UNIVERSIDADE FEDERAL DE PELOTAS

Programa de Pós-Graduação em Biotecnologia

Dissertação

Efluente da parboilização do arroz: Bioremediação e produção de biomassa

Gustavo Waltzer Fehrenbach

Pelotas, 2019

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Dissertação apresentada ao Programa de Pós-Graduação em Biotecnologia da Universidade Federal de Pelotas, como requisito parcial à obtenção do título de Mestre em Ciências (área do Conhecimento: Biotecnologia).

Orientador: Prof. Dr. Fábio Pereira Leivas Leite

Pelotas, 2019

Universidade Federal de Pelotas / Sistema de Bibliotecas
Catalogação na Publicação

F296e Fehrenbach, Gustavo Waltzer

Efluente da parboilização do arroz : bioremediação e produção de biomassa / Gustavo Waltzer Fehrenbach ; Fábio Pereira Leivas Leite, orientador. — Pelotas, 2019.

54 f. : il.

Dissertação (Mestrado) — Programa de Pós-Graduação em Biotecnologia, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, 2019.

1. Efluente. 2. Levedura. 3. Microalga. 4. Isolamento. 5. Viabilidade. I. Leite, Fábio Pereira Leivas, orient. II. Título.

CDD : 660.62

Elaborada por Ubirajara Buddin Cruz CRB: 10/901

BANCA EXAMINADORA

Prof. Dr. Fábio Pereira Leivas Leite
Universidade Federal de Pelotas – Centro de Desenvolvimento Tecnológico

Prof. Drª. Daniela Isabel Brayer Pereira
Universidade Federal de Pelotas – Instituto de Biologia

Prof Dr. Claudio Martin Pereira de Pereira
Universidade Federal de Pelotas – Centro de Ciências Químicas, Farmacêuticas e de Alimentos

Aos meus pais e irmão, principalmente a ti minha mãe. Fosse a força que me faltou em momentos de dor e a esperança nas horas de desespero. À vida, dedico.

Agradecimentos

Primeiramente, agradeceria a Deus. Faço isso na representação das pessoas que mais me aproximam ao sentimento e personificação dele, meus pais. Suspeito em uma próxima vida receber benção tão grandiosa quanto a existências destas duas pessoas que estão todos os dias no meu coração e pensamento. Vocês mostram de forma indireta todos os valores, caráter e amor que, nas formas mais simples, podemos sentir. Vocês justificam cada passo da minha caminhada e, poderia dizer, que sempre serão meu destino. A vocês, meu mais sincero obrigado e agradecimento, que a vida e nossa família possa enchê-los de bençãos, saúde, alegrias e razões para acordarem ainda mais felizes. Resumo meu carinho por cada um com um sincero amo vocês.

Aos meus colegas e amigos do Laboratório de Microbiologia (Lab 4); Diogo (Laboratório de Bioquímica e Bioprospecção) pela parceria nas longas quantificações de fósforo; Michel Gerber e Jéssica pela estrutura e pronta disposição do laboratório para realização de análises; Diego Gil de Los Santos que estimulou o questionamento e inter-relacionalização dos problemas previamente a realização dos experimentos; Daniel Arsand, ex orientador e um amigo que sempre está disposto a ajudar, obrigado por sempre acreditar no meu potencial; prof. Luciana Bica e sua equipe pelo imediato suporte e ajuda nas realizações de experimentos de fitotoxicidade; prof. Eduardo pela parceria que se consolidará na etapa seguinte deste projeto. E Aline, pelo compromisso, disciplina e amizade, obrigado por todos os momentos compartilhados, te “orientar” me obrigou a saber muito mais sobre tudo, fico feliz por termos crescido juntos nessa caminhada.

Ao meu orientador Prof. Fábio Leivas Leite, por aceitar um desconhecido engenheiro na sua equipe, permitindo que eu fizesse uso da criatividade e conhecimento para solucionarmos o problema apresentado nesse trabalho e direcionamento cirúrgico em muitos momentos.

Ao Programa de Pós-Graduação em Biotecnologia e toda sua equipe. A Coordenação de Aperfeiçoamento de Pessoal de Nivel Superior (CAPES) pela bolsa de estudo.

“Senhor!

Fazei de mim instrumento de tua paz!

Onde houver ódio, que eu leve o amor

Onde houver ofensa, que eu leve o perdão

Onde houver discórdia, que eu leve a união

Onde houver dúvida, que eu leve a fé

Onde houver erro, que eu leve a verdade

Onde houver desespero, que eu leve a esperança

Onde houver tristeza, que eu leve a alegria

Onde houver trevas, que eu leve a luz

Ó Mestre, fazei que eu procure mais consolar que ser consolado,

compreender que ser compreendido, amar que ser amado.

Pois é dando que se recebe,

é perdoando que se é perdoado

e é morrendo que se vive para a vida eterna”

Oração de São Francisco

Resumo

FEHRENBACH, Gustavo Waltzer. **Efluente da parboilização do arroz: Bioremediação e produção de biomassa.** 2019. 54f. Dissertação (Mestrado) – Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

A parboilização do arroz é um processo hidrotérmico que demanda elevado volume de água, gerando efluente de alta complexidade e potencial poluidor. O desenvolvimento de novos métodos de tratamento torna-se necessário, visto a limitação das técnicas atuais na tratabilidade deste efluente. O objetivo desse trabalho foi avaliar o potencial da bioremediação e obtenção de biomassa no efluente da parboilização do arroz (EP), utilizando como agentes microbiológicos a levedura *Saccharomyces cerevisiae* e microalga *Chlorella* spp. isolada da lagoa pós-tratamento terciário. Foram avaliados parâmetros físico-quimicos: fósforo (P), nitrogênio total (NTK) e demanda química de oxigênio (DQO); e parâmetros microbiológicos: viabilidade celular e biomassa. Os cultivos com *S. cerevisiae* foram realizados em frascos aletados de 1 L, 180 rpm, 28 $^{\circ}$C e pH 5.5, e apresentaram 17,28% de remoção de fósforo, 74% de NTK, 55% de DQO, 1.79 x 10^9 UFC.mL^{-1} de viabilidade celular e 1,52 g.L^{-1} de biomassa em 48 h. A aplicação de *S. cerevisiae* na redução dos parametros físico-químicos é uma alternativa de baixo custo para auxiliar no tratamento e redução da complexidade do EP com geração de biomassa de ampla aplicabilidade como co-produto. Os cultivos com a microalga *Chlorella* spp. isolada foram realizados em reator cilindrico *air lift* com aeração por bomba, 16 h de luz (2000 lux), pH 7 e 28 $^{\circ}$C, e apresentaram remoções máximas de 82,48% de DQO e 92,96% de TKN gerando 3,1 g.L^{-1} de biomassa após 18 dias de cultivo. As concentrações de P oscilaram mas não houve remoção durante o cultivo. Ainda assim, esses resultados sugerem que a aplicação da microalga *Chlorella* spp. é uma alternativa para redução das concentrações de DQO e TKN no EP com geração de biomassa de aplicação comercial. A dispensabilidade de suplementação e adequação do meio, além da simplicidade de cultivo e monitoramento, destacaram o potencial do microorganismos utilizados no tratamento do EP, gerando 1,52 g.L^{-1} de *S. cerevisiae* e 3,1 g.L^{-1} de *Chlorella* spp. e taxas de remoção suficientes para adequar o EP a niveis regulamentados ou reduzir a complexidade do efluente que é encaminhado para estação de tratamento de efluentes. Novos estudos serão realizados utilizando *S. cerevisiae* e *Chlorella* spp. em maior escala e nível de controle de parâmetros, a fim de otimizar remoções, modelar o processo de tratamento e o aumento de biomassa.

Palavras-chave: Efluente, levedura, microalga, isolamento, viabilidade

Abstract

Fehrenbach, Gustavo Waltzer. **Parboiled rice effluent: bioremediation and biomass production.** 2019. 54f. Dissertação (Master Degree) – Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Rice parboilization is a hydrothermal process that demands high volume of water, generating effluent of high complexity and polluting potential. The development of new treatment methods is necessary, considering the limitations of current techniques in the treatment of this effluent. The objective of this work was evaluate the bioremediation potential and biomass production in the effluent of rice parboilization (PE), using as microbiological agents the *Saccharomyces cerevisiae* yeast and microalga *Chlorella* spp., isolated from the pond after tertiary treatment. Physicochemical parameters were evaluated: phosphorus (P), total nitrogen (TKN) and chemical oxygen demand (COD); and microbiological parameters: cell viability and biomass. Cultures with *S. cerevisiae* were carried out in baffled flasks of 1 L, 180 rpm, 28 ºC and pH 5.5, and presented 17.28% of phosphorus removal, 74% NTK, 55% COD, 1.79 x 10^9 CFU.mL^{-1} of cell viability and 1.52 g.L^{-1} of biomass in 48 h. The application of *S. cerevisiae* in the reduction of physicochemical parameters is a low-cost alternative to assist in the treatment and reduction of PE complexity with the generation of biomass of wide applicability as a co-product. The cultures with microalgae *Chlorella* spp. were realized with 2000 lux, pH 7 and 28 ºC, and showed maximum removals of 82.48% of COD and 92.96% of TKN, generating 3.1 g.L^{-1} of biomass after 18 days of cultivation. P concentrations oscillate but there was no removal during cultivation. Nevertheless, these results suggest that the application of microalga *Chlorella* spp. is an alternative to reduce the concentrations of COD and TKN in the PE with generation of commercially biomass. The absence of supplementation and suitability of the medium, besides the simplicity of cultivation and monitoring, highlighted the potential of the microorganisms used in the treatment of PE, generating 1.52 g.L^{-1} of *S. cerevisiae* and 3.1 g.L^{-1} of *Chlorella* spp. and removal rates sufficient to match the PE to regulated levels or reduce the complexity of the effluent that is routed to effluent treatment station. Further studies will be performed using *S. cerevisiae* and *Chlorella* spp. on a larger scale and level of parameter control in order to optimize removals, model the treatment process and increase biomass.

