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FERNANDO DE GODOI SILVA

**ESTRATÉGIAS INOVADORAS NO CONTROLE DE TOXINAS
NATURAIS: LEVEDURA RECOMBINANTE PARA
BIODEGRADAÇÃO DE MICROCISTINAS E MÉTODO PARA
MONITORAMENTO DE AFLATOXINAS EM QUEIJO MINAS
FRESCAL**

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em Ciência de Alimentos da Universidade
Estadual de Londrina - UEL, como requisito
parcial para a obtenção do título de Doutor em
Ciência de Alimentos

Orientadora: Prof^a. Dr^a. Elisa Yoko Hirooka

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Dedico esta tese à minha família, Aparecida, Paulo e Larissa, pelo carinho, paciência e apoio em todos os momentos. Esta conquista foi possível graças ao incentivo de vocês.

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RESUMO

Toxinas naturais, contaminantes inevitáveis em alimentos e água, constituem perigo crescente ao mundo globalizado com impacto direto à saúde humana, prejuízos econômicos e, restrição na disponibilidade de alimentação segura à população. Medidas de controle visam constante monitoramento e procedimentos de descontaminação para assegurar o abastecimento, seja de água ou de matéria-prima agropecuária com segurança ratificada a níveis oficialmente recomendados. Cianotoxinas e micotoxinas, metabólitos secundários microbianos, se destacam pela prevalência de contaminação e toxicidade. Microcistinas (MCs), cianotoxinas hepatotóxicas de ocorrência principal em água são produzidas por cianobactérias. Por outro lado, as aflatoxinas (AFs), micotoxinas de *Aspergillus* spp., com ênfase na AFB₁, são cancerígenos comprovados do grupo 1 (IARC) alvos de regulamentação pelo agronegócio internacional. Visando contribuição ao controle de MCs e AFs, este trabalho apresenta três tópicos: (i) Avanço na tecnologia de DNA recombinante e desenvolvimento de alternativa para biodegradação de MCs por cepa GRAS de *Saccharomyces cerevisiae* baseada na expressão de gene *mlrA* códon-otimizado para levedura. (ii) Caracterização da microcistinase A recombinante perante biodegradação de MCs. (iii) Disponibilização de ferramentas de controle de toxinas naturais, com foco no monitoramento de AFs e análise em amostras de queijo Minas frescal. No primeiro tópico, o gene *mlrA*, codificador da enzima microcistinase A que lineariza e reduz a toxicidade de MCs, foi confeccionado com base no genoma de *Sphingosinicella microcystinivorans* cepa B9 para recombinação gênica e expressão em *S. cerevisiae*. Um primeiro plasmídeo contendo promotor e terminador de *S. cerevisiae* e sítio múltiplo de clonagem foi utilizado para expressão. O segundo plasmídeo contendo regiões de homologia para o locus *URA3* foi usado para integração cromossomal de *mlrA*. O sucesso nas técnicas de expressão plasmidial e integração cromossomal do gene foram confirmadas por PCR. A levedura recombinante PE-2 com expressão plasmidial de *mlrA* biodegradou 69,6%

(205ng/mL) de MCs em 72 horas e 83% (267ng/mL) em 120 horas de cultivo, comprovando a eficácia desta alternativa biotecnológica. No experimento seguinte (tópico ii) avaliou-se a atividade da microcistinase A gerada a partir do primeiro experimento. De início foram testados diferentes métodos de lise da parede celular, obtendo extrato concentrado em enzima. Na avaliação da cinética enzimática foi obtida taxa máxima de degradação de MC de 3,09mg/L/h e constante de Michaelis-Menten de 2,81μM. Alta atividade da enzima foi observada em pH neutro e em temperatura na faixa 35-57,5°C. Em pH alcalino e temperaturas >65°C a enzima foi inativada. Condições ótimas de atividade foram definidas em pH 6,3 e 42,2°C. Em suma, a levedura recombinante gerada demonstrou alto potencial de expressão da microcistinase A, cujos dados fornecem base científica-tecnológica a avanços na biodegradação de MCs. No último experimento (tópico iii) foi desenvolvido e validado um método de UPLC-FLR para detecção simultânea de AFM₁ e AFB₁ em queijo minas frescal. Os resultados da validação foram satisfatórios para todos os parâmetros avaliados: linearidade ($R^2 > 0,99$), exatidão (recuperação média: 81-105% para AFM₁; 80-109% para AFB₁), precisão (RSD < 18%), e baixo limite de quantificação (0,03μg/kg para AFM₁ e 0,31μg/kg para AFB₁). Ainda que nenhuma das 20 amostras analisadas de queijo minas frescal tenha apresentado contaminação detectável, salienta-se que o método validado, implementado como ferramenta de monitoramento, contribui para garantir a segurança e agregar valor a este produto essencialmente nacional. Assim, o trabalho executado atingiu os objetivos, propondo novas opções ao controle de toxinas naturais de ocorrência universal em dois recursos interdependentes – água e alimentos, essenciais ao desenvolvimento e saúde humana. Os dados aqui apresentados poderão fundamentar avanços em estudos, promover aplicações práticas e aprimorar os métodos de controle de toxinas naturais, visando reduzir impactos destes contaminantes na saúde pública.

Palavras-chave: microcistinase A; recombinação gênica, biodegradação de microcistinas; aflatoxinas; cromatografia líquida.

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ABSTRACT

Natural toxins of food and water pose an emerging threat to the globalized world with direct impacts on human health, economic losses, and reduced availability of safe food for the population. Control measures aim to implement constant monitoring and decontamination procedures to ensure the supply of both water and agricultural products, within safety levels recommended by official standards. Cyanotoxins and mycotoxins, microbial secondary metabolites, stand out due to their prevalence of contamination and toxicity. Microcystins (MCs), hepatotoxic cyanotoxins primarily found in water, are produced by cyanobacteria. On the other hand, aflatoxins (AFs), mycotoxins from *Aspergillus* spp., particularly AFB₁, are Group 1 carcinogens (IARC) and regulated by the international agribusiness sector. Aiming to contribute to the control of MCs and AFs, this work presents three topics: (i) Advances in recombinant DNA technology and the development of an alternative for the biodegradation of MCs by a GRAS strain of *Saccharomyces cerevisiae* based on the expression of the *mlrA* gene codon-optimized for yeast. (ii) Characterization of the recombinant microcystinase A for MCs biodegradation. (iii) Provision of tools for controlling natural toxins, focusing on the monitoring of AFs and analysis in samples of Minas frescal cheese. In the first topic, the *mlrA* gene, encoding the enzyme microcystinase A, which linearizes and reduces the toxicity of MCs, was synthesized based on the genome of *Sphingosinicella microcystinivorans* B9 for genetic recombination and expression in *S. cerevisiae*. A first plasmid, containing promoter and terminator from *S. cerevisiae* and a multiple cloning site, was used for expression. The second plasmid, containing homologous regions for the URA3 locus, was used for chromosomal integration of *mlrA*. Successful plasmid expression and chromosomal integration of the gene were confirmed by PCR. The recombinant yeast with plasmidial expression of *mlrA* biodegraded 69.6% (205 ng/mL) of MCs in 72 hours and 83% (267 ng/mL) in 120 hours of cultivation, proving this biotechnological alternative was effective. In the following experiment (topic ii), the activity of

microcystinase A generated from the first experiment was evaluated. Initially, different methods for cell wall lysis were tested to obtain an enzyme-concentrated extract. Enzyme kinetics evaluation demonstrated maximum MC degradation rate of 3.09 mg/L/h and Michaelis-Menten constant of 2.81 μ M. High enzymatic activity was observed at neutral pH and temperatures ranging from 35-57.5°C. Alkaline pH and temperatures >65°C inactivated the enzyme. Optimal activity conditions were pH 6.3 and 42.2°C. In summary, the generated recombinant yeast demonstrated high potential for expressing microcystinase A, providing a scientific and technological basis for advancing the biodegradation of MCs. In the last experiment (topic iii), a UPLC-FLR method was developed and validated for the simultaneous detection of AFM₁ and AFB₁ in Minas frescal cheese. The validation results were satisfactory for all parameters evaluated: linearity ($R^2 > 0.99$), accuracy (mean recoveries: 81-105% for AFM₁; 80-109% for AFB₁), precision (RSD < 18%), and low limit of quantification (0.03 μ g/kg for AFM₁; 0.31 μ g/kg for AFB₁). Although none of the 20 samples of Minas Frescal cheese analyzed showed detectable levels of contamination, it is noteworthy the validated method, implemented as a surveillance tool, contributes to ensure the safety and add value to this essentially national product. Thus, this work achieved its objectives by proposing new options for controlling naturally occurring toxins in two interdependent resources—water and food—essential for human development and health. The results presented here may support advanced studies, promote practical applications, and enhance methods for controlling natural toxins, aiming to reduce the impact of these contaminants on public health.

Keywords: microcystinase A; gene recombination; biodegradation of microcystins; aflatoxins; liquid chromatography.

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LISTA DE ABREVIATURAS E SIGLAS

5-FOA	Ácido 5-fluoroorótico
AF(s)	Aflatoxina(s)
AFB ₁	Aflatoxina B ₁
AFB ₂	Aflatoxina B ₂
AFG ₁	Aflatoxina G ₁
AFG ₂	Aflatoxina G ₂
AFM ₁	Aflatoxina M ₁
ANOVA	Análise de variância
ANVISA	Agência Nacional de Vigilância Sanitária
AOAC	Association of Official Analytical Collaboration
BSA	Bovine serum albumin
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
DAD	Detector de arranjo de diodos
DTT	Ditiotreitol
E172	Ácido glutâmico na posição 172 da estrutura da microcistinase A
EC	European Commission
EDTA	Ácido etilenodiamino tetra-acético
EFSA	European Food Safety Agency
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
FLD/FLR	Detectores de fluorescência
G418	Geneticina
GSH	Glutathione
GST	Glutathione-S-transferase
GRAS	Generally recognized as safe
H205	Histidina na posição 205 da estrutura da microcistinase A
H260	Histidina na posição 260 da estrutura da microcistinase A
HPLC	Cromatografia líquida de alta eficiência
IARC	International Agency for Research on Cancer
IN	Instrução normativa

INMETRO	Instituto Nacional de Metrologia, Normalização e Qualidade Industrial
IPTG	Isopropil β -D-tiogalactopiranosídeo
K _m	Constante de Michaelis-Menten
LB	Meio de cultivo Luria Broth
LioAc	Acetato de lítio
LMT	Limite máximo tolerado
LOD	Limite de detecção
LOQ	Limite de quantificação
MAT	Mating type locus
MBP	Maltose-binding protein
MC(s)	Microcistina(s)
MC-LA	Microcistina-leucina-alanina
MC-LF	Microcistina-leucina-fenilalanina
MC-LR	Microcistina-leucina-arginina
MC-RR	Microcistina-arginina-arginina
MC-YR	Microcistina-tirosina-arginina
MFC	Minas frescal cheese
MPL	Maximum permitted level
N264	Asparagina na posição 264 da estrutura da microcistinase A
OD	Optical density
OMS	Organização Mundial da Saúde
PCR	Polymerase chain reaction
PEG	Polietilenoglicol
PP1a	Protein phosphatase 1a
PVDF	Fluoreto de polivinilideno
R ²	Coeficiente de determinação
RDC	Resolução da diretoria colegiada
RSD	Relative standard deviation
SDS-PAGE	Eletroforese em gel de poliacrilamida com dodecil-sulfato de sódio
TE	Solução tampão de Tris e EDTA
UHPLC	Cromatografia líquida de ultra eficiência
UHT	Ultra high temperature

UPLC	Cromatografia líquida de ultra performance
MS/MS	Espectrometria de massas em tandem
USDA	United States Department of Agriculture
UV	Ultravioleta
Vis	Visível
Vmax	Velocidade máxima de reação
WHO	World Health Organization
YNB	Yeast Nitrogen Base
YPD	Yeast Peptone Dextrose

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1 INTRODUÇÃO

A presença de contaminantes em alimentos e água constitui uma grave ameaça à segurança alimentar e à saúde. No caso de toxinas naturais, estas são produzidas por diferentes organismos, e podem causar desde leve casos de intoxicação até ao desenvolvimento de câncer, considerando o contaminante envolvido (González-Jártin *et al.*, 2024).

Dentre as toxinas de relevância para humanos encontramos as microcistinas, heptapeptídeos cíclicos produzidos pelo metabolismo secundário de cianobactérias, especialmente do gênero *Microcystis*. Estas toxinas contaminam a água após eventos de floração de cianobactérias, e penetram na cadeia alimentar, expondo os humanos aos seus efeitos principalmente por meio da ingestão de água e alimentos contaminados (Pham *et al.*, 2020). Microcistinas são potentes substâncias hepatotóxicas estáveis em condições ambientais, levando a OMS a definir como limite de segurança a concentração máxima de 1 µg/L de MC-LR em água de consumo humano e ingestão diária tolerável de 0,04 µg/kg (WHO, 2002; Cheng *et al.*, 2021). No Brasil, a portaria nº888 de 4 de maio de 2021, do Ministério da Saúde, estabelece procedimentos de controle e vigilância para a água de consumo e seu padrão de potabilidade, determinando, entre outros parâmetros, o valor máximo permitido de 1 µg/L para o somatório de todas as variantes de microcistinas (BRASIL, 2021).

Além dos riscos imediatos decorrentes da intoxicação aguda, as microcistinas são consideradas como substâncias promotoras de câncer pertencentes ao grupo 2B de possíveis carcinogênicos humanos pela Agência Internacional de Pesquisa em Câncer (IARC, 2010). Com relação a processos usados no controle de microcistinas, algumas técnicas físico-químicas tradicionalmente aplicadas ao tratamento de água podem apresentar eficácia limitada na remoção destas toxinas, além de alto custo e resultar na formação de resíduos desconhecidos e tóxicos (Pham; Dang, 2018; Weir; Wood; Zimmer-Faust, 2021). Por outro lado, o controle biológico, exercido principalmente por enzimas microcistinases, demonstra bons resultados, com custo reduzido, elevada eficácia e sem gerar compostos de maior toxicidade (Kumar *et al.*, 2019). Microcistinases são enzimas que naturalmente degradam microcistinas e atuam em sequência resultando em pequenos peptídeos.

A primeira enzima desta via de degradação é codificada pelo gene *mlrA* e catalisa a linearização de microcistinas cíclicas, reduzindo o potencial tóxico e estabilidade das toxinas (Dexter *et al.*, 2020).

A partir da tecnologia de DNA recombinante foi possível aperfeiçoar a expressão da microcistinase A e conduzir novos estudos com o intuito de definir aplicações adequadas para a enzima. A maior parte dos microrganismos que naturalmente expressam o gene *mlrA* são bactérias, e por esse motivo, os estudos de expressão heteróloga do gene são comumente conduzidos em linhagens de *Escherichia coli* ou outras bactérias (Moolwang *et al.*, 2021). No entanto, o uso de leveduras para esta finalidade, principalmente de *Saccharomyces cerevisiae*, pode representar uma alternativa viável e eficaz. Considerando as características próprias da levedura, há uma ampla disponibilidade de técnicas de manipulação genética, aliadas ao fácil cultivo e elevada resistência do microrganismo (Valério *et al.*, 2014). Ademais, algumas linhagens são consideradas seguras para aplicação em alimentos (GRAS – *Generally Recognized as Safe*), o que induz a um grande potencial científico e de aplicação industrial (Lopes *et al.*, 2017). Deste modo, a obtenção de linhagens recombinantes de microrganismos com expressão do gene *mlrA* e seu efetivo emprego no biocontrole de microcistinas parece ser uma opção promissora.

Outro grupo de toxinas que representa atualmente uma grande ameaça à saúde humana é o das aflatoxinas. Estes compostos produzidos por fungos, principalmente *Aspergillus flavus* e *Aspergillus parasiticus*, são contaminantes de cereais e leguminosas. As aflatoxinas afetam os humanos diretamente através de alimentos contendo toxinas pré-formadas, e também a partir do consumo de seus metabólitos transmitidos aos produtos de origem animal (Mahato *et al.*, 2019). A Aflatoxina B₁, a mais prevalente e tóxica dentre as aflatoxinas, após ser consumida na alimentação animal é parcialmente convertida em Aflatoxina M₁ e transmitida para o leite de vaca (Benkerroum, 2020). Ambas as toxinas têm alto potencial hepatotóxico e são classificadas pelas IARC (*International Agency for Research on Cancer*) como substância do Grupo 1, carcinogênica para humanos (OSTRY *et al.*, 2017). As aflatoxinas apresentam estrutura química que as confere estabilidade, o que agrava o risco da presença destes contaminantes em alimentos, uma vez que o processamento não reduzirá significativamente as suas concentrações (Iha *et al.*, 2013). Considerando que a descontaminação de aflatoxinas envolve processos complexos e impraticáveis em alimentos, as medidas de mais efetivo controle se

baseiam no monitoramento da contaminação (Wang *et al.*, 2019). Com base no risco, alguns alimentos como leite e queijo têm limites máximos tolerados de AFM₁ estabelecidos em 0,5 e 2,5 µg/kg, respectivamente, pela ANVISA (Brasil, 2022). Assim, para garantir que os alimentos obedecem a estas determinações legais e são seguros para consumo, frequente monitoramento deve ser realizado, o que demanda maior investimento no desenvolvimento e disponibilização de métodos práticos de análise (Shabeer *et al.*, 2022).

Independentemente da origem ou do tipo de toxina, o controle destes compostos em água e alimentos é essencial, e a redução de riscos aos humanos demanda esforços direcionados ao monitoramento de contaminantes e aplicação de possíveis medidas de descontaminação. As políticas públicas envolvidas devem estabelecer critérios de segurança para cada composto com base no risco. Programas de vigilância devem ser promovidos, e recursos empenhados para o desenvolvimento, aprimoramento e implantação dos métodos de descontaminação, a fim de assegurar a saúde da população (Thompson; Darwish, 2019).

Neste sentido, a presente tese teve como objetivo promover avanços científicos em métodos de controle de algumas toxinas naturais. A primeira parte do trabalho buscou construir uma cepa recombinante de *Saccharomyces cerevisiae* com a expressão de gene *mlrA* e consequente produção de microcistinase A. Diferentes técnicas de DNA recombinante foram testadas para esta finalidade. Além disso, a partir da levedura recombinante, os perfis de atividade e estabilidade da enzima obtida foram avaliados para determinar condições adequadas para a sua aplicação. A última parte do trabalho buscou desenvolver e validar um método de UPLC-FLR para detecção simultânea de AFB₁ e AFM₁ em queijo minas frescal. O método foi utilizado para análise destas toxinas em amostras de queijo. Os experimentos aqui realizados produziram resultados que podem ser úteis a novos estudos e para a aplicação prática no controle de toxinas naturais em água e alimentos.

2 OBJETIVOS

2.1 OBJETIVO GERAL

- Promover avanços científicos e de potencial tecnológico para o controle de toxinas naturais, com foco na biodegradação de microcistinas empregando tecnologia de DNA recombinante e método de monitoramento de aflatoxinas em queijos frescos.

2.2 OBJETIVOS ESPECÍFICOS

- Construir linhagem recombinante de *Saccharomyces cerevisiae* GRAS com expressão do gene *mlrA* baseado no genoma de *Sphingosinicella microcystinivorans* B9, como alternativa para o biocontrole de microcistinas.
- Investigar a expressão de *mlrA* por células da levedura recombinante, a partir de diferentes técnicas, como expressão plasmidial e integração cromossomal.
- Avaliar o potencial de biodegradação das linhagens recombinantes de levedura produtora de microcistinase A.
- Extrair microcistinase A expressa por levedura recombinante e proceder uma caracterização parcial da enzima, incluindo possíveis alterações estruturais, perfil de atividade e estabilidade da enzima em diferentes condições de temperatura e pH.
- Implementar e validar método de detecção de aflatoxinas em queijo minas frescal por UPLC-FLR.
- Investigar a contaminação por aflatoxinas em amostras de queijo minas frescal por meio de método previamente desenvolvido e validado.

3 REVISÃO DE LITERATURA

3.1 TOXINAS NATURAIS CONTAMINANTES DE ALIMENTOS E ÁGUA

Toxinas naturais são compostos de estruturas e funções diversas, produzidas por diferentes organismos e microrganismos, geralmente para conferir vantagens à sobrevivência destes seres (Nwaji *et al.*, 2023). Dentre os potenciais produtores de toxinas naturais, encontramos muitas espécies de animais, plantas, fungos, bactérias e algas, resultando em uma ampla variedade de substâncias que podem afetar os humanos através da alimentação e promover danos à saúde (Picardo *et al.*, 2019). Existem mais de 20 mil compostos bioativos reconhecidos como tóxicos para os seres humano, com diferentes modos de ação e níveis de severidade em casos de intoxicação (Hansen; Hilscherova; Bucheli, 2021).

Destacam-se entre os variados tipos de toxinas naturais, as cianotoxinas e as micotoxinas. Cianotoxinas são produzidas por vários gêneros de cianobactérias e consideradas importantes contaminantes de água. A exposição humana se dá principalmente pela ingestão direta, contato com água contaminada, ou através de alimentos que bioacumularam as toxinas, como no caso de uso de água contaminada para irrigação. Os efeitos da intoxicação na saúde humana são variados, e a depender do tipo de toxina envolvida, resultam em afecções hepáticas, neurológicas e dermatológicas (Mutoti *et al.*, 2023). Quanto às micotoxinas, estas são metabólitos secundários produzidos por fungos filamentosos e frequentemente encontradas como contaminantes de alimentos. A exposição humana às micotoxinas se dá principalmente por meio da ingestão de alimentos contaminados direta ou indiretamente. A intoxicação humana resulta em várias desordens de saúde dependentes, dentre outros fatores, da toxina envolvida e causando desde leves sintomas gastrointestinais ao desenvolvimento de câncer (González-Jártin *et al.*, 2024).

Além da clara ameaça à saúde de animais e humanos, toxinas naturais causam importantes perdas econômicas e sociais. Apesar da dificuldade de se mensurar precisamente os danos causados por estes compostos, alguns poucos estudos reportaram estimativas de perdas devido às micotoxinas, ficotoxinas e cianotoxinas. Os custos gerados englobam o impacto direto na saúde pública, perda de produtividade animal e humana, incluindo os investimentos necessários para o

monitoramento e controle destes contaminantes (Anderson *et al.*, 2000; Hamilton *et al.*, 2014; Ajani; Harwood; Murray, 2017; Udomkun *et al.*, 2017).

Diante disso, programas de monitoramento estão sendo desenvolvidos para algumas toxinas em nível nacional e internacional. Tais ferramentas baseiam-se na análise de produtos e na determinação da concentração de contaminantes em alimentos e água. Os programas objetivam garantir que os alimentos consumidos apresentem níveis seguros de contaminação, definidos, por exemplo, pelo *Codex Alimentarius*. Limites de segurança são definidos com base em evidências científicas e na avaliação de risco considerando a toxina envolvida e os alimentos mais predispostos à contaminação (Thompson e Darwish, 2019). Neste mesmo sentido, as recomendações do CODEX STAN 193-1995 (última revisão em 2009) foram direcionadas à prevenção da contaminação na origem, busca por medidas de controle e descontaminação, e para o estabelecimento e manutenção de planos de vigilância a fim de evitar a comercialização dos alimentos contaminados fora dos padrões de segurança.

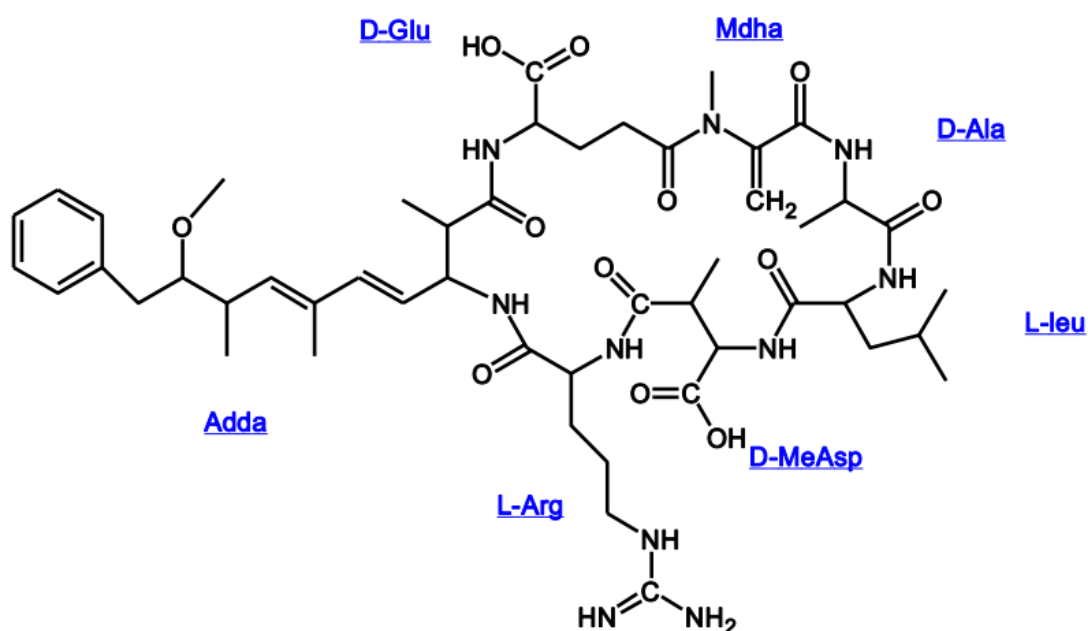
3.2 MICROCISTINAS

As microcistinas são heptapeptídeos cíclicos, produtos do metabolismo secundário de cianobactérias. Dentre os gêneros produtores de microcistinas, destacam-se a *Anabaena* sp., *Aphanizomenon* sp., *Oscillatoria* sp., e principalmente *Microcystis* sp., sendo a espécie *Microcystis aeruginosa* uma das principais relacionadas a eventos de floração e acumulação de toxinas. A contaminação de fontes de água por microcistinas se torna mais abundante após a floração (*bloom*) das cianobactérias, com multiplicação celular exacerbada e liberação das toxinas (Zhou *et al.*, 2021). O processo de floração é favorecido em temperaturas elevadas, naturalmente ocorrendo durante o verão em áreas temperadas, com possibilidade de ocorrer durante todo o ano em regiões tropicais e subtropicais (Pham *et al.*, 2020). Outro fator determinante para a floração das cianobactérias é a disponibilidade de nutrientes na água. A eutrofização de lagos e de outras fontes de água fornece condições para a multiplicação intensa destes microrganismos, e consequentemente resulta em maior chance de contaminação e de altos níveis acumulados de microcistinas. A combinação da crescente eutrofização de fontes de água, com o aumento da temperatura média ocasionado pelas mudanças climáticas globais, deve contribuir para um aumento na ocorrência de eventos de floração de cianobactérias, agravando o impacto das microcistinas na sociedade (Lürling; Van Oosterhout; Faasen, 2017).

Mais de 250 análogos de microcistinas já foram identificados, com os mais diversos efeitos na saúde humana. A principal e mais estudada variante de microcistina é a MC-LR. Devido a alta prevalência e potencial tóxico deste congênera, comumente as informações relativas à toxicidade de MC-LR são extrapoladas para todo o grupo de microcistinas (Preece *et al.*, 2021). Quimicamente, as microcistinas são formadas por sete aminoácidos que se fecham em um ciclo, o que confere elevada estabilidade à estrutura. Dois dos aminoácidos que formam a molécula são variáveis, os de posição 2 e 4. O nome dado a cada análogo de microcistina depende dos aminoácidos que ocupam estas posições (Birbeck *et al.*, 2019). Na Figura 1, publicada por Wang, Jiao e Rao (2021) foi ilustrada a estrutura da MC-LR, com os aminoácidos L-Leucina (L) na posição 2 e L-Arginina (R) na posição 4.

Apesar de a ingestão de água ser a principal via de contaminação para humanos, as microcistinas também podem ser transmitidas através de alimentos contaminados. A irrigação de cultivos agrícolas com água contaminada resulta na acumulação de toxinas em raízes, folhas e demais tecidos vegetais (Haida *et al.*, 2024). Peixes, moluscos e outros animais criados em ambiente contaminado bioacumulam microcistinas, que são transmitidas através da cadeia alimentar, podendo atingir os humanos (WHO, 2020). Adicionalmente, o contato direto com águas recreacionais também representa uma forma de exposição que deve ser considerada (Massey *et al.*, 2018).

Figura 1. Estrutura química da MC-LR.



Fonte: Wang, Jiao e Rao (2021).

Casos de intoxicação aguda em humanos são raros, uma vez que seria necessária a exposição a concentrações elevadas de microcistinas, normalmente encontradas somente em águas com evidente floração de cianobactérias. Ainda assim, em 1996 em Caruaru-PE, no Brasil, ocorreu o maior surto de intoxicação por microcistinas já relatado. Neste evento, 131 pessoas que faziam tratamento de doenças renais em uma clínica foram expostas às toxinas através da água utilizada na hemodiálise. A origem da contaminação foi um reservatório hídrico onde foi observada a floração de cianobactérias, e de onde a água contaminada foi coletada

e usada no abastecimento da clínica sem o devido tratamento. Os pacientes relataram mal-estar, sintomas neurológicos e gastrointestinais, dor abdominal e hepatomegalia. O surto resultou na morte de 52 pessoas nos meses seguintes à contaminação (Azevedo *et al.*, 2002).

Mais frequentemente, a exposição humana às microcistinas ocorre em baixos níveis de contaminação, porém, durante longos períodos. Como efeitos da exposição crônica, normalmente são observados sinais de lesão hepática, com alterações nos níveis séricos de enzimas e redução no metabolismo de lipídeos, resultando em doenças hepáticas crônicas (Zhao *et al.*, 2020). Alterações na função renal e o desenvolvimento de câncer também são associados com a exposição crônica às microcistinas (Yao *et al.*, 2023; Lad *et al.*, 2022). De acordo com a IARC (2010), as microcistinas são consideradas substâncias promotoras de câncer e classificadas como possivelmente carcinogênicas para humanos (grupo 2B), principalmente devido ao seu potencial de inibição de proteínas fosfatase 1 e 2a. Considerando a séria ameaça que estas toxinas trazem à saúde humana, a OMS definiu como limite de segurança a concentração máxima de 1 µg/L de MC-LR em água de consumo humano, e ingestão diária tolerável de 0,04 µg/kg de peso corporal (WHO, 2002; Cheng *et al.*, 2021).

Dados os riscos que as microcistinas representam para a saúde pública, e a existência de limites de segurança estabelecidos para estes contaminantes, frequente monitoramento dos níveis de contaminação deve ser realizado para garantir a inocuidade de alimentos e água de consumo. Métodos cromatográficos, imunoenzimáticos, e ensaios de inibição de proteína fosfatase 1a (PP1a) estão entre os principais disponíveis para a detecção e quantificação destas toxinas (He *et al.*, 2022). Somam-se aos métodos de detecção, os procedimentos de descontaminação com o intuito de promover um efetivo controle de microcistinas e minimizar os impactos à população.

3.2.1 Métodos de Controle de Microcistinas

O principal objetivo do controle de microcistinas é assegurar que a água utilizada para consumo humano, fins agrícolas, industriais e recreacionais esteja livre de níveis perigosos de toxinas. Para que este controle seja efetivo, o monitoramento deve ser frequente em fontes de água susceptíveis à multiplicação

de cianobactérias e contaminação por microcistinas. Ao mesmo tempo, os métodos de tratamento de água devem incluir técnicas capazes de reduzir as concentrações de microcistinas a níveis seguros (Massey; Yang, 2020). Contudo, devido às características próprias da molécula, e considerando as dificuldades de se proceder abrangente e rotineiro monitoramento destas toxinas, o controle efetivo acaba sendo uma tarefa complexa.

