UNIVERSIDADE FEDERAL DE VIÇOSA

JEFERSON SILVA CUNHA

USO DA TECNOLOGIA DE ULTRASSOM NO PROCESSAMENTO DA CICLODEXTRINA GLICOSILTRANSFERASE: EFEITO NA ATIVIDADE, ESTABILIDADE E MACROESTRUTURA

VIÇOSA – MINAS GERAIS

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Dissertação 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 *Magister Scientiae*.

Orientador: Bruno Ricardo de Castro Leite Junior

Coorientadora: Alline Artigiani Lima Tribst

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Bruno Ricardo de Castro Leite Junior

Orientador

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RESUMO

CUNHA, Jeferson Silva, M. Sc., Universidade Federal de Viçosa, fevereiro de 2024. **Uso da tecnologia de ultrassom no processamento da ciclodextrina glicosiltransferase: efeito na atividade, estabilidade e macroestrutura.** Orientador: Bruno Ricardo de Castro Leite Júnior. Corientadora: Alline Artigiani Lima Tribst.

As maltodextrinas podem ser convertidas em β-ciclodextrina (β-CD) através da ciclodextrina glicosiltransferase (CGTase). No entanto, a CGTase apresenta limitações devido aos altos custos de produção e à baixa estabilidade enzimática na produção de β-CD. Este estudo explora a tecnologia de ultrassom (US) como uma solução potencial para potencializar a performance enzimática. Investigando o impacto do US na atividade, estabilidade e conformação estrutural da CGTase, os experimentos utilizaram um banho ultrassônico (25 kHz, 38 W/L) com pH variado (4,0 e 6,0), temperaturas (25, 55, 80°C), e tempos (5-120 min). Os resultados revelaram que o pré-tratamento ultrassônico aumentou significativamente a atividade da CGTase. Notavelmente, em pH 6,0, foram observados aumentos máximos de 93% (pré-tratamento a 25°C/30min), 68% (pré-tratamento a 25°C/30min) e 31% (pré-tratamento a 25°C/60min) a 25°C, 55°C e 80°C, respectivamente. Da mesma forma, o pré-tratamento em pH 4,0 durante 5 min/55°C mostrou atividade relativa aumentada de 28%, 21% e 16% a 25, 55 e 80°C. Os níveis de atividade aumentados permaneceram estáveis após 24 horas de armazenamento a 8°C. Os pré-tratamentos ultrassônicos induziram alterações conformacionais na CGTase, evidentes em um aumento de até 11% na intensidade de fluorescência intrínseca, e resultaram em modificações macroestruturais, incluindo redução do tamanho de partícula (até 85%) e índice de polidispersão (45,8%). Estes resultados são interessantes e demonstram que sob condições específicas de sonicação (pH, tempo e temperatura) é possível ativar a CGTase para produzir compostos industrialmente relevantes.

Palavras-chave: ciclodextrina glicosiltransferase; tecnologia emergente; atividade enzimática; estabilidade enzimática; sonicação; β-ciclodextrina.

ABSTRACT

CUNHA, Jeferson Silva, M.Sc., Federal University of Viçosa, February of 2024. **Uso da tecnologia de ultrassom no processamento da ciclodextrina glicosiltransferase: efeito na atividade, estabilidade e macroestrutura.** Advisor: Bruno Ricardo de Castro Leite Júnior. Advisor: Alline Artigiani Lima Tribst.

Maltodextrins can be converted into β-cyclodextrin (β-CD) through cyclodextrin glycosyltransferase (CGTase). However, CGTase poses limitations due to high production costs and low enzyme stability in β-CD production. This studyexplores ultrasound (US) technology as a potential solution to enhance enzyme performance. Investigating the impact of US on CGTase activity, stability, and structural conformation, the experiments utilized an ultrasonic bath (25 kHz, 38 W/L) with varied pH (4.0 and 6.0), temperatures (25, 55, 80°C), and times (5-120 min). Results revealed that ultrasonic pretreatment increased CGTase activity significantly. Notably, at pH 6.0, maximum increases of 93% (pre-treated at 25°C/30min), 68% ((pre-treated at 25°C/30min), and 31% (pre-treated at 25°C/60min) were observed at 25°C, 55°C, and 80°C, respectively. Similarly, pretreatment at pH 4.0 for 5 min/55°C showed increased relative activity of 28%, 21%, and 16% at 25, 55, and 80°C. The enhanced activity levels remained stable after 24 hours of storage at 8°C. Ultrasonic pretreatments induced conformational changes in CGTase, evident in an up to 11% increase in intrinsic fluorescence intensity, and resulted in macrostructural modifications, including reduced particle size (up to 85%) and polydispersion index (45.8%). These results are interesting and demonstrate that under specific sonication conditions (pH, time, and temperature) it is possible to activate CGTase to produce industrially relevant compounds.

Keywords: cyclodextrin glycosyltransferase; emerging technology; enzymatic activity; enzymatic stability; sonication; β-cyclodextrin.

LISTA DE ILUSTRAÇÕES

CAPÍTULO 1

CAPÍTULO 2

LISTA DE TABELAS

SUMÁRIO

INTRODUÇÃO GERAL

As ciclodextrinas (CDs) constituem oligossacarídeos cíclicos caracterizados por um domínio externo hidrofílico e um núcleo hidrofóbico. Estas substâncias encontram aplicação nos setores alimentício, cosmético e farmacêutico (MATENCIO et al., 2020). O interesse significativo direcionado às CDs na indústria alimentícia é atribuído à sua capacidade potencial para diversas aplicações, incluindo o carreamento de substâncias bioativas nos alimentos (SANTOS, BUERA & MAZZOBRE, 2017).

A produção das CDs é realizada de forma majoritária por via enzimática. A ciclodextrina glicosiltransferase (CGTase) se trata de uma enzima utilizada especialmente para a produção industrial de CDs, pela reação de ciclização através da degradação do amido e açúcares similares (maltodextrinas). As CGTases são sintetizadas por uma gama de bactérias, particularmente cepas de *Bacillus* (ASTRAY; MEJUTO; SIMAL-GANDARA, 2020).

Embora o potencial de uso das CDs seja crescente, sua produção industrial em larga escala via ação enzimática é desafiadora. Isso se deve alto custo de produção e baixa estabilidade das enzimas (GRAEBIN et al., 2016). Deste modo, com o objetivo de contornar essas limitações e levando em consideração a relevância das CDs, diferentes alternativas podem ser desenvolvidas para minimizar essas limitações, como imobilização (WANG et al., 2018) e uso de diferentes tecnologias não convencionais (MA et al., 2015).

De fato, existe um campo de pesquisa preocupado em desenvolver técnicas que melhorem as propriedades das enzimas. Dentre elas, destaca-se a tecnologia de ultrassom (US). O US, que produz cavitação causadas pelos ciclos de compressão-expansão das ondas, pode ser utilizado para potencializar a ação enzimática (MA et al., 2015), conforme verificado para pectinase, xilanase, celulase e proteases (DALAGNOL et al., 2017; PACHECO et al., 2023).

Embora vários estudos demonstraram o potencial da aplicação do US na melhoria da performance enzimática, trabalhos que descreviam o efeito do US na CGTase para geração de β-ciclodextrinas (β-CDs) são escassos na literatura científica. Diante disso, identificou-se uma lacuna no conhecimento técnico-

científico que precisa ser abordada para otimizar o desempenho da CGTase na síntese de β-CDs. Portanto, esse trabalhou demonstrou que o US foi capaz de promover a ativação da CGTase em diferentes condições de processamento, ocasionando modificações em sua estrutura conformacional e preservando a estabilidade do biocatisador.

OBJETIVOS

Objetivo geral

Investigar a tecnologia de ultrassom como pré-tratamento da ciclodextrina glicosiltransferase (Toruzyme®) em condições ótimas e não ótimas de temperatura e pH, visando a produção específica de β-CDs, bem como avaliar seu efeito na estrutura conformacional e estabilidade desta enzima sob condições de ativação.

Objetivos específicos

- Estabelecer a temperatura ótima de atividade da CGTase utilizando maltodextrina (DE=10) como substrato;
- Analisar como a atividade da CGTase é influenciada pela temperatura, pH e tempo de pré-processamento ultrassônico, mantendo potência e frequência constantes;
- Avaliar a atividade enzimática após o processamento ultrassônico em condições ótimas e não ótimas de temperatura combinadas com a condição ótima de pH;
- Selecionar condições de ativação e examinar como o ultrassom impacta a estabilidade da atividade enzimática após o armazenamento a 8°C por 24 horas;
- Analisar o efeito do ultrassom na macroestrutura da CGTase (análises de fluorescência intrínseca, tamanho de partículas e índice de polidispersão) em condições de ativação.

CAPÍTULO I

REFERENCIAL TEÓRICO

1. CICLODEXTRINAS E APLICAÇÃO NA INDÚSTRIA DE ALIMENTOS

Em 1891, A. Villiers identificou celulosinas, dextrinas cristalinas provenientes da hidrólise do amido de batata por *Bacillus amylobacter*. Posteriormente, Schardinger e sua equipe descobriram novas dextrinas resultantes da degradação enzimática bacteriana do amido, dando origem às CDs, como α–ciclodextrina, β–ciclodextrina (celulosina), e γ–ciclodextrina identificada em 1935 por Freudenberg e Jacobi. Ao longo dos anos, foram descobertos diversos tipos de dextrinas cíclicas e concomitante vários métodos inovadores foram criados para promover a purificação das ciclodextrinas. Além disso, também foram desenvolvidas algumas técnicas para melhorar a solubilidade dessas substâncias, como a substituição de grupos hidroxila (VARAN et al, 2017).

Ciclodextrinas (CDs) são moléculas cíclicas comumente compostas por 6 (α-ciclodextrina), 7 (β-ciclodextrina) ou 8 (γ-ciclodextrina) monômeros de glicose.Sua conformação assemelha-se a um cone truncado, devido à estrutura de cadeira da glicopiranose (Figura 1). A superfície externa é hidrofílica devido aosgrupos hidroxilas, enquanto a cavidade central, revestida pelos resíduos de glicose, confere características hidrofóbicas ao interior da molécula de CD(VARAN et al., 2017).

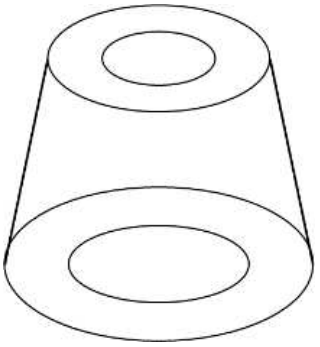

Figura 1. Forma das ciclodextrinas.

Fonte: autor.

A capacidade das CDs para criar complexos de inclusão deriva de suas cavidades hidrofóbicas, que podem acomodar moléculas também hidrofóbicas. As moléculas hóspedes devem ter um tamanho suficientemente pequeno para caber dentro da cavidade, cuja profundidade é constante, mas o diâmetro varia entre diferentes CDs. Adicionalmente, as CDs exibem distintas solubilidades em água (VARAN et al., 2017). Assim, as propriedades físico-químicas encontram-se na Tabela 2.

Tabela 2. Propriedades físico-químicas das ciclodextrinas.