Keywords: Effluent, yeast, microalgae, isolation, viability

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Lista de Abreviaturas e Símbolos

CONAMA: Conselho Nacional do Meio Ambiente

CONSEMA: Conselho Estadual do Meio Ambiente

DBO: demanda bioquímica de oxigênio

DQO: demanda química de oxigênio

EP: efluente da parboilização

N-NH3: nitrogênio amoniacal

N-NO3: nitrogênio nitrato

NTK: nitrogênio total Kjedahl

PE: parboiled effluent

SST: sólidos suspensos totais

Sumário

1 INTRODUÇÃO GERAL

O arroz (*Oryza sativa L.*) é o principal alimento de mais da metade da população mundial, considerado o segundo cereal mais importante para alimentação humana, superado apenas pelo trigo. No Brasil o consumo médio de arroz por habitante é aproximadamente 25 kg por ano. Não somente parte do hábito alimentar, o Brasil é um dos principais produtores: em 2016/2017 foi responsável por 11,963 milhões de toneladas, correspondente a 3,8% da produção mundial (Statista, 2018; CONAB, 2016; Brasil, 2015).

Após a colheita na lavoura, o arroz é encaminhado às indústrias para armazenagem, passando por etapas de seleção e secagem até que se atinja 12-13% de umidade, permitindo o armazenamento para posterior destino e beneficiamento. No Brasil, aproximadamente 25% da produção é destinada a indústria de parboilização,caracterizada por etapas de encharcamento, gelatinização e secagem complementar, a fim de obter uma maior concentração de nutrientes no interior do grão (Paraginsky et al, 2014).

O processo de parboilização requer elevado volume de água, gerando 1-4 m^3 de efluente por tonelada de arroz processado (Kumar et al, 2016; De Los Santos et al, 2012). O efluente da parboilização (EP) apresenta elevadas concentrações de fósforo (P), nitrogênio (NTK), demanda química de oxigênio (DQO) e demanda bioquímica de oxigênio (DBO), devido a solubilização dos nutrientes do arroz. Este aumento na complexidade da matriz do EP é um desafio para as estações de tratamento, devido ao significativo potencial poluidor e risco ambiental. Quando lançado acima dos padrões regulamentados acaba por reduzir os níveis de oxigênio dissolvido nos corpos hídricos, ocasionando a mortandade dos seres vivos no meio aquático (Felipi & Zanoteli, 2005). Atualmente as indústrias contam com estações de tratamento de efluentes, permitindo que esta cadeia produtiva opere com reduzido impacto ambiental. No entanto, as tecnologias atuais tem se apresentado insuficientes na redução dos níveis de fósforo, além do elevado custo para tratamento e adequação do efluente.

O lançamento do EP tratado em corpos hídricos somente é realizado após adequação aos parâmetros ambientais a niveis iguais ou inferiores aos estabelecidos e/ou atingir a eficiência de remoção mínima. No Rio Grande do Sul, o CONSEMA é o orgão responsável pela fiscalização e monitoramento, e estabelece na resolução CONSEMA nº 355/2017 os limites para emissão de efluentes em águas superficiais, inferiores a legislação federal (CONAMA). Os tratamentos convencionais não são suficientes para redução de fósforo a concentrações iguais e/ou inferiores á legislação, porém a eficiência de remoção é respeitada, permitindo que o efluente seja lançado aos corpos hídricos. A pesquisa por novas tecnologias para o tratamento de efluentes é necessária para redução de custos nas estações de tratamento, redução do risco ambiental e otimização da cadeia produtiva.

O uso de agentes microbiológicos na atenuação, transformação e/ou eliminação de contaminantes é denominado bioremediação (Dyvia et al, 2015). Esta técnica tem sido amplamente pesquisada devido a capacidade adaptativa dos micro-organismos, sustentabilidade do processo e lucrabilidade. Em efluentes industriais o uso de micro-organismos é realizado a décadas no polimento e remoção de nutrientes em sistemas de lodo ativado, reatores anaeróbios e *wetlands*. A biomassa de levedura cervejeira *Saccharomyces cerevisiae* é um resíduo do processo fermentativo industrial aplicado no tratamento de efluentes, biosorção e como agente bioacumulador (Ferreira et al, 2010; Safariková et al, 2005). O isolamento de micro-organismos em estações de tratamento e locais de acidente ambiental permite a obtenção de isolados adaptados as condições adversas locais. Dessa forma, também foi avaliado a aplicação de isolado microalgal no tratamento do EP.

Este trabalho teve como objetivo avaliar a aplicação de *Saccharomyces cerevisiae* e um isolado microalgal no tratamento do EP, visando a redução de parâmetros ambientais, viabilidade celular e produção de biomassa, a fim de dispor à indústria um método aplicável e rentável de tratamento.

2 REVISÃO BIBLIOGRÁFICA

2.1 Arroz parboilizado e efluente

A parboilização é um processo hidrotérmico realizado para gelatinizar o amido do grão e transferir vitaminas hidrossolúveis e sais minerais presentes no tegumento e no embrião para o interior do endosperma (Balbinoti et al, 2018). Após este processo os grânulos de amido estão fundidos irreversivelmente, reduzindo a quebra de grão, aumentando a resistência a pragas e estendendo o tempo de prateleira (Buggenhout et al, 2013; Heinemann et al, 2005). O arroz parboilizado foi primeiramente obtido através do simples processo de imersão em água, realizado a noite e posterior secagem ao sol. Esse processo foi aprimorado e hoje aproximadamente 25% da produção mundial de arroz é destinada á parboilização (Paraginsky et al, 2014).

O arroz passa por operações de limpeza e seleção antes de entrar no processo de parboilização, padronizando o cereal a ser processado. Em seguida é encaminhado para parboilização, composta por 2 etapas centrais: encharcamento e gelatinização.

Na etapa de encharcamento ocorre a umectação dos grânulos de amido através da entrada de água (60-70ºC) no grão previamente seco ao processamento. A temperatura da água é fator determinante para difusão da água no arroz e temperaturas superiores a 60ºC contribuem para redução de contaminação por bactérias anaeróbias, *Staphylococcus*, leveduras e bactérias acido-láticas, visto a duração do processo de imersão ser, em muitas indústrias, de 48 h (Dutta & Mahanta, 2014; Sridhar & Manohar, 2003). O teor de umectação é um dos fatores controlados em função das características de absorção de água, determinantes também para duração do encharcamento que normalmente está entre 6 e 9 horas – periodo médio para o teor de umidade do grão atingir 30-33% e ser encaminhado para gelatinização.

Após atingir o teor de umidade necessário, o arroz é encaminhado para gelatinização onde é aquecido em autoclave em condições específicas

segundo a variedade e que estão entre 10-30 min de duração, empregando pressões de vapor de 0,4 a 1,2 kgf.cm^2 a temperatura de 105-120^{o}C.

Por fim, o arroz é seco para permitir o descasque do arroz e conservação do produto. A secagem ocorre em 2 etapas: primeiramente, o arroz tem sua umidade reduzida a 18-20% em temperaturas elevadas para redução rápida do elevado teor de umidade pós autoclave (PAGE, 2019); em seguida, a segunda etapa de secagem é feita de forma intercalada em secadores ou por secagem natural, sob temperaturas inferiores á primeira etapa e umidade próxima a 13%. Com isso, a umidade interna do grão é padronizada e o grão é deixado em descanso para que a cariopse fique translúcida e com textura endurecida. Se bem sucedido, no fim da parboilização o arroz apresentará as trincas nos grãos soldadas, quebra reduzida e teor nutritivo superior ao arroz polido (PAGE, 2019; Heinemann et al, 2005).

Das etapas da parboilização, o encharcamento é considerado o ponto crítico (Amato et al, 1989) de maior risco ambiental devido ao requerimento de água, geração de efluente (1 - 4 m^3 por ton de arroz) e complexidade (Kumar et al, 2016). A Tabela 1 apresenta as características fisico-químicas do efluente gerado no processo de parboilização de 113 indústrias de arroz em West Bengal, Índia (Mukherjee et al, 2016).

Tabela 1: Características físico-quimicas do efluente da parboilização do arroz em West Bengal, Índia (Adaptado de Mukherjee et al, 2016).

Parâmetro	Faixa	Média
pH	3.8 - 6,7	5 ± 0,78
Condutividade elétrica (mS cm^{-1})	1,1 - 3,4	2.6 ± 0,76
P (mg. L^{-1})	3,99 - 72,05	39,24 ± 16,09
N-NH_3 (mg. L^{-1})	51,2 - 153,9	104,0 ± 19,7
NO_3-N (mg. L^{-1})	1,18 - 6,74	4,11 ±1,67
Oxigênio dissolvido (mg. L^{-1})	0,6 - 66,4	10,4 ±18,0
DBO (mg. L^{-1})	114,8 - 415,2	305,22 ± 71,19
DQO (mg. L^{-1})	218,0 - 1706,0	1130,51 ± 298,61
SST (mg. L^{-1})	6050 - 6550	6258 ± 186,74
Açúcar redutor (mg. L^{-1})	20,68 - 570,46	184,47 ± 155,35
Lignina oxidável (mg. L^{-1})	5,0 - 40,0	18,53 ± 9,39
Cor (cor cloroplatinato)	250,08 - 700,95	533,21 ±147,81

Fonte: Mukherjee et al, 2016

A similaridade no processo de parboilização de indústrias instaladas em países em desevolvimento gera efluentes com características semelhantes observado nos trabalhos de Gerber et al (2018), Mukherjee et al (2016) e De los Santos et al (2012). Os autores ressaltam a problemática na redução das concentrações de fósforo, nitrogênio e matéria orgânica, e segundo Queiroz & Koetz (1997), são parâmetros responsáveis pela dificil tratabilidade do efluente.

