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Faculdade de Ciências Biológicas e Ambientais - FCBA
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# AVALIAÇÃO DO POTENCIAL BIOTECNOLÓGICO DE LEVEDURAS INDUSTRIAIS

Valkirea Matos Nascimento

Dourados-MS
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**VALKIREA MATOS NASCIMENTO**

# AVALIAÇÃO DO POTENCIAL BIOTECNOLÓGICO DE LEVEDURAS INDUSTRIAIS

Tese apresentada à Universidade Federal da Grande Dourados (UFGD), como parte dos requisitos exigidos para obtenção do título de DOUTOR EM BIOTECNOLOGIA E BIODIVERSIDADE.
Área de Concentração: Biotecnologia e Biodiversidade

Orientador: Prof. Dr. Gustavo Graciano Fonseca.
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Por

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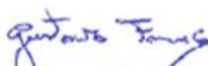

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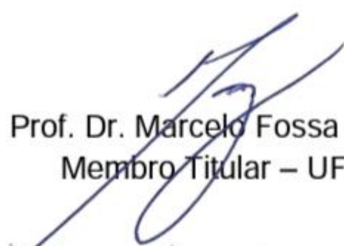

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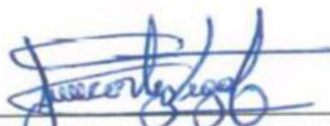

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# LISTA DE SIGLAS E ABREVIATURAS

Ace ........................................................................................................................ acetato
ANOVA ........................................................................................... análise de variância
B1 e B6 ................................................................................................................ vitaminas
C ........................................................................................................................... carbono
CAPES ..................................... Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CAT-1 ............................................................................... isolado 1 da Usina Catânduva
CEL ........................................................................................................................ células
CNPq .................................... Conselho Nacional de Desenvolvimento Científico e Tecnológico
$CO_2$ ............................................................................................... dióxido de carbono
DNS ....................................................................................... ácido 3,5-dinitrosalicílico
DT .................................................................................................. tempo de duplicação
EDTA ........................................................................... ácido etilenodiamino tetra-acético
ETH ou Eth ............................................................................................................ etanol
$t_0$ ..................................................................................................................... tempo inicial
$t_f$ ...................................................................................................................... tempo final
T .................................................................................................................... temperatura
FEC ........................................................................... fase exponencial de crescimento
Fig. ........................................................................................................................ figura
*FLO1* e *FLO9* ......................................................... genes que codificam para a floculina
FT858 ............................................................................ isolado 858 da Empresa Fermentec
GAL ................................................................................................................... galactose
GLC ....................................................................................................................... glicose
GLI ou Gly ............................................................................................................ glicerol
Gpr1 .................................................................................... receptor acoplado à proteína G
Km ........................................................................................... *Kluyveromyces marxianus*
LAC ....................................................................................................................... lactato
MCS ................................................................................................ biomassa celular seca
NADPH ................................................................. fosfato de nicotinamida adenina dinucleotídeo
$OD_{600nm}$ ..................................................................................... densidade óptica a 600 nm
p ................................................................................................................... probabilidade
$P_{CEL}$ ....................................................................................... produtividade máxima de células
PE-2 .................................................................................................. Isolado 2 da Usina Pedra

$P_{ETH}$ ....................................................................................produtividade máxima de etnol
pH..........................................................................................................potencial hidrogeniônico
RID..........................................................................................................refratômetro diferencial
S ........................................................................................................................... substrato
$S_0$ ........................................................................................ concentração inicial de substrato
$S_{final}$ .................................................................................... concentração final de substrato
Sc .............................................................................................*Saccharomyces cerevisiae*
SUC............................................................................................................................ sacarose
*SUC2* ............................................................................ gene que codifica a invertase extracelular
T ........................................................................................................................ temperatura
TFA ........................................................................................................... ácido trifluoracético
UFGD....................................................................... Universidade Federal da Grande Dourados
UPLC ....................................................................... cromatógrafo líquido de ultra performance
UV......................................................................................................................... ultravioleta
YPD ................................................................................................ meio levedura-peptona-dextrose
$Y_{X/S}$ ....................................................................... fator de conversão de substrato a células
X..................................................................................................... concentração de biomassa
$X_0$ ........................................................................................ concentração inicial de biomassa
$X_f$ ............................................................................................ concentração final de biomassa
$X_{max}$ ..................................................................................... concentração máxima de biomassa
$\mu_{max}$ .................................................................. velocidade específica máxima de crescimento
$\mu_S$ ou $q_s$ .............................................................. velocidade específica de consumo de substrato
[ ] ............................................................................................................ concentração

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**RESUMO**

NASCIMENTO, Valkirea Matos. Universidade Federal da Grande Dourados, março de 2021. "**Avaliação do potencial biotecnológico de leveduras industriais**". Orientador: Prof. Dr. Gustavo Graciano Fonseca. Co-orientador: Prof. Dr. Fernando Araripe Gonçalves Torres.

*Sacharomyces cerevisiae* CAT-1 destaca-se como a linhagem com maior capacidade fermentativa comprovada até o momento. Contudo *S. cerevisiae* FT858 foi recentemente apresentada como uma potencial fermentadora alcoólica. Assim, o objetivo deste estudo foi estudar a fisiologia de *S. cerevisiae* FT858 em termos do consumo de substratos, formação de metabólitos extracelulares, crescimento celular e atividade de invertase em diferentes fontes de carbono a fim de compará-la com *S. cerevisiae* CAT-1. *S. cerevisiae* FT858 apresentou parâmetros cinéticos de crescimento muito próximos aos obtidos com *S. cerevisiae* CAT-1 em condições de cultivo semelhantes (aerobiose, temperatura, agitação, substrato e concentração). Porém houve um aumento de 10% no rendimento de sacarose em etanol obtido com *S. cerevisiae* FT858 quando comparado com *S. cerevisiae* CAT-1 a 37°C. A concentração dos substratos interferiu diretamente na produção de invertase tanto para *S. cerevisiae* FT858 quanto para *S. cerevisiae* CAT-1. A expressão enzimática sofreu forte regulação por meio da concentração de glicose no meio de cultura. Contudo apenas *S. cerevisiae* CAT-1 apresentou comportamento constitutivo para a enzima invertase. Num segundo estudo um total de 14 linhagens de leveduras, incluindo *S. cerevisiae* CAT-1, também foram avaliadas em termos de parâmetros cinéticos e comparadas. De maneira geral leveduras do gênero *Kluyveromyces* apresentaram maior potencial respiratório enquanto leveduras do gênero *Saccharomyces* maior capacidade fermentativa. Entre outros resultados, destaca-se que *Kluyveromyces marxianus* NRRLy 2415 foi a maior produtora de biomassa (23,17±0,000 C-X), enquanto a *S. cerevisiae* BG-1 foi a menor produtora (3,05±0,000 C-X).

**Palavras-chave**

Parâmetros cinéticos; fermentação; repressão catabólica; açúcares; invertase.

## ABSTRACT


NASCIMENTO, Valkirea Matos. Federal University of Grande Dourados, March 2021. **“Evaluation of the biotechnological potential of industrial yeasts"**. Advisor: Prof. Dr. Gustavo Graciano Fonseca. Co-adviser: Prof. Dr, Fernando Araripe Gonçalves Torres.

*Sacharomyces cerevisiae* CAT-1 stands out as the strain with the highest fermentation capacity proven to date. However, *S. cerevisiae* FT858 was recently presented as a potential alcoholic fermenter. Thus, the aim of this work was to evaluate the physiology of *S. cerevisiae* FT858 in terms of substrate consumption, formation of extracellular metabolites, cell growth and invertase activity in different carbon sources to compare it with *S. cerevisiae* CAT-1. *S. cerevisiae* FT858 presented kinetic growth parameters very close to those obtained with *S. cerevisiae* CAT-1 under similar culture conditions (aerobiosis, temperature, agitation, substrate and concentration). However, there was a 10% increase in sucrose yield into ethanol obtained with *S. cerevisiae* FT858 when compared to *S. cerevisiae* CAT-1 at 37 °C. The concentration of the substrates directly interfered in the production of invertase for both *S. cerevisiae* FT858 and CAT-1. The enzymatic expression underwent strong regulation through the concentration of glucose in the culture medium. However, only *S. cerevisiae* CAT-1 showed a constitutive behavior for the enzyme invertase. In a second study, a total of 14 yeast strains, including *S. cerevisiae* CAT-1, were also evaluated in terms of kinetic parameters and compared. In general, yeasts of the genus *Kluyveromyces* showed greater respiratory potential while yeasts of the genus *Saccharomyces* had a greater fermentative capacity. Among other results, it is noteworthy that *Kluyveromyces marxianus* NRRLy 2415 was the largest producer of biomass (23.17 ± 0.000 C-X), while *S. cerevisiae* BG-1 was the smallest producer (3.05 ± 0.000 C-X).



**Keywords**

Kinetic parameters; fermentation; catabolic repression; sugars; invertase.

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## CAPÍTULO 1

# 1 INTRODUÇÃO GERAL

## 1.1 Apresentação

Esta tese apresenta as atividades realizadas pela aluna Valkirea Matos Nascimento durante o seu doutorado pelo Programa de Pós-Graduação em Biotecnologia e Biodiversidade da Universidade Federal da Grande Dourados (UFGD).

O texto foi estruturado de modo que cada capítulo venha corresponder a uma publicação, exceto esta introdução geral (Capítulo 1) e as conclusões gerais (Capítulo 5). Assim, este trabalho foi organizado da seguinte forma:

- Capítulo 2. *Saccharomyces cerevisiae* CAT-1: Uma breve revisão;
- Capítulo 3. Efeitos da fonte de carbono na fisiologia e atividade de invertase da levedura *Saccharomyces cerevisiae* FT858;
- Capítulo 4. Avaliação fisiológica de 14 linhagens de leveduras industriais através de parâmetros cinéticos.

## 1.2 Objetivos

Os objetivos do presente trabalho foram:

- Revisar a literatura acerca das leveduras utilizadas em plantas industriais de etanol, com ênfase em *Saccharomyces cerevisiae* CAT-1;
- Estudar a fisiologia de *Saccharomyces cerevisiae* FT858 em termos do consumo de substratos, formação de metabólitos extracelulares, crescimento celular e atividade de invertase em diferentes fontes de carbono;
- Investigar e comparar a fisiologia de 14 linhagens de leveduras industriais em termos de parâmetros cinéticos e produção de metabólitos.

# CAPÍTULO 2

## 2 *Saccharomyces cerevisiae* CAT-1: UMA BREVE REVISÃO

**RESUMO**

*Saccharomyces cerevisiae* PE-2, CAT-1 e FT858 são responsáveis por 70% de todo o etanol produzido no Brasil. Contudo, *S. cerevisiae* CAT-1 tem se mostrado até então mais eficiente especialmente em altas concentrações de açúcar quando comparada a outras leveduras. Estas leveduras têm sido pouco estudadas dada a sua importância econômica. Desta forma, o objetivo deste trabalho foi revisar estudos com *S. cerevisiae* CAT-1. Apesar de esforços relacionados ao sequenciamento do genoma e à estudos proteômicos com esta levedura, há ainda lacunas importantes que não foram preenchidas a respeito de sua fisiologia quanto ao transporte de açúcares, metabolismo e regulação. Este conhecimento é imprescindível para o crescimento e fortalecimento de diversos setores produtivos no país.

### 2.1 Introdução

A fermentação é um dos mais antigos bioprocessos relatados na História da humanidade, onde com o passar dos tempos, foi aprimorada e estudos levaram à identificação das culturas microbianas e o controle das condições dos processos, levando a fermentação à nível industrial. O processo é amplamente utilizado nas indústrias de alimentos, farmacêutica, química e de biocombustíveis. Sendo as leveduras as principais fábricas celulares de produtos biotecnológicos no mundo, e a *Saccharomyces cerevisiae* a principal representante, e devido à sua importância nos processos biotecnológicos de escala industrial a espécie é amplamente estudada (25, 23, 42).

No Brasil, a produção de etanol é uma das maiores atividades industriais envolvendo o microrganismo. A eficiência deste é possível através do controle de variáveis operacionais como a temperatura, pH, níveis de álcool, concentrações de substrato e biomassa (25). Portanto, a escolha de uma linhagem de levedura é um passo importante para um processo fermentativo eficiente, pois este depende de diferentes características microbianas como velocidade de transformação dos açúcares em etanol,

resistência à altas concentrações de açúcares e etanol e à alterações de pH e temperatura, estabilidade genética e insensibilidade à antibióticos (2,38).

Neste contexto, linhagens de leveduras foram investigadas para melhorar a produção de etanol e, através de pesquisas, foram descobertas algumas linhagens que se adaptaram de forma natural ao processo utilizado no país, tornando-se amplamente utilizadas em diversas usinas. Dentre estas encontra-se a *S. cerevisiae* CAT-1 (34).

## 2.2 Evolução adaptativa de leveduras em plantas industriais de etanol

Na produção de etanol combustível, linhagens de *S. cerevisiae* são os principais iniciadores do processo fermentativo. Como o processo ocorre em meio não asséptico, facilita a contaminação por linhagens de levedura selvagens. Com o monitoramento ao longo do tempo verificou-se que alguns destes microrganismos persistiam ou predominavam os fermentadores, mostrando-se mais adaptados ao processo em relação a cultura inicial (6, 15, 28).

O processo de fermentação no Brasil utiliza densidades de células de levedura muito altas em modo semi-contínuo alimentado em batelada para fermentar caldos contendo altas concentrações de açúcar, produzindo alta concentração de etanol com alto rendimento (90 - 92% do máximo teórico) e produtividade (cada ciclo de fermentação dura 6 - 10 h), após cada ciclo é feito a reciclagem de células (3). Este procedimento consiste em um tratamento ácido usado como estratégia para controle de contaminantes. O processo envolve a mistura da massa celular (suspensão viscosa) obtida após a centrifugação do vinho com uma solução de ácido sulfúrico-água (pH 2,0 - 2,5) por um período de 1 - 2 h e posteriormente as células de levedura são reintroduzidas nos tanques para fermentação subsequente durante toda a safra de 6 a 9 meses (13).

Durante esta etapa, uma pressão seletiva (evolução adaptativa) é imposta à população de levedura, levando as cepas com maior tolerância às condições estressantes oferecidas pelo processo persistirem por vários ciclos. As leveduras se adaptam aos fatores adversos encontrados no ambiente, ajustando sua fisiologia e atividades metabólicas para evitar perda substancial de viabilidade na cultura (32, 31).

Observando as diferenças de comportamento e através do monitoramento dos microrganismos durante o processo industrial é possível então selecionar linhagens selvagens que apresentam boas características de fermentação, como alta produção de

etanol, baixo teor de formação de glicerol e ácidos orgânicos, baixa formação de espuma, alta viabilidade, entre outras (6, 15).

Pereira *et al*. (32) mostraram que a grande biodiversidade encontrada em ambientes de destilaria podem ser uma importante fonte de linhagens robustas tolerantes ao estresse para processos biotecnológicos, como também possíveis hospedeiros para serem usados em programas de melhoria por meio da engenharia metabólica.

Devido aos maiores níveis de estresse oferecidos a levedura durante a produção de etanol combustível, as linhagens isoladas deste tipo de processo, apresentam uma capacidade de fermentação superior aos outros isolados industriais, como *Kluyveromyces marxianus*, *S. cerevisiae* isolado de cerveja e cepas de laboratório (33).

### 2.3 *Saccharomyces cerevisiae* CAT-1

No Brasil, desde o início da década de 1990, cepas de leveduras têm sido investigadas para melhorar a produção de etanol (34). Neste contexto, após 12 anos estudando e isolando leveduras nos processos industriais, Basso *et al*. (5) selecionaram 14 linhagens que apresentavam alto desempenho fermentativo em escala laboratorial, e dentre estas poucas linhagens apresentaram a capacidade de serem reintroduzidas no processo industrial. Neste estudo, duas linhagens de *S. cerevisiae* CAT-1 e PE-2 apresentaram uma elevada capacidade de implantação industrial e maior competitividade em relação às cepas contaminantes do processo, podendo representar uma média de 45% a 54% da biomassa de leveduras, ou até mesmo, em algumas destilarias, representar a biomassa total ao final de uma temporada de fermentação, e estas tornaram-se disponíveis comercialmente.

Três linhagens *S. cerevisiae* selecionadas de processos são responsáveis por 70% de todo o etanol produzido no Brasil: PE-2, CAT-1 e FT858 (11, 27). Destas, *S. cerevisiae* CAT-1 apresenta uma capacidade de fermentação muito eficiente, especialmente em altas concentrações de açúcar e mostra elevado potencial para a produção de destilados a partir de cereais, quando comparada a outras leveduras (5, 3). Em pesquisa de fermentação em co-cultivo com *S. cerevisiae* CAT-1 e PE-2, verificou-se que *S. cerevisiae* CAT-1, a partir da fase exponencial, apresentou taxa populacional mais elevada que *S. cerevisiae* PE-2, confirmando sua robustez e maior resistência as condições de estresse do processo (37).

As características micro e macromorfológicas de *S. cerevisiae* CAT-1 foram avaliadas por Camargo *et al*. (12). A levedura apresenta forma arredondada, tamanho

grande e se reproduz por brotamento. Suas colônias apresentam pigmentação branca, com bordas irregulares e textura suave e brilhante.

Por ser *S. cerevisiae* CAT-1 uma das cepas mais utilizadas em usinas brasileiras até a atualidade, Babrzadeh *et al.* (3) sequenciaram seu genoma no intuito de elucidar os motivos de sua robustez ao processo. No estudo em questão, verificou-se que a linhagem tem um alto grau de heterozigose, ausência dos genes da *flocculina FLO1* e *FLO9* (possível responsável pela falta de floculação e o fenótipo de formação de espuma) e amplificação nos pares de genes teloméricos *SNO2/SNO3* e *SNZ2/SNZ3*, os quais são demonstrados como necessários para a utilização eficiente do açúcar por meio de seus efeitos no metabolismo da piridoxina (vitamina B6) e da tiamina (vitamina B1). Além disso, foi identificada a presença de outros genes correlacionados com a capacidade de cepas industriais fermentarem maltose e maltotriose.

Além das caraterísticas citadas acima, *S. cerevisiae* CAT-1 apresentou amplificação do gene *SEO1* (que codifica uma permease da subfamília do transportador alantoato) e *ADH7* (que codifica uma álcool desidrogenase de cadeia média dependente de NADPH com ampla especificidade de substrato), como também outros genes que estão aparentemente envolvidos na resistência a materiais tóxicos. Para surpresa dos pesquisadores, a cepa CAT-1 não apresentou amplificação do gene *SUC2*, que codifica a invertase extracelular responsável pela hidrólise e fermentação da sacarose, como também apresenta características semelhantes em relação ao gene *SUC* as demais linhagens *S. cerevisiae* (3).

Em *S. cerevisiae* CAT-1 foi detectado apenas a presença do gene *IRA2*. Os genes *IRA1* e *IRA2* estão envolvidos na resposta a diferentes estresses ambientais em leveduras. São inibidores da via Ras-cAMP-PKA que tem papel fundamental na resposta transcricional à presença de açúcares fermentáveis (3). *IRA2* codifica um regulador da via que está envolvido na síntese do glicogênio. Uma vez que a via cAMP também regula a via glicolítica com efeitos secundários na expressão dos genes *HXT*, o gene pode funcionar como um supressor em virtude do seu impacto na sinalização de cAMP (9).

Estudos indicam que essas evoluções adaptativas positivas em *S. cerevisiae* CAT-1 podem não estar relacionadas apenas a alterações genéticas, mas sim nos processos de transcrição e sinalização de disponibilidade de substratos (28). A robustez da linhagem tem sido muito investigada, e através de estudos de proteoma percebeu-se que dependendo das condições de cultivo a levedura apresenta diferenças qualitativas e

quantitativas na expressão de proteínas, principalmente naquelas que desempenham papéis importantes na via da glicólise (2).

Em geral, na evolução adaptativa, quando os microrganismos se deparam com uma nova fonte de carbono, eles evoluem os genes estruturais ou regulatórios que estão relacionados ao metabolismo do mesmo. No entanto, estudo com *S. cerevisiae* frente ao substrato galactose não identificou alterações nos genes envolvidos no metabolismo do carboidrato. O que foi observado foi que as mudanças fenotípicas são consequências de mutações em sistemas regulatórios em proteínas envolvidas na via de sinalização Ras/PKA (21).

