ARIANA DE SOUZA SOARES

EFEITO DA TECNOLOGIA DE ULTRASSOM NA ATIVIDADE DE ENZIMAS COMERCIAIS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, para obtenção do título de *Doctor Scientiae*.

Orientador: Afonso Mota Ramos

Coorientadores: Bruno Ricardo de Castro Leite Júnior
                    Pedro Esteves Duarte Augusto

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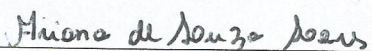ARIANA DE SOUZA SOARES

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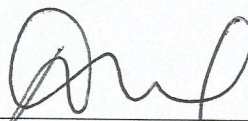título de *Doctor Scientiae*.

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RESUMO

SOARES, Ariana de Souza Soares, D.Sc., Universidade Federal de Viçosa, maio de 2020, **Efeito da tecnologia de ultrassom na atividade de enzimas comerciais.** Orientador: Afonso Mota Ramos. Coorientadores: Bruno Ricardo de Castro Leite Júnior e Pedro Esteves Duarte Augusto.

É crescente a utilização de enzimas em diversos segmentos, tais como têxtil, médico, farmacêutico e de alimentos. Reações enzimáticas apresentam alta eficiência, especificidade, não são tóxicas e, portanto, tem grande importância industrial. Entretanto, o uso de enzimas, em larga escala, é limitado devido ao alto custo e baixa estabilidade. A tecnologia de ultrassom vem sendo proposta para modificar o desempenho enzimático, e, portanto, pode ser uma alternativa para minimizar essas limitações. Nesse contexto, esse estudo objetivou avaliar o efeito do ultrassom na atividade de enzimas comercias, visando melhorias no seu desempenho. Para o estudo, utilizou-se um banho ultrassônico operando a 25 kHz (invertase e lipase) e 40 kHz (amiloglicosidade), com potência volumétrica de 22W/L (invertase e lipase) e 9,5W/L (amiloglicosidade). Em um primeiro momento, avaliou-se o efeito do processamento ultrassónico, como um pré-tratamento da amiloglicosidade, na atividade e estabilidade enzimática. A atividade foi avaliada sob condições ótimas e não ótimas de temperatura e pH e sua estabilidade foi avaliada durante o armazenamento a 8 ° C. Para a invertase, foi avaliado o efeito em três situações: a reação (invertase atuando no substrato sob processamento ultrassonográfico), o substrato isolado (sacarose) e a enzima isolada (invertase). O processamento por ultrassom foi realizado sob diferentes condições de pH e temperatura. Por fim, o efeito do ultrassom foi estudado no desempenho da lipase na hidrólise da gordura do leite de cabra. O processamento foi realizado em diferentes condições de temperatura, avaliando o efeito em três abordagens: lipase, creme de cabra e reação. O ultrassom foi capaz de aumentar, reduzir ou não alterar a atividade enzimática da amiloglicosidade, dependendo das condições aplicadas. Em diferentes condições de ultrassom, principalmente a 35 e 65 ° C, a atividade enzimática não se alterou. O aumento da atividade de até 15% ocorreu em condições não ideais de pH e temperatura (pH 3,5 ou 5,5 / 80 ° C). A reação assistida por ultrassom promovida pela invertase foi acelerada em relação ao processamento convencional. Esse processo aumentou a taxa de hidrólise da sacarose em 33% a 40 ° C e 30% a 30 ° C. O ultrassom como método de pré-tratamento não provocou a hidrólise da sacarose, mas reduziu ligeiramente a atividade da invertase, independentemente do pH e da temperatura. Como

pré-tratamento, o ultrassom não induziu a hidrólise do creme de cabra, mas aumentou levemente a atividade da lipase (até 12%), dependendo da temperatura e do tempo de processamento. A hidrólise de gordura assistida por ultrassom apresentou maior taxa de conversão (12 - 28%) quando comparada ao processamento convencional em diferentes temperaturas. Portanto, a tecnologia de ultrassom surge como uma alternativa interessante para melhorar o desempenho dessas enzimas e acelerar as reações enzimáticas.

Palavras-chave: Enzimas. Ultrassom. Pré-tratamento. Reação assistida. Ativação.

ABSTRACT

SOARES, Ariana de Souza Soares, D.Sc., Universidade Federal de Viçosa, May, 2020, **Effect of ultrasound technology on trade enzyme activity.** Advisor: Afonso Mota Ramos. Co-advisors: Bruno Ricardo de Castro Leite Júnior and Pedro Esteves Duarte Augusto.

The use of enzymes in several segments is growing, such as textiles, medical, pharmaceutical, food. Enzymatic reactions have high efficiency, specificity, are non-toxic, and therefore have great industrial importance. However, the use of enzymes, on a large scale, is limited due to high cost and low stability. The ultrasound technology has been proposed to modify the enzymatic performance, and, therefore, it can be an alternative to minimize these limitations. In this context, this study aimed to evaluate the effect of ultrasound on the activity of commercial enzymes, in order to improve the performance of these enzymes. For the study, an ultrasonic bath was used, operating at 25 kHz (invertase and lipase) and 40 kHz (amyloglucosidase), with volumetric power of 22.0W/L (invertase and lipase) and 9.5 W/L (amyloglucosidase). At first, the effect of ultrasonic processing, as a pre-treatment of amyloglucosidase, on the enzymatic activity and stability was evaluated. The activity was evaluated under optimal and not optimal conditions of temperature and pH and its stability was evaluated during storage at 8 ° C. For invertase, the effect was evaluated in three situations: the reaction (invertase acting on the substrate under ultrasound processing), the isolated substrate (sucrose) and the isolated enzyme (invertase). Ultrasonic processing was carried out under different pH and temperature conditions. Finally, the effect of ultrasound was studied on the performance of lipase in the hydrolysis of fat from goat's milk. The processing was carried out under different temperature conditions, evaluating the effect in three approaches: lipase, goat cream and reaction. Ultrasound was able to increase, reduce or not change the enzymatic activity of amyloglycosity, depending on the conditions applied. Under different ultrasound conditions, mainly at 35 and 65 ° C, the enzyme activity did not change. The increase in activity (up to 15%) occurred under non-ideal conditions of pH and temperature (pH 3.5 or 5.5 / 80 ° C). The ultrasound-assisted reaction promoted by invertase was accelerated in relation to conventional processing. This process increased the rate of sucrose hydrolysis by 33% at 40 ° C and 30% at 30 ° C. Ultrasound as a pre-treatment method did not promote sucrose hydrolysis, but slightly reduced invertase activity, regardless of pH and the temperature. As a pre-treatment, ultrasound did not

induce hydrolysis of the goat's cream, but slightly increased the lipase activity (up to 12%), depending on the temperature and processing time. Ultrasound-assisted fat hydrolysis showed a higher conversion rate (12 - 28%) when compared to conventional processing at different temperatures. Therefore, ultrasound technology appears as an interesting alternative to improve the performance of these enzymes and accelerate enzymatic reactions.

Keywords: Enzymes. Ultrasound. Pre-treatment. Assisted reaction. Activation.

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INTRODUÇÃO GERAL

Enzimas são proteínas que podem ser aplicadas em vários segmentos industriais, tais como na área química, médica, farmacêutica, têxtil, de detergentes, de papel, de couro, de biocombustível, de alimentos e bebidas. Nos alimentos, podem ser encontradas naturalmente ou adicionadas para o desempenho de alguma função catalítica específica, visando à obtenção de produtos com diferentes aplicações e funcionalidades. Apesar da elevada qualidade dos produtos obtidos por ação enzimática, o uso de enzimas na indústria de alimentos apresenta barreiras, que são o alto custo e a baixa estabilidade. Diante disso, algumas tecnologias foram desenvolvidas com o intuito de minimizar essas limitações, sendo o ultrassom uma alternativa.

O ultrassom (US) é uma tecnologia emergente que se baseia na propagação de ondas sonoras com frequências maiores que o limite da audição humana (> 20 kHz). Sua aplicação na indústria de alimentos tem evoluído ao longo dos anos, sendo utilizada para melhorar as operações de transferência de calor e massa e para auxiliar no processamento e conservação de alimentos, como nas técnicas de emulsificação, filtração, desidratação, congelamento, extração e inativação microbiana. Uma das aplicações emergentes do US é a modificação do desempenho enzimático, que pode causar ativação dependendo das condições de processo e da estrutura da enzima.

Essa tecnologia pode ser usada em três abordagens: como um pré-tratamento para a enzima, como um pré-tratamento para o substrato ou auxiliando a reação. A cavitação acústica, que é o principal efeito do ultrassom, gera uma grande quantidade de energia, o que resulta na propagação de ondas de choque e forças de cisalhamento, causando forte turbulência nos arredores. Esses efeitos mecânicos reduzem a barreira limitante de difusão entre enzima e substrato, o que aumenta a transferência de massa, e, portanto, aumenta a velocidade da reação enzimática. Além disso, pode provocar modificações estruturais na enzima e / ou substrato, expondo os sítios ativos da enzima ou aumentando a área superficial dos substratos, o que também pode contribuir para melhorar o desempenho de algumas enzimas.

Considerando-se que o ultrassom é uma técnica promissora para melhorar a eficiência de enzimas, e que o aumento da atividade enzimática é crucial para minimizar suas limitações e proporcionar ampliação do mercado, é importante avaliar o efeito do ultrassom em diferentes condições de processo em enzimas de importância comercial para a indústria de alimentos.

OBJETIVOS

Objetivo Geral

       Avaliar o efeito da tecnologia de ultrassom na atividade de enzimas comerciais visando ativações em condições de interesse industrial.

Objetivos Específicos

- ✓ Determinar a temperatura e pH ótimo de atividade das enzimas Amiloglicosidase, Invertase e Lipase;

- ✓ Avaliar o efeito da temperatura, pH e tempo do processo de ultrassom durante o pré-tratamento ultrassônico da enzima (potência e frequência constantes) na atividade enzimática;

- ✓ Avaliar a atividade enzimática em condições ótimas e não ótimas de pH e temperatura da enzima após o processo de ultrassom;

- ✓ Avaliar o efeito da temperatura de processo de ultrassom durante o pré-tratamento ultrassónico do substrato;

- ✓ Avaliar o efeito da temperatura de processo durante a hidrólise enzimática sob ultrassom;

- ✓ Determinar os parâmetros cinéticos enzimáticos após a aplicação do ultrassom como um pré-tratamento e durante a hidrólise enzimática.

CAPÍTULO 1

REFERENCIAL TEÓRICO

1. Enzimas

As enzimas são na maioria das vezes proteínas produzidas por organismos vivos e apresentam a função de catalisar reações bioquímicas indispensáveis para a sobrevivência dos mesmos (PARKIN, 1993; OLEMPSKA-BEER et al., 2006). Elas diminuem a energia de ativação necessária para que as reações ocorram, e por isso, aceleram os processos (WHITAKER, 2003a).

De acordo com a reação catalisada, elas são divididas em 6 classes (WHITAKER, 2003a). As Oxidoredutases, catalisam a oxidação ou redução do substrato por transferência de elétrons; Transferase, responsável pela transferência de grupos químicos; Hidrolases, catalisam reações de hidrólise de ligações covalentes; Liase, responsável pela adição de grupos a duplas ligações ou remoção de grupos deixando dupla ligação; Isomerase, causam rearranjo de um ou mais grupos do substratos sem alterar a composição atômica do produto e Ligases, catalisam a união de duas moléculas com gasto de energia (WHITAKER, 2003a).

Podem ser obtidas a partir de animais, plantas e micro-organismos. A fonte microbiana é preferida devido a sua facilidade para manipulação genética, grande disponibilidade devido à ausência de sazonalidade, capacidade de produção em massa, além de geralmente serem mais estáveis (RANA, WAIA, GAUR, 2013; ANBU et al., 2015).

Uma das características mais importantes das enzimas é a sua especificidade na reação com determinado substrato com consequente formação de um produto conhecido. (WHITAKER, 2003b). Essa especificidade se deve a sua forma, a qual apresenta encaixes na superfície, conhecidos como sítios ativos, em que os substratos se ligam. Os sítios de ligação entre a enzima/substrato são dados por um arranjo tridimensional dos aminoácidos de uma determinada região da molécula (BELITZ, GROSCH, SCHIEBERLE, 2009). A reação enzimática ocorre pela formação do complexo enzima-substrato, e posterior dissociação, com liberação do produto e da enzima em sua forma nativa (DOBLE, KRUTHIVENTI, GAIKAR, 2004).

As enzimas podem ser aplicadas em vários segmentos industrias, como na química, médica, farmacêutica, têxtil, detergentes, papel, couro, alimentos para animais, biocombustível, alimentos e bebidas e etc. (KIRK, BORCHET, FUGLSANG, 2002; CHOI, HAN, KIM, 2015). Elas são exploradas em vários processos devido a suas

propriedades únicas, como alta especificidade, alta taxa de catálise, capacidade de desempenho com rendimento melhorado e menor geração de resíduos. No entanto, o segmento de enzimas alimentares e de bebidas compreende o maior segmento de enzimas industriais, gerando receitas de quase US $ 1,2 e 1,8 bilhões em 2011 e 2016 respectivamente (PATEL, SINGHANIA, PANDEY, 2016).

A indústria de alimentos representa um dos setores econômicos onde as enzimas encontraram uma grande variedade de aplicações, podendo ser utilizadas para controlar a qualidade dos alimentos (a presença ou ausência de algumas enzimas tem um grande impacto no controle de qualidade do produto final), para modificar as propriedades do alimento, como as propriedades físico-químicas e reológicas por exemplo, e ser utilizadas como aditivos alimentares (AGUILAR et al., 2008).

São aplicadas no processamento de diversos alimentos como leite, queijos, vinhos, cerveja, sucos de frutas, produtos de panificação, óleos e gorduras (LI et al., 2012; PATEL, SINGHANIA, PANDEY, 2016). Exemplos de enzimas comerciais importantes e com grande aplicação na indústria de alimentos são a Amiloglicosidade, Invertase e Lipase.

A amiloglicosidase, é uma exoenzima, extracelular, que hidrolisa as ligações glicosídicas α - 1,4 e α - 1,6; a partir das extremidades não redutoras de amido e dextrina, produzindo glicose (LIN, FELDBERG, CLARK, 1993). A ação é lenta no ataque inicial à amilose, pois, por atuar apenas nas extremidades não redutoras não consegue penetrar no interior da estrutura helicoidal da amilose (TORRES, LEONELL, MISCHANLL, 2012). A principal aplicação é a sacarificação do amido, visando a produção de glicose aplicada na indústria de alimentos como adoçante. Outra aplicação, é a obtenção de matéria prima para produção de etanol que pode ser utilizado na produção de perfumes, remédios e bebidas alcoólicas (KUMAR, SATYANARAYANA, 2009; TRIBST, CRISTIANINI, 2012a). Mais recentemente, vem sendo aplicada na indústria de suco, com o objetivo de reduzir a teor de amido que pode estar presente, por ser característica da própria fruta, ou pela utilização de frutas imaturas misturadas a frutas maturas devido a grandes volumes de frutas que são processadas na produção do suco (TRIBST, CRISTIANINI, 2012a). Nessas condições, a polpa contém amido em quantidades suficientes para causar turbidez ou até mesmo gelatinizar durante o processamento (RIBEIRO et al., 2010).

A invertase hidrolisa a ligação α- 1,2 glicosídica da sacarose, produzindo uma mistura equimolar de glicose e frutose. Essa mistura de monossacarídeos é chamada de açúcar invertido (STURM, 1999). Sua principal aplicação na indústria é a produção de

xarope de açúcar não cristalizável, já que a frutose é mais doce que a sacarose possibilitando o aumento considerável do gosto doce sem a cristalização do material. Esse xarope é utilizado na fabricação de doces e bebidas, sendo, portanto, uma das enzimas mais utilizadas na indústria de alimentos na área de confeitos, doces, chocolates e cookies. Também é utilizada para a fabricação de mel artificial, agentes plastificantes utilizados nas indústrias de cosméticos, farmacêuticas e de papel, além de eletrodos enzimáticos para detecção de sacarose (KULSHRESTHA et al., 2013; NADEEM et al., 2015).

A lipase catalisa a hidrólise de triacilgliceróis em glicerol e ácidos graxos, a síntese de ésteres de vários álcoois e ácidos graxos (esterificação) e reações de transesterificação (GANDHI, 1997). Devido ao fato de catalisarem diversas reações, a lipase tem um potencial enorme de aplicações em diferentes setores, como alimentos, detergentes, farmacêutico, couro, têxtil, cosmético, produção de biodiesel, indústria de papel, e tratamento de águas residuais (NAVVABI et al., 2018). Na indústria de alimentos são empregadas para diversos fins, como: na melhoria da qualidade do pão por meio de alterações nos lipídios da farinha; no aprimoramento do sabor da manteiga, queijo e margarina; aumento do aroma e aceleração do processo fermentativo em cervejas; desenvolvimento de aromas e redução do conteúdo de gordura em carnes. Além disso, as lipases são úteis para aumentar ácidos graxos poli-insaturados em óleos vegetais e para melhorar a digestibilidade dos lipídios naturais (PASTORE, COSTA, KOBLITZ, 2003; NAVVABI et al., 2018).

Industrialmente, as reações enzimáticas podem ser utilizadas para substituir algumas reações químicas e apresentam como vantagens alta eficiência catalítica, que muitas vezes é superior aos catalizadores químicos; não são tóxicas; alto grau de especificidade, o que garante que a reação catalisada não será perturbada por reações indesejáveis, resultando no produto final desejado, o que permite rendimentos maiores e redução de custos de material; operam em condições suaves de temperatura, pressão e pH, resultando em encomia de energia e custos (KRAJEWSKA, 2004; PATEL, SINGHANIA, PANDEY, 2016). Por outro lado, o uso de enzimas como catalisadores para processos industriais em grande escala é limitado por seu alto custo de produção e estabilização no armazenamento. Durante o uso, a sua estabilidade diminui devido a alterações no pH, temperatura, mudanças de conformação resultantes de fricção, pressão osmótica impostas pelos arredores de seu uso e efeito acumulativo de todos esses fatores em função da duração do uso (KOTWAL, SHANKAR, 2009; PATEL, SINGHANIA, PANDEY, 2016).