Fósforo e nitrogênio são os nutrientes mais importantes em requerimento quantitativo em plantas. No solo, fósforo está presente em alta concentração, representando 0,05% dos minerais, porém devido à baixa solubilização e fixação ao solo apenas 0,1% é diretamente metabolizável (Illmer et al, 1995). A Tabela 2 apresenta as formas nas quais o fosfato se apresenta na natureza, natureza química e solubilidade.

Tabela 2*:* Principais estruturas de fosfato existentes nos ecossistemas, classificadas quanto à sua natureza química e solubilidade (Adaptado de EMBRAPA, 2008).

Fosfato	Formas solúveis	Formas insolúveis
Inorgânico	PO_4^{3-}, HPO_4^{2-}, H_2PO^{4-}, H_3PO_4, ortofosfatos	Complexo fosfato-argila
	$Fe_2(H_2PO_4)_3$, monohidrogênio fosfato férrico	Complexo metal hidróxido
	$Ca(H_2PO_4)_2$ dihidrogênio fosfato de cálcio	Minerais, ex. apatita $Ca_{10}(OH)_2(PO_4)_6$
Orgânico	Compostos orgânicos dissolvidos	Fósforo complexado à matéria orgânica

Fonte: Gomes et al, 2000

O fósforo presente no solo está sujeito a diversas reações fisico-químicas e biológicas, onde uma pequena parte está diretamente disponível para absorção pelas plantas e micro-organismos. A absorção do fósforo no solo ocorre através da solubilização, como ocorre com os ortofosfatos através de vias de alta afinidade na epiderme de raízes e fungos (Bucher, 2007). A forma predominante do fósforo na fração inorgânica é adsorvido em superficies minerais e na forma orgânica absorvida ou adsorvida em biomassa ou compostos orgânicos do solo. Dessa forma, o fósforo presente no efluente pode acumular no ambiente, visto que não será diretamente metabolizado quando lançado ao meio ambiente.

As indústrias de arroz parboilizado empregam sistemas de lodo ativado e reatores anaeróbios para redução de nitrogênio e fósforo em suas estações de tratamento (Gaboardi et al, 2018), custosos e incapazes remoção total do fósforo. O uso de sistemas biológicos torna-se uma opção, visto que mecanimos de assimilação de fósforo em efluentes foram identificados em bactérias no lodo ativado, armazenando montantes de ortofosfato nas células na forma insolúvel de polifosfato, sendo esta a forma majoritária de armazenamento de fósforo nos microorganismos (Egle et al, 2016).

2.2 Leveduras

As leveduras são micro-organismos unicelulares eucarióticos distribuídos na natureza em folhas, flores, solo, água e animais de sangue quente (Martini, 1993). São fungos da classe dos ascomicetos e filo *Ascomycota*. Apresentam membrana celular definida, pouco espessa em células jovens e rígida em adultas. As leveduras não formam filamentos, são imóveis, quimio-heterotroficos e aeróbios facultativos. Se destacam pela habilidade de converter açúcares em etanol através da fermentação e são amplamente utilizadas na indústria alcooleira, fermentados e panificação (Oca et al, 2016).

O ciclo de vida das leveduras é sincronizado com o crescimento dos brotos, que ao atingirem o tamanho de células maduras são separadas da célula mãe. A formação de brotos pode ocorrer nas células mãe e filha até mesmo antes da separação celular (Oca et al, 2016). O crescimento das leveduras ocorre em quatro etapas características: lag, exponencial, estacionária e declínio. A fase lag é comumemente chamanda de adaptativa, pois é caracterizada pela adaptação da levedura ao meio de cultura e ocorre após a inoculação. Nesta etapa não ocorre divisão celular, apenas crescimento em tamanho, síntese de RNA, enzimas, coenzimas e atividades fisiológicas. Após adaptação, a levedura entra na fase exponencial e multiplica-se em taxa geométrica e constante, com o menor tempo de geração e dependente da disponibilidade de nutriente no meio. Na fase estacionária, a levedura tem sua taxa de multiplicação e morte próximas, não ocorrendo aumento no número de células - esta fase ocorre principalmente pela escassez de nutrientes no meio e acúmulo de produtos tóxicos. Após consumo de todo substrato e acúmulo de produtos tóxicos, a levedura entra na fase de declínio e a taxa de morte celular supera de multiplicação.

A levedura *Saccharomyces cerevisiae* é a espécie mais estudada e explorada, amplamente empregada na fermentação de açúcares do arroz, trigo, cevada, milho e açúcares presentes na farinha para produção de pães. O cultivo de leveduras em escala laboratorial é realizado em frascos, erlenmeyers e biorreatores, permitindo um eficiente controle de pH, temperatura, aeração e concentração de nutrientes. Os principais elementos químicos assimilados em

biomassa de levedura são: carbono, hidrogênio, nitrogênio, fósforo e enxofre (Piper et al, 2002).

A facilidade em mapear um gene de origem de determinado fenótipo para uma região do genoma de *S. cerevisiae* contribuiram para utiização das leveduras como modelo de célula eucariota, permitindo a realização de diversos estudos, sendo o primeiro organismo eucarioto com genoma sequenciado (Alberghina e Cirulli, 2010). Além disso, outros fatores foram importantes para escolha das leveduras nestes estudos: mecanismo simples de replicação, recombinação, divisão celular e conservação do metabolismo entre leveduras e eucariotos de maior porte (mamíferos) (Bostein e Fink, 2011). É estimado que 31% dos genes das leveduras são homólogos aos humanos (Herskowitz, 1988).

Saccharomyces cerevisiae é a principal levedura aplicada na indústria de bebidas e alimentos e utilizada como modelo de micro-organismo eucarioto (Bostein e Fink, 2011). Apresenta um tempo de duplicação próximo a 90 minutos e atinge densidade celular máxima de 2×10^8 $UFC.mL^{-1}$ em meio padrão *Yeast Peptone Dextrose* (Kovačević, 2015). Evidências moleculares em jarras retiradas das tumbas do reio Escorpião I, um dos primeiros reis do Egito, datam o uso de *S. cerevisiae* na fermentação do vinho em 3150 a.c (Cavalieri et al, 2003).

Além das aplicações em alimentos e genética, a levedura *S. cerevisiae* tem sido aplicada como ingrediente de alimentos funcionais, alimentação animal e tratamento de efluentes com elevada concentração de metais (Ferreira et al, 2010).

2.3 Microalgas

As algas são organismos fotossintetizantes presentes em rios, lagos, oceanos e efluentes. Apresentam rápida reprodução e toletantes a diferentes condições ambientais, faixas de pH, salinidade e temperatura (Khan et al, 2018). São classificadas quanto aos pigmentos em Chlorophyta (algas verdes), Rhodophyta (algas vermelhas) e Phaeophyta (algas pardas) e tamanho (macroalgas e microalgas). Microalgas são a base da cadeia trófica dos bentos e ocupam importante papel na composição do plâncton. Apresentam elevado valor nutritivo pois acumulam matéria orgânica na forma de lipídeos, proteínas, carboidratos, hidrocarbonetos, pigmentos e pequenas moléculas (Ruiz-Martinez et al, 2012).

Microalgas são fontes de diversos bioprodutos aplicados nos mais variados setores. Vitaminas e carboidratos extraídos de microalgas tem sido aplicados na alimentação humana e animal (Cuellar-Bermudez et al, 2015); pigmentos bioativos como clorofila, β-caroteno, carotenóides, ficobiliproteínas e astaxantina tem sido aplicadas em terapias tumorais, disordens neurais e doenças; ácidos graxos apresentam valor nutricional e aplicação médica. Seu potencial adaptativo e baixo requerimento nutritivo estimularam a aplicação em estações de tratamento de efluentes domésticos (Ibraheem, 1998), suinocultura (Pouliot et al, 1986), processamento de alimentos (Rodrigues e Oliveira, 1987) e metais pesados como cádmio, mercúrio e arsênio (Cai-XiaoHua et al, 1995).

O crescimento microalgal depende da interação entre fatores biológicos e físicos-químicos (Raven, 1988). Os fatores biológicos estão relacionados com a interação com outros organismos e as taxas metabólicas de cada espécie de microalga. Moawad (1968) relatou que os fatores ambientais favoráveis ao crescimento algal são desfavoráveis para a sobrevivência de coliformes e organismos patogênicos. Os fatores físico-quimicos que afetam de forma direta o crescimento são: temperatura, pH, luz, nutrientes e agitação/aeração.