Santos *et al.* (38) realizaram estudos proteômicos comparativo entre as *S. cerevisiae* CAT-1 e PE-2, demonstrando que a expressão mais forte de proteínas envolvidas na resposta ao estresse em *S. cerevisiae* CAT-1 permite que a linhagem consiga lidar melhor com as mudanças do meio de cultivo durante a fermentação. Em *S. cerevisiae* CAT-1 detectaram-se níveis de Tps3 75% maior (proteína envolvida na síntese de trealose) e uma capacidade de acumular trealose duas vezes maior. A trealose é responsável pelo aumento da capacidade da levedura em suportar o estresse da fermentação, estabilizando membranas e proteínas, evitando a desnaturação proteica e a agregação das proteínas desnaturadas.

## 2.4 Fisiologia de *S. cerevisiae* CAT-1

A fisiologia de uma espécie pode variar dependendo da cepa e da fonte de carbono utilizada no processo biotecnológico. Beato *et al.* (8) conduziram um estudo de avaliação fisiológica de 20 linhagens de *S. cerevisiae* (selvagens, industriais e de laboratório) no qual concluíram que cepas industriais têm maior capacidade fermentativa e que os estudos fisiológicos realizados para uma cepa não podem ser atribuídos a todas as cepas dessa espécie, pois cada linhagem apresenta uma resposta diferente às condições oferecidas.

Por mais que *S. cerevisiae* tenha sua fisiologia bem estudada em nível laboratorial, poucos são os estudos que abordam a fisiologia de cepas brasileiras de etanol combustível (24, 15, 8, 28, 27). Como os processos biotecnológicos envolvendo leveduras podem utilizar matérias primas complexas, e a utilização de um microrganismo que seja capaz de utilizar o substrato disponível é de fundamental importância para a eficiência do processo, é importante o conhecimento da fisiologia do microrganismo que será empregado (17, 28).

Desta forma, as condições estressoras mais encontradas no processo de etanol combustível são baixo pH (3,0), alta concentração de etanol (8% m/v), alta temperatura (40°C) e alta concentração de ácido acético (10 g $L^{-1}$). Pesquisas com cepas isoladas de ambiente industriais concluíram que não existe uma cepa que seja a mais tolerante a todas as condições de estresse testadas, ou seja, o que define a eficiência da levedura no processo é como ela reage ao conjunto de fatores oferecidos (16, 8).

Della-Bianca e Gombert (16) realizaram a avaliação das tolerâncias destes fatores estressantes em cepas *S. cerevisiae* isoladas de ambientes industriais (dentre elas *S. cerevisiae* CAT-1). Foi observado que as condições de estresse que mais distinguiram a robustez das cepas de etanol combustível das de laboratório e de panificação foram o calor e o baixo pH. Apesar disto, *S. cerevisiae* CAT-1 não demostrou tolerância em pH 2,5. Outro estudo também relatou menor tolerância de CAT-1 em crescer sob condições de estresse por ácido acético comparado a outras leveduras *S. cerevisiae* (8).

Basso *et al.* (5) relataram a não prevalência de *S. cerevisiae* CAT-1 nos fermentadores após sofrerem vários ciclos de reciclagem de células no processo industrial em que a linhagem foi empregada em conjunto com outras duas linhagens industriais (*S. cerevisiae* PE-2 e BG-1) e fermento de panificação. Após 122 dias de operação apenas *S. cerevisiae* PE-2 foi detectada. Mesmo que *S. cerevisiae* CAT-1 não predomine nos biorreatores ao longo do processo, a fermentação em co-cultura com *S. cerevisiae* CAT-1 e PE-2, apresenta maiores rendimentos em etanol em relação a cultivos com cultura pura. Provavelmente por apresentar melhor adaptação aos estresses oferecidos durante a fase inicial de crescimento (37).

Entretanto, *S. cerevisiae* CAT-1 apresentou uma maior termotolerância entre cepas industriais em ensaios pontuais em placas YPD a 40°C (16). Outro estudo demostrou que a linhagem apresenta uma maior produtividade em etanol e maior velocidade de crescimento máxima quando cultivada em temperatura de 37°C em relação a cultivos em 30°C (28).

Quanto à tolerância a etanol, cepas industriais demostram ausência de fenótipo de aglomeração após exposição a 8% (m/v) de etanol (8). Em pesquisa realizada em placas com elevada concentração de etanol, *S. cerevisiae* CAT-1 e outras leveduras industriais apresentaram crescimento, porém foi observado que as colônias formadas eram menores quando comparadas a cepas de laboratório, revelando que cepas industriais tem menor tolerância ao crescimento celular na presença de etanol em placas. Os autores ainda ressaltam que esta análise não seja totalmente confiável para

pesquisa de tolerância em etanol dentro de um processo biotecnológico, uma vez que no processo este aumento da concentração é gradativo e a levedura vai se adaptando as alterações ao longo do processo (16).

### 2.5 Relação de *S. cerevisiae* CAT-1 com diferentes substratos

Leveduras de uma forma geral detectam a disponibilidade e a qualidade dos nutrientes dispostos ao ambiente externo por meio de múltiplas redes de sinalização que permite o ajuste do metabolismo e perfil transcricional para uma rápida adaptação às mudanças nutricionais. A obtenção de energia pelas células se dá por meio da fermentação de açúcares glicose, frutose, sacarose, galactose, melibiose, maltose, entre outros, ou através da oxidação de uma variedade de produtos de fermentação, como glicerol, etanol e lactato (43).

Estes microrganismos preferem uma fonte de carbono fermentável a qualquer outra metabolizada por oxidação. Elas preferem glicose ou frutose a outros mono-, di- e trissacarídeos, mesmo para aqueles di- ou trissacarídeos, como sacarose, rafinose, e trealose, que pode ser convertida diretamente em glicose ou frutose (43, 17, 28).

Camargo *et al.* (12) testaram as capacidades de assimilação e fermentação de uma variedade de substratos em *S. cerevisiae* CAT-1 e outras leveduras isoladas. O teste foi realizado em tubos de ensaio e os substratos testados foram glicose, frutose, lactose, galactose, sacarose, maltose, amido, manitol, rafinose, celobiose e xilose. Destes a linhagem assimilou todos os substratos, porém não houve fermentação em lactose, amido, manitol, celobiose e xilitol.

Os efeitos da fonte de carbono e a interação entre elas sob a fisiologia de *S. cerevisiae* CAT-1 foram avaliados através de parâmetros cinéticos por Nascimento e Fonseca (28). Neste estudo concluiu-se que o crescimento da linhagem é dependente da temperatura e da natureza do açúcar ou mistura de açúcares utilizados como fonte de carbono. Além disso, os cultivos com frutose como única fonte de carbono apresentaram maiores rendimentos de substrato em etanol nesta fonte ($Y_{ETH/S}$) do que em glicose, seguido por sacarose e maltose. Nos cultivos com uma mistura binária de açúcares, foi evidente a repressão catabólica entre os substratos. Outro estudo também avaliou a fisiologia da linhagem em cultivos com frutose como única fonte de carbono e os resultados obtidos corroboram com o estudo acima citado (39).

O maior rendimento de frutose em etanol se dá porque a glicose é primeiramente metabolizada por múltiplas vias em glicose-6-fosfato e dividida em porções para o

crescimento da biomassa ou convertida em frutose-6-fosfato. Em contraste, a frutose é diretamente metabolizada em frutose-6- fosfato e, em seguida, irreversivelmente fosforilada em frutose-1,6-bifosfato (42, 28).

Apesar de a linhagem apresentar bons resultados com relação a utilização de maltose como substrato (3, 12, 28), muitas das aplicações industriais de leveduras *Saccharomyces* dependem da fermentação eficiente de hidrolisados de amido ricos em α-glicosídeos maltose e maltotriose. Duval *et al.* (17) concluíram que *S. cerevisiae* CAT-1 e outras linhagens industriais, por mais que utilizem e fermentem a maltose de maneira eficiente, quando cultivadas na presença de maltotriose apresentam uma taxa de crescimento muito lenta.

Segundo Hong *et al.* (21), apesar da semelhança estrutural entre galactose e glicose, *S. cerevisiae* cresce a metade da taxa em galactose comparado com a glicose. Em cultivos quimicamente definidos com galactose como única fonte de carbono, *S. cerevisiae* CAT-1 apresentou uma taxa de crescimento em galactose aproximadamente 65% da taxa de crescimento em glicose (28). Dados que sugerem que a linhagem seja ligeiramente mais eficiente frente a este substrato comparada a outras linhagens da espécie.

Apesar da abundância de glicose na natureza, *S. cerevisiae* possui a característica de crescimento rápido em sacarose. Isso pode estar relacionado à maior afinidade por sacarose do receptor acoplado à proteína G (Gpr1), que pode aumentar o fluxo glicolítico por meio da ativação da via de sinalização do cAMP (8).

Para o consumo do substrato sacarose a levedura apresenta dois mecanismos, sendo predominante a hidrólise por uma invertase extracelular, produzindo glicose e frutose, que entram na célula por difusão facilitada através de transportadores específicos e/ou não-específicos. O outro mecanismo é o transporte ativo da sacarose e hidrólise intracelular (7).

O processo bioquímico envolvido consiste na oxidação incompleta da sacarose, que sofre hidrólise pela enzima invertase resultando em glicose e frutose, que por sua vez, entram na via glicolítica e sofrem inúmeras reações, sendo convertidas em piruvato, que é descarboxilado pela ação da enzima piruvato descarboxilase, produzindo acetaldeído e liberando $CO_2$. O acetaldeído é então reduzido à etanol através da enzima álcool desidrogenase (29, 12, 36).

## 2.6 Potenciais aplicações de *S. cerevisiae* CAT-1

Quando um microrganismo é isolado de algum processo mostrando-se eficiente, geralmente torna-se muito utilizado em processos semelhantes ao de origem. Partindo disto, *S. cerevisiae* CAT-1 tem sido muito empregada em processos de geração de etanol (6, 15, 11, 37, 18). Apesar disto, existem outras possíveis aplicações para a linhagem conforme demostrado na Tabela 2.1.

Visando a seleção de novas linhagens que apresentem características semelhantes ou superiores ao desempenho demonstrado pela *S. cerevisiae* CAT-1 em plantas de etanol combustível, a linhagem tem sido utilizada como padrão referência de levedura industrial em uma variedade de estudos (17, 8, 31, 12, 39, 41). Através desta comparação é possível selecionar cepas com características até superiores a *S. cerevisiae* CAT-1. Como é o caso da linhagem LBGA-01, uma nova cepa termotolerante capaz de fermentar a 40 °C, sendo mais resistente a estressores como sacarose, furfural e etanol do que *S. cerevisiae* CAT-1 (34).

Já no processo de fermentação de bebidas, a *S. cerevisiae* CAT-1 foi investigada para fabricação de bebidas fermentadas derivadas de substratos a base de milho. O estudo revelou que, embora todas as demais cepas do estudo pertencessem à espécie *S. cerevisiae*, elas mostraram um padrão diferente de produção de compostos voláteis, e *S. cerevisiae* CAT-1 foi a única cepa que produziu o composto heptil éster, que está relacionado às notas florais (41). O estudo ainda reforça a importância de estudar e caracterizar o desempenho da cepa em substratos específicos em bioprocessos diferentes.

Na fabricação de whisky a linhagem mostrou um desempenho de fermentação equivalente e uma tolerância superior ao estresse em relação a uma levedura padrão de destilação de whisky escocês comercial. Além disto, os destilados produzidos com a cepa apresentavam perfis de sabor aceitáveis e não exibiam características sensoriais atípicas (1).

A produção de bioetanol a partir de matérias-primas lignocelulósicas tem se tornado foco nos últimos anos. Um dos principais açúcares obtidos da fração hemicelulósica é a xilose que não é consumida naturalmente por *S. cerevisiae*. Partindo disto, através da engenharia metabólica, muitas são as pesquisas em busca da construção de uma linhagem de *S. cerevisiae* capaz de fermentar xilose (35, 14).

**Tabela 2.1.** Diferentes aplicações da linhagem *S. cerevisiae* CAT-1.

| Aplicação | Referência |
|---|---|
| **Padrão de referência** | |
| Seleção de novas linhagens para produção de etanol celulósico | (31) |
| Avaliação da capacidade fermentativa de leveduras | (18) |
| Avaliação fisiológica de leveduras utilizando frutose como única fonte de carbono | (39) |
| Prospecção de leveduras para produção de bioetanol e indústria de alimentos | (12) |
| Fermentação de substrato a base de milho | (41) |
| Avaliação da fisiologia de leveduras isoladas de diferentes biomas | (8) |
| Produção de invertase | (27) |
| Na caracterização linhagens termotolerante isolada durante a produção de etanol no Brasil | (34) |
| **Engenharia metabólica** | |
| Utilizada como hospedeiro de genes, modificação genética para o consumo de xilose | (14) |
| Cepa projetada para a fermentação de xilose | (35) |
| **Outras aplicações** | |
| Biocatalisadores na biotransformação de chalconas | (40) |
| Fermentação em Co-cultura | (37) |
| Potencial no biocontrole da mancha preta nos citros | (19) |
| Produção de frutooligossacarídeos (nistose) | (4) |
| Produção de bioetanol a partir de resíduos de mamão | (10) |
| Vinho de frutas produzido a partir de cagaita | (30) |
| Avaliação da capacidade de fermentar substratos maltose e maltotriose. | (17) |
| Produção de álcool para whisky escocês | (1) |

Neste contexto, a maioria das cepas de *S. cerevisiae* projetadas para fermentação de xilose são selecionadas pela facilidade de manipulação genética e não pela robustez. Estudos apontam que o desenvolvimento de cepas eficientes em fermentação pode ser uma estratégia útil para gerar cepas mais eficientes em relação ao metabolismo da xilose e tolerância ao inibidor, consequentemente cepas mais resistentes ao processo (22).

Desta forma, *S. cerevisiae* CAT-1 foi testada como hospedeira de genes para fermentação de xilose, onde apresentou resultados positivos por ser mais adaptada as condições oferecidas (35, 14).

A linhagem também tem sido aplicada na química verde como biocatalisadores na biotransformação de chalconas (40), na produção de frutooligossacarídeos (nistose) (4), apresenta potencial para uso no biocontrole da mancha preta dos citros, através da produção de compostos voláteis (19), na produção de bioetanol a partir de resíduos de mamão (10) e na produção de vinho de frutas a partir de cagaita (30).

### 2.7 Atividade de invertase em *S. cerevisiae* CAT-1

As invertases são hidrolases glicosídicas que catalisam a clivagem catalítica de sacarose (α-D-glucopiranosil-β-D-frutofuranosídeo) em dois monossacarídeos, *i.e.*, glicose e frutose. O nome oficial da enzima invertase é β-frutofuranosidase (EC.3.2.1.26) (26). Em *S. cerevisiae*, a invertase é codificada pelo gene *SUC2*, o qual é responsável por dois transcritos da enzima, uma invertase citoplasmática constitutiva (expressa em níveis baixos) e uma invertase periplasmática, cujos níveis são regulados pela glicose (20).

Fernandes *et al.* (18) utilizaram *S. cerevisiae* CAT-1 como padrão industrial para avaliação de capacidade de fermentação de leveduras. Durante o estudo percebeu-se que a linhagem apresentou degradação intensa da sacarose antes de consumir a glicose livre do meio. Após realizar análise sobre a atividade da enzima periplasmática da linhagem, concluiu-se que a taxa de produção de invertase foi a mesma para as três fontes de carbono testadas (glicose, frutose e sacarose). O estudo concluiu que independente da natureza do açúcar ou concentração, a levedura produziu as mesmas quantidades de invertase, concluindo que a invertase periplasmática é uma enzima constitutiva em *S. cerevisiae* CAT-1.

Já outro estudo revelou que a concentração dos substratos interferiu diretamente na produção de invertase em *S. cerevisiae* CAT-1 e outra levedura industrial. A expressão enzimática sofreu forte regulação através da concentração de glicose no meio de cultura e *S. cerevisiae* CAT-1 também apresentou comportamento constitutivo para a enzima invertase, uma vez que a atividade enzimática foi observada apenas para *S. cerevisiae* CAT-1 em cultivos com glicose como única fonte de carbono (27). Outra característica da invertase produzida por *S. cerevisiae* CAT-1 é a atividade da transferase, resultando na produção de frutooligossacarídeos (nistose) (4).

## 2.8 Considerações finais

Dentre as leveduras isoladas em usinas de produção de açúcar e álcool no Brasil, *S. cerevisiae* CAT-1 é a linhagem com maior capacidade fermentativa comprovada até o momento. Apesar de esforços relacionados ao sequenciamento do genoma e a estudos proteômicos com esta levedura, há ainda lacunas importantes que não foram preenchidas a respeito de sua fisiologia quanto ao transporte de açúcares, metabolismo e regulação. Além disso, estudos comparativos com outras leveduras industriais são importantes para a compreensão da robustez e resistência desta linhagem. A elucidação e sistematização deste conhecimento é imprescindível para a manutenção do país na vanguarda tecnológica da fermentação alcoólica de cana-de-açúcar.

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# CAPÍTULO 3[1]

## 3 EFFECTS OF THE CARBON SOURCE ON THE PHYSIOLOGY AND INVERTASE ACTIVITY OF THE YEAST *Saccharomyces cerevisiae* FT858


### Abstract

There are few published studies on *Saccharomyces cerevisiae* FT858 fermentative efficiency. The aim of this work was to evaluate the biotechnological potential of *S. cerevisiae* FT858 through kinetic growth parameters, and the influence of the concentration of the substrate on the synthesis of the invertase enzyme. Invertases have a high biotechnological potential and their production through yeast is strongly influenced by the sugars in the medium. FT858 has an excellent biotechnological potential compared to yeast reference *S. cereviseae* CAT-1, as it presented a low glycerol yield on substrate ($Y_{GLY/S}$) and a 10% increase in ethanol yield on sucrose in cultures with sucrose at 37 °C. The substrate concentration directly interfered in invertase production and the enzymatic expression underwent strong regulation through glucose concentration in the culture medium and *S. cerevisiae* CAT-1 presented constitutive behavior for the invertase enzyme.


### 3.1 Introduction

Microorganisms, in general, can undergo evolutionary mutations induced by adverse conditions in the environment in which they live, seeking to guarantee their survival. Thereby, some strains of the yeast *Saccharomyces cerevisiae* were isolated from industrial ethanol plants, showing high fermentative efficiency, as they evolved to withstand the stress conditions offered during this process. Thus, some of these strains became commercially available, being responsible for much of the Brazilian's ethanol production, as is the case of *S. cerevisiae* CAT-1 and FT858 (Basso et al. 2011; Della-Bianca et al. 2013; Borges et al. 2015).

Yeasts exhibit different behaviors depending on the bioprocess to which they are subjected. Cell growth and fermentation are related not only to the presence of the sugar, but also to the availability of nutrients in the medium (Santos et al. 2018).

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[1] Artigo publicado. **3 Biotech**, 2020 (https://doi.org/10.1007/s13205-020-02335-w). Seguindo as normas disponíveis em: https://www.springer.com/journal/13205/submission-guidelines (Apêndice 4).

Through the monitoring of microorganisms during the industrial process, it is possible to select wild strains capable of surviving and dominating fermentation. These strains can present good fermentation characteristics, such as high ethanol productivity, low glycerol and organic acids formation, low foaming exigence, high viability, among others (Basso et al., 2011; Della-Bianca et al. 2013). Currently, only three *S. cerevisiae* strains selected from industrial processes are responsible for 70% of all ethanol produced in Brazil: PE-2, CAT-1 and FT858 (Borges et al. 2015).

Although the FT858 strain, as well as CAT-1, were isolated from an industrial environment, there are few published studies on its fermentative efficiency. Santos et al. (2018) evaluated the strain in different cultivation conditions and found that it has high fermentative capacity. In another study, the efficiency of the FT858 strain was compared with two other industrial strains, with this strain presenting higher ethanol production in short fermentation periods and higher sugar consumption (Morales et al. 2014).

Invertases have high biotechnological potential because they can act as hydrolyzing agents in different processes. Invertase is widely used in food and chemical industries, mainly as an additive for the production of inverted sugar. In addition, it is also important in the production of fuel ethanol, lactic acid and glycerol, medicines, paper, and as enzymatic electrodes for the detection of sucrose (Barbosa et al. 2018).

The production of invertases through yeasts is strongly influenced by the sugars in the medium. However, the nature and concentration of the carbon source are not the only important variables to be controlled in the process. To obtain the highest possible yield, conditions must be optimized in terms of nitrogen sources, pH, temperature, size and age of the inoculum (Qureshi et al. 2017; Barbosa et al. 2018).

