that strains of *Aspergillus* showed higher invasion capacity than *Penicillum* strains, that can explain the higher BG activity found in this work.

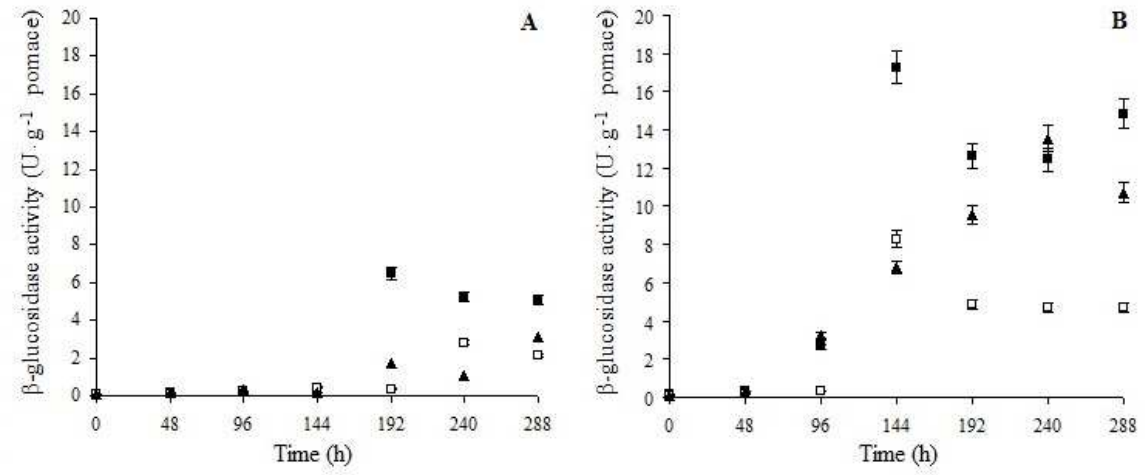

Figure 1. Kinetics of BG activities in SSC of grape pomace with the 3 fungi strains. A) cultures with moisture-saturated air injection. B) cultures without moisture-saturated

air injection; (■) *A. niger* BLAn1; (□) *P. chrysosporium* BLPc1; (▲) *Penicillium* sp. BLPen1. Results are the mean of duplicates.

Surprisingly, the lower productivities were found in the cultivation columns with injection of moisture-saturated air. This lower productivity of enzyme can be correlated with the moisture in the system. Reduction in enzyme activity was also observed by Delabona *et al.* (2013) who described an increase in endoglucanase production by *A. niger* P47C3, from 31.2 to 78.3 IU · g^{-1} of residue, when moisture content of wheat bran was reduced from 70 to 50 % (w/w). The effect was similar for another strain, *A. fumigatus* P40M2, showing a 1.9-fold increase in enzyme production under the same comparative conditions. Singhania *et al.* (2007) found that higher initial moisture levels had a negative effect on cellulase production by *Trichoderma reesei* grown on wheat bran in SSC.

This reduction in enzyme activity when forced air was passed through the substrate, associated with higher initial moisture content could be associated to a reduction in substrate porosity, leading to a limitation in the oxygen transfer (DELABONA *et al.*, 2013). In our work, the moisture of the system with moisture-saturated air injection was maintained around 60 % (mass fraction, wet basis) along the cultivation time. However, in the other system, the moisture was as low as 23 % (mass fraction, wet basis) at the end of cultivation. In aerobic fungi cultures, this limitation can be liked to the inhibition of the enzyme production. Furthermore, the moisture in the columns reactors increased substrate compaction, which could be an additional hindrance for mycelial penetration and microbial degradation, also making more difficult the removal of heat and CO_2 generated during bioprocess.

Concerning the overall results for BG activity obtained in our research, it is difficult to compare with results reported in the literature, because there is lack of standardization of the methodologies on enzyme activity determination, as well as the conversion of the many substrates characteristics. Comparatively, however, wheat bran appeared as the best suited substrate, with reported activities of BG of 33 U · g^{-1} dry substrate at 96 h of cultivation (BANSAL *et al.*, 2012). In the solid-state tray cultivation of wheat bran by *A. niger*, the BG activity was 21.69 IU · g^{-1} dry substrate at 96 h of incubation period (DHILLON *et al.*, 2011). It is important to note that wheat bran will incorporate a lower amount of water as substrate.