As microcistinas são moléculas estáveis em condições ambientais que mantêm sua estrutura e potência por longos períodos. Em condições naturais a degradação destas toxinas ocorre lentamente, com tempo de meia-vida estimado em 10 semanas (Opoku-Duah *et al.*, 2022). Sob temperaturas de até 40°C e em pH próximo à neutralidade, as microcistinas mantêm-se inalteradas durante várias semanas (Harada *et al.*, 1996; Le *et al.*, 2021).

Com relação às técnicas convencionalmente aplicadas ao tratamento de água, a eficácia no controle de microcistinas é limitada. Quando a contaminação ainda está restrita ao interior das cianobactérias, é possível que os procedimentos usuais de remoção de microrganismos eliminem a maior parte das células, evitando assim a contaminação da água. Por outro lado, eliminar as toxinas já liberadas na água após a lise celular é um processo mais complexo, e por este motivo, o uso de algicidas não é indicado para eliminar os microrganismos após casos de floração, uma vez que a lise celular liberaria as toxinas para o meio (Hitzfeld *et al.*, 2000; Weir; Wood; Zimmer-Faust, 2021).

Alguns métodos tradicionalmente aplicados no tratamento de água como coagulação, floculação, sedimentação e filtração demonstram resultados positivos na eliminação de células de cianobactérias. No entanto, estas etapas podem apresentar restrições de uso e a mesma eficácia não é observada para remoção de microcistinas extracelulares. Durante os processos há risco considerável de lise celular, liberando mais toxinas na água (Pham; Dang, 2018). Etapas adicionais incluídas no tratamento de água como ultrafiltração por membranas e adsorção de moléculas em carvão ativado aumentam a remoção de microcistinas de água. No entanto, não há atualmente um único método completamente eficaz na remoção das toxinas, sendo os melhores resultados obtidos com a combinação de diferentes técnicas (Sengul; Ersan; Tufekçi, 2018).

Outras técnicas de natureza físico-química utilizadas no tratamento de água apresentam resultados variáveis. A fotocatalise resulta em altas taxas de remoção,

mas, assim como no uso de membranas filtrantes, a técnica demanda muita energia e tem elevados custos de manutenção. Por outro lado, o uso de ozônio e cloração em condições específicas pode apresentar boa atividade na remoção de microcistinas, mas também resultar na formação de outros compostos tóxicos. Portanto, as alternativas físico-químicas para redução na contaminação de microcistinas são em alguns casos eficazes, mas encontram limitações que requerem a busca de soluções mais seguras e sustentáveis (Kumar *et al.*, 2018).

Alternativamente, métodos biológicos de controle de microcistinas apresentam adequada eficácia com custos relativamente menores e menor risco da formação de subprodutos tóxicos. Estes métodos se sustentam na capacidade de alguns seres vivos, principalmente bactérias, de inibir o crescimento de cianobactérias e degradar microcistinas (Mohamed *et al.*, 2021). Especificamente na biodegradação de microcistinas, enzimas naturalmente produzidas por microrganismos são capazes de catalisar reações que modificam a estrutura das microcistinas gerando produtos de menor toxicidade ou não tóxicos. Apesar das vantagens apresentadas pelos métodos biológicos em comparação com os físico-químicos, as taxas de reação normalmente são menores, ou seja, requerem maior tempo para obter significativo nível de degradação, o que pode implicar custos adicionais de implantação de sistemas efetivos de tratamento (Kumar *et al.*, 2019). Ainda assim, estes métodos são promissores e foco de muitas pesquisas na área de biocontrole de microcistinas.

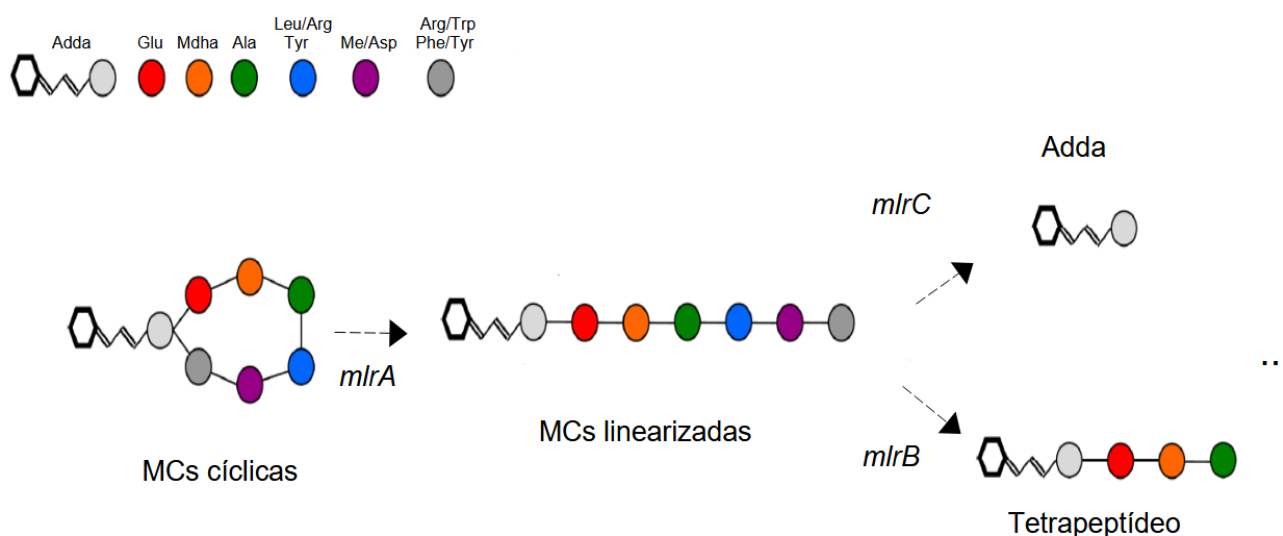
3.2.2 Microcistinases e a Biodegradação de Microcistinas

Alguns microrganismos possuem naturalmente genes que codificam enzimas associadas à biodegradação de microcistinas. Entre as bactérias que apresentam essa capacidade se destacam *Sphingomonas* spp., *Sphingopyxis* spp., *Novosphingobium* spp., *Bacillus* spp., *Acinetobacter* spp. e *Pseudomonas* spp. A maior parte dos processos já descritos de controle biológico de microcistinas em água envolve bactérias que expressam peptidases codificadas pelo *cluster* de genes *mlr* (He *et al.*, 2022; Santos *et al.*, 2024).

O primeiro gene deste sistema, denominado *mlrA*, codifica uma peptidase (microcistinase A) que foi descrita pela primeira vez por Bourne *et al.* (1996) e desencadeia o processo de biodegradação de microcistinas. A ação da

microcistinase catalisa a hidrólise da ligação peptídica entre Adda e o aminoácido variável adjacente da estrutura cíclica da toxina. Como resultado, microcistinas linearizadas são formadas e ficam expostas às outras enzimas do *cluster mlr*, produzindo peptídeos menores e de reduzida toxicidade (Alqhtani, 2011). Na Figura 2, é apresentada a parte inicial da via de biodegradação de microcistinas por *mlrABCD*.

Figura 2. Representação parcial da via de biodegradação de microcistinas por microcistinases – *mlrABCD*.



Fonte: adaptado de Dziga *et al.* (2013).

As microcistinas cíclicas são linearizadas pela microcistinase A, e então convertidas em um tetrapeptídeo pela enzima codificada por *mlrB*. Em ambas as etapas ocorre hidrólise da cadeia peptídica. No caso da *mlrB*, o alvo da enzima é a ligação entre o primeiro aminoácido (alanina) e o segundo aminoácido (variável) da cadeia linear. Na sequência, a enzima *mlrC* atua na quebra do tetrapeptídeo em peptídeos menores ou aminoácidos isolados, enquanto *mlrD* parece ter função de auxiliar no transporte e eliminação das microcistinas e dos produtos da biodegradação (Haida *et al.*, 2024). Outros dois genes, *mlrE* e *mlrF*, foram reportados no genoma da bactéria *Sphingopyxis* sp. linhagem C-1, como enzimas que atuam na sequência de degradação de microcistinas (Okano *et al.*, 2015). Posteriormente, Jin *et al.* (2018a) e Wang *et al.* (2018) relataram os mesmos genes em *Sphingosinicella microcystinivorans* B9 e *Novosphingobium* sp. THN1. Ainda que

esta via de degradação siga uma sequência em comum, diferentes produtos podem ser gerados. A depender da microcistina envolvida, da cepa bacteriana, e consequentemente, da presença dos genes específicos *mlr*, variados compostos intermediários são obtidos.

Independentemente dos produtos finais gerados por esta via de biodegradação, o início do processo é sempre dependente da microcistinase A. Essencial para desencadear a biodegradação de microcistinas, esta enzima é encontrada em bactérias como uma proteína integral da membrana plasmática que forma uma cavidade aberta para o espaço periplasmático. Acredita-se que a atividade catalítica da enzima seja determinada pelos resíduos de aminoácidos E172, H205, H260 e N264 (Yu *et al.*, 2021). Com relação a classificação da enzima, os dados disponíveis atualmente ainda são controversos. Nos primeiros estudos publicados sobre a enzima, Bourne *et al.* (2001) sugeriram que *mlrA* codifica uma metaloprotease. Por outro lado, estudos mais recentes questionaram essa classificação, indicando que a enzima apresenta similaridade com prenil-endopeptidases do tipo CAAX II (Xu *et al.*, 2019; Sun *et al.*, 2021). Assim, fica evidente que o tema ainda carece de estudos, para aprofundar os conhecimentos acerca da enzima e definir com maior certeza as suas características estruturais e químicas, além de aspectos relacionados à atividade e estabilidade enzimática.

As microcistinas lineares produzidas a partir da ação da microcistinase A têm reduzida toxicidade, estimada em até 2100 vezes menor que as toxinas cíclicas (Dziga *et al.*, 2012; Dexter *et al.*, 2018). Em outros estudos, esta redução foi estimada próxima de 160 vezes menor quando comparada à toxina original (Bourne *et al.*, 1996). Os testes usados para esta análise geralmente comparam a inibição de proteínas fosfatase entre microcistinas cíclicas e lineares. Ainda que não haja um consenso sobre a exata toxicidade de microcistinas lineares, não resta dúvidas que a microcistinase A tem papel fundamental na redução de riscos associado às microcistinas, por gerar compostos de reduzido potencial tóxico e menor estabilidade (Thenuwara; Westrick; Isailovic, 2024).

Como exemplo de espécie bacteriana que naturalmente expressa as microcistinases, destaca-se a *Sphingosinicella microcystinivorans* B9. Esta bactéria isolada do lago Tsukui, no Japão, apresenta notável capacidade natural de degradação de microcistinas. O sequenciamento do genoma completo da B9 publicado por Jin *et al.* (2018a), confirmou a presença do *cluster mlrCADBEF*

(conforme a ordem dos genes). Os mesmos autores relataram que o gene *mlrA* da B9 possui 100% de similaridade com o gene de *Rhizobium* sp. TH e 89% para *mlrA* da linhagem C1 de *Rhizobium* sp. Como a descoberta da B9 é relativamente recente, poucos estudos foram conduzidos até este momento, e os dados sobre a bactéria ainda são escassos. No entanto, os resultados de experimentos já publicados demonstraram que a bactéria tem grande potencial de aplicação. Harada *et al.* (2004) comprovaram avançada degradação de MC-LR evidenciado pelo isolamento de resíduos do aminoácido Adda após o uso de extratos obtidos de cultivos de B9. Também, Tsuji *et al.* (2006) utilizaram células de B9 imobilizadas em resina de poliéster e verificam total degradação das MC-LR e MC-RR adicionadas aos ensaios. Em outro estudo, 98% das microcistinas foram degradadas após 96 horas de incubação com a B9 (Kuriama *et al.*, 2012). Ainda que poucos dados estejam disponíveis, é comprovada e expressiva a ação da *Sphingosinicella microcystinivorans* B9 na degradação de microcistinas, o que fomenta novos estudos para viabilizar a aplicação da própria bactéria ou de seu potencial genético no controle de toxinas.

3.2.3 DNA Recombinante e Manipulação do Gene *mlrA*

Técnicas de DNA recombinante podem ser usadas para a expressão heteróloga de genes com o propósito de aperfeiçoar a expressão, aumentar o rendimento de proteína produzida e promover avanços no conhecimento (Khan *et al.*, 2016). Para estudar genes e proteínas pouco conhecidos, a possibilidade de transferir sua produção para outros microrganismos pode promover significativos avanços em menor tempo. Quando o hospedeiro escolhido tem reconhecida aptidão para a engenharia genética, uma maior disponibilidade de técnicas descritas de manipulação do DNA proporciona maiores chances de sucesso na expressão heteróloga do gene. Ainda neste sentido, para que a abordagem de recombinação de DNA seja efetiva, é necessário considerar vários aspectos, como a seleção de hospedeiro, tipos de vetores usados, método de transformação e marcadores usados para seleção das células transformadas. Todas estas condições são importantes para que a estratégia escolhida de recombinação gênica obtenha os resultados esperados. (Deckers *et al.*, 2020; Pouresmaeil; Azizi-Dargahlou, 2023).

Com relação aos hospedeiros, as bactérias são muito utilizadas para recombinação gênica devido à baixa complexidade e menores custos associados ao seu uso. Além disso, há um grande quantidade de estudos publicados sobre recombinação gênica nestes microrganismos, o que fornece os dados necessários para definir estratégias de sucesso na expressão heteróloga de genes. Como espécie bacteriana muito utilizada na produção de proteínas recombinantes se destaca a *Escherichia coli* (Kleiner-Grote; Risse; Friehs, 2018). Por outro lado, apesar de apresentarem maior complexidade celular, os fungos podem induzir modificações pós-traducionais, como glicosilação, enovelamento ou pontes dissulfeto, que podem interferir na função, localização e estabilidade da proteína, sendo estas desejáveis em alguns casos. Considerando especificamente o uso de leveduras, características como, baixo custo de produção, facilidade e segurança na manipulação, e elevada produção de biomassa, estimulam que células de *Saccharomyces cerevisiae*, por exemplo, sejam frequentemente empregadas em técnicas para recombinação gênica (Popova *et al.*, 2023).

S. cerevisiae é o microrganismo eucarioto mais bem estudado e empregado na engenharia genética. Muitas alternativas de técnicas de manipulação e expressão de genes heterólogos nestes microrganismos estão descritas na literatura. O interesse por esta levedura se baseia na segurança e facilidade de seu cultivo, além de características como metabolismo eficiente e alta resistência. Além do mais, algumas linhagens de *S. cerevisiae* são consideradas como GRAS para aplicação em alimentos, demonstrando assim adequado potencial para uso na indústria de alimentos (Valério *et al.*, 2014; Lopes *et al.*, 2017).

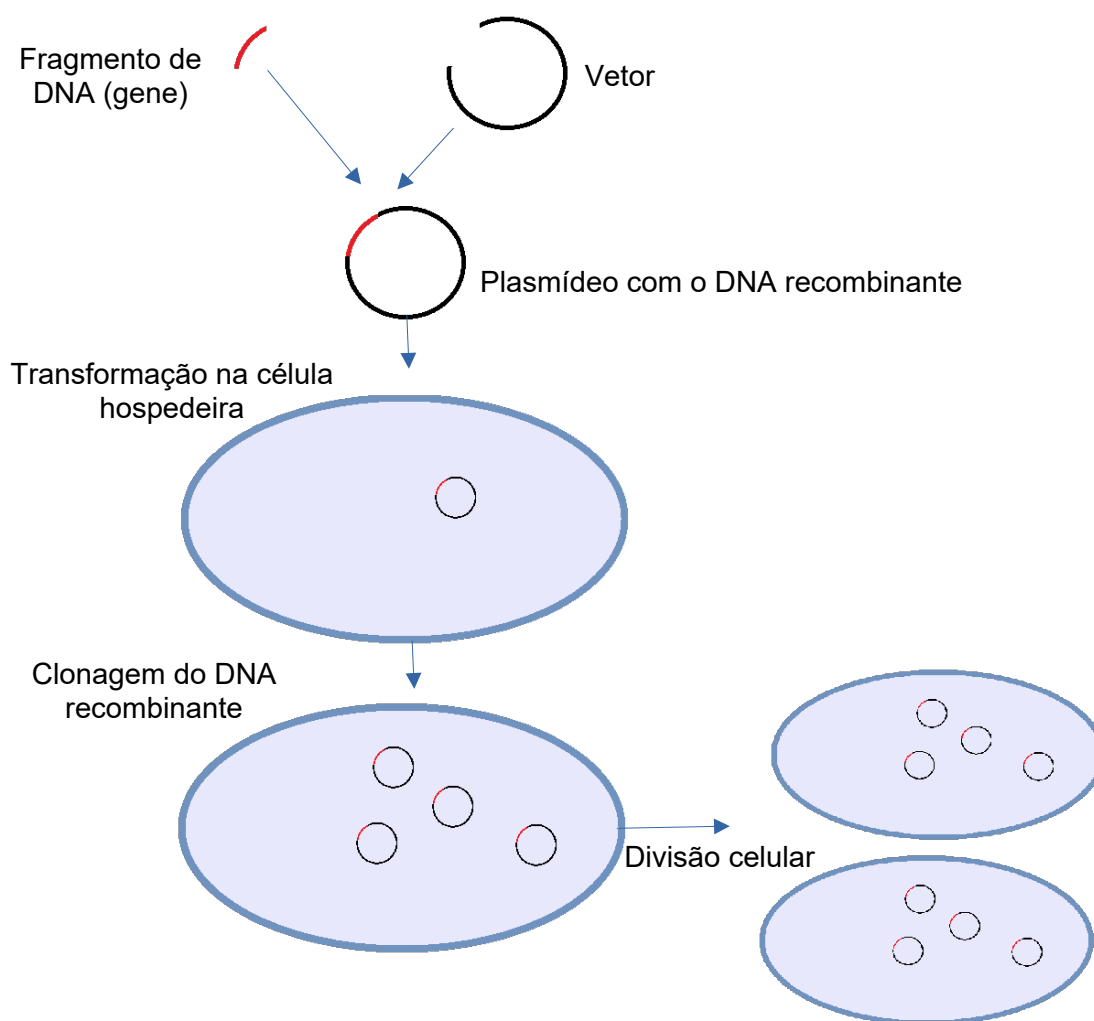
A partir da tecnologia de DNA recombinante, e por meio do uso de plasmídeos foi possível disseminar no meio científico a expressão de proteínas heterólogas. Estas estruturas são utilizadas como vetores de clonagem, expressão ou sinalização do gene de interesse (Nora *et al.*, 2019). Na Figura 3 foi representada a clonagem de um determinado gene, uma das técnicas utilizadas na tecnologia de DNA recombinante. Para recombinação no DNA cromossomal em eucariotos, técnicas tradicionais ainda são muito empregadas considerando as altas taxas de sucesso. Estas técnicas são baseadas principalmente no pareamento de regiões homólogas e integração do DNA recombinante (Yang; Blenner, 2020).

Com relação à expressão heteróloga do gene *mlrA*, a maioria dos estudos descreveram experimentos conduzidos em bactérias como hospedeiros. Este fato é

esperado uma vez que o gene é natural, segundo a maior parte dos relatos atuais, de espécies bacterianas. Neste sentido, trabalhos publicados por Moolwang *et al.* (2021) e Liu *et al.* (2020), demonstraram efetiva expressão de *mlrA* em células de *E. coli*. Ainda que o hospedeiro escolhido tenha sido o mesmo em ambos estudos supracitados, é importante destacar que diferentes níveis de proteína são obtidos por diferentes linhagens de hospedeiros e distintas estratégias de recombinação.

Um dos objetivos da expressão de *mlrA* em outros organismos é obter maior rendimento na produção da enzima, seja para estudos aprofundados ou aplicação direta na degradação de microcistinas. Dziga *et al.* (2012) conseguiram expressar com sucesso *mlrA* em diferentes linhagens de *E. coli* e *Staphylococcus aureus*, resultando em um considerável aumento na expressão do gene.

Figura 3. Exemplo de clonagem gênica.



Fonte: adaptado de Ijaz e Haq (2019).

Até o atual momento, a expressão da microcistinase A em leveduras somente havia sido relatada por Broman *et al.* (2017), com confirmação que a enzima foi produzida por células de *S. cerevisiae*. A partir da comprovação de que a levedura é capaz de expressar estas enzimas funcionais, novos estudos devem indicar linhagens mais apropriadas, e definir técnicas melhoradas de DNA recombinante, para possivelmente implementar leveduras no biocontrole de microcistinas. De um modo geral, o conhecimento acerca da microcistinase A ainda é escasso, e para se aprofundar nos estudos e estabelecer aplicações práticas desta enzima, o uso das tecnologias de DNA recombinante parece ser o caminho mais efetivo.

3.3 AFLATOXINAS

Aflatoxinas são micotoxinas produzidas por *Aspergillus spp.* e contaminantes de uma ampla variedade de culturas agrícolas. *Aspergillus flavus* e *Aspergillus parasiticus* são as principais espécies associadas à colonização de plantas e produção de aflatoxinas. A contaminação de cereais e outros cultivos agrícolas por estas toxinas representa uma grave ameaça à saúde, e que também implica severos prejuízos econômicos (Jallow *et al.*, 2021).

Cereais podem ser contaminados ainda no campo, isto é, durante o crescimento da planta, ou no pós-colheita, nas etapas de armazenamento, transporte e processamento dos grãos. Entre os fatores que influenciam no risco de infecção fúngica de plantas e consequente produção de aflatoxinas, se destacam temperatura, umidade e a composição do substrato (Yunes *et al.*, 2020). Em estudo conduzido por Medina *et al.* (2017) foi demonstrado que a interação de alta temperatura com atividade de água elevada resulta em expressiva produção de aflatoxinas em grãos de milho contaminados por *A. flavus*. Considerando as condições ambientais de temperatura média e umidade relativa do ar, observa-se que as regiões de clima tropical e subtropical são as mais favoráveis ao crescimento de fungos produtores de aflatoxinas (Brown, 2018). Uma preocupação crescente no atual cenário de mudanças climáticas consiste no maior risco de exposição às aflatoxinas. Maiores temperaturas médias e eventos climáticos extremos devem expor os cultivos agrícolas de uma área muito mais ampla que a atual ao estresse térmico e desbalanço hídrico. Estas condições adversas reduzem o potencial de defesa das plantas ao ataque fúngico, e amplificam os prejuízos decorrentes da contaminação por aflatoxinas (Yu *et al.*, 2022). Levando em conta que o Brasil já apresenta na maior parte de seu território condições climáticas favoráveis à proliferação de fungos produtores de micotoxinas, a tendência é que o problema causado por aflatoxinas se acentue com as mudanças climáticas (Dos Santos *et al.*, 2021).

Durante o crescimento das plantas recomenda-se que sejam aplicadas boas práticas agrícolas, diminuindo o risco de ataque fúngico e contaminação de grãos. Estas práticas envolvem medidas que partem da escolha de variedades mais resistentes ao estresse hídrico e ao ataque de pragas, descarte de grãos ardidos, até o processo de secagem e armazenamento (Xu *et al.*, 2022). Após a colheita, os

grãos ainda podem ser contaminados caso sejam mantidas condições adequadas à proliferação fúngica. Processos inadequados de seleção e secagem de grãos mantêm as condições propícias ao crescimento fúngico. Além disso, a estocagem por longos períodos em ambiente com temperatura e umidade elevadas, também favorecem a proliferação de *Aspergillus spp.* e podem culminar em expressiva contaminação dos grãos por aflatoxinas (Waliyar *et al.*, 2015).

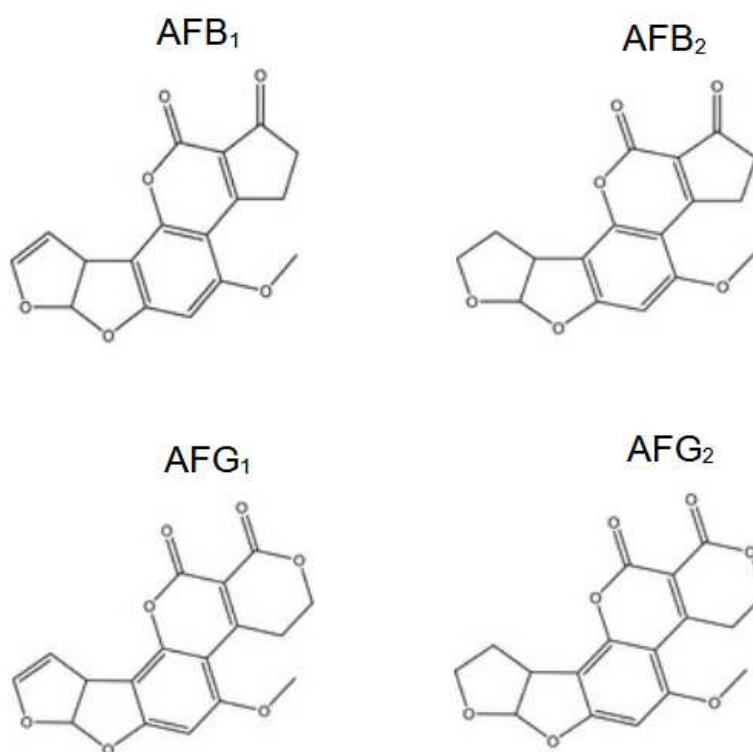
A grande preocupação quanto às aflatoxinas e sua relação com a saúde humana está no fato de que estes compostos possuem alto potencial tóxico e estão associadas à carcinogênese. Como agravante, apesar de ser um contaminante comum de cereais, aflatoxinas já foram detectadas em oleaginosas, frutas secas, carne, leite e derivados. Ou seja, a exposição humana às aflatoxinas por meio da dieta pode ocorrer através de uma grande variedade de alimentos. Além do risco no consumo direto, a alimentação contaminada quando destinada aos animais, expõe os humanos ao risco de ingestão das toxinas e de seus metabólitos tóxicos por meio de produtos de origem animal (Mahato *et al.*, 2019).

Quimicamente, as aflatoxinas são moléculas derivadas de policetídeos formadas pelo metabolismo secundário de *Aspergillus spp.* (Payne; Brown, 1998; Kumar *et al.*, 2017). São conhecidas mais de 18 aflatoxinas, entre toxinas de ocorrência natural e derivadas de processos metabólicos. Os principais análogos conhecidos são AFB₁, AFB₂, AFG₁ e AFG₂ (Figura 4). AFB₁ é a mais prevalente e também a de maior toxicidade. A partir do metabolismo hepático de AFB₁ é produzida AFM₁, outra aflatoxina de importância, sobretudo devido à contaminação presente em leite e derivados (Benkerroum, 2020). Este análogo hidroxilado pode ser excretado no leite, urina e estar presente em tecidos e outras secreções. A presença de AFM₁ no leite de vaca e materno alerta para graves riscos de intoxicação em crianças e recém-nascidos (Barzoki *et al.*, 2023; Dogan; Afacan; Ozdemir, 2022).

Apesar de não ser comum em humanos, a exposição à elevadas concentrações de aflatoxinas em um curto período de tempo pode causar intoxicação aguda. Nestes casos, observa-se extensa lesão hepática, o que leva a sintomas como febre, náuseas, vômitos, dor abdominal, distúrbios gastrointestinais, edema, confusão mental, e morte em cerca de 25% dos casos de aflatoxicose aguda (Dhakal e Hashimi, 2020). Por outro lado, a exposição crônica está relacionada a sinais pouco específicos como imunossupressão, deficiência no crescimento,

teratogenicidade e neurotoxicidade. Além disso, a ingestão de doses sub-letais de aflatoxinas por longos períodos está associada ao desenvolvimento de câncer, principalmente de carcinoma hepatocelular (Dabuo *et al.*, 2022). As aflatoxinas foram classificadas pela IARC como substâncias carcinogênicas para humanos do grupo 1 (OSTRY *et al.*, 2017), o que reforça a necessidade de se monitorar a presença destes contaminantes em alimentos.

Figura 4. Estrutura química das quatro principais aflatoxinas encontradas na natureza.



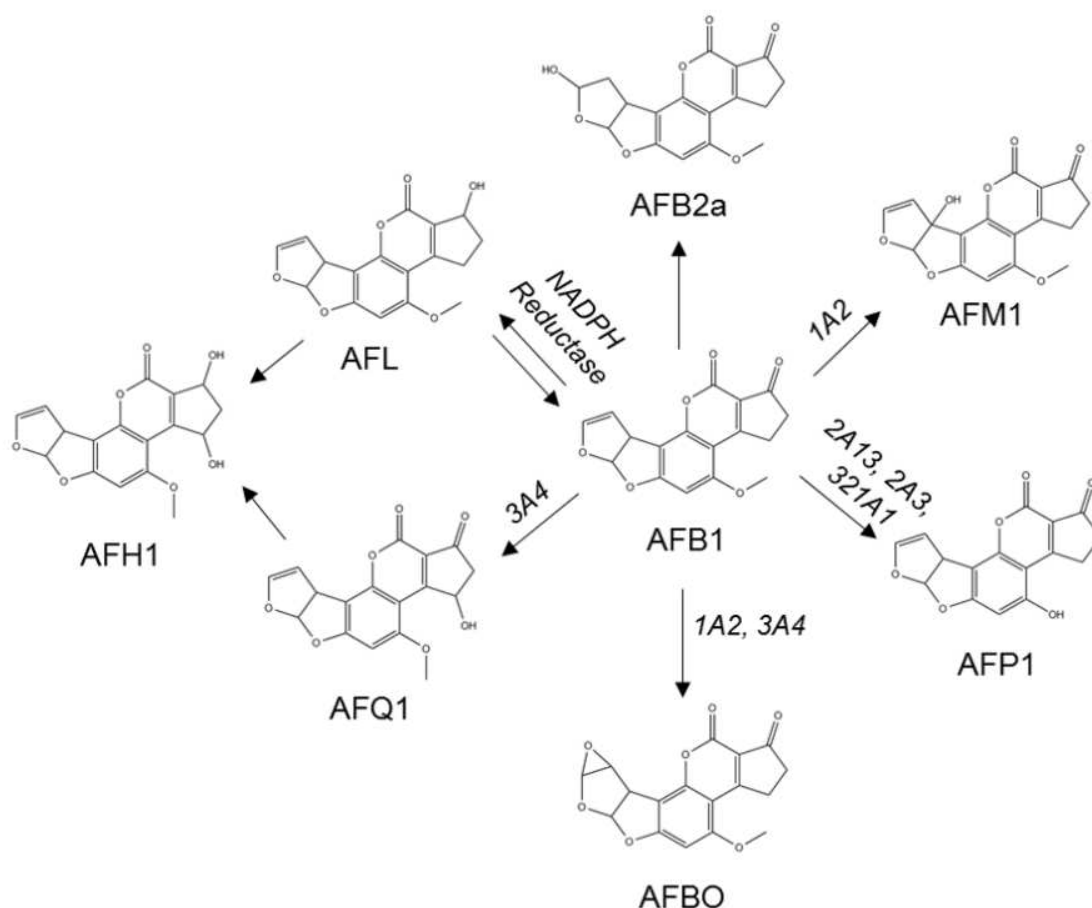
Fonte: adaptado de EFSA (2020).

