UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
ESCOLA DE ENGENHARIA
DEPARTAMENTO DE ENGENHARIA QUÍMICA
PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA QUÍMICA

DANIELE MISTURINI ROSSI

PRODUÇÃO BIOTECNOLÓGICA DE HIDROGÊNIO, 1,3-PROPANODIOL E ETANOL UTILIZANDO GLICEROL RESIDUAL PROVENIENTE DA SÍNTESE DE BIODIESEL

Porto Alegre, 2012

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Tese submetida ao Programa de Pós-Graduação em Engenharia Química da UFRGS como um dos requisitos à obtenção do grau de Doutor em Engenharia Química.

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À Comissão Examinadora, abaixo assinada, aprova a Tese "Produção biotecnológica de hidrogênio, 1,3-Propanodiol e etanol utilizando glicerol residual proveniente da síntese de biodiesel", elaborada por Daniele Misturini Rossi, como requisito parcial para obtenção do Grau de Doutor em Engenharia

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RESUMO

O glicerol é gerado em grandes quantidades durante a produção de biodiesel e tem se tornado um substrato potencialmente atrativo para a produção bacteriana de produtos de valor agregado, tais como 1,3-Propanodiol (1,3-PD), etanol, hidrogênio e outros. O presente trabalho teve como objetivo a produção de hidrogênio, 1,3-PD e etanol utilizando glicerol residual obtido da síntese do biodiesel. A primeira parte do trabalho visou à produção de hidrogênio utilizando glicerol residual. Nesta etapa, o consórcio microbiano ambiental foi submetido a diferentes tratamentos (ácido, básico, choque térmico, congelamento e descongelamento e dessecação). Os resultados mostraram que a maior produção de hidrogênio ocorreu com o consórcio tratado com dessecação e choque térmico, com produções de 34,2 % mol e 27,3 % mol, respectivamente. A otimização da produção de hidrogênio foi realizada utilizando planejamento experimental e alcançou uma produção máxima de 45 % mol. A próxima etapa do projeto foi isolar as bactérias presentes no consórcio microbiano ambiental. Experimentos em biorreatores foram realizados utilizando glicerol puro e glicerol residual e os resultados mostraram que houve a completa degradação do glicerol pelas bactérias do consórcio e também a produção de 1,3-PD, hidrogênio e ácidos. Nesta etapa, 32 bactérias foram isoladas e testadas para a produção de bioprodutos em experimentos em aerobiose e anaerobiose. Quatro bactérias foram selecionadas e o seqüenciamento do gene foi identificado por 16S rRNA identificou três dos isolados como *Klebsiella pneumoniae* e um isolado como *Pantoea agglomerans*. Dentre as quatro bactérias, uma cepa, nomeada como *K. pneumoniae* BLh-1, foi selecionada para os trabalhos posteriores. Para otimização das condições de cultivo, utilizou-se a seleção de variáveis através do delineamento experimental Plackett Burman (P-B) avaliando a produção de 1,3-PD. Sete variáveis foram estatisticamente significativas e um novo planejamento fracionário 2^{7-3} foi realizado com a finalidade de otimizar o meio de produção. Experimentos em biorreatores mostraram que a produção em bioreator com pH controlado com o meio otimizado no planejamento alcançou uma produção máxima de 23,80 g/L de 1,3-PD com produção de 12,30 g/L de etanol. Os ensaios com alimentação de glicerol residual mostraram que altas taxas de alimentação causam acúmulo de glicerol e diminuem a produtividade de 1,3-PD e etanol. Os estudos utilizando fluorescência 2D e modelo quimiométrico foram aplicados para avaliar as mudanças metabólicas nos cultivos aeróbios e anaeróbios. Os resultados mostraram que houve diferença nas regiões de NADH e riboflavinas ambas associadas ao metabolismo do microorganismo.

Palavras-chaves: glicerol, 1,3-propanodiol, etanol, planejamento Plackett-Burman, planejamento fracionário, hidrogênio.

ABSTRACT

Glycerol is generated in large amounts during biodiesel production and it is becoming a potentially attractive substrate for bacterial production for value-added products such as 1,3-propanediol (1,3-PD), ethanol, hydrogen, among others. This work aimed at studying the production of hydrogen, 1,3-PD and ethanol using the raw glycerol obtained by biodiesel synthesis. The first steps were to produce hydrogen using raw glycerol. At this step, the environmental microbial consortium was submitted to different pretreatments (acid, base, heat shock, dry heat and desiccation, freezing and thawing). The results showed that dry heat and desiccation had the higher hydrogen production followed by heat shock with production of 34.2 % mol and 27.3 % mol, respectively.The optimization of hydrogen production was performed using experimental design and had a maximum hydrogen production of 45 % mol. The next spet of this project was to isolate the bacteria present in an environmental microbial consortium. Experiments in bioreactors with the microbial consortium using raw and pure glycerol were carried out and the results showed that there was a complete glycerol degradation by microbial bacteria with productions of 1,3-PD, hydrogen and acids. In this step, 32 bacteria were isolated and tested separately for by-products production under aerobic and anaerobic conditions. Four bacteria were selected and sequence gene realized by 16 S rRNA, three of them were identified as *Klebsiella pneumoniae* and one isolate identified as *Pantoea agglomerans*. Among the four bacteria, the strain *K. pneumoniae* BLh-1 were selected for further experiments. Plackett Burman (P-B) experimental procedure was used for assessing the production of 1,3-PD. Seven variables were statistically significant and a new 2^{7-3} fractional design was conducted with the aim of optimizing culture conditions. Bioreactors experiments using the optimized culture conditions showed that production in bioreactor with controlled pH reached a maximal 1,3-PD production of 23.80 g/L with 12.30 g/L of ethanol. The fed-batch experiments showed that high feeding rates caused glycerol to accumulated in the medium and the1,3-PD and ethanol productivity decreased. The 2D fluorescence metodology was applied in order to study the anaerobic-aerobic changes on *K. pneumoniae* metabolism. The results showed a significant difference on NADH and riboflavin fluorescence that are fluorescence regions associated to metabolism.

Keywords: glycerol, 1,3-propanediol, ethanol, Plackett-Burman design, fractional factorial design, hydrogen.

CAPÍTULO I - INTRODUÇÃO

O desenvolvimento de pesquisas para a produção de bio-combustíveis alternativos têm sido bastante significativo nos últimos anos, devido principalmente à preocupação com a exaustão das reservas de combustíveis fósseis, como por exemplo, o petróleo. Desta forma, a produção de biodiesel e bioetanol têm emergido como uma das estratégias mais viáveis no que diz respeito a fontes não convencionais de energia. Dentre os combustíveis renováveis mais promissores destaca-se o biodiesel. Este produto é, em geral, obtido a partir da transesterificação de óleos vegetais com álcoois (metanol e etanol), usando catálise básica ou pela esterificação desses materiais na presença de catalisadores ácidos. O grande aumento do volume de biodiesel leva também à formação de bioprodutos secundários, entre eles, o glicerol.

O glicerol é gerado em grandes quantidades durante a síntese de biodiesel. Logo, com o aumento evidente da produção deste biocombustível no Brasil e no mundo, o glicerol vai passar a ser um subproduto excedente. Este grande aumento de biodiesel já surtiu efeito no preço do glicerol, o qual caiu drasticamente nos últimos anos. Uma alternativa para utilização deste subproduto é a sua conversão em bioprodutos como 1,3-propanodiol (1,3-PD) (monômero básico na indústria de polímeros), PHAs (polihidroxialcanoatos), ácido cítrico, bioplásticos, produção de enzimas como lipases, hidrogênio e etanol.

Estudos baseados nas transformações químicas e biológicas estão sendo propostos para converter o glicerol em produtos atrativos para o mercado. A conversão biológica tem gerado bastante interesse nos últimos anos, principalmente, na produção de bioprodutos. O glicerol poderia ser utilizado como fonte de carbono em bioconversões microbianas para a produção de uma grande gama de químicos, os quais poderiam ser usados como produtos finais ou como precursores para a produção de outros químicos.

O escopo deste projeto de doutorado é o de dar destino ao glicerol residual gerado na produção de biodiesel através da sua utilização em bioprocessos para a produção de bioprodutos, com especial atenção ao hidrogênio, 1,3-PD e etanol. Desta forma, pretende-se agregar valor ao processo de síntese de biodiesel, pela recuperação do glicerol, pela produção de produtos de valor agregado, além de contribuir para a formulação de processos ambientalmente mais limpos.

Diante deste contexto, o **objetivo principal** deste projeto foi a produção microbiana de hidrogênio, 1,3-PD e etanol utilizando o glicerol residual obtido da síntese de biodiesel.

Os objetivos **específicos** foram:

- ✓ Avaliar a degradação do glicerol residual por um consórcio microbiano ambiental;
- ✓ Utilizar diferentes tratamentos no consórcio microbiano ambiental e avaliar a produção de hidrogênio;
- ✓ Isolar os microorganismos presentes no consórcio microbiano ambiental;
- ✓ Testar os isolados e verificar a produção de bioprodutos de interesse;
- ✓ Identificar os microorganismos selecionados;
- ✓ Otimizar as condições de cultivo para a produção de 1,3-PD e hidrogênio utilizando ferramentas estatísticas;
- ✓ Estudar a produção de 1,3-PD e etanol utilizando várias estratégias de operação em biorreatores submersos;
- ✓ Estudar a operação em batelada alimentada em biorreatores submersos com a finalidade de aumentar a produção de etanol e/ou 1,3-PD;
- ✓ Aplicar a técnica de fluorescência 2D e modelagem para avaliar as diferenças metabólicas no cultivo de *K. pneumoniae* BLh-1;

Esta tese foi desenvolvida, em sua maioria, no laboratório de Biotecnologia (BiotecLab) do Instituto de Ciência de Tecnologia de Alimentos (ICTA) da Universidade Federal do Rio Grande do Sul (UFRGS). Parte deste trabalho foi desenvolvida durante o estágio de doutorado realizado no Laboratório de Tecnologia Química da Universidade de Leibniz, na Alemanha, sob orientação do Prof. Bernd Hitzmann. O estágio no exterior foi de 5 meses, compreendendo o período de 09/2010 a 01/2011.

Este trabalho está estruturado em capítulos. O Capítulo I traz a introdução do trabalho juntamente com os objetivos. O Capítulo II traz os embasamentos teóricos pertinentes à análise dos resultados. Os Capítulos III, IV,V,VI, VII e VIII descrevem as metodologias utilizadas na condução dos experimentos e os resultados obtidos; estes capítulos estão apresentados na forma de artigos científicos que foram publicados, aceitos e/ou submetidos para publicação. As considerações finais sobre o tema desta tese estão apresentadas no Capítulo IX. Ao final, estão incluídos apêndices relevantes para complementação do trabalho.

CAPÍTULO II - REVISÃO BIBLIOGRÁFICA

2.1 Biodiesel e glicerol

Biodiesel é um combustível biodegradável derivado de fontes renováveis como óleos vegetais e gorduras animais que, em presença de um catalisador, reagem quimicamente com um álcool, como o metanol. Existem diferentes espécies de oleaginosas no Brasil das quais se podem produzir o biodiesel, entre elas mamona, dendê, girassol, babaçu, soja e algodão (MNE, 2007). O óleo vegetal é um triglicerídeo, ou seja, é um tri éster derivado do glicerol. Sob ação de um catalisador básico, ou mesmo ácido, e na presença de metanol ou etanol, o óleo sofre transesterificação formando três moléculas de ésteres metílicos ou etílicos dos ácidos graxos e liberando o glicerol (Figura 1).

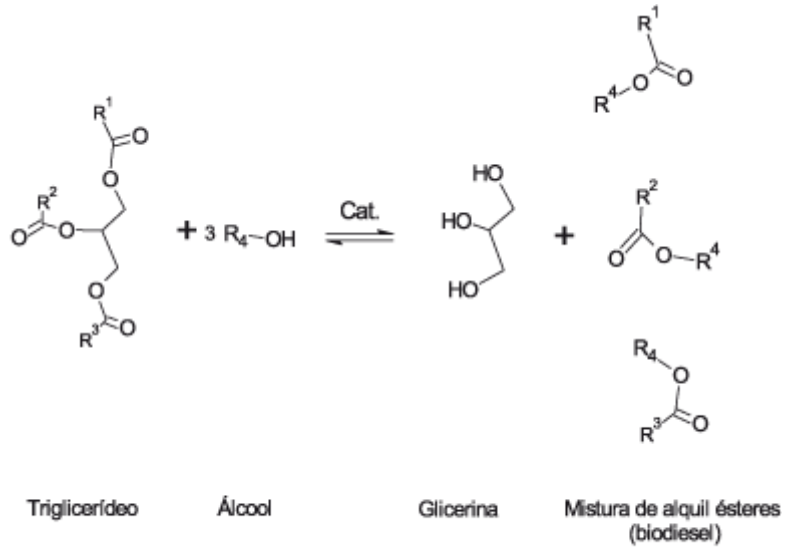


Figura 1- Produção de biodiesel a partir da transesterificação de óleo vegetal (LÔBO e FERREIRA, 2009).

Após a reação de transesterificação que converte a matéria graxa em ésteres (biodiesel), a massa reacional final é constituída de duas fases, separáveis por decantação e/ou por centrifugação. A fase mais pesada é composta de glicerina bruta, impregnada dos excessos utilizados de álcool, de água e de impurezas inerentes à matéria-prima. A fase menos densa é constituída de uma mistura de ésteres metílicos ou etílicos, conforme a natureza do álcool originalmente adotado, também impregnado de

excessos reacionais de álcool e de impurezas (SBRT, 2007). A glicerina bruta emergente do processo, mesmo com suas impurezas convencionais, já constitui um subproduto vendável. No entanto, o mercado é muito mais favorável à comercialização do glicerol purificado, quando o seu valor é realçado. A purificação da glicerina bruta é feita por destilação a vácuo, resultando em um produto límpido e transparente, denominado comercialmente de glicerol ou glicerina destilada.

A União Européia produz mais de 1,35 milhões de toneladas de biodiesel, em cerca de 40 unidades de produção. Isso corresponde a 90% da produção mundial de biodiesel. O governo garante incentivo fiscal aos produtores, além de promover leis específicas para o produto, visando melhoria das condições ambientais através da utilização de fontes de energia mais limpas. A tributação dos combustíveis de petróleo na Europa, inclusive do óleo diesel mineral, é extremamente alta, garantindo a competitividade do biodiesel no mercado. No mercado internacional, o biodiesel produzido tem sido usado em veículos de passeio, transporte de estrada e off-road, frotas cativas, transporte público e geração de eletricidade. O maior país produtor de biodiesel é a Alemanha, responsável por cerca de 42 % da produção mundial. Sua produção é feita a partir da colza, produto utilizado principalmente para nitrogenização do solo. A extração do óleo gera farelo protéico à ração animal. O óleo é distribuído de forma pura, isento de mistura ou aditivos, para a rede de abastecimento de combustíveis compostas por cerca de 1700 postos (BIODIESEL.BR, 2010).

No Brasil, o Programa Nacional de Uso e Produção de Biodiesel-PNPB, foi criado em janeiro de 2005. Com objetivos de caráter social, econônico e ambiental, o PNPB faz parte dos grandes programas e metas do governo (GÓES, ARAÚJO e MARRA, 2010). Segundo o boletim mensal de biodiesel de outubro de 2011, existem 65 plantas produtoras de biodiesel autorizadas pela ANP, correspondendo a uma capacidade autorizada de 17.862,95 m^3 /dia (ANP, 2011). As matérias-primas utilizadas para a produção de biodiesel no Brasil compreendem principalmente, o óleo de algodão (4,95 %), gordura bovina (13 %) e o óleo de soja com mais de 78 % da produção (ANP, 2011). De acordo com a resolução nº 6, de 16 de setembro de 2009 (ANP, 2009), já está em uso desde janeiro de 2010 a adição de cinco porcento, em volume, de biodiesel ao óleo diesel, conhecido como B5. Também, de acordo com a resolução da ANP de 12 de janeiro de 2011 (ANP, 2011), já está estabelecido o uso do B6 a B20 para uso experimental em frotas cativas ou em equipamento industrial específico. Este cenário

indica que a viabilização comercial do biodiesel passa pelo consumo deste volume extra de glicerol que está sendo gerado, buscando aplicações de larga escala e agregando valor à cadeia produtiva.

A glicerina ou glicerol, é um triol conhecido como 1,2,3-propanotriol. Vários níveis e designações de glicerol estão disponíveis comercialmente. Eles diferem um pouco em seu conteúdo de glicerol e em outras características, tais como cor, odor e impurezas. A chamada glicerina loira é normalmente utilizada para designar a glicerina oriunda dos processos de produção do biodiesel, onde a fase glicerinosa sofreu um tratamento ácido para neutralização do catalisador e remoção de ácidos graxos eventualmente formados no processo. Em geral, esta glicerina contém cerca de 80% de glicerol, além de água, metanol e sais dissolvidos (MOTA, SILVA e GONÇALVES, 2009; ARRUDA, RODRIGUES e FELIPE, 2007). A tabela 1 apresenta as caractéristicas físico-químicas do glicerol.

O glicerol é usado na preparação de diversos produtos tais como remédios, produtos de uso pessoal, comida, bebida, tabaco, resinas alquídicas, poliol-poliéster, celofane e explosivos. As características físicas, químicas e nutricionais do glicerol dependem do tipo de ácido graxo (gordura animal ou óleo vegetal) e do tipo de catálise empregada na produção do biodiesel. Entretanto, o seu uso é condicionado ao seu grau de pureza, que deve estar usualmente acima de 95% (SBRT, 2007).

Tabela 1-Propriedades físico-químicas do glicerol

Massa molar	92,09 g/mol
Densidade (glicerol 100%) 25ºC	1,262 Kg/m3
Viscosidade 20ºC	939 cps
Ponto de ebulição (101.3 KPa)	290ºC
Ponto de fusão	18ºC
Ponto de inflamação	177ºC
Tensão superficial 20ºC	63,4 mN/m
Calor específico (glicerol 99.94%) 26ºC	2,435 J/g
Calor de evaporação 55ºC	55ºC 88,12 J/mol
Calor de dissolução	5,8 KJ/mol
Calor de formação	667,8 KJ/mol
Condutividade térmica	0,28 W/(m.K)

Fonte: ARRUDA, RODRIGUES e FELIPE, 2007.

Na indústria de alimentos, o glicerol é utilizado como aditivo alimentar em função de suas propriedades estabilizantes, antioxidantes, sequestrantes, emulsificantes e umectantes. Também, é considerado como um agente crioprotetor em microorganismos,

pois não permite a formação de cristais de gelo na célula, mantendo a estabilidade da parede celular e vitalidade da mesma durante o processo de congelamento para sua conservação (ARRUDA, RODRIGUES e FELIPE, 2007).

A utilização do glicerol em processos microbianos é de grande importância, visto que é um composto considerado fundamental dentro do sistema metabólico de microorganismos, atuando como precursor de numerosos compostos e como regulador de vários mecanismos bioquímicos intracelulares. Entretanto, o principal problema para a utilização do glicerol residual em processos fermentativos é a grande quantidade de sais de cloreto presentes, oriundos da produção do biodiesel via catálise básica, o qual pode inibir o crescimento microbiano e diminuir a formação de produtos (ITO *et al.*, 2005). Por outro lado, o glicerol contém elementos nutricionais como fósforo, magnésio, cálcio, enxofre, nitrogênio e sódio que são possíveis de serem utilizados pelos microorganismos para o seu crescimento durante processos fermentativos.

A conversão microbiológica do glicerol a vários produtos e sua utilização como fonte de carbono para o crescimento microbiano, tem sido foco de diversos estudos. O glicerol tem sido amplamente utilizado na produção de 1,3-PD, o qual é um monômero usado na produção de biopolímeros. Existem na literatura diversas publicações utilizando glicerol puro para a produção de 1,3-PD (BARBIRATO *et al*., 1995; BIEBL *et al*., 1992; DECKWER, 1995; HIMMI, BORIS e BARBIRATO, 1999; ZHENG *et al*., 2008) ácido cítrico (LEVINSON, KURTZMAN e KUO, 2007; PAPANIKOLAOU *et al*., 2008) e 2,3 butanodiol (BIEBL *et al*., 1999; ZENG, BIEBL e DECKWER, 1990). Porém, existem ainda poucos trabalhos na literatura utilizando glicerol residual da síntese de biodiesel. Um dos primeiros trabalhos foi publicado por Papanikolaou *et al*. (2000). Os autores realizaram cultivos batelada e contínuos com um novo isolado de *Clostridium butyricum* F2b e obtiveram durante cultura contínua, 35 g/L de 1,3-PD. Ito *et al*. (2005) estudaram a produção de hidrogênio e etanol por *Enterobacter aerogenes* HU-101 utilizando glicerol presente no efluente da síntese de biodiesel com 41 % de pureza e demonstraram um alto rendimento e alta taxa de produção dos bioprodutos desejados. Papanikolaou *et al*. (2008), utilizando glicerol residual, produziram 1,3-PD e ácido cítrico usando *Clostridium butyricum* F2b e *Yarrowia lipolytica*, respectivamente. Os autores obtiveram uma produção de 43,5 g/L de 1,3-PD em cultura contínua em dois estágios e 62,5 g/L de ácido cítrico com altas concentrações de glicerol no meio.

2.2 Produção de 1,3-Propanodiol, Etanol e Hidrogênio

Sabe-se que glicerol é fermentado por bactérias anaeróbias facultativas a 1,3-PD, etanol, 2,3 butanodiol, ácido acético e lático. Entre estas substâncias, 1,3-PD tem recebido muita atenção devido a sua ampla aplicação em polímeros, cosméticos, alimentos, adesivos, lubrificantes, laminados e solventes (HOMANN *et al*., 1990; SAXENA *et al*., 2009). Antigamente, 1,3-PD tinha um mercado negligenciável comparado a outros produtos devido à falta de disponibilidade e qualidade suficiente.

A maior parte do 1,3-PD produzido comercialmente tem sido obtido, principalmente, através da conversão química da acroleína pela DuPont ou através do óxido de etileno pela Shell (Figura 2). A DuPont utiliza acroleína na síntese, o qual sofre uma hidratação catalítica a 3-hidroxipropanol seguida por hidrogenação para produzir 1,3-PD. A Shell produz 1,3-PD pela hidroformilação do óxido de etileno seguido por hidrogenação. No primeiro caso, o rendimento não excede 40% e está em torno de 80% no segundo caso. Os problemas destes processos convencionais são as altas pressões aplicadas nas etapas de hidroformilação e hidrogenação, seguidas de altas temperaturas, uso de catálise de alto custo e liberação de intermediários tóxicos (SAXENA *et al*., 2009). Diante deste fato, a utilização de microorganismos para a produção de 1,3-PD poderia diminuir significativamente os custos de produção.

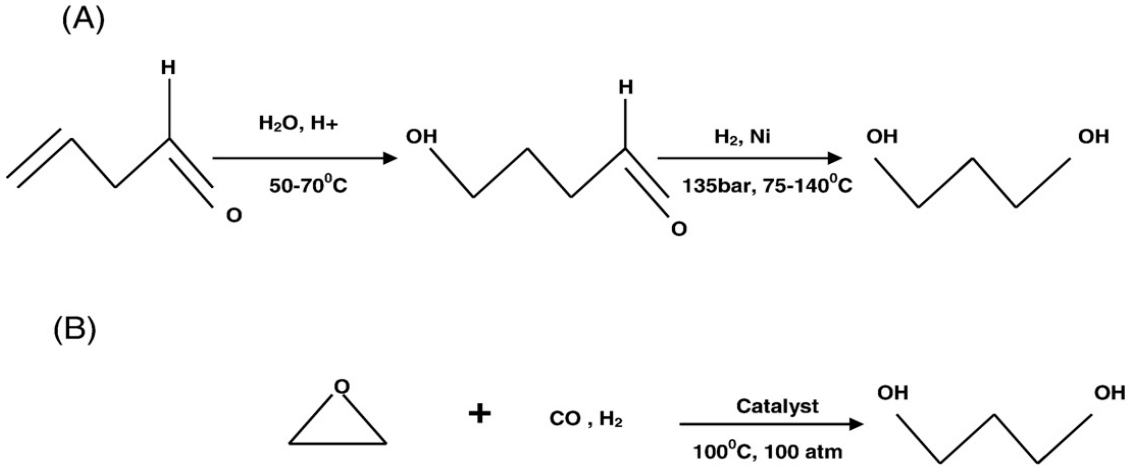


Figura 2- Síntese química de 1,3-PD. (A) síntese da acroleína e (B) síntese com óxido de etileno (SAXENA *et al*., 2009).

Para competir com a indústria petroquímica, propriedades vantajosas e especiais dos polímeros baseados em 1,3-PD são necessárias. A fácil biodegradabilidade de polímeros baseados em 1,3-PD poderia ser de particular interesse. É esperado que

plásticos naturais, baseados em 1,3-PD, sejam facilmente biodegradáveis quando comparados com polímeros puramente sintéticos (DECKWER, 1995).

O 1,3-PD merece destaque como monômero para produção de poliésteres, poliéteres e poliuretanos, entre eles um novo tipo de poliéster, politrimetileno tereftalato, conhecido como PTT. Este polímero é biodegradável e encontra grande potencial na indústria têxtil e carpetes, apresentando excelentes propriedades como boa resiliência e alta resistência a corantes de baixa geração estática.

1,3-PD é um típico produto da fermentação do glicerol e não tem sido encontrado em conversões anaeróbicas de outros substratos orgânicos. Somente poucos organismos, a maioria deles bactérias, são capazes de formá-lo. Estes organismos incluem enterobactérias do gênero *Klebsiella (K. pneumoniae), Enterobacter (E. agglomerans), Citrobacter (C. freundii*), *Lactobacilus* (*L. brevis* e *L. buchneri*) e espécies de *Clostridiuns* dos grupos *C. butyricum* e *C. parteurianum* (CHEN e LIN, 2003; CHENG *et al*., 2004; BARBIRATO, SOUCAILLE e BORIES, 1996; BIEBL *et al*., 1992). A Figura 3 mostra os caminhos do metabolismo do glicerol em *K. pneumoniae.*

O transporte do glicerol através da membrana celular constitui a primeira etapa para o seu metabolismo. O glicerol é um dos poucos substratos que atravessa a membrana celular por difusão, facilitada por proteínas localizadas nas camadas mais internas da membrana plasmática, as permeases (RIVALDI *et al*., 2009). Glicerol é fermentado por um processo de dismutação envolvendo dois modos paralelos. Através do modo oxidativo, glicerol é desidrogenado pela enzima glicerol desidrogenase a dihidroxiacetona (DHA) o qual é então posteriormente metabolisada a piruvato. Através do modo redutivo paralelo, glicerol é desidratado pela enzima glicerol dehidratase B_{12} dependente para formar 3-hidroxipropionaldeído (3-HPA), o qual é então reduzida a 1,3-PD pela 1,3-PD oxidoredutase.

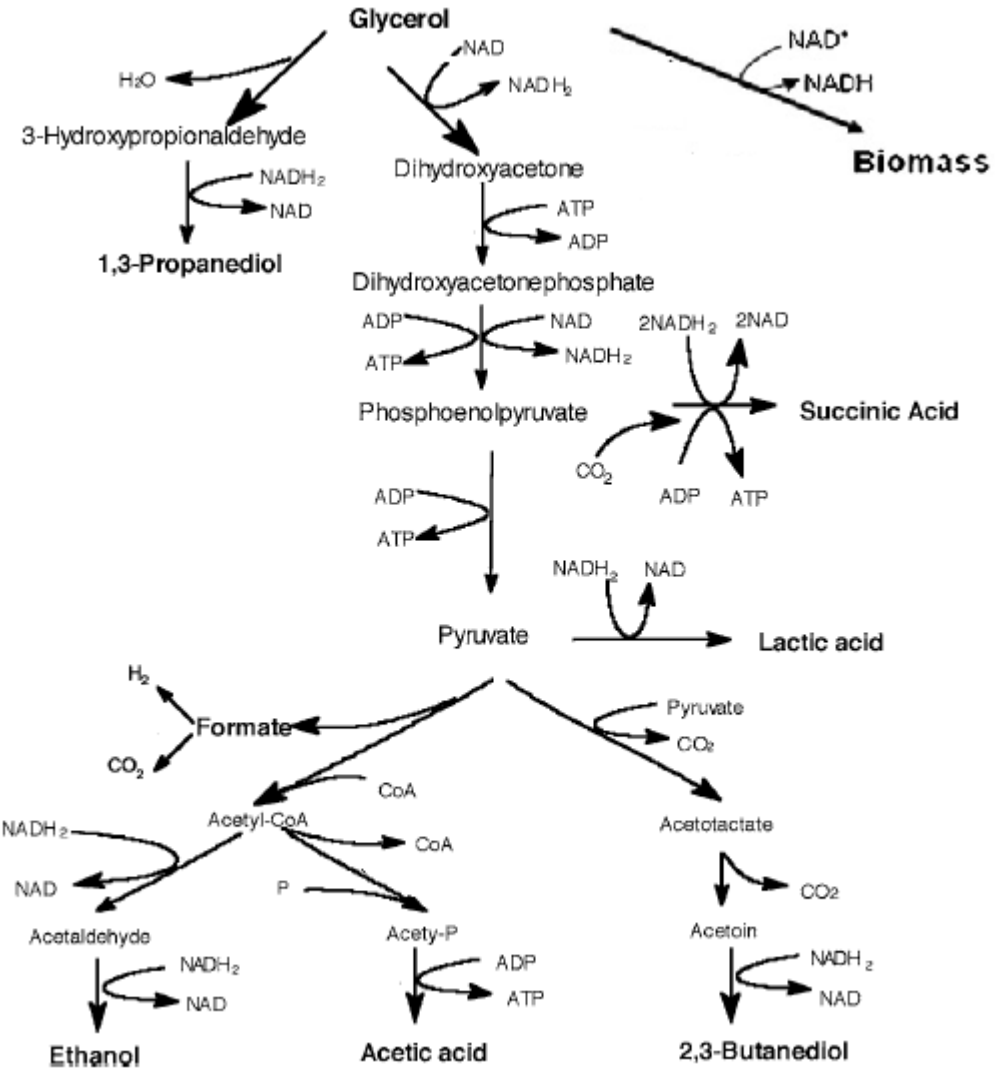


Figura 3- Rotas bioquímicas da fermentação do glicerol (HUANG, GONG e TSAO, 2002)

Os genes destas enzimas estão em um regulon chamado *dha*. O papel fisiológico do 1,3-PD é regenerar os equivalentes redutores ($NADH_2$) os quais são liberados da formação de dihidroxiacetona (DHA) e durante posterior oxidação da dihidroxiacetona fosfato (DHAP) bem como da biossíntese (ZENG, BIEBL e DECKWER, 1997). Sob condições anóxicas estritas, o $NADH_2$ que é produzido durante a glicólise e produção de biomassa são regenerados principalmente para a redução de glicerol a 1,3-PD, mas, por outros simples passos de fermentação, forma piruvato, acetato, hidrogênio, CO_2, butirato, etanol, lactato e outros. A composição dos bioprodutos difere entre os microorganismos, mas estão relacionadas também as condições do processo.

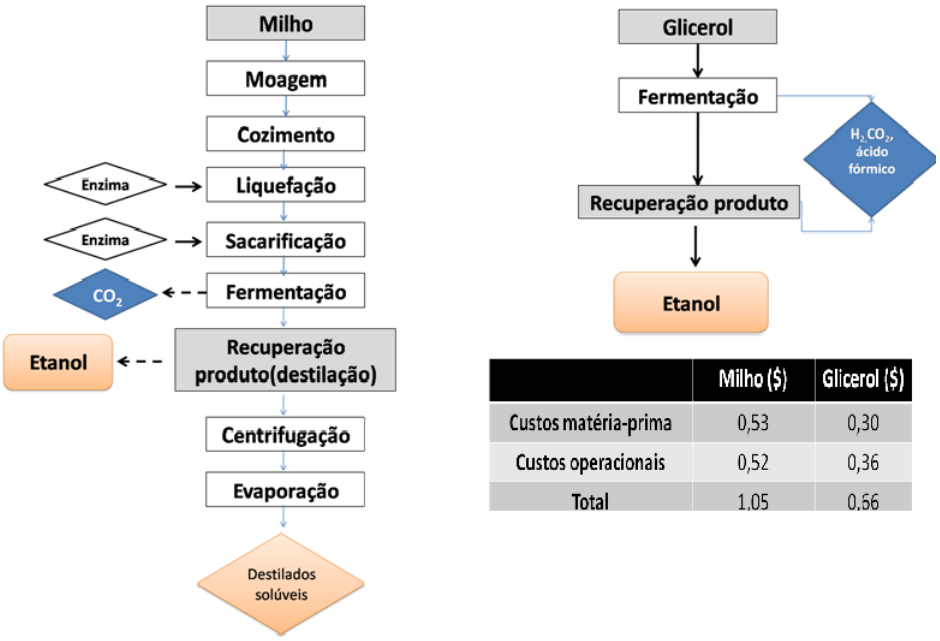
Hidrogênio é uma fonte de combustível limpo, com alta produção de energia (122 KJ/g). Ele está sendo considerada uma fonte promissora de combustível, pois não

produz nenhum gás de efeito estufa sendo água o único produto resultante de sua combustão (LAY, LEE e NOIKE, 1999; CHEONG e HANSEN, 2006). Apesar de seu potencial como combustível "limpo e verde", 95 % do hidrogênio é produzido de gás sintetizado, usando fontes não renováveis de combustíveis fósseis, tais como gás natural, óleo e carvão (DAS e VEZIROGLU, 2001, DUNN, 2002). As formas convencionais de produção de hidrogênio apresentam balanço energético desfavorável, uma vez que para a produção de hidrogênio via eletrólise consome-se de 4,5 a 5 KWh/m^3. Para gerar hidrogênio em eletrolisadores industriais de alta eficiência e na forma de vapor de metano são necessárias altas quantidades de energia para propiciar temperaturas de 970 a 1110 K e pressões na faixa de 3,5 Mpa (STOJIC *et al*., 2003; KOTHARI, BUDDI e SAWHNEY, 2008).

A geração biológica de hidrogênio como um bioproduto do metabolismo de microganismos é uma nova área de desenvolvimento tecnológico, oferecendo uma produção a partir de diversos recursos renováveis (CHEONG e HANSEN, 2006). A obtenção biológica de hidrogênio pode ocorrer através de processos fotossintéticos e fermentativos, sendo a fermentação uma técnica mais simples comparada aos processos fotossintéticos e apresenta bons rendimentos e altas taxas de produção. A dificuldade da fermentação está na seleção deculturas de microorganismos que não contenham nenhum tipo de bactéria consumidora de hidrogênio, por exemplo, metanogênicas e sulfuredutoras (HAWKES *et al*., 2002, VARDAR-SCHARA, MAEDA, WOOD, 2008).

