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AVALIAÇÃO DA PRODUÇÃO BIOTECNOLÓGICA DE BUTANOL A PARTIR DE SORGO SACARÍNEO

DISSERTAÇÃO DE MESTRADO

Luiz Jardel Visioli

Santa Maria, RS, Brasil
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AVALIAÇÃO DA PRODUÇÃO BIOTECNOLÓGICA DE BUTANOL A PARTIR DE SORGO SACARÍNEO

Luiz Jardel Visioli

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Orientador: Prof. Dr. Marcio Antonio Mazutti
Co-orientador: Prof. Dr. Marcio Schwaab

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AVALIAÇÃO DA PRODUÇÃO BIOTECNOLÓGICA DE BUTANOL A PARTIR DE SORGO SACARÍNEO

Elaborada por
Luiz Jardel Visioli

como requisito parcial para obtenção do grau de
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COMISSÃO EXAMINADORA:

Marcio Antonio Mazutti, D. Sc.
(Presidente/Orientador)

Wagner Luiz Priamo, D. Sc. (IFRS)

Ayrton Figueiredo Martins, D.Sc. (UFSM)

Santa Maria, 17 de Fevereiro, 2014.

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RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Engenharia de Processos
Universidade Federal de Santa Maria

PRODUÇÃO DE BIOBUTANOL A PARTIR DE SORGO SACARÍNEO POR MEIO DE PROCESSOS BIOTECNOLÓGICOS

AUTOR: LUIZ JARDEL VISIOLI
ORIENTADOR: MARCIO ANTONIO MAZUTTI
Data e Local de Defesa: Santa Maria, 17, fevereiro de 2014.

A produção de butanol a partir de processos fermentativos é de fundamental importância para aumentar a oferta mundial de biocombustíveis e permitir que estes substituam o uso de combustíveis fósseis. A principal dificuldade em relação a esta produção ocorre devido a não viabilidade econômica dos processos de produção aplicados. Os aspectos que mais contribuem para isto são a inibição pelo produto a baixas concentrações, baixa produtividade e uso de substratos caros. Este trabalho está dividido em quatro artigos científicos que estão voltados a questões envolvidas com a produção deste biocombustível. Os dois primeiros fazem uma revisão da literatura científica sobre o tópico, já os últimos são trabalhos científicos de desenvolvimento de metodologias e processos. O artigo 1 traz um ponto de vista em relação ao desenvolvimento do processo, desde o ano de 1980, através da análise das patentes registradas sobre produção de butanol no mundo. Além disso, a partir dos resultados é possível prever, parcialmente, como a tecnologia deverá avançar nos próximos anos. O artigo 2 faz uma revisão dos artigos científicos publicados sobre o fermentação butílica nos últimos tempos. A principal característica do mesmo é apontar os principais problemas relacionados à produção, mostrando a importância dada ao substrato utilizado, o micro-organismo e os processos de separação. No artigo 3 uma metodologia para determinação de solventes no meio de fermentação é desenvolvida. Esta técnica propõe uma relação linear entre a variação da densidade, a concentração de açúcar e a concentração de solventes. Com sua aplicação o cromatógrafo pode ser dispensado e há somente a necessidade de um densímetro. O ajuste se mostrou bastante promissor e aparentemente capaz de predizer os resultados. Por fim, no artigo 4 é desenvolvido um processo para a produção de biobutanol via fermentação por clostridium a partir de sorgo sacaríneo. Butanol é produzido a partir do substrato sendo necessário um acréscimo pequeno de extrato de levedura e triptona com apenas 12,5% de volume de inóculo, com pH inicial ajustado em 5,5. Para a execução dos experimentos em meio anaeróbio foram elaborados aparatos alternativos e de baixo custo, que demonstraram ser eficientes na sua função. O principal ponto observado durante o trabalho é que é possível produzir biobutanol a partir de sorgo sacaríneo utilizando artefatos fabricados no laboratório para manutenção do meio anaeróbio.

Palavras-chave: produção de biobutanol, metodologia de análise, sorgo sacaríneo, fermentação clostridial.

ABSTRACT

Thesis for the degree of Master of Science
Post-Graduation Program in Process Engineering
Federal University of Santa Maria

BIOBUTANOL PRODUCTION FROM SWEET SORGUM BY MEANS OF BIOTECHNOLOGICAL PROCESSES

AUTHOR: LUIZ JARDEL VISIOLI
ADVISOR: MARCIO ANTONIO MAZUTTI
Date and Local: Santa Maria, 17, February de 2014.

The biobutanol production by fermentative process has a great importance to increase the global supply of biofuel and becomes these able to replace the use of fossil fuel. The main difficulty associated to this production occurs due the not economic viability of applied production process. The aspects that have more contribution to this are the product inhibition at low concentration, low titer and the use of expensive substrates. This work is divided in four scientific articles which are focused in question involved to this solvent production. The first two are review papers about the topic, whereas the last two are research papers related to development of analytical methods and production process. The first paper reports to the main process development since 1980 year, by analyses of registered patents in relation to butanol production worldwide. The second paper presents a review from scientific articles about butyric fermentation published in recent years. The central characteristic of it is show the main troubles related to production, exhibiting the importance of the used substrate, as well as the choice of microorganism and separation process. Third paper presents a methodology to solvents determination from fermentation medium. This technique proposes a linear relationship between the density variation, sugar and solvents concentrations. The method proposed showed good results being promising to predict the ABE concentration in an easy and fast procedure. Fourth paper reports the development of the process to production of biobutanol by clostridial fermentation from sweet sorghum juice. Butanol is produced from substrate and small addition of yeast extract and tryptone, using 12.5% of initial inoculums size, at initial pH pH value equal to 5.5. In this work was demonstrated the possibility to produce biobutanol from sweet sorghum.

Keywords: biobutanol production, methodology of analysis, sweet sorghum, clostridial fermentation.

LISTA DE TABELAS

ARTIGO 1

ARTIGO 2

ARTIGO 3

ARTIGO 4

APÊNDICES

LISTA DE FIGURAS

ARTIGO 1

ARTIGO 2

ARTIGO 3

ARTIGO 4

APÊNDICES

LISTA DE APÊNDICES

SUMÁRIO

1 INTRODUÇÃO

O constante crescimento do preço do petróleo seja pela diminuição das reservas internacionais ou por questões geopolíticas, traz à tona a necessidade de se produzir combustíveis de origem renovável. Para alguns autores da área, além de uma questão energética, a produção de biocombustíveis utilizando substratos regionais, é uma questão de soberania nacional (SORENSEN, 2008). Outra importante razão para a produção e uso de biocombustíveis é a redução da emissão de gases causadores do efeito estufa (TAYLOR, 2008). É discutido que se a produção deste tipo de combustível não utilizar suprimentos de origem fóssil a emissão de carbono para atmosfera pode ser zerada.

Uma fonte importante para a produção de biocombustíveis é a fermentação. No Brasil, etanol é produzido em grande quantidade pela fermentação do caldo de cana de açúcar. No entanto, a produção de butanol surge como uma alternativa interessante, atraindo muita atenção, principalmente, em relação à pesquisa e ao desenvolvimento de processos industriais. A busca pela viabilização da produção de biobutanol vem sendo fomentada, sobretudo devido às propriedades deste biocombustível. Entre estas se destacam ser menos volátil, menos corrosivo, ter maior capacidade energética e principalmente ser um combustível muito similar à gasolina (QUERSHI e EZEJI, 2008).

A fermentação butílica é normalmente realizada em meio submerso. Uma das principais dificuldades desta fermentação é o manuseio do inóculo. O micro-organismo normalmente utilizado tem morte celular se entrar em contato com o oxigênio. Outra grande dificuldade com relação à produção de butanol são os aparatos necessários para que ela ocorra de forma adequada. Para o manuseio e mistura do pré-inóculo ao meio de fermentação é necessário um ambiente fechado com condições de anaerobiose. Comercialmente, uma caixa de luvas pode ser adquirida, no entanto, tem preço bastante elevado e é interessante o desenvolvimento de um artefato mais acessível. A intolerância à presença de oxigênio durante a fermentação constitui uma grande dificuldade para a produção industrial deste combustível.

A produção de biobutanol se dá através da fermentação de açucares por bactérias do gênero clostridium. Clostridium é um gênero de bactérias gram-positivas que tem por principal característica seu caráter anaeróbico. Os principais micro-organismos utilizados na produção de butanol são*: C. acetobutylicum, C. beijerinckii, C. butilicum, C. pasteurianum e*

C. saccharobutylicum (ZHENG *et al.*, 2009). A fermentação butílica produz dois co-produtos principais, a acetona e o etanol, ficando conhecida como fermentação ABE.

Algumas outras dificuldades são constatadas durante a produção de biobutanol utilizando estas bactérias. O que mais afeta a viabilidade do produto é a inibição do micro-organismo pelo produto à baixa concentração (menor que 20 g/L), além do alto custo para separação dos produtos do meio de fermentação. Como qualquer outro tipo de fermentação, o custo dos biocombustíveis está muito associado ao custo da matéria-prima utilizada para a fermentação. No entanto, o substrato tem de ser ofertado em quantidade significativa e com disponibilidade, preferencialmente, durante todo o ano. Alguns autores (QURESHI *et al.*, 2010 e AL-SHORGANI *et al.*, 2012) afirmam que este fator é o principal a ser superado a fim de viabilizar a produção do biobutanol.

Além da preocupação com o custo final do bicombustível produzido é necessário analisar a viabilidade da implementação de uma planta industrial para sua produção. QUERSHI e colaboradores (2007) descrevem como prioritário o uso de resíduos agroindustriais para esta viabilização. Estes resíduos são, normalmente, ricos em celulose e esta seria a fonte de açúcar para a fermentação. Entretanto, quando se fala neste tipo de matéria-prima deve-se atentar para o fato de que mais uma etapa de hidrólise será necessariamente acrescentada ao processo, aumentando seu custo final. Ao mesmo tempo é de estrita importância que a matéria-prima esteja disponível em grande quantidade e durante todo o ano. Isto dificilmente acontecerá com um resíduo uma vez que sua produção está associada a outro produto e, portanto, a geração deste resíduo não ocorrerá de acordo com a planta de bicombustível instalada (o que gerará problemas operacionais futuros). Surge então a necessidade de uma rica fonte de açúcares que esteja adaptada ao clima local, sendo o sorgo sacaríneo uma alternativa muito atraente.

O sorgo sacaríneo (*Sorghum bicolor (L.) Moench*) é fonte de biomassa e açúcares. Seu caldo contém quantidades similares de glicose e sacarose e ambas podem ser utilizadas na produção de butanol. Além disso, o bagaço é composto por grande quantidade de carboidratos (celulose e hemicelulose) propiciando alguma aplicação adicional (YU *et al.*, 2012). Quando comparado a outras fontes tradicionais de açúcar, o sorgo também, pode ser considerado uma alternativa. Em relação à beterraba, sua colheita é muito mais simples e menos onerosa, pois suas reservas de açúcar não se encontram sob o solo. No caso brasileiro sua utilização poderia ser questionada devido ao amplo cultivo da cana de açúcar. No entanto, ele apresenta melhor adaptação à climas mais frios, que é o caso da região sul do país. Outra importante vantagem está no plantio. O sorgo tem germinação a partir de sementes, o que facilita o manejo e reduz

custos de produção. Já a colheita é muito similar a da cana tornando possível se utilizar a mesma tecnologia existente para o processo (WHITFIELD *et al.*, 2012).

Na região sul do Brasil, o clima dificulta o plantio de cana de açúcar com alto desempenho. No entanto, como o sorgo pode se desenvolver em climas com invernos mais rigorosos do que aqueles tolerados pela cana, se torna muito atrativo para, principalmente, o Rio Grande do Sul. MARCHEZAN e SILVA (1984) relatam bons resultados com o cultivo de diversas cultivares de sorgo em Santa Maria comprovando a viabilidade da cultura na região central do Rio Grande do Sul. A Embrapa também vem investindo no desenvolvimento de novas variedades da cultura com objetivo de ampliar a área usada para produção de energia e desenvolver uma cultura para a entressafra de cana de açúcar. Para o Rio Grande do Sul, esta seria uma boa oportunidade de entrar de vez no mercado de bioenergia via fermentação e colaborar para a diversificação da matriz energética nacional.

Depois de realizar a fermentação e produzir o butanol sua quantificação é de fundamental importância, assim como as dos co-produtos acetona e etanol. A principal forma descrita para esta determinação é a utilização de cromatografia gasosa. As condições cromatográficas usadas são bastante variadas, no entanto, como os compostos a serem determinados têm ligação carbono hidrogênio, o detector mais adequado é o de ionização de chama.

No entanto, este trabalho busca, além de um processo para produção de butanol, formas de reduzir o custo. Para isso é importante que se analisem todas as possibilidades em todas as etapas envolvidas. No que diz respeito à quantificação dos produtos de fermentação, uma propriedade física simples pode auxiliar em uma análise preliminar. A densidade de produtos e do meio inicial é bastante distinta e isso pode gerar uma mudança na densidade da mistura durante a fermentação. A dificuldade da técnica está em conseguir relacionar esta variação com a concentração dos produtos. Em caso positivo, um simples densímetro pode ser suficiente para as medidas e não haveria necessidade de analise instrumental, diminuindo ainda mais o investimento. Todavia, a técnica não é capaz de quantificar os produtos individualmente, mas somente a concentração total de solventes ABE.

Para que se possam abordar as principais questões referentes à produção de biobutanol a partir de sorgo sacaríneo, brevemente discutidas neste capítulo introdutório, o trabalho foi dividido da seguinte forma. A primeira parte do trabalho consta de uma abrangente revisão bibliográfica. A revisão está ainda subdividida em dois artigos de revisão, publicados em periódicos internacionais da área. O primeiro artigo consta de uma revisão das patentes registradas nesta área desde 1980 e traz uma importante visão do desenvolvimento

tecnológico no período. Este manuscrito já se encontra publicado no periódico Recent Patents on Engineering. O segundo artigo aborda o desenvolvimento científico do tópico, levando em conta os artigos acadêmicos publicados nos últimos anos que abordam a produção de butanol via processos fermentativos. Para uma maior clareza na leitura, o artigo está dividido de acordo com os aspectos fundamentais envolvidos nesta produção. Este manuscrito encontra-se submetido à revista Sustainable Chemical Processes. Conforme já comentado, a revisão bibliográfica foi abrangente, visto que é de fundamental importância um amplo conhecimento em relação ao assunto.

Com relação aos resultados experimentais obtidos durante o desenvolvimento do mestrado, também há uma divisão em dois artigos científicos. Esta divisão se tornou necessária em razão da distinção entre os assuntos abordados em cada um deles. No primeiro se encontra o desenvolvimento de uma metodologia analítica alternativa para a estimação da concentração de solventes. A metodologia propõe a utilização das medidas de densidade do meio (e suas variações) para a predição do teor de solventes total. O manuscrito se encontra submetido no periódico Bioprocess and Biosystems Engineering. Já o segundo artigo científico resultante da pesquisa relata o desenvolvimento de um processo de produção de butanol a partir de sorgo sacaríneo usando fermentação clostridial. Neste trabalho, são relatadas as etapas de otimização das variáveis testadas ao longo do trabalho. Outro aspecto fundamental é mostrar algumas alternativas desenvolvidas para o manejo de inóculo e meio de fermentação a fim de reduzir o investimento necessário à pesquisa. Este último manuscrito está submetido ao periódico Journal of Food Process Engineering.

2 OBJETIVOS

O objetivo principal deste trabalho é a análise de um processo de produção de butanol a partir de sorgo sacaríneo por meio de processos fermentativos usando a bactéria *clostridium beijerinckii* NRRL B-592. Para melhor alcançar este objetivo, foram traçados alguns objetivos específicos:

- Analisar o desenvolvimento de novas tecnologias ao longo dos últimos anos através de uma prospecção das patentes registradas na área;
- Apresentar os principais estudos que vem sendo realizados a fim de aprofundar o conhecimento e condensar as informações em um único volume;
- Compreender as principais dificuldades encontradas pelos pesquisadores em relação à produção butanol;
- Desenvolver os aparatos necessários para a manipulação e fermentação anaeróbicas com os equipamentos disponíveis e com baixo custo total;
- Propor um método físico para a estimação da composição da mistura produzida na fermentação que possa ser uma alternativa ao uso de cromatografia;
- Realizar experimentos de fermentação a fim de verificar a influência de algumas das principais variáveis envolvidas no processo em questão.

3 REVISÃO BIBLIOGRÁFICA

ARTIGO 1- TECHNOLOGICAL PROSPECTION FOR BIOBUTANOL PRODUCTION

Luiz J. Visioli, Heveline Enzweiler, Raquel C. Kuhn, Marcio Schwaab, Marcio A. Mazutti*

Department of Chemical Engineering - Federal University of Santa Maria, Av. Roraima, 1000, Santa Maria, RS, 97105-900, Brazil

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Abstract

This work reviewed the patents related to biobutanol production from biomass, giving the reader an overview of the topic. The main characteristics and trends about biobutanol production were compiled and discussed. It was pointed out that the future production of biobutanol will be driven by the selection of a new microorganism or the development of an engineered strain which will be able to overproduce butanol. In addition, a microorganism that produces low content of acetone and ethanol and tolerates high butanol concentration in the medium is highly desirable, since it is the main bottle-neck in the process nowadays.

Keywords: biobutanol, technological prospection, fermentation, bioreactor design, process and products, microorganisms, separation.

Introduction

Patents are important instruments for the technological development of nations, since they encourage governs and inventors to invest in research and creative work with practical application [1, 2]. Besides, patents are able to indicate the future direction that a research topic may take and what still demands improvement [2]. In 2009, Kharkwal et al. [3] reviewed the inventions and relevant papers regarding microbial production of butanol, demonstrating the importance of patents in science development.

The production of biobutanol was firstly reported by Louis Pasteur, in 1861, [4] and has been studied since 1912, when Chaim Weizmann discovered the bacteria *Clostridium acetobutylicum*. The research in this area was enhanced during and/or near the Second World War, when the fuel demand substantially raised. Some patents were published in this period [5-8], but, after the Second World War, few patents have been published [9, 10] due to the increase of feed-stock costs and the advancement of the petrochemical industry.

The research about biobutanol production has increased over the last years due to the need of an efficient and viable renewable fuel (biofuel) as an alternative to fossil fuels [11]. The world production of biobutanol is projected to increase from 25 million gal, in 2008, to more than an order of magnitude until 2020, what will be accompanied by an increasing trend in prices of butanol, leveraging the production of biobutanol [12]. Regarding its properties,

biobutanol can be compared to gasoline, but it also has carbon neutral, with no net addition of greenhouse gases into the atmosphere [13].

The technical and economical viability of the industrial production of biobutanol depends on some factors, such as the use of cheap and abundant substrates, the development of an efficient pre-treatment of feedstock [14, 15], the development of engineered microorganism to increase butanol concentration [16], the development of methods of disruption of unwanted metabolic pathway or the pathway creation in other bacteria (e.g. *E. Coli*) [17], the development of strategies for in situ separation [18] and its optimization to decrease the costs of the process [19] and to increase the energetic efficiency during the downstream [20]. Furthermore, it is very important to operate the industrial plant at optimized conditions in order to make the production of biobutanol economically feasible [21].