3.3.1 Metabolismo da Aflatoxina B₁

Devido à maior prevalência e elevada toxicidade da AFB₁, o metabolismo deste composto é o melhor compreendido entre as aflatoxinas. Na Figura 5 foram demonstrados alguns possíveis produtos do metabolismo hepático da AFB₁. O processo de biotransformação da toxina é mediado por enzimas do citocromo P450 e uma grande variedade de moléculas pode ser formada. Acredita-se que os epóxidos gerados a partir da AFB₁ sejam os principais responsáveis pelo potencial carcinogênico da toxina. As moléculas de AFB₁-exo-8,9-epóxidos são muito reativas

e se ligam covalentemente na posição N7 de resíduos de guanina, levando à depurinação e carcinogênese (Okechukwu *et al.*, 2024).

Figura 5. Metabolismo hepático da AFB₁ por enzimas do citocromo P450.



Fonte: Rushing e Selim (2019).

Segundo Rushing e Selim (2019), metabólitos hidroxilados também são gerados a partir do sistema P450, como parte do processo de detoxificação. A adição de hidroxilas à molécula de AFB₁ facilita o transporte e eliminação através de secreções e excreções. A partir de hidroxilação são produzidas Aflatoxina M₁ (AFM₁) Aflatoxina Q₁ (AFQ₁) e Aflatoxicol (AFL). Destes metabólitos, AFM₁ se destaca como uma grave ameaça à saúde pública por ser frequentemente excretado no leite. Estudos estimam que 0,3% a 6,2% da AFB₁ ingerida por vacas leiteiras é transferida na forma de AFM₁ para o leite. Outras espécies animais demonstram taxas de conversão e transmissão para o leite diferentes (Creppy, 2002; Ketney; Santine; Oancea, 2017). Embora o potencial carcinogênico de AFM₁ seja estimado 10 vezes menor que o de sua molécula precursora, esta toxina ainda é carcinogênica e uma

ameaça à saúde humana, demandando controle da contaminação, especialmente em leite e derivados (Magoha *et al.*, 2016; Kortei *et al.*, 2022).

3.3.2 Aflatoxinas em Produtos de Origem Animal

Além de importantes prejuízos na produtividade, a contaminação por aflatoxinas na alimentação animal pode resultar na transmissão de resíduos destas toxinas para alimentos de origem animal. As cadeias produtivas nas quais os animais podem ser expostos às aflatoxinas por longos períodos, como ocorre nas produções de leite e ovos, apresentam um risco maior de transferência de metabólitos de aflatoxinas para os alimentos (Feddern *et al.*, 2013).

Produtos de origem animal contaminados por resíduos de aflatoxinas contribuem para o aumento da ingestão diária destas toxinas. Consequentemente, os impactos na saúde humana são maiores quando a exposição indireta é frequente. Considerando que leite é o produto de origem animal com o maior nível de transferência de aflatoxinas, os laticínios são os que apresentam maior prevalência de contaminação e riscos associados (Peles *et al.*, 2019). Neste sentido, limites máximos tolerados (LMTs) foram definidos para AFM₁ em laticínios, conforme apresentado na Tabela 1. Estes limites foram estabelecidos pela ANVISA na RDC nº722 e IN nº160, ambas publicadas em 1º de julho de 2022.

Tabela 1. LMTs para aflatoxinas em alimentos e matéria-prima no Brasil.

Micotoxina	Alimento/matéria-prima	LMT (µg/kg)
AFM ₁	Leite fluido	0,5
	Leite em pó	5
	Queijos	2,5

*Valores definidos pela RDC nº722 de 01 de julho de 2022 e IN nº160 de 01 de julho de 2022 – ANVISA.

A regulamentação visa determinar padrões de segurança para aflatoxinas nos alimentos com maior prevalência de contaminação. No Brasil, dentre os alimentos de origem animal, apenas alguns laticínios possuem regulamentação oficial para aflatoxinas. Do mesmo modo, a legislação europeia que regulamenta os LMTs de aflatoxinas em alimentos não apresenta critérios definidos para alimentos de origem

animal, com exceção do leite. A concentração máxima de $0,05\mu\text{g/kg}$ de AFM_1 em leite cru, beneficiado ou destinado à produção de derivados constitui a única regulamentação destes contaminantes em alimentos de origem animal (*Commission Regulation* – EC – nº2023/915 - 2023).

Outros países possuem recomendações oficiais para níveis máximos aceitáveis de aflatoxinas em alimentos distintos dos previstos na legislação brasileira. Ainda assim, a maioria não especifica valores para produtos de origem animal. Nos Estados Unidos, o FDA (*Food and Drug Administration*) estabeleceu no ano de 2000, os limites de ação para a contaminação por aflatoxinas em alimentos. No leite, foi limitada à $0,5\mu\text{g/kg}$ a concentração máxima de AFM_1 , enquanto que, para outros alimentos não previstos em regulamentações específicas, determinou-se o limite máximo de $20\mu\text{g/kg}$ de aflatoxinas totais. Portanto, considerando as recomendações do FDA, $20\mu\text{g/kg}$ seria a concentração máxima aceitável de aflatoxinas em alimentos de origem animal, exceto para leite.

A preocupação relacionada à presença de aflatoxinas em leite e derivados não se baseia somente na prevalência do contaminante, mas também nos hábitos de consumo destes produtos, que frequentemente compõem a dieta de crianças, que são mais susceptíveis aos efeitos da intoxicação (Milicevic *et al.*, 2021). Também é importante destacar que o processamento térmico aplicado aos alimentos não é suficiente para degradar ou diminuir a toxicidade das aflatoxinas, uma vez que estes compostos são muito estáveis e resistentes às altas temperaturas e procedimentos empregados em alimentos (Mahato *et al.*, 2019). Iha *et al.* (2013) analisaram amostras de leite pasteurizado e UHT comercializados no Brasil, detectando AFM_1 em 83% das amostras. O beneficiamento do leite mostrou-se incapaz de eliminar AFM_1 . Além disso, os autores avaliaram o potencial de degradação de AFM_1 no processamento do leite para produção de queijo e iogurte. Houve apenas 3,2% de redução no conteúdo de AFM_1 com a produção de queijo e 6% em iogurte. Estes dados demonstram que os processos de tratamento térmico e produção de derivados não são capazes de reduzir consideravelmente os riscos da contaminação por aflatoxinas presentes no leite. Portanto, reitera-se a importância de monitorar e prevenir a contaminação inicial dos alimentos e da matéria-prima, para garantir a segurança do produto final.

Ainda que os laticínios demonstrem ser os produtos de origem animal com maior prevalência de contaminação por aflatoxinas, com destaque para AFM_1 , a

presença de aflatoxinas em outros alimentos não pode ser desconsiderada. Na Tabela 2 são apresentados exemplos de experimentos que detectaram aflatoxinas em produtos de origem animal.

Tabela 2. Análises de aflatoxinas em produtos de origem animal.

Produto	Toxina	Amostras positivas	Concentração (µg/kg)	Método de análise	Referência*
Carne bovina e de aves, miúdos e embutidos	AFs	Variável conforme o produto	2,82 – 288,00	HPLC-FLD	1
Carnes processadas	AFs	37,5%	0,30 - 52,93	HPLC-FLD	2
Queijo	AFM ₁	80%	0,11 - 14,43	UHPLC-FLR	3
Leite	AFM ₁	100%	0,04-0,55	ELISA	4
Queijo	AFM ₁	-	0,08-0,24		
Ovo	AFB ₁	73% (clara) 79% (gema)	0,43 – 1,21	UPLC-MS/MS	5
Ovo	AFB ₁	48,0%	0,05 - 3,85	ELISA	6

AFs: Aflatoxinas totais (somatório de AFB₁+AFB₂+AFG₁+AFG₂);

*Referências: 1. Elbarbary *et al.* (2023); 2. Elzupir e Abdulkhair (2020); 3. Mudannayake *et al.* (2024); 4. Zebib, Abate e Woldegiorgis (2023); 5. Omar, Haddad e Fakhiri (2023); 6. Silva *et al.* (2022).

Elbarbary *et al.* (2023) analisaram amostras de produtos cárneos de diferentes espécies (bovina e aves), miúdos e embutidos comercializados no Egito. As quatro principais aflatoxinas foram avaliadas e dentre elas, AFB₁ foi a mais detectada (63% das amostras). As concentrações médias de aflatoxinas totais encontradas variaram de 2,82µg/kg em carne bovina congelada até 288µg/kg em fígado de aves. A presença das toxinas foi maior em miúdos e embutidos cárneos em comparação com a carne comercializada in natura. Embora a maior parte dos produtos avaliados tiveram contaminações abaixo de 20µg/kg, como recomendado pelo FDA, a alta prevalência de produtos contaminados alertam para possíveis risco à saúde da população. Ainda com relação a produtos cárneos, Elzupir e Abdulkhair

(2020) detectaram diferentes níveis de contaminação por aflatoxinas em carnes processadas na Arábia Saudita. No estudo, AFB₁ também foi a toxina de maior prevalência, e a amostra de maior contaminação resultou na elevada concentração de 52,93µg/kg da toxina.

A presença de aflatoxinas em ovo também já foi comprovada. Em um estudo conduzido na Jordânia, Omar, Haddad e Fakhiri (2023) detectaram AFB₁ em ovos comercializados em várias regiões do país. As concentrações médias de toxina encontradas foram baixas, porém a prevalência de amostras positivas foi acima de 70%. Da mesma forma, em análises realizadas no Brasil por Silva *et al.* (2022) foi detectada a presença de AFB₁ em 48% das 400 amostras de ovos coletadas diretamente de granjas produtoras. A concentração média da toxina nas amostras positivas foi de 1,01µg/kg.

Com relação à contaminação por AFM₁, os dados de investigação da toxina em leite e derivados evidenciam a alta prevalência de amostras positivas. Mudannayake *et al.* (2024) e Zebib, Abate e Woldegiorgis (2023) avaliaram amostras de leite e queijos consumidos, respectivamente no Sri Lanka e na Etiópia, sendo que ambos indicaram mais 80% de amostras contaminadas pela toxina. Portanto, os resultados descritos em estudos de diferentes regiões e para os mais variados produtos de origem animal, demonstraram que o risco de contaminação destes alimentos por aflatoxinas é real e demanda maior monitoramento para a redução de riscos à saúde humana.

3.3.3 Detecção de Aflatoxinas em Alimentos

O monitoramento de contaminantes em alimentos deve ser realizado com frequência, e para tal, os métodos analíticos utilizados devem ser adequados, específicos e sensíveis aos analitos de interesse (Beyene *et al.*, 2019). Considerado o grande potencial tóxico e o evidente risco que as aflatoxinas representam ao nível de saúde pública, a análise da contaminação destes compostos em alimentos visa garantir a obediência aos limites de segurança estabelecidos em lei. Atualmente, a RDC nº722 de 01 de julho de 2022 da ANVISA constitui a regulamentação brasileira vigente aplicada ao controle das concentrações de aflatoxinas em alimentos.

Uma grande variedade de técnicas pode ser utilizada na detecção de aflatoxinas, estando os métodos cromatográficos e imunoenzimáticos entre os mais

empregados para esta função (Mahato *et al.*, 2019). As análises de cromatografia líquida utilizam detectores de absorção em UV/Vis, fluorescência ou espectrometria de massas, e são técnicas de referência para a quantificação de aflatoxinas, devido ao atendimento a parâmetros de validação metodológica, como robustez e boa sensibilidade (SILVA *et al.*, 2018). As aflatoxinas são moléculas naturalmente fluorescentes sob luz ultravioleta, apresentando os análogos B₁ e B₂ fluorescência na cor azul, enquanto G₁ e G₂ possuem fluorescência na cor verde. Esta característica físico-química justifica o amplo uso de detectores de fluorescência para a análise de aflatoxinas (Zhang e Banerjee, 2020).

Alternativamente, métodos imunoenzimáticos podem ser empregados para a detecção de aflatoxinas. As técnicas apresentam elevada especificidade e se baseiam na interação entre anticorpo e antígeno. Algumas vantagens destes métodos estão na facilidade de execução, baixo custo e elevada sensibilidade, permitindo a detecção do analito em concentrações muito baixas (Azri *et al.*, 2018).

A seleção do método de análise para aflatoxinas deve considerar a capacidade de detecção do analito em concentrações suficientemente baixas com o objetivo de avaliar se as amostras cumprem com os LMTs definidos em lei. Além do mais, espera-se que a técnica utilizada produza resultados com rapidez e precisão, sendo assim efetivamente útil para o monitoramento de aflatoxinas em alimentos (Miklós *et al.*, 2020).

3.3.4 Controle de Aflatoxinas em Alimentos

A prevenção do crescimento fúngico, e consequentemente, da contaminação dos alimentos por aflatoxinas é a medida mais eficiente para minimizar os prejuízos econômicos e os efeitos da ingestão destes compostos na saúde animal e humana. No entanto, alguns métodos podem ser aplicados no controle de aflatoxinas para reduzir a contaminação (Sipos *et al.*, 2021).

As estratégias de controle da contaminação por aflatoxinas incluem métodos físicos, químicos e biológicos (Wu *et al.*, 2009). Os processos físicos, normalmente são utilizados no tratamento de grãos após a colheita, por técnicas que incluem a separação de grãos, moagem, remoção da casca, limpeza, processamento térmico, irradiação ou a combinação de vários destes processos. Adsorventes de micotoxinas também são frequentemente utilizados, especialmente na composição de rações de

aves e suínos, como forma de reduzir a absorção entérica destes contaminantes (Filazi *et al.*, 2017).

Alguns agentes químicos demonstram ação na degradação de aflatoxinas, no entanto estes têm baixa aplicabilidade em alimentos. Processos como a amonização podem ser muito agressivos e resultar na formação de novos compostos tóxicos e reduzir o valor nutricional do alimento. Assim, os métodos físicos e químicos têm efeitos limitados e a aplicação em alimentos pode resultar em perdas nutricionais, custo elevado de processamento e baixa eficácia (Peng *et al.*, 2018).

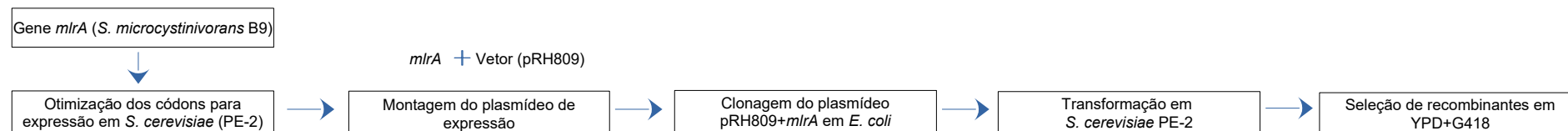
Como alternativa, métodos biológicos têm ganhado destaque pela boa eficiência e praticidade, além de produzirem menor volume de resíduos de impacto ambiental (WANG *et al.*, 2019). Alguns destes estudos se baseiam no controle biológico do crescimento de espécies aflatoxigênicas de *Aspergillus* spp. (Tejero *et al.*, 2021). Outros são direcionados para a capacidade que alguns microrganismos, como bactérias ácido lácticas e leveduras, têm de adsorver aflatoxinas, reduzindo assim a contaminação dos alimentos (Dogi *et al.*, 2017; Hashemi; Amiri, 2020). Independentemente do método de descontaminação utilizado, os esforços aplicados ao controle de aflatoxinas em alimentos devem incluir as medidas preventivas e monitoramento frequente. Somente a combinação destas ações em várias frentes pode resultar na efetiva mitigação de riscos à população.

4 MATERIAL E MÉTODOS

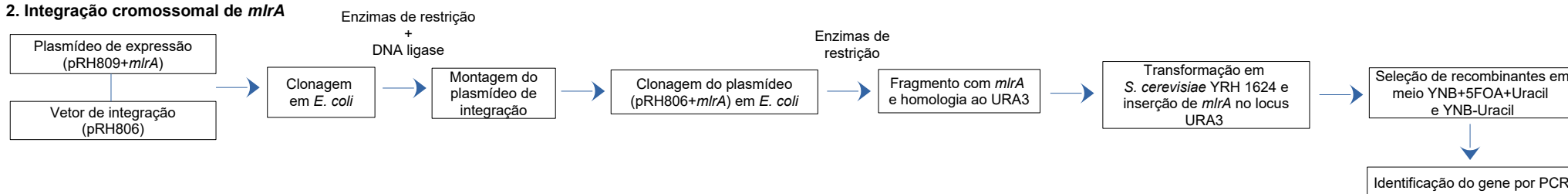
As atividades executadas nesta tese foram descritas detalhadamente nos capítulos adiante e ilustradas nas Figuras 6, 7 e 8.

Figura 6. Representação esquemática das atividades executadas no Capítulo 1: Microcystin-Detoxifying Recombinant *Saccharomyces cerevisiae* Expressing the *mlrA* Gene from *Spingosinella microcystinivorans* B9.

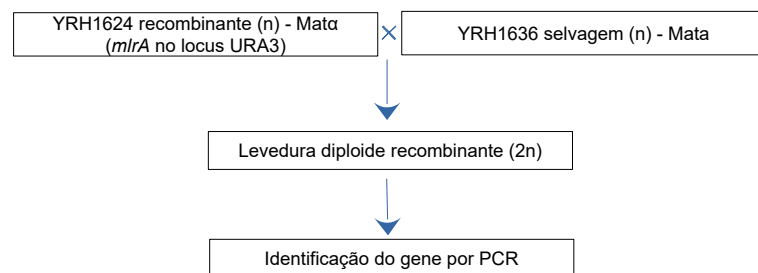
1. Expressão plasmidial de *mlrA*



2. Integração cromossomal de *mlrA*



3. Obtenção de diploides recombinantes



4. Testes de degradação de MCs com leveduras recombinantes

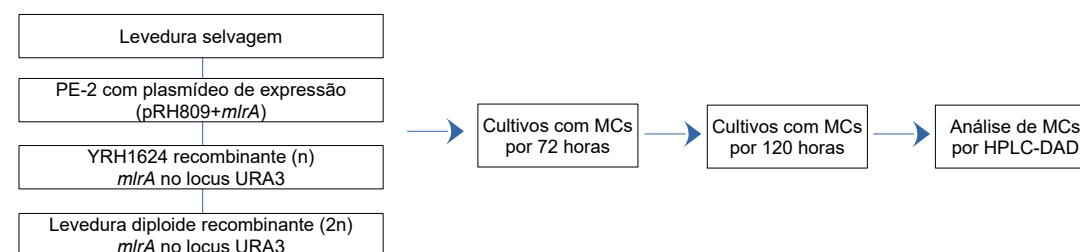
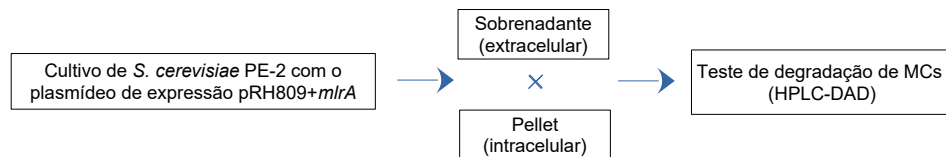
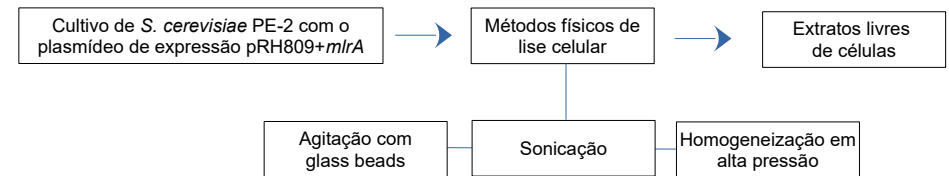


Figura 7. Representação esquemática das atividades executadas no Capítulo 2: Advanced Assessment of a Yeast-Expressed Recombinant Microcystinase A: Enzyme Properties, Activity, and Stability.

1. Determinação da localização da microcistinase A



2. Preparo de extratos proteicos a partir da levedura recombinante



3. Avaliação do perfil de atividade da microcistinase A expressa por levedura recombinante

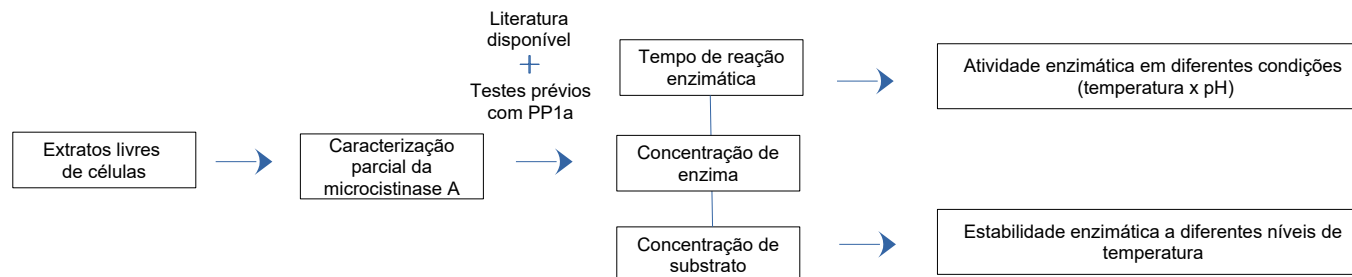
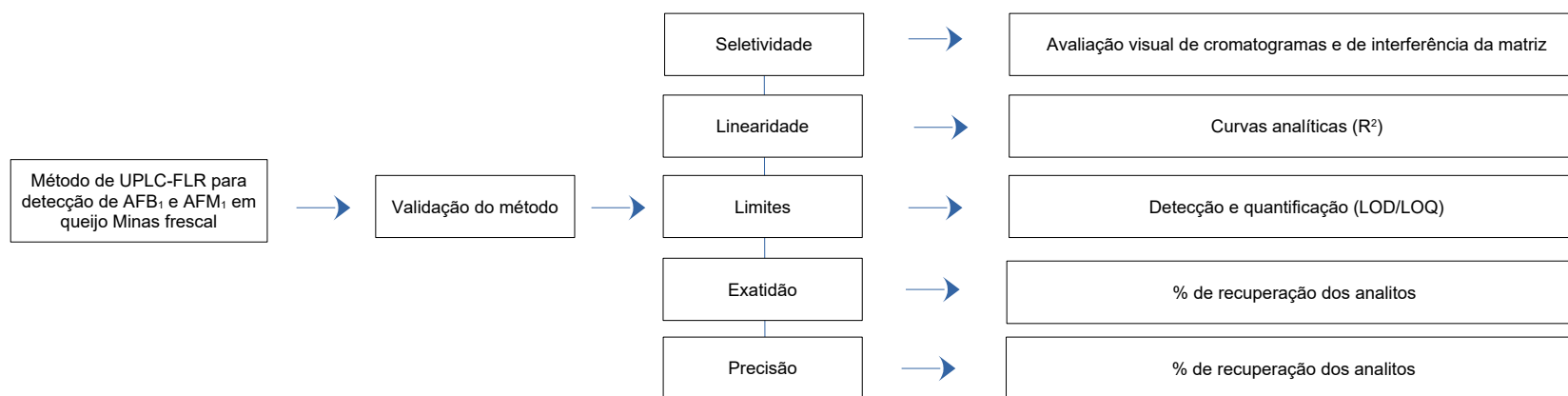


Figura 8. Representação esquemática das atividades executadas no Capítulo 3: Simultaneous Detection of AFB₁ and AFM₁ in Minas Frescal Cheese by UPLC-FLR Analysis: Method Development and Validation.

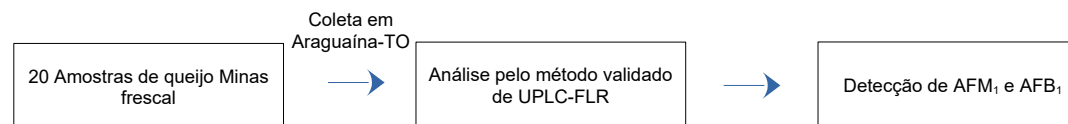
1. Padronização do método de UPLC-FLR para AFM₁ e AFB₁



2. Validação intralaboratorial do método



3. Aplicação do método para monitoramento de AFM₁ e AFB₁ em queijo minas frescal



5 RESULTADOS

Os resultados obtidos nos experimentos realizados foram descritos em capítulos no formato de artigo científico, conforme abaixo:

5.1 Capítulo 1. Microcystin-Detoxifying Recombinant *Saccharomyces cerevisiae* Expressing the *mlrA* Gene from *Sphingosinicella microcystinivorans* B9.

Este artigo foi publicado na revista *Microorganisms* em fevereiro de 2023. Disponível através do DOI: 10.3390/microorganisms11030575.

5.2 Capítulo 2. Advanced Assessment of a Yeast-Expressed Recombinant Microcystinase A: Enzyme Properties, Activity, and Stability.

5.3 Capítulo 3. Simultaneous Detection of AFB₁ and AFM₁ in Minas Frescal Cheese by UPLC-FLR Analysis: Method Development and Validation.

5.1 CAPÍTULO 1:

MICROCYSTIN-DETOXIFYING RECOMBINANT SACCHAROMYCES CEREVISIAE EXPRESSING THE MLRA GENE FROM SPHINGOSINICELLA MICROCYSTINIVORANS B9



Article

Microcystin-Detoxifying Recombinant *Saccharomyces cerevisiae* Expressing the *mlrA* Gene from *Sphingosinicella microcystinivorans* B9

Fernando de Godoi Silva, Daiane Dias Lopes, Ronald E. Hector, Maikon Thiago do Nascimento, Tatiana de Ávila Miguel, Emília Kiyomi Kuroda, Gisele Maria de Andrade de Nóbrega, Ken-Ichi Harada and Elisa Yoko Hirooka

Special Issue

Microbial Biodegradation of Toxic Pollutants

Edited by

Dr. Miroslav Pátek and Prof. Dr. Tomas Cajthaml





Article

Microcystin-Detoxifying Recombinant *Saccharomyces cerevisiae* Expressing the *mlrA* Gene from *Sphingosinicella microcystinivorans* B9

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Abstract: Contamination of water by microcystins is a global problem. These potent hepatotoxins demand constant monitoring and control methods in potable water. Promising approaches to reduce contamination risks have focused on natural microcystin biodegradation led by enzymes encoded by the *mlrABCD* genes. The first enzyme of this system (*mlrA*) linearizes microcystin structure, reducing toxicity and stability. Heterologous expression of *mlrA* in different microorganisms may enhance its production and activity, promote additional knowledge on the enzyme, and support feasible applications. In this context, we intended to express the *mlrA* gene from *Sphingosinicella microcystinivorans* B9 in an industrial *Saccharomyces cerevisiae* strain as an innovative biological alternative to degrade microcystins. The *mlrA* gene was codon-optimized for expression in yeast, and either expressed from a plasmid or through chromosomal integration at the *URA3* locus. Recombinant and wild yeasts were cultivated in medium contaminated with microcystins, and the toxin content was analyzed during growth. Whereas no difference in microcystins content was observed in cultivation with the chromosomally integrated strain, the yeast strain hosting the *mlrA* expression plasmid reduced 83% of toxins within 120 h of cultivation. Our results show microcystinase A expressed by industrial yeast strains as a viable option for practical applications in water treatment.

Keywords: microcystinase; heterologous expression; biodegradation; public health



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1. Introduction

Cyanobacteria (blue-green algae) are prokaryotes well adapted for growth in eutrophic environments, which are able to produce compounds harmful to animals and humans, especially during massive growth or blooms [1]. Among its bioproducts, microcystins (MCs) are considered of greatest concern due to their prevalence and hepatotoxic effects [2]. Besides liver damage, exposure to MCs is associated with several other disorders in humans, depending on the duration and dose of exposure [3]. Moreover, microcystin-leucine-arginine (MC-LR) is the most potent variant among MC congeners (MC-LR, MC-RR, MC-YR, MC-LA, MC-LF), and it is classified as a possible carcinogen to humans by the International Agency of Research on Cancer [4,5]. Considering the risks posed by MC-contaminated water, the World Health Organization (WHO) set a provisional maximum concentration at 1.0 µg.L⁻¹ MC-LR in drinking water, and a tolerable daily intake of 0.04 µg of MC-LR per kg of body mass per day for humans [6,7].

These toxins can pass through food webs, accumulating microcystins in fish tissue and agricultural crops, mainly in leafy vegetables irrigated with contaminated water [2,8]. The general structure of microcystins consists of a cyclic heptapeptide, which confers some stability to certain physical and chemical treatment processes [9]. In water treatment, methods usually applied have limited effectiveness for microcystin control, such as chlorination, UV, ozonation, carbon adsorption, and membrane filtration. Despite possible effects on MC removal or degradation, some of these physical and chemical processes may release other cytotoxins [10]. Therefore, new methods of controlling MCs that are effective, safe, and applicable in practice are needed.

The MC degradation pathway led by microcystinases encoded by the *mlrABCD* complex is the most important and well-known biological tool for the environmental control of these toxins [11]. The first enzyme involved in MC degradation is encoded by the *mlrA* gene, cleaving the Adda–Arg bond in the MC-LR cyclic structure (Figure 1). Available data on the linear MC structure show toxicity is reduced approximately 2100-fold when compared to cyclic MC-LR [12,13]. In addition, the linearized toxin is less stable and active sites are more susceptible to enzymatic degradation [14]. Recent studies have focused on the heterologous expression of the *mlrA* gene in other bacterial species with the purpose of improving the efficiency of natural MC biodegradation systems. Dexter et al. (2021) described experiments that used this strategy and most instances obtained efficient degradation of MCs [15]. As the enzyme encoded by *mlrA* is highly effective and acts as a sustainable biological alternative to MCs degradation, its engineering and heterologous expression may lead to promising increased production and enhanced activity. Nevertheless, further studies are recommended to refine reported results through different technical approaches and to test alternatives to conventional expression in bacteria strains.

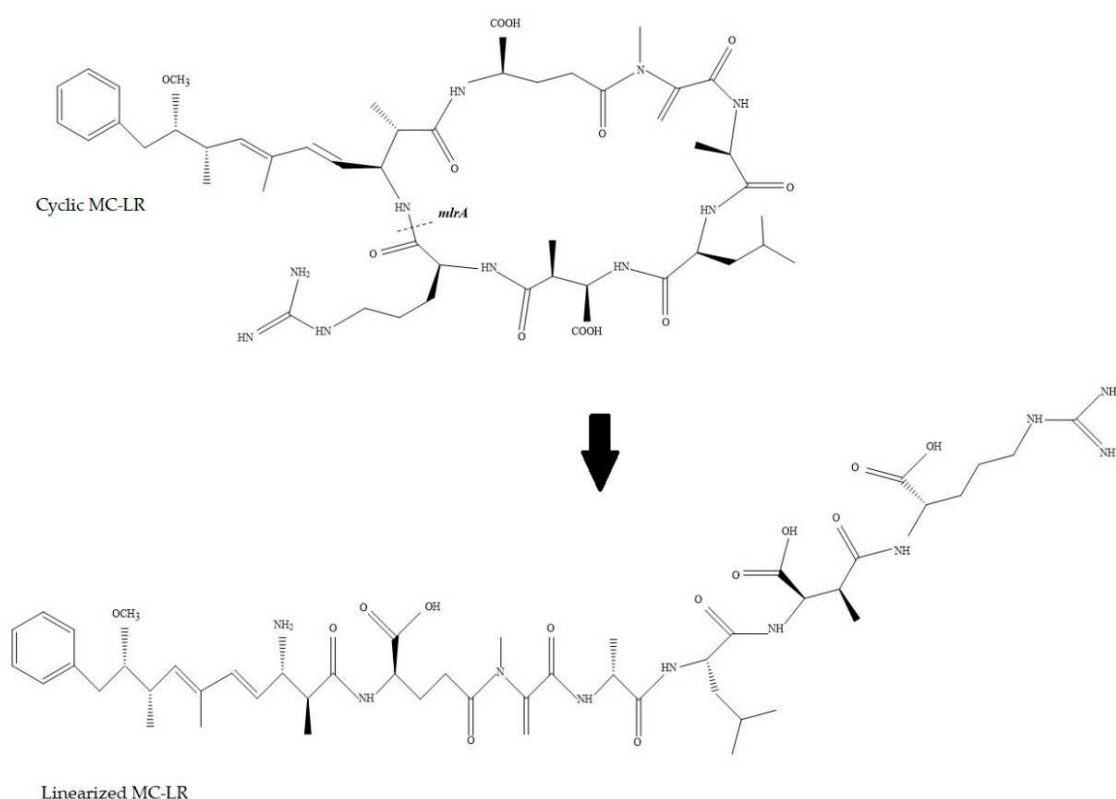


Figure 1. Linearization of Microcystin-LR by microcystinase A.

