
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
(MICROBIOLOGIA APLICADA)

**Produção de 1,3-propanodiol por *Klebsiella pneumoniae* e
Escherichia coli: Otimização de meio e clonagem de genes.**

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Tese de doutorado apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas (Microbiologia Aplicada).

**Rio Claro
2015**

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Produção de 1,3-propanodiol por *Klebsiella pneumoniae* e *Escherichia coli*: Otimização de meio e clonagem de genes.

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547.29 Avila Neto, Paulo Marcelo
A958p Produção de 1,3-propanodiol por *Klebsiella pneumoniae* e
Escherichia coli : otimização de meio e clonagem de genes. /
Paulo Marcelo Avila Neto. - Rio Claro, 2015
107 f. : il., figs., gráfs., tabs.

Tese (doutorado) - Universidade Estadual Paulista,
Instituto de Biociências de Rio Claro
Orientador: Jonas Contiero

1. Fermentação. 2. Glicerol. 3. Biodiesel. 4. Planejamento
experimental. 5. Engenharia metabólica. 6. Genética de
bactérias. I. Título.


CERTIFICADO DE APROVAÇÃO

TÍTULO: Produção de 1,3-propanodiol por *Klebsiella pneumoniae* e *Escherichia coli*: Otimização de meio e clonagem de genes

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Data da realização: 11 de março de 2015.

Dedicatória

Aos meus pais, que sempre me apoiaram e incentivaram a estudar e seguir em busca dos meus objetivos.

AGRADECIMENTOS

A Deus, meu Pai do céu, que escutou meus desabafos e me orientou sempre, me ajudando no escuro. A meus pais e meu irmão Adriano, que me apoiaram sempre nos estudos e financeiramente, quando a bolsa falhava, quando a coisa apertava, dando amor e carinho, e incentivando sempre a fazer o melhor. Ao meu orientador Jonas Contiero, um amigo, que faz de tudo para que alunos possam produzir mais, alcancarem os objetivos e crescerem profissionalmente. À UNESP, pelas instalações e pela minha formação acadêmica no mestrado e doutorado. À FAPESP pela bolsa e reserva técnica (Processo FAPESP 2011/01974-3), e pelo estágio BEPE no exterior (Processo FAPESP 2011/21620-1). À Universidade do Tennessee e ao Prof.º Cong Trinh pela oportunidade de estágio e o aprendizado em clonagem e engenharia de bactérias e leveduras. Aos amigos de laboratório: Fabrício e Cacá, Kate Blanco, Roberta Lovaglio, Luciana Coelho, Daiane, Vinícius Silva e Kenny U. Sato. Ao “*manolo*” Lorenzo Briganti, pelo ano mais intenso e louco, pela amizade e as viagens pelos EUA. Obrigado João Paulo, pelo apoio e alegrias que me deu. À Paula Martins, ao Prof.º Henrique Ferreira, à Lillian e ao Giordanni, pelo apoio na molecular. Ao Michael Wierzbicki, Donovan Layton, Adam Thompson e à Morgan, pelo apoio dado, conhecimento passado, e amizade. Aos outros amigos que contribuíram indiretamente para essa empreitada, Adriano Uemura, Tulio Capelini, César Terrasan, Ivan Dinis, Marcelo Vitor, Rodrigo Araújo, James Webster, Kevin Massey, John Keck, obrigado pela amizade, festas, desabafos e apoio quando precisei. Vocês vão pra sempre morar no meu coração. Adam Haas, Debbie O’Steen, Katherine Harris e Tom Albretch, moramos 5 meses juntos, mas fomos e somos uma família, obrigado! Aos vizinhos, Orlando e Cristina Vasques, obrigado por cuidar de mim e da minha casa quando estive fora. Gostaria de desculpar-me se esqueci de alguém, me desculpe, mas cada um sintam-se abraçado, beijado e eternamente agradecido por menor que seja sua contribuição nesse período que passamos juntos.

“What the result of these investigations will be the future will tell; but whatever they may be, and to whatever this principle may lead, I shall be sufficiently recompensed if later it will be admitted that I have contributed a share, however small, to the advancement of science.”

Nikola Tesla, 1888.

RESUMO

O glicerol de biodiesel pode ser utilizado para produção de 1,3 propanodiol por bactérias. Nesse estudo sobre a produção de 1,3 propanodiol por *Klebsiella pneumoniae* GLC29 foi possível otimizar o meio de cultura para a produção de 1,3-propanodiol utilizando técnicas estatísticas de planejamento experimental e superfície de resposta, onde foram experimentados 11 possíveis variáveis no meio de cultura e verificou-se a influência de apenas 5 (glicerol, extrato de levedura, sulfato de amônio, vitamina B12 e Fumarato). Posteriormente, verificou-se a produção desse composto em bioreatores de 0,75 litros, tanto em batelada como batelada alimentada exponencial, onde foi possível atingir 23.6 g/L de 1,3-propanodiol em batelada usando glicerol puro, 27 g/L de 1,3-propanodiol em batelada usando glicerol bruto de biodiesel e 29.9 g/L de 1,3-propanodiol usando glicerol bruto de biodiesel por batelada alimentada exponencial. Além da otimização, foi possível amplificar e clonar todos os genes responsáveis à produção de 1,3-propanodiol de *Klebsiella pneumoniae* GLC29 em um plasmídeo usando a técnica *Gibson Assembly*, um método recente de montagem isotermal. Os genes foram expressos em cepas de *Escherichia coli*, e foi então verificada a produção de 1,3-propanodiol em frascos e bioreatores, onde foi possível atingir 11 g/L de 1,3-propanodiol produzido por *E. coli* SZ63 contendo o plasmídeo pSB1C3-dhaB123TFG em batelada alimentada.

Palavras Chave: Glicerol, Biodiesel, Planejamento Experimental, Engenharia Metabólica, Genética de Bactérias

Financiamento: FAPESP Processos FAPESP 2011/01974-3 e 2011/21620-1.

ABSTRACT

Biodiesel glycerol can be used for the production of 1,3-propanediol by bacteria. In this study on the production of 1,3-propanediol by *Klebsiella pneumoniae* GLC29 was possible to optimize media culture the production of 1,3-propanediol using statistical experimental design techniques and response surface methodology, where 11 variables were tested and only 5 were found significant (glycerol, yeast extract, ammonium sulfate, vitamin B12 and fumarate). Subsequently, production of 1,3-propanediol was verified in 0,75 liter reactors, both in batch and exponential fed-batch. It was possible to achieve 23.6 g/L of 1,3-propanediol in batch cultures using pure glycerol, 27 g/L of 1,3-propanediol in batch cultures using biodiesel glycerol, and 29.9 g/L of 1,3-propanediol in exponential fed-batch using biodiesel glycerol. Also, it was possible to amplify and clone all the genes responsible for the production of 1,3-propanediol by *Klebsiella pneumoniae* GLC29 in a plasmid, using Gibson assembly, a recent isothermal assembly methodology. The genes were expressed in *Escherichia coli* strains, and it was then verified the production of 1,3-propanediol in flasks and bioreactors, in which it was possible to reach 11 g/L 1,3-propanediol produced by *E. coli* SZ63 with the plasmid pSB1C3-dhaB1234TFG in fed-batch cultures.

Keywords: Glycerol, Biodiesel, Experimental Design, Metabolic Engineering, Genetic of Bacteria.

Funding: FAPESP Processes 2011/01974-3 and 2011/21620-1.

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1. INTRODUÇÃO

Em 2005, o governo brasileiro autorizou o uso comercial do biodiesel, o qual passou a ser adicionado ao diesel comum a uma proporção de 2% (B2). Em 2008, tornou-se obrigatório o seu uso, e a partir de 2010 a proporção passou a ser de 5% (B5) (FARIA *et al.*, 2007, BRASIL, 2005). Somente com o uso do B2, foi gerado um mercado de 800 milhões de litros de biodiesel por ano (ALMEIDA; PINTO, 2007), sendo que com a B5, a produção seria de dois bilhões de litros por ano (BULHÕES, 2007). Em 2014, passou a ser obrigatório o uso da B7, gerando um mercado de 3,4 bilhões de litros anualmente. E com a crescente produção de biocombustíveis, é esperada uma grande disponibilidade de glicerol, pois este é um subproduto da produção do biodiesel. A utilização de óleos vegetais, como o de mamona e de canola para a produção do biodiesel gera para cada 10 kg de biodiesel, 1 kg de glicerol (YAZDANI; GONZALES, 2007). Isto significa uma produção atual de 340 milhões de litros de glicerina por ano no Brasil em 2015.