Propriedade/Tipo de ciclodextrina	α	β	γ
Quantidade de monômeros de glicose	6	7	8
Massa molar (g/mol)	972	1135	1297
Diâmetro central da cavidade (Å)	4,7 a 5,3	6,0 a 6,5	7,5 a 8,3
Comprimento da cavidade (Å)	8	8	8
Solubilidade em água a 25 °C (g/100 mL)	14,5	1,85	23,2

Fonte: VARAN et al., 2017.

Portanto, em virtude da capacidade das CDs hospedar moléculas hóspedes, a indústria alimentícia tem demonstrado interesse nesses compostos, uma vez que apresentam diversas aplicações úteis com propósitos específicos em produtos alimentícios.

As CDs podem ser usadas na biossíntese de compostos bioativos, como polifenóis/estilbenos e flavonoides, a partir de culturas de células vegetais para atuarem como elicitores, proporcionando maiores rendimentos. Isto é, as CDs são reconhecidas pelas células que estimulam o sistema de defesa da planta, desencadeando cascatas de transdução de sinal que envolvem diversas vias complexas de sinalização intracelular. Como uma resposta importante das plantas, os elicitores levam à superprodução e acúmulo de metabólitos secundários (GARCÍA-PÉREZ et al., 2019). Adicionalmente, o uso de CDs pode

ser explorado em outros tipos de organismos, como as cianobactérias, visando aumentar a produção de produtos antifúngicos (SHISHIDO et al., 2015), bem como seu uso pode ser combinado com enzimas (BONNET et al., 2010). A produção ou extração de compostos bioativos através do uso de CDs é reportada na Figura 2.

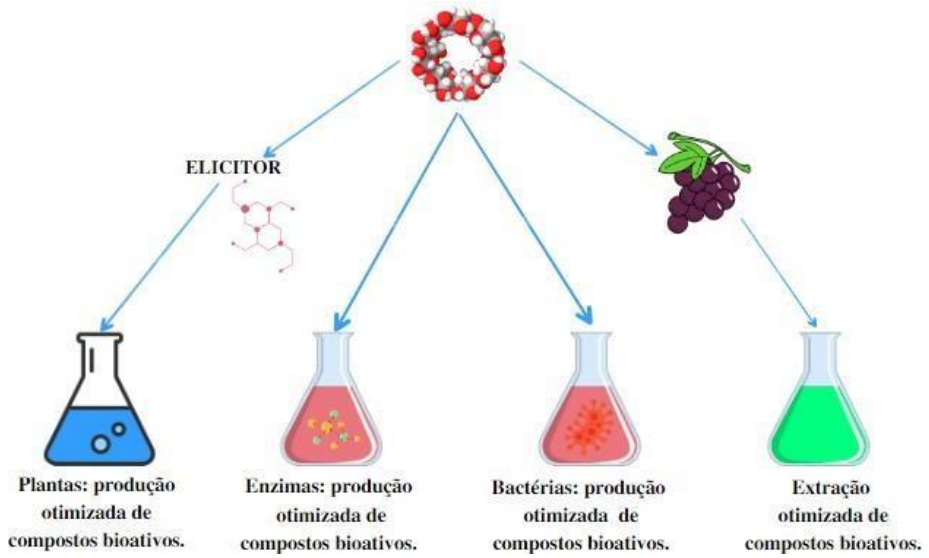

Figura 2. Representação gráfica da produção e/ou extração de compostos bioativos usando CDs.

Fonte: Adaptado de Matencio et al., 2020.

Adicionalmente, as CDs podem ser usadas para sequestrar compostos indesejados. Como exemplo, ALONSO et al. (2019) desenvolveu um estudo visando a redução do colesterol em leite pasteurizado não homogeneizado a 4 °C, com a adição de β-CD, e os resultados indicaram uma remoção variando de 65,0 a 95,0%. Os resultados mais promissores foram alcançados com 0,6% de β-CD. A remoção do colesterol com o uso de ciclodextrinas é exemplificada na Figura 3.

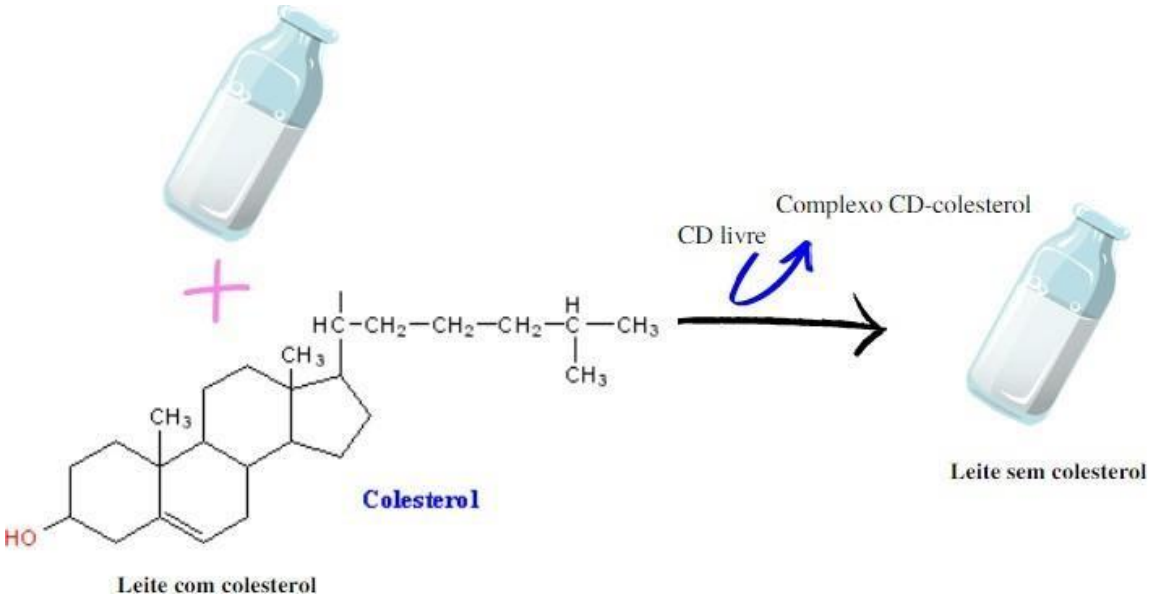

Figura 3. Esquema representativo da remoção de colesterol por meio da inclusão molecular por β-CD.

Fonte: autor.

Em outras aplicações de uso tecnológico em alimentos, refere-se às α-ciclodextrinas, que geralmente são usadas como transportadores e estabilizadores para moléculas hóspedes volumosas. Por exemplo, o ácido cinâmico (AC), um ácido orgânico natural presente em frutas e especiarias, conhecido por sua capacidade antimicrobiana contra bactérias deteriorantes e patogênicas. No entanto, sua baixa solubilidade em água é um entrave para sua aplicação. Neste caso, a produção do complexo de inclusão α-CD:AC melhoroua solubilidade e reduziu significativamente as populações de *E. coli* O157:H7 e *Salmonella entérica* suspensos em cidra de maçã (TRUONG et al., 2010). Em relação à ꞵ–CD, é comumente empregada como estabilizantes em emulsões de diferentes formulações alimentícias, como sobremesas lácteas e recheios à base de gordura (SAOKHAM; LOFTSSON, 2017).

2. ENZIMAS

As enzimas são macromoléculas com propriedades de catalisadores biológicos, que aceleram a velocidade de uma reação ao reduzirem a energia de ativação. Além disso, desempenham funções cruciais em diversas produções industriais, exercendo papéis essenciais nos setores de alimentos, rações,

produtos farmacêuticos, corantes, tratamento de água, têxteis, cosméticos, couros, biocombustíveis, entre outros (WANG et al., 2018).

As principais fontes de enzimas são animais, plantas e microrganismos, com destaque para a preferência pela fonte microbiana. Isso se deve à facilidade de manipulação genética, disponibilidade contínua, capacidade de produção em massa e maior estabilidade em diferentes condições do meio (ANBU et al., 2015).

A especificidade das enzimas na interação com substratos é uma característica crucial, resultando na formação de produtos conhecidos. Essa especificidade decorre da presença de sítios ativos em sua superfície, nos quais os substratos se conectam. Os sítios ativos são determinados pelo arranjo espacial dos aminoácidos em um local específico da molécula (BELITZ; GROSCH; SCHIEBERLE, 2009). Após a ligação do substrato nessa região, que configura a formação do complexo enzima//substrato, ocorre a liberação tanto do produto quanto da enzima em seu estado nativo.

Diferentemente dos métodos convencionais que utilizam ácido-base e altas temperaturas, a hidrólise enzimática destaca-se pela sua eficiência e especificidade. Adicionalmente, condições mais suaves de temperatura e pressão podem ser empregadas nas reações enzimáticas, sem a necessidade de utilizar solventes químicos prejudiciais ao meio ambiente (WANG et al., 2018).

Em 1956, em decorrência dos progressos no isolamento e identificação de novas enzimas, a União Internacional de Bioquímica estabeleceu a Comissão Internacional de Enzimas. Essa comissão tinha como objetivo criar critérios para a nomenclatura e classificação das enzimas, visando padronizar a terminologia utilizada por diferentes pesquisadores ao estudarem uma mesma enzima. Nesse processo, levando em consideração a natureza das reações que catalisam, as enzimas foram categorizadas em sete classes (Tabela 1) (NELSON; COX, 2022).

Tabela 1. Classes das enzimas.

Classe	Reação catalisada
Transferases	Transferência de grupos funcionais.
Liases	Rompimento de ligações específicas (como C-O e C-C); adição de grupos ou quebra nas ligações duplas.
Hidrolases	Reações de hidrólise.
Oxidorredutases	Promovem a transferência de e^- (elétrons).
Ligases	Síntese de ligações específica (como C-C e C-N) a partir de reações de condensação.
Isomerases	Transferência de grupos no interior da molécula, resultando na formação de isômeros.
Translocases	Facilita o deslocamento de outra molécula, geralmente através de uma membrana celular.

Fonte: NELSON; COX, 2022.

Industrialmente, dentre as enzimas comerciais usadas, destacam-se as ciclodextrinas glicosiltransferase. Essas enzimas extracelulares são capazes de realizar a conversão do amido e são produzidas por vários tipos de microrganismos, principalmente por bactérias como *Bacillus sp, Micrococcus luteus*, *Breviabacterium*, *Klebseilla pneumonia, Thermococcus sp* e *Thermoanerobacter sp*. As archaea e os fungos também podem produzir CGTase (LIM et al., 2021).

A CGTase catalisa quatros reações enzimáticas, caracterizadas como ciclização, acoplamento, desproporção e hidrólise, representadas pela Figura 4.