Yeasts are commonly manipulated by molecular engineering techniques aiming to increase the productivity of industrial strains or set new applications, mainly in food, chemical, and pharmaceutical areas. Among reported yeast species, *Saccharomyces cerevisiae* is the

most widely studied due to its robustness and safety: it is classified as generally recognized as a safe (GRAS) microorganism [16]. Additionally, this microorganism grows in simple medium, providing high cell-density, and can produce post-translational modifications to expressed proteins, such as glycosylation [17]. These characteristics coupled with its well-characterized genetics motivate innovative academical and industrial applications of recombinant yeast [18].

Therefore, this work developed recombinant *S. cerevisiae* PE-2 strains for the heterologous expression of the *mlrA* gene from the *Sphingosinicella microcystinivorans* B9 strain. To date, this is the first attempt to express this gene in a widely used industrial yeast strain. The gene was codon-optimized for yeast expression and two different strategies were used: plasmid expression and chromosome integration of the *mlrA* gene into the *URA3* locus. Plasmid-hosting strains were selected considering their resistance to geneticin (G418). Gene integration into the yeast chromosome was confirmed by growth on selective media and by PCR amplification of the *mlrA* gene. The results demonstrated the feasibility of heterologous expression of microcystinases in yeast cells and provide a basis for further experiments to enhance strategies for controlling microcystins.

2. Materials and Methods

2.1. Strains, Plasmids and Reagents

Saccharomyces cerevisiae strains used in this work were: PE-2 (Brazilian diploid ethanol-producing yeast; Fermentec, Piracicaba, Brazil), YRH1624 (*MAT* α spore isolated from PE-2), and YRH1636 (*MAT* α spore isolated from PE-2). To generate haploid yeast strains YRH1624 and YRH1636, *S. cerevisiae* strain PE-2 was sporulated and 4-spore tetrads were dissected to YPD plates as described in Lopes et al. (2017) [19]. The mating type for each strain was determined as described in Section 2.7.

Expression plasmid pRH809 contains the *KanMX* marker for resistance to G418, the *Ashbya gossypii* *TEF* (*AgTEF*) promoter, and *S. cerevisiae* *RPL41B* terminator. The integration plasmid pRH806 contains the *AgTEF* promoter, multi-cloning site, and *S. cerevisiae* *ADH1* terminator, flanked by 400 to 500 bp of homology to the PE-2 *URA3* gene to target integration to the *URA3* locus. *Escherichia coli* competent NEB10 β cells (NEB, Ipswich, MA, USA) were used for transforming and cloning the plasmids. Geneticin G418 (200 $\mu\text{g.mL}^{-1}$), ampicillin (100 $\mu\text{g.mL}^{-1}$), and chloramphenicol (100 $\mu\text{g.mL}^{-1}$) were used for selection of microorganisms and prevention of contamination during cultivation. The restriction enzymes *SpeI*, *Sall*, *XhoI*, *SacI*, *BssHII*, and *T4* DNA ligase were purchased from New England Biolabs (NEB; Ipswich, MA, USA).

Sphingosinicella microcystinivorans B9 strain was provided by Dr. Ken-Ichi Harada [20]. Tests previously performed by part of our research group confirmed this strain's potential and high efficiency in MCs degradation. Additionally, *Microcystis* sp. TAC95 was cultivated and cyanobacterial extracts were prepared as described by Kuriama et al. (2012) [21]. Extracts were analyzed on HPLC-DAD and quantified by calibration curves prepared with standard MC-LR purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and ammonium acetate HPLC grade used on MCs analyses were purchased from Sigma-Aldrich (St. Louis, MO, USA) and JT Baker (Phillipsburg, NJ, USA), respectively.

In general, yeast strains were cultivated in YPD (1% yeast extract, 2% dextrose, 2% peptone), while *E. coli* were grown in LB medium (0.5% yeast extract, 1% tryptone, 1% NaCl). Selection of yeast strains with *mlrA* gene integrated to their chromosomes was performed with Yeast Nitrogen Base (YNB—Difco; Sparks, MD, USA) supplemented with 2% dextrose and amino acids (50 $\mu\text{g.mL}^{-1}$ tyrosine, 50 $\mu\text{g.mL}^{-1}$ serine, 50 $\mu\text{g.mL}^{-1}$ valine, 50 $\mu\text{g.mL}^{-1}$ isoleucine, 50 $\mu\text{g.mL}^{-1}$ phenylalanine, 50 $\mu\text{g.mL}^{-1}$ aspartic acid, 50 $\mu\text{g.mL}^{-1}$ proline, 100 $\mu\text{g.mL}^{-1}$ arginine, and 100 $\mu\text{g.mL}^{-1}$ threonine). Uracil (175 $\mu\text{g.mL}^{-1}$) and 5-fluoroorotic acid (1 mg.mL^{-1} ; 5-FOA; Zymo Research, Irvine, CA, USA) were added to YNB plates to select cells with mutation within the *URA3* locus.

2.2. Construction of the Expression Plasmid (pRH809+mlrA)

The *mlrA* expression vector (pRH809) has *AmpR* and *KanMX* markers, which confer ampicillin and geneticin (G418) resistance to the host, besides *AgTEF* promoter and *RPL41B* terminator regions. Codon-optimization of the *mlrA* gene for expression in *S. cerevisiae* cells was based on the *S. microcystinivorans* B9 strain genome deposited in GenBank (accession number AP018711) [20]. Adaptations were made manually based on a codon usage frequency table, and the final gene sequence was evaluated using an online tool for codon-optimization, resulting in an adequate expression level (www.genscript.com (accessed on 1 August 2019)).

A 1061 bp DNA fragment containing the codon-optimized *mlrA* gene and homology regions to the pRH809 vector was purchased from Fastbio (Ribeirão Preto, Brazil) and used to construct the expression plasmid pRH809+*mlrA*. pRH809 vector was digested with *SpeI* and *Sall* enzymes and the fragments were assembled using NEBuilder Hi-Fi DNA Assembly Kit (NEB) according to the developers' instructions. The constructed expression plasmid was transformed into competent *E. coli* NEB10 β cells following the developers' protocols (NEB). Bacterial cloning was performed during overnight incubation at 37 °C and continuous agitation (250 rpm) in LB medium added with 100 $\mu\text{g}\cdot\text{mL}^{-1}$ of ampicillin. pRH809+*mlrA* plasmid was extracted on QIAprep Spin MiniPrep Kit (Qiagen; Venlo, Netherlands), and the DNA concentration was measured on Nanodrop 2000/2000c Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The constructed expression plasmid had a fragment of its DNA amplified using M13 primers (M13F-5'GTAAACGACGGCCAG3'; M13R-5'CAGGAAACAGCTATGAC3'), and analyzed by sequencing, confirming no mistakes in the assembled fragment. Part of the content of extracted pRH809+*mlrA* plasmid was used to transform PE-2 yeast cells, while the other was used to construct the integration vector.

2.3. pRH809+mlrA Transformation into PE-2 *S. cerevisiae* Strain

PE-2 yeasts were transformed using a standard lithium acetate method with some modifications [22]. Yeasts were grown in YPD medium overnight at 30 °C and constant agitation at 180 rpm. An aliquot was calculated and transferred to an additional flask to complete a 25 mL final volume of YPD medium, corresponding to the optical density (OD) of 0.1 at 600 nm. OD measurement was performed on the Anthos Zenyth 200 rt Spectrophotometer (Biochrom, Holliston, MA, USA). The yeast was incubated for 4 h at 30 °C and 220 rpm. Then, 10 mL of the medium was transferred to a 50 mL conical tube and centrifuged at $3500\times g$ for 5 min. The supernatant was discarded and the pellet was suspended in 1 mL of a solution A (10X Tris-EDTA Buffer—TE, 1 $\text{mol}\cdot\text{L}^{-1}$ LioAc and water—1:1:8). The mixture was moved to a microcentrifuge tube and cells were pelleted at $10,000\times g$ for 30 s. The supernatant was discarded and the process of suspension in solution A and centrifugation was repeated. Next, the pellet was suspended in 50 μL of solution A, and 1 μg of the plasmid (pRH809+*mlrA*) and 50 μg of DNA carrier (salmon sperm) were added. A total of 300 μL of solution B (10X TE Buffer, 1 $\text{mol}\cdot\text{L}^{-1}$ LioAc and 50% of polyethylene glycol—PEG—1:1:8) was added to the tube. The mixture was incubated at 30 °C for 30 min, 42 °C for 15 min, and kept at room temperature for 10 min. Then, the tube was centrifuged at $10,000\times g$ for 1 min, the supernatant was discarded, and the pellet was suspended in 500 μL of sterile water. This last process was repeated once. Next, 100 μL of the suspended pellet was spread on YPD + G418 (200 $\mu\text{g}\cdot\text{mL}^{-1}$) agar plates and incubated for 48 h at 30 °C. Only transformed cells were able to grow in the presence of G418.

2.4. Construction of the Integration Vector

The entire process to construct the integration vector is summarized in Figure 2.

Originally, the pRH806 vector was constructed with an *AmpR* marker and two regions of homology to *S. cerevisiae* *URA3* locus. This plasmid was transformed and cloned into competent *E. coli* cells, extracted, and quantified as described previously for the pRH809+*mlrA* plasmid. Plasmids pRH806 and pRH809+*mlrA* were both digested with *SacI*

and XhoI restriction enzymes. Fragments were analyzed by 0.8% agarose gel electrophoresis (Figure 3a). The longest pRH806 fragment (vector containing the *URA3* homology regions) and the shortest pRH809+*mlrA* fragment (insert with *mlrA* gene) were excised from the gel and purified with Pure Link Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen, Waltham, MA, USA). The concentration of DNA in purified fragments was quantified on Nanodrop 2000/2000 c at 260 nm. The new pRH806+*mlrA* plasmid was assembled with T4 DNA ligase. The integration plasmid was transformed and cloned into *E. coli* competent cells, then extracted and quantified exactly as performed in the previous steps. The assembled vector (pRH806 plasmid with *URA3* homology region—*AgTEF* promoter—codon-optimized *mlrA*—*RPL41B* terminator—*URA3* homology region) was used to integrate the expression cassette into the *URA3* locus of *S. cerevisiae*. The vector was digested with the BssHII restriction enzyme in the regions of homology. To confirm fragment size and the efficiency of digestion, a 5 μ L aliquot of the digestion product was analyzed in 0.8% agarose gel electrophoresis (Figure 3b). Two bands with a length of approximately 3000 bp confirmed the initial size of the plasmid and digestion by the enzyme. The remaining 75 μ L from the restriction process were purified on Pure Link PCR Purification Kit (Invitrogen) and used to transform YRH1624 yeast cells.

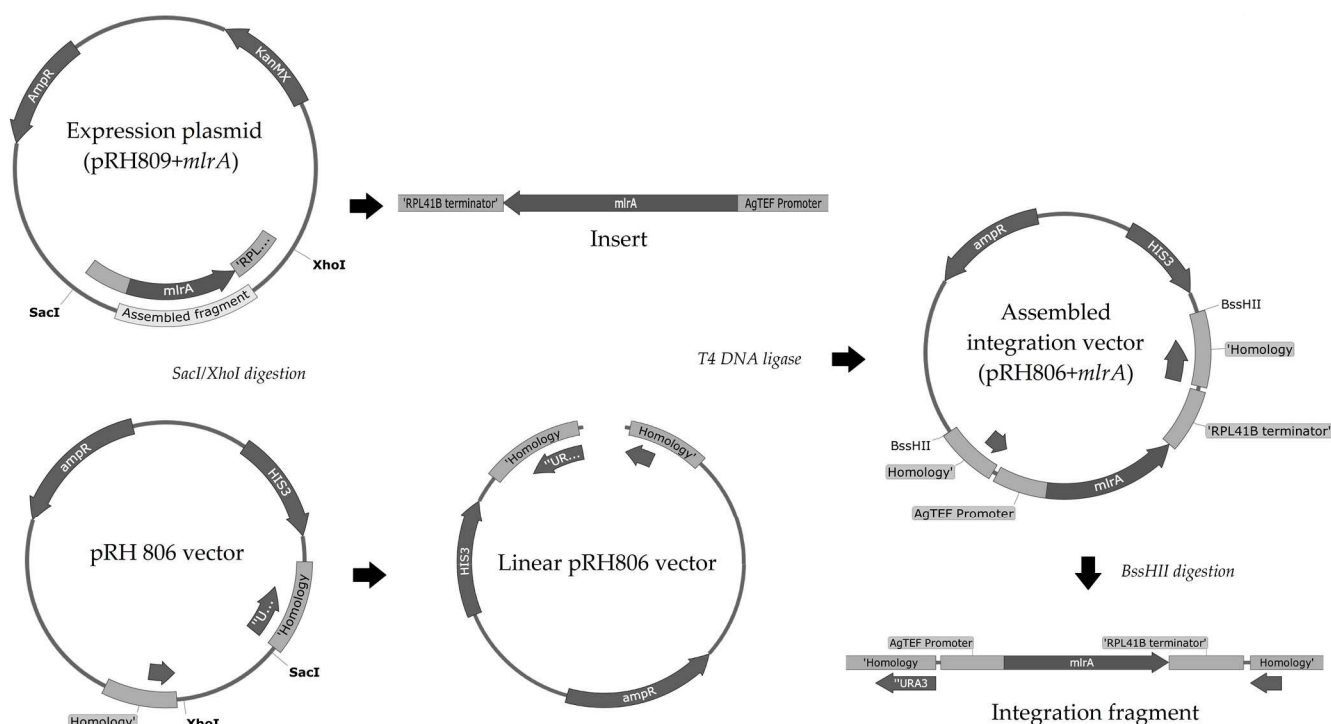


Figure 2. Construction map of the integration vector (pRH806+*mlrA*): Expression plasmid (pRH809+*mlrA*) was assembled as mentioned in the text (in Section 2.2). Both pRH809+*mlrA* and pRH806 vectors were digested with *SacI* and *XhoI*. Insert fragment (1901 bp) and the linear pRH806 vector (4610 bp) were ligated with T4 DNA Ligase. The assembled vector was digested with *BssHII*, and the integration fragment (2925 bp) was transformed into yeast cells. By crossing over with the homology regions, *mlrA* gene was integrated at the *URA3* locus.

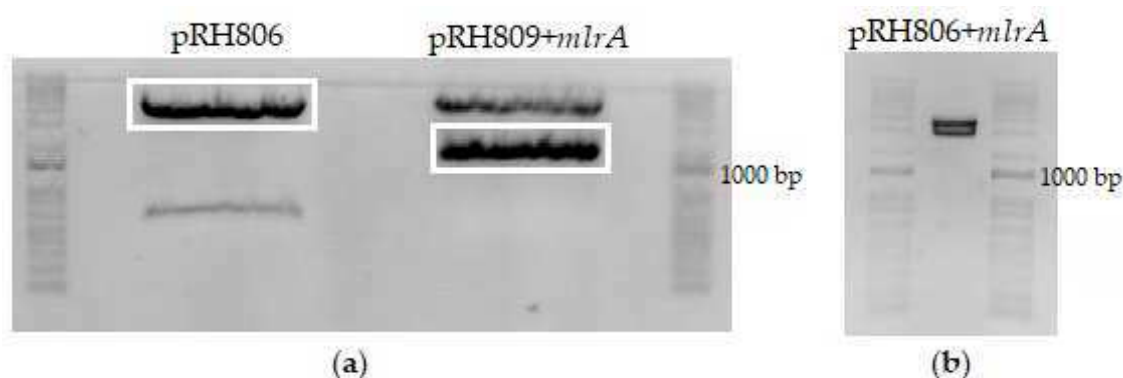


Figure 3. Electrophoresis analyses of procedures applied to construct the integration vector (pRH806+*mlrA*). (a)—*SacI* and *XhoI* digestion products from plasmids pRH809+*mlrA* (expression plasmid containing the *AgTEF* promoter-*mlrA*-*RPL41B* terminator) and pRH806 (vector to chromosomal integration). Bands marked containing 4610 bp and 1906 bp were excised, purified, and ligated to construct the new pRH806+*mlrA* plasmid. (b)—pRH806+*mlrA* vector digested with *BssHII* enzyme. Two DNA fragments were produced. The shortest fragment (2925 bp) contains *URA3* homology regions and the integrating expression cassette.

2.5. Transformation into YRH1624 *S. cerevisiae* Strain and *mlrA* Integration into *URA3* Locus

Transformation steps were carried out as described for pRH809+*mlrA*. After colonies grew on YPD agar plates, replica plates were performed on YNB+5-FOA+Uracil and YNB-Uracil plates. After 48 h at 30 °C, only *URA3* mutant colonies were able to grow in the presence of 5-FOA. Furthermore, *URA3* disrupted cells did not grow in the absence of Uracil supplementation. Then, colonies that grew on YNB+5-FOA+Uracil plates but did not grow on YNB-Uracil plates were selected and replicated on new plates to assess the mutation in *URA3* and the stability of such cells. The replication process was repeated 5 times. At the final of five replications, colonies considered recombinant at the *URA3* locus were confirmed by PCR amplification of the integrated *mlrA* gene.

The recombinant haploid YRH1624+*mlrA* strain (*MAT* α) was mated with YRH1636 wild-type strain (*MAT* α) as described by Treco and Winston (2008) [23]. Freshly grown yeasts from each strain were mixed on a YPD plate and incubated at 30 °C for 4 h. Then, the mixture was streaked on a YNB-Uracil plate and incubated at 30 °C for 48 h. YRH1636 wild-type and diploid strains were able to grow in the absence of uracil. Several colonies randomly selected were analyzed by PCR to confirm they were diploid and the integration of *mlrA* into their genome.

2.6. PCR Amplification and Identification of *mlrA* Integration

Integration of the *mlrA* gene into the *URA3* locus was confirmed by the amplification of a DNA fragment containing part of the integrated *mlrA* gene and a sequence outside of the homology region in the complementary strand of chromosome V. Primers were purchased from Integrated DNA Technologies (IDT, Coralville, IO, USA) and designed to amplify the 738 bp fragment. The assay was performed following instructions of developers with adjustments: mixture consisted of 1X Buffer, 5 mmol.L⁻¹ MgCl₂, 200 μ mol.L⁻¹ of each Deoxynucleotide triphosphates, 1 μ mol.L⁻¹ of each primer, 1.25 U of Dream Taq Hot Start DNA Polymerase (Thermo Scientific, Waltham, MA, USA) and 1 μ L of DNA as template. DNA extraction was performed on the PureLink Genomic DNA Mini Kit (Invitrogen). ddH₂O was added up to 25 μ L of final reaction volume. Sterile water was used as template for negative control. The amplification was carried out in a Veriti Thermal Cycler 9901 (Applied Biosystem, Thermo Scientific). Primer sequences and the amplification conditions are shown in Table 1. PCR products were analyzed by electrophoresis on 1.2% agarose gel stained with SYBR Safe DNA Gel Stain (Invitrogen).

Table 1. Primers sequences and PCR conditions to assess *mlrA* gene on integrated yeast strains and mating type *.

MlrA Fragment Amplification		
Primers	Sequences	
mlrA F	5' TTA TGG TGA AGG ATA AGT TTT GAC C 3'	
mlrA R	5' TTA GTT TGC CGC TAT GCC TC 3'	
PCR Conditions		
35 cycles	Initial denaturation	95 °C/3 min
	Denaturation	95 °C/30 s
	Annealing	50 °C/1 min
	Extension	72 °C/1 min
	Final extension	72 °C/10 min
Mating type		
Primers	Sequences	
MATa	5' ACT CCA CTT CAA GTA AGA GTT TG 3'	
MATalpha	5' GCA CGG AAT ATG GGA CTA CTT CG 3'	
MATlocus	5' AGT CAC ATC AAG ATC GTT TAT GG 3'	
PCR Conditions		
35 cycles	Initial denaturation	95 °C/10 min
	Denaturation	95 °C/30 s
	Annealing	50 °C/30 s
	Extension	72 °C/1 min
	Final extension	72 °C/2 min

* PCR amplification of the *mlrA* integrated gene was performed with Dream Taq Hot Start DNA Polymerase (Thermo Scientific), while Mating type amplification was performed with Gold Taq Hot Start Polymerase (Cellco). PCR products were analyzed on 1.2% agarose gel electrophoresis.

2.7. Mating-Type PCR Testing

Yeast strains used for this experiment were tested to assess their mating type. PCR amplification confirmed the selection of opposite mating types to mate recombinant and wild-type strains, as well as selecting diploid recombinant colonies. One amplification reaction consisted of: mixture of 1X Buffer, 200 $\mu\text{mol.L}^{-1}$ of each Deoxynucleotide triphosphates, 1 $\mu\text{mol.L}^{-1}$ of each primer, 1.25 U of Gold Taq Hot Start Polymerase (Cellco; São Carlos, SP, Brazil), 1 μL of DNA as template and ddH₂O up to 25 μL of final volume. Sterile water was added as template for negative control. The amplification process and primer sequences were shown in Table 1. Electrophoresis analysis of the PCR products were performed as described in the previous section for the *mlrA* gene integration.

2.8. MC Degradation Test

Prior to MC degradation tests, *Microcystis* sp. extracts were analyzed on HPLC-DAD and compared to standard curves performed with MC-LR. The retention time for both standard MC-LR and extracts peaks was the same. Possibly, most of the microcystin content in the extracts was composed of the MC-LR variant, however, no confirmation was carried out. Therefore, the toxins in extracts were regarded as MCs.

Initially, yeast strains were grown in YPD medium overnight at 30 °C and constant agitation at 180 rpm. Then, aliquots of grown medium were calculated considering an initial OD (600 nm) of 0.1 for 10 mL test volume. Calculated amounts were transferred to sterile tubes and centrifuged at 3500 $\times g$ for 5 min. Supernatant was discarded and the pellet was resuspended in the degradation test medium (10 mL). One test sample consisted of 10 mL final volume containing: 1 mL of YPD, 10 μL of chloramphenicol (final concentration

of 100 $\mu\text{g.mL}^{-1}$), approximately 300 ng.mL^{-1} of equivalent MC-LR and sterile water. G418 (200 $\mu\text{g.mL}^{-1}$) was added to cultivations with the expression plasmid (pRH809+*mlrA*). Tests were performed in triplicate. The incubation conditions were the same used for yeast growth: 30 °C and 180 rpm. Aliquots (600 μL) were collected from all the samples within 0, 24, 48, and 72 h of cultivation, and centrifuged at $10,000\times g$ for 5 min. The supernatant was filtered on PVDF 0.22 μm , 13 mm hydrophilic syringe filters (Millex, Darmstadt, Germany) and stored at $-20\text{ }^{\circ}\text{C}$ until analyses on HPLC-DAD. The degradation test was performed with the three different recombinant strains, one wild-type strain, and a negative control (without any yeast). An additional test was performed at 120 h of cultivation with the strain host of the expression plasmid (pRH809+*mlrA*) and the wild type. Only these strains were selected for the extended test, since strains with chromosomally integrated *mlrA* were not able to degrade MCs on the preliminary tests.

2.9. HPLC-DAD Analyses of MCs

MCs analyses were performed on a Shimadzu HPLC System with a DGU-20As Degasser, LC-20AD Pump, CTO-20AC Oven, and a SPD-M20A Diode Array Detector (Shimadzu do Brasil, Barueri, SP, Brazil). Chromatographic separation was achieved at 40 °C using a Luna C18 column (Phenomenex; $4.6\times 250\text{ mm}\times 5\text{ }\mu\text{m}$). The mobile phase consisted of an isocratic mixture of 20 mmol.L^{-1} ammonium acetate and Acetonitrile (70:30; v:v). The flow rate was 1.0 mL.min^{-1} and 20 μL of samples were injected into the HPLC system. MCs were detected at their maximum absorbance of 238 nm. The full chromatographic run was 10 min and the peak corresponding to MCs was observed at 6.36 min. All samples were analyzed in duplicate.

2.10. Statistical Analyses

Analyses of variance (ANOVA) and Tukey test were performed using R Studio Version 2022.02.3 + 492 (www.rstudio.com (accessed on 5 September 2022)) to assess differences on microcystin mean concentrations with tested yeasts. Assays were performed in triplicate and a 95% confidence interval ($p < 0.05$) was used on statistical analysis.

3. Results

The *mlrA* gene used in this study originated in the bacterial strain *S. microcystinivorans* B9. The gene was codon-optimized for expression in *S. cerevisiae*. *S. microcystinivorans* B9 naturally has the *mlr* gene cluster in its genome, and it was made available for tests carried out by part of our research group. Although available reports have demonstrated great potential for cyanotoxins degradation [21], to date, few studies are available on the evaluation of this bacterium to control MCs, and the heterologous expression of *mlrA* from the B9 strain has not been assessed previously. Therefore, the *mlrA* gene from *S. microcystinivorans* B9 was selected to be expressed for the first time in an industrial *S. cerevisiae* strain (PE-2) using both plasmid-based expression and chromosomal integration.

The *S. cerevisiae* strain PE-2 has been shown to have multiple mutations in the *HO* endonuclease gene, which is required for mating type switching, likely rendering it non-functional [24]. To generate haploid strains of PE-2, the diploid PE-2 strain was first sporulated, and individual spores were isolated and tested to determine their mating type and stability of the mating type. Strains YRH1624 and YRH1636, haploids derived from PE-2, were shown to maintain stable MAT α and MATa mating types, respectively, indicating that the *HO* endonuclease in strain PE-2 is not functional (Figure 4a). YRH1624 strains with integrated *mlrA* were then successfully obtained and collected after repeated cultivation on selective medium (as described in Section 2.5). Integration of the *mlrA* gene into the *URA3* locus was confirmed by PCR amplification. Next, recombinant MAT α colonies were mated with YRH1636 wild-type (MATa) to obtain a recombinant diploid. After mating, colonies were randomly selected to assess mating type and presence of *mlrA* at the *URA3* locus (Figure 4). Diploid strains with integrated *mlrA* were then used in MC degradation tests.

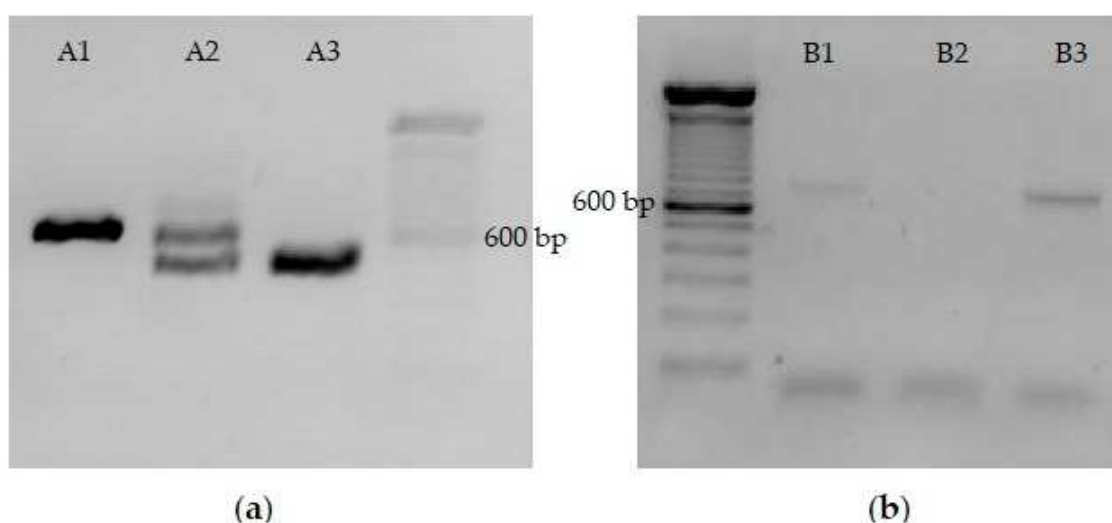


Figure 4. Electrophoresis analyses assessing mating type and *mlrA* integrated gene on yeasts (a) Mating type of tested yeasts: A1—haploid wild-type YRH1636 (*MATa*); A2—diploid strain obtained from YRH1624 recombinant strain mating with YRH1636 wild type; A3—haploid integrated YRH1624 strain (*MATalpha*). (b) *mlrA* gene PCR amplification: B1—diploid+*mlrA* integrated strain; B2—YRH1636 (wild type); B3—YRH1624+*mlrA* integrated strain.

The recombinant yeast strains and a YRH1636 wild-type strain were first used to test MC degradation during 72 h of cultivation. Data are shown in Table 2. As expected, no difference was observed in the concentration of MCs over time for the negative control and wild-type strain, however, none of the recombinant strains with chromosomally integrated *mlrA* reduced the toxins' content. On the other hand, the yeast strain hosting the *mlrA* expression plasmid (pRH809+*mlrA*) reduced MC concentration significantly, even after only 24 h of cultivation.

Table 2. MCs degradation test during 72 h of yeast growth *.

Time (h)	Microcystins Concentration (Mean $\pm$ Standard Deviation—ng.mL ⁻¹)				
	Negative Control	Wild-Type Yeast	pRH809+ <i>mlrA</i> Host	Integrated Strain (n)	Integrated Strain (2n)
0	295 $\pm$ 12.9 ^{Aa}	295 $\pm$ 12.9 ^{Aa}	295 $\pm$ 12.9 ^{Aa}	295 $\pm$ 12.9 ^{Aa}	295 $\pm$ 12.9 ^{Aa}
24	298 $\pm$ 10.9 ^{Aa}	291 $\pm$ 17.3 ^{Aa}	200 $\pm$ 70.0 ^{Bb}	286 $\pm$ 18.7 ^{Aa}	285 $\pm$ 25.9 ^{Aa}
48	300 $\pm$ 21.4 ^{Aa}	299 $\pm$ 18.5 ^{Aa}	153 $\pm$ 46.7 ^{Bc}	285 $\pm$ 17.0 ^{Aa}	290 $\pm$ 17.7 ^{Aa}
72	292 $\pm$ 14.4 ^{Aa}	294 $\pm$ 19.2 ^{Aa}	90 $\pm$ 50.6 ^{Bd}	284 $\pm$ 11.5 ^{Aa}	286 $\pm$ 22.8 ^{Aa}

* Different uppercase letters mean differences in row (by yeast strains). Different lowercase letters mean differences in column (on time). Negative control was performed without any yeast strain. Wild-type is YRH1636 yeast. PE-2 hosts the expression plasmid (pRH809+*mlrA*). Haploid (n) integrated strain is YRH1624+*mlrA*. Diploid (2n) integrated strain was obtained from YRH1624+*mlrA* mating with wild-type YRH1636.

Although the strains with integrated *mlrA* did not reduce MC concentration, the approach using the expression plasmid was efficient. Within the first 24 h of growth MC concentration in the medium was reduced by 32.2%. At the end of 72 h, the total MC content was reduced by 69.6%. As only the PE-2 strain hosting the pRH809+*mlrA* plasmid showed the ability to degrade MCs, an extended test with this strain was performed for 120 h of cultivation and the recombinant yeast was compared to the wild type (Figure 5).