A conversão desse glicerol em outras moléculas é um desafio e uma alternativa para reduzir os custos da produção do biodiesel (PAPANIKOLAOU *et al.*, 2002). Todavia, novas aplicações estão sendo procuradas para grandes volumes de glicerol, pois este, não pode ser utilizado para uso direto em alimentos e cosméticos sem um processo de limpeza e refino (PACHAURI; HE, 2006).

Sabe-se que o glicerol pode ser utilizado como fonte de carbono em bioprocessos para fabricação de moléculas de alto valor agregado, a partir da fermentação (ALMEIDA; PINTO, 2007). Dentre as principais espécies estudadas estão os gêneros *Clostridium*, *Citrobacter*, *Klebsiella* e *Pseudomonas* (CÁRDENAS *et al.*, 2006; CHENG *et al.*, 2004; DECKWER, 1995; GALDEANO-VILLEGAS *et al.*, 2007; GONZÁLEZ-PAJUELO *et al.*, 2006; LEE *et al.*, 2001; PACHAURI; HE, 2006; SOLAIMAN *et al.*, 2006; YAZDANI; GONZALES, 2007;).

Uma das aplicações estudadas é a utilização do glicerol residual do biodiesel para produção de 1,3-propanodiol por *Clostridium butyricum* em anaerobiose. Vias alternativas para utilização do glicerol em larga escala estão também sendo estudadas em relação à sua transformação em ácido cítrico e óleo de origem unicelular (PAPANIKOLAOU; AGGELIS, 2002; RYMOWICZ *et al.*, 2006).

Este trabalho foi estruturado com uma revisão bibliográfica em português, e dois capítulos em inglês contendo a otimização de meio de cultura para produção por *K. Pneumoniae* GLC29 e a clonagem e expressão de seus genes em um plasmídeo para *E. coli*

para produção de 1,3-propanodiol.

2. REVISÃO DA LITERATURA

2.1 Biodiesel

A maior parte de toda a energia consumida no mundo provém de combustíveis fósseis, como o petróleo, carvão e o gás natural. É quase unânime na literatura que essas fontes são limitadas e com previsão de esgotamento no futuro. Há uma tendência de crescimento contínuo do consumo, a uma taxa média de 3% ao ano no mundo desde 1985. Entretanto as reservas de petróleo, comercialmente exploráveis, são descobertas a taxas menores que o consumo. As reservas mundiais de petróleo em 2005 totalizavam 1,147 trilhões de barris, e o consumo anual deste combustível fóssil em torno de 80 milhões de barris. Estima-se que as reservas mundiais de petróleo se esgotarão por volta do ano de 2046, isto sem levar em consideração a taxa de crescimento no consumo (RATHMANN *et al.*, 2005).

A busca por fontes alternativas tem sido objeto de estudo no mundo todo. Neste contexto, os biocombustíveis surgem como alternativa para substituir os combustíveis derivados do petróleo, e sua demanda vem crescendo rapidamente, sendo a utilização de biomassa para sua produção uma alternativa promissora (DA SILVA *et al.*, 2009). Estudos já apontam que, a utilização da biomassa para fins energéticos vem tendo uma participação crescente perante matriz energética mundial, levando a estimativa de que até o ano de 2050 deverá dobrar o uso mundial de biomassa disponível (FISCHER, 2001).

Atualmente, 46% da matriz energética brasileira provém de fontes renováveis, enquanto que a média mundial é de 13,6%. Certamente a utilização de biocombustíveis tende a aumentar uma vez que a disponibilidade de combustíveis fósseis é limitada (DA SILVA *et al.*, 2009).

Assim como o etanol pode substituir a gasolina em motores com ciclo Otto (ou com partida faísca elétrica), o biodiesel pode substituir o diesel em motores de ignição por compressão (DA SILVA *et al.*, 2009), sendo que o biodiesel tem 85% do poder energético do diesel de petróleo (CÁRDENAS, 2006).

O biodiesel é um combustível não fóssil formado a partir de alquil-monoésteres derivados da transesterificação de óleos e gorduras. A adição de alcoóis de baixa massa

molar, como o metanol e o etanol e um catalisador ácido ou básico (MA; HANNA, 1999) resulta na transformação dos triglicerídeos em moléculas menores de ésteres de ácidos graxos.

Vários são os benefícios e motivações de caráter ambiental no uso do biodiesel. O biodiesel pode reduzir em 78% as emissões de gás carbônico, permite que seja feito um ciclo fechado de carbono, ou seja, o CO₂ emitido de sua queima é reabsorvido pela cultura de oleaginosas, e ainda pode diminuir em 90% as emissões de fumaça, inclusive as emissões de óxido de enxofre, causador da chuva ácida e de irritações das vias respiratórias (CARVALHO, 2006). Além disso, possui características melhores que o diesel comum, como alto número de octano; teor médio de oxigênio; maior ponto de fulgor; menor emissão de partículas HC, CO e CO₂; caráter não tóxico e biodegradável, além de ser proveniente de fontes renováveis (FARIA *et al.*, 2007; FERRARI *et al.*, 2005).

O biodiesel somente se insere na matriz energética brasileira a partir de 2005, com a criação do marco regulatório, Lei 11.097/2005, que torna facultativo seu uso, em uma mistura de 2% ao diesel comum, a partir da sua publicação. Após 2008, o uso da mistura de 2% se torna obrigatória e a de 5%, que estava prevista por lei para iniciar em 2013 (LIMA, 2007; RATHMANN *et al.*, 2005), foi antecipada para janeiro de 2010. Atualmente, o Brasil consome cerca de 35 milhões de toneladas por ano de óleo diesel, e com o uso da B2, isto trouxe uma economia ao país de U\$160 milhões em importação de petróleo. Com o uso da B5, essa economia chegou a U\$400 milhões (FERREIRA; CRISTO, 2006). Além disso, o país contém grandes plantações de oleaginosas e, conseqüentemente, pode fazer uso dessa diversidade para produção de biodiesel a partir de plantas como palma, babaçu, soja, girassol, amendoim, mamona e dendê (GERIS *et al.*, 2007; FERRARI, 2005).

Além de fontes oleaginosas cultiváveis para produção do biodiesel, está sendo investigada a produção de biodiesel de óleo de origem unicelular por algas e leveduras, produzindo biocombustível a partir de biomassa que não compromete fontes de alimentos (YAZDANI; GONZALES, 2007). Outra possibilidade é a utilização de óleo de fritura de restaurantes e lanchonetes. Se estima que haja um mercado de 30 mil toneladas desse resíduo que pode ser aproveitado para fabricação do biodiesel (PARENTE, 2003).

2.2 Glicerol

O glicerol é conhecido desde 1779, quando foi descoberto por Scheele no processo de saponificação do azeite de oliva. Foi também observado por Pasteur no processo de

fermentação etanólica. O glicerol, ou 1,2,3-propanotriol ($\text{OH-CH}_2\text{-CH(OH)-CH}_2\text{-OH}$), é um poliálcool, atóxico, de sabor adocicado, incolor, viscoso, higroscópico, com ponto de fusão de 17,8 °C, ponto de ebulição de 290 °C e solúvel em álcool e éter, e insolúvel em hidrocarbonetos. (ARRUDA *et al.*, 2007). O glicerol é utilizado amplamente nas indústrias alimentícias, farmacêutica, têxtil, química e de cosméticos. Como exemplo pode se citar o papel do glicerol na produção de resinas e poliésteres devido à sua reatividade poli-funcional e também como lubrificante na indústria alimentícia e têxtil (PACHAURI; HE, 2006). Outra característica do glicerol é seu papel como osmorregulador, importante mecanismo que ocorre nas células como reação a fatores ambientais (ARRUDA *et al.*, 2007). Este mecanismo é utilizado pela levedura *Sacharomyces cerevisiae*, em resposta a stress hiper-osmótico (redução da água extracelular) (WANG *et al.*, 2001).