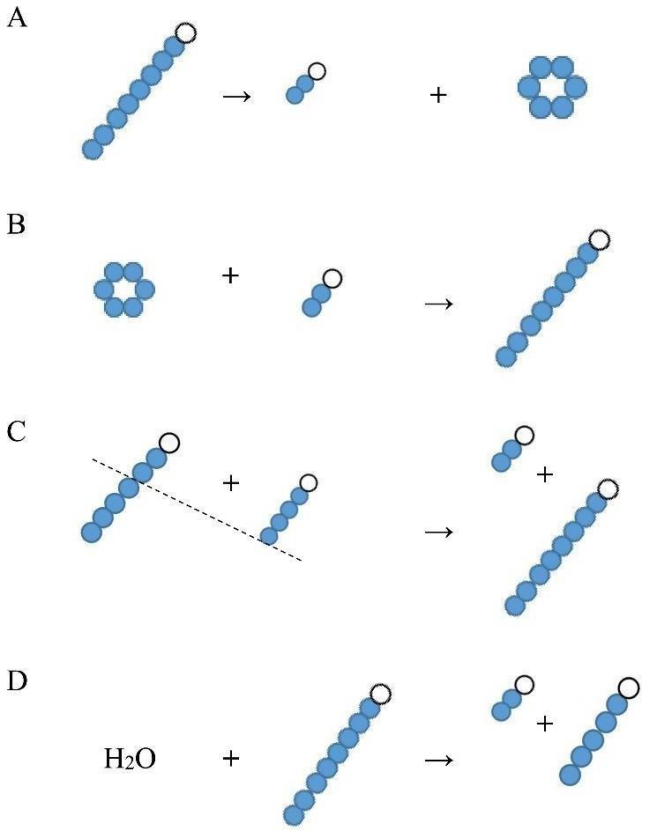

Figura 4. Esquema das reações catalisadas por CGTase: A) Ciclização B) Acoplamento C) Desproporcionamento D) Hidrólise.

Fonte: LIM et al., 2021.

Como os outros membros da família das glicosil hidrolases (família 13), a CGTase pode catalisar reações de hidrólise, clivando as ligações na molécula de amido, que é seguida pela reação do produto de clivagem com água, resultando em uma nova extremidade redutora. No entanto, a CGTase geralmente tem atividade de hidrólise menor e catalisa principalmente três reações de transglicosilação/ciclização (clivagem de uma ligação α-glicosídica em amilose ou amido e subsequente formação de um CD), acoplamento (clivagem de uma ligação α-glicosídica de um anel CD e transferência do maltooligossacarídeo resultante para um substrato aceitador) e desproporção (clivagem de uma ligação α-glicosídica de um maltooligossacarídeo linear e transferência de uma parte para um substrato aceitador). A atividade de ciclização da CGTase é uma reação de transglicosilação intramolecular, onde cadeias lineares e longas do amido são quebradas e formam estruturas de anéis

cíclicos de oligossacarídeos, denominados ciclodextrinas. As ciclodextrinas podem ser divididas em α, β e Y (VAN DER VEEN et al., 2000).

3. APLICAÇÃO DO ULTRASSOM EM REAÇÕES ENZIMÁTICAS

As ciclodextrinas são obtidas através da conversão enzimática da molécula de amido ou substratos similares. A ação enzimática é um método popular e comum devido à sua alta seletividade, especificidade do substrato e condições de reação com temperatura e pressão de trabalho mais amenas. No entanto, as enzimas apresentam um custo elevado e baixa estabilidade. Portanto, a intensificação das reações enzimáticas é crucial para aumentar a performance enzimática (SOARES et al., 2020).

Dessa forma, diferentes abordagens estão sendo desenvolvidas para contornar essas limitações, sendo a tecnologia de ultrassom (US) uma delas. A aplicação estratégica do US pode ser empregada para promover o aumento da atividade e estabilidade de enzimas, otimizando, assim, o processo em larga escala (DALAGNOL et al., 2017). Adicionalmente, a inserção do US nos processos tem atraído consideravelmente a atenção das indústrias de alimentos nos últimos anos, pois é uma tecnologia relativamente econômica, ecologicamente correta e de fácil operação (WANG et al., 2018).

A tecnologia de US emprega ondas mecânicas acima da audição humana (>20 kHz), conhecidas como ultrassônicas. A cavitação acústica é considerada seu efeito primário e é caracterizada pelo surgimento de microbolhas que crescem e se colapsam no líquido, induzindo modificações físicas e químicas em compostos submetidos ao processamento ultrassônico (WANG et a., 2018; PACHECO et al., 2023). Sendo assim, o ultrassom pode exercer seus efeitos na estrutura e morfologia de uma enzima e/ou em um polímero de carboidrato (PACHECO et al., 2023).

Especificamente, quando a energia vibracional do ultrassom passa pelo meio como uma onda mecânica, inicia-se um movimento contínuo do tipo onda e sucessivos ciclos de expansão e compreensão são formados. No ciclo de expansão, é gerada uma pressão negativa que supera a resistência à tração do líquido, formando cavidades. Devido à absorção da energia acústica, as

cavidades aumentam de tamanho até alcançarem seu limite crítico seguido de implosão e liberação da energia absorvida e, concomitantemente, há um aquecimento da área circundante a uma temperatura momentânea próxima a 5500°C. Além da alta temperatura, a alta pressão, tensão de cisalhamento, turbulência e agitação são características distintas em todo o processamento ultrassônico (WANG et al., 2018).

O US pode ser utilizado em três abordagens em relação às reações enzimáticas: como pré-processamento do biocalisador, como pré-processamento do material que será hidrolisado (substrato) ou na reação assistida (WANG et al., 2018). Além disso, sua aplicação pode ocorrer de duas maneiras: através do método direto ou indireto. No método indireto, um banho ultrassônico é utilizado e a sonicação é realizada a partir das paredes do recipiente que contém a amostra. Já no método direto, uma sonda ultrassônica é colocada em contato direto com a amostra e oferece uma potência ultrassônica muito maior do que a fornecida pelo ultrassom de banho (GOULA et al., 2017). A Figura 5 demonstra os métodos de processamento com o US.

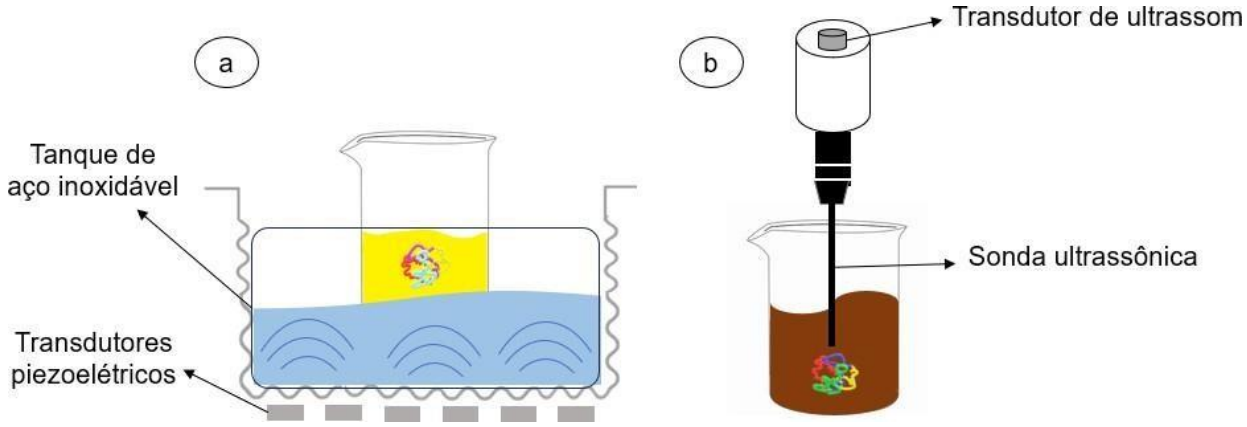

Figura 1. Métodos de processamento por US: A) Banho ultrassônico; B) Ultrassom de sonda.

Fonte: autor.

3.1. EFEITO DO ULTRASSOM EM ENZIMAS

O US é utilizado para ativar ou inativar enzimas, dependendo das condições aplicadas, e sua aplicação é influenciada por fatores como a

geometria e o tipo de sistema, densidade de energia acústica, frequência e tempo (MAWSON et al., 2011).

É difícil esclarecer o mecanismo específico de ativação ou inativação de uma enzima, uma vez que vários efeitos químicos e físicos ocorrem simultaneamente, afetando a conformação nativa da enzima e alterando as interações responsáveis pela estabilidade da proteína (interações hidrofóbicas e de van der Waals, forças eletrostáticas, ligações de hidrogênio, etc) (WANG et al., 2018). No entanto, é certo que as mudanças na atividade enzimática dependem principalmente da composição de aminoácidos e da conformação específica da proteína (WANG et al., 2018).

É reportado na literatura diversos estudos que objetivaram potencializar a performance de diferentes enzimas, como proteases (PACHECO et al., 2023) e amilase (LEAES et al., 2013). A Tabela 3 demonstra o incremento na atividade enzimática alcançado para algumas enzimas pré-processadas por US.

Tabela 3. Condições de processamento ultrassônico que levaram à ativação de diferentes enzimas.

Enzima alvo	Parâmetros ultrassônicos	Principal resultado	Referência
Alcalase®	40 °C, 60 min, 38 W/L e 20 kHz	Aumento de 15,6% na atividade enzimática (AE)	GUIMARÃES et al. (2023)
Brauzyn® e Neutrase®	25 °C, 60 min, 23,8 W/L e 40 kHz	Aumento de 20,8% e 22,7% na AE	PACHECO et al. (2024)
Flavourzyme®	25 °C, 90 min, 23,8 W/L e 40 kHz	Aumento de 22,4%	PACHECO et al. (2024)
Amiloglucosidase	23°C, 5 min, 9.5 W L^{-1} e 40 kHz	Aumento de 15% na AE	SOARES et al. (2021)
Lipase	25°C, 45 min, 22W/L e 25kHz	Aumento de 12% na AE	SOARES et al. (2020)

Poligalacturonase	15min, 4.5W/mL e 22kHz	Aumento de 12 % na AE 20.98%	MA et al. (2015)
Pepsina	60min, 300W, 40kHz	Aumento de 14.55% na AE	YU et al. (2014)

Para exemplificar, Subhedar & Gogate (2014) trataram a celulase com um sonicador de sonda (17,33 W/cm^2, 20 kHz) por 30 min para analisar os efeitos ultrassônicos em sua atividade enzimática. Após o tratamento ultrassônico, a atividade enzimática obteve um aumento de 23,4%, indicando que a maioria das moléculas de celulase na solução sofreu alterações conformacionais, com a exposição de novos sítios ativos. Por outro lado, Wang et al. (2012) mostraram que um maior incremento na atividade (<18,17%) da mesma enzima foi alcançada quando a amostra foi tratada com US a 24 kHz, 15 W por 10 min. A aplicação do US teve como resultado a observação de um ligeiro aumento na quantidade de triptofano na superfície da celulase. Este aumento foi acompanhado por uma deformação parcial na estrutura da α-hélice do biocatalisador.

Em um estudo desenvolvido por Oliveira et al. (2018) foi elucidado o impacto do US na sacarificação utilizando amiloglucosidase. Os resultados apontaram um aumento no rendimento de glicose para todas as combinações enzima-amido, demonstrando um efeito duplo do US tanto na enzima quanto no substrato.