A produção de hidrogênio por fermentação tem sido tratada com pouca atenção comparada à produção por microorganismos fotossintéticos, que tem sido extensivamente estudada (DAS e VEZIROGLU, 2001). A produção de etanol a partir de microorganismos, por sua vez, é amplamente estudada, utilizando diversas fontes de carbono, como: açúcares de cana e de beterraba, amidos provenientes de grãos e biomassa lignocelulósica (um complexo de vários polissacarídeos). Os estudos da produção de hidrogênio também estão mais focados na utilização de açúcares como a glicose. Poucos estudos de produção de etanol e hidrogênio a partir do glicerol têm sido encontrados na literatura. Com o aumento da produção de biodiesel no mundo, a produção de etanol a partir do glicerol irá se tornar interessante, já que o etanol produzido poderá ser utilizado na transesterificação do biodiesel contribuindo para o desenvolvimento de processos ambientalmente mais limpos.

A produção de etanol apresenta uma excelente oportunidade de ilustrar as vantagens da fermentação do glicerol. A Figura 4 compara a produção de etanol a partir do glicerol e a produção de etanol à partir de açúcares derivados do milho em termos do tipo de produção necessária e dos custos operacionais e de matérias-primas associadas.



	Milho ($)	Glicerol ($)
Custos matéria-prima	0,53	0,30
Custos operacionais	0,52	0,36
Total	1,05	0,66

Figura 4- Comparação da produção de etanol à partir de açúcares derivados do milho com produção de etanol à partir da fermentação do glicerol. A tabela compara a matéria-prima e os custos do processo (YAZDANI e GONZALES, 2007).

Pode-se verificar que a produção de etanol através de açúcares derivados de milho é mais complexa e com isso, o capital é maior. Por outro lado, os custos operacionais são quase 40 % menores na produção de etanol da fermentação do glicerol, mesmo quando nesta análise não for dado crédito à produção de ácidos e hidrogênio coproduzidos com etanol. Embora esta comparação relate a produção de etanol, as mesmas vantagens de custos podem ser realizadas para a produção de outros combustíveis e químicos do glicerol (YAZDANI e GONZALES, 2007).

O trabalho desenvolvido por Ito *et al*. (2005), é um dos únicos artigos voltados especificamente para a produção de etanol e hidrogênio a partir do glicerol proveniente da produção de biodiesel. Neste trabalho, os autores utilizaram o microorganismo *Enterobacter aerogenes* HU-101, isolado de um lodo metanogênico, em um meio contendo resíduo de biodiesel diluído com um meio sintético para aumentar a taxa de utilização de glicerol, seguido da adição de extrato de levedura e triptona, a fim de

acelerar a produção de etanol e hidrogênio. Os resultados com fermentações batelada mostraram que os rendimentos de etanol e hidrogênio com glicerol bruto (0,67 mol/mol glicerol e 0,71 mol/mol glicerol, respectivamente) foram menores quando comparadas com ensaios feitos com glicerol comercial (0,89 mol/mol glicerol e 0,86 mol/mol glicerol, respectivamente) devido, principalmente, à grande quantidade de sais contidos no glicerol bruto, o qual pode inibir o crescimento celular.

Estudos utilizando o microorganismo *K. pneumoniae* para a produção de 1,3-PD têm mostrado uma significativa produção de etanol como bioproduto. Mu *et al*. (2006), utilizaram *K. pneumoniae* DSM 2026, para a produção de 1,3-PD com glicerol puro e glicerol derivado da síntese química e da síntese catalisada por enzima, em fermentações em shaker e batelada alimentada. Os resultados obtidos pelos autores, mostraram que houve uma significativa produção de etanol, tanto no cultivo com glicerol comercial (10,3 g/L) como com o glicerol derivado da síntese química (11,9 g/L). Liu e Fang. (2007) estudaram a produção de hidrogênio utilizando resíduo de biodiesel contendo glicerol com *K. pneumoniae* DSM 2026. Os autores fizeram uma otimização que aumentou em cinco vezes a produção de hidrogênio (57,6 mL/50 mL de meio) comparado com o meio padrão (11,6 mL/50 mL de meio) após 24 horas de fermentação. Experimentos em batelada foram conduzidos com o meio otimizado e apresentaram uma elevada produção de hidrogênio (117,8 mmol/L) e rendimento de 0,53 mol/mol de hidrogênio.

2.3 Espectroscopia de fluorescência 2D

Sensores de fluorescência são instrumentos ópticos os quais têm sido usados nos últimos 15 anos para diferentes aplicações em biotecnologia, por exemplo, medida de biomassa, caracterização de reatores e monitoramento de bioprocessos (MAROSE *et al*., 1998). Os sensores são geralmente baseados na medida do NAD(P)H fluorescente a 450 nm após a excitação a 360 nm. Os primeiros sensores disponíveis para detecção eram limitados a região de NAD(P)H causando uma limitada aplicação. Nesta região, outras substâncias podem fluorescer, como riboflavina e piridoxina, podendo causar uma sobreposição de espectros.

O aperfeiçoamento dos sensores permitiu que as medidas de fluorescência fossem realizadas em uma ampla faixa de espectros dando muito mais informação do bioprocesso pelo uso de diferentes filtros, os quais conduziram a fluorescência 2D (MAROSE *et al*., 1998, LINDEMANN *et al*., 1998). A matriz resultante está

apresentada na Figura 5. Fluorescência 2D é um método limitado a medida de compostos fluorescentes, porém, este tem sido aplicado, principalmente, para o monitoramento de biomassa. Biomassa contem um número de fluoróforos naturais como NAD(P)H, flavinas e aminoácidos aromáticos (por ex. triptofano), os quais produzem picos característicos em um espectro de fluorescência bem resolvido (ÖDMAN *et al*., 2009).

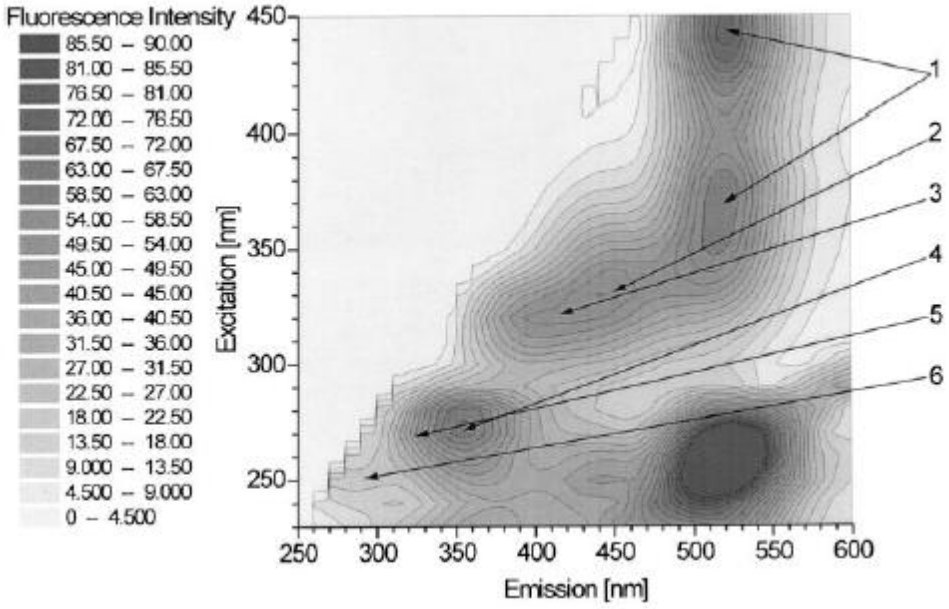


Figura 5- Espectro de fluorescência 2D de fluoróforos biogênicos (1-FMN, FAD e riboflavina; 2-NADH; 3-piridoxina; 4-triptofano; 5-tirosina; 6-fenilalanina). Cada contorno representa uma determinada intensidade de fluorescência. Adaptado de Lindemmann *et al*., 1998.

Um dos instrumentos mais utilizados para a medida de espectofotometria 2D é chamado de Bio View sensor (DELTA Light & Optics, Denmark). Este é utilizado para aplicações industriais no monitoramento online de bioprocessos e utiliza até 16 diferentes filtros para excitação e emissão, permitindo medidas de comprimento de onda na faixa de 290 nm a 550 nm para excitação e 310 a 590 nm para emissão. A Figura 6 mostra o esquema de montagem do spectrofotômetro de fluorescência 2D.

O Bio View sensor usa uma lâmpada de Xe para excitação. A luz passa pelo filtro de excitação e é direcionada para um condutor óptico, o qual guia a luz através de uma entrada até o biorreator. Esta entrada é protegida para separar o caldo de fermentação do dispositivo óptico. Dentro do biorreator os fluoroforos são irradiados com a luz de comprimento de onda selecionado e a luz fluorescente é coletada via um segundo cabo de luz para um filtro emissor. Os dados são tranferidos para o computador onde é analisado através de um software.

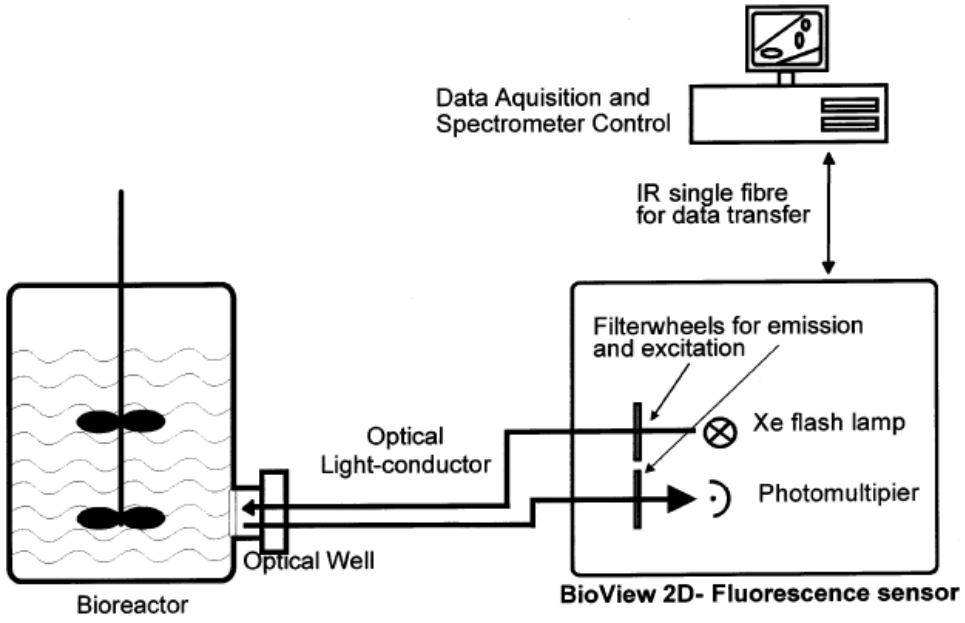


Figura 6- Esquema de funcionamento do spectofotometro de fluorescencia 2D. Adaptado de Lindemmann *et al*., 1998.

Métodos quimiométricos tais como, análise dos componentes principais (PCA), componentes principais de regressão (PCR), e regressão por métodos quadrados (PLS) são úteis para a análise quantitativa dos dados espectrofotométricos (BOEHL *et al*., 2003; RHEE e KANG, 2007). Estas técnicas têm sido usadas para analisar mudanças metabólicas em *E. coli* recombinante (JOHANSSON e LÍDEN, 2006), para caracterizar o estudo de *Claviceps purpurea* (BOEHL *et al*., 2003), monitorar as variáveis de estado na produção da proteína heteróloga produzida por *Pichia pastoris* (SURRIBAS *et al*., 2006) e para monitorar processos online de fermentações com *E.coli* recombinante (RHEE e KANG, 2007). Estes modelos são baseados na correlação entre as trajetórias analíticas disponíveis na tabela de dados de fluorescência, isto é, o consumo de substrato pode ser modelado baseado nos sinais relacionados ao crescimento celular. Tais modelos, baseados em medidas indiretas, conduzem a uma informação fisiológica bem definida, onde a correlação entre substratos e produtos é constante. Entretanto, predições imprecisas podem ocorrer em situações onde essas correlações mudam em todo o processo. Em muitos processos microbianos, o metabolismo do microorganismo passa por uma ou mais fases durante o cultivo como resultado, por exemplo, de limitação de oxigênio ou acumulação de compostos inibidores ao crescimento, como etanol. Estes tipos de processos aumentam a complexidade do monitoramento de processos batelada (ÖDMAN *et al*., 2009). Arnold *et al*. (2001) usaram um modelo

segmentado, onde múltiplos modelos de calibração foram aplicados em diferentes intervalos de tempo durante o processo, permitindo assim, estudar melhor a fermentação

2.4 Utilização de ferramentas estatísticas

O planejamento experimental é baseado em princípios estatísticos onde os pesquisadores podem extrair do sistema em estudo o máximo de informações úteis, fazendo um número mínimo de experimentos. Os métodos utilizados são ferramentas poderosas com os quais vários objetivos específicos podem ser alcançados. Podem-se fabricar produtos com melhores características, diminuir o tempo de seu desenvolvimento, aumentar à produtividade de processos, minimizar a sensibilidade de produtos as variações de condições ambientais e assim por diante (NETO, SCARMINO e BRUNS, 2001).

A metodologia do planejamento fatorial, associada à análise de superfície de resposta, é uma ferramenta fundamentada na teoria estatística, que fornece informações seguras sobre o processo, minimizando o empirismo que envolve técnicas de tentativas e erro (RODRIGUES e IEMMA, 2005). O planejamento fatorial e a metodologia de superfície de resposta permitem considerar simultaneamente vários fatores em diferentes níveis e as interações entre eles, utilizando um reduzido número de experimentos.

Os métodos utilizados independem da natureza do problema a que são aplicados. Eles servem para estudar reações químicas, sistemas biológicos, processos mecânicos e também varrer todas as possíveis escalas de interesse, desde uma reação em bancada até um processo industrial operando em larga escala (NETO, SCARMINO e BRUNS, 2001).

INTRODUÇÃO AOS CAPÍTULOS III, IV, V, VI, VII E VIII

Os Capítulos III, IV, V, VI, VII e VIII estão apresentados em forma de artigos científicos, seguindo as normas propostas pelo Programa de Pós-Graduação em Engenharia Química (PPGEQ).

Estes capítulos trazem os materiais e métodos utilizados no desenvolvimento de cada artigo, juntamente com os resultados obtidos e as conclusões pertinentes a cada etapa de trabalho.

O primeiro artigo (Capítulo III - "Comparison of different pretreatments methods for hydrogen production using environmental microbial consortia on residual glycerol from biodiesel"), foram utilizados diferentes tratamentos ao consórcio microbiano ambiental com a finalidade de verificar a produção de hidrogênio. Neste trabalho, também está apresentado o isolamento de bactérias do consórcio microbiano que foram utilizadas em trabalhos posteriores. Este artigo está publicado no periódico ***International Journal of Hydrogen Energy***, 36(8): 4814-4819, 2011. Doi:10.1016/j.ijhydene.2011.01.005.

O segundo artigo (Capítulo IV - "The optimization of biohydrogen production by bacteria using residual glycerol from biodiesel synthesis") foi realizada a otimização da produção de hidrogênio através de ferramentas estatísticas. Este trabalho foi realizado com a bactéria selecionada na etapa de isolamento. Este artigo está publicado no periódico ***Journal of Environmental Science and Health***, **Part A**, 46(13): 1461-1468. Doi 10.1080/10934529.2011.609036.

O terceiro artigo (Capítulo V - "Bioconversion of residual glycerol from biodiesel synthesis into 1,3-propanediol and ethanol by isolated bacteria from environmental consortia") foram mostradas detalhadamente as etapas de isolamento das bactérias presentes no consórcio microbiano ambiental para testar a habilidade de produção de 1,3-propanodiol e etanol, bem como ensaios em "shaker" e biorreatores com a cepa selecionada. Este artigo está publicado no periódico ***Renewable Energy***, 39 (1): 223-227, 2012. Doi: 10.1016/j.renene.2011.08.005.

No quarto artigo (Capítulo VI– "Efficient and optimized conversion of residual glycerol from biodiesel synthesis into 1,3-propanediol and ethanol by a new strain of *Klebsiella pneumoniae"*) foi realizada a otimização da produção de 1,3-propanodiol utilizando ferramentas estatísticas. Com as condições otimizadas foram realizados experimentos em bioreator batelada e batelada alimentada com a finalidade de verificar o aumento da produção de 1,3-propanodiol em condições otimizadas e controladas. Este artigo foi submetido para publicação no periódico ***Bioprocess and Biosystems Engineering*** em outubro de 2011.

O quinto artigo (Capítulo VII – "Residual glycerol metabolism by *Klebsiella pneumoniae*: pool of metabolites under anaerobiosis and microaerobiosis as a function of feeding rates" aborda o estudo das diferentes taxas de alimentação em biorreatores batelada alimentada em condições de microaerobiose e anaerobiose. Este estudo teve por objetivo estudar a influência da concentração de glicerol no meio com relação aos produtos formados mostrando assim, as diferenças metabólicas do microorganismo em diferentes condições. Este artigo foi submetido para publicação no periódico ***Journal of Biotechnology*** em dezembro de 2011.

O sexto artigo (Capítulo VIII – "Chemometric modeling and two-dimensional fluorescence data for a new isolated of *Klebsiella pneumoniae* bioprocess characterization using raw glycerol as carbon source") aborda o estudo de biorreatores batelada em condições de anaerobiose e aerobiose com a finalidade de estudar o metabolismo do microorganismo em estudo para produção de 1,3-propanodiol. Este artigo foi desenvolvido no Technisch Institut für Chemie, Hannover, Alemanha durante o estágio de curta duração realizado no período de setembro 2010 a janeiro de 2011. Este artigo foi aceito para publicação no periódico ***Journal of Industrial Microbiology and Biotechnology*** em 09 de dezembro de 2011. Doi:10.1007/s/0295-011-1075-8.

CAPÍTULO III- COMPARISON OF DIFFERENT PRETREATMENTS METHODS FOR HYDROGEN PRODUCTION USING ENVIRONMENTAL MICROBIAL CONSORTIA ON RESIDUAL GLYCEROL FROM BIODIESEL

Artigo publicado no periódico: ***International Journal of Hydrogen Energy***, 36(8):4814-4819, 2011. Doi:10.1016/j.ijhydene.2011.01.005.

Comparison of different pretreatment methods for hydrogen production using environmental microbial consortia on residual glycerol from biodiesel

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ABSTRACT

The pretreatment of environmental microbial consortia by five methods (acid, base, heat-shock, dry heat and desiccation, freezing and thawing) was conducted in order to evaluate their applicability for the selection of hydrogen-producing bacteria capable of using residual glycerol from biodiesel synthesis as substrate. Results showed that substrate degradation rates of consortia pretreated with dry heat and desiccation and heat shock were higher compared with controls during the fermentation using glycerol, with degradation rates as high as 65%. The maximal hydrogen and biomass productions were obtained by dry heat and desiccation: 34.19% mol and 4,340 mg/L, respectively. Dry heat and desiccation followed by heat shock are simple pretreatments methods that can be used to improve the biotechnological production of hydrogen. DNA sequencing performed to identify the bacteria strains present in the consortium showed that they belonged to the genus *Klebsiella* and *Pantoea*.

Keywords: Biotechnological production of hydrogen; Biodiesel; Residual glycerol; Environmental microbial consortia.

1- Introduction

Research on alternative energy sources has gained renewed interest due to the growing awareness that the accumulated carbon dioxide in the atmosphere is a potential cause of climate change [1]. Combustion of hydrogen produces no greenhouse gases and has a high-energy yield of 122 kJ/g, which is 2.75-fold higher than hydrocarbon fuels. Thus using hydrogen as a clean fuel seems to be a promising technology [2,3]. Current production of hydrogen can be achieved by either physico-chemical or biological methods. Biological production of hydrogen from complex natural and residual substrates such as sugar-rich wastewaters, cellulose, municipal solid waste, sugarcane juice, corn pulp, and paper have been reported [4,5]. Biological hydrogen production may be more effective if organic wastewater or other industrial wastes are employed as raw materials in this process.

The production of alternative fuels such as biodiesel and ethanol has dramatically increased over the last few years. Due to the increasing production of biodiesel, a glut of crude glycerol has resulted and the price has plummeted over the past few years. Therefore, it is imperative to find alternative uses for glycerol. Recent research has been conducted showing the possibilities of using residual glycerol in biotechnological processes, especially for the production of 1,3-propanediol, ethanol, and organic acids [6-10]. However, few investigations have focused on its conversion into hydrogen. The anaerobic conversion of substrates, such as, glucose, starch and sucrose into hydrogen is a complex biochemical process. Several fermentative bacteria produce hydrogen, which functions as an intermediate energy carrier and can be used in fuel cells [11]. In this process, bacterial hydrogenases liberate hydrogen to dispose of excess electrons. Bacteria that use these pathways are strict anaerobes (*Clostridia*, methanogenic bacteria, archaea), facultative anaerobes (*Escherichia coli, Enterobacter, Citrobacter*) and some aerobes (*Alcaligenes, Bacillus*) [5]. Hydrogen production is usually carried out using mixed cultures of uncharacterized bacteria and with unsterilized substrates since these processes are more practical and robust than those using pure, well define bacteria [11,12]. The pretreatment methods reported for enriching hydrogen-producing bacteria consortia are: heat-shock, use of acids or bases, aeration, freezing and thawing, chloroform, sodium 2-bromoethanesulfonate or its acid, and iodopropane [13]. These reports focus on the use of sugar sources such as glucose, sucrose, or starch for the production of hydrogen, but none of them employed residual glycerol from biodiesel as a carbon source. Therefore, the objective of this study was to investigate the

influence of different pretreatment methods (acid, base, heat-shock, dry and desiccation, and freezing and thawing) for selecting and conditioning an environmental-isolated consortium of bacteria for their use in producing hydrogen from residual glycerol resulting from biodiesel synthesis. Finally, DNA sequencing was performed to identify the bacteria strains present in the consortium capable of metabolizing raw glycerol and converting it into hydrogen.

3 Materials and methods

3.1 Raw (residual) glycerol

The raw (or residual) glycerol was supplied from a biodiesel producing and refining facility in the South of Brazil (Passo Fundo, RS, Brazil). The raw glycerol quality and composition was strictly controlled corresponding to 80.9% glycerol, 6.4% ash, 6.6% NaCl, and 11.6% moisture with a pH of 7.26. One single batch was used throughout this research.

3.2 Environmental microbial consortium and its pretreatments

The environmental microbial consortium was collected from the bottom portion of an upflow anaerobic sludge blanket reactor (UASB) at a local soybean treatment plant (Esteio, RS, Brazil). The concentration of the volatile suspended solids (VSS) of the environmental microbial consortium was 4,600 mg/L.

Five physico-chemical treatments were applied to the consortium. The heat-shock pretreatment was carried out by boiling the sludge at 100 °C for 15 min. The acid and base pretreatments were performed by respectively adjusting the pH of the samples to 3.0 or 10.0 using 1 M HCl or NaOH and maintaining this pH for 24 h. The dry heat and desiccation was performed by storing the sample for 2 h in a drying oven at 105°C followed by desiccation in a desiccating jar for 2 h. The freezing and thawing was performed by exposing the samples to –10°C for 24 h, followed by a thawing process for 6 h at 30°C. Control experiments were carried out with the same environmental microbial consortium without applying any further pretreatments. All experiments were run as duplicates. The results were plotted as the mean of the two experiments showing the standard deviation.

3.3 Cultures

The main substrate solution consisted of organic and inorganic nutrients. It had a total chemical oxygen demand (COD) of 30.7 g/L O_2, mainly from glycerol. The medium used for the pretreatment tests was composed of 30 g/L of raw glycerol added of the following

nutrients (in g/L): 4 $(NH_4)_2SO_4$; 0.125 K_2HPO_4, 0.12 $MgSO_4.7H_2O$; 0.01 $MnSO_4.H_2O$; 0.025 $FeSO_4.7H_2O$; 0.005 $CuSO_4.5H_2O$; 0.125 $CoCl_2.6H_2O$. A trace-element solution was added at 0.1% (v/v) to medium and contained (in g/L): 0.1 $MnCl_2.4\ H_2O$; 0.06 H_3BO_3; 0.0037 $CuSO_4.5H_2O$; 0.2 $CoCl_2.6\ H_2O$; 0.025 $NiCl_2.6\ H_2O$; 0.035 $Na_2MoO_4.\ 2\ H_2O$; 0.14 $ZnSO_4\ 7H_2O$; 8 $NaHCO_3$; and 0.9 ml HCl 37%. Batch runs were conducted in 60 mL glass bottles with a working volume of 30 mL, pH adjusted to 7.0 with 1 M HCl or NaOH, inoculated with a 10% (v/v) environmental microbial consortium, corresponding to an initial cell concentration (measured as VSS) of 460 mg/L. Each bottle was flushed with nitrogen gas to provide oxygen-free conditions, capped with a rubber stopper and placed into a reciprocal shaker at 150 rpm, 35 ^oC for 36 h. These same culture conditions and procedures were used to cultivate the isolated bacteria from the consortium. In these experiments, the collected gas samples at 36 h were analyzed for hydrogen.

3.4 Isolation, sequencing of 16S rDNA genes and genetic analysis of bacteria in the consortium

The isolation and identification of the bacteria present in the microbial consortium was carried out in order to acquire information on the most relevant genera present in it. The samples were collected at the end of cultivation, serially diluted into 0.1% sterile peptone water and 0.1 mL of these cell suspensions were spread onto nutrient agar (NA) and incubated at 35°C. The most probable number (MPN) method was used to enumerate the bacteria cells present in the microbial consortium. After incubation for 24 and 48 h, colony counts were determined and representative colonies were sub cultured into LB (Luria Bertani) liquid medium and stored in glycerol (1:1) at -20°C for posterior identification. Cellular morphologies were determined by bright field microscopy of Gram-stained preparations.

The molecular identification of the isolates were conducted at the ACTGene Laboratory (Biotechnology Centre, UFRGS, Porto Alegre, RS, Brazil) using the automatic sequencer ABI-PRISM 3100 Genetic Analyzer equipped with 50 cm capillaries and POP6 polymer (Applied Biosystems, USA). DNA templates (30 to 45 ng) were labeled with 3.2 pmol of the primer 5'-NNNNNNNNNNNNN-3' and 2 μL of BigDye Terminator v3.1 Cycle Sequencing RR-100 (Applied Biosystems, USA) to a final volume of 10 μL. Labeling reactions were performed in a GeneAmp PCR System 9700 (Applied Biosystems, USA) thermocycler with a initial denaturing step of 96 ºC for 3 min followed by 25 cycles of 96 ºC for 10 sec, 55 ºC for

5 sec, and 60 ºC for 4 min. Labeled samples were purified by isopropanol precipitation followed by 70% ethanol rinsing. Precipitated products were re-suspended in 10 μL formamide, denatured at 95 ºC for 5 min, ice-cooled for 5 min and electroinjected in the automatic sequencer. Sequencing data were collected using the software Data Collection v1.0.1 (Applied Biosystems, USA), programmed with the following parameters: Dye Set "Z"; Mobility File "DT3100POP6 {BDv3}v1.mob"; BioLIMS Project "3100_Project1"; Run Module 1 "StdSeq50_POP6_50cm_cfv_100"; and Analysis Module 1 "BC-3100SR_Seq_FASTA.saz".

3.5 Analytical methods

The soluble metabolites were analyzed using a Shimadzu high performance liquid chromatography system (Shimadzu Corp., Japan) with a RID-10A refractive index detector. The stationary and mobile phases were an Aminex HPX-87H column (300 x 7.8 mm) (Bio-Rad, USA) and 0.005 mol/L H_2SO_4 solution at 0.8 mL/min, respectively. The column temperature was controlled at 65 °C. The hydrogen production was analyzed using a gas chromatograph (Agilent 6890N, USA) equipped with a thermal conductivity detector (TCD) and a PoropaK Q column (mesh 80/100, 6ft, 1/8 in). The temperatures of the column and the TCD detector were 80º C and 150 ºC, respectively. The COD (Chemical Oxygen Demand) was measured according to the closed reflux colorimetric method and volatile suspended solids (VSS) were analyzed according to the procedures described in APHA standard methods [14].

4 Results and discussions

4.1 Effect of different pretreatments on hydrogen production by environmental microbial consortium

Fig. 1 illustrates the effect of different pretreatments methods on the hydrogen production by environmental microbial consortium. Hydrogen production was higher for the dry heat and desiccation (34.2% mol), and for the heat shock (27.3% mol) treatments, while the base pretreatment did not show any hydrogen production.

Biological hydrogen production by dark fermentation processes shares many common features with methanogenic anaerobic digestion. Typical anaerobic mixed cultures cannot produce hydrogen once it is rapidly consumed by methane-producing bacteria. Therefore, the most effective way to enhance hydrogen production in anaerobic microbial cultures is to

restrict methanogenesis by allowing hydrogen to become an end product of the metabolic flow. Pretreatment of cultures have been used to selectively enrich specific groups of bacteria [12]. Spore forming hydrogen producing bacteria such as *Clostridium* will form endospores as a result of bacterial stress when environmental conditions are harmful (high temperature, desiccation, nutrient limitation, extreme acidity and alkalinity), while methanogenic bacteria will have no such capabilities, preventing the competitive growth of these bacteria, which are hydrogen consumers [15].

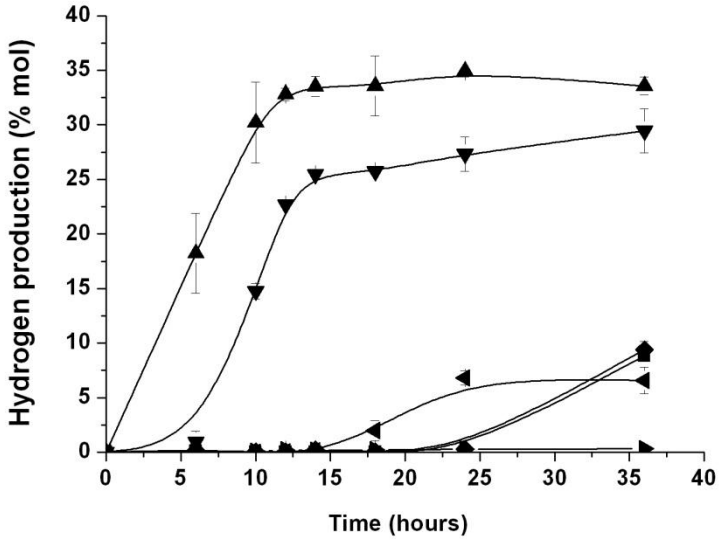

Figure 1- Kinetics of hydrogen production of environmental microbial consortium according to different methods of pretreatment using glycerol as the sole carbon source. Treatments: (▲) dry heat and desiccation, (▼) heat shock, (■) control, (▼) acid, (♦) freezing and thawing, (►) base. Results are the mean of duplicates with standard deviation.

Wang and Wan [13] described the use of five methods of sludge pretreatment (heat shock, acid, base, aeration and chloroform) in order to induce hydrogen production by a consortium collected from anaerobic digested sludge at Beijing Sewage treatment plant. The results showed that the higher hydrogen production (215.4 mL) was obtained using heat shock pretreatment with temperature of 35°C, initial pH=7.0 and glucose as carbon source. Cheong and Hansen [15] have also carried out a study comparing five pretreatment methods (acid, sodium 2-bromoethanesulfonate, wet and heat shock, dry heat and desiccation, and freezing and thawing) for enriching hydrogen producing bacteria from cattle manure sludge growing on glucose. These authors found that the acid pretreatment was the more efficient.

The hydrogen production from samples pretreated with base or acid methods were similar to those of the control, possibly because extreme pH might have suppressed both

methanogenic and hydrogen producing bacteria. Although endospore-forming bacteria such as *Clostridia* show high pH tolerance, facultative anaerobes such as *Enterobacter* and *Klebsiella* species have shown a very restricted optimal pH range between 5.0 and 6.0 for the production of hydrogen [4,16]. Studies with enterobacteria isolated from sludge showed optimal pH range for hydrogen production between 6 and 6.5 [17-19].

Table 1 shows the yields of hydrogen production after 36 h of cultivation for different pretreatments. The results showed that heat shock produced the highest yield of hydrogen followed by dry heat and desiccation, while acid and base pretreatments produced lower yields than the control. The hydrogen production has been studied using glucose as usual substrate and hydrogen yields in continuous culture typically can range anywhere from 0.7 to 4 mol H_2/mol glucose depending on bacterial community, temperature, retention time and other factors [5,20,21].

Table 1- Results of hydrogen Yields, pH, VSS and efficiency of utilization of glycerol for the different pretreatments at 36 h of cultivation. Data represent the average of duplicates with standard deviation.

Pretreatment method	Hydrogen Yields (% mol/g glycerol)	pH	VSS (mg/L)	Efficiency of utilization of glycerol (%)
Heat shock	5.27 ± 2.27	4.75 ± 0.04	1,535 ± 7.07	65.70 ± 5.45
Dry heat and desiccation	3.13 ± 1.91	4.84 ± 0.01	4,340 ± 27.98	65.10 ± 5.69
Acid	0.49 ± 0.23	4.58 ± 0.01	500 ± 28.28	48.17 ± 1.17
Base	0.03 ± 0.01	5.90 ± 0.01	1,277 ± 95.49	23.89 ± 2.25
Freezing and thawing	1.34 ± 0.22	4.76 ± 0.01	1,955 ± 44.76	60.22 ± 0.31
Control	1.20 ± 0.04	4.71 ± 0.08	1,148 ± 67.88	48 ± 2.41

Glycerol is usually used for the production of 1,3-propanediol by *Klebsiella pneumoniae* and this microorganism can also convert glycerol to hydrogen at high rates and yields [22,23]. Li and Fang [17] investigated the hydrogen production using biodiesel wastes with *Klebsiella pneumoniae*. Their results showed hydrogen yields, hydrogen evolution rate, and production of 0.53 mol/mol glycerol, 17.8 mmol/L/h, and 117.8 mmol/L, respectively. Ito et al. [8] conducted a study on hydrogen and ethanol production from waste discharged from biodiesel manufacturing process using *Enterobacter aerogenes* HU-101. Their results showed that yields of hydrogen from glycerol (1.2 mol/mol) exceeded the theoretical maximal yield of H_2 (1.0 mol/mol) with 1.7 g/L glycerol. However, the yields of hydrogen, ethanol and acetate decreased when the concentration of biodiesel wastes increased. Fountoulakis and Manios [24] studied the effects of raw glycerol on the performance of a single-stage anaerobic reactor treating different types of organic waste to produce methane

and hydrogen. The 1% (v/v) addition of raw glycerol to the feed increased the methane production rate from 479 mL/d to 1210 mL/d. In relation to hydrogen production, the authors showed that raw glycerol had a significant positive effect on the anaerobic fermentation with hydrogen enhanced of 2.9 mmol H_2/g glycerol when this substrate was used.