A temperatura de cultivo é geralmente próxima a observada no ambiente nativo da microalga – para organismos de clima polar, as temperaturas são mantidas inferiores a 10 ºC; clima temperado entre 10-25 ºC e tropical superior a 20 ºC. Intensidade de luz e duração dos ciclos claro/escuro são fatores

limitantes no cultivo de microalgas, afetando diretamente a fotossíntese, composição bioquímica e rendimento da biomassa. O intervalo ótimo de pH para a maioria das microalgas é entre 7-9, porém há espécies resistentes e com ótimo de atividade em condições extremas. As microalgas não são capazes de crescer em condições de alta e baixa iluminação, exigindo a determinação experimental da melhor condição para a microalga em uso a fim de maximizar a assimilação de CO_2 (Krzemińska et al, 2014). Em relação aos nutrientes, as microalgas requerem principalmente fósforo e nitrogênio, além dos macronutrientes (sódio, magnésio, cálcio e potássio), micronutrientes (molibdato, manganês, boro, cobalto, ferro e zinco) e elementos traço (Khan et al, 2018). Para manter o cultivo homogêneo e distribuir de forma uniforme as células, CO_2, nutrientes, luz e trocas gasosas, é necessário o emprego de motores com eixo para agitação e/ou aeradores (Zeng et al, 2011).

O cultivo de microalgas é realizado em condições específicas para estimular o armazenamento de matéria orgânica favorecendo o acúmulo de, por exemplo, lipídeos, carboidratos e proteínas (Frumento et al, 2013). O cultivo de microalgas pode ser realizado em sistemas abertos (Figura 1a) (raceway e circular) e fechados (Figura 1b) (placas, tubulares, tanques agitados e fotobioreatores *airlift*). A escolha do sistema dependerá do espaço disponível para instalação, parâmetros de produção/remoção, cepa utilizada, investimento, exigência de controle sob condições de cultivo, nível de esterilidade e outros fatores.

Figura 1: Sistemas de cultivo de microalga. a) Cultivo de microalga em tanques abertos tipo raceways. b) Cultivos fechados em fotobioreatores do tipo *air lift*. Adaptado de: https://microbioengineering.com/rw22-101;http://www.plant-biotech.net/bryotechnology_gallery/#3 (b)

O fotobiorreator *airlift* é um dos principais reatores que utiliza agitação pneumática. São ideais para cultivo em laboratório, visto a facilidade de operação, compacto, baixo custo, elevadas taxas de transferência de massa e calor, baixa sedimentação e fácil remoção/reposição de sólidos (Menna, 2010).

Os fatores biológicos e físico-químicos necessários para desenvolvimento de algas são específicos de cada cepa, permitindo ou não o desenvolvimento no ambiente, rios, estações de tratamento e locais contaminados (Lee et al, 2014). A coleta de organismos em áreas contaminadas é uma das estratégias das técnicas de bioremediação, pois permite o isolamento de cepas adaptadas ao meio e, que possivelmente, serão capazes de remediar essas concentrações quando seu crescimento for favorecido. Dessa forma, essa técnica tem sido explorada a fim de facilitar a descoberta de micro-organismos viáveis para uso, reduzindo o tempo com processos de *screening*. Além disso, o isolamento de cepas adaptadas permite aplicação no tratamento de outros resíduos de característica e condições semelhantes.

3 HIPÓTESES E OBJETIVOS

3.1 Hipótese

Os micro-organismos selecionados são capazes de bioremediar as concentrações de fósforo, nitrogênio e matéria orgânica no efluente da parboilização do arroz gerando biomassa.

3.2 Objetivo Geral

Remover fósforo, nitrogênio e matéria orgânica do efluente da parboilização do arroz utilizando *Saccharomyces cerevisiae* e *Chlorella* spp. gerando biomassa com viabilidade celular e interesse comercial.

3.3 Objetivos Específicos

- Isolamento e identificação de microalgas da estação terciária de tratamento de efluentes;
- Avaliar a produção de biomassa e viabilidade celular para os cultivos utilizando *S. cerevisiae* em efluente da parboiloização do arroz;
- Avaliar a produção de biomassa para os cultivos utilizando *Chlorella* spp. em efluente da parboilização do arroz;
- Avaliar a remoção de P, DQO e NTK no efluente da parboilização do arroz utilizando *Saccharomyces cerevisiae* e *Chlorella* spp.;

Manuscrito 1

Jornal: Applied Microbiology and Biotechnology

Environmental biotechnology or Biotechnological products and process engineering

Springer - ISSN: 0175-7598 (Print) 1432-0614 (Online)

IF: 3,34

4.1 Manuscrito 1: *Saccharomyces cerevisiae* on the parboiled rice effluent: Bioremediation and biomass production

Article

***Saccharomyces cerevisiae* on the parboiled rice effluent: Bioremediation and biomass production**

Gustavo Waltzer Fehrenbach[1] · Aline Sitowski[1] · Diogo La Rosa Novo[2] · Diego Gil de Los Santos[3] · Fábio Leivas Leite*[1]

*Fábio Leivas Leite E-mail: fleivasleite@gmail.com;fabio_leite@ufpel.edu.br

[1] Centro de Desenvolvimento Tecnológico, Biotecnologia, Universidade Federal de Pelotas; 96160-000. Pelotas, RS, Brazil.

[2] Centro de Ciências Químicas, Farmacêuticas e de Alimentos – Universidade Federal de Pelotas; 96160-000. Pelotas, RS, Brazil.

[3] Instituto Federal Sul-riograndense, Campus Pelotas, 96015-360. Pelotas, RS, Brazil.

Received: xx May 2019 / Revised: xx May 2019 / Accepted: xx May 2019

Abstract

Yeasts are widely employed in food industry such as beverage and probiotics. Recently, new applications as effluent and waste treatment are being studied. Parboiled effluent (PE) represents a risk to environment due to high concentration of nutrient and organic matter. The potential of *Saccharomyces cerevisiae* bioremediation of phosphorus (P), total Kjedahl nitrogen (TKN) and chemical oxygen demand (COD) in parboiled rice effluent was evaluated. At 48 hours of culture we obtained removals of 17%, 74% and 55% of P, TKN and COD, respectively. The removals were followed by a biomass production of 1.52 g. L^{-1} with high cell viability of 1.79 x 10^9 CFU. mL^{-1}. Moreover, *Saccharomyces cerevisiae* tolerated the variations on parameters and maintained removals and cell viability during a study of 2 years with sampling every 3 months. These results suggest that *S. cerevisiae* is an alternative for phosphorus, nitrogen and organic matter removals and biomass production, reducing the complexity of parboiled effluent matrix and producing a marketable yeast biomass.

Keywords *Saccharomyces cerevisiae* · Parboiled effluent · bioremediation · biomass · cell viability

1. Introduction

Rice is one of the most produced cereal in volume, holding a unique social and economic importance. In 2017 the world rice production was 483 million of tons (Statista, 2018) and Brazil had a production (2016/2017) of 11.963 million of tons corresponding to 3.8% of world production (CONAB, 2017).

The products obtained from rice productive chain are assigned to human and animal feeding (Streck et al, 2018), probiotic production (Zhuang et al, 2018) and alcoholic beverages (Hittinger et al, 2018). Parboiled rice is obtained by serial processes of polishing, cooking and rice drainage, producing by the end of the process high volumes of effluent (±2 L per kg of rice). This effluent shows high concentrations of nitrogen, phosphorus, chemical oxygen (COD) and biologic oxygen demand (BOD) representing a risk to environment. If not handled properly, this effluent can lead to environmental contamination and aquatic damage, posteriorly reaching humans due to water reuse in irrigation and consumption (Felipi & Zanoteli, 2005).

The complexity of parboiled effluent (PE) matrix increases the costs with effluent treatment and consequently of final product. Gerber et al (2016) and Mukherjee et al (2016) reported similar concentrations of total nitrogen (23.5 – 153.9 mg. L^{-1}), phosphorus (3.99 – 72.05 mg. L^{-1}), BOD (350.3 - 1148 mg. L^{-1}) and COD (218 - 1706 mg. L^{-1}) in parboiled rice industries in Brazil and India, respectively. In Brazil, phosphorus receives more attention due to strict limits of discharge and inefficiency of actual techniques on removal under the limits stablished by environmental laws (Pan et al, 2018). The nutrient concentration in the discharged effluent not only favors the development of harmful algae as alters food network in aquatic ecosystems, limiting the dissolved oxygen and leading the reduction of aquatic life (Conley et al, 2009). In soil, industrial effluents are anthropogenic sources of distinct nutrients, may lead to environmental disruptions (Liu et al, 2015), where, for example, the enhanced nitrogen deposit can cause a nutrient imbalance and soil acidification.

Effluent treatment process is normally a sequence of physical, chemical and biologic treatments. The necessity of environmental protection and costs reduce are pushing the development of new technologies. Effluent is started to be treated as a potential bioproduct to many industrial sectors such as: production of lactic acid (Watanabe et al, 2012), bioethanol (Watanabe et al, 2009), bacteria biomass (Bastos et al, 2013) and microalgal biomass (Mukherjee et al, 2016). Besides that, the absence of toxic metal contaminants (Gerber et al, 2016) and high nutrient concentration motivates the

exploration of PE for different uses.

Yeasts are unicellular fungi used as biocatalytic to food industry, and generally some recognized as safe (GRAS). Due to versatile, yeasts are being employed in many effluent and waste treatment as vinasse (dos REIS et al, 2018), landfill leachate (Wichitsathian et al, 2004) and effluents with heavy metals (Machado et al, 2010). Yeast biomass generated along the treatment can also be applied as source of B-complex vitamins, microorganism substrate, food ingredients and animal feeding (Ferreira et al, 2010). The objective of this study was to culture *Saccharomyces cerevisiae* on effluent of parboiled rice industry and evaluate the removal of elements that might represent a risk to environment and produce biomass with high cell viability.