Oliveira et al. (2017) investigaram o papel do ultrassom na atividade de quatro alfa-amilases disponíveis comercialmente de fontes fúngicas e bacterianas. Os autores constataram que o US pode ser aplicado tanto para inativação da alfa amilase (enzimas do *Aspergillus oryzae* e *Bacillus amyloliquefaciens*) quanto para ativação (enzimas do *Bacillus licheniformis*). Similarmente, Souza et al. (2013) também avaliaram o impacto da sonicação de um banho ultrassônico a 40 kHz na atividade de uma enzima amilase comercial. Para mensuração da atividade enzimática, o pH foi mantido fixo (4,5) e uma ampla faixa de temperatura foi testada (30-100 °C), com e sem sonicação. Os resultados constataram que em temperaturas mais baixas (fora da temperatura

ideal da enzima) a sonicação pode potencializar a reação enzimática. Entretanto, a uma temperatura mais alta (que está mais próxima das condições ideais da enzima) a sonicação reduziu a atividade da enzima.

Pacheco et al. (2023) estudaram o impacto do processamento ultrassônico na atividade da Alcalase. Os autores reportaram que atividade da enzima aumentou 10,3% quando foi processada nas seguintes condições: 40 kHz, 23,8 W/L, 25 °C e 120 min. Os autores sugeriram que esse incremento foi devido às mudanças conformacionais na estrutura terciária e secundária (hélice α, folha β, volta β) promovidas pela cavitação ultrassônica.

Portanto, a utilização do US em reações enzimáticas revela um potencial significativo para aplicações industriais, proporcionando melhorias no desempenho enzimático quando aplicado isoladamente (Tabela 3) (WANG et al., 2018). Por fim, até onde sabemos, o efeito do ultrassom ainda não havia sido elucidado para a CGTase e, assim, este estudo tornou-se oportuno, tendo como principal objetivo estudar o impacto da aplicação do US na atividade, estabilidade e estrutura conformacional desta enzima.

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CAPÍTULO 2

Manuscript: Use of ultrasound to improve the activity of cyclodextrin glycosyltransferase in the producing of β-cyclodextrins: Impact on enzyme activity, stability and insights into changes on enzyme macrostructure

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CAPÍTULO 2

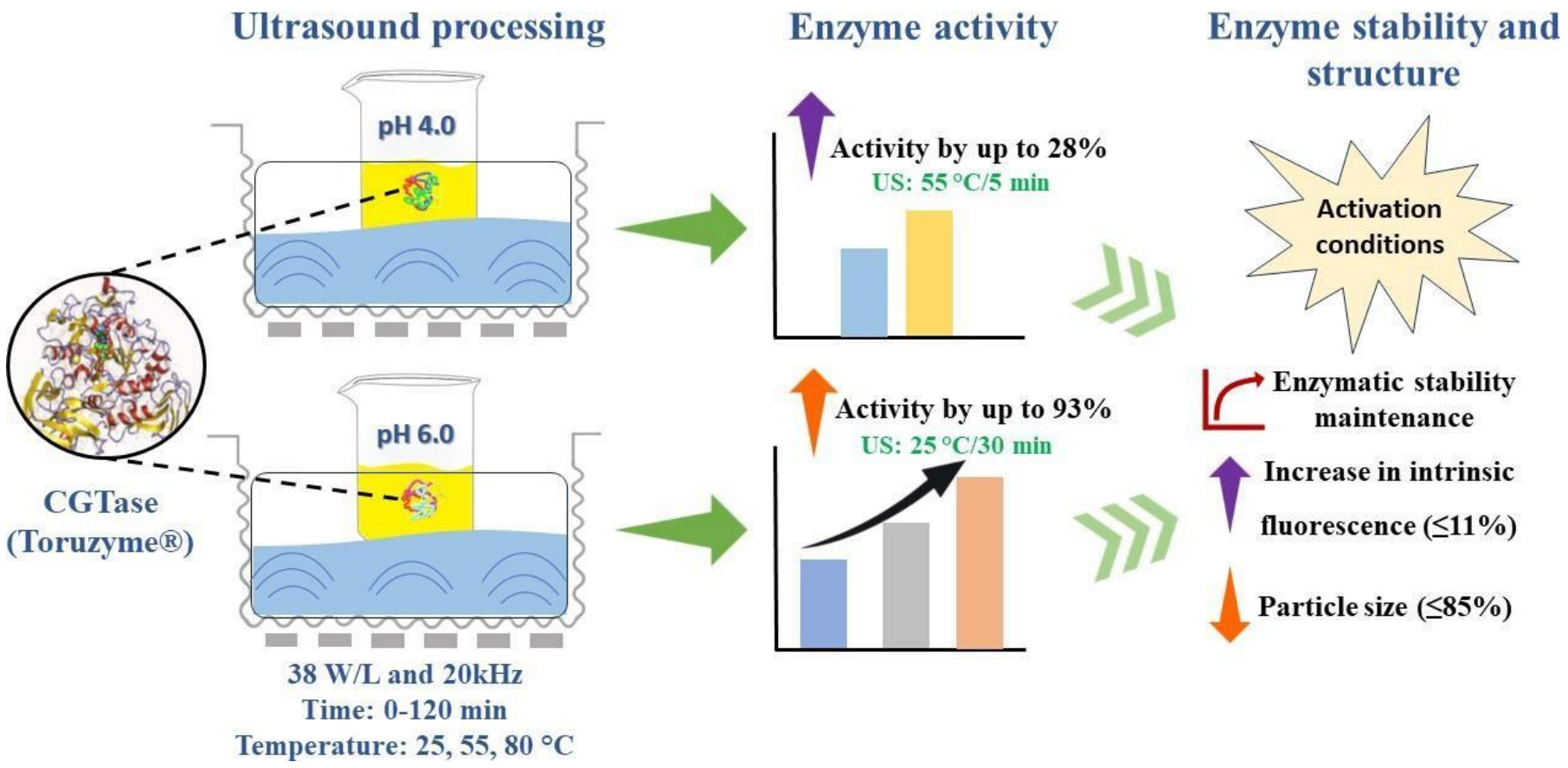
Use of ultrasound to improve the activity of cyclodextrin glycosyltransferase in the producing of β-cyclodextrins: Impact on enzyme activity, stability and insights into changes on enzyme macrostructure

Abstract

This work explored the impact of ultrasound (US) on the activity, stability, and macrostructural conformation of cyclodextrin glycosyltransferase (CGTase) and how these changes could maximize the production of β-cyclodextrins (β-CDs). The results showed that ultrasonic pretreatment (20 kHz and 38 W/L) at pH 6.0 promoted increased enzymatic activity. Specifically, after sonication at 25 °C/ 30 min, there was a maximum activity increase of 93% and 68% when biocatalysis was carried out at 25 and 55 °C, respectively. For activity measured at 80 °C, maximum increase (31%) was observed after sonication at 25 °C/ 60 min. Comparatively, US pretreatment at low pH (pH=4.0) resulted in a lower activity increase (max. 28%). These activation levels were maintained after 24 hours of storage at 8°C, suggesting that changes on CGTase after ultrasonic pretreatment were not transitory. These pretreatments altered the conformational structure of CGTase, revealed by an up to 11% increase in intrinsic fluorescence intensity, and resulted in macrostructural modifications, such as a decrease in particle size and polydispersion index (up to 85% and 45.8%, respectively). Therefore, the sonication of CGTase under specific conditions of pH, time, and temperature (especially at pH 6.0/ 30 min/ 25 °C) promotes macrostructural changes in CGTase that induce enzyme activation and, consequently, higher production of β-CDs.

Keywords: cyclodextrin glycosyltransferase; emerging technology; enzymatic activity; enzymatic stability; sonication; β-cyclodextrin.

Graphic Abstract



1. Introduction

Cyclodextrins (CDs) are non-reducing cyclic molecules composedprimarily of six (α-CD), seven (β-CD), or eight (γ-CD) glucose monomers, linked through α- (1,4) bonds (Matêncio et al., 2020). Among these oligosaccharides, β- CDs stand out for their use in the food and pharmaceutical industry, mainly as bioactive compound carriers (Santos, Buera, & Mazzobre, 2017).

CDs are produced from starch or similar substrates through the cyclization reaction, one of four reactions mediated by cyclodextrin glycosyltransferase (CGTase). For most CGTases, the main products are α- and β-CDs (Li et al., 2007).

Considering that CDs are exclusively produced by enzyme reaction, the main challenges of β-CD large-scale production are related to the low stability and high cost of CGTase (Graebin et al., 2016). In this scenario, strategies to improve the efficiency and stability of the commercial preparation of CGTase from *Thermoanaerobacter* sp. (Toruzyme® 3.0 L, a commercial enzyme used worldwide for the production of CDs) has been developed, mainly focused on enzyme immobilization techniques through various approaches (Schöffer et al., 2013; Schöffer et al., 2017; Gimenez et al., 2019; Ogunbadejo & Al-Zuhair, 2023). However, based on our current knowledge, no prior studies investigated the effect of physical processing, specifically ultrasound (US), on the properties of this enzyme. Therefore, establishing ultrasonic process conditions that can enhance CGTase activity is essential for industrial application.

The US is an unconventional technology that operates based on the transmission of sound waves at frequencies above the range of human hearing (>20 kHz) (Huang et al., 2017). Under specific conditions, this technology can

alter the enzyme conformation due to the effects of acoustic cavitation phenomenon generated during sonication, leading to improvements in enzyme activity/stability (Hou et al., 2019; Pacheco et al., 2023). Several studies have demonstrated that ultrasonic pretreatment was able to activate enzymes (Huang et al., 2017; Wang et al., 2018), such as cellulase, pectinase, xylanase, lipase, and proteases (Dalagnol et al., 2017; Soares et al., 2020; Guimarães et al., 2023).

The effects of US on enzymes may be influenced by experimental conditions, such as enzyme source, US frequency, and treatment time, pH, and temperature (Oliveira et al., 2017). Thus, a simultaneous and systematic evaluation of US parameters that affect CGTase activity can contribute to identifying conditions capable of favoring the performance and stability of this enzyme.

Given the above considerations, the present study aimed to investigate the effects of US under both optimal and non-optimal pH and temperature conditions as a pretreatment of Toruzyme®, evaluating how the sonicationprocess can be able to modulate CGTase activity/stability due to conformational/structural alterations. These results can help to optimize the large-scale production of β-CDs, making the process more sustainable, energy-efficient, and with less risk of microbial contamination.

2. Materials and Methods

CGTase (Toruzyme® 3.0 L, Novozymes, Paraná, Brazil) and Maltodextrin (DE 10, Gemacom Tech, Guarani, Brazil) were used as enzyme and substrate, respectively. The reagents dibasic sodium phosphate (Na_2HPO_4), sodium phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$), sodium carbonate (Na_2CO_3), and

sodium bicarbonate ($NaHCO_3$) were purchased from Dinâmica Química Contemporânea (Indaiatuba, Brazil). Phenolphthalein ($C_{20}H_{14}O_4$) was obtained from Anidrol (Diadema, Brazil). All chemicals and reagents used in this study were of analytical grade.

Different approaches were used to clarify the influence of US on CGTase activity. Initially, the optimum temperature of the enzyme was determined. Subsequently, CGTase was processed by US varying time (0-120 min), temperature (25, 55 and 80 °C) and pH (4.0 and 6.0). After ultrasonic processing, CGTase activity was determined at different temperatures (25, 55 and 80 °C) at pH 6.0. For each temperature evaluated, the US condition that promoted the maximum activation of the CGTase was selected to evaluate the enzyme stability, as well as to evaluate the macrostructural characteristics of the CGTase activated by US. This information is summarized in the flowchart of this study (Figure 1).