Based on these aspects, the objective of this review is to present the technological prospection of biobutanol production worldwide since 1980. For this purpose, a search for patents has been done on an international basis at Spacenet.com website and also at inpi.gov.br – Brazilian Inventions. In both cases, the key words used were Butanol and "bacterial" or "fermentation", and "butanol" and "production process", being the main results compiled in graphic and/or tables, showing the evolution and future trends of biobutanol production.

Recent advantaces on the biobutanol production

The recent advances in the biotechnological area are evident from a search in patents database worldwide. For the production of biobutanol it is not different, since technological bottlenecks have been studied along the years to show the economical viability in the production of this important and efficient biofuel [11, 22]. The distribution of the published patents over the years, as shown in Fig. (1), is an interesting way to discuss how science is evolving [1]. Fig. (1) discriminated 120 different international patents that discuss biobutanol production from the fermentation of biomass.

Fig. (1) reveals that the production of biobutanol has in-creased from 1980s, although the number of registered patents in early 2008 was low. According to García et al. [15, 17], many pilot plants were constructed and some researchers continued their investigations, mainly in France, Austria, South Africa and Russia. The lack of interest in the production of

biobutanol during this period was associated with difficulties to obtain better performance and yield, low production cost of petrochemical butanol and the high cost of substrate [17]. In addition, the worldwide appeal for biofuel, or at least something superior than ethanol, decreased considerably [23]. Fig. (1) also shows an increase in the number of patents related to biobutanol production in the last five years, and this clearly demonstrates that the area is growing quickly [24]. When compared with the increase between 1984 and 1992, the current interest in the topic is larger. This is due to high price of crude oil and the increasing concern over global warming, which have renewed the interest in biotechnological production of butanol, not only as a chemical, but also as an alternative fuel. It led to an increase in the number of companies that are developing biobutanol processes [17].

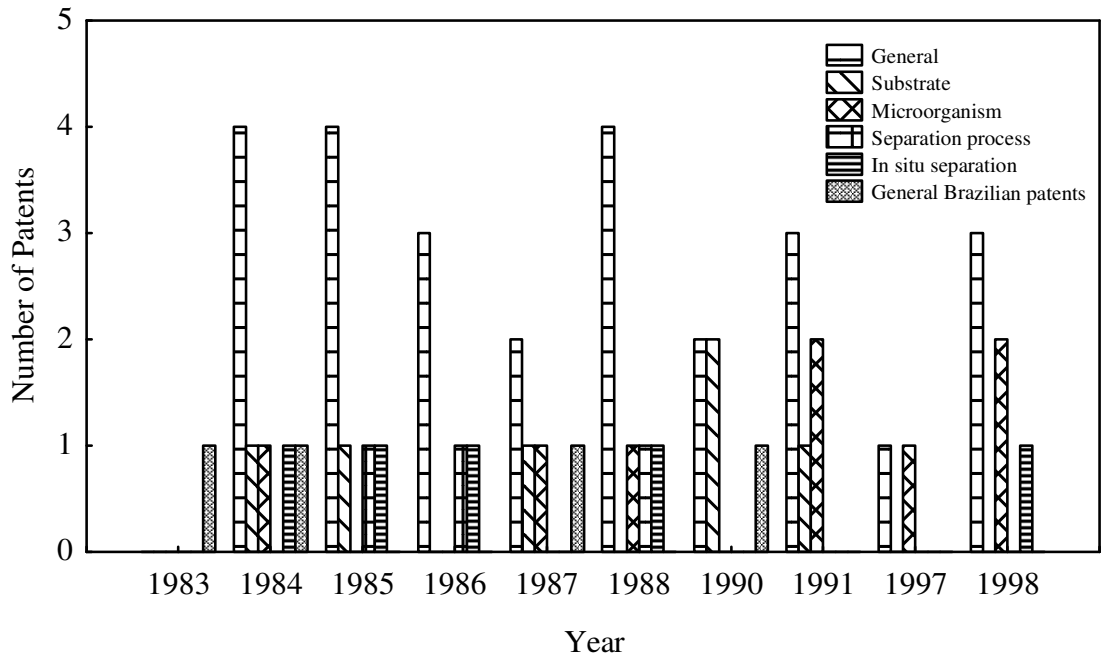

Figure 1a- Evolution of patents published concerning whole process, microorganism, separation process, *in situ* separation and Brazilian patents over the years between 1980 and 2000.

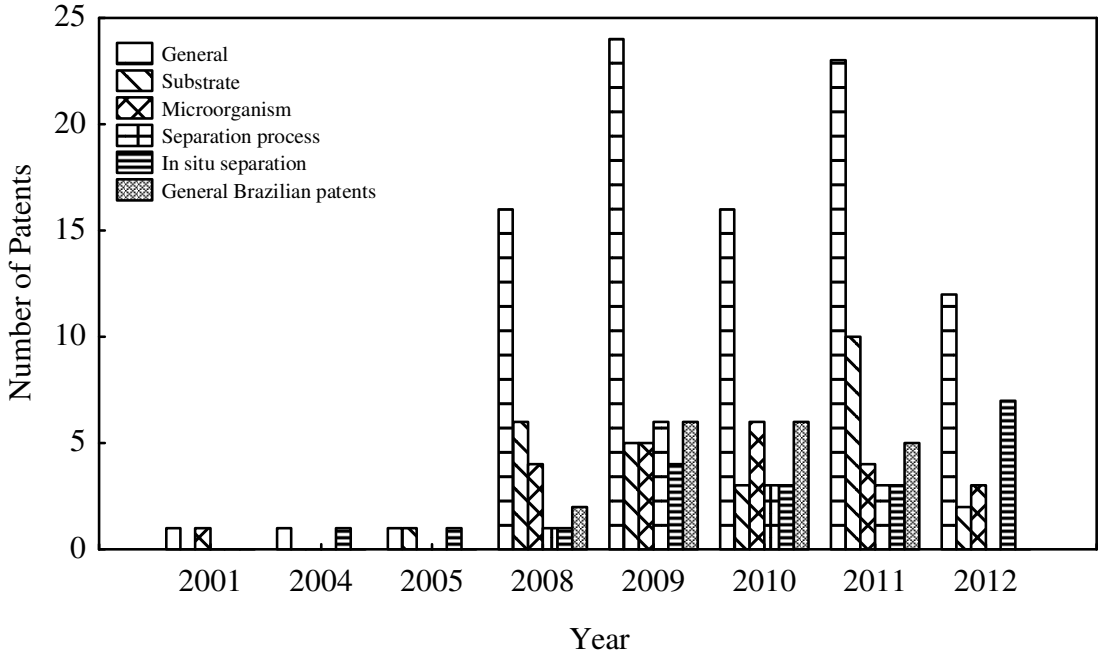

Figura 1b- Evolution of patents published concerning whole process, microorganism, separation process, *in situ* separation and Brazilian patents over the years and between 2000 and 2012.

Another important characteristic that needs to be investigated is the distribution of patents according to different aspects that influence the production of biobutanol, such as, substrate, microorganism, separation process, in situ separation and the entire process. Substrate and microorganism are the main focus of the deposited patents (28% and 27%, respectively), whereas the inventions about in situ separation are responsible for 20% of patents. In fact, substrate and microorganism influence the process performance, so the majority of researches and deposited patents are related to these subjects. Concerning substrate, Jurgen et al. [25] stated that, for the production of butanol fuel for transportation purposes it should be produced from lignocellulosic biomass. In addition, the choice of low cost feedstocks is necessary to make the fermentation economically feasible, since the feedstocks impact the production cost greatly [26]. Concerning microorganism, most patents are focusing on the development of an engineered bacteria, aiming to overcome the microbial growth inhibition by biobutanol itself [27], as well as the low titer (due to a greater production of solvents) generated by bacteria from clostridium strains [28].

Table 1 shows the main patents registered since 1980 (it must be observed that only the most relevant inventions were considered in Table (1), presenting information about the year of

invention, classification, the applicant(s) reported in the register and a brief description of the patent.

From Table 1 it is possible to observe that about 21% of the granted/deposited patents are from four companies: Butamax Advanced Biofuels LLC® (a company formed by two large world companies: BP® and DuPont®) [68], GEVO Inc® from USA, Institute of Process Engineering (IPE), from China, and Institut Francais du Petrole®, from France. Butamax Advanced Biofuels LLC® had a major number of patents (11 registers from 120 selected), followed by Institut Francais du Petrole® (with 9 registers), IPE (6 patents) and GEVO Inc® (5 patents). However, Butamax Advanced Bio-fuels LLC® is a new company, being born in 2009 [68], and, in the last two years, is the leader in deposits of patents. So this company will be likely the most important applicant of biobutanol technologies worldwide.

Table 1: Principal patents registered since 1980, classification and a brief description.

Ref.	Year	Classification	Applicant	Kind of butanol	Description
[29]	2012	*in situ* separation	BUTAMAX TM ADVANCED BIOFUELS	Iso butanol	Product is removed during the fermentation by extraction into a water-immiscible organic extraction agent in the presence of at least one osmolyte.
[30]	2012	*in situ* separation	BUTAMAX TM ADVANCED BIOFUELS	Iso butanol	Product is removed during the fermentation by extraction into a water-immiscible organic extraction agent in the presence of at least one electrolyte.
[31]	2012	*in situ* separation	BUTAMAX TM ADVANCED BIOFUELS	Iso butanol	Method for recovering butanol from a fermentation medium using two extractions with immiscible organic extraction agent.
[32]	2012	Substrate	GUANGXIACADEMY OF SCIENCES	n-Butanol	Use o cassava as substrate to butanol production.
[33]	2011	Separation process	BUTLER III EUGENE	n-Butanol	The method describes a process to remove butanol from fermentation broth without harm the cells, increasing the production.
[34]	2011	Substrate	GUANGZHOU INST ENERGY CONV CAS	ND	Method for producing butanol by co-fermenting cellobiose, pentose and hexose as substrate.
[35]	2011	Substrate	CHENGDU INST BIOLOGY CAS	ND	The invention reports the use of duckweed as a raw material.
[36]	2011	Microorganism	GUANGXIACADEMY OF SCIENCES	ND	The invention relates to a strain that tolerates 30 g L^{-1} butanol in a culture medium and fermentation liquor.
[37]	2011	Substrate	INST PROCESS ENG CAS	n-Butanol	The invention discloses a method for producing butanol by continuous solid state fermentation of restaurant-kitchen garbage.
[38]	2011	Entire process	UNIV NANJING	ND	The invention discloses a method for continuously producing bio-butanol by fermentation, separation and coupling of a static bed.
[39]	2011	*in situ* separation	METABOLIC EXPLORER SA	n-Butanol	The present invention relates a process for the recovery of a solvent, in particular butanol, from a fermentation broth comprising an isothermal gas stripping step.
[40]	2010	Microorganism	GEVO INC.	n-Butanol	The invention discloses a recombinant microorganism expressing at least a heterologous enzyme of an NADH-dependent pathway

					for conversion of a carbon source to n-butanol.
[41]	2010	*in situ* separation	UNIV NANJING	ND	The invention relates a process for *in situ* separating acetone, butanol and ethanol by coupling biomass fermentation and pervaporation.
[42]	2010	Substrate	JIANSHE WANG	n-Butanol	The invention discloses a method for producing biological butanol with straw-like materials or agricultural and forestry wastes.
[43]	2010	Substrate	UNIV BEIJINGCHEMICAL	n-Butanol	Method for producing butanol by hydrolyzing and fermenting waste crops rich in hemicellulose.
[44]	2010	Microorganism	DSM IP Assets B.V.	n-Butanol	The invention relates the engineered pathway to produce butanol.
[45]	2010	Microorganism	BUTAMAX TM ADVANCED BIOFUELS	Both	Bacteria containing non-native pathways for butanol production increased the tolerance to butanol when the content of unsaturated trans fatty acids increased in the membrane
[46]	2010	Entire process	DU PONT/ BUTAMAX TM ADVANCED BIOFUELS	n-Butanol	A method for the production of 1-butanol by fermentation using a microbial production host . The method employs a reduction in temperature during the fermentation process that results in a more robust tolerance of the production host to the butanol.
[47]	2010	Entire process	STICHTING DIENST LANDBOUWKUNDI	n-Butanol	The invention relates a process for the combined production of butanol and hydrogen from biomass, comprising the steps of fermenting biomass to obtain butanol in a first reaction mixture.
[48]	2010	Entire process	JIANGSU LIANHAI BIOLOG TECHNOL	n-Butanol	The method describes the butanol production using flour and deeply-processed waste water as main materials.
[49]	2009	Microorganism	COBALT TECHNOLOGIES INC	n-Butanol	A method and system for high-yield fermentation using Clostridium species having tolerance of a concentration of butanol of up to 15%.
[50]	2009	*in situ* separation	NIPPON CATALYTIC CHEM IND	n-Butanol	The method purpose to recovery 1-butanol by a pervaporation separation membrane method using a PV membrane having a specific membrane thickness in the temperature region.
[51]	2009	Microorganism	DU PONT/ BUTAMAX TM ADVANCED	Both	The invention relates to the fields of microbiology and genetic engineering. More specifically, altered membrane cyclopropane fatty acid composition was found to play a role in butanol

			BIOFUELS		tolerance in bacteria which are not natural butanol producers
[52]	2009	Microorganism	DU PONT/ BUTAMAX TM ADVANCED BIOFUELS	Both	Microorganisms demonstrating high tolerance to alcohols, particularly butanols have been isolated.
[53]	2009	Separation process	UNIV HEBEI TECHNOLOGY	ND	The method purpose the use of distillation towers to rectify the products of fermentation.
[54]	2009	Entire process	UNIV TSINGHUA	n-Butanol	The invention provides a process for producing butanol by intermittent oxygen supply, material adding and fermentation of microbe.
[55]	2009	Substrate	GUANGZHOU INST ENERGY CONV CAS	n-Butanol	The invention provides a method for producing acetone-butanol by adopting fermentation and taking the byproduct of wheat starch slurry or wheat starch which is obtained after extracting wheat gluten from the wheat as a main raw material.
[56]	2009	Entire process	GEVO INC.	n-Butanol	Metabolically-engineered yeast and methods of producing n-butanol.
[57]	2009	In Situ separation	GEVO INC.	ND	This invention is directed to methods for recovery of C3-C6 alcohols from dilute aqueous solutions, such as fermentation broths.
[58]	2009	Entire process	RELIANCE LIFE SCIENCES PVT LTD	n-butanol	The present invention provides a process for production of high yields of butanol by *Clostridium acetobutylicum* ATCC 10132. The process can be completed in a shorter span of time, using batch process through manipulation of various process parameters.
[59]	2008	IN SITU separation	TETRAVITAE BIOSCIENCE INC	n-Butanol	Methods and systems are provided for the separation of solvents, including, but not limited to, butanol, from a fermentative solventogenesis reaction medium.
[60]	2008	Entire process	DU PONT; BRAMUCCI MICHAEL G; FLINT DENNIS; MILLER JR EDWARD S; NAGARAJAN	n-Butanol	A method for the production of 1-butanol by fermentation using a microbial production host is disclosed. The method employed a reduction in temperature during the fermentation process that results in a more robust tolerance of the production host to the butanol product.

			VASANTHA; SEDKOVA NATALIA; SINGH MANJARI; VAN DYK TINA K		
[61]	2008	Microorganism	GEVO INC.	n-Butanol	The invention disclosed a recombinant microorganism expressing at least a heterologous enzyme of an NADH-dependent pathway for conversion of a carbon source to n-butanol.
[62]	2008	Entire process	ADVANCED BIOFUELS INC	n-Butanol	The invention described a method for solvent production that used a recombinant bacteria and yeast to produce biobutanol from lignocellulosic and other plant-based feedstocks.
[63]	2005	In Situ separation	UNIV ILLINOIS	n-butanol	The invention used the gas stripping to remove the butanol produced by fermentation from the medium. This generated a continuous process for production of solvents. Furthermore the concentration of substrate and microorganism were controlled to the continuity of process.
[64]	1988	IN SITU separation	UNIV VERMONT	n-Butanol	The method is carried out by culturing a butanol-producing microorganism in a culture medium containing a fluorocarbon.
[65]	1986	IN SITU separation	CENEDELLA RICHARD J	n-Butanol	The process of extracting butyric acid and normal butanol from microbial fermentation broth, comprising contacting an aqueous solution of a microbial fermentation broth with vinyl bromide how extract.
[66]	1985	IN SITU separation	KAO CORP	n-Butanol	Obtainament of butanol from a glucose raw material by fermentation method, to recover butanol efficiently and easily, by using a specific alcohol as an extraction agent.
[67]	1984	IN SITU separation	CHIYODA CHEM ENG CONSTRUCT CO	ND	To recover efficiently and easily butanol, by extracting the butanol from a fermentation liquor with a higher alcohol based extracting agent in producing the butanol by the fermentation method

Substrate

The choice of substrate is an important aspect to be evaluated during the development of any Bioprocess, due to the impact on the process viability, mainly under the economic aspect [26]. In case of biobutanol, there are several alternative substrates reported in the patents. Fig.(1) presents a chronological evolution of the patents concerning the development of substrate for the production of biobutanol.

From Fig.(1) it is observed that the majority of inventions have been reported in the last five years, following the same trend for general patents, since the selection of an appropriate substrate is one of the main bottlenecks to be solved in the industrial production of butanol. Besides, many of the feedstocks that can be used as substrate for butanol production usually require a pretreatment, what can produce fermentation inhibitors. Consequently, substrate selection is a very important issue to be evaluated in this area, explaining the high number of inventions concerning this topic of biobutanol production [69]. The main classes of substrate reported were lignocellulosic materials, with approximately 38% of the registers, starches and sugars with 22% and 28% of patents, respectively.

It was possible to observe an increase in the use of cellulosic and starch-rich raw material for the production of biobutanol. According to Jurgens et al. [25] lignocellulosic materials are good sources of substrate for biobutanol production, since they are cheaper than sugar and starch sources and more abundant around the world, besides, they do not compete with the food chain. On the other hand, foodstuff wastes are also considered important sources of carbon [70], but the low availability can limit their use. Even though more expensive than other substrates, sugar material has a great application in the production of biobutanol, since it does not require pretreatment and it is an abundant raw-material, especially in Brazil due to large scale production of sugarcane juice [71].

Charles et al. [32] reported the use of cassava as a substrate to produce biobutanol. Although cassava is used to human feed in many countries worldwide, its surplus production can be directed to biofuel production. Other substrates, such as hydrolyzed straw [34], cellulosic, lignocellulosic and hemicellulosic [43] duckweed [35], restaurant-kitchen garbage [37] and wheat processing residue [55] are also reported for the production of biobutanol.