4.2 Effect of treatments on the production of other soluble metabolites

Table 2 summarizes the effects of the different pretreatments on the production of several soluble metabolites. The major soluble metabolites formed in the cultures treated by heat shock and dry heat and desiccation were acetic and butyric acids, and 1,3-propanediol. Control, acid, base and freezing and thawing pretreatments did not show any acetic acid production. Acetic and butyric acids are the main fermentation products of pyruvate from *C. butyricum*, while 1,3-PD is produced by enterobacteria species such as *K. pneumoniae* and *Clostridium* [25,26].

Table 2- Effect of pretreatment on soluble metabolites. Data represent the average of duplicates with standard deviation.

	Soluble metabolite concentrations (g/L)		
Pretreatment method	**Acetic acid**	**Butyric acid**	**1,3-Propanediol**
Heat-shock	1.59 ± 0.14	2.04 ± 0.40	4.61 ± 1.05
Dry heat and desiccation	1.86 ± 0.03	2.53 ± 0.21	4.55 ± 0.12
Acid	–	1.29 ± 0.21	3.76 ± 0.46
Base	–	–	2.30 ± 0.46
Freezing and thawing	1.89 ± 0.33	1.48 ±0.54	6.31 ±1.56
Control	2.07± 0.04	1.31± 0.72	4.34± 0.55

(–) not detected

Results from studies using glucose as substrate for consortia fermentation have shown similar results to the ones presented in this study. In the Wang and Wan [13] study the main soluble metabolites were ethanol, acetic acid, butyric and propionic acids showing that two different microbial metabolisms were present: mixed and ethanol fermentation. Khanal et al. [27], studied the effects of pH using a composting pile as seed source and sucrose and starch as organic substrates for hydrogen production. The authors detected propionate, acetate and butyrate as the major soluble metabolites. Liu and Fang [17] using biodiesel wastes as substrates to produce hydrogen *K. pneumoniae* DSM 2026 cultures reported a production of 6.7 g/L of 1,3-propanediol.

4.3 Effect of treatments on substrate degradation rate

Table 1 shows the effect of different pretreatment methods on the rate of substrate degradation after 36h of cultivation. The results showed that the substrate degradation rate of the environmental microbial consortium pretreated by dry heat and desiccation, heat shock, and freezing and thawing, in this order, were higher than that of the control, while the substrate degradation rate of the pretreatments with acid and base, were negatively affected. The pretreatments by dry heat and desiccation and heat shock produced a maximal substrate degradation efficiency of 66% of an amount of 30 g/L of raw glycerol. Sakai and Yagishita. [18], studying the hydrogen and ethanol production using glycerol-containing wastes discharged from a biodiesel fuel production plant using a bioelectrochemical reactor with thionine, showed that glycerol at a concentration of 10.12 g/L was almost completely consumed (8.54 g/L) under pH=6.5 and 7.0. Wang and Wan [13], comparing different pretreatment methods (acid, base, heat shock, aeration and chloroform), for enriching hydrogen producing bacteria from digested sludge using glucose as a substrate, showed a degradation efficiency of 97.2% with digested sludge pretreated by heat shock.

4.4 Effect of treatments on the microbial growth and final pH of cultivation

The VSS assay was used in this study in order to estimate biomass. Table 1 shows the effects of the different pretreatment methods on biomass determined at 36 h of cultivation. The results showed that the higher biomass corresponding of 4,340 mg/L was obtained with the pretreatment by dry heat and desiccation. The biomass of base pretreated sludge was lower than the control indicating that this treatment inhibited the growth of the microbial consortia contributing thereby to a lower hydrogen production and yields.

One of the key process parameter in the production of hydrogen is the system pH because it may directly affect the hydrogenase activity as well as the metabolic pathway [5]. Table 1 shows the effects of different pretreatment methods on the final pH of cultures at 36 h of cultivation and, except for the base treatment that finished at pH=5.9, all others produced a significant drop on pH to 4.5, reflecting the organic acid production shown before. These results were expected and similar to studies with glucose [13,28,29]. The influence of pH has been recognized as a key factor in determining the outcome of hydrogen fermentation. The pH is related to three important facts: growth limitation of methanogens, hydrogen production performance, and regulation of shift to solventogenesis [30]. Maintaining pH in the acidophilic range of 5.5 -6.0 is ideal for effective hydrogen production due to repression

over methanogenic bacteria, indirectly promoting hydrogen producers within the system [12]. The optimal pH for hydrogen production is in the range of 5- 7 [4,5,26,27]. Van Ginkel and Sung [4] studied the biohydrogen production as a function of pH and substrate concentration using compost from potato and soybean soil as natural inocula and applying heat shock pretreatment. The authors showed that the highest rate of hydrogen production occurred with pH of 5.5 with a conversion efficiency of 46.6 mL H_2/(g COD/L).

4.5 Analysis of the bacterial community present in the consortium

Only four different colonies were isolated from the cultivations, as assumed from the microscopic observation after Gram dying and cell morphology in the plates. They were all Gram-negative. Based on 16S rRNA sequence, three isolates were identified as *Klebsiella pneumoniae* (96% of confidence), while the fourth was identified as *Pantoea agglomerans* (93% of confidence). The *Klebsiella* isolates could not be considered as being the same strain, since they always produced very distinctive colony morphologies when plated. Therefore, strains were labeled as *K. pneumoniae* BLh-1, BLh-2, and BLh-3, and as *P. agglomerans* BL1. Some microorganisms have been isolated and evaluated for hydrogen production, including photosynthetic bacteria and anaerobic dark fermentative bacteria such as *Clostridium* sp., *Enterobacter* sp., *Bacillus* sp., and *Klebsiella* sp. [5,20,27,31,32]. Wu et al. [32] isolated a *Klebsiella* sp. HE1 from sewage sludge, which produced 0.92 mol H_2/ mol of sucrose, used as the carbon source for cultivation. However, few studies have been reported for the use of residual glycerol as a carbon source to produce hydrogen instead of defined media using common sugars as substrates. The isolated strains were individually cultivated under the same conditions described above in a simple experiment to check their ability to metabolize residual glycerol and the results shown in Table 3 clearly demonstrate their high potential to use glycerol as the sole carbon source and to produce hydrogen.

Table 3- The hydrogen production by the four isolated strains from the consortium. Data represent the average of duplicates with standard deviation[a].

Strain	Hydrogen Yields (% mol/g glycerol)	Efficiency of utilization of glycerol (%)[b]
K. pneumoniae BLh-1	31.16 ± 2.53	>99%
K. pneumoniae BLh-2	18.95 ± 4.87	>99%
P. agglomerans BL1	17.86 ±1.57	>99%
K. pneumoniae BLh-3	17.15 ± 2.41	>99%

[a]Experimental conditions were the same as for cultures with the consortium; hydrogen yields and glycerol utilization measured as a single sample at 36 h of cultivation.

[b]Glycerol was not detected after 36 h; results reflect the sensitivity of HPLC detection.

5 Conclusions

This research shows that residual glycerol can be used as substrate for the production of hydrogen in substitution of other, more expensive carbon sources as glucose, sucrose or starch. The pretreatments methods (acid, base, heat shock, dry heat and desiccation, freezing and thawing), used for selective enrichment of hydrogen production using an environmental anaerobic consortium showed considerable influence on hydrogen production and substrate degradation. The physical pretreatments using heat showed the best results on hydrogen production and maximal substrate degradation rate. DNA techniques used to identify bacterial strains present in the consortium showed that they belonged to the genera *Klebsiella* and *Pantoea*, the last one shown for the first time as a hydrogen producer from glycerol. The results showed that glycerol could be efficiently used in the production of hydrogen by replacing the traditional sources of carbon such as sugars. The use of this waste could then reduce production costs of hydrogen using an environmental friendly process.

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CAPÍTULO IV – THE OPTIMIZATION OF BIOHYDROGEN PRODUCTION BY BACTERIA USING RESIDUAL GLYCEROL FROM BIODIESEL SYNTHESIS

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The optimization of biohydrogen production by bacteria using residual glycerol from biodiesel synthesis

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ABSTRACT

In this research the production of hydrogen by *Klebsiella pneumoniae* BLb01 using residual glycerol discharged from a biodiesel fuel production plant was investigated. *K. pneumoniae* BLb01 was isolated from a bacteria-rich sludge of an upflow anaerobic sludge blanket reactor (UASB) of a soybean processing plant. A Plackett–Burman design (P-B) and Response Surface Methodology (RSM) were employed to determine the optimal condition for enhanced hydrogen production. The maximal hydrogen production, which was 45.0 mol % and with 98% of glycerol degradation, was achieved with the optimized medium with the following composition: 30 $g.L^{-1}$ glycerol; 3 $g.L^{-1}$ yeast extract 3 $g.L^{-1}$ K_2HPO_4; 1 $g.L^{-1}$ KH_2PO_4; temperature 39 ºC and pH 9.0. These results show the ability of this new strain of effectively converting residual glycerol into value-added energy products.

Keywords: Hydrogen production, Biodiesel, Residual Glycerol, *Klebsiella pneumoniae*.

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1 Introduction

Worldwide biodiesel production has been on an exponential growth profile over the past several years. The biodiesel production process generates glycerol, also known as glycerin, as the main by-product. For every ton of biodiesel produced, approximately 100 kg of crude glycerol (also referred in the literature as “raw” or “residual” glycerol) is generated[1]. Crude glycerol has a very low commercial value because of the impurities it contains. Moreover, as the demand and production of biodiesel grows, the amount of crude glycerol generated is becoming a problem, requiring environmentally and economically sound destinations[2]. One possible option for using glycerol is the biotechnological production of hydrogen. The demand for hydrogen is growing due to the technical advances in fuel cell industry, allowing for easier ways to use it as fuel. Hydrogen is thought to be an ideal and efficient energy carrier of the future due to its high conversion efficiency, recyclability and nonpolluting nature. It can be produced by thermochemical, electrochemical and bioconversion processes. However, approximately 95% of the hydrogen currently produced is from fossil fuel-based feedstock. [3] Therefore, technologies for the production of hydrogen from renewable and cleaner sources must be developed. [4,5]

Before a suitable bioconversion process is established, bacteria with high hydrogen-producing efficiency should be obtained. Many types of microorganisms have been isolated and evaluated for hydrogen production, including photosynthetic and anaerobic dark fermentative bacteria. [6,7] Among these, some were reported to grow anaerobically on glycerol as the sole carbon and energy source, such as *Clostridium butyricum*, *C. tyrobutyricum*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *E. aerogenes.* [8-12] However, bacterial hydrogen production is still restricted due to the low yields of hydrogen production. Consequently, the screening of high-producing strains is a significant step for the development of commercial biohydrogen production processes.[9]

The optimization of fermentation conditions and, in particular, the nutritional and environmental parameters, is very important in order to boost any biotechnological process. Statistical methods are believed to be an effective and powerful approach for rapidly screening key factors from a multivariable system in order to optimize fermentation conditions. [13] For instance, it has been reported that for biohydrogen production essential micronutrients for bacterial metabolism during fermentation are required such as sodium, magnesium, zinc, and iron. [14]

Besides the biohydrogen production in biotechnological processes, ethanol can also be obtained. Ethanol is a widely used biofuel, being a product of high commercial value. Therefore, biological production of ethanol has as much interest as the production of biohydrogen. [2]

The aim of the present research was to isolate a good hydrogen-producer bacterium capable of bioconverting residual glycerol from biodiesel synthesis and to optimize the medium composition as well as culture conditions for hydrogen production. The optimal cultivation conditions and the effect of nutrition components for the fermentation process were investigated using the statistical tools Plackett–Burman design and Response Surface Methodology (RSM).

2 Materials and methods

2.1 Residual Glycerol and Chemicals

Residual glycerol was supplied by a biodiesel plant (Passo Fundo, Brazil), with a composition of 82.82% of glycerol, 5.50% ash, 5.91% NaCl, 11.20% moisture, 0.48% monoacylglycerols and pH 7.26. All other chemicals used in this research were of analytical or chromatographic grade and were supplied by Sigma-Aldrich (St. Louis, MO, USA).

2.2 Bacteria Isolation

The bacterial consortium used as a source for the microbial isolation was collected from the bottom portion of an upflow anaerobic blanket reactor from a soybean processing plant (Esteio, Brazil). After collecting, the samples were kept at 4°C for posterior use. Prior to use, the sludge was heated at 95°C for 20 minutes in order to inhibit the methanogenic bacteria and then inoculated at 10% (v/v) into a 2.0 L bioreactor (Biostat-B. Braun, Germany) with a working volume of 1.8 L. The operational conditions of the bioreactor were: initial pH 7.0, temperature of 35°C and 250 rpm, run for 48 hours. In order to ensure anaerobic conditions, pure nitrogen gas was sparged into the vessel. The synthetic medium devised for bacteria isolation had the following composition (in $g.L^{-1}$): $(NH_4)_2SO_4$ 4; K_2HPO_4 0.0125; $MgSO_4.7H_2O$ 0.12; $MnSO_4.H_2O$ 0.01; $FeSO_4.7H_2O$ 0.025; $CuSO_4.5H_2O$ 0.005; $CoCl_2$. $6H_2O$ 0.125; residual glycerol 70. Cultivation samples were collected at varying intervals, appropriately diluted in 0.1% sterile peptoned water and 0.1 mL of either 1:10 or 1:100 dilutions were spread onto nutrient agar (NA) and McConkey agar plates and incubated at 35°C under anaerobic conditions. After incubation for 24 to 48 h, colonies

counts were carried out and representative isolated colonies were subcultured and submitted to identification. Cellular morphologies were determined by bright field microscopy of Gram-stained preparations.

2.3 Batch Flask Experiments

Screened strains were cultured in anaerobic conditions in order to verify hydrogen and ethanol production and in aerobic conditions aiming to determine ethanol production. The medium used for batch flask fermentation had the following composition (in $g.L^{-1}$): $(NH_4)_2SO_4$ 4; K_2HPO_4 0.52; KH_2PO_4 0.25; $MgSO_4.7H_2O$ 0.2; yeast extract 1.5; bacteriological peptone 1; 1 mL of trace elements solution; residual glycerol 30. The trace element solution contained (in $g.L^{-1}$): $MnCl_2.4\ H_2O$ 0.1; H_3BO_3 0.06; $CuSO_4.\ 5H_2O$ 0.0037; $CoCl_2.6\ H_2O$ 0.2; $NiCl_2.6\ H_2O$ 0.025; $Na_2MoO_4.2\ H_2O$ 0.035; $ZnSO_4.7H_2O$ 0.14; HCl 37% 0.9 mL L^{-1}. The pre-inoculum was prepared in 125 mL Erlenmeyer flasks containing 50 mL of medium, which was inoculated and incubated at 35°C in a rotary shaker at 150 rpm overnight. The aerobic experiments were carried out in 125 mL Erlenmeyer flasks with 50 mL culture medium added. The anaerobic cultures were carried out in 60 mL serum bottles tightly sealed with 30 mL of culture medium added. The bacteria were inoculated at 5% (v/v) with a hypodermic syringe. The flasks were immediately closed with rubber lids and sealed with aluminum taps. In order to assure anaerobic conditions, the medium employed was pre-heated for 20 minutes, cooled at room temperature and bubbled with nitrogen. The samples were centrifuged at 3,500 *g* at 4°C for 15 min and the supernatant was filtered and frozen for posterior chromatographic analysis.

2.4 Sequencing of 16S rDNA Genes and Genetic Analysis

Samples were sequenced in the ACTGene Laboratory (*Centro de Biotecnologia*, UFRGS, Porto Alegre, RS, Brazil) using an automatic sequencer ABI-PRISM 3100 Genetic Analyzer equipped with 50 cm capillaries and POP6 polymer (Applied Biosystems). DNA templates (30 to 45 ng) were labeled with 3.2 pmol of the primer 5'-NNNNNNNNNNNNN-3' and 2 μL of BigDye Terminator v3.1 Cycle Sequencing RR-100 (Applied Biosystems) in a final volume of 10 μL. Labeling reactions were performed in a GeneAmp PCR System 9700 (Applied Biosystems) termocycler with a initial denaturing step of 96ºC for 3 min followed by 25 cycles of 96ºC for 10 sec, 55ºC for 5 sec and 60ºC for 4 min. Labeled samples were purified by isopropanol precipitation followed by 70% ethanol rinsing. Precipitated products were suspended in 10 μL formamide, denatured at 95ºC for 5 min, ice-

cooled for 5 min and electroinjected in the automatic sequencer. Sequencing data were collected using the software Data Collection v1.0.1 (Applied Biosystems) programmed with the following parameters: Dye Set "Z"; Mobility File "DT3100POP6 {BDv3}v1.mob"; BioLIMS Project "3100_Project1"; Run Module 1 "StdSeq50_POP6_50cm_cfv_100"; and Analysis Module 1 "BC-3100SR_Seq_FASTA.saz".

2.5 Plackett-Burman Design

The Plackett-Burman (P-B) is an effective technique for picking individual most important factors from a long list of possible interfering factors. [15,16] The influence of eleven variables on hydrogen production was investigated using the methodology of Plackett-Burman generating 20 experimental trials. Each independent variable was tested at a high (+1) and a low (-1) level, as shown in Table (1).

Table 1- P-B Experimental design matrix with hydrogen production as response

	Variables[a]/levels[b]											
Run.	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	**Hydrogen (mol%)**
1	–1	+1	+1	+1	+1	+1	+1	–1	–1	+1	+1	13.1
2	–1	–1	–1	–1	+1	+1	+1	+1	–1	–1	–1	2.9
3	–1	–1	+1	+1	–1	–1	+1	+1	–1	–1	+1	2.8
4	–1	+1	–1	–1	–1	–1	+1	–1	–1	+1	–1	8.0
5	–1	+1	–1	+1	–1	+1	–1	+1	–1	+1	–1	17.2
6	–1	–1	+1	–1	–1	+1	–1	–1	–1	–1	+ 1	24.9
7	–1	–1	–1	+1	+1	–1	–1	–1	–1	–1	–1	17.6
8	–1	+1	+1	–1	+1	–1	–1	+1	–1	+1	+1	28.9
9	+1	+1	+1	–1	+1	–1	–1	–1	+1	+1	+1	12.5
10	+1	–1	–1	+1	+1	–1	–1	+1	+1	–1	–1	18.2
11	+1	–1	+1	–1	–1	+1	–1	+1	+1	–1	+1	15.6
12	+1	+1	–1	+1	–1	+1	–1	–1	+1	+1	–1	18.1
13	+1	+1	–1	–1	–1	–1	+1	+1	+1	+1	–1	17.8
14	+1	–1	+1	+1	–1	–1	+1	–1	+1	–1	+1	23.9
15	+1	–1	–1	–1	+1	+1	+1	–1	+1	–1	–1	29.8
16	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	29.0
17	0	0	0	0	0	0	0	0	0	0	0	19.6
18	0	0	0	0	0	0	0	0	0	0	0	18.0
19	0	0	0	0	0	0	0	0	0	0	0	20.0
20	0	0	0	0	0	0	0	0	0	0	0	19.6

[a] X_1 glycerol: (–1) = 5 g.L^{-1}, (+1) = 35 g.L^{-1}, X_2 initial pH: (–1) = 4 , (+1) = 8; X_3 Temperature: (–1) = 25°C, (+1) = 37°C; X_4 yeast extract: (–1) = 0 g.L^{-1}, (+1) = 5 g L^{-1}; X_5 peptone: (–1) = 0 g.L^{-1}, (+1) = 5 g.L^{-1}; X_6 NH_4Cl: (–1) = 0 g.L^{-1}, (+1) = 5 g.L^{-1}; X_7 :$(NH_4)_2SO_4$: (–1) = 0 g.L^{-1}, (+1) = 5 g.L^{-1}; X_8: KH_2PO_4: (–1) = 0 g.L^{-1}, (+1) = 3 g.L^{-1}; X_9: K_2HPO4: (–1) = 0 g.L^{-1}, (+1) = 3 g.L^{-1}; X_{10}: $FeSO_4.7H_2O$: (–1) = 0 g.L^{-1}, (+1) = 0.1 g.L^{-1}; X_{11}: $MgSO_4.7H_2O$: (–1) = 0 g.L^{-1}, (+1) = 0.2 g.L^{-1}; [b] (–1) lower level; (+1) higher level; (0) central point.

The hydrogen production was estimated after 24 hours of cultivation and the variables whose confidence levels were greater than 95% were considered to have a significant influence on hydrogen production.

2.6 Fractional factorial Design (FFD)

According to the P-B design, nine variables had significant effect on hydrogen production. The variables having the most significant effect on hydrogen production were then identified using a 2-level fractional factorial design (FFD). Six variables were considered for the FFD design and are listed in Table 2.

Table 2- The two levels of medium components used in the fractional factorial design.

Run	X_1 (g L^{-1})	X_2 (pH)	X_3(°C)	X_4 (g L^{-1})	X_5 (g L^{-1})	X_6 (g L^{-1})	H_2 (mol%)
1	(30) –1	(7) –1	(31) –1	(3) –1	(1) –1	(1) –1	17.0
2	(50) +1	(7) –1	(31) –1	(3) –1	(5) +1	(1) –1	24.3
3	(30) –1	(9) +1	(31) –1	(3) –1	(5) +1	(5) +1	22.5
4	(50) +1	(9) +1	(31) –1	(3) –1	(1) –1	(5) +1	14.4
5	(30) –1	(7) –1	(37) +1	(3) –1	(5) +1	(5) +1	37.5
6	(50) +1	(7) –1	(37) +1	(3) –1	(1) –1	(5) +1	30.7
7	(30) –1	(9) +1	(37) +1	(3) –1	(1) –1	(1) –1	32.8
8	(50) +1	(9) +1	(37) +1	(3) –1	(5) +1	(1) –1	38.5
9	(30) –1	(7) –1	(31) –1	(7) +1	(1) –1	(5) +1	14.1
10	(50) +1	(7) –1	(31) –1	(7) +1	(5) +1	(5) +1	13.8
11	(30) –1	(9) +1	(31) –1	(7) +1	(5) +1	(1) –1	9.4
12	(50) +1	(9) +1	(31) –1	(7) +1	(1) –1	(1) –1	7.7
13	(30)–1	(7) –1	(37) +1	(7) +1	(5) +1	(1) –1	25.5
14	(50) +1	(7) –1	(37) +1	(7)+1	(1) –1	(1) –1	26.7
15	(30) –1	(9) +1	(37) +1	(7) +1	(1) –1	(5)+1	40.2
16	(50) +1	(9) +1	(37) +1	(7) +1	(5) +1	(5) +1	36.6
17(C)	(40) 0	(8) 0	(34) 0	(5) 0	(3) 0	(3) 0	15.7
18(C)	(40) 0	(8) 0	(34) 0	(5) 0	(3) 0	(3) 0	16.9
19(C)	(40) 0	(8) 0	(34) 0	(5) 0	(3) 0	(3) 0	15.0
20 (C)	(40) 0	(8) 0	(34) 0	(5) 0	(3) 0	(3) 0	15.4

X_1 = Glycerol: (–1) = 30 g.L^{-1}, (+1) = 50 g.L^{-1}, (0) = 40 g.L^{-1}; X_2 = pH: (–1) = 7 , (+1) = 9, (0) = 8
X_3 = Temperature: (–1) = 31°C, (+1) = 37°C, (0) =34°C; X_4 = Yeast extract: (–1) = 3 g.L^{-1}, (+1) = 7 g L^{-1}, (0) = 5 g L^{-1}; X_5 = KH_2PO_4: (–1) = 1 g.L^{-1}, (+1) = 5 g.L^{-1}, (0) = 3 g.L^{-1}; X_6 = K_2HPO_4: (–1) = 1 g.L^{-1}, (+1) = 5 g.L^{-1}, (0) = 3 g.L^{-1}

The other three variables were fixed in their optimal values. According to the 2-level six variable concepts, a complete matrix would have been based on 2^6, demanding 64 experimental runs, which would not be feasible. On the other hand, a design of 2^{6-2} FFD, with a resolution IV, reduces the runs to only 16. Table 2 shows the design matrix covering six variables to evaluate their effect on hydrogen production and also gives the response

expressed as hydrogen (mol %). Each independent variable was investigated at a high (+1) and a low (−1) level. Runs of the central point were included in the matrix and statistical analysis was used to identify the effect of each variable on hydrogen production. The variables having major effects on hydrogen production were identified for the isolates on the basis of confidence levels above 95% ($p < 0.05$).

2.7 Response Surface Methodology (RSM)

Optimization of selected culture conditions by P-B design in order to maximize hydrogen production was determined by an RSM. A complete 2^3 factorial including 6 axial points and four repetitions on the central point, with a total of 18 experiments, was carried out. Table 3 presents the codified variables employed in the planning as well as the results of hydrogen production in mol %. The following medium components and conditions were kept constant: glycerol, 30 $g.L^{-1}$; pH, 9.0, and yeast extract, 3 $g.L^{-1}$.

The second order polynomial equation employed was represented by:

$$Y = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_{ij} x_i x_j + \Sigma\beta_{ii} x_i^2 \qquad (1)$$

Where Y is the response, β_0 is a constant, β_i is the linear effect coefficient, β_{ii} is the quadratic effect coefficient, β_{ij} is the interaction effect coefficient, x_i and x_j are the codified levels of the variables X_i and X_j. Equation 1 was employed to plot the response surfaces for the variables.

The tested variables were codified according to the equation 2:

$$x_i = \left(\frac{X_i - X_0}{\Delta X_i}\right) \qquad (2)$$

Where x_i is the codified value, X_i is the real value of the independent variable, X_0 is the real value of the central point, and ΔX_i is the difference between the points.

Table 3- Codified values, real variables and hydrogen production response (mol %) for response surface methodology.

	Variables[a]/levels			
Run	**X_1(Temperature)**	**X_2 (K_2HPO_4)**	**X_3(KH_2PO_4)**	**H_2 (mol %)**
1	(37) – 1	(2.2) – 1	(2.2) – 1	40.3
2	(37) – 1	(2.2) – 1	(5.8) + 1	41.0
3	(37) – 1	(5.8) + 1	(2.2) – 1	39.8
4	(37) – 1	(5.8) + 1	(5.8) + 1	40.0
5	(45) + 1	(2.2) – 1	(2.2) – 1	33.7
6	(45) + 1	(2.2) – 1	(5.8) + 1	35.1
7	(45) + 1	(5.8) + 1	(2.2) – 1	9.7
8	(45) + 1	(5.8) + 1	(5.8) + 1	25.2
9	(35) – 1.68	(4) 0	(4) 0	39.6
10	(47) + 1.68	(4) 0	(4) 0	0.6
11	(41) 0	(1) – 1.68	(4) 0	45.6
12	(41) 0	(7) + 1.68	(4) 0	45.6
13	(41) 0	(4) 0	(1) – 1.68	48.2
14	(41) 0	(4) 0	(7) + 1.68	42.4
15	(41) 0	(4) 0	(4) 0	44.8
16	(41) 0	(4) 0	(4) 0	37.9
17	(41) 0	(4) 0	(4) 0	45.9
18	(41) 0	(4) 0	(4) 0	41.6

[a] X_1 -Temperature:(–1) = 37°C, (+1) = 45°C, (0) = 41°C; X_2 – K_2HPO_4:(–1) = 2.2 g.L^{-1}, (+1) = 5.8 g.L^{-1}, (0) = 4.0 g.L^{-1}; X_3 – KH_2PO_4 :(–1) = 2.2 g.L^{-1}, (+1) = 5.8 g.L^{-1} (0) = 4.0 g.L^{-1}

2.8 Analytical Methods

The composition of the cultivation broth was analyzed using a Shimadzu high performance liquid chromatography system (Shimadzu Corp.) with a RID-10A refractive index detector. The stationary and mobile phases were an Aminex HPX-87H column (300 x 7.8 mm) (Bio-Rad, USA) and 0.005 mol L^{-1} H_2SO_4 solution at 0.8 mL.min^{-1}, respectively. The column temperature was controlled at 65°C. The hydrogen production was analyzed by a gas chromatograph (Agilent model 6890N) equipped with a thermal conductivity detector (TCD) and a PoropaK Q column (mesh 80/100, 6ft, 1/8 in). The column temperature was 80ºC and the TCD detector was kept at 150ºC. The culture samples were centrifuged at

3,500 *g* for 15 min in order to pellet the cell, followed by filtration using cellulose acetate membrane filters (pore size ϕ 0.22 μm, Sartorius-Germany).

2.9 Data Analysis

Experimental designs and data analyses were carried out using Statistica 7.0 (Statsoft, Tulsa, UK, USA).

3 Results and discussion

3.1 Screening and Isolation of Microorganisms

Isolation in bioreactor using residual glycerol as carbon source was carried out to select the hydrogen-producing bacteria. Thirty-two types of colonies were grown, isolated and further purified on LB agar plates. Cells were microscopically observed after Gram dyeing. All the strains were gram-negative rods bacteria except for one strain that was gram-positive. Four among the thirty-two selected bacteria, under both aerobic and anaerobic conditions, presented ability to produce hydrogen and degrade glycerol. Table 4 shows the four hydrogen producer strains. For the anaerobic conditions, strains 1 (31.2 mol%) and 5 (19.0 mol%) presented the highest hydrogen production and almost complete glycerol consumption after 24 h incubation.

Table 4- Products formed and remaining glycerol, aerobic an anaerobic conditions after 24 hours of culture starting from 30 $g.L^{-1}$ of glycerol.

Strain	Anaerobic Conditions			Aerobic Conditions	
	Glycerol($g\ L^{-1}$)	**Ethanol($g\ L^{-1}$)**	**H_2 (mol %)**	**Glycerol ($g\ L^{-1}$)**	**Ethanol ($g\ L^{-1}$)**
1	14.7 ± 0.7	0.8 ± 0.02	31.2 ± 2.5	3.7 ± 0.6	2.5 ± 0.2
5	2.1 ± 0.4	1.9 ± 0.3	18.9 ± 4.9	11.1 ± 3.8	2.4 ± 0.5
31	12.2 ± 0.2	1.2 ± 0.7	17.9 ± 1.6	12.0 ± 3.6	0.5 ± 0.5
33	11.9 ± 0.9	1.8 ± 0.5	17.2 ± 2.4	15.2 ± 4.0	1.0 ± 0.4

For both aerobic and anaerobic conditions, the production of ethanol, a value-added product, was also observed. In the case of ethanol, strains 1 and 5, were those that presented the best yields, 2.5 $g.L^{-1}$ and 2.4 $g.L^{-1}$, respectively. Despite the low observed values, production of ethanol will be investigated and optimized in a future work.

3.2 Strain Identification

The four new isolated hydrogen-producing strains (1, 5, 31, 33) were identified based on the approach of 16S rDNA genes. The 16S rDNA showed that the strains 1, 5, and 31 were identified as *Klebsiella pneumonie* with 96% of confidence for strain 1 and 5 and 85 % of confidence for strain 31. The strain 33 had 93% confidence for *Pantoea agglomerans.*

3.3 Screening of Culture Conditions

Strain 1, labeled as *K. pneumonie* BLb01 (named after as *K. pneumonie* BLh-1), was selected for experiments aiming the optimization of culture conditions for hydrogen production. P-B experimental design was used to evaluate the impact on hydrogen production of the variables: glycerol, temperature, yeast extract, peptone, NH_4Cl, $(NH_4)_2SO_4$, KH_2PO_4, K_2HPO_4, $FeSO_4.7H_2O$ and $MgSO_4.7H_2O$. Table 1 shows the P-B experimental design for 20 runs, with two levels of concentration for each variable and the corresponding hydrogen production (mol %), which has varied from 2.8 to 29.8 mol %. The variation reflects the importance of optimization in order to attain higher hydrogen production. The variables and their ranges were chosen based on the literature. Table 5 shows the statistical analysis of the studied variables.

Table 5- Estimated effects for hydrogen production from Plackett-Burman design.

Variables	Parameters	Effect	p-value
Mean/interaction	-	17.88000	0.00000
X_1	Glycerol	3.434	0.00465
X_2	pH	2.413	0.01263
X_3	Temperature	12.248	0.00010
X_4	Yeast extract	6.196	0.00082
X_5	Peptone	1.126	0.08719
X_6	NH_4Cl	2.636	0.00988
X_7	$(NH_4)_2SO_4$	-0.074	0.87828
X_8	K_2HPO_4	2.946	0.00721
X_9	KH_2PO_4	2.608	0.01017
X_{10}	$FeSO_4.7H_2O$	-3.203	0.00568
X_{11}	$MgSO_4.7H_2O$	-1.916	0.02365

Standard error = 0.449; p-values < 0.05, R^2 = 0.98.

The growth kinetics has been monitored for 48 h, but the highest hydrogen production was always observed after 24 h, therefore the value in Table 1 refers to 24 hours of growth. Nine variables were found to be significant at the 95% level. Residual glycerol showed positive effect on hydrogen production. This result shows that the strain *K. pneumonie* BLb01 has great ability to degrade glycerol at high concentrations. There are few works in

the literature reporting the use of residual glycerol as a carbon source in hydrogen production. [7,17]

Most of the papers use glucose as carbon source. [8,18-19] In addition to the carbon source, the nitrogen source is an important parameter for hydrogen production. Peptone and yeast extract were used as nitrogen sources but only the latter presented positive effect.

Hydrogen-producing bacteria are quite sensitive to pH fluctuations because it can result in changes of their metabolic pathway. Zuo et al. [20] performed a series of experiments to investigate the effects of several factors on anaerobic bio-hydrogen producing process and the results showed that the nature of substrate and its concentration, temperature, and initial pH could affect the anaerobic biohydrogen production at different levels. The initial pH and temperature had positive effect on hydrogen production showing that higher values of initial pH are necessary once a pH drop from 4.5 to 5.0 is verified after 24 hours. Changes in pH values can lead the microorganisms to different metabolic routes yielding different products. During hydrogen production, fermentation pathway may shift organic acids production to alcohol production when pH is decreased to 4.5 or below. [21]

The variables glycerol, temperature, pH, yeast extract, K_2HPO_4 (X_8), KH_2PO_4 (X_9) have positive effects and will be further discussed in the FFD design. The variables $FeSO_4.7H_2O$ (X_{10}) and $MgSO_4.7H_2O$ (X_{11}) have negative effects and were excluded because the lower level was 0 g L^{-1}. The variable NH_4Cl had positive effect but was set at 5 g.L^{-1} because that is a high value.

3.4 Fractional Factorial Design (FFD)

Table 2 shows the hydrogen responses in mol % after 24 hours culture. Each independent variable was evaluated in high (+1) and low level (–1). Central point analyses were included in the matrix and statistical analysis was employed to identify the effect of each variable on hydrogen production.

The fractional factorial design lead to an hydrogen production varying from 7.7 to 40.2 mol%. It can be observed that these values are higher than those from the previous experiment (Table 1). The central points (runs 17, 18 19 and 20) presented good experiment reproducibility. Table 6 shows the statistical analyses of the studied variables using fractional factorial design. The variables with the most effect over hydrogen production were identified for the corresponding isolate with confidence level higher than 95% ($p < 0.05$).

Table 6- Estimated effects for hydrogen production resulting from fractional factorial design.

