KELLY CRISTINA DOS REIS

BIOMASS AND VOLATILE METABOLITES PRODUCTION OF INDUSTRIAL INTEREST FROM VINASSE BY YEASTS

LAVRAS – MG

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Thesis presented to the Federal University of Lavras, as requirements part of the Graduate Program in Agricultural Microbiology, concentration area in Agricultural Microbiology to obtain the title of Doctor.

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"Ao homem pertencem os
planos do coração, mas
do Senhor vem a
resposta da língua."

Provérbios 16:1

"Um pouco de ciência nos afasta de Deus".
Muito, nos aproxima."

Louis Pasteur

RESUMO

Países como Brasil e México possuem bebidas destiladas típicas e mundialmente conhecidas, cachaça e tequila. A produção destes destilados é similar e apresentam um processo de produção que gera o resíduo liquido denominado vinhoto. Esse resíduo possui alto grau poluidor e necessita de alternativas para o seu aproveitamento. Propostas do uso direto e tratamento do vinhoto são conhecidas, mas ineficientes e aplicáveis à longo prazo. É neste contexto que o uso do vinhoto pode ser a alternativa viável para a economia e ao meio ambiente. Dessa forma, foram testadas leveduras de diferentes substratos do Brasil e do México quanto à capacidade de produzirem biomassa e compostos voláteis de interesse industrial a partir do vinhoto. Modelos estatísticos foram utilizados, para avaliar diferentes condições de cultivo na otimização da produção de biomassa. A quantidade de biomassa foi avaliada por peso seco. As análises de fatores nutricionais / anti-nutricionais foram realizadas na biomassa do isolado que apresentou maior produtividade. As leveduras que apresentaram bom desempenho foram avaliadas quanto a produção de compostos voláteis utilizando vinhoto de tequila e cachaça. A composição/quantificação dos compostos voláteis foram realizadas por HS-GC-FID (*Headspace* em Cromatografia gasosa acoplada ao Detector de ionização em Chama). Os resultados indicaram que o uso do vinhoto pode ser uma maneira econômica para a produção de "single cell protein" e compostos voláteis, aliados à redução de parâmetros poluidores. Leveduras selecionadas no aproveitamento do resíduo, quando em condições são ajustadas, pode ser uma alternativa promissora na produção produtos de alto valor agregado.

Palavras-chave: Bioaroma, resíduo, biorrefinaria

ABSTRACT

Brazil and Mexico have typical and world-known distilled drinks, cachaça and tequila. The production of these distillates is similar and both generate a liquid residue, known as vinasse. This residue presents high polluting degree and needs alternatives for its use. Proposals of direct use and treatment of vinasse are known, but inefficient and applicable in the long run. It is in this context, the use of vinasse could be the viable alternative for the economy and the environment. Yeasts from different substrates from Brazil and Mexico were tested for their ability to produce biomass and volatile compounds of industrial interest from this residue. Statistical models were used to evaluate different cultivation conditions in the optimization of biomass production. The biomass generated was evaluated by dry weight. Analyzes of nutritional and anti-nutritional factors were performed Yeasts showed best performance were used in the evaluation for the production of volatile compounds using tequila and cachaça vinasse. Composition and quantification of the volatile compounds were carried out by HS-GC-FID (Headspace in Gas Chromatography coupled to the Flame Ionization Detector). The results indicated that the use of vinasse can be an economically viable way for production of single cell protein and volatile compounds, along with the reduction of pollutant parameters. The main volatile compound detected was 2-phenylethanol. So, yeasts growth, when conditions are adjusted, can be a promising alternative in the production of high added value products.

Key words: Bioflavour, Residue, Biorefinery

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO GERAL

Os avanços tecnológicos têm provocado impactos no meio ambiente representados na forma de resíduos liberados na natureza sem seu devido tratamento. Nas indústrias produtoras de cachaça e tequila, um dos principais subprodutos é o vinhoto. A otimização dos processos para viabilizar a melhor utilização desses subprodutos e o desenvolvimento de outros processos que aumentem o seu valor tem sido objeto de atenção de indústrias do setor.

O vinhoto é um resíduo líquido caracterizado por um odor forte, coloração escura, baixo pH e elevados valores de DBO (demanda bioquímica de oxigênio) e DQO (demanda química de oxigênio). Esse resíduo, quando lançado em rios e lagos, provoca um alto grau de poluição comprometendo a sobrevivência dos organismos presentes nesse ambiente.

A fim de se eliminar a carga poluidora desse resíduo, inúmeras alternativas tecnológicas para o aproveitamento desses resíduos vêm sendo estudados, oferecendo muitas vantagens ao produtor. Entre as alternativas para o aproveitamento pode-se destacar: fertirrigação, geração de biogás através da digestão anaeróbica, produção de biomassa microbiana para suplementação animal (SCP - "*Single Cell Protein*"), o uso na construção civil (fabricação de tijolos), compostos voláteis (flavorizantes) de interesse industrial, entre outros.

Países da América e Europa, já têm desenvolvido pesquisas visando a utilização do vinhoto e assim, a redução do poder poluidor desse resíduo, além da geração de produtos de valor agregado.

A fim de auxiliar nas pesquisas, a microbiota de processos fermentativos tem sido estudada, devido a boa fonte de isolados microbianos com características de relevância industrial. As leveduras já têm sido utilizadas em processos envolvendo aproveitamento de resíduos e são rotineiramente utilizadas nesses processos biotecnológicos, por apresentarem tamanho celular e habilidade em crescerem em diversos ambientes e meios.

Gêneros como a *Candida*, *Saccharomyces*, *Kluyveromyces*, *Pichia*, *Rhodotorula*, têm sido cultivadas em águas residuais, hidrolisados e polpas, resíduos agroindústrias, para a produção de proteínas unicelulares (SCP) para suplementação animal e produção de metabólitos voláteis, entre outros.

2 REFERENCIAL TEÓRICO

2.1 Resíduos Agroindustriais

O gerenciamento inadequado dos resíduos tem proporcionado problemas de contaminação no solo e águas, pois apresentam grande concentração de matéria orgânica (MATOS, 2005; KARTHIKEYAN et al., 2010). Os resíduos, ao serem lançados em corpos hídricos, provocam redução na concentração de oxigênio dissolvido causando prejuízo aos ecossistemas aquáticos (YOKOMIZO et al., 2009).

A descarga de resíduos agroindustriais em rios provoca a diminuição da penetração de luz solar, diminuindo assim a atividade de fotossíntese e de oxigênio dissolvido (BHATIA & GOYAL, 2014).

Alguns problemas relacionados aos resíduos agroindustriais têm incentivado a utilização e a bioconversão de resíduos em produtos de alto valor agregado (CHAPLA et al., 2010). O uso desses resíduos, além de representarem uma matéria-prima de baixo custo, é também uma forma de reduzir o nível alarmante o efeito da degradação ambiental (GOLDEMBERG & LUCON, 2007).

Os resíduos gerados, assim como ocorre em águas residuais provenientes da indústria, necessitam de tratamentos adequados a fim atender os padrões de legislação ambiental quanto ao seu descarte (LAUFENBERG et al., 2003; SOCCOL & VANDENBERGHE, 2003).

Existem estudos para aplicações desses resíduos provenientes da agroindústria, como a utilização de fonte de carbono em bioprocessos para obtenção de produtos de maior valor agregado (BLANDINO et al 2001). Dentre os produtos destacam-se a produção de etanol, proteínas, enzimas, ácidos orgânicos, aminoácidos,

metabólitos secundários biologicamente ativos entre outros (HAHN-HAGERDAL, 2006; VENDRUSCOLO et al., 2008).

2.2 Cana-de-açúcar e Agave

A cana-de-açúcar é uma planta da família das gramíneas (Poaceae), originária do sudeste asiático (GUPTA et al., 2010). A família Poaceae, também representada por milho, arroz e outras gramíneas, tem como principal característica a forma da inflorescência (espiga), o crescimento do caule em colmos e as folhas com lâminas de sílica em suas bordas (LANDELL et al., 2014). O cultivo de cana-de-açúcar ocorre principalmente em países tropicais e subtropicais (UNICA, 2017).

Atualmente o Brasil é o maior produtor mundial de cana-de-açúcar, sendo o interior paulista a região que apresenta maior cultivo, devido ao clima favorável (MOZAMBANI et al., 2006; LIMA et al., 2011).

O cultivo dessa planta apresenta alto interesse econômico, pois é a principal matéria-prima de diversos produtos como açúcar (sacarose), álcool (etanol) e destilado (cachaça), além de alguns subprodutos como fertilizantes (vinhoto) e também geração de energia elétrica obtida através da queima de bagaço (UNICA, 2017).

Os biocombustíveis surgiram como fontes renováveis e, por isso, recebem uma atenção especial crescente (LANDELL et al., 2005). A sua produção à partir da cana tem se destacado, devido ao seu cultivo ter crescido de forma impressionante no Brasil, a ponto de se tornar uma cultura agrícola de grande importância para economia do país (UNICA, 2017).

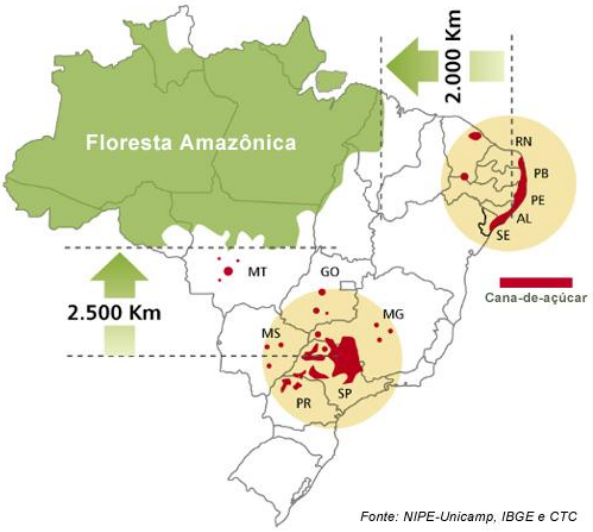

Figura 1. Mapa da produção de cana-de-açúcar no Brasil

No entanto, a produção em excesso desse resíduo faz com que seja necessário pensar em uma forma do seu aproveitamento (SPADOTTO, 2017).

O agave faz parte da família Agavaceae e pertence à classe de monocotiledôneas. Atualmente, existem aproximadamente 640 espécies que fazem parte do gênero entre elas se encontra o *Agave tequilana* Weber (BAUTISTA JUSTO et al., 2001). Devido a sua diversidade, o gênero Agave, está entre os mais conhecidos que faz parte dessa família (VÁZQUEZ-GARCÍA, 2007).

Agave são plantas perenes que geralmente adotam forma de roseta devido a disposição de suas folhas que crescem de forma espiral (VÁZQUEZ-GARCIA, 2007). As folhas são suculentas e caracterizadas por serem grossas, fibrosas, duras e terminação afilada e quase sempre com a margem espinhosa (BAUTISTA JUSTO et al., 2001). O *Agave tequilana* Weber Var. *azul* é a planta que faz parte dessa família utilizada na produção da Tequila, particularmente, não

suporta solos arenosos, argilosos, salinos e apresenta tolerância a acidez do solo, porém apresenta preferência a solos com pH neutro (pH 7) (BAUTISTA JUSTO et al., 2001).

Essa planta tolera falta de chuva, considerando que a umidade é retida no solo, porém quando plantadas em solos secos a floração é precoce (6 a 7 anos), mas nestas condições a concentração de açúcar é limitada, enquanto que em climas temperados se favorece a concentração de açúcar, porém a planta prolonga a floração até 8 a 9 anos (VÁZQUEZ-GARCÍA, 2007).

Figura 2. Mapa da produção de agave tequilero (*Agave tequilana* Weber) no México.

A produção de agave tequilero coexiste com o desenvolvimento da indústria de Tequila. A Tequila elaborada a partir do *Agave tequilana* Weber e o aproveitamento do agave, devido à raiz indígena, fazem com que Tequila seja caracterizada como símbolo da identidade nacional importante e a bebida mexicana mais conhecida no mercado mundial (CTR, 2016). No processo de produção da Tequila, assim como no de Cachaça, a produção gera resíduos sólidos e líquidos.

Nas figuras 3 e 4, são apresentados os fluxogramas de produção da Cachaça e de Tequila, respectivamente, os principais resíduos gerados e os produtos formados na biorefinaria.