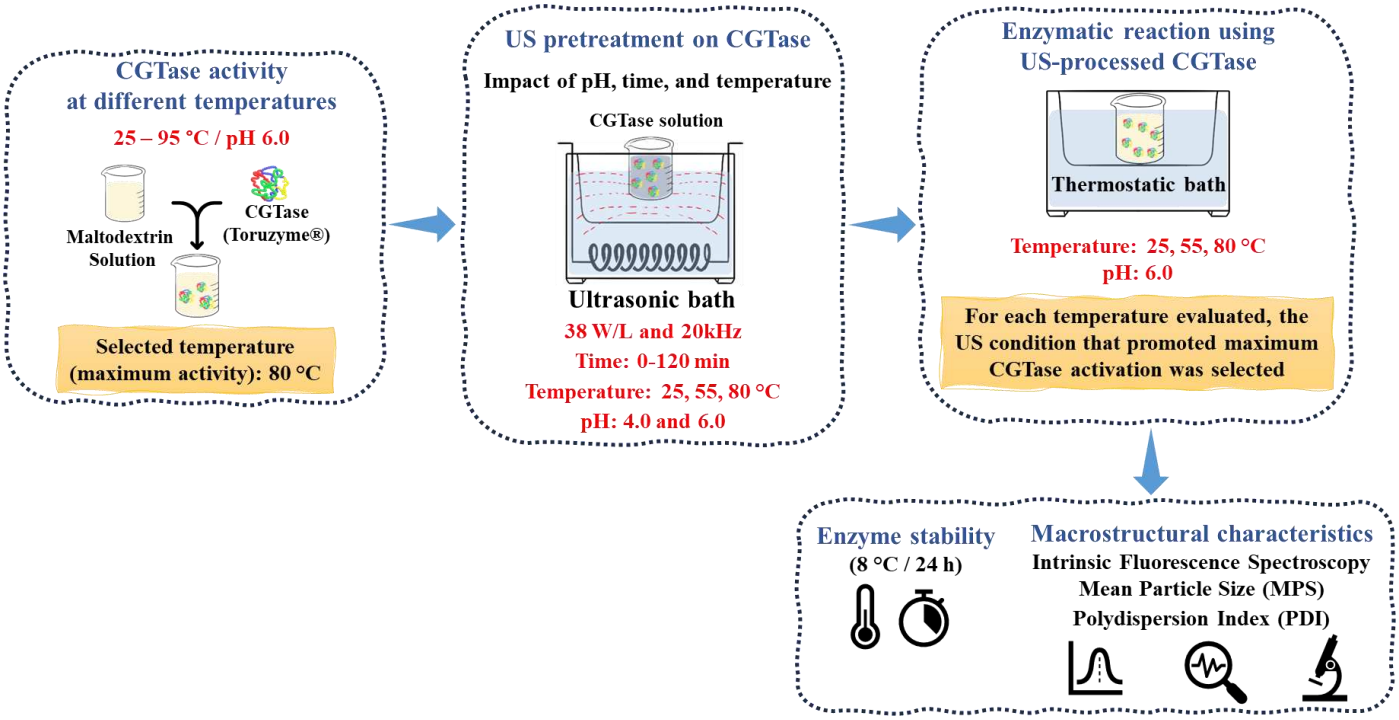


Figura 1. Flow chart of the work.

2.1. Evaluation of CGTase activity in maltodextrin at different temperatures

The activity of CGTase at different temperatures was determined at pH 6.0 and the procedures were carried out according to Rojas et al. (2019). This pH was chosen considering the optimal pH for CGTase activity as described in the literature (Kim et al., 1997; Gimenez et al., 2019; Rojas et al., 2019), as well as in the information obtained in the CGTase (Toruzyme®) technical sheet provided by the manufacturer. For this, β-CD production rates were evaluated using as substrate a maltodextrin solution (1.0% w/v, produced from corn starch, which was prepared from 10 mM sodium phosphate buffer, pH 6.0). In a thermostatic bath, 30 µL of the enzyme preparation (1% v/v, diluted in 10 mM sodium phosphate buffer pH 6.0) was mixed with 15 mL of maltodextrin solution. The reaction was carried out at 25, 55, 60, 70, 75, 80, 90, and 95 °C. These temperature levels were selected for two reasons: (1) 25°C was chosen as a room temperature (which is desirable to reduce the cost of process); (2) range between 55 - 95 ºC was chosen considering the importance of determining optimal temperature and percentage of activity decrease at sub-optimal conditions.During the reaction, aliquots of 1 mL were taken at 10-minute intervals for 30 minutes. To inactivate CGTase, 20 µL of HCl (3 M) was added directly tothe samples, and the mixture was subsequently incubated for 5 min in a boiling bath.The quantification of β-CD produced was performed by the colorimetric dye quenching method using phenolphthalein, as described by Tardioli et al. (2006). Absorbance was measured at 550 nm using an SP-22 spectrophotometer (Biospectro, Curitiba, Brazil). Blank was defined as the sample collected at 0 min and mixed with phenolphthalein solution.

The temperature exhibiting the maximum activity was defined as the optimal temperature, corresponding to an enzymatic activity of 100%. The relative enzyme activity (REA) was determined using equation 1 (Eq. 1).

$$REA(\%) = \left(\frac{activity\_non\_optimal\_condition}{activity\_optimal\_condition}\right) \cdot 100 \qquad Eq.1$$

2.2. Effect of US pretreatment on CGTase activity: Impact of pH, time, and temperature

A 150 mL beaker containing 100 mL of enzyme preparation (1% v/v diluted in 10 mM sodium phosphate at pH 4.0 or 6.0) was immersed in an ultrasonic bath (Unique, model USC 2800 A, Indaiatuba, Brazil) with nominal capacity of 9.5 L, internal dimensions of 30 × 24 × 15 cm, equipped with five 20 kHz transducers below the tank, nominal power of 450 W and volumetric power of 38 W/L. The beaker was positioned in the region with the highest intensity ultrasonic (determined by the aluminum foil method) (Vinatoru, 2015). The processes were performed at different temperatures (25, 55, and 80 °C) and time (0 to 120 minutes). To regulate the sample temperature in the ultrasonic bath, a heat exchanger was coupled inside the ultrasonic bath, and an external thermostatic bath ensured water recirculation. Enzymatic activity was determined immediately after collecting 5 mL aliquots at 5, 15, 30, 45, 60, 90, and 120 minutes of sonication and measured at temperatures of 25, 55, and 80 °C at pH 6.0 in a thermostatic bath, as described in section 2.1. The control was represented by an aliquot (5 mL) collected at time 0 of sonication (sample not subjected to US processing), and its activity was evaluated under the same conditions used for the processed samples.

The quantification of relative enzymatic activity after the application of ultrasound, referred to as REA_E, was determined by the ratio between the activity of the samples subjected to US and the activity of the untreated enzyme, under the same temperature and pH condition, as expressed in equation 2 (Eq. 2).

$$REA_E\ (\%) = \left(\frac{US\_treated\_sample\_activity}{Non\_treated\_sample\_activity}\right) \cdot 100 \qquad Eq.2$$

2.3. Enzyme stability and macrostructural characteristics changes induced by the US treatment on CGTase under activation conditions

From the results of section 2.2., it was selected 3 sonication conditions that resulted in higher activity increase (25 °C/30 min or 60 min at pH 6.0 and 55 °C/5 min at pH 4.0) for additional evaluations regarding stability, particle characteristics, and tertiary structure.

2.3.1. CGTase stability

To investigate the activation reversibility, the enzyme activity was assessed after the ultrasonic treatment and after 24 hours of storage at 8°C (Tribst & Cristianini, 2012). The stability of REA_E during storage was evaluated by considering the activity post-storage period with the activity immediately afterthe US treatment, maintaining identical conditions, following the methodology shown in section 2.1.

2.3.2. Mean Particle Size (MPS) and Polydispersion Index (PDI)

The MPS and the PDI of each enzyme solution were evaluated and calculated using the Zetasizer Nano-ZS device, following the procedures shown by Pacheco et al. (2024). The PDI was determined from the ratio between the

mean particle size and the standard deviation for each identified peak (Soares et al., 2019). The experimental parameters of the equipment were configured as follows: refractive index of 1.330, scattering angle of 173°, and viscosity of 0.8872 cP.

2.3.3. Intrinsic Fluorescence Spectroscopy

Intrinsic fluorescence spectra of CGTase were evaluated at room temperature (25 ± 1 °C) employing a SpectraMax M5 fluorescence spectrophotometer (Molecular Devices, San Diego, USA), following the protocol shown by Ai et al. (2019). A 300 µL aliquot of each enzyme preparation, subjected to ultrasonic processing or untreated, was introduced into StakMax™ microplate readers. Tryptophan fluorescence (Trp) emission spectra at λ in the range of 305–450nm were acquired by scanning the sample with an excitation wavelength λex = 295nm.

2.4. Experimental design and statistical analysis

The study was executed following a completely randomized design (CRD). The procedures were replicated three times independently, and the analyses were conducted in triplicate for each processing repetition (n=9). Data for REA, REA_E, MPS, and PDI were subjected to one-way ANOVA, followed by Tukey's post hoc test for multiple comparisons at a 95% confidence level (Statistical Analysis System - SAS Institute, USA; version 9.2). Data were presented as mean and standard deviation.

3. Results

3.1. CGTase activity at different temperatures

Figure 2 shows the Toruzyme® activity at different temperatures and a fixed pH of 6.0. The results showed maximum activity between 80 and 95 °C ($p>0.05$). The optimal activity of Toruzyme® has already been reported at high temperatures, confirming its great thermostability (Norman & Jorgensen, 1992; Matte et al., 2012; Koga et al., 2021). Therefore, it was decided to work at 80 °C in the subsequent steps of this study because operating at temperatures as low as possible allows energy savings for large-scale processes. In addition, at this temperature, the substrate/product solubility is high and the cyclodextrins formed are not degraded, which possibly contributes to an increase in the productivity and yield of β-CDs (Kim et al., 1997; Matte et al., 2012). The evaluation of REA under non-optimal conditions showed that the enzyme had low activity below 75 °C, with a gradual reduction in REA with temperature decreased, reaching the lowest activity (6.3%) at 25 ºC (Figure 2).