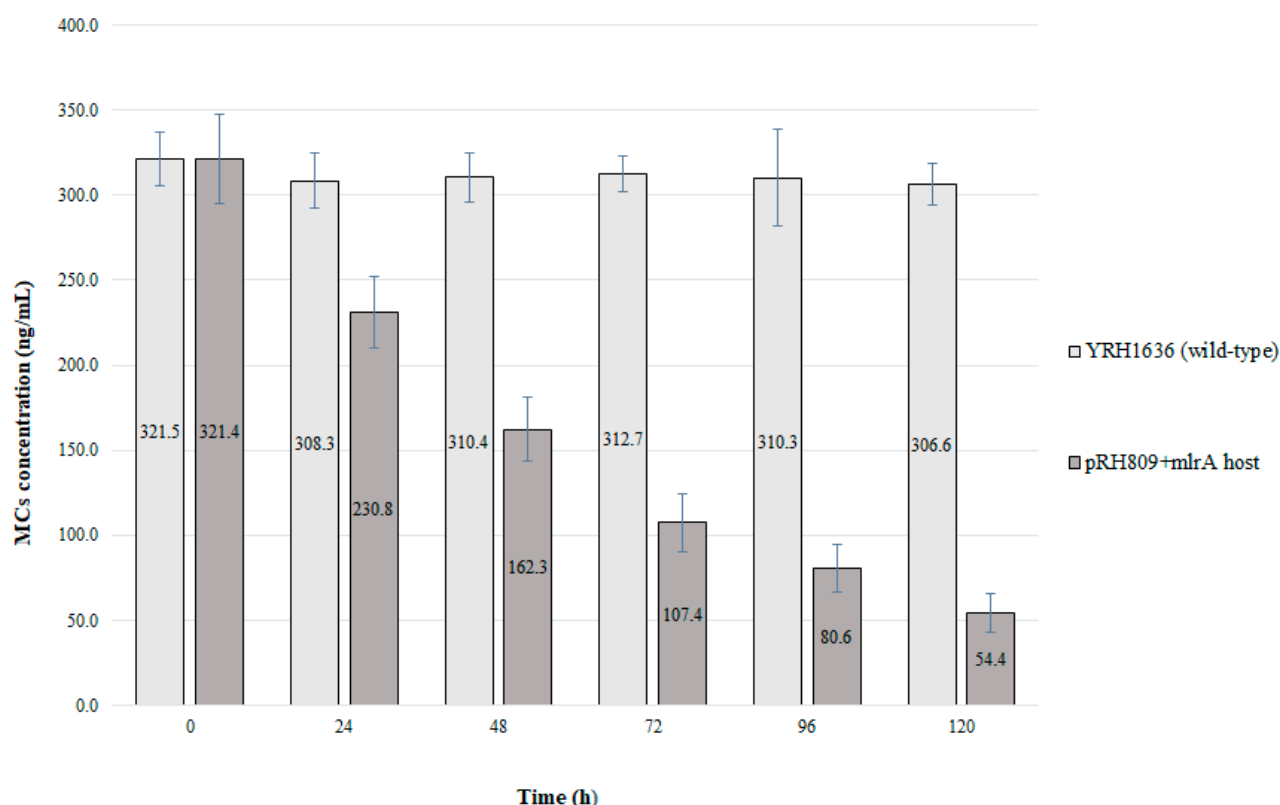


Figure 5. Time course of MC degradation test. Negative control consisted of the wild-type yeast (YRH1636). For the negative control, there was no difference among the mean concentrations of MCs during all the time of cultivation. For the strain with pRH809+*mlrA*, MC content differed among all the analyzed times ($p < 0.05$).

Prolonged cultivation of the strain with pRH809+*mlrA* reinforced the efficient reduction in the concentration of MCs. During the first 24 h of cultivation, toxin content was reduced by 28.2%. After 120 h of cultivation, about 267 ng.L^{-1} of MCs were reduced from the medium, which represents degradation of more than 83% of total MCs. The wild-type yeast strain did not reduce the concentration of MCs from the medium.

In addition to the reduction in MC concentrations observed by our results, analyses of the chromatograms suggested that part of the original cyclic toxin was being transformed into linearized MC. Chromatograms of the recombinant and wild-type yeasts were combined from 0 to 120 h of cultivation. In Figure 6, HPLC-DAD results are shown for elution times from 5.5 to 7.5 min.

At 6.36 min, there was a peak corresponding to cyclic MC, which was confirmed with a MC-LR standard. During the cultivation of the recombinant yeast (PE-2+pRH809+*mlrA*), a significant reduction in the peak area was observed at 6.36 min and a new peak appeared at 6.87 min. Considering the expected activity of microcystinase A, it is suggested that this new peak consists of linearized MCs. The area of the new peak at 6.87 min (with 120 h of cultivation) was quantified as 246.7 ng.mL^{-1} on the MC-LR calibration curve. This concentration added to the remaining 55.4 ng.mL^{-1} of cyclic MCs sums to 302.1 ng.mL^{-1} , which is very close to the MCs concentration at the beginning of the test. Chromatograms from wild-type yeast and yeast with integrated *mlrA* showed no significant difference in the 6.36 min peak area. Moreover, no peak was observed at 6.87 min for these strains.

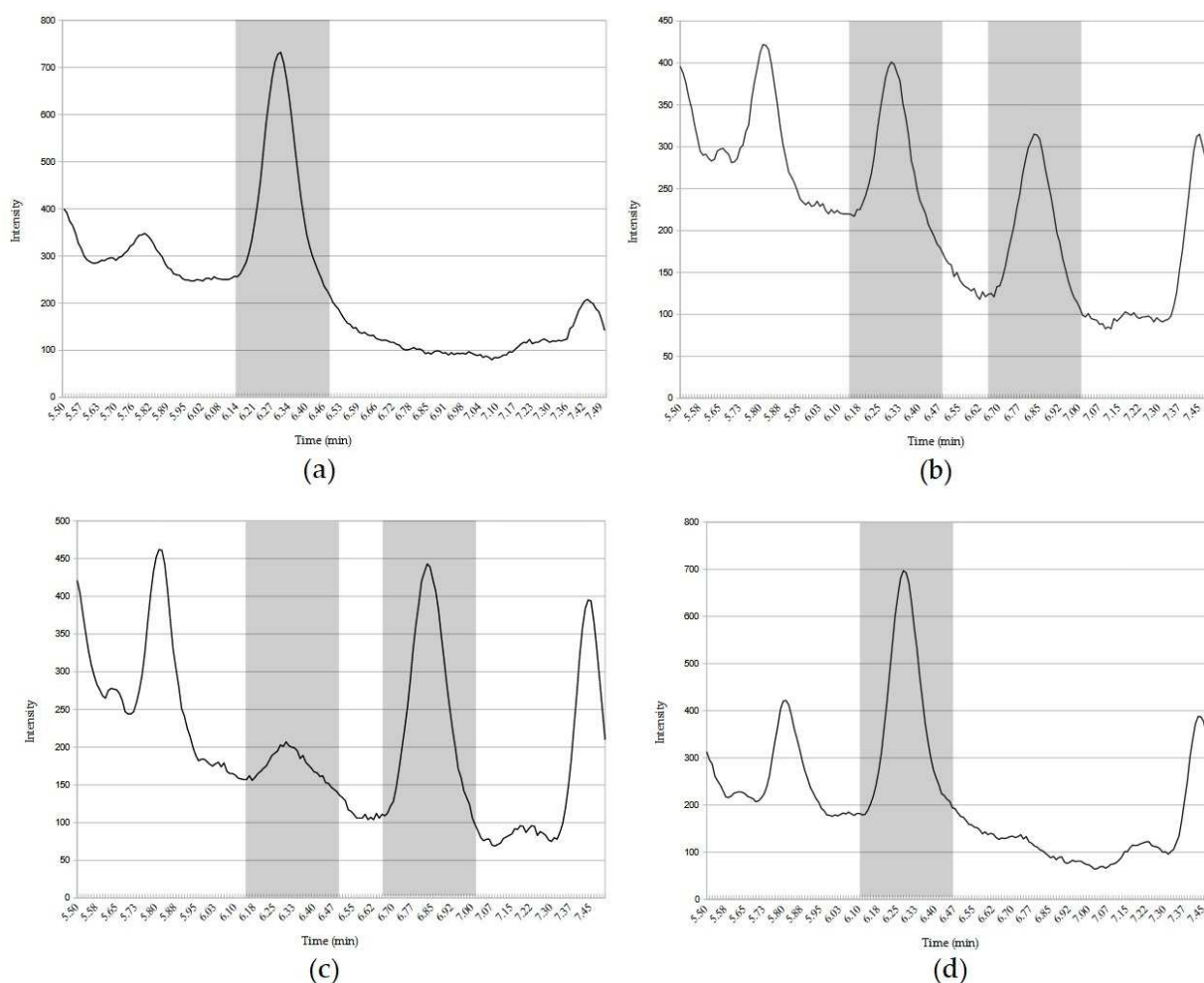


Figure 6. Combined chromatograms of yeast strains cultivated with 300 ng.mL^{-1} MCs in medium. Highlighted peak at 6.36 min represents MCs extracts confirmed by comparison with MC-LR standard. The second highlighted peak area on 6.87 min is suggested to be the linearized MC. (a)—initial point/YPD medium added with MCs before yeast inoculation (0 h); (b)—recombinant yeast (PE-2+pRH809+*mlrA*) culture extracts after 48 h of cultivation; (c)—recombinant yeast (PE-2+pRH809+*mlrA*) culture extracts after 120 h of cultivation; (d)—wild yeast (YRH1636) culture extract after 120 h of cultivation.

4. Discussion

Two strategies were used by this study to heterologously express the *mlrA* gene in *S. cerevisiae*: plasmid-based expression and chromosomal integration. The first tested approach was successful and resulted in a significant decrease in MC content. On the other hand, the integrated recombinant strains did not show degradation activity when cultured with MCs. Chromosomal integration of target genes is a strategy used to overcome possible problems of plasmid-based overexpression. Advantages of chromosomal integration include increased stability of expression and eliminating the need for antibiotics used for plasmid maintenance [25]. However, no MC degradation was observed for strains with integrated *mlrA*.

PCR amplification of the *mlrA* gene in selected strains indicated that the *mlrA* gene was correctly integrated at the *URA3* locus (Figure 4). One possibility for the lack of expression is the orientation of integrated *mlrA* gene. Previous studies reported transcriptional interferences in *S. cerevisiae* resulting from promoter occlusion or transcriptional collision [26,27]. Steiner and Philippsen (1994) described that the *AgTEF* promoter contains two upstream activation sequences (UASrpg) that bind Rap1p, a protein that has been shown to act as a

roadblock to upstream transcription by inducing its termination [28]. Due to the presence of Rap1p binding sites in the *AgTEF* promoter, and its orientation to the remaining *URA3* gene, promoter occlusion is not likely. Conversely, the orientation of the integrated *mlrA* gene with respect to the *URA3* promoter suggests that transcript collision could be the cause of poor expression. Another hypothesis for the inability of the strain with integrated *mlrA* to degrade MCs is that the gene copy number compared to the plasmid-based strain was too low. Nonetheless, to date, it has not been possible to define exactly what is causing the inhibition of *mlrA* expression in integrated strains or their inability to reduce MCs content. Further studies should be carried out to assess the possibilities that might be affecting the gene expression, in addition to considering solutions, such as using different promoters, changing integration orientation, increasing the copy number of integrated gene, and targeting a different locus for *mlrA* chromosomal integration.

Whilst yeast with integrated *mlrA* did not reduce MCs, efficient degradation of the toxin was observed for cells hosting the expression plasmid (pRH809+*mlrA*). These results reinforce the ability of microcystinase A to be expressed in *S. cerevisiae* cells and indicate the enzyme's potential to degrade such contaminants. The objective of this work was to provide a novel biological method to reduce MC toxicity and its impact on public health. The *mlrA* gene encodes the first enzyme of the cluster *mlrABCD* related to the natural degradation of microcystins. This enzyme hydrolyzes the Adda–Arg bond in cyclic structure of MC-LR, reducing its stability and toxicity [14]. A codon-optimized version of the *mlrA* gene sequence from *S. microcystinivorans* B9 strain was used in our study. *S. microcystinivorans* B9 has proved to be efficient in the natural degradation of microcystins, as well as showing the degradation of MCs with immobilized bacteria, and further activity for different amino acid-containing compounds during bacterial growth [29–31]. Other studies confirmed the potential of MC degradation by B9 strain as one of the most efficient among tested microorganisms, reducing 95% of the initial concentration of toxins ($1.0 \mu\text{g.mL}^{-1}$) after 96 h of cultivation [20]. Despite the reported positive results for such bacteria, it is important to note that the genome of *S. microcystinivorans* B9 has other genes involved in the degradation of MCs. Thus, to study *mlrA* activity in the degradation of MCs, and enhance its potential for further applications, engineering and expressing it in different biological systems is required.

Different strategies are available for expressing the *mlrA* gene, presenting distinct potentials of enzyme production and activity. For example, Dexter et al. (2018) expressed the gene in cyanobacteria, resulting in an increased production of the enzyme compared to the native bacteria that hosts *mlrA* and prolonged enzymatic activity under semi-natural conditions of water contamination [32]. Wang et al. (2017) expressed the *mlrA* gene from *Novosphingobium* sp. THN1 in *E. coli* and evaluated its enzyme activity on microcystin–arginine–arginine (MC-RR) degradation. The tests were performed with intact recombinant cells and cell-free extracts. After 56 h of incubation, both treatments reduced more than 70% of MC-RR, however, different trends were observed. Cell-free extracts reduced microcystin concentration at an almost constant rate, while the intact bacteria degraded 73.8% in just 8 h. After this period, the degradation rate was reduced, and a plateau was reached. It was suggested that with the lower concentration of MCs in the medium, the enzyme activity was also reduced [33]. A similar degradation plateau was also seen in a study using probiotic bacteria to remove MC-LR. Using live bacteria, Nybom et al. (2007) showed a maximum MC reduction of approximately 70% with most conditions tested resulting in 40% of MC-LR removed [34]. In each case, the degradation slowed significantly after the first 5 h. This behavior was not observed in our work, in which the reduction in MCs occurred at an almost constant rate of 30% per day and reached 83%.

Heterologous expression of the *mlrA* gene has been performed in *E. coli* strains by most of the available research. Zhu et al. (2016) expressed the *mlrA* gene from *Rhizobium* sp. in *E. coli* cells and $8.3 \mu\text{g.L}^{-1}$ of MC-LR was degraded within 10 h of incubation following lag phase [35]. On the other hand, Moolwang et al. (2021) worked with purified *mlrA* recombinant enzyme expressed in *E. coli* BL21 strains. A complete degradation of

30 $\mu\text{g}\cdot\text{mL}^{-1}$ of MC-LR was reported within 30 h of incubation [36]. In addition, Liu et al. (2020) overexpressed the *mlrA* gene in K12 B1 strain reducing intracellular and extracellular MC content. Furthermore, *Microcystis aeruginosa* growth in BG11 medium was inhibited, which represents a useful biological tool in the control of toxigenic cyanobacteria [37]. In all the studies mentioned above, expression of the *mlrA* gene was induced with IPTG (isopropyl- β -D-thiogalactoside), and experiments reported by Moolwang et al. (2021) and Liu et al. (2020) were performed with the purified enzyme [35–37]. In contrast, here, a constitutive promoter was used, which does not require adding a reagent for induction and MC degradation was assessed during yeast growth.

Genetic engineering aiming to couple the efficient biodegradation of toxins by recombinant enzymes with desired characteristics of yeasts represents a promising alternative to the future development of practical applications for the *mlrA* enzyme. Working with *S. cerevisiae* strains has several advantages that can result in significant advances for research and industrial purposes. The yeast is considered a valuable model organism for genetic engineering due to its highly efficient metabolism, pH tolerance, safety, ease of cultivation, and resistance to some toxic compounds, including MC-LR [38]. Furthermore, as it is the most well-studied eukaryotic microorganism, numerous genetic tools are extensively described for *S. cerevisiae* engineering [19,39].

To date, only one report was found regarding the heterologous expression of an *mlrA* gene in *S. cerevisiae*. Broman et al. (2017) evaluated the expression of a codon-optimized *mlrA* gene from *Sphingomonas* sp. ACM-3962 in yeast cells. The *GAL1* promoter was used and expression was induced with 2% galactose. Enzymes were purified and applied to MC-LR degradation tests. Enzymatic activity was observed as a decrease in MC-LR intensity over time and results confirmed the possibility of engineering *mlrA* and expressing it in yeast cells [40]. Nevertheless, it is important to highlight fundamental differences in the conceptualization and objectives of both studies. The strategies here used a constitutive promoter, and optionally a gene chromosomal integration aiming to provide constant enzyme production and improve stability of the cells. Additionally, the enzymatic activity was measured by the reduction in MCs concentration during yeast cultivation. In addition, this is the first time that a widely used industrial *S. cerevisiae* strain was applied to express a *mlrA* gene.

In the absence of microcystinase A, glutathione and cysteine conjugates of MC are common metabolites seen as the first step in detoxification of cyclic MC [41]. Since shifted or decreased MCs peaks were not observed in cultures with the negative control and wild yeast (Figure 6 d), we do not believe significant levels of GSH-MCs were formed with our strains. The activity of microcystinase A expressed in yeast was observed as the reduction in MC content (Figure 6). A new peak appearing only in cultures of the yeast hosting the *mlrA* expression plasmid likely represents the enzymatic product (i.e., linear MCs). Other studies support this hypothesis, such as Fionah et al. (2022) in which the appearance of a peak corresponding to linearized MC occurred after the expression of *mlrA*: the identity of the new compound as linear MC was confirmed by LC-MS [42]. A new peak in microcystinase assays was also reported by Cai et al. (2022). After LC-MS analyses, it was confirmed to be linearized MC [43]. Although the discussion above strongly suggests that the new peak observed in our study is linearized MCs, it was not confirmed by secondary analysis.

Alternatively, the linearization of cyclic MC can be associated to different physical and chemical methods. The association of microcystinase A activity with H_2O_2 treatment, reduced simultaneously the toxicity of MC and the growth of cyanobacteria [44]. Wu et al. (2019) immobilized the enzyme in L-cystein graphene oxide and observed the degradation of 83% of nodularins within just one hour of incubation [45]. These are reported alternatives that can be used to spread *mlrA* applicability and increase the degradation of cyanotoxins. Here, MCs were solely treated with the *mlrA* enzyme, so our results offer proof of concept that *S. cerevisiae* is an appropriate host to produce active microcystinase A.

5. Conclusions

Expression of the codon-optimized *mlrA* gene in *S. cerevisiae* strains was assessed by two different strategies: using an expression plasmid and gene integration at the *URA3* locus. Chromosomal integration of the *mlrA* gene at the *URA3* locus was successfully achieved, and both haploid and diploid strains were generated with the integrated gene. Although presence of the gene was confirmed, MCs were not degraded by these strains. However, the PE-2 based strain containing the expression plasmid (pRH809+*mlrA*) was highly efficient at degrading MCs, reducing MC concentration by 83% after 120 h. The results demonstrate that industrial *S. cerevisiae* strains can be used as host for synthesizing microcystinase A. Further studies are warranted to improve the activity of the recombinant enzyme and define suitable methods for its practical application.

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5.2 CAPÍTULO 2:

**ADVANCED ASSESSMENT OF A YEAST-EXPRESSED
RECOMBINANT MICROCYSTINASE A: ENZYME
PROPERTIES, ACTIVITY, AND STABILITY**

Abstract

Microcystins (MCs) are toxins produced by cyanobacteria and important contaminants of water. As noted threats for human and the public health, effective strategies to control microcystins impact must couple monitoring and toxin removal techniques. In this sense, the biocontrol based on the activity of the enzyme microcystinase A emerge as a sustainable and functional alternative for microcystins biodegradation. Aiming to diversify and enhance the production and activity of this enzyme, it was heterologously expressed in *Saccharomyces cerevisiae* PE-2 strain. The enzyme originally from the bacteria *Sphingosinicella microcystinivorans* B9 was expressed in yeast cells with successful production of active enzymes. Still, further investigation into the recombinant enzyme is needed to determine its properties when expressed by yeast cells, especially regarding the possibility of post-translational modifications on the protein. Moreover, the evaluation of activity and stability characteristics would provide a basis for optimized enzymatic assays. Therefore, this study aimed to obtain microcystinase A expressed by yeast cells and conduct a partial characterization of its profile. Different methods to disrupt the cells were applied to release the intracellular enzyme for testing. Then, cell-free extracts were used to assess the enzymatic activity with different times of incubation and protein concentrations. Enzyme kinetics analysis demonstrated the maximum biodegradation rate of 3.09mg/L/h and K_m constant of 2.81 μ M of MCs. Higher microcystinase A activity was observed with temperatures in the range 35°C-57.5°C. At 65°C, the enzyme was quickly denaturated. Also, alkaline pH seemed to strongly inhibit the enzyme activity. In general, assays carried out in mild-acid or neutral pH (6-7) resulted in more than 70% of MCs degradation. Statistical analyses defined 42.2°C and pH 6.3 as optimal conditions for enzyme activity. The enzyme was fully active after 2 hours at 20°C, but progressive inactivation was promoted in higher temperatures. One hour at 50°C reduced 40% of the enzyme activity, whilst complete inactivation occurred within 30 minutes at 65°C. The results of this study provide a valuable foundation about characteristics of this recombinant microcystinase A, supporting the development of further applied studies focusing microcystin biocontrol.

Keywords: microcystinase A; microcystin control; enzyme activity; thermal stability; yeast.

1. Introduction

Environmental toxins can affect severely the safety of water sources and pose as an important threat to human and animal health. In the case of microcystins contamination, the ingestion and direct contact with the toxins can cause symptoms of intoxication, mainly resulting in liver disease, but also related to cancer promotion, eventually leading to death [1]. These toxins are produced by cyanobacteria especially after bloom events, when the increased content of nutrients in water promotes the proliferation of cyanotoxigenic microorganisms. Human activities have led to frequent eutrophication of water sources, which, combined with the effects of climate change, exacerbates water contamination by microcystins, making it an emerging worldwide concern [2]. With this imminent threat, constant surveillance is required to prevent human intoxication. Furthermore, new research must focus on the development of methods to control microcystins levels.

The effective control of microcystins in water is complicated due to structural characteristics of these compounds. Naturally presented as cyclic toxins, they demonstrate high stability in a large range of pH values, high temperature, and in the presence of sunlight [3]. A provisional guideline of 1 µg/L of MC-LR in drinking water was established by the World Health Organization (WHO, 2020) as an international standard for the water quality [4]. Brazilian regulations on water potability align with WHO limit, however the established level of 1 µg/L is designated for the total concentration of all microcystins variants [5]. Although different treatments can be applied to remove cyanobacteria or reduce toxins levels from water, physical-chemical methods face limitations primarily due to high implementation costs and sustainability concerns. The conventional water treatment cycle requires complementary techniques for effective microcystins removal, such as oxidation, adsorption or membrane filtration. Nevertheless, these methods can introduce chemical substances and generate residues of complicated management, along with the additional risk of lysis of cyanobacteria cells and the consequent release of microcystins into the water [6]. To enhance the efficiency of microcystins removal from water, innovative methods should be explored taking into account both economic and environmental feasibility. Among known techniques used to reduce the toxicity of microcystins, a biological method based on the natural microcystinase A enzyme is highlighted. This enzyme encoded by the gene *mIra* cleaves the cyclic structure,

linearizing the microcystins and resulting in compounds of reduced stability and toxicity estimated as 2,100 lower than the original structure [7,8].

With the objective of improving the expression and better investigate the activity of the *mlrA* enzyme, it has been heterologously expressed in different microorganisms, mainly in bacteria as reported by Dexter *et al.* (2020) [9]. Despite well-defined mechanism of action, enzymes expressed in different biological models can present distinct properties with influence on its activity. The approaches for heterologous expression, types of host cells, efficiency on gene transcription and translation, variations in protein location, post translational modifications, and interactions with the host cellular machinery can impact the gene expression and the enzyme functionality [10].

Recently, the *mlrA* gene was also expressed in a *Saccharomyces cerevisiae* strain using the original gene sequence from *Sphingosinicella microcystinivorans* B9 strain with codon adaptations for expression in yeast [11]. The successful DNA recombination technology allowed to obtain active microcystinase A produced by eukaryotic cells. Post-translational modifications are frequently observed in *Saccharomyces cerevisiae* heterologously expressed proteins, resulting in differences on the enzyme location, functionality and stability [12]. Among the modifications commonly observed in this yeast genus, glycosylation is frequently reported, leading to proteins with increased molecular weight, though this does not always correlate with differences in enzyme activity. [13].

Considering the absence of data about microcystinase A expressed in yeast, it is essential to generate and disseminate basic knowledge about the recombinant enzyme to substantiate new research and practical advancements targeting its potential for the biocontrol of microcystins. Likewise reported by Sun *et al.* (2021), the understanding of this enzyme characteristics, activity and its tolerance to environmental conditions were not completely elucidated, demanding new studies to supplement future applications [14].

Therefore, this study investigated the activity of this specific recombinant *mlrA* enzyme originated from *Sphingosinicella microcystinivorans* B9 and expressed in an industrial strain of *Saccharomyces cerevisiae*. The enzyme characteristics were assessed regarding the gene expression by yeast cells, and the possibility of post-translational modifications added to the recombinant protein. Cell-free extracts were obtained through different extraction methods applied to disrupt cell walls and

release the enzymes. Several assays were performed with cell-free extracts in order to define enzyme characteristics, mainly related to its kinetics, the effect of temperature and pH on the enzymatic activity and stability. The provided results should disseminate information on aspects related to microcystinase A activity and the influence of its expression by yeast cells, allowing further advances in studies that aim to apply the enzyme potential for microcystins biocontrol.

2. Material and Methods

The recombinant *Saccharomyces cerevisiae* PE-2 hosting the *mlrA* gene was constructed with the cooperation of USDA and State University of Londrina (Brazil) researchers. The process of cell construction was performed at the State University of Londrina and it was entirely described by Silva *et al.* (2023a) [11]. Following procedures in order to extract the enzyme and conduct the characterization of its activity were carried out at the Department of Food Technology and Bioprocess Engineering of the Max Rubner-Institut (Karlsruhe, Germany) in 2022 and 2023.

2.1. Yeast Cultivation and Enzyme Expression

The recombinant *Saccharomyces cerevisiae* PE-2 strain hosting the plasmid with the *mlrA* gene was cultivated in Yeast Potato Dextrose medium (2% peptone, 1% yeast extract, 2% dextrose) under incubation at 30°C and uninterrupted agitation (100rpm) in an Orbital Incubator INFORS AG CH 4103 (Minitron, Bottmingen, Switzerland). The medium was supplemented with 0.1% of geneticin (G418). As the recombination protocol used plasmidial expression of the gene, it was necessary to add the antibiotic during the yeast cultivation to avoid the loss of the introduced genetic structure within consecutive yeast generations.

To obtain high concentrations of the target enzyme, yeast cells were grown until reaching the maximum optical density (OD), approximately 6.0, measured by absorbance at 600nm in a spectrophotometer Specord 200 (Analytic Jena, Germany). The concentration of yeast per mL of cultured medium was not estimated, as models available in literature rely on cell counts during the log phase of growth, while our culture had already reached a constant OD, indicating it had entered the stationary phase of growth. Considering the sole report on microcystinase A expression in yeast cells, it has been suggested the enzyme is located in the

intracellular region which would require the existence of specific transporters for the intake and ulterior release of toxins by the cells [15]. To verify the hypothesis that the *mlrA* gene was translated as an intracellular protein either by our constructed recombinant yeast, an enzymatic preliminar trial was performed after yeast cultivation. After yeast cultivation, the cells were harvested using a Thermo Scientific Sorvall LYNX 6000 Centrifuge (Germany) set at 130 x g and 4°C for 10 minutes. The supernatant was collected to test the effect of the enzyme activity only in the presence of extracellular content, i.e., without cell mass. Meanwhile, collected pellets were resuspended in the same amount of fresh YPD medium and used to test the enzyme activity in the presence of the yeast cells. Both samples were mixed to predetermined concentrations of microcystins. After one hour of incubation (37°C/100 rpm), the toxins were analyzed and the differences verified on their concentrations to estimate the fraction of cultured medium containing microcystinase A.

2.2. Yeast Cells Lysis Protocols

As the aim of this experiment was to study the microcystinase A characteristics when produced by recombinant yeast cells, it was necessary to disrupt the cells membranes to liberate these intracellular enzymes for testing. Without the presence of living cells, tests using fixed concentrations of enzyme would allow for a clearer interpretation of its profile and activity, free from cellular interferences. Then, different mechanical methods were assessed to the lysis of cell membranes. The objective here was to define a suitable technique that results in cell-free extracts with high concentration of proteins combined to effective enzymatic activity. Eight different protocols were tested considering the availability of equipments and procedures performed at Max Rubner-Institut, as described below:

- (i) – Glass beads I
- (ii) – Glass beads II
- (iii) – Sonication I
- (iv) – Sonication II
- (v) – Sonication III
- (vi) – Sonication IV
- (vii) – High pressure homogenization I
- (viii) - High pressure homogenization II

(i) Glass beads I: yeast cultivated medium with maximum OD_{600nm} was centrifuged for 10 min at 23450 x g and 4°C. The supernatant was discarded, the cellular content (pellet) was washed with cold water and centrifuged again in the same conditions. Once more, the supernatant was discarded and the pellet weight was measured. Three parts of cold 50mM Tris+DTT buffer (pH 7.4) were added to one part of pellet. Then, 4 parts of chilled acid-washed glass beads (425-600µm, Sigma) were mixed to the samples. The tubes were agitated for 1 minute on a vortex mixer and placed on ice for 2 minutes to prevent protein denaturation. Mixing and cooling steps were repeated 5 times. After that, the tubes were placed on ice until the glass beads settle. Then, the supernatant containing the cell-free extract was decanted to a new flask. An additional step was performed adding 4 parts of the same buffer to the mixture of cells and glass beads, gently inverting the tube for 10 times. Following, the glass beads were settled again and the supernatant was collected and pooled with the first fraction of cell-free extracts. Pooled extracts were centrifuged at 23450 x g and 4°C for 60 min and the supernatant was applied to tests of total protein concentration and microcystinase A activity.

(ii) Glass beads II: this method followed the same procedures of the first glass beads method with additional repetitions of vortex mixing and cooling steps. Instead of 5 times, the process was repeated for 15 times to test the effect of increased agitation on the content of proteins in extracts, and the remaining enzyme activity.

(iii) Sonication I: all the sonication protocols were conducted in a Sonoplus Ultrasonic Homogenizer (Bandelin, Germany) using a probe MS72 specific for cell disruption and 75% amplitude. Prior to the extraction, the cells were grown until maximum OD_{600nm} and the cells were harvested as described for the glass beads methods. Then, the pellets were resuspended in 5mL of 50mM Tris+DTT buffer (pH 7.4) and applied to 4 different sonication protocols. In this first one, the cells were broken with 4 cycles of sonication. One cycle consisted of 1 minute under sonication followed by 1 minute without sonication (0.5 duty cycle), with samples resting on ice to avoid denaturation caused by the produced heat. After the 4 cycles of sonication, the samples were centrifuged at 23450 x g and 4°C during 30 min, the supernatant was collected (cell-free extracts) and used to measure total protein content and microcystinase A activity.

(iv) Sonication II: cells were disrupted by the combination of 10 repetitions of the following 0.5 duty cycle: 2 minutes under sonication and 2 minutes without sonication, with samples cooling down on ice. Then, the samples were centrifuged ($23450 \times g/4^{\circ}\text{C}/30 \text{ min}$) and the supernatant was collected for testing.