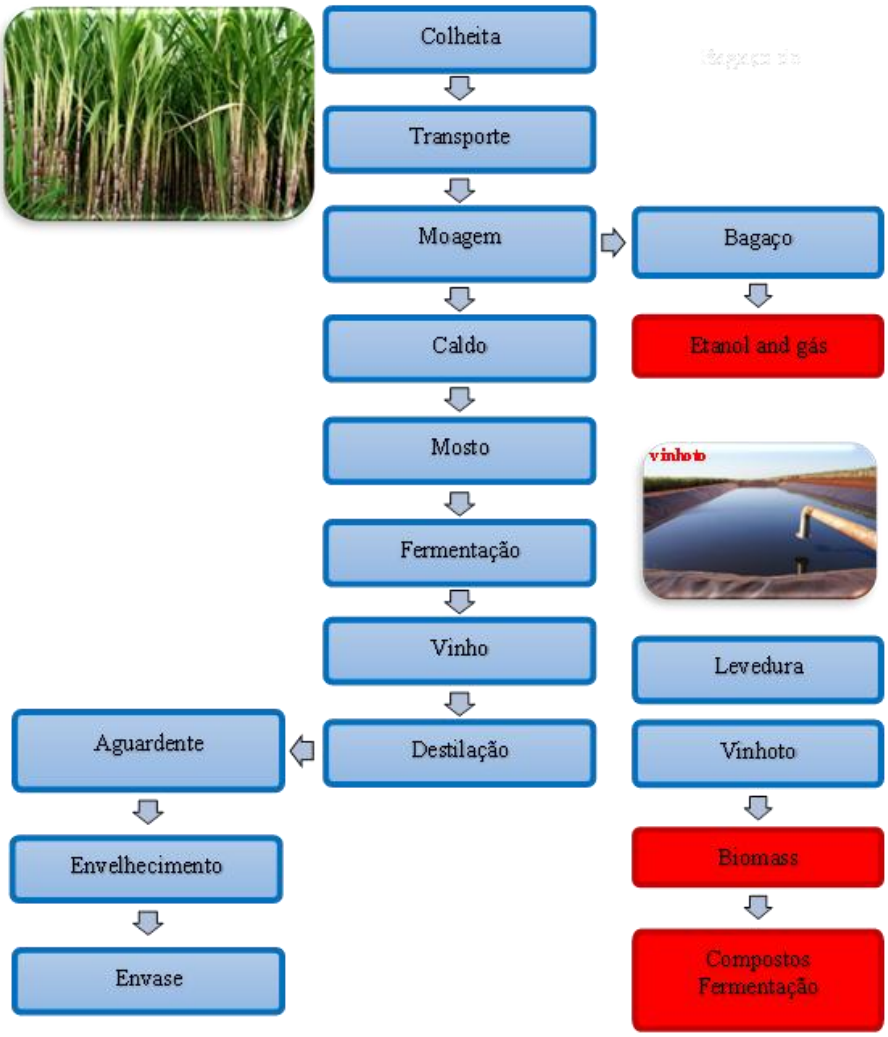

Figura 3. Fluxograma de produção de cachaça e principais resíduos gerados. Nas caixas em vermelho os produtos oriundos de um processo de biorefinaria.

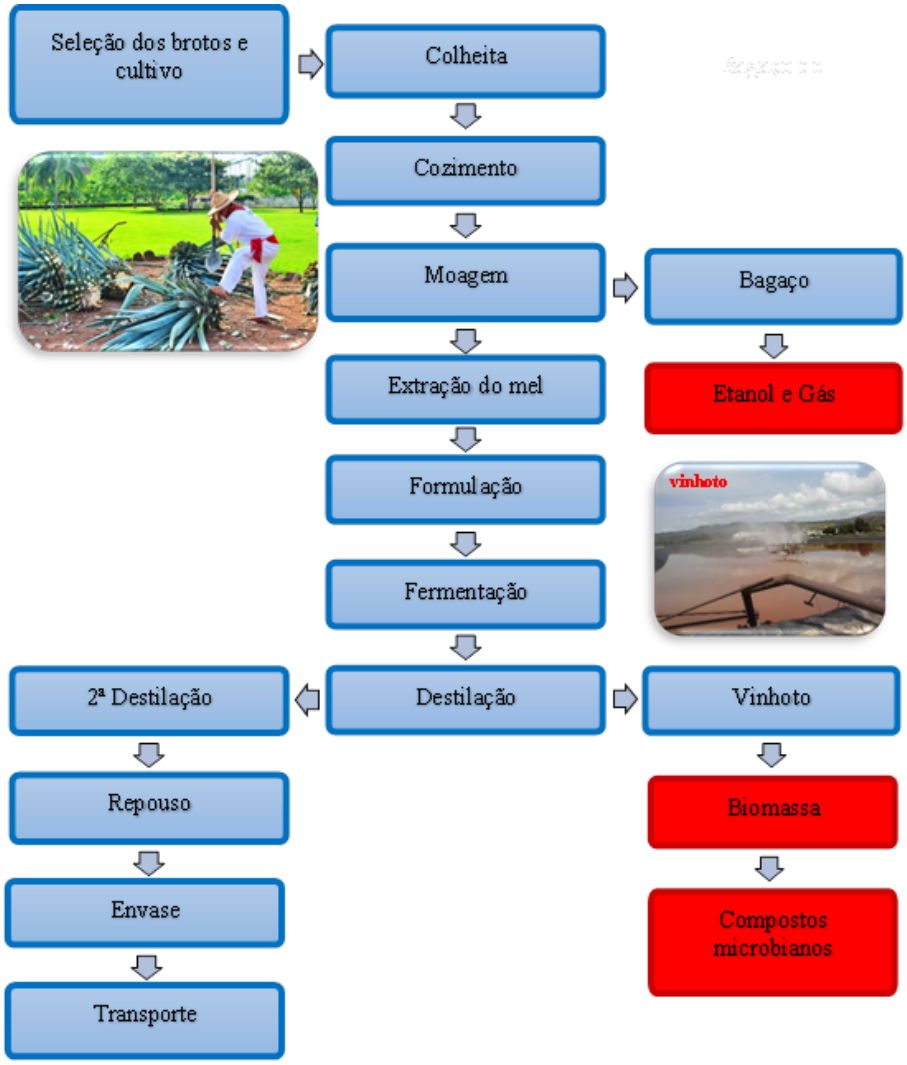

Figura 4. Fluxograma de produção de tequila e os principais resíduos gerados. Nas caixas em vermelho os produtos oriundos de um processo de biorefinaria. Na foto observa-se a pinha de agave que será utilizada na produção de tequila, sendo as folhas descartadas.

2.3 Vinhoto proveniente da produção de Cachaça e Tequila

2.3.1 Características Gerais do Vinhoto

O vinhoto, vinhaça ou restilo, é o resíduo líquido proveniente da destilação de caldo de cana-de-açúcar e pinha de agave após a fermentação do mosto no processo de obtenção de etanol e bebidas alcoólicas destiladas (OHGREN et al., 2006; BUITRÓN & CARVAJAL, 2010).

É um material com aproximadamente 6% de constituintes sólidos, onde se destaca a matéria orgânica em maior quantidade e presença de minerais em quantidade considerável como, potássio (K), Cálcio (Ca) e Magnésio (Mg) (RODRIGUES FILHO & OLIVEIRA, 2002). O vinhoto apresenta alto poder poluente e alto poder corrosivo, sendo considerado como o principal efluente das destilarias e álcool presentes nas indústrias sucroalcooleiras e tequileras (OHGREN et al., 2006; BUITRÓN & CARVAJAL, 2010). Esse resíduo possui composição química variável, dependendo da qualidade da matéria-prima utilizada, das etapas de fermentação, das condições industriais e do clima (SILVA et al., 2007).

A elevada produção nas indústrias implica em grandes volumes de vinhoto, sendo que nos alambiques de cachaça, são gerados, aproximadamente 9 litros de vinhoto para cada 1 litro de cachaça produzida e 7 a 10 litros de vinhoto por litro de tequila (CEDEÑO, 1995; CARDOSO, 2001, VAN HAANDEL, 2005).

2.3.2 Potencial Poluidor do Vinhoto

O vinhoto apresenta-se, de forma geral, uma cor escura e odor típico, pH ácido (em torno de 3,5) e elevado conteúdo orgânico, acarretando grande problemas ambientais, necessitando de tratamento

antes do descarte no ambiente. O constituinte principal do vinhoto é a matéria orgânica e sua riqueza nutricional está relacionada com a origem do mosto (SILVA et al., 2007).

Esses compostos orgânicos fazem com que o vinhoto se caracteriza como efluente de destilaria com alto poder poluente e alto valor fertilizante, sendo que o poder poluente é aproximadamente 100 vezes maior que o esgoto doméstico, devido ao elevado nível de demanda bioquímica de oxigênio (DBO) (SILVA et al., 2007).

O vinhoto gerado na produção de cachaça e tequila apresenta DBO de 35.000 a 50.000 mg/L e 25.000 a 60.000mg/L, respectivamente (KUMARI & PHOGAT, 2010; LÓPEZ-LÓPEZ et al, 2010). A carga de poluentes orgânicos em vinhoto apresenta também valores elevados de demanda química de oxigênio (DQO), entre 70.000-150.000 mg/L, podendo constatar que o vinhoto apresenta concentração elevada e cujo despejo, sem tratamento prévio, pode causar impacto ambiental grave (MENDEZ-ACOSTA, 2010).

A temperatura do vinhoto, quando liberado no meio ambiente, gira em torno de 50-100°C, se não resfriada antes de sua descarga em corpos d'água, provoca o aumento da temperatura da água e redução do oxigênio dissolvido, provocando a morte de peixes e outros organismos aeróbios (JIMENEZ et al., 2003).

A disposição inadequada desse resíduo representa um grande problema ambiental devido volume produzido, a alta concentração de matéria orgânica e sais dissolvidos, alta temperatura, coloração escura e presença de compostos tóxicos (JIMENEZ et al., 2003; PANT & ADHOLEYA, 2007). Na Tabela 1, pode ser observada a composição química do vinhoto usado para produção de biomassa microbiana visando à suplementação de alimentação animal (SILVA et al., 2011).

Tabela 1. Composição química do vinhoto derivado de caldo de cana utilizado para produção e biomassa microbiana para suplementação animal.

Parâmetros	**Valores**
Açúcares Totais (%)	0.18
Glicose (%)	0.14
Sacarose (%)	0.04
Solubilidade (%)	6.51
Teor de Água (%)	98.0
Proteína (%)	0.33
Cinzas (%)	0.24
Fibra Bruta (%)	0
Extrato de éter (%)	0.04
Carotenóides Totais (mg $100g^{-1}$)	0.003
Clorofila (mg $100g^{-1}$)	0.32
Nitrato (g $100g^{-1}$)	31.0
Pectina Total (mg $100g^{-1}$)	91.4
Pectina Solúvel (mg $100g^{-1}$)	5.9
Tanino (mg $100g^{-1}$)	80.47
Celulose (mg $100g^{-1}$)	0
Lignina (mg $100g^{-1}$)	0
Hemicelulose (mg $100g^{-1}$)	0
Acidez Total (v v^{-1})	0.4
Demanda Química de Oxigênio (DQO) (mg L^{-1})	57.500
Demanda Bioquímica de Oxigênio (DBO) (mg L^{-1})	1.900
Oxigênio dissolvido (mg L^{-1})	1.0
Sólidos totais (mg L^{-1})	24.560
pH	7.27

A composição química do vinhoto de tequila (Tabela 2) apresenta uma composição menos variável uma vez que a produção de

tequila é restrita a uma região do México com características de solo e ambientes específicos (Cedeño, 1995)

Tabela 2. Composição do vinhoto gerado durante o processo de destilação da Tequila.

Parâmetros	**Valores**
Lipídeos (mgL^{-1})	10–100
DQO Total (mgL^{-1})	60.000 a 100.000
DQO solúvel (mgL^{-1})	40.000 a 80.000
DBO Total (mgL^{-1})	35.000 a 60.000
DBO solúvel (mgL^{-1})	25.000 a 50.000
Sólidos Totais (mgL^{-1})	25.000 a 50.000
Sólidos suspensos Totais (mgL^{-1})	2.000 a 8.000
Sólidos suspensos (mgL^{-1})	10–500
Sólidos suspensos voláteis (mgL^{-1})	1.990 a 7.500
Sólidos dissolvidos totais (mgL^{-1})	23.000 a 42.000
Sólidos sedimentáveis (mgL^{-1})	10–900
Alcalinidade Total (mgL^{-1})	<6,00
Acidez Total (mgL^{-1})	1.500 a 6.000
Acidez fixa (mgL^{-1})	1.480 a 5.800
Acidez volátil (mgL^{-1})	20–200
Ca (mgL^{-1})	200–1.100
Mg (mgL^{-1})	100–300
K (mgL^{-1})	150–650
Fosfatos (mgL^{-1})	100–700
Nitrogênio Total (mgL^{-1})	20–50
Nitrogênio amoniacal (mgL^{-1})	15–40
Nitrogênio Orgânico (mgL^{-1})	5.0–10
Açúcares redutores totais (% w)	0.5–2.0
Açúcar (% w)	0.4–1.0

(conclusão)

Cu (mgL^{-1})	<3.0
Fe (mgL^{-1})	<45
Ni (mgL^{-1})	<0.02
Zn (mgL^{-1})	<1.0

A matéria orgânica é um dos componentes mais importantes do vinhoto, porém a presença de elementos como enxofre, potássio e em menor proporção, nitrogênio e magnésio, faz com que seja interessante a busca pelo aproveitamento desse resíduo, pois é uma fonte considerável de nutrientes (SILVA et al., 2007). Sendo assim, o conhecimento da composição química do vinhoto é fundamental para a orientação correta da sua utilização.