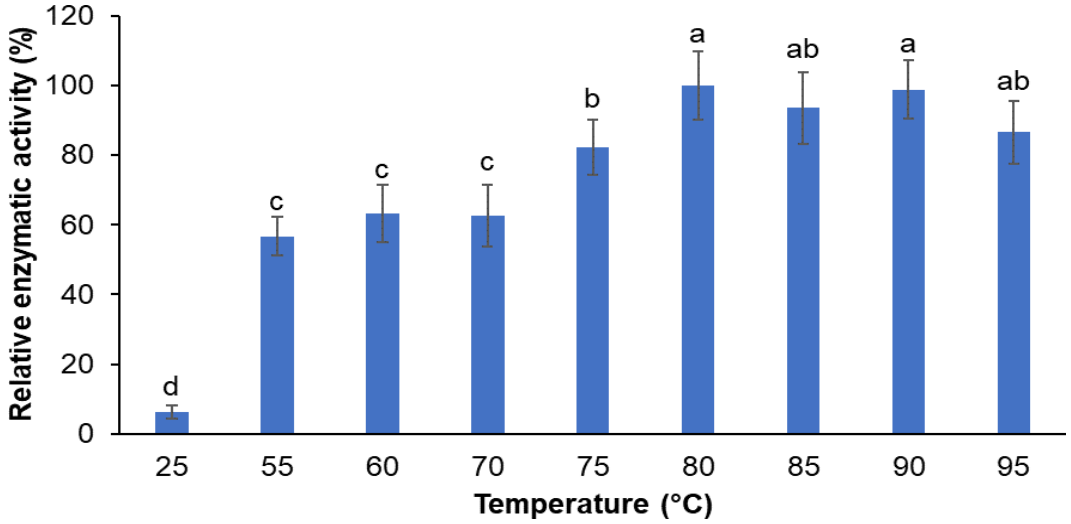


Figure 2. Influence of temperature on the relative enzyme activity (REA) of CGTase in maltodextrin. *The temperature exhibiting the highest activity (80ºC) was identified*

as optimal, corresponding to 100% of the enzyme activity. The REA was determined using Eq. (1) for all other temperature values. [a-d] *Distinct letters mean differences in the relative enzyme activity at different temperatures according to post-hoc Tukey's test at 95% confidence ($p<0.05$).*

3.2. Effect of US pretreatment on CGTase activity: Impact of pH, time, and temperature

Figure 3 demonstrates the REA_E measured at optimal pH (pH=6.0) and temperatures of 25, 55, and 80 °C, using CGTase pretreated by US at pH 6.0 and different temperatures (25, 55, and 80 °C), for up to 120 min. The results revealed that sonication was able to enhance, diminish, or maintain the CGTaseactivity, contingent upon the process conditions.

Specifically, maximum increases were observed when the enzyme was sonicated for 30 min at 25 °C and the activity measured at 25 °C and 55 °C (93% and 68% increment, respectively ($p<0.05$). In addition, the greatest activity increase (31%, $p<0.05$) at the optimal temperature (80 °C) was reached after CGTase sonication for 60 min at 25 °C (Figure 3).

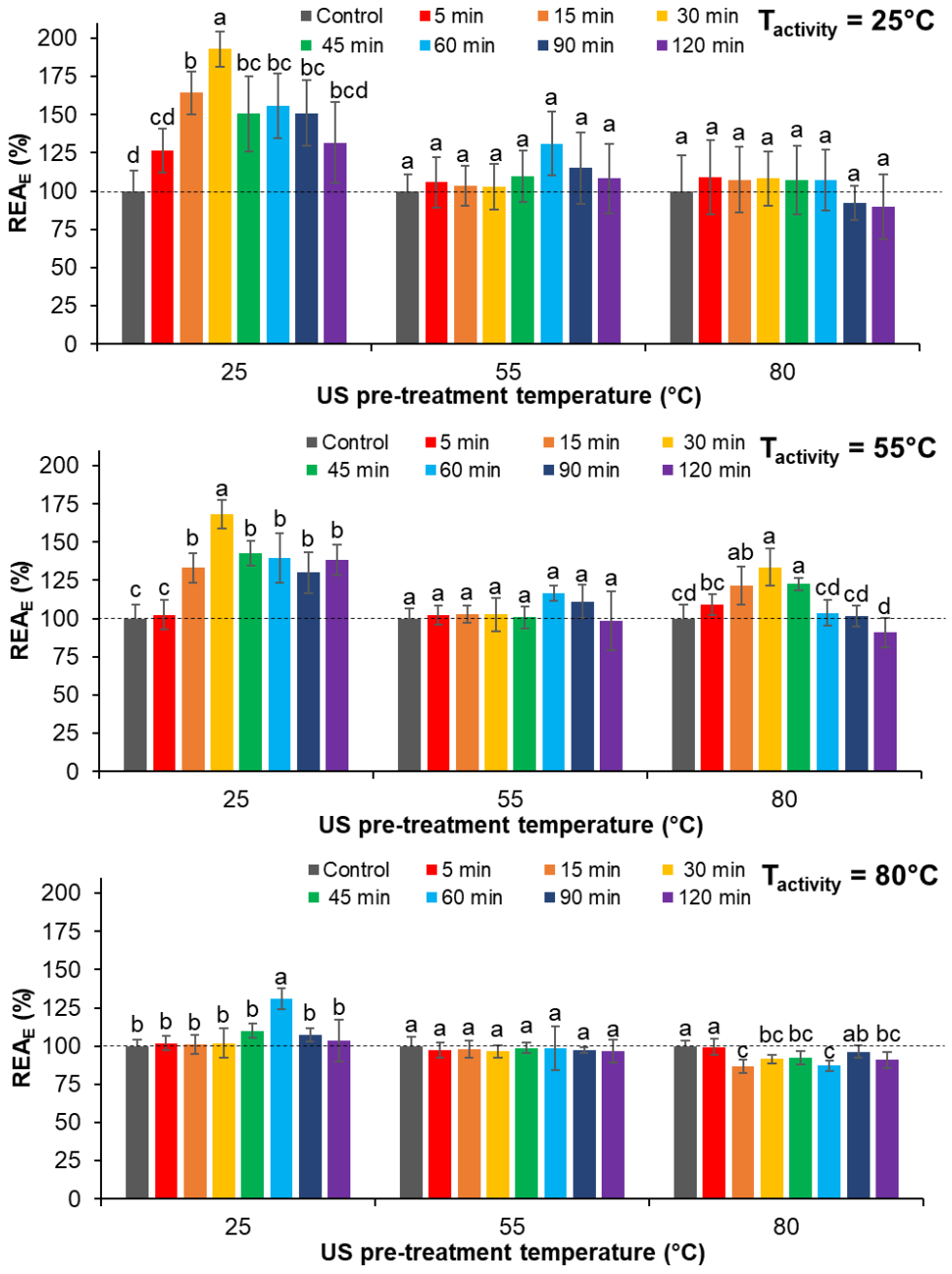


Figure 3. Relative activity after US pretreatment of the enzyme (REA_E) measured at 25, 55 and 80 °C/pH 6 in maltodextrin using US pre-treated CGTase for up to 120 min at different temperatures 25, 55 and 80°C in pH 6.0 (Eq. (2)). *[a-d] Distinct letters observed between the control sample and US pre-treated samples at different time points under the same temperature denote significant differences according to the post-hoc Tukey's test at 95% confidence ($p<0.05$). US: ultrasound.*

It is noteworthy that the results obtained at 55 °C of the reaction using the enzyme sonicated for 30 min at 25°C are promising because they exceeded the CD concentration obtained using native enzyme at optimal temperature (10% difference in the production of β-CDs). From an industrial point of view, this resultis important, as it may provide time and energy savings due to the lower working temperature for catalysis (Calsavara et al., 2011). On the other hand, despite the greater gains promoted by the US when the activity was measured at 25 °C, the activity of CGTase at this temperature is very low (6.3% REA), limiting the β-CD production process.

Moreover, considering that pH affects the three-dimensional configuration of an enzyme and that physical processes carried out in enzymes with different initial configuration can affect their final configuration (Tribst et al., 2012), this work evaluated the impact of CGTase (1% v/v) sonication at pH 4.0 under the same processes times and temperatures previously evaluated.

Figure 4 demonstrates the REA_E measured at optimal pH (6.0) and temperatures of 25, 55, and 80 °C, using CGTase pretreated by US at pH 4.0 under temperatures of 25, 55, and 80 °C for up to 120 min. Similar to the observed after CGTase sonication at pH 6.0, these results showed that enzyme activity can be altered or not after sonication at non-optimal pH, but the positive impact was at least 50% lower than observed after sonication at pH 6.0. The increase in activity (maximum obtained after CGTase sonication at 55 °C/ 5 min) was lower than 30% for activities measured at 25 and 55 °C and <16% for activity measured at 80 °C.

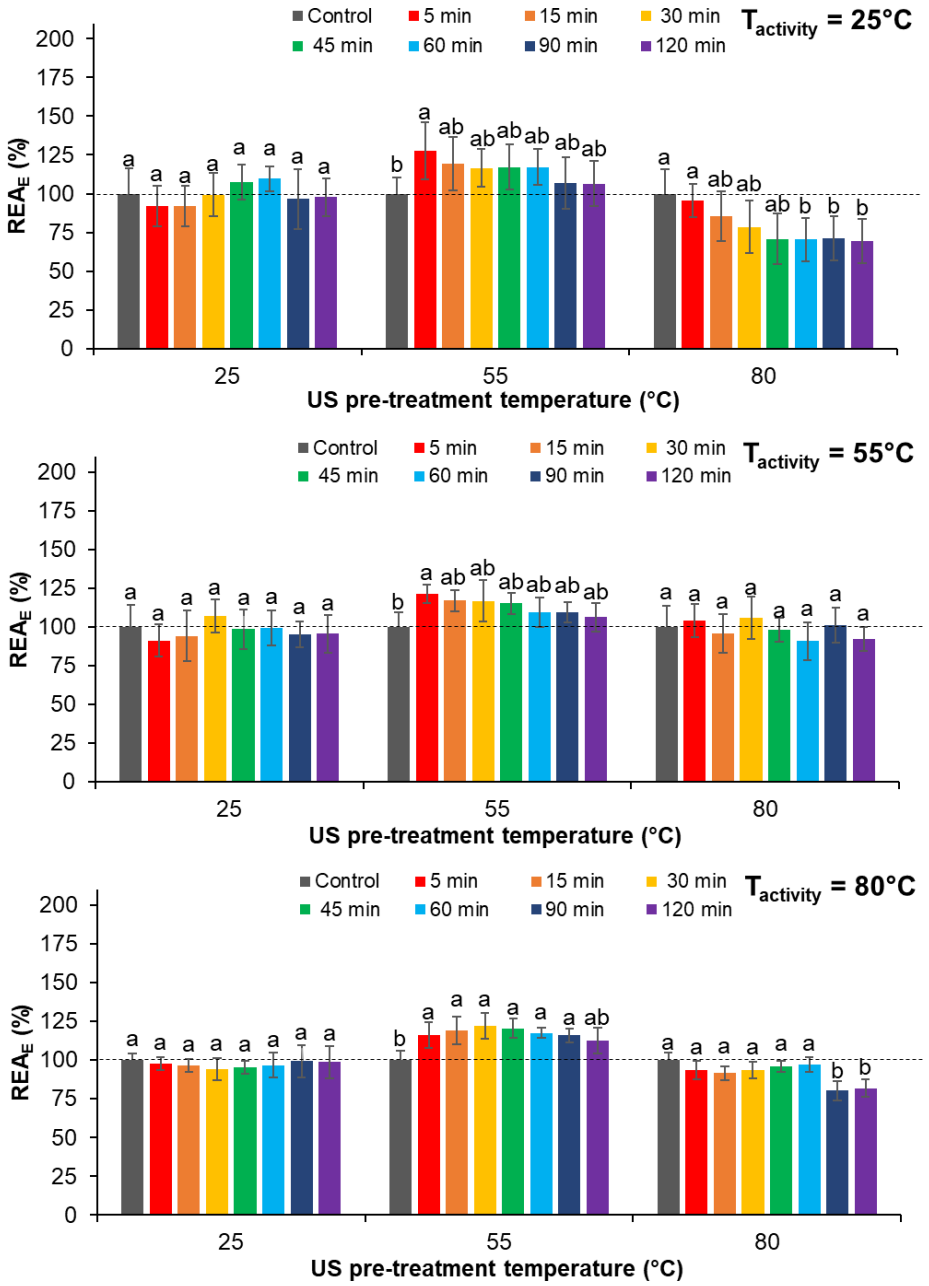


Figure 4. Relative activity after US pretreatment of the enzyme (REA_E) measured at 25, 55 and 80 °C/pH 6 in maltodextrin using US pre-treated CGTase for up to 120 min at different temperatures 25, 55 and 80°C in pH 4.0 (Eq. (2)). $^{a\text{-}d}$ *Distinct letters observed between the control sample and US pre-treated samples at different time points under the same temperature denote significant differences according to the post-hoc Tukey's test at 95% confidence ($p<0.05$). US: ultrasound.*

3.3. Evaluation of enzymatic stability and macrostructural changes induced by the US pretreatment on CGTase under activation conditions

For industrial use of CGTase, it is imperative to explore the enzyme's stability post-ultrasonic treatment and ascertain the reversibility of the modifications induced by US. The results in Figure 5 showed that CGTase solutions prepared at pH 4.0 and 6.0 and subjected or not to the sonication process under activation conditions maintained its catalytic activity after 24 h of refrigerated storage ($p>0.05$).