(v) Sonication III: cells were broken using a 0.8 duty cycle - 192 seconds under sonication followed by 48 seconds with samples resting on ice. The sonication cycle was repeated 10 times. Afterwards, the samples were centrifuged ($23450 \times g/4^{\circ}\text{C}/30\text{min}$) and the supernatant was collected for testing.

(vi) Sonication IV: this method was used to disrupt cells with only one cycle of uninterrupted sonication (1.0 duty cycle) performed for 10 minutes. Then, the samples were centrifuged ($23450 \times g/4^{\circ}\text{C}/30\text{min}$) and cell-free extracts collected for testing.

(vii) High Pressure Homogenization I: two methods used a GEA Panda Plus 2000 High Pressure Homogenizer (GEA, Germany) to disintegrate the cells membranes following the equipment instructions. Yeast cultivation and the procedures carried out prior to the extraction protocol were the same described for all the other methods. The only difference in this case was the volume of cultivation. Due to specific requirements of the equipment, a larger cultivation volume (250mL) was necessary for extraction in the homogenizer. The cells harvested from the cultivated medium were resuspended in 100mL of 50mM Tris+DTT (pH 7.4) and poured into the equipment. Samples were only once injected, and cells broken under 1,500 bar of pressure, followed by collection and centrifugation ($30240 \times g/4^{\circ}\text{C}/30\text{min}$). The supernatant containing cell-free extract was used for testing total protein content and microcystinase A activity.

(viii) High Pressure Homogenization II: the preparation steps were conducted in the same way of the preceding method, however, here the sample was injected twice in the equipment. In the first injection, cells were broken under 1,500 bar of pressure, while 1,700 bar was set on the second injection. The homogenized sample was collected, centrifuged ($30240 \times g/4^{\circ}\text{C}/30\text{min}$) and the supernatant applied for testing.

The concentration of total protein in all the cell-free extracts was measured by Bradford method at OD595nm, using a BSA standard curve for quantification. All the samples were analyzed in triplicates. The extract with the lowest concentration of total proteins was used as a reference to dilute other extracts in 50mM Tris+DTT buffer (pH 7.4) and compare the activity of microcystinase A among extracts. Cell-free extracts were previously diluted to 0.4mg/mL of total protein and incubated at 37°C for 60 minutes and agitation of 100 rpm with microcystins.

Microcystins used in this experiment were obtained from *Microcystis* sp. cultivations by adaptations of the technique described by Kuriama *et al.* (2012) [16]. The NIES TAC95 strain was cultivated in sterile ASM-1 medium at 27°C, under illumination ($20\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) with a 12-hour photoperiod and constant agitation. After reaching the density of 10^6 cells per mL, the culture was centrifuged ($3500 \times g$ for 15min at 4°C). The collected biovolume was frozen and thawed three consecutive times, then sonicated to lyse the cyanobacteria cells (Unique Maxiclean 800, Unique, Brazil). The extracts were sequentially vacuum filtered through 0.45 μm and 0.2 μm cellulose ester membranes and diluted in ultrapure water to an approximate concentration of 2mg/mL of microcystins.

Temperature and pH conditions used to test the activity of microcystinase A were selected taking into consideration the published data about this enzyme produced by different hosts [14,7]. Also, previous trials were conducted using a protein phosphatase 1a (PP1a) inhibition assay adapted from the method described by Almeida *et al.* (2006) [17]. A starting point for some of the factors evaluated by this study, such as the buffer, time, and other experimental conditions for microcystinase A reaction were selected based on these preliminar PP1a inhibition assays.

The assays for microcystinase A testing were prepared with 300 μL of final volume as follows: 30 μL of the cell-free extracts mixed to 15 μL of *Microcystis* sp. extracts (containing 2 $\mu\text{g}/\text{mL}$ of microcystins) and 255 μL of Tris buffer+DTT (pH 7.4). After reaction, the samples were heated in a water bath set at 100°C during 10 minutes to stop the enzymatic reaction, and promptly cooled on ice. Then, the samples were centrifuged at $19320 \times g$ and 4°C for 10 minutes, the supernatant was filtered in 0.45 μm cellulose membrane filters (Phenex, Phenomenex, USA) and maintained in vials at 4°C until injection in the HPLC system. These analyses aimed to identify the most suitable method for producing extracts with high protein concentrations while preserving microcystinase A activity.

Afterward, to support the hypothesis that the removal of microcystins occurs due to the enzyme produced by the recombinant yeast, negative control samples were extracted from native yeast cells without the *mlrA* gene. Aliquots of wild-type and recombinant yeast cell-free extracts were analyzed using SDS polyacrylamide gel electrophoresis (SDS-PAGE) and compared to identify the target protein. The extracts were diluted, adequately prepared with Roti-Load buffer and injected in a 12.5% polyacrylamide separation gel. Coomassie brilliant blue was used as stain for the gels and the ladder (Bio-Rad, USA) covered the range of 10-250kDa proteins.

2.3. Chromatographic Analyses of MCs

All the chromatographic analyses of microcystins were performed in a HPLC Agilent 1260 Infinity equipment coupled to a Hewlett Packard 1100 Series Diode Array Detector (G1315A model), and a G1316A Oven (Agilent Technologies, USA). A Luna C18 column (4.6mm × 250mm, 5 µm; Phenomenex, USA) was used for the separation of the extract compounds and microcystins were acquired with an isocratic mobile phase consisting of the mixture of 20mM Ammonium acetate and acetonitrile in the proportion of 78:22 (v/v). Microcystins were detected considering their maximum absorbance at 238 nm. The chromatographic method was adapted from Silva *et al.* (2023a) [11]. Cyclic microcystins were acquired at 5.4 minutes while the linearized toxin peak appeared at 5.1 minutes.

2.4. Enzymatic Reaction Over Time

A test was conducted with cell-free extracts containing 0.4 mg of total proteins per mL, 2 µg of microcystins per mL and 50mM Tris+DTT buffer (pH 7.4) to complete 1 mL of final volume. Enzymatic reaction occurred at 37°C and 100 rpm for up to 130 minutes. The total extension of this test was defined based on preliminar results obtained through the analysis of microcystinase A activity and its effect observed on the inhibition of PP1a. Therefore, aliquots were collected with 15, 30, 60, 80, 100, 115 and 130 minutes. Collected samples were heated (100°C for 10 minutes) to stop the enzymatic reaction, cooled and prepared for the HPLC analysis as described in the section 2.3. Analyses were performed in triplicate. The results would allow exploring the enzyme activity through time setting a suitable time for reaction, considering its effectiveness and the convenience of analysis.

2.5. Total Protein Concentration in Cell-free Extracts and Its Effect on Microcystinase A Activity

The effect of different concentrations of total protein in cell-free extracts was assessed considering the rate of MCs enzymatic degradation. Extracts with total protein content measured by Bradford method were diluted in the range of 0.25mg/mL-1.5mg/mL with 50mM Tris buffer + DTT, pH 7.4. Microcystins were added to the reaction in the concentration of 2µg/mL. The analyses were performed in triplicate and the enzymatic reaction occurred at 37°C and 100 rpm for 60 minutes. Procedures performed after the enzymatic assay, including sample preparation and HPLC-DAD analysis followed the same methods described in the sections above.

2.6. Enzyme Kinetics

To comprehend the enzyme kinetics, assays with several substrate concentrations (*Microcystis* sp. extracts with microcystins) were performed. For the reaction, predetermined conditions were applied: 0.4 mg/mL of total protein in cell-free extracts diluted in 50mM Tris buffer (pH 7.4), incubation at 37°C and 100 rpm of agitation during 60 minutes. Microcystins were added from 0.2µg/mL to 6.5µg/mL. The velocity of reaction (V_0) considered the amount of substrate (S) degraded, which means the concentration of cyclic microcystins converted into linear toxin within one hour of enzymatic reaction. Michaelis-Menten model was used on the representation of the enzyme kinetics and the constant values were calculated based on the Lineweaver-Burk plot. Double-reciprocal transformations of the reaction rate ($1/V_0$) and substrate concentration ($1/[S]$) were used on the graph and curve construction. K_m was calculated based on the slope of the curve and V_{max} was obtained from the y-intercept ($1/V_{max}$).

2.7. Microcystinase A Activity Assessment: Temperature and pH Conditions

An experiment was planned considering combinations of distinct conditions of temperature and pH. Temperatures of incubation were tested from 20°C to 65°C with an interval of 7.5°C between different levels. Meanwhile, the activity of the enzyme was analyzed with buffer pH values in the range of 5.0 to 10.0. Control samples were analyzed by injecting buffer at different pH values added of MCs into the HPLC-DAD

system in order to assess whether the pH would affect the retention time of toxins. For all the assays, 50mM Tris buffer was used with the necessary pH adjustments made with the addition of 1M HCl or 5N NaOH. Assays evaluating microcystinase A activity across all combinations of temperature and pH were conducted in triplicate. Cell-free extracts with 0.4mg/mL of total protein were mixed to 2µg/mL of MCs and buffer up to complete the final volume of 300µL. Samples were incubated during 60 minutes with continuous agitation (100 rpm) at different temperatures. The following procedures to stop the enzymatic reaction, sample preparation and chromatographic analyses were done exactly as described in the previous sections 2.2. and 2.3.

2.8. Temperature Stability Test

To investigate how temperature conditions could affect the stability of the recombinant microcystinase A, and consequently, reduce its activity, 4 temperature levels (20°C, 35°C, 50°C and 65°C) were applied to an additional pre-incubation step. Aliquots of cell-free extracts were pre-incubated for 15, 30, 60 or 120 minutes at the different levels of temperature. Then, the microcystinase A assay was performed at the same conditions established for other tests (37°C/100rpm/60min) and with the same concentration of substrate (2µg/mL). Finally, the pre-incubated cell-free extracts were added to the reaction at the final concentration of 0.4mg/mL of total protein. Degradation rates obtained by the pre-incubated samples were compared to the control samples that were not submitted to any additional pre-incubation step. Thereby, the thermal stability of microcystinase A was demonstrated as the percentage of activity maintained after time-dependent exposure to different levels of temperature.

2.9. Statistical Analyses.

All the statistical analyses conducted in this experiment, including mean comparisons, ANOVA, Tukey test, and the construction of contour and response plots, were performed using R Studio Software Version 2022.02.3 + 492 (www.rstudio.com - Accessed on 18 May 2024).

3. Results and Discussion

A preliminar test using cultivated YPD medium with and without cells showed that only in the presence of the recombinant cells the enzymatic reaction was observed, which reflected on the reduction of microcystins concentration. This result reinforces the hypothesis that the recombinant *mlrA* enzyme, when heterologously expressed by yeast cells, it is not secreted into the extracellular medium, remaining intracellular as described by Valério *et al.* (2014) and Broman *et al.* (2017) [18,15]. Nevertheless, the whole process involved in the transport of toxins into the yeast cells and the release of the catabolites to the external medium are not well defined. Therefore, to make the enzyme available for testing and evaluate its properties, regardless of cellular interferences, it was necessary to define an efficient protocol to disrupt cell membranes and release part of the enzyme to cell-free extracts.

The results of 8 different methods evaluated to extract proteins from the yeast cells and the estimation of microcystinase A activity obtained with diluted cell-free extracts were presented on Table 1:

Table 1. Total protein content and microcystinase A activity measured with cell-free extracts obtained from different methods for cells lysis.

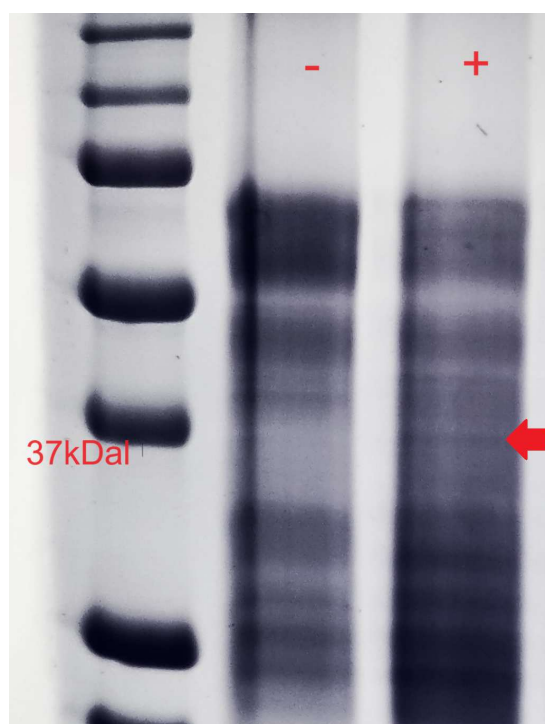
Method	Total protein concentration* (mean±sd – mg/ml)	Total protein concentration in extracts used for tests (mg/ml)	% of cyclic MC degradation
(i) Glass beads I	1.35±0.17	0.4	26.43
(ii) Glass beads II	3.49±0.09		41.33
(iii) Sonication I	0.45±0.20		25.41
(iv) Sonication II	1.83±0.26		32.43
(v) Sonication III	1.52±0.25		59.56
(vi) Sonication IV	1.55±0.24		70.24
(vii) High Pressure Homogenization I	2.64±0.24		55.55
(viii) High Pressure Homogenization II	1.84±0.7		43.56

*Bradford method was used to quantify the total protein content in extracts.

Only mechanical methods were tested to break yeast cells. These methods have the advantages of ease of application and low cost, meanwhile the lack of selectivity on the released protein may result in a more complex process of purification [19]. In our case, the reduced selectivity of the method was not a problem, since the recombinant enzyme targets a very specific site of the toxin structure, thus, other enzymes in the extract should not be of great importance to measure microcystinase A activity [20]. This characteristic permitted the evaluation of the enzyme activity simply with cell-free extracts, eliminating the need for additional purification steps.

Regarding the protein content in the extracts, the protocols that used glass beads for extended time (ii) and high pressure homogenization (vii) yielded extracts with concentrations higher than 2.5mg/mL. However, the activity of microcystinase A extracted with these methods was reduced compared to other approaches, such as two of the sonication protocols (v, vi). Milder methods resulted in either lower total protein concentration and reduced enzyme activity. An effective procedure to obtain sufficient microcystinase A for testing should combine a high yield of extracted proteins with functional enzyme activity. Mukherjee *et al.* (2020) tested different methods to extract proteins from *Saccharomyces cerevisiae* cells, obtaining low yield with glass beads and sonication. Although the authors described hot-SDS and alkali treatment as the most efficient methods, it was also mentioned that these methods resulted in significant protein denaturation, which is an undesirable effect for studies that aim to evaluate active and functional proteins [21]. On the other hand, Liu *et al.* (2013) assessed the effect of sonication factors on the total protein content of disrupted cells and, similarly to our results, the release of proteins in extracts was increased when higher duty cycles were applied [22]. So, considering the best activity for the enzyme was observed with cell-free extracts obtained by the fourth sonication method (vi), besides the good practicality of this protocol, the method was chosen as the most suitable among those tested to release active enzymes and comply with the purposes of the experiment. Furthermore, the presence of the recombinant protein was also analyzed in yeast extracts by SDS-PAGE. Figure 1 shows the SDS-PAGE results of two cell-free extracts obtained from *Saccharomyces cerevisiae* cells by the chosen extraction method (vi).

Figure 1. SDS-PAGE with the protein profiles of cell-free extracts obtained from: (-) wild yeast without the *mlrA* gene; (+) recombinant *Saccharomyces cerevisiae* PE-2 strain expressing the *mlrA* gene.



The left column (-) demonstrated the profile of proteins extracted from a wild-type yeast which does not host the *mlrA* gene on its genome. The second column (+) shows the profile of proteins extracted from recombinant *Saccharomyces cerevisiae* yeast producing microcystinase A. In our study, the strategy used to express the enzyme was to assemble *mlrA* as a constitutive gene that does not require any specific substance for its expression. Either, no tag was added to the gene in order to avoid any possible negative effect on the enzyme activity. Undesirable effects as reduced activity, lower affinity to the substrate and decreased thermal resistance were reported as possible problems caused by proteins tagging [23]. As the expression of microcystinase A in eukaryotic cells represents an innovative advance on the biocontrol of MCs, there is an evident lack of data on this approach. Therefore, this study also tried to unveil basic characteristics of the expressed protein, such as the molecular weight and any possible structural modifications. Considering solely the SDS-PAGE analysis, it seemed no structural modification was observed on the enzyme, maintaining 36-37kDal of molecular weight, which was the expected length based on the sequence of amino acids of the gene. The band representing the expressed microcystinase A was highlighted in Figure 1.

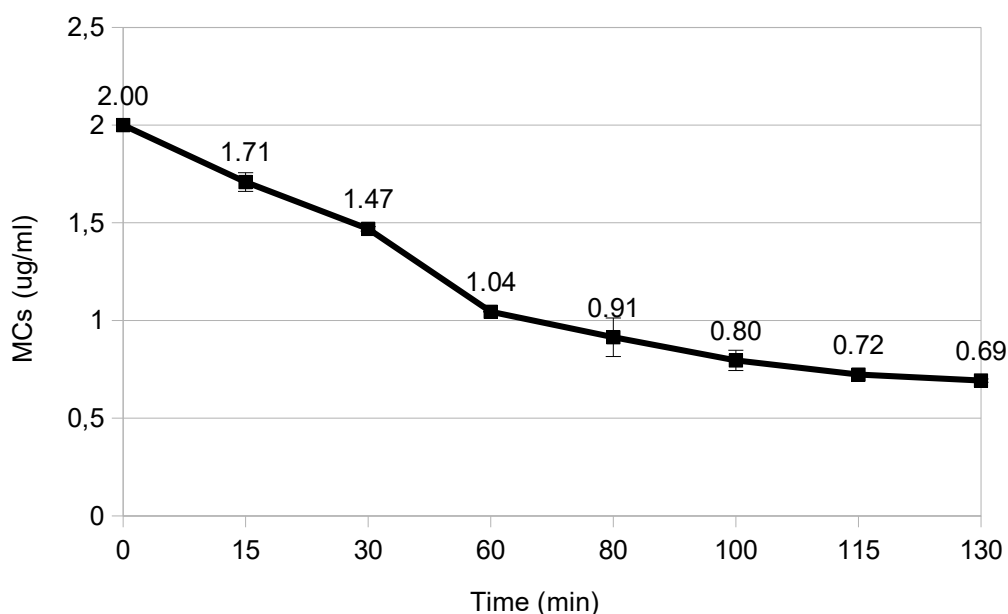
Taking into account the length of heterologously expressed *mlrA* gene, different SDS-PAGE results were reported, from 25kDal described by Dziga *et al.* (2012) and Sun *et al.* (2021), around 42kDal by Maseda *et al.* (2012) and Fionah *et al.* (2022), up to 78kDal by Liu *et al.* (2020) [24,14,25,26,27]. For all these experiments, tags were used to facilitate the process of purification, and no negative effects were reported on the enzyme activity. Such basic information are important in the comparison of enzymatic activity results, since the differences on the strategies applied for the gene expression, including distinct host microorganisms, might affect the enzyme structure and activity.

Subsequent experiments were performed to define suitable conditions for the microcystinase A reaction. It is important to note that the available literature on the enzyme and preliminary tests conducted with a PP1a inhibition assay were considered in the planning of these tests. PP1a is known to be strongly inhibited by MC-LR, allowing the development of screening assays to detect microcystins based on rates of the phosphatase inhibition [28]. Our preliminar tests with MCs-contaminated samples treated with microcystinase A, showed reduced inhibition of PP1a, which means a decrease in MCs content. These results confirmed the ability of the extracted microcystinase A to linearize cyclic microcystins, and provided a basis for the subsequential tests, demonstrating the selected buffer and the initial conditions set for enzymatic reaction as adequate to investigate microcystinase A activity.

In Figure 2 it was shown the degradation of MCs during 130 minutes of reaction. On the first minutes of enzymatic reaction, an accelerated degradation rate was observed, with 0.29µg/mL degraded within the first 15 minutes, followed by 0.24µg/mL in the next 15 minutes. Almost 50% of the total toxins were degraded with one hour of reaction. Afterward, the reaction rate was reduced. Between 100 and 115 minutes of reaction the content of toxin was reduced by 0.08µg/mL, whilst the last 15 minutes demonstrated the lowest rate of degradation, only 0.03µg/mL. The decrease on the rate of enzymatic activity with longer times may have occurred due different reasons, such as, reduced availability of substrate, or protein denaturation with prolonged exposure to temperature and pH conditions. Initial toxin concentration and pH of the medium are among main factors that can interfere in the activity of MCs degrading enzymes [29,30]. Considering the effective reduction of the MCs concentration and the convenience of analysis in relative short time, 60 minutes of

reaction was set as an adequate time for the enzymatic evaluation. Despite it was not possible to determine exactly the reason for the decrease in the activity rate over time, the exposure of the enzyme to adverse temperature or pH conditions could promote intense denaturation with longer times of reaction.

Figure 2. Cyclic MCs concentration during microcystinase A reaction*.



*The concentration of total proteins in cell-free extracts was 0.4mg/mL. MCs were added as substrate in the concentration of 2µg/mL. Incubation was set at 37°C and 100 rpm.

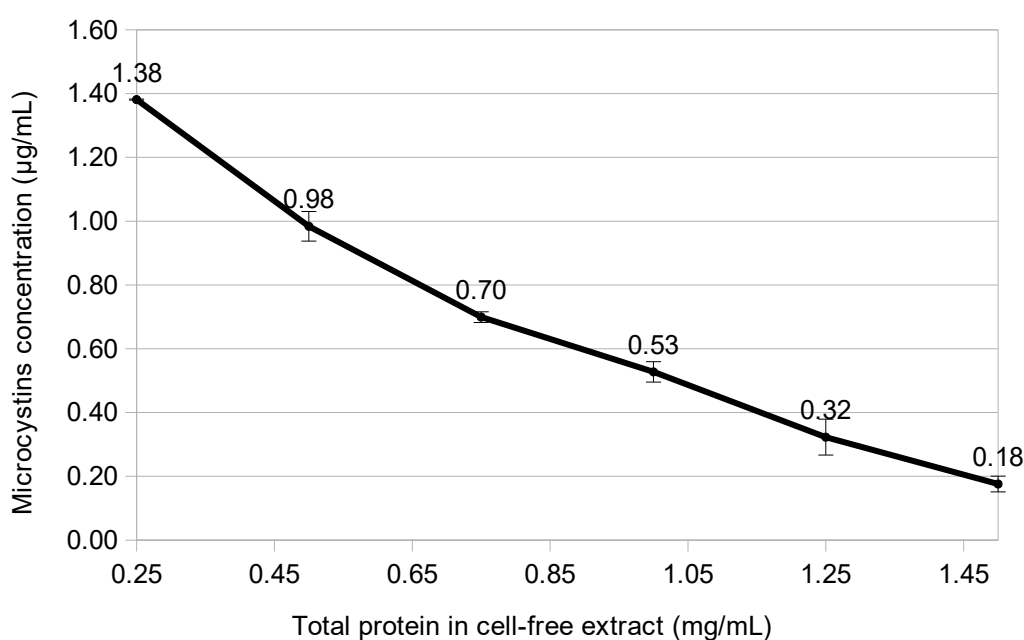
Xu *et al.* (2020) used purified *mlrA* enzyme to degrade MC-LR and observed the highest activity of the enzyme on the first 10 minutes of reaction, while the degradation rate was considerably reduced with time and lower concentrations of toxin. The effect of the purified enzyme was quickly demonstrated with almost 20µg/mL of MC-LR degraded within 10 minutes, in contrast to our results, of approximately 0.3µg/mL degraded in 15 minutes. These authors used 0.06mg of pure enzyme per mL in parallel to 0.4mg/mL of total proteins in our cell-free extracts [31]. The clear difference on the results of MC-LR degradation described by Xu *et al.* (2020) may be due the strategies used, that probably allowed them to get enhanced levels of protein expression and easier purification steps. The *mlrA* gene was expressed in *Escherichia coli* cells with induction by IPTG and the protein was bound to a GST-tag. At the final of the purification process, the enzyme had reduced molecular weight without loss of its catalytic activity. Also, the specific content of the

enzyme microcystinase A used by their assays was certainly higher than the present in our cell-free extracts. As it can be observed on the SDS-PAGE gel (Figure 1), the total protein content is composed of several protein, in which microcystinase A represents a small part.

Different approaches must be taken into consideration when comparing the degradation rates of MCs. With cell-free extracts we could reduce the concentration of MCs by approximately the half in only 1 hour of reaction ($0.96\mu\text{g/mL/h}$). On the other hand, Shen *et al.* (2019) obtained a reduction of 88% from the total amount of toxins ($14\mu\text{g/L}$) in experiments with the bacteria *Lysinibacillus boronitolerans* CQ5 strain. The highest level of microcystins removal was observed only with 244 hours of cell growth [32]. Regarding the application of living cells to these sort of tests, the optimal conditions necessary for the growth of microorganisms might be different of those that result in efficient enzymatic reaction. Besides that, the cell density and the cellular machinery interferences could influence the rate of toxin removal.

In the following sequence, the effect of different concentrations of total protein in cell-free extracts on the degradation of microcystins was investigated (Figure 3).

Figure 3. Cyclic MCs after microcystinase A reaction with different concentrations of total protein in cell-free extracts*.



*The enzyme assay was conducted at 37°C and 100 rpm for 1 hour. MCs were added as substrate in the concentration of $2\mu\text{g/mL}$.

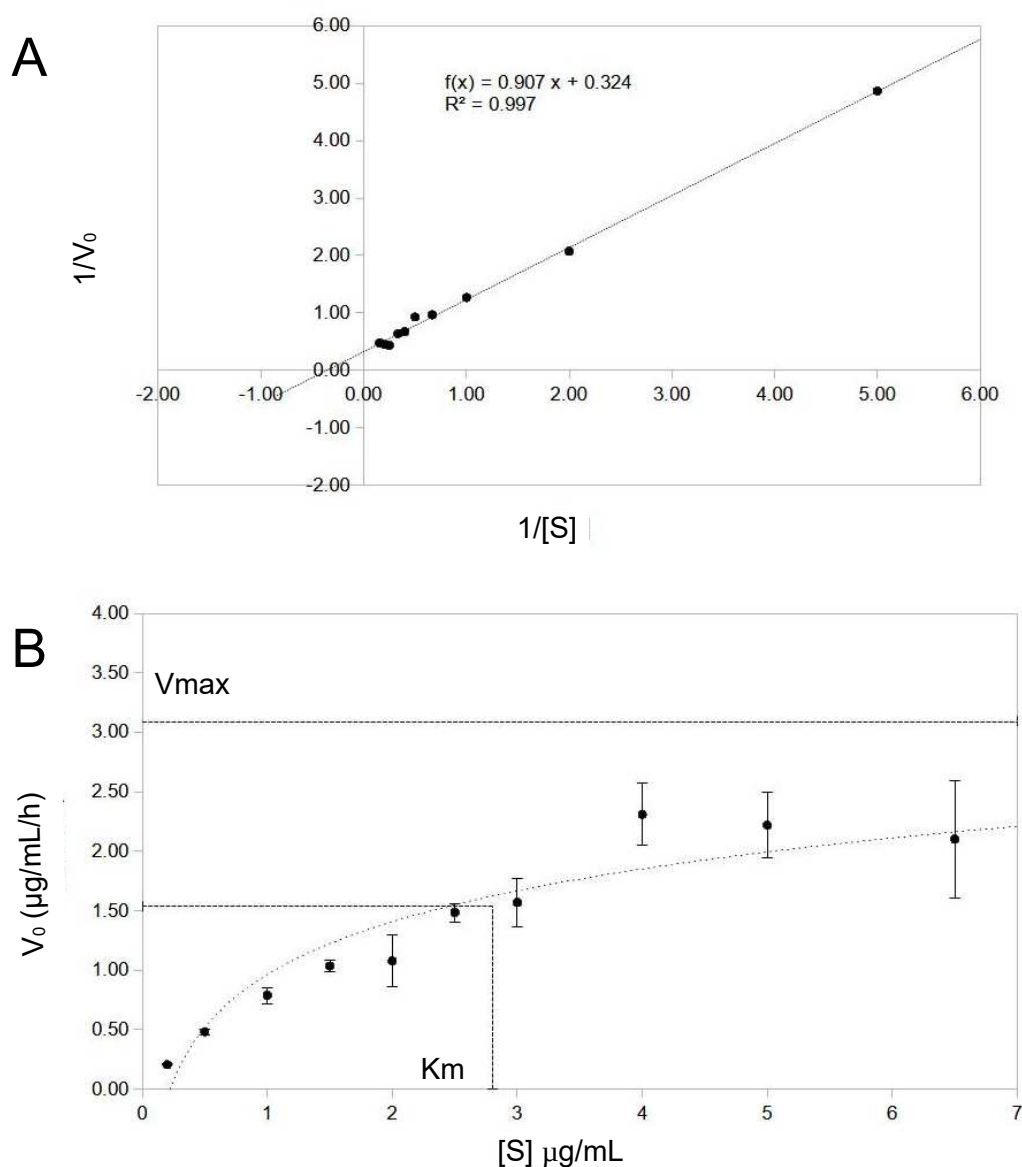
As expected, higher levels of proteins increased the rate of degradation. With 1.5 mg of proteins per mL of cell-free extract, within 1 hour of incubation at 37°C and 100 rpm, the concentration of MCs was reduced from 2.0 to 0.18 μg per mL, more than 90% of reduction. Even with the most diluted samples (0.25mg/mL), it was observed more than 30% of toxin degradation, and with 0.5mg/mL of total proteins in cell-free extracts, it was possible to reduce half of the microcystins concentration. Therefore, to analyze the enzyme activity in a range that allows to discern slight variations on the toxin concentrations, and to better utilize the prepared cell-free extracts, the experiments proceeded with 0.4mg/mL of proteins in extracts.

Cai *et al.* (2022) also tested different concentrations of microcystinase A for the degradation of MC-LR. Similarly to our results, increasing the enzyme concentration had a positive effect on the degradation rates. Using 0.1mg/mL of the enzyme, around 8 μg /mL of MC-LR were degraded in 20 minutes of reaction. When the concentration of enzyme was duplicated to 0.2mg/mL, it was possible to degrade around 16 μg of toxins per mL. It is important to mention these authors used an enzyme previously purified. so, even the lower concentration of proteins submitted to tests when compared to our cell-free extracts, it probably represented a higher amount of microcystinase A [7]. Regarding the cell-free extracts used in our experiment, although the presence of the microcystinase A is double-confirmed by electrophoresis and by the evidence on substrate (MCs) depletion, there is a large number of other proteins that were also extracted. Thus, the content of the target enzyme was not measured. Nevertheless, the simple extraction method used in our experiment enabled to disrupt yeast cells and release sufficient amount of active enzyme for tests, conveniently meeting the objectives of this investigation and producing reliable data for further studies.

In the next, different concentrations of substrate were added to enzyme assays and evaluated considering their respective reaction rates. These data were used to determine properties of the recombinant microcystinase A kinetics. The representation of Michaelis-Menten equation with calculated parameters, and its derivation on the Lineweaver-Burk plot were illustrated in Figure 4. The results demonstrated the maximum velocity of MCs degradation by the enzyme was 3.09 μg per mL of solution within one hour of reaction, or 3.09 mg/L/h. Km constant was calculated to be 2.8 μg of MCs per mL (or 2.81 μM), at which half of the maximum velocity of enzymatic reaction is achieved. These values were obtained with the

predetermined concentration of 0.4mg of total proteins per mL of cell-free extracts, as defined by previous tests.