Algumas formas foram desenvolvidas visando minimizar essas limitações, como a imobilização de enzimas, utilização de engenharia genética e de proteína (IYER, ANANTHANARAYAN, 2008, PATEL, SINGHANIA, PANDEY, 2016; LONGWELL, LABANIEH, COCHRAN, 2017). Além disso, tecnologias não convencionais como alta pressão, micro-ondas e ultrassom começaram a ser estudadas para ativação e estabilização enzimática (TRIBST, CRISTIANINI, 2012 a; TRIBST, CRISTIANINI, 2012b; SZABO, CSIZA, 2013; YU et al., 2014; YADAV, HUDE, TALPADE, 2015; DALAGNOL et al., 2017).

2. Tecnologia de Ultrassom

Ultrassom é uma tecnologia considerada como uma alternativa ao processamento convencional de alimentos. Baseia-se na propagação de ondas sonoras ao longo do produto, com frequências superiores a 20 kHz. Em contraste, as ondas sonoras com frequência na faixa de 16 Hz a 18 kHz estão na faixa de sons percebidos pelo ouvido humano e abaixo de 16 Hz estão na faixa infrassônica (RASTOGI, 2011).

O sistema de produção de ondas ultrassônicas é composto basicamente por um gerador, transdutor e um emissor (MULET et al., 2003; LEADLEY, WILLIAMS, 2006). O gerador produz energia elétrica ou mecânica e o transdutor converte essa energia na energia do som em frequência ultrassônica. Os principais transdutores são os de fluidos, magnetostrictivos e piezoelétricos (são os mais utilizados e eficientes). O acoplador ou emissor, é responsável por emitir as ondas do transdutor para a amostra, sendo mais comuns os sistemas de banhos ou sondas. Em banhos ultrassônicos, os transdutores são fixados na parte inferior do tanque, e a energia ultrassonora é transmitida através de um líquido, ocorrendo muita dispersão de energia, enquanto que nos sistemas de sonda, esta fica em contato direto com a amostra e são utilizadas para amplificar o sinal ultrassônico gerado a partir de um transdutor (MULET et al., 2003; LEADLEY, WILLIAMS, 2006, GOGATE, KABADI, 2009). Porém, a aplicação de banhos a nível industrial é mais vantajoso, pois, além do baixo custo, sistemas com sonda tem difícil aplicação, pois a intensidade diminui exponencialmente ao se afastar da sonda, além de apresentar um alto grau de desgaste, limitando sua aplicação (GOGATE, KABADI, 2009).

O ultrassom é um tema atraente na indústria de alimentos. Suas aplicações são divididas em duas categorias distintas de acordo com a energia gerada pelo campo sonoro. Podem ser classificados em sistemas de baixa e alta energia, sendo que essa energia pode ser quantificada pela potência sonora (W), intensidade do som (W/m^2) e potência

volumétrica (W/m^3) (BERMÚDEZ-AGUIRRE, 2017). O de baixa energia utiliza intensidade inferior a 1 W/ cm^2 e frequências maiores que 100 kHz. Esse sistema não tem energia suficiente para provocar alteração nas propriedades físico-químicas do material por onde propaga, portanto é não destrutivo. Na indústria de alimentos é geralmente usado para obter informações sobre as propriedades físico-químicas dos alimentos, como composição, estrutura e estado físico. Já o de alta energia, utiliza intensidade superior a 1 W/cm^2 (10 -1000 W/cm^2) e frequências entre 20 kHz a 100 kHz. Dessa forma, provoca alterações nas propriedades físico-químicas do material por onde passa, tendo várias aplicações na área de alimentos (MCCLEMENTES, 1995, KNOR et al., 2004; ERCAN, SOYSAL, 2013; BERMÚDEZ-AGUIRRE, 2017) como melhoria de tecnologias de desidratação (GARCIA-PEREZ et al., 2009; RAHAMAN et al., 2019), filtração (KYLLONEN, PIRKONEN, NYSTROM, 2005; WEN, SUI, HUANG, 2008), extração (CHEMAT, LUCHESI, 2006; ZHU et al., 2017), emulsificação (JAFARI, HE, BHANDARI, 2007; XIONG et al., 2019), congelamento (ZHENG, SUN 2006; COLUCCI, et al., 2018), além de atuarem na inativação microbiana (DRAKOPOULOU et al., 2009; HUANG et al., 2017) e inativação/ativação enzimática (HUANG et al., 2017; ROJAS et al., 2017; SOARES et al., 2019).

Quando uma onda sonora passa através de um líquido, cria-se ciclos alternados de compressão e de expansão. Após alguns ciclos, mais precisamente no ciclo de expansão, onde a pressão é menor do que a pressa de vapor do líquido, bolhas são geradas devido ao abaixamento local da pressão, o que leva a sua vaporização. A cavitação, nada mais é do que a formação e evolução dessas bolhas, sendo que existem dois tipos de cavitação, a estática e transiente (BERMÚDEZ-AGUIRRE, 2017). A estática é caracterizada por bolhas duradouras, que permanecem estáveis por muitos ciclos de compressão e expansão, sendo comum em ultrassom de baixa intensidade. A cavitação transiente, comum em processos de alta intensidade, ocorre quando as bolhas aumentam de tamanho ao longo de vários ciclos de compressão e expansão, até chegar em um tamanho crítico, em que a energia do ultrassom não é mais capaz de manter a pressão de vapor estável dentro das bolhas, como consequência, o vapor condensa e as bolhas colapsam violentamente, gerando regiões locais de altas pressões (100 MPa) e temperatura (5000K), que produzem por sua vez, forças de cisalhamento e turbulência na zona de cavitação. A combinação desses fatores tem uma variedade de efeitos no sistema aplicado (KNOR et al., 2004; PATIST, BATES, 2008; SORIA, VILLAMIEL, 2010; KENTISH, 2017).

A aplicação de ultrassom também pode levar a formação de espécies químicas reativas. As condições extremas de temperatura e pressão geradas durante a cavitação transiente podem levar a sonólise das moléculas de água, promovendo a formação de radicais hidroxil, sendo que esses radicais podem reagir com outras substancias químicas presentes no meio (BHASKARACHARYA, KENTISH, ASHOKKUMAR, 2009).

A utilização do ultrassom em tecnologia de alimentos tem evoluído muito com o passar dos anos, por ser uma técnica promissora no processamento e na conservação, com maiores rendimentos, tempos de processamento mais curtos, redução dos custos de operação e manutenção, melhoria dos atributos de qualidade, e redução de patógenos (PATIST, BATES, 2008; BERMÚDEZ-AGUIRRE, 2017). Uma das maiores vantagens do uso do ultrassom na indústria de alimentos comparado com outras técnicas é sua ação eficiente, sendo considerada uma técnica segura e não poluente, devido ao fato de se tratar de uma tecnologia, com sustentabilidade ambiental, além do baixo custo (CHEMAT, ZILLE-HUMA, KHAN, 2011).

3. **Aplicação da tecnologia de ultrassom em enzimas**

Tendo em vista o colapso cavitacional ocasionado pelas ondas ultrassônicas, o qual proporciona aumento da pressão e temperatura em regiões determinadas, muitos estudos começaram a ser desenvolvidos, sugerindo que os efeitos mecânicos e químicos gerados durante a sonicação desempenham um papel importante na inativação de enzimas (ISLAM, ZHANG, ADHIKARI, 2014; O'DONNELL et al., 2010). Na literatura, existem muitos trabalhos com o propósito de inativar enzimas indesejáveis em alimentos processados, como é o caso da pectinametilesterase em sucos de tomate (RAVIYAN *et al.,* 2005), pêra (SAEEDUDDIN et al., 2015), maçã (ABID et al., 2014) e cenoura (JABBAR et al., 2015); peroxidase (POD) em água de coco (ROJAS et al., 2017), agrião (CRUZ, VIEIRA, SILVA 2006); polifenoxidase (PPO) em suco de goiaba (CHENG et al., 2007), morango (SULAIMAN et al., 2015), abacaxi (COSTA et al., 2013), proteases e lipases em leite (VERCET et al., 2001; ARROYO et al., 2017), entre outros.

A cavitação acústica pode causar inativação através de três mecanismos, que podem atuar sozinhos ou combinados. O primeiro é puramente térmico, devido às elevadas temperaturas alcançadas durante a cavitação transitória. O segundo é devido aos radicais livres gerados durante a sonólise da água, que podem reagir com alguns resíduos de aminoácidos que participam da estabilidade enzimática ou na função catalítica com consequente alteração na atividade. O terceiro, devido a forças de cisalhamento que são

criadas, que assim como a elevada temperatura pode levar a modificações na estrutura da enzima (VERCET et al., 2001; ERCAN, SOYSAL, 2013).

Alguns trabalhos relataram que, apesar do objetivo do estudo ser a inativação, o processo foi capaz de ativar, inativar ou não alterar a atividade das enzimas estudadas. Cheng *et al.* (2007) relataram um aumento na atividade da enzima PPO em suco de goiaba ultrassônicado (35 kHz, por 30 min) em comparação com o controle. Eles observaram um aumento na atividade enzimática possivelmente devido às condições de processamento empregadas. Eles utilizaram um banho ultrassônico para estudos de inativação. Entretanto, os banhos de sonicação são geralmente de baixa potência para evitar danos de cavitação nas paredes do tanque e, conseqüentemente, a densidade de energia acústica é baixa devido ao grande volume de processamento. No entanto, um baixo nível de potência de ultrassom, pode levar a mudanças na conformação, enquanto que níveis de potência maiores inativam enzimas devido à desnaturação. Cruz *et al.* (2006) relataram aumento da atividade de POD no agrião devido a sonicação em baixas temperaturas, o que poderia estar relacionado com a mudança de conformação da enzima, promovendo uma maior interação enzima-substrato.

Os mecanismos de inativação/ativação enzimática são diretamente ou indiretamente dependentes de variáveis de processamento, como o tipo do sistema utilizado e geometria, frequência, densidade de energia acústica e tempo (RASO et al., 1999; O'DONNELL et al., 2010). Além disso, os mecanismos de inativação/ativação ultrassônica são específicos da enzima e dependem da composição de aminoácidos e da estrutura conformacional da enzima (OZBEK, ULGEN, 2000; ISLAM, ZHANF, ADHIKARI, 2014).

Portanto, o ultrassom pode ter efeitos positivos na atividade enzimática e pode ser usado para acelerar reações. A aplicação do ultrassom em reações enzimáticas mostra grande potencial para aplicações industriais. Essa tecnologia pode atuar em três alvos em relação às reações enzimáticas (tabela 1): enzima e substrato isoladamente e / ou sistema de reação mista (WANG et al., 2018), portanto, diferentes fatores podem explicar as alterações na performance da enzima devido a aplicação do ultrassom, sendo importante a discussão de cada um deles.

Tabela 1 -Modificação ultrassónica nos diferentes alvos.

Enzima	Alvo	Parâmetros	Resultado	Referência
Celulase	Enzima	6W/ml, 40kHz, 30°C, 80 seg	Aumento de 39% na atividade enzimática	Nguyen, Le (2013)
Dextranase	Enzima	40W, 24kHz, 25°C, 15 min	Aumento de 14% na atividade enzimática	Bashari et al. (2013)
Lipase	Enzima	22W/L, 25kHz, 25°C, 45 min	Aumento de 12% na atividade enzimática	Soares et al. (2020)
Xilanase	Substrato	23W/L, 40kHz, 30°C, 5 min	Aumento de 25% na atividade enzimática	Dalagnol et al. (2017)
α-amilase	Reação	$242,5W/cm^2$, 40kHz, 10 min	Aumento de 24% na atividade enzimática	Oliveira et al. (2017)
Invertase	Reação	22W/L, 25kHz, 40°C	Aumento de 33% na taxa de hidrolise da sacarose.	Soares et al. (2019).
Lipase	Reação	22W/L, 25kHz, 25°C	Aumento de 28% na taxa de hidrolise do creme do leite de cabra.	Soares et al. (2020)

3.1 Efeito do ultrassom nas enzimas

A tecnologia de ultrassom pode afetar a estrutura enzimática devido a mecanismos físicos e / ou químicos (ROJAS, 2017). Tais mecanismos podem alterar a conformação nativa da enzima que depende de interações hidrofóbicas, ligação de hidrogênio, interações de van der Waals e forças eletrostáticas para estabilizar a estrutura molecular tridimensional das proteínas globulares (CHEMAT, ZILLE-HUMA, HKAN, 2011). Cada quantum de energia adicionado ao sistema resulta em uma mudança conformacional, que pode aumentar ou diminuir a atividade enzimática exigindo um estudo caso a caso para compreendê-lo (ROJAS, HELMEISTER, AUGUSTO, 2016). Geralmente, um tratamento ultrassônico de baixa intensidade e curta duração é mais benéfico para melhorar a atividade enzimática, enquanto a exposição prolongada pode resultar em perda progressiva da estabilidade e atividade das enzimas (DELEGADO-POVEDANO, CASTRI, 2015).

Yu et al. (2014) relataram mudanças conformacionais da enzima pepsina, obtendo um aumento de sua atividade (14%) após 60 minutos de tratamento utilizando um banho

ultrassônico operando a 40 kHz, 28 W/L e 25°C. Eles observaram mudanças na estrutura secundária, apresentando menor teor de folha-β e maior β-voltas, após 60 minutos de aplicação.

Subhedar, Gogate (2013) também observaram mudanças na estrutura da celulase. A atividade enzimática aumentou cerca de 24% com a aplicação do ultrassom de sonda após 30 minutos de processo (20 kHz, 17,3 W/cm², 50 °C). A intensidade de fluorescência da celulase tratada com ultrassom diminuiu em comparação com a da celulase não tratada, o que confirmou claramente que, o tratamento ultrassônico diminuiu o número de triptofano na superfície da celulase. A estrutura secundária também foi alterada, sendo observado uma diminuição de 12,4% do conteúdo de α-hélice, e um aumento de 29,6% de bobina aleatória.

A aplicação do ultrassom de sonda, operando a 22kHz, 4,5W/ml, 30 °C após 15 minutos aumentou a atividade da pectinase em 21% (MA et al., 2015). O tratamento alterou a estrutura da enzima, diminuindo a quantidade de triptofano na superfície da pectinase, além de aumentar o conteúdo de folha-β.

Yu et al. (2013), demostraram aumento na atividade da tirosinase de 17,2%, após 150 minutos de tratamento em um ultrassom de banho (40kHz, 31W/L, 25°C). Ao analisarem a estrutura da enzima, perceberam mudanças na conformação, com aumento do conteúdo de folha-β.

3.2 Efeito do ultrassom no substrato

O ultrassom pode promover ruptura de macromoléculas (CHEMAT, HUMA, KHAN, 2011). Dessa forma, a diminuição no tamanho das partículas pode aumentar a transferência de massa, além de aumentar a área superficial do substrato, podendo dessa forma acelerar a reação (WANG et al., 2018).

Dalagnol et al. (2017), avaliaram o efeito da aplicação do ultrassom de banho (40kHz, 23W/L, 30°C) como um pré-tratamento na celulose (10 min) e xilanase (5 min). Analisando-se, a atividade enzimática os autores relataram aumento de 17% e 25% na atividade da celulase e xilanase, respectivamente.

Wang et al. (2017), observaram que a aplicação do ultrassom (22kHz, 7,2W/mL, 35°C) durante a hidrolise do amido pela amiloglicosidase, promoveu mudanças na estrutura do amido (aumento da solubilidade e diminuição do tamanho das partículas), o que contribui para acelerar o processo de hidrolise do amido (25,8%).

3.3 Reação enzimática assistida por ultrassom

A reação enzimática é uma etapa crítica durante muitos processos, pois os métodos enzimáticos convencionais muitas vezes requerem tempo significativamente longo. O uso de outras técnicas para auxiliar as reações enzimáticas atrai muito interesse, sendo o ultrassom uma alternativa (SINGH, 2015).

Durante a cavitação, há uma liberação significativa de energia, resultando na propagação de ondas de choque e forças de cisalhamento, com forte turbulência nos arredores da implosão das bolhas. Esses efeitos mecânicos reduzem a barreira limitante de difusão entre enzima e substrato, aumentando a transferência de massa durante o processo (WANG et al., 2018) e, consequentemente, acelerando a reação.

Sun et al. (2015), avaliou a hidrólise enzimática do xilano pela ação da xilanase assistida por um ultrassom de banho (40KHz, 200W, 50 °C, 30 minutos), e observaram que a combinação do ultrassom e enzima aumentou em 50% o conteúdo de xilooligossacarídeos em comparação com a reação realizada apenas com a enzima.

Ma et al. (2016) avaliaram o efeito do ultrassom de sonda (22Khz, 4,5W/ml, 20 °C, 10 minutos) na hidrólise de pectina catalisada por pectinase, e também observaram que o processo foi acelerado em relação à hidrólise convencional, com um aumento de 25% na velocidade da reação.

Wang et al. (2017) investigou o impacto sinérgico da amiloglicosidade e do ultrassom de sonda operando a 22Khz, 7,2 W/L, 35°C por 40 minutos na hidrólise do amido. Os autores relataram intensificação da hidrolise enzimática por ultrassom, com aumento de 33% na velocidade da reação.