Variable	Parameters	Effect	p value
X_1	Glycerol	- 7.798	0.004652*
X_2	pH	2.493	0.010925*
X_3	Temperature (ºC)	19.090	0.000027*
X_4	Yeast extract	- 4.5421	0.001947*
X_5	K_2HPO_4	4.001	0.002822*
X_6	KH_2PO_4	4.395	0.002144*

* Significant effects at 95% confidence; p = Statistical probability

In the present experiment, all the analyzed variables were significant for the hydrogen production. On the other hand, glycerol presented a negative effect, indicating that increasing the concentration from 30 $g.L^{-1}$ to 50 $g.L^{-1}$ is not advantageous since concentrations above 30 $g.L^{-1}$ of substrate inhibit bacteria growth. This behavior can be justified by the presence of impurities in the glycerol resulting from the biodiesel production like fat acids salts and free fat acids or simply by saturation of the medium by glycerol causing microorganism deaths. [22] In this second optimization experiment the studied pH range was wider than the one used in the first experiment with P-B optimization and the variable pH showed a positive effect indicating the need of a high initial value of pH since it is reduced during the fermentation process. The yeast extract presented a negative effect. This compound, used as a source of nitrogen, is one of the necessary ingredients for microorganism growth. The use of high concentrations was not significant for microorganism growth besides being not interesting from the economic point of view. The other studied variables presented positive effect.

Temperature variation from 31°C to 37°C increased hydrogen production, which is in agreement with literature data for other substrates. Lin and Chang [23] evaluated the effect of temperature in the range 15°C to 34°C and achieved the maximal hydrogen production at 34°C using hexoses as substrates. Yu et al.[24] evaluated the effect of temperature in the range 15°C to 55°C and achieved the maximal hydrogen production at 55°C using the effluent of a wine processing plant as substrate. Phosphates presented positive effect, which is relevant for the culture medium since they are necessary to the hydrogen production due to their buffering capacity. [25]

3.5 Response Surface Methodology (RSM)

Data for hydrogen production under the conditions established in the RSM design are presented in Table 3. Values varied from 0.6 to 48.2 mol% after 24 hours of cultivation. The central points presented a small variation indicating good reproducibility for the process.With the exception of X_3 (KH_2PO_4) and the temperature interactions with KH_2PO_4 (X_1, X_3) and K_2HPO_4 with KH_2PO_4 (X_2, X_3), all parameters were highly significant allowing the elaboration of a model with the codified variables according to the reduced Equation 3.

$$H_2 = 43.16 - 9.02X_1 - 8.79X_1^2 - 2.59X_2 - 4.05X_1X_2 \qquad (3)$$

The regression coefficient (R^2=0.8727) showed that 87.27% of the variability could be explained by the model. The F value (22.29) is higher than the theoretical F (3.18), confirming the statistical significance of the model. The experimental design using response surface methodology proved to be useful to evaluate those constituents of the medium that affect the hydrogen production. The resulting models were significant allowing building the response surfaces and defining the regions of interest. Through the response surface analysis (Fig. 1 and 2), the maximal values determined for hydrogen production (mol %) corresponded to the temperature around 39 °C. On the other hand, phosphate concentrations (K_2HPO_4 e KH_2PO_4) inside the studied range (1 to 7 $g.L^{-1}$) had no effect in the response. These results, in operational and economical terms, indicate that phosphates can be used in lower concentrations.

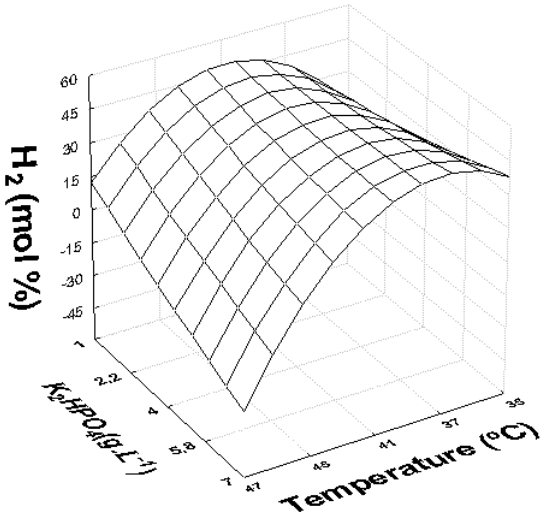

Figure 1- Contour plot for hydrogen production (mol %), interaction between T(ºC) and K_2HPO_4

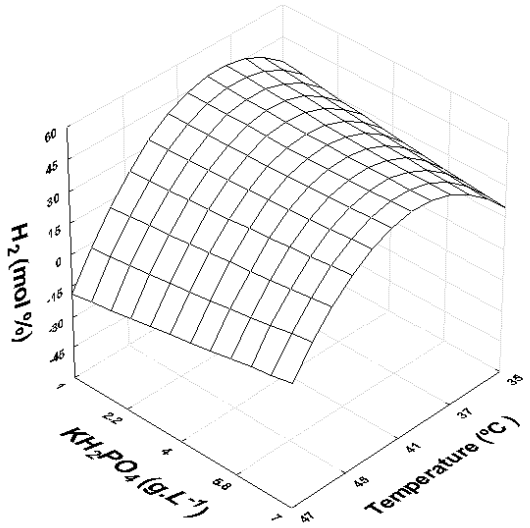

Figure 2- Contour plot for hydrogen production (mol %), interaction between T(ºC) and KH_2PO_4.

3.6 Validation of the Models

According to the statistical design, the optimized medium was prepared with the following composition: glycerol 30 g L^{-1}; yeast extract 3 g L^{-1}; K_2HPO_4 3 g L^{-1}; KH_2PO_4 1 g L^{-1}; temperature 39 ºC and pH=9,0. The validation of the model was carried out by solving Equation 3 and comparing the predicted value (45.3 mol %) with the experimental value (45.0 mol %), indicating that the model is valid in the studied range.

4 Conclusions

The present work shows that *Klebsiella pneumoniae* BLb01 isolated from sludge presented a high capacity for residual glycerol degradation (98%) with a very good production of hydrogen. The use of a Plackett-Burman design, Fractional Factorial Design and Response Surface Methodology allowed the optimization model for working conditions in fermentation experiments, leading to a considerable increase in hydrogen production (29.8 to 45.0 mol %). These results contribute to promote the use of biohydrogen obtained through a low cost process. In addition to obtain a value-added product the proposed bioprocess is associated with the degradation of glycerol generated in excess in the biodiesel synthesis, contributing to the environmental preservation.

Acknowledgements

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CAPÍTULO V – BIOCONVERSION OF RESIDUAL GLYCEROL FROM BIODIESEL SYNTHESIS INTO 1,3-PROPANEDIOL AND ETHANOL BY ISOLATED BACTERIA FROM ENVIRONMENTAL CONSORTIA

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Bioconversion of residual glycerol from biodiesel synthesis into 1,3-propanediol and ethanol by isolated bacteria from environmental consortia

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ABSTRACT

Residual (raw) glycerol originated from biodiesel synthesis is becoming of great environmental and economical concern due to its ever-growing surplus. In the present study, several bacteria strains were isolated and characterized for their ability to convert this raw glycerol into 1,3-propanediol (1,3-PD) and ethanol. The best producers of both 1,3-PD and ethanol were identified by 16S rDNA sequences to be *Klebsiella pneumoniae* and *Pantoea agglomerans* strains. Batch bioreactor cultivations under anaerobic and aerobic conditions were carried out in order to access the kinetics of glycerol consumption and product formation. Results showed that one isolated, *Klebsiella pneumoniae* BLh-1, was able to simultaneously produce up to 9.4 g/L of 1,3-PD with yields of 0.41 mol product mol^{-1} glycerol, and 4.9 g/L of ethanol with yields of 0.14 mol product mol^{-1} glycerol under anaerobic conditions, showing great potential for bioprocesses.

K**eywords:** 1,3-propanediol, ethanol, raw glycerol, biodiesel, bacteria consortium.

1 Introduction

Biodiesel is produced by transesterification of vegetable oils or animal fats, with ethanol or methanol (alcoholysis), catalyzed by alkali, generating about 10 % (v/v) of glycerol as the main by-product. Biodiesel has been widely used in Germany, which currently is the largest producer and consumer of this biofuel. In Brazil, the addition of 2 % biodiesel (B2) in the diesel is mandatory since 2008, and this amount is expected to increase to 5 % (B5) by 2013. Brazil will become a major producer and consumer of biodiesel in the context of a strong strategy for biofuel production, traditionally with ethanol, and the exceptional good conditions for cultivating oleaginous plants for oil extraction [1]. Therefore, large surpluses of glycerol will be generated that require new commercial uses.

Glycerol is used as an ingredient in cosmetics, paint, automotive industry, foods, tobacco, pharmaceutical products, pulp and paper, leather and textiles, among many other applications [2]. More recently, it is being studied as a possible substitute for traditional carbohydrates, such as sucrose and glucose, in some industrial fermentation processes in order to obtain value-added products, among them 1,3-propanediol (1,3-PD), ethanol, hydrogen, and organic acids [1,3,4].

1,3-PD is an important bulk chemical with many possible applications in polymers, cosmetics, foods, adhesives, lubricants, laminates, solvents, antifreeze, and in medicine [5,6,7]. For example, a 1,3-PD-based new polyester, polytrimethylene terephthalate (PTT), has several unique properties for the production of fibers. The biotechnological production of 1,3-PD from glycerol has been demonstrated for some bacteria. A number of microorganisms, such as *Lactobacillus lycopersici* and *Bacillus subtillis*, can ferment sugars producing glycerol, but are unable to further convert glycerol into 1,3-PD [2]. However, bacteria of the genera *Klebsiella*, *Enterobacter*, *Citrobacter*, *Lactobacillus*, and some species of *Clostridium*, have shown to ferment glycerol or mixtures of glycerol and sugars, into 1,3-PD [8,9,10]. The metabolic reactions proposed for glycerol fermentation basically follow two possibilities. In the oxidative pathway, glycerol is converted to dihydroxyacetone by glycerol dehydrogenase (GDH), and subsequently phosphorylated by adenosine triphosphate–dependent dihydroxyacetone kinase. Dihydroxyacetone-phosphate undergoes normal glycolysis to form pyruvate, which is further converted into various organic acids (lactic acid, acetic acid, succinic acid), 2,3-butanediol, hydrogen, CO_2, and ethanol. Glycerol is also converted through the reductive pathway to 3-hydroxypropionaldehyde (3-HPA) by the coenzyme B12-dependent glycerol dehydratase (GDHt). 3-HPA is then converted by a

$NADH_2$-linked oxidoreductase to 1,3-PD, which is then excreted from the cell. The physiologic function for the transformation of glycerol into 1,3-PD is probably to regenerate the oxidized form of the reducing equivalents, NAD^+, to be used in the energy-producing pathways of glycerol degradation [11].

Ethanol is primarily produced from sugarcane, maize, and sugar beet in countries such as Brazil, USA and in Europe, respectively. With the expected surplus of glycerol from biodiesel, ethanol production from it will become extremely attractive, since ethanol could be used in the esterification of biodiesel, contributing to develop cheaper and environmentally cleaner processes. For instance, ethanol production from glycerol is calculated to cost almost 40 % less in comparison with ethanol from maize [12]. Therefore, more work is in need if the vast amounts of surplus glycerol are to be cost-effectively converted into commercial products. The aim of this research is to study the isolation of new strains of bacteria capable of efficiently use and convert raw glycerol into biotechnological products such as 1,3-PD and ethanol, under both aerobic and anaerobic conditions.

2 Materials and methods

2.1 Residual raw glycerol

The raw glycerol was supplied by a biodiesel manufacturing plant (Passo Fundo, Brazil). The biodiesel was obtained by transesterification of degummed soybean oil, using methanol as the alcohol. The raw glycerol composition was (in weight based) 82.8 % glycerol (including 0.5 % of monoacylglycerols), 6.0 % NaCl, 11.2 % moisture, and pH 4.84.

2.2 Bacterial isolation and degradation of residual glycerol using a microbial consortium

The bacterial consortium was collected from the sludge of an upflow anaerobic bacterial blanket reactor at a local soybean treatment plant (Esteio, Brazil). The concentration of the volatile suspended solids (VSS) of the sludge was 4.6 g/L. Microbial samples were collected, immediately refrigerated, and transported to the laboratory for further studies. In order to isolate the bacteria and to determine their capability of using glycerol as carbon source, fermentations were conducted using 2 L bioreactors (Biostat-B. B. Braun, Germany). Prior to inoculum, the bacterial consortium was boiled for 20 minutes at 95°C to inhibit methanogenic bacteria and then inoculated at 10 % (v/v) into the bioreactors. The operational conditions of the bioreactors were: initial pH 7.0, temperature 35°C, agitation speed of 250

rpm for 48 hours. To ensure anaerobic conditions nitrogen was bubbled into the culture broth. The synthetic medium contained the following components (in g/L): 4 $(NH_4)_2SO_4$, 0.0125 K_2HPO_4, 0.12 $MgSO_4.7H_2O$, 0.01 $MnSO_4.H_2O$, 0.025 $FeSO_4.7H_2O$, 0.005 $CuSO_4.5H_2O$, 0.125 $CoCl_2.6H_2O$, and 70 g of raw glycerol. Fermentations run at these same conditions were carried out with pure glycerol (> 99 % purity) and were used as controls. For cell isolation, samples were collected every 3 hours and were serially diluted in 0.1 % (v/v) sterile peptone water; subsequently, 0.1 mL of these cell suspensions were spread onto nutrient agar (NA) and McConkey agar and incubated at 35°C. After incubation for 24 and 48 h, colony counts were determined and representative colonies were sub cultured into LB (Luria Bertani) liquid medium and stored in glycerol (1:1) at -20°C for posterior identification. Cellular morphologies were determined by bright field microscopy of Gram-stained preparations.

2.3 Sequencing of 16S rDNA genes and genetic analysis

The molecular identification of the isolates were conducted at the ACTGene Laboratory (Biotechnology Centre, UFRGS, Porto Alegre, RS, Brazil) using the automatic sequencer ABI-PRISM 3100 Genetic Analyzer armed with 50 cm capillaries and POP6 polymer (Applied Biosystems-USA). DNA templates (30 to 45 ng) were labeled with 3.2 pmol of the primer 5'-NNNNNNNNNNNNN-3' and 2 μL of BigDye Terminator v3.1 Cycle Sequencing RR-100 (Applied Biosystems-USA) in a final volume of 10 μL. Labeling reactions were performed in a GeneAmp PCR System 9700 (Applied Biosystems-USA) thermocycler with a initial denaturing step of 96 ºC for 3 min followed by 25 cycles of 96 ºC for 10 sec, 55 ºC for 5 sec, and 60 ºC for 4 min. Labeled samples were purified by isopropanol precipitation followed by 70 % ethanol rinsing. Precipitated products were re-suspended in 10 μL formamide, denatured at 95 ºC for 5 min, ice-cooled for 5 min and electroinjected in the automatic sequencer. Sequencing data were collected using the software Data Collection v1.0.1 (Applied Biosystems-USA), programmed with the following parameters: Dye Set "Z"; Mobility File "DT3100POP6 {BDv3}v1.mob"; BioLIMS Project "3100_Project1"; Run Module 1 "StdSeq50_POP6_50cm_cfv_100"; and Analysis Module 1 "BC-3100SR_Seq_FASTA.saz".

2.4 Kinetics of batch flask fermentations

Screened strains capable of using glycerol as carbon source were cultured under both anaerobic and aerobic conditions to verify their production of 1,3-PD and ethanol. The

medium (M1) used for batch flask fermentation contained (in g/L): 4 $(NH_4)_2SO_4$, 0.52 K_2HPO_4, 0.25 KH_2PO_4, 0.2 $MgSO_4.7H_2O$, 1.5 yeast extract, 1 bacteriological peptone, 1 mL trace elements solution containing (in g/L): 0.1 $MnCl_2.4\ H_2O$, 0.06 H_3BO_3, 0.0037 $CuSO_4.5H_2O$, 0.2 $CoCl_2.6H_2O$, 0.025 $NiCl_2.6\ H_2O$, 0.035 $Na_2MoO_4.2\ H_2O$, 0.14 $ZnSO_4.7H_2O$, 0.9 mL HCl 37%, added with 30 g/L of raw glycerol. Inocula were prepared by transferring one loopfull of cells into 125 mL flasks filled with 50 mL of M1 and then incubated at 37 °C in a rotary shaker at 150 rpm for 12 hours. The anaerobic experiments were conducted in 60 mL serum bottles with 30 mL working volume sealed with butyl rubber stoppers. The medium was previously boiled for 20 min, cooled with continuous bubbling of nitrogen and then sterilized (121°C, 15 min). The isolated bacteria were inoculated as a 5 % (v/v), 1 unit of OD (600 nm), with the help of a hypodermic syringe, and incubated at 37 °C in a rotary shaker at 150 rpm for 24 hours. The aerobic experiments were conducted using 125 mL flasks with 30 mL working volume and incubated at 37 °C in a rotary shaker at 150 rpm for 24 hours. Samples from cultivations were collected and immediately centrifuged at 2,800 *g* at 4°C for 15 min and the supernatant was filtered and frozen for posterior chromatography analyses.

2.5 Kinetics of batch bioreactor fermentations

One of the strains, designed as *K. pneumoniae* BLh-1, showed a remarkably good production of 1,3-PD and ethanol and was thus selected for further investigations. As pre-inocula, selected colonies were transferred into 500 mL flasks filled with 200 mL M1 and incubated at 37°C in a rotary shaker at 150 rpm, 12 hours. The 2 L bioreactors (Biostat-B.Braun, Germany), with working volumes of 1.5 L, were inoculated with 10 % (v/v), 1 unit of OD (600 nm) of the pre-inocula cultures. Experiments under anaerobic conditions were performed at 37°C, 250 rpm and pH 7.0. Nitrogen was used to sparge the culture broth. The aerobic cultivations were performed at 37°C, 350 rpm, 1 vvm of air. The pH was controlled at 7.0 by automatic addition of either 10 M NaOH or 1 M H_3PO_4. All bioreactor experiments were run in duplicates.

2.6 Analytical methods

The composition of fermented media were analyzed using a Shimadzu HPLC (Shimadzu Corp., Japan) equipped with a RID-10A refractive index detector. The stationary and mobile phases were an Aminex HPX-87H column (300 x 7.8 mm,Bio-Rad, USA) and 0.005 $mol.L^{-1}$ H_2SO_4 solution at 0.8 $L.min^{-1}$, respectively. The column temperature was

controlled at 65°C. The culture samples were prepared by centrifugation at 3,500 *g* for 15 min to pellet the cell, followed by filtration using 0.22 μm pore size cellulose acetate membrane filters (Sartorius, Germany). Biomass concentration was gravimetrically determined and expressed as dry cell weight. For the HPLC analyses, standards of HPLC grade of glycerol, 1,3-PD, ethanol, acetic acid, and lactic acid were used (> 99.5 purity).

3 Results and discussion

3.1 Testing the consortium capability of degrading glycerol

We used a direct approach to immediately identify bacteria capable of metabolize residual glycerol from biodiesel by cultivating environmental consortia in bioreactors with raw glycerol as the sole carbon source. Results shown in figure 1 compare the abilities of bacteria to grow in either raw or pure glycerol. Pure glycerol was exhausted in 32 hours with a production of 22.8 g/L of 1,3-PD, with yields ($Y_{p/s}$) of 0.40 mol product mol^{-1} glycerol. For raw glycerol, consumption was completed within 32 hours of cultivation with approximately the same amounts of 1,3-PD being produced (19.9 g/L, $Y_{p/s}$ 0.35 mol product mol^{-1} glycerol). Small amounts of organic acids were also detected in cultures, especially acetic and butyric acids, possibly indicating the presence *Klebsiella* and *Citrobacter* (acetic producers) and species of *Clostridium*, known to form butyric acid as a byproduct.

Although some works reported the use of environment-isolated consortia, they were mainly concerned with the production of hydrogen, using sugars as carbon sources [13, 14, 15]. In these processes, known as *dark fermentation*, a large group of anaerobic fermentative bacteria are present [16]. These bacteria have been identified as strict anaerobes (*Clostridium* species), facultative anaerobes (*Enterobacter, Citrobacter, Klebsiella*), and a few aerobes (*Alcalligenes, Bacillus*) [10,17,18]. Our results showed that some of these species were present in the microbial consortia and were capable of efficiently converting raw glycerol into 1,3-PD.

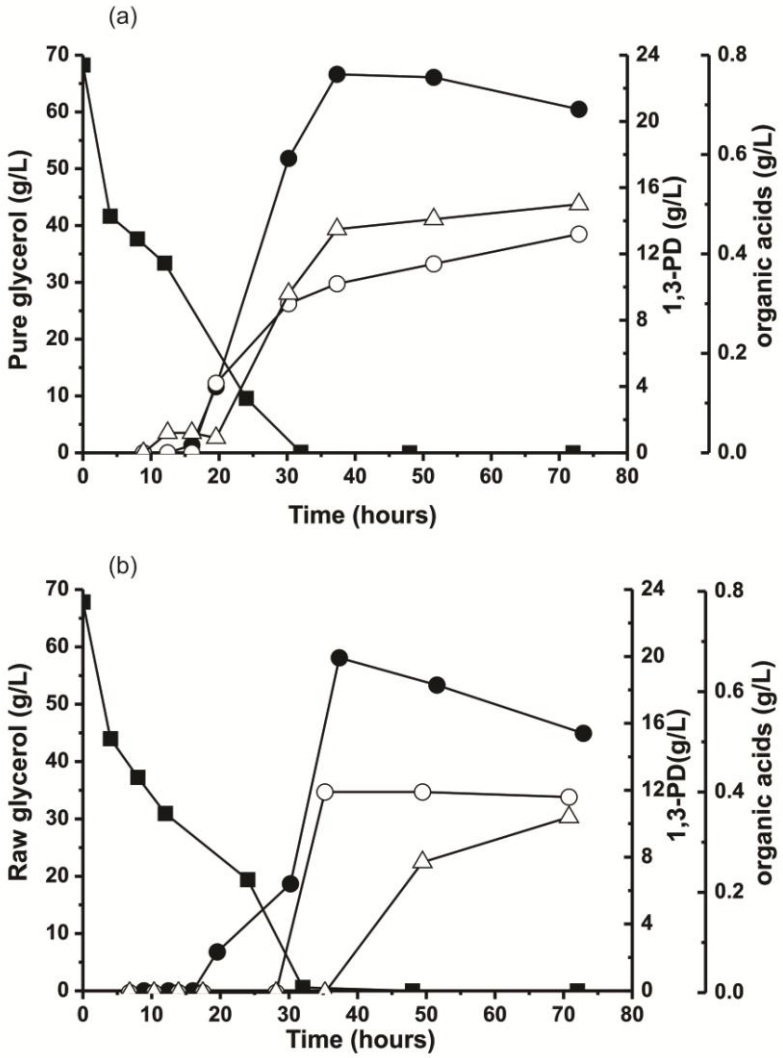

Figure 1- (a) Kinetics of anaerobic cultivation of the microbial consortium on pure glycerol, and (b) on raw glycerol (- ■ -) substrate (pure glycerol); (-●-) 1,3-PD; (-○-) acetic acid; (-Δ-) butyric acid.

3.2 Screening and isolation of 1,3-PD producers

Bioreactor experiments using raw glycerol were carried out in order to isolate the potential producers of 1,3-PD and ethanol. Thirty-two different colonies were recovered from bioreactor cultivations and plated onto LB agar plates. Cells were microscopically observed after Gram dying, and all but one of the isolates were Gram-negative rods. In the next step, the isolated strains were tested for their ability to produce 1,3-PD under both aerobic and anaerobic conditions in shaker flasks, separately. Four of them presented the ability to consume glycerol and produce 1,3-PD and ethanol as main products. Figure 2 shows the results of 1,3-PD and ethanol production of four strains under anaerobic and aerobic conditions.

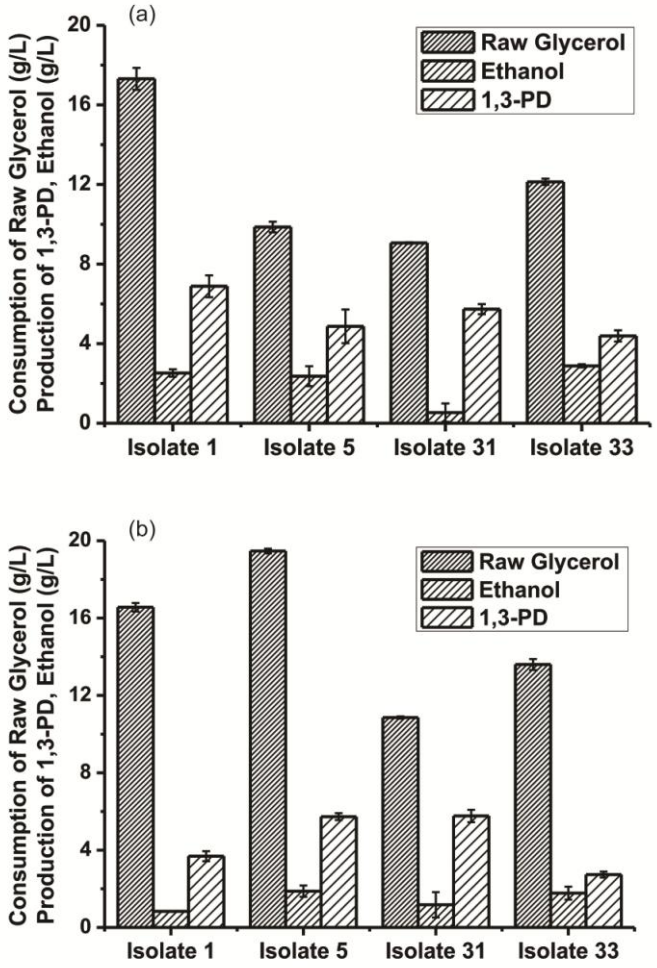

Figure 2- Profile of raw glycerol consumption and 1,3-PD and ethanol production by isolated bacteria from screening; (a) anaerobiosis, (b) aerobiosis, both at 24 hours of cultivation. Results are mean of three experiments.

The highest 1,3-PD production (6.88 g/L) under anaerobic conditions was achieved for strain 1. Glycerol was almost depleted by two strains after only 24 hours of cultivation, indicating that these bacteria have a remarkable affinity for glycerol. Under aerobic conditions, strains 5 and 31 were the best producers of 1,3-PD and ethanol.

Homman et al. [5] isolated bacteria from a consortium and selected six that were characterized as *Citrobacter freundii, Klebsiella oxytoca,* and *Klebsiella pneumoniae*. The selected bacteria produced in shaker cultures between 9.3 and 13.1 g/L of 1,3-PD, and 0.2 to 3.6 g/L of ethanol using 20 g/L of pure glycerol at pH 7.0 under anaerobic conditions. Hao et al. [19] isolated eight strains from soil samples and two were characterized as *Klebsiella pneumoniae* with 99 % of confidence value, while another six were characterized as *Citrobacter freundii* with 98 and 99 % of confidence value using 16S rDNA identification. The isolates were able to produce 1,3-PD under aerobic conditions using 30 g/L of pure

glycerol. The authors showed a production of up to 11 g/L of 1,3-PD and 2.5 g/L of ethanol in shaker cultivation. It is noteworthy to point that in these researches the authors worked with pure glycerol at low concentrations.

3.3 Strain identification

The four newly isolated 1,3-PD strain producers were identified based on 16S rDNA genes as *Klebsiella pneumonie* (>96 % of confidence for strain 1 and 5 and >85 % of confidence for strain 31), while strain 33 was identified as *Pantoea agglomerans* (>93 % confidence).

The best producer of 1,3-PD, strain 1, renamed as *Klebsiella pneumonie* BLh-1, was selected for further experiments in batch bioreactor cultivations.

3.4 Batch bioreactor cultivations

Figure 3 shows the kinetics of batch cultivation of *K. pneumonie* BLh-1under anaerobic conditions. The specific growth rate (μ) was 0.38 h^{-1} and the 1,3-PD yield was 0.41 mol product mol^{-1} glycerol. The experiments under aerobic conditions (Figure 4) showed a lower production of 1,3-PD when compared to anaerobic fermentation and the glycerol was almost completely consumed after 12 hours. The specific growth rate (μ) was practically the same, 0.37 h^{-1} with 1,3-PD yields of 0.3 mol product mol^{-1}glycerol. The maximal production of 1.3-PD was 9.4 g/L in 28 hours under anaerobic conditions and 6.2 g/L at 12 hours for aerobic cultivation.

The highest concentrations of ethanol were found for the anaerobic cultivation (Figure 3b), with 4.9 g/L and yields of 0.14 mol product mol^{-1} glycerol. Predictably, aerobic production of ethanol was lower compared with anaerobic conditions, while acetic acid formation increased until 15 hours up to 5.3 g/L. During the stationary phase of the aerobic cultivation (Figure 4b) there was some products consumption, which was not observed for the anaerobic cultivation (Figure 3b).

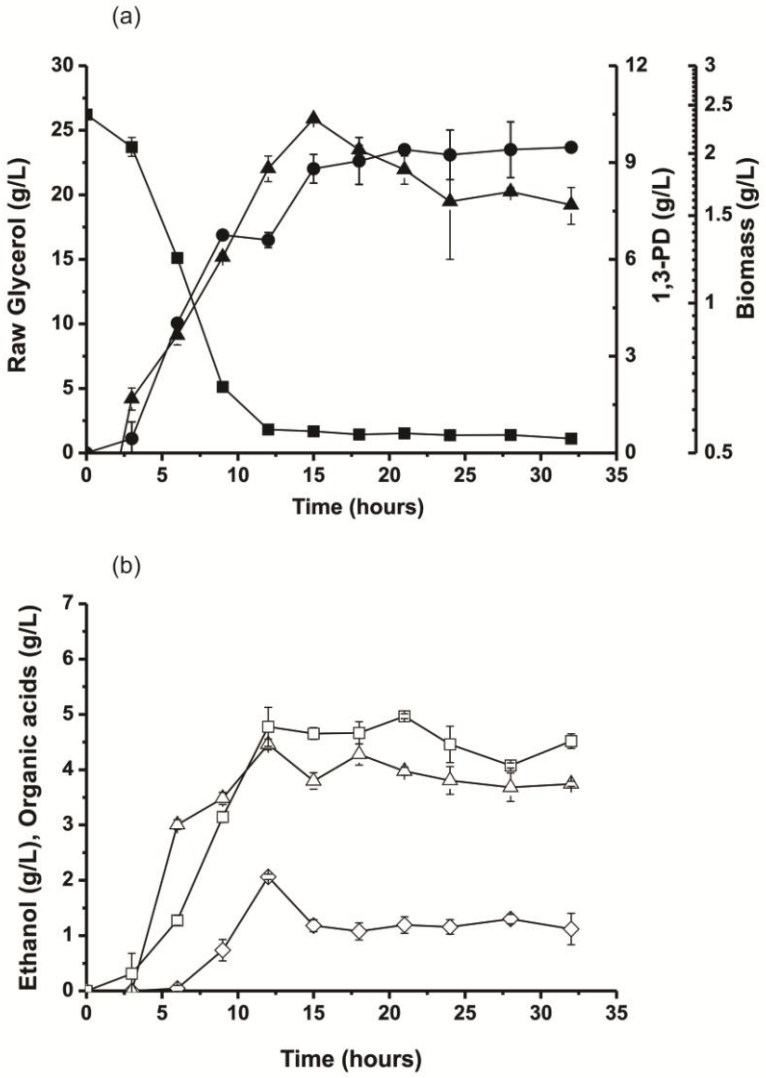

Figure 3- Batch anaerobic cultivation of *K. pneumoniae* BLh-1:(a) (-■-) glycerol; (-●-) 1,3-PD; (-▲-) biomass. (b) (-□-) ethanol; (-Δ-) acetic acid; (-◊-) lactic acid. Results are mean of two experiments.

The results presented in this work showed that the *K. pneumoniae* BLh-1 was able to degrade raw glycerol with the production of 1,3-PD in a similar way to results reported in other works where pure glycerol was used [19, 5]. For instance, Zhang et al. [20] studied the rapid conversion of pure glycerol to 1,3-PD by a *Klebsiella pneumoniae* strain isolated from soil. The authors obtained a maximal production of 12.2 g/L of 1,3-PD in batch fermentation with total consumption of 20 g/L of glycerol in 8 hours, with a productivity of 1.53 g 1,3-PD/L/h. Hao et al. [19] studied the 1,3-PD production under aerobic conditions using *K. pneumoniae* strain TUAC01, also isolated from soil. The authors obtained a maximal production of 13.7 g/L of 1,3-PD in batch fermentation with total consumption of 30 g/L of pure glycerol in 10 hours and yields of 0.5 mol product mol^{-1} glycerol.

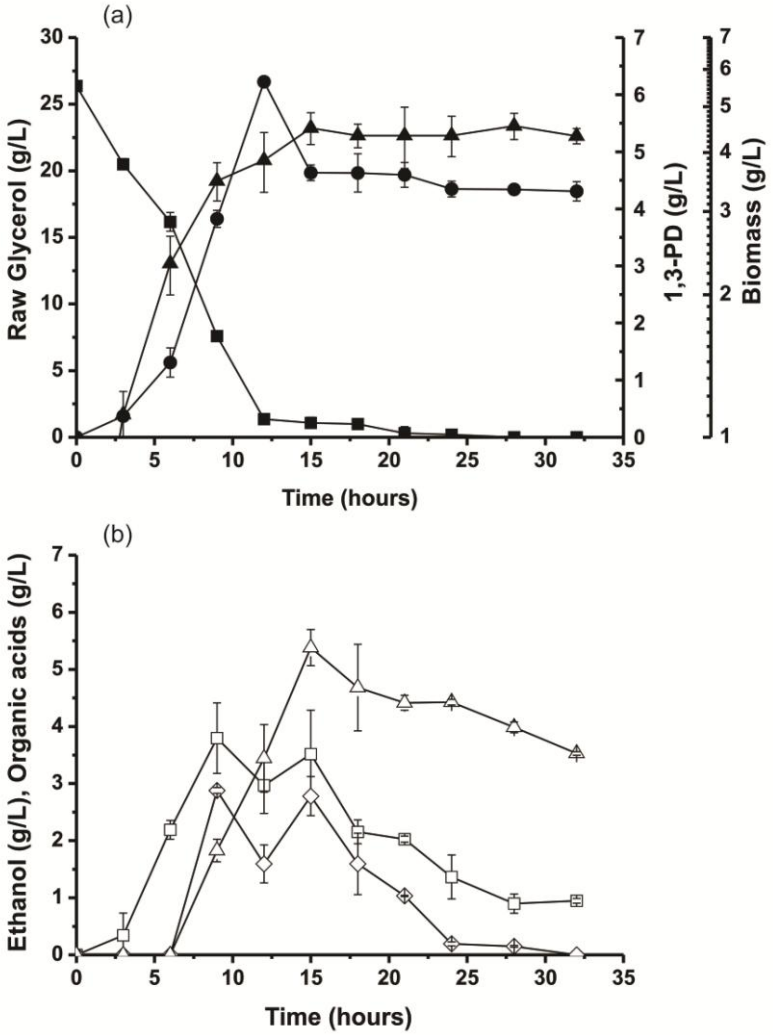

Figure 4- Batch aerobic cultivation of *K. pneumoniae* BLh-1: (a) (-■-) glycerol; (-●-) 1,3-PD; (-▲-) biomass. (b) (-□-) ethanol; (-Δ-) acetic acid; (-◊-) lactic acid. Results are mean of two experiments

Ethanol was the main byproduct of glycerol fermentation compared with organic acids (Figure 3b). This result shows the ability of *Klebsiella* species to form more important byproducts such as ethanol and, in smaller proportions, lactate and acetate. Mu et al. [21] studied the production of ethanol by *K. pnemoniae* using crude glycerol from biodiesel preparations showing a final production of 11.9 g/L. Jarvis et al. [22] demonstrated that formate and ethanol are the major products of glycerol fermentation by *Klebsiella planticola* isolated from animal rumen. Ito et al. [23] showed that *E. aerogenes* could produce ethanol from biodiesel wastes containing glycerol, with all the glycerol consumed in 24 hours, yielding 1.0 mol product mol^{-1} glycerol. Studies with *K. pnemoniae* showed that when glycerol concentration is culture limiting, cell mass formation is optimized and ethanol is

produced. Inversely, when glycerol concentrations are high in the medium, 1,3-PD is produced instead of ethanol. One of the reasons for this physiological difference could be that carbon flux from oxidation of glycerol is channeled into glycolysis, which leads to the formation of pyruvate-derived by-products such as ethanol, which competes with the biosynthesis of 1,3-PD for NADH [24].