2. Material and methods

2.1 Microorganism and experimental conditions

Saccharomyces cerevisiae (*S. cerevisiae*) was kindly supplied by the Biotechnology Center of the Federal University of Pelotas (UFPel), Brazil. Yeast inoculum was prepared transferring a colony of *S. cerevisiae* from yeast malt agar (Sigma-Aldrich®) to liquid yeast malt (YM) and cultivated at 180 rpm in shaker (Certomat BS-TA), at 28 ˚C for 16 h. YM (positive control for cell viability and biomass) and parboiled effluent (PE) (experimental media for cell viability, biomass, chemical oxygen demand, total Kjedahl nitrogen and phosphorus) were inoculated with *S. cerevisiae*: both medias were inoculated with 10 % (v/v) of *S. cerevisiae* at ~ 1.2 x 10^4 CFU. mL^{-1}. A negative control for parameters removal and biological growth was prepared in PE without yeast inoculum (PE-). All experiments were made in baffled flasks of 1 L containing 200 mL of medium at initial pH of 5.5.

2.2 Sampling and storage of PE

PE was obtained from a local industry at the city of Pelotas – Brazil (31º 46' 19" S, 52º 20' 33" W). Samples were collected from the process of parboiled rice in sterilized plastic vials. The PE samples were sterilized by autoclaved for 25 min and stored at 4 ºC until used.

2.3 Cell viability

Yeast cell viability and growth (CFU. mL^{-1}) was evaluated at 0, 12, 24, 36, 48 and 72 h in yeast malt (YM) and parboiled effluent (PE). A triplicate sampling of 500 µL from YM and PE at each interval was used to determine colony-forming unit (CFU). Serial dilutions were

made in sterile distilled water and each dilution spread onto YM agar plate. The plates were incubated at 28 °C for 48 h to counting.

2.4 Biomass determination

Biomass quantification (g) was made collecting a sample of 10 mL from PE and YM cultures at 0, 12, 24, 36, 48 and 72 hours and transferring to sterile vials. The samples were centrifuged at 2500 *g* (Kubota - KR 600) for 15 min and pellet washed two times with sterile water before let dry at 60 °C and weighted in analytical balance (Shimadzu - ATY224) until constant weight was obtained, indicating the absence of humidity.

2.5 Analytical determination of phosphorus, total nitrogen and chemical oxygen demand

Triplicate samples of 500 µL from YM, PE and PE- were collected for phosphorus (P), total nitrogen (TKN) and chemical oxygen demand (COD) determination at 0, 24 and 48 h. Samples were stored frozen until analysis, then at room temperature (25 °C) were centrifugated (Kubota - KR 600) at 3000 *g* for 5 min and supernatant used for analysis.

Phosphorus was determined by colorimetric method of phosphate vanadiummolibdate (NBR 12772, 1992), transferring 2 mL of molibdateantimonium (LabSynth) to 10 mL volumetric flask followed by respective sample and dilution. Analytical curve was prepared adding different volumes of phosphate (PO_4^{3-}) standard 1000 mg. L^{-1} on 1, 2.5, 5, 7.5 and 10 mg. L^{-1} concentrations. The determinations were made in triplicate and verified on UV-VIS (Kasuaki – IL592) at 400 nm, quantifying the colorimetric intensity of complex antimoniumphosphatemolibdate. Phosphorus (mg. L^{-1}) was obtained converting phosphate results through molecular weight.

Total nitrogen was determined by HI93767B-50 Hanna® TKN kit, transferring 500 µL and potassium persulfate to digestion tube and heated in a heated block (Hanna® HI839800) at 105 °C for 30 min. Then, at room temperature sodium metabisulfite was added and mixed. After 3 min of reaction, total nitrogen reagent was added and tube left reacting for 2 min. The last step was transfer 2 mL from tube to tube containing colorimetric reagent and the color was analyzed in photometer Hanna® HI8339902.

COD was determined by HI93754C-25 Hanna® COD kit, transferring 200 µL of sample to digestion tubes containing dichromate and digestion was realized at 150 °C for 2 h. Colorimetric reaction was analyzed in photometer Hanna® HI8339902.

2.6 Statistical analysis

The results were analyzed on *Statistica* software version 10 (Statsoft) by *student's t-test* comparing means and $p < 0.05$ was considered significant. All experiments were done, at least three times in triplicates.

3. Results

The results of cell viability for PE and YM at 0, 12, 24, 36, 48 and 72 h are shown in Figure 1. The growth tax of *S. cerevisiae* in PE on intervals of 12 h were 102.45 (0-12 h), 0.23 (12-24), 0.308 (24-36 h), 0.916 (36-48 h) and 4.16 (48-72 h) $CFU.mL^{-1}h^{-1}$; and in YM: 224.83 (0-12 h), 0.341 (12-24 h), 0.066 (24-36 h), 0.091 (36-48 h) and 0.175 (48-72 h) $CFU.mL^{-1}h^{-1}$. The maximum cell viabilities obtained were: 9×10^{10} CFU. mL^{-1} at 72 h for PE and 2.8×10^{8} at 48 h for YM. Statistical difference ($p \leq 0.05$) was observed between PE and YM at 24 ($p = 0.0036$), 48 ($p = 0.0015$) and 72 h ($p = 0.0170$) signaled on Figure 1.

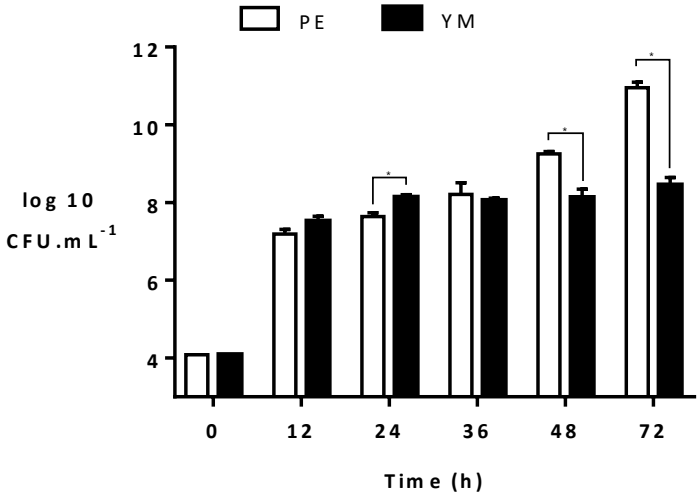

Figure 1: Cell viability in PE and YM. The data represents the number of viable cell of *S. cerevisiae* in Colony formed unit (CFU) (mean +/- SD) at 0, 12, 24, 36, 48 and 72 h in Parboiled Effluent (PE) and Yeast Malt (YM). Asterisks (*) means statistical difference ($p \leq 0.05$) between PE and YM at same time point.

The biomass (g) results at 12, 24, 36 and 48 h for PE culture were 0.31, 0.91, 1.06 and 1.52 g. L^{-1} and 1.45, 3.03, 4.25 and 4.9 g. L^{-1} for YM.

The removal rates (%) of phosphorus (P), total nitrogen (TKN) and chemical oxygen demand (COD) for 48 h of culture in PE and PE- (negative control) are shown in Table 1. The parameters concentrations were compared at 0 and 48 h on each culture and statistical difference was obtained for removals in PE for all parameters (COD, TKN and P); TKN removal was statistically significant in PE-.

Table 1: Removal rates (%) of P, TKN and COD in parboiled effluent inoculated with *S. cerevisiae* and parboiled effluent. Standard deviations are presented by ± SD and *p* values of removals between 0 and 48 h are showed.

Medium	% P ± SD	*P*	% COD	*P*	% TKN	*P*
PE	17.28 ± 1.13	0.0161	55.96 ± 1.17	0.0009	73.45 ± 5.75	0.0179
PE-	0.63 ± 0.108	0.7721	12.67 ± 0.89	0.2558	23.07 ± 1.44	0.0041

Saccharomyces cerevisiae growth curve results in PE at 0, 2, 4, 6, 8, 10 and 24 h are presented on Figure 3. 1. Cell viability increased 40, 2.01, 0.44, 1.68, 2.5 and 11.66 $CFU.mL^{-1}.h^{-1}$ between 0-2, 2-4, 6-8, 8-10 and 10-24, respectively.

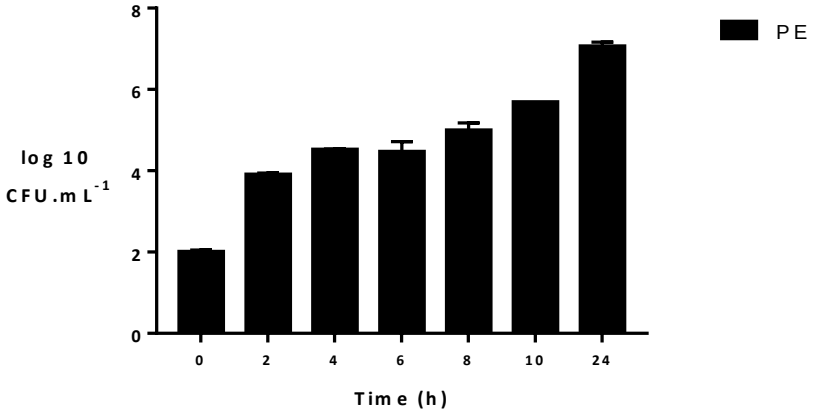

Figure 2: Dynamic of *Saccharomyces cerevisiae* growth (mean +/- SD) at 0, 2, 4, 6, 8, 10 and 24 h in parboiled effluent.