Microorganism

The inventions related to microorganisms are the second position regarding the number of inventions since 1980. Thus, it is very important to discuss the theme, in order to provide the reader with the perception of what level the technological development has reached in this topic. Fig.(1) presents the chronological profile of published patents that have been related to microorganism, demonstrating a considerable increase in number of patents over the last years. The majority of patents concerning the use/development of microorganisms for the production of biobutanol describe the characteristics of a new engineered microorganism during fermentation [56, 61, 72-75]. From this analysis, it is possible to conclude that the microbial modification is fundamental for the industrial production of biobutanol, as long as, from genetic modification, it is possible to increase butanol titer (in relation to other solvents) [49, 76, 77], decrease the inhibition of microorganism by butanol [36,78] and create a microorganism with a higher oxygen tolerance [54], facilitating the process operation during fermentation [16, 79]. Although there is a predominance of patents using genetically modified microorganism, there is a patent that reports the increase in butanol tolerance by using a solid support to immobilize the microorganism [80], creating a barrier for the contact between butanol and cell wall, minimizing the inhibition [49].

An important factor showed in this review is the production of iso or n-butanol during fermentation. The native butanol producer is *Clostridium sp*, and these bacteria produce n-butanol [44]. Therewith, recombinant microorganism that has been engineered to have a *clostridium* pathway will produce n-Butanol [46]. On the other hand, Flint and van Dyk [45] reported the production of iso-butanol by a *clostridium* engineered pathway, introduced in a *Pseudomonasputida*, to improve the tolerance of the microorganism to butanol in the medium. According to Charles et al. [31], iso-butanol just can be produced by biological synthesis using a recombinant host.

Some important patents about microorganism in this field are better discussed below. Contag et al. [49] reported the production of biobutanol using *Clostridium* species having tolerance up to 15% (150 g L^{-1}). The authors used immobilized and mutant bacteria. Su and Yang [36] patented strain of *Pediococcus acidilactici N-30* that is able to grow in a medium with 30 g L^{-1} of butanol. Bramucci et al. [52] reported the isolation of a facultative aerobic microorganism of the genus *Enterococcus* that has a tolerance to 2.5% w/v butanol growing

on a solid medium. Buelter et al. [61] developed a recombinant microorganism that activates a heterologous enzyme of a NADH-dependent pathway for conversion of carbon source to n-butanol through the production of one or more metabolic intermediates. These reduce the production of byproducts during fermentation**.**

Separation process

The separation process of biobutanol, water and co-products from fermentation broth is an important aspect in the production from biomass [81]. This review will report separately the final separation or rectification and in situ separation, since they are different processes and both have a great importance in the production of this biofuel. Fig.(1) presents the chronological publication of patents in this field.

From Fig.(1), it can be observed that the total number of inventions related to separation process is smaller than the ones related to microorganism or substrate. It occurs because the separation process is not a limiting step of biobutanol production, although it is considered a restrictive step to the economical feasibility of the production [20, 53]. At the moment, the low butanol concentration in the fermented broth is the major drawback [11]. However, the interest in the separation process becomes essential due to the necessity of reduction of the energetic costs associated with the separation of butanol.

From the patents presented in Fig.(1), some of them deserve more attention. Butler III [33] proposed a vessel to remove the excess of butanol during fermentation, avoiding product inhibition. The main problem solved by this invention is the removal of biobutanol without harming the cells. On the other hand, Feng and Wang [82] reported a scheme of distillation towers that increases the purity of products and decreases the energy costs of separation. Similar invention is also reported by Li et al. [53], who stated that the patented distillation equipment has a better performance in operation, decreasing the separation energy costs.

In situ separation

For a better understanding during the reading, this review divides the separation process and in situ separation, since the last one is widely used to decrease the inhibition byproduct during fermentation [18].

In the last five years the number of patents has significantly increased, especially since 2012 (Fig.1), when eight patents have been registered. This trend shows the importance of in situ separation, since it allows an increase in production of butanol during the fermentation stage and also helps the following separation stage [83]. Besides that, in situ separation is an excellent alternative to make the industrial production of this biofuel feasible, mainly due to the promising results obtained. Furthermore, some authors [18, 81] reported that this technology also reduces the separation cost (mainly with energy) to rectify the products at a down-stream process.

Some important patents in this field are reported below. Charles and Ranjan [29] used a non ionic organic compound to remove butanol from aqueous medium during fermentation, increasing the production due to in situ extraction of butanol. Furthermore, the separation of an organic compound is easier due to its immiscibility with water. In the same direction, another invention reports the use of water immiscible organic compounds (C12 to C22 fatty alcohols, fatty acids, fatty esters, fatty aldehydes, and mixtures thereof) to remove butanol from fermentation medium [31]. Jiang et al. [41] and Eita et al. [50] proposed the use of pervaporation to remove butanol from the fermentation medium, enhancing the efficiency of ABE process. Other works reported the use of vinyl bromide to extract any butyric acid and normal butanol [65], fluorocarbon and the use of higher alcohol as solvent for in situ butanol extraction [67].

Brazilian patents

In this section, patents published in Brazil were considered to evaluate the role of the country concerning the development of technologies for biobutanol production. During the search for national patents, the number of inventions found was considerably lower than the

international data-base. Fig.(1) presents the chronological distribution of patents published in Brazil since 1980.

From Fig.(1), it is possible to observe that the Brazilian scenario is very similar to worldwide, where the majority of inventions were registered in the last five years. The main categories patented in Brazil, are related to the development of microorganism (43% of patents) entire process (30% of the patents) and substrate (17% of patents). It is important to emphasize that in worldwide inventions, the use of different substrates showed to be more important than in Brazilian patents, because Brazil is the greatest producer of sugarcane, and this is a cheaper and a good source of sugar for the production of butanol [84]. On the other hand, these data show a possibility to future advances in this aspect of diversifying the feedstocks used for the production of biobutanol, including starch-rich materials and lignocellulosics [85].

Regarding to the applicant of the Brazilian patents, it is possible to observe that Butamax Advanced Biofuels LLC® is the leader of the market. This company holds about 35% of all inventions linked to biobutanol production. This can be explained because the greater part of national patents, about this matter, has been registered in the last four years, or the company's lifetime. Table (2) shows a brief description of the patents registered in Brazil.

Concluding remarks

From this review it was possible to verify that great part of the inventions have been published in the last five years. Furthermore, in the year 2011, it was verified the greatest number of registers. However, it is expected a pronounced increase in the number of patents for the next years, since biobutanol has been considered a promising biofuel and is attracting attention of multinational companies. From the analysis of data presented in this paper it could be seen that the great interest of universities and companies are in the development of engineered microorganisms able to increase the production of butanol in the medium as well as the resistance of this microorganism to the toxic effects of products. In terms of separation of the production of biobutanol there is a preference for in situ separation. Substrate will always be important in the development of the process, since it impacts the final production costs. The research on the production of biobutanol for the next years, will be guided by the selection of a new microorganism or the development of an engineered strain able to

overproduce butanol, as well as decrease the production of acetone and ethanol and tolerate the high butanol concentration in the medium, since this is the main bottleneck in the process nowadays.

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Table 2: Principal patents registered in Brazil since 1980, classification and brief description.

Ref.	Year	Classification	Applicant	Kind of butanol	Description
[86]	2011	Microorganism	BUTAMAX ADVANCED BIOFUELS LLC	Both	Increasing tolerance to butanol in yeast has been accomplished by decreasing activity of Pdr5p encoded by an endogenous PDR5 gene.
[87]	2011	Substrate	BP Biofuels UK Ltd	ND	The invention reports the use of lignocellulosic as a raw material to production of butanol.
[88]	2011	Microorganism	BUTAMAX ADVANCED BIOFUELS LLC	Iso Butanol	Bacteria that are not natural butanol producers were found to have increased tolerance to butanol when the membrane content of unsaturated trans fatty acids was increased.
[89]	2011	Entire process	The Regents Of The University Of Colorado	Both	The invention describes a method of production for butanol from bacterial fermentation.
[90]	2010	Entire Process	Stichting Dienst Landbouwkundig Onderzoek	n-Butanol	The invention relates to a process for the combined production of butanol and hydrogen from biomass.
[91]	2010	IN SITU separation	BUTAMAX ADVANCED BIOFUELS LLC	Both	A method of making butanol from at least one fermentable carbon source that overcomes the issues of toxicity resulting in an increase in the effective titer, the effective rate, and the effective yield of butanol production.
[92]	2010	Microorganism	BUTAMAX ADVANCED BIOFUELS LLC	ND	Yeast cells with a reduced general control response to amino acid starvation were found to have increased tolerance to butanol in the growth medium.
[93]	2010	IN SITU separation	BUTAMAX ADVANCED BIOFUELS LLC	ND	The present invention relates to a two stage process to control the butanol concentration from fermentation broth.
[94]	2009	Entire process	E.I DU PONT DE MOURS AND COMPANY	n-Butanol	A method for the production of 1-butanol by fermentation using a microbial production host is disclosed. The method employs a reduction in temperature during the fermentation process that results in a more robust tolerance of the production host to the butanol product.
[95]	2009	Microorganism	Biofuelchem CO., LTD	n-Butanol	Disclosed herein is a method for producing butanol in yeast having the

					ability to biosynthesize butanol using butyryl-CoA as an intermediate.
[96]	2009	Microorganism	Gevo, Inc.	n-Butanol	There are disclosed metabolically-engineered yeast and methods of producing n-butanol.
[97]	2009	Entire process	Metabolic Explorer	n-Butanol	The present invention provides a method for the biological production of n-butanol at high yield from a fermentable carbon source.
[98]	2008	Microorganism	E.I. DU PONT DE NEMOURS AND COMPANY	Iso Butanol	Methods for the fermentative production of four carbon alcohols are provided. Specifically, butanol, preferably 2-butanol is produced by the fermentative growth of the recombinant bacteria expressing a 2-butanol biosynthetic pathway.

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ARTIGO 2- RECENT ADVANCES ON BIOBUTANOL PRODUCTION

Luiz J. Visioli, Heveline Enzweiler, Raquel C. Kuhn, Marcio Schwaab, Marcio A. Mazutti*

Department of Chemical Engineering - Federal University of Santa Maria, Av. Roraima, 1000, Santa Maria, RS, 97105-900, Brazil

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Abstract

Recent studies have shown that butanol is a potential gasoline replacement that can also be blended in significant quantities with conventional diesel fuel. However, biotechnological production of butanol has some challenges such as low butanol titer, the usage of low cost feedstocks and product inhibition. Regarding this fact, the work reviewed the main technologies being developed to make biobutanol technically and economically feasible. The last studies integrating continuous fermentation processes with efficient product recovery techniques and the usage of mathematical models as tools for process, scale-up, optimization and control were presented.

Keywords: biobutanol; engineered microorganism; separation; fermentation.

Introduction

During the last decade there has been an increasing interest in the production of chemicals and fuels from renewable resources. Reasons for this trend include growing concerns about global warming and climatic change, volatility of oil supply, the increasing price of crude oil and the existing legislation restricting the usage of nonrenewable energy sources. Furthermore, the generation of biofuels may improve the local employment opportunities and contribute to the reduction of CO_2 emissions [1-3]. Among several liquid alternative fuels, biobutanol has been shown as a great promise because of its very similar properties to gasoline [4] and, in comparison with ethanol, it has some attractive characteristics such as carbon chain length, volatility, polarity, combustion value and a less corrosive [5] octane rating [6]. It can also be a substitute for gasoline without the need to alter any current vehicle or engine technologies [7], it has less ignition problems (heat of vaporization of butanol is less than half of ethanol, an engine running on butanol should be easier to start in cold weather than the one running on ethanol or methanol) [8].

Commercial butanol fermentation processes have been developed by some companies [2]. However, there is an expectative to increase the number of companies devoted to biobutanol production worldwide as well as the development of new technologies aiming to increase yielding [9]. A difficulty faced in butanol fermentation is the inhibition caused by

products. Butanol concentration around 20 g/L inhibits microbial growth [5]. Another difficulty in butanol production is the fact that clostridium species are strictly anaerobes [10] and the anaerobic condition is maintained with sprinkling of oxygen free before the beginning of fermentation and the reactor needs to be maintained closed during the process [11].

The cost of the plant for butanol production largely depends on the price of feedstock and it is extremely sensitive to any price fluctuation [12]. Thereby, the commodity price is still very dependent of feedstock price and an expensive raw material generates an expensive product. Agricultural residues and wastes demonstrated to be cheaper than other sources [13]. However, the hydrolysis of these materials can generate fermentation inhibitors and it is another problem to be resolved [14].

Another important point in butanol production is the separation techniques and their application, mainly in separation in situ or continuous recovery [15]. Distillation is the unit operation widely used in separation of aqueous solution from butanol fermentation. However, the problem in this process is the formation of an azeotrope that generates great energy cost [16]. Furthermore, the alternative methods are reported with the objective to promote a cheaper and efficient separation. More recently, mathematical models have been developed to help the design of process as well as simulate the behavior of process in industrial scale without the need to carry out experiments to optimize the operational condition of the reactor [17-19].

Although there are excellent reviews available in the literature concerning butanol production [12, 20-29], this review is focused on the presentation of the main technologies being developed to make biobutanol technically and economically feasible in a way that it complements the existing literature about the topic. For this purpose, the last studies reporting the microorganism used in butyric fermentation are reviewed, promising to integrate continuous fermentation processes with efficient product recovery techniques and the usage of mathematical models as tools for process, optimization and control.

Microorganism

According to Liu et al. [30] most microbial strains employed for butanol fermentation are the mesophiles *Clostridium acetobutylicum* and *Clostridium beijerinckii,* where *Clostridium acetobutylicum* is reported for the usage in acetone–butanol–ethanol (ABE)

fermentation, which are the major products obtained in the process [7]. Furthermore, *Clostridium acetobutylicum* was the first bacterium that was used for ABE fermentation [2]. However, another different *clostridium sp.* also were reported, for example; *C. pasteurianum* [31]*, C. sporogenes* [32]*, C. saccharoperbutylacetonicum* [33] *and C. saccharobutylicum* [26]. The main strains used for biobutanol production are reported in Table 1.

ABE fermentation is one of the oldest known industrial fermentations with a history of more than 100 years [4]. However, this fermentation has not had a boom because butanol is highly toxic to microorganisms that catalyze its production, and for this reason less than 13 g/L of butanol are produced during batch fermentation. In general, in fermentation carried out to Clostridia sp, the solvent (ABE) production is 15–25 g/L and the yield is 0.25–0.4 g ABE/g sugar [2]. Substrate inhibition is not a major concern in ABE fermentation when glucose is used as a carbon source [53]. However, Chen et al. [54] reported an inhibition of butanol production at high substrate concentrations (decreasing the butanol yield) and Ezeji et al. [55] reported that clostridium sp. showed a catabolic inhibition to sugar concentration higher than 162 g/L.

The pH of the fermentation broth, initially at 6.8–7.0, drops to 4.5–5.0 during the acidogenic phase. This phase is associated with the fast growth of cells and the secretion of the carboxylic acids, acetate, and butyrate [4]. According to Napoli et al. [34], the pH range for this fermentation is between 4.0 and 5.0. In the same work the temperature used was 35ºC. Li et al. [53] verified that the pH (maintained over fermentation) of 4.3 is the optimal for butanol production using *C. acetobutylicum* This pH was reported before by Bahl et al. [56]. On the other hand, Qureshi [14] reported that the pH inside the reactor is self controlled at approximately 5.2 ± 0.2 during the solventogenic stage of *C. beijerinckii*. These different range of pH are due to different clostridium species used in the process.

The metabolism of Clostridia strains has two distinct phases, acidogenesis and solventogenesis. The acidogenesis is characterized by substrate conversion into acids (acetic and butyric acids), exponential cell growth with ATP formation. This is a fundamental step of fermentation, without which the number of viable cell would be greatly reduced making the normal solvents production difficult. The solventogenesis phase is characterized by conversion of substrate and acids into solvents (ABE) [23, 34]. Solventogenic clostridia can utilize a wide range of carbon sources, such as starch, sucrose, glucose, fructose, galactose, cellobiose, xylose, arabinose, glycerol, and syngas, as fermentation substrates for the production of acetone, butanol, and ethanol. According to Jang et al. [57] it is very important to have a better understanding of the genes that are the basis for performing system-level

metabolic engineering for the development of superior butanol, producing strains to improve biomass conversion [4], increase oxygen tolerance, increase the cell density, prolong cell viability, direct the utilization of cellulosics and mainly high solvent tolerance and high butanol selectivity [58]. Genetic modification of Clostridium is widely used by inserting some heterogenetic genes or over expressing or knocking out/down some relative endogenous genes to improve butanol production. Some researchers are working with genetic tools which are being used to manipulate their metabolism by introducing the genes that are responsible for butanol production from *Clostridium acetobutylicum* into *E. coli* and yeast (commonly *Saccharomyces cerevisiae*) [24]. However, this manipulation can increase a concentration of butanol in the medium, without inhibition by products and this is able to increase the titer of butanol production. The detailing of genetic engineering ways to butanol production can be observed on some papers e. g. [59, 60].

According to Lütke-Eversloh and Bahl [61], the engineering in the strains of genus clostridia can be made by the following ways; disruption of pathway that synthesizes the unwanted products. In other studies, the pathway of acetone production was disrupted. Therewith, the rate of butanol production increases from 71% to 80% [62]. In the same way, Sullivan [8] proposed to change the pathway of formation of acetate and butyrate (acidogenic stage).

Isar and Rangaswamy [50] reported an increase in the tolerance of solvents from 18 g/L to 25 g/L using *Clostridium beijerinckii* solvent adapted strain. It indicated that the strain has adapted to butanol and become solvent tolerant in the absence of any mutation carried out to bring this change about.

In the study of Abd-Alla and El-Enany [41], the authors mentioned an alternative to maintain the anaerobic medium during the clostridial fermentation. The culture of *Bacillus Subtilis* DSM 4451 was applied to maintain strict anaerobic conditions for *C. acetobutylicum* ATCC 824 in the growing and for subsequent ABE production. Thus, fermentation does not need N_2 flushing to remove the oxygen so the cost decreased. The highest butanol production obtained was 21.7 g/L (that is similar to tha reported to clostridium production) just using the consortium of microorganism to maintain the anaerobic medium. However, there should be warning about the products generated by the *Bacillus* and about their consumption of substrate, because they can become competitors during the process.

The main trend observed about the microorganism used in the butyric fermentation is the attempt to create an engineered microbe to overcome the clostridium limitation. The increase of solvent tolerance, butanol titer, and oxygen traces tolerance are the most modified

characteristic at the developed microorganism. However to the future the useful microorganism, probably, needs to have all these characteristics to be viable for industrial process application.

Substrates

The prices of substrates influence the economic competition with the petrochemical industry [34]. The cost of feedstock represents over 70% of total production cost of biobutanol [63]. In the beginning of butanol fermentation, substrates based in sugars and starch were used, but these are expensive and the process becomes unviable. One of the strategies to lower biobutanol production cost is to use cheap and renewable feedstock, such as lignocellulosic materials (e.g. agricultural waste, paper waste, wood chips), which are abundant and sustainable. The production of alcohol using lignocelluloses follows an integrated process involving basically three steps: pre-treatment, hydrolysis and fermentation [7]. The main substrates used for biobutanol production are reported in Table 1.