The same yeast can present different behaviors depending on the type and variety of substrate used during a process (Batistote et al. 2010; Santos et al. 2013; Fonseca et al. 2013; Nascimento and Fonseca 2019). In addition, the transcriptional profile of the yeast *S. cerevisiae* is strongly affected by glucose, where the expression levels of about 40% of the genes are regulated, up or down, when this sugar becomes available to cells growing in non-fermentable carbon sources (Zaman et al. 2008; Gancedo et al. 2015).

Thus, the aim of this work was to evaluate the biotechnological potential of *S. cerevisiae* FT858 through kinetic growth parameters, and the influence of the concentration of the substrate on the synthesis of the invertase enzyme.

## 3.2 Material and methods

### 3.2.1 Microorganisms and maintenance

The yeasts *Sacharomyces cerevisiae* FT858 and CAT-1 were utilized in this work. The first strain was kindly provided Prof. Dra. Margareth Batistote from the State University of Mato Grosso do Sul (UEMS) while the second one by the São Fernando Sugar and Alcohol Distillery, Dourados, MS, Brazil. The yeasts were maintained on YPD agar (15 g $L^{-1}$ agar, yeast extract, 10 g $L^{-1}$, peptone, 20 g $L^{-1}$, glucose, 20 g $L^{-1}$) (Camargo et al. 2018).

### 3.2.2 Cultivation medium

The mineral medium contained per liter of distilled water: $(NH_4)_2SO_4$, 5.0 g; $KH_2PO_4$, 3.0 g; $MgSO_4.7H_2O$, 0.5 g; trace elements (EDTA, 15 mg; $ZnSO_4.7H_2O$, 4.5 mg; $MnCl_2.2H_2O$, 0.84 mg; $CoCl_2.6H_2O$, 0.3 mg; $CuSO_4.5H_2O$, 0.3 mg; $Na_2.MoO_4.2H_2O$, 0.4 mg; $CaCl_2.2H_2O$, 4.5 mg; $FeSO_4.7H_2O$, 3.0 mg; $H_3BO_3$, 1.0 mg; KI, 0.1 mg). The pH was adjusted to 6.0 with KOH before autoclaving (121°C, 20 min). Then 1 mL of sterile-filtered vitamin solution prepared in demineralized water was added, to a final concentration per liter of D-biotin, 0.05 mg; calcium pantothenate, 1.0 mg; nicotinic acid, 1.0 mg; myo-inositol, 25 mg; thiamine HCl, 1.0 mg; pyridoxin HCl, 1.0 mg; and para-aminobenzoic acid, 0.20 mg (Verduyn et al. 1992). The sole carbon sources used were glucose, fructose, galactose, and sucrose. They were sterilized separately and added aseptically to the medium to a final concentration of 10 or 100 g $L^{-1}$ (Nascimento and Fonseca 2019).

### 3.2.3 Culture conditions for physiology studies

The inoculum was prepared by transferring a loopful of cells from a YPD plate to a 250 mL Erlenmeyer flask containing 125 mL of mineral medium added of each sole carbon source (glucose, fructose, galactose, or sucrose) at 10 g $L^{-1}$. After 12 h growth on an orbital shaker (200 rpm) at 30 or 37 °C, cultivations started by adding a certain volume of the pre-culture to 500 mL Erlenmeyer flasks containing 250 mL of mineral medium, so that the initial cell concentration in the flask was 0.1 optical density unit at 600 nm ($OD_{600nm}$) (Biospectro sp-220) (Silva et al. 2019). All cultivations were carried out aerobic in flasks stoppered with cotton, in triplicate.

### 3.2.4 Culture conditions for the evaluation of the invertase activity

The inoculum was prepared as described in the previous section, except in Erlenmeyer flasks (50 mL flasks with 25 mL of mineral medium) and the only carbon source (glucose, or sucrose) at 10 or 100 g $L^{-1}$. After 12 h growth on an orbital shaker (200 rpm) at 30 or 37 °C, cultivations started by adding a certain volume of the pre-culture to 125 mL Erlenmeyer flasks containing 50 mL of mineral medium, so that the initial cell concentration in the flask was 0.1 optical density unit at 600 nm ($OD_{600nm}$) (Biospectro sp-220). All cultivations were carried out in triplicate.

### 3.2.5 Sampling and sample preparation

Samples were taken every 30 min. (maximum of 4 mL per sample) and placed in an ice bath. Out of these, 2 mL were used for optical density measurement ($OD_{600nm}$) (Biospectro sp-220), followed by adequate dilutions (when necessary) and the remaining 2 mL were centrifuged (17,609 x *g*, 5 min., 5 °C). The supernatant was frozen at -80 °C for further determination of the concentration of sugars and extracellular metabolites. The sedimented fraction was utilized to determine the biomass concentration (Fonseca et al. 2013).

### 3.2.6 Biomass concentration and pH

The biomass pellet obtained after sample centrifugation was dried in an oven (105 °C) until constant weight. The dried cell mass (g $L^{-1}$) was obtained by the quotient of the difference of weighing by the volume of centrifuged medium. Biomass concentration (X) was also indirectly determined via OD measurements performed with a spectrophotometer (Biospectro sp-220) at 600 nm. For this purpose, the measured absorbance values were converted into mass values using a linear relationship (OD units per gram dry cell mass) determined for each experiment. The pH was obtained by potentiometric measurements (Hanna) (Fonseca and Nascimento 2019; Silva et al. 2019).

### 3.2.7 Sugars and extracellular metabolites

Glucose, fructose, galactose, glycerol, ethanol, and organic acids were separated by using an UPLC Agilent 1290 with a Rezex ROA - Organic Acid $H^+$ ion-exclusion column (8%) (Phenomenex). The column was eluted at 55 °C using water acidified with trifluoroacetic acid (TFA) at 0.005 M as mobile phase, at a flow rate of 0.6 mL $min^{-1}$.

The volume injected was 20 μL, and the run was performed isocratically. These compounds were detected by a UV-absorbance detector at 254 nm connected in series with an Agilent 1260 Differential Refractometer (RID) coupled to a data acquisition module (Nascimento and Fonseca 2019). Sucrose was measured by the same procedure described previously, with changes in temperature and flow (25 °C; 0.3 mL $min^{-1}$) (Barbosa et al. 2018).

### 3.2.8 Kinetic parameters

The kinetic parameters were determined as described elsewhere (Fonseca et al. 2013; Nascimento and Fonseca 2019; Silva et al. 2019). The exponential growth phase (EGP) was identified as the linear region on an ln (X) vs. time plot for batch cultivation data. The maximum specific growth rate ($\mu_{max}$) was determined as the slope of this linear region and the doubling time (DT) by the ln (2) quotient by $\mu_{max}$. The biomass yield on substrate ($Y_{X/S}$) was determined as the slope of the line on an X vs. S plot, exclusively including points belonging to the EGP. The specific rate of substrate consumption ($r_S$) was calculated by the quotient of $\mu_{max}$ by $Y_{X/S}$. The maximum biomass concentration ($X_{max}$) was indicated by the maximum dried cell mass concentration or $OD_{600\ nm}$ observed in each experiment. The product (ethanol, glycerol or acetic acid) yield on substrate ($Y_{Eth/S}$; $Y_{Gly/S}$; $Y_{Ace/S}$) was determined as the slope of the line on a P vs. S plot. The maximum cell ($P_{Cel}$) and ethanol ($P_{Eth}$) productivities were obtained according to Eqs. 3.1 and 3.2, respectively:

$$P_{Cel} = \frac{(X_f - X_0)}{(t - t_0)} \tag{3.1}$$

$$P_{Eth} = \frac{(P_f - P_0)}{(t - t_0)} \tag{3.2}$$

where $X_f$ is the final biomass concentration (g $L^{-1}$) and $X_0$ is the initial biomass concentration (g $L^{-1}$), while $P_f$ is the final ethanol concentration (g $L^{-1}$) and $X_0$ is the initial ethanol concentration (g $L^{-1}$).

### 3.2.9 Enzyme extracts

Every 2 h, an Erlenmeyer flask was removed from the shaker for sampling and 20 mL of the culture medium was centrifuged at 1,500 × *g* for 5 min. The cell mass was resuspended in 5 mL of acetate buffer and centrifuged again to eliminate impurities. The procedure was repeated and after the last centrifugation, the cell mass was

resuspended with 10 mL of buffer. The extracts were used for the invertase determination assays (Barbosa et al. 2018).

### 3.2.10 Invertase activity

The reaction mixture was composed of 0.9 mL of 0.1 M sodium acetate buffer with pH 5.0, containing 10 g $L^{-1}$ sucrose, and 0.1 mL of enzymatic solution. The mixture was incubated for 10 min at 50 °C. The enzymatic reaction was intermitted by adding 1 mL of DNS (3,5-dinitrosalicylic acid) and 8 mL of distilled water. The product (reducing sugar) was quantified by spectrophotometry (540 ηm), using the DNS method (Miller 1959). An invertase unit was defined as the amount of enzyme required to release 1 μmol of product (glucose or fructose) per minute of reaction.

### 3.2.11 Statistical analysis

The Microsoft Excel 2010 program was utilized to calculate the analysis of variance (ANOVA). The Tukey test was used to determine differences between the variables temperature and substrate, in the 95% confidence interval.

## 3.3 Results and discussion

### 3.3.1 Physiological evaluation of *S. cerevisiae* FT858 on different substrates and temperatures

The kinetic parameters related to cell growth during cultivations with *S. cerevisiae* FT858 are shown in Table 3.1. The experiments were carried out on glucose, fructose, sucrose, and galactose, as the only carbon source (10 g $L^{-1}$) at 30 and 37 °C. Fig. S1 shows the growth kinetics, metabolite formation and sugar consumption of these cultivations.

Comparing the maximum specific growth rates ($\mu_{max}$), it is possible to observe that these values were not influenced by the increase in temperature in substrates glucose, fructose and sucrose. Moreover, the difference was not significant ($p>0.05$) between cultivations with glucose or fructose. The highest $\mu_{max}$ was obtained with sucrose at 37 °C (0.46 ± 0.017 $h^{-1}$) and the lowest with galactose at 30°C (0.28 ± 0.002 $h^{-1}$) (Table 3.1). Other studies carried out with industrial yeast strains revealed an increase in growth rates for cultivations at 37 °C (Della-Bianca and Gombert 2013; Nascimento and Fonseca 2019), which was not observed here with *S. cerevisiae* FT858.

*S. cerevisiae* FT858 presented the lowest affinity for the substrate galactose since they had the lowest $\mu_{max}$ values at 30 and 37 °C (0.28 ± 0.002 $h^{-1}$; 0.31 ± 0.001 $h^{-1}$, respectively) (Table 3.1). Close related values were reported for *S. cerevisiae* CAT-1 under the same culture conditions (0.28 ± 0.001 $h^{-1}$; 0.35 ± 0.000 $h^{-1}$) (Nascimento and Fonseca 2019). It occurs because the galactose catabolism by the cell involve more metabolic reactions in relation to the other studied substrates. Other factors, such as the transport system and accumulation of intermediates in the metabolic pathway, are responsible for slowing cell growth (Ostergaard et al. 2000; Ideker et al. 2001; Bro et al. 2005; Fonseca et al. 2013; Nascimento and Fonseca 2019).

Regarding the substrate consumption rate ($r_S$), the influence of the temperature increase is perceived in the cultures with glucose, fructose and galactose, where at higher temperatures higher $r_S$ were obtained. The sucrose substrate did not show any significant difference ($p>0.05$) (3.13 and 3.49 $h^{-1}$) (Table 3.1). The behavior of this parameter is related not only to temperature, but also to the substrate transport system used by yeast.

The transport of common monosaccharides (glucose or fructose) in *S. cerevisiae* is carried out by the process of facilitated diffusion (Bisson et al. 2016). The increase in temperature promotes the acceleration of metabolism and the uptake of available substrates, and as a result, the increase in $r_S$. In Fig. S1, it is observed that the cultures carried out at 37 °C reached the stationary phase in less time. It was more evident in the cultivations with glucose and fructose.

Since the galactose catabolism pathway is more complex than the other evaluated substrates, as much as the temperature promotes the acceleration of the yeast metabolism, it becomes limited by the increase in the concentration of metabolic intermediates that inhibit the flow through the Leloir pathway (Hong et al. 2011).

Similar behavior also occurs in the cultivations with sucrose. Since the predominant mechanism of sucrose consumption in *S. cerevisiae* occurs after the hydrolysis of sucrose by extracellular invertase, which produces glucose and fructose that enter the cell through facilitated diffusion transporters (Basso et al. 2011). Thus, the consumption of this substrate is also limited by the catabolic repression in the expression of invertase, generated by the concentration of glucose released in the culture medium. The dynamics of this process regulates the consumption of sucrose by yeast throughout the cultivation, as can be seen in Fig. 3.1 (Alipourfard et al. 2019; Nascimento and Fonseca 2019).

The products formed with the most significant concentrations during the cultivations were ethanol, glycerol, and acetate (Fig. S1; Table 3.1). The product yield on substrate ($Y_{P/S}$) were calculated for ethanol ($Y_{ETH/S}$), glycerol ($Y_{GLY/S}$) and acetate ($Y_{ACE/S}$) (Table 3.1).

With the increase in temperature, only the cultivations with glucose and fructose showed a significant difference ($p>0.05$) for the $Y_{GLY/S}$ values. For all evaluated substrates, *S. cerevisiae* FT858 showed lower $Y_{GLY/S}$ values when compared to those obtained with the *S. cerevisiae* CAT-1 (Nascimento and Fonseca 2019).

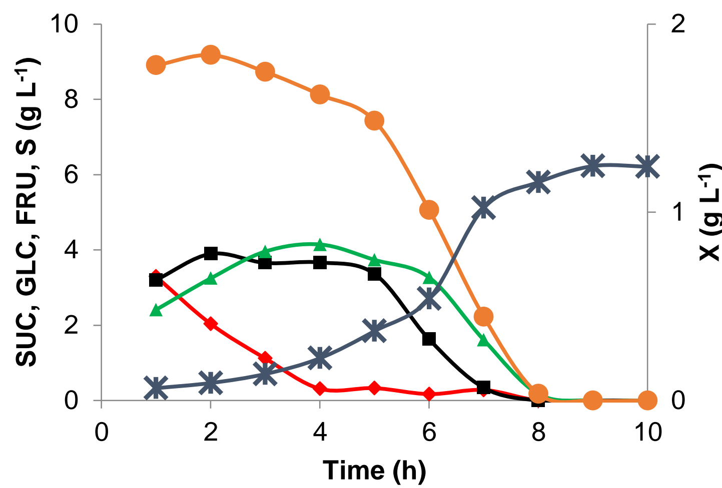


**Figure 3.1.** Kinetics of substrate consumption and cell growth during cultivation of *S. cerevisiae* FT858 with sucrose 10 g $L^{-1}$ at 30 °C. (⨯) X (biomass, g $L^{-1}$); (●) S (total substrate, g $L^{-1}$); (♦) SUC (sucrose, g $L^{-1}$); (▲) FRU (fructose, g $L^{-1}$); (■) GLC (glucose, g $L^{-1}$).

*S. cerevisiae* CAT-1 and other yeast strains, have the characteristic of producing less glycerol because of its great resistance at some adverse conditions found in the process (Basso et al. 2008). A major concern during industrial fermentation processes is that the cells convert the sugar consumed into the production of glycerol, which is related to the stress caused to the cells during fermentation (Basso et al. 2011; Borges et al. 2015). In this context, *S. cerevisiae* FT858 is more efficient than *S. cerevisiae* CAT-1 because it presents lower $Y_{GLY/S}$ values.

**Table 3.1** Kinetic parameters of cultures performed with *S. cerevisiae* FT858 under different culture conditions.

| $S_o$ (10 g $L^{-1}$) | T (°C) | $\mu_{max}$ ($h^{-1}$) | $X_{max}$ (g $L^{-1}$) | DT (h) | $r_S$ ($h^{-1}$) | $Y_{X/S}$ ($g_{MCS}$ $g_S^{-1}$) | $Y_{ETH/S}$ ($g_{ETH}$ $g_S^{-1}$) | $Y_{GLY/S}$ ($g_{GLY}$ $g_S^{-1}$) | $Y_{ACE/S}$ ($g_{ACE}$ $g_S^{-1}$) | $P_{CEL}$ ($g_{DCM}$ $L^{-1}$ $h^{-1}$) | $P_{ETH}$ ($g_{ETH}$ $L^{-1}$ $h^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| GLC | 30 | $0.40\pm0.001^{bcA}$ | $1.70\pm0.017^{cA}$ | $1.75\pm0.006^{cdA}$ | $2.68\pm0.008^{bcdB}$ | $0.15\pm0.001^{cA}$ | $0.39\pm0.004^{cB}$ | $0.03\pm0.002^{bcB}$ | $0.01\pm0.005^{abA}$ | $0.14\pm0.001^{abA}$ | $0.40\pm0.001^{cdA}$ |
| | 37 | $0.39\pm0.016^{cA}$ | $1.13\pm0.000^{fB}$ | $1.78\pm0.074^{cA}$ | $3.84\pm0.193^{aA}$ | $0.10\pm0.002^{eB}$ | $0.46\pm0.013^{bA}$ | $0.07\pm0.003^{aA}$ | $0.00\pm0.000^{abA}$ | $0.12\pm0.000^{cB}$ | $0.43\pm0.011^{bcA}$ |
| FRU | 30 | $0.37\pm0.001^{cA}$ | $1.75\pm0.008^{cA}$ | $1.85\pm0.004^{cA}$ | $2.54\pm0.143^{cdB}$ | $0.15\pm0.008^{cA}$ | $0.39\pm0.004^{cA}$ | $0.04\pm0.001^{bB}$ | $0.01\pm0.001^{abA}$ | $0.13\pm0.001^{bA}$ | $0.35\pm0.009^{dA}$ |
| | 37 | $0.37\pm0.012^{cA}$ | $1.34\pm0.009^{dB}$ | $1.86\pm0.058^{cA}$ | $3.96\pm0.023^{aA}$ | $0.09\pm0.002^{eB}$ | $0.39\pm0.007^{cA}$ | $0.07\pm0.004^{aA}$ | $0.00\pm0.000^{abA}$ | $0.13\pm0.006^{bA}$ | $0.47\pm0.040^{abA}$ |
| SUC | 30 | $0.43\pm0.001^{abA}$ | $1.71\pm0.013^{cA}$ | $1.62\pm0.003^{deA}$ | $3.13\pm0.066^{abcA}$ | $0.14\pm0.003^{cA}$ | $0.50\pm0.002^{abA}$ | $0.03\pm0.004^{bcdA}$ | $0.01\pm0.000^{bA}$ | $0.14\pm0.001^{aA}$ | $0.45\pm0.008^{bcB}$ |
| | 37 | $0.46\pm0.017^{aA}$ | $1.22\pm0.030^{eB}$ | $1.50\pm0.054^{eA}$ | $3.49\pm0.574^{abA}$ | $0.12\pm0.004^{dB}$ | $0.52\pm0.017^{aA}$ | $0.05\pm0.011^{bA}$ | $0.00\pm0.001^{bA}$ | $0.13\pm0.003^{bcB}$ | $0.53\pm0.020^{aA}$ |
| GAL | 30 | $0.28\pm0.002^{dB}$ | $1.94\pm0.027^{aA}$ | $2.46\pm0.017^{aA}$ | $1.43\pm0.016^{eB}$ | $0.20\pm0.004^{aA}$ | $0.36\pm0.002^{cA}$ | $0.02\pm0.001^{dA}$ | $0.01\pm0.001^{aA}$ | $0.13\pm0.001^{bcA}$ | $0.26\pm0.002^{eB}$ |
| | 37 | $0.31\pm0.001^{dA}$ | $1.86\pm0.002^{bA}$ | $2.21\pm0.007^{bB}$ | $1.87\pm0.023^{deA}$ | $0.17\pm0.003^{bB}$ | $0.39\pm0.032^{cA}$ | $0.02\pm0.001^{cdA}$ | $0.01\pm0.001^{abB}$ | $0.13\pm0.003^{bA}$ | $0.33\pm0.001^{dA}$ |

GLC glucose, FRU fructose, SUC sucrose, GAL galactose, S substrate, T temperature, $\mu_{max}$ maximum specific growth rate, $X_{max}$ maximum cell concentration, DT doubling time, $r_S$ specific rate of substrate consumption, $Y_{X/S}$ biomass yield on substrate, $Y_{ETH/S}$ ethanol yield on substrate, $Y_{GLY/S}$ glycerol yield on substrate, $Y_{ACE/S}$ acetate yield on substrate, $P_{CEL}$ maximum cell productivities, $P_{ETH}$ maximum ethanol productivities, DCM: dry cell mass. Equal small letters in the same column do not present significant difference ($p > 0.05$). Equal capital letters for the same substrate do not show significant difference ($p > 0.05$).