4. Discussion

The complexity of actual systems for wastewater treatment of parboiled effluent (PE) represents a risk for environment and cost to rice industry due to, demand of higher substrate removals, thus, stimulating research for new technologies. In the industry, wastewater treatment plant has a minimum retention time of ±15 days and in general composed by a pumping tank; a hydrodynamic sieve; an equalization/neutralization tank; an upflow anaerobic sludge blanket (UASB) reactor; and tertiary system for polishing (Gerber et al., 2018). In order to improve the removal of substrates and organic matter, we proposed a method for PE treatment.

Bioremediation systems use microorganisms to degrade, reduce hazardous waste, and removal contaminating substrate on effluent stations (Divya et al, 2015). *Saccharomyces cerevisiae* is widely employed in bioremediation process (dos REIS et al, 2018) so our

hypotheses was that using *S. cerevisiae* in PE we will be able to reduce environmental contaminants, P, COD, TKN, and have biomass production with viable cells.

Yeast cell multiplication occurs when carbohydrates are available in aerobic wort, allowing the oxidization of pyruvate to provide energy (Ratledge, 1991). To measure the *S. cerevisiae* growth, we employed the spread plate method that allowed the quantification of actively dividing/growing cells in the medium. PE is a complex residue that can be toxic or not sustain yeast growth. Cell viability is also a parameter to investigate, since many *S. cerevisiae* applications such as probiotic require a viable cell (Posteraro et al, 2005).

In this study we observed that *S. cerevisiae* cultured in PE as growth media kept a cell viability (CFU. mL^{-1}), similar a well stablish yeast media, YM, at 0, 12, 24, 36, 48 and 72 h. No toxicity for *S. cerevisiae* was observed in PE media, allowing its growth with no difference to YM (positive control for cell viability) at 12 h of experiment. At 24 h, the control medium YM showed a significant higher cell count than the PE, probably due to concentration of nutrients and carbon source easier consumed than in PE (Fig.1). Gaboardi et al (2018) and Muller et al (2007) related the consumption of approximately 90% of sucrose on first 6 hours of *S. boulardii* growth, leading to a reduced growth rate. One can suggest that similar effect might happen in PE, where that simpler sucrose sources present in the effluent are metabolized at beginning of lag phase. In order to investigate this fact, we repeated the experiment setup for PE and analyzed cell growth collecting samples at 0, 2, 4, 6, 8, 10 and 24 h (Fig 3).

Interesting, after the consumption of sucrose, another energy source was probably used in PE media, since after the 6 h we observed a steady cell growth until 72 h (Fig. 2, 3). However, was out of the scope of this study analyze the possible metabolic route used by *S. cerevisiae* in PE media. The growth in YM was stopped at 24 hours, and the number of viable cells was constant until the end of the experiment at 72 hours.

In PE media the cell growth increased from 2 to 4 h reaching 3.4×10^4, however at 6 h a reduction in cell growth was observed (2.9×10^4), then, growth continuously until 72 hours. The growth in PE media was statistically superior at 36, 48 and 72 h, allowing to assimilate more complex nutrients present on parboiled effluent.

Biomass is the amount of viable and dead cells generated during the microorganism multiplication. The *S. cerevisiae* biomass is the second major by-product from brewing industry and is highly dependent of type and availability of nutrient, strain and conditions of operation (Ferreira et al, 2010). Watanabe (2009) report the economic viability of *S. cerevisiae* biomass for animal nutrition and distillery industry. In this study, the PE media allowed a linear biomass increase (R^2= 0.9506) of *S. cerevisiae*, reaching 1.52 g. L^{-1} of

biomass after 48 h of grow, whereas for YM a 4.9 g. L^{-1} was obtained. One might expect this result since the organic substance was 2.87 folds higher and protein 7.27 folds higher for YM. Furthermore, the nutrients in YM are available at adequate concentrations. Other authors obtained similar results using a bioreactor culture for biomass production: Gaboardi et al (2018) obtained 3.8 g. L^{-1} with 1% of sucrose supplementation in parboiled effluent, and Gil de los Santos et al (2012) obtained 2.1 g. L^{-1} a different yeast, *Pichia pastoris* X-33, supplementing parboiled rice effluent with 15 g. L^{-1} of biodiesel-derived glycerol. In this study we observed that PE allowed the growth of *S. cerevisiae* without supplementation, reducing the substrate concentration and producing a yeast biomass of high cell viability as product with no extra cost. We expect to increase the biomass results with new studies as evaluating the culture in bioreactor and principally increasing oxygenation, a critical parameter to achieve good growth in low carbon sources that uses respiratory growth (Olivares-Marin et al, 2018).

Rice industry generates high volumes of parboiled effluent per day with non-valuable destination due to direct destination to effluent treatment station. Our partner industry generates an average volume of 750.000 L. day^{-1} of parboiled effluent that could produce 1.140 kg of *S. cerevisiae* biomass and reducing the contaminating substances assisting the effluent treatment.

The analytical determination of P, COD and TKN in parboiled effluent at 0 h showed average concentrations of 150, 574.5 and 137.5 mg. L^{-1}, respectively. With the cultivation of *S. cerevisiae* in this PE we observed a great potential for desired substance removal, reaching removals of 17.28% for phosphorus, 73.45% for TKN and 55.96% for COD. Brazilian environmental laws stablish the limits to the discharge of treated industrial effluent in rivers, so is necessary to obtain an efficient removal of P, COD, and TKN. In this study, *S. cerevisiae* allowed the reduction of TKN to a level 15 mg. L^{-1} higher than the allowed (20 mg. L^{-1}), 47 mg. L^{-1} under the concentration for COD (300 mg. L^{-1}), 117 mg. L^{-1} and 57% higher for P (75% or 3 mg. L^{-1}). Phosphorus had the lower removal in PE, probably due to medium composition and concentration in inadequate proportion to promote phosphorus uptake by yeast cells. Hattingh (1963) suggests that concentrations of COD: N: P were at maximum 81: 4: 1 for optimal removal of phosphorus in sludge - in our study this proportion was ~4: 1: 1, reducing the bioremediation of phosphorus. The retention of inorganic phosphorus is not only a need of industrial sector to respect limits stablished by environmental laws but also comes as an opportunity to reduce the massive application of TKN fertilizers on the soil. Furthermore, clean phosphate rock reserves are estimated to end until 2059 (Natasha Gilbert, 2009) and new methodologies to recover this mineral are being developed in effluents and waste. The biomass obtained can be used as source of phosphorus for agricultural uses; animal feeding due to high protein concentration and probiotic effects; grow medium and/or supplement and source of specific vitamins and

nutrients (Suarez and Guevara, 2018; Broadway et al, 2015; Ferreira et al, 2010).

On this way, to increase the phosphorus removal its necessary a more profound study on parameters applied during the treatment such as pH, temperature, O_2 concentration. The modification of PE with supplementation and residues from other industries might improve removal rates and obtain more coproducts suggesting a new application for PE (Gil de Los Santos et al, 2012). Related works applying the yeast *Saccharomyces boulardii* on PE supplemented with 1% sucrose obtained 78%, 49% and 74% of P, TKN and COD removal in 48 h. Also, one might consider that this option can be followed by complications with logistic operations, costs and complexity, treatment process, environmental authorizations, dependence of external materials and partners.

In this study we identified a variation of pH, COD, TKN and P in PE collected during 2 years with interval of 3 months. These variations on parameters were also verified by Queiroz and Koetz (1997) and Mukherjee et al (2016) and occur mostly due to seasonally and process variations. On this scenario, the microorganism had to tolerate, endure and adapt to maintain its industrial applicability. *S. cerevisiae* showed robustness and maintained proportional removals to parameters variations, high cell viability and wasn't negative influenced. Our process can directly be applied to support the nutrient removal on effluent treatment station and yeast biomass can be used to fish feeding (Lara-Flores et al, 2002; Rumsey et al, 1991), as source of nutrients for *Lactobacillus* (Champagne et al, 2003; Rakin et al, 2004; Rakin et al, 2007) and animal feeding (Broadway et al, 2015).

5. Conclusion

On this study, bioremediation potential of *Saccharomyces cerevisiae* in parboiled effluent treatment was able to remove 17.28% of phosphorus, 74% of TKN and 55% of COD, and high cell viability (1.79×10^9 CFU. mL^{-1}) and 1.52 g. L^{-1} of biomass were also obtained. These results suggest that *S. cerevisiae* is a promising alternative for bioremediation of PE and yeast biomass production, reducing the complexity of parboiled effluent matrix and producing a widely applicable yeast.

6. Funding

We would like to thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for G.W.F scholarship.

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Manuscrito 2

Jornal: **Biochemical Engineering Journal**

Elsevier - ISSN: 1369-703X

IF: 3,26

https://www.journals.elsevier.com/biochemical-engineering-journal

4.2 Manuscrito 2: Bioremediation and biomass production by *Chlorella* spp. isolated from parboiled rice effluent

Bioremediation and biomass production by Chlorella spp*. isolated from parboiled rice effluent*

Gustavo Waltzer Fehrenbach, Aline Sitowski, Claudio Martin Pereira de Pereira, Fábio Leivas Leite*

*Corresponding author.
Email address: fleivasleite@gmail.com; fabio_leite@ufpel.edu.br

Abstract

The parboiling of rice is a process of high-water demand, generating 2 L of effluent per kilogram of processed rice. This effluent is recognized by high concentrations of P, COD and TKN, and is a potential environmental contaminant if not treated correctly. Bioremediation methods are replacing or being applied to support treatment stations due to non-expensive, polishing potential and sustainable alternative. On this paper we isolate a *Chlorella* spp. from tertiary treatment station of rice effluent and cultivated in parboiled effluent. The cultures were realized on *air lift* reactors with 3 L of PE and a control medium with Watanabe, 28ºC, pH 7, 16:8 and 2000 lux for 18 d. *Chlorella* spp. showed a highest biomass concentration in PE of 3.35 g. L^{-1}, 39-95% of TKN removal and 66-82% of COD removal. These results suggest that PE could be used as culture medium to produce microalgae biomass and bioproducts of high commercial value, in addiction to reduction of COD and TKN levels and environmental risk.