O efeito do ultrassom na estrutura e atividade enzimática têm sido objetivo de várias pesquisas. Durante muito tempo seu principal objetivo era a inativação enzimática, no entanto, alguns estudos mostraram que ele pode melhorar a performance enzimática, tanto por acelerar o transporte dos substratos e enzimas durante as reações, quanto por atuar sobre a estrutura da enzima e do substrato (MASON et al., 2011). Porém, embora alguns efeitos sejam conhecidos para certas enzimas, pode ser diferente para outras, devido a diferenças na sequência de aminoácidos e estrutura enzimática, sendo importante um estudo caso a caso. (DALAGNOL et al., 2017).

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CAPÍTULO 2

Ultrasound Processing of Amyloglucosidase: Impact on Enzyme Activity, Stability and Possible Industrial Applications

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Abstract

This study evaluated the effect of ultrasound processing as a pre-treatment of amyloglucosidase on the enzymatic activity and stability. The activity was evaluated under optimal (65°C / pH = 4.5) and non-optimal conditions of temperature and pH and its stability was evaluated during storage at 8°C. The enzyme solution was processed at 9.5 W/L, 40 kHz, 23°C and at pH 3.5, 4.5, and 5.5, for up to 120 minutes. The activity was measured at 35, 65 and 80°C. The US process was able to increase, reduce or not alter the enzymatic activity, depending on the conditions applied. These modifications depended on the pH of the enzyme solution, the ultrasound processing time and the activity temperature. In different ultrasound conditions, mainly at 35 and 65°C, the enzyme activity did not change, demonstrating that this technology can be used for other purposes, such as microbial inactivation, without affecting the enzyme. The activity increase (up to 15%) occurred under non-optimal pH and temperature conditions (pH 3.5 or 5.5 / 80°C), suggesting that ultrasound promoted stabilization and enzymatic protection. This result is interesting in the starch saccharification, which requires the enzymatic reaction at high temperatures. Therefore, such results can increase the application range of this enzyme in different industrial applications.

Keywords: Ultrasonics, pre-treatment, enzymatic solutions, enzymatic activity, activation.

1. Introduction

A*myloglucosidase*/ glucoamylase (AMG) (EC 3.2.1.3) is an enzyme that hydrolyzes α-1,4 and α-1,6 glycosidic bonds from the non-reducing ends of starch and dextrins, producing glucose (Lin, Feldberg & Clark, 1993). Its main application is the starch saccharification, for the production of glucose for different industries: from the production of food glucose to obtaining raw material for ethanol production, which can be used in the production of perfumes, medicines, alcoholic beverages and others (Tribst & Cristianini, 2012). In addition, the AMG use in the juice industry is increasing, in order to reduce the starch content in certain fruits (Ribeiro, Henrique, Oliveira, Macedo & Fleuri, 2010).

However, the use of enzymes for large-scale industries presents two obstacles: high production cost and low stability under process conditions that use for example, high temperature, diverse pH values, affecting enzymatic activity. (Patel, Singhania & Pandey, 2016). Thus, several alternatives are being developed to minimize these limitations, such as enzyme immobilization, genetic engineering (Patel, Singhania & Pandey, 2016) and emerging technologies, such as high pressure, microwaves and ultrasound, which began to be studied to promote enzymatic activation and stabilization (Dalagnol, Silveira, Baron, Manfroi & Rodrigues, 2017; Mazinani & Yan, 2016; Tribst & Cristianini, 2012).

The ultrasound (US) is a technology which consists of using acoustic energy, with frequencies higher than 20 kHz. US has been proposed in food processing and preservation, as well as improving operations involving heat and mass transfer. (Chemat, Huma & Khan, 2011). A new focus on the ultrasound application is its effect on enzyme structure and activity, with several studies and different approaches (Huang et al., 2017; Islam, Zhang & Adhikari, 2014). However, there is still a need for studies of US processing of AMG.

In fact, most of the studies in the literature use ultrasound to promote the inactivation of undesirable food enzymes (Arroyo, Kennedy, Lyng & Sullivan, 2017; Sulaiman, Soo, Farid & Silva, 2015). On the other hand, ultrasound technology can not only inactivate, but also activate certain enzymes, depending on the process conditions (Nadar & Rathod, 2017). Since various enzymes catalyze desirable reactions, it is of interest to improve or increase enzymatic efficiency. Moreover, depending on the process conditions, ultrasound technology may not affect the enzymatic activity. In this case, the application of ultrasound aims to ensure the microbiological quality of the enzymatic solution, without interfering with the speed of the reaction (Soares et al., 2019), since

several studies have demonstrated its ability to inactivate microorganisms (Betts, Williams & Oakley, 2014; Huang et al., 2017).

Furthermore, ultrasound can promote enzymatic reactions on different approaches: assisting the reaction, as a pre-treatment to the enzyme or as a pre-treatment to the substrate (Wang et al., 2018). In the literature, most studies evaluate the effect of ultrasound during the enzymatic reaction. For instance, the works of Leaes et al.(2013), Wang et al. (2017), and Oliveira, Pinheiro, Fonseca, Cabrita e Maia (2018) studied the amyloglucosidase reaction assisted by ultrasound, but the effect of this technology as a pre-treatment of enzymatic solution was not evaluated. This can be an interesting approach for the enzyme production to enhance the further application. Moreover, it is important to evaluate the effect of ultrasound on the enzyme isolated, since the application of this technology could lead to an increase in enzymatic activity or to ensure microbiological quality of commercial enzyme solutions.

Therefore, the need to better understand the effect of ultrasound technology on the activity of enzymes of commercial interest, under different conditions, is highlighted. Consequently, the objective of this work was to evaluate, for the first time, the effect of ultrasound processing as pre-treatment of AMG solution on the further enzyme activity under optimal and non-optimal conditions of pH and temperature, as well as on the stability of the enzymatic solution under activation conditions during storage at 8 °C.

2. Material and methods

2.1 Ultrasound reactor

During the experiments, an ultrasonic bath (Unique, model USC 2800 A, Indaiatuba, Brazil) with a frequency of 40 kHz and a volumetric power of 9.5 W/L (determined following the method described by Tiwari, Muthukumarappan, O'Dnnell e Cullen, (2008)) was used. The temperature was controlled using a stainless-steel heat exchanger coupled to the ultrasonic bath and to an external bath, to maintain the temperature at 23 during the entire process.

2.2 Determination of amyloglucosidase activity and optimum pH and temperature conditions

Amyloglucosidase produced by Aspergillus *niger* was obtained from Prozyn Biosolution (Butantã, Brazil).

To determine the optimum pH and temperature of the enzyme, the AMG activity was performed at pH values of 3.5, 4.0, 4.5, 5.0 and 5.5, and at temperatures of 50, 55, 60, 65 and 70 °C (in a water bath). Amyloglucosidase activity was determined according to Leaes et al. (2013) with some modifications. The reducing groups released by the enzyme action were determined using the 3,5-dinitrosalicyclic acid (DNS) method proposed by Miller (1959).

To this end, 0.5 mL of the enzymatic solution (0.01% (w/v) in 0.05 M sodium acetate buffer at different pHs) was added to 0.5 mL of the soluble potato starch solution (Êxodo, São Paulo, Brazil at 1% (w/v)) in 0.05 M sodium acetate buffer at respective pHs. The mixture was incubated for 10 minutes at different temperatures and pHs. Subsequently, 1.0 mL of the DNS reagent was added to stop the reaction, and the mixture was placed and kept in boiling water for 5 minutes. After being cooled in ice water, the samples were placed in glass cuvettes to read the absorbance at 540 nm using a visible UV-1800 spectrophotometer (Shimadzu, São Paulo, Brazil). Activity was calculated using the standard glucose curve. The enzyme unit (U) was defined as the amount of enzyme that catalyzes the release of one μmol of glucose per minute of reaction.

The standard glucose curve was obtained using solutions containing 1 to 10 g/L of glucose diluted in 0.05 M sodium acetate buffer (pH 3.5, 4.0, 4.5, 5.0, 5.5). For this, 1.0 mL of the glucose solutions was added with 1.0 mL of the DNS reagent and kept in boiling water for 5 minutes. Subsequently, after cooling in ice water, the absorbance of the samples was measured at 540 nm, similarly to the methodology described for the amyloglucosidase samples.

The condition with the highest activity (pH and temperature) was established as optimum with 100% of the enzymatic activity. The relative enzymatic activity was calculated based on the optimum condition.

2.3 Effect of ultrasonic processing on enzyme activity

The AMG solution was processed using the ultrasound technology, in different conditions. Then, after ultrasound processing, the enzyme activity and stability were evaluated. This approach simulated a possible procedure for the enzyme producer, similarly to those proposed by Tribst, Augusto e Cristianini (2013): the enzyme solution pre-treatment would simulate a possible procedure to obtain a different product by enhancing its properties (for example: increase of the enzyme activity, thermal resistance, stability or activity at specific conditions, or even guarantee microbial stability without using additives). For instance, this approach has been studied for other technologies, such

as the high pressure homogenization (Tribst, Augusto & Cristianini, 2013) or high hydrostatic pressure (Leite Júnior, Tribst & Cristianini, 2017). However, the use of ultrasound with this objective is rare in the literature. Moreover, this approach is different to the proposal of reaction assisted by ultrasound, such as those described by Leaes et al. (2013), Wang et al. (2017), and Oliveira, Pinheiro, Fonseca, Cabrita e Maia (2018).

The ultrasonic bath was filled with 6.5 L of the enzyme solution (0.1 g/L, in 0.05 mol/L sodium acetate buffer) at different pHs (3.5, 4.5 and 5.5) and processed at temperature of 23°C. After 5, 15, 30, 60, 90 and 120 minutes of process, 10 mL of the enzymatic solution were collected, and the enzymatic activity was performed. The activity was determined at temperatures of 35, 65 and 80°C (in a water bath). As a control, 10 mL of an unprocessed aliquot was collected, and its activity was analyzed under the same conditions as the processed samples.

After ultrasound processing the enzymatic activity analysis was performed according to the procedures described in section 2.2 and the relative enzymatic activity after processing was calculated considering the non-processed samples under the same conditions of pH and temperature.

2.4 Evaluation of enzymatic stability

The evaluation of enzymatic stability was performed in the conditions that ultrasound resulted in activation. The enzymatic activity was measured after storing the enzyme solutions under refrigeration (8°C) for 24 hours, according to Tribst and Cristianini (2012), in order to assess whether the activations are reversible. The relative enzymatic activity after storage was calculated considering the activity after the storage processing and right after ultrasonic processing, at the same pH and temperature.

2.5 Experimental design and statistical analysis

A completely randomized (CRD) design was conducted. The procedures were performed with three repetitions in each process condition. The analyses were performed in triplicate for each repetition of the process, totaling nine readings for each condition evaluated, with the results expressed as mean ± standard deviation.

The analysis of variance (ANOVA) was performed to compare the effects of different treatments (processed and unprocessed samples), and the Tukey test was used to determine the difference between them with a 95% confidence level. Statistical analyses were performed using the Statistical Analysis Systems program (Statistical Analysis System - SAS Institute, Cary, North Carolina, EUA), version 9.2.

3. Results and discussion

3.1 Determination of amyloglucosidase activity and optimum pH and temperature conditions

The AMG activity at different temperature and pH is shown in Figure 1. The optimal condition of the enzyme (higher activity) was determined at a temperature of 65 °C and pH 4.5. At this condition the activity was 32688.8 U/g of enzyme, which was considered as 100%. These results are similar to those found by Tribst and Cristianini (2012) .
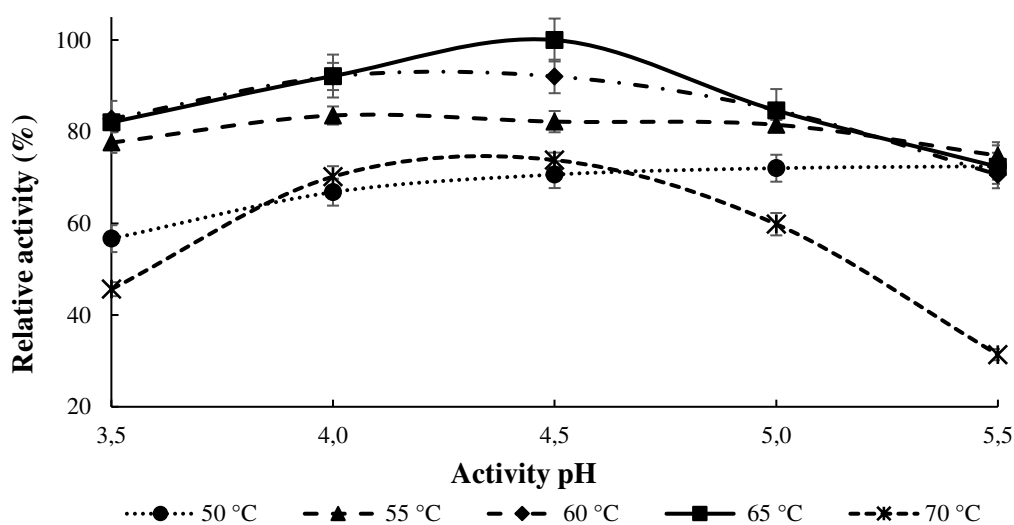
Figure - 1 Effect of pH and temperature on AMG activity. Vertical bars represent standard deviation.

Temperature and pH variation resulted in significant changes in enzyme activity, promoting a reduction of 43.3% at 50°C (pH 3.5) and 68.6% at 70°C (pH 5.5). In general, the AMG activity was almost constant in the evaluated pH range, when at temperatures below 65°C. It indicates that the high temperature associated with extreme pH (in relation to the optimum conditions) promotes the loss of enzymatic activity (Liu et al., 2015; Vandersall, Cameron, Nairn, Yelenosky & Wodzinski, 1995). Liu et al. (2015) demonstrated that heating at temperatures $\geq$ 70°C promotes unfolding of the α-helix, which is often correlated with the loss of activity. In addition, at high temperatures, tryptophan residues were partially exposed, indicating changes in the tertiary structure of the enzyme.

After defining the optimum conditions of AMG activity (pH 4.5 and 65°C, respectively), three conditions of pH were chosen for processing: 4.5, 3.5 and 5.5. In each one, three conditions of temperature were selected for analysis: 65, 35 and 80°C.

Table 1 shows the results of the unprocessed AMG activity, performed under different pH and temperature conditions. It was found that temperature promoted significant changes in the activity. In temperatures higher than the optimum condition (increase from 65 to 80°C), a lower activity was observed in the different pH values (reduction of 79.7% (pH 4.5) to 90.3% (pH 5.5)). Regarding pH, the greatest reductions on the AMG activity were observed by increasing the pH from 4.5 (optimum) to 5.5 (reduction from 27.7% (at 65°C) to 65.6% (at 80°C)), with more drastic structural changes.

Table 1- Enzymatic activity (U/g) of unprocessed amyloglucosidase under different pH and temperature conditions.

Process and analysis pH	Analysis Temperature (°C)		
	35	65	80
3.5	10759.7 ± 299.5 b,B	26804.8 ± 662.0 b,A	5363.7 ± 18.1 b,C
4.5	12474.5 ± 295.6 a,B	32668.8 ± 772.0 a,A	6648.3 ± 78.4 a,C
5.5	11289.7 ± 356.8 b,B	23634.1 ± 158.9 c,A	2288.2 ± 37.2 c,C

Different capital letters indicate a significant difference ($p < 0.05$) between the different temperature of the samples and different lowercase letters indicate a significant difference ($p < 0.05$) between the different pH of the sample. Mean ± standard deviation of nine replicates.

3.2 Effect of ultrasonic processing on enzyme activity

In order to evaluate the effect of ultrasound on AMG activity, the enzymatic solutions were processed with ultrasound under the same pH conditions as the control samples (pH 3.5, 4.5, 5.5, 9.5 W/L, 40 kHz, 23 °C for up to 120 minutes). Subsequently, the AMG activity of processed and unprocessed was measured at the temperatures of 35, 65 and 80 °C.