Figure 4. Enzyme kinetics: (A) Lineweaver-Burk plot; (B) Graphical representation of substrate concentration vs. reaction rate.



Considering the available data on the kinetics of similar enzymes, diverse levels for these parameters have been published. Yang and Blenner (2020) also tested microcystinase extracted from bacteria that were able to degrade MCs, and obtained a maximum degradation rate of 51.7 µg/mL/h for MC-LR [33]. On the other

hand, Weir, Wood and Zimmer-Faust (2021) calculated the values of V_{max} and K_m for a purified linearized microcystinase, and determined the V_{max} of 5.6 μg of MC-LR per L per hour and the Michaelis-Menten constant of around 10 μM of MC-LR. In this case, purified enzyme was used in the concentration of 0.76 mg/mL. Despite the higher concentration and the pure form of the enzyme, the V_{max} calculated represented a lower rate of degradation [20]. This difference can be explained by specific characteristics of the enzyme and its catalytic process using linearized MCs as substrate, instead of cyclic toxins. In addition, increased K_m value indicated lower affinity between enzyme and substrate, demanding higher levels of substrate to reach half of the maximum reaction rate. Although cell-free extracts were used in our study instead of purified enzymes, the low K_m value suggests that unespecific binding did not occur at a significant level, indicating high affinity between enzyme and substrate. In addition, the maximum rate of MCs removal (>3.0 mg/mL) demonstrated the enzyme expressed in yeast cells was functional and might be useful in further researches aiming an effective application of its potential.

Massey *et al.* (2022) presented a maximum rate of 5 $\mu\text{g/mL/h}$ of MC-LR degradation with *Sphingopyxis* sp., a result quite close to ours, despite the differences expected when working with the whole cells and with cell-free extracts [34]. Microcystin-degrading bacteria can completely degrade microcystins into smaller peptides, likely involving additional enzymes that enhance the degradation process. However, the main biological system related to this natural process is triggered by microcystinase A. Here, we worked specifically with the *mIra* enzyme originally copied from the *Sphingosinicella microcystinivorans* B9 bacteria and expressed in a *Saccharomyces cerevisiae* PE-2 strain. Regarding the B9 strain and its role in the biodegradation of MCs, Tsuji *et al.* (2006) proved the potential of the strain to degrade MCs, mainly MC-LR and MC-RR, with complete removal of 600 μg of both toxins in 1 day of experiment [35]. When using cell-free extracts or the pure enzyme obtained from these microorganisms it is possible to reduce the time of reaction and optimize conditions in order to achieve the maximum potential on toxin degradation. In this sense, our study aimed to investigate whether the expression of the enzyme by yeast cells would determine structural and functional modifications, besides providing basic knowledge on its activity under experimental conditions.

When immobilized microorganisms were applied to testing microcystins degradation, the observed rates were widely varied, from 1.04 $\mu\text{g/L/h}$ of [Dha7]MC-

LR degraded by *Novosphingobium* sp. KKU25s, to the very high levels, such as 650mg/L/h of MC-LR degraded by *Sphingopyxis* sp. YF1 [36,37,38]. Although data on the specific kinetics of microcystinase A are scarce, the limited results regarding similar enzymes, microorganisms or biological alternatives for the toxin removal, indicate promising options to be explored for the control of microcystins. As another example, Zhang, Tang and Jiang (2023) reported the removal of 200µg/L of MC-LR using an adsorption method, with high effectiveness within a few minutes of reaction. In addition to the physical removal method, a biodegradation assay was performed and the reaction plot observed was completely different, in spite of the same final degradation result. In the biodegradation assay, the reduction in toxin concentration was progressive, in a relatively constant rate. The combination of both methods enhanced the result of the isolated tests [39].

In any case, as most of the tested methods applied to control microcystins are in experimental phase, further investigation is required, especially for methods with proven efficiency, such as the microcystinase A assays. In this regard, to verify the potential of this recombinant microcystinase A on the degradation of microcystins, the enzyme activity was assessed considering different conditions of temperature and pH. The results of the experiment were shown on Table 2.

It was observed with the lowest tested temperature (20°C) a maximum degradation rate of 0.61 µg/mL of MCs with pH 6. Alkaline conditions reduced the enzyme activity and no degradation was observed with pH 9 and 10. At the other extreme, with 65°C incubation, only a low reaction rate was observed at neutral and mild-acid conditions. Temperatures in the range of 42.5°C-57°C showed degradation rates above 70% in pH 6 or 7. The highest rate of MCs degradation was 1.79µg/mL/h achieved with the enzymatic reaction performed at 50°C and pH 7.

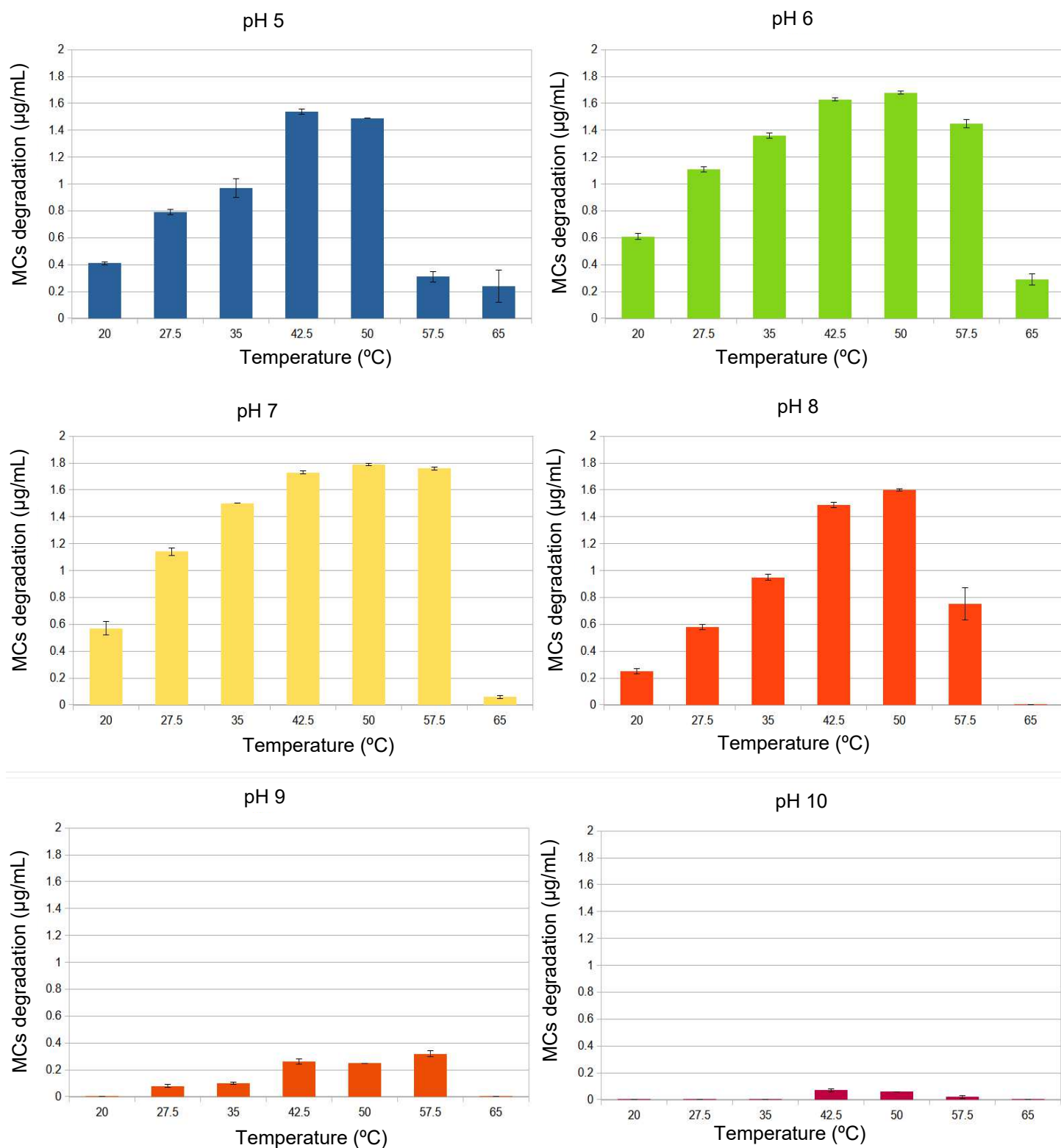
Cai *et al.* (2022) reported the degradation rate of 1.0µg/mL/min using 0.4mg of microcystinase A under optimal conditions (30°C, pH 7.2 and 20µg/mL of MC-LR) [7]. In our study, cell-free extracts were used, in which the concentration of 0.4 mg/mL is referred to total proteins in extract, including microcystinase A. Under approximate reaction conditions (27.5°C and pH 7), the degradation rate observed in our tests was 1.14 µg/mL/h, 60 times lower. Nevertheless, it is important to highlight that the optimal conditions described for enzyme activity differed significantly from those observed in our experiment. Additionally, a reduced reaction rate is anticipated when comparing cell-free extracts to assays conducted with pure enzymes.

Table 2. Effect of temperature and pH on microcystinase A activity*.

MCs Degraded by Microcystinase A												
T (°C)	pH 5		pH 6		pH 7		pH 8		pH 9		pH 10	
	Mean±SD (µg/mL)	%	Mean±SD (µg/mL)	%	Mean±SD (µg/mL)	%	Mean±SD (µg/mL)	%	Mean±SD (µg/mL)	%	Mean ±SD (µg/mL)	%
20	0.41±0.01 ^{Bd}	20.5	0.61±0.02 ^{Ae}	30.5	0.57±0.05 ^{Ad}	28.5	0.25±0.02 ^{Cf}	12.5	0 ^{Dc}	-	0 ^{Da}	-
27.5	0.79±0.02 ^{Bc}	39.5	1.11±0.02 ^{Ad}	55.5	1.14±0.03 ^{Ac}	57	0.58±0.02 ^{Ce}	29	0.08±0.01 ^{Dbc}	4	0 ^{Da}	-
35	0.97±0.07 ^{Cb}	48.5	1.36±0.02 ^{Bc}	68	1.5±0.00 ^{Ab}	75	0.95±0.02 ^{Cc}	47.5	0.1±0.01 ^{Db}	5	0 ^{Ea}	-
42.5	1.54±0.02 ^{Ca}	77	1.63±0.01 ^{Ba}	81.5	1.73±0.01 ^{Aa}	86.5	1.49±0.02 ^{Cb}	74.5	0.26±0.02 ^{Da}	13	0.07±0.01 ^{Ea}	3.5
50	1.49±0.00 ^{Ca}	74.5	1.68±0.01 ^{Ba}	84	1.79±0.01 ^{Aa}	89.5	1.60±0.01 ^{Ba}	80	0.25±0.00 ^{Da}	17.5	0.06±0.00 ^{Ea}	3
57.5	0.31±0.04 ^{De}	15.5	1.45±0.03 ^{Bb}	72.5	1.76±0.01 ^{Aa}	88	0.75±0.12 ^{Cd}	37.5	0.32±0.02 ^{Da}	16	0.02±0.01 ^{Ea}	1
65	0.24±0.12 ^{Ae}	12	0.29±0.04 ^{Af}	14.5	0.06±0.01 ^{Be}	3	0 ^{Bg}	-	0 ^{Bc}	-	0 ^{Ba}	-

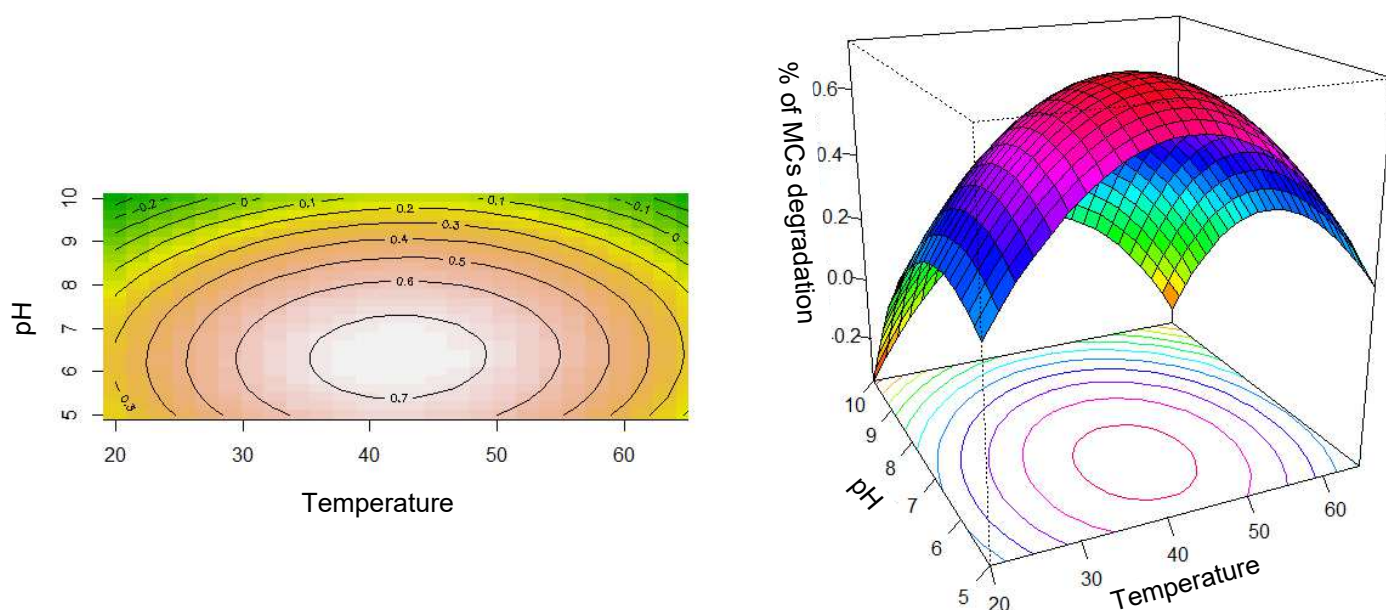
*Degradation values are referred to the mean and standard deviation of triplicate analysis of MCs quantified by HPLC-DAD. Initial concentration of MCs added to tests was 2µg/mL. Different capital letters represent differences on the columns (pH) while small letters considered the effect of temperature in different lanes. Tukey test was used to differentiate the means with the significance level of $P < 0.05$.

To complement the analysis of temperature and pH effects on the degradation of MCs by the recombinant microcystinase A, the results were also presented in Figure 5 with the toxin degradation rates divided by pH level. In general, the activity of the enzyme was higher with the increase of pH, from 5 to 7. However, a strong inhibition of the enzyme was noticeable in alkaline environment. Higher activity was observed with reaction performed around 42.5°C, with rates above 1.4µg/mL/h when the pH of buffer was in the range of 5-8. Moreover, close to neutrality (pH values 6 and 7), the enzyme could maintain its activity in a high level, decreasing at least 50% of the toxins concentration in a large range of temperatures of incubation (27.5°-57°C).



Through statistical analysis, the optimal conditions of temperature and pH were calculated for the recombinant microcystinase A and the plots were illustrated in Figure 6 below:

Figure 6. Countour (left) and surface response plots (right) estimated for the combined evaluation of temperature and pH values on the percentage of MCs degraded by the recombinant microcystinase A.

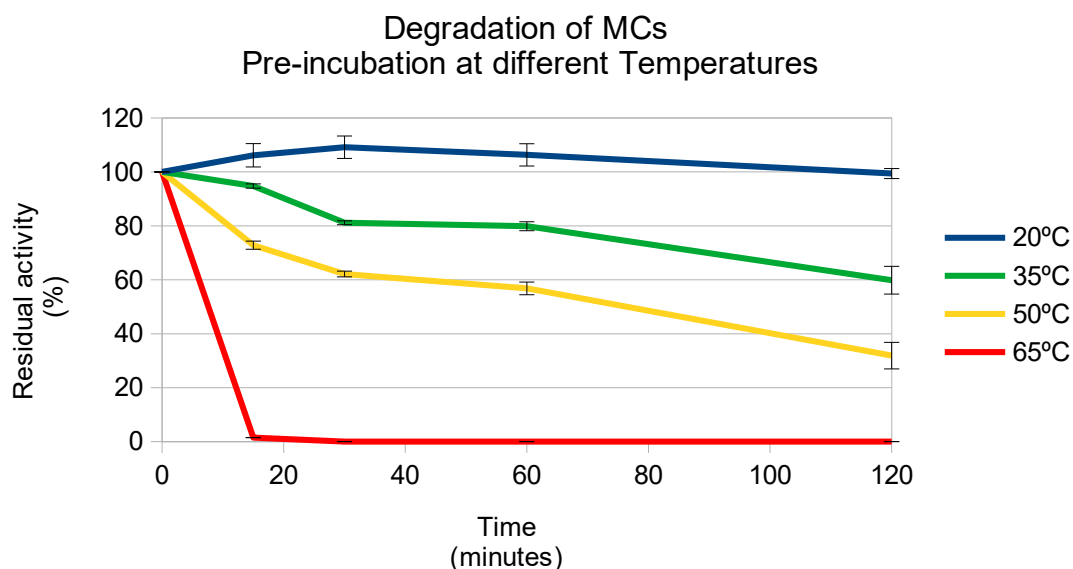


Optimal temperature and pH were defined at 42.2°C and pH 6.3, respectively. Sun *et al.* (2021) tested the *mlrA* expressed in *E. coli* cells, and obtained for the recombinant enzyme the optimal conditions of 40°C and pH 7.4 [14]. Differently, Massey *et al.* (2022) reached the optimal degradation rate of 3µg/mL/h of MC-LR applying *Sphingopyxis* sp. YF1 cells incubated at 30°C and pH 7 [34]. The maximum rate of degradation reported by these authors was similar to ours (3.09µg/mL/h), however, as their idea was to use a living microorganism on the control of MCs, optimal pH and mainly the temperature conditions were distinguished. As discussed previously, tests performed with living cells are expected to have different results, since the requirements for cell growth, metabolism and enzymatic activity may be different.

Thus, a last experiment was carried out to assess the thermal stability of the recombinant microcystinase A. Pre-incubation steps were performed during variable

times and the degradation rates were compared to a control assay that was not submitted to pre-incubation. Results were shown in Figure 7.

Figure 7. Thermal stability of microcystinase A: residual activity for MCs degradation after pre-incubation at different levels of temperature.



The control sample (without pre-incubation) showed after 1 hour of enzymatic reaction at 37°C, pH 7.4 and 100 rpm a reduction of 67.6% of the toxins, which means a linearization of 1.35µg/mL of MCs. When cell-free extracts containing the recombinant microcystinase A were pre-incubated at different temperatures, the activity of the enzyme was modified, mostly reduced with longer exposure to higher temperatures. At 20°C the enzyme remained stable and no reduction was observed on the enzymatic activity throughout the 120 minutes of pre-incubation. With higher temperatures employed to the pre-incubation step, increasing the time under adverse conditions, the activity of the enzyme decreased. Even so, after 60 minutes at 35°C, the activity of the enzyme was still around 80% of the control. These results suggested the enzyme is moderately tolerant to environmental conditions. Also, if it is maintained under lower temperatures it can retain almost full activity. Obviously, extended investigation is needed to define precisely the limits of the enzyme activity when exposed to those temperatures for many hours or days.

On the other hand, at very high temperatures, above the conditions normally found in nature, the enzyme showed very low residual activity. Only in the assays with the shortest time of exposure (15 min) at 65°C the enzyme demonstrated 1% of

activity compared to the control. The results are in accordance with the data presented in Table 2, a very low degradation rate occurred at 65°C, without additional pre-incubation. When the exposure of the enzyme to higher temperatures was longer, protein denaturation was accelerated and no enzyme activity was observed.

Likewise, Liu *et al.* (2020) observed a low reduction on *mlrA* activity after one hour of incubation at temperatures below 35°C, and high stability at room temperature, maintaining 50% of the activity after 11 days at 25°C. However, residual enzymatic activity was reported after 1 hour of incubation at 80°C [27]. The main reason for such high thermal stability might be the use of a MBP (malto binding protein) tag. The authors expressed the *mlrA* in *Escherichia coli* cells, resulting in a fused protein with 78kDal of molecular weight, while the *mlrA* enzyme has approximately 36kDal. Also, contrasting rates of degradation were observed for the enzyme in alkaline conditions when compared to our results. Gomari *et al.* (2020) described the MBP tag improves the solubility and stability of target proteins. Adding these proteins to the desirable gene can be effective to improve the yield of the purification process [40]. However, target proteins tagging can raise the costs of the process, and result in possible interferences on the protein structure and function [41]. Therefore, we believe the main reason for the differences on thermostability and activity in higher values of pH described by Liu *et. al* (2020) occurred due the use of the MBP tag [27].

In our study, since its initial conceptualization, the aim was to express continuously active microcystinase A in *S. cerevisiae* cells. To avoid any possible reduction on the functionality of the recombinant enzyme an affinity tag was not added to the target gene. Obviously, this resulted in a complicated process for the complete enzyme purification; hence, cell-free extracts were prepared to perform this characterization. The use of the extracts were adequate as the high specificity of the enzyme to its substrates allowed assessing its activity in the presence of other proteins without important interferences. Still, characteristics concerning possible structural modifications added to the enzyme expressed by yeast were investigated, and aspects of the enzymatic activity and stability at different temperature and pH conditions were elucidated.

4. Conclusions

Microcystinase A heterologously expressed in *Saccharomyces cerevisiae* was extracted from cells and evaluated regarding the enzyme properties and activity. One sonication method was defined as suitable to liberate the intracellular enzyme to cell-free extracts retaining the target protein in active form. Apparently, no post-translational modification was observed with noticeable effect on the enzyme location, activity or stability. The enzyme kinetics analysis reached the maximum velocity of reaction of 3.09 mg/L/h of MCs and the K_m value of 2.81 μ M of MCs. High level of enzymatic activity was achieved with temperatures in the range of 35°C to 57.5°C, especially with pH values close to neutral conditions. Alkaline pH seemed to have a strong inhibitory effect on the enzyme. The optimal conditions of the microcystinase A activity were statistically defined at 42.2°C and pH 6.3. At low temperatures the enzyme showed stability, while long exposure to high temperatures (above 50°C) reduced drastically its activity. The results presented here provide a valuable basis for planning applied studies and may lead to further practical applications of the enzyme in the biocontrol of microcystins .

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5.3 CAPÍTULO 3:

**SIMULTANEOUS DETECTION OF AFB₁ AND AFM₁ IN
MINAS FRESCAL CHEESE BY UPLC-FLR ANALYSIS:
METHOD DEVELOPMENT AND VALIDATION**

Abstract

Aflatoxins are mycotoxins contaminants of dairy products, especially after Aflatoxin B₁ ingestion by cows and consequent excretion of its hydroxylated metabolite, AFM₁, into the milk. These toxins are thermally stable, harmful for human and have proven capacity to develop cancer. The processes used in cheese manufacturing are unable to significantly reduce aflatoxins content; therefore, this product may be a source of contamination. Among Brazilian types of cheese, Minas Frescal Cheese (MFC) is highlighted due to its ease of production, high yield and pleasant flavour. Despite its popularity, data on the investigation of aflatoxins in MFC are scarce. Moreover, a few studies have described efficient methods to analyze these toxins in fresh cheese, providing alternatives for products monitoring and compliance with official safety limits. Then, to provide an affordable and reliable option for aflatoxins analysis in MFC, this study developed and validated a UPLC-FLR method for simultaneous detection of AFM₁ and AFB₁. Satisfactory results were obtained for all the parameters evaluated in the validation: selectivity, linearity ($R^2 > 0.99$), accuracy (recoveries of 81-105% for AFM₁ and 80-109% for AFB₁) and precision (relative standard deviation $< 18\%$). Limits of detection and quantification were as low as 0.01 µg/kg and 0.03 µg/kg for AFM₁, and 0.1 µg/kg and 0.031 µg/kg for AFB₁. The method has proven to be a sensitive, reliable and convenient alternative to detect aflatoxins contamination in MFC, including the conventional analysis of AFM₁ in dairies, and the assessment of direct contamination by AFB₁. Furthermore, twenty samples of MFC purchased in Araguaína-TO (Brazil) were analyzed by the method, however none resulted positive for aflatoxins. Despite the absence of contaminated samples, larger sampling is indicated to better evaluate the risks of aflatoxins contamination in MFC. Conversely, the analytical method complied with all the requirements to compose a practical tool applicable on the surveillance of aflatoxins in fresh type cheese.

Keywords: aflatoxin; surveillance; public health; minas frescal cheese; UPLC-FLR.

1. Introduction

Minas Frescal Cheese (MFC) is one of the most popular dairy products in Brazil, characterized as a soft white cheese with high water activity obtained from pasteurized milk in a simple process [1]. The Brazilian Ministry of Agriculture, Livestock and Food Supply defined this product in specific regulations as a fresh cheese produced by enzymatic coagulation of milk with rennet, other coagulating enzymes or acid lactic bacteria. Resulting from the manufacturing process, this semi-fat cheese with very high moisture content is produced and intended for fresh consumption [2,3]. The popularity of this kind of cheese lies on the pleasant sensorial aspects, low content of fat and sodium, and large offering since the production technology is easy, resulting in high yield in a short time [4].

The nutritive composition of MFC, combined with its high moisture content, makes this type of dairy product an ideal medium for microbial growth, with the risks of contamination present at several stages of production [5]. Besides biological contamination, the ingredients of dairy production can be affected by chemical contaminants. Among them, aflatoxins can occur in milk as important threats to human consumers. These mycotoxins are secondary metabolites of *Aspergillus flavus* and *A. parasiticus*, and contaminants of animal feed and food. Human intoxication are mainly related to cancer development, after chronic exposure to the toxins [6].

Aflatoxins can be transmitted to milk after the ingestion of contaminated feed by the animals. Part of the AFB₁ ingested is transformed into derivatives and can reach animal tissues and their products, such as milk and eggs. The main metabolite formed from AFB₁ hydroxylation is AFM₁, usually found in milk [7]. A carry-over from 0.3 to 6.2% is estimated from the AFB₁ ingested in animal feed to AFM₁ in cows milk [8,9]. Additionally, aflatoxins are transferred to cheese on the manufacturing of the dairies with contaminated milk, besides direct contamination of the product after aflatoxigenic molds growth and toxins liberation [10].

Considering the risks associated to these toxins for human health, AFB₁ and its metabolite, AFM₁, were classified as carcinogenic substances by the International Agency for Research on Cancer [11]. Both toxins are highly toxic and mainly related to the development of liver cancer. Apart from their toxicity, these mycotoxins have a common chemical structure that provide them high stability to the thermal processing of milk, and other procedures commonly used on dairy production. In this sense,

aflatoxins contaminating milk maintain their toxic potential in cheese, and pose as a hazard that must be considered [12].

Brazilian regulations set a maximum permitted limit of AFM₁ in milk and cheese at 0.5 µg/kg and 2.5 µg/kg, respectively [13]. There is no specific tolerable limit for AFB₁ detected in milk or in any dairy product. These standards were defined based on the risks of human exposure to food with probable contamination. Nevertheless, only with frequent surveillance it is possible to define if the products are safe and comply with those established limits [14]. In this respect, the development of practical and reliable alternatives for the detection of aflatoxins in different types of cheese is essential to allow a wider monitoring and ensure the quality and safety of dairies for human consumption.

Therefore, this experiment aimed to develop a simple and practical method capable of performing the simultaneous analysis of aflatoxins B₁ and M₁ in Minas Frescal cheese, encompassing direct and indirect contaminations of cheese. Extraction procedures and chromatographic conditions of the UPLC coupled to a fluorescence detector were set for a convenient analysis with reliable results. The method was submitted to a single-laboratory validation. Finally, it was applied to investigate AFM₁ and AFB₁ contamination in 20 samples of MFC. This study provided a useful and valid alternative for the routine monitoring of aflatoxins as contaminants of this popular cheese of Brazil, and possibly, for other types of fresh cheese with similar characteristics.

2. Material and Methods

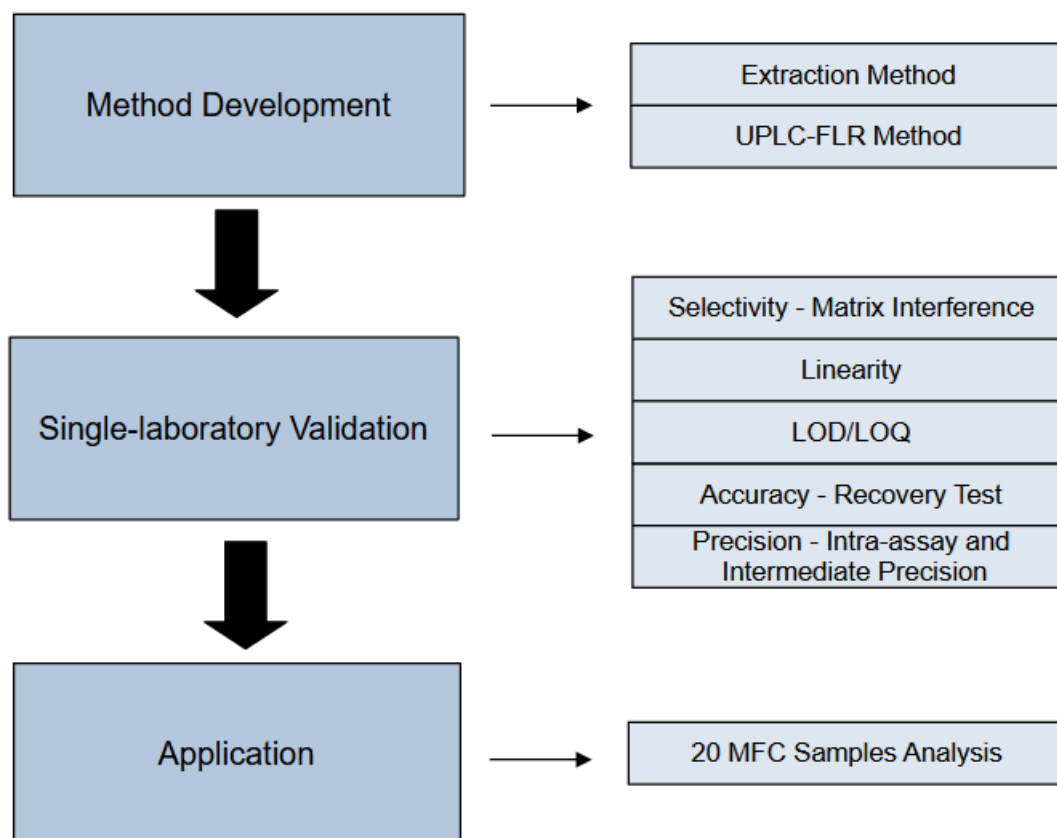
2.1. Sampling

Twenty samples of MFC were purchased from street markets in the city of Araguaína, Tocantins, Brazil. The samples were collected by researchers of the Federal University of Northern Tocantins and frozen after collection. In this conditions, the samples were sent to the Laboratory of Immunoreagents and Molecular Techniques of the Departament of Food Science and Technology - State University of Londrina, Brazil. Since the reception of the samples they were stored frozen until analyses.

2.2. Experimental Planning

Overall, the activities planned and performed by this study followed the sequence shown in the Figure 1:

Figure 1. Schematic experimental planning.