The molasses sources are used to biobutanol production. However, these kinds of substrate are more expensive than agricultural residues. On the other hand, it is molasses can be used directly in the fermentation without any or few pretreatment. Thus, it is not possible assert that the cellulosic residues will be ever cheaper than molasses one. van der Merwe et al. [64] report an analyses of energy efficiency and economics of biobutanol production process and the substrate selected to this were sugarcane molasses because the authors consider that it is can generated a not expensive process. Another important point to be analyzed related to substrate choice is the availability through the year. The major source to this kind of raw material are the agricultural residues and wastes, such as rice straw, wheat straw, wood (hardwood), byproducts left over from the corn milling process (corn fiber), annual and perennial crops, waste paper [14] and sweet sorghum [65]. These raw materials consist of three types of polymers: cellulose, hemicellulose, and lignin. Cellulose has strong physical-chemical interaction with hemicelluloses and lignin. Cellulose, a linear glucose polymer (that is broken in the hydrolysis), is a highly ordered polymer formed of cellobiose representing about 50% of the wood mass. Hemicellulose is a short, highly branched heteropolymer formed mainly of xylose, plus glucose, mannose, galactose and arabinose and sometimes uronic acids. Lignin is made up of phenylpropanoid units derived from the corresponding p-

hydroxycinnapyl alcohols. Lignin is hydrophobic and highly resistant to chemical and biological degradation [66]. *Clostridium beijerinckiii* is being explored as a promising strain to produce biobutanol from cellulosic materials [26].

The problems in the usage of cellulosic or lignocellulosic materials for butanol production are the processes for production of these hydrolysates, resulting in the generation of chemical byproducts that inhibit cell growth and fermentation. Such inhibitors include salts, furfural, hydroxymethyl furfural, acetic, ferulic, glucuronic, r-coumaric acids, and phenolic compounds. Lignocellulosic materials are difficultly converted to sugars by directly biological methods because a lignin network covers the layer of cell walls [39]. Furthermore, the hydrolytic process can generate significant amount of waste and hence increase the cost of butanol [36]. Moreover, for good fermentation of any substrates (mainly cellulosic and starchy after a hydrolysis treatment) there is the need of nutritional supplementation. Lee et al. [67] reported the usage of KH_2PO_4, K_2HPO_4, ammonium acetate, para-aminobenzoic acid, thiamin, biotin, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, $FeSO_4 \cdot 7H_2O$, NaCl, yeast extract as supplement for biobutanol production.

The pretreatment of starchy and cellulosic material is a limiting step and needs to be optimized for a satisfactory production of butanol. Liu et al. [30] pretreated wheat bran using sulfuric acid at high temperature and, after neutralization with $Ca(OH)_2$, used *C. beijerinckii 55025* for butanol production. This procedure increased the cost of butanol produced, but it can considerably decrease if a large amount and cheap source of raw material is used. Lépiz-Aguilar [35] used HCl 1M combined with high temperature during 2h or enzymatic hydrolysis (using α-amylase and β-glucoamylase) to hydrolyze the cassava flour. The best results in terms of butanol production were 23.98 and 13.78 $g.L^{-1}$ using enzymatic and acid hydrolysis, respectively. Qureshi et al. [7] studied the pretreatment of wheat straw with a mix of enzymes (cellulose, β-glucosidase and xylanase) at pH 5.0, 45 ºC for 72h and 80 rpm, obtaining a butanol production of 12.0 g/L. However, the hydrolysis of starchy and cellulosic raw material can be made by any other ways (any examples of main steps of hydrolysis are briefly showed in table 1). Some few technologies to hydrolyze the raw material were reported, such as microwave-assisted pre-treatment processes, steam explosion, ozonolysis, oxidative delignification, pulsed-electric-field pretreatment [68-70].

Qureshi et al. [36] believed that barley straw can be used for butanol production. However, there may be the presence of inhibitory chemicals in this substrate and it is necessary a pretreatment (with lime called overliming) for an effective fermentation. After the pretreatment, the production of butanol was higher than while using glucose as substrate.

Similarly, Qureshi et al. [37] evaluated corn stover and switchgrass hydrolyzed as substrate to butanol production. The production of butanol using corn stover hydrolyzed was similar to the one presented in a previous work [36] using barley, whereas using switchgrass hydrolyzed the production was lower.

Al-Shorgani et al. [44] reported the formation of inhibitors during the acid pretreatment of cellulosic raw material (rice bran and de-oiled rice bran). Similarly to others studies, the authors used overliming treatment and extraction of inhibitors with nonionic polymeric adsorbent resin. The usage of these procedures improved butanol production, productivity and yield. Qureshi et al. [71] concluded that the formation of fermentation inhibitors after hydrolysis of cellulosic raw material is substrate and pretreatment dependent. Thus, it is necessary a specific study for each substrate and treatment.

Lin et al. [51] reported the use of corn straw as raw material for butanol production after hydrolysis using alkali pre-treated of corn straw. The sugar concentration obtained in the hydrolysis was between 42-44 g/L and this represents approximately 400 grams of sugar per 1 kg of corn straw, producing 6.54 g/L (65g/kg of corn straw) of butanol in the fermentation. Using another residue from corn production (corncob), Zhang et al [48] reported production of 16 g/L of solvents using the enzymatic hydrolyzed corncob pre-treated and detoxified with $Ca(OH)_2$. These two papers have a great important to viability of process because discuss the use of two residues generated by the same crop.

Several authors have stated that the biobutanol production only will be feasible industrially if a low cost substrate could be employed. However is important to consider the total cost involved in the substrate utilization. In these scenarios, the tendency is the diversification of substrates and the use of regional crops (molasses, starch or cellulosic one) to butanol production decreasing the associated cost.

Bioreactors for biobutanol production

According to Kumar and Gayen [26], the operation of bioreactors for biobutanol production can be accomplished in batch, fed-batch, and continuous modes. Continuous processes offer various advantages such as reduction in sterilization and re-inoculation time, superior productivity, and reduction in butanol inhibition, but this reactor presents high product recovery costs because of low concentration of biofuel [26]. Fed-batch fermentation

is started with a low substrate concentration. When the fermentation culture consumes the substrate, more substrate is added to maintain the fermentation process while not exceeding the detrimental substrate level [53]. The usage of a continuous packed bed reactor (PBR) is reported as an alternative for fermentation using immobilized microorganism (this work has an immobilized *Clostridium acetobutylicum*) [34]. Lu et al. [38] used a fibrous bed bioreactor (FBB). This reactor is interesting because the microorganism is immobilized in the bed enabling a process to recover products in situ without losses of cells. However, the reactor that is the most reported is the batch (as can be verified in table 1). This preference can be explained because it has an easy handling, maintain the anaerobic medium, controller the temperature, pH, and taking samples, furthermore, this is the reactor that shows few difficulties to coupling a separation unit.

Mariano et al. [15] reported the usage of 14 liters bioreactor using 7 liters of medium. This is a batch reactor and the anaerobic medium was maintained by oxygen free nitrogen. Parekh et al. [72] reported the use of a pilot-scale of 200 liter with 200 liters of medium using corn step water how raw material and was obtained 17.8 g/L of butanol. The same size of bioreactor was used by Lee et al. [59]. These bioreactors are larger than the other reported, thus, these study is very important to predict the behavior of clostridial fermentation after the scale up.

The influence of pH has been recognized as a key factor in determining the outcome of ABE fermentation. The pH can be shifted from an initial value to another value during the fermentation and thus the production can be increased. According to [45] with the pH control, the fermentation period decreased from 40 to 32 h and concentration, yield and productivity increased.

Separation

The separation process used in the purification of biobutanol from the fermentation broth is the distillation. However, butanol-water system at 101.3 kPa has an azeotrope at 55.5 wt% butanol. The greatest difficulty in this process is that butanol is only soluble in water up to 7.7 wt% butanol and, because the azeotrope occurs above this solubility limit, two liquid phases are formed at the azeotrope – the upper phase contains 79.9 wt% butanol while the lower phase contains 7.7 wt% butanol [16], which boils at a lower temperature [10].The

recovery of low concentration of butanol by traditional distillation is energy intensive and thus, economically infeasible [73].

Mariano et al. [74], Secuianu et al. [75] and Ezeji [3] reported some of the most commonly used techniques to continuously remove butanol from the fermentation broth, namely adsorption, gas stripping, ionic liquids, liquid-liquid extraction, pervaporation, aqueous two-phase separation, supercritical extraction, and flash fermentation. Adsorption should allow separation of butanol from the bulk aqueous fermentation broth. Hydrophobic adsorbents potentially show high selectivity for butanol over water [5]. In adsorption, alcohol is preferentially transferred from the feed liquid to a solid adsorbent material [16].

Dhamole et al. [76] used non-ionic surfactant to decrease butanol toxicity and its separation from the non-ionic surfactant micelle aqueous solution by cloud point extraction. Thus, the fermentation is not inhibited and butanol concentration is increased in the micelles.

Ezeji [3] used gas stripping for *in situ* separation because it is a simple technique that is free of emulsion formation and it does not require membrane or expensive chemicals. The production of ABE was increased from 17.6 g L^{-1} to 232.8 g L^{-1} when the authors used gas stripping in the fermentation of 500 g L^{-1} of sugar using the strain *C. beijerinckii* BA101. Moreover, gas stripping was more selective in removing butanol than acetone and ethanol [38]. Gas stripping is more efficient when butanol concentration in the fermentation broth is higher than 8 g L^{-1} [49]. According to Ezeji et al. [40], the production of ABE increased from 18.6 g L^{-1} to 81.3 g L^{-1}, whereas sugar consumption increased about 487% (compared with the control of the same substrate without gas stripping) using gas stripping in the fermentation of liquefied corn starch with *C. beijerinckii* BA101.

Pervaporation technique using membranes with high product selectivity is one of the most promising alternatives to conventional distillation. Without heating energy, the pervaporation membrane process enables to efficiently separate and concentrate the product in a single step, and to maintain the productivity of microorganism as a result of preventing product inhibition [77]. Yen et al. [78] tested a new composite of membrane for pervaporation with 5% and 10% of carbon nanotubes in a membrane of poly(ether-block-amide), and it has increased the productivity and yield about 20%, compared to using just a poly(ether-block-amide) membrane.

In the perstractive separation, the fermentation broth and the extractant are separated by membrane. The membrane contactor provides surface area where the two immiscible phases can exchange butanol, thus, the toxicity of solvent for the cells does not occur [79].

According to Qureshi and Maddox [52], the consumption of lactose in ABE fermentation using perstractive separation increased from 28.6 g L^{-1} to 227 g L^{-1}.

The removal of butanol or ABE from fermentation broth by liquid–liquid extraction is considered an important technique. Usually, a water-insoluble organic extractant is mixed with the fermentation broth. The main problem concerns the use of this technique is related to the toxicity of the solvent to the cells [79]. The major limitation is that the extractant with high partition coefficient often leads to microbial toxicity because of direct contact between the fermentation broth and the extractant [80].

The membrane-assisted extractive fermentation, two phases of extractant and fermentation broth are separated by a porous membrane. The membrane can be either hydrophilic or hydrophobic and the interface is immobilized by the impregnation of its pores with one of the two phases depending on the membrane affinity. Thus, the microbial toxicity of the extractant can be reduced. Tanaka et al. [80] reported an increase in the glucose consumption from 66% to 100% due to the absence of inhibition for butanol in the medium.

Mariano et al. [15] reported the usage of a cyclic vacuum applied in a bioreactor during fermentation. Vacuum is a good form to remove butanol of the fermentative medium, which is a good form to decrease product inhibition. Furthermore, in this study the continuous and intermittent vacuum has been tested, and with the usage of intermittent vacuum it has been observed a reduction of 39% in the energy expenditure without product inhibition because of low butanol concentration. Moreover, this process is a pre-concentration of aqueous solution of butanol which decreases the energy expenditure in the purification of butanol.

The same authors reported the use of a flash fermentation to in situ butanol recovery. Flash fermentation is a good form to decrease butanol concentration from fermentation broth. In this technology, a partial separation of the solvents and water occurs in the flash tank separator, where the liquid fraction returns to the fermentor and the vapor fraction (after condensation) plus the purge and permeate streams will compose the final stream that is sent to distillation. Thus, butanol concentration is ever less of critical concentration (inhibition by product) [17]. Furthermore they develop a mathematical model to predict the behavior of the process. This consisted of a batch fermentation reactor, and a vacuum flash vessel (besides a filter to remove any solids before it gets in the flash vessel). The schematic design of the process is showed in the Figure 1. For the development of the model it has been considered the mass differential equations of the bath reactor assuming constant volume, however, it is

added a relationship for the removal of butanol from the medium. The objective of this work was to demonstrate that a flash was able to be used to decrease product inhibition [17].

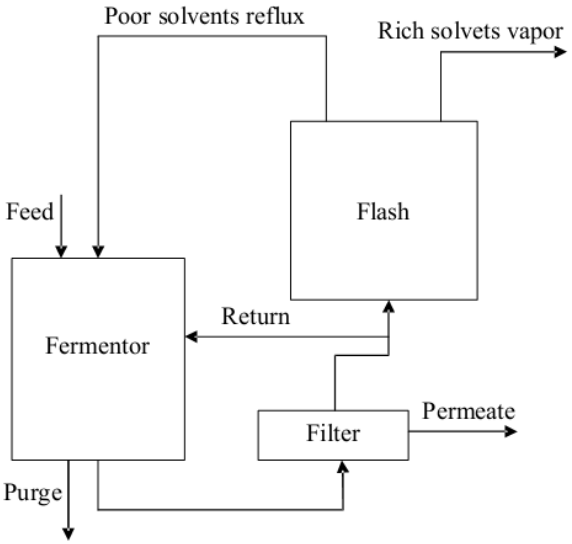

Figure 1: schematic design of flash fermentation process

The Figure 1 scheme demonstrates the cyclic process that can be generated using this technology. The volatile compounds are removed in the flash tank and the poor solvents flow is refeed to the fermentor. The feed is used to control the sugar concentration in the reactor, the purge is used to control the level and remove old cells to allow its renovation and the filter is used to prevent that solids enter in the flash tank.

Mariano et al. [81] proposed a mathematical model of a continuous flash fermentation and used this model to optimize the process using response surface techniques. In another work, Mariano et al. [82] used the same model proposed by Mariano et al. [81], but the process was optimized using the method of particle swarm optimization to obtain the best operating conditions for butanol production.

The same authors proposed the utilization of a servo control in flash fermentation [18]. This work was carried out because in previous studies it has been proved that this process can be used to improve butyric fermentation. So it becomes necessary to control the removal of butanol, since natural oscillations can occur during the dynamic process. The mathematical modeling is similar to the one used in [17] because these works are a sequence, but, in this case some few changes in the differential equation was accomplished, due to alteration of

reaction volume. The objective of the control was to keep sugar and butanol concentrations constant in the fermentor. The controller was efficient to regulate the operating conditions. Thus, the usage of a controller in flash fermentation is able to enlarge the process, and suit this to an industrial application.

Similarly, Liu et al. [19] propose a mathematical model to simulated process consisted of a fermentor, a gas striping, and a purification process for the condensed from gas striping. The objective was to simulate a process to produce 150.000 tons of butanol per year with purity of 99 wt%, and evaluate the energy demand of all parts of this process. The authors concluded that ABE fermentation has lesser liquid fuel production (energy basis) using corn as a substrate than the ethanol production process. However, this scenario can change very fast with the development of the process and genetic engineering.

Clearly, the use of in situ separation techniques to butanol production is tendency. This demonstrated that is able to solve the product inhibition problem of this fermentation. Furthermore, it can be considered a pre-separation process and decrease the quantity to be purified posteriorly. The use of mathematical models to simulate the behavior of a fermentation process linked to any of these separation process is important to predict the how useful the arrangement will be. Therewith is possible represent experimentally just the best theoretical conditions and predict the adjustment necessary to scale up the process to industrial application.

Table 1: Microorganism, substrate, Yield/Production and main aspects in the butanol production reported.

Microorganism	Substrate	Yield/Production	Technology	Reference
C. acetobutylicum (immobilized)	Cheese whey (lactose)	Yield: 15% to 0.54 h^{-1} of dilution and 28% to 0.97 h^{-1} of dilution	Reactor (PBR) with immobilized clostridium	[34]
C. beijeirinckii ATCC 55025	Hydrolysate of wheat bran	Yield: 32% / Production: 8.8 g L^{-1} of biobutanol	Acid Hydrolyses	[30]
C. beijerinckii	Cassava flour	Production: 23.98 g.l^{-1} of butanol	Enzymatic treatment with yield of 9.12% to Reducing sugar	[35]
C. beijerinckii P260	Wheat straw	Yield: 42%	Acid pretreatment and enzymatic hydrolysis	[14]
	Barley straw	Yield: 43%/ Production: 26,64g L^{-1} of total solvents	Dilute sulfuric acid hydrolysis/ overliming	[36]
	Corn stover	Yield: 43%/ Production: 18.04g L^{-1} of total solvents	Acid and enzymatic steps of hydrolysis/ overliming	[37]
	Switchgrass	Yield: 37%/ Production: 8.91g L^{-1} of total solvents		
	Glucose	Production: 17.54 g L^{-1} of butanol	Intermittent vacuum application	[10]
C. saccharobutylicum DSM 13864	Sago starch	Yield: 29%	Free microorganism fermentation	[26]
C. acetobutylicum	Cassava bagasse	Yield: 32%/ Production: 76.4 g L^{-1} of butanol	Hydrolyze by enzymes fibrous bed bioreactor / Gas stripping	[38]
	Palm empty fruit bunches	Production: 1.262g L^{-1} of butanol	Acid pretreatment/ enzymatic hydrolysis	[39]
C. beijerinckii BA101	Liquefied corn starch	Butanol production: 81, 3 g L^{-1} (with gas stripping)/ 18.6 g L^{-1} (without gas stripping)	Bath reactor/ gas stripping/ enzymatic hydrolyses	[40]
C. acetobutylicum ATCC 824 and Bacillus subtilis DSM 4451	Spoilage date palm fruits	Yield: 42%/ production: 21.56 g L^{-1} of Solvents	Bacterial consortium (anaerobic conditions)	[41]

C. beijerinckii NCIMB 8052	Tropical maize stalk juice	Production: 0.27 g-butanol/g-sugar	Optimization of pH, agitation, sugar concentration	[42]
C. acetobutylicum ATCC824	Sugar maple Hemicellulosic	Production: 7 g L^{-1} of butanol	Alkali pretreatment/ acid hydrolyses/ overliming	[43]
C. saccharoperbutylacetonicum N1-4	Rice bran	Yield: 57% to sugar generated.	Acid hydrolysis	[44]
	De-oiled rice bran	Yield: 44% to sugar generated.	Acid pretreatment/ Enzymatic Hydrolysis	
C. acetobutylicum XY16	Glucose	Production: 20.3 g L^{-1} of butanol	pH steps in the fermentation	[45]
C. sporogenes BE01	rice straw	Production of 3.49 g/L and 5.32g/L of butanol and total solvents respectively	Acid pretreatment/ Enzymatic Hydrolysis/ Overliming	[32]
C.saccharoperbutylacetonicum N1-4	rice straw	Maximum butanol production of 6.6 g/L and butanol yield 0.2 g/g of total sugar.	Absence of pretreatment/ Enzymatic hydrolysis/ Non-sterile conditions	[33]
C. pasteurianum	Glycerol	Maximum butanol production of 8.8 g/L and butanol yield 0.35 g/g of glycerol at initial substrate concentration of 25 g/L.	Immobilized cells/ Bath fermentation	[31]
C. acetobutylicum NCIM 2337	Rice straw	Butanol production of 13.5 g/L and butanol yield 0.34 g/g of total sugar generated.	Acid treatment with shear stress	[46]
C. acetobutylicum MTCC 481	Rice straw	Butanol production of 1.72 g/L.	Steam explosion	[47]
		Butanol production of 1.6 g/L	Acid treatment	
		Butanol production of 2.1 g/L	Acid pre-treatment/ Enzymatic hydrolysis	

C beijerinckii NCIMB 8052	Corncob	Butanol production of 8.2 g/L	Alkali pre-treatment/ enzymatic hydrolysis/ overliming	[48]
C. acetobutylicum JB200	Glucose	Yield: 21%/ Production: 172 g L^{-1} of solvents	Gas stripping	[49]
C. beijerinckii ATCC 10132	Glucose	Production: 20 g L^{-1} of butanol	Bath reactor	[50]
C. acetobutylicum CICC 8008	Corn straw	Production: 6.20 g L^{-1} of butanol	Enzymatic hydrolysis/ bath reactor	[51]
C. acetobutylicum P262	Whey permeate medium	Yield: 44%/ Production: 98.97 g L^{-1} of solvents	Perstraction/ bath reactor	[52]

Concluding remarks

In this review, the aspects in the process of biobutanol production have been discussed, which included the importance of microorganism as well as the limitations imposed to the process, substrates that can be used in the fermentation and the impact in the cost generated by their use, types of bioreactors used in the process, separation techniques with special attention to in situ separation allowing to decrease butanol inhibition and, finally, the development of mathematical models to represent the in situ separation techniques. The development of microorganism for butanol production has been very reported over the last years. It is interesting because it proves the importance of this product to the world. The engineering of clostridial bacterium appear in many works and the results obtained showed an increase in biobutanol production. On the other hand, the changes in the bacterium are directed to decrease acetone production as well as increase the resistance of microorganism to high concentration of butanol with consequent improvement in the usage of sugar for biobutanol production. Concerning substrate, there is a trend to use lignocellulosic materials, but the inhibitor substances generated during hydrolysis imposed difficulties for the industrial usage of this raw material for biobutanol production. *In situ* separations, associated with low energy expenditure during the removal of biobutanol are the technologies that will predominate in the future.