Figure 2 shows the relative enzymatic activity after AMG processing, demonstrating that the US process promoted changes in amyloglucosidase activity: ultrasound increased, reduced or did not change the enzyme activity. These results were

dependent on the pH of the enzymatic solution, the ultrasound time applied and the temperature of the enzymatic activity.
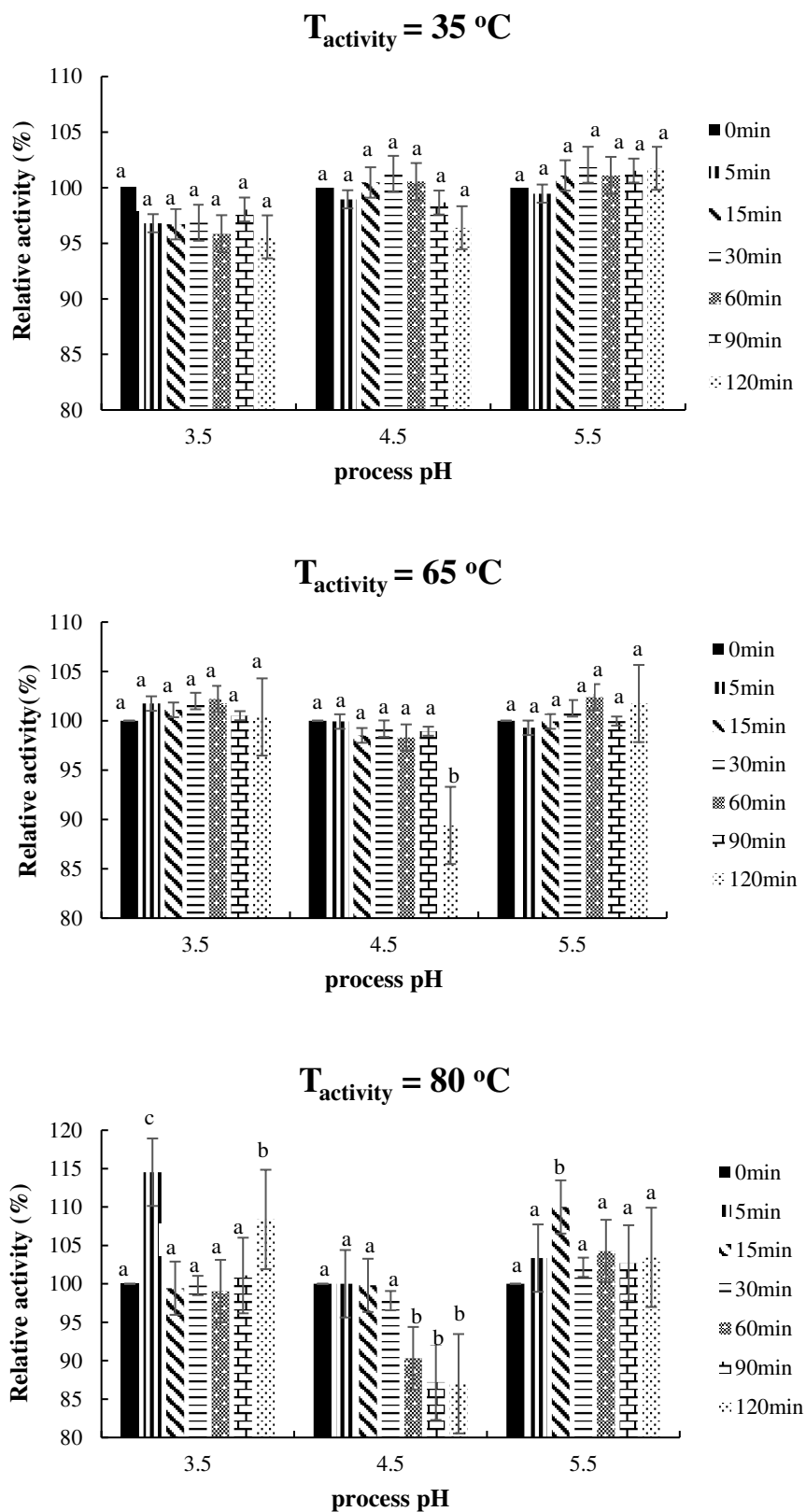
Figure - 2 Relative enzymatic activity after AMG processing (REAP) at different temperatures immediately after the ultrasonic process at 23°C at different pH values and

processing time. Equal letters between the unprocessed sample and processed samples at different times at the same pH and temperature did not differ statistically at 5% probability by the Tukey test.

Ultrasound can be used throughout three targets concerning the enzymatic reactions: mixed reaction system, enzyme and substrate (Wang et al., 2018). Therefore, different factors can explain the changes on enzyme activity due to ultrasound.

Firstly, ultrasound can change the enzyme molecule. The physical (high temperature and pressure) and chemical (free radical formation) effects of the ultrasonic process promote changes in the enzyme's conformational structure, leading to modification in enzymatic activity. (Rojas, Trevilin, Funcia, Gut & Augusto, 2017). The physical (high temperature and pressure) and chemical (free radical formation) effects of the ultrasonic process promote changes in the enzyme's conformational structure, leading to modification in enzymatic activity. Therefore, the energy added to the system by US process can result in a change in the interactions responsible for the native conformation of the enzyme, as electrostatic forces, Hydrogen bonging, Van der Waals interactions (Chemat, Huma & Khan, 2011). Each conformational change can increase or decrease the enzymatic activity.

In fact, Yu, Zeng and Lu (2013) applied ultrasound as a pre-treatment and reported a change in the secondary structure of tyrosinase, with increased β-sheet content, which resulted in an increase in the enzyme activity by a better structural stabilization, activation of isoenzymes or exposure of new active sites. In another study, also applying the ultrasound as a pre-treatment, the pepsin activation was correlated with changes in the secondary structure showing the lower β-sheet content and higher β-turn, as well as changes in the tertiary structure showing an increase in fluorescence intensity of tryptophan (Yu, Zeng, Zhang, Liao & Shi, 2014). However, in the same study, a reduction was observed in the activity of α-amylase and papain notwithstanding the same process conditions. Therefore, it is not possible to establish a rule about the effect of ultrasound process on enzymes, since the enzymes present different characteristics and resistances due to several factors such as differences in the amino acid sequence and in the enzymatic structure (Islam, Zhang & Adhikari, 2014), as well as the various ultrasonic processing conditions. Consequently, it is important to evaluate the effect of ultrasonic processing on different enzymes of interest. No study applied the ultrasound as a pre-treatment in the AMG enzyme.

Secondly, ultrasound can change the substrate. In fact, ultrasound can promote the depolymerization of starch with the release of glucose (Chemat, Huma & Khan, 2011), which can facilitate the interaction enzyme-substrate.

Finally, ultrasound can enhance the reaction, reducing the limiting barrier of diffusion between enzyme and substrate, which increases the mass transfer in the reaction. Acoustic cavitation, which is the main effect of ultrasound, generates a large amount of energy, which results in acoustic streaming transmitted to the system (Nadar & Rathod, 2017; Wang et al., 2018).

For instance, Leaes et al. (2013), Wang et al. (2017), and Oliveira, Pinheiro, Fonseca, Cabrita and Maia (2018) studied the amyloglucosidase reaction assisted by ultrasound, but not the effect of this technology as a pre-treatment of enzymatic solution. However, as the reaction and modification of both enzyme and substrate happens simultaneously, it is difficult to describe how ultrasound enhances the enzymatic reaction – highlighting the importance of the present work.

Furthermore, there are other important differences between the results obtained in the present work and those of the study of Leaes et al. (2013) and Oliveira, Pinheiro, Fonseca, Cabrita and Maia (2018).

Leaes et al. (2013) verified higher products formation during hydrolysis in the ultrasonic bath up to 70 °C, whereas, we verified an increase in the enzymatic activity when measured at 80 °C after the ultrasonic bath processing at 23 °C. These results show that the effects of ultrasound on the AMG are different when the enzyme solution is processed or when the reaction is performed under ultrasound.

In this context, the study of Oliveira, Pinheiro, Fonseca, Cabrita and Maia (2018) was conducted using a probe ultrasound. Despite the positive result, the scale up of probe applications at the industrial level is difficult, limiting its relevance for this purpose (Gogate & Kabadi, 2009), in special due to the intense wear and acoustic field distribution across the reactor. In addition, the authors observed that the US increased products formation when combined with lower temperatures (40-60 °C), indicating that the effect of ultrasound on the AMG is dependent of the equipment type, the conditions applied and the form applied (only in the enzymatic solution or in the reactions carried out under ultrasound). In fact, even the same amount of added ultrasonic energy, though considering different systems, can result in opposite behaviour (Rojas, Trevilin & Augusto, 2016), due to differences on the acoustic field distribution.

Complementarily, from the industrial point of view, it is difficult to perform the reaction under ultrasound, which would be expensive due to the processed volume,

limiting the process viability. In this way, the pre-treatment by ultrasound only in AMG can be an alternative to increase the economic viability of the process aiming at higher compound production with less economic and technological cost.

The main application of amyloglucosidase is the starch saccharification, in which the starch needs to be preheated at higher temperatures (95-105°C) and the pH needs to be adjusted to 5.8-6.5 to promote the liquefaction by α-amylase activity (Crabb & Mitchinson, 1997). Consequently, it would be interesting to activate the enzyme at high temperatures and pHs, resulting in time and energy savings. In addition, another possible application of amylases is together with acidic pectinolytic enzymes, in order to promote juice clarification (Kothari, Kulkarni, Maid & Baig, 2013; Lee, Yusof, Hamid & Baharin, 2006). In this context, the activation of amyloglucosidase at lower temperatures is also important, since pectinases perform better at temperatures of 30-50°C (Kashyap, Vohra, Chopra & Tewari, 2001).

When the AMG activity was evaluated at 35 and 65°C, the ultrasound processing did not cause any changes in its activity, regardless of the pH ($p > 0.05$, Figure 2), except when the enzyme was processed at pH 4.5 for 120 minutes, and the activity was evaluated at 65°C. In this case, a reduction of 10% ($p<0.05$) in its activity was observed (Figure 2). On the other hand, when the activity was evaluated at 80 °C, there were changes in the enzyme activity after applying the ultrasound in the three evaluated pHs ($p <0.05$, Figure 2). Samples processed at pH 3.5 showed a 15% and 8% increase after 5 and 120 minutes of ultrasound application, respectively. At pH 4.5, there was a decrease in the relative activity, with a maximum reduction of 13%. Finally, at pH 5.5, there was a 15% increase in activity after 15 minutes of pre-treatment ($p <0.05$, Figure 2).

In this context, US process at 23 °C may have promoted stabilization/enzymatic protection, reducing the loss of activity at high temperatures (80 °C). This activation may be interesting when the enzyme is applied to the saccharification of starch. It allows the enzymatic process to be carried out at a temperature higher than the optimal temperature, resulting in energy and time savings and, thus, meeting the industrial demand.

Moreover, according to Svensson, Pedersen, Svnedsen and Esen (1982), *Aspergillus niger* produces two forms of amyloglucosidase (isoenzymes), both found in commercial enzymatic solutions, in different proportions. These forms are designated as amyloglucosidase I and II, and they have different characteristics, such as amino acid composition, molecular weight, carbohydrate content and amide grouping, resulting in different properties (Svensson, Pedersen, Svendsen & Esen, 1982). Therefore, the possible presence of two isoenzymes in the commercial solution used in this study may

also explain the difference found in AMG behavior, such as activations at different times of ultrasound (5 and 120 minutes) in the same process / activity condition (pH = 3.5 temperature 80 ºC).

Finally, the maintenance of AMG activity in several conditions after ultrasonic processing is also an interesting result. Several studies have already demonstrated the microbial inactivation capacity of ultrasound (Betts, Williams & Oakley, 2014; Huang et al., 2017). Therefore, ultrasound could be used to ensure the microbiological quality of the commercial enzymatic solution during storage, without interfering in the efficiency of the reaction performed later (Soares et al., 2019). In fact, amyloglucosidase can be commercialized as solution, which facilitates its application but results in stability problems. Microbiological control is often guaranteed with the use of preservatives, such as potassium sorbate and sodium benzoate. Although these preservatives have been accepted as safe, they have been avoided by the consumer, resulting in the need to develop new approaches to ensure the enzyme solution microbiological stability without using additives. Among several emerging technologies studied for this purpose, ultrasound technology can be an alternative (Soares et al., 2019).

It is important to determine the enzymatic stability in order to assess whether the activations caused by the US processing are transient or permanent to identify possible ways to maintain the enzymatic activation after the process. However, most studies have not addressed this feature. The enzymatic stability of the AMG activated by the US process, under different processing conditions, was evaluated after one day of storage at 8°C. The REAS results are shown in Figure 3.

The results obtained in Figure 3 show that the unprocessed enzyme presented no significant difference in activity during storage at pHs 3.5 and 5.5, indicating that AMG was stable when kept in solution at 8°C for 24 h. However, the processed enzymes show a decrease in activity after storage, returning to close to 100% activity.

Therefore, we can conclude that although ultrasound provides an increase in AMG activity, these changes were reversible. Equal activity for the processed and unprocessed enzymes indicates that the processed AMG returned to its native configuration after a rest period. Consequently, if ultrasound is proposed as a method to increase AMG activity, this should be performed just before the application.

T$_{activity}$ = 80°C pH = 3.5

Tactivity = 80° pH = 5.5
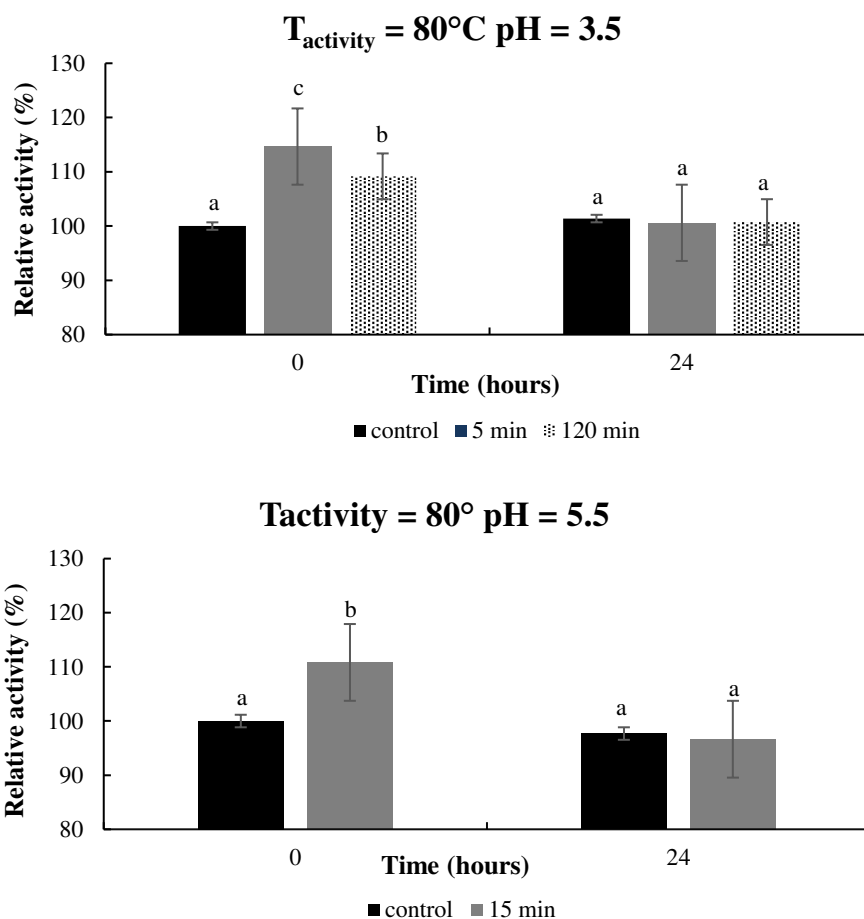
Figure - 3 Amyloglucosidase activity activated by the US process after 24h of storage at 8°C (REAS). Equal letters between unprocessed and processed samples at different analysis times at the same pH and temperature did not differ statistically at 5% probability by the Tukey test.

4. Conclusions

Ultrasound was able to increase, maintain or decrease amyloglucosidase activity after processing. The changes depended on the pH of the enzyme solution, processing time and activity temperature. Activations occurred at 80 ºC, suggesting that US promoted stabilization. This result is interesting in the application of starch saccharification, which requires the active enzyme at high temperatures. Therefore, the results show that US can increase AMG activity, especially in non-optimal pH and temperature conditions, increasing the range of applications of this enzyme in the industry. However, further studies need to be performed to increase enzymatic stability of the activated enzymes during storage.

Acknowledgments

5. References

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CAPÍTULO 3

Ultrasound assisted enzymatic hydrolysis of sucrose catalyzed by invertase: investigation on substrate, enzyme and kinetics parameters

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Abstract

This work studied the ultrasound technology as an alternative to enhance the reaction of sucrose inversion. To achieve it, the effect of ultrasound processing was evaluated in three situations: the reaction (invertase acting on substrate under ultrasound processing), the isolated substrate (sucrose) and the isolated enzyme (invertase). Ultrasound (25 kHz, 22 W/L) processing was conducted under different conditions of pH and temperature. The ultrasound assisted reaction was accelerated in relation to the conventional processing. This process increased the sucrose hydrolysis rate up to 33% at 40 °C, and 30% at 30 °C. The ultrasound as a pre-treatment method did not promote sucrose hydrolysis, but slightly reduced the invertase activity, independently of the pH and temperature of evaluation. Sonication also increased Vmax (increased of 23 %) and maintained constant Km, indicating that the ultrasound accelerated mass transfer during the reaction, but did not directly affected the enzyme. In addition, the invertase catalytic efficiency enhanced 27% under sonication. Therefore, ultrasound technology emerges as an interesting alternative to improve the invertase performance, accelerating enzymatic reaction.

Keywords: ultrasonics; invert sugar; emerging technology; enzymatic activity.

1. Introduction

Invertase / β-fructofuranosidase (EC.3.2.1.26) catalyzes the hydrolysis of sucrose, producing an equimolar mixture of glucose and fructose, which is called "invert sugar" (Kulshrestha, Tyagi, Sindhi, & Yadavilli, 2013; Nadeem et al., 2015). Fructose is sweeter than sucrose, and both glucose and fructose are more soluble than sucrose. Therefore, when compared with sucrose, invert sugar can increase sweetness without crystallization. Consequently, this syrup is widely used in sweets and beverages manufacture, and invertase is the most used enzyme in the confectionery, sweets, chocolates and cookies areas. Moreover, invertase is also used for the manufacture of artificial honey, plasticizers with application in the cosmetics, pharmaceutical and paper industries, as enzyme electrodes for sucrose detection, as well as for production of fructo-oligosaccharides (FOS) (Chand Bhalla, Bansuli, Thakur, Savitri, & Thakur, 2017; Nadeem et al., 2015).

Sucrose may also be hydrolyzed in acidic medium (pH 2-3) at elevated temperatures (70-85 °C) to produce invert sugar. However, acid hydrolysis has the disadvantages of undesirable product formation, high energy, low efficiency and high corrosive power of the equipment (Vitolo, 2004) In this way, the production of invert sugar by enzymatic action is replacing the acid hydrolysis, since the reaction is conducted under milder conditions of temperature and pH (Vitolo, 2004), resulting in a product of enhanced quality. Therefore, due to several applications, invertase is an enzyme of great industrial importance.

However, the use of enzymes as catalysts for large-scale industrial processes can be limited by their high production cost and low stability (Patel, Singhania, & Pandey, 2016). In fact, different alternatives are being developed to minimize these limitations, such as enzymes immobilization, use of genetic engineering (Patel, Singhania, & Pandey, 2016), and use of different emerging technologies. In this context, emerging technologies such as high hydrostatic pressure, microwave and ultrasound began to be studied for enzyme activation and stabilization (Dalagnol, Silveira, Baron, Manfroi, & Rodrigues, 2017; Rejasse, Lamare, Legoy, & Besson, 2007; Tribst & Cristianini, 2012).