O glicerol pode ser produzido por saponificação de óleos e gorduras, assim como a sua produção a partir de petróleo utilizando cloração em altas temperaturas. Ambas as rotas não são mais utilizadas para produção em grande escala (ARRUDA *et al.*, 2007). Como subproduto da transesterificação para a fabricação do biodiesel, um triglicerídeo reage com o álcool na presença de uma base ou ácido forte, e produz uma mistura de ésteres de ácidos graxos e glicerol (GERIS *et al.*, 2007). A fase mais densa 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. O glicerol produzido pode ser retirado por centrifugação (MA; HANNA, 1999), ou pela adição de água (HAJÉK *et al.*, 2006). Durante a transesterificação, são produzidos 10% de glicerol (m/m) em proporção ao total de biodiesel produzido (MU *et al.*, 2006; PAPANIKOLAOU *et al.*, 2002).

Entretanto, é economicamente inviável a utilização do glicerol residual obtido a partir da produção do biodiesel na indústria alimentícia, farmacêutica e cosmética, uma vez que este exige purificação como branqueamento, desodorização e trocador de íons para remover qualquer traço de outros elementos tornando o processo oneroso (PACHAURI; HE, 2006). Alguns processos geram de forma tão impura que acaba não tendo aceitação comercial. Nesse caso, a glicerina torna-se um efluente da planta, exigindo adequado tratamento, o que demanda investimentos adicionais, com os custos de descarte atribuídos à produção do biodiesel (FERREIRA; CRISTO, 2006; YAZDANI; GONZALEZ, 2007).

O aproveitamento do glicerol residual do biodiesel no uso industrial pode tornar a produção do biodiesel competitivo no mercado de combustíveis, reduzindo o custo de

produção do mesmo (ARRUDA, 2007). Mesmo assim, a inserção da cadeia produtiva do biodiesel na matriz energética brasileira deverá gerar um aumento significativo da oferta interna de glicerina (RATHMANN, *et al.*, 2005). Também, o preço do glicerol vem se tornando competitivo com o dos açúcares usados na produção de químicos via fermentação microbiana (DHARMADI *et al.*, 2006), e esta fonte de carbono está se tornando uma promissora e abundante alternativa devido à produção do biodiesel (DA SILVA *et al.*, 2009), sendo esperado cerca de 700 a 900 milhões de toneladas de glicerol disponíveis em 2010 (VOLLENWEIDER *et al.*, 2004), e estando o preço do glicerol bruto do biodiesel entre 60 a 80 euros por tonelada no mercado europeu (RYMOWICZ *et al.*, 2008). A disponibilidade de glicerol no mercado tem sido tão grande que a Dow Chemical e a Procter & Gamble Chemicals desativaram em 2006 suas plantas de produção de glicerol sintético (MAERVOET *et al.*, 2011).

Recentemente novas aplicações do glicerol vêm sendo descobertas, como substrato para fermentações bacterianas com a finalidade de se obter metabólitos de alto valor agregado como polímeros biodegradáveis, ramnolipídios, biossurfactantes, dentre outros (ARRUDA, 2007). Apesar de vários micro-organismos serem capazes de utilizar o glicerol na presença deceptor final de elétrons (no caso o oxigênio), poucos são capazes de fermentá-lo (YAZDANI; GONZALES, 2007).

2.3 Utilização do glicerol para obtenção de produtos de alto valor agregado

O glicerol começa a ser metabolizado já na membrana celular. Algumas espécies de bactérias apresentam em sua membrana canais facilitadores de glicerol (GlpF) e aquagliceroporinas, que conduzem tanto água, como também glicerol. Os canais facilitadores de glicerol que fazem a fosforilação do glicerol são descritas em *Escherichia coli*, *Bacillus subtilis*, *Klebsiella*, *Shigella*, *Nocardia*, *Enterococcus*, *Lactobacillus lactis*, e espécies de *Pseudomonas*.

Com grande disponibilidade, o glicerol residual do biodiesel torna-se interessante como fonte de carbono para fermentação. Um dos subprodutos que podem ser obtido a partir de fermentação utilizando o glicerol como fonte de carbono é o 1,3- propanodiol (1,3-PDO) produzido por algumas espécies de bactérias da família *Enterobacteriaceae*, e pelas espécies de *Clostridium butyricum*, *Citrobacter freundii* e *Klebsiella pneumoniae* (HOMANN *et al.*, 1990), ácido succínico utilizando *Anaerobiospirillum succiniciproducens* (YAZDANI;

GONZALES, 2007; LEE *et al.*, 2001), butanol pela fermentação por *Clostridium pasteurianum*, ácido propiônico por *Propionibacteria acidipropionici*, ácido acético, ácido láctico, 1,3-butanodiol (DECKWER, 1995), dihidroxiacetonas, hidrogênio e poli-hidroxicarboxilatos (PACHAURI; HE, 2006; SOLAIMAN *et al.*, 2006; ASHBY *et al.*, 2005).

A conversão do glicerol para ácido succínico por *Anaerobiospirillum succiniciproducens* também é descrita e a sua produção pode ser obtida junto com a pequena formação de ácido acético. A máxima produção realizada através de fermentação alimentada de glicerol e extrato de levedura resultou em uma produção máxima de 49 g/L de ácido (YAZDANI; GONZALES, 2007).

Alguns micro-organismos têm a capacidade de produzir metabólitos menos tóxicos como alcoóis e glicóis para escapar da diminuição progressiva do pH do meio conforme produzem ácidos orgânicos. É o caso de *Clostridium acetobutanicum* que forma ácidos acético e butírico em pH neutro e logo após a acidificação do meio, passa a produzir acetona e butanol. O mesmo acontece com as bactérias do gênero *Klebsiella*, que são capazes de adaptar a formação de metabólitos segundo o pH do meio, como *Klebsiella pneumoniae*, que a partir da fermentação do glicerol, troca da produção de ácido acético para 2,3-butanodiol assim que o pH atinge abaixo de 5,5 (BIEBL *et al.*, 1998).

2.3.1 Produção microbiana de 1,3-propanodiol

O maior destaque está na produção microbiana de 1,3-propanodiol (1,3-PDO). O 1,3-PDO contém dois grupos hidroxila na posição α e γ , cuja estrutura e fórmula é $\text{CH}_2\text{OHCH}_2\text{CH}_2\text{OH}$. É um líquido incolor, viscoso em temperatura e pressão normais, inflamável, sabor um pouco doce, com uma toxicidade muito baixa, e é miscível com água e etanol (SONG *et al.*, 2010). O 1,3-PDO é conhecido há mais de 100 anos, e é amplamente empregado para produção de polímeros do tipo poli-trimetileno-tereftalatos (PTT). O PTT é conhecido por suas propriedades elásticas, empregado amplamente na fabricação de resinas, adesivos, tintas aquosas, laminados, revestimentos, molduras, poliéster alifático, e anticongelantes. O 1,3-PDO é conhecido por ser uma molécula bifuncional, e pode ser usado em aplicações semelhantes as do etilenoglicol, propilenoglicol, 1,3-butanodiol e 1,4-butanodiol. Além disso, como os passos de produção de polietileno (PET) e PTT são análogos, logo é possível converter plantas industriais de PET já existentes para produção de PTT, a um custo de

cerca de 10-20% da construção de uma nova planta. O PTT apresenta a mesma resistência que o PET com a vantagem de ter uma cristalização mais rápida e menor ponto de fusão e menor temperatura para moldagem (MAERVOET, 2011).

Existem vários estudos com *Klebsiella pneumoniae*, *Citrobacter freundii*, *Clostridium butyricum* e *Clostridium acetobutylicum*, que são capazes de converter o glicerol residual do biodiesel em 1,3 PDO. Dentre estas bactérias, se destaca a *Clostridium butyricum* que também é capaz de produzir diidroxiacetona, etanol, acetato e butirato (Figura 1). A ampla variedade de aplicações do 1,3 PDO permite estimar que em 2020 possa ocorrer um mercado potencial de 230 mil toneladas por ano (CÁRDENAS *et al.*, 2006).