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4 RESULTADOS

ARTIGO 3- DEVELOPMENT OF A FAST AND LOW COST METHOD FOR ACETONE-BUTANOL-ETHANOL QUANTIFICATION IN INDUSTRIAL FERMENTATIONS

Luiz J. Visioli, Eliana A. Alves, Aline C. M. Trindade, Raquel C. Kuhn, Marcio Schwaab, Marcio A. Mazutti

Department of Chemical Engineering - Federal University of Santa Maria, Av. Roraima, 1000, Santa Maria, RS, 97105-900, Brazil

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Abstract

In this work is developed a procedure based on mixture density to determine the concentration of acetone-butanol-ethanol solvents (ABE) instead of instrumental methods based on chromatography. For this purpose, several calibration points were prepared using different culture media to ensure real fermentation conditions and mixture densities determined for ABE concentration ranging from 0 to 25 g L^{-1} at three different sugar concentrations, namely 10.0, 35.0 and 60.0 g L^{-1}. The results obtained demonstrated a linear relation of density with ABE and sugar concentration. The main contribution of this work was to determine the ABE concentration in a simple, fast and low cost way.

Keywords: Butanol determination, reduction cost, biofuel, fermentation.

Introduction

Biofuel production is one of the key in development of a sustainable economy [1]. Bioethanol is the biofuel most used in the world, however butanol has better proprieties, mainly the energy content that is higher than ethanol [2]. In this scenario biobutanol can be attractive to replace the gasoline supply due to its great similarity to this fossil fuel [3]. Furthermore biobutanol can be obtained from many different raw materials including a lot of residues of food industries and agriculture [4].

The biobutanol production was in focus in the last years. According to Qureshi et al. (2007) there is the trend to increase quickly the biobutanol production, mainly in countries in accelerated development as is the case of China and Brazil. On the other hand, research has been carried to produce industrially biobutanol using lignocellulosic biomass. These raw materials can decrease the cost of this biofuel [5]. The importance of biobutanol as alternative fuel can be verified by the great number of patents registered in the last years worldwide [6].

The perspective of expansion in the biobutanol production generated the necessity of an efficient and cheaper method to quantify the products from fermentative broth that are (mainly) butanol, acetone and ethanol (known as ABE fermentation) in different titers depending of the strain used [7]. Nowadays this determination is made by gas chromatography [8; 9] in the great majority of the studies. Liquid chromatography (especially

HPLC-RID) also can be used, although less frequent. However, determination of butanol using chromatography is limiting for industrial scale due to high cost associated with equipments, accessories and chemicals.

An alternative cost effective is the determination based on density variation in the media. The propriety that is very different between products and initial broth to fermentation is the apparent density. The ABE solvents have a density near 0.8 g cm^{-3} and the broth has a density higher than 1.0 g cm^{-3} (water density + dissolved solids). This shows a difference higher than 20% in the density between the solvents and fermentation media, in a manner that is possible to associate the decreasing in density of broth with solvent production in a simple and fast way.

Based on these aspects, the main objective of this work is to develop and validate a methodology to determine the concentration of ABE solvents based on the variation of density of the fermented media. In the first step of the work, synthetic solutions diluted in distilled water with different concentrations of ABE solvents were used to develop the method. In the second step, different concentrations of ABE solvents were diluted in sweet sorghum juice at different sugar concentration to simulate the real process condition. The main contribution of this technique is the elimination of requirement of chromatography on the analyses and therewith becomes cheaper and faster the process making it accessible to more researchers/companies worldwide.

Materials and Methods

Chemicals

The solvents (acetone, ethanol and butanol) and chemicals used were from Sigma-Aldrich® all the reactants used have a purity PA . The sweet sorghum was kindly provided by micro distillery of Federal University of Santa Maria-Brazil, and was farmed in the geographic coordinates 29° 41′ 29″ south, 53° 48′ 3″ west.

Method development

It was used as base for method development a linear relation between acetone-butanol-ethanol (ABE) concentration and mixture density. In this way, a calibration curve is obtained using several ABE concentrations at different fermentation media, in order to prove the prediction capacity of the method.

Determination of ABE in water

Calibrations points were prepared at concentrations ranging from 0 to 25 $g.L^{-1}$ for each solvent separately. In a second moment, all solvents were mixed in a molar relation of 6:3:1 for butanol, acetone and ethanol respectively. Afterwards, samples at concentrations ranging from 0 to 25 $g.L^{-1}$ of ABE solvents were prepared. For each calibration point, it was determined the density of the water and solution containing ABE at determined concentration. The difference between the values of densities was used for calibration purposes, conforms Eq. 1:

$$ABE = a + b \cdot \Delta\rho \quad (1)$$

where ABE is the solvent concentration ($g\,L^{-1}$), $\Delta\rho$ is the difference of densities between water and after the addition of solvents ($g\,cm^{-3}$) and a,b are parameters to be estimated.

Determination of ABE in RCM media

To ensure real fermentation conditions, ABE solvents were mixed in a molar relation of 6:3:1 for butanol, acetone and ethanol, respectively in Reinforced Clostridium Medium (RCM) [10], which contains ($g\,L^{-1}$): yeast extract 3.0, meat extract 10.0, peptone 10.0, soluble

starch 1.0, L-cysteine hydrochloride 0.5, sodium acetate 3.0, agar 0.5 and NaCl 5.0. Afterwards, samples at concentrations ranging from 0 to 25 g L^{-1} of ABE solvents were prepared and density determined. In this step, it was verified the effect of sugar concentration in the problem and observe if still is possible to determine ABE concentration, because the sugar concentration influences greatly the solvent density, since sugar concentration alters during the fermentation due to growth of microorganism, being necessary to know its value to predict the ABE concentration by mixture density. For this purpose, tests were carried out using three different sugar concentrations, namely 0, 30.0 and 60.0 g L^{-1} using anhydrous glucose. In the same way that previous section, or each calibration point, it was determined the density of the RCM media and solution containing ABE at determined concentration. The difference between the values of densities was used for calibration purposes, taking into account that ABE concentration is dependent of density variation and sugar concentration, conforms Eq. 2:

$$ABE = a + b \cdot S + c \cdot \Delta\rho \quad (2)$$

where ABE is the solvent concentration ($g.g^{-1}$), S is sugar concentration ($g.L^{-1}$), $\Delta\rho$ is the difference of densities between RCM media and after the addition of solvents ($g.cm^{-3}$) and a, b, c are parameters to be estimated.

Determination of ABE in sweet sorghum as fermentation media

To ensure real fermentation conditions, ABE solvents were mixed in a molar relation of 6:3:1 for butanol, acetone and ethanol, respectively using sweet sorghum as fermentation medium at different sugar concentrations (10.0, 35.0 and 60.0 g L^{-1}) containing (g L^{-1}): yeast extract 2.0, KH_2PO_4 0.5, $K_2HPO_4.3H_2O$ 0.65, $MgSO_4.7H_2O$ 0.2, $FeSO_4.7H_2O$ 0.01, $MnSO_4.H_2O$ 0.01, NaCl 0.01, sodium thioglycolate 1.0 and tryptone 1.0 [8]. Afterwards, samples at concentrations ranging from 0 to 25 g L^{-1} of ABE solvents were prepared and density determined. For each calibration point, it was determined the density of the sweet sorghum media and after the addition of ABE at determined concentration. The difference

between the values of densities was used for calibration purposes, taking into account that ABE concentration is dependent of density variation and sugar concentration, conforms Eq. 3:

$$ABE = a + b \cdot S + c \cdot \Delta\rho \quad (3)$$

where ABE is the solvent concentration (g g^{-1}), S is sugar concentration (g L^{-1}), Δρ is the difference of densities between sweet sorghum media and after the addition of solvents (g cm^{-3}) and a, b, c are parameters to be estimated.

Measurement of mixture density

The densities of the solutions were measured with an Anton Paar DMA 4500 oscillating U-tube densimeter equipped with automatic temperature correction and operated under the static mode. The sample was injected three times to ensure that the strap sample is completely free of contamination, which can affect the density measured. So the sample was injected and the syringe was maintained attached to inlet of densimeter in order to prevent the occurrence of air bubbles. The density was set at the temperature of 20 ± 0.01ºC and the time of analyses can be considered the time to achieve this temperature.

Parameter Estimation

All the parameters were estimated using the algorithm of Levenberg–Marquardt of software Statistica 7.0 (Statsoft Inc., Tulsa, OK, USA). The estimation of the model parameters consisted to minimize the sum square residues (SSR) amongst predicted and experimental data.

Results and discussion

Estimation of ABE in water

The results presented in this section will give support for the next step because will be demonstrated the relationship between concentration of solvents and density. Figure 1 shows the influence of concentration of each solvent separately on mixture density. It is seen a linear relation between concentration of each solvent and density. The coefficient of determination of the three fits are greater than 0.999 and the probability of the parameter to be significant is very close of 100 % for parameters of each fit. Furthermore, the visual analyses demonstrate the good agreement among predict and experimental data.

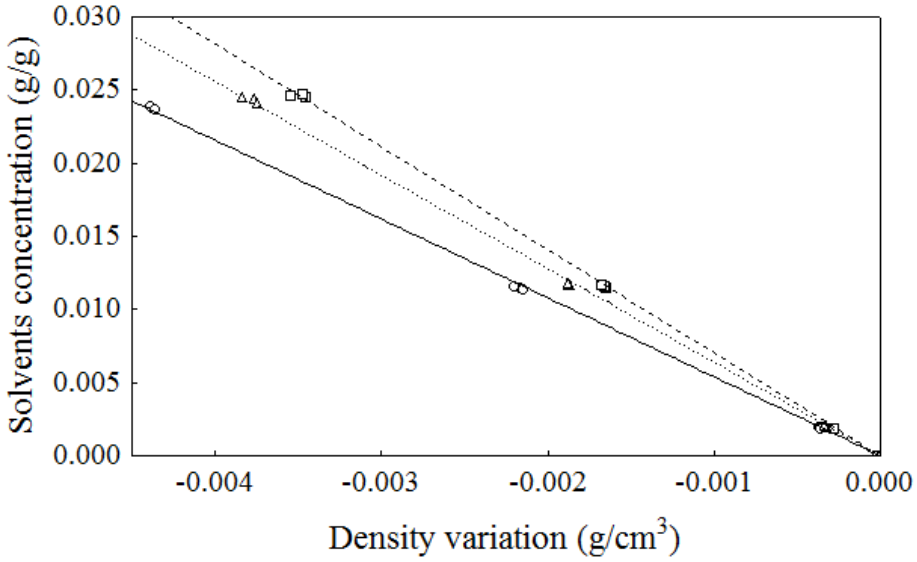

Figure 1: Relationship of experimental data of density variation (g/cm^3) and solvent concentration (g/g) of butanol (Δ), acetone (□) and ethanol (○) solution using distilled water as medium and its respective linear fits (….), (----), (——).

The results presented in Figure 1 demonstrated that is possible to develop a method based on mixture density to determine the ABE concentration in fermentation media. However, in real fermentation all compounds will be dissolved in liquid media, being necessary to verify if a linear relation is also maintained in this case. For this reason, it was tested the method to quantify ABE solvents considering ABE molar proportion of 6:3:1 and the results are presented in Figure 2.

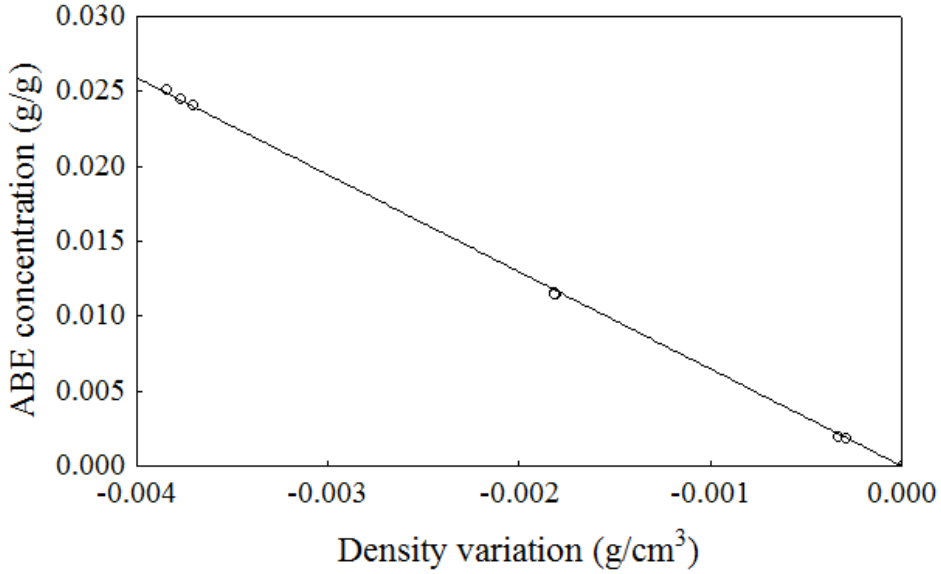

Figure 2: Relationship of experimental data of density variation (g/cm^3) and solvent concentration (g/g) of butanol, acetone and ethanol (○) in respective molar proportion 6:3:1solution using distilled water as medium and its respective linear fit (—).

The linear relation among ABE concentration and mixture density is maintained for three solvents. In the same way that was observed in the use of individual solvent, the coefficient of determination was greater than 0.999 and the probability of the parameter been significant is very close of 100 %. This facts increase the possibility of a good fit to represent the density variation using a real medium during the biobutanol production by fermentation. However, this method does not allow the estimation of these compounds separately, since all them respond in the same manner to different concentrations. By other hand, is possible to obtain an approximate estimation for the concentration of each compound, since a same microorganism produce a fixed relation among them.

Estimation of ABE in RCM media

The results presented in Figures 1 and 2 are related to a mixture of water and ABE solvents in different concentrations. However, real fermentations present other compounds in the media. By example, if synthetic compounds are used to formulate the culture media there is the presence of salts and, mainly, sugar that alters the density of mixture. For this reason, it was changed distilled water by reinforced clostridium medium (RCM), which is largely applied in the butyric fermentation for the growth of the clostridium sp., the most used bacteria to butanol production from fermentation [11].

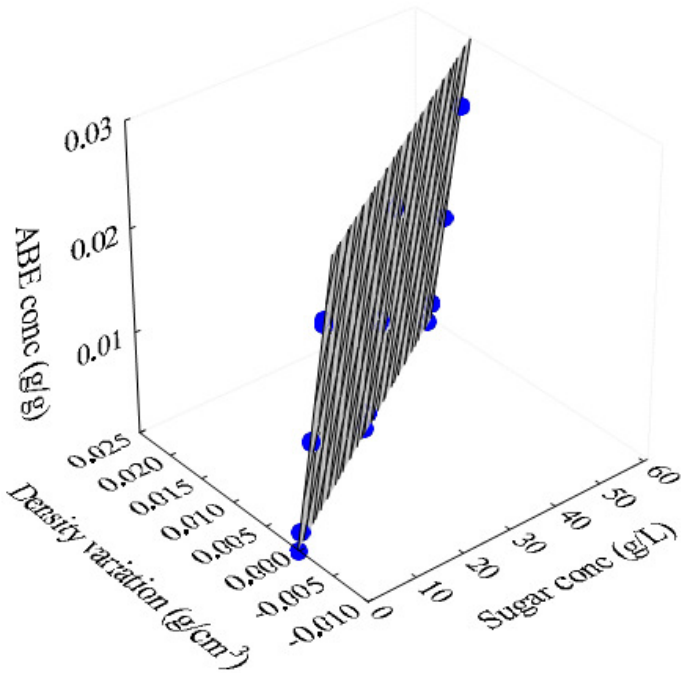

Figure 3: Relationship of experimental data of density variation (g/cm^3), solvent concentration (g/g) of ABE in respective molar proportion 6:3:1solution and sugar concentration (●) using RCM as medium and its respective linear fit model.

Figure 3 shows the results obtained for different ABE and sugar concentrations. A perfect linear adjustment is verified for all calibration points, indicating that is possible to predict the ABE concentration in function of density variation and sugar concentration. The determination coefficient is greater than 0.99 and the probability of the parameter been significant is very close of 100% as well as the calibration of solvents in water. It is evident that the insertion of sugar concentration as a variable to be measured in the analyses is adequate to become this technique applicable. Parameter values, standard error, significance level and confidence interval are presented in Table 1. The p-level of each parameter are very close to zero, indicating that these parameters are statistically significant. This proves that the model proposed could be very useful as a less expensive way to solvents concentration estimation in clostridial fermentation for butanol production.