The highest $Y_{ETH/S}$ values were achieved with sucrose at both temperatures (average of 0.51 $g_{ETH}/g_{substrate}$) (Table 3.1). Only for the glucose substrate $Y_{ETH/S}$ showed a significant difference ($p<0.05$) between 30 and 37 °C. The substrates fructose and galactose, at both temperatures, and glucose at 30 °C, the difference was not significant ($p>0.05$) between them. The $Y_{ETH/S}$ obtained in this study for all tested substrates were higher than those reported in the literature for *S. cerevisiae* CAT-1 (Nascimento and Fonseca 2019). It is important to underline that a 10% increase in $Y_{ETH/S}$ was obtained when sucrose was used as substrate ($Y_{ETH/S}$ = 0.53 $g_{ETH}$ $g_{substrate}^{-1}$ for *S. cerevisiae* FT858 against $Y_{ETH/S}$ = 0.44 $g_{ETH}$ $g_{substrate}^{-1}$ reported for *S. cerevisiae* CAT-1) at 37 °C. The ethanol productivity ($P_{ETH}$) was higher for the *S. cerevisiae* FT858 cultivations with fructose and sucrose at 37 °C (0.47 ± 0.040 and 0.53 ± 0.020 $g_{ETH}$ $L^{-1}$ $h^{-1}$, respectively).

### 3.3.2 Influence of the substrate concentration on the invertase production by *S. cerevisiae* FT858 and CAT-1

For a better evaluation of the biotechnological potential of *S. cerevisiae* FT858, the activity of the invertase enzyme produced in cultures with glucose or sucrose as the only carbon source, at low (10 g $L^{-1}$) and high (100 g $L^{-1}$) concentrations was investigated. Results obtained with *S. cerevisiae* CAT-1 were utilized as comparative parameter.

Growth kinetics, metabolite formation and substrate consumption were obtained for *S. cerevisiae* FT858 and CAT-1 during cultivations with 100 g $L^{-1}$ carbon source (Fig. S2). The kinetic growth parameters of these cultivations were compared with those obtained here with 10 g $L^{-1}$ carbon source for *S. cerevisiae* FT858 and the data reported in the literature (Nascimento and Fonseca 2019) for *S. cerevisiae* CAT-1 at the same carbon source concentration (Table 3.2).

Cultivations were carried out up to 24 h. As only the concentration of the carbon source was changed, while the concentrations of the other relevant components of the mineral medium (*e.g.* nitrogen source) were maintained the same, the conditions were not sufficient to sustain the cell growth until the total consumption of the carbon source at the high concentration (Table 3.2; Fig. S2). Therefore, for these experiments, only the growth parameters were calculated.

As *S. cerevisiae* FT858 is a yeast strain isolated from an ethanol industrial plant, its fermentative capacity is strongly related to the enzymatic activity of invertase. Since

the cleavage of sucrose into glucose and fructose is the fundamental step in sucrose metabolism (Basso et al., 2008; Marques et al., 2016).

Table 3.2 shows that the strains showed very different behaviors in relation to the production of biomass in cultivations with 100 g $L^{-1}$ substrate. With the glucose substrate, *S. cerevisiae* CAT-1 presented a lower $X_{max}$ (2.37 g $L^{-1}$) and a higher $\mu_{max}$ (0.41 $h^{-1}$) compared with *S. cerevisiae* FT858 ($X_{max}$ = 2.88 g $L^{-1}$; $\mu_{max}$ = 0.35 $h^{-1}$). In sucrose, the opposite was observed in relation to $X_{max}$. For *S. cerevisiae* CAT-1 it reached 2.85 g $L^{-1}$, which was superior to the 2.29 g $L^{-1}$ found for *S. cerevisiae* FT858. The $\mu_{max}$ were very close (0.38 $h^{-1}$ for *S. cerevisiae* CAT-1 and 0.37 $h^{-1}$ for *S. cerevisiae* FT858) (Table 3.2).

Production of invertase by *S. cerevisiae* FT858 and CAT-1 was evaluated from glucose and sucrose in order to investigate the effect of the concentration of these substrates on the production of this enzyme (Fig. 3.2).

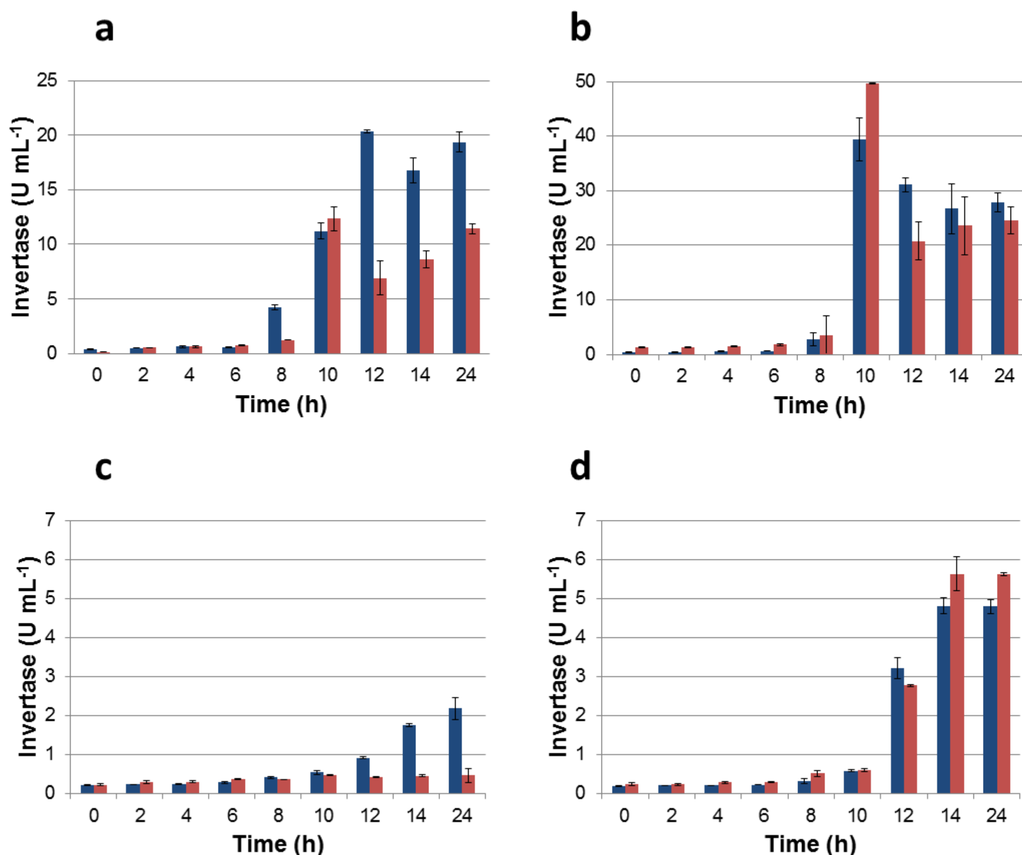


**Figure 3.2.** Invertase production by industrial yeasts *S. cerevisiae* CAT-1 and FT858 during cultures with 10 g $L^{-1}$ and 100 g $L^{-1}$ substrate (**a** glucose 10 g $L^{-1}$, **b** sucrose 10 g $L^{-1}$, **c** glucose 100 g $L^{-1}$, **d** sucrose 100 g $L^{-1}$). Blue bars correspond to *S. cerevisiae* CAT-1 and red bars correspond to *S. cerevisiae* FT858.

**Table 3.2** Comparison of kinetic growth parameters of cultivations with *S. cerevisiae* FT858 and CAT-1 in high and low substrate concentration.

| $S_o$ | Strain | [ ] ($g\ L^{-1}$) | $\mu_{max}$ ($h^{-1}$) | $X_{max}$ ($g\ L^{-1}$) | DT (h) | $P_{CEL}$ ($g_{DCM}\ L^{-1}\ h^{-1}$) | $S_{final}$ residual ($g\ L^{-1}$) | Reference |
|---|---|---|---|---|---|---|---|---|
| GLC | FT858 | 10 | $0.40{\pm}0.001^{c}$ | $1.70{\pm}0.017^{d}$ | $1.75{\pm}0.006^{c}$ | $0.14{\pm}0.001^{b}$ | $0^{c}$ | This work |
| | | 100 | $0.35{\pm}0.002^{e}$ | $2.88{\pm}0.028^{a}$ | $1.95{\pm}0.009^{a}$ | $0.12{\pm}0.001^{c}$ | $39.8{\pm}2.522^{b}$ | This work |
| | CAT-1 | 10 | $0.44{\pm}0.007^{ab}$ | $2.00{\pm}0.012^{e}$ | $1.58{\pm}0.025^{de}$ | $0.08{\pm}0.000^{a}$ | $0^{c}$ | Nascimento and Fonseca 2019 |
| | | 100 | $0.41{\pm}0.001^{c}$ | $2.37{\pm}0.032^{b}$ | $1.71{\pm}0.004^{c}$ | $0.10{\pm}0.001^{d}$ | $50.5{\pm}1.784^{a}$ | This work |
| SUC | FT858 | 10 | $0.43{\pm}0.001^{b}$ | $1.71{\pm}0.013^{d}$ | $1.62{\pm}0.003^{d}$ | $0.14{\pm}0.001^{ab}$ | $0^{c}$ | This work |
| | | 100 | $0.37{\pm}0.000^{de}$ | $2.29{\pm}0.012^{c}$ | $1.87{\pm}0.001^{b}$ | $0.09{\pm}0.001^{d}$ | $39.3{\pm}1.412^{b}$ | This work |
| | CAT-1 | 10 | $0.42{\pm}0.014^{a}$ | $2.00{\pm}0.030^{d}$ | $1.64{\pm}0.055^{e}$ | $0.08{\pm}0.001^{b}$ | $0^{c}$ | Nascimento and Fonseca 2019 |
| | | 100 | $0.38{\pm}0.010^{d}$ | $2.85{\pm}0.007^{a}$ | $1.86{\pm}0.052^{b}$ | $0.12{\pm}0.000^{c}$ | $54.5{\pm}3.634^{a}$ | This work |

GLC glucose, SUC sucrose, S substrate, [ ] substrate concentration, $\mu_{max}$ maximum specific growth rate, $X_{max}$ maximum cell concentration, DT doubling time, $P_{CEL}$ maximum cell productivities, DCM: dry cell mass, equal letters in the same column there is no significant difference ($p > 0.05$).

It was observed that, at the concentration of 10 g $L^{-1}$ (Fig. 3.2a,b), the yeasts only presented considerable enzymatic activity after 8 h of cultivation. For glucose, the greatest activity was observed with 12 h for CAT-1 (20.4 ± 0.15 U $mL^{-1}$) and with 10 h for *S. cerevisiae* FT858 (12.3 ± 1.06 U $mL^{-1}$). For sucrose, the greatest activities were observed in 10 h of culture for both strains (*S. cerevisiae* CAT-1 = 39.4 ± 3.93 U $mL^{-1}$; *S. cerevisiae* FT858 = 49.6 ± 0.11 U $mL^{-1}$). From this time on, a considerable reduction in activity was observed, but maintained stable between 20 and 30 U $mL^{-1}$ up to 24 h of cultivation.

In the cultures with 100 g $L^{-1}$ of the carbon sources (Fig. 3.2c,d), the two strains showed a very low invertase activity. There was no invertase activity for *S. cerevisiae* FT858 in glucose. On the other hand, *S. cerevisiae* CAT-1 presented little activity after 12 h of cultivation. In sucrose there was a greater production of invertase. However, still at very low levels. *S. cerevisiae* FT858 showed higher activity compared to *S. cerevisiae* CAT-1 (5.62 ± 0.03 and 4.81 ± 0.20 U $mL^{-1}$, respectively). In a previous study with *S. cerevisiae* FT858, substrate concentration affected the fermentative behavior of the yeast, leading to a greater loss of viability and ethanol concentration (Santos et al. 2018).

The invertase activity was measured in whole cells. Therefore, only periplasmic and regulated invertases were considered, where the *SUC2* gene is glucose repressed at a concentration equal or superior to 20 g $L^{-1}$ and induced at a concentration below to 2 g $L^{-1}$ (Gancedo et al. 2015). Thus, in both substrates at 10 g $L^{-1}$, the enzymatic activity was more evident when the cultivations tend to reach the stationary phase, *i.e.*, when the carbon source is practically exhausted from the medium. For the *S. cerevisiae* FT858 cultivations started with 10 g $L^{-1}$ glucose, at 8 h of growth, the concentration of glucose was 4 g $L^{-1}$ while at 10 h it dropped to approximately 0.1 g $L^{-1}$. This indicates that only after the decrease in glucose concentration the expression of the *SUC2* gene is induced.

The gene repression generated by the glucose concentration was also observed in cultures with 10 g $L^{-1}$ sucrose. It occurred because the levels of glucose / fructose in the medium increased over time, regulating the production of invertase by the yeast. At 6 h of cultivation with *S. cerevisiae* FT858, the glucose concentration was 2.6 g $L^{-1}$, while at 8 h it was 0.4 g $L^{-1}$. At 10 h, time that the highest Invertase activity was observed, all sugars had already been consumed.

It has been reported that in *S. cerevisiae*, when glucose is not abundant or even absent in the medium, *SUC2* expression occurs at a baseline level. In an environment

rich in sucrose, the basal level of the invertase generates a glucose / fructose pool around the cells, causing the maximum expression of *SUC2*. In addition to this sugar pool, changes in concentration throughout the cultivation were also related to gene expression. When the glucose / fructose concentration accumulates above a limit, *SUC2* is repressed, leading to the consumption of the hexoses already available. This invertase expression dynamics, through the glucose balance, is what optimizes the sucrose consumption by the yeast (Marques et al. 2016).

When considering acceptable enzyme activity values above 1 U $mL^{-1}$, in the cultivations with the carbon source at 100 g $L^{-1}$ (Fig. 3.2c,d) there was a subtle invertase activity for *S. cerevisiae* CAT-1 in glucose, and for both strains in sucrose. The hydrolytic activity of invertases occurs predominantly in sucrose concentrations of 50 g $L^{-1}$ or less (Barbosa et al. 2018). Moreover, for both strains, the invertase activity became more apparent after 12 h, when the cultures approached the concentration of 50 g $L^{-1}$. Due to the osmotic stress, to which the yeasts were exposed, there was a great repression of invertase expression in these cultivations due to the increase in the concentrations of glucose / fructose in the medium.

There are indications that the invertase produced by *S. cerevisiae* CAT-1 is of a constitutive nature. It is suggested because the invertase activity was still evident in an extremely rich medium, with glucose as the sole carbon source, which was not observed for *S. cerevisiae* FT858.

Another aspect observed was the different dynamic behavior of the sucrose catalysis. In the cultivation times that showed higher invertase activity (12, 14 and 24 h), the glucose concentration for *S. cerevisiae* FT858 remained regulated and close to 15 g $L^{-1}$. For *S. cerevisiae* CAT-1, this concentration fluctuated considerably, reaching values very close to 20 g $L^{-1}$, which would suppress the expression of *SUC2* (Gancedo et al. 2015). After 14 h of cultivation *S. cerevisiae* FT858 increased invertase activity in relation to *S. cerevisiae* CAT-1.

There are several signaling pathways involved in the response to nutrients in *Saccharomyces* spp. The Rgt1 network also participates in controlling the expression of hexose transporter genes according to the level of glucose availability, so that cells express only the transporters with the greatest affinity for available glucose (Zaman et al. 2008). Gancedo et al. (2015), in their study of *SUC2* expression in *S. cerevisiae* observed that both Rgt1 and Mth1 play an important role in blocking invertase

transcription in the absence of glucose, suggesting that the main mechanism is the binding of the Rgt1 complex -Mth1 into an Rgt1 binding site on the *SUC2* promoter.

### 3.4 Conclusion

It was concluded that the culture conditions directly interfered in the yeast physiology. *S. cerevisiae* FT858 has an outstanding biotechnological potential, since the kinetic growth parameters observed were very close to those obtained with *S. cerevisiae* CAT-1 under similar cultivation conditions. It underlines the low glycerol yield on substrate ($Y_{GLY/S}$) (even at 37 °C) and a 10% increase in the yield of sucrose into to ethanol obtained with *S. cerevisiae* FT858 when compared to *S. cerevisiae* CAT-1 at 37 °C. The concentration of the substrates directly interfered in the production of invertase for both *S. cerevisiae* FT858 and CAT-1. The enzymatic expression underwent strong regulation through the concentration of glucose in the culture medium. *S. cerevisiae* CAT-1 presented constitutive behavior for the invertase enzyme.

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# CAPÍTULO 4

## 4 AVALIAÇÃO FISIOLÓGICA DE 14 LINHAGENS DE LEVEDURAS INDUSTRIAIS ATRAVÉS DE PARÂMETROS CINÉTICOS

**Resumo**

Leveduras sofrem mutações evolutivas que tornam linhagens de uma mesma espécie mais eficientes para um determinado processo do que outro. Desta forma, leveduras personalizadas já estão surgindo no mercado da indústria de etanol combustível, visando à melhoria do desempenho na fermentação. Parâmetros cinéticos são uma importante ferramenta para a avaliação da fisiologia e comparação de microrganismos. Neste estudo foram avaliadas 14 linhagens de leveduras industriais, sendo 5 pertencentes ao gênero *Kluyveromyces* (*K. lactis* CBS 2359, *K. marxianus* ATCC 26548, *K. marxianus* CBS 6556, *K. marxianus* PYCC 3886 e *K. marxianus* NRRLy 2415) e 9 ao gênero *Saccharomyces* (*S. kluyveri* FM 479, *S. kluyveri* Y 708, *S. cerevisiae* ATCC 32167, *S. cerevisiae* BG-1, *S. cerevisiae* CAT-1, *S. cerevisiae* PG-2, *S. cerevisiae* SA-1, *S. cerevisiae* UR-1 e *S. cerevisiae* PK 113-7D). Potenciais aplicações biotecnológicas foram destacadas considerando-se o maior potencial respiratório das leveduras do gênero *Kluyveromyces* e a maior capacidade fermentativa das leveduras do gênero *Saccharomyces*.

### 4.1 Introdução

Com o passar dos anos, algumas linhagens de leveduras foram isoladas de plantas industriais, por apresentarem melhores resultados em seus processos, uma vez que evoluíram para resistir às condições de estresse oferecidas (2, 5, 4). Tornaram-se então amplamente utilizadas dentro dos respectivos processos em que se destacaram e, apesar disto, pouco são os estudos comparativos de dados fisiológicos destas linhagens industriais (15, 14, 7).

A composição de uma levedura pode sofrer variações de acordo com o modo em que é cultivada. Além da condição de aerobiose ou anaerobiose, outros fatores também são responsáveis por isto, como o excesso ou déficit de carbono ou de qualquer um de seus outros nutrientes vitais (nitrogênio, fósforo, potássio, etc.), mudanças na temperatura, pH, entre outros. A célula responde ao ambiente controlando sua expressão

genética e a atividade enzimática dentro dela (17). Partindo deste princípio, os microrganismos são capazes de sofrer mutações evolutivas induzidas por condições adversas do meio em que vivem, buscando garantir a sua sobrevivência (4, 23, 15).

Como exemplo sobre a variação fisiológica dentro de um grupo de leveduras de uma mesma espécie temos o estudo realizado por Lane *et al*. (12), onde 13 linhagens da espécie *Kluyveromyces marxianus* foram submetidas a iguais condições de cultivo (aerobiose e tipo de substrato) e situações de estresse (termotolerância e halotolerância), e como resultado, as linhagens apresentaram respostas fisiológicas diferentes entre si. Confirmando que as mutações evolutivas tornam linhagens de uma mesma espécie mais eficientes para um determinado processo do que outro.

De acordo com Walker e Basso (23), assim como na indústria do vinho, leveduras personalizadas já estão surgindo no mercado da indústria de etanol combustível, visando à melhoria do desempenho na fermentação. Desta forma, são oferecidas a indústria linhagens de leveduras mais adaptadas às condições e particularidades de cada planta industrial, aumentando sua produtividade. Esta personalização é baseada na compreensão da fisiologia da levedura, e pode envolver engenharia genética para gerar linhagens com maior tolerância ao estresse e/ou melhoria da produtividade industrial.

Neste contexto, o estudo fisiológico de linhagens industriais é de fundamental importância, pois esse tipo de informação é fundamental para preparar futuras tentativas de melhoramento genético, e consequentemente, melhoria do processo industrial aos quais estão envolvidas (5). Além de possibilitar indicativos de novas aplicações biotecnológicas para o microrganismo em questão. Assim, o objetivo deste trabalho foi comparar 14 linhagens de leveduras industriais em uma mesma condição de cultivo através de parâmetros cinéticos e produção de metabólitos.