Ultrasound (US) is an emerging technology that relies on the propagation of sound waves with frequencies higher than the audible limit of human hearing (> 20 kHz). US has been proposed to improve heat and mass transfer operations and to assist food processing and preservation (Huang et al., 2017). One of the emerging applications of US process is the modification of enzyme performance, which may cause enzymatic activation or inactivation (Huang et al., 2017; Nadar & Rathod, 2017). There are many

works available in the literature that focus on the efficiency of ultrasound to promote enzymatic inactivation (Arroyo, Kennedy, Lyng, & Sullivan, 2017; Saeeduddin et al., 2015; Sulaiman, Soo, Farid, & Silva, 2015; Vercet, Burgos, Crelier, & Lopez-buesa, 2001). However, further studies are needed to demonstrate the ability of the ultrasound to potentiate different enzymes of industrial interest. More specifically, there is a lack of information regarding the effect of ultrasound on invertase activity and its reaction.

Ultrasound can be used in three approaches in relation to the enzymatic reactions: as a pre-treatment to the enzyme, as a pre-treatment to the substrate or assisting the reaction, i.e., in the mixed reaction system (Wang et al., 2018). The majority of studies available in the literature focus on analyzing the assisted reaction. Some examples are the works with amyloglucosidase (Oliveira, Pinheiro, Fonseca, Cabrita, & Maia, 2018), alpha-amylase (Oliveira, Correia, Segundo, Fonseca, & Cabrita, 2017), cellulase (Szabó & Csiszár, 2013), xylanase (Sun, Zhang, Xiao, & Jin, 2015) and pectinase (Ma et al., 2016). All observed that ultrasound accelerated the reaction rate. However, by evaluating only the reaction, it is impossible to fully explain the effects of ultrasound, since this technology can present a simultaneous effect on both the enzyme and the substrate. In addition, most of those studies use an ultrasound probe. Although positive, the ultrasound probe application at industrial level (scale up) is difficult, since the intensity decreases exponentially in moving away from the horn (Gogate & Kabadi, 2009). In addition, it presents a high degree of wearing, limiting its relevance for this purpose.

Only one work evaluated the effect of ultrasound on invertase. Sakakibara, Wang, Takahashi, Takahashi, and Mori (1996) evaluated the ultrasound assisted enzymatic reaction of invertase and showed a higher formation of products during the hydrolysis. However, the authors have not evaluated the effect of ultrasound on the sucrose inversion, as well on the isolated enzyme. As the reaction and possible modifications of the enzyme and the substrate occur simultaneously, it is difficult to describe how ultrasound increased the enzymatic reaction. Furthermore, it is interesting to evaluate the effect of this technology on the enzyme isolated, once this can be an approach to desirably change the activity and/or control microorganisms in commercial enzymatic solutions. In addition, the reaction conducted by Sakakibara, Wang, Takahashi, Takahashi, and Mori (1996) was carried out at fixed temperature (25 °C) and pH (4.0), limiting the available information. Temperature and pH are factors that influence enzymatic activity, and temperature is a limiting factor in the effect of ultrasound (Patist & Bates, 2008).

Therefore, the objective of this work was to evaluate the effect of ultrasound as a pre-treatment to the invertase activity under optimal and non-optimal conditions of pH

and temperature, as well as the effect of this technology assisting the enzymatic hydrolysis of sucrose by invertase. To describe the obtained results, the effect of US technology on sucrose hydrolysis were also evaluated.

2. Material and Methods

Figure 1 present a flow chart of the present work. Different approaches were performed to elucidate the effect of ultrasound on reaction and invertase activity.

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┌─────────────────────────────────────────────────────────┐
│  Enzyme characterization (optimum conditions of reaction) │
│  45, 50, 55, 60 and 65 ° C / pH (4.0, 4.5, 5.0, 5.5, 6.0).│
└─────────────────────────────────────────────────────────┘
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ENZYME + SUBSTRATE	SUBSTRATE	ENZYME
Effect of ultrasound on the reaction	Effect of ultrasound on the substrate	Effect of ultrasound on the enzyme
(30, 40, 55 °C; pH = 5.0)	(30, 40, 55 °C/ pH = 5.0)	(Process: 23 °C; pH = 4.0, 5.0, 6.0) (Reaction: 40, 55, 65 °C; pH = 4.0, 5.0, 6.0)

Kinetics of sucrose hydrolysis	Kinetics of Michaelis-Menten

Kinetics of enzymatic inativation

Figure 1 - Flow chart of the present work.

Firstly, the objective of the work was to evaluate the effect of ultrasound during the enzymatic reaction. Thus, the reaction was conducted at the optimum temperature of the enzyme (55 °C), previously determined, a condition in which the industries produce invert sugar. In addition, the reaction was conducted at 40 °C and 30 °C, since lower temperatures can improve the effects of ultrasound, result in energy savings and increase product quality.

Then, to ensure the substrate hydrolysis was exclusively promoted by invertase, the effect of ultrasound as a pre-treatment on the substrate was conducted under the same conditions (temperature and time) as the enzymatic reaction under US.

Finally, the effect of ultrasound was evaluated as a pre-treatment to the enzyme, in order to evaluate if this technology would promote enzymatic activation or inactivation. The activity was conducted at the enzyme optimum temperature (55 °C), at 40 °C (temperature at which there was an acceleration of the enzymatic reaction promoted by the ultrasound) and at 65 °C, in order to evaluate the effect of extreme temperatures, since activations usually occur in conditions that are not ideal of the enzyme.

2.1 Ultrasound reactor

An ultrasonic bath (Unique, model USC 2800 A, Indaiatuba, Brazil) was applied as reactor, with a volumetric capacity of 9.5 L, equipped with five transducers arranged below the vat, at a frequency of 25 kHz and nominal power of 450 W. A stainless steel heat exchanger was coupled to the ultrasonic bath and to an external water bath to control the temperature of the samples, preventing overheating. The actual volumetric power delivered to the enzyme solution was 22 W/L, which was measured following the calorimetric method described by Tiwari, Muthukumarappan, O'Donnell, and Cullen, (2008).

2.2 Enzyme characterization: determination of invertase activity, optimum pH and temperature

Commercial invertase from *Sacharomyces cerevisiae* was kindly donated by Novozymes (Araucária, Brazil).

To determine the enzyme optimum conditions, the invertase activity was evaluated at pH values of 4.0; 4.5; 5.0; 5.5 and 6.0, and at temperatures of 45, 50, 55, 60, and 65 °C. Invertase activity was measured according to Li, Wang, Ling and Liao (2017), with some modifications, following the method of reducing sugars by the reaction with 3,5-dinitrosalicylic acid (DNS), proposed by Miller (1959).

For this, 0.2 mL of the enzymatic solution (0.02 g/L in 0.05 mol/L sodium acetate buffer at pH 4.0; 4.5; 5.0; 5.5 and 0.05 mol/L sodium phosphate buffer at pH 6.0) was added to 0.8 mL of the sucrose solutions (Neon, São Paulo, Brazil at 35 g/L in 0.05 mol/L sodium acetate and sodium phosphate buffer at respective pHs). The mixture was incubated for 5 minutes at different temperatures. Subsequently, 1.0 mL of the DNS reagent was added to stop the reaction, and the mixture was placed and kept in boiling water for 5 minutes. After cooling in ice water, the samples were placed in glass cuvettes to read the absorbance at 540 nm using a visible UV-1800 spectrophotometer (Shimadzu, São Paulo, Brazil). Activity was calculated using the standard glucose curve. One Katal

was defined as the amount of enzyme that catalyzes the release of one mol of glucose per second of reaction (NCIUB, 1979).

The standard glucose curve was obtained using solutions containing 1 to 10 g/L of glucose diluted in 0.05 mol/L sodium acetate and phosphate buffer (pH 4.0, 4.5, 5.0, 5.5, 6.0). For this, 1.0 mL of the glucose solutions was added with 1.0 mL of the DNS reagent and kept in boiling water for 5 minutes. Subsequently, after cooling in ice water, the enzyme activity was determined similarly to the described.

The condition (pH and temperature) with the highest activity was established as optimum, with 100% of the enzymatic activity. The relative enzymatic activity (REA) was calculated using Equation 1, for all the other pH and temperature values:

$$\text{REA (\%)} = (\text{activity}_{\text{non-optimal condition}}/ \text{activity}_{\text{optimal condition}}) \times 100 \qquad \text{(Equation 1)}$$

2.3 Effect of ultrasound on the enzymatic reaction

A volume of 6.5 L of sucrose solution (600 g/L, previously diluted in 0.05 mol/L sodium acetate at pH 5.0) and enzyme (previously diluted in 0.05 mol/L sodium acetate at pH 5.0 using the ratio of 0.1 g of enzyme to each 1 kg of sucrose solution) was added directly into the ultrasonic bath tub and processed at temperatures of 30, 40 and 55 °C. After different times of processing (0.083 – 9 hours), 0.1 mL of the solution was collected, diluted in acetate buffer, and DNS reagent was added to stop the reaction. Then, the concentration of reducing sugars was quantified, according to 2.2.

As the control treatment, the reaction (250 mL) was performed in a water bath, under the same conditions of pH and temperature as the samples processed, but without ultrasound.

2.3.1 Determination of invertase kinetic parameters
2.3.1.1 Kinetics of sucrose hydrolysis

The sucrose hydrolysis used in the current work was based on the first-order kinetics, where the reaction rate was demonstrated by the increase in the amount of reducing sugar released by sucrose (Ma et al., 2016), as described on Equation 2:

$$\ln (C_\infty - C_t) = -k_{\text{reaction}} \, t + \ln C_\infty \qquad \text{(Equation 2)}$$

Where:

C_t = Concentration of reducing sugar (g/L) at time t;

C_∞ = Ultimate concentration of reducing sugar (g/L);

t = Time of enzymatic reaction (h);

$k_{reaction}$ = rate constant reaction hydrolysis (h^{-1}) at given temperature.

2.3.1.2 Parameters of Michaelis-Menten Model

The effect of ultrasound treatments on enzymatic kinetic was described using the Michaelis-Menten model (Goody & Johnson 2011). (equation 3).

$$V = \frac{V_{max} \times [S]}{K_m + [S]} \qquad \text{(Equation 3)}$$

The reaction rate (V) at each substrate concentration [S] was measured through the slope of the linear part of reducing sugars curve versus reaction time and the Michaelis-Menten constant (K_m) and maximum reaction rate (V_{max}), were attained by nonlinear regression.

Sucrose solutions with different initial concentrations (15 – 200 g/L) and the invertase solutions (0.1 g of enzyme to each 1 kg of sucrose solution) were incubated at 40 °C/ pH = 5.0 for 10 minutes (condition in which the ultrasound assisted reaction obtained an increase in the rate of hydrolysis), with or without ultrasound treatment, and the concentration of reducing sugars was quantified according to the section 2.2.

2.4 Effect of ultrasound on the substrate

A volume of 6.5 L of sucrose solution (600 g/L, previously diluted in 0.05 mol/L sodium acetate at pH 5.0) was added directly into the ultrasonic bath tub and processed at temperatures of 30, 40 and 55 °C. After different times of processing (1, 2, 3, 5, 7, 9 hours), 1 mL of the solution was collected and 1mL DNS reagent was added. The concentration of reducing sugars was quantified according to the section 2.2.

2.5 Effect of ultrasound on invertase activity

A volume of 6.5 L of the enzyme solution (0.02 g/L, previously diluted in 0.05 mol/L sodium acetate and sodium phosphate buffer) at different pHs (4.0; 5.0 and 6.0) was added directly into the ultrasonic bath and processed at a constant temperature of 23 °C at 25 kHz and 22 W/L for different periods. After 3, 5, 10, 15, 20, 30, 45 and 60 minutes of process, 10 mL of the enzymatic solution were collected and the enzymatic activity was performed immediately. The activity was determined at temperatures of 40, 55 and 65 °C. As a control, an unprocessed aliquot (10 mL) was collected, and its activity was performed under the same conditions as the processed samples.

The enzymatic activity was performed according to the procedure described in section 2.2. The relative enzymatic activity after processing (REAP) was calculated considering the activity of the samples processed by ultrasound in relation to the non-processed ones, under the same conditions of pH and temperature, according to Equation 4:

$$REAP^y_x (\%) = (Activity_{processed}/ Activity_{unprocessed}) \times 100 \quad (Equation\ 4)$$

Where:

Y = pH of the solution under ultrasound processing;

X = Temperature of enzymatic activity measurement.

The first order kinetics (Equation 5) was used to describe the changes on the enzymatic activity over the ultrasonic processing time (Fujikawa & Itoh, 1996).

$$\ln(A/A_0) = - k_{inactivation}\, t \quad (Equation\ 5)$$

where:

A = Activity$_{processed}$ (U/g) at time t;

A_0 = Activity$_{unprocessed}$ (U/g);

t = Ultrasonic processing time (minutes);

$k_{inactivation}$ = Rate constant of inactivation (min^{-1}) at given temperature and pH.

2.6 Experimental design, mathematical regressions and statistical analysis

A completely randomized design (CRD) was conducted. The procedures were performed with three repetitions in each process condition. The analyses were performed in triplicate for each repetition of the process, totaling nine readings for each condition evaluated, with the results expressed as mean ± standard deviation.

The parameters of each mathematical model were determined by non-linear regression using the software Curve Expert Professional (version 2.6.5, Hyams Development, Chattanooga, USA).

The analysis of variance (ANOVA) was performed to compare the effects of different treatments (processed and unprocessed samples), and the Tukey test was used to determine the difference between them with a 95% confidence level. Statistical analyses were performed using the Statistical Analysis Systems (SAS) program (Statistical Analysis System - SAS Institute, Cary, North Carolina, USA), version 9.2.

3. Results and Discussion

3.1 Enzyme characterization: determination of invertase activity at different pH and temperatures

Figure 2 shows the invertase activity at different pH and temperatures. The enzyme optimal condition (the highest activity) was obtained at pH 5.0 and 55 °C. In this condition, the activity was 12.08 kat/g of enzyme, which was considered as 100% residual activity (REA). These results are similar to those found by Bergamasco, Bassetti, de Moraes, and Zanin (2000).
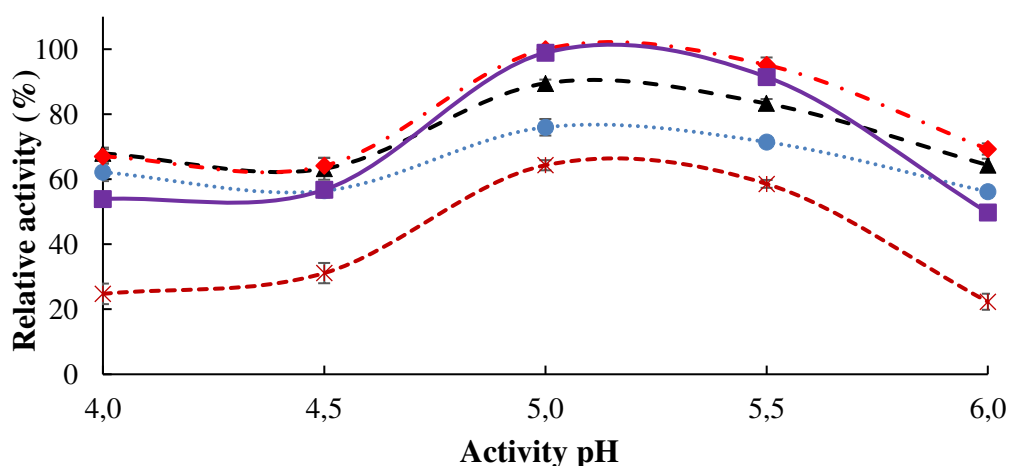
Figure 2 - Effect of pH and temperature on Invertase activity. Vertical bars represent standard deviation. (⋯●⋯ 45 °C); (– ▲ – °C); (– ◆ – 55 °C); (─■─ 60 °C); (– ✳ – 65 °C)

Temperature and pH variation resulted in significant changes in enzyme activity, promoting a reduction of 40.3 % at 45 °C (pH 4.0) and 77.7 % at 65 °C (pH 6.0). In general, higher temperatures associated with extreme pH, in relation to the optimum conditions, promotes activity loss.

After defining the invertase optimum conditions (pH 5.0 and 55 °C), the process conditions were chosen to evaluate the effect of ultrasound assisting the enzymatic hydrolysis of sucrose, as well as the effect of this technology as a pre-treatment for invertase activity, as described in the next sections.

3.2 Effect of ultrasound on the enzymatic reaction

In industrial processing, the invert sugar reaction is carried out at 55 °C and pH 5.0 (optimum conditions), with a proportion of 0.1 g of enzyme for each 1 kg of sucrose

solution at 600 g/L. In this way, it would be interesting to increase the reaction rate at lower temperatures, resulting in time and/or energy savings.

In order to evaluate the effect of ultrasound on the sucrose hydrolysis by invertase, the enzymatic reaction was carried out not only at the optimal conditions (55 °C, pH = 5.0), but also at lower temperatures (30 and 40 °C).