2.3. Aflatoxins Extraction Method

The method used for the extraction of AFM₁ and AFB₁ from MFC samples was adapted from procedures described by Skrbic, Antic and Zivancev (2015) [15] for white cheese analysis. Five grams of ground and homogenized cheese samples were mixed with 20mL of a solution composed of 84% of acetonitrile (Honeywell, USA) and 16% of water (v/v). Tubes were agitated in a Orbital Shaker (Quimis, Brasil) at 300 rpm during one hour. Then, the mixture was centrifuged (Centrifuge 5810R, Eppendorf, Germany) at 2660 x g for 5 minutes. An aliquot (3mL) of the extract was collected and mixed to an equal volume of hexane (Synth, Brasil) at 300rpm for 5 minutes. This stage was adapted from Pattono et al. (2013) for the removal of fat from cheese [16]. Next, the tubes were placed on the bench for the

separation of layers, the top tier containing hexane was discarded, and an aliquot of 2mL from the remaining solution was transferred to another tube and dried under N₂ flow in a water bath at 40°C. To concentrate the toxins, the dried extract was resuspended in 1mL of the mobile phase (H₂O:ACN, 76:24, v/v), then, filtered in PVDF 0.22µm filter (Millex, Merk, Germany) and placed in glass vials for the UPLC-FLR analysis. Samples that were not promptly analyzed were stored at freezer temperatures.

2.4. Chromatographic Method for the Analysis of AFM₁ and AFB₁

The chromatographic analysis of AFM₁ and AFB₁ were carried out in a UPLC equipment (Acquity UPLC, Waters, USA) coupled to a Fluorescence Acquity UPLC FLR Detector. Five microliters of the extracted samples were injected in the system. The mobile phase consisted of water and acetonitrile (76:24, v/v) in an isocratic run. Separation of the analytes was conducted in a HSS C18 (2.1mm x 100mm, 1.8µm, Waters, USA) column at the flow rate of 0.4mL/min. The oven temperature was set at 30°C. A complete run had 9 minutes, with AFM₁ retention time of 2.92 minutes and AFB₁ acquired at 7.16 minutes. The detection of the analytes occurred under 365nm of excitation and 429nm of emission wavelengths. No derivatization procedures were necessary to be performed by this experiment.

2.5. Method Validation

A single-laboratory validation was carried out for the UPLC-FLR method used in the acquisition of aflatoxins B₁ and M₁ from MFC. The validation aimed to verify parameters of the method to define the extent of reliability of the produced results. Standards of both analytes used in this validation were purchased from Sigma-Aldrich (Merk, Germany). The parameters assessed were: selectivity, linearity, limits of detection and quantification, accuracy and precision. All tests followed the Brazilian Guidelines for the Validation of Analytical Methods as outlined in RDC 166/2017 by the Brazilian Health Regulatory Agency (ANVISA). Additionally, the criteria for accepting results from the parameter assessments considered the orientative document from the National Institute of Metrology, Standardization and Industrial Quality's (INMETRO), as well as AOAC publications [17,18,19]. Statistical analysis were performed using R Studio Software Version 2022.02.3 + 492 (www.rstudio.com - Accessed on 5 September 2022).

2.5.1. Selectivity

This parameter was assessed by observing the peaks obtained for AFM₁ and AFB₁ in the presence of other compounds from the matrix, ensuring that the areas of the analytes are accurately delimited without the overlapping of nonspecific peaks. Besides that, the matrix effect on the signal generated for both analytes was verified. For each toxin, 3 analytical curves were prepared with the mobile phase mixture used as diluent, and 3 other curves were diluted with matrix extract. The same concentrations of aflatoxins were added in all the curves. The mean slopes of the curves and their respective standard deviation values were used to statistically compare them using t student test. Whether differences are confirmed, resulting in amplification or reduction of aflatoxins signals, their quantification must be performed on matrix-matched calibration curves.

2.5.2. Linearity

The linearity of the method was assessed in the range of 0.05-5 µg/kg for AFM₁ and 0.1-10 µg/kg for AFB₁. For each analytical curve, 7 different concentrations of the analytes were prepared within the mentioned ranges. The analysis were carried out in triplicate and the linearity of the analytical curves was verified by the analysis of the coefficient of determination (R^2).

2.5.3. Limits of Detection and Quantification

The limits of detection and quantification of AFM₁ and AFB₁ of the UPLC-FLR method were calculated based on the standard deviation values of the lowest levels of each toxin in the analytical curves. The equations used for the determination of limits were:

LOD: 3.3 x standard deviation of the lowest level of the analyte/slope of the curve

LOQ: 10 x standard deviation of the lowest level of the analyte/slope of the curve

2.5.4. Accuracy

This parameter evaluation was conducted by the analysis of recovery tests. MFC samples were added of 0.25, 1 and 3 $\mu\text{g/kg}$ of AFM_1 , while AFB_1 was supplemented in the levels of 0.5, 2 and 6 $\mu\text{g/kg}$. These artificially contaminated samples underwent the extraction procedure and were analyzed using the UPLC-FLR method. The analytes peak areas were used to quantify toxins, and the rate of recovery was calculated on the percentage of observed toxin in relation to the expected result, as follows:

$$\text{Recovery (\%)}: \text{concentration observed } (\mu\text{g/kg}) / \text{concentration expected } (\mu\text{g/kg}) \times 100$$

The tests were carried out in triplicate and the results expressed as the mean recovery values for both toxin level.

2.5.5. Precision

Two different analyses were performed to assess the precision of the method: repeatability and intermediate precision test. On the repeatability test, seven replicates of each level of added toxin were analyzed and the relative standard deviation (RSD) obtained from these samples was used in the precision verification. For the intermediate precision test, the same procedure of the repeatability test was performed, however during three different days, completing a total of 21 replicates. Likewise, the RSD was considered in the evaluation of intermediate precision. For AFM_1 analysis, MFC samples were artificially contaminated with 0.1, 0.5 and 2.5 $\mu\text{g/kg}$ of toxin. AFB_1 was added in the levels of 0.31, 1.25 and 5 $\mu\text{g/kg}$. The extraction method and chromatographic analysis were the same for all the tests and followed the procedures described on previous sections. Additionally, the same analyst carried out the precision analysis.

2.6. Sample analysis

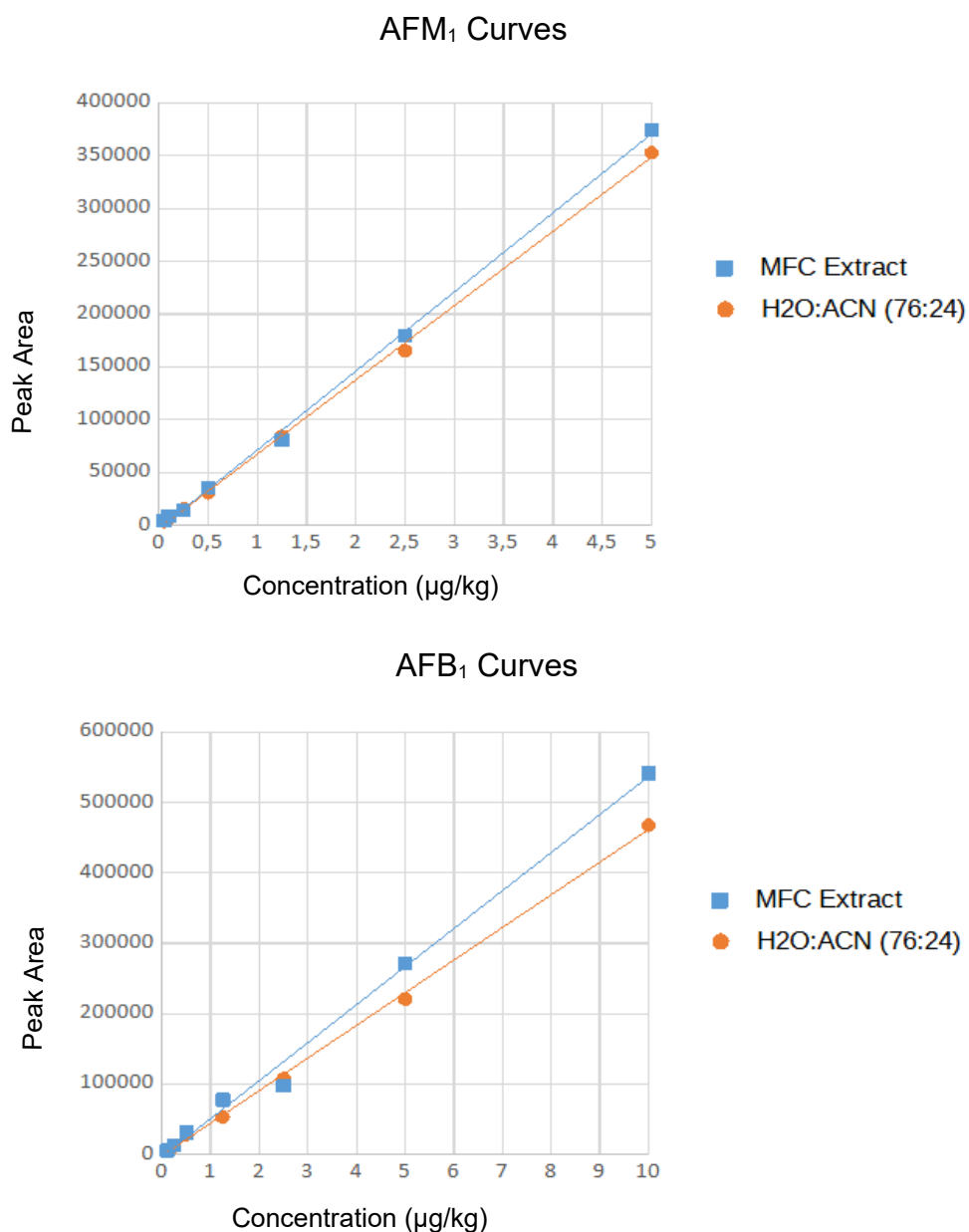
Twenty samples of MFC purchased from street markets of Araguaína-Tocantins were analyzed regarding their contamination with AFM_1 and AFB_1 by the method previously developed and validated. All the samples were analyzed in triplicates.

3. Results

The results of tests performed to evaluate different parameters for the method single-laboratory validation were described and either presented in Figure 2 and Table 1 below. Firstly, to assess the selectivity of the method, two approaches were used: viewing of chromatograms to differentiate analytes peaks in the presence of matrix interferences, and the estimation of matrix interferences on the signal of the analytes. On the method development, the content of each solvent in the mobile phase, and other conditions, such as, oven temperature and flow rate were adjusted to result in the complete separation of AFM₁ and AFB₁ from other compounds. The adaptations enabled the isolation of peaks, allowing for precise area definition and accurate quantification. Then, the final method demonstrated adequate selectivity without the overlap of peaks, which was essential for the validity of the analyses. One second analysis used to verify the selectivity of the method aimed to determine the level of matrix interferences on the quantification of the analytes. The results were shown in Figure 2.

The lines presented in the figure represent the means of three curves diluted in both MFC extracts and in the mobile phase mixture. Different plots were presented for each analyte. At the top plot, it was observed that the slopes of the AFM₁ curves prepared in both solutions were relatively similar, while for AFB₁ (bottom plot), analytical curves diluted in MFC extract showed increased values of the slope. Numerically, AFM₁ curves prepared in the matrix extract resulted in a slope value 6% higher than the those diluted in the mixture of water and acetonitrile. This difference was considered statistically insignificant by t test. Therefore, the standard curve diluted in the mobile phase could be used to quantify AFM₁ from the extracted samples without significant differences on the quantification of this specific analyte. On the other hand, for the AFB₁ curves, the slope values of curves prepared in matrix extract were 16% higher. The t test analysis confirmed the differences. Then, to quantify AFB₁ extracted from MFC cheese samples, the analytical curve used should be prepared in the matrix extract in order to avoid errors due to the interferences of the matrix. Although no significant matrix interferences were observed on the AFM₁ quantification, it was decided to use analytical curves performed in MFC extracts for both toxins. Thus, errors in the AFB₁ quantification would be prevented, and eventually low discrepancies on the AFM₁ measurement could also be attenuated.

Figure 2. Matrix interferences analysis: comparison of means of three curves for AFM₁ and AFB₁ diluted in H₂O:ACN (76:24) and in MFC extracts.



The linearity of the developed method was verified with the analytical curves for AFM₁ and AFB₁ prepared in MFC extracts. Analytes concentrations were added in the range of 0.05-5.0 µg/kg for AFM₁ and 0.1-10.0 µg/kg for AFB₁. By the coefficient of determination (R^2) it was demonstrated the linear trend of the method for the mentioned intervals of concentration of both toxins, with values above 0.99. AFM₁ analytical curve was defined at $y=73795.51x-2178.31$, while, AFB₁ curve was $y=50491.28x-2508.16$. Also based on the curve results, the limits of detection and

quantification were calculated for both analytes. These limits and the results of accuracy (recovery test) and precision parameters evaluation were shown in Table 1.

Table 1. Single-laboratory validation of the UPLC-FLR method for the analysis of AFM₁ and AFB₁ in Minas Frescal Cheese.

	LOD (µg/kg)	LOQ (µg/kg)	Toxin (µg/kg)	Recovery (%)			Precision (%)		
				Mean	Min	Max	Toxin (µg/kg)	RSD (1 day)	RSD (3 days)
AFM ₁	0.01	0.03	0.25	89.17	77.85	101.31	0.1	11.18	17.52
			1	105.04	74.98	128.53	0.5	3.3	8.11
			3	81.36	77.79	83.18	2.5	2.78	7.47
AFB ₁	0.1	0.31	0.5	101.85	85.27	125.80	0.31	8.72	8.98
			2	108.85	92.16	118.98	1.25	2.47	6.7
			6	80.03	70.70	92.79	5	4.37	5.95

RSD: relative standard deviation.

Considering the AFM₁ analytical curve, the lowest concentration of analyte that could be detected was defined at 0.01ng/g. The limit of quantification calculated was 0.03ng/g. On the other hand, with the AFB₁ curve, these limits were set at 0.1ng/g and 0.31ng/g. These results indicated the sensitivity of the method was around 10-fold higher for AFM₁ than AFB₁.

On the accuracy parameter evaluation, a recovery test was performed for three different levels of each toxin. The results showed satisfactory recovery rates for all the analytes in all the tested levels. For AFM₁ added samples, the mean recoveries of toxin were from 81% to 105%. Similar recoveries were observed for samples fortified with AFB₁, with mean values in the range of 80-109%.

Then, the precision of the method was assessed by two different modes: intra-assay and intermediate precision test. The RSD obtained from samples analyzed by the same analyst, in the same equipment and conditions, and within a short time (1 day of analysis), and through different days of analyses (3 days) showed low deviation of results. Considering AFM₁ added samples, the intra-day assay presented RSD values from 2.8% to 11.2%, while the analysis of three different days resulted in

RSD in the range of 7.5-17.5%. For samples with AFB₁ adding, the method also demonstrated high precision, with RSD lower than 9% in both intra-assay and intermediate precision tests. Then, the intermediate precision results obtained for the two analytes indicate the method is able to maintain the same response through different days of analysis, since all the precision results were within recommended limits for analytical methods validation.

Therefore, all the parameters evaluated demonstrated the method is reliable for the detection and quantification of AFM₁ and AFB₁. The results complied with the recommended criteria published by AOAC (2016) and INMETRO (2020) for the validation of analytical methods. Moreover, the evaluation of parameters was fully performed in accordance with the official guidelines set by the Brazilian Health Regulatory Agency [17,18, 19].

Afterwards, twenty samples of MFC purchased from street markets of Araguaína-TO, Brazil were analyzed by the aforementioned developed and validated method. This part of the study aimed to detect and quantify aflatoxins contamination of this popular type of cheese. The results could provide information about the risks associated to mycotoxins in this dairy product. However, none of the samples resulted in quantifiable contamination for any of the analyzed toxins.

4. Discussion

4.1. Method Development and Single-laboratory Validation

The entire method employed here, from the extraction of analytes to their final quantification, was adapted to use feasible equipment and reagents. The approach aims for the simple and effective acquisition of target toxins within a short analysis time. To extract aflatoxins from MFC, different techniques were tested and good results were obtained with the method described by Skrbic, Antic and Zivancev (2015) for soft cheese with modifications [15]. To improve the acquisition of aflatoxins on the chromatographic analysis, an additional step aiming to increase the concentration of toxins was added to the method. Therefore, the extracts were 2-fold concentrated and the analysis considered this factor for the final quantification of aflatoxins. Another modification made to the original method was the addition of a hexane simple extraction stage. This solvent is widely used for the extraction of lipids, due to its very low polarity [20]. In our experiment, this additional step reduced

the number of undesirable peaks, enhanced the selectivity of the method and improved the recovery of the toxins. Considering the chemical structures of AFB₁ and AFM₁, both are insoluble in nonpolar solvents [21,9]. Therefore, the use of hexane in the extraction minimized potential interferences from nonpolar molecules, with the analytes remaining in the polar portion of the extract. Even with the additional steps, the extraction method remains simple and practical, as it utilizes only common solvents and equipments in quick and easy procedures. Furthermore, the method clearly demonstrated its effectiveness in extracting toxins at very low concentrations without the necessity for costly materials, such as immunoaffinity columns.

On the single-laboratory validation, the method showed adequate selectivity with the separation of the analytes in well-delimited peaks, allowing their accurate quantification. This result agrees with Marson *et al.* (2020) affirmation that the selectivity for chromatographic analysis are proven when no other substance is eluting at the same time of the target analytes [22]. A simple comparison of chromatograms generated for matrix extracts with and without the addition of the analytes allows to assess the selectivity of the method.

The matrix interference test confirmed the mean slope of AFB₁ curves prepared in MFC extracts was significantly higher than the mean slope of analytical curves diluted in the mobile phase mixture. That means, the signal of AFB₁ increased in the presence of other compounds from the matrix. This effect observed on the response of an analyte in the presence of the matrix, resulting in the increase or suppression of its signal, may lead to mistakes on the detection and inaccurate quantification of the analyte [23]. To overcome these potential mistakes, the analytical curve considered for the quantification of AFB₁ was prepared with MFC extract. Likewise, the subsequent validation tests were conducted on matrix extract. In spite of the lower and statistically negligible matrix effect for AFM₁ signal, the same approach was applied to both analytes, using a matrix-matched curve for the quantification of the toxins.

Considering the linearity and the limits of detection and quantification calculated for the method, these parameters demonstrated satisfactory values, within official guidelines recommendations. Also, the sensitivity of the method was adequate for the analysis of toxins naturally found in residual levels. For both aflatoxins, the curves were linear on the working ranges, and LOD and LOQ were as low as, or even lower, than those presented by other studies, as shown in Table 2:

Table 2. Studies investigating AFM₁ contamination in MFC.

Reference	Aflatoxin analyzed	LOD	LOQ	Samples analyzed	Positive samples	Mean contamination
Gonçalves (2021a)	M ₁	0.02	0.06	28	8 (28.6%)	0.11 µg/kg
Oliveira (2011)	M ₁	0.01	0.03	24	6 (25%)	0.14µg/kg
De França (2024)	M ₁	0.07	0.7	89	10 (11.2%)	N.M.
Barroncas (2019)	M ₁	0.06	N.M.	25*	0	0
Our results	M ₁	0.01	0.03	20	0	0
	B ₁	0.10	0.31	20	0	0

*Including MFC./ N.M.: not mentioned.

In this respect, Jaksic *et al.* (2021) analyzed AFM₁ by an ELISA method with limit of detection determined at 0.025µg/kg, which demonstrated high sensitivity [24]. Immunoenzymatic techniques are known to detect minimal levels of the analytes due the high affinity of specific antibodies to their antigens. Still, our results for AFM₁ were even lower, reinforcing the method great capacity to detect this mycotoxin at very low concentrations.

The method's performance on accuracy and precision assessments were also adequate. Our results were within acceptable levels for both paraments, considering the limits suggested by AOAC (2016) and INMETRO (2020) [18,19]. Regarding the low levels of aflatoxins tested (below 10ppb of concentration), recovery values were considered acceptable if they fell within the range of 60-115%. For both analytes, and in the three levels of concentration, the mean recoveries observed were in accordance to these regulations. In addition, to verify method's precision, repeatability and intermedite precision tests were performed. For analytes concentrations lower than 10ppb, the maximum RSD values acceptable for repeatability and intermediate precision tests are 21% and 32%, respectively. As shown in Table 1, our method demonstrated satisfactory RSD in both tests, which implies the method is precise.

In comparison to other studies that developed methods to investigate AFM₁ contamination in MFC, De França *et al.* (2024) showed recoveries in the range of 75.6-102.8% and RSD below 13% [25]. Zebib, Abate and Woldegiorgis (2022) aimed to detect AFM₁ in different dairy products observing recoveries from 89 to 96%, and

for the precision assessment, the RSD was below 5% in intra-day, and below 12% for inter-day assays [6]. Both studies had results in accordance to AOAC recommendations, and are similar to those demonstrated by our method. Therefore, considering all the evaluated parameters were in accordance to international and Brazilian guidelines of analytical validation, the method used by this experiment proved to be adequate for the detection and quantification of AFM₁ and AFB₁ extracted from MFC. Furthermore, the developed method allowed to get a quick and practical response on the exploring of aflatoxins in soft cheese, without complex and expensive processes.

4.2. AFM₁ and AFB₁ Contamination of MFC Samples

Aflatoxin M₁ presence in milk occurs as a result of the ingestion by dairy cows of feed contaminated with AFB₁. When this aflatoxin is consumed, a part of it is converted into AFM₁ by hydroxylation and excreted on milk in a carry-over rate that may be as high as 6.5% [26]. The prevalence of AFM₁ contamination in milk is related to the conditions of the cattle feeding, and depending on the AFB₁ abundance, both toxins can be excreted in milk, although AFB₁ contamination reports in milk are scarce. A few studies demonstrated that AFB₁ may be present in processed cow's milk and in human breast milk [27,28]. However, as these findings are unexpected and the observed concentrations are quite low, the main concern of risks associated to AFB₁ in dairy products are related to the growth of molds and direct contamination. On the other hand, milk and cheese contamination with AFM₁ is frequently documented [29].

The available literature regarding aflatoxins transmission from milk to cheese presents some controversial information and the whole process was not completely understood. Chavarria *et al.* (2017) reported that around 70% of the AFM₁ in the milk was released to the whey, due the high affinity of the toxin to α -lactoalbumin [30]. On the other hand, Khaneghah *et al.* (2021) emphasized the great capacity of AFM₁ binding to casein, the main protein in the cheese. In any case, the concentration of the toxin per gram of cheese is frequently higher, in consequence of aggregation of solids of several liters of milk for curd formation [10]. Considering the process of soft-cheese production, the concentration of AFM₁ increases by 1.71-fold compared to the levels of toxin in naturally contaminated milk [31]. Other studies showed an increment of 2.5-fold in the concentration of AFM₁ in MFC from contaminated milk [32]. For hard

cheese, the concentration factor may reach very high values, varying from 3.8 to 9.6-fold higher than the contamination found in milk [33].

MFC is a type of cheese with very high moisture content and short shelf life correlated to the initial quality of the raw milk. In spite of the influence of the quantity of microorganisms in milk, generally MFC retains its normal characteristics for approximately 21 days, if stored under refrigeration [34,35,36]. The short stability of this product makes it as suitable for consumption only for a few weeks, however, this aspect also reduces the risks of direct contamination of MFC by AFB₁, since mold growth and subsequent toxin production occur under specific combinations of temperature and time. Temperature seems to play a key role in the production of aflatoxins by *Aspergillus* spp., with studies defining 35°C as the best condition for maximum contamination [37]. In this respect, Casquete *et al.* (2017) observed that *Aspergillus* species growing on cheese samples did not produce aflatoxins at 15°C. The toxin accumulation started to be detected for colonies that grew at 20°C, and a significant increase in contamination was observed at temperatures above 25°C [38]. Therefore, considering that MFC is stored at low temperatures, the potential of *Aspergillus* spp. growth on the cheese surface with significant production of aflatoxin is minimum. Then, it is assumed the threat of aflatoxins contamination in MFC is mostly related to the presence of AFM₁ in the milk, although additional investigation may be useful to detect eventual cases of direct contamination.

In our study, all the samples had negative results for AFM₁ and AFB₁ contamination. This means, if present, toxins concentrations were below detectable levels, 0.01µg/kg for AFM₁ and 0.1µg/kg for AFB₁. In any case, if contaminated the samples would have very low levels of toxins, below Brazilian official permitted level for AFM₁ in cheese, representing no risk for human consumption [13].

In contrast to our results, Silva *et al.* (2023b) detected AFM₁ in all the samples of four different types of cheese analyzed in Pernambuco, Brazil. The mean concentration of the toxin was 0.05µg/kg, which represents a low level of contamination, also in accordance to the Brazilian guidelines. Nevertheless, the high incidence of positive samples indicates the milk used by dairy industries of this region might be contaminated with aflatoxins. Frequent surveillance on the quality and contaminants of milk, besides early investigation on the conditions of the cow's feed was recommended in order to reduce the prevalence of dairy products contaminated with aflatoxins [39].

Specifically regarding MFC samples, other published studies investigated the presence of aflatoxins in this sort of product, and their results were diverse, from completely negative sampling to reports of high prevalence of contaminated samples (data summarized on Table 2). In all the studies presented, the toxins were analyzed by chromatographic methods with fluorescence detection. It should be noted that differences in the results may arise due to distinct extraction procedures, equipment and method performances, as well as divergences in data interpretation.

Gonçalves *et al.* (2021^a) reported AFM₁ in 8 of 28 MFC samples (28.6%), with mean contamination of 0.11 µg/kg [40]. In Brazil, the Maximum Permitted Level (MPL) of AFM₁ in cheese is 2.5 µg/kg [13]. Therefore, despite the considerable prevalence of positive samples, all of them would be considered safe based solely on their compliance with official regulations. Other countries or regions did not define specific limits for aflatoxins in cheese, but include milk intended for the manufacture of milk-based products. As an example, the European Union determined the MPL of 0.05 µg/kg of AFM₁ in milk, preventing cheese production with highly contaminated milk [41]. Similarly to the study above, Oliveira *et al.* (2011) observed the mean concentration of 0.14 µg/kg of AFM₁ in MFC positive samples. The prevalence of positivity was 25% and no sample showed toxin levels above Brazilian MPL [42]. Both studies demonstrated the contamination of MFC is present, and although the level of contamination was low, the prevalence of positive samples indicates increased risk associated to the sum of aflatoxins ingested in the diet.

In a recent experiment, De França *et al.* (2024) analyzed 89 samples of MFC purchased in São Paulo, Brazil and only 4 samples resulted in AFM₁ contamination above the LOQ (0.7 µg/kg). The accurate concentrations of toxin in positive samples were not mentioned. Other 6 samples showed traces of AFM₁, with the measured concentrations in the range between LOD and LOQ (0.07 µg/kg-7 µg/kg). This data reinforces the need of developing new methods and enhancing those already used for detection of aflatoxins, in order to provide alternatives to obtain reliable results of this toxins at residual levels [25]. Considering the LOD and LOQ calculated for our method, it is likely that a larger number of positive samples could be encountered using a method with enhanced sensitivity.

On the other hand, Barroncas *et al.* (2019) evaluated the presence of AFM₁ in different types of cheese, including MFC, from the Amazon region. Similarly to our results, the authors observed that none of the analyzed samples had detectable

levels of aflatoxin contamination. The authors mentioned as a possible reason for the absence of positive samples the practices of free range and mostly grass-fed cows in this region [14]. The same can be assumed for our results, considering the proximity of the regions, and the tradition of producing dairy cattle under extensive system.

Then, considering available literature on aflatoxins M₁ and B₁ contamination on MFC, some studies indicated a low prevalence of positive samples for the hydroxylated metabolite. In general, the concentrations detected of AFM₁ were within Brazilian and international regulations. AFB₁ does not seem to represent a real risk of contamination in MFC, whilst AFM₁ prevalence suggests it must be monitored in a higher frequency to guarantee that this contaminant levels are maintained low, in order to prevent impacts on human health.

5. Conclusions

The method developed and validated in this experiment proved to fulfill its purpose of providing accurate and precise results with high selectivity and sensitivity for the measurement of AFM₁ and AFB₁ contamination in minas frescal cheese. All the parameters evaluated presented satisfactory results, indicating the method may be a useful alternative to the monitoring of aflatoxins in dairy products. In respect to the analysis of 20 samples of MFC purchased from street markets of Araguaína, Brazil, all the samples were negative to both contaminants. Further investigation must be carried out with larger sampling, and possibly considering products from other regions and different types of cheese, to better estimate the real situation of aflatoxins contamination in fresh cheese consumed in Brazil.

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6 CONCLUSÃO

O presente trabalho promoveu avanços científicos e tecnológicos aplicáveis ao controle de toxinas naturais e cumpriu com os objetivos planejados em sua idealização. Foram realizados experimentos em duas frentes principais: (1) biodegradação de microcistinas com base na tecnologia de DNA recombinante e expressão de microcistinase A por levedura; e (2) desenvolvimento de método de análise prático e confiável para monitoramento de aflatoxinas em alimentos de origem animal, com foco em queijo minas tipo frescal.

A construção de uma cepa industrial GRAS de *Saccharomyces cerevisiae*, capaz de produzir microcistinase A ativa por meio de expressão plasmidial foi concluída com sucesso. A cepa obtida representa uma promissora alternativa para aplicação futura no biocontrole de microcistinas. Testes realizados durante o cultivo celular comprovaram efetiva degradação de microcistinas, superior a 80% em 120 horas. Por outro lado, as estratégias utilizadas para integração cromossomal de *mlrA*, apesar de confirmadas por técnicas moleculares, não resultaram em significativa atividade enzimática. Novos estudos devem ser conduzidos para definir as causas do baixo nível de expressão e estabelecer outros protocolos que viabilizem obter cepas de levedura com efetiva expressão de microcistinase A, também a partir da integração de *mlrA* ao cromossomo.

Considerando a levedura com expressão plasmidial, a comprovada produção e atividade enzimática permitiram avanços no conhecimento acerca do potencial de aplicação da enzima. Na sequência dos experimentos realizados, extratos celulares gerados a partir da levedura recombinante, forneceram microcistinase A em altas concentrações para investigação das propriedades enzimáticas. Os dados demonstraram que a expressão em *S. cerevisiae* provavelmente não gerou modificações pós-traducionais na estrutura da proteína recombinante. A atividade da enzima se manteve elevada em uma ampla faixa de temperatura (35-57,5°C), e as maiores taxas de reação foram observadas em pH próximo à neutralidade. Além disso, foi possível estabelecer aspectos relativos à cinética enzimática e limitantes para a atividade quanto às condições de temperatura e pH de ensaios. Portanto, as informações aqui geradas são essenciais para fundamentar novos estudos e aprimorar as alternativas de aplicação da enzima, ou da própria levedura, como ferramentas efetivas ao biocontrole de microcistinas.

O último tópico abrangido por esta tese envolveu o desenvolvimento de um método de UPLC-FLR para detecção de AFM₁ e AFB₁ em queijo minas tipo fresco. Considerado a popularidade do produto, a possibilidade que este seja fonte de aflatoxinas, e a escassez de dados destas toxinas no alimento, disponibilizar alternativas para a detecção dos contaminantes é indispensável para garantir amplo monitoramento. Assim, o desenvolvimento deste método simples, rápido, sensível e eficaz na detecção de ambas toxinas, amparado pelos resultados da validação intralaboratorial, representa mais uma útil opção ao monitoramento de aflatoxinas em queijo minas fresco. Além disso, a considerar as características próprias do tipo de queijo, é possível que o método também seja aplicado a produtos similares, se verificada essa possibilidade por meio de novos estudos. Na aplicação do método, nenhuma das 20 amostras de queijo minas fresco produzidas em Araguaína-TO resultaram em contaminação detectável de aflatoxinas. Do ponto de vista da saúde pública estes resultados são positivos, porém somente com uma vigilância frequente e ampla podemos assegurar a ausência de riscos associados a contaminação por aflatoxinas neste produto.

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