2.3.3 Alternativas para o aproveitamento do Vinhoto

O problema provocado pelo poder poluidor do vinhoto e seu lançamento indevido em rios, juntamente com a exigência legal fez com que as indústrias optassem por outras formas de descarte desse resíduo (CONAMA, 2005). Entre esses estudos estão a utilização agrícola do resíduo "*in natura*" para produção de fertilizantes (MADEJÓN et al., 2001; VACCARI et al., 2005); produção de biogás através da biodigestão anaeróbica (WILKIE et al. 2000); produção de proteínas unicelulares através da fermentação aeróbica para alimentação animal (RAJOKA et al., 2006; SILVA et al, 2011; PIRES et al., 2016).

Como foco deste trabalho, será dada ênfase no aproveitamento para produção de biomassa e de compostos microbianos de forma mais detalhada dentro do conceito de biorefinaria. Entre os vários conceitos está a **biorefinaria verde** (JERING & GÜNTHER, 2010),

que é o ramo que visa à produção de compostos via fermentação. Utiliza-se o resíduo usado para produção de biomassa microbiana, proteína e aminoácidos, além de compostos voláteis (JERING & GÜNTHER, 2010).

2.3.3.1 Produção de gás metano pelo processo de digestão anaeróbica

A digestão anaeróbica é um processo fermentativo que tem como finalidade a remoção de matéria orgânica, formação de gás e a produção de biofertilizantes mais ricos em nutrientes, portanto é uma alternativa promissora para alguns casos de poluição por resíduos (HOSSEINI & WAHID, 2014).

A tecnologia da digestão anaeróbica da vinhaça colabora diretamente para o desenvolvimento sustentável, além de dar um destino mais eficiente e adequado aos resíduos é também uma forma de geração de energia, o que tem atraído muitos produtores a pesquisar e a investir mais neste setor (ALBANEZ et al., 2016). Na biodigestão anaeróbica da vinhaça, ocorre uma redução significativa de carga orgânica, garantindo segurança em sua aplicação na fertirrigação sem alterar suas características nutricionais (OLIVEIRA, 2009).

O processo de biodigestão desse resíduo consiste no processamento de sua carga orgânica, este processo gera biogás e vinhaça biodigerida com reduzida carga orgânica, no entanto, sem alterações em suas propriedades fertilizantes (SZYMANSKI et al., 2010). A produção de gás pela biodigestão da vinhaça em usinas ou destilarias tem sido objetos de estudos e tentativas de viabilização comercial, porém surgiu o interesse de utilização do biogás para geração de energia elétrica (OLIVEIRA, 2009).

A digestão anaeróbica que ocorre no processo de produção de gás consiste na degradação da matéria orgânica, na ausência de oxigênio, por bactérias metanogênicas e este processo ocorre em duas etapas (BARROS et al., 2011). A primeira com a formação de ácidos orgânicos pela atuação das bactérias acidogênicas (BOUALLOGUI et al., 2003). Na segunda etapa os ácidos são transformados em produtos gasosos principalmente metano e gás carbônico (LASTELLA et al., 2002).

2.3.3.2 Produção de Proteínas Microbiana ("Single Cell Protein")

Single Cell Proteín (SCP) se refere à produção de proteínas unicelulares que podem ser cultivadas em diferentes fontes de carbono (NASSERI et al., 2011). O instituto de Tecnologia de Massachusetts criou, há aproximadamente 50 anos, criou o termo "*Single Cell Proteín*" (SCP) para descrever a idéia de proteína unicelular (SCP) admitida de forma universal para designar as células microbianas cultivadas para serem destinadas à alimentação humana e animal (GIBRIEL el al., 1981).

O uso de microrganismos com interesses nutricionais na alimentação se iniciou no final da 2ª Guerra Mundial, denominado "*Single Cell Proteín* (SCP)" (GIBRIEL et al., 1981). Essa denominação se refere a células microbianas, mortas e secas, como leveduras, bactérias e fungos filamentosos, que podem ser cultivadas em diferentes fontes de carbono (NASSERI et al., 2011).

Microrganismos como bactérias (*Lactobacillus, Alcaligenes*), leveduras (*Saccharomyces, Candida, Kluyveromyces, Pichia, Torulopsis*) e fungos (*Aspergillus, Penicilium*) são considerados como fontes de proteína. Entre as espécies de leveduras, *S. cerevisiae* e *C.*

utilis são amplamente utilizadas para o consumo humano (BEKATOROU et al., 2006). A segurança do uso de produtos de origem microbiana para alimentação animal e humana depende da seleção dos microrganismos, do substrato, processo e características do microrganismo (NASSERI et al., 2011).

As leveduras apresentam importância industrial que vai além da fermentação tradicional (PIRES et al., 2016). Atualmente, produtos da biotecnologia a partir do uso de leveduras têm afetado vários setores comerciais de suma importância, como indústrias alimentícias, de bebidas, biocombustível, produção de enzimas industriais, produtos agrícolas entre outros (PRETORIUS et al., 2003; SILVA et al., 2011).

Espécies como a *Candida*, *Saccharomyces*, *Kluyveromyces*, *Pichia*, *Rhodotorula*, sozinhas ou em culturas mistas com outras leveduras, têm sido cultivadas em águas residuais de processo, hidrolisados e polpas, em resíduos lácteos, em resíduos da indústria sucroalcooleira e da pesca (BEKATOROU et al., 2006; YADAV et al., 2014).

Vários desses resíduos têm sido usados para produção de SCP para alimentação animal (SILVA et al., 2011; PIRES et al., 2016). Na fermentação microbiana, que os parâmetros de grande relevância para avaliação econômica de tais processos biotecnológicos são o rendimento celular para um determinado substrato (RAJOKA et al., 2006). Além disso, são avaliadas a velocidade específica máxima de crescimento, taxa de consumo de substrato, rendimento específico de produto e a taxa de formação de produto (RAJOKA et al., 2006).

A fabricação de ração animal, a partir da vinhaça, também é uma possibilidade estudada durante os anos 80 (CORAZZA & SALLES FILHO, 2000). O resíduo deve ser tratado para a redução do

nível de potássio, podendo ser utilizado como ração de bovinos, suínos, aves e na piscicultura (SILVA et al., 2011; PIRES et al., 2016).

Em ruminantes, por exemplo, a ração feita da vinhaça não pode ultrapassar 10% da alimentação diária, já em suínos ela não deve ultrapassar de 2 a 3%. As pesquisas, realizadas desde a década de 1970, buscavam a redução de DBO e aumento da aceitabilidade (CORAZZA & SALLES FILHO, 2000).

Estudos realizados por Chara & Suárez (1993), uma alternativa para o uso do vinhoto é a sua concentração de sólidos de 60% como suplemento alimentício para ruminantes. Em outros países como Rússia e Eslováquia existem fábricas de proteína para animais, utilizando vinhoto.

Freire & Cortez (2000), a produção de biomassa por diferentes espécies de fungos cultivados em vinhoto in natura e vinhoto concentrado de cana-de-açúcar e de mandioca. A redução de DBO é de, aproximadamente, 83% e produção de 17,2g de biomassa seca/litro de vinhoto com um teor de proteína bruta variando de 35 a 50% e a utilização de fungo filamentoso é mais adequada devido à facilidade de recuperação com o auxilio de peneiras.

Cazetta & Celligoi (2005) realizaram um estudo, onde avaliaram a capacidade de crescimento e síntese de lipídeos e proteínas por *Saccharomyces cerevisiae*, *Rhodotorula mucilaginosa* e *Yarrowia lipolytica* em melaço a 10% e vinhoto bruto de cana-de-açúcar. Em vinhoto bruto a levedura que apresentou maior crescimento foi *R. mucilaginosa* com 7,05 g/L e o maior teor protéico da biomassa foi obtido por *S. cerevisiae* (50,35%).

Silva et al (2011) avaliaram 7 diferentes variáveis (pH, temperatura, concentração de vinhoto, glicose, extrato de levedura, fosfato de potássio e peptona) que podem afetar a produção e produtividade da SCP bem como a quantidade de fatores antinutricionais, ou seja, presença de altos conteúdos de ácidos nucleicos. Usando o modelo estatístico experimental Plackett-Burman (1946), foi observado que o cultivo com as leveduras dos gêneros *Pichia, Candida* e *Saccharomyces* foram realizados com 50% do volume do meio de cultivo composto de vinhoto, o que representa que, um grande volume de vinhoto que pode ser usado com consequente diminuição do descarte em cursos d'água. Além disso, este estudo representou um avanço na utilização do vinhoto como substrato que pode ser usado de forma ainda mais otimizada em um conceito de biorefinarias.

2.3.4 Produção de Compostos Voláteis por Via Biotecnológica

Os processos biotecnológicos têm sido difundidos em vários segmentos industriais, oferecendo novos produtos e processos que apresentam menor agressão ao meio ambiente (ESTCHMANN et al., 2002). Atualmente, os setores que têm maior benefício com o desenvolvimento desta tecnologia são as indústrias farmacêuticas (Antibióticos, vacinas), alimentícia (gomas xantanas, flavorizantes), indústria química (etanol, ácido cítrico) (SCHRADER et al., 2004).

Avanços na tecnologia enzimática, engenharia genética, monitoramento de bioprocessos e técnicas de recuperação de produtos proporcionaram novas oportunidades em potencial para produção de compostos voláteis de interesse industrial, como por exemplo, produção de aromas (PENG et al., 2009).

Em virtude das vantagens esperadas, surge um grande interesse por esta área de pesquisa, onde se resulta no desenvolvimento de bioprocessos como alternativa para substituir síntese química (REID, 2003).

A síntese química de compostos voláteis por mais que apresente vantagens, possui também alguns obstáculos, pois envolve vários passos que resultam em alto custo de produção (DAMASCENO et al., 2001). Além disso, provoca o excesso de carga de resíduos não biodegradáveis, além de produzir misturas racêmicas dando origem a compostos de aroma com propriedades inadequadas (DAMASCENO et al., 2001; ESTCHMANN et al., 2002; KOVACHEVA et al., 2010).

Diversos incentivos impulsionam o desenvolvimento de processos fermentativos de produção de aromas, como a possibilidade de condições brandas de reação, seus produtos são reconhecidos como substâncias naturais (SCHRADER et al., 2004). A alta especificidade de biocatalisadores faz com que o uso de matérias-primas seja de baixo custo e também apresente facilidade na sua aquisição, porém apresenta a desvantagem referente ao alto custo de produção e baixo rendimento (VERSINI et al., 2009).

A produção de bioaromas através de processos biotecnológicos pode ser feita por bioprodução e biotransformação (PANDEY et al., 2000; DAMASCENO et al., 2003). A bioprodução (síntese do novo) ocorre por vias fermentativas com uso de açúcares e aminoácidos, onde a cultura microbiana pode ser melhorada pela otimização das condições de cultivo (DAMASCENO et al., 2003). A biotransformação catalisa a transformação do substrato em um único passo (PANDEY et al., 2000).

2.3.4.1 Produção de Compostos Voláteis por Leveduras

Os primeiros relatos de microrganismos produtores de compostos voláteis de interesse industrial ocorreram na década de 70, onde *Sporobolomyces odorus* foi avaliada para a produção de aromas, resultando na identificação de compostos como metanol, etanol, álcool isobutírico, acetaldeído, acetato de etila entre outros (TAHARA & MIZUTANI, 1975). Drawert & Barton (1978) relataram o processo de biossíntese de citronelol, linalol a partir da glicose por *Kluyveromyces lactis*.

Estudos realizados com *Saccharomyces cerevisiae* relataram que é uma levedura facilmente encontrada e já testada na produção de 2-feniletanol, que é um composto que pode ser obtido quimicamente, porém quando a purificação desse composto gerado gera resíduos nocivos à saúde e ao meio ambiente (ESTCHMANN et al., 2002).

Os processos biotecnológicos são uma boa alternativa para a tradicional extração ou destilação de compostos como 2-feniletanol, visto que esses compostos quando obtidos de forma natural estão presentes em baixa concentração (STARK et al., 2002; SCHRADER et al., 2004). HUANG, LEE & CHOU (2001) verificaram que o crescimento e a produção de 2-feniletanol por *Pichia fermentans* melhoraram com o aumento da velocidade de agitação, apresentando melhores resultados quando sua agitação utilizada foi de 250rpm, obtendo 524,4 mg/L.

Ozyilmaz & Gezer (2010) estudaram a produção de ésteres de aroma por *Candida rugosa* e lipase de pâncreas de suínos. A lipase de *Candida rugosa* e de pâncreas de suínos foram imobilizadas e foram usadas na produção de três ésteres de aroma muito importantes na

indústria: acetato de isoamila (aroma de banana), valerato de etila (aroma de maçã verde) e acetato de butila (aroma de abacaxi).