Keywords: Chlorella, Biomass, Effluent, Bioremediation, Cell viability

1. Introduction

Rice is one of the most important and widely consumed cereal worldwide. Food and Agriculture Organization (FAO) and United States Department of Agriculture (USDA) forecast a world rice production between 487.8 and 513 million tons for 2018/19; Brazil production is estimated to raise 136,000 tons to 8.16 million tons for the same period based on a 20-year trend updated in 2017/18 by Government of Brazil report (USDA, 2018) and is projected to be the largest exporter of rice in South America. Parboiled rice is one product obtained from rice productive chain and nearly 25% of Brazilian rice is designated to this sector (Paraginsky et al, 2014). The process to obtain parboiled rice demands a high volume of water and generates an average volume of 2 L of parboiled

effluent (PE) per kg of rice (Gaboardi et al, 2018). PE is recognized as potential contaminant due to high levels of COD, TKN, P and absence of effective treatment to reduce these parameters and therefore the risk to the environment.

The conventional system for parboiled effluent treatment is composed by physicochemical and anaerobic/aerobic steps, demanding a high volume of chemicals, complex monitoring and operational costs. Biological techniques are a trend due to necessity of nutrient reuse, costs reduction, environmental protection and sustainability – aspects that are harder to obtain applying complex chemical compounds on the treatment plant.

Alternatives for PE treatment are arising with several researches on biological treatment systems as cyanobacteria and microalgae (Queiroz et al, 2007; Bastos et al, 2014; Abinandan et al, 2015; Mukherjee et al, 2016), yeast (Gil de los Santos et al, 2012) and wetlands (Gerber, 2002). Microalgae are photosynthetic microorganisms useful on treatment stations due to rapidly grow, ability in incorporate difficult-removal nutrients such as phosphorus and nitrogen, widely application of generated microalgae biomass, CO_2 fixation and energy recovery (De la Noue and De Pauw, 1988; Brenan and Owende, 2010; Abdel-Raouf et al, 2012). They have been applied in a many variety of residues as piggery effluent (Martin et al, 1985a; Pouliot et al, 1986), agricultural wastes (Phang and Ong, 1988), effluent from food processing factories (Rodrigues and Oliveira, 1987), toxic minerals (Soeder et al, 1978; Cai-XiaoHua et al, 1995) and also on parboiled effluent (Mukherjee et al, 2016).

The isolation of wild microorganisms from effluent treatment stations or contaminated sites is interesting due to adaptation to nutrient levels and environment, increasing the bioremediation potential. Besides that, exogenic microorganisms generally fail or shows low activity on bioremediation process since the environment is different from controlled conditions (Díaz-Ramírez et al, 2003; Venosa and Zhu, 2003). Therefore, the tertiary treatment station of parboiled rice effluent represents an adequate niche for microalgae development due to nutrient concentrations and exposure to sun. The objective of this study was isolate promising microalgae for removals of total Kjedahl nitrogen and organic carbon demand and biomass production in parboiled rice effluent.

2. Material and Methods

2.1 Effluent collection and storage

PE was obtained from a local industry at the city of Pelotas – Brazil (latitude 31.646790, longitude 52.340367). Samples were collected from the process of parboiled rice in sterilized plastic vials. The PE samples were sterilized by autoclaved for 25 min and stored at 4 °C until used.

2.2 Microalgae isolation and experimental conditions

Samples from the last pound of rice effluent treatment station where collected in sterile vials of 50 mL and immediately transported at room temperature to Microbiology Laboratory – UFPEL (Brazil), stored at 2 °C. BG11 agar plates (Himedia®) were prepared to isolate microalgae and cyanobacteria from treatment station: 60 µL of collected sample were transferred to each of a triplicate BG11 agar plate and incubated for 7 days, 28 °C, light/dark cycle (16:8) and 2000 lux. Colonies with different morphology were then selected and transferred to different Erlenmeyer flasks containing 200 mL of Watanabe, consisting of 1.25 $g.L^{-1}$ KNO_3 (NEON), 1.25 $g.L^{-1}$ KH_2PO_4 (Synth), 20 mg. L^{-1} $MgSO_4$ (Synth), 20 mg. L^{-1} $FeSO_4$ (Synth) and 1 $ml.L^{-1}$ of A5 solution (2.9 $g.L^{-1}$ H_3B_3O, 1.81 $g.L^{-1}$ $MnCl_2$, 0.08 $g.L^{-1}$ $CuSO_4$, 0.018 $g.L^{-1}$ $3(NH_4)O_7MoO_3$ and (Synth) 0.11 $g.L^{-1}$ $ZnCl_2$), cultivated for 7 days at 28 ºC, light/dark cycle (16:8) of 2000 lux and pH 7. The mediums were centrifuged at 2000 g (Kubota - KR 600) for 10 min and obtained pellet inoculated in Erlenmeyer flasks with 200 mL of PE with pH 7 and cultivated for 7 days, 28 °C, light/dark cycle (16:8) and 2000 lux.

The microalgae with higher cell number in Neubauer chamber was selected and culture centrifuged at 2000 g (Kubota - KR 600) for 10 min. The pellet of biomass was weighty on analytical balance (Shimadzu - ATY224) and inoculum prepared in PE with 0.5 $g.L^{-1}$ of biomass and 20% v/v. The experiments were carried out in 4 L column photo-reactor with 3 L of PE of culture for 18 days, 28 °C, light/dark cycle (16:8), pH 7, 2000 lux and air injection. The regime of operation was maintained fed-batch since the volume lost with evaporation was replaced. Every 48 h the pH was measured and calibrated to 7. A culture in Watanabe medium was realized for grow and biomass control at same experimental conditions as PE.

2.2 Analytical determination of total nitrogen and chemical oxygen demand

Triplicate samples of 1 mL from PE and Watanabe were collected for total Kjedahl nitrogen (TKN) and chemical oxygen demand (COD). TKN and COD were determined at 0, 2, 6, 10, 14 and 18 d. Samples were stored frozen until analysis, when at room temperature (25 °C) were centrifugated (Kubota - KR 600) at 3000 g for 5 min and supernatant used for analysis.

Total nitrogen was determined by HI93767B-50 Hanna® TKN kit, transferring 500 µL and potassium persulfate to digestion tube and heated in a heated block (Hanna® HI839800) at 105 °C for 30 min. Then, at room temperature sodium metabisulfite was added and mixed. After 3 min of reaction, total nitrogen reagent was added and tube left reacting for 2 min. The last step was transfer 2 mL from tube to tube containing colorimetric reagent and the color was analyzed in photometer Hanna® HI8339902.

COD was determined by HI93754C-25 Hanna® COD kit, transferring 200 µL of sample to digestion tubes containing dichromate and digestion was realized at 150 °C for 2 h. Colorimetric reaction was analyzed in photometer Hanna® HI8339902.

2.3 Biomass

Biomass quantification (g) was made collecting a triplicate sample of 10 mL from each media at intervals of 48 h and transferring to sterile vials. The samples were centrifuged at 2000 g (Kubota - KR 600) for 10 min and pellet washed two times with sterile water before let dry at 60 ºC until constant weight. The dried pellets were weighted in analytical balance (Shimadzu - ATY224).

2.4 Statistical analysis

The results were analyzed on *Statistica* software version 10 (Statsoft) by *student's t-test* comparing means and $p < 0.05$ was considered significant. All experiments were done, at least three times in triplicates.

3. Results

3.1 Microalgae isolation

The first isolation step resulted in a wide variety of cells in BG11 and obtained cells posteriorly cultivated in Watanabe liquid medium. From primary isolates, only two isolates of different morphology were viable and grow in parboiled effluent. Figure 1

shows the selected isolate that was in higher number at cell counting and used afterwards in photo-column reactor.

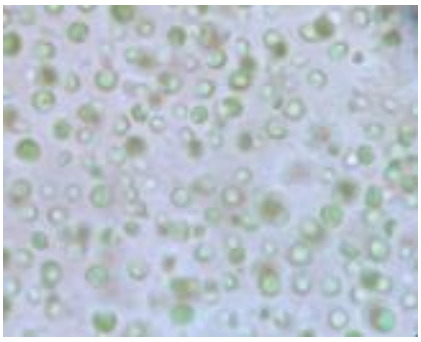

Figure 1: Isolated microalgae from tertiary effluent treatment station of rice industry and cultivated in parboiled effluent on column photo-reactor culture.

3.2 Biomass production

Chlorella sp. biomass in PE was compared to control medium Watanabe (Figure 2). A 4-order polynomial grow (R^2= 0.9945) suited the biomass results in PE and was higher than Watanabe from 0 to 12 d. Statistical difference ($p<0.05$) was observed at 0 (p= 0.0109), 2 (p= 0.0138) and 4 (p= 0.0019). From 12 to 18, Watanabe biomass was higher with no statistical significance. The maximum biomass obtained for PE was at 18 d (3.15 $g.L^{-1}$) and higher biomass tax between the days 0-2 (21.25 $mg.h^{-1}$) and 10-12 (14.27 $mg.h^{-1}$). The increasement of biomass in control medium Watanabe was linear (R^2= 0.9543) and reached the highest concentration of 3.35 $g.L^{-1}$ at 18 d.