Figure 3 shows the sucrose hydrolysis by invertase at different temperatures, with or without ultrasonic application. Without the application of ultrasound, the degree of sucrose conversion was 72 % at 30 °C, and 82 % at 40 °C, while it was 100 % at the enzyme optimum temperature (55 °C). Ultrasound resulted in a faster and higher hydrolysis process at temperatures of 30 and 40 °C, with a conversion of 79 % and 91 %, respectively. However, no change was observed in the process at 55 °C.
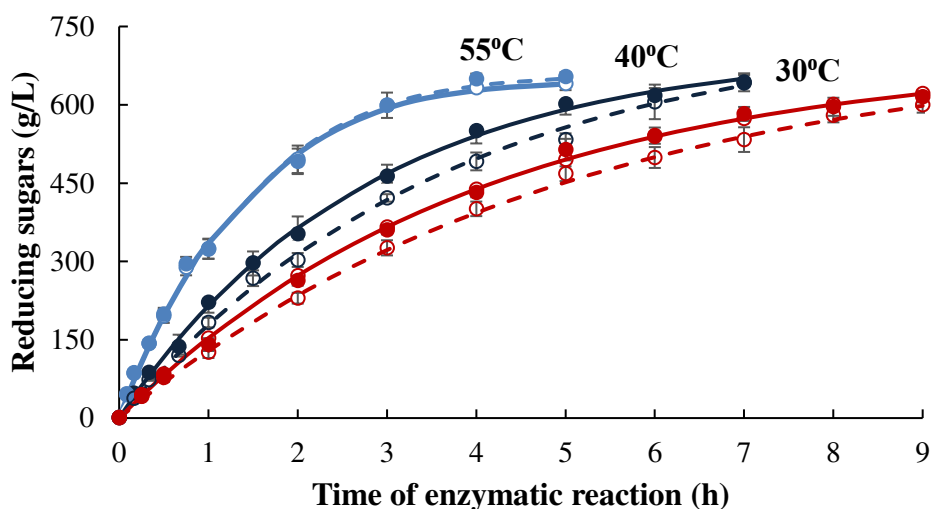
Figure 3 - Sucrose hydrolysis by invertase at different temperatures (30, 40, 55°C) at pH = 5.0, with and without ultrasound application. The dots are the experimental values (open circles and dashed line are the reaction without ultrasound, and filled circles and continuous line are the reaction with ultrasound), the vertical bars are the standard deviation for each condition and the curves are the adjusted model of Equation 2.

Table 1 shows the kinetic parameters, based on Equation 2, for the enzymatic reaction performed at different temperatures, with and without ultrasound. For the evaluated conditions, the hydrolysis kinetics fitted well the first order kinetic model ($R^2>$ 0.99).

Regardless the application of ultrasound, $k_{reaction}$ increased as the temperature rose, until the enzyme optimum temperature (55 °C). Although ultrasound did not influence the reaction under optimal temperature (55 °C), this technology increased the catalytic

activity of invertase under non-optimal temperatures: the reaction hydrolysis rate ($k_{reaction}$) increased 33 % at 40 °C and 30 % at 30 °C. It is worth notice this result is interesting, since low operation temperature is preferred in industry due to energy and cost saving, as well as better product quality

Table 1 - Hydrolytic kinetics of invertase on sucrose at different temperatures with and without ultrasound, based on Equation 2.

Treatment	$k_{reaction}$ (h^{-1})				C_∞ (g/L)		
with ultrasound - 55°C	0.64	±	0.05	a	697	±	12
without ultrasound - 55°C	0.65	±	0.03	a	684	±	14
with ultrasound - 40°C	0.36	±	0.02	b	706	±	35
without ultrasound - 40°C	0.27	±	0.02	c	749	±	17
with ultrasound - 30°C	0.26	±	0.02	c	696	±	31
without ultrasound - 30°C	0.20	±	0.02	d	720	±	25

* Mean ± standard deviation of nine replicates; Equal letters among treatments did not differ statistically at 5% probability by the Tukey test. $k_{reaction}$= rate constant reaction hydrolysis (h^{-1}) at given temperature. C_∞ = Ultimate concentration of reducing sugar (g/L).

Different facts can explain the obtained results. Firstly, at optimal conditions, by definition, the reaction is faster, facilitated, which normally hinder any improvement by other technology. On the other hand, at non-optimal conditions, any addition of energy can be perceived, which can explain the positive effect of ultrasound. Moreover, temperature affects the propagation of ultrasonic waves, as well as the cavitation phenomenon (Patist & Bates, 2008).

Temperature affects the vapor pressure, surface tension and the viscosity of the liquid medium (Muthukumaran, Kentish, Stevens, & Ashokkumar, 2006; Patist & Bates, 2008). Higher temperatures increase the number of cavitation bubbles. However, the collapse is dampened by the higher vapor pressure, thereby decreasing the cavitational intensity (Patist & Bates, 2008). On the other hand, by increasing temperature, the viscosity and surface tension are decreased, facilitating the formation of cavitation bubbles and allowing a more violent collapse. Therefore, there is an optimum temperature at which the viscosity is low enough to form violent cavitation bubbles, but the temperature avoids the damping effect by a high vapor pressure (Patist & Bates, 2008).

Ma et al. (2016) evaluated the effect of ultrasound on pectinase-catalyzed pectin hydrolysis. They also observed that the process was accelerated in relation to the

conventional hydrolysis. When the reaction was conducted at 50 °C, an increase in reaction rate (k) was not observed. However, similarly to this work, at lower temperatures, ultrasound increased the reaction rate: the reaction rate increase reached 25 % when the reaction was performed at 20 °C, demonstrating once again the importance of temperature in ultrasound processing.

Finally, ultrasound can act on three targets in relation to the enzymatic reactions: enzyme, substrate and mixed reaction system (Wang et al., 2018) . Acoustic cavitation, which is the main effect of ultrasound, generates a large amount of energy, which results in propagating shockwaves and shear forces, causing strong turbulence within the surroundings. These mechanical effects reduce the limiting barrier of diffusion between enzyme and substrate, which increases the mass transfer during the reaction.

However, another factor that can explains the increase in the product formation is the structural modifications of enzymes and/or substrates: the enzymatic reaction can be accelerated by increasing the surface area of substrates and/or exposing active sites of enzyme (Nadar & Rathod, 2017; Wang et al., 2018). Therefore, the effect of ultrasound on the enzyme and substrate, isolated, must be evaluated, as described in the next sections.

3.3 Effect of ultrasound on the substrate

Ultrasound can promote the rupture of macromolecules (Chemat, Huma, & Khan, 2011). To evaluate the effect of ultrasound on sucrose, this sugar was previously diluted with buffer (pH = 5.0) and then processed at 30, 40, 55 °C, while the amount of reducing sugars was quantified. Even after 9 h of ultrasound processing (a longer evaluation than the described processes in this work), there was no increase in the concentration of reducing sugars. It clearly indicates that the ultrasound did not promote sucrose hydrolysis. Therefore, we can guarantee that any hydrolysis of sucrose during enzymatic reaction in this study is exclusively related with the invertase action.

3.4 Effect of ultrasound on enzyme activity

In order to evaluate the effect of ultrasound as a pre-treatment to invertase, the enzymatic solutions were processed with ultrasound (22.0 W/L, 25 kHz, 23 °C) under the pH conditions of 4.0; 5.0 and 6.0, for up to 60 minutes. Subsequently, its activity was measured at the temperatures of 40, 55 and 65 °C.

Figure 4 shows the relative enzymatic activity after invertase processing (REAP). Ultrasound reduced or did not change the enzyme activity, depending on the conditions of processing and evaluation (reaction).

pH of process - 5.0

pH of process - 4.0

pH of process - 6.0
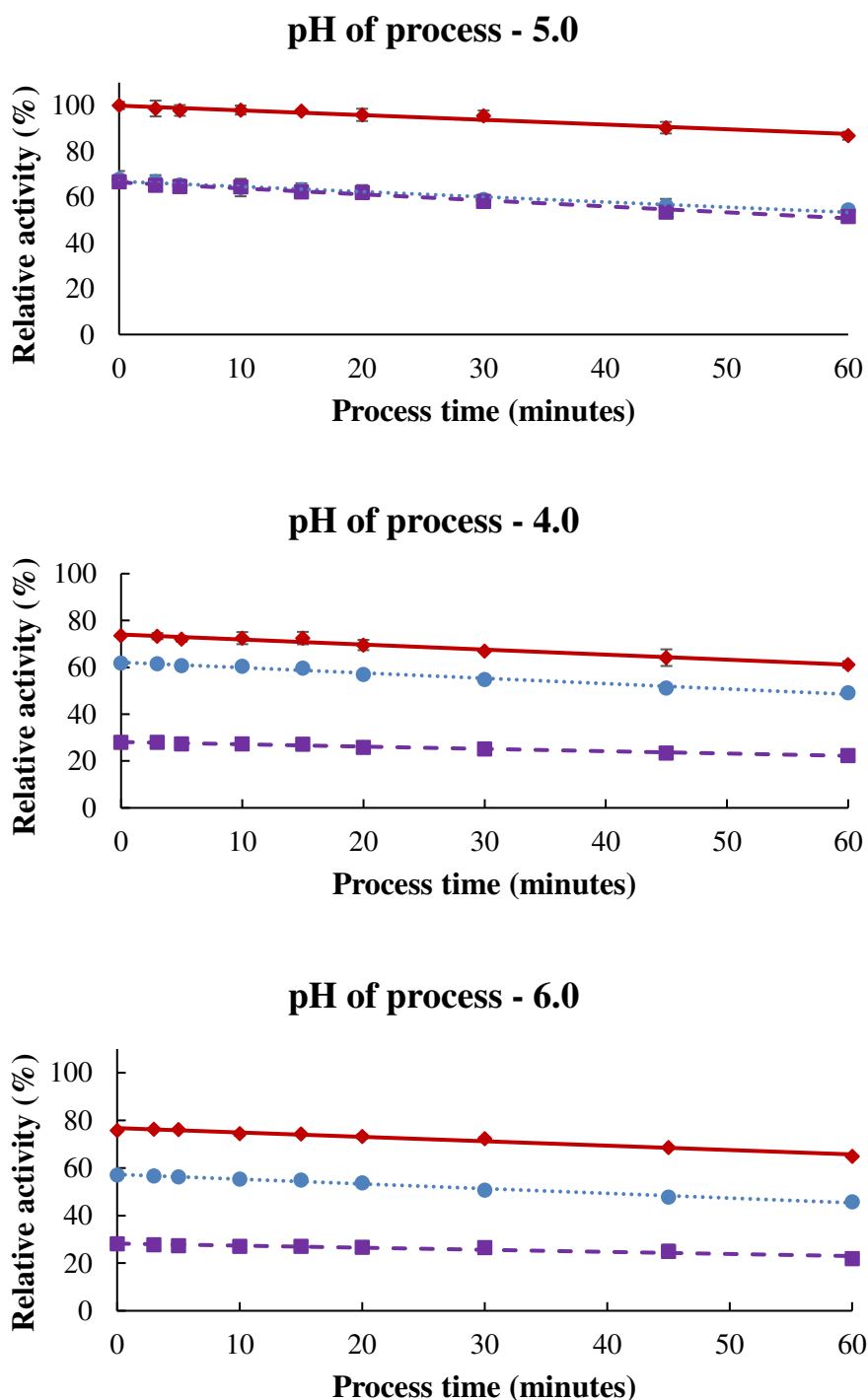
Figure 4 - Relative enzymatic activity after invertase processing (REAP) at different temperatures immediately after the ultrasonic process at different pH values and processing time. 100% of activity refers to the native form under optimal conditions (55 °C/ pH = 5.0). Vertical bars represent standard deviation. Curves are the model of Equation 5. (·····●····· 40°C); (——◆—— 55°C); (– ■ – 65°C).

When the enzyme was evaluated at its optimum temperature (55 ° C), a maximum activity loss of only 13.2 % was obtained even after 1 hour of processing. The pattern of

activity loss was the same for the three evaluated pH values. On the other hand, under non-optimal conditions, the enzyme lost activity at shorter processing times, with higher kinetic parameter as show in Table 2 (fitted well to the first order kinetic model $R^2 >$ 0.96). It demonstrated the enzyme resistance to the ultrasound processing at optimum conditions, as well as the small resistance of its conformational structure at non-optimum conditions.

Table 2 – Invertase inactivation under ultrasonic processing. Parameters of first order kinetic (Equation 5).

pH of process	Temperature (°C)	$k_{inactivation}$ (min^{-1})	
	40	0.0041 ± 0.0003	a
4.0	55	0.0027 ± 0.0003	b
	65	0.0039 ± 0.0002	a
	40	0.0038 ± 0.0003	a
5.0	55	0.0024 ± 0.0003	b
	65	0.0041 ± 0.0002	a
	40	0.0039 ± 0.0002	a
6.0	55	0.0026 ± 0.0003	b
	65	0.0037 ± 0.0004	a

* Mean ± standard deviation of nine replicates; Equal letters did not differ statistically at 5% probability by the Tukey test. $k_{inactivation}$ = Rate constant of inactivation (min^{-1}) at given temperature and pH.

The changes in activity are correlated with the physical (high temperature and pressure) and chemical (formation of free radicals) effects of ultrasound on the conformational structure of the enzyme (Rojas, Trevilin, Funcia, Gut, & Augusto, 2017). Therefore, each quantum of energy added to the system by the US process can result in a change in the enzyme native conformation, which depends on hydrophobic interactions, hydrogen bonding, van der Waals interactions and electrostatic forces to stabilize the three-dimensional molecular structure of globular proteins (Chemat et al., 2011). Each conformational change can increase or decrease the enzymatic activity.

Although ultrasound promoted significant changes on the enzymatic activity, this inactivation was not drastic, with a maximum reduction of 15.1 % even after 1 hour of ultrasonic processing. In fact, sonication at room temperature has minor effects on enzyme activity (O'Donnell, Tiwari, Bourke, & Cullen, 2010; Sulaiman et al., 2015), and when the goal is enzyme inactivation, there is a need to use thermosonication or

manothermossonication (Bermúdez-Aguirre, 2017). On the other hand, this small variation on the enzyme activity can be useful on the ultrasonic assisted reaction.

The maintenance of invertase activity in several conditions after ultrasonic processing is also an interesting result. Several studies have already demonstrated the microbial inactivation capacity of ultrasound (Betts, Williams, & Oakley, 2014; Huang et al., 2017). Therefore, ultrasound could be used to ensure the microbiological quality of the commercial enzymatic solution during storage, without interfering in the efficiency of the reaction performed later. In fact, invertase can be commercialized as solution, which facilitates its application but results in stability problems. Microbiological control is often guaranteed with the use of preservatives, such as potassium sorbate and sodium benzoate. Although these preservatives have been accepted as safe, they have been avoided by the consumer, resulting in the need to develop new approaches to ensure the enzyme solution microbiological stability without using additives. Among several emerging technologies studied for this purpose, ultrasound technology can be an alternative.

3.5 Parameters of Michaelis-Menten of reaction

The Michaelis-Menten plots are shown in Figure 5, while the values of K_m and V_{max} are presented in Table 3 ($R^2 > 0.98$).
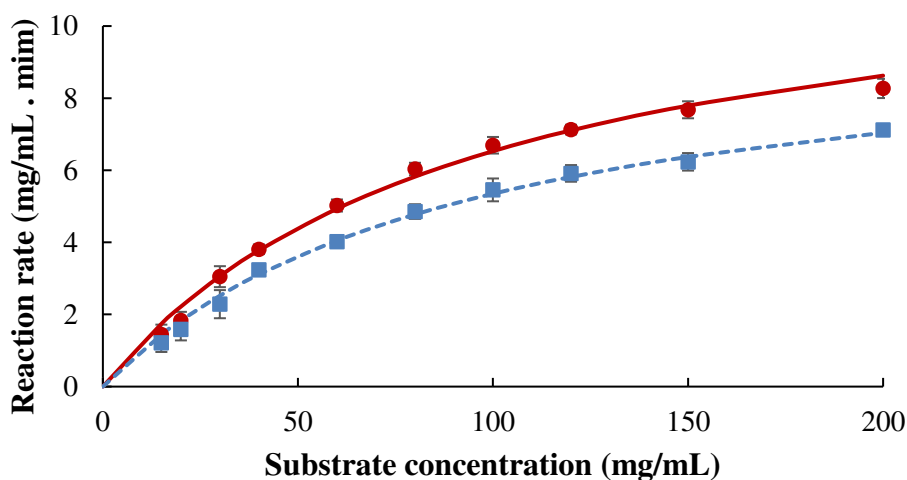
Figure 5 - Plots of the Michaelis-Menten parameters: the reaction rate as a function of the of substrate concentration for conventional reaction and ultrasound assisted reaction. Vertical bars represent standard deviation. Curves are the model of Equation 3.
(--■-- Without ultrasound); (—●— With ultrasound)

Table 3 - Kinetic parameters (from Equation 4, $R^2 > 0.98$) of the invertase during hydrolysis reactions with and without ultrasound.

Sample	V_{max} (mg mL^{-1} min^{-1})	K_m (mg mL^{-1})	V_{max} / K_m (min^{-1})
With ultrasound	12.7 ± 0.9 a	94 ± 4 a	0.14
Without ultrasound	10.3 ± 0.7 b	93 ± 5 a	0.11

* Mean ± standard deviation of nine replicates; Equal letters did not differ statistically at 5% probability by the Tukey test. K_m = Michaelis-Menten constant. V_{max} = maximum reaction rate.

Ultrasonic treatment increased V_{max} by 23.3 %, indicating intensification on the binding of enzyme-substrate complex and an accelerated hydrolysis. On the other hand, K_m was statistically equal for both methods. A possible decrease in K_m would indicate an increase in the affinity between invertase and sucrose, possibly due to a better exposure of the active site of the invertase under ultrasound (Dalagnol et al., 2017). In section 3.4, when the invertase solution was pre-processed with ultrasound, no increase in the enzymatic activity was observed. Therefore, both results are in agreement (no variation of K_m and on the enzyme activity on section 3.4), and we can infer that ultrasound did not expose more active sites of the enzyme.

Once ultrasound also did not alter the substrate (section 3.3), we can propose that the reaction enhancement (section 3.2) can be mainly due to the strong turbulence generated during cavitation. It promotes flow, increasing mass transfer through the reactor, thus reducing the limiting diffusion barrier between enzyme and substrate, and therefore, accelerating the process.