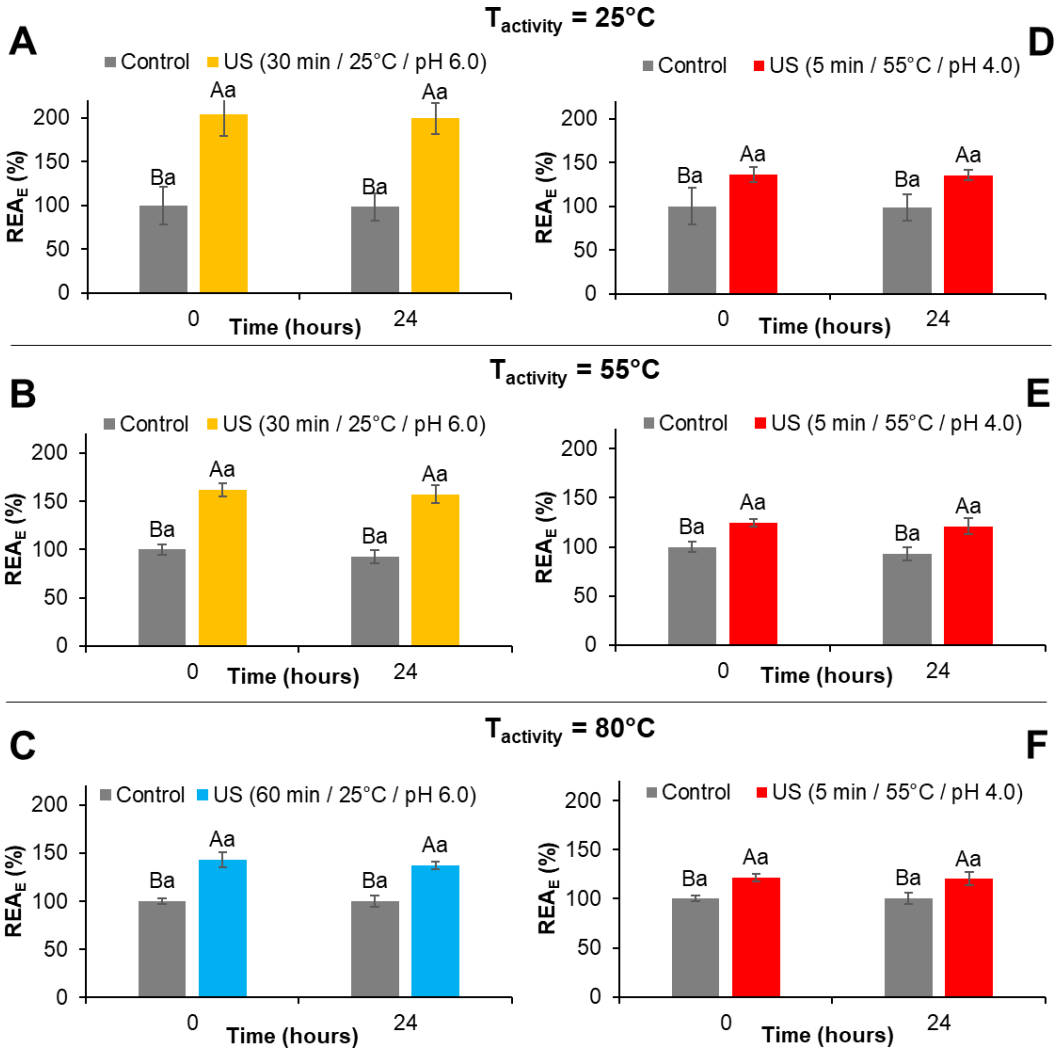


Figure 5. Stability of REA_E after 24h of storage at 8°C measured at 25°C (A and D), 55 °C (B and E) and 80 °C (C and F) using US-activated CGTase. *US: ultrasound.* [a-b] *Distinct capital letters observed between samples (control and US-activated CGTase) at the same time and distinct lowercase letters observed between times (0 and 24 hours) for the same sample denote significant differences according to the post-hoc Tukey's testat 95% confidence ($p<0.05$).*

Maintaining the structural integrity of the enzyme molecule is vital to ensure its catalytic function. To study the effect of US treatment on the molecular macrostructure of CGTase, particle size, polydispersity index, and fluorescence spectrum analyses were investigated (Figure 6).

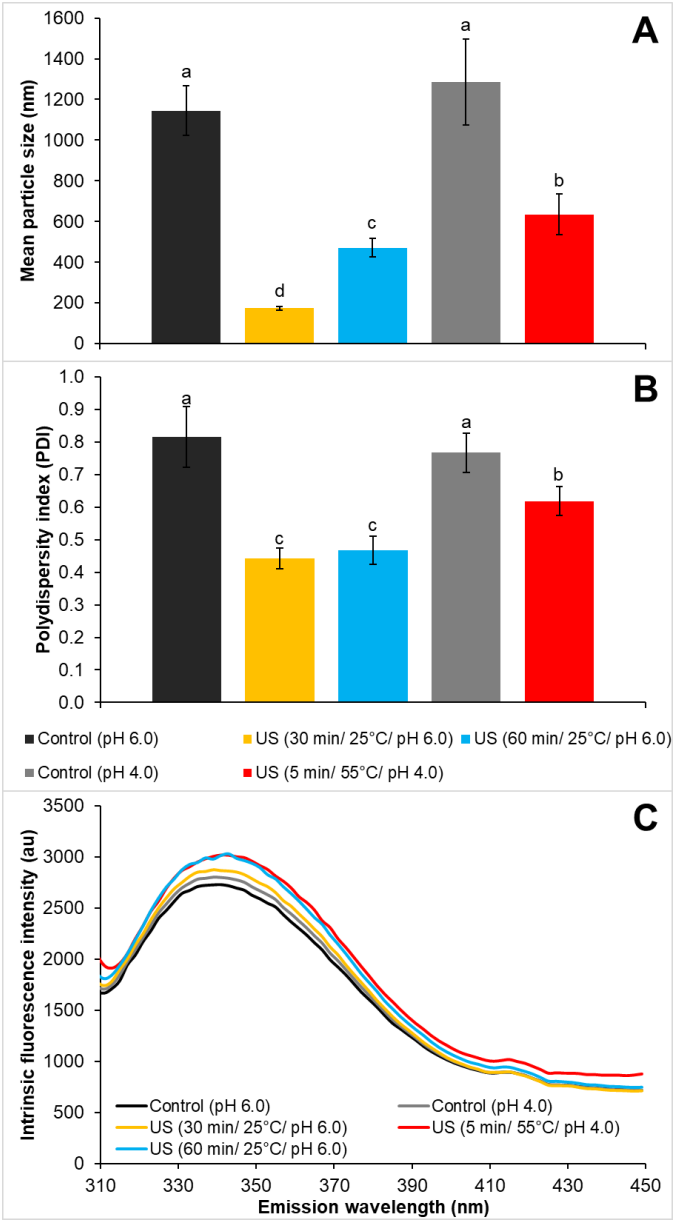


Figure 6. Mean particle size (MPS) (A), polydispersity index (PDI) (B), and Intrinsic fluorescence spectra (C) of US-activated CGTase. *Distinct letters observed between samples denote significant differences according to the post-hoc Tukey's test at 95% confidence ($p<0.05$).*

The mean particle size of the native and US-pretreated CGTase under activation is shown in Figure 6A. Native Toruzyme® at pH 6.0 and 4.0 had the highest sizes, 1145 and 1285 nm, respectively. After ultrasonic treatment under activation conditions, a decrease in mean particle size ($p>0.05$) was observed, reaching the smallest size after sonication at 30 min/ 25°C/ pH 6.0 (85%

reduction). Similarly, all polymer polydispersion index values decreased when CGTase was processed under activation conditions, with a maximum reduction of ~45.8% for the US process condition at pH 6.0 (Figure 6B).

The intrinsic fluorescence spectra of CGTase in both its native and US-pretreated states under activation conditions are shown in Fig.6C. The spectra of the untreated and US treated enzymes (at pH 4.0 and 6.0) exhibited a single peak, with the maximum fluorescence wavelength (λmax) recorded at ~340 nm, but with observable differences in the fluorescence intensity peak, especially for samples processed by US at pH 6.0/ 60 min/ 25°C or pH 4.0/ 5 min/ 55 °C, indicating increments of 11% and 8%, respectively.

4. Discussion

Ultrasound technology has been explored as a strategy for enzyme activation or inactivation according to the conditions used, such as the type of system used, geometry, time, temperature, frequency, acoustic energy density, and pH (Mawson et al., 2010; O'donnell et al., 2010).

Cavitation is the main phenomenon during sonication and occurs due to the formation, growth, and collapse of microbubbles within the reaction system caused by temperature and pressure differences (More et al., 2022). The mechanical and shear forces of cavitation (Delgado-Povedano & de Castro, 2015) strongly depend on the intensity and duration of the ultrasonic treatment (Zhu et al., 2015) and its consequences in the structure of a biocatalyst in linked to the amino acid composition and enzyme structure (Petrović et al., 2018). Additionally, shock waves and microjets can contribute to changes in enzymatic activity (Munir et al., 2019) by promoting the disruption of hydrogen bonds and

van der Waals interactions, which consequently impacts the enzymatic conformation (Huang et al., 2017; Wang et al., 2018).

After sonication of CGTase at pH 4.0 and 6.0, the results highlighted that activation was more noticeable when the treatment was carried out at optimal pH (pH = 6.0) and activity was measured at sub-optimal temperature (25 and 55°C). Therefore, the CGTase structure was more resistant to physical modifications induced by US when the solution pH was adjusted below the ideal pH, which can be explained by the reconfiguration of the enzyme's three-dimensional structure, which possibly increased the interactions (electrostatic and hydrophobic) between protein structures, hindering the activation gains promoted by ultrasound (Abadía-García et al., 2016; Jiang et al., 2022).