Para um grande número de bactérias, incluindo *Citrobacter*, *Clostridium*, *Enterobacter*, *Klebsiella* e algumas espécies de *Lactobacillus*, uma consequência do crescimento anaeróbico em glicerol é a geração de excesso de redutores na forma de NADH. A regeneração de NAD requer a formação de um subproduto para servir como um acceptor de elétrons. Vias enzimáticas para a oxidação e redução do glicerol foram incorporadas e usadas quando o glicerol está presente, mas fontes de carbono alternativas como glicose estão ausentes (NAKAMURA; WHITED, 2003).

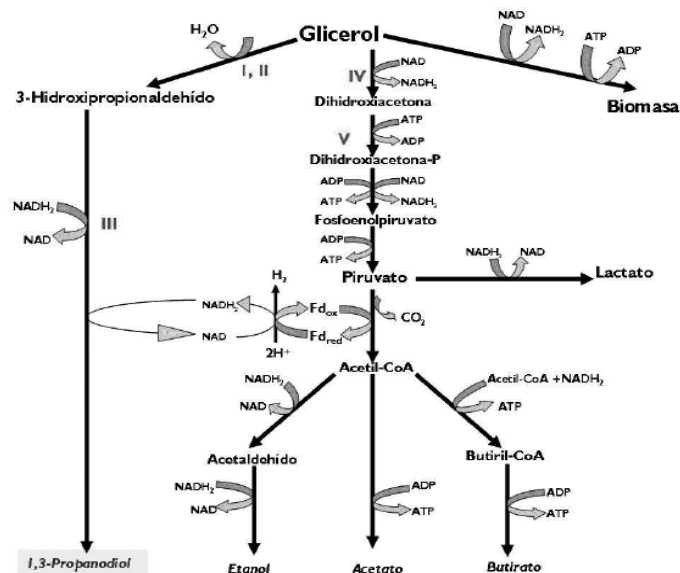


Figura 1 - Rota de assimilação de glicerol por *Clostridium butyricum*. I - Glicerol desidratase; II - ativador da enzima glicerol desidratase; III - 1,3-propanodiol oxidoreductase; IV - glicerol desidrogenase; V - diidroxiacetona kinase; Fd - Ferredoxina. (De YAZDANI; GONZALES 2007).

A produção do 1,3-propanodiol está ligada a um processo oxidativo do glicerol. O glicerol entra na célula por transportador facilitado de glicerol GlpF, ou por difusão (MAERVOET *et al.*, 2011). Quando entra na célula, pode seguir por duas vias. Na via oxidativa, sofre desidrogenação por uma glicerol desidrogenase (glyDH) ligada a uma coenzima NAD^+ , gerando uma diidroxiacetona (DHA) e NADH_2 . A DHA é então fosforilada por uma diidroxiacetona quinase, dependente de ATP (Figura 2). Através do processo paralelo, a via fermentativa, o glicerol é desidratado pela de glicerol desidratase dependente de coenzima B12, para formar 3-hidroxi propionaldeído (3- HPA). O 3-HPA, tóxico a célula, é então reduzido para 1,3-PDO, pela 1,3-propanodiol desidrogenase dependente de NADH_2 . (YAZDANI; GONZALES, 2007). Em *K. Pneumoniae* (Figura 2), a taxa de reação global da via reductora é limitada, em primeiro lugar porque essa reação é mediada por uma coenzima, a cianocobalamina (vitamina B12).

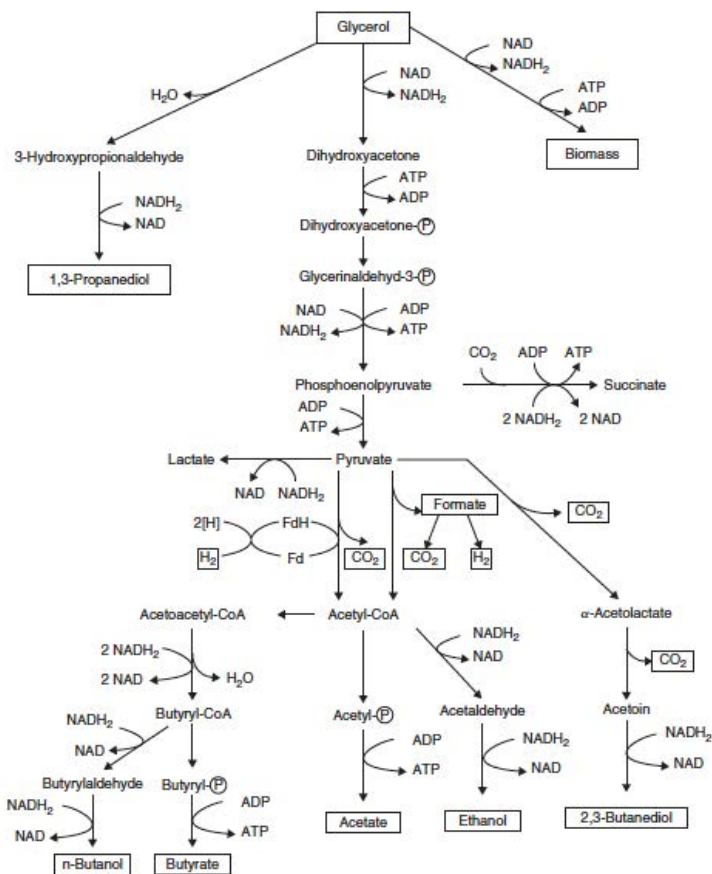


Figura 2 - Fermentação do Glicerol em *K. pneumoniae* (SONG *et al.*, 2010).

Além disso, pode haver inibição pelo substrato, formando uma ligação irreversível da ligação Co-C da cobalamina com a apoenzima, formando uma alquilcobalamina. Contudo,

fatores de reativação, complexo de proteínas dhaF e dhaG fazem troca da cobalamina inativada, por uma nova vitamina B12, na presença de íons de magnésio (Mg^{2+}) e com consumo de ATP. Então, para evitar a baixa atividade da enzima, deve haver o controle da quantidade de glicerol e adição de vitamina B12 ao meio (YAMANISHI *et al.*, 2012; SHIBATA, *et al.*, 2002; KAJIURA *et al.*, 2001, DANIEL *et al.*, 1998)

2.4 Engenharia metabólica na produção de 1,3-propanodiol

Com o uso da engenharia metabólica, é possível manipular as vias de síntese de certos produtos e, reduzir ou até eliminar a formação de subprodutos indesejáveis (CHENG; LIU; LIU, 2005). Forage e Lin (1982) verificaram os genes responsáveis pela produção de 1,3-PDO em *Klebsiella pneumoniae*. Com isso, identificaram o complexo de genes responsáveis pela via da diidroxiacetona (dha), que codificam enzimas no metabolismo do glicerol, como glicerol desidratase (*dhaB1*, *dhaB2* e *dhaB3*), 1,3-propanodiol oxireductase (*dhaT*), glicerol desidrogenase (*dhaD*), diidroxiacetona quinase (*dhaK*) e um gene regulatório (*dhaR*).

DuPont & Genencor International, Inc. modificaram geneticamente *Escherichia coli* de modo que este organismo pudesse produzir 1,3-PDO partir de glicose. Uma vez que *E. coli* selvagem não pode produzir glicerol a partir da glicose de forma eficiente, sete genes tiveram que ser introduzidos no genoma. Dois desses genes (*dar1* e *gpp2*), originados de *Saccharomyces cerevisiae*, e 5 genes (*dhaB1*, *dhaB2*, *dhaB3*, *dhaBX* e *orfX*) a partir de *Klebsiella pneumoniae*. Desta forma, fornece 1,3-propanodiol a uma produtividade de 3,5 g/L.h, uma concentração de 135 g/L, e um rendimento de 0,62 mol/mol.