Georgetti *et al.* (2009) studied the BG-producing *A. awamori* in order to enhance the antioxidant activity and the mobilization of free phenolic compounds of soybean flour by SSC. The highest enzymatic activity reported was 1 000 U · mL^{-1} in the aqueous extract. The BG activity is also dependent on the strain. Correia *et al.* (2004) investigated the solid-state cultivation of a mixture of pineapple waste and soy flour as the substrate using *Rhizopus oligosporus*. After 240 h, according to different amounts of substrate (5 g of pineapple pomace/5 g of soy flour and 9 g of pineapple pomace/1 g of soy flour), the BG activities peaked at 60 and 120 U · mg^{-1} protein, respectively.

Since the injection of moisture-saturated air was not favorable to BG production, further studies were conduced with the omission of this operation.

3.2 Total phenolic content and antioxidant activities

The time course variations in TPC and antioxidant activities of cultivated grape pomace extracts are shown in Figures 2 and 3, respectively. The control grape pomace extract (without inoculation) was also evaluated under same conditions and presented 21.55 mg GAE · g^{-1} pomace of TPC, 9.13 mmol TEAC · g^{-1} pomace, and 178.34 mmol TEAC · g^{-1} pomace, measured by DPPH and ABTS methods, respectively. The results showed that there is a reduction in TPC recovery for the cultivations of *P. chrysosporium* BLPc1 and *Penicillium* sp. BLPen1 during the initial stages of growth, stabilizing the activities at the end of the runs. For *A. niger* BLAn1, TPC increased along the cultivation time, reaching 21.15 mg GAE · g^{-1} pomace, suggesting the higher BG activity on grape pomace by this strain.

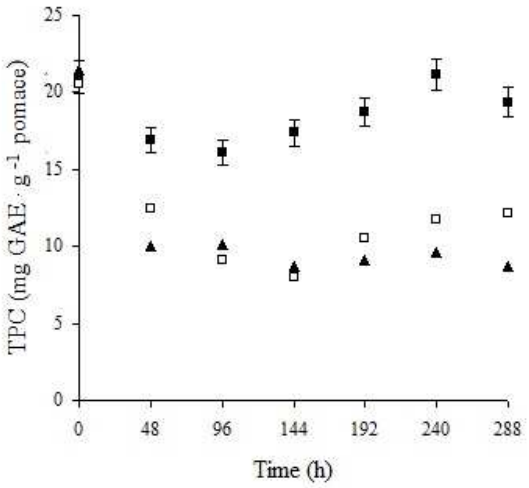

Figure 2. Variation in TPC of cultivated grape pomace extracts during 288 h of cultivation. (■) *A. niger* BLAn1; (□) *P. chrysosporium* BLPc1; (▲) *Penicillium* sp. BLPen1. Results are the mean of duplicates.

Concerning the antioxidant activities, they increased along the time of cultivation. *A. niger* BLAn1 and *Penicillium* sp. BLPen1 showed the highest antioxidant activities (16.30 mmol TEAC · g^{-1} pomace and 10.57 mmol TEAC · g^{-1} pomace) at 240 h, whereas *P. chrysosporium* BLPc1 presented 11.35 mmol TEAC · g^{-1} pomace at 288 h (by DPPH methodology). Results followed the same profile when the ABTS assay was used, with *A. niger* BLAn1 and *Penicillium* sp. BLPen1 showing maximal activities of 158.61 mmol TEAC · g^{-1} pomace and 102.55 mmol TEAC · g^{-1} pomace at 240 h, whereas for *P. chrysosporium* BLPc1, the antioxidant capacity reached 104.68 at 192 h. These results suggest that fungi β-glucosidases might be hydrolyzing β-glucosidic linkages, generating free phenolic forms, therefore enhancing free radical-scavenging potential, according to enzyme production.