## 4.2 Material e métodos

### 4.2.1 Microrganismos e manutenção

Para este estudo foram utilizadas 14 linhagens de leveduras industriais, sendo 5 pertencentes ao gênero *Kluyveromyces* (*K. lactis* CBS 2359, *K. marxianus* ATCC 26548, *K. marxianus* CBS 6556, *K. marxianus* PYCC 3886 e *K. marxianus* NRRLy 2415) e 9 ao gênero *Saccharomyces* (*S. kluyveri* FM 479, *S. kluyveri* Y 708, *S. cerevisiae* ATCC 32167, *S. cerevisiae* BG-1, *S. cerevisiae* CAT-1, *S. cerevisiae* PG-2, *S. cerevisiae* SA-1, *S. cerevisiae* UR-1 e *S. cerevisiae* PK 113-7D). As leveduras foram

mantidas em ágar YPD (ágar 15 g $L^{-1}$, extrato de levedura, 10 g $L^{-1}$; peptona, 20 g $L^{-1}$; glicose, 20 g $L^{-1}$) (3).

### 4.2.2 Meio de cultura

Para a realização dos cultivos foi utilizado o meio mineral, que continha por litro de água destilada: $(NH_4)_2SO_4$, 5,0 g; $KH_2PO_4$, 3,0 g; $MgSO_4.7H_2O$, 0,5 g; 1 mL de elementos-traço (preparada em água desmineralizada, contendo por litro: EDTA, 15 g; $ZnSO_4.7H_2O$, 4,5 g; $MnCl_2.2H_2O$, 1 g; $CoCl_2.6H_2O$, 0,3 g; $CuSO_4.5H_2O$, 0,3 g; $Na_2MoO_4.2H_2O$, 0,4 g; $CaCl_2.2H_2O$, 4,5 g; $FeSO_4.7H_2O$, 3,0 g; $H_3BO_3$, 1,0 g; KI, 0,1 g), com pH final de 6,0 e autoclavado (121°C, 15 min), posteriormente adicionou-se ao meio 1 mL de solução filtro-esterilizada de vitaminas (preparada em água desmineralizada, contendo por litro: D-biotina, 0,05 g; pantotenato de cálcio, 1,0 g; ácido nicotínico, 1,0 g; mio-inositol, 25 g; cloreto de tiamina, 1,0 g; piridoxina, 1,0 g; e ácido para-aminobenzóico, 0,20 g) (22). A fonte de carbono utilizada foi glicose na concentração de 10 g $L^{-1}$, autoclavada separadamente e adicionada assepticamente ao meio.

### 4.2.3 Condições de cultivo

Os cultivos foram realizados em frascos Erlenmeyer de 50 mL, contendo 5 mL de meio mineral. Para o pré-inoculo transferiu-se uma alçada da levedura contida na placa de Petri para um frasco tipo Erlenmeyer de 50 mL com 5 mL de meio mineral, acrescido da fonte de carbono, e assim levados a incubador rotativo orbital a 200 rpm, a 30°C durante 12 h. Após este período, fez-se o inóculo em um novo frasco. A este novo frasco foi adicionado, a fonte de carbono e uma alíquota do pré-inóculo de modo que a densidade óptica inicial ($OD_{600nm}$) fosse 0,1. Todos os ensaios foram realizados em duplicata (16).

### 4.2.4 Amostragens e preparo das amostras

As fases exponenciais de crescimento de cada linhagem foram identificadas previamente através do monitoramento da leitura da densidade óptica ($OD_{600nm}$). Os cultivos foram conduzidos até que cada linhagem atingisse a metade de sua fase exponencial, então foram interrompidos e os volumes dos frascos centrifugados (17.609 *g*, 5 min., 5°C). O sobrenadante foi congelado a -80°C para posterior determinação das

concentrações de açúcares e metabólitos extracelulares. A fração sedimentada foi utilizada para a determinação da concentração de biomassa (8).

### 4.2.5 Determinação da biomassa e pH

Para a análise da biomassa, o sedimento obtido após centrifugação da amostra foi seco em estufa (105°C) até peso constante. A massa celular seca ($g\ L^{-1}$) foi obtida pelo quociente entre a diferença de peso por volume de meio centrifugado. Biomassa (X) também foi determinada indiretamente através de medições de DO realizadas com um espectrofotômetro a 600 nm. Para este fim, os valores de absorbância medidos foram convertidos em valores de massa utilizando uma relação linear (unidades DO por grama de biomassa seca) determinada para cada experimento. O pH foi obtido através de aferições potenciométricas (8, 16).

### 4.2.6 Determinação da concentração de açúcares e metabólitos extracelulares

Glicose, etanol, glicerol e ácidos orgânicos foram determinados por UPLC Agilent 1290, equipado com coluna Rezex ROA – Organic Acid $H^+$ (8%) (Phenomenex). A corrida foi realizada de forma isocrática com fase móvel ácido trifluoroacético (TFA) a 0,005 M, a uma vazão de 0,6 mL $min^{-1}$, com temperatura de 55ºC e o volume injetado foi de 20 µL. Estes compostos foram detectados por um detector refratômetro diferencial Agilent 1260 (RID), acoplado a um módulo de aquisição de dados (16).

### 4.2.7 Determinação de parâmetros cinéticos

Os parâmetros cinéticos foram determinados conforme descrito na literatura (8, 16). A fase exponencial de crescimento (FEC) foi identificada como a regressão linear da plotagem do ln (DO) em função do tempo para os dados de cultivo. A velocidade específica de crescimento máxima ($\mu_{max}$) foi determinada como a inclinação desta reta e a concentração máxima de biomassa ($X_{max}$) foi obtida através de curva de calibração.

## 4.3 Resultados e discussão

Os cultivos com as 14 linhagens de leveduras quando atingiram a metade da fase exponencial de crescimento foram interrompidos para análise dos parâmetros e metabólitos formados, e estes estão dispostos na Tabela 4.1.

Com relação à velocidade de crescimento especifica ($\mu_{max}$) pode-se observar que a maior parte das linhagens apresentaram velocidades próxima entre si (variando entre 4 e 5 $h^{-1}$). Apenas duas linhagens apresentaram taxas mais baixas, *K. marxianus* PYCC 3886 (3,40 ± 0,015 $h^{-1}$) e *S. cerevisiae* PK 113-7D (3,71 ± 0,048 $h^{-1}$). Quanto ao consumo do substrato (residual de glicose/10) percebe-se também que as linhagens apresentaram praticamente o mesmo consumo, a maioria apresentando uma concentração residual de glicose próxima de 10 C-glicose/10 quando atingiram a metade da fase exponencial. A linhagem *K. marxianus* PYCC 3886, mostrou um maior consumo do substrato, onde a sua concentração residual de glicose foi de apenas 8,107±1,154 C-glicose/10.

*K. marxianus* NRRLy 2415 foi a maior produtora de biomassa, apresentando uma concentração de 23.17±0.000 C-X, enquanto a *S. cerevisiae* BG-1 foi a menor produtora (3.05±0.000 C-X). Das 7 linhagens pertencentes a espécie *S. cerevisiae*, 5 apresentaram concentração de biomassa próximas de 6.00 C-X. Já as linhagens da espécie *K. marxianus* apresentaram concentrações variadas entre si. Estudos de diferenças fisiológicas em linhagens de *K. marxianus*, também relatam que diferentes linhagens desta espécie apresentaram parâmetros diferentes mesmo quando submetidas a iguais condições de cultivo (12, 18).

**Tabela 4.1.** Parâmetros cinéticos e produção de metabólitos dos cultivos realizados com 14 linhagens de leveduras industriais.

| **Linhagem** | **pH** | **μ max** | **Residual Glc/10** | **Biomassa** | **Metabólitos** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | **C-Glicose** | **C-X** | **C-Etanol** | **C-Citrato** | **C-Piruvato** | **C-Succinato** | **C-Lactato** | **C-Fumarato** | **C-Acetato** | **C-Glicerol** |
| *S. kluyveri* FM 479 | 5,70 | 4,86±0,002 | 9,83±0,251 | 12,12±0,000 | 3,305±1,375 | - | 17,247±1,133 | - | 0,063±0,037 | 3,208±1,152 | 2,614±0,212 | 0,003±0,000 |
| *S. kluyveri* Y 708 | 5,75 | 5,44±0,005 | 9,97±0,538 | 16,40±0,000 | 4,301±1,395 | - | 12,282±1,292 | - | 0,070±0,025 | 3,506±0,901 | 4,093±0,231 | 0,004±0,001 |
| *K. lactis* CBS 2359 | 6,05 | 5,09±0,021 | 11,06±0,145 | 7,37±0,000 | 20,832±10,930 | - | 5,488±0,789 | - | - | 1,569±0,335 | 0,180±0,050 | 0,001±0,000 |
| *K. marxianus* ATCC 26548 | 5,70 | 5,66±0,003 | 9,01±0,720 | 13,37±0,000 | 1,564±0,323 | 0,142±0,142 | 8,418±2,408 | - | 0,148±0,073 | 22,039±6,508 | 2,623±0,953 | 0,002±0,001 |
| *K. marxianus* CBS 6556 | 5,95 | 5,39±0,036 | 11,41±0,710 | 6,69±0,000 | 1,534±0,253 | - | 3,263±0,957 | - | 0,053±0,018 | 7,025±0,961 | 0,373±0,187 | 0,000±0,000 |
| *K. marxianus* PYCC 3886 | 5,95 | 3,40±0,015 | 8,107±1,154 | 8,91±0,000 | 2,829±1,160 | 1,019±0,336 | 8,550±7,240 | - | 0,015±0,037 | 87,699±36,964 | - | 0,005±0,003 |
| *K. marxianus* NRRLy 2415 | 5,90 | 4,81±0,009 | 10,01±0,819 | 23,17±0,000 | 1,477±0,153 | - | 3,334±1,078 | 0,029±0,019 | - | 7,958±0,573 | 0,698±0,331 | 0,001±0,000 |
| *S. cerevisiae* ATCC 32167 | 6,00 | 4,61±0,049 | 9,31±0,726 | 5,78±0,000 | 6,450±1,140 | - | 9,002±1,714 | - | 0,113±0,059 | - | 0,472±0,109 | 0,005±0,001 |
| *S. cerevisiae* BG-1 | 6,05 | 4,97±0,010 | 10,30±0,422 | 3,05±0,000 | 47,514±1,115 | - | 5,327±1,007 | - | - | - | 0,288±0,065 | 0,003±0,000 |
| *S. cerevisiae* CAT-1 | 6,00 | 4,45±0,030 | 9,32±1,049 | 6,50±0,000 | 15,440±1,995 | - | 16,354±3,382 | - | 0,040±0,016 | - | 0,596±0,257 | 0,006±0,001 |
| *S. cerevisiae* PG-2 | 6,05 | 4,93±0,015 | 9,84±0,428 | 6,10±0,000 | 16,199±1,310 | - | 6,016±0,763 | - | - | - | 0,330±0,069 | 0,005±0,001 |
| *S. cerevisiae* SA-1 | 6,00 | 4,24±0,067 | 10,34±0,493 | 6,11±0,000 | 4,322±0,953 | - | 8,625±2,901 | - | 0,135±0,091 | - | 0,245±0,136 | 0,003±0,001 |
| *S. cerevisiae* UR-1 | 5,95 | 4,79±0,007 | 10,04±0,376 | 7,11±0,000 | 48,844±0,975 | - | 10,617±1,517 | - | 0,058±0,012 | - | 0,372±0,069 | 0,007±0,001 |
| *S. cerevisiae* PK 113-7D | 6,00 | 3,71±0,048 | 10,78±0,514 | 4,13±0,000 | 5,759±2,110 | - | 5,633±1,451 | - | 0,040±0,017 | - | 0,268±0,081 | 0,003±0,001 |

$\mu_{max}$: velocidade específica máxima de crescimento; C: carbono; Glc: glicose; X: biomassa

As maiores concentrações de biomassa foram obtidas pelas linhagens da espécie *K. marxianus* e *S. kluyveri.* A espécie *K. marxianus* já é muito conhecida por sua alta conversão de substrato à biomassa (11, 8, 10). Já a espécie *S. kluyveri,* apesar de pertencer ao gênero *Saccharomyces*, mostra comportamento diferente de linhagens de *S. cerevisiae*, pois apresenta altas taxas de conversão de substrato em célula e comportamento mais respiratório, enquanto *S. cerevisiae* apresenta comportamento respiro-fermentativo (9, 20) sob estas condições.

A levedura *S. cerevisiae*, em baixas concentrações de glicose no meio de cultivo, consome glicose através de respiração ou fermentação. A presença de oxigênio induz à cadeia respiratória e à produção de biomassa, mas simultaneamente diminui a cinética de fermentação de açúcar e produção de etanol (19). Este tipo de consumo foi observado com as linhagens de levedura *S. kluyveri* e *K. marxianus* aqui estudadas, onde apresentaram uma maior produção de biomassa em relação a produção de etanol.

Já as baixas concentrações de oxigênio, em *S. cerevisiae,* resultam na repressão catabólica das primeiras enzimas do Ciclo dos Ácidos Tricarboxílicos, causando o chamado "efeito Pasteur", resultando no direcionamento do metabolismo celular à produção de etanol. Durante o cultivo, com o consumo dos açúcares, uma grande quantidade de dióxido de carbono é produzida, criando uma condição semi anaeróbica, favorecendo a fermentação. Porém, mesmo na presença de oxigênio, se a concentração de açúcar for superior a um valor crítico, a respiração torna-se impossível e o microrganismo apenas metaboliza os açúcares pela via fermentativa (fenômeno conhecido como o “efeito Crabtree”) (19). Este tipo de comportamento mostrou-se evidente para a maioria das linhagens da espécie S. cerevisiae, onde apresentaram produção de etanol superior a formação de biomassa.

É importante ressaltar que as células de *S. cerevisiae,* mesmo que metabolizem os acucares via fermentativa, ainda necessitam de oxigênio, ao menos durante as primeiras horas após a inoculação, para reações que resultam na biossíntese de esteróis, ácidos graxos insaturados e fosfolipídios, necessários na formação das membranas celulares e continuação do crescimento (19).

Os metabólitos detectados nos cultivos foram etanol, citrato, piruvato, succinato, lactato, furamato, acetato e glicerol. Poucos cultivos produziram citrato e succinato, quando presentes apresentavam-se em baixas concentrações. Os demais metabólitos foram produzidos por praticamente todas as linhagens, com exceção de lactato para *K.*

*lactis* CBS 2359 e *K. marxianus* NRRLy 2415, e acetato para *K. marxianus* PYCC 3886.

As concentrações de glicerol para todas as linhagens foram baixas. A presença em concentrações mais elevadas deste metabólito é relacionada a uma resposta a algum estresse oferecido ao microrganismo durante o cultivo (8, 16). Como os cultivos foram interrompidos na metade da fase exponencial visando que nenhuma situação de estresse interferisse na produção de metabólitos, nenhum estresse promoveu a produção de glicerol.

A maior concentração de lactato foi obtida com a linhagem *K. marxianus* ATCC 26548 (0,148 ± 0,073 C-Lactato). Apenas metade das linhagens estudadas apresentaram o metabólito fumarato, todas do gênero *Kluyveromyces* e apenas duas linhagens do gênero *Saccharomyces*, sendo elas pertencentes a mesma espécie.

A produção de etanol pode ser observada para todas as linhagens sendo as menores concentrações obtidas pelas linhagens da espécie *K. marxianus*. Já as maiores concentrações foram observadas por *S. cerevisiae* UR-1, *S. cerevisiae* BG-1 e *K. lactis* CBS 2359 (48,844 ± 0,975; 47,514 ± 1,115; 20,832 ± 10,930 C-etanol respectivamente) (Tabela 4.1). Como todos os cultivos foram realizados sem um grau pré-determinado de limitação de oxigênio, e com as mesmas condições de agitação, frasco e volume útil de cultivo, estes dados podem ser indicativos que estas as linhagens sofreram um efeito Crabtree mais forte que as demais (18). Kiers *et al.* (11) em seu estudo com *K. lactis* CBS 2359 constatou que mesmo a linhagem sendo conhecida como Crabtree-negativa a fermentação aeróbia pode ocorrer devido a uma aeração inadequada ou por outras limitações do meio de cultivo. Dados estes reafirmados recentemente por Dias *et al*. (6).

Com base nos parâmetros cinéticos obtidos neste estudo já é possível sugerir linhagens específicas a um determinado processo biotecnológico, de forma que este processo obtenha produtividade elevada. Porém, para uma indicação mais assertiva, mais parâmetros devem ser avaliados afim contribuir ao novo mercado de personalização de leveduras (23).

## 4.4 Conclusão

Este estudo permitiu avaliar os parâmetros cinéticos das leveduras industriais para comparação entre linhagens de mesma espécie ou espécies diferentes com outras leveduras cultivadas em condições semelhantes. As observações aqui sublinham algumas potenciais aplicações biotecnológicas que, com maiores investigações, podem

levar a aplicações industriais. Leveduras do gênero *Kluyveromyces* apresentaram maior potencial respiratório enquanto leveduras do gênero *Saccharomyces* maior capacidade fermentativa.

### 4.5 Referências


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# CAPÍTULO 5

## 5 CONCLUSÕES GERAIS

*Sacharomyces cerevisiae* CAT-1 tem sido apresentada como a linhagem com maior capacidade fermentativa comprovada até o momento. Apesar disto, não desponta isoladamente nesta condição, os resultados aqui obtidos com *S. cerevisiae* FT858 indicaram que esta linhagem tem um excelente potencial biotecnológico, uma vez que os parâmetros cinéticos de crescimento observados foram muito próximos aos obtidos com *S. cerevisiae* CAT-1 em condições de cultivo semelhantes (aerobiose, temperatura, agitação, substrato e concentração).

As condições de cultivo interferiram diretamente na fisiologia das leveduras. Destaca-se um aumento de 10% no rendimento de etanol a partir de sacarose obtido com *S. cerevisiae* FT858 quando comparado com *S. cerevisiae* CAT-1 a 37 °C.

A concentração dos substratos interferiu diretamente na produção de invertase tanto para *S. cerevisiae* FT858 quanto para CAT-1. A expressão enzimática sofreu forte regulação por meio da concentração de glicose no meio de cultura. Contudo apenas *S. cerevisiae* CAT-1 apresentou comportamento constitutivo para a enzima invertase.

Outras linhagens também foram avaliadas em termos de parâmetros cinéticos e comparadas. De maneira geral leveduras do gênero *Kluyveromyces* apresentaram maior potencial respiratório enquanto leveduras do gênero *Saccharomyces* maior capacidade fermentativa.

Estudos mais aprofundados e detalhados são importantes especialmente para a comparação das linhagens industriais brasileiras e identificação de novos potenciais biotecnológicos para estas leveduras.