4 Conclusions

Although the utilization of raw glycerol in fermentation offers a notable advantage in relation to the use of pure glycerol, the majority of the studies for microbial 1,3-PD production have been conducted using pure glycerol, with only a few reports on the potential use of raw glycerol from biodiesel production for 1,3-PD. The results obtained in the present work with raw glycerol compared well with those for pure glycerol showing that this by-product could be used with a great potential to produce 1,3-PD, as well as ethanol, in further experiments with optimized conditions. The study also showed that was possible to isolate new bacteria strains capable to produce 1,3-PD and ethanol from natural microbial consortia. One of the isolated strains, identified as *K. pneumoniae* BLh-1, presented good productions of 1,3-PD and ethanol under anaerobic cultivations, with yields of 0.41 and 0.14 mol product mol^{-1} glycerol for 1,3-PD and ethanol, respectively. The study of biochemical pathways of glycerol are an important tool to understand all the factors influencing cell physiology and gene regulation in order to improve the by-products syntheses. Therefore, further studies with this new isolated strain are granted to improve these two metabolites production.

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CAPÍTULO VI – EFFICIENT AND MAXIMIZED CONVERSION OF RESIDUAL GLYCEROL FROM BIODIESEL SYNTHESIS INTO 1,3-PROPANEDIOL BY A NEW STRAIN OF *KLEBSIELLA PNEUMONIAE*

Artigo submetido ao periódico: ***Bioprocess and Biosystems Engineering*** em outubro de 2011.

Efficient and maximized conversion of residual glycerol from biodiesel synthesis into 1,3-propanediol by a new strain of *Klebsiella pneumoniae*

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ABSTRACT

A stepwise strategy was devised to maximize the simultaneous bioconversion of residual glycerol from biodiesel synthesis into 1,3-propanediol (1,3-PD) by a new strain *Klebsiella pneumoniae*. This strain is unique in its ability to convert glycerol simultaneously into 1,3-PD and ethanol. The influence of glycerol concentration, pH, temperature, nitrogen and mineral sources were investigated using the Plackett–Burman (P–B) statistical design to find the best medium composition. Seven variables were statistically significant at 95% significance and a new 2^{7-3} fractional factorial design (FFD) was applied to further refine the optimization of culture conditions. Results showed that the ideal medium composition and culture conditions for the simultaneous syntheses of 1,3-PD and ethanol are glycerol 65 g/L, yeast extract 5 g/L, peptone 5 g/L, $(NH_4)_2SO_4$ 7 g/L, K_2HPO_4 7 g/L, and temperature of 37 ºC. Scaled-up experiments in batch bioreactors under controlled pH produced up to 23.80 g/L of 1,3-PD and 12.30 g/L of ethanol, while in fed-batch cultivations a three-fold increase of 1,3-PD production (36.86 g/L) was obtained compared with the results of the FFD design.

Keywords: Raw glycerol, 1,3-propanediol, ethanol, *Klebsiella penumoniae*, fermentation experimental design.

1 Introduction

Glycerol is generated in large amounts during the synthesis of biodiesel, with 10% of the oil being converted to this by-product. The utilization or disposal of this glycerol is complex, expensive, and it is becoming an important cost factor of the related industry [1]. There are several approaches to convert glycerol into high value-added products by both biotechnological and chemical processes. For instance, glycerol has found many applications in the cosmetic, paint, automotive, food, tobacco, pharmaceutical, pulp and paper, leather and textile industries [2,3]. On the other hand, the biological conversion of glycerol could help circumvent the disadvantages of chemical catalysis (e.g., low product specificity, use of high pressure and/or temperature etc.), while offering the opportunity to synthesize a large range of products and functionalities [4,5]. So far, the main biotechnological use of the residual glycerol has been its biotransformation into 1,3-propanediol (1,3-PD), a glycol of great importance for the textile and chemical industry. This chemical can be utilized as a monomer for the synthesis of polyesters and polyurethanes, as a component in the plastic polytrimethyleneterephthalate (PTT) synthesis, and in the production of polyester resins for the paint industry, among other uses [6,7]. Reported bacterial strains capable of producing 1,3-PD belong to genera *Klebsiella (K. pneumoniae)*, *Enterobacter (E. agglomerans)*, *Citrobacter (C.freundii*), *Lactobacillus* (*L. brevis* and *L. buchneri*), and *Clostridium* (*C. butyricum* and *C. parteurianum*) [8,9,10,11,12]. In the absence of an external oxidant, glycerol is metabolized through a dismutation process involving two possible pathways. Glycerol can be transformed to dihydroxyacetone by glycerol dehydrogenase (GDH) and subsequently phosphorylated by adenosine triphosphate–dependent dihydroxyacetone kinase. Dihydroxyacetone-phosphate undergoes normal glycolysis to form pyruvate, which is further converted into various organic acids and alcohols. Alternatively, glycerol is converted to 3-HPA by the coenzyme B12-dependent glycerol dehydratase (GDHt). 3-HPA is then converted by a $NADH_2$-linked oxidoreductase to 1,3-PD, which is then excreted from the cell. Therefore, it has been postulated that these bacteria can either produce ethanol or 1,3-PD, depending on the culture conditions, along with other metabolites, such as organic acids and H_2.

Recently, a new strain of *K. pneumoniae* was isolated, studied and characterized in our group [13] with the unique ability to convert glycerol into 1,3-PD and ethanol simultaneously. In this context, the aim of the present work was to investigate the production of 1,3-PD and

ethanol from residual glycerol by medium and culture conditions optimization using this new isolated *K. pneumonie* strain.

2 Materials and methods

2.1 Residual raw glycerol

The raw glycerol was supplied by a local biodiesel manufacturing plant (Passo Fundo, Brazil), which uses soybean oil as the main raw material. The biodiesel composition was determined to be (as mass fraction): 82.8 % of glycerol, 5.5 % ash, 5.9 % NaCl, 11.2 % moisture, 0.5 % monoacylglycerols, and pH 4.8. The biodiesel production in this plant is obtained by the transesterification of degummed soybean oil using methanol or sodium methylate.

2.2 Microorganism and inocula preparation

K. pneumoniae BLh-1 was isolated from microbial consortia and it has been identified and characterized in a previous work [13]. Certified stocks of *K. pneumoniae* BLh-1 are kept at microbiology culture collection of BiotecLab (UFRGS, Brazil). The culture was maintained on Luria–Bertani (LB) agar slant at 4 °C and subcultured every 4 weeks. Inocula were prepared by transferring one loopfull of cells into 125 mL flasks filled with 50 mL of medium and incubated at 37 °C in a rotary shaker at 150 rpm for 12 h. The medium contained (g/L): 4 $(NH_4)_2SO_4$, 0.52 K_2HPO_4, 0.25 KH_2PO_4, 0.2 $MgSO_4.7H_2O$, 1.5 yeast extract, 1 bacteriological peptone, 1 mL trace element solution (containing in g/L: 0.1 $MnCl_2.4\ H_2O$, 0.06 H_3BO_3, 0.0037 $CuSO_4.5H_2O$, 0.2 $CoCl_2.6H_2O$, 0.025 $NiCl_2.6\ H_2O$, 0.035 $Na_2MoO_4.2\ H_2O$, 0.14 $ZnSO_4.7H_2O$, 0.9 mL HCl 37 %), added with 30 g/L of glycerol (as raw glycerol). The inocula were adjusted to optical density (OD) 1.0 at 600 nm. This procedure was used as the standard preparation for all experiments.

2.3 Cultivation procedures

Shaker anaerobic experiments were conducted using 60 mL serum bottles with 30 mL working volume sealed with butyl rubber stoppers. Previous to inoculation, media were boiled for 20 min and cooled with continuous bubbling of pure nitrogen to remove any traces of oxygen. The bacterium was inoculated into flasks as 5 % (volume fraction) of a 1 OD cell concentration using a hypodermic syringe and incubated at 37 °C in a rotary shaker at 150 rpm for 24 h. Media composition, pH, and temperature varied according to the experimental design described in Table 1.

2.4 Experimental design

2.4.1 Placket-Burman design (P-B design)

Experimental designs were carried out for 1,3-PD as the sole dependable variable, since ethanol cannot be efficiently produced without pH control, which is impossible in agitated flasks. Nevertheless, the obtained values for ethanol production were included in Table 1.

P-B design was employed to determine the nutrients and conditions that could have a significant effect on 1,3-PD production. Eleven real variables and four dummy variables were screened in 20 trials, with four replicates at the central point. Each independent variable was tested at high (+1) and low (-1) levels. The minimal and maximal ranges selected for the eleven parameters are shown in Table 1, where each column represents an independent variable. Variables confidence levels > 95 % were considered to have significant influence on 1,3-PD production.

2.4.2 Fractional factorial design (FFD)

According to the P-B design, seven variables had significant effect on 1,3-PD production. These variables were then further optimized using a 2-level fractional factorial design. The selected variables for the FFD are listed in the Table 2. The other variables were fixed according to the values obtained in the P-B design. Each independent variable was investigated at a high (+1) and a low (−1) level. Four runs of the central point were included in the matrix and statistical analysis was used to identify the effect of each variable on 1,3-PD production. The variables with confidence levels > 95 % were considered to have significant influence on 1,3-PD production.

Table 1- Placket-Burman design with the codified, real variables and results for 1,3-PD production.

	Variables/levels															
Run	**X_1**	**X_2**	**X_3**	**X_4**	**X_5**	**X_6**	**X_7**	**X_8**	**X_9**	**X_{10}**	**X_{11}**	**D_1**	**D_2**	**D_3**	**D_4**	**1,3-PD**
	(g/L)	**(pH)**	**(ºC)**	**(g/L)**	**(g/L)**	**(g/L)**	**(g/L)**	**(g/L)**	**(g/L)**	**(g/L)**	**(g/L)**					**(g/L)**
1	(5) -1	(4) -1	(25) -1	(0) -1	(5) 1	(5) 1	(5) 1	(3) 1	(3) 1	(0.1) 1	(0) -1	-1	-1	-1	1	1.48
2	(35) 1	(4) -1	(25) -1	(0) -1	(0) -1	(0) -1	(0)) -1	(3) 1	(3) 1	(0.1) 1	(0.2)1	1	1	-1	-1	1.50
3	(5) -1	(8) 1	(25) -1	(0) -1	(0) -1	(5) 1	(5) 1	(0) -1	(0) -1	(0.1) 1	(0.2) 1	1	-1	1	-1	1.21
4	(35) 1	(8) 1	(25) -1	(0) -1	(5) 1	(0) -1	(0) -1	(0) -1	(0) -1	(0.1) 1	(0) -1	-1	1	1	1	9.25
5	(5) -1	(4) -1	(37) 1	(0) -1	(5) 1	(0) -1	(5) 1	(0) -1	(3) 1	(0) -1	(0.2) 1	-1	1	1	-1	0.64
6	(35) 1	(4) -1	(37) 1	(0) -1	(0) -1	(5) 1	(0) -1	(0) -1	(3) 1	(0) -1	(0) -1	1	-1	1	1	0.60
7	(5) -1	(8) 1	(37) 1	(0) -1	(0) -1	(0) -1	(5) 1	(3) 1	(0) -1	(0) -1	(0) -1	1	1	-1	-1	1.36
8	(35) 1	(8) 1	(37) 1	(0) -1	(5) 1	(5) 1	(0) -1	(3) 1	(0) -1	(0) -1	(0.2) 1	-1	-1	-1	-1	6.88
9	(5) -1	(4) -1	(25) -1	(5) 1	(5) 1	(5) 1	(0) -1	(3) 1	(0) -1	(0) -1	(0) -1	1	1	1	-1	0.99
10	(35) 1	(4) -1	(25) -1	(5) 1	(0) -1	(0) -1	(5) 1	(3) 1	(0) -1	(0) -1	(0.2) 1	-1	-1	1	1	5.79
11	(5) -1	(8) 1	(25) -1	(5) 1	(0) -1	(5) 1	(0) -1	(0) -1	(3) 1	(0) -1	(0.2) 1	-1	1	-1	1	2.14
12	(35) 1	(8) 1	(25) -1	(5) 1	(5) 1	(0) -1	(5) 1	(0) -1	(3) 1	(0) -1	(0) -1	1	-1	-1	-1	6.51
13	(5) -1	(4) -1	(37) 1	(5) 1	(5) 1	(0) -1	(0) -1	(0) -1	(0) -1	(0.1) 1	(0.2) 1	1	-1	-1	1	1.90
14	(35) 1	(4) -1	(37) 1	(5) 1	(0) -1	(5) 1	(5) 1	(0) -1	(0) -1	(0.1) 1	(0) -1	-1	1	-1	-1	6.15
15	(5) -1	(8) 1	(37) 1	(5) 1	(0) -1	(0) -1	(0) -1	(3) 1	(3) 1	(0.1) 1	(0) -1	-1	-1	1	-1	2.17
16	(35) 1	(8) 1	(37) 1	(5) 1	(5) 1	(5) 1	(5) 1	(3) 1	(3) 1	(0.1) 1	(0.2) 1	1	1	1	1	9.67
17(C)	(20) 0	(6) 0	(31) 0	(2.5) 0	(2.5) 0	(2.5) 0	(2.5) 0	(1.5) 0	(1.5) 0	(0.05) 0	(0.1) 0	0	0	0	0	5.68
18(C)	(20) 0	(6) 0	(31) 0	(2.5) 0	(2.5) 0	(2.5) 0	(2.5) 0	(1.5) 0	(1.5) 0	(0.05) 0	(0.1) 0	0	0	0	0	5.73
19(C)	(20) 0	(6) 0	(31) 0	(2.5) 0	(2.5) 0	(2.5) 0	(2.5) 0	(1.5) 0	(1.5) 0	(0.05) 0	(0.1) 0	0	0	0	0	5.89
20 (C)	(20) 0	(6) 0	(31) 0	(2.5) 0	(2.5) 0	(2.5) 0	(2.5) 0	(1.5) 0	(1.5) 0	(0.05) 0	(0.1)0	0	0	0	0	5.93

[a] X_1 raw glycerol: (-) = 5g/L, (+) = 35 g/L, X_2 initial pH : (-)= 4 , (+) = 8; X_3 Temperature: (-) = 25°C, (+) = 37°C; X_4, yeast extract: (-) = 0 g/L, (+) = 5 g/L; X_5 peptone: (-) = 0 g/L, (+) = 5 g/L; X_6 NH_4Cl: (-) = 0 g/L, (+) = 5 g/L; X_7 $(NH_4)_2SO_4$: (-) = 0 g/L, (+) = 5 g/L; X_8 KH_2PO_4: (-) = 0 g/L, (+) = 3 g/L; X_9 K_2HPO4: (-) = 0 g/L, (+) = 3 g/L; X_{10} $FeSO_4.7H_2O$: (-) = 0 g/L, (+) = 0.1 g/L; X_{11} Mg $FeSO_4.7H_2O$: (-) = 0 g/L, (+) = 0.2 g/L. [b] (-) low level; (+) high level ; (0) central point.

Table 2- Factorial Fractional design showing codified, real and results for 1,3-PD production.

	Variables[a] /Levels[b]							
Run	**X_1**	**X_2**	**X_3**	**X_4**	**X_5**	**X_6**	**X_7**	**1,3-PD (g/L)**
1	(35) -1	(7) -1	(5) -1	(5) -1	(3) -1	(3) -1	(0.1) -1	8.87
2	(65) 1	(7) -1	(5) -1	(5) -1	(7) 1	(3) -1	(0.3) 1	13.08
3	(35) -1	(9) 1	(5) -1	(5) -1	(7) 1	(7) 1	(0.1) -1	6.78
4	(65) 1	(9) 1	(5) -1	(5) -1	(3) -1	(7) 1	(0.3) 1	9.79
5	(35) -1	(7) -1	(9) 1	(5) -1	(7) 1	(7) 1	(0.3) 1	10.73
6	(65) 1	(7) -1	(9) 1	(5) -1	(3) -1	(7) 1	(0.1) -1	8.16
7	(35) -1	(9) 1	(9) 1	(5) -1	(3) -1	(3) -1	(0.3) 1	6.29
8	(65) 1	(9) 1	(9) 1	(5) -1	(7) 1	(3) -1	(0.1) -1	5.33
9	(35) -1	(7) -1	(5) -1	(9) 1	(3) -1	(7) 1	(0.3) 1	10.61
10	(65) 1	(7) -1	(5) -1	(9) 1	(7) 1	(7) 1	(0.1) -1	10.99
11	(35) -1	(9) 1	(5) -1	(9) 1	(7) 1	(3) -1	(0.3) 1	10.86
12	(65) 1	(9) 1	(5) -1	(9) 1	(3) -1	(3) -1	(0.1) -1	7.10
13	(35) -1	(7) -1	(9) 1	(9) 1	(7) 1	(3) -1	(0.1) -1	10.22
14	(65) 1	(7) -1	(9) 1	(9) 1	(3) -1	(3) -1	(0.3) 1	11.20
15	(35) -1	(9) 1	(9) 1	(9) 1	(3) -1	(7) 1	(0.1) -1	6.73
16	(65) 1	(9) 1	(9) 1	(9) 1	(7) 1	(7) 1	(0.3) 1	8.13
17(C)	(50) 0	(8) 0	(7) 0	(7) 0	(5) 0	(5) 0	(0.2) 0	9.45
18(C)	(50) 0	(8) 0	(7) 0	(7) 0	(5) 0	(5) 0	(0.2) 0	8.43
19(C)	(50) 0	(8) 0	(7) 0	(7) 0	(5) 0	(5) 0	(0.2) 0	10.27
20 (C)	(50) 0	(8) 0	(7) 0	(7) 0	(5) 0	(5) 0	(0.2) 0	9.51

[a] X_1 raw glycerol: (-) = 35g/L, (+) = 65 g/L, X_2 initial pH : (-)= 7 , (+) = 9; X_3 yeast extract: (-) = 5 g/L, (+) = 9 g/L; X_4 peptone: (-) = 5 g/L, (+) = 9 g/L; X_5 $(NH_4)_2SO_4$: (-) = 3 g/L, (+) = 7 g/L; X_6 K_2HPO4: (-) 0.1 g/L, (+) = 0,3 g/L; X_7 $FeSO_4.7H_2O$: (-) = 3 g/L, (+) = 7 g/L. [b] (-) low level; (+) high level ; (0) central point.

2.5 Batch and fed-batch bioreactor cultivations

The batch experiments were performed in 2L bioreactors (Biostat-B.Braun, Germany), fully equipped and controlled for temperature, pH, and feeding. The anaerobic cultivations were operated at 37 °C, 250 rpm and initial pH 7.0 for uncontrolled pH experiments. Nitrogen was sparged for 30 min before inoculation to assure anaerobic conditions. Bioreactors were inoculated with a 10 % (volume fraction) of a 1 OD cell concentration of overnight-grown cultures at 37 °C and 150 rpm.

The fed-batch experiments were conducted in 5L bioreactors (Biostat-B.Braun, Germany), with 3L of initial volume using the optimized medium and under the optimized conditions defined in the FFD design. Glycerol (as raw glycerol) was fed at 10 g/h at a constant flow rate of 0.4 $mL.min^{-1}$, with feedings started after 8 h of batch growth. Same procedures of inoculation and anaerobic conditions were used as in the batch experiments. The pH was controlled at 7.0 by the automatic addition of 10 M NaOH or 1 M H_3PO_4. All experiments were conducted in triplicates.

2.6 Analytical methods

The composition of fermentation broth was analyzed using a modular Shimadzu HPLC (Shimadzu Corp., Japan) equipped with a RID-10A refractive index detector. The stationary and mobile phases were an Aminex HPX-87H column (300 x 7.8 mm, Bio-Rad, USA) and 0.005 mol/L H_2SO_4 solution at 0.8 mL/min, respectively. The column temperature was controlled at 65°C. The culture samples were prepared by centrifugation at 3 500 *g* for 15 min to pellet cells, followed by filtration using cellulose acetate membrane filters (pore size 0.22 μm, Sartorius, Germany). Biomass concentration was estimated by cell dry weight.

2.7 Data Analysis

All experimental designs and results analyses were carried out using Statistica 7.0 (Statsoft, Tulsa, UK, USA). The significance of the regression coefficients ant the associated probabilities, *p(t)* were determined by Student's *t-test*. The variance explained by the model is given by the multiple determination coefficients, R^2.

3 Results and discussion

3.1 Plackett-Burman design

The P-B experimental design was used to evaluate the influence of glycerol, temperature, yeast extract, peptone and other inorganic sources of nitrogen, and several salts on 1,3-PD production by *K. pneumoniae* BLh-1. The variables and their ranges were chosen based on the literature [14,15,16,17]. Table 1 shows the P-B experimental design for each variable and the corresponding 1,3-PD production (g/L), which varied between 0.60 and 9.67 g/L. This variation reflects the importance of primary optimization in the way to achieve higher 1,3-PD productions. In Table 3 is presented the statistical analysis of the studied variables on 1,3-PD production.

Table 3- Effect estimates for 1,3-PD production from the result of Plackett-Burman design

Variables	Parameters	Effect	t-value	p<0.05
X_1	Raw glycerol	4.30	71.07	6.0E-06*
X_2	pH	2.51	41.53	3.1E-05*
X_3	Temperature	0.06	1.03	0.3782
X_4	Yeast extract	1.55	25.57	0.0001*
X_5	Peptone	2.05	33.82	5.7E-05*
X_6	NH_4Cl	0.00	0.00	1.0000
X_7	$(NH_4)_2SO_4$	0.92	15.22	0.0006*
X_8	KH_2PO_4	-1.10	-18.19	0.0003*
X_9	K_2HPO_4	0.18	2.97	0.0590*
X_{10}	$FeSO_4.7H_2O$	1.05	17.36	0.0004*
X_{11}	$MgS\ O_4.7H_2O$	0.15	2.51	0.0864

* Significance effect > 95 % confidence. Standard error: 0.1469; R^2 = 0.9086

Seven variables were found to be significant at 95 % significant level. The coefficient of determination (R^2) was calculated to be 0.9086 implying that 90.86 % of experimental data could be explained by the model. Raw glycerol showed a positive effect on 1,3-PD production, which increased steadily with glycerol. This result shows that *K. pneumoniae* BLh-1 has the ability to degrade raw glycerol present in the medium at high concentrations. The variables NH_4Cl, KH_2PO_4, and $MgSO_4.7H_2O$ had no significant influence on 1,3-PD production. The variable K_2HPO_4 was considered significant because its value was close to the 95 % of confidence level and therefore included in the next experimental design. The variation of temperature had shown no effect on 1,3-PD production. Usually, enterobacteria species have their optimal growth temperature ranging from 25°C to 40°C [18], range that was tested in this study. Therefore, the temperature was fixed at 37 °C as the optimal. According to the results presented in Table 3 and the statistical analysis, it was possible to select the main variables that have influence on 1,3-PD production as: raw glycerol, initial pH, yeast extract, peptone, K_2HPO_4, $(NH_4)_2SO_4$, and $FeSO_4.7H_2O$. These variables were further studied in a FFD design.

3.2 Fractional factorial design (FFD)

The seven variables (raw glycerol, pH, yeast extract, peptone, $(NH_4)_2SO_4$, K_2HPO_4 and $FeSO_4.7H_2O$) were studied in a new fractional factorial design 2^{7-3} with resolution IV. This statistical approach was chosen over the more commonly used central composite design (CCD), because it allowed reducing the amount of trials (from 128 to 16), without losing accuracy. The temperature was set at 37°C. Table 2 shows the 2^{7-3} fractional design with the results for 1,3-PD production. Productions of 1,3-PD were higher when compared with the P-

B design, with values ranging from 5.33 g/L to 13.08 g/L. The variables that showed significant effects on 1,3-PD production were pH, yeast extract, and K_2HPO_4. The pH range studied on the FFD was wider than in the P-B design, showing that high pH decreases the production of 1,3-PD. These results are in agreement with other studies showing a range of pH between 6 and 8 as the best for 1,3-PD production using *K. pneumoniae* species [16,15,11]. Therefore, the pH 7.0 was selected for further studies. Yeast extract is used as minerals and vitamins sources and is one of the necessary nutrients for microbial growth. According to the FFD design, the yeast extract had a negative effect indicating that high concentrations of this compound decrease the production of 1,3-PD. This is an important outcome because yeast extract is the most expensive medium component, and its concentration could be set at a low value indicated by the central point (5 g/L). Raw glycerol had no significant effect, but the maximal production occurred in the trials 2 and 14, both with the highest levels (65 g/L), subsequently this variable was set at 65 g/L. The $FeSO_4.7H_2O$ had no significant effect at 95 % confidence level. The $(NH_4)_2SO_4$ positively influenced the production of 1,3-PD, but its level was fixed in 7 g/L because higher concentrations could produce some growth inhibition. Peptone had a positive effect and its concentration was set at 5 g/L since this is also an expensive medium component. Table 4 presents the estimated effects for the production of 1,3-PD parameters of the fractional factorial design 2^{7-3}.

Table 4- Effect estimates for 1,3-PD production from the result of factorial fractional design.

Variables	Parameters	Effects	t-value	p- value
X_1	Raw glycerol	0.33	0.88	0.4396
X_2	pH	-2.85	-7.56	0.0047*
X_3	Yeast extract	-1.41	-3.73	0.0333*
X_4	Peptone	0.85	2.25	0.1090
X_5	$(NH_4)_2SO_4$	0.92	2.43	0.0926
X_6	$FeSO_4.7H_2O$	-0.12	-0.33	0.7590
X_7	K_2HPO4	2.06	5.46	0.0120*

* Significance effect > 95 % confidence.
Standard error: 0.5705; $R^2 = 0.9571$.

The coefficient of determination (R^2) was calculated to be 0.9571 for 1,3-PD. This implied that 95.71 % of the response for 1,3-PD production was explained by the model. The optimal conditions for 1,3-PD production were defined as: raw glycerol, 65 g/L; pH, 7.0; temperature, 37°C; yeast extract, 5 g/L; peptone, 5 g/L; $(NH_4)_2SO_4$, 7 g/L; K_2HPO_4, 7 g/L; and 1 mL/L of trace-elements. Experimental analyses were performed in quadruplicate in order to validate the model and the average of the experiments was 12.54 g/L (data not shown). The experimental value differed only 4.78 % of the maximal value found on FFD

design that was 13.08 g/L (Table 2, trial 2). According to the results from FFD design, further experiments using response surface methodology were not necessary.

There are few studies using experimental design to address the production of 1,3-PD. Oh et al. [15] used response surface methodology (RSM) in order to optimize the 1,3-PD production using raw glycerol with *K. pneumoniae* ATCC 700721. The authors used a central composite design 2^5 where the variables were: pH, glycerin, cultivation time, temperature, and $(NH_4)_2SO_4$. The optimized conditions were: 35 g/L crude glycerin, 8 g/L $(NH_4)_2SO_4$, pH 7.4, 8 h of culture at 37 °C, with a production of 13.74 g/L in batch bioreactor fermentations. The highest 1,3-PD production found in FFD design in this study was 13.08 g/L, which is remarkably high, considering that the experiments were performed in shaker and without pH control.

3.3 Batch and fed-batch cultivations

Using the optimized parameters obtained in the experimental designs, the scaling-up of 1,3-PD and ethanol productions were carried out in bioreactors, where other important environmental parameters could be tested. Both batch and fed-batch cultivations were tested. First, batch experiments were carried out in order to investigate the influence of pH control on kinetics and products, with results depicted in Figure 1 (uncontrolled pH) and Figure 2 (controlled pH). Under uncontrolled pH operation, the maximal production of 1,3-PD was 14.4 g/L, with some glycerol remaining after 32 h of cultivation, indicating that the pH, which dropped below 5 after 32 h due to the production of acetic and lactic acids, is an important physiological parameter for this bioprocess.

Ethanol was inhibited in this pathway, with low concentrations being produced (Fig.1b). However, under controlled pH cultivations (Fig. 2a), 1,3-PD production reached around 24 g/L, with very high yields of conversion (0.46 mol/mol) and productivity of 1.0 g/L.h. Zhang et al. [16] studied the rapid conversion of glycerol to 1,3-PD with a newly isolated strain of *K. pneumoniae* XJ-Li. The authors optimized the temperature and pH for production of 1,3-PD and found that the maximal concentration of 1,3-PD (12.2 g/L) in batch fermentation was obtained at pH 8.0 and 40 °C, with remarkable decreases of this product and biomass below pH 8.0.

Sattayasamitsathi et al. [19] studied the statistical optimization for the simultaneous productions of 1,3-PD and 2,3-butanediol using raw glycerol with a selected strain isolated from wastewater samples. The strain was identified as *K. pneumoniae* SU6. The authors developed a mathematical model to investigate the influences of each medium composition

and their interactions on the production of 1,3-PD and 2,3-BD under micro-aerobic and anaerobic cultivation. They reported maximal concentrations of 1,3-PD and 2,3-BD under micro-aerobic cultivation, higher than those obtained under anaerobiosis. The maximal yield of 1,3-PD (24.98 g/L) was achieved in the medium containing 200 g/L crude glycerol (mass fraction 27 %), 1.96 g/L yeast extract, 2.87 g/L ammonium phosphate and 2.16 g/L sodium fumarate. In this research, the batch production of 1,3-PD was similar compared with the cited works that were optimizating 1,3-PD production.

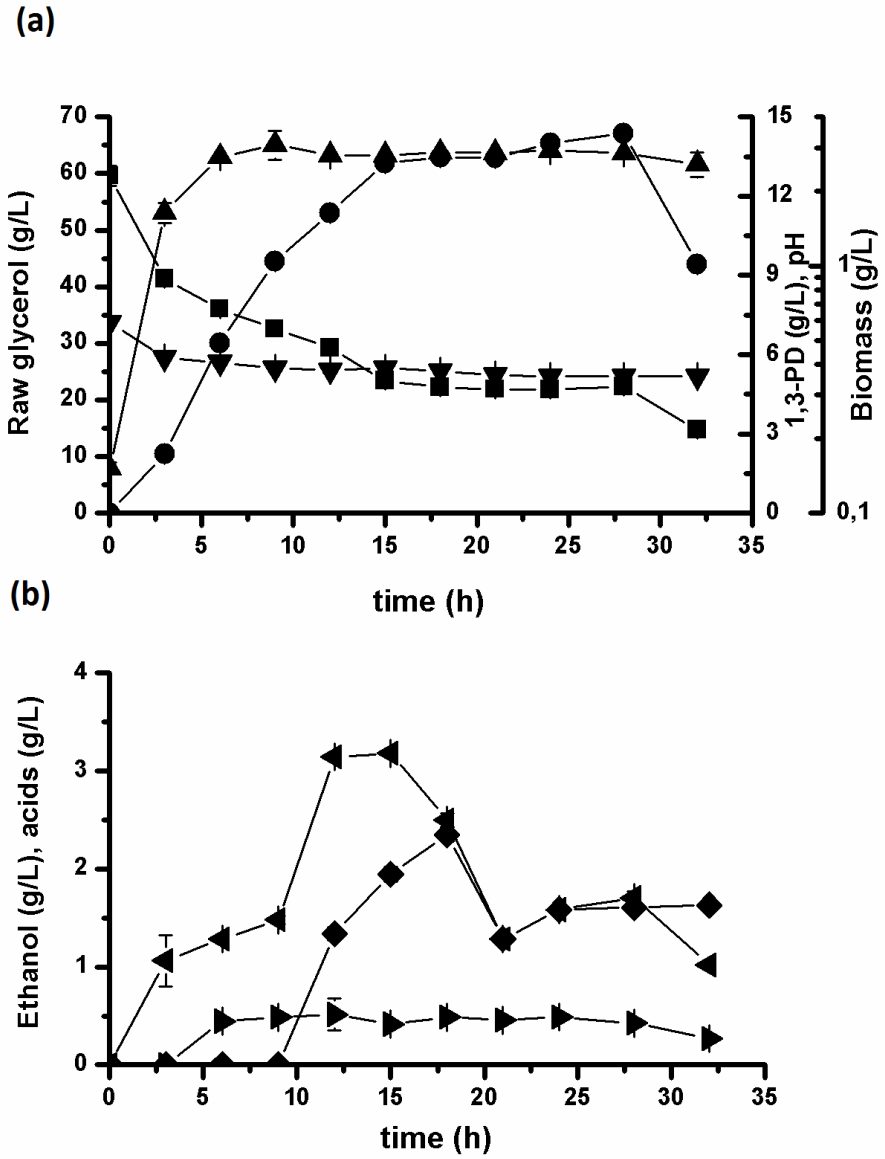

Figure 1- Batch cultivations with *K. pneumoniae* BLh-1 without pH control (a): (-■-) glycerol; (-●-) 1,3-PD; (-▲-) biomass; (-▼-) pH; (b): (-►-) ethanol; (-◄-) acetic acid; (-♦-) lactic acid. Results represent the mean of duplicates.