2.4 Custo Benefício na produtividade de biomassa microbiana e compostos voláteis por vias biotecnológicas

O mercado consumidor tem procurado produtos de qualidade e baixo custo e é neste contexto que os produtos gerados a partir de matéria-prima proveniente dos resíduos agroindustriais, como o vinhoto, tornou-se uma alternativa econômica (PIRES et al., 2016).

A produtividade de biomassa das leveduras com o uso do vinhoto, por exemplo, é economicamente viável devido o baixo custo, porém existe a necessidade de suplementação de oxigênio (LUTOSLAWSKI et al., 2011). O valor comercial de produtos baseados em leveduras como *S. cerevisiae* varia de US $ 0,36 a US $ 9,60 por grama usando outras fontes. Em contraste, o custo para produzir 1 g de biomassa microbiana foi de US $ 0,20 (PIRES et al., 2016).

A produção de compostos voláteis por vias químicas já está bem estabelecida, porém a purificação destes compostos requerem elevados custos que é de aproximadamente US$ 5000 kg^{-1} (KOVACHEVA et al., 2010). Além do elevado custo, ocorre a geração de resíduos nocivos à saúde e ao meio ambiente (ESTCHMANN et al., 2002).

O uso de resíduos como substratos para a produção de compostos voláteis de aroma frutal tem sido uma forma de reduzir o custo com a produção (BICAS et al., 2010) e os processos biotecnológicos vêm para se unir à isso, representando assim uma boa alternativa na produção destes compostos (SCHRADER et al., 2004).

O custo de alguns compostos voláteis como 2 Feniletanol apresentam um valor de 50% mais barato que valor do composto quando comparado com a produção por vias químicas (CHREPTOWICZ et al., 2016). O valor comercial destes compostos varia de US$ 50 a US$ 300 kg^{-1} quando produzidos quimicamente. Em contraste, esse valor se reduz a metade quando utilizados resíduos agroindustriais, como o vinhoto, nesses processos, mostrando que esta é uma tendência crescente na biotecnologia.

3. CONSIDERAÇÕES GERAIS

O uso do vinhoto em bioprocessos, com a capacidade de gerar produtos de valor agregado como o uso de proteínas microbianas na alimentação animal e humana e, produção de compostos voláteis, como novos produtos naturais na indústria alimentícia e cosméticos, têm se mostrado uma solução viável para o problema criado com a geração de efluente altamente poluente derivado da agroindústria.

Os resíduos agroindustriais podem apresentar uma fonte de nutrientes, carboidratos que poderiam ser utilizados em processos de fermentação. A agroindústria gera para cada litro de destilado, aproximadamente, 10L de vinhoto para Tequila e 8L de Vinhoto. Esses resíduos têm sido um grande problema para os órgãos ambientais e indústrias, porém são ricos em carboidratos, proteínas, nutrientes e considerados recursos renováveis e baratos.

Tendo em vista ao que foi exposto, este trabalho abordou a produção de proteínas microbianas e metabólitos voláteis de interesse industrial por leveduras utilizando vinhoto cana e agave como substratos. Tornou-se necessária a seleção de leveduras capazes de utilizar esse resíduo como substrato para a produção dos produtos citados. Além da utilização de técnicas de cultivo para redução de custo e tempo, porém com maiores detalhes e precisão nas respostas da produção destes compostos, sendo ferramentas que auxiliaram na eficiência dos resultados.

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SEGUNDA PARTE – ARTIGOS

ARTIGO 1

Biological treatment of vinasse with yeast and simultaneous production of single cell protein for feed supplementation

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Abstract

Vinasse is the final residue of the bioethanol production, which presents low pH value (≤ 3), high Biochemistry Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) in the range of 3,000 to 9,000mgL-1 characterizing it as highly polluting. Although vinasse is a highly polluting effluent, it might be used for SCP production and others add value products due to high carbon source content. The aim of this work was to propose an aerobic biological treatment to the use of vinasse in a fermentative process by different yeasts for SCP production. The optimization of the process was done by Central Composite Rotational Design (CCRD). Out of ten yeasts from CCMA (Culture collection of Agriculture Microbiology, Lavras, Brazil), *Saccharomyces cerevisiae* (CCMA 0187 and CCMA 0188), *Candida glabrata* (CCMA 0193) and *Candida parapsilosis* (CCMA 0191) presented the higher biomass production of 306 mgL-1, 312 mgL^{-1}, 388 mgL-1 and 306 mgL^{-1}. The microbial biomass presented a low antinutritional value and, on average, 46.85% of the protein. Biological treatment was promising since there was reduction of toxicity in 55.8 and 46.9 % Of BOD and COD, respectively. These results indicated the potential of the use of yeasts for vinasse treatment, and concomitantly a good alternative for biomass production.

Key words: Spirit production waste; add-value products; yeast; friendly-environment.

1. Introduction

The growing productive cycle of industrialized products, commodities and agroindustry's has increased and generated the

accumulation of liquid and solid waste. The release of these wastes in the environment without treatment, cause pollution and depletion of natural resources (España-Gamboa et al. 2011; Fuess & Garcia 2014).

Vinasse is one of the most polluting liquid wastes (Ohgren et al. 2006; Buitrón & Carvajal 2010). It is estimated that for each litre of distillate produced are generated from 6 to 17 litres of vinasse, depending on the beverage produced (Van Haandel 2005; Cabello et al. 2009; CRT 2016). This waste is generated by the production of bioethanol, which is produced in several countries, including China (813 million gallons / year), European Union (1,387), Brazil (7,093) and United States as the main producer with 14.806 million gallons / Year (FRG 2016). Vinasse is also residue from the production of spirit called cachaça in Brazil (1.2 billion litres cachaça / year) (IBRAC 2016). In Brazil, approximately 320 billion litres of vinasse (CONAB 2016) are generated per annum. The distilled spirit Tequila from agave also generated high vinasse production (227.2 million litres of tequila / year) (CRT 2016). Generating some 2,272 million litters of vinasses (CNIT 2016).

Vinasse presents physical-chemical characteristics that characterize it as a highly polluting residue. Generally, it is characterized by a strong odor, dark coloration, pH value < 3 and high values of BOD $\geq$ 5000mgL^{-1} and COD $\geq$ 12000mgL^{-1} (WHO 1995; COPAM 1998; Furry et al. 2006; Silva et al. 2011).

Some alternatives regarding the possible forms of treatment and reuse have been tested by several countries such as Colombia (Ortegón et al. 2016), Mexico (López-López et al. 2010), Spain (Bueno et al. 2009), Egypt (Haggag et al. 2015), China (Jiang et al. 2012), Iran (Sadeghi et al. 2016) and Brazil (Silva et al. 2011; Campos

et al. 2014; Pires et al. 2016). One of the economic and profitable alternatives to the use of vinasse is the production of microbial protein (*SCP - Single Cell Protein*) (Dhanasekaran et al. 2011; Gao et al. 2012; Garcia et al. 2014; Pires et al. 2016) using yeast strains. The yeasts are microorganisms commonly present in must and vinasse being, therefore adapted to the physical-chemical conditions of this residue. The selection of strains to obtain SCP should be based on the presence of nutritional and anti-nutritional factors that will be influenced by the composition of the culture medium (Bekatorou et al. 2006). Yeasts also present high proteins, lipids, vitamins, carbohydrates and amino acids and low nucleic acid contents (Anupama & Ravindra 2000; Pires et al. 2016).

The use of vinasse to produce SCP as supplementation in animal feed has already been reported by Rajoka et al. 2006; Silva et al. 2011; Nitayavardhana et al. 201; Pires et al. 2016. Besides production, it is important that the biomass produced have high quality for food supplementation. Using the vinasse as substrate can also lead to the reduction of pollutant parameters present in the vinasse and its direct disposal in the water is possible (Campos et al. 2014), after confirming low toxicity using bioindicator organisms (APHA 2005).

The aim of this work was to propose an aerobic vinasse biological treatment with concomitant yeast biomass production (SCP) destined to the supplementation of animal feed as value added product.

2. Material and Methods

2.1 Vinasse

Fresh vinasse was provided by a cachaça producer from the State of Minas Gerais (MG), Brazil, during the 2014 harvesting. Samples were collected immediately after distillation of fermented sugar cane juice, transported, and stored in aseptic tanks at -20 °C until use. Upon use, filtered and sterilized at 121°C/20 min. Before starting the experiment, the physicochemical characteristics of vinasse were determined according to standard procedure (item 2.2).

2.2 Physicochemical analysis vinasse

Physicochemical analysis of fresh and spent vinasse were done specially that indicate organic contaminated. Total sugars, Glucose, Sucrose, BOD, COD, Electric conductivity, Dissolved Oxygen, Turbidity, Colour, Dissolved oxygen (DO), Dissolved solids, Sediments solid and Total nitrogen were determined according to the American Public Health Association (APHA 2005). Manganese, copper, zinc, and iron were analysed by atomic absorption spectrometry (Malavolta et al. 1997).

2.3 Microorganisms and inoculum concentration

Yeasts were obtained from the Culture Collection of Agricultural Microbiology, Federal University of Lavras – CCMA/UFLA, Lavras, MG, Brazil. The isolates were: (i) *Saccharomyces cerevisiae* CCMA 0137, CCMA 0185, CCMA 0186 e CCMA 0187 (formerly UFLA CA15, UFLA CA76, UFLA CA93, UFLA CA155) from cachaça fermentation; (ii) *Saccharomyces cerevisiae* CCMA 0188, CCMA O189 e CCMA 0190 (formerly PE2, CAT1, VR1) from alcohol fuel production; (iii) *Candida parapsilosis* CCMA 0191 (formerly UFLA YCN448), *Pichia anomala* CCMA

0192 (formerly UFLA CAF70) and *Candida glabrata* CCMA 0193 (formerly UFLA CAF119), both isolated from coffee fermentation. These isolated were selected according to results reported by Silva et al. (2011).

Yeast were grown in YW broth 0.6% yeast extract, 0.6% peptone and 1.2% glucose (w/v). Cell were centrifuged at 10,000 g/ 5min, washed with sterile distilled water and once the population reached 10^8 CFU mL^{-1}, isolates were inoculated at a 1:10 ratio with respect to the total volume of culture medium plus fresh vinasse inoculated into 250mL flasks containing 100mL culture medium plus fresh vinasse by according to CCRD condition. Cultures were incubated with shaking (150rpm) at 28°C and samples every 24h for pH and viable cell count readings, by the Neubauer chamber with methylene blue dye and then analyzed by fluorescence microscopy using staining methods propidium iodide (PI) dye (Chan et al., 2011). For each yeast mixture, 80 µl aliquots of yeast suspension were mixed with 20 µl of a 20 µg/mL PI stock solution to give a final concentration of 40 µg/mL PI. Blotted stained yeast samples and images were acquired using epifluorescence microscopy with Apotome system (excitation 525 nm and emission 595 nm). Cultivation was ended once cells entered stationary phase or died, usually after 48h. Microbial biomass were determined at the end of the incubation.

2.4 Central Composite Rotational Design (CCRD)

Previous work (Silva et al., 2011) showed that peptone and vinasse concentration have influence over biomass production, so both variables were performing in CCRD. Eight CCRD experiments were

generated from 2 independent variables, presenting one central point and two axial points, being X_1 = peptone concentration (0.18, 1.0, 3.0, 5.0 and 5.82 v/v) and X_2 = vinasse (21.8, 30.0, 50.0, 70.0 and 78.2 gL^{-1}).

The validation was performed by testing the best conditions obtained in the optimization as comparing them with the values predicted by the model. The experiment was done in triplicate, based on five points under conditions of interest whiting the surface and applying the same experimental procedures used to build the models. Statistical analyzes and graphs were performed using Design Expert® version 8.0 software (Stat-Ease Inc., Minneapolis, MN, EUA).

2.5 Nutritional characteristics of biomass production

The biomass produced in culture medium containing 70% vinasse was evaluated in terms of nitrogen and protein content, DNA/RNA ratio, and amino acid composition. Nitrogen content was determined by the Kjeldahl (APHA, 1992) method and crude protein was estimated using a conversion factor of 6.25 (N X 6.25). Microbial DNA and RNA content was determined using the Insta-GeneTM kit (BioRad, Hercules, CA) according to the manufacturer's instructions, and quantified at 260 and 280 nm, respectively, using a NanoDrop®ND-1000 (Thermo Fisher Scientific, Waltham, MA). Total amino acid composition was determined using a Shimadzu HPLC (Kyoto, Japan) equipped with a fluorescence RF-20A/20AXS detector, Shimpack Amino-Na separation column, Trap Ammonia Shim-pack IS C-30Na column, and EX detection at 350 nm. Mobile phase A consisted of a 99.5% ethanol aqueous solution of sodium citrate dihydrate, trisodium salt p. a. (0.2 N), and perchloric acid, pH

3.22. Mobile phase B consisted of an aqueous solution of sodium citrate dihydrate, trisodium salt p. a. (0.2 N), sodium hydroxide p. a., and boric acid (0.2 M), pH 10.0. A 0.6 mL/min flow rate was used during 45 min. Amino acids were quantified by normalizing the peak area of each amino acid to a standard peak area. The latter was derived under the same conditions as the samples. Amino acid content was expressed in grams of amino acid per 100 g of protein.