## APÊNDICES

**Apêndice 1** Material suplementar do Capítulo 3.

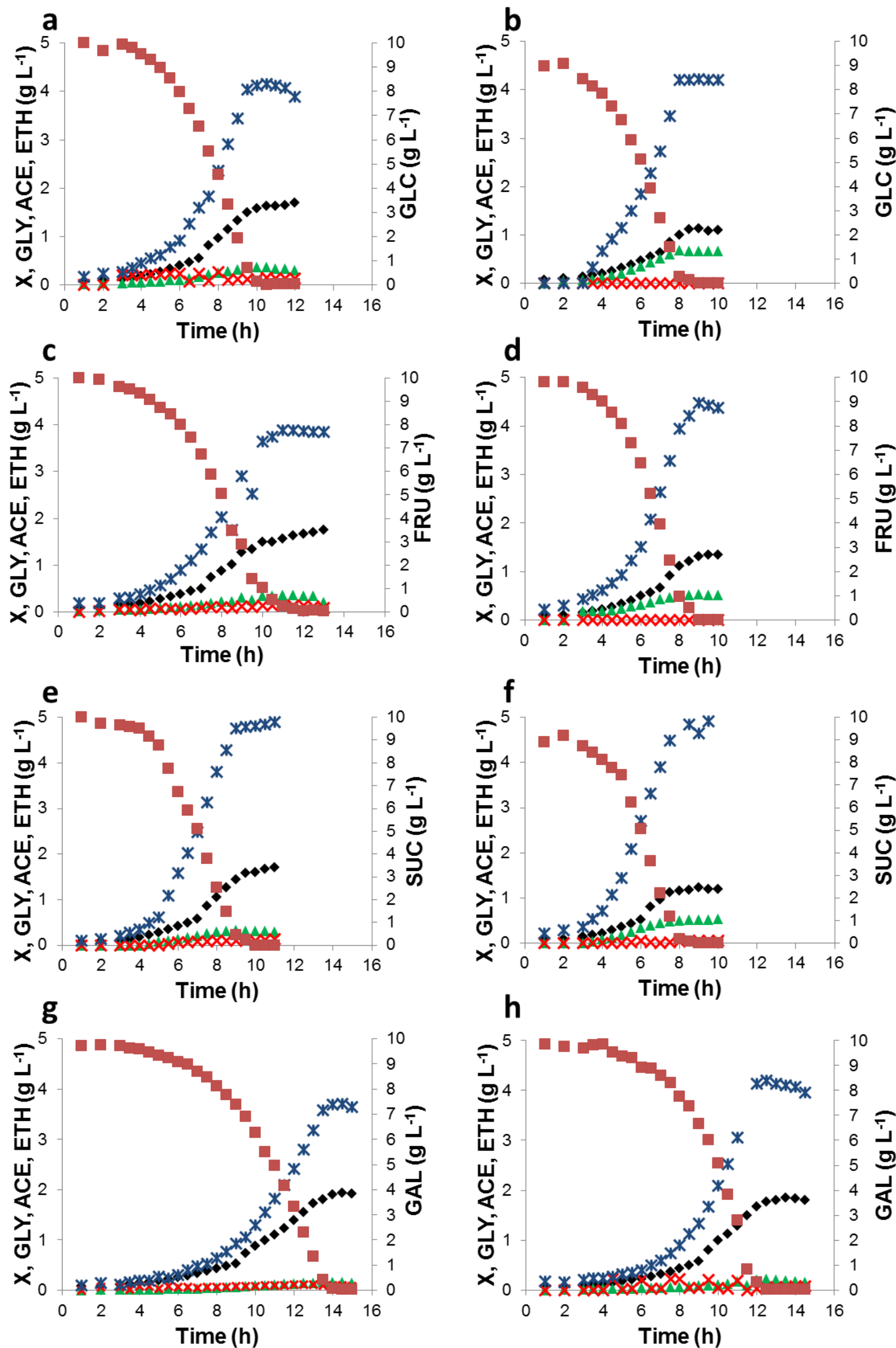


**Figure S1.** Growth kinetics, metabolite formation and sugar consumption during cultivations with **a** glucose/30 °C, **b** glucose/37 °C, **c** fructose/30 °C, **d** fructose/37 °C, and sucrose/30 °C, **f** sucrose/37 °C, **g** galactose/30 °C, **h** galactose/37 °C. (♦) X (biomass, g $L^{-1}$); (■) GLC (glucose, g $L^{-1}$) / FRU (fructose, g $L^{-1}$) / SUC (sucrose, g $L^{-1}$) / GAL (galactose, g $L^{-1}$); (▲) GLY (glycerol, g $L^{-1}$); (×) ACE (acetic acid, g $L^{-1}$); (x̶) ETH (ethanol, g $L^{-1}$).

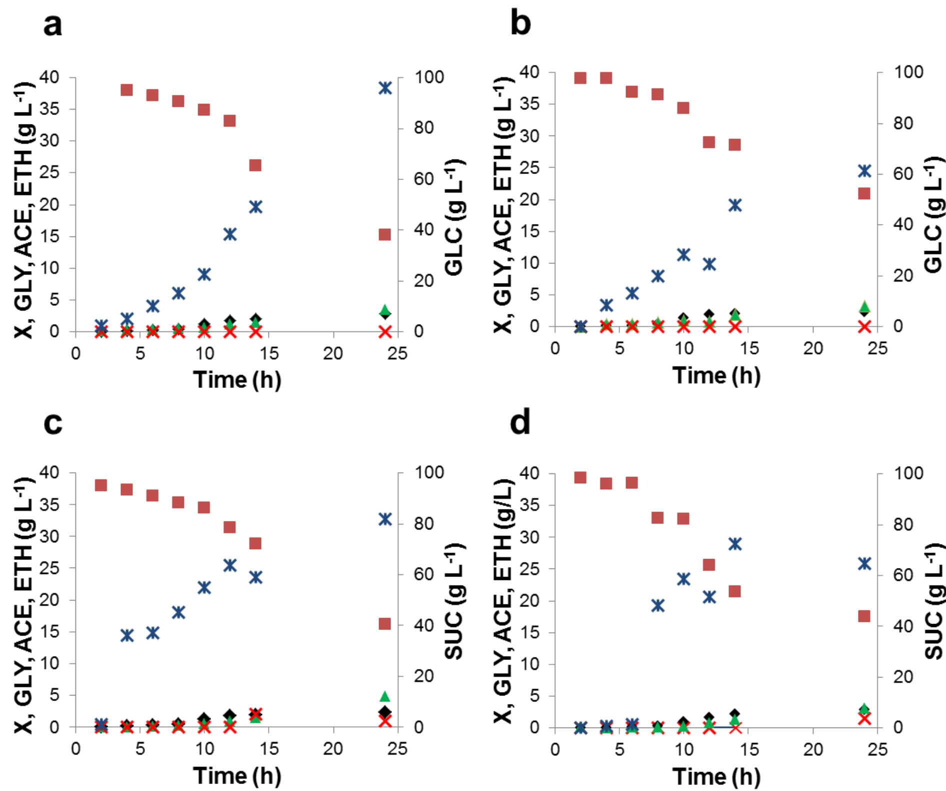


**Figure S2.** Growth kinetics, metabolite formation and substrate consumption of industrial yeasts *S. cerevisiae* CAT-1 and FT858 during cultivation with 100 g $L^{-1}$ substrate (**a** glucose/FT858, **b** glucose/CAT-1, **c** sucrose/FT858, **d** sucrose/CAT-1). (♦) X (biomass, g $L^{-1}$); (■) GLC (glucose, g $L^{-1}$) / SUC (sucrose, g $L^{-1}$); (▲) GLY (glycerol, g $L^{-1}$); (×) ACE (acetic acid, g $L^{-1}$); (x̶) ETH (ethanol, g $L^{-1}$).

**Apêndice 2** Dados experimentais referentes às cinéticas de crescimento com *S. cerevisiae* FT858 em fonte única de carbono (Capítulo 3).

**Glicose / 30°C**

| Tempo (h) | X (g $L^{-1}$) | GLC (g $L^{-1}$) | Gly (g $L^{-1}$) | Ace (g $L^{-1}$) | Eth (g $L^{-1}$) |
|---|---|---|---|---|---|
| 0 | 0,064 | - | - | - | - |
| 1 | 0,070 | 10,141 | 0,021 | 0,000 | 0,170 |
| 2 | 0,087 | 9,668 | 0,034 | 0,000 | 0,229 |
| 3 | 0,127 | 9,924 | 0,042 | 0,210 | 0,265 |
| 3,5 | 0,152 | 9,804 | 0,049 | 0,210 | 0,350 |
| 4 | 0,185 | 9,528 | 0,056 | 0,208 | 0,448 |
| 4,5 | 0,234 | 9,303 | 0,064 | 0,224 | 0,554 |
| 5 | 0,284 | 8,966 | 0,078 | 0,227 | 0,617 |
| 5,5 | 0,346 | 8,526 | 0,100 | 0,231 | 0,785 |
| 6 | 0,412 | 7,977 | 0,103 | 0,239 | 0,917 |
| 6,5 | 0,484 | 7,291 | 0,143 | 0,069 | 1,267 |
| 7 | 0,557 | 6,564 | 0,169 | 0,237 | 1,602 |
| 7,5 | 0,853 | 5,525 | 0,213 | 0,093 | 1,823 |
| 8 | 0,991 | 4,549 | 0,250 | 0,263 | 2,359 |
| 8,5 | 1,192 | 3,326 | 0,282 | 0,111 | 2,908 |
| 9 | 1,378 | 1,919 | 0,323 | 0,119 | 3,438 |
| 9,5 | 1,528 | 0,700 | 0,354 | 0,128 | 4,034 |
| 10 | 1,599 | 0,152 | 0,363 | 0,127 | 4,119 |
| 10,5 | 1,631 | 0,009 | 0,373 | 0,151 | 4,159 |
| 11 | 1,628 | 0,036 | 0,343 | 0,143 | 4,119 |
| 11,5 | 1,661 | 0,039 | 0,333 | 0,144 | 4,065 |
| 12 | 1,708 | 0,035 | 0,308 | 0,135 | 3,894 |

**Glicose / 37°C**

| Tempo (h) | X (g $L^{-1}$) | GLC (g $L^{-1}$) | Gly (g $L^{-1}$) | Ace (g $L^{-1}$) | Eth (g $L^{-1}$) |
|---|---|---|---|---|---|
| 0 | 0,065 | - | - | - | - |
| 1 | 0,068 | 8,969 | - | - | - |
| 2 | 0,106 | 9,087 | 0,046 | - | - |
| 3 | 0,144 | 8,426 | 0,068 | - | - |
| 3,5 | 0,175 | 8,142 | 0,095 | - | 0,338 |
| 4 | 0,198 | 7,858 | 0,122 | - | 0,675 |
| 4,5 | 0,233 | 7,300 | 0,167 | - | 0,914 |
| 5 | 0,309 | 6,742 | 0,211 | - | 1,153 |
| 5,5 | 0,381 | 5,934 | 0,280 | - | 1,500 |
| 6 | 0,471 | 5,126 | 0,350 | - | 1,847 |
| 6,5 | 0,536 | 3,910 | 0,436 | - | 2,283 |
| 7 | 0,625 | 2,695 | 0,522 | - | 2,719 |
| 7,5 | 0,843 | 1,486 | 0,602 | - | 3,461 |
| 8 | 1,001 | 0,276 | 0,681 | - | 4,202 |
| 8,5 | 1,113 | 0,138 | 0,674 | - | 4,212 |
| 9 | 1,132 | - | 0,667 | - | 4,223 |
| 9,5 | 1,070 | - | 0,668 | - | 4,211 |
| 10 | 1,104 | - | 0,669 | - | 4,200 |

**Frutose / 30°C**

| Tempo (h) | X (g $L^{-1}$) | FRU (g $L^{-1}$) | Gly (g $L^{-1}$) | Ace (g $L^{-1}$) | Eth (g $L^{-1}$) |
|---|---|---|---|---|---|
| 0 | 0,067 | - | - | - | - |
| 1 | 0,073 | 9,988 | 0,022 | 0,000 | 0,189 |
| 2 | 0,090 | 9,929 | 0,030 | 0,009 | 0,195 |
| 3 | 0,132 | 9,608 | 0,044 | 0,055 | 0,291 |
| 3,5 | 0,151 | 9,535 | 0,050 | 0,058 | 0,301 |
| 4 | 0,176 | 9,348 | 0,058 | 0,024 | 0,375 |
| 4,5 | 0,218 | 9,066 | 0,070 | 0,061 | 0,458 |
| 5 | 0,262 | 8,722 | 0,084 | 0,059 | 0,569 |
| 5,5 | 0,312 | 8,458 | 0,096 | 0,060 | 0,695 |
| 6 | 0,381 | 7,991 | 0,110 | 0,058 | 0,889 |
| 6,5 | 0,440 | 7,443 | 0,133 | 0,073 | 1,100 |
| 7 | 0,503 | 6,730 | 0,154 | 0,081 | 1,345 |
| 7,5 | 0,728 | 5,863 | 0,191 | 0,094 | 1,693 |
| 8 | 0,862 | 5,047 | 0,225 | 0,103 | 2,023 |
| 8,5 | 1,030 | 3,461 | 0,227 | 0,090 | 1,760 |
| 9 | 1,308 | 2,895 | 0,308 | 0,120 | 2,907 |
| 9,5 | 1,344 | 1,408 | 0,253 | 0,095 | 2,528 |
| 10 | 1,518 | 1,024 | 0,346 | 0,129 | 3,647 |
| 10,5 | 1,520 | 0,504 | 0,343 | 0,128 | 3,741 |
| 11 | 1,613 | 0,228 | 0,352 | 0,132 | 3,876 |
| 11,5 | 1,638 | 0,126 | 0,358 | 0,134 | 3,875 |
| 12 | 1,700 | 0,038 | 0,349 | 0,128 | 3,873 |
| 12,5 | 1,711 | 0,065 | 0,340 | 0,125 | 3,843 |
| 13 | 1,753 | 0,041 | 0,203 | 0,079 | 2,515 |

**Frutose / 37°C**

| Tempo (h) | X (g $L^{-1}$) | FRU (g $L^{-1}$) | Gly (g $L^{-1}$) | Ace (g $L^{-1}$) | Eth (g $L^{-1}$) |
|---|---|---|---|---|---|
| 0 | 0,063 | - | - | - | - |
| 1 | 0,087 | 9,798 | - | - | 0,222 |
| 2 | 0,100 | 9,798 | - | - | 0,298 |
| 3 | 0,151 | 9,575 | 0,179 | - | 0,438 |
| 3,5 | 0,186 | 9,291 | 0,179 | - | 0,523 |
| 4 | 0,215 | 9,006 | 0,179 | - | 0,608 |
| 4,5 | 0,271 | 8,547 | 0,209 | - | 0,771 |
| 5 | 0,340 | 8,088 | 0,240 | - | 0,933 |
| 5,5 | 0,403 | 7,279 | 0,280 | - | 1,222 |
| 6 | 0,485 | 6,470 | 0,321 | - | 1,510 |
| 6,5 | 0,557 | 5,204 | 0,374 | - | 2,069 |
| 7 | 0,661 | 3,937 | 0,427 | - | 2,628 |
| 7,5 | 0,912 | 2,459 | 0,465 | - | 3,283 |
| 8 | 1,104 | 0,981 | 0,503 | - | 3,939 |
| 8,5 | 1,210 | 0,490 | 0,515 | - | 4,207 |
| 9 | 1,286 | - | 0,526 | - | 4,475 |
| 9,5 | 1,328 | - | 0,522 | - | 4,425 |
| 10 | 1,338 | - | 0,518 | - | 4,374 |

**Sacarose / 30°C**

| Tempo (h) | X (g L$^{-1}$) | SAC (g L$^{-1}$) | Gly (g L$^{-1}$) | Ace (g L$^{-1}$) | Eth (g L$^{-1}$) |
|---|---|---|---|---|---|
| 0 | 0,060 | - | - | - | - |
| 1 | 0,061 | 9,992 | - | - | 0,099 |
| 2 | 0,079 | 9,729 | - | - | 0,140 |
| 3 | 0,114 | 9,636 | - | - | 0,205 |
| 3,5 | 0,138 | 9,567 | - | - | 0,280 |
| 4 | 0,174 | 9,497 | - | - | 0,355 |
| 4,5 | 0,219 | 9,139 | 0,037 | - | 0,483 |
| 5 | 0,273 | 8,781 | 0,075 | - | 0,611 |
| 5,5 | 0,343 | 7,759 | 0,106 | 0,033 | 1,093 |
| 6 | 0,411 | 6,736 | 0,137 | 0,066 | 1,576 |
| 6,5 | 0,480 | 5,911 | 0,169 | 0,073 | 2,025 |
| 7 | 0,583 | 5,086 | 0,202 | 0,080 | 2,473 |
| 7,5 | 0,854 | 3,807 | 0,250 | 0,085 | 3,134 |
| 8 | 1,045 | 2,528 | 0,298 | 0,091 | 3,794 |
| 8,5 | 1,266 | 1,482 | 0,310 | 0,096 | 4,278 |
| 9 | 1,441 | 0,437 | 0,323 | 0,101 | 4,762 |
| 9,5 | 1,575 | 0,218 | 0,319 | 0,103 | 4,787 |
| 10 | 1,592 | - | 0,315 | 0,104 | 4,813 |
| 10,5 | 1,662 | - | 0,308 | 0,122 | 4.,851 |
| 11 | 1,700 | - | 0,301 | 0,140 | 4,890 |
| 11,5 | 1,711 | - | - | - | - |

**Sacarose / 37°C**

| Tempo (h) | X (g $L^{-1}$) | SAC (g $L^{-1}$) | Gly (g $L^{-1}$) | Ace (g $L^{-1}$) | Eth (g $L^{-1}$) |
|---|---|---|---|---|---|
| 0 | 0,06 | - | - | - | - |
| 1 | 0,07 | 8,902 | - | - | 0,210 |
| 2 | 0,10 | 9,175 | - | - | 0,282 |
| 3 | 0,14 | 8,728 | - | - | 0,357 |
| 3,5 | 0,18 | 8,426 | 0,046 | - | 0,533 |
| 4 | 0,22 | 8,123 | 0,091 | - | 0,709 |
| 4,5 | 0,28 | 7,775 | 0,136 | - | 1,077 |
| 5 | 0,35 | 7,426 | 0,180 | - | 1,445 |
| 5,5 | 0,44 | 6,244 | 0,258 | 0,029 | 2,079 |
| 6 | 0,53 | 5,062 | 0,336 | 0,058 | 2,713 |
| 6,5 | 0,81 | 3,643 | 0,391 | 0,029 | 3,309 |
| 7 | 0,97 | 2,225 | 0,447 | - | 3,905 |
| 7,5 | 1,12 | 1,199 | 0,478 | 0,021 | 4,481 |
| 8 | 1,16 | 0,173 | 0,508 | 0,042 | 5,058 |
| 8,5 | 1,18 | 0,086 | 0,512 | 0,050 | 4,848 |
| 9 | 1,22 | - | 0,515 | 0,057 | 4,639 |
| 9,5 | 1,20 | - | 0,528 | 0,061 | 4,908 |

**Galactose / 30°C**

| Tempo (h) | X (g $L^{-1}$) | GAL (g $L^{-1}$) | Gly (g $L^{-1}$) | Ace (g $L^{-1}$) | Eth (g $L^{-1}$) |
|---|---|---|---|---|---|
| 0 | 0,078 | - | - | - | - |
| 1 | 0,079 | 9,716 | 0,008 | 0,000 | 0,099 |
| 2 | 0,088 | 9,750 | 0,014 | 0,067 | 0,137 |
| 3 | 0,111 | 9,726 | 0,017 | 0,066 | 0,115 |
| 3,5 | 0,126 | 9,610 | 0,019 | 0,020 | 0,164 |
| 4 | 0,144 | 9,580 | 0,021 | 0,073 | 0,191 |
| 4,5 | 0,164 | 9,468 | 0,023 | 0,074 | 0,185 |
| 5 | 0,195 | 9,334 | 0,024 | 0,030 | 0,278 |
| 5,5 | 0,218 | 9,243 | 0,026 | 0,079 | 0,234 |
| 6 | 0,252 | 9,090 | 0,028 | 0,069 | 0,309 |
| 6,5 | 0,287 | 8,981 | 0,033 | 0,065 | 0,400 |
| 7 | 0,338 | 8,691 | 0,040 | 0,051 | 0,473 |
| 7,5 | 0,378 | 8,462 | 0,045 | 0,057 | 0,521 |
| 8 | 0,425 | 8,127 | 0,052 | 0,063 | 0,637 |
| 8,5 | 0,474 | 7,778 | 0,060 | 0,069 | 0,772 |
| 9 | 0,525 | 7,385 | 0,072 | 0,076 | 0,922 |
| 9,5 | 0,742 | 6,914 | 0,082 | 0,082 | 1,060 |
| 10 | 0,873 | 6,255 | 0,094 | 0,088 | 1,302 |
| 10,5 | 0,987 | 5,485 | 0,103 | 0,095 | 1,547 |
| 11 | 1,105 | 4,956 | 0,117 | 0,103 | 1,824 |
| 11,5 | 1,233 | 4,146 | 0,124 | 0,103 | 2,088 |
| 12 | 1,389 | 3,313 | 0,134 | 0,107 | 2,416 |
| 12,5 | 1,547 | 2,293 | 0,139 | 0,106 | 2,799 |
| 13 | 1,733 | 1,349 | 0,146 | 0,106 | 3,180 |
| 13,5 | 1,807 | 0,413 | 0,157 | 0,103 | 3,574 |
| 14 | 1,908 | 0,066 | 0,161 | 0,097 | 3,686 |
| 14,5 | 1,930 | 0,041 | 0,161 | 0,095 | 3,701 |
| 15 | 1,913 | 0,038 | 0,148 | 0,086 | 3,640 |

**Galactose / 37°C**

| Tempo (h) | X (g $L^{-1}$) | GAL (g $L^{-1}$) | Gly (g $L^{-1}$) | Ace (g $L^{-1}$) | Eth (g $L^{-1}$) |
|---|---|---|---|---|---|
| 0 | 0,07 | - | - | - | - |
| 1 | 0,08 | 9,856 | 0,000 | 0,000 | 0,390 |
| 2 | 0,08 | 9,756 | 0,017 | 0,000 | 0,185 |
| 3 | 0,11 | 9,695 | 0,022 | 0,000 | 0,192 |
| 3,5 | 0,12 | 9,805 | 0,028 | 0,000 | 0,192 |
| 4 | 0,13 | 9,855 | 0,029 | 0,000 | 0,192 |
| 4,5 | 0,14 | 9,512 | 0,031 | 0,229 | 0,285 |
| 5 | 0,17 | 9,348 | 0,036 | 0,022 | 0,308 |
| 5,5 | 0,20 | 9,297 | 0,039 | 0,026 | 0,361 |
| 6 | 0,23 | 8,907 | 0,042 | 0,217 | 0,410 |
| 6,5 | 0,28 | 8,862 | 0,049 | 0,034 | 0,487 |
| 7 | 0,33 | 8,603 | 0,055 | 0,038 | 0,733 |
| 7,5 | 0,37 | 8,296 | 0,063 | 0,229 | 0,729 |
| 8 | 0,44 | 7,736 | 0,070 | 0,233 | 0,926 |
| 8,5 | 0,50 | 7,351 | 0,078 | 0,046 | 1,112 |
| 9 | 0,57 | 6,670 | 0,089 | 0,044 | 1,370 |
| 9,5 | 0,80 | 6,021 | 0,102 | 0,210 | 1,660 |
| 10 | 1,00 | 5,068 | 0,120 | 0,041 | 2,087 |
| 10,5 | 1,13 | 3,814 | 0,131 | 0,037 | 2,535 |
| 11 | 1,29 | 2,799 | 0,173 | 0,196 | 2,957 |
| 11,5 | 1,49 | 0,833 | 0,093 | 0,000 | 3,552 |
| 12 | 1,68 | 0,336 | 0,212 | 0,030 | 4,025 |
| 12,5 | 1,77 | 0,045 | 0,220 | 0,043 | 4,182 |
| 13 | 1,80 | 0,039 | 0,211 | 0,058 | 4,095 |
| 13,5 | 1,86 | 0,038 | 0,199 | 0,070 | 4,045 |
| 14 | 1,84 | 0,037 | 0,181 | 0,075 | 4,020 |
| 14,5 | 1,81 | 0,036 | 0,166 | 0,074 | 3,945 |

**Apêndice 3** Dados experimentais referentes às cinéticas de crescimento com *S. cerevisiae* FT858 e CAT-1, com substratos glucose e sacarose nas concentrações de 1 e 10%. Cinéticas realizadas para obtenção dos extratos enzimáticos (Capítulo 2).