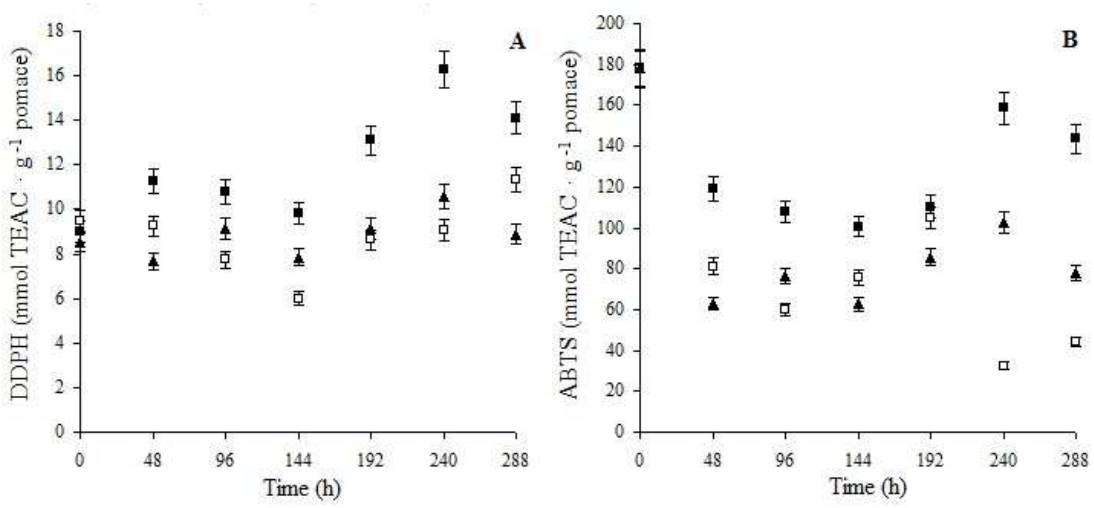

Figure 3. Variation in antioxidant activity of cultivated grape pomace during 288 h of cultivation. A) DPPH methodology; B) ABTS methodology; (■) *A. niger* BLAn1; (□) *P. chrysosporium* BLPc1; (▲) *Penicillium* sp. BLPen1. Results are the mean of duplicates.

Ajila *et al.* (2012) studied the content variation and liberation of phenolic compounds, and the increase in antioxidant activities during SSC of apple pomace using *P. chrysosporium*. The peak of TPC and their free radical-scavenging potential

showed to be correlated with the increase in the BG activity, demonstrating that the enzyme played an important role in the release of polyphenolic aglycones from apple residue. The TPC increased 2-fold compared to non-cultivated samples, as measured in the ethanolic extract.

Starzyńska-Janiszewska *et al.* (2008) reported that scavenging capacity against the ABTS•$^{+}$ radical was higher in SSC grass pea with *Rhizopus oligosporus* than in non-cultivated samples, which correlated well with the phenolic compounds that were present in the substrate. These authors also reported differences in the results of DPPH and ABTS assays, because the bioactive compounds in their samples showed antioxidant properties and react with the DPPH• radical, but they were not capable of scavenging the ABTS•$^{+}$ radical (STARZYŃSKA-JANISZEWSKA *et al.*, 2008; MARTÍNEZ-ÁVILA *et al.*, 2011).

Georgetti *et al.* (2009) found that the polyphenol content in the cultivated soybean extracts was 2 to 3 times higher than in the non-cultivated substrate. The cultivated extracts presented a scavenging activity toward the DPPH• radical. Zheng and Shetty (2000) reported that the cultivation of *Lentinus edodes* on cranberry pomace as substrate was capable to convert the polyphenolic compounds in total free phenolics at yields of 0.5 mg $\cdot$ g^{-1} of pomace, whereas the BG activity was about 9 U $\cdot$ g^{-1} pomace in long run fermentations lasting 50 days. The ability of *Rhizopus oligosporus* to produce enhanced levels of free phenolics from the cultivated mixture of pineapple residue and soy flour was evaluated by Correia *et al.* (2004). These authors found that during early stages of growth, high antioxidant activity correlated with low phenolic content and low BG specific activity. The authors went on to show that the decrease in antioxidant activity after 6 days of cultivation was possibly caused by the cleavage of lignin-like compounds via fungal-mediated degradation (CORREIA *et al.*, 2004). Similarly, the decrease in TPC and antioxidant activity in our work might be attributed to the polymerization and lignification of the released polyphenolics by the lignifying and tannin-forming peroxidases, manganese peroxidase and others enzymes produced by the *P. chrysosporium* and *A. niger* as previously reported by Vazquez-Duhalt *et al.* (1994) and Aissam *et al.* (2005). However, some bioactive compounds present in the grape pomace of our work were liberated by enzyme clivage, leading an increase of free radical-scavenging potential at the end of the cultivation. Fungi, in general, synthesize bioactive compounds with an ecological function, varying from protective action against lethal photooxidation to

protection against environmental stress. These bioactive compounds can also act as cofactors in enzyme reactions that are needed for biosynthetic functions (DEMAIN, 2000; MAPARI *et al.*, 2005).

4 Conclusions

The fungus *A. niger* BLAn1 presented the highest enzyme production of β-glucosidase. The TPC and antioxidant activities increased along the fermentation time, peaking at the end of runs for the 3 studied strains. Surprisingly, our results shown that the expected injection of moisture-saturated air through the pomace, believed important to remove physiological heat and produced CO_2, was detrimental to BG activity. The BG production on grape pomace by these new strains of fungi, never used in bioprocesses before, points to the potential of this system as possible ways for enzyme production, thereby releasing free phenolics and improving the antioxidant activity.