2.6 Toxicity analysis

The toxicity of spent vinasse was evaluated using the microcrustacean *Daphnia similis* in samples of fresh and spent vinasse. The residue is considered suitable for environment disposal when the survival rate of *D. similis* is more than 50% (APHA, 2005). Six concentrations of fresh and spent vinasse (1, 2.5, 5, 10 and 50%) and distilled water (as control positive) were used. Five young bodies (6 to 24 hours old) were exposed the vinasse and the negative control (100% vinasse) at 20 ± 2°C in 12/12 h photoperiod. Each concentration was observed immobility and/or mortality of individuals after exposure period of 48 hours.

From the immobility and/or mortality of the test organism data were used to calculate LC50 using the method of Spearman-Karber" Trimmed Spearman-Karber Method (Hamilton et al., 1977) estimating in toxicity bioassays". Four replications, captaining twenty individuals for each concentration were analysed. The relationship between fresh and spent vinasse was used to calculate the percentage of reduction of toxicity using the formula proposed by Isidori et al. (2003):

$$\%TR = 1 - \frac{LC_{50}fresh}{LC_{50}spent} .100$$

%TR: toxicity reduction

LC_{50}fresh: Lethal concentration with fresh vinasse

LC_{50}spent: Lethal concentration with spent vinasse

3 Results

3.1 Central Composite Rotational Design (CCRD)

The 10 strains used in this work (Table 1) were evaluated for biomass production, and four strains (*S. cerevisiae* CCMA0187, *S. cerevisiae* CCMA0188, *C. parapsilosis* CCMA0191 and *C. glabrata* CCMA0193) showed the highest biomass production.

pH value, total soluble solids and viable cell counts (data not shown) were monitored throughout the treatment period in CCRD design experiments. pH value ranged from 3.5 to 6 in all treatments. After 48h, there was a 2 log reduction in yeasts viability. Maximum cell growth was achieved during *C. parapsilosis* CCMA 0191 growth, reaching 2.6 log CFU mL^{-1}. Biomass production ranged from 41mgL-1 (Assay 8) to 388mgL^{-1} (Assay 4), reflecting the influence of the combination of peptone and vinasse concentration over the biomass (Table 1). *C. parapsilosis* strain CCMA0191 showed the highest biomass production (388mgL^{-1}) and was selected for the optimization process using the CCRD. The experimental design was 2^2, with 11 trials being conducted, including four axial tests and another three central points. The best condition was observed in assay 4 (peptone, 5gL^{-1} and vinasse, 70vv^{-1}) showing maximum biomass production of 388mgL^{-1} (Table 1). The model fit was assessed by the coefficient of determination R^2. The regression equation obtained indicated

R^2=0.9938, with their predicted and fitted values of 0.9591 and 0.9875, respectively, suggesting an adequate fit of the model to quadratic experimental data and indicating that the model can explain 99.28% of the variability in response. The experimental results were modelled with a polynomial equation of second order to explain the dependence of the microbial growth on the two analyzed factors (peptone and vinasse concentrations).

$$Y_{CCMA0191} = +241.00+43.51* X_1+45.17* X_2+20.50* X_1* X_2+24.44* X_1^2+12.19* X_2^2$$

Where Y is the estimated biomass production and X_1 and X_2 are coded values, respectively, for peptone (gL^{-1}) and vinasse (vv^{-1}) concentrations, so the biomass production can be estimated on the basis of quadratic effect on both factors. The statistical significance of the model was checked by F-test (Table 2). The analysis of variance (ANOVA) for the biomass production showed that the regression model was significant, which was described by the mathematical model based on the significant variables. The mathematical model described the biomass production based on the significant variables.

Table 2. ANOVA analysis of CCRD for the experimental results of the *C. parapsilosis* CCMA 0191 grown for 5 days.

Factor		Effect	F value	P value Prob > F	Significance
Regression model		241.00	159.53	<0.0001	***
X_1	[Peptone]	43.51	329.40	<0.0001	***
X_2	[Iron]	45.17	354.99	<0.0001	***
X_1X_2		20.50	36.56	0.0018	***
X_1^2		24.44	73.34	0.0004	***
X_2^2		12.19	18.24	0.0079	***
Lack of fit			5.23	0.1647	NS

(***) Significant at >99.9% (for 0.0001 <P value <0.001); NS: not significant (NS P <0.05 was considered to be non-significant).

Regression analysis between X_1 and X_2 (Table 2), evaluated after 5 days of growth was significant for the confidence interval of 95% (<0.05). As noted, X_1 and X_2 factors showed positive effects (values of 43.51 and 45.17).

The combination of peptone and vinasse concentrations induced a greater yeast biomass production, which was also noted by the quadratic effect of X1 and X2. The analysis of variance showed the existence of significant differences between the effects caused by each factor analyzed. The prediction of the optimal operating conditions for the biomass production was determined experimentally using response surface methodology (RSM). The interaction effect of the parameters that significantly affected biomass production by C. parapsilosis CCMA191 is shown in Figure 2. The curve in the response surface was plotted against two independent variables (peptone and vinasse concentrations) for the predicted response Y (biomass production).

A RSM showed the model proposed for the biomass production after 5 days of testing. Table 2, indicated that the maximum biomass production with higher peptone (A) and vinasse concentrations values (B) (Figure 1). The optimal conditions for biomass production (214.9 mgL-1) was obtained with 5gL-1 peptone and 70vv-1 of vinasse concentration 70vv-1. The validation was conducted with new experiments carried out under the optimum conditions shown in the CCRD. The biomass yield obtained was 494 mgL-1 for C. parapsilosis CCMA 0191, confirming the proposed model.

Table 1. Two variable central composite rotational design (CCDR) and their responses to the biomass production (mgL^{-1}) during 120 hours of cultivation at 28°C and 150rpm. **The higher microbial biomass production is in bold.**

ASSAYS	[Peptone] (gL^{-1})	[Vinasse] (v/v)	Biomass (gL^{-1})									
			CCMA 0188 *Saccharomyces cerevisiae*	**CCMA 0137** *Saccharomyces cerevisiae*	**CCMA 0185** *Saccharomyces cerevisiae*	**CCMA 0190** *Saccharomyces cerevisiae*	**CCMA 0189** *Saccharomyces cerevisiae*	**CCMA 0186** *Saccharomyces cerevisiae*	**CCMA 0187** *Saccharomyces cerevisiae*	**CCMA 0191** *Candida parapsilosis*	**CCMA 0192** *Pichia anomala*	**CCMA 0193** *Pichia anomala*
1	1.0 (-1)	30 (-1)	130	150	165	34	128	111	106	215	166	206
2	5.0 (+1)	30 (-1)	220	151	124	49	75	139	238	254	237	259
3	1.0 (-1)	70 (+1)	143	166	64	96	220	125	139	267	167	279
4	5.0 (+1)	70 (+1)	**312**	286	191	160	118	94	**306**	**388**	304	**306**
5	0.28 (-1.41)	50 (0)	224	109	5	55	108	49	93	200	290	262
6	5.82 (+1.41)	50 (0)	300	204	92	129	151	202	221	353	225	264
7	3.0 (0)	21.8 (-1.41)	128	83	130	117	100	155	185	220	270	251
8	3.0 (0)	78.2 (+1.41)	233	41	94	105	96	214	253	324	248	221
9	3.0 (0)	50 (0)	84	154	254	103	99	190	196	244	201	291
10	3.0 (0)	50 (0)	123	183	160	100	69	165	169	242	147	277
11	3.0 (0)	50 (0)	67	135	60	147	90	185	190	237	119	292

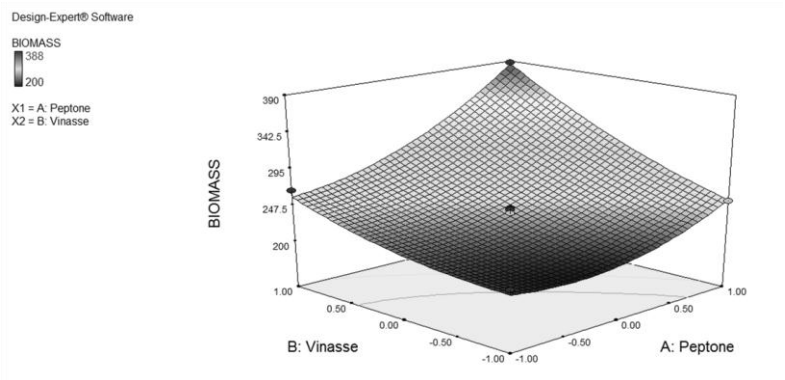

Figure 1. Effect of peptone and vinasse concentrations by *C. parapsilosis* CCMA0191(coded levels) Response surface for the biomass production during the central composite rotational design-CCRD

3.2 Physicochemical analysis from vinasse

The physical-chemical analysis of fresh vinasse sample (Table 3) revealed the low content of total sugars (0.013%), glucose (0.015%), mineral components among others. The COD/BOD rate indicated that vinasse is a biodegradable waste so the action of microorganism is possible. Due to the low concentration of these nutrients, it was necessary to add glucose, yeast extract and potassium phosphate. From the parameters pre-established by COPAM, spent vinasse showed near values to stablished by legislation for pH, COD and BOD parameters (Table 3).

Of the 10 isolates tested, four strains were able to minimize COD and BOD present in the vinasse. Spent vinasse displayed 3.9% higher DO, and 55.8% and 46.9% lower BOD and COD when *C. parapsilosis* CCMA0191 grown, but still kept above the levels allowed by COPAM (2008), and CONAMA (2005) and WHO (1995) regulates standards worldwide. The minerals concentrations were low in fresh vinasse and weren't detected in spent vinasse (Table 3). Values of turbidity, color, total nitrogen, dissolved solids, sediment

solids decreased in spent vinasse (Table 3). The reduction of BOD and COD were used here to measure the efficiency in the wastewater treatment.

Table 3. Physico-chemical parameters assessed from vinasse before and after the treatment by Environmental Brazilian Legislation (COPAM, 2008). In bold letters the parameters into the limits established by COPAM.

Parameters	Limits COPAM	Fresh vinasse	Spent vinasse				Efficiency of treatment (%)
			CCMA 0187	**CCMA 0188**	**CCMA 0191**	**CCMA 0193**	
pH	**6.00 – 9.00**	3.5	5.0	5.5	6.5	6.0	-[1]
Total sugars (%)	-	0.013	-	-	-	-	-
Glucose (%)	-	0.015	-	-	-	-	-
Sucrose (%)	-	0.000	-	-	-	-	-
BOD (mgL^{-1})	**60 mgL^{-1} or reduction of 60%**	3960	1850	1860	1750	1825	55.8[2]
COD (mgL^{-1})	**180 mgL^{-1} or reduction of 60%**	9397	4500	4558	4989	4490	46.9[2]
COD:BOD	-	2.37	2.43	2.45	2.85	2.46	-
Electric conductivity (μScm^{-1})	-	950	-	-	-	-	-
Dissolved Oxygen (mgL^{-1})	-	7.6	8.0	8.3	8.0	7.9	-[1, 2]
Turbidity (NTU)	-	18.3	10.4	13.9	11.9	10.1	44.9
Color (Ptco)	-	1562	1500	1519	1450	1486	7.05
Dissolved solids (mgL^{-1})	-	16143	8899	9765	8757	8001	50.44
Sediment solids (mgL^{-1})	-	0.5	0	0	0	0	0
Total Nitrogen (mgL^{-1})	-	390.2	109.7	113.9	105.7	103.0	73.6[2]
Copper (mgL^{-1})	**1mgL^{-1}**	0.01	0.00	0.00	0.00	0.00	-
Manganese (mgL^{-1})	**1mgL^{-1}**	0.014	0.00	0.00	0.00	0.00	-

Zinc mgL^{-1})	**5mgL^{-1}**	0.001	0.00	0.00	0.00	0.00	-
Iron (mgL^{-1})	**15mgL^{-1}**	0.015	0.00	0.00	0.00	0.00	-

Joint deliberation COPAM / CERH-MG No. 1 of May 05, 2008, section II, art 29. [1] unique parameter that showed values in spent vinasse; [2] Value referring to yeast that presented the best result

The cell viability was done by fluorescence microscopy during the treatment period (Figure 2). Each fluorescence image is an overlay of the fluorescence signal from FL channel 1 (viable), presented using red false color. The counted yeast cells were presented by the software, which clearly distinguishes the viable cells and nonviable cells. The viable cells of the yeast were effectively stained with high fluorescence intensities. In average, 45% of visualized cells died after 5 days of treatment (Software Apotome). *S. cerevisiae* CCMA0186 showed the highest death rate in 5 days of culture, while *C. parapsilosis* CCMA 0191 was the one that presented greater amount (65%) of live cells (Figure 2).