**Glicose / 1%**

| Tempo (h) | CAT-1 | | FT858 | |
|---|---|---|---|---|
| | X (g $L^{-1}$) | Invertase (U $mL^{-1}$) | X (g $L^{-1}$) | Invertase (U $mL^{-1}$) |
| 0 | 0,056 | 0,350 | 0,056 | 0,120 |
| 2 | 0,080 | 0,500 | 0,089 | 0,534 |
| 4 | 0,167 | 0,609 | 0,180 | 0,609 |
| 6 | 0,395 | 0,549 | 0,388 | 0,737 |
| 8 | 1,116 | 4,226 | 0,873 | 1,241 |
| 10 | 1,508 | 11,203 | 1,265 | 12,331 |
| 12 | 1,775 | 20,359 | 1,500 | 6,907 |
| 14 | 1,819 | 16,772 | 1,600 | 8,591 |
| 24 | 2,004 | 19,374 | 1,986 | 11,413 |

**Sacarose / 1%**

| Tempo (h) | CAT-1 | | FT858 | |
|---|---|---|---|---|
| | X (g $L^{-1}$) | Invertase (U $mL^{-1}$) | X (g×$L^{-1}$) | Invertase (U $mL^{-1}$) |
| 0 | 0,052 | 0,297 | 0,053 | 1,316 |
| 2 | 0,062 | 0,402 | 0,084 | 1,342 |
| 4 | 0,143 | 0,526 | 0,185 | 1,489 |
| 6 | 0,300 | 0,609 | 0,409 | 1,808 |
| 8 | 0,805 | 2,726 | 0,940 | 3,556 |
| 10 | 1,511 | 39,398 | 1,366 | 49,624 |
| 12 | 1,815 | 31,098 | 1,526 | 20,731 |
| 14 | 1,908 | 26,702 | 1,674 | 23,552 |
| 24 | 1,999 | 27,773 | 1,931 | 24,580 |

**Glicose / 10%**

| Tempo (h) | CAT-1 | | FT858 | |
|---|---|---|---|---|
| | X (g $L^{-1}$) | Invertase (U $mL^{-1}$) | X (g $L^{-1}$) | Invertase (U $mL^{-1}$) |
| 0 | 0,035 | 0,218 | 0,048 | 0,218 |
| 2 | 0,048 | 0,222 | 0,069 | 0,283 |
| 4 | 0,097 | 0,227 | 0,129 | 0,288 |
| 6 | 0,214 | 0,275 | 0,262 | 0,362 |
| 8 | 0,420 | 0,410 | 0,457 | 0,358 |
| 10 | 1,324 | 0,533 | 1,275 | 0,463 |
| 12 | 1,871 | 0,905 | 1,735 | 0,406 |
| 14 | 2,074 | 1,745 | 2,035 | 0,450 |
| 24 | 2,368 | 2,169 | 2,885 | 0,458 |

**Sacarose / 10%**

| Tempo (h) | CAT-1 | | FT858 | |
|---|---|---|---|---|
| | X (g $L^{-1}$) | Invertase (U $mL^{-1}$) | X (g $L^{-1}$) | Invertase (U $mL^{-1}$) |
| 0 | 0,048 | 0,178 | 0,045 | 0,231 |
| 2 | 0,049 | 0,196 | 0,061 | 0,222 |
| 4 | 0,090 | 0,196 | 0,113 | 0,275 |
| 6 | 0,165 | 0,213 | 0,231 | 0,288 |
| 8 | 0,305 | 0,314 | 0,428 | 0,502 |
| 10 | 1,003 | 0,577 | 1,269 | 0,585 |
| 12 | 1,644 | 3,214 | 1,725 | 2,764 |
| 14 | 2,139 | 4,807 | 1,871 | 5,629 |
| 24 | 2,855 | 4,785 | 2,291 | 5,617 |

**Apêndice 4** 3 BIOTECH – Specific Instructions for Authors (Updated 19 August 2019)

**3 BIOTECH – Specific Instructions for Authors (Updated 19 August 2019)**


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# 3 BIOTECH – Specific Instructions for Authors

(updated 19 August 2019)

## 1. JOURNAL OVERVIEW

**3 BIOTECH is a peer-reviewed e-journal** and all accepted manuscripts will be published online at the journal's website with volume and article numbers shortly after receipt of authors corrected galley proofs. The manuscripts will be published under **Continuous Article Publishing** (CAP). CAP aims to speed up the process from acceptance of articles to final online publication without the need for articles to be placed in a waiting line. This ensures that all new articles in 3 BIOTECH are immediately available for researchers in relevant indexing and abstracting databases.

1.1 Aims and Scope:

3 BIOTECH publishes papers related to the study of the Environment and Biodiversity with application in biotechnology in the three life sciences disciplines: (i) Medical and Biomedical Sciences, (ii) Agricultural Sciences and (iii) Environment, and hence the title of the journal.

3 BIOTECH recognises that the Biotechnology of these three life science sectors relies heavily on the use of complex scientific tools.Therefore, 3BIOTECH aims to present information on techniques and methods used in biotechnology and to address problems and benefits associated with such tools, methods and techniques as applied to particular biotechnology applications. 3 BIOTECH will appeal to scientists and engineers in both academia and industry focused on the safe and efficient application of Biotechnology to Medicine, Agriculture and the Environment.

Papers in all areas of prokaryotic and eukaryotic biotechnology are welcome. Examples of areas covered by 3 BIOTECH, but not limited to, include genomics, metagenomics, metabolomics and transcriptomics, proteomics, secondary metabolites, biosynthetic pathway modulations in tissue culture and demonstration of their functional role in plants, cancer and stem cell research, nanotechnology, genetic engineering and cloning, bioremediation and biodegradation, bioinformatics and system biology, biomarkers and biosensors, biodiversity, biofuels, biodiscovery and taxonomy, engineered / novel enzyme with high end biotechnological applications, biorobotics and biotoxins, analytical biotechnology, infectious diseases and vaccines, immunology, structural biology, synthetic biology and natural medicines.

1.2 Exclusions to the scope:

Submissions that are considered to be routine reports or contain limited, research-specific content, will not be accepted. Some typical examples of manuscripts with limited scope include the following:

*Bioactive natural products:* Manuscripts describing bioactive products from chemically characterized extracts from medicinal plants, animals or microbes, and in the formulation and delivery systems of natural products, are welcome. Manuscripts should include *in vitro* and *in vivo* cytotoxic studies. However, manuscripts describing uncharacterised extracts or partially characterised extracts with studies of general activities e. g. antioxidant properties, antimicrobial activities etc will not be considered.

*Plant tissue / cell culture*: Manuscripts describing original/novel research on application of plant cell, tissue, and organ culture for germplasm conservation (cryopreservation), genetic transformation, protoplast culture, somatic hybridization, hairy root culture, *in vitro* polyploidization and mutation, as well as for enhanced production of bioactive compounds / secondary metabolites are welcome. However, simple/routine protocols *in vitro* propagation or description of the effect of plant growth regulators on micro-propagation will not be considered.

*Molecular Modelling:* Manuscripts describing *in silico* bioinformatic analysis without supporting experimental data will only be considered if complex issue(s) are solved and knowledge in this area is significantly advanced.

*Statistical Approaches in Designing Experiments:* Manuscripts which are based entirely on the use of statistical methods e.g. Response Surface Methodology (RSM) to optimise growth, increase production etc. will not be considered.

*Enzymes*: Manuscripts describing enzymes that have not been demonstrably purified to homogeneity and which have not been molecularly and functionally characterised will not be considered.

*Genomes*: Bioinformatics *in silico* analysis without experimental evidence of the biotechnological potential of the organism will not be accepted.

*Basic scientific data and analysis*: Manuscripts that contain basic scientific information but without experimentally demonstrated evidence of a biotechnological application(s) will not be considered.

*Examples of Routine reports with limited research content include*:

- Report on isolation and characterization of microbe without any biotechnological applications.
- Report on identification of microbes using only phenotypic/biochemical-physiological traits.
- Synthesis of metal nanoparticle using plant/microbial extract and production optimization.
- Physio-chemical and biological characterization of environmental samples.

*Molecular marker-assisted breeding*: Routine genetic diversity assessment involving a small number of accessions / genotypes / populations of local / limited interest and use of routine molecular markers such as RAPD, ISSR, ISR etc. will not be considered.

## 2. EDITORIAL PROCESS

3 BIOTECH accepts biotechnology-related papers which are original, unpublished and not under simultaneous consideration by another journal.

### 2.1 Internal Editorial Review:

3 BIOTECH receives a large volume of submissions and to avoid unnecessary delays and to assist in providing authors with a timely response on their manuscripts, each manuscript undergoes a stringent editorial quality and format assessment using a set of criteria by members of the Editorial Board. A list of the assesement criteria for which a decision is usually reached within 3 weeks is provided below:

- Compliance of the journal's scope
- Plagiarism
- Compliance of the journal's format and style
- Quality of the abstract
- Relevance of the work to biotechnology: Demonstration of the potential benefit of the bioproduct / microbe in a practical situation (proof of concept).
- A high standard of presentation: Overall presentation such as clarity, readability (flow) of the manuscript, appropriate use of the English language, and extent of typographical errors.
- Appropriateness of the design and methodology and whether the description is adequate for others to replicate the work.
- The quality of the data and the presentation of the data.
- The quality of the figures and the description of the figure legends. All legends should have enough description for a reader to understand the figure without readers having to refer back to the main text of the manuscript.

- The conclusions are reliable and significant
- Creation of new knowledge or extension / improvement in knowledge when compared to other peer-reviewed published works in the same research area; demonstartion of novelty
- New versions of previously rejected papers is not permitted unless indicated otherwise in the journal's decision letter.

**2.2 External Peer Review:**

If the manuscript meets the journal's preliminary standards described above than it is forwarded to an experienced member of the Editorial Board. The Editor will invite a minimum of two qualified external reviewers for the review of the manuscript. Reviewers are selected from a database of experts and may also include reviewers nominated by the authors. The member of the Editorial Board advises the EiC on the reviewers' reports and a final decision to accept or reject a manuscript is then made. The journals rejection rate for the period 2012 to 2018 has varied between 70 – 76%.

The peer review is a double-blind review process in which the authors and reviewers identities are not revealed to each other during the review process. This process is different to the traditional single blind process, in which the reviewer's identity is not revealed to the authors.

Final Decision: We attempt to reach a final decision on peer reviewed manuscripts within 8 – 10 weeks after submission. If an offer of publication is made which is subject to revision, then the authors are asked to submit a revised version of the manuscript within 4 weeks. A second round of evaluation starts after re-submission. Articles are usually published online within 4 weeks after acceptance.

## 3. EDITORIAL POLICIES

**3.1 General:**

In the event the required information is not provided, the manuscript will be returned to the authors for correction and this may lead to significant delays in reviewing the manuscript.

All files should be prepared using a word processor (.doc, .docx or .rtf format) and not as pdf files.

All text should be 12-point font size (Times New Roman, Arial, or Courier) with double spaced lines and 2.0 cm margins.

**3.2 Language:**

Manuscripts should be in English (consistent with either British or American spelling). Authors are strongly advised to ensure that the manuscript is written in clear and concise language, is intelligible to a broad readership and is of a publishable standard prior to submission. Manuscripts that are deficient in this respect may be rejected and returned to the author without peer review.

To help authors avoid receiving negative comments from referees or editors about the poor use of the English language in their manuscripts, and for authors who are unsure of correct English usage, at least one of the following steps should be considered:

- Have the manuscript reviewed for clarity by a colleague whose native language is English or by a colleague who has a good command of the English language
- Use one of the many English language-editing services, preferably with experience in editing scientific manuscripts, that are available. The names of some editing services are listed below:
  - Inter-Biotec (http://www.inter-biotec.com/)
  - Inter-Biotec also provides a free online writing course (http://www.inter-biotec.com/biowc/biowc.html) to help biomedical scientists whose first language is not English to write and publish their papers in English-language journals.
  - SPI Professional Editing Services (http://www.prof-editing.com/)

◦ Write Science Right (http://www.writescienceright.com/)

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**3.3 Use of Human and / or Animal Subjects:**

The corresponding author must confirm that all research studies conducted using either vertebrates or higher invertebrates were performed in accordance with the relevant government's regulatory guidelines and regulations, and must include at an appropriate place in the article, a statement which includes details of the authority approving the experiments. In addition, for experiments involving human subjects, authors must state the committee approving the experiments, and include in their submission a statement confirming that informed consent was obtained from all subjects.

**3.4 Conflict of Interest (COI) and Author Contributions:**

All listed authors in the manuscript are required to include a statement on Conflict Of interest (COI) and Author Contribution as decribed below under sections 3.4.1 and 3.4.2 respectively. Both these sections must be included in the manuscript after the section "Acknowledgement".

3.4.1 Conflict Of interest (COI): A conflict of interest (COI) is defined as a competing financial interest which could have the potential to influence behaviour, content or perception and undermine the objectivity, integrity or value of a publication. Public knowledge of such work after it has been published would cause embarrassment. Some examples of COI include stocks or shares in companies that may gain or lose financially through publication; consultation fees or other forms of remuneration from organisations that may gain or lose financially; patents or patent applications whose value may be affected by publication.

3 BIOTECH takes the issue of COI seriously and therefore the authors are required to declare in their manuscript with either one of the following statements:

- The authors declare that they have no conflict of interest in the publication
- The authors declare that they have a conflict of interest [in which the case authors should supply an appropriate statement describing the conflict of interest].

3.4.2 Author Contributions: Submission of a paper implies that all authors have seen and approved the manuscript and its contents, and that they are aware of the responsibilities connected to the authorship. Consequently, all listed authors are required to include a statement of their contributions in this section.

**3.5 Changes to the list or order of authors names:**

Submission of a manuscript implies that all authors have seen and approved the manuscript and its contents, and that they are aware of the responsibilities connected to the authorship. Therefore all authors are advised to carefully check the list and order of authors before submission of the manuscript. Changes to the authors names or the order can only be requested during the revision and acceptance stages of the review process by submitting a "Change of authorship request form" citing sufficient justification. However, there can be no guarantee that the requested changes will be accepted. A change in the list or the order of the authors in the list will not be accepted at the galley proof stage or after publication of the manuscript. Requests for a change of the corresponding author will not be accepted at any stage after submission of the manuscript.

**3.6 Scientific Integrity – Plagiarism, use of 3rd Party Images & Imagine Manipulation:**
The members of the Editorial Board are committed to maintaining a very high standard of scientific integrity of published scientific reports in the journal and authors are reminded of their responsibility to avoid all misrepresentations in the reports of their work. The credibility of a research project is determined by promoting objectivity and avoiding misrepresentation in written component of the manuscript and in the assignment of credit to the researchers involved in the project.

The journal will investigate suspected cases that violate research ethics and instances of scientific fraud and misrepresentations, inappropriate manipulation of graphics files, redundant publications, and plagiarism. Depending on the outcomes of these investigations, the journal may decide to publish errata or corrigenda and, in cases of serious scientific misconduct, ask authors to retract their paper, or impose a retraction on them.

3.6.1Plagiarism: CrossCheck is a multi-publisher initiative to screen submitted content for originality. 3 BIOTECH uses CrossCheck to detect instances of plagiarism, overlapping and similar text in submitted manuscripts and published work. To find out more about CrossCheck visit http://www.crossref.org/crosscheck.html. 3 BIOTECH is a member of the Committee on Publication Ethics (http://publicationethics.org/). COPE provides a forum for publishers and Editors.

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3.6.3 Electronic Manipulation of Images: If a digital image figure has been subjected to significant electronic manipulation, it can misrepresent data, present unrepresentative data or result in a loss of meaningful signals. No specific feature(s) of an image should be enhanced, obscured, moved, removed, or introduced. If images from different parts of the same gel, or from different gels, fields, or exposures have been grouped, then this must be made explicit in the text of the figure legend or in the Methods section.

Members of the Editorial Board reserve the right to request original versions of figures from the authors of a paper under consideration, or after publication, if concerns arise. If the original data cannot be produced, the acceptance of the manuscript may be withdrawn. The journal also reserves the right to retract published papers in which data has been misrepresented and / or electronic image manipulation has affected the interpretation of the data. The journal also reserves the right to bring such matters to the attention of the funding agencies and institutions to which the authors are associated.

The following publication is a good reference for acceptable practices: Rossner M, Yamada KM (2004). What's in a picture? The temptation of image manipulation. J Cell Biol 166: 11-15

**3.7 Availability of Published Material**
By publishing a paper in 3 BIOTECH, the authors agree that they will make freely available any of the organisms, viruses, cells, nucleic acids, antibodies, reagents, data and associated protocols that were used in the reported research that are not available commercially, to colleagues for academic research without preconditions.

**3.8 Submission to Public Databases**
3 BIOTECH will only review and publish manuscripts if the authors agree that all data which cannot be published in the journal (e.g. nucleotide sequences, structural data, or data from large-

scale experiments) will be freely available in one of the public databases. The sequence data, separate from the manuscript, must be submitted to a Public Database and the data released for review. The reference to the data e.g. gene sequence, metagenome / genome sequence data, crystal structure data etc must be included under a separate heading “Accession Numbers” before the “Acknowledgements” section. Examples of some databases are given below:

3.8.1 Sequence Data

All sequence data should be submitted in electronic form to any one of the three major collaborative databases given below:

- DNA Data Bank of Japan - DDBJ (http://www.ddbj.nig.ac.jp)
- European Bioinformatics Institute - EMBL (http://www.ebi.ac.uk)
- GenBank - (http://www.ncbi.nlm.nih.gov).

The data must be released before submission to 3 BIOTECH so that the data can be reviewed by members of the Editorial Board and reviewers.

3.8.2 Structural Data: 3BIOTECH accepts and follows the recommendations of the International Union of Crystallography (IUCr) with regard to the deposition and release of macromolecular structural data. These guidelines are set out in the article by the IUCr Commission on Biological Macromolecules in Acta Crystallographica (2000), D56, 2.