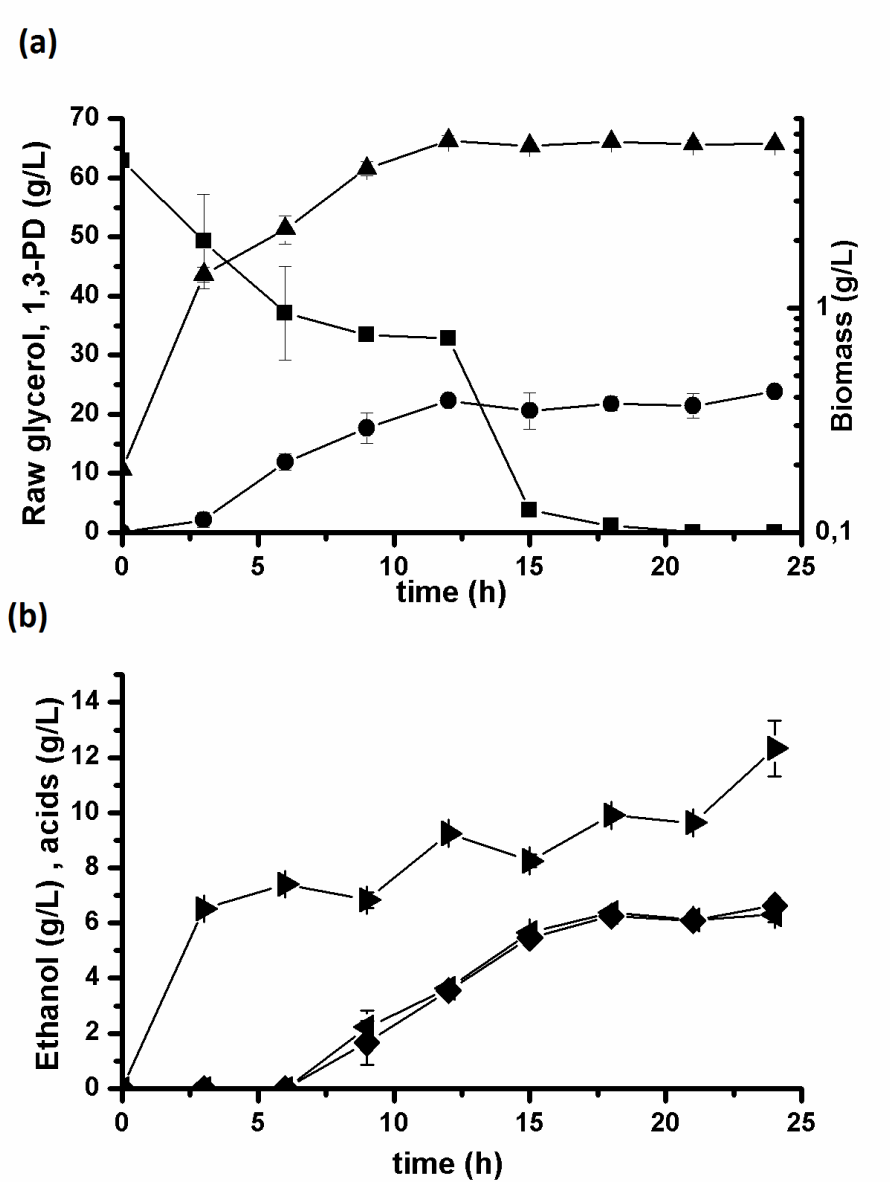

Figure 2- Batch cultivations of *K. pneumoniae* BLh-1 in bioreactors with controlled pH (a): (-■-) glycerol; (-●-) 1,3-PD; (-▲-) biomass; (b): (-►-) ethanol; (-◄-) acetic acid; (-♦-) lactic acid. Results represent the mean of duplicates.

In order to test whether the controlled feeding of glycerol could exert any influence over the production of 1,3-PD and ethanol, a set of fed-batch bioreactor experiments were run, with Figure 3 representing the kinetics of triplicates. By controlling the growth rate of cells, 1,3-PD production reached a maximal of 37 g/L in 48 h of cultivation, approximately 3 times higher when compared with optimized medium in FDD (13.10 g/L, run 2) and 2 times higher when compared with the batch, controlled pH experiments. Ethanol production was also enhanced, reaching 18 g/L compared to batch fermentation (12.30 g/L).

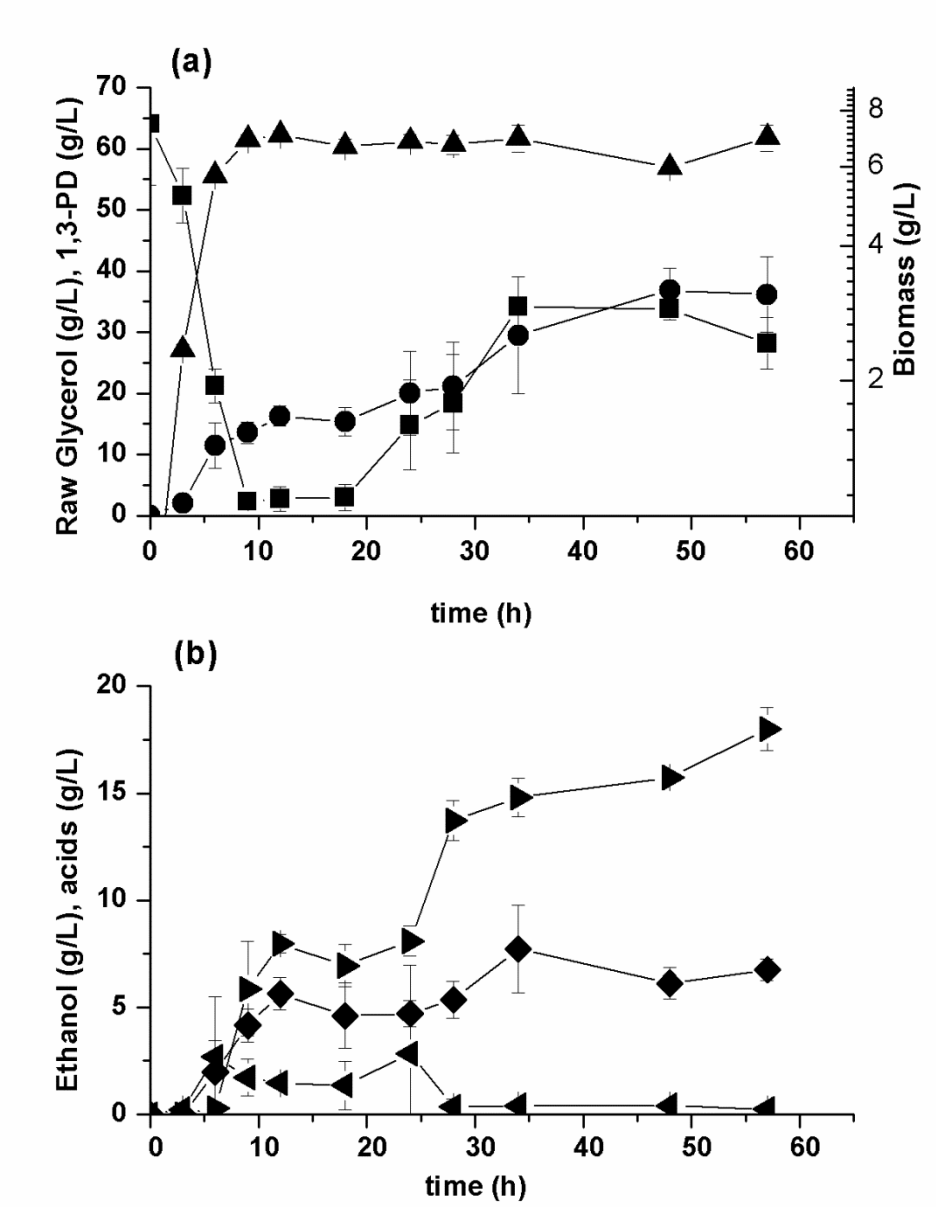

Figure 3- Fed-batch cultivation of *K. pneumoniae* BLh-1 in bioreactors (a): (-■-) glycerol; (-●-) 1,3-PD; (-▲-) biomass; (b): (-►-) ethanol; (-◄-) acetic acid; (-♦-) lactic acid. Results represent the mean of triplicates.

Assimilation of glycerol by *Klebsiella* is an oxido-reduction associated process that directly competes for NADH. The carbon flux from glycerol oxidation is channeled into glycolysis generating biomass and pyruvate-derived byproducts (such as ethanol, lactic acid, and 2,3-butanediol), while the reductive metabolism of glycerol generates 1,3-PD [20,21,22]. The physiologic purpose for the transformation of glycerol to 1,3-PD is to regenerate the oxidized form of the reducing equivalents, NAD^+, to be used in the energy-producing pathways of glycerol degradation [14]. In previous studies using *K.pneumoniae* BLh-1, Rossi et al. [13] had already observed the potential for 1,3-PD and ethanol production of this strain. The authors showed a 1,3-PD and ethanol production of 9.4 g/L and 4.96 g/L, respectively,

without optimized culture conditions. Wu et al. [23] studied the glycerol conversion into hydrogen, ethanol, and diols using a *Klebsiella* sp. HE1. Their results on batch fermentation showed maximal ethanol and 1,3-PD productions of 12.2 g/L and 12.5 g/L, respectively, at pH 6.0 and 50 g/L of glycerol at 35ºC. Oh et al. [24] used a mutant strain of *K. pneumoniae* obtained by γ-irradiation and named GEM 167, for ethanol production. The mutant increased the ethanol production up to 21.5 g/L with 0.25 g/L.h of productivity compared to control strain, *K. pneumoniae* Cu. Compared to cited literature, the results presented in this work confirmed the great capacity of the isolated *K.pneumoniae* BLh-1 for the simultaneous production of 1,3-PD and ethanol, shown for the first time.

4 Conclusion

The present study demonstrated that the use of P-B and FDD experimental designs could be used in combination to determine significant variables and best conditions for 1,3-PD production using the newly isolated strain of *K. pneumoniae* BLh-1. The results obtained for cultivation using raw glycerol from biodiesel synthesis as the sole carbon source showed that it is feasible to use this abundant, cheap surplus by-product in an industrially important bioprocess. Furthermore the batch and fed-batch cultivations have shown that the production of 1,3-PD could be coupled with that of ethanol and showing a fast and efficient glycerol conversion by this bacterial strain. These results showed that *K. pneumoniae* BLh-1 could be an excellent biological platform to be explored for the production of 1,3-PD, ethanol and other value-added byproducts. Further experiments are granted in order to investigate in deep the physiology and molecular mechanisms of glycerol conversion by *K. pneumoniae* BLh-1.

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CAPÍTULO VII – RESIDUAL GLYCEROL METABOLISM BY *KLEBSIELLA PNEUMONIAE*: POOL OF METABOLITES UNDER ANAEROBIOSIS AND MICROAEROBIOSIS AS A FUNCTION OF FEEDING RATES

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Residual glycerol metabolism by *Klebsiella pneumoniae*: pool of metabolites under anaerobiosis and microaerobiosis as a function of feeding rates

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ABSTRACT

The metabolism of residual glycerol from biodiesel synthesis uptake by *Klebsiella pneumoniae* BLh-1 was investigated in this study. Batch and fed-batch cultivations were performed in bioreactors under anaerobic and microaerobic conditions. Results of batch cultivations showed that the main product was 1,3-propanediol (1,3-PD) in both conditions, although the higher yields and productivities (0.46 $mol.mol^{-1}$ glycerol and 1.22 $g.L^{-1}.h^{-1}$, respectively), were obtained under anaerobic condition. Large amounts of ethanol were also produced under batch anaerobic condition, peaking at 12.30 $g.L^{-1}$. Microaerobic batch cultivations were characterized by faster growth kinetics, with higher biomass production, but lower conversions of glycerol into 1,3-PD, with yields and productivities of 0.33 $mol.mol^{-1}$ glycerol and 0.99 $g.L^{-1}.h^{-1}$, respectively. The fed-batch cultivations were carried out in order to test the effects of feeding of glycerol on cells. Fed-batch under anaerobiosis showed that 1,3-PD and ethanol concentrations increased with the feeding rate, with maximal productions of 26.12 $g.L^{-1}$ and 19.2 $g.L^{-1}$, respectively. The microaerobic conditions diverted the bacterium metabolism to an elevated lactic acid formation, reaching 59 $g.L^{-1}$ in higher feeding rates of glycerol, with a lower production of ethanol.

Keywords: raw glycerol, *Klebsiella pneumoniae*, 1,3-propanediol, cultivation.

1 Introduction

Biodiesel, which is a mixture of methyl esters of fatty acids, is mainly produced from vegetable oils by transesterification with methanol. As a by-product, 1 mol of glycerol is produced for every 3 mols of methyl esters, which is equivalent to approximately a mass fraction of 10 % of the total product (Karinen and Krausen 2006). The generated glycerol is usually contaminated with chemicals such as salts and methanol, water, and has a low pH, which makes this by-product of the biodiesel industry of little use to the more sophisticated industries of cosmetics, paints, automobiles, foods, tobacco, pharmaceuticals, pulp and paper, leather, and textiles, where glycerol is an important ingredient. It also finds limited use as a feedstock for the production of various chemicals (Wang et al. 2001). Therefore, new uses for this abundant industrial residue must be devised in order to reduce the cost of biodiesel production.

Since pure glycerol is both a cell metabolite and a carbon substrate for growth, the residual glycerol from biodiesel could be postulated as a substrate for microorganisms that are able to grow on glycerol as the sole carbon and energy source. Several strains of *Citrobacter freundii* (Homann et al. 1990; Daniel et al. 1995), *Klebsiella pneumoniae* (Forage and Lin 1982; Biebl et al. 1998; Németh et al. 2003), *Clostridium butyricum* (Biebl et al. 1992; Himmi et al. 1999), and *Lactobacillus reuteri* (Talarico et al. 1990), have shown the ability to grow on glycerol. The microbial bioconversion of glycerol into various compounds has been recently investigated, with particular focus on the production of 1,3-propanediol (1,3-PD), which can be applied as a basic ingredient of polyesters (Zheng et al. 2008; Oh et al. 2008; Cheng et al. 2004). The industrial production of 1,3-PD has attracted attention as an important monomer to synthesize a new type of polyester, polytrimethylene terephthalate, which finds great potential in textiles and carpets. 1,3-PD can also be used in other fields such as cosmetics, in replacing fossil oil-based glycols, drugs production, lubricants, among others (Zeng and Biebl 2002; Sauer et al. 2008).

Sauer et al. (2008) elegantly revised the metabolic reactions proposed for the bioconversion of glycerol, which is presented in Figure 1. There are two main routes, depending on the environmental conditions and strain characteristics.

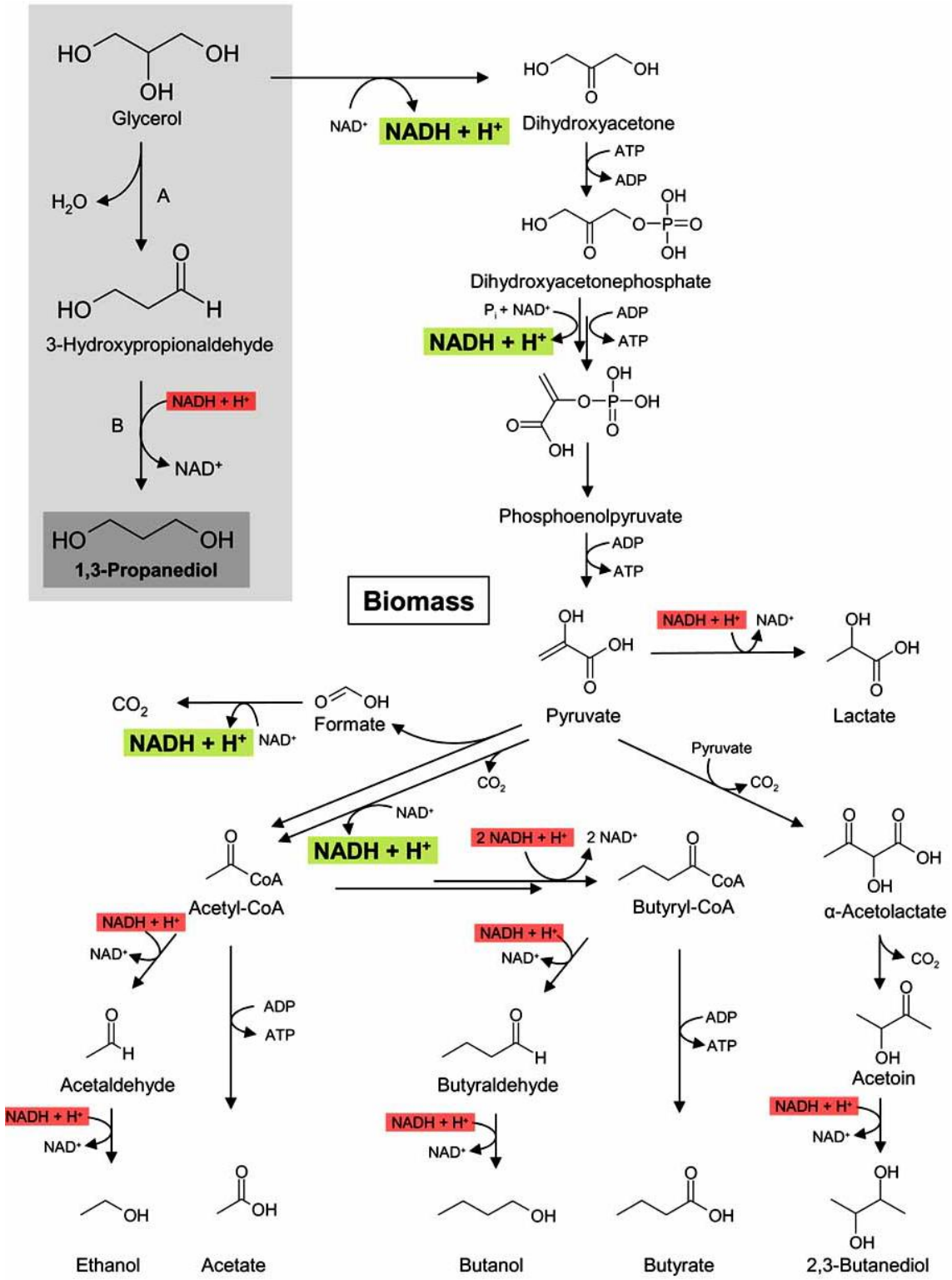

Figure 1: Metabolic pathways involved glycerol dissimilation by *K. pneumoniae* according to Sauer et al. (2008).

In the oxidative pathway, glycerol is converted to dihydroxyacetone by glycerol dehydrogenase (GDH); and is subsequently phosphorylated by adenosine triphosphate–dependent dihydroxyacetone kinase. Dihydroxyacetone-phosphate undergoes normal

glycolysis to form pyruvate, which is further converted into various organic acids (lactic acid, acetic acid, succinic acid), 2,3-butanediol, hydrogen, CO_2, and ethanol. Glycerol is also converted through the reductive pathway to 3-hydroxypropionaldehyde (3-HPA) by the coenzyme B12-dependent glycerol dehydratase (GDHt). 3-HPA is then converted by a $NADH_2$-linked oxidoreductase to 1,3-PD, which is then excreted from the cell. The physiologic function for the transformation of glycerol into 1,3-PD is probably to regenerate the oxidized form of the reducing equivalents, NAD^+, to be used in the energy-producing pathways of glycerol degradation (Huang et al. 2002). Questions arise whether these pathways could be reproduced under closer industrial cultivation conditions such as in bioreactor-grown cells and with residual glycerol instead of its pure form.

K. pneumoniae is a metabolically versatile bacterium that is able to grow both in the presence and absence of oxygen and has been widely investigated as a 1,3-PD producer under anaerobic conditions (Biebl et al. 1998, Cheng et al. 2004). Some authors also reported the production of 1,3-PD under microaerobic or low aerobic conditions (Zhang and Xiu 2009; Chen et al. 2003; Cheng et al. 2004). However, other important biotechnological products could be obtained from glycerol bioconversion, among them ethanol, by far the most important biofuel, and organic acids, but very little information on the basic metabolism of their production has been reported so far. Therefore, the goals of this study were to investigate the metabolism of residual glycerol from biodiesel synthesis by *K. pneumoniae* BLh-1, a newly isolated strain, in bioreactor cultivations under anaerobic and microaerobic conditions, and the effects of glycerol feeding in fed-batch cultivations.

2 Material and Methods

2.1 Residual raw glycerol

The raw, or residual, glycerol was supplied by a soybean biodiesel manufacturing plant (Passo Fundo, Brazil) and contained (as mass fraction): 82.4 % glycerol, 5.73 % ash, 6.17 % NaCl, 11.5 % moisture, 0.4 % monoacylglycerols, pH 5.13. The biodiesel production is obtained by transesterification of degummed soybean oil using methanol or sodium methylate.

2.2 Microorganism maintenance and inoculum preparation

K. pneumoniae BLh-1 was isolated and characterized in a previous work (Rossi et al. 2012) and was used in this research. Cultures were maintained on Luria–Bertani (LB) agar slant at 4°C and it was subculture every 4 weeks. Inocula were prepared by transferring one loopfull of cells into 125 mL flasks filled with 50 mL of medium and then incubated at 37°C in a rotary shaker at 150 rpm overnight. The medium contained (in $g.L^{-1}$): 5 yeast extract, 5 peptone, 7 $(NH_4)_2SO_4$, 7 K_2HPO_4, 1 mL trace elements solution (containing in $g.L^{-1}$: 0.1 $MnCl_2.4\ H_2O$, 0.06 H_3BO_3, 0.0037 $CuSO_4.5H_2O$, 0.2 $CoCl_2.6H_2O$, 0.025 $NiCl_2.6\ H_2O$, 0.035 $Na_2MoO_4.2\ H_2O$, 0.14 $ZnSO_4.7H_2O$, 0.9 mL HCl 37 %), and 65 of raw glycerol. The inocula preparations were adjusted to optical density (OD) 1.0 at 600 nm. This procedure was used as the standard inocula preparations for all experiments.

2.3 Batch cultivations

The batch experiments were performed in a 2L stirred-tank bioreactor (Biostat B model, B. Braun Biotech International, Germany), fully equipped with pH, temperature, agitation (2 six flat-blade turbines), aeration, and flow controls. The anaerobic bioreactors were operated at 37°C, 250 rpm and controlled pH 7.0. Nitrogen was sparged for 30 min before inoculation to ensure anaerobic conditions. The microaerobic bioreactors were operated at 37°C, 300 rpm, controlled pH 7.0 and 0.4 vvm of air. The bioreactors were inoculated with a volume fraction 10 % of an inoculum grown overnight at 37°C and 150 rpm in 500 mL Erlenmeyer flask filled with 200 mL of medium. The inoculum was standardized O.D 1.0 (600 nm). All experiments were performed in duplicates and the medium used was the same used for the inocula preparations, described above.

2.4 Fed-Batch strategies

The same bioreactors were used for the fed-batch experiments. Different glycerol concentrations were used for feeding under anaerobic and microaerobic conditions. The feeding rates of glycerol were: 2.5 $g.h^{-1}$, 5.0 $g.h^{-1}$, 7.5 $g.h^{-1}$, and 10.0 $g.h^{-1}$. Cultivations were started in batch mode to allow cell accumulation and adaptation and then feeding was started at 9 h of cultivation and fed at a constant flow rate of 0.3 $mL.min^{-1}$ during 24 h. Bioreactors were inoculated with a volume fraction 10 %, OD = 1 (600 nm) of the preinocula cultures. Experiments were conducted at 37°C, 250 rpm, and controlled pH 7.0 with nitrogen sparged for 30 min for anaerobic conditions before the cultivation.

The microaerobic bioreactors were operated at 37 °C, 300 rpm, controlled pH 7.0, and 0.4 vvm of air. The pH was controlled by the automatic addition of 10 M NaOH or 1 M H_3PO_4.

2.5 Analytical methods

The composition of the cultivation broth was analyzed using a Shimadzu HPLC (Shimadzu Corp.) equipped with a RID-10A refractive index detector. The stationary and mobile phases were an Aminex HPX-87H column (300 x 7.8 mm, Bio-Rad, USA) and 0.005 $mol.L^{-1}$ H_2SO_4 solution at 0.8 $mL.min^{-1}$, respectively. The column temperature was controlled at 65°C. The culture samples were prepared by centrifugation at 3 500 *g* for 15 min to pellet the cell, followed by filtration using cellulose acetate membrane filters (pore size 0.22 μm, Sartorius, Germany). Biomass concentration was estimated by measuring optical density (600 nm) and cell dry weight method.

3 Results

3.1 Batch cultivations

The *K. pneumoniae* BLh-1 strain was isolated from an environment microbial consortium and has shown to be an efficient producer of 1,3-PD and ethanol, both under anaerobiosis and aerobiosis in agitated flask experiments (Rossi et al. 2012). In this work, the scaling-up of the cultivations to bioreactors, allowed for a more accurate analysis of its metabolism. The time courses of 1,3-PD, glycerol, biomass and other metabolites concentrations in batch cultivations of *K. pneumoniae* BLh-1 under anaerobic and microaerobic conditions are shown in Fig 2a and 2b, respectively. Glycerol was rapidly consumed and it was exhausted after 15 h of cultivation in anaerobic condition, producing 23.8 $g.L^{-1}$ and 12.3 $g.L^{-1}$ of 1,3-PD and ethanol, respectively. Other metabolites such as lactic acid and acetic acid were produced in lower concentrations. Under microaerobic conditions, glycerol was exhausted after 12 h of cultivation, with higher and faster cell production, but with lower productions of 1,3-PD, ethanol, and other metabolites. In Table 1 are compared the kinetic parameters for anaerobic and microaerobic batch cultivations. 1,3-PD was the main end product for both conditions, followed by ethanol and their yields and productivities were higher under anaerobiosis.

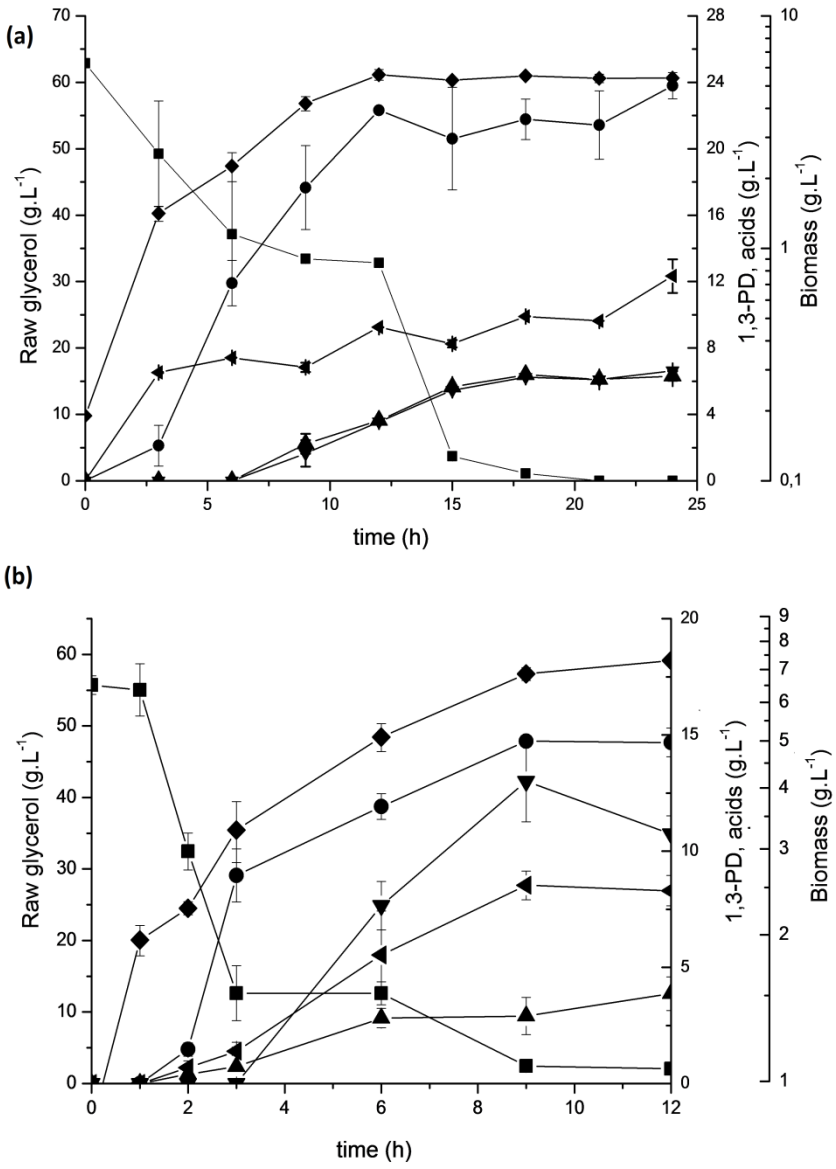

Figure 2: Batch cultivations of *K. pneumoniae* BLh-1 under anaerobic conditions (a) and under microaerobic cultivation (b). The symbols represent: (-■-) raw glycerol; (-●) 1,3-PD; (-▲-) acetic acid; (-▼-) lactic acid; (-◄-) ethanol; (-♦-) biomass. Results represent the mean of duplicates.

Table 1- Kinetic parameters of batch cultivation under anaerobic and microaerobic conditions.

	$C_{1,3\text{-PD}}$ ($g.L^{-1}$)	C_{ETOH} ($g.L^{-1}$)	$Y_{X/S}$ ($g.mol^{-1}$)	$Y_{1,3\text{-PD}}$ ($mol.mol^{-1}$)	Y_{ETOH} ($mol.mol^{-1}$)	Y_{lactic} ($mol.mol^{-1}$)	Y_{acetic} ($mol.mol^{-1}$)	$Q_{1,3\text{-PD}}$ ($g.L^{-1}.h^{-1}$)	Q_{ETOH} ($g.L^{-1}.h^{-1}$)
Batch									
Anaerobic (24h)	23.80	12.30	7.64	0.46	0.40	0.10	0.16	0.99	0.51
Micro-aerobic (12h)	14.67	8.30	11.22	0.33	0.30	0.20	0.11	1.22	0.69

3.2 Fed-batch cultivations

Fed-batch experiments were conducted in order to study the effect of controlled feeding rates of raw glycerol on the metabolism of *K. pneumoniae* BLh-1. Figure 3 shows the kinetics of anaerobic cultivations with different glycerol feedings. The cultivation using 2.5 $g.h^{-1}$ of raw glycerol (Fig.3a and 3b) showed fast glycerol consumption until 10 h remaining below 10 $g.L^{-1}$ after that. The maximal ethanol and 1,3-PD productions were 15.9 $g.L^{-1}$ and 20.4 $g.L^{-1}$, respectively. When feeding increased to 5.0 $g.h^{-1}$ (Fig.3c and 3d) during cultivation, almost all glycerol was consumed until 15 h and the main metabolites increased at a low rate with maximal productions of 19.8 $g.L^{-1}$ of ethanol in 28 h and 20 $g.L^{-1}$ of 1,3-PD in 48 h. Cultivations with feeding rates of 7.5 $g.h^{-1}$ (Fig. 3e and 3f) and 10.0 $g.h^{-1}$ (Fig. 3g and 3h) showed little increase in ethanol production, but 1,3-PD had a 35 % increased production, reaching 27.3 $g.L^{-1}$ at feeding rate of 10.0 $g.h^{-1}$, after 60 h of cultivation. However, at the feeding rate of 10.0 $g.h^{-1}$, glycerol accumulated in the bioreactors, suggesting that feeding was higher than the maximal cell growth rate. The results obtained for the feeding rate of 2.5 $g.h^{-1}$ shows that lactic and acetic acids (Fig. 3b) were not produced during the course of fed-batch cultivation, indicating that under this condition, cell metabolism was entirely unrepressed. Except for the feeding rate of 10.0 $g.h^{-1}$, the feeding strategies were able to avoid the production of lactic acid, the main competitor with ethanol production.

Under the microaerobic conditions (Figures 4a-4h) a surprisingly high production of lactic acid was observed when increasing the feeding rates of glycerol, reaching 59 $g.L^{-1}$ in 40 h of cultivation (Fig. 4f and 4h). Glycerol consumption was complete for the feeding rates of 2.5, 5.0, and 7.5 $g.h^{-1}$, but not for 10.0 $g.h^{-1}$, again suggesting feeding was higher than the maximal growth rate of *K. pneumoniae* BLh-1. The highest 1,3-PD production was 28.2 $g.L^{-1}$ with 10.0 $g.L^{-1}$ feeding of glycerol. The yields of conversion of glycerol into 1,3-PD were similar in both conditions. The highest 1,3-PD yields were 0.49 $mol.mol^{-1}$ glycerol under anaerobiosis and 0.46 $mol.mol^{-1}$ glycerol under microaerobic conditions (Table 3). The highest 1,3-PD and ethanol productivities were 2.6 and 1.17 $g.L^{-1}.h^{-1}$, respectively, both in anaerobic cultivations (Table 2).

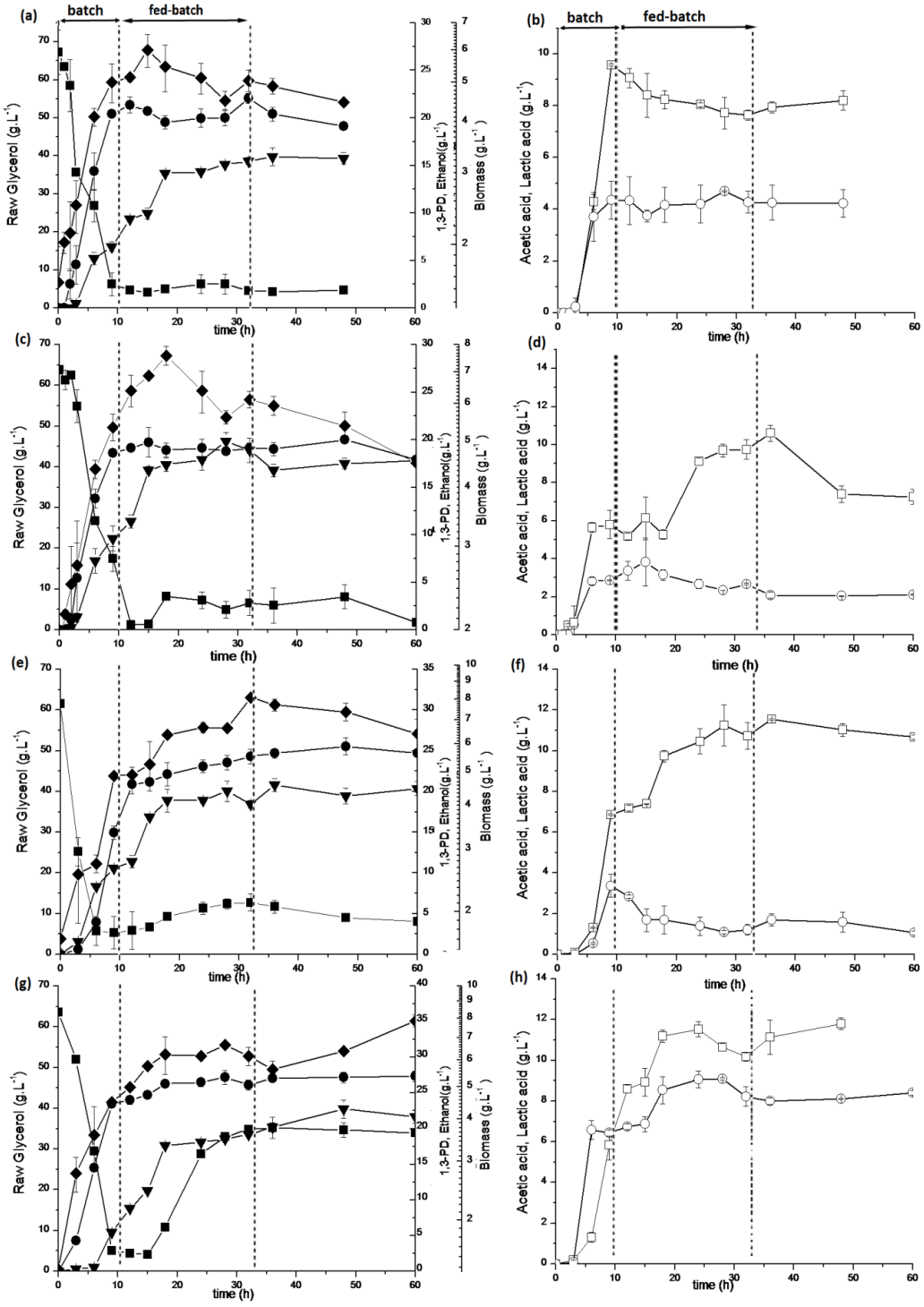

Figure 3:Fed-batch cultivations of *K. pneumoniae* BLh-1 under anaerobic conditions. 3a and 3b: 2.5 $g.h^{-1}$ fed raw glycerol, 3c and 3d: 5 $g.h^{-1}$ fed raw glycerol, 3e and 3f: 7.5 $g.h^{-1}$ fed raw glycerol, 3g and 3h: 10 $g.h^{-1}$ fed raw glycerol. The symbols represent:(-■-) raw glycerol; (-●-) 1,3-PD; (-○-) acetic acid; (-▼-) ethanol;(-□-) lactic acid; (-♦-) biomass. Results represent the mean of duplicates.