Table 1: Confidence intervals, standard errors, p-levels, final objective function of the adjustment of experimental data using RCM as medium and parameter value estimated to Equation 2.

Parameter	Estimate	Standard error	p-level	Low conf. limit	Up conf. limit
A	0.00124	0.000290	0.0002	0.00065	0.00184
B	0.00195	0.000033	<0.0001	0.00188	0.00202
C	-5.36712	0.089532	<0.0001	-5.55082	-5.18341
Objective function			0.0000186		

Estimation of ABE in sweet sorghum as fermentation media

Industrial fermentations are carried out using low cost raw material and, by this reason, the proposed method was applied to determine ABE concentration in fermentation media based on the use of sweet sorghum, since this medium can be used in butyric fermentation [12]. Calibration points at different ABE and sugar concentrations were prepared in a similar way that for determination of ABE in RCM media and the variation of density determined in each case. Figure 4 shows the experimental results as well the linear fitting proposed. As can be seen, there is a good agreement among experimental and fitting data, showing that the variation of density and sugar concentration can be used to predict ABE concentration in a simple, fast and low cost way. The determination coefficient was greater than 0.99. Parameter values, standard error, significance level and confidence interval are

presented in Table 2, where is confirmed that the linear model satisfactorily represent the experimental behavior. Other important characteristic demonstrated is that the parameter "c" for RCM and sweet sorghum media are statically equal, indicating that the decrease of density in both cases is very similar. This parameter is not dependent of the medium proprieties but is dependent of the ABE concentration. This is expected and shows that for similar media is possible to have a preliminary estimative of ABE production for a fixed sugar concentration.

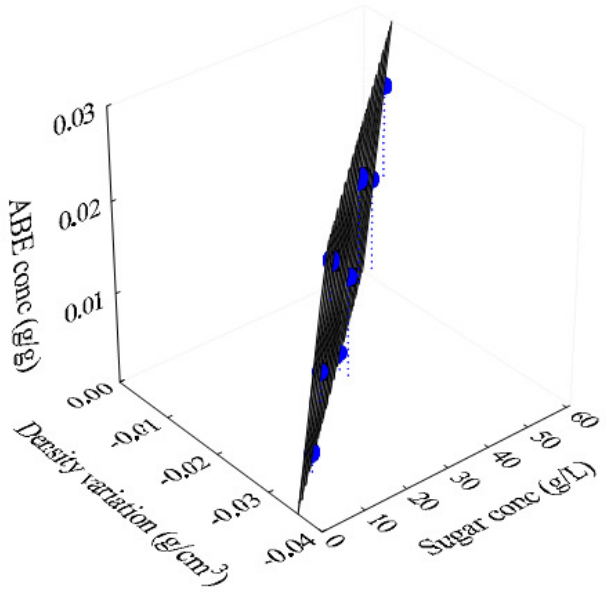

Figure 4: Relationship of experimental data of density variation (g/cm^3), solvent concentration (g/g) of ABE in respective molar proportion 6:3:1solution and sugar concentration (●) using sweet sorghum as medium and its respective linear fit model.

Table 2: confidence interval, standard error and p-level and final objective function to the adjustment of experimental data using sweet sorghum as medium showed in figure 4. And parameter value estimated to equation 2.

Parameter	Estimate	Standard error	p-level	Low conf. limit	Up conf. limit
a	-0.19421	0.005367	<0.0001	-0.20529	-0.18314
b	0.00320	0.000084	<0.0001	0.00303	0.00337
c	-5.46423	0.141423	<0.0001	-5.75611	-5.17235
Objective function			0.0000323		

Conclusion

In this work was developed a method for estimation of ABE solvents concentration in fermentation media based on mixture density. The results obtained demonstrated a linear relation among density and ABE concentration in water. For real fermentation media, it was necessary to take into account the concentration of sugar, where the method presented satisfactory results for synthetic and industrial fermentation media. The main contribution of this work was to determine the ABE concentration in a simple, fast and low cost way, unlike to classic chromatographic procedures.

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ARTIGO 4- EVALUATION OF BIOBUTANOL PRODUCTION USING SWEET SORGHUM BY *CLOSTRIDIUM BEIJERINCKII* NRRL B-592

Luiz J. Visioli, Eliana A. Alves, Aline C. M. Trindade, Raquel C. Kuhn, Marcio Schwaab, Marcio A. Mazutti

Department of Chemical Engineering - Federal University of Santa Maria, Av. Roraima, 1000, Santa Maria, RS, 97105-900, Brazil

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Abstract

In this work the production of biobutanol by *Clostridium beijerinckii* NRRL B-592 using sweet sorghum juice as carbon source was evaluated. Operational variables as pH and initial inoculum size as well as supplementation of industrial media with yeast extract and tryptone were considered. Maximum butanol obtained was 2.12 $g\,L^{-1}$ using 12.5% of inoculum size, 0.05 g/100 mL of tryptone and 0.1 g/100 mL of yeast extract and initial pH of 5.5. The main contribution of this work was to show a systematic procedure for development of a low cost industrial media for biobutanol production from sweet sorghum.

Keywords: Biobutanol production, cost reduction, biofuel, clostridium fermentation, manipulation apparatus.

Introduction

The increase in oil prices and the necessity to diversify the energetic chain have generated the interest in the production of renewable biofuels worldwide (Qureshi and Ezeji 2008). Ethanol and butanol production from fermentation are largely reported as good alternatives to replace the fossil fuels consumption. Although the industrial production of ethanol by fermentation is consolidated, mainly in Brazil, biobutanol industry are slowly reappearing after ceased operation by the end of 1960s (Qureshi *et. al*. 2007). Butanol presents better properties than ethanol, making it a candidate to replace gasoline and it can be considered as a bulk chemical precursor for the production of chemicals (Lu *et. al.* 2012). In this scenario the researches on biobutanol production are very important to optimize, cheapen and becomes easier this production (Mariano *et. al.* 2010).

The anaerobic fermentation of a sugar source by strains of *Clostridium spp*. is the main biotechnological route for butanol production. However, this fermentation produces significant amounts of ethanol and acetone becoming known for ABE fermentation (Jones

and Woods 1986). The most reported clostridium strains to butanol production are: *acetobutylicum, saccharobutylicum, beijerinckii, butylicum, aurantibutyricum* and *tetanomorphum* (Qureshi and Ezeji 2008). The anaerobic condition maintenance during the fermentation is indispensable, increasing the operation cost of the process (Ezeji *et. al*. 2013).

The economics of the biobutanol production are largely dependent on the cost of the fermentation substrate (García *et. al.* 2011). In this sense, it is very important to find a cheap and available raw material to be used in this fermentation. Residues are an alternative defended for many authors (Qureshi and Ezeji 2008). However this kind of substrate has limited availability and presents great variability on the characteristics. The sweet sorghum can be an attractive sugar source to biobutanol production. Sweet sorghum (*Sorghum bicolor* (L.) *Moench*) is a high biomass and sugar-yielding crop. Its juice contains similar quantities of glucose and sucrose and the productivity is high (Yu *et. al.* 2012).

However, one of the difficulties in the use of industrial media is the fact that bacteria are much sensitive to any variability of culture media. Based on this aspect, this work is focused on the production of biobutanol by *Clostridium beijerinckii* NRRL B-592 using sweet sorghum juice as raw material. Operational variables as pH and initial inoculum size as well as supplementation of industrial media with yeast extract, and tryptone were evaluated by means of experimental methodology.

Material and Methods

Microorganism, culture maintenance, inoculum preparation and microorganism manipulation

The microorganism used in this study was *Clostridium beijerinckii* NRRL B-592 kindly provided by United States Department of Agriculture (USDA) -Agricultural Research Service (ARS). The microorganism was shipped lyophilized in the vacuum medium. The reactivation of the strain was done using the Reinforced Clostridium Medium (RCM) as cellular grown medium (Khamaiseh *et. al.* 2012). This procedure was carried out during 48-62 hours at 37ºC. The temperature used is that reported as the optimum to cellular grow to

Clostridium beijerinckii NRRL B-592 by ARS in its home page. The microorganism grow was carried out in a sealed test-tube to prevent the oxygen entering.

The microorganism was stored in RCM medium at temperature of 4-7ºC. After the period of storage the microorganism was grown again in the RCM without necessity of heat shock. The inoculums were prepared using 10% (v/v) of seed solution of microorganism from storage. This grown occurred in a sealed test-tube during 24 hours at 37ºC and then was peaked again to test-tube using 10% of grown solution, and this last grown too for 24 hours and is ready to be a fermentation inoculum.

The RCM medium used in the procedures was made using regents from Sigma-Aldrich® all these with PA purity content. This medium contains (g L^{-1}) yeast extract 3.0, meat extract 10.0, peptone 10.0, soluble starch 1.0, L-cysteine hydrochloride 0.5, sodium acetate 3.0, agar 0.5, NaCl 5.0 and pH 6.8. After the preparation the used in the inoculation was autoclaved at 121ºC for 20 minutes.

To anaerobic conditions maintenance several special manipulation procedure was necessary during inoculation. So as to inoculum preparation to fermentation begins was done into an anaerobic chamber (glove box), hand-made in the laboratory, using a polypropylene stuff box, a neoprene glove, Polyvinyl chloride connections and glue to reduce the necessary to implement the fermentation. Before of the start the inoculation a flux of nitrogen (99.9% of purity provided from Air liquid®) was turned on and maintained during 20 minutes to ensure oxygen-free in the box. The medium was sparged to remove oxygen traces before inoculation.

Fermentations

The fermentation process was carried out using sweet sorghum as sugar source. Sorghum juice was provided by the micro distillery of the Federal University of Santa Maria - Brazil, and was farmed in the geographic coordinates 29° 41′ 29″ south, 53° 48′ 3″ west. The sugar concentration of the in nature sorghum juice was determined using a Dinitrosalisylic acid method (DNS) methodology described by Miller (1959), which was maintained constant at 60 g L^{-1} for all fermentations. The biobutanol production medium containing sweet sorghum juice as carbon source was supplemented with (g L^{-1}): KH_2PO_4 0.5, $K_2HPO_4.3H_2O$ 0.65, $MgSO_4.7H_2O$ 0.2, $FeSO_4.7H_2O$ 0.01, $MnSO_4.H_2O$ 0.01, NaCl 0.01, and sodium

thioglycolate 1.0 (Liu *et. al.* 2010). All the regents used were from Sigma-Aldrich® all these with PA purity content. The medium also was supplemented with tryptone, yeast extract and corn steep liquor, which were used as independent variables in the experimental design and will be better presented below. Oxygen-free nitrogen was sparged in the medium during 15 minutes. Afterwards, the fermentation media was autoclaved at 121ºC for 20 minutes.

Biobutanol production was carried out in 250 mL conical flasks containing 100 mL culture medium with concentrations of supplements, initial inoculum concentration and pH defined in the experimental design. Fermentation was started with defined inoculum concentration (5-20%) and pH at 150 rpm (Tecnal® shaker model TE-420) at 37°C for 96 hours. The fermented broth was centrifuged at 4000 rpm at 4°C by 15 min (Ependorf®, model 5804R). After this process the sample was stored at -15ºC to further analysis.

To evaluate the effects of initial pH, inoculum size, concentrations of tryptone and yeast extract a Placket-Burman design with eight fermentations plus three central points (PB8) was conceived. Table 1 presents the range investigated for each independent variable. Based on the analysis of PB8, a second Placket-Burman design with eight fermentations plus three central points (PB8) was conceived to evaluate the effects of initial pH, inoculum size, concentrations of tryptone and yeast extract, where the evaluated range for each variable is presented in Table 2. All the results were analyzed using the software Statistica® 7.0 (Statsoft Inc., Tulsa, OK, USA), considering a significance level of 90%.

Analytical procedures

Determination of solvents (acetone-butanol-ethanol) was carried out by gas chromatography (Shimadzu, GC – 17A) using a capillary column DB-Wax (30m x 0.25mm x 0.25μm) with polyethylene glycol as the stationary phase. The injection was done in a split injector (ration 1:20) at temperature of 220ºC, where samples 0.2 μL were injected. The detector of flame ionization (FID) was maintained at temperature of 250ºC and its flame operating with hydrogen and synthetic air. The column was operated according to the following column temperature gradient programming: 40°C (8 min); 20°C/min up to 180°C and maintained at this temperature for 10 min. Nitrogen was the carrier gas with a flow rate of 0.8 ml/min. Methyl ethyl ketone (MEK) was used as internal standard (Qureshi *et. al*. 2007).

Results and discussion

Table 1 presents the results referring to butanol production obtained in the PB8. As can be seen, the production occurred only at some specific experimental conditions at low concentrations. However, the production occurred mainly in the runs carried out at initial pH of 6.0, indicating that the range evaluated for pH was not appropriated for this microorganism. This was confirmed by analysis of effects, expressed in the form of Pareto chart presented in the Fig. 1, where pH presented negative effect on butanol production at a significance of 89% ($p<0.11$).

Table 1. Matrix of the first PB8 to evaluate the influence of process variables on biobutanol production.

Run	Initial pH	Inoculum (%)	Tryptone (g/100mL)	Yeast extract (g/100mL)	Butanol production (g/kg)
1	8 (1)	5 (-1)	0 (-1)	0.4 (1)	0.00
2	8 (1)	20 (1)	0 (-1)	0 (-1)	0.00
3	8 (1)	20 (1)	0.2 (1)	0 (-1)	0.00
4	6 (-1)	20 (1)	0.2 (1)	0.4 (1)	0.79
5	8 (1)	5 (-1)	0.2 (1)	0.4 (1)	0.06
6	6 (-1)	20 (1)	0 (-1)	0.4 (1)	0.00
7	6 (-1)	5 (-1)	0.2 (1)	0 (-1)	0.17
8	6 (-1)	5 (-1)	0 (-1)	0 (-1)	0.68
9	7 (0)	12.5 (0)	0.1 (0)	0.2 (0)	0.00
10	7 (0)	12.5 (0)	0.1 (0)	0.2 (0)	0.00
11	7 (0)	12.5 (0)	0.1 (0)	0.2 (0)	0.05

Based on the results obtained in the first experimental design a second PB8 was conceived. The variables investigated were the same, being altered the range. The results obtained in the second PB8 design are presented in Table 2. The highest production was 2.12 ± 0.07 $g.L^{-1}$ at central point of the PB8. In other runs, the production was low. Data of Table 2 were used to compute the main effects of independent variables, which are presented in Figure 2. As can be seen, all variables investigated were not significant in the range studied. However, the condition of central point is not considered in the calculation of the

effects. In this sense, there is the indicative that central the condition of central point is the best for biobutanol production.

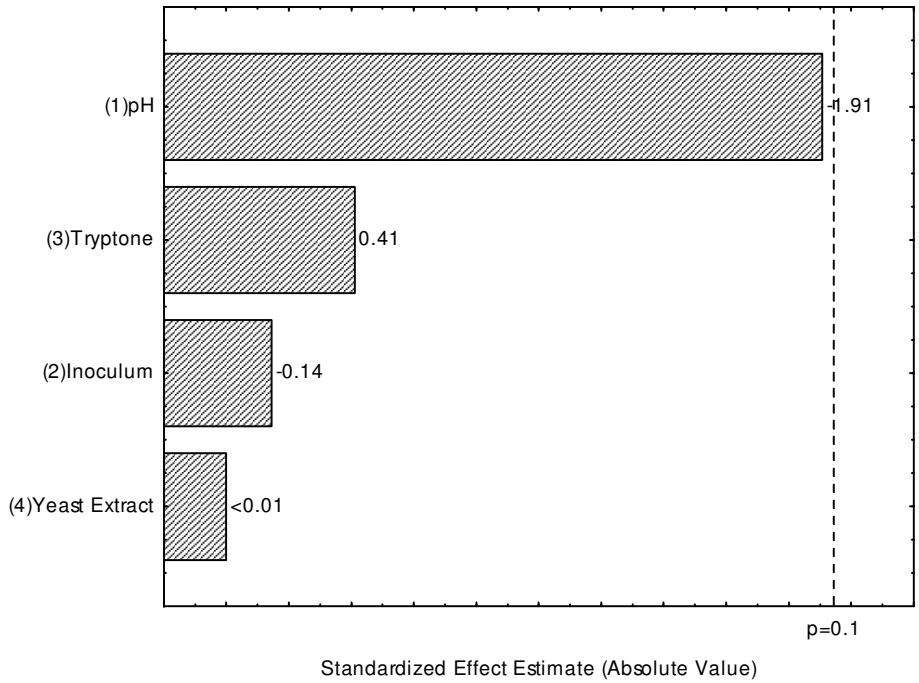

Figure 1. Effects of independent variables of the first PB8 in the production of biobutanol presented in the form of Pareto chart.

Table 2. Matrix of the second PB8 to evaluate the influence of process variables on biobutanol production.

Run	Initial pH	Inoculum (%)	Tryptone (g/100mL)	Yeast extract (g/100mL)	Butanol production (g/kg)
1	6 (1)	5 (-1)	0 (-1)	0.2 (1)	1.64
2	6 (1)	20 (1)	0 (-1)	0 (-1)	0.10
3	6 (1)	20 (1)	0.1 (1)	0 (-1)	0.28
4	5 (-1)	20 (1)	0.1 (1)	0.2 (1)	0.00
5	6 (1)	5 (-1)	0.1 (1)	0.2 (1)	0.67
6	5 (-1)	20 (1)	0 (-1)	0.2 (1)	0.00
7	5 (-1)	5 (-1)	0.1 (1)	0 (-1)	0.00
8	5 (-1)	5 (-1)	0 (-1)	0 (-1)	0.00
9	5.5 (0)	12.5 (0)	0.05 (0)	0.1 (0)	2.13
10	5.5 (0)	12.5 (0)	0.05 (0)	0.1 (0)	2.18
11	5.5 (0)	12.5 (0)	0.05 (0)	0.1 (0)	2.05

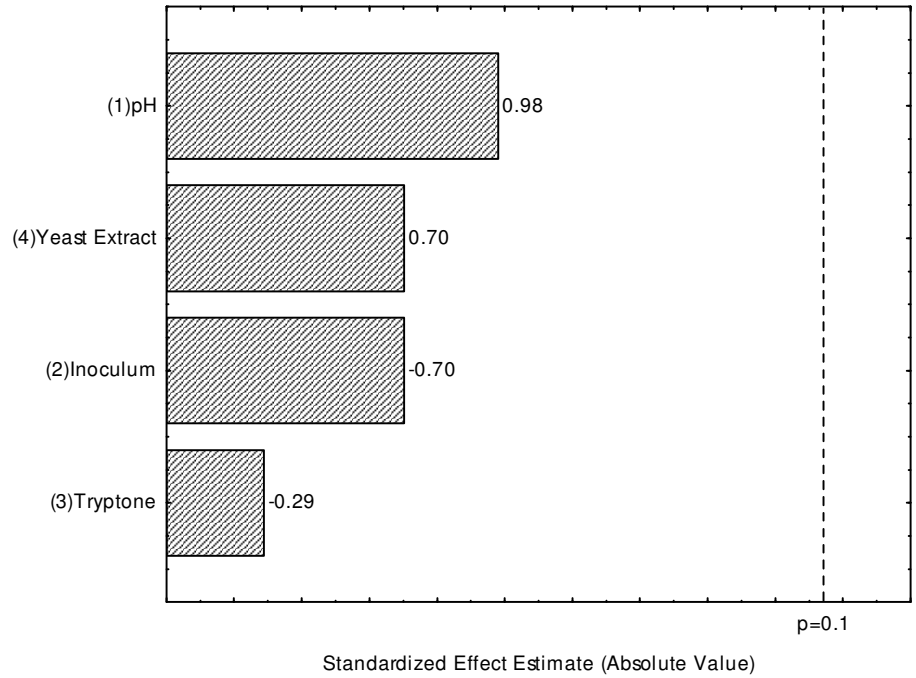

Figure 2. Effects of independent variables of the second PB8 in the production of biobutanol presented in the form of Pareto chart.