The catalytic efficiency (V_{max}/K_m) of invertase enhanced 27.2 % under sonication condition. Once again, the results indicated that combination of invertase and US improved the process, with product formation faster and more efficiently.

The most important industrial application of invertase is the production of invert sugar. This syrup is used in food industry in beverages, confectionery, sweets, chocolates, jelly, cookies among others (Nadeem et al., 2015). Ultrasound enhanced the sucrose hydrolysis reaction at temperatures of 30 and 40 °C, which may be of interest to the industry. In addition, ultrasound offers the advantage of relatively low cost when compared to other technologies (Freitas et al., 2014), such as high pressure, and it can be considered a "green" technology without generation of chemical compounds (Dalagnol

et al., 2017). The use of ultrasonic bath allows the application on an industrial scale (Freitas et al., 2014; Patist & Bates, 2008).

Therefore, in the face of all this evidence, we suggest the possibility of using this technology to enhance industrial processing. However, more studies need to be developed to elucidate the association of enzymes with this technology for industrial applications, as well as a strict cost and viability study must be conducted for each case.

4. Conclusions

This work was the first to evaluate the effect of ultrasound in the invertase reaction, as well as a pre-treatment in the substrate and enzyme. We demonstrated the ultrasound did not favor sucrose hydrolysis and invertase activity. We can thus affirm that the positive effects of ultrasound are related with the turbulence and mass transfer during the reaction. Ultrasound enhanced the sucrose enzymatic hydrolysis. The reaction temperature was an important parameter, once high temperatures reduced the effect of ultrasound. The enzyme reaction rate increased by 30 % in the presence of ultrasound at 30 and 40 °C. The sonication also increased Vmax and maintained constant Km, confirming that ultrasound provided an increased rate of enzymatic hydrolysis. Therefore, the results extend the use of ultrasound, demonstrating that this process improves the enzymatic hydrolysis of the invertase, result that may be interesting at the industrial level.

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CAPÍTULO 4

Effect of ultrasound on goat cream hydrolysis by lipase: Evaluation on enzyme, substrate and assisted reaction

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CAPÍTULO 4

Effect of ultrasound on goat cream hydrolysis by lipase: Evaluation on enzyme, substrate and assisted reaction

Abstract

The effect of ultrasound on lipase performance was studied in goat cream hydrolysis. Ultrasound (25 kHz, 22 W/L), was carried out on lipase and/or goat cream under different conditions (pH 6.0; temperature of 25, 40, 55 °C; up to 5 hours). As a pre-treatment, ultrasound did not induce goat cream hydrolysis, but slightly increased lipase activity (up to 12%), depending on the temperature and processing time. The fat hydrolysis assisted by ultrasound had higher hydrolysis rate (12 - 28%) when compared to conventional processing at different temperatures. These results were related with the acceleration of mass transfer during ultrasound reaction, as well as the modifications promoted by ultrasound on the enzyme activity. Therefore, ultrasound was demonstrated as an interesting alternative to improve the goat cream hydrolysis by lipase.

Keywords: goat milk, cream fat, enzyme, ultrasonic, fatty acid.

1. Introduction

Goat milk is an attractive food due to its high nutritional value, easy digestion and hypoallergenicity (Clark & Mora García, 2017). Consequently, consumer interest in goat's milk and dairy products is growing, with new perspectives in the food industry (Verruck, Dantas, & Prudencio, 2019). However, there are still some difficulties in the acceptance goat's milk derivate, which are associated to unpleasant flavor and odor characteristics due to the high content of short chain fatty acids, such as capric, caprylic and caproic (Verruck et al., 2019).

On the other hand, the goat milk composition can be extremely interesting from an industrial point of view, aiming to obtaining short chain fatty acids. These acids are used as flavoring in the food industry (Lee, Chua, Yeoh, & Ngoh, 2014), meeting the growing demand for replacement of artificial additives (Marriott, 2012).

The chemical hydrolysis uses high temperature and pressure, which leads to high operating costs (Posorske, 1984). Moreover, it has low specificity, with formation of undesirable products needing purification steps (De Castro, Mendes, Dos Santos, & De Aguiar, 2004) or limiting applications in food products. Therefore, enzymatic hydrolysis promoted by lipase (E.C. 3.1.1.3) has been replacing chemical hydrolysis.

The enzymatic reaction is carried out under milder conditions, with greater specificity and simplified process, resulting in a better quality product (De Castro et al., 2004; Freitas, Bueno, Perez, & De Castro, 2008). However, the use of enzymes in industry has two important barriers: high production costs and low stability (Chapman, Ismail, & Dinu, 2018).

Therefore, other possibilities are being developed to minimize these barriers, such as enzyme immobilization, genetic engineering and using emerging technologies to enhance the enzyme and/or reaction performance. Among the emerging technologies, high pressure processing (Tribst, Ribeiro, & Cristianini, 2017), high pressure homogenization (Tribst, Augusto, & Cristianini, 2013), microwaves (Mazinani & Yan, 2016) and ultrasound (Soares et al., 2019) were already proposed to promote enzyme activation and stabilization.

Ultrasound (US) consists of using acoustic energy, with frequencies higher than 20 kHz, to cause physicochemical changes on products, increasing their quality, safety or improving the process (Chemat, Huma, & Khan, 2011; Huang et al., 2017). One of the emerging applications for this technology is the modification of enzyme performance. In fact, ultrasound is able to promote enzyme activation (Oliveira, Correia, Segundo,

Fonseca, & Cabrita, 2017) and hydrolysis acceleration (Subhedar, Babu, & Gogate, 2015) under specific power, time and temperature conditions (Nadar & Rathod, 2017).

US can act on three targets in relation to the enzymatic reactions: enzyme, substrate and/or mixed reaction system (Wang et al., 2018). However, the effect of US on goat cream hydrolysis promoted by lipase has not been studied yet. Therefore, the objective of this work was to evaluate the effect of US as a pre-treatment on lipase activity and goat cream, separately and under different temperatures, as well as the US assisted enzymatic reaction.

2. Material and Methods

Figure 1 shows the experimental design of the present work. Different approaches were carried out to elucidate the effect of ultrasound on lipase, cream and assisted reaction. The assays were carried out at the optimum pH of the enzyme (previously determined in section 2.2).
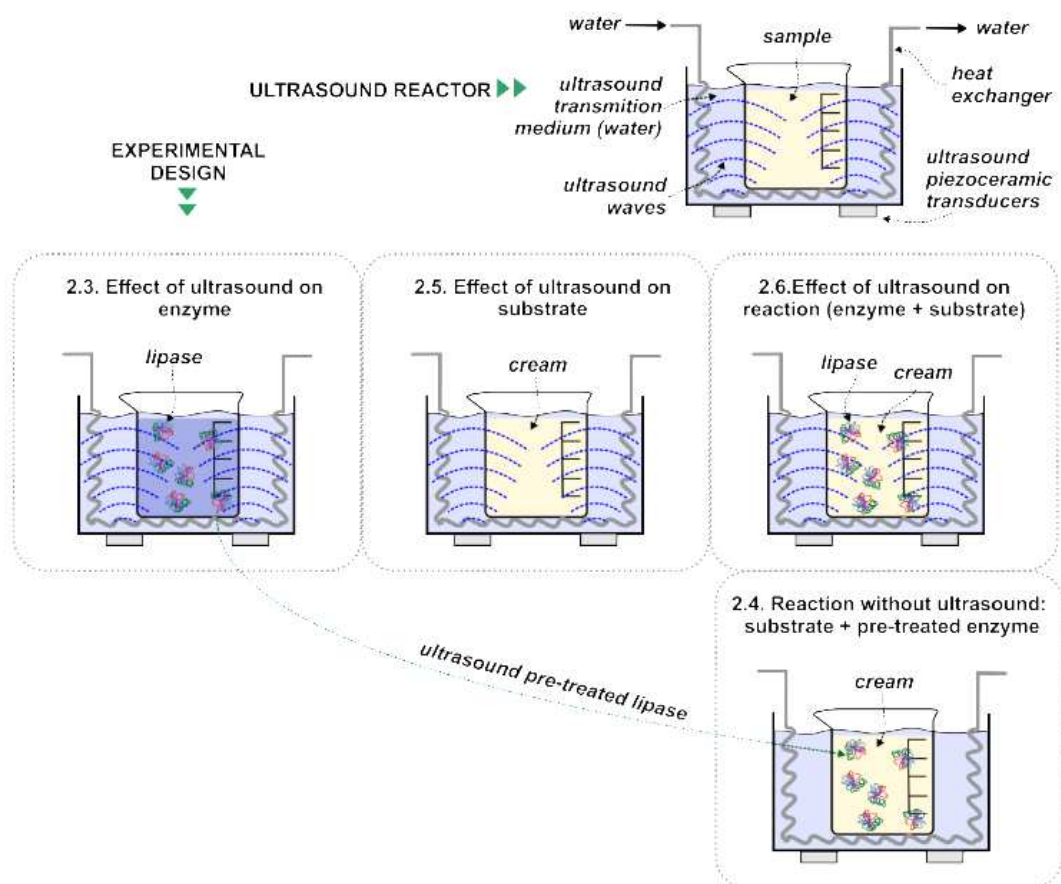
Figure 1 - Experimental design of this work, with the article section numbers.

Firstly, to evaluate the effect of US as a pre-treatment on the enzyme, the process was carried out at different temperatures (25, 40 and 55 °C) and processing times (up to 300 min), followed by lipase activity determination (section 2.3). Then, US-activated enzymes (US pre-treated enzymes with higher activation) were used to evaluate the effect of cream hydrolysis at 25, 40 and 55°C, without US, up to 300 min (section 2.4). Finally, the effect of US on goat cream hydrolysis (section 2.5) and the effect of US assisted reaction (section 2.6) was evaluated under the same conditions (25, 40 and 55 °C for up to 300 min).

2.1 Lipase and goat milk cream

Commercial liquid lipase from *Aspergillus oryzae* was donated by Novozymes (Araucária, Brazil). Fresh goat's milk was purchased from 45 healthy animals located in the goat sector of the Federal University of Viçosa (Viçosa, Brazil). Immediately after milking, the milk was centrifugated (Hanil Scientific, model combi 514R, Gimpo, Rep. of Korea) at 10,956 g for 15 min at 5°C to obtain the cream, with the fat content standardized to 37% (w/v). Subsequently, the cream was pasteurized (85°C/5 min) in a water bath and refrigerated at 1°C until processing and analyzes (limit of 96 h).

2.2 Lipase optimum conditions

The lipase activity was evaluated at the temperatures of 25, 35, 45, 55 e 65°C, and at pH values of 5.4; 6.0; 6.6 to determine the enzyme optimum conditions. Lipase activity was measured according to Pastore, Costa, & Koblitz (2003), with some modifications.

For this, 1g of goat cream was added to 4 mL of sodium acetate buffer (pH 5.4 and 0.1 mol/L) or sodium phosphate buffer (pH 6.0; 6.6 and 0.1 mol/L). Then, 0.4 mL of the enzyme solution (50 mL/L in 0.1 mol/L sodium acetate buffer pH 5.4 or 0.1 mol/L sodium phosphate buffer pH 6.0 and 6.6) was added. The mixture was incubated for 30 min at different temperatures (25-65 °C). Subsequently, the reaction was stopped by the addition of 15 mL of acetone/ethanol (1:1) and lipase activity was measured by titrating the fatty acids released with 0.1 mol/L KOH solution using phenolphthalein as an indicator. A control containing the same reaction medium received the enzyme solution only after the acetone/ethanol mixture was added, being titrated to determine the initial acidity of the goat cream. For the determination of enzyme activity, acidity was expressed as palmitic acid (Verruck et al., 2019) and one unit of activity was defined as the amount of enzyme required to release 1 µmol of fatty acid per minute from the reaction.

The pH and temperature condition with the highest activity was established as optimal, with 100% of the enzymatic activity. The relative enzyme activity (REA) was calculated using Eq. (1) for all other temperature and pH values:

$$\text{REA (\%)} = (\text{activity}_{\text{non-optimal condition}} / \text{activity}_{\text{optimal condition}}) \times 100 \quad (1)$$

2.3 Effect of ultrasound process on lipase activity

To evaluate the effect of the ultrasound pre-treatment on lipase, 50 mL of the enzyme solution (50 mL/L, diluted in 0.1 mol/L phosphate buffer pH 6.0) was added to a glass beaker and submitted to processing in an ultrasound bath (Unique, model USC 2800 A, Indaiatuba, Brazil, with internal dimensions of 30 x 24 x 15 cm, capacity 9.5 L, equipped with five transducers arranged below the vat, frequency 25 kHz and volumetric power of 22W/L, measured according to the calorimetric method described by O'Donnell, Tiwari, Bourke, & Cullen (2010)). The US bath was previously filled with a volume of 6.5 L of distilled water, and the beaker containing the sample was positioned at the point of maximum exposure to ultrasonic intensity (previously determined by the aluminum foil method (Vinatoru, 2015)). The temperature of the samples was controlled using a stainless-steel heat exchanger inside the ultrasonic bath with the recirculating water provided by an external bath.

The enzyme was processed at 22 W/L at three temperatures (25, 40 and 55°C) up to 300 min. After, 5, 15, 30, 60, 90, 120, 150, 180, 240 and 300 min of processing, 2 mL samples were collected and lipase activity was determined on goat cream according to the procedure described in section 2.2 at optimum pH (pH 6.0) and temperature (55 °C). As a control, an aliquot of 2 mL was collected at 0 min and the activity was carried out under the same conditions.

The relative enzyme activity after processing (REAP) was calculated considering the activity of the samples processed by ultrasound in relation to the non-processed ones, under the same temperature condition, according to Eq. (2):

$$\text{REAP (\%)} = (\text{Activity}_{\text{processed}} / \text{Activity}_{\text{non-processed}}) \times 100 \quad (2)$$

2.4 Goat cream hydrolysis using ultrasound pre-treated lipase

The evaluation of cream hydrolysis by US pre-treated lipase was carried out at 25, 40 and 55 °C up to 300 min. The US processing conditions (25°C/45 min and 40°C/60 min) were chosen considering the maximum enzymatic activation according to the results of section 2.3.

A volume of 200 mL of the cream solution (40 g of goat cream previously diluted in 160 mL of 0.1 mol/L phosphate buffer pH 6.0) was added with 16 mL of the enzyme solution (50 mL/L previously diluted in phosphate buffer 0.1 mol/L pH 6.0) pre-treated by ultrasound at 25°C/45 min and 40°C/60 min. Hydrolysis was carried out in a thermostatic bath at different temperatures (25, 40 and 55 °C), and samples were collected up to 300 min for fatty acid quantification, according to section 2.2.

The cream hydrolysis kinetics was evaluated using the first order kinetics (Eq. 3), where the reaction rate was demonstrated by the increase in released fatty acids (Wang et al., 2017):

$$\ln (C_\infty - C_t) = -K\, t + \ln C_\infty \quad (3)$$

Where:

C_t = Concentration of fatty acid (mg/g cream) at time t;

C_∞ = Final concentration of fatty acid (mg/g cream);

t = Time of enzymatic reaction (h);

K = rate reaction (h^{-1}) at given temperature. In this case, $K = K_1$, which represents the goat cream hydrolysis promoted by the US pre-treated lipase.

2.5 Effect of ultrasound on the hydrolysis of goat cream without lipase

A volume of 200 mL of the cream solution (40 g of goat cream, diluted in 160 mL of 0.1 mol/L phosphate buffer pH 6.0) was added to a glass beaker and placed in the ultrasound bath exactly as described on section 2.3. The cream without lipase was processed at 22 W/L at 25, 40 and 55°C for up to 300 minutes and the fatty acid released was quantified according to section 2.2.

2.6 Ultrasound assisted goat cream hydrolysis by lipase at different temperatures

A volume of 200 mL of cream solution (40 g of goat cream diluted in 160 mL of 0.1 mol/L phosphate buffer pH 6.0) and 16 mL of enzyme solution (50 mL/L previously diluted in 0.1 mol/L phosphate buffer pH 6.0) were added to a glass beaker and placed in the ultrasound bath exactly as described on section 2.3. The US assisted reaction was carried out at three temperatures (25, 40 and 55°C) up to 300 min. Aliquots of 5.4 mL were collected and the fatty acid was quantified according to section 2.2. For a control treatment (US non-assisted reaction), the reaction was carried out in a thermostatic bath, under the same conditions of pH and temperature. The kinetic parameters of goat cream

hydrolysis assisted or not assisted by US were obtained using the first order kinetic (Eq. 3, where K = K_2, which represents the goat cream hydrolysis reaction rate by lipase assisted or not assisted by US).

2.7 Experimental design and statistical analysis

The experiment was carried out using a completely randomized design (CRD). The processes were conducted with three repetitions, and analyses in triplicate for each repetition of the process. The results were expressed as mean ± standard deviation.

ANOVA was applied for data analysis and interpretation to compare the effects of different treatments, processed and unprocessed samples. When there was significant difference, the averages were compared by the Tukey test at 95% of probability (Statistical Analysis System - SAS Institute, Cary, NC, USA; version 9.2).

The parameters of each mathematical model were determined by non-linear regression using the software Curve Expert Professional (version 2.6.5, Hyams Development, Chattanooga, USA).

3. Results and Discussion

3.1 Lipase activity: optimum temperature and pH

Lipase activity at different temperature and pH is shown in Figure 2. The enzyme optimum condition was obtained at 55 °C and pH 6.0, similarly to results of Falony, Armas, Mendoza, & Hernández (2006), for lipase from *Aspergillus Niger*. In this condition, the activity was 390 U/mL of enzyme, which was considered as 100% REA. In addition, it was observed a strong reduction on lipase activity (70%) at pH 6.6 and temperature of 65 °C, probably due to denaturing effects.