Devido ao fato de *E. coli* ter bom crescimento em glicerol, vários pesquisadores vem tentando modificá-la geneticamente para produzir 1,3-PDO, valorizando assim o glicerol como fonte de carbono. Ma *et al.* (2009) inseriu 2 genes em *E. coli* e expressando os dois genes na mesma direção, conseguiu produzir 11,3 g/L de 1,3-PDO a partir de 40 g/L de glicerol. Zhang *et al.* (2006) construiu um plasmídeo similar em *E. coli*, e usando delineamento experimental, a partir de concentrações fixadas em 61,8 g/L de glicerol, 6,2 g/L de extrato de levedura e 49 mg/L de vitamina B12, afirma ter conseguido uma produção de 41,3 g/L de 1,3-PDO. Zeng *et al.*, afirmam ter conseguido o máximo com mutante de *E. coli* quando usaram os genes *dhaB1* e *dhaB2* de *C. butyricum*, que apresenta rápida conversão e não é dependente de vitamina B12, e usaram fermentação em dois estágios e

reciclo de células, o primeiro a 30 °C para obtenção de alta densidade celular, e o segundo para produção, passando a cultura para 42 °C e anaerobiose, chegando ao final com 104 g/L de 1,3-PDO, a uma produtividade de 2,61 g/L.h.

3. JUSTIFICATIVA

A produção a partir de glicerol bruto pode contribuir para redução da poluição ambiental e valorização comercial desta fonte de carbono e baratear a produção do 1,3-propanodiol. Além disso, a engenharia metabólica para produção de 1,3-PDO pode levar a uma maior produtividade e rendimento do que a bactéria selvagem. Utilizando cepas com baixo gasto de energia de manutenção celular e baixa produção de metabólitos secundários, optou-se a utilização de *E. coli* por ser bem conhecida, com vetores de expressão bem definidos e de fácil manipulação. Apesar de *K. pneumoniae* já possuir o “maquinário” necessário a produção de 1,3-PDO, a sua patogenicidade é limitante para uso em escala industrial.

4. OBJETIVOS

Geral: O objetivo desse trabalho é avaliar os subprodutos do processo fermentativo a partir do glicerol, principalmente sua conversão a 1,3-propanodiol, e a clonagem e expressão de genes de *K. pneumoniae* GLC29 em *Escherichia coli* para a produção de 1,3-propanodiol.

Objetivos específicos:

4.1 Realizar a clonagem dos genes responsáveis para produção de 1,3-PDO (*dhaB1*, *dhaB2*, *dhaB3*, *dhaT*, *dhaF* e *dhaG*) em diferentes cepas de *E. coli* para obtenção de mutantes.

4.2 Realizar fermentações em diferentes concentrações de Glicerol, Extrato de levedura, sulfato de amônia, fumarato e vitamina B12, utilizando Delineamento Composto Central Rotacional (DCCR).

4.3 Avaliar a produção de 1,3-PDO utilizando glicerol bruto de biodiesel pela cepa *K. pneumoniae* GLC29 e por *E. coli* mutante, e determinar parâmetros cinéticos em batelada e batelada alimentada;

CHAPTER I

Media optimization for 1,3-propanediol production by *K. pneumoniae* GLC29 using experimental design and surface response methodology.

5. INTRODUCTION

The use of vegetable oils for the biodiesel production generates 1 kg of byproduct glycerol for each 10 kg of biodiesel obtained (YAZDANI; GONZALES, 2007). Estimative on global biodiesel market shows it will reach 37 billion gallons, and will reach a 42% annual growth, representing a feedstock of 4 billion gallons of crude glycerol (GANESH *et al.*, 2012).

Glycerol conversion to other molecules is a challenge and an alternative to reduce the costs from biodiesel production (PAPANIKOLAOU *et al.* 2002). However, new applications are being studied for large volumes of biodiesel glycerol, since it can't be used in food and cosmetics industries without a cleaning and refining process (PACHAURI, HE, 2006). However, it is economically impracticable the use of residual glycerol from biodiesel in the food industry, pharmaceuticals and cosmetics, since it requires purification as bleaching, deodorization and ion exchange treatment to remove any traces of other elements, which makes it a very costly process (PACHAURI, HE, 2006). Some processes generate residual glycerol so impure that it does not have any commercial acceptance. In this case, the glycerol becomes an industrial plant effluent, demanding adequate treatment, which requires additional investments, and its disposal costs are attributed to the production of biodiesel (FERREIRA; CRISTO, 2006; YAZDANI; GONZALEZ, 2007). Additionally to this excess of waste glycerol, soap industries and alcohol beverage manufacturing units also have large amounts of glycerol being produced, so there is a need to dispose or use it (KAUR *et al.*, 2012).

It has been known that glycerol may be used as carbon source in bioprocesses (ALMEIDA; PINTO, 2007), and several species among the genera *Clostridium*, *Citrobacter*, *Klebsiella* and *Pseudomonas* (CÁRDENAS *et al.*, 2006; CHENG *et al.*, 2004; DECKWER, 1995; GALDEANO-VILLEGAS *et al.*, 2007; GONZÁLEZ-PAJUELO *et al.* 2006, Lee *et al.* 2,001; PACHAURI, HE, 2006; SOLAIMAN *et al.* 2,006, YAZDANI, GONZALES, 2007;) are being studied for the biotransformation of glycerol from biodiesel to specialty chemicals. One promising solution is the use of biodiesel glycerol to produce 1,3-propanediol by *Klebsiella pneumoniae* and *Clostridium butyricum* (PAPANIKOLAOU; AGGELIS, 2002; RYMOWICZ *et al.* 2006).

The production of 1,3-propanediol is connected to an oxidative process of glycerol. Glycerol enters the cell by *glpF* (glycerol facilitated transport), or by diffusion (MAERVOET *et al.*, 2011). When it enters the cell, it can follow two routes. At the first, suffers oxidative dehydrogenation by a NAD⁺ dependent glycerol dehydrogenase, becoming dihydroxyacetone

(DHA). DHA is next phosphorylated to dihydroxyacetone phosphate by an ATP-dependent DHA kinase. (OH *et al.* 2012; YAZDANI; GONZALES, 2007). Through the parallel process, glycerol is dehydrated to form 3-hydroxypropionaldehyde (3-HPA) by glycerol dehydratase (EC 4.2.1.30), in *K. pneumoniae* case, B12-dependent, composed of 3 peptides, encoded by *dhaB1*, *dhaB2*, and *dhaB3*. Then, 3-HPA is reduced to 1,3-PDO by 1,3-PDO oxidoreductase (EC 1.1.1.202) linked to NADH (YAZDANI; GONZALES, 2007).

In *K. pneumoniae* (Fig. 2), the overall reductive reaction rate is limited, firstly because this reaction is mediated by cyanocobalamin (vitamin B12). Furthermore, substrate inhibition may occur, with an irreversible binding of cobalamin with the enzyme to form alkylcobalamines. However, reactivation factors, encoded by genes *gdrA* and *gdrB* (or *dhaF* and *dhaG*), swap the inactivated cobalamin for a new molecule of vitamin B12, requiring the presence of magnesium ions (Mg^{2+}) and with consumption of 1 ATP. The resultant Apo enzyme rebinds coenzyme B12, and glycerol conversion to 3-HPA resumes. To avoid low activity of the enzyme, the amount of glycerol should be controlled and vitamin B12 to the medium should be added (YAMANISHI *et al.* 2012; NAKAMURA; WHITED, 2003; SHIBATA *et al.*, 2002; KAJIURA *et al.* 2001, DANIEL *et al.* 1998). As a consequence of the normal catalytic cycle with glycerol, the coenzyme B12 is occasionally rendered inactive (B12-inact). The B12-inact remains tightly bound to the dehydratase and catalysis ceases. An auxiliary enzyme, glycerol dehydratase reactivase, facilitates the dissociation of the B12-inact and glycerol dehydratase (EC 4.2.1.30). The resultant apoenzyme rebinds and glycerol conversion to 3-HPA resumes (NAKAMURA; WHITED, 2003).