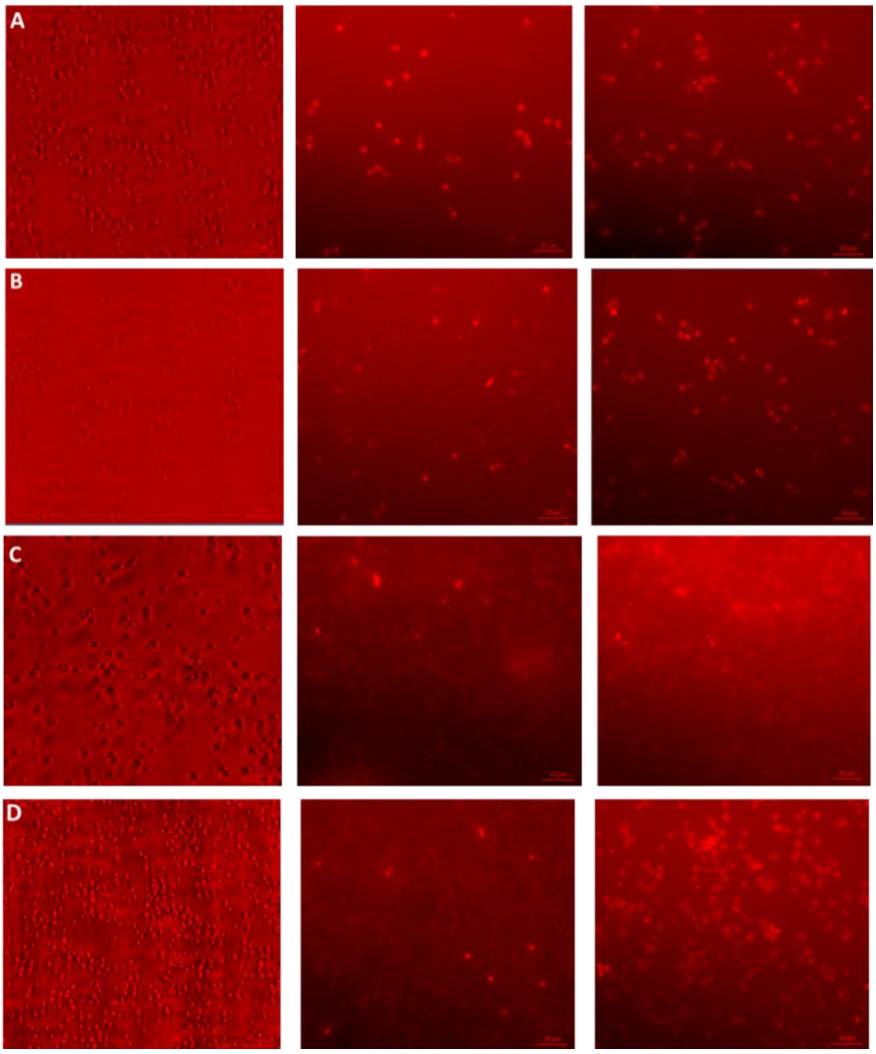

Figure 2. Yeast viability assay stained with Propidium Iodide (PI) as described. The four samples showed live yeast (left panel), live/dead yeasts (central panel) and dead (right panel). A: Samples of *S. cerevisiae* strains CCMA0186 B: *S. cerevisiae* CCMA0188 C: *C. parapsilosis* CCMA0191 D: *C. glabrata* CCMA0193. PI fluorescence monitors dead cells.

3.3 Nutritional quality of biomass

The amino acids content from yeast biomass were compared with reference standard FAO/WHO/UNU (1989) (Table 4). Maximum biomass nutritional analysis from *C. parapsilosis* CCMA 0191

showed 55.01% protein, while *C. glabrata* CCMA 0193 showed highest anti nutritional components (nucleic acid ≅ 2.76%) (Table 4). Protein composition analysis revealed the presence of almost all essential amino acids except threonine, valine, leucine, phenylalanine and tryptophane for the *S. cerevisiae* CCMA 0188 and leucine and tyrosine for the *C. parapsilosis* CCMA 0191 (Table 4). In addition, nine non-essential amino acids were quantified, and proline was the most abundant (17.58%). The concentration of lysine was high in all yeasts strains, but *C. parapsilosis* CCMA 0191 (40.38%) and *S. cerevisiae* CCMA 0188 (30.7%) showed the highest values (Table 4).

Table 4. Protein profile, nucleic acid and total amino produced from *S. cerevisiae* CCMA0188, *S. cerevisiae* CCMA 0187, *C. parapsilosis* CCMA 0191 and C.*glabrata* CCMA 0193 in cultivation using vinasse as substrate.

Compounds	**CCMA0188**	**CCMA 0187**	**CCMA 0191**	**CCMA 0193**
Protein (g 100g^1 of dry biomass)	38.2	51.7	**55.01**	42.5
Nucleic acids (DNA + RNA) (g 100g^1 of protein)	2.12	1.08	2.16	**2.76**
Essential amino acids (g 100g^1 of protein)				
Lysine	**30.7**	17.82	**40.38**	6.23
Threonine	n.d	1.58	3.07	0.78
Valine	n.d	2.00	3.15	2.19
Isoleucine	10.5	1.69	3.81	3.79
Leucine	n.d.	0.53	n.d.	0.11
Phenylalanine	n.d	5.42	1.26	1.73
Tryptophane	n.d	1.0	1.02	0.99
Nonessential amino acids (g 100g^1 of protein)				
Aspargic acid	1.28	1.10	4.43	4.34
Serine	6.49	3.85	5.97	4.03
Glutamic	5.07	2.19	6.86	10.33
Proline	3.41	7.33	17.58	11.25
Glycine	n.d	0.68	17.19	13.22
Alanine	5.42	5.64	9.07	8.67
Tyrosine	9.66	0.83	n.d.	0.02

Histidine	n.d	4.92	13.73	1.26
Arginine	n.d	0.31	2.33	1.4

n.d. = non detected; **BOLD: The highest microbial biomass production**

3.4 Toxicity analysis

The toxicity tests were performed from fresh and spent vinasse treated by four yeast The LC50 with juveniles had the sensitivity range between 4.72mgL^{-1} and 7.77mgL^{-1} concentration to the dilution water used was the water that kept the organisms in the laboratory, showing a difference between the yeasts used the treatment of vinasse (Table 5). The positive control (distilled water) was found to have 100% survival. The concentration of 100% vinasse (negative control) was observed the mortality of 100% of population. The low pH value of the fresh vinasse (3.5) was also an interferent in the assay, since the micro crustaceans showed pH sensitivity.

Table 5. Lethal concentration LC50 48h of the fresh and spent vinasse for the *D. similis* young form.

STRAINS	**Positive Control** Distilled water [Dead]%	**Negative Control** Spent vinasse [Dead]%	**LC50 48h** *Daphnia similis*	**CI95%**		**%TR**
				Less limit	**High limit**	
S. cerevisiae CCMA0186	0	62	5.72	5.20	6.29	39.26
S. cerevisiae CCMA 0188	0	59	7.77	6.64	9.10	44.8
C. parapsilosis CCMA 0191	0	59	6.23	6.02	6.44	41.10
C. glabrata CCMA 0193	0	51	4.72	3.84	5.81	34.35
Mean	**0**	**59.25**	**6.11**	**-**	**-**	**-**

CI= Confidence interval; TR= Toxicity Reduction

4 Discussion

Disposal of effluents into the environment is controlled by governmental law. In Brazil CONAMA (2005) and COPAM (2008), in the United States DEC (Department of Environmental Conservation) (2016), Mexico NMX (Norma Mexicana) (2012) and World Health Organizations (WHO, 1995). Although there is no consensus on the best parameters to evaluate to describe the pollutant degree of a residue, the most common are COD and BOD values (Arvanitoyannis, 2008). The biological treatment with the four selected yeasts strains was able to reduce the COD and BOD values of vinasse (COD / BOD 2.5). The total solids content is a parameter that influences the BOD and COD values, and its reduction may have corroborated by the decrease of these parameters. However, the COD value after the treatment still presented values higher than the allowed one. It is possible that the solids concentration may have influenced the lower COD reduction besides the presence of oxidizable inorganic compounds.

According to the norms established by the COPAM / CERH-MG Joint Legislative Decree No. 1 of May 5, 2008, BOD values should reach 60mgL-1 or be reduced by 60% after treatment. In this study, a maximum reduction was very close to the desired (55.8%) when cultivated vinasse with C. parapsilosis CCMA 0191. These results indicated that after some adjustments, such as the use of physicochemical methods in subsequent stages to reduce the organic load, might be possible to reach the minimum standard established by the legislation to be discarded in the environment. A study conducted by Campos et al. (2014), showed that the biological treatment using physicochemical methods in 8 stages was possible to obtain the

reduction in 96.7% of BOD. Lopez-Lopez et al. (2010) also observed that the treatment with microorganisms is an alternative for the use of pollute vinasse, however the steps suggested were far more complex than the one proposed here. Considering the other physicochemical parameters evaluated the vinasse treatment with 4 yeasts strains showed efficiency, however *C. parapsilosis* CCMA 0191 was the best one. The vinasse treatment could combine with the good production and high quality of yeast biomass.

From the physical-chemical characteristics of the spent vinasse was possible to calculate the maximum permissible flow of the effluent to be discarded in water bodies. For example, in a river with a water volume of $45m^3\ s^{-1}$, the vinasse can be introduced with flow of around $0.8\text{-}1m^3s\text{-}1$, without exceeding the established limits of color and turbidity (Tigini et al. 2011). However, due to the ecotoxicity demonstrated by the lethality of *Daphnia similis*, a micro-cracking bioindicator (Buratini et al., 2004), spent vinhoto should be released at a concentration of less than 6%. Daphnia crustaceans are widely used in toxicity testing because of their wide distribution in rivers and ponds, and because their importance in the food chain (APHA, 2005). The D. similis species is today the most widely used bioindicator organism due to its high sensitivity to toxic by-products (Vesela and Vijverberg, 2007). Campos et al., (2014) proposed joint physical-chemical and biological treatment for the vinhoto that presented a toxic effect to Daphnia sp similar to the one found in this study, reinforcing that the biological treatment is important and necessary to increase the efficiency of vinasse treatment. Silva et al., (2011); Pires et al., (2016) also studied vinasse as substrate for yeast growth. Usually, vinasse presents a chemical composition (toxic effect) that

stresses the yeasts cells inducing pseudomycelia formation (Silva et al., 2011), and causing death after 96 hours of exposure. However, even with the reduction of viable cells, the production of biomass is economically advantageous due to low cost of production and high nutritional value indicated for animal feed supplementation (Nytayavardhana et al., 2013).

The addition of peptone at different concentrations to vinasse interfered in the performance for the biomass production of 4 selected strains (CCMA 0188, CCMA 0187, CCMA 0193 and CCMA 0191). It was expected because yeasts require certain carbon and nitrogen concentration, and their interaction are important parameters in the regulation of cell growth (Schineper et al., 2004). Another advantage of the use of yeasts is the increase of pH, without chemical additives, not generating additional costs to the industries and, meets the requirements of agencies that recommend pH values of 6.0 to 9.0 for final effluent, as required by the Council (CONAMA, 2005) and the World Health Organization (WHO, 1995).

The use of cheap and abundant raw material for the production of SCP using microorganisms is still one of the best and cheapest technologies possible for the commercialization of SCP (Pires et al., 2016). As a dietary supplement, microbial biomass should contain levels of nutritional compounds (eg total nitrogen) and low levels of anti-nutrients (eg, Nucleic Acid $\leq$ 10% dry mass) (Silva et al., 2011; Pires et al., 2016, FAO, 2006).

Microbial production efficiency and microbial flow are determinants of the amount of microbial protein that reaches the animals small intestine (Cavalcante et al., 2006). The synthesis of microbial proteins, due to the excellent balance of amino acids, proved

that the production of microbial biomass (SCP) for feeding is an alternative for the use in the diet (Sumar et al., 2015).

Amino acid composition is the most important factor in the definition of food protein quality, followed by protein digestibility and bioavailability of its amino acids (Wolfe et al., 2016). Most non-ruminant animals require the ingestion of essential amino acids such as isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (FAO / WHO / UNU, 1989). The determination of amino acids constituent of the protein present in the microbial biomass revealed the presence of all the essential amino acids except for the absence of methionine and cysteine suggesting a possible adequate and economically interesting destination for the biomass produced with vinasse as substrate. Considering the quality of SCP, it is necessary to determine the amount of protein and amino acid extracted from it, to verify if this protein can be destined animal diet (Ahmadi et al., 2010).