One important aspect of this result is that the greatest butanol production using *Clostridium beijerinckii* NRRL B-592 and sweet sorghum (60 g/L of total sugars) requires low concentration of yeast extract and tryptone (a half than was reported by Liu *et. al.* 2010), which are expensive nutrient for industrial fermentation. This result demonstrates that the sweet sorghum can be used for clostridium culture.

Conclusion

In this work was evaluated the production of biobutanol by *Clostridium beijerinckii* NRRL B-592 using sweet sorghum juice as carbon source. Maximum butanol obtained was 2.12 $g.L^{-1}$ using 12.5% of inoculums size, 0.05 g/100 mL of tryptone and 0.1 g/100 mL of yeast extract and initial pH of 5.5. The main contribution of this work was to show a systematic procedure to development of a low cost industrial media for biobutanol production from sweet sorghum.

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5 DISCUSSÃO

Devido ao fato deste trabalho estar disposto em formato de quatro artigos científicos (dois trazendo revisões da bibliografia e dois resultados obtidos) se torna importante uma breve discussão dos principais tópicos observados no trabalho como um todo. O primeiro artigo que compõe a revisão bibliográfica traz uma visão de como os registros de novas tecnologias vem ocorrendo nos últimos anos no que diz respeito à produção de biobutanol a partir de processos fermentativos. Já o segundo artigo traz uma abordagem mais acadêmica do que vem sendo desenvolvido em relação às principais dificuldades deste processo.

Um dos pontos mais significativos durante as pesquisas bibliográficas para os dois artigos está relacionado à importância do substrato na produção de butanol. De acordo com muitos autores, entre eles QURESHI *et al*. (2010) e AL-SHORGANI *et al*. (2012), este pode ser o fator que mais afeta o preço final do biocombustível. A importância dada à matéria precursora para o álcool é confirmada pela análise das patentes registradas. A maior fração se refere a substratos alternativos que podem ser usados para este processo.

A inibição pelo produto também é considerada muito importante para a viabilização da produção. Para superar este gargalo são observadas diversas patentes propondo desenvolvimento de micro-organismo "engenheirado" e de tecnologias de remoção dos produtos *in situ* do fermentador. Da mesma forma, este aspecto tem destaque nos trabalhos acadêmicos recentes, nos quais também é observado desenvolvimento de novos microorganismos e de formas remover os solventes do meio.

No entanto, o tópico fundamental abordado na maioria dos artigos e patentes consultados é a redução de custos para a viabilidade econômica final. Pode-se afirmar que praticamente todos os autores concordam que se os custos não forem reduzidos o biobutanol não se tornará competitivo nem mesmo ao longo prazo. Desta forma, todos os aspectos relatados na revisão bibliográfica podem ser considerados meios para esta redução.

Com relação aos artigos científicos, o terceiro deles propõe uma forma de análise para quantificar a produção de solventes durante a fermentação. Já o quarto descreve o processo de produção de biobutanol, usando a bactéria *clostridium beijerinckii* NRRL B-592, a partir de sorgo sacaríneo.

O método para estimar a produção de solventes na fermentação butílica com uso de densímetro surgiu da tentativa de evitar o uso de cromatografia. Além do menor tempo gasto

com a análise e do menor custo total de cada amostragem, o procedimento demonstrou ser capaz de predizer a concentração os solventes resultantes da fermentação com boa precisão. A grande limitação do método é que não há como quantificar individualmente cada um dos produtos, mas sim, como um todo (ABE). Com isso esta metodologia é mais recomendada para uma análise preliminar do processo, ou para acompanhamento de um processo já bem conhecido.

Para a produção de butanol a partir de sorgo sacaríneo foi aperfeiçoada uma caixa de luvas para manutenção de meio anaeróbio, além de um arranjo para manutenção de meio anaeróbico durante a fermentação (conforme apresentado nos anexos 1 e dois deste trabalho). Estes aparatos experimentais foram de fundamental importância durante o desenvolvimento da pesquisa e sem os mesmos ela seria impossível.

Já a otimização das variáveis resultou em um ponto ótimo de operação sem acréscimo de água de maceração de milho, com baixo acréscimo de extrato de levedura (0,1 g/100 mL) e triptona (0,05 g/100 mL) com pH 5,5 e 12,5% de inóculo. Este resultado demonstra que o sorgo é promissor como substrato para a fermentação butílica já que não é possível diminuir os custos com aditivos necessários em outros substratos reportados na literatura (LIU *et al*., 2010). Além disso, como a estimação dos parâmetros não resulta em nenhuma variável significativa, deve-se considerar a possibilidade de um processo sem adição de extrato de levedura ou triptona. Estes resultados tornam promissora a possibilidade de aumento de escala no processo, já que, podem gerar um processo com custo final e reduzido.

6 CONCLUSÕES

A fermentação anaeróbia de sorgo sacaríneo com o microorganismo *Clostridium beijerinckii* NRRL B-592 é capaz de produzir biobutanol. Ainda que as concentrações obtidas durante as fermentações estejam em um patamar bastante inferior àquele apresentado na literatura (utilizando outras fontes de açúcar) há uma produção razoável do álcool. Algumas conclusões a que se chegou ao decorrer do trabalho foram:

- A caixa de luvas construída em laboratório foi eficiente para o manuseio de inóculo e meio de fermentação mantendo ambiente anaeróbico. A caixa apresenta fácil manuseio e limpeza e pode ser mantida durante os períodos sem uso como um ambiente fechado e estéril. De modo semelhante os arranjos com o erlenmeyer, rolha, agulha oca e balão funcionaram bem impedindo a entrada de oxigênio, mas permitindo a saídas dos gases gerados pela fermentação. A grande redução de custos com o desenvolvimento destes aparatos foi fundamental para a produção de biobutanol, além de aumentar atratividade do processo avaliado;

- O desenvolvimento de uma metodologia para a estimação da concentração de da mistura de solventes durante a fermentação utilizando o densímetro foi possível. A metodologia pode ser uma alternativa ao uso da cromatografia em casos específicos, já que não possibilita a determinação da concentração de cada um dos solventes separadamente. A análise é bastante rápida propiciando estimação da concentração dos solventes de várias amostras em um curto espaço de tempo. A interpretação dos resultados não requer muito esforço já que a relação funcional entre densidade, concentração de açúcar e de solventes é linear. Esta proposta está em concordância com o objetivo indireto deste trabalho, que é reduzir, sempre que possível, os custos totais do processo;

- A avaliação do processo de produção de butanol resultou na não necessidade de uso de água de maceração de milho e pouca necessidade de extrato de levedura e triptona, pH inicial em 5,5 e 12,5% de inóculo crescido por 24 horas em meio RCM. O ponto de operação encontrado demonstrou que o sorgo sacaríneo é um promissor meio para esta fermentação. A não necessidade de uso de fontes complementares de nitrogênio demonstra que provavelmente o caldo de sorgo fornece quantidade suficiente do mesmo. O uso de apenas 5% de inóculo para a fermentação também é um resultado importante, pois colabora na redução total de custo do processo.

7 SUGESTÕES

Por falta de tempo e equipamentos algumas sugestões para a continuidade do trabalho se tornam importantes. Seria importante analisar a influência de variáveis fixadas neste trabalho sobre a produção de butanol. Como as principais variáveis podem ser consideradas: a temperatura, a agitação, concentração inicial de açúcar. Por outro lado, como a produção de butanol pelo microorganismo *Clostridium beijerinckii* NRRL B-592 não foi alta, fica como sugestão para que sejam avaliados outros microorganismos para identificar espécies mais eficientes na produção de butanol.

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APÊNDICE A- CONSTRUÇÃO DA CAIXA DE LUVAS PARA MANIPULAÇÃO ANAERÓBICA DO MICROORGANISMO.

Para o manuseio correto de microorganismos do gênero *clostridium*, que tem como uma das suas principais características serem estritamente anaeróbicos tornou-se necessário a confecção de um equipamento que pudesse fornecer uma atmosfera livre de oxigênio. A alternativa menos onerosa para isto foi adaptar uma caixa plástica comercial de polipropileno com capacidade aproximada de 50 litros (dimensões de 46x35x30 cm), de forma semelhante ao reportado em MONTIPÓ (2012) tornando possível a manipulação de objetos no seu interior sem que se tenha contato com o ambiente externo. A forma encontrada para a manipulação dentro da caixa foi acoplar duas luvas na face frontal da caixa, sendo assim possível ter os movimentos da mão no seu interior. Para isso foram usadas luvas de neopreno, que é um tipo de borracha adequado para o manuseio de substâncias abrasivas e, portanto, é suficientemente inerte para as manipulações necessárias neste trabalho.

A maior dificuldade na elaboração da caixa foi à forma como as luvas foram fixadas aos orifícios feitos na face da caixa. Além disso, devido ao uso constante, as luvas são danificadas com frequência, e se faz necessária uma forma prática para a substituição das mesmas. O polipropileno é um plástico muito liso e com isso é extremante difícil que uma cola de qualquer espécie seja eficiente em fixar algo à face da caixa. Assim foram colocadas duas placas de alumínio (também com o orifício para a entrada das luvas), uma na parte interna e outra na externa da caixa e ambas fixadas por rebites do mesmo material. Para que pequenos orifícios oriundos de imperfeições nas placas metálicas não persistissem no projeto final da caixa foi aplicada uma pequena camada de cola de silicone acético (com finalidade somente de dar uma perfeita vedação).

Depois de fixadas as chapas metálicas foram coladas, com cola epóxi, duas extremidades de joelhos de tubulação de PVC com 100 mm de diâmetro interno. O uso de joelho foi considerado mais adequado devido às rebarbas existentes nas suas extremidades, que garante uma maior área para a colagem. Novamente, para garantir a não existência de orifício por onde o ar possa passar foi aplicada uma fina camada de silicone acético sobre a cola epóxi. Depois de completamente terminado o processo de secagem de todas as colagens as luvas foram fixadas. Para fixá-las e ao mesmo tempo permitir sua substituição foram

utilizadas braçadeiras metálicas que pressionam as luvas contra a parede externa do tubo PVC proeminente a face da chapa metálica.

Para a tampa da caixa de luvas ter uma vedação suficiente e não permitir a entrada de oxigênio foram feitos furos na tampa e na rebarba da caixa e colocados parafusos. Dessa forma a tampa fica fixa e fazendo a pressão contra a caixa, mas ainda pode ser facilmente removida quando necessário. Mesmo assim poderia entrar na caixa e então foi colocada uma borracha de vedação entre a tampa e a caixa, de forma que com a pressão dos parafusos, a caixa ficasse completamente vedada. Para que o oxigênio seja eliminado de dentro é necessário um fluxo constante de gás inerte. Neste caso foi escolhido o nitrogênio e sua entrada ocorre na parte superior da face lateral esquerda da caixa. Para permitir a saída dos gases foi feito um pequeno orifício, praticamente estrangulado, para permitir uma leve pressão positiva no interior da caixa. Para melhor entendimento da forma como a caixa foi construída pode observá-la concluída na Figura A1.

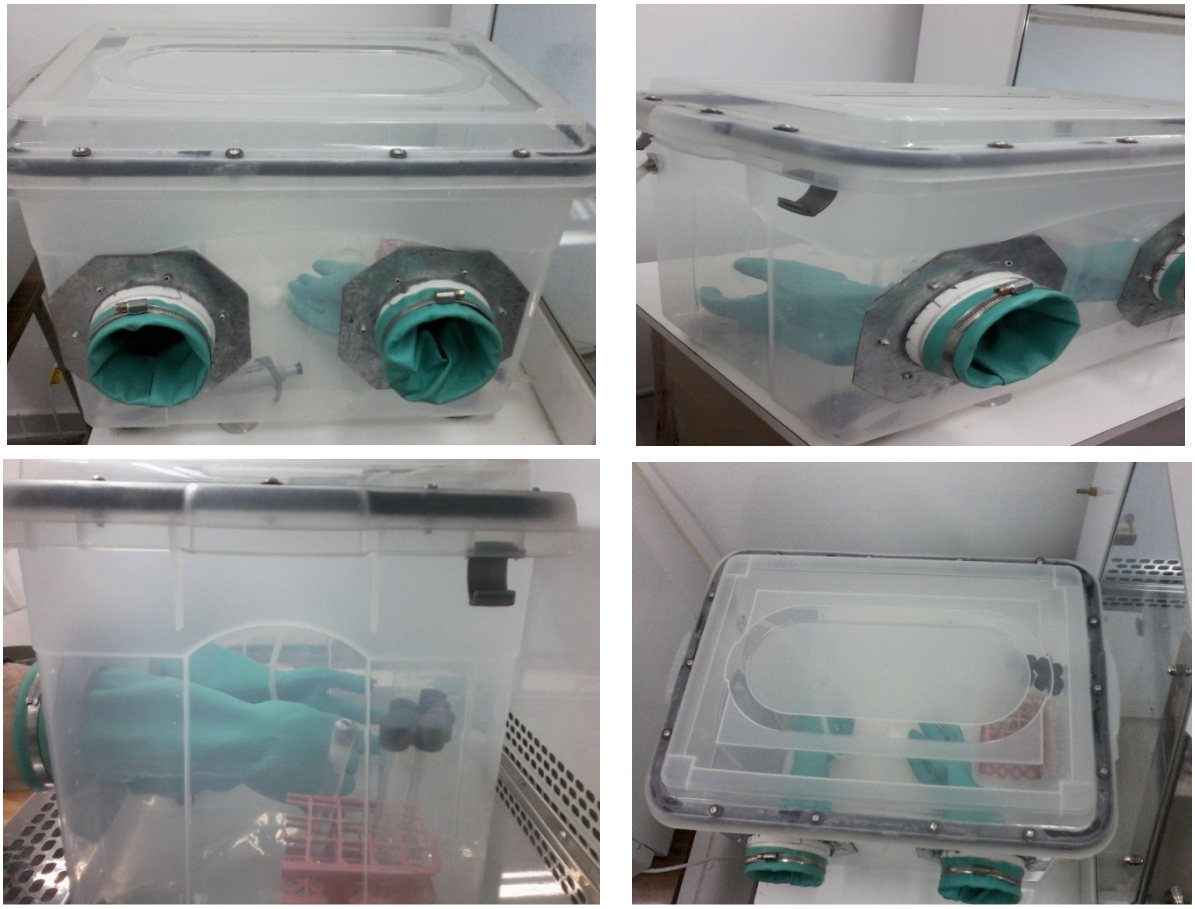

Figura A1: Caixa de luvas construída para inoculação em meio anaeróbico.

Na Figura A1 pode-se ter uma boa visão de como ficou a versa final da caixa. Pode-se observar que as braçadeiras de fixação das luvas são de fácil acesso para quando uma troca for necessária. Também dá para notar que a amplitude de movimento das luvas permite o manuseio de qualquer material em qualquer região da caixa. Na vista superior aparecem os parafusos de fixação da caixa e na vista frontal é possível ter uma idéia de como é a visão do operador durante a manipulação.

O procedimento para a manipulação é bastante simples. Os equipamentos, meios e recipientes necessários, tudo já devidamente esterilizado na autoclave, são colocados no interior da caixa. Então a mesma é fechada, os parafusos são apertados e o fluxo de nitrogênio é iniciado. Para remover o oxigênio existente no interior da caixa o fluxo de nitrogênio é mantido por 15 minutos antes de se começar a inoculação. Enquanto a caixa está aberta e depois durante o tempo de aguardo para remoção do oxigênio a caixa é mantida dentro de uma câmara de fluxo laminar a fim de reduzir o risco de contaminação. A Figura A2 mostra como a caixa fica no interior da câmara de fluxo que é onde os procedimentos são realizados.

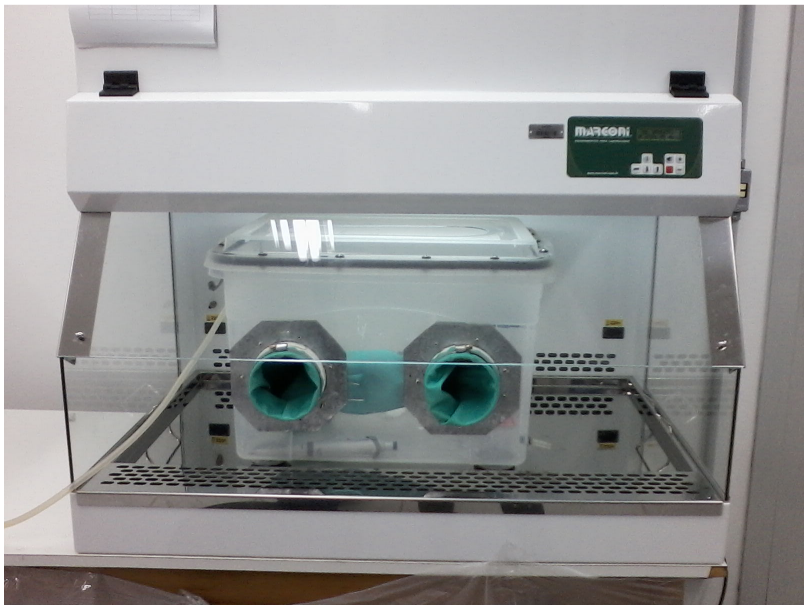

Figura A2: Caixa de luvas no interior da câmara de fluxo laminar pronta para realização de procedimentos de inoculação.

APÊNDICE B- APARATO DESENVOLVIDO PARA FERMENTAÇÃO ANAERÓBICA.

Para a produção de butanol a partir de fermentação clostridial é estritamente necessário que o meio anaeróbio seja mantido no interior do fermentador. Para isso torna-se indispensável pensar em um artefato que possa ser completamente lacrado ainda no interior da caixa de luvas e que se mantenha assim até o final da fermentação. A primeira proposta testada foi o uso direto de um erlenmeyer de 250 mL como reator e uma rolha de silicone para vedação. A rolha é colocada no orifício do erlenmeyer ainda dentro da caixa e o conjunto contendo meio e inóculo é retirado e colocado no shaker para fermentar.