Acknowledgements

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6 DISCUSSÃO GERAL

O presente trabalho foi desenvolvido com o intuito de obter e difundir informações a respeito do bagaço de uva, resíduo pouco explorado e prejudicial ao meio-ambiente se descartado incorretamente. Tal resíduo apresenta-se como produto de alto valor agregado quando submetido a processos de recuperação dos compostos antioxidantes que estão presentes em sua matriz.

Nesse sentido, no primeiro capítulo, foram abordados os principais aspectos referentes ao bagaço de uva e suas propriedades bioativas. Além disso, tratou-se das diferentes extrações desses compostos presentes em frutos, hortaliças e seus resíduos. Apresentou-se ainda uma revisão a respeito do cultivo em estado sólido sobre resíduos da agro-indústria, uma nova abordagem para a recuperação dos compostos antioxidantes dos mesmos.

O capítulo II foi elaborado com o primeiro artigo intitulado "*An eco-friendly design for bioactive compounds extraction from grape pomace*". Esse estudo teve como objetivos principais avaliar as condições ótimas de extração dos compostos bioativos do bagaço de uva utilizando solventes à base de etanol e água e surfactante Tween 80, além de relacionar o conteúdo de polifenóis totais e a atividade antioxidante das amostras ao longo de 6 horas de extração.

A extração sólido-líquido apresentou-se como um processo viável para recuperação dos compostos antioxidantes do resíduo da agro-indústria. As condições ótimas para essa operação foram: temperatura, 75 ºC; concentração de etanol na mistura etanol/água, 28,8 %, concentração de surfactante Tween 80, 5 %. O conteúdo total de polifenóis obtido foi de 21,55 mg GAE/g bagaço seco, valor acima daqueles relatados na literatura. Em relação à atividade antioxidante dos extratos, recuperou-se 9,13 mmol de TEAC/ g bagaço seco e 178,34 mmol TEAC/g bagaço seco, pelas metodologias com os radicais DPPH• e $ABTS^{\bullet+}$ respectivamente.

As variáveis avaliadas na extração afetaram significativamente ($p < 0,05$) o conteúdo de polifenóis totais, porém somente a temperatura e concentração de etanol influenciaram a atividade antioxidante. Os resultados sugerem que o etanol pode ser utilizado como uma alternativa aos solventes de grau não-alimentício e ambientalmente incorretos, além de verificar que o uso do surfactante auxilia na

extração sólido-líquido. Ainda, pode-se identificar relação entre a quantidade de fenólicos e a potencial sequestrante de radicais livres ao longo do processo.

Já o segundo artigo intitulado "*Enhanced antioxidant activity of free phenolic compounds of grape pomace cultivated with three different β-glucosidase fungi producers*", apresentado no capítulo III, teve como principal objetivo estudar o cultivo em estado sólido do bagaço de uva com três fungos filamentosos – *Aspergillus niger* BLAn1, *Phanerochaete chrysosporium* BLPc1 e *Penicillium* sp. BLPen1 – em relação à produção enzimática de β-glicosidase e à recuperação dos compostos bioativos e seu poder antioxidante.

Os melhores resultados encontrados estiveram entre 144 e 240 horas de cultivo. As maiores atividades de β-glicosidase foram 17,26 ± 0,47, 8,29 ± 0,07 e 13,54 ± 2,82 U/g bagaço para os fungos *A. niger* BLAn1, *P. chrysosporium* BLPc1 e *Penicillium* sp. BLPen1. No sistema de cultivo com injeção forçada de ar umidificado, menores atividades enzimáticas foram identificadas para todos os micro-organismos. O conteúdo de polifenóis totais alcançou valores de 21,15, 12,13 e 10,08 mg GAE/g bagaço e a maior atividade antioxidante encontrada foi de 16,30, 11,35 e 10,57 mmol TEAC/g bagaço (pela metodologia com o radical DPPH•) e de 158,61, 104,68 e 102,55 mmol TEAC/g bagaço (pela metodologia com o radical $ABTS^{\bullet+}$) para BLAn1, BLPc1 e BLPen1.

Portanto, ficou comprovada a relação entre hidrólise enzimática dos fenólicos glicosilados e atividade antioxidante, à medida que com o aumento da atividade de β-glicosidase, houve aumento do potencial sequestrante de radicais livres dos extratos cultivados. Nesse sentido, tal estratégia apresenta-se como uma proposta interessante e aplicável para atribuir valor a esse resíduo vinícola tão inutilizado e prejudicial ao meio-ambiente.

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