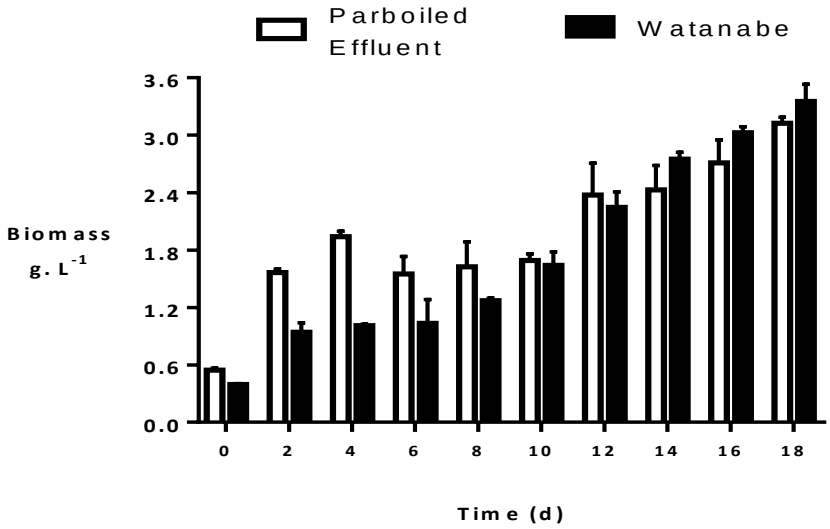

Figure 2: Biomass production in parboiled effluent and Watanabe medium. The data represents the biomass (mean +/- SD) at 0, 2, 4, 6, 8, 10, 12, 14, 16 and 18 d in parboiled effluent and Watanabe inoculated with *Chlorella* spp.

3.4 Parameters removal

Total Kjedahl nitrogen (TKN) and carbon organic demand (COD) removals at 2, 6, 10, 14 and 18 d were compared to 0 d concentration and presented in Figure 3. The maximum removals occurred at day 6 for COD (82.48%) and at day 14 for TKN (94.9%).

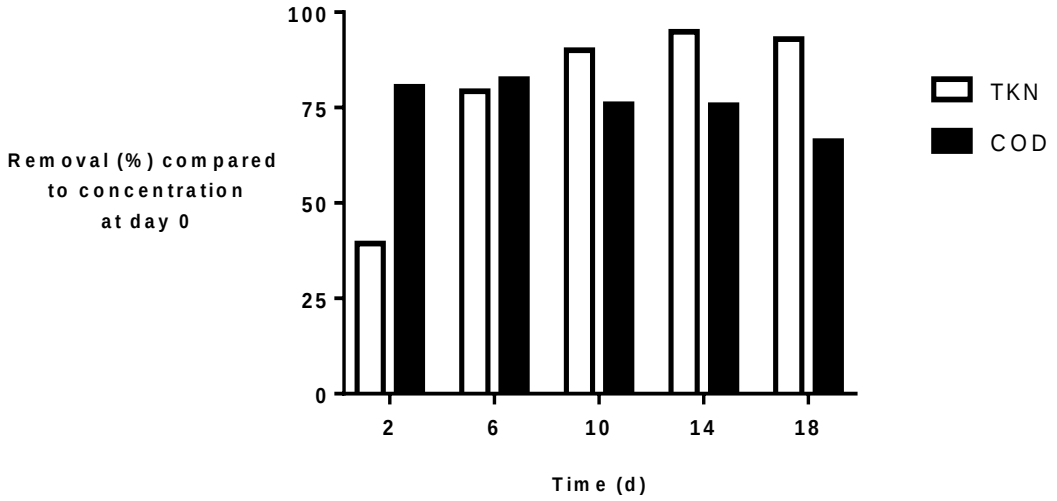

Figure 3: Total Kjedahl nitrogen and carbon organic demand removals (%). The removals are related to 0 d concentration of TKN and COD, not cumulative, at 2, 6, 10, 14 and 18 d.

4. Discussion

A wide variety of cells were first obtained in BG 11 plates from tertiary effluent treatment station. Nonetheless, isolated cells were under lower concentrations of nitrogen, phosphorus and organic matter than in parboiled effluent (PE). To select resistant and viable strains, a culture in PE at pH 7 was realized, obtaining 2 strains. This culture in PE also could lead to higher growth as reported by Mukherjee et al (2016) that obtained better results with pre-acclimatized microalgae and cyanobacteria. The isolate was selected quantitatively and morphological compared to algae bank (Guiry, 2019), identified as *Chlorella* spp. The objective of this research was obtaining a strain able to grow and produce biomass in PE medium, reducing environmental parameters levels, and the identification of obtained strain was not focused.

Chlorella species are primary producers in different ecosystems, easily adapted, versatile in mass cultivation systems and widely applicated biotechnologically (Huss et al, 1999; Safi et al, 2014; Krienitz and Bock, 2012). The *Chlorella* biomass is a product of economic interest and a low production cost is essential to make the process viable. Waste-grown microalgae shows as potential solution for remediation of high nutrient levels in industrial effluent and non-expensive system to produce biomass, when compared to standard mediums. *Chlorella* spp. maximum biomass is generally between 0.3 and 0.5 g. L^{-1} when cultivated in standard medium as BG11 (Li et al, 2010). The biomass obtained in parboiled effluent (PE) is presented on Figure 2 and reached a maximum of 3.15 g. L^{-1} at 18 d in a fed-batch culture and was higher than observed by many authors. Moreover, the highest yield rate obtained on this work was between 0-2 (21.25 mg. h^{-1}) and 10-12 d (14.27 mg. h^{-1}). In 18 d of *Chlorella ellipsoidea,* Yeang et al (2011) obtained 0.425 g. L^{-1} of biomass in the domestic secondary effluent of activated sludge treatment with low concentrations of phosphorus and nitrogen; Kim et al (2010) obtained a maximum of 0.13 g. L^{-1} of *Chlorella vulgaris* biomass in wastewater effluent with 9 d.

The PE was collected from a local industry that generates a diary volume of 750.000 L of effluent. CONSEMA is the environmental agency at state level on Rio Grande do Sul, stablishing more restrictive limits than federal environmental agency CONAMA, that regulates, in addition to other duties, effluent and emission limits. The discharge limits for COD and TKN stablished by CONSEMA (Resolution nº 355/2017) for this

effluent volume are: 300 mg. L^{-1} and 20 mg. L^{-1}, respectively. *Chlorella* spp. was able to reduce COD levels under 300 mg. L^{-1} in all sampled days. The COD was probably assimilated by microalgae during cellular reproduction, since the higher grow tax observed between 0-2 d was followed by high COD removals. The decrease on biomass at day 4 shows a change of organic source, as microalgae restart to accumulate biomass followed by a constant (82-75%) COD removal. TKN levels were highly consumed by *Chlorella* spp. and 1 mg. L^{-1} higher than discharge limit (20 mg.L^{-1}) after 14d and 94.9% of efficiency.

Converting by the maximum biomass obtained on our study, this volume could produce 2.362,5 kg of *Chlorella* spp. biomass. However, more researches are needed to upscale, improve nutrient removal and also direct the *Chlorella* spp. metabolism to accumulate specific substrates. Mature cells of *Chlorella vulgaris* can accumulate a total protein content between 42-58% of biomass dry weight, depending on growth conditions (Morris et al, 2008; Servaites et al, 2012), and are comparable or even better emulsifying agents than commercial ingredients (Ursu et al, 2014). In optimal conditions, *C. vulgaris* can reach 5-40% lipids per dry weight of biomass, with high potential to be applied on biodiesel (Becker, 1994). In low concentrations of nitrogen, total carbohydrates can reach 12-55% dry weight (Branyikova et al, 2011; Choix et al, 2012). Many other products can be extracted from *Chlorella* biomass as pigments (chlorophyll, carotenoids, astaxanthin, cantaxanthin and others), minerals (potassium, magnesium, zinc and others) and vitamins (B1, B2, B3, B5, B6, B7, B9, B12, E and A) (Yeh and Chang, 2011; Panahi et al, 2012; Singh and Gu, 2010; Chacón-Lee and González-Mariño, 2010; Kitada et al, 2009). Thus, *Chlorella* sp. biomass can be a co-product from rice productive chain due to wide application and industrial interest.

The removal levels correspond with industrial necessities, since the limits are respected in a process that generates a secondary product with high value and low retention time.

5. Conclusion

We successfully isolated two strains from the tertiary treatment station of parboiled rice effluent and evaluated the biomass of chosen microalgae due to cell number and over repro- duction, morphologically identified as *Clorella* sp. The biomass yield reached a high level and presents as coproduct. More studies are necessary to analyse biotic and

physical factors, nutrient uptake and grow parameters to improve biomass yield and treatment potential.

6. Funding

We would like to thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the master scholarship and research funding.

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5. CONCLUSÃO GERAL

Pode se concluir que a levedura *Saccharomyces cerevisiae* apresenta potencial bioremediador em efluente da parboilização do arroz, reduzindo as concentrações P, NTK e DQO e gerando uma biomassa de alta viabilidade. O isolado de *Chlorella* spp. obtido da estação terciária de tratamento de efluentes gerou elevada concentração de biomassa, reduzindo as concentrações de DQO e NTK em curto intervalo de tempo.

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