A vedação foi claramente eficiente, pois houve desenvolvimento do microrganismo e a fermentação começou a ocorrer com sucesso. No entanto, aproximadamente 24 horas após o inicio do processo a pressão dentro do reator subiu de tal forma que rolha foi removida da abertura. O aumento da pressão ocorreu devido à produção de gases durante o crescimento do microorganismo e produção do butanol. Com a abertura livre o meio anaeróbio é perdido e a fermentação é imediatamente interrompida. Outro fator importante a ser observado é que não se tem conhecimento se o aumento da pressão no erlenmeyer inibe ou não a produção de solventes. Então o esquema proposto para vencer esta dificuldade deve prever a necessidade de alivio de pressão e não apenas uma forma de impedir que a rolha saia.

Como a vedação do primeiro esquema proposto foi eficiente é possível partir do princípio de que uma rolha pode ser usada desde que se acople um alívio de pressão eficiente. Para permitir a saída dos gases do interior do erlenmeyer a idéia foi perfurar a rolha com uma agulha oca com um êmbolo de vedação removível. Desta forma a rolha poderia ser colocada na abertura do erlenmeyer dentro da caixa e o conjunto poderia ser retirado já vedado. Para permitir a saída dos gases sem entrada de oxigênio durante a fermentação a proposta foi colocar um balão sobre o conjunto rolha e agulha. Depois disto o êmbolo de vedação pode ser removido manualmente (dentro do balão) e os gases podem expandi livremente no volume da bexiga. Quando a pressão interna atinge um valor levemente positivo os gases podem escapar entre a boca do balão e a rolha. O comportamento do sistema é similar ao de uma válvula de diafragma. O desenho esquemático de como o aparato foi pensado pode ser observado na Figura B1.

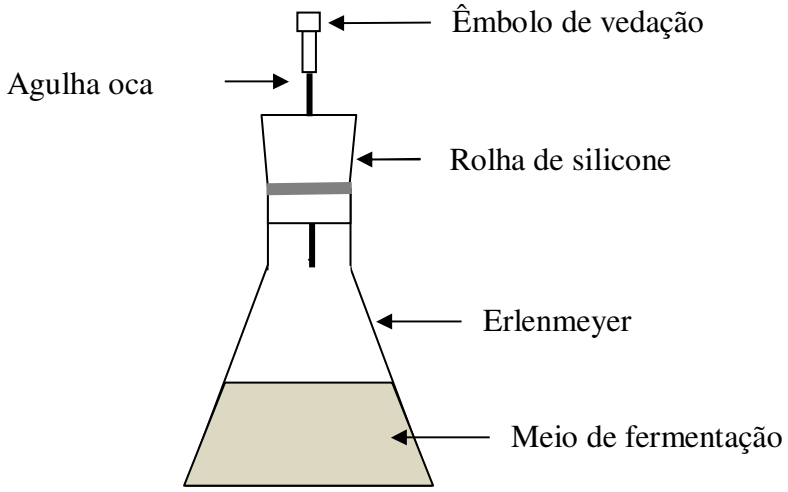

Figura B1: Desenho esquemático do aparato utilizado para a produção anaeróbica de biobutanol.

Figura B2: Aparato utilizado para a produção anaeróbica de biobutanol.

Na figura B1 não foi possível demonstrar o balão acoplado cuja boca envolve a rolha de silicone. Para melhor visualização de como este funciona é possível observar fotos reais do aparato durante a fermentação na Figura B2. Nesta mesma figura é possível observar alguns balões se inflando o que é o sinal de que está ocorrendo produção de gases e conseqüentemente a fermentação também está se desenvolvendo.

APÊNDICE C- DESENVOLVIMENTO DO MÉTODO CROMATOGRÁFICO.

Foi desenvolvida uma metodologia de análise cromatográfica para a separação dos produtos da fermentação butílica, acetona, etanol e butanol, e também, do padrão interno usado para a quantificação dos produtos, metil-etil-cetona. A coluna utilizada para a separação foi J&W DB-WAX com 30 m de comprimento, 0,25 mm de diâmetro e 0,25 μm de espessura da camada de fase estacionária, está é composta por polietileno glicol. A razão de split usada na injeção foi 1:20. A fase móvel utilizadas foi nitrogênio com uma vazão de 0.8 mL/min. O injetor foi mantido a 220 ºC. A temperatura inicial da coluna foi de 40 ºC mantido por 8 minutos. Então, a temperatura foi elevada, a uma taxa de 20 ºC/min até 180 ºC, permanecendo nesta temperatura por 10 minutos. Como detector foi utilizado um detector de ionização de chamas (FID), mantido a temperatura de 250 ºC.

A separação dos solventes (acetona, etanol, butanol e metil-etil-cetona) pode ser observada na Figura C1. Os tempos de retenção médios para cada um dos compostos foi: Acetona, 4,8 min, metil-etil-cetona, 6,3 min, etanol, 7,6 min e butanol, 12.5 min.

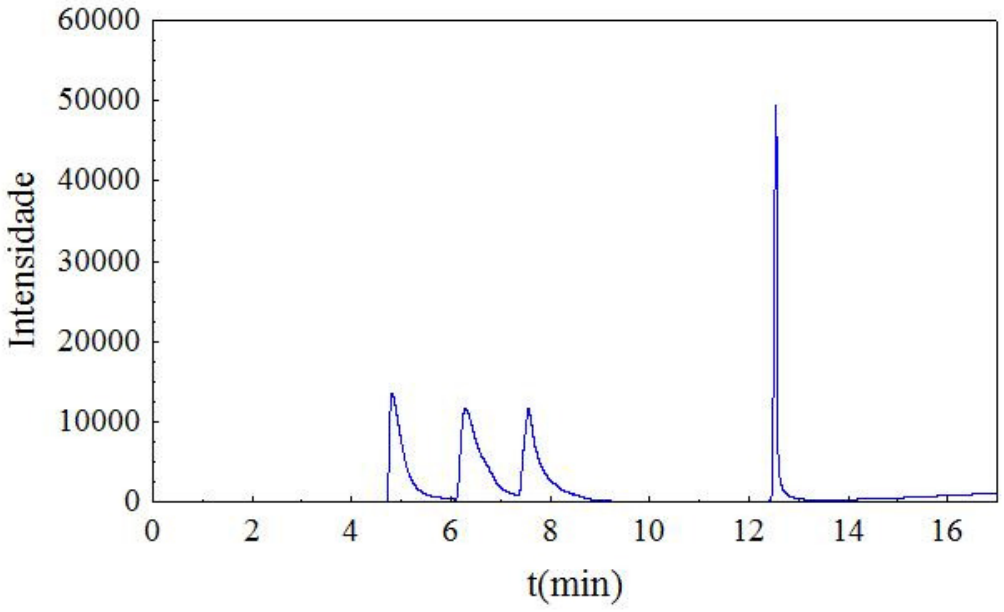

Figura C1: Separação dos solventes, acetona, metil-etil-cetona, etanol, butanol, respectivamente.

A fim de demosntrar a eficácia do método cromatográfico em separar os produtos de fermentação a partir de amostras de mosto fermentado. A Figura C2 mostra a separação dos produtos de uma fermentação de 96 horas. A separação a partir do mosto de fermentação se comporta exatamente da mesma forma que para os padrões em água. Isso significa que o meio de fermentação não interfere na análise.

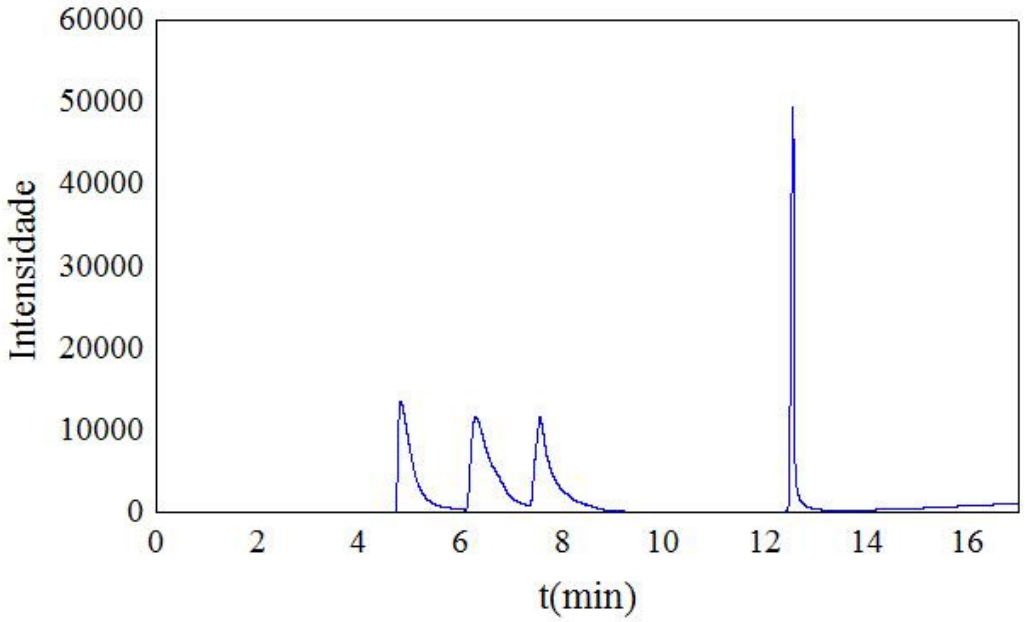

Figura C2: Separação dos solventes, acetona, metil-etil-cetona, etanol, butanol a partir de amostra fermentada por 96 horas.

APÊNDICE D- CALIBRAÇÃO E VALIDAÇÃO DO MÉTODO CROMATOGRÁFICO.

A calibração do método cromatográfico para os solventes produzidos durante a fermentação foi feita levando em conta as relações de resposta para detector FID proposta por DIETZ (1967). Esta relação possibilita que a partir de uma calibração confiável para um composto possa-se inferir calibração para os demais compostos. Assim neste caso apenas o butanol foi calibrado e para os demais compostos foi adotado a inferência.

Para a calibração do butanol foi feita a partir de uma solução aquosa adotando como valor real os valores da massa da água, do padrão interno, metil-etil-cetona (MEK) e do butanol. A massa é adotada, pois a balança apresenta uma maior confiabilidade do que as pipetas volumétricas. Conforme SCHWAAB e PINTO (2011) é mais eficiente, quando se trata de estimar parâmetros da reta, fazer soluções padrões nas extremidades e no centro da faixa possível das variáveis. Como neste, teoricamente, a reta passa pela origem, não há necessidade de se ter padrões no nível inferior. Desta forma a concentração utilizada como máxima para butanol foi de 25 g/kg e a intermediária foi de 12,5 g/kg, cada uma delas repetida três vezes. Os valores de massa utilizados na calibração são apresentados na Tabela D1, assim como os valores das áreas dos picos cromatográficos para cada um dos compostos.

Tabela D1: Massas utilizadas na preparação das amostras de calibração [g], áreas cromatográficas do butanol e do MEK e razões mássica e de áreas entre butanol e MEK (butanol/MEK).

Amostra	Massa água	Massa butanol	Massa MEK	Área butanol	Área MEK	Razão massa	Razão área
1	24,7457	0,3246	0,1322	427910	134737	2,46	3,18
2	24,8484	0,3118	0,1329	639361	177350	2,35	3,61
3	24,7451	0,3501	0,1297	639052	172111	2,70	3,71
4	24,9604	0,6303	0,1309	715272	100924	4,82	7,09
5	24,7277	0,6256	0,1317	912184	135582	4,75	6,73
6	24,7079	0,6395	0,1298	742137	110462	4,93	6,72

Os valores das razões calculados na Tabela D1 são utilizados na estimação dos parâmetros da calibração. O modelo ajustado pode ser observado na Equação D1.

$$R_{massa} = a * R_{área} \tag{D1}$$

Onde, R_{massa} é a razão mássica exposta na tabela e $R_{área}$ é a razão de áreas também apresentada na tabela. A estimação levou aos resultados; para o parâmetro, $a = 0{,}707$, a função objetivo, (mínimos quadrados) $F_{obj} = 0{,}160236$, e a variância do parâmetro determinado no ajuste, $Var_a = 0{,}0001805$. O gráfico do ajuste pode ser visto na Figura D1 junto com as curvas limites de confiança da predição. Para o cálculo das curvas inferior e superior de confiança da predição do modelo são utilizados princípios abordados em SCHWAAB e PINTO (2007). A equação D2 mostra o modelo adotado para calcular estes limites.

$$R_{massa} = a * R_{área} \pm \sqrt{Var_a * R_{área}^2 + \frac{F_{obj}}{GL}} * t_{(95\%,GL)} \tag{D2}$$

Onde GL são os graus de liberdade da estimação e $t_{(95\%,GL)}$ é o t de Student com 95% de confiança para os graus de liberdade do problema. Neste caso o número de graus de liberdade é cinco, já que a calibração tem seis pontos experimentais e apenas um parâmetro. O valor do t de Student para este caso é 2,571. Na Figura D1 são apresentados os limites da confiança do modelo e também o ajuste dos dados experimentais apresentados na Tabela D1.

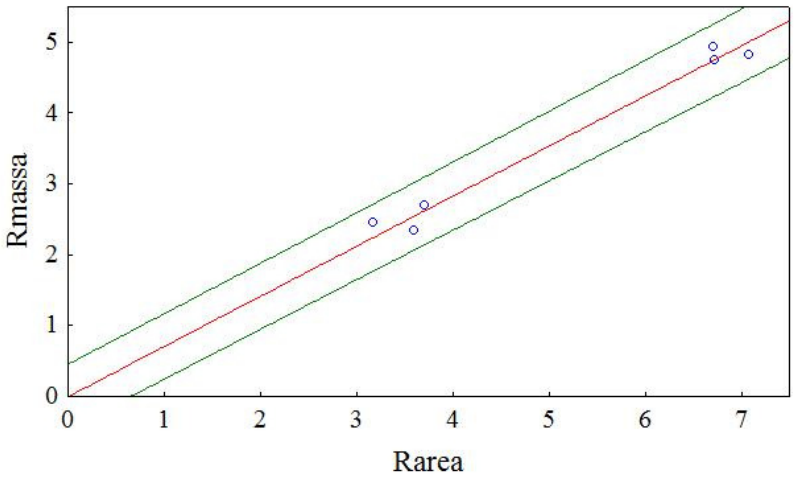

Figura D1: ○ pontos experimentais, ─ Ajuste linear para a calibração, ─ Limite inferior e superior de certeza da predição com 95% de confiança.

Para a validação desta calibração foi feita a análise em triplicata de uma amostra com razão mássica de 3,62. Os resultados da razão de áreas para os três experimentos foram, 4,7; 5,1 e 4,6. O resultado predito pelo modelo foi de 5,1 o limite superior de 5.8 e o inferior de 4,4. Como todos os valores medidos na validação se encontram entre os limites inferior e superior da predição, pode-se afirmar com 95% de confiança estatística que a calibração está apta para predizer a razão entre as massas a partir de uma razão de áreas medida.

APÊNDICE E- TESTE DO USO DO MICRO-ORGANISMO *CLOSTRIDIUM BEIJERINCKII* B-597 PARA A PRODUÇÃO DE BIOBUTANOL A PARTIR DE SORGO SACARÍNEO.

Anteriormente à avaliação do micro-organismo *Clostridium beijerinckii* B-592 foi realizado um teste inicial cinético com a bactéria *Clostridium beijerinckii* B-597. Para estes testes foram realizadas fermentações de 24, 48, 72, 96 e 120 horas de duração com a concentração do meio de fermentação fixada nas seguintes condições: 60 g/L de açúcares redutores totais provenientes do sorgo sacaríneo, 2,0 g/L de Extrato de Levedura, 1 g/L de triptona, 0,5 g/L de KH_2PO_4, 0,5 g/L de K_2HPO_4, 1 g/L de tioglicolato de sódio, 0,2 g/L de $MgSO_4.7H_2O$, 0,01 g/L de $MnSO_4.H_2O$, 0,01 g/L de $FeSO_4.7H_2O$, 0,01 g/L de NaCl, o pH inicial foi ajustado para 6,8. As condições, de temperatura, agitação e recipiente nas quais a fermentação foi realizada são idênticas aquelas descritas durante este trabalho para todas as demais fermentações.

Os resultados desta fermentação, em relação a produção de butanol, estão compilados na tabela E1.

Tabela E1: Produção de butanol a partir de sorgo sacaríneo utilizado o micro-organismo *Clostridium beijerinckii* B-597.

Tempo (h)	Concentração de butanol (g/kg)
0	0
24	0,3
48	7,3
72	6,8
96	8,4
120	7,4

A tabela E1 reporta resultados bastante promissores para a produção de butanol, obtendo-se até 8,4 g/kg do produto no tempo de 96 horas (tempo considerado durante o trabalho como suficiente para cessar o processo de fermentação). O micro-organismo *Clostridium beijerinckii* B-597 deveria ter sido utilizado durante toda a investigação realizada nesta dissertação, devido ao seu melhor desempenho em relação ao *Clostridium beijerinckii* B-592. No entanto, o mesmo foi armazenado por mais de 15 dias a 7 ºC em meio RCM e após

este período a bactéria não apresentou mais nenhum crescimento celular, inviabilizando seu uso posterior.

APÊNDICE F- TESTE DO USO DE ÁGUA DE MACERAÇÃO DE MILHO PARA A PRODUÇÃO DE BUTANOL.

O maior objetivo deste trabalho foi à análise e desenvolvimento de um processo fermentativo para a produção de butanol com o menor custo possível. Água de maceração de milho é um resíduo da indústria de amido de milho. Este resíduo apresenta alta concentração de proteínas que são fundamentais como substrato durante o processo fermentativo. Como resíduo o custo com a utilização deste material é baixo, não afetando demais a viabilidade do processo. O uso da mesma poderia minimizar a necessidade de utilização de aditivos, de alto custo, ao processo, como triptona e extrato de levedura.

Para testar a influência da adição da água de maceração de milho no processo foi realizado um planejamento de experimento do tipo Plackett Burman para cinco variáveis. No total foram realizados 15 experimento independentes, sendo três réplicas no ponto central. A forma como os dados foram tratados neste planejamento é idêntico aquela utilizada durante os demais experimentos e devidamente descrita durante o trabalho. As variáveis estudadas e suas respectivas faixas foram: pH de 6-8, inóculo de 5-20%, extrato de levedura 0-0,4 g/100mL, triptona 0-0,2 g/100mL e água de maceração de milho de 0-5 g/100mL.

Os experimentos não apresentaram nenhuma variável significativa. No entanto, em todas as fermentações onde se utilizou a água de maceração de milho não ocorreu produção de butanol. Além disso, na presença deste complemento o micro-organismo *Clostridium beijerinckii* B-592 produziu concentrações de até 22 g/L de etanol, o que demonstra que provavelmente a presença de água de maceração modifica o metabolismo da bactéria. Com isso, não é possível produzir butanol, por *Clostridium beijerinckii* B-592, com o acréscimo de água de maceração de milho ao meio fermentativo.