6. MATERIAL AND METHODS

6.1 Strain studied and maintenance of isolates

The microorganism *K. pneumoniae* CLG29 was previously isolated by Da Silva (2014), and kept at -86 °C in 20% glycerol solution or lyophilized. Subcultures were performed regularly to maintain the viability of the cultures. Cultures were reactivated in test tubes with 5 mL of Luria Berthani (LB) and incubated at 37° C for 12-24h.

6.2 Sequencing

DNA from the 16S region was amplified from genomic DNA extracted from the isolated *Klebsiella pneumoniae* GLC29 using primers 8F and 1492R and Taq polymerase in a standard PCR reaction. The isolated DNA was treated with Big Dye Polymerase kit prior to sequencing. Sequences were aligned using MEGA 5.0 and ClustalW softwares using the Neighbor-Joining method (SAITOU; NEI, 1987).

6.3 Fermentation by *K. pneumoniae* GLC29

K. pneumoniae GLC29 was grown in rotatory shaker at 37 °C and 100 rpm for 8-24h in 125 mL Erlenmeyer flasks containing 50 mL of synthetic medium (g/L): (NH₄)₂PO₄ 5.0, K₂HPO₄ 1.5; NaCl 1.0, and 1 mL of trace elements stock solution (DA SILVA, 2014) was added per liter of media prepared, which composition was (g/L): EDTA 0.5, CaCl₂.2H₂O 0.5, CoCl₂.6H₂O 0.16; (NH₄)₆Mo₇O₂₄.4H₂O 0.1, CuSO₄.5H₂O 0.16; FeSO₄.7H₂O 0.5, MnSO₄.H₂O 0.5, ZnSO₄.7H₂O 0.22, NiCl₂.6H₂O 0.03, H₃BO₃ 0.12. Carbon and nitrogen sources, along with salts and vitamins concentrations were determined in the experimental design.

Experimental designs and batch cultures in fermenter vessels were performed with media components as optimized in the experimental designs discussed in results section. Synthetic Media for those experiments were the same as described above.

6.4 Detection of produced metabolites

Periodically, samples of 1 mL were collected from the cultures and centrifuged at 10,000 g for 10 minutes. The cell-free supernatant was filtered (0.22 µm) and analyzed by high performance liquid chromatography (HPLC) using ion exchange column Phenomenex Rezex ROA (300 mm x 7.8 mm) at a temperature of 60 °C and 0.005 M of H₂SO₄ water solution as mobile phase at 0.5 mL/min. External standards used were ethanol, 1,3-propanediol, propionic acid, acetic acid, 2,3-butanediol and glycerol.

6.5 Obtaining the absorbance curve and Dry Mass

Microbial growth was quantified by reading the optical density and correlated to dry

cell mass by simple linear correlation. Successive dilutions of cell suspension and readings were made using optical spectrophotometry at 600 nm (DO 600 nm) with readings in the range of 0.2 to 0.8 absorbance. The Total Dry Mass was multiplied by the inverse of the dilutions made, and correlated with the absorbance values obtained and verified using linear correlation and Pearson's correlation coefficient.

6.6 Optimization using Experimental Design

Traditional techniques for multivariate optimization systems are not only time consuming but also don't show interactions among the factors tested. They require a large number of experiments to determine optimal points, which are usually not reproducible or statistically significant. Design of experiments (DoE) is an alternative to improve processes and to investigate correlations and synergistic or antagonistic interactions between factors tested collectively, minimizing the number of experiments, and it is possible to analyze the results using a response surface method (IMANDI *et al.*, 2007; RYMOWICZ; CIBIS, 2006). Techniques of fractional factorial design and full factorial design to screen factors that influence microbial growth and production of 1,3-Propanediol were used. Five replicates of each the center point were performed to determine experimental error and calculate ANOVA. The significant level $p < 0.05$ or $p < 0.1$ for screening methods was used. Pareto chart of effects were analyzed, and the absolute value of the standardized ($p < 0.05$ or $p < 0.1$) effect represented in bars was calculated by dividing each coefficient by its standard error (Coef/SE Coef).

Experimental designs were made to screen possible significant variables in the culture medium. Eleven variables were chosen to be screened and a Plackett-Burman Factorial DoE was executed with 5 central points and 4 dummy variables, which were used to calculate experimental error. Then, with less significant variables, it was performed a Box, Hunter & Hunter Factorial DoE and two Central Composite non factorial Surface response Designs with full resolution to observe interactions between the variables. Samples were taken every 8, 24 and 48 hours from the fermentation flasks. Experiments were performed always in replicates or triplicates, accordingly to the limitations imposed by the number of experiments and data obtained from ANOVA.

7. RESULTS AND DISCUSSION

7.1 Strain Identification

The evolutionary history was inferred using the Neighbor-Joining method (Saitou; Nei, 1987). The optimal tree with the sum of branch length = 0.08674627 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (FELSENSTEIN J., 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (TAMURA *et al.*, 2004) and are in the units of the number of base substitutions per site. The analysis involved 20 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1225 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (TAMURA *et al.*, 2011). Based on the relationship with the consensus to the other *Klebsiella pneumoniae* strains and its relative species, it is proposed that the bacteria isolated by Da Silva (2014) is a *Klebsiella pneumoniae*.

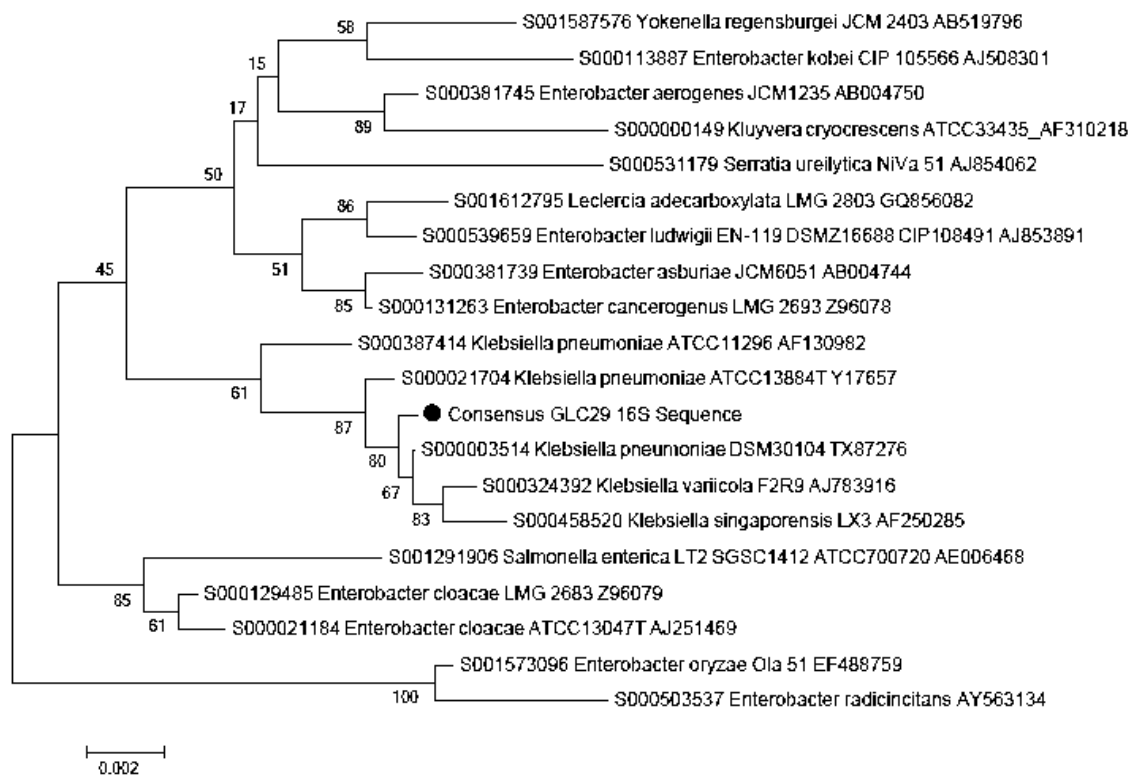


Figure 3 - Evolutionary relationships of taxa from strain *Klebsiella pneumoniae* GLC29 (closed circle).