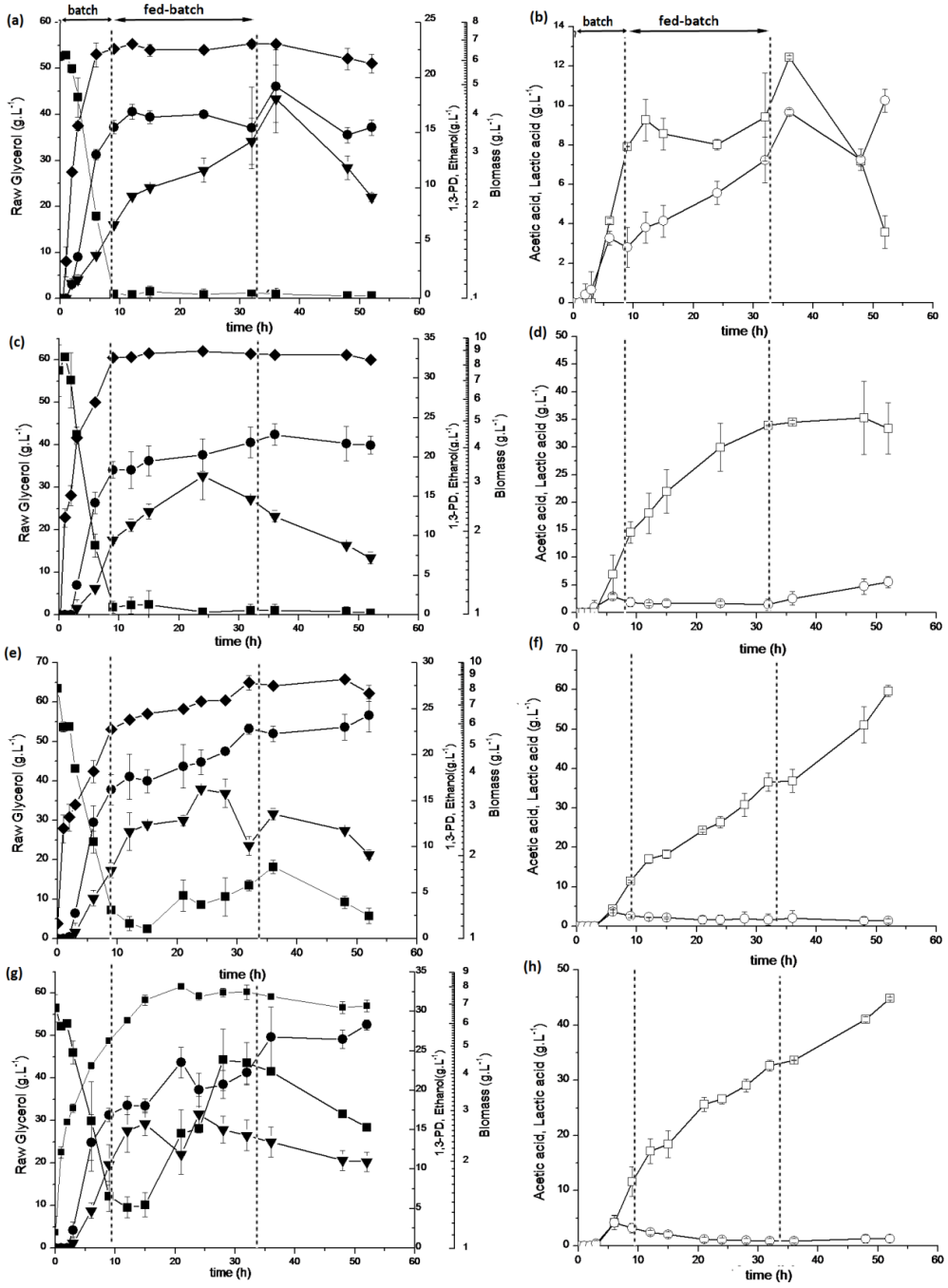

Figure 4: Fed-batch cultivations of *K. pneumoniae* BLh-1 under microaerobic conditions. 4a and 4b: 2.5 $g.h^{-1}$ fed raw glycerol, 4c and 4d: 5 $g.h^{-1}$ fed raw glycerol, 4e and 4f: 7.5 $g.h^{-1}$ fed raw glycerol, 4g and 4h: 10 $g.h^{-1}$ fed raw glycerol. The symbols represent: (-■-) raw glycerol; (-●-) 1,3-PD; (-○-) acetic acid; (-▼-) ethanol; (-□-) lactic acid; (-♦-) biomass. Results represent the mean of duplicates.

Table 2- Kinetic parameters from anaerobic cultivation under different feeding rates.

	Time cultivation (h)	$C_{1,3\text{-PD}}$ ($g.L^{-1}$)	C_{ETOH} ($g.L^{-1}$)	C_{HAC} ($g.L^{-1}$)	C_{HLAC} ($g.L^{-1}$)	$Y_{X/S}$ ($g.mol^{-1}$)	$Y_{1,3\text{-PD}}$ ($mol.mol^{-1}$)	Y_{ETOH} ($mol.mol^{-1}$)	Y_{HLAC} ($mol.mol^{-1}$)	$Q_{1,3\text{-PD}}$ ($g.L^{-1}.h^{-1}$)	Q_{ETOH} ($g.L^{-1}.h^{-1}$)	Q_{HLAC} ($g.L^{-1}.h^{-1}$)
Feeding rate glycerol ($g.h^{-1}$)												
2.5	9*	20.41	6.45	4.35	9.56	5.11	0.40	0.21	0.16	2.26	0.71	1.06
	32**	22.05	15.45	4.27	7.64	0.11	0.05	0.45	-	0.07	0.39	-
5.0	9	18.61	9.6	2.86	5.80	6.94	0.49	0.39	0.10	2.12	1.06	0.64
	32	19.13	18.89	2.67	9.75	0.81	0.007	0.22	0.04	0.02	0.41	0.17
7.5	9	14.93	10.57	3.37	6.86	5.23	0.31	0.36	0.12	1.66	1.17	0.76
	32	24.29	18.43	1.21	10.72	2.83	0.10	0.14	0.04	0.4	0.34	0.17
10.0	9	23.44	5.46	6.43	5.84	4.83	0.48	0.18	0.10	2.6	0.6	0.64
	32	26.12	19.20	8.22	10.17	1.25	0.03	0.22	0.04	0.11	0.6	0.18

*The parameters were calculated from 0-9h of cultivation, ** The parameters were calculated from 9-32 h of cultivation.

Table 3-Kinetic parameters from microaerobic cultivation under different feeding rates.

	Time cultivation (h)	$C_{1,3\text{-PD}}$ ($g.L^{-1}$)	C_{ETOH} ($g.L^{-1}$)	C_{HAC} ($g.L^{-1}$)	C_{HLAC} ($g.L^{-1}$)	$Y_{X/S}$ ($g.mol^{-1}$)	$Y_{1,3\text{-PD}}$ ($mol.mol^{-1}$)	Y_{ETOH} ($mol.mol^{-1}$)	Y_{HLAC} ($mol.mol^{-1}$)	$Q_{1,3\text{-PD}}$ ($g.L^{-1}.h^{-1}$)	Q_{ETOH} ($g.L^{-1}.h^{-1}$)	Q_{HLAC} ($g.L^{-1}.h^{-1}$)
Feeding rates glycerol ($g.h^{-1}$)												
2.5	9*	13	6.65	2.81	7.92	10.30	0.36	0.26	0.15	1.72	0.74	0.88
	32**	15.44	14.22	7.24	9.41	0.56	0.08	0.40	0.04	0.10	0.33	0.06
5.0	9	18.38	9.45	1.87	14.55	10	0.40	0.34	0.15	2.04	1.05	1.05
	32	21.85	14.70	1.42	33.9	0.24	0.05	0.14	0.25	0.15	0.23	0.84
7.5	9	16.17	7.41	2.56	11.39	7.52	0.35	0.26	0.21	1.79	0.82	1.26
	32	22.80	10	1.69	36.54	2.33	0.07	0.05	0.23	0.28	0.11	1.09
10.0	9	16.87	10.55	3.22	11.63	8.44	0.46	0.46	0.26	1.87	1.17	1.29
	32	22.20	14.27	0.9	32.66	1.86	0.05	0.06	0.18	0.23	0.16	0.91

*The parameters were calculated from 0-9h of cultivation, ** The parameters were calculated from 9-32 h of cultivation

4 Discussion

Results in this research show that for batch bioreactor cultivations, *K. pneumoniae* BLh-1 is able to simultaneously produce 1,3-PD and ethanol as main metabolites, under both anaerobiosis and microaerobiosis conditions (Fig. 2). The main metabolite product was 1,3-PD under both conditions, with yields and productivities higher in anaerobiosis. Ethanol was mainly produced in anaerobiosis and lactic acid under microaerobiosis. Acetic acid was produced in low concentrations. As expected, under microaerobic conditions, biomass production was enhanced, while 1,3-PD and ethanol formation were reduced. Chen et al. (2003) suggested that dissolved oxygen activates the gene expression system for the enzymes of the citric acid cycle (CAC) in *Klebsiella*, with more energy available through the production of ATP for cell growth. Cheng et al. (2004) studied the production of 1,3-PD by *K. oxytoca* M5al (which was previously classified as *K. pneumoniae*) under different aeration strategies, using 50 $g.L^{-1}$ of glycerol in batch cultivations. The authors showed that yields and productivities of 1,3-PD reduced proportionally with aeration and were also lower than values for anaerobic cultivations. The authors reported the highest yield of 0.54 $mol.mol^{-1}$ glycerol and productivity of 0.62 $g.L^{-1}.h^{-1}$ obtained under anaerobiosis, while in aerobic cultures (0.4 vvm, 150 rpm), the yield and productivity were 0.46 $mol.mol^{-1}$ glycerol and 0.57 $g.L^{-1}.h^{-1}$, respectively. Comparatively, under the conditions of this work, yields of 0.33 $mol.mol^{-1}$ and productivities of 0.99 $g.L^{-1}.h^{-1}$ (microaerobic) and 1.22 $g.L^{-1}.h^{-1}$ (anaerobic) were observed for 1,3-PD. However, it is important to observe that different strains will certainly vary in their metabolisms. Furthermore, in all preceding works authors used chemically pure glycerol, while the entire research described in this paper was carried out using raw glycerol from biodiesel synthesis.

Fed-batch cultivations of *K. pneumoniae* BLh-1 were run to allow the control of cell growth rate, and therefore to study the effect of feeding of raw glycerol in the general metabolism of this bacterium. According to results presented on Table 2, under anaerobic condition the ethanol production was increased with the feeding of glycerol, suggesting that its formation, as well as of organic acids, was linked to the consumption of reducing equivalents necessary to 1,3-PD production. This metabolism sink of NAD/NADH contributed to the small increase of 1,3-PD concentration during fed-batch cultivations. According to Zhang et al. (2006), the NADH required for the biosynthesis

of 1,3-PD is mainly regenerated from NAD^+ along with the oxidation of glycerol. Ethanol is one of the major byproducts and its formation competes for NADH, thus unfavorable for the production of 1,3-PD by *K. pneumoniae.*

Cheng et al. (2005) studied the multiple growth inhibition of *K. pneumoniae* M5a1 in 1,3-PD fermentation. Fed-batch experiments have shown that 1,3-PD fermentation by *K. pneumoniae* proceeded in two phases; in phase I, cells grew to a maximal concentration and 1,3-PD was rapidly produced; during phase II, byproducts accumulated reaching up to 6 $g.L^{-1}$ acetate, 6 $g.L^{-1}$ lactate, and 10 $g.L^{-1}$ of ethanol at 28 h, with considerable reductions in cell growth and 1,3-PD production. The authors then performed batch fermentations adding a mixture of all inhibitory byproducts – to concentrations similar of that obtained in the fed-batch cultivations - in order to examine whether these metabolites were responsible for impairing cell growth and 1,3-PD productivity, their results confirming dramatically decreases for both. A previous work (Costa et al. 2011) with *K. pneumoniae* BLh-1 showed that it produces hydrogen along with ethanol. Zhang et al. (2008) studied the metabolic flux and robustness of glycerol metabolism in *K. pneumoniae* DSM2026 and showed that the fluxes of ethanol and hydrogen decreased with increasing dilution rates on continuous anaerobic cultivations, suggesting that the formation of ethanol and hydrogen strongly impaired the production of 1,3-PD, caused by either the consumption or the no-formation of reducing equivalents required for the synthesis of 1,3-PD.

Usually, lactic acid was not investigated in other studies. The critical concentration of lactate to cause cell growth inhibition of *K. pneumoniae* was assessed to be 19 and 26 $g.L^{-1}$ under anaerobic and aerobic conditions, respectively (Cheng et al. 2005). However, it was shown in this work that lactic acid was the main metabolite produced under microaerobic conditions and had rapidly increased with the feeding rate of glycerol (Table 3 and Fig. 3). Menzel et al. (1997) studied the *K. pneumoniae* DSM 2026 in continuous fermentation to enhance 1,3-PD concentration and productivity. The authors showed that with a low glycerol concentration in the feeding medium, biomass and products increased with increasing glycerol concentration. Further increases of glycerol resulted in substrate excess. Under conditions of excess of glycerol in the medium, biomass concentration decreased markedly, while 1,3-PD increased and leveled off at relatively high concentrations of residual glycerol. However, ethanol was drastically reduced, while lactic acid (150 mmol.L-1), and 2,3-butanediol (300 mmol.L-

1) were found in considerable concentrations in the culture. Yang et al. (2007) reported that *K. oxytoca* M5al is an excellent 1,3-PD producer, but showed that this strain produces high concentrations of lactic acid. To overcome this disadvantage, the authors used metabolic engineering in order to eliminate lactic acid production by deleting ldhA gene coding for lactate dehydrogenase (LDH) in *K. oxytoca* M5al. The authors were able to increase 1,3-PD production, its productivity, and the molar conversion rate from glycerol to 1,3-PD compared to the wild type strain. The authors also showed that the fed-batch cultivation of the recombinant strain, using sucrose as a co-substrate under microaerobic conditions, improved the synthesis of 1,3-PD, obtaining 83.53 $g.L^{-1}$ with no lactic acid production. Zheng et al. (2008), studying the physiologic mechanism of 1,3-PD synthesis in fed-batch cultivations of *K. pneumoniae* CGMCC 1.6366, have found that the dominant flux distribution was shifted from acetate to 1,3-PD formation during early-exponential phase and then to lactate synthesis in the late-exponential growth phase.

Acetic acid production was lower in both cultivations, not exceeding 9 $g.L^{-1}$ under anaerobic conditions at high glycerol feeding (Fig.3h). Acetic acid does not compete for reducing equivalents, since its excretion from cells generates energy in the form of ATP, and the maximal supply of NADH for 1,3-PD synthesis is provided if acetic acid is the exclusive metabolite of the oxidative branch of glycerol pathway (Zeng and Biebl. 2002). According to Zheng et al. (2008), while the excretion of acetate generates energy in the form of ATP, excretion of other metabolites, for example, lactic acid and ethanol, consume reducing equivalents. Although the production of acetic acid can generate additional ATP, studies showed that its production can cause cell growth inhibition and this is deleterious to 1,3-PD production, which is growth associated (Zhang et al., 2006). Cheng et al. (2006) studied the 1,3-PD production by glycerol fermentation using the broth from *C. krusei*, used to actually produce glycerol. The authors showed that acetic acid production was responsible for low cell growth and 1,3-PD productivity in fed-batch fermentation. In a previous work, the same authors (Cheng et al. 2005) showed that acetic acid could adversely affect the growth of *K. oxytoca* M5a1.

In conclusion, the results obtained in this study showed the ability of *K. pneumoniae* BLh-1 to metabolize residual glycerol from biodiesel synthesis without any purification and to be an important producer of 1,3-PD and ethanol, simultaneously. The use of different feeding rates showed the different glycerol biodegradation pathways of

K. pneumoniae BLh-1 under different aeration conditions. Further studies are granted in order to investigate in more details this new wild strain. The use of metabolic engineering, determination of nucleotide pools and enzymes analyses could help to improve the knowledge of intracellular metabolic fluxes and the understanding of how different pathways interact.

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CAPÍTULO VIII – CHEMOMETRIC MODELING AND TWO-DIMENSIONAL FLUORESCENCE ANALYSIS OF BIOPROCESS WITH A NEW STRAIN OF *KLEBSIELLA PNEUMONIAE* TO CONVERT RESIDUAL GLYCEROL INTO 1,3-PROPANEDIOL

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Chemometric modeling and two-dimensional fluorescence analysis of bioprocess with a new strain of *Klebsiella pneumoniae* to convert residual glycerol into 1,3-propanediol

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ABSTRACT

The goal of this study was to show that the metabolism of *Klebsiella pneumoniae* under different aeration strategies could be monitored and predicted with an application of chemometric models and fluorescence spectroscopy. Multi-wavelength fluorescence was applied for on-line monitoring of process parameters on *K. pneumoniae* cultivations. Differences observed in spectra collected under aerobiosis and anaerobiosis can be explained by the different metabolic states of the cells. To predict process variables such as biomass, glycerol, and 1,3-PD, chemometric models were developed on the basis of the acquired fluorescence spectra, which were measured continuously. Although glycerol and 1,3-PD are not fluorescent compounds, the results showed that this technique could be successfully applied to the on-line monitoring of variables in order to understand the process and thus improve 1,3-PD production. The root mean square errors of predictions were 0.78 units, 10 g/L, and 2.6 g/L for optical density, glycerol, and 1,3-PD, respectively.

Keyword: On-line bioprocess monitoring; 2D-fluorescence spectroscopy; chemometrics; *Klebsiella pneumonia*; raw glycerol.

1 Introduction

Different spectroscopic techniques have been studied for their potential applications in monitoring and controlling various biological and chemical processes [1, 2]. Among these spectroscopy methods, 2D-fluorescence spectroscopy has received considerable attention for the non-invasive monitoring of many biological processes. Using a multi-wavelength excitation/emission spectrofluorometer, the fluorescence of fluorophors like NADH, amino acids such as tyrosine and tryptophan, as well as vitamins like pyridoxine and riboflavin, can be simultaneously measured [3,4].

Fluorescence sensors are optical measuring instruments that have been used in the last 15 years for different application fields in biotechnology as, for example, detection of biomass, characterization of reactors, and particularly for bioprocess monitoring [5-8]. In general, optical sensors measurements are ideal for monitoring bioprocesses because they can be performed *on line* and *in situ*, and do not interfere with cells or media. [9]. The potential of the application of 2D-fluorescence spectroscopy has been investigated by the cultivation of various microorganisms [5,10]. Marose et al. [5] correlated the off-line cell mass concentration during *Saccharomyces cerevisiae* cultivation with the culture fluorescence in the different regions of protein, NAD(P)H, riboflavin, and pyridoxine. This method for the on-line detection of biomass is especially suitable if the cells cannot be counted or solid particles in the medium exclude the method of weighing biomass. Even metabolic changes such as anaerobic–aerobic transitions were detected by the 2D-fluorescence spectroscopy. Changes in the relation of oxidized to reduced form of NADH, $FADH_2$ during the aerobic–anaerobic transitions are detected in the region of NADH fluorescence (ex 350/em 450 nm) and FAD/FMN (ex 450/em 530 nm).

Chemometric methods such as the principal component analysis (PCA), the principal components regression (PCR), and the partial least square (PLS) regression are useful for the quantitative analysis of the spectroscopic data [2,3]. These techniques have been used to analyze metabolic changes in recombinant *E. coli* [11] to study the bioprocess characterization of *Claviceps purpurea* [1], monitoring the state variables in heterologous protein production by *Pichia pastoris* [12], monitor online process in recombinant *E. coli* fermentations [2], determination of cell mass and the antibiotic polymyxin B in *Bacilus polymyxa* [13] and to estimate the biomass, glucose and ethanol in *Saccharomyces cerevisiae* cultivation [14].

Klebsiella pneumoniae is a Gram-negative facultative bacterium that has been widely investigated due to its wide substrate tolerance and use, and, concerning this work, its high yields and productivities of 1,3-PD. This chemical has acquired industrial importance in recent years because of its wide range of applications, such as in the synthesis of polyesters, polyethers, and polyurethanes [15, 16]. The production of 1,3-PD by *K. pneumoniae* has been reported in several works, most of which using pure glycerol as the carbon source for cell growth and product synthesis [17-20]. However, raw glycerol, a by-product from biodiesel synthesis that is becoming an abundant and very cheap raw material, could be used as well in this bioprocess [21-23]. Glycerol metabolism by *K. pneumoniae* can be followed through the reductive and/or oxidative pathways. In anaerobiosis, cells will take up glycerol, which is first converted to 3-hydroxipropanaldehyde (3-HPA) by glycerol dehydratase (GDHt) and then reduced to 1,3-PD by 1,3-propanediol oxidoreductase (PDOR). Under conditions leading to the oxidative pathway, glycerol is dehydrogenated by NAD^+- dependent glycerol dehydrogenase (GDH) to dihydroxyacetone (DHA), which is then phosphorylated by ATP-dependent dihydroxyacetone kinase (DHAK) before entering glycolysis. The main products of these metabolic pathways are ethanol, acetic acid, lactic acid, 2,3-butanediol (2,3-BD), while providing energy and reducing equivalents ($NADH_2$) for biomass and 1,3-PD synthesis [16, 22,24].

Although some works have reported the *K. pneumoniae* production of 1,3-PD and tried to elucidate the mechanisms of the enzymatic pathway [24,25,26] so far none has used the 2D-fluorescence method for following the *K. pneumoniae* fermentation kinetics. Although not new, 2D-fluorescence could be applied in this process in order to better elucidate the metabolic changes that are involved during *K. pneumoniae* cultivation. In this context, the aims of this research are to evaluate the use of *in situ* 2D-fluorescence spectroscopy combined with chemometric modeling for the estimation of metabolic changes of *K. pneumonia*. Metabolites formed during fermentation using raw glycerol from biodiesel synthesis as the sole carbon source, in especial 1,3-PD, were evaluated.

2 Materials and methods

2.1 Chemicals and substrate

All chemicals used in this research were of analytical grade and purchased from Sigma-Aldrich (St. Louis, USA). The raw glycerol used as the sole carbon source for cell cultivations was supplied by a biodiesel manufacturing plant (Passo Fundo, Brazil) and had a composition of (mass fraction) glycerol, 82.4 %; ashes,5.73 %; NaCl, 6.17 %; moisture, 11.49 %; and pH 5.13, monoacylglycerols, 0.39 %.

2.2 Microorganism maintenance and inocula preparation

K. pneumoniae BLh-1 was isolated from the sludge of a soybean processing plant (Esteio, Brazil). This strain was identified by 16S rDNA gene sequencing and identified as *K. pneumoniae* as reported elsewhere [27]. Stocks of this bacterium are kept at Microbiology Culture Collection of BiotecLab (UFRGS, Brazil). Working stocks of cultures were maintained frozen at -18 ºC in (volume fraction) 20 % glycerol suspensions, while for immediate use, cells were maintained on Luria–Bertani (LB) agar slant at 4 °C and subcultured every 2 weeks. Inocula were prepared by transferring one loopfull of cells into 500 mL flasks filled with 200 mL of medium and then incubated at 37°C in a rotary shaker at 150 rpm overnight. Inocula were standardized as 1 OD unit at 600 nm. The medium contained (in g/L): 5 yeast extract, 5 peptone, 7 $(NH_4)_2SO_4$, 7 K_2HPO_4, 1 mL trace-elements solution, and 65 of raw glycerol. The trace-elements solution contained (in g/L): 0.1 $MnCl_2.4H_2O$, 0.06 H_3BO_3, 0.0037 $CuSO_4.5H_2O$, 0.2 $CoCl_2.6H_2O$, 0.025 $NiCl_2.6H_2O$, 0.035 $Na_2MoO_4.2H_2O$, 0.14 $ZnSO_4.7H_2O$, and 0.9 mL HCl (volume fraction 37 %).

Batch cultivation experiments were performed in a fully equipped 10 L bioreactor (Biostat-B.Braun-Germany). The anaerobic cultivations were run at 37 °C and stirrer speed of 250 rpm. Nitrogen was added to ensure anaerobic conditions. In all cultivations the pH was controlled at 7.0 by automatic addition of either 10 M NaOH or 1 M H_3PO_4. The aerobic cultivations were carried out with an airflow rate of 0.5 vvm (volumes of air/volume of culture). The bioreactors were inoculated with 10 % volume fraction of inoculum. Anaerobic experiments were performed in triplicates, while aerobic cultivations were run in duplicates.

2.3 *Off-line* analysis

The composition of the cultivation broth was analyzed using a Merck LaChrom 7000 series liquid chromatograph (VWR GmbH, Darmstadt, Germany) equipped with a RID-10A refractive index detector. The stationary and mobile phases were an Aminex HPX-87H column (300 x 7.8 mm, Bio-Rad, USA) and 0.005 mol/L H_2SO_4 solution at 0.7 mL/min, respectively. The column temperature was controlled at 65 °C. The culture samples were prepared by centrifugation at 3,500 *g* for 10 min to pellet the cell, followed by filtration using cellulose acetate membrane filters (pore size 0.22 μm, Sartorius, Germany). Biomass concentration was estimated by measuring optical density (600 nm) and cell dry weight method, which consisted in filtering (Millipore cellulose nitrate membranes, 0.45 μm pore diameter) 10 mL of sampled culture and dried to constant weight at 80 ºC in an oven.

2.4 Fluorescence spectroscopy

The fluorescence measurements were performed with the BioView® sensor (DELTA Light & Optics, Denmark). Marose et al. [5] presented one of the first and well-detailed works on the use the BioView® sensor for measuring the cell growth and metabolism change of cells of several bacteria and *S. cerevisiae*. The BioView® sensor is equipped with a xenon flash lamp for the excitation light. The sensor uses two different filter wheels with 16 different filters for excitation and emission and a photomultiplier for detection of the emission light. The liquid light guide is connected to a quartz window of the bioreactor. The measurements were carried out in steps of 20 nm in the excitation wavelength ranging from 270 to 550 nm and the emission wavelength ranging from 310 to 590 nm. During the cultivation 2D-fluorescence spectra were continuously collected.

2.5 Chemometric modeling

The chemometric modeling was performed using MATLAB® 7.4.0.287 (R2007a) (The Math Works™). For every process variable, a single partial least square model (PLS-1 model) was calculated based on the data of two anaerobic cultivations. The centered data of the fluorescence spectra were used as independent variables and the values of the process variables determined off-line were the dependent variables. Leave-one-out cross-validation was used for validation of the chemometric models and to determine the number of components for each model, which were used to predict

external data and the error of prediction was calculated. The comparison by root mean square error of validation (RMSEV) of predictions of the left out values with the values determined off-line was performed using one to ten principal components. The first principal component is dominated by the fluorescence area of FMN/FAD coenzymes (riboflavin, mainly 450/510, 430/530, and 470/530). The second principal component is also dominated by the fluorescence area of FMN/FAD coenzymes (pyridoxine, mainly 430/490, 410/490, and 390/490). The third principal component is dominated by the fluorescence area of NADH and protein. Finally, the fourth principal component is dominated by the fluorescence area of all principal components mentioned above. The minimal RMSEV decides how many principal components are used for prediction of process variables of a new cultivation run. Here the root of the mean square error of predicted and measured values (RMSEP, equation 1) is used as degree for the quality of the model.

$$\boldsymbol{RMSEP} = \sqrt{\boldsymbol{MSEP}} = \sqrt{\frac{\sum_{i=1}^{n}(y_i - \hat{y})^2}{n}} = \sqrt{\frac{\sum_{i=1}^{n} e_i^2}{n}} \qquad (1)$$

3 Results and discussion

3.1 Bioreactor cultivations

During the cultivations of *K. pneumoniae* BLh-1, the fluorescence signal was recorded continuously and the process variables (biomass, glycerol consumption, and products formation) were analyzed off-line. The time courses of these process variables during the cultivations are shown in Fig. 1. The anaerobic cultivation of *K. pneumoniae* (Fig. 1a) showed a quick increase in biomass and product formation up to 10 hours. The highest 1,3-PD concentration was 26.84 g/L. Rapid glycerol consumption was observed, being completely depleted by 15 h, showing the exceptional ability of this strain to metabolize raw glycerol. The aerobic kinetics was similar to the anaerobic cultivation concerning biomass and glycerol consumption (Fig.1b). However, 1,3-PD production was significantly lower, reaching a maximal of 17.67 g/L. Other by-products were produced in the both cultivations such as lactic acid, acetic acid, and ethanol, again suggesting the potential use of this strain combined with raw glycerol for other

industrial bioprocesses. Table 1 shows the kinetics parameters for *K. pneumoniae* BLh-1.

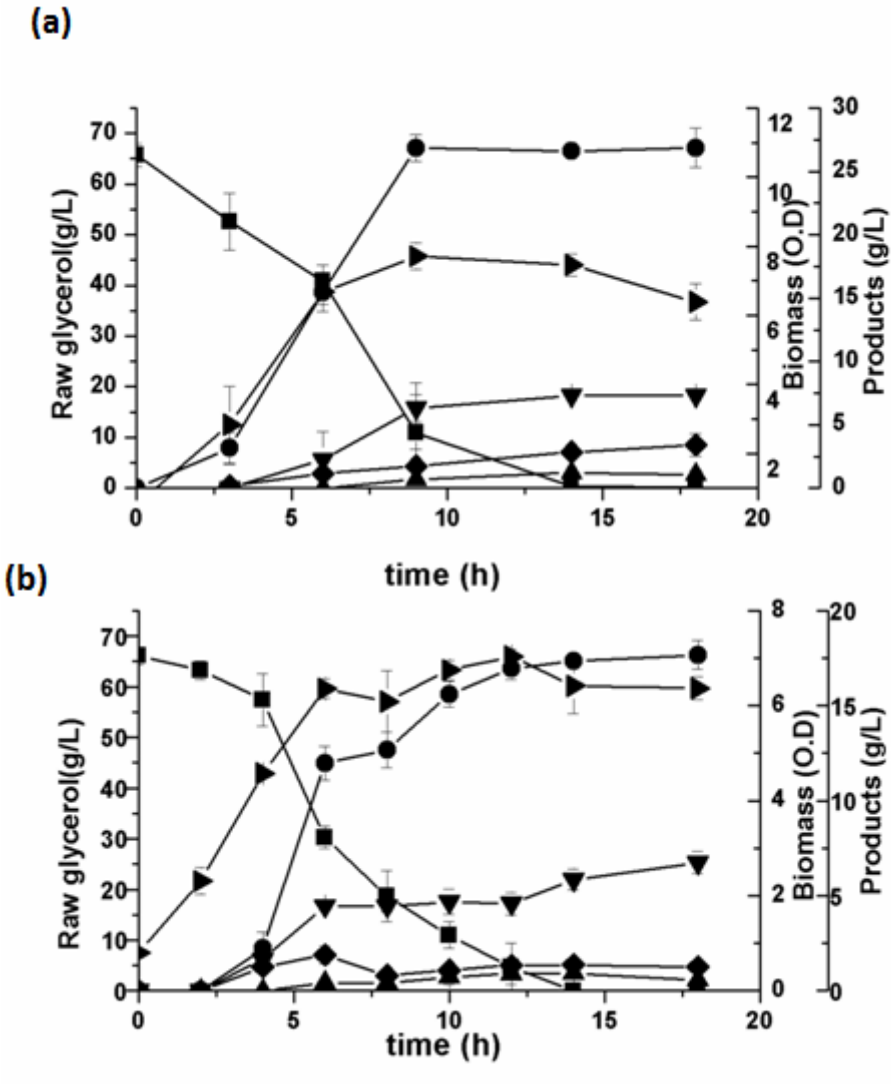

Figure 1- Batch fermentation for *K. pneumoniae* BLh-1. (a) Anaerobic cultivation; (b) aerobic cultivation. (■) Raw glycerol, (●) 1,3-PD, (▲) acetic acid, (▼) lactic acid, (►) biomass, (♦) ethanol. Results represent the mean of triplicates (anaerobic) and duplicates (aerobic).

Table 1- Kinetic parameters for different aeration conditions using *K. pneumoniae* BLh1.

Condition	**$C_{1,3\text{-PD}}$ (g/L)**	**$Y_{x/s}$ (g/mol)**	**$Y_{1,3\text{-PD/s}}$ (mol/mol)**	**$Y_{EtOH/s}$ (mol/mol)**	**$Y_{acetic/s}$ (mol/mol)**	**$Y_{lactic/s}$ (mol/mol)**	**Productivity (1,3-PD) (g/L.h)**
Anaerobiosis	26.84	4.78	0.51	0.11	0.03	0.11	1.49
Aerobiosis	17.67	8.83	0.32	0.04	0.02	0.10	0.98

The yield and productivity were higher for anaerobic cultivation compared to aerobic cultivation. The main by-product was lactic acid with similar yields in both

cultivations. These results compare well with other reports found in the literature using glycerol as carbon source. For instance, Zhang et al. [18] studied the rapid conversion of pure glycerol to 1,3-PD by a *K. pneumoniae* strain isolated from soil. The authors obtained a maximal production of 12.2 g/L of 1,3-PD in batch fermentation with molar yield and productivity of 0.75 and 1.53 g/L.h, respectively. The main by-product was acetate with little accumulations of ethanol and lactate. Cheng et al. [28] studied the 1,3-PD production using *K. pneumoniae* M5aL using pure glycerol at 50 g/L with different aeration strategies. The authors showed that the higher 1,3-PD yield of 0.54 mol/mol was obtained using N2 in batch fermentation with 0.62 g/L.h of productivity. With airflow rates of 0.6 vvm, 1,3-PD yield was 0.41 mol/mol with productivity of 0.57 g/L.h. They also observed that the highest concentrations of acetic acid and ethanol were found in the anaerobic batch fermentation. Increasing the airflow rate, acetic acid and ethanol formation decreased while lactic acid formation increased. Oh et al. [23] using raw glycerol with *K. pneumoniae* ATCC 700721 for producing 1,3-PD using experimental design, obtained a final production of 13.74 g/L in batch fermentations and 1,3-PD yield of 0.47 mol/mol.