4 Conclusions

The proposal of a joint biological treatment of the vinasse with the production of SCP as added-value product was satisfactory and compensatory from the economic and environmental point of view. Some modifications in the biological treatment process should be carried out, such as the use of physicochemical methods in subsequent stages to increase the reduction of organic load. The use of immobilized cells could be another possibility to increase cell viability and consequently biomass productivity and reduction of the organic load. The biological treatment suggested here is of low cost and low technological complexity being easily to reproduce on a large scale.

The effiency of our proposal could be increased in subsequent cycles with the constant exposure of *C. parapsilosis* CCMA 0191 to vinasse.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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ARTIGO 2

Aromatic/Volatile compounds production from tequila and cachaça vinasses by yeast biological treatments

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Abstract

The vinasse is a waste produced from the elaboration process of distilled beverages, such as tequila and sugar cane spirit (cachaça). The presence of organic compounds (acids, alcohols and sugars), minerals and residual nitrogen (amino acids, peptides and nitrogen salts) make the vinasse a hazardous liquid waste to the environment highly harmful to the fauna, flora, micro fauna and micro flora of rivers and lagoons. In order to develop a biological process to reduce the environmental damage of the vinasse, a biological treatment performed by different yeast strains (*Saccharomyces cerevisiae*, *Candida parapsilosis* e *Candida glabrata*) was carried out. *C. parapsilosis*. A higher reduction of chemical and biochemical oxygen demand (COD and BOD) was observed with all the yeasts tested in tequila vinasse than in cachaça vinasse. On the contrary, for all the yeasts, a higher production of volatile compounds was observed in cachaça vinasse than in tequila vinasse. *C. parapsilosis* produced the highest concentration of 2 phenylethanol (four times) than the other yeast strains. These results indicated that the environmental damage of vinasse can be reduced by biological treatment with yeasts, with aroma compounds production. Which could have economical potential, in particular for the tequila industry.

Key words: Waste, add-value products, Flavouring compounds

1. Introduction

In the Latinamerican countries such as Brazil and Mexico, the production of traditional distilled beverages such as Cachaça and Tequila are economically important industries. At the end of the elaboration process, after distillation process, a liquid waste with an

acid pH (3 to 5) named vinasse is produced, which is responsible for the contamination of soils and water (Rodrigues Filho & Oliveira, 2002; Ohgren et al., 2006) when it is disposed without treatment (Beltran et al., 2005; Silva et al., 2007; España-Gamboa et al., 2011; Silva et al., 2011; Pires et al., 2016).

It is estimated that for each liter of cachaça generates from 4 to 8L of vinasse (Novaes, 2000; Van Haandel, 2005), approximately 320 million of liters of vinasse (IBRAC, 2016; CONAB, 2016). In relation to the production of tequila (CRT 2016), 7 to 10Lof vinasse are generated for 1L of tequila. In 2016, 250.5 million of liters of tequila were produced, generating 2,250 million liters of Tequila vinasse (Consejo Regulador del Tequila, CRT 2016). This waste is maily composed by water and organic matter, which is directly related to the waste origin (Cedeño, 1995; Van Haandel, 2005). The high content of organic matter results in the high value of the chemical oxygen demand (COD), which varies from30,000 to 150,000 mgL^{-1} as well as the biochemical oxygen demand (BOD) varying from 1,900 – 100,000 mgL^{-1} O_2L^{-1}) (Iñiguez & Peraza, 2007; Campos et al., 2014).

Therefore, different efforts have been performed to reduce the environmental damage of the vinasse, such as in the production of the single cell protein (SCP) by aerobic fermentation (Rodrigues et al., 2011; Silva et al, 2011; Pires et al., 2016). The use of yeasts in the biological treatments of the vinasse has been studied due to its fast growth and stress resistance to the vinasse composition than others microorganisms (Silva et al., 2011).

In our knowledge, this is the first report of volatile compounds production by yeasts from the cachaça and tequila vinasses. This product has interesting economical perspectives for the cosmetic and

food industries and have already been used by industries because of the natural origin. In bio-route are used in synthesis of aromas for new microbial processes (fermentation) of natural precursors using microbial cells. The impact of the biological treatment in the reduction of the vinasse pollution was also evaluated.

2. Material and Methods

a. Vinasse

Fresh vinasse was provided by the cachaça producer from the State of Minas Gerais (MG), Brazil and the tequila producer from the State of Jalisco, México, during the 2014 harvesting. Samples were collected immediately after distillation of fermented sugar cane juice, transported, and stored in aseptic tanks at -20 °C until use. Upon use, filtered and sterilized at 121°C/20 min. Before starting the experiment, the physicochemical characteristics of both vinasse source was determined according to standard procedure (item 2.2).

2.2 Physicochemical analysis of vinasse

Physicochemical analysis of vinasses were carried out to determine the composition. Total sugars, glucose, sucrose, BOD, COD, electric conductivity, turbidity, color, dissolved oxygen (DO), dissolved solids, solid sediments and total nitrogen were determined according to the American public health association (APHA, 2012). Manganese, copper, zinc, and iron were analyzed by atomic absorption spectrometry. The soluble solids content was determined using a portable refractometer to determine sugars (Instrutherm, model RT -30 ATC) and pH (HI 2210 pH meter - Hanna instruments). The biomass was evaluated by dry (60°C) constant weight.

2.3 Microorganisms and inoculum concentration

Yeasts were obtained from the culture collection of Agricultural Microbiology Department, Federal University of Lavras – CCMA/UFLA, Lavras, MG, Brazil. The isolated yeasts used were: (i) *Saccharomyces cerevisiae* CCMA 0187 from cachaça fermentation; (ii) *Saccharomyces cerevisiae* CCMA 0188 from alcohol fuel production; (iii) *Candida parapsilosis* CCMA 0191 and *Candida glabrata* CCMA 0193, both isolated from coffee fermentation. These isolated yeasts were selected according to the results reported by Silva et al. (2011).

Yeast were grown in YW broth (0.6% yeast extract, 0.6% peptone and 1.2% glucose w/v). Cell were centrifuged at 10,000 g/ 5min, washed with sterile distilled water and once the population reached 10^8 CFU mL^{-1}, isolates were inoculated at a 1:10 ratio considering to the total volume of culture medium. Fresh vinasse was added into 250mL flasks containing 100mL culture medium by according Silva et al., 2011 with modifications. Cultures were incubated at 28°C and 150 rpm during 120 h and samples were taken each 24h, pH and viable cell count readings (Neubauer chamber with methylene blue dye) were carried out.

2.3 Volatiles compounds analysis

2.3.1 Extraction of volatiles by headspace-SPME

Vinasse samples from cachaça and tequila were added in vials for headspace analysis. A carboxen/poly (dimethylsiloxane) (DVB/CAR/PDMS) type 75µm SPME fiber (Supelco Co., Bellefonte, PA, USA) was used to extract volatile constituents. Five mL of vinasse was placed in a 15mL hermetically sealed flask, and heated

for 15 min at 60°C, to reach sample headspace equilibrium. Then, volatile compounds were extracted by placing the SPME fiber in the headspace for 30min at 60°C. For compound desorption, the fiber was placed in the GC injection port heated at 240°C for 5min..

2.3.2 HS-SPME/GC analysis

The analysis of volatile compounds was carried out before and after biological treatment for each type of vinasse, in the case of cachaça it was performed using a gas chromatografh (GC), Shimadzu model 17A equipped with an FID (flame ionization detector) and a capillary DB wax column (30m X 0.25mm i.d. X 0.25µm) (J&W Scientific, Polsom, Calif., U.S.A.) and in the case of tequila a Hawlett Packard GC 6890 series was used with the same column.

For both GC devices, the same method was applied, the oven temperature was maintained at 40°C for 5 min, raised to 120°C by increments of 6°C/min, and then maintained at 200°C for 8 min. Injector and detector temperatures were kept at 240 and 250°C, respectively. The carrier gas (N_2) was maintained at a flow tare of 2mL/min. volatiles compounds were identified by comparing the retention times of the compounds with those of standard compounds injected under the same conditions (Arellano et al., 2008).

The volatile compounds quantified were acetaldehyde, ethanol, ethyl acetate, methanol, ethyl butyrate, 1-propanol, isobutanol, isoamyl acetate, butanol, 3-methyl-1-butanol, ethyl hexanoate, ethyl lactate, phenethyl acetate and 2-phenylethanol using external standards. As relative percentages of individual compounds were calculated from the total area of volatiles in the chromatograms.

3 Results and Discussion

3.1 Physicochemical analysis from vinasse

The fresh vinasse from cachaça and tequila were characterized before the biological treatment, a low content of total sugars (0.013% and 0.74%) and glucose (0.015% and 0.45%) was found for cachaça and tequila respectively, as well as very low minerals (Table 1). The high values of BOD and COD present in the fresh vinasse 14,070mgL^{-1} and 43,932mgL^{-1}, respectively, for the tequila vinasse and 3,960mgL^{-1} and 9,397mgL-1, respectively, for the cachaça vinasse, in particular from the tequila process showed the necessity of a treatment before being liberated to the environment, as they have high levels of pollutants and they can interfere in the equilibrium of the aquatic life (Arvanitoyannis, 2008).

The pH varied from 3.5 to 5-6.5 with the all tested yeasts after the biological treatments, which were between the allowed limits of Mexican (NMX) and Brazilian (COPAM) regulations (Table 1). The increase of pH without chemical additives, do not generate additional costs to the industries and these values are also recommended by other international regulations (WHO, 1995). Dissolved oxygen (DO) is one of the most important parameters in determination of the water body quality (Young et al., 2012) and it has an impact on the pullulants of the effluent (Araújo et al., 2004). After the biological treatment with all the yeasts tested the concentration has higher than 5 mgL^{-1}. Thus, the DO at end of treatment was similar to in freshwater (Young et al., 2012).

The biological treatment with all the yeasts reduced the BOD and COD, with a higher efficiency for the tequila vinasse (Figure 1). According to the government environmental regulations in Brazil and

Mexico (COPAM, 2008; NMX, 2012), the BOD and COD values must suffer a reduction of 60% after the treatment. The biological treatment with *C. parapsilosis* CCMA 0191 presented the highest efficiency in both vinasses, however it i (Figure1).

Campos et al. (2014) obtained a higher BDO reduction (96.7%), after physic-chemical and biological treatment of cachaça vinasse. Nevertheless, in this work only a biological treatment was applied, which could be economically and ecologically more suitable, in particular for tequila vinasse.

In relation to the other physic-chemical parameters analyzed, there was a decrease in the nitrogen, solids and ion contents with the biological treatment using all the yeaststst, and one more time it was more efficient in tequila vinasse than cachaça vinasse (Table 1). The biological treatment efficiency for COD and BOD reduction was more related to the vinasse composition (Satyawali & Balakrishnan, 2008, Campos et al., 2014). than the yeast strains used, the most effective yeast was treatment C. parapsilosis CCMA 0191.

Table 1. Physico-chemical parameters assessed from fresh and treated tequila and cachaça vinasse, according by environmental brazilian legislation (COPAM, 2008) and environmental mexican legislation (NMX, 2012). In bold letters the parameters into the limits established by COPAM and NMX.

Parameters	Limits NMX	Limits COPAM	Fresh vinasse		Treated vinasse								Efficiency of treatment (%)	
					CCMA 0187		CCMA 0188		CCMA 0191		CCMA 0193		T	C
			Tequila (T)	Cachaça (C)	T	C	T	C	T	C	T	C		
pH	**5.00-9.00**	**6.00 – 9.00**	4.5	3.5	6.0	5.0	6.0	5.5	6.5	6.5	6.5	6.0	-[1]	-[1]
Total sugars (%)	-	-	0.74	0.013	-	-	-	-	-	-	-	-	-	-
Glucose (%)	-	-	0.45	0.015	-	-	-	-	-	-	-	-	-	-
Sucrose (%)	-	-	0.00	0.00	-	-	-	-	-	-	-	-	-	-
COD:BOD	-	-	3.12	2.37	2.76	2.43	3.06	2.45	2.96	2.85	2.73	2.46	-	-
Electric conductivity (μScm^{-1})	-	-	1020	950	-	-	-	-	-	-	-	-	-	-
Dissolved Oxygen (mgL^{-1})	**>5mgL^{-1} or >60%**	-	7.3	7.6	8.6	8.0	8.5	8.3	9.0	8.0	8.7	7.9	-[1,2]	-[1, 2]
Turbidity (NTU)	-	-	22.9	18.3	14.1	10.4	16.6	13.90	14.03	11.87	14.1	10.09	38.7	44.9
Color (Ptco)	-	-	3001	1562	2907	1500	2900	1519	2899	1450	2878	1486	51.7	7.05
Dissolved solids (mgL^{-1})	-	-	23197	16143	12007	8899	12375	9765	12002	8001	12257	8757	65.5	50.44
Sedimented solids (mgL^{-1})	**1mgL^{-1}**	-	0.5	0.5	0	0	0	0	0	0	0	0	0	0
Total Nitrogen (mgL^{-1})	-	-	818.2	390.2	395	109.7	398	113.9	377	105.7	381	103.0	53.9	73.6[2]
Copper (mgL^{-1})	**5mgL^{-1}**	**1mgL^{-1}**	0.017	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-
Manganese (mgL^{-1})	**1mgL^{-1}**	**1mgL^{-1}**	0.009	0.014	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-
Zinc mgL^{-1})	**5mgL^{-1}**	**5mgL^{-1}**	0.026	0.001	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-
Iron (mgL^{-1})	**10mgL^{-1}**	**15mgL^{-1}**	0.014	0.015	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-