After determining the optimal temperature and pH of lipase (55 °C and pH 6.0), the process conditions were chosen to evaluate the effect of ultrasound on lipase activity and goat cream, as well as in the reaction assisted by US - as shown in the next sections.
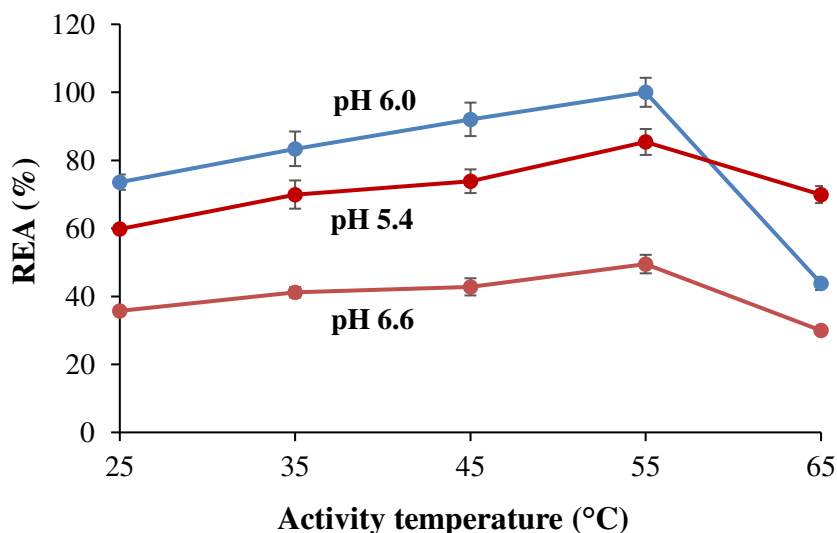
Figure 2. Effect of temperature and pH on relative enzyme activity (REA) in goat cream. Vertical bars represent standard deviation.

3.2 Effect of ultrasound on lipase activity

Figure 3 shows the relative enzyme activity carried out at optimum pH and temperature after processing by US (REAP) at 25, 40 and 55 °C for up to 300 min. Ultrasound was unable to increase the activity of lipase after processing at 55 °C, while a slight, but significant activity increase (12%) was observed after US processing at 25 °C (for 45 min) and 40 °C (for 60 min).

These effects can be explained as consequences of the collapsing cavitation bubbles induced by ultrasound, since it lead to extreme localized temperature, pressure and strong shear forces, which can alter the enzyme conformation (Wang et al., 2018).

Temperature affects the vapor pressure, surface tension and the viscosity of the liquid medium, which affects both the propagation of ultrasonic waves, as well as the cavitation phenomenon (Patist & Bates, 2008). Higher temperatures increase the number of cavitation bubbles. However, the collapse is dampened by the higher vapor pressure, thereby decreasing the cavitational intensity. On the other hand, by increasing temperature, the viscosity and surface tension are decreased, facilitating the formation of cavitation bubbles and allowing a more violent collapse (Patist & Bates, 2008). Therefore, each product has an optimum temperature at which the viscosity is low enough to form violent cavitation bubbles and avoid the dampening effect by a high vapor pressure (Patist & Bates, 2008).

In this way, the absence of lipase activity changes after US processing at 55 °C can probably be explained by the reduced cavitation intensity at high temperatures.

Moreover, at the optimum temperature (55 °C), the enzyme structure may be more resistant, which helps explain the lower US effect at 55 °C.
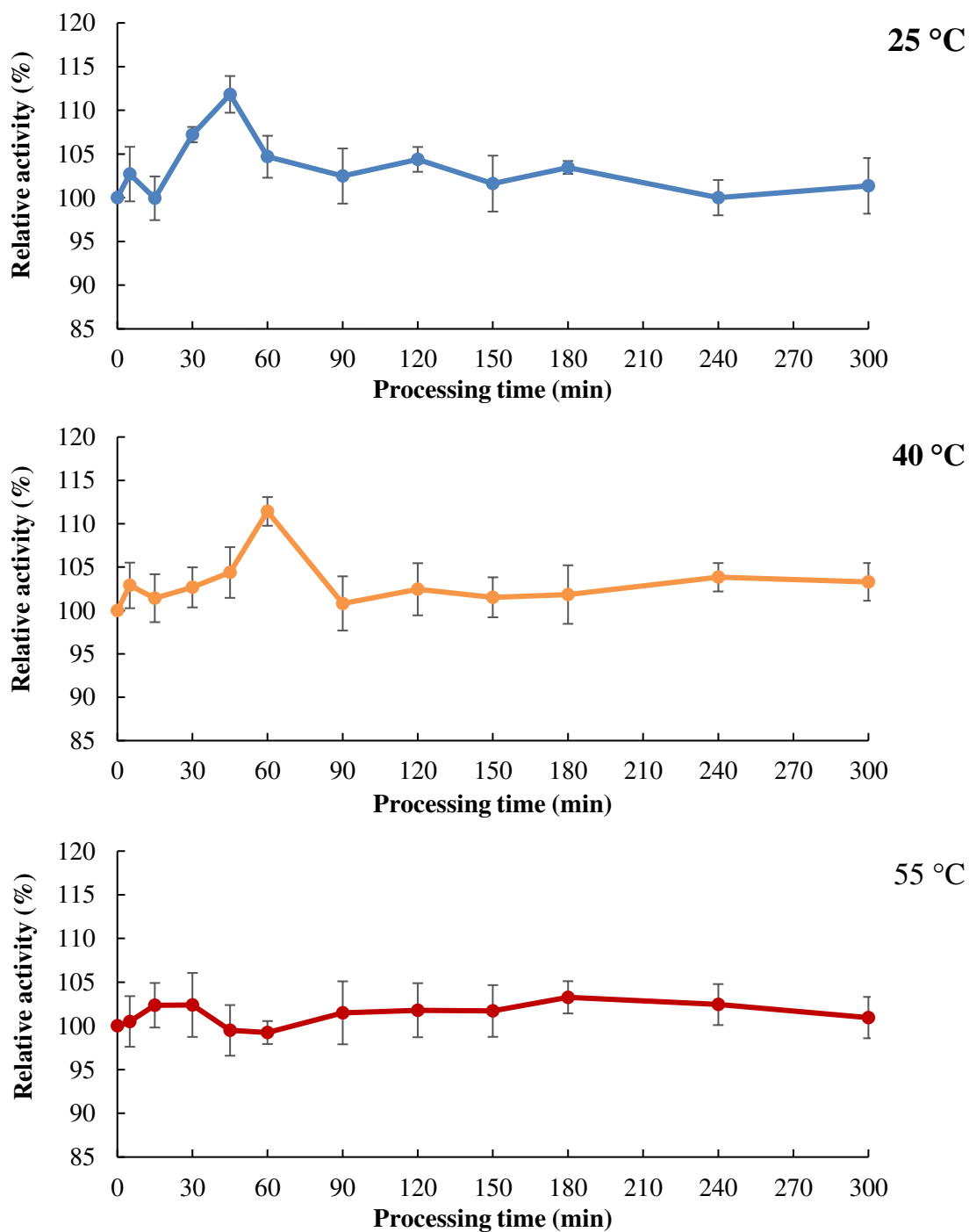
Figure 3. Relative enzyme activity measured at 55 °C / pH 6.0 in goat milk cream (REAP, Eq. 2) using lipase pre-treated by ultrasound for up to 300 min at different temperatures (25 °C, 40 °C and 55 °C).

Prior results showed that US (40 kHz, 28 W/L, 25°C) activated pepsin (14%, after 60 min of treatment) but inactivated α-amylase (86%) and papain (26%) under the same

process conditions (Yu, Zeng, Zhang, Liao, & Shi, 2014). It reinforces that it is not possible to predict the impact of US on enzymes from results previously obtained in other systems. In fact, each system (enzyme, ultrasound reactor and processing conditions) leads to specific energy distributions and, consequently, possible molecular changes and enzyme activities (Islam, Zhang, & Adhikari, 2014). This can explain the results here described. Therefore, the process conditions that activated lipase were selected to evaluate the hydrolysis of the goat cream - as shown in the next section.

3.3 Goat cream hydrolysis using ultrasound-processed lipase

Lipase pre-treated by US (22.0 W/L, 25 kHz, pH 6.0) at the conditions for maximum activation (25 °C / 45 min and 40 °C / 60 min) was used to determine the kinetic parameters of goat cream hydrolysis at 20, 40 and 55 °C (Table 1). The hydrolysis data showed good fit to the first order kinetic model ($R^2 > 0.96$).

Table 1. Parameters of first order kinetic model (Eq. 3) adjusted to goat cream hydrolysis by US pre-treated lipase.

Pre-treatment enzyme	Reaction temperature (°C)	K_1 (h^{-1})	C_∞ (mg/g)
Control		0.91 ± 0.02 e	147 ± 3 b
US - 25°C/ 45 min	25	1.02 ± 0.02 d	147 ± 2 b
US - 40°C/ 60 min		1.01 ± 0.04 d	148 ± 1 b
Control		1.17 ± 0.03 c	151 ± 2 a,b
US - 25°C/ 45 min	40	1.32 ± 0.05 b	152 ± 1 a,b
US - 40°C/ 60 min		1.27 ± 0.01b	154 ± 1a,b
Control		1.32 ± 0.05 b	156 ± 1 a
US - 25°C/ 45 min	55	1.50 ± 0.01 a	156 ± 2 a
US - 40°C/ 60 min		1.47 ± 0.05 a	157 ± 2 a

Mean ± standard deviation; Equal letters among treatments (Hydrolysis reaction conducted at different temperatures by the processed and unprocessed enzyme) did not differ statistically at 5% probability by the Tukey test. K_1 = rate reaction hydrolysis (h^{-1}) at given temperature. C_∞= Final concentration of fatty acid (mg/g cream).

As expected, an increase ($p<0.05$) in the K_1 parameter was observed with the increase of reaction temperature, with higher hydrolysis rate at 55 °C (optimal of the enzyme). Furthermore, K_1 was also increased when ultrasound was used as a pre-treatment of the enzyme ($p<0.05$). A maximum increase in K_1 due to ultrasound ranged between 9% and 13%.

In addition, equal K_1 values were observed for ultrasound pre-treated lipase at 40 °C and non-treated one at 55 °C. This highlights that US pre-treatment allows to carry out the cream hydrolysis at lower temperature, which can save energy for heating and cooling the cream, reduce the need for insulation and possibly reducing undesirable thermal effects.

After 300 min of hydrolysis, the final fatty acid concentration (C_∞) ranged from 147 to 157 mg/g of cream (these values represent about 50% hydrolysis of triacylglycerols). The reactions carried out at 55 °C showed a slight, but significantly higher C_∞ (~ 6%) compared to the reactions carried out at 25 °C ($p>0.05$). However, no differences were found between the reactions carried out with US pre-treated lipases and non-treated ones in relation to C_∞. In this context, it is emphasized that regardless of the enzyme used (pre-treated or non-treated by US), there is a limit on the final concentration of fatty acid produced.

The overall assessment of these results shows that US can be an interesting strategy for improving lipase performance under optimal and non-optimal conditions, allowing a broader use in the food industry. However, ultrasound can also affect the substrate (goat cream) and/or the reaction (goat cream and lipase reacting under ultrasound). Therefore, to clarify the best strategy, we also evaluated both strategies, as described as follows.

3.4 Effect of ultrasound on the hydrolysis of goat cream without lipase

After evaluating the impact of ultrasound on lipase activity, and before evaluating the impact of ultrasound in the reaction (cream + lipase), it was evaluated if this technology was able to cause cream hydrolysis by itself, i.e., in the absence of enzyme. The results, however, showed no increase in cream fatty acid concentration even after 5 h of US processing. Therefore, ultrasound was not able to cleavage the cream triacylglycerol in the evaluated conditions and, consequently, it was ensured that the goat cream fat hydrolysis promoted throughout our study is due exclusively to the lipase action.

3.5 Ultrasound assisted reaction of goat cream hydrolysis by lipase

Table 2 shows the kinetic parameters of ultrasound assisted reaction of goat cream hydrolysis by lipase, at different temperatures. Again, the data fitted well to the first order model ($R^2 > 0.97$).

The US assisted enzymatic reaction showed a higher hydrolysis rate (K_2) (p<0.05) if compared to the conventional process: K_2 increased 12% at 55 °C, 23% at 40°C and 28% at 25°C (Table 2). The higher US effect was observed at lower temperatures, similarly to the results of Ma et al. (2015). These authors observed the reduction from 50 to 30 °C increased the ultrasound effect from 12% to 36% in the polygalacturonase hydrolysis. In addition, the reaction assisted by US carried out at 40 °C showed a higher K_2 when compared to the conventional reaction at 55 °C (p>0.05 – Table 2), which can be interesting in order to conduct reaction at lower temperatures, as described before.

Table 2 - Parameters of first order kinetic model (Eq. 3) adjusted to hydrolytic of lipase on goat cream at different temperatures under ultrasound (US) process.

Hydrolytic kinetics	Reaction temperature (°C)	K_2 (h^{-1})			C_∞ (mg/g)			
without US	25	0.87	±	0.03 d	155	±	4	a
under US		1.11	±	0.03 c	153	±	3	a
without US	40	1.13	±	0.06 c	151	±	1	a
under US		1.39	±	0.06 a	152	±	2	a
without US	55	1.29	±	0.03 b	156	±	3	a
under US		1.44	±	0.09 a	157	±	3	a

* Mean ± standard deviation of nine replicates; Equal letters among treatments (Enzymatic hydrolysis reaction conducted at different temperatures with or without ultrasound application) did not differ statistically at 5% probability by the Tukey test. K_2=rate reaction hydrolysis (h^{-1}) with or without ultrasound at given temperature. C_∞= Final concentration of fatty acid (mg/g of cream).

After 5 hours of reaction, the fatty acid concentration (C_∞) ranged from 151-157 mg/g of cream. No differences were verified between US assisted reaction and conventional process, for all the temperatures.

During cavitation, there is a significant release of energy, resulting in propagating shockwaves and shear forces, with strong turbulence within the surroundings of bubble implosion. These mechanical effects not only can change the enzyme structure and activity (as discussed on section 3.2), but they also reduce the limiting barrier of diffusion between enzyme and substrate, increasing mass transfer during processing (Wang et al., 2018). Consequently, the reaction is accelerated.

Furthermore, both ultrasonic waves propagation and cavitation are affected by temperature, as discussed before. This helps to explain the bigger effect of ultrasound when the reaction was performed at lower temperatures. In addition, it may be suggested that part of the fat is crystallized in the reaction carried out at 25 °C (Nunes, De Paula, De Castro, & Dos Santos, 2010). Therefore, as a positive effect of US, cavitation bubbles may favor the breakage of these crystals allowing better lipase action during the reaction under US. The effect of ultrasound processing on goat milk and cream microstructures, however, should be deeply evaluated in future works.

Finally, comparing results from Tables 1 and 2, similar hydrolysis rates (K_1 and K_2) were observed at each reaction temperature (differences less than 8%). In addition, the final concentration of fatty acids (C_∞) was also similar.

Consequently, from an industrial perspective, it is probably more advantageous to carry out US processing of the enzyme solution (smaller scale for a shorter period) than performing the reaction under US (large volumes for long periods).

Therefore, the overall evaluation of the results showed that US can improve the goat cream hydrolysis. Additionally, US enhanced the lipase activity, which can expand its industrial application. The adoption of this "green" technology in industrial plants is relatively simple, have low cost in comparison with other technologies, does not generate chemical wastes and allows the application on an industrial scale using ultrasonic bath (Freitas et al., 2014; Soares et al., 2019). However, a study of the industrial viability needs to be developed to elucidate the real advantage of using US in the hydrolysis reaction of goat cream by lipase.

4. Conclusion

The effect of ultrasound on goat cream hydrolysis by lipase was evaluated for the first time. We demonstrated that ultrasound did not cause cream hydrolysis, but increased lipase activity by 12% under specific conditions (25 °C / 45 min and 40 °C / 60 min). In addition, the ultrasound assisted hydrolysis rate was increased (12% at 55°C, 23% at 40°C and 28% at 25°C), and lower reaction temperatures showed the greatest ultrasound

effects. Therefore, the results extend the use of ultrasound, demonstrating that this technology can be used to improve the lipase performance for free fatty acids obtaining from goat milk cream.

Acknowledgments

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CONCLUSÃO GERAL

A avaliação geral dos resultados mostrou que a tecnologia de ultrassom pode melhorar o desempenho das enzimas, sendo uma importante alternativa para minimizar suas limitações e proporcionar ampliação do mercado.

O aumento da atividade (até 15%) da amiloglicosidase ocorreu na temperatura de 80 ° C, sendo interessante na sacarificação do amido, que requer a reação enzimática a altas temperaturas.

A aplicação do ultrassom aumentou a taxa de hidrólise da sacarose em até 33% quando a reação foi realizada a 30 e 40°C, o que está relacionado à turbulência e aumento da transferência de massa durante a reação.

Como pré-tratamento, a tecnologia aumentou a atividade da lipase (até 12%) e durante a hidrólise da gordura assistida por ultrassom apresentou maior taxa de conversão (12 - 28%) quando comparada ao processamento convencional, sugerindo que o ultrassom atuou na estrutura da enzima e acelerando a transferência de massa.

A inclusão dessa tecnologia "verde" em planta industrial é simples, apresenta baixo custo em comparação com outras tecnologias e permite a aplicação em escala industrial utilizando banho ultrassônico, podendo ser útil para a indústria de alimentos. No entanto, mais estudos precisam ser desenvolvidos para elucidar a associação de enzimas com essa tecnologia para aplicações industriais, bem como um estudo rigoroso de custo e viabilidade para cada caso.