7.2 Culture Optimizations of *Klebsiella pneumoniae* GLC29

7.2.1 Placket-Burman Fatorial DoE

Eleven variables were chosen for culture media screening, based on the literature. We chose to experiment salts which might have influence on the known enzymes glycerol dehydratase (EC 4.2.1.30) and 1,3-propanediol oxidoreductase (EC 4.2.1.30) based on Brenda website database (SCHOMBURG *et al.*, 2013). Variables and decoded levels are show in Table 1.

Table 1 – Culture Optimizations of *Klebsiella pneumoniae* GLC29 - Placket-Burman Design: decoded levels of the independent variables. Variables D1 to D4 are dummies.

Variable	Levels		
	-1	0	+1
(1) Glycerol (g/L)	30.0	45.0	60.0
(2) Yeast Extract (g/L)	1.0	3.0	5.0
(3) (NH ₄) ₂ SO ₄ (g/L)	0.0	1.5	3.0
(4) NaNO ₃ (g/L)	0.0	1.5	3.0
(5) Fumarate (mM)	0.0	2.5	5.0
(6) Vitamin B12 (µg/L)	0.0	125.0	250.0
(7) Inoculum (%)	1.0	5.0	10.0
(8) CaCl ₂ (mM)	1.0	3.0	5.0
(9) MgSO ₄ (mM)	4.0	7.0	10.0
(10) ZnCl ₂ (mM)	1.0	3.0	5.0
(11) MnSO ₄ (mM)	1.0	3.0	5.0
(12) D1	0.0	0.0	0.0
(13) D2	0.0	0.0	0.0
(14) D3	0.0	0.0	0.0
(15) D4	0.0	0.0	0.0

As results, we collected 4 significant ($p < 0.1$) dependent variables: cell dry weight (DCW, g/L), 1,3-propanediol (1,3-PDO, g/L), consumed glycerol (g/L) and calculated yield ($\text{g}_{1,3\text{-PDO}}/\text{g}_{\text{glycerol}}$), shown on figures 4 to 7. Table 2 shows the results from this experimental design, and in bold, experiment number 7 was the most significant in production.

Figure 4 shows the Pareto chart of effects for dry cell weight, screening 11 variables. Pareto chart of the effects are used to determine the magnitude of an effect, displaying the absolute value of the effects a reference line on the chart.

Table 2 - Coded variables and results from Plackett-burman experimental design (screening variables).

Run	Independent Variables															Dependent Variables			
	Glycerol	YE	(NH ₄) ₂ SO ₄	NaNO ₃	Fumarate	Vit. B12	Inocule	CaCl ₂	MgSO ₄	ZnCl	MnSO ₄	D1	D2	D3	D4	DCW g/L	1,3-PDO g/L	Rem.Gly g/L	Yield g _p /g _s
1	-1	-1	-1	-1	1	1	1	1	1	1	-1	-1	-1	1	0,46	2,25	16,65	0,17	
2	1	-1	-1	-1	-1	-1	-1	1	1	1	1	1	1	-1	0,31	0,50	30,8	0,42	
3	-1	1	-1	-1	-1	1	1	-1	-1	1	1	1	-1	1	0,60	1,92	20,80	0,21	
4	1	1	-1	-1	1	-1	-1	-1	-1	1	-1	-1	1	1	0,003	0,17	29,00	0,18	
5	-1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	1	1,00	3,39	8,11	0,15	
6	1	-1	1	-1	-1	1	-1	-1	1	-1	-1	1	-1	1	0,83	0,80	26,36	0,22	
7	-1	1	1	-1	-1	-1	1	1	-1	-1	-1	1	1	-1	1,71	13,1	6,53	0,56	
8	1	1	1	-1	1	1	-1	1	-1	-1	1	-1	-1	-1	0,72	0,61	25,80	0,14	
9	-1	-1	-1	1	1	1	-1	1	-1	-1	-1	1	1	1	0,42	1,52	45,8	0,17	
10	1	-1	-1	1	-1	-1	1	1	-1	-1	1	-1	-1	1	0,65	0,93	57,14	0,32	
11	-1	1	-1	1	-1	1	-1	-1	1	-1	1	-1	1	-1	0,41	0,15	1,47	0,002	
12	1	1	-1	1	1	-1	1	-1	1	-1	-1	1	-1	-1	0,36	0,83	52,04	0,11	
13	-1	-1	1	1	1	-1	-1	-1	-1	1	1	1	-1	1	0,58	0,51	55,39	0,11	
14	1	-1	1	1	-1	1	1	-1	-1	1	-1	-1	1	-1	0,55	1,26	59,19	1,56	
15	-1	1	1	1	-1	-1	-1	1	1	1	-1	-1	-1	1	0,79	6,11	37,16	0,26	
16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0,48	1,07	56,83	0,33	
17(C)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0,42	0,88	42,67	0,38	
18(C)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0,44	0,73	43,60	0,53	
19(C)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0,45	0,68	41,99	0,22	
20(C)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0,44	0,6	39,22	0,10	

YE: Yeast Extract, Vit. B12: Vitamin B12, Rem. Gly.: Remaining Glycerol, DCW, Dry Cell Weight.

Any effect that extends beyond this reference line is potentially important. Glycerol, $(\text{NH}_4)_2\text{SO}_4$, NaNO_3 , fumarate, inoculum percentage, zinc chloride, were considered significant, and R^2 , which measures the obtained data adjusted to the calculated model, was 0.952, which means a fit of 95.2%. No dummy variable (D1 to D4) were significant, which was expected for a valid design.

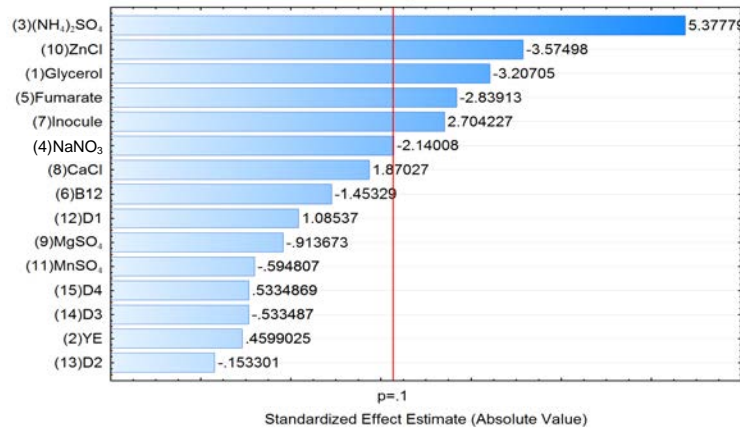


Figure 4 - Culture Optimizations of *Klebsiella pneumoniae* GLC29 – Pareto chart of effects for Dry Cell Weight at 72 h culture (DCW) in Placket-Burman DoE. Variables with p -value < 0.1 are considered significant.

Table 3 shows the ANOVA results for dependent variable DCW, and significant variables are shown in bold. Calculated F-value was higher than the F-value table.

Table 3 - ANOVA Variable: Dry Cell Weight g/L; $R^2=0.952$; R^2 Adjusted: 0.77 (PE Placket-Burman 11 variables)

Variables	SS	df	MS	F	p
(1) Glycerol (g/L)	0.27	1.00	0.27	10.29	0.03
(2) Yeast Extract (g/L)	0.01	1.00	0.01	0.21	0.67
(3) $(\text{NH}_4)_2\text{SO}_4$ (g/L)	0.75	1.00	0.75	28.92	0.01
(4) NaNO_3 (g/L)	0.12	1.00	0.12	4.58	0.10
(5) Fumarate (mM)	0.21	1.00	0.21	8.06	0.05
(6) Vitamin B12 ($\mu\text{g/L}$)	0.05	1.00	0.05	2.11	0.22
(7) Inoculum (%)	0.19	1.00	0.19	7.31	0.05
(8) CaCl_2 mM	0.09	1.00	0.09	3.50	0.13
(9) MgSO_4 (mM)	0.02	1.00	0.02	0.83	0.41
(10) ZnCl (mM)	0.33	1.00	0.33	12.78	0.02
(11) MnSO_4 (mM)	0.01	1.00	0.01	0.35	0.58
(12) D1	0.03	1.00	0.03	1.18	0.34
(13) D2	0.00	1.00	0.00	0.02	0.89
(14) D3	0.01	1.00	0.01	0.28	0.62
(15) D4	0.01	1.00	0.01	0.28	0.62
Error	0.10	4.00	0.03		
Total SS	2.20	19.00			

SS: Sum of Squares; df Degrees of freedom; MS Mean Squared; F: Calculated F value for the F-test.; p: p value

It's noticeable that ammonium sulfate was very important to cell growth, while inoculum concentration had also positive effect even after 72 h. Zinc chloride from 1 mM, glycerol from 3% to 6%, NaNO_3 and fumarate addition were not beneficial to cell growth (Fig. 4), but parameters with negative effect can be influenced by negative interactions. Other parameters had no significant effect on cell growth, and do not need to be added to promote growth.