Joint deliberation COPAM / CERH-MG No. 1 of May 05, 2008, section II, art 29. NMX-AA-026-SCFI-2010; NMX-AA-034-SCFI-2001; NMX-AA-004-SCFI-2000; NMX-AA-028-SCFI-2001; NMX-AA-012-SCFI-2001; NMX-AA-030/1-SCFI-2001[1] unique parameter that showed values in spent vinasse; [2] Value referring to yeast that presented the best result

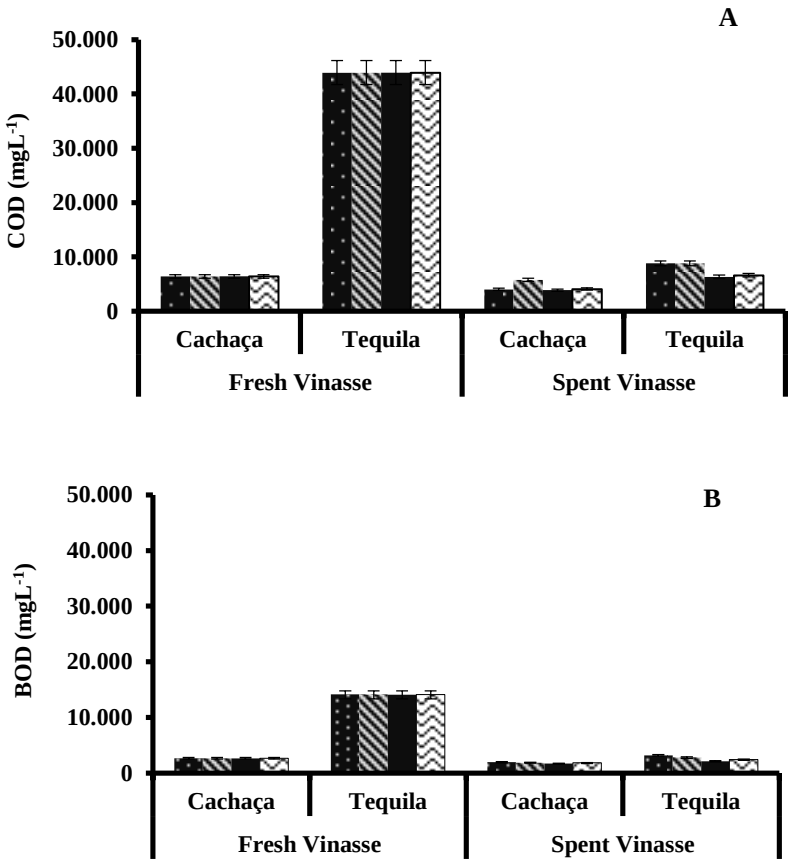

Figure 1. Values of the COD **(A)** and BOD **(B)** fresh and spent vinasse of the Cachaça and Tequila using *Saccharomyces cerevisiae CCMA0188* ( ), *Saccharomyces cerevisiae* CCMA 0187 ( ), *Candida glabrata* CCMA 0193( ) and *Candida parapsilosis* CCMA0191 ( )

3.2 Biomass Analysis

The biomass production was similar in the cachaça and tequila vinasse 0.409 mgL^{-1} and 0.426 mgL^{-1} for *S. cerevisiae* CCMA 0188, 0.286 gL^{-1} and 0.326 gL^{-1} for *S. cerevisiae* CCMA0187, 0.494 gL^{-1} and 0.509 gL^{-1} for *C. parapsilosis* and 0.462 gL^{-1} and 0.453 gL^{-1} for *P. anomala* CCMA0193, respectively. As observed, the non-*Saccharomyces* presented better performance, being the biomass of *C. parapsilosis* CCMA 0191. Cazzeta & Celligoi (2005) in a work with vinasse from the ethanol production, presented the production of $5gL^{-1}$

of biomass, and the authors added molasses as carbon source and fermentation was carried out in 50hs of cultivation. However, even the lowest biomass production, the production of volatiles does not suffer interference (Table 2), concluding that the formation of these compounds does not have direct correlation to the growth. However, even the smaller biomass production, the production of volatile did not suffer interference (Table 2) concluding that the formation of these compounds does not have direct correlation to the growth.

3.3 Volatiles compounds produced by vinasse

Sixteen compounds, including alcohols (7 compounds), aldehyde (1) and esters (8) were detected in the tequila and cachaça vinasse samples (Table 2). In the tequila vinasse the alcohols were predominant (281.6mgL^{-1}), followed by esters (68,7 mgL^{-1}) and acetaldehyde (30,2 mgL^{-1}) (Table 2). In the cachaça vinasse alcohols (162,3 mgL^{-1}) and esters (49,6 mgL^{-1}) were predominant, therefore the volatile compounds production was higher in tequila vinasse than cachaça vinasse, which could be also correlated with theorigin and composition of vinasse (Table 2). In the case of major compounds, for acetaldehyde the production varied from 12 to 30 mgL^{-1} for all the tested yeasts. For the higher alcohols production, there were differences in function of the compound produced and the yeast type used in the biological treatment, non-saccharomyces yeasts produced more 2-phenyetanol (2-PE) and butanol than *Saccharomyces* strains, in particular those treated with *C. parapsilosis* (Table 2). On the contrary, the *Saccharomyces* strains produced more amyl alcohols than non-*Saccharomyces* strains. It is interesting the production of 2-phenylethanol, which was the highest compound produced in tequila vinasse. It has been found that other microorganisms produce this

compound such as *Pichia fermentans*, *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* among others, under various culture conditions, however optimization is required (Huang et al., 2000; Etschmann et al., 2004; Hua et al., 2010). 2-phenylethanol can be extracted naturally from rose petal, in the form of rose essential oil containing approximately 60% of this compound. The lack of considerable quantity of flowers require the acquisition of this compound chemically, however the purification demand high costs (US $ 5200kg^{-1} (Kovacheva et al., 2010) and the generation of harmful waste to the health and environment (Estchmann et al., 2002). By this reason, biotechnical process with the sub products utilization, they represent a good alternative to the production of this alcohol (Schrader et al., 2004), which can achieve a saving of 50% in the 2-PE value (Chreptowicz et al., 2016).

Beside the alcohols, esters are other compounds of great importance in the food and cosmetic industry. The esters could be responsible of the fruity flavors and aromas of beverages and perfumes (Park et al., 2009). They have a low threshold of smell, in other words, just some milligrams can be detected (Chan & Su, 2008). Esters such as ethyl acetate and phenetyl acetate were already reported as compounds of microbial origin (Medeiros et al., 2000; Searens et al., 2010).

Table 2. Volatile compounds (mgL^{-1}) extracted from Cachaça and Tequila vinasse samples after 120hs of fermentation

Compounds/ Yeasts	mgL^{-1}							
	CCMA0188 [a]		**CCMA 187** [b]		**CCMA 193** [c]		**CCMA 191** [d]	
	T	C	T	C	T	C	T	C
Major Compounds								
Aldehydes								
Acetaldehide	14.6	12.0	9.94	23.07	30	14.72	26	25.68
Álcohols								
2-PhenylEthanol	33.45	50.0	23.92	53.97	62.4	64.2	281.6	162.3
Butanol	10.5	17	15.62	8.6	17.7	23.4	32.1	31.5
Amyl Álcohols	24.19	14.3	12.68	9.74	5.83	4.70	8.88	9
Esters								
Phenetyl acetate	19.12	32.42	43.0	39.0	57.6	43.5	68.7	49.6
Ethyl lactate	8.99	16.3	15.2	25.0	20.82	31.6	21.0	40.8
Ethyl acetate	14.03	32.42	11.0	39.0	12.2	43.5	19.1	49.62
Ethyl hexanoate	12.61	10.02	14.9	19.0	18.5	19.3	11.9	24.3
Minor Compounds								
Alcohols								
Ethanol	2.92	1	2.56	0.51	1.05	0.3	4.16	1.57
Methanol	5.05	1	3.63	0.52	1.01	0.48	2.62	2.17
1-Propanol	3.15	1.5	2.42	0.4	1.92	1.50	1.75	2.11
Isobutanol	6.21	0	3.21	0.3	2.7	0.68	2.98	2.01
Esters								
Ethyl butirate	2.03	0.9	3	1.9	4.39	2.5	5.24	3.0
Isoamyl acetate	nd[e]	nd	nd	nd	nd	nd	nd	nd
Ethyl octanoate	nd	nd	nd	nd	nd	nd	nd	nd
Ethyl decanoate	nd	nd	nd	nd	nd	nd	nd	nd

[a] CCMA0188 = *Saccharomyces cerevisiae;* [b] CCMA0187 = *Saccharomyces cerevisiae*; [c] CCMA0193 = *Candida glabrata;* [d] CCMA0191 = *Candida parapsilosis;* [e] nd = not detected; T = Tequila; C = Cachaça

The levels produced in this work varied principally in function of the type of vinasse and type of yeasts used, the concentration produced of phenylethyl acetate was the highest ester concentration observed after biological treatment, its production was higher in tequila vinasse with non-*Saccharomyces* yeast strains than in cachaça vinasse with *Saccharomyces* yeasts, one more time *C. parapsilosis*

was the higher producer, which could be related with the levels of 2-phenylethanol produced (Table 2). This same strain *C. parapsilosis* CCMA 0191 was already reported as a higher producer of phenylethyl acetate in coffee fermentation (Evangelista et al., 2014), however concerning the production of this volatile compound from vinasse have not been reported. In the case of ethyl acetate, it was the second ester produced and the non-*Saccharomyces* yeasts produced also more ethyl lactate in cachaça vinasse than the *Saccharomyces* yeasts in tequila vinasse (Table 2). The microbial production of ethyl acetate has been also observed in yeast species but only *Pichia anomala*, *Candida utilis* and *Kluyveromyces marxianus* produce this ester in larger amount (Löser et al., 2012). This compound is a simple ester that can be chemically produced, from the reaction of acetic acid with ethanol (Searen et al., 2010) or of being from microbial origin (Mantzouridou & Paraskevopoulou (2013) and it is utilized as a compound in the formulation of artificial essences of apple, pear, pineapple among others (Cristiani & Monnet, 2001) and, it was already reported as a producer of bio aroma as pineapple (Zhang et al., 2010). Ethyl acetate is widely used in the formulation to the production of candies and pharmaceutical products. The same behavior was observed for the ethyl lactate produced, the non-*Saccharomyces* yeasts produced more ethyl lactate in the vinasse of cachaça than the *Saccharomyces* yeasts in the tequila vinasse, this ester has also been reported in microorganisms such as *Saccharomyces cerevisiae*, *Kluyveromyces marxianus* and *Pichia kluyveri* (Duarte et al., 2010; Segura-García et al., 2015), but it is the first time that this compound is analyzed in residue of the production of cachaça and tequila by aerobic digestion. Finally, the ester

produced in lower concentration was ethyl hexanoate, which confers the typical pineapple and green apple aroma (Tokitomo, 2007; Duarte et al., 2013). Morais & Silva (2011). One more time, this compound was more produced when utilized the cachaça vinasse (24.3 mgL^{-1}). The same was observed to the ethyl acetate detected in greater concentration from the cachaça vinasse (49.62 mgL^{-1}). Considering the production of minor compounds, the yeast strain *C. parapsilosis* CCMA0191 was the principal responsible for the ethanol production (4.165mgL^{-1}) in tequila vinasse. It has been reported that the ethanol production was a clear consequence of the sugar fermentation by the yeasts preferentially in anaerobic conditions during the cooling of the agave pines, a phenomena already observed by Cedeño (1995). As an oxygenation of the vinasses was produced by the flask shaking, this could be the reason because of the low ethanol levels observed. Therefore, this fermentation strategy is suitable for aroma production from cachaça vinasse and tequila vinasse.

Other compounds in concentrations inferior to 5.0 mgL^{-1} were also detected as methanol, 1-propanol, isobutanol, e ethyl butyrate specially using the tequila vinasse.

4. Conclusions

The vinasse can be used as substrate for to the production of aroma compounds (fruity), which could be an alternative of sustainable reuse of this industrial sub product, enabling a more friendly environment disposal of vinasses.

Considering the volatile compound production yield, the tequila vinasse presented greater reduction of BOD and COD after the cultivation with *C. parapsilosis* CCMA0191 and it can be used to the

2-phenylethanol production, while ethyl acetate production can be performed from the cachaça vinasse. Therefore, the biological yeast treatment of vinasses could be a biotechnological ad-value alternative for the production of these compounds in comparison with the hazardous chemical process.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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