On the other hand, regarding activity measurement, the greater improvement observed at sub-optimum temperatures may be explained considering that, in these conditions, the interaction between enzyme-substrate is a challenge due to the low amount of energy and, consequently, any improvement in active site exposure caused by US leads to an expressive improve in the enzyme performance (Pastit & Bates, 2008). Conversely, at optimal temperature, the catalysis is naturally favored and the impact of positive changes on the enzyme configuration tends to be less perceived.

Comparatively, the US promoted activity increments of similar magnitude to those obtained for other enzymes under sub-optimal temperatures and short and moderate processing times. To exemplify, Nguyen & Le (2013) observed that under process conditions at 6 W/mL, 40 kHz, 30 °C, and 80 seconds, cellulase activity increased by 38% compared to the enzyme without ultrasonic treatment

and Bashari et al. (2013) observed that US treatment of dextranase at 40 °C, 25 kHz, 40 W for 15 min resulted in a 13.43% increase in enzyme activity.

On the other hand, when sonication was applied at high temperatures for long time, it was possible to observe a reduction in CGTase activity (as observed after ultrasonic pretreatment of CGTase at pH 6.0 for ≥15 min at 80 °C with the determination of activity at 25 °C (Fig 3); or US pretreatment at pH 4.0 for 120 min at 80 °C with the determination of activity at not ideal (25 °C) and ideal (80 °C) temperatures (Fig 4)). These results can be explained by the formation of protein aggregates at high temperatures after prolonged ultrasonic treatment. According to Yu et al. (2013), aggregates can be produced by exposing the cysteine residues of proteins to reaction with highly reactive superoxide radicals formed in the sonication of water, thus leading to disulfide bonds between proteins. Consequently, the active sites on the enzyme surface are obstructed within the aggregates, thus reducing the possibility of the enzyme binding to the substrate (Pacheco et al., 2023).

Several authors report that exposure to ultrasonic processing can reduce enzymatic activity (Rojas et al., 2016; Soares et al., 2019; Wang et al., 2020). Pacheco et al. (2024) observed that the activity of a commercial protease (Brauzyn®) was reduced by 28% after ultrasonic treatment (23.8 W/L, 40 kHz) at 55 °C and 60 min. Guimarães et al. (2023) also reported a gradual reduction in Alcalase® activity after 60 min, with the highest level of decay (44%) being obtained when performing sonication (38 W/L, 20 kHz) at 60 °C for 180 min. Comparatively, the reduction in CGTase activity in more severe conditions was up to 31% ($p<0.05$), demonstrating that each enzyme responds differently to ultrasonic parameters.

Fluorescence spectrometry is an analysis that allows inferences about the three-dimensional structure of proteins, since different spectrometric profiles indicate changes in the tryptophan, phenylalanine and lysine microenvironment (Jin et al., 2019). The results of this analysis (Figure 6C) showed that the US increased the fluorescence intensity of CGTase, indicating that this technology modified the fluorescence of tryptophan residues within the evaluated microenvironment. Therefore, under activation conditions, the results demonstrated that the ultrasound induced the molecular unfolding of the enzyme (Abadía-García et al., 2016; Qian et al., 2023), releasing hydrophobic interactions of the protein molecules and increasing hydrophobic regions exposure, mainly at low treatment temperatures (60 min/25 °C/pH 6.0) or short treatment times (5 min/55 °C/pH 4.0). Prior studies have also reported that US caused a change in the exposure of tryptophan residues from other enzymes, such as dextranase and glucoamylase (Bashari et al., 2013; Meng et al., 2018).

These changes on enzyme conformation can be attributed to the consequences of the cavitation phenomenon (Pastit & Bates, 2008), whose energy is capable of breaking weak interactions in protein structure and inducing changes in the tertiary and secondary structure of enzymes (Hou et al. 2019; Pacheco et al., 2023).

The exposure of active sites that were previously trapped within the enzyme three-dimensional structure is one of the most described reasons to explain the increase of biocatalyst activity after sonication (Wang et al., 2012; Mehrabani et al., 2022; Fadimu et al., 2022), as seen in Figure 3 and 4. However, considering the characteristics of CGTase, another explanation may be based on changes in the preferred route of enzyme conversion. CGTase is an amylolytic

enzyme capable of catalyzing four distinct types of reactions: cyclization, coupling, disproportionation, and hydrolysis (Leemhuis et al., 2010). The CGTase hydrolytic activity is considerably lower than its transglycosylation activity, due to hydrophobicity and the presence of phenylalanine (Phe) residues at positions 183 and 259 (Van der Veen et al., 2001; Mathew & Adlercreutz, 2012). Nakamura et al. (1994) also highlighted the fundamental role of Phe-183 and Phe-259 in CGTase cyclization activity, emphasizing their cooperative engagement in substrate binding during cyclization. Therefore, it is presumed that the ultrasonic treatment under the selected conditions led to the highest cyclization activity. In addition, according to Fujiwara et al. (1992), aromatic amino acid residues with hydrophobic properties at position 255 in the CGTase of *B. stearothermophilus* (a different strain to the one studied in this study) are also important for cyclization. Thus, it is hypothesized that the conformational changes resulting from the ultrasonic treatment of CGTase (corroborated by the intrinsic fluorescence results) have favored this fact.

Additionally, changes in CGTase macrostructural characteristics were confirmed by changes in the MPS and PDI results (Figure 6). The evaluation of these parameters revealed the influence of US on CGTase morphology and size distribution. As mentioned, CGTase pretreated by US under activation conditions showed lower MPS values ($p<0.05$), proving that the particles subjected to shear suffered collisions and had their average sizes reduced as a result (Lorenzetti et al., 2020). Similarly, Nadar & Rathod (2017) described a similar behavior for lipase, attributing it to the disintegration of the molecular aggregates of the enzyme into smaller fragments due to the disruption of van der Waals forces and hydrophobic interaction through the sonication process. These authors also

suggested that the increase in the surface area of the particles associated with the decrease in their size favored greater activity of the enzyme. Therefore, the intrinsic fluorescence data of CGTase also converge with the MPS results, indicating the disruption of protein aggregates and probable exposure of aromatic amino acids. Consequently, PDI data also decreased ($p<0.05$) (Figure 6B). The PDI characterizes the distribution of molecular weights in a polymer; the lower its value, the more uniform the molecular weight distribution (Ai et al., 2019). Thus, the dispersion was favored by a group of smaller and homogeneous particles, which enhances the action of the enzyme due to greater dispersion in solution (Selvaraj et al., 2022).

The structural changes in macrostructural characteristics promoted by US under specific conditions were beneficial in enhancing CGTase activity. However, it is necessary to understand whether these phenomena are transient or irreversible. Figure 5 reports that the native and US-treated enzymes demonstrated a stabilizing effect after 24 h of the process, i.e., the CGTase functionality was not disturbed, and REA_E was maintained, demonstrating that these changes were irreversible. In fact, by changing the protein conformation, ultrasonic treatment may change the content of free sulfhydryl groups (Wang et al., 2018). This suggests the possibility of stabilizing disulfide bonds in the three-dimensional structure, contributing to the preservation of enzymatic activity. Similarly, previous results also showed that sonication (4.5 W/mL for 15min) caused a 21% increase in polygalacturonase activity and that this increase remained stable after 24 h of storage (Ma et al., 2015).

This study is pioneering, as it brought to light the use of an unconventional technology (high intensity (38 W/L) and low frequency (20 kHz) ultrasound) to

modulate the enzymatic activity of CGTase, demonstrating that the parameters used in the ultrasonic process (pH, time and temperature) need to be carefully selected. According to the results presented, it was possible to observe that the US can be applied as a pretreatment of Toruzyme® to optimize the enzymatic conversion process of maltodextrin into β-CDs, ensuring higher productivity and yield.

The industrial interest of CGTase has been growing due to the in-deep knowledge regarding their structure and mechanisms. The improvement of the properties of CGTases through non-conventional technologies, such as the US, has been expanding, but some gaps still need to be overcome, such as the evaluation of the formation of α-CD and γ-CD and the combination of chemical treatments with US. Furthermore, the impact of pH and salt concentration on US-activated CGTase activity, as well as the impact of freeze-drying should be determined before commercial use. Despite these drawbacks, the results of this research play a fundamental role in this process, since the established US conditions can be applied to improve CGTase activity, with guaranteed stability. In further studies, the effect of these selected process can be further explored regarding the formation of different CDs as well as changes in the enzyme regarding its secondary and tertiary structure.

5. Conclusion

In this work, we studied the effects of ultrasonic treatments under different pH, time and temperature on the activity, particle size, polydispersity, and fluorescence spectrometry of a commercial cyclodextrin glycosyltransferase (Toruzyme®) and observed that different processing approaches were able to change the behavior of the enzyme. Activation conditions were found applying

ultrasound at pH 4.0 and 6.0 and activation levels were more noticeable (<93%) when activity was measured at non-ideal temperatures (25 and 55° C). Specifically, sonication at pH 6.0/30 min/25 °C had the most promising result, as it allowed the reduction of the reaction temperature from 80 to 55 °C with a β-CD production almost 10% higher than that observed in the traditional process (using enzyme not sonicated). Intrinsic fluorescence results suggested that enzyme activation is probably explained by changes in the conformation of CGTase. The MPS and PDI results (reduction of 85% and 45.8%, respectively) also suggested better accessibility of the enzymes to the substrate due to broken of protein agglomerates. Based on these results, it is possible to conclude that ultrasonic bath processing can be a promising strategy to positively modulate CGTase activity, allowing greater conversion of maltodextrin into β-CD and requiring low energy consumption, with expected reduction on the CD cost production.

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CONCLUSÕES GERAIS

As β-ciclodextrinas são oligossacarídeos cíclicos que possuem uma cavidade hidrofóbica interna e uma superfície externa hidrofílica, tornando-as moléculas interessantes com várias aplicações em diferentes campos, sendo seu uso focado especialmente na inclusão de moléculas hóspedes, liberação controlada de fármacos, síntese de nanomateriais, além de mascarar o odor e sabor indesejáveis e etc. A obtenção de β-CDs é realizada exclusivamente pelo uso de enzimas (CGTases), o que impulsiona o uso de tecnologias para otimizar o processo, visando potencializar a performance enzimática. O ultrassom destaca-se como um método promissor para tal finalidade.

Neste trabalho, verificou que o processamento ultrassônico da Toruzyme®, uma CGTase comercial, foi capaz de modular a atividade da enzima em diferentes condições de reação. Dependendo das condições do processo, o ultrassom foi capaz de aumentar, diminuir ou manter a atividade enzimática. Para os resultados favoráveis, isto é, àqueles que promoveram incrementos na atividade, os resultados estão possivelmente atrelados às mudanças conformacionais do biocatalisador promovidas pela cavitação acústica.

Os incrementos na atividade (ativação enzimática) foram corroborados pela análise de tamanho das partículas e fluorescência intrínseca. As mudanças estruturais se mantiveram após período de repouso, demonstrando que o ultrassom além de promover ganho na atividade, também mantém a estabilidade enzimática. Portanto, o uso do ultrassom é uma estratégia benéfica para o processamento da CGTase, podendo subsidiar a otimização do processo de obtenção de β-CDs em larga escala.