Structures of biological macromolecules must be submitted to a publicly available and recognized database, such as Protein DataBank (http://www.rcsb.org./pdb/home/home.do), Biological Magnetic Resonance Databank (http://www.bmrb.wisc.edu/), NDB (http://ndbserver.rutgers.edu). Manuscripts reporting new three-dimensional structures of small molecules from crystallographic analysis should include a .cif file and a structural figure with probability ellipsoids for publication as Supplementary Information. These files must have been checked using the IUCR's CheckCIF routine (http://checkcif.iucr.org/) and a PDF copy of the output must be included at submission, together with a justification for any alerts reported. Crystallographic data for small molecules should be submitted to the Cambridge Structural Database (http://www.ccdc.cam.ac.uk/). In the case of low-resolution structures for which only a chain trace is reported, a set of C-alpha positions and structure-factor amplitudes may be sufficient.

3.8.3 Microarray Data: Data from microarray experiments should be submitted to either the ArrayExpress (http://www.ebi.ac.uk/arrayexpress) or GEO (http://www.ncbi.nlm.nih.gov/geo) or CIBEX (http://cibex.nig.ac.jp/index.jsp) databases. Microarray data should be described according to MIAME guidelines (http://www.mged.org/Workgroups/MIAME/miame.html).

3.8.4 Other data sets: 3BIOTECH strongly recommends deposition of other types of data sets into appropriate public repositories that are at an earlier stage of development. Examples of such repositories that facilitate sharing large data sets, some of which can offer the option of anonymous referee access to data before publication, include:

*Proteomics data:* PRIDE (http://www.ebi.ac.uk/pride/), PeptideAtlas (http://www.peptideatlas.org/), Tranche (http://www.proteomecommons.org)

*Protein interaction data:* IMEx consortium of databases including DIP, IntAct and MINT (http://www.imexconsortium.org/)

*Chemical compound screening and assay data:* PubChem (http://pubchem.ncbi.nlm.nih.gov/)

*Cryoelectron Microscopy:* Structures of biological macromolecules solved by electron microscopy

must be submitted to the EMDB database at http://www.ebi.ac.uk/msd/Deposition.html. For a brief description of the database, see Tagari et al (2002) Trends Biochem Sci 27: 589.

**3.9 Nomenclature**

3.9.1 Microbes: The genus, species, and sub-species / variey names should be written in italics; strain number sand culture collection numbers and sources of all strains under investigation should be given in the Methods section.

The scientific names should be given in full (e.g., *Escherichia coli*) in the title, in the abstract, and when first mentioned in the body of the manuscript. Thereafter, the appropriate abbreviation of the full generic name of the microbe should be reduced to conform with the Rules of Nomenclature (except in tables and figure legends) as suggested in the International Code of Nomenclature of Bacteria (Lapage, S.P., Sneath, P.H.A., Less el, E.F., Skerman, V.B.D., Seeliger, H.P.R. and Clark, W.A. International code of nomenclature of bacteria (1990 Revision). American Society for Microbiology, Washington, D.C., 1992). Alternatively, use the validation lists published in the International Journal of Systematic and Evolutionary (IJSEM) and / or published in the Microbiology List of Prokaryotic names with Standing in Nomenclature at the URL http://www.bacterio.cict.fr/

In case of usage of symbols that do not conform to those that have previously appeared in the literature, their aliases may be obtained from the approved nomenclature in the Human Gene Nomenclature Database (Genew) [www.gene.ucl.ac.uk/nomenclature/guidelines.html] and LocusLink, to allow retrieval of all the information available for each gene.

3.9.2 Genetics: Genes, mutations, genotypes, and alleles should also be indicated in italics but the protein product of a gene should be in Roman type; phenotypes should not be italicized.

For human genetics nomenclature, use the HUGO database. (a) In case of usage of symbols that do not conform to those that have previously appeared in the literature, their aliases may be obtained from the approved nomenclature in the Human Gene Nomenclature Database (Genew) [www.gene.ucl.ac.uk/nomenclature/guidelines.html] and LocusLink, to allow retrieval of all the information available for each gene. (b) It is sometimes advisable to indicate the synonyms for the gene the first time it appears in the text. Gene prefixes such as those used for oncogenes or cellular localization should be shown in Roman: v-fes, c-MYC

For bacterial genetics nomenclature follow Demerec et al (1966) Genetics 54: 61-76; J Bacteriol (first issue of each year); Microbiol Mol Biol Rev (1998) 62:814-984 (*Escherichia coli* K-12); Microbiol Rev (1988) 52:485-532 (*Salmonella typhimurium*); Microbiol Rev (1985) 49:158-179 (*Bacillus subtilis*); Annu Rev Microbiol (1986) 40:79-105 (Pseudomonas); Microbiol Rev (1982) 46:426-570 (*Neurospora crassa*); Nature (1997) 387 (6632 Suppl):67-73 (*Saccharomyces cerevisiae*).

For plant genes follow the recommendations of the International Society for Plant Molecular Biology Commission on Plant Gene Nomenclature, which are posted regularly on the public databases and published annually in Plant Molecular Biology Reporter, starting with the December 1993 issue.

Chemistry/Biochemistry: For guidance in the use of biochemical terminology follow the recommendations issued by the International Union of Biochemistry and Molecular Biology (IUBMB; http://www.chem.qmw.ac.uk/iubmb/); International Union of Pure and Applied Chemistry (IUPAC; http://www.chem.qmw.ac.uk/iupac/index.html).

For enzyme nomenclature use Enzyme Handbook (1990) Springer, Berlin Heidelberg New York; Enzyme Nomenclature (1992) Academic Press, London New York.

**3.10 Taxonomy**

3.10.1 Microbes: When a new bacterial name is proposed, an international authority on nomenclature should be contacted, and the name approved. All microbes described as taxonomical novel species and / or if the scientific content of the manuscript is essentially dependent on the strain, than the culture(s) must be deposited in an internationally recognized culture collection. Publication of an article in 3 BIOTECH is subject to the understanding that authors will distribute freely any strains, clones, or antibodies described therein for use in academic research. Genes used to identify isolates must be submitted to EMBL/GenBank/DDBJ, the data released and the accession number reported in the manuscript.

When a new fungal name is proposed, an international authority on nomenclature should be contacted, and the name approved. Mycobank numbers must be added for new species and taxonomic changes. See http://www.mycobank.org/

3.10.2 Biological Material: If the biological material (e.g. enzyme) has not been identified as to species, the manuscript will not be considered for publication unless a special protocol has been followed. Thus, a voucher specimen of the organism should be deposited with a recognized taxonomist for the particular group of organisms in question. The taxonomist should then assign to specimen an identifying number unique to the organism so that any additional collections of the same organism would bear this same number. The number will be retained until the organism is completely identified. The taxonomist should write a brief taxonomic description to be included in the manuscript, which should state how the organism relates morphologically to known species.

Herbarium: In a separate paragraph, experimental biological material should be reported as authenticated if cultivated or from a natural habitat, and the herbarium deposit cite and voucher number should be recorded.

Authors who purchase dried 'herbal remedies' or other materials from companies must make provision for their proper deposit in a herbarium, for access by future workers. When a commercially available extract is obtained, the extraction procedure from the organism of origin must be specified. The identification of the extract should be supported by an HPLC trace of known secondary metabolite constituents of the organism, which should be included in the manuscript.

**3.11 Phylogeny:** For phylogeny, only bootstrap values ≥ 95 should be included at the internodes. The accession number of the sequences should be given in brackets after the genus / species / strain number and all type culture strain numbers should be indicated by a superscript T. The full names of the abbreviations of the culture collections and the unit of the scale bar should be included in the figure legend.

**3.12 Abbreviations**

In general, abbreviations use should be restricted to a minimum. Abbreviations should be restricted to SI symbols and those recommended by the IUPAC. Abbreviations must be defined in parentheses after their first mention in the text. Standard units of measurement and chemical symbols of elements may be used without definition in the body of the paper.

## 4. TYPES OF MANUSCRIPTS

The following six (6) categories of manuscripts will be accepted. Authors should note that the format, word counts and page length (approximate) restrictions apply for each manuscript type as described below. Authors are also advised to peruse section 5, below, and organise their manuscripts

for submission so that it meets the requirements for the double blinded review process

**4.1 Original Articles:**

Original articles should describe complete research work and should not include preliminary research.

*Structure:* Full length research papers follow the AIMRAD standard structure. with the following headings: Abstract, Introduction, Methods, Results and Discussion followed by References and Acknowledgement.

- Abstract
- Manuscript Body
  - Introduction: The introduction should be on a separate page and it should be in context to the work being presented and should clearly state the purpose and objectives of the research. The introduction should be succinct and provide only the necessary background information, rather than a comprehensive treatise of the specific field. It should not contain subheadings.
  - Methods: This section should not be extensively descriptive but should contain sufficient detail so that, in conjunction with cited references, all experimental procedures can be reproduced by others. Essential technical detail or full descriptions of materials that are not of immediate importance for the understanding of the manuscript may be removed into Supplementary information, based upon the advice of peer-reviewers. Laboratory chemical and biochemical supply firms should be indicated and commercial companies and institutions who may have provided analytical services should be included in this section. Usage of statistical tools in experimental design is highly encouraged.
  - Results & Discussion: Results and Discussion can be presented under separate headings or as a combined heading. This can be further divided into sub-headings. The presentation of experimental detail in the Results & Discussion section(s) should be kept to a minimum. Reiteration of information that is made obvious in tables, figures, or reaction schemes should be avoided. Within the discussion, brief speculation on the implications of the reported findings may be included if appropriate. Discussion should be in depth and should be written in such way that it should prominently highlight the novelty or new advancement in the present knowledge based on framed objectives or research questions.
  - Conclusions: If an optional conclusion section is used, its content should not substantially duplicate the abstract.
- Accession numbers
- Acknowledgements
- Conflict of Interest and Author Contributions.
- References
- Tables and Figures

**4.2 Review Articles:**

Review papers are normally invited, but prospective authors are encouraged to contact the members of the Editorial Board to discuss possible contributions. Review papers will describe particular topics of current interest or controversy within the scope of 3 BIOTECH. Review papers should be a critique / critical evaluation of the topic and not a mere regurgitation of information.

*Structure*: The review should include the following headings but should not include an "Experimental" section:

- Abstract
- Main text arranged under subheadings

- Should end with a Conclusion section.
- Acknowledgements
- Author contributions and conflict of interest statement
- References
- Tables and Figures

**4.3 Short Research Reports:**

Short Research Reports should describe important preliminary findings from innovative research that deserve immediate dissemination. Research reports should be of high scientific quality and should not present poorly elaborated research and basic scientific data.

*Structure*: Short Research Reports should contain the following headings only:

- Abstract
- Main text consisting of introduction, methods, results and discussion without any headings
- Accession numbers
- Acknowledgements
- Author contributions and conflict of interest.
- References
- Tables / Figures

**4.4 Protocols & Methods:**

Protocols submitted to the journal are proven experimental procedures that authors have successfully used in their laboratories and reported as part of their research work in a peer-reviewed journal. The submitted protocol should not be in press or under consideration by any other journal. As protocols are constantly evolving, subsequent modifications made by the authors to improve the protocol are acceptable for submission as long as there is no direct repetition of text between this protocol and previous publications of the method, as this would constitute plagiarism.

*Structure*: Protocols should contain the following headings only:

- Abstract
- Main text consisting of introduction, methods, results and discussion without any headings which must include a detailed protocol and the description must be detailed so that it can be easily reproduced.
- Accession numbers (if relevant)
- Acknowledgements
- Author contributions and conflict of interest statement.
- References
- Tables / Figures

**4.5 Genome / Metagenome Research Reports:**

Genome Reports are short research reports describing (a) high-quality draft (< 70 contigs) and completely assembled and annotated genome sequences of microorganisms and viruses or metagenomes of significance / relevance to biotechnology, in the fields of agriculture, the environment, or human health AND (b) the genome sequence data / analysis is supported by relevant biotechnological experimental evidence using whole cells or biomolecules.

*Structure*: Genome Research Reports should include the following seven (7) sectional headings

- Title Page:
- Abstract:
- Genome Reports do not have any sub-headings,
    - Brief introduction
    - Methods, Results, Discussion and Conclusion should be combined into one. For this,

it is preferable that an experimental method is described and is than followed by the results of this method. A second method is described which is than followed by the results of the second method and so on. The last paragraph(s) is reserved for discussions and conclusions.

  - A MixS Table is compulsory: Authors should comply with community metadata standards the "Minimal Information about any (X) Sequence" (MixS) when submitting their sequence data to GenBank, ENA or DDBJ. A MixS checklist can be found at http://gensc.org/projects/mixs-gsc-project/ and MIxS standard information can be found at http://www.ebi.ac.uk/ena/submit/mixs-checklists. A MIxS table has to be included in the manuscript.

- Accession Number(s): The assembled genome and all relevant sequences must be deposited in NCBI's GenBank, ENA or DDBJ and all accession numbers for the data obtained must be provided. This data must be made publicly accessible at the time of submission to 3 BIOTECH.
- Acknowledgements
- Author contributions and Conflict of Interest statement
- References:

**4.6 New Research Reports:**

The New Research Reports category

- should be original and novel.
- Will focus only on new diseases and new diagnostic molecular markers & methods of plant, humans, insects and animals.
- will be a repository of research records that will support researchers, field workers and diagnostic personnel undertaking work related to biotechnology.
- is not intended to be an interim / preliminary report prior to publication of a full length report.

*Structure*:

- The words "First Report" must be included in the manuscript title.
- No sub-headings should be used
- An Abstract is not required
- A maximum of 800 words (up to 2 pages) is allowed.
- Accession numbers (if relevant)
- Acknowledgements
- Author contributions and conflict of interest statement
- References (maximum of up to 6 is allowed)
- Tables and Figures (A combined total of up to 2 is allowed)

## 5. ORGANISING MANUSCRIPTS FOR SUBMISSION

The journal uses a double-blind review review process in which both the author(s) and reviewer(s) identities are not revealed to each other during the review process and this requires that author anonymity remains when preparing a manuscript for submission. For the authors identity to remain anonymous, the following seven (7) files must be prepared and submitted in the same order via the online Editorial Manager at https://www.editorialmanager.com/bitc/default.aspx.

The contents of each file is described below:

File 1: Anonymity File which contains 6 sections (6 to 7 pages)
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File 3: Body of manuscript (continuous text, multi-page file)
File 4: Table (each table is on a new page, multi-page file)
File 5: Figure (each figure is on a new page, multi-page file)

File 6: Legend to Figures (one to two page file)
File 7: Supplementary Data (multi-page file)

**<u>File 1 Anonymity File</u>**: This file contains six (6) sections: 1. Manuscript Title and Author information, 2. Accession numbers, 3. Acknowledgments, 4. Statements (Conflict of Interest and Author Contributions), 5. A list of 6 reviewers and 6. A mandatory cover letter. Further description is given below:

<u>1. Manuscript Title and Author Information</u>: This section contains three parts: Title of the manuscript, (part A), Author names and affiliations (part B) and Running Title (part C).

A. The title of the manuscript:

- should be concise (no more than 30 words) and accurately reflect the content of the manuscript.
- non-standard abbreviations are not allowed
- should be typed with all words in boldface capitals, double spaced and centered.
- serial titles are not acceptable.

B. Author names and affiliations: All authors who have contributed to the work should be listed below the manuscript title. For each author the following information is required.

- The full name of the authors with first name, initial(s) and surname / family name. The corresponding author's names must be asterisked.
- The full department / institute addresses with country of residence. Superscript numbers should be used to indicate the department, institution, city with postal code and country where the work was carried out, for each author.
- A valid INSTITUTE email address, an ORCID or Scopus ID, or a web link e.g. Research Gate, Google Scholar, must be provided as a means of verification.
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C. Running Title: A short title of the manuscript should be written

<u>2. Accession Numbers</u>:
The molecular data accession numbers for16S rRNA gene, other rRNA genes, ITS, WGS, SRA etc) or culture collection numbers for new taxa should be reported here.

<u>*3. Acknowledgements:*</u>
Funding source(s), acknowledging the assistance of colleagues should be reported here.

<u>*4. Statements:*</u>
The following statements should be included on a single page:

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**<u>OR</u>**
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spectroscopic, analytical or crystallographic services who are not co-authors, and any financial support that has been received. Editors and referees should not be acknowledged.

5. Nomination of Six (6) Potential Reviewers:
Up to 3 pages may be used for this section. Authors must provide the following information for nominated reviewers. In the event that the information is not provided, the manuscript will be returned to the author for correction and this may lead to significant delays in processing the manuscript.

*Selection Criteria:*

- Conflict of interest: None of the reviewers have a conflict of interest in reviewing the manuscript i. e they are not former supervisors, students, or recent collaborators, family members of any of the listed authors.
- Experience & Expertise: All reviewers must have expertise in the relevant category of the manuscript.
  - At least three (3) author nominated reviewers are experts in the field of the research described in the manuscript AND
  - at least two (2) nominated reviewers are international experts and are not from the home country of any of the author(s) AND
  - one author nominated reviewer has experience / expertise in the broad field of biotechnology

*Reviewer Information:*

- The name and title of the reviewer
- The reviewers Department, full name of the Institute, city and country
- The valid institutional email address
- Verification of the reviewer's expertise using their ORCID or Scopus ID, or a web link to their publication record.
- At least five (5) Keywords that describe the experience and expertise of the reviewers

6. Mandatory Cover Letter:
The letter can be of up to two (2) pages. The cover letter must include detailed information as set out below. In the event that the information is not provided, the manuscript will be returned to the author for correction and this may lead to significant delays in processing the manuscript.

*(a) Author verification:* A list of all authors together with the full department / institute addresses and valid institute email addresses. If institute email addresses are not available than a web link, ORCID or Scopus ID must be provided as a means of verification. The corresponding author should be asterisked.

*(b) Significance of the Work:* A brief paragraph explaining the significance of the work. A statement of the novelty of the work and how the work differentiates it from other similar work reported in the literature.

*(c) Originality of the manuscript:* A statement that the submitted manuscript is original, unpublished and not under simultaneous consideration by another journal.

**File 2. Abstract & Keywords:** The file is limited to only one single page and should include an abstract and key words:

- The abstract should be a summary of the scope of the work and the findings in no more than the words specified for each manuscript category described above.
- The abstract should convey the main finding of the paper and explain the ground-breaking

significance of the field together with a summary of the results.

- The abstract must exclude an introduction and must provide enough information that will allow a reader to understand the key findings of the research without the reader having to refer back to the main text.
- Non-standard abbreviations should be avoided and reference citations within the abstract are not allowed
- Up to five (5) keywords, which do not appear in the title, should be given in alphabetical order, immediately below the abstract.

**File 3 The Manuscript:** This file can have multiple pages but must include the following information.

- Depend on the type of manuscript selected for submission, the content of the file would include introduction. Methods, Results and Conclusions.
- References.

**File 4 Tables:** The table file may contain multiple pages and should contain all the tables.

- Each table should be written on a separate page.
- All tables should be numbered.
- Tables should be cited in the text in consecutive numerical order.
- For each table, supply a table caption (title) explaining the components of the table.
- Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.
- Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included below the table body.

**File 5 Figures:** The figure file may contain multiple pages and should contain all the figures.

- Each figure or each panel of figures must be on a separate page
- All figures must be numbered as reflected in the main body of the manuscript.

**File 6 Legend to Figures:** This file usually contains a single page with descriptions of all the figure legends

- All figure legends should be numbered according to the figure numbers
- A concise title of the figure legend should be provided.
- The legend should contain adequate information for the reader to gain an understanding of the figure without having to refer to the manuscript text.
- Any symbols or abbreviations used in the figure must be defined in the legend, even if this has already been done in the main text.
- Experimental details should, where possible, be given in the Methods section, and not in

**File 7 Supplementary Data:** Supplementary data can be included in this filename

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   ii. e.g. write "In our previous work [2]" and not "In the previous work of Author's_own_name et al. [2]"
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## 6. REVISION

If a manuscript is returned to the authors for amendments and the authors fail to resubmit the revision within the time frame indicated in the decision letter than it will be regarded as withdrawn. Additional time for revision can be granted upon request, at the editors' discretion. Only a single round of revision will be permitted.

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b. Clearly indicate in detail at the designated place on the web form and/or cover letter all changes / corrections that have been made to address the reviewers' recommendations / suggestions.

c. Revised manuscripts may be returned to authors for further modification of the scientific content and/or for shortening and language corrections.

## 7. PUBLICATION

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Authors will be sent an email with a link to download the proof. In the interest of speed, corrections should be returned within 48 hours. Only typographic corrections and other minor changes may be made in a galley proof and substantive changes will require editorial approval and may delay publication of the article.

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