The production of 1,3-PD is clearly associated with cell growth and its physiological role is to regenerate the oxidized form of the reducing equivalents, NAD^+, to be used in the energy-producing pathways of glycerol degradation [22]. Therefore, accumulation of lactic acid and ethanol, will not only inhibit cell growth, but also consume much more equivalents of NADH, directly competing with the formation of 1,3-PD. On the other hand, the formation of acetic acid by cells is independent of NADH [29,30], thus not interfering in the conversion of glycerol to 1,3-PD. In the metabolic flux of *K. pneumoniae*, the pathway of glycerol to acetic acid provides part of the energy (ATP) necessary for growth, but in its undissociated form, acetic acid is considered the main inhibitory metabolite [29,31,32].However, the results obtained in this work showed that lactic acid produced during the course of fermentation was the main inhibitor of 1,3-PD production, both under aerobiosis and anaerobiosis (Fig. 1).

3.2 Fluorescence data

Fluorescence spectra that were gathered during the anaerobic and aerobic cultivations of *K. pneumoniae* are shown on Fig. 2a and 2b, respectively, while the subtraction of anaerobic-aerobic spectra showing the differences during the cultivation is shown on Fig. 2c. The Fig. 2a and 2b show areas related to fluorescence at ex/em

450/530 nm and ex/em 370/470 nm. These areas are due to the biogenic fluorophors flavine (FAD, FMN) and NADH NADH [1,5]. As previously reported, fluorescence in the region NADH, riboflavin and pyridoxine can be used to estimate the cell growth [5,33]. NADH is involved in redox reactions, carrying electrons from one reaction to another. NADH and $FADH_2$ transfer electrons to O_2 during the oxidative phosphorylation, where ATP is formed. Without oxygen the relation of the oxidized to reduced form is shifted toward the reduced form. NADH can no longer be oxidized via the oxidative phosphorylation and is accumulated. This effect can be visualized on Fig. 2a in a region of NADH fluorescence (ex/em 370/450 nm). When the oxidative phosphorylation is stopped the $FMNH_2$ pool increases. Since the reduced coenzyme does not fluoresce, the FMN fluorescence must decrease under anaerobic conditions.

During the first 12 h of cultivation an increase of NADH fluorescence was observed in both cultivations; after that, there was a rapid depletion of glycerol (Fig.3a). These data are showing that this compound is related to the metabolic state of *K. pneumoniae*. The data for riboflavin (Fig.3b) show the differences in intensity between anaerobic and aerobic cultivations. Under anaerobiosis, a negative intensity was observed in the region of riboflavin (ex/em 450/530 nm), while for the aerobic cultivation a positive intensity for this metabolite was observed (Fig. 2a and 2b). This fact is also related to cell metabolism, since riboflavin is used in a wide variety of redox reactions and is related to cell respiration. Flavins can act as oxidizing agents because of their ability to accept a pair of hydrogen atoms and the reduction of isoalloxazine ring (FAD, FMN oxidized form) yields the reduced forms of the flavoproteins ($FMNH_2$ and $FADH_2$). The study of this parameter is very important in order to increase the production of 1,3-PD, which is growth associated. The highest values of 1,3-PD were obtained around 12 h of cultivation when the NADH was at its highest concentration (Fig.3a). It has been postulated that the synthesis of 1,3-PD requires NADH as coenzyme and the NADH generated from glycerol determines the yields of 1,3-PD [20]. The conversion of the glycerol to 1,3-PD is a chemical reduction, serving as a sink for reducing equivalents, which are generated by oxidation of the second part of glycerol for energy provision and biomass formation [34].

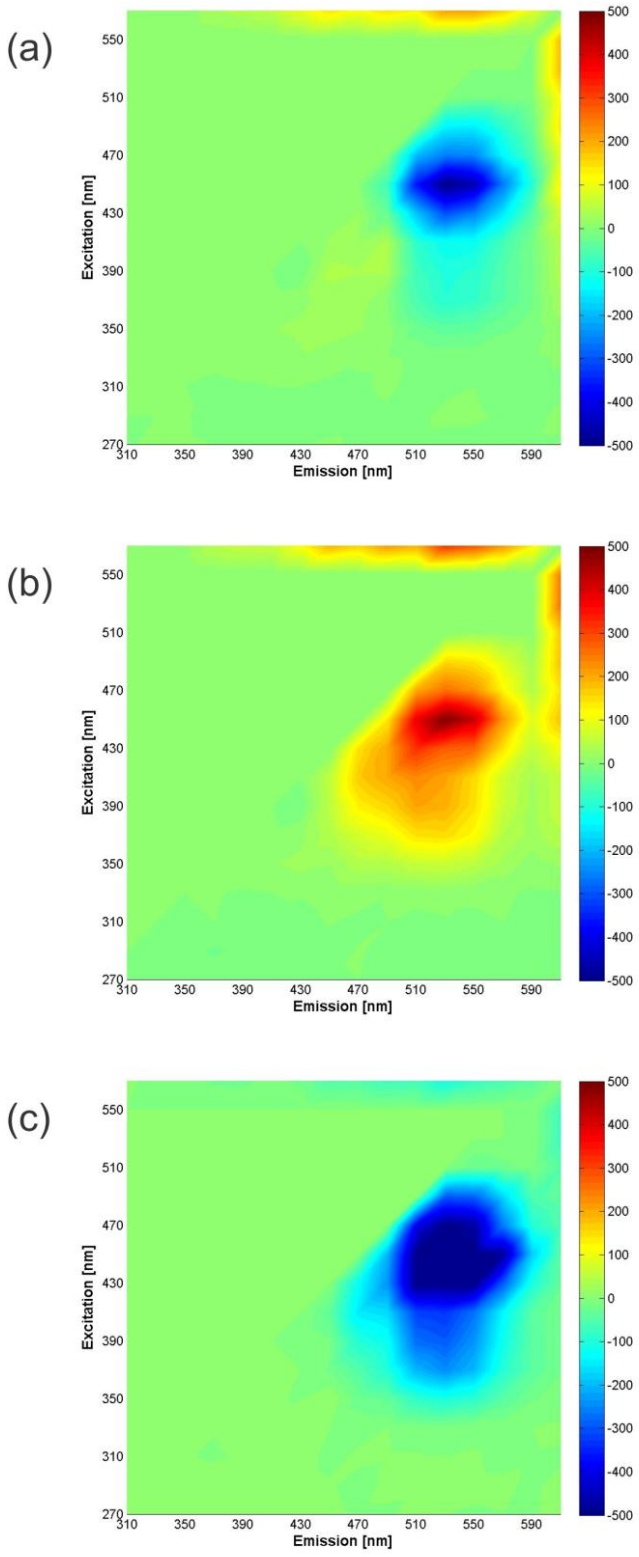

Figure 2- Fluorescence spectrum of *K. pneumoniae* BLh-1 cultivation. (a) Anaerobic cultivation; (b) aerobic cultivation; (c) subtraction spectrum.

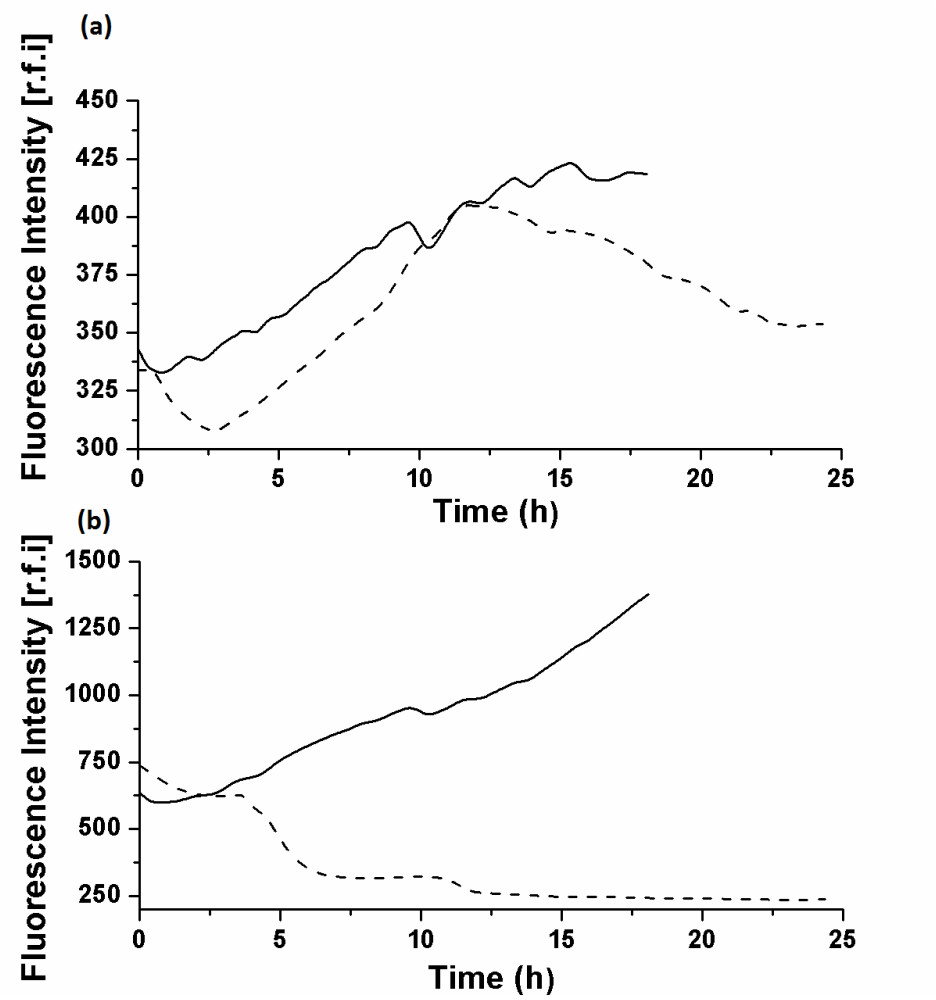

Figure 3- Courses of fluorescence intensity during *K. pneumoniae* BLh-1 cultivation. (a) NADH intensity in the region of ex/em 370-470 nm; (b) Riboflavin intensity in the region of ex/em 450-530 nm. Solid lines (-) represent aerobic fluorescence intensity and dashed lines (--) represent anaerobic fluorescence intensity.

Chemometric evaluations of fluorescence data recorded during cultivation of *K. pneumoniae* BLh-1 were carried out by PLS modeling process variables using the fluorescence spectra as well as the off-line measurements from the cultivations. The PLS models for the glycerol, 1,3-PD, and biomass were based on one, two, and four principal components, respectively. The chemometric models were used to perform predictions of the process variables of the second anaerobic batch cultivation of *K. pneumoniae* BLh-1. Figure 4 shows the predicted courses of the optical density, glycerol and 1,3-PD in comparison to off-line measurements, and the RMSEP of predicted values in respect with off-line measurements are presented in Table 2. The obtained results of predictions represent the kinetics of the off-line measurements of all three variables in a satisfactory way.

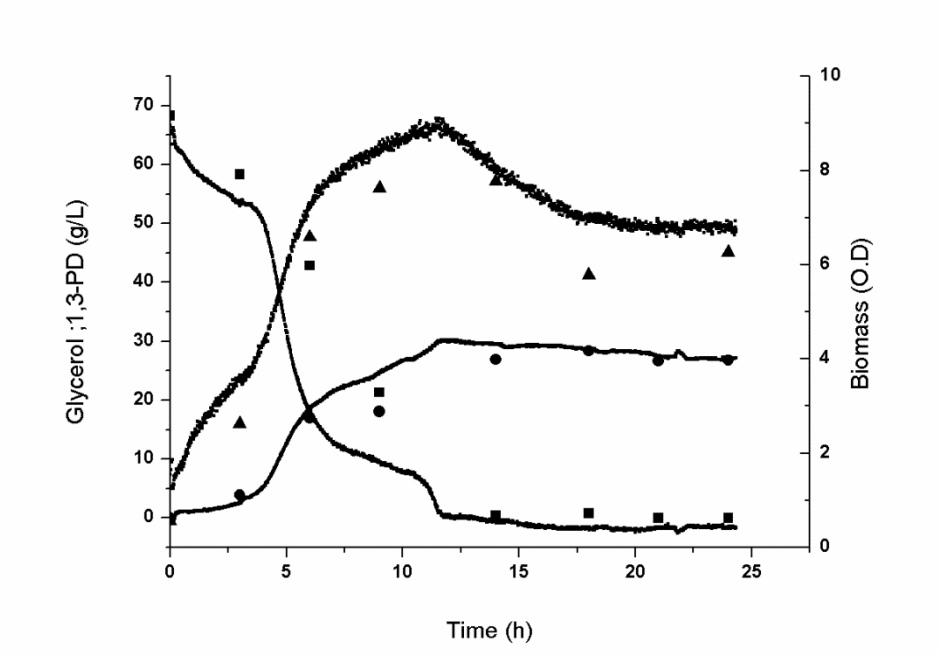

Figure 4- Comparison of predicted and measured values on *K. pneumoniae* BLh-1 cultivation for biomass, glycerol and 1,3-PD. Solid lines (-) represent predicted values and symbols are off-line values: (■) Raw glycerol, (●) 1,3-PD and (▲) biomass.

Table 2- Root mean squared error of prediction (RMSEP) for biomass, glycerol, and 1,3-PD for batches of *K. pneumoniae* BLh-1.

Process variable	RMSEP	Mean error
Optical density	0.78 (units)	10 %
Glycerol	10 (g/L)	14 %
1,3-PD	2.6 (g/L)	9 %

Although glycerol and 1,3-PD are not fluorescing substances, they can be predicted from the fluorescence spectra due to their correlation with the fluorophor concentration in the culture broth. Most likely, the prediction error would be smaller, if the database for calibration would have been bigger. According to Ödman et al. [14] the batch variation in the data has an important impact on the calibration. Therefore, if more batch data were included in the calibration, the variation in the chosen number of components would be reduced. Lantz et al. [13] studied the determination of cell mass and polymyxin using multi-wavelength fluorescence in cultures of *Bacillus polymyxa*. The authors conducted batch cultivations varying the medium content of phosphate and nitrogen in order to achieve different biomass-polymyxin ratios under three different growth conditions. PLS regression was used to establish the mathematical relationships between off-line determined cell mass and polymyxin concentrations with on-line

fluorescence data. The authors showed that when the same fluorescence data were used to compute a calibration model, the correlation was only 0.55. However, when separate models were performed for each type of cultivation high correlations and low RMSECV (0.4-0.5 g/L) values were found. Ödman et al. [14] studied on-line estimation of biomass, glucose, and ethanol in *Saccharomyces cerevisiae* using in situ-multi-wavelength fluorescence and standard monitoring sensors. Batch cultivations were performed at high (190-305 g/L) or low (21-25 g/L) glucose gravity conditions. The authors showed that the best models using high gravity cultivation had RMSEPs of 1.0 g/L CDW, 1.8 g/L ethanol, and 5.0 g/L consumed glucose, corresponding to 4 %, 2 % and 2 % concentration intervals, respectively. Values obtained for low gravity models were 0.3 g/L CDW, 0.7 g/L ethanol, and 1 g/L consumed glucose, corresponding to 4%, 8% and 4% concentration intervals, respectively.

4 Conclusion

It has been demonstrated that 2D-fluorescence and chemometric models can be used for quantitative prediction of biomass, substrate, and product formation during batch cultures of *K. pneumoniae* BLh-1. Using the fluorescence spectra the metabolic state of the cells could be followed. Glycerol and 1,3-PD, which are not fluorescing compounds, could be successfully determined applying fluorescence in order to monitorate the process. The higher than expected predicted error could be a consequence of the exceptionally fast metabolism of *K. pneumonia* BLh-1 towards glycerol consumption, biomass formation, and 1,3-PD production associated to this metabolism. The 2D-fluorescence measurements produced earlier, more extensive, and as reliable as information about the metabolic changes of cultures, when compared with the traditional off-line analytical methods. Another advantage of the 2D-fluorescence method is that it does not introduce sampling effects in the results since it is noninvasive. The combination of fluorescence and chemometric models is suitable for on line bioprocess monitoring and process prediction thereby creates an effective tool for bioprocess improvement.

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CAPÍTULO IX- CONSIDERAÇÕES FINAIS

Este projeto de doutorado permitiu a realização de um trabalho completo onde a etapa fundamental foi o de dar destino a um subproduto gerado atualmente em grandes quantidades, o glicerol. A partir da utilização deste substrato pode-se verificar a produção de produtos de grande valor agregado como o hidrogênio, o etanol e o 1,3-PD.

Foi um trabalho bastante abrangente com várias etapas, começando com os estudos em um consórcio microbiano para verificar a capacidade de degradação do glicerol pelos microorganismos nele presentes, o isolamento e a seleção de microganismos potencialmente produtores de 1,3-PD, etanol e hidrogênio, a otimização das condições de cultivo para produção de hidrogênio e 1,3-PD, a fermentação batelada em biorreatores submersos e a fermentação batelada alimentada. Além disso, pode-se avaliar as diferenças metabólicas envolvidas nas fermentações permitindo um estudo mais aprofundado da bactéria selecionada em questão.

Na primeira etapa deste trabalho foram utilizados diferentes tratamentos no consórcio microbiano ambiental com a finalidade de verificar a produção de hidrogênio utilizando glicerol residual como única fonte de carbono. Existem na literatura relacionada, vários trabalhos utilizando glicose ou resíduos lignocelulósicos para a produção de hidrogênio, porém, nenhuma publicação até o momento utilizando apenas o glicerol residual foi encontrada. Os resultados apresentados mostraram que a utilização do tratamento por dessecação e tratamento térmico foram os mais efetivos para a produção de hidrogênio e que o glicerol residual sofreu uma degradação significativa comparada as fontes de açúcares utilizados em outros trabalhos. Este estudo permitiu mostrar que as bactérias presentes no consórcio microbiano foram capazes de degradar o glicerol residual e produzir bioprodutos de interesse.

O próximo passo foi otimizar as condições de cultivo para a produção de hidrogênio. Nesta etapa, foram utilizadas ferramentas estatísticas que foram importantes para que se fizesse um estudo completo das principais variáveis que poderiam afetar a produção de hidrogênio utilizando um número mínimo de experimentos. Os resultados mostraram-se bastante promissores com uma produção de hidrogênio após a validação do modelo de 45 % mol, valor quase duas vezes maior, comparado à primeira etapa da otimização, que produziu 29 % mol de hidrogênio.

O isolamento foi à etapa fundamental do trabalho, com a seleção de cepas capazes de degradar o glicerol e produzir hidrogênio, 1,3-PD e etanol, principais produtos de interesse. As fermentações realizadas em biorreatores permitiram o isolamento de 32 bactérias. Estas foram fermentadas isoladamente e quatro apresentaram bons reultados de utilização de glicerol e formação de produtos. As condições de fermentação também foram importantes durante esta etapa, mostrando que, em anaerobiose, a produção de 1,3-PD e etanol foram maiores comparados aos ensaios aeróbios. A identificação dos isoldados por seqüenciamento do gene 16S rRNA mostrou que, dos quatro isolados, três foram identificados como *Klebsiella pneumoniae* que, segundo a literatura, são os maiores produtores de 1,3-PD utilizando glicerol como fonte de carbono.

O planejamento experimental Plackett-Burman foi utilizado com o objetivo de selecionar as variáveis importantes na produção dos bioprodutos. Este tipo de planejamento é indicado quando se quer estudar várias variáveis ou se tem pouco conhecimento do processo. Os resultados apresentados mostraram que a otimização das condições de produção de 1,3-PD foi eficiente, com produções aumentadas em até três vezes, comparados aos primeiros ensaios em shaker. Os ensaios em biorreatores batelada mostraram que, em condicões de pH controlado, houve um aumento na produção de 1,3-PD e etanol comparado aos ensaios sem controle de pH mostrando que este influencia diretamente na produção dos metabólitos de interesse.

As estratégias de alimentação em biorreatores batelada alimentada foram realizadas com a finalidade de se estudar melhor a diferença de metabolismo frente às diferentes condições de aeração, como também, estudar o aumento da produção dos produtos de interesse. Nesse estudo, pode-se verificar que as altas concentrações de alimentação levaram a um acúmulo de glicerol no meio e apenas um sensível aumento nas produções de 1,3-PD e etanol em ambas as condições de aeração. Pórem, os cultivos microaerófilos mostraram um grande aumento da produção de ácido lático à medida que foi aumentando a concentração de glicerol. Os resultados apresentados neste estudo permitiram conhecer melhor o metabolsimo da cepa em estudo, mostrando que a produção dos bioprodutos (ácido lático, etanol, acético) compete diretamente com a produção de 1,3-PD. Estudos mais detalhados, com a análise de NADH e as enzimas envolvidas se fazem necessários para aprimorar os estudos acerca do seu metabolismo.

Por fim, os estudos realizados utilizando fluorescência 2D e modelagem quimiométrica foram de suma importância para o melhor conhecimento do metabolsimo

da *K. pneumoniae* BLh-1. Esta ferramenta permitiu estudar a nível fisiológico as mudanças envolvidas durante as diferentes estratégias de aeração. A utilização de equivalentes redutores NADH pode ser acompanhada durante a fermentação mostrando um aumento do consumo de NADH até a completa utilização do glicerol. Pode-se verificar também a importância de cofatores, como a riboflavina, durante as diferentes condições de aeração. Estes estudos também permitiram a modelagem das principais variáveis do processo, mostrando que os dados off-line e os preditos pelo modelo conseguiram descrever o sistema dos principais produtos de fermentação.

Em conclusão, os estudos realizados neste trabalho demonstraram que o isolado obtido tem um grande potencial para a produção de produtos de grande valor agregado partindo-se da utilização de um subproduto da indústria de produção de biodiesel. Estes resultados são importantes do ponto de vista ambiental, visto que a crescente produção de biodiesel irá consequentemente, produzir cada vez mais glicerol residual e este excesso poderá se tornar um problema ambiental se não for dado um correto destino a este resíduo. Além disso, os resultados mostraram que a produção de etanol e 1,3-PD, monômero usado na produção de polímeros, foram bastante promissores e são de grande interesse econômico e industrial podendo, em fututros trabalhos, ser produzido em maior escala com posterior separação dos produtos do caldo de fermentação. A produção de hidrogênio como uma fonte de energia também aparece como uma alternativa à produção de energia limpa. Enfim, este trabalho conseguiu demonstrar que o glicerol residual pode ser utilizado para a produção de importantes bioprodutos que são promissores do ponto de vista econômico e também do ponto de vista ambiental.

CAPÍTULO X – PERSPECTIVAS

O trabalho realizado permitiu o estudo da produção de hidrogênio, 1,3-PD e etanol utilizando glicerol residual oriundo da síntese de biodiesel. Os resultados obtidos demostraram que outros estudos podem ser realizados para aumentar a produção e produtividade destes produtos. Portanto, o avanço nos estudos acerca destes produtos pode ser realizado, entre eles destacam-se:

Quanto à produção de hidrogênio:

Estudar a produção de hidrogênio em biorreatores contínuos com sistema de analisador de gases para uma melhor avaliação da produção dos gases formados;

Estudar a produção de hidrogênio em biorreatores de leito fixo a diferentes taxas de diluição com a finalidade de aumentar a produtividade;

Avaliar o estudo de biorreatores contínuos de leito fixo com células imobilizadas. Este estudo poderá ser realizado utilizando-se diferentes suportes.

Quanto à produção de 1,3-PD e etanol:

Estudar a produção dos produtos utilizando células imobilizadas em biorreatores de leito fixo e fluidizado;

Estudar com maiores detalhes a etapa de biorreatores batelada alimentada utilizando outros tipos de alimentação e diferentes vazões de alimentação, podendo-se desenvolver o trabalho para reatores contínuos com diferentes taxas de diluição;

Estudar o metabolismo da *K.pneumoniae* BLh-1 utilizando engenharia metabólica e genética. Desta forma, será possível inibir uma rota em favorecimento de outra aumentando, com isso, a produção de um determinado produto;

Estudar a etapa de recuperação dos produtos de fermentação.

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APÊNDICE

APÊNDICE - RESULTADOS NÃO APRESENTADOS NOS ARTIGOS CIENTÍFICOS

SELEÇÃO DE BACTÉRIAS PRODUTORAS DE 1,3-PD E ETANOL

Esta primeira etapa do projeto de doutorado teve como objetivo selecionar bactérias capazes de produzir 1,3-PD e etanol. Com isso, foram testadas bactérias cedidas pelo Hospital de Clínicas de Porto Alegre. Nesta etapa, 30 bactérias do gênero *Klebsiella* foram selecionadas para o estudo após uma ampla pesquisa na literatura citando o gênero como os maiores produtores de 1,3-PD e também de hidrogênio (BIEBL *et al*., 1992; DECKWER, 1995; WANG *et al*., 2001). Este trabalho foi apresentado em forma de pôster no 15º ENQA (Encontro Nacional de Química Analítica) e 3º CIAQA (Congresso Iberoamericano de Química Analítica) em 2009.

Os resultados obtidos com as bactérias testadas foram promissores, porém estes não foram utilizados no decorrer do projeto por apresentarem resultados inferiores as bactérias isoladas no consórcio microbiano ambiental e, principalmente, por sua patogenicidade.

A seguir segue os resultados apresentados no 15º ENQA e 3º CIAQA.

Seleção de bactérias degradadoras de glicerol utilizando análise de produtos em cromatografia líquida de alta eficiência

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Palavras Chaves: glicerol, biodiesel, biodegradação.

1 Introdução

O glicerol, glicerol ou 1,2,3- propanotriol, constitui o maior resíduo gerado no processo de produção de biodiesel, sendo que 10 % do volume total de biodiesel correspondem o glicerol. Estima-se que com o incremento do volume de biodiesel, o glicerol co-produzido aumentará de 83 para 330 milhões de L/ano até 2010[1]. Com isso, torna-se necessário buscar alternativas para a utilização deste produto evitando que se torne um problema no futuro devido a sua acumulação. Várias estratégias baseadas nas transformações químicas e biológicas estão sendo propostas para converter o glicerol residual em produtos com maior valor. A conversão biológica tem gerado bastante interesse nos últimos anos, principalmente, na produção de bioprodutos, entre eles, o 1,3-propanodiol, etanol, 2,3 butanodiol, ácido acético e lático[2].

O objetivo do presente trabalho foi selecionar bactérias capazes de degradar glicerol usando como ferramenta a análise de produtos através de métodos cromatográficos. Bactérias provenientes da UTI do Hospital de Clínicas de Porto Alegre, RS, foram utilizadas neste trabalho.

2 Materiais e métodos

Os experimentos foram realizados em Erlenmeyers de 125 mL com 50 mL de meio. As condições de operação foram: T: 37°C, pH 7,0, 120 rpm. O meio de cultivo utilizado continha: 30 g de glicerol, 4 g (NH4)2SO4; 0,125 g K2HPO4; 0,12 g MgSO4.7H2O; 0,12 g MgSO4.7H2O; 0,025g FeSO4.7H2O; 0,005 g CuSO4.5H2O; 0,125 g CoCl2.6H2O. As concentrações dos produtos formados foram determinadas utilizando métodos cromatográficos de análise. O equipamento utilizado foi um HPLC da marca Perkin Elmer equipado com uma coluna Aminex HPX-87H (Bio-Rad) e detector de índice de refração. As condições de trabalho foram: fluxo 0,8 mL/min.; temperatura do forno: 65°C; fase móvel: ácido sulfúrico 0,005 M.

3 Resultados e discussão

Os resultados mostram que houve a formação principalmente de etanol, 1,3 propanodiol e acido lático. A figura 1 mostra a quantidade de glicerol remansecente no meio após 24h e as figuras 2 e 3 mostram a formação de produtos em aerobiose e anaerobiose, respectivamente.

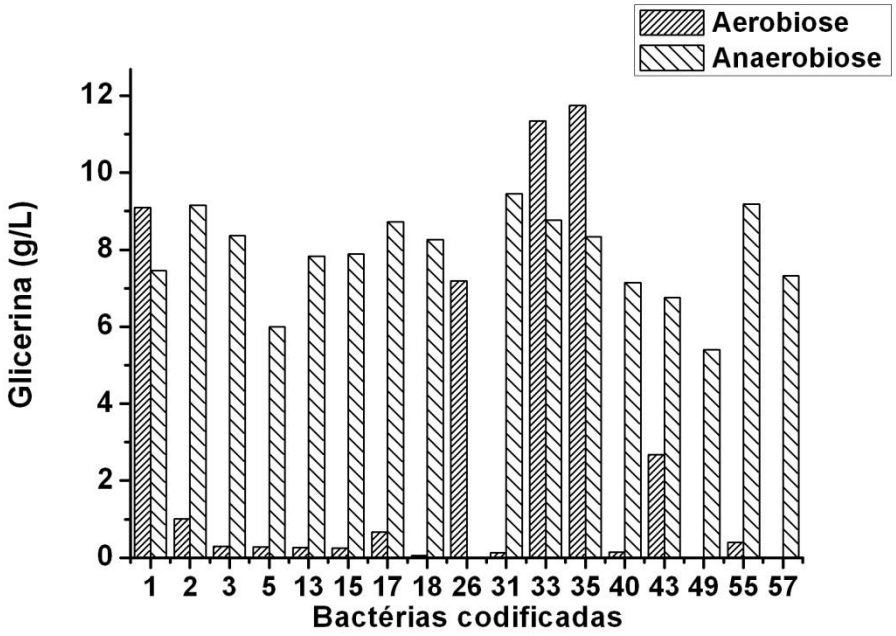

Figura 1- Glicerol remanscente no meio após 24 horas de cultivo

Em relação à formação de produtos, ambos os cultivos apresentaram similares produções de 1,3-PD, com uma produção máxima de 4,28 g/L em aerobiose pelo isolado 43 e 3,87 g/L pelo isolado 5 em anaerobiose. Os cultivos em aerobiose apresentaram também a formação de etanol e ácido acético diferindo do cultivo anaeróbio o qual apresentou a produção apenas de etanol. A maior produção de etanol foi de 3 g/L com o isolado 5 em anaerobiose.

Os resultados apresentados acima estão de acordo com a literatura com etanol e ácido acético como principais bioprodutos da fermentação com glicerol.

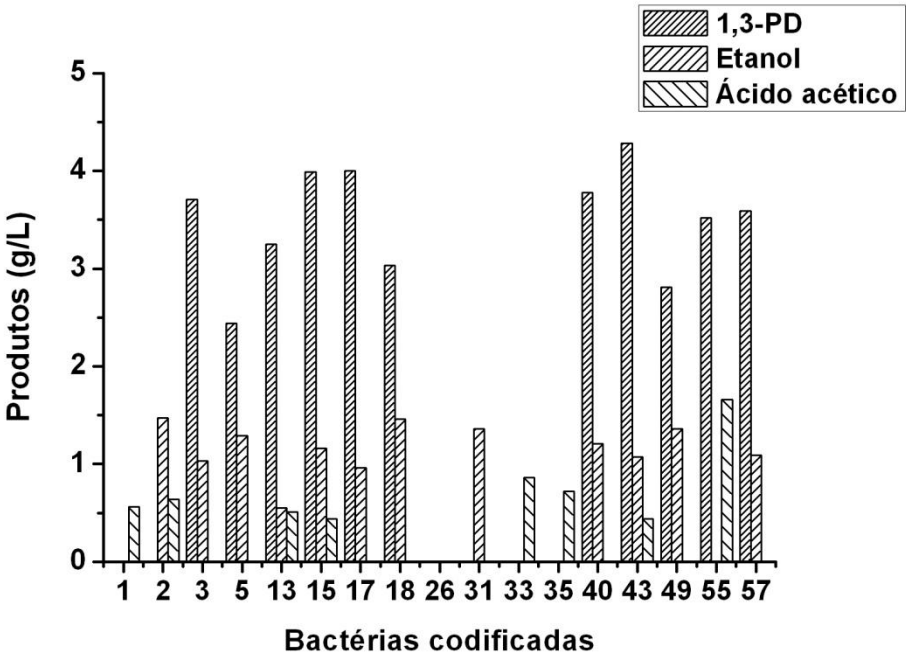

Figura 2-Formação de produtos em aerobiose após 24 horas de cultivo

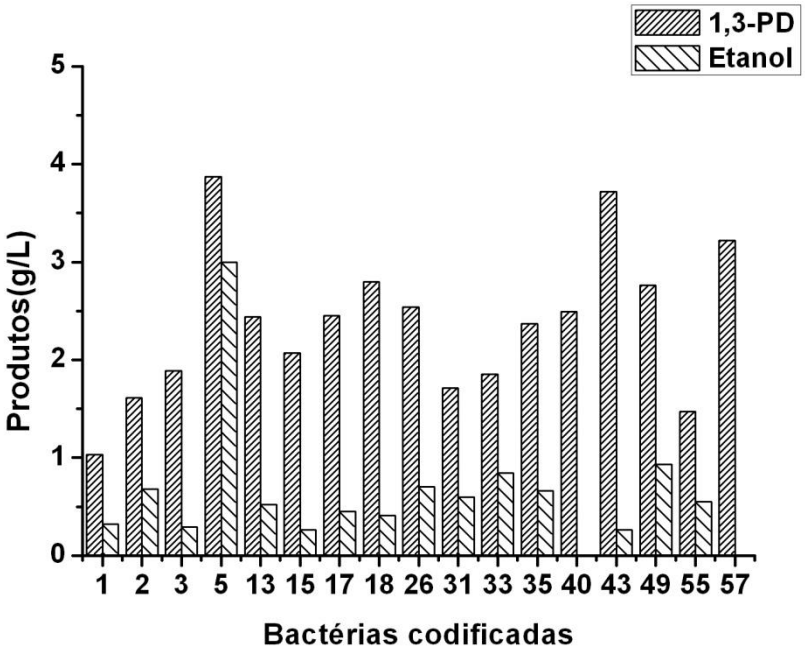

Figura 3- Formação de produtos em anaerobiose após 24 horas de cultivo

4 Conclusão

De acordo com os resultados obtidos, pode-se concluir que as bactérias selecionadas têm potencial para degradar o glicerol. Esta etapa foi um importante passo para futuros testes com glicerol residual utilizando-as na biodegradação deste resíduo e gerando importantes bioprodutos.

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