Figure 5 illustrates the effect of the variables tested for 1,3-propanediol production. Glycerol from 3% to 6%, manganese sulfate, vitamin B12 and fumaric acid addition had negative effect on the 1,3-propanediol biosynthesis. But as considered above, these results are not conclusive, since negative effects can be obtained by negative interactions between factors and further experiments are necessary.

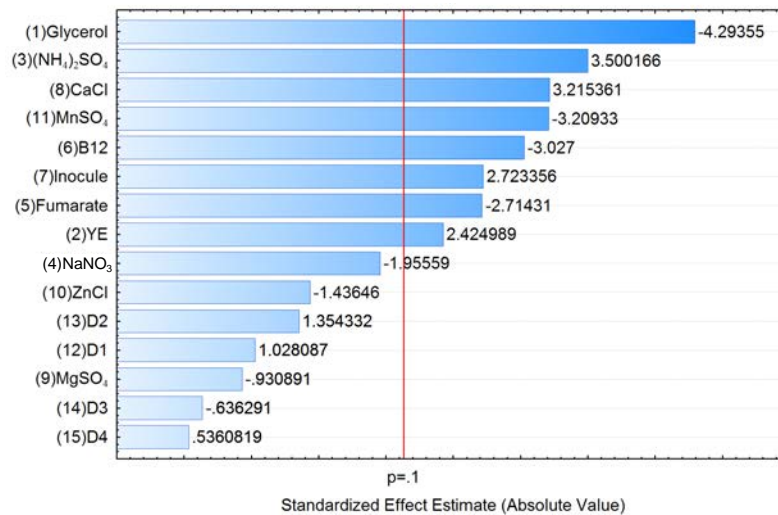


Figure 5 - Culture Optimizations of *Klebsiella pneumoniae* GLC29 -Pareto chart of effects for 1,3-propanediol production at 72 h in Plackett-Burman DoE. Variables with p-value<0.1 were considered significant.

However, ammonium sulfate, calcium chloride, yeast extract and inoculum had positive impact on total 1,3-propanediol produced. Therefore, it is suggested that they must be kept for additional studies. Furthermore, NaNO_3 , MgSO_4 and ZnCl_2 had no significant impact on 1,3-PDO synthesis, so their removal from the media is recommended for the production. Glycerol, although had a negative influence on the total production, is the main carbon source and new experiments with lower concentrations were performed on the subsequent experiments. ANOVA results for dependent variable 1,3-propanediol production are shown on table 4, and significant variables are shown in bold. Calculated F-value was higher than the F-value table.

Table 4 - ANOVA of the Variable 1,3-Propanediol g/L; $R^2 = 0.95$; R^2 Adjusted: 0.80 (PE Placket-Burman 11 var) DV: 1,3-Propanediol g/L

Variables	SS	df	MS	F	p
(1) Glycerol (g/L)	32.47	1.00	32.47	18.43	0.01
(2) Yeast Extract (g/L)	10.36	1.00	10.36	5.88	0.07
(3) (NH₄)₂SO₄ (g/L)	21.58	1.00	21.58	12.25	0.02
(4) NaNO ₃ (g/L)	6.74	1.00	6.74	3.82	0.12
(5) Fumarate (mM)	12.98	1.00	12.98	7.37	0.05
(6) Vitamin B12 (µg/L)	16.14	1.00	16.14	9.16	0.04
(7) Inoculum (%)	13.06	1.00	13.06	7.42	0.05
(8) CaCl₂ mM	18.21	1.00	18.21	10.34	0.03
(9) MgSO ₄ (mM)	1.53	1.00	1.53	0.87	0.40
(10) ZnCl (mM)	3.63	1.00	3.63	2.06	0.22
(11) MnSO₄ (mM)	18.14	1.00	18.14	10.30	0.03
(12) D1	1.86	1.00	1.86	1.06	0.36
(13) D2	3.23	1.00	3.23	1.83	0.25
(14) D3	0.71	1.00	0.71	0.40	0.56
(15) D4	0.51	1.00	0.51	0.29	0.62
Error	7.05	4.00	1.76		
Total SS	168.21	19.00			

SS: Sum of Squares; df Degrees of freedom; MS Mean Squared; F: Calculated F value for the F-test.; p: p value

Glycerol consumption was also analyzed, since the remaining glycerol is not economically feasible. The effect of the variables on the remaining glycerol is shown on Table 6. Thus, yeast extract and MgSO₄ favored glycerol consumption, while NaNO₃, glycerol and ZnCl did not. Other factors had no significant influence in residual glycerol. Interestingly, MgSO₄, which had no significant influence on 1,3-propanediol production nor on Dry Cell Weight (DCW) had significant influence on glycerol consumption, suggesting that it might be driving carbon flux to a competitive metabolic pathway.

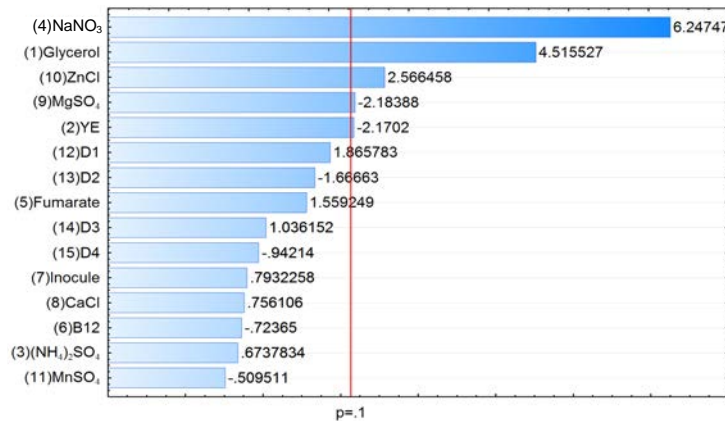


Figure 6 - Culture Optimizations of *Klebsiella pneumoniae* GLC29 - Pareto chart of effects for remaining glycerol after 72 h in Placket-Burman DoE. Variables with p-value < 0.05 were considered significant.

Table 5 - ANOVA results for the independent variable Remaining Glycerol g/L; $R^2=0.95$; Adjusted R^2 : 0.79 (PE Plackett-Burman 11 var)

Variables	SS	df	MS	F	p
(1) Glycerol (g/L)	1318.54	1.00	1318.54	20.39	0.01
(2) Yeast Extract (g/L)	304.56	1.00	304.56	4.71	0.10
(3) $(\text{NH}_4)_2\text{SO}_4$ (g/L)	29.36	1.00	29.36	0.45	0.54
(4) NaNO_3 (g/L)	2523.98	1.00	2523.98	39.03	0.00
(5) Fumarate (mM)	157.22	1.00	157.22	2.43	0.19
(6) Vitamin B12 ($\mu\text{g/L}$)	33.86	1.00	33.86	0.52	0.51
(7) Inoculum (%)	40.69	1.00	40.69	0.63	0.47
(8) CaCl_2 (mM)	36.97	1.00	36.97	0.57	0.49
(9) MgSO_4 (mM)	308.42	1.00	308.42	4.77	0.09
(10) ZnCl (mM)	425.94	1.00	425.94	6.59	0.06
(11) MnSO_4 (mM)	16.79	1.00	16.79	0.26	0.64
(12) D1	225.11	1.00	225.11	3.48	0.14
(13) D2	179.62	1.00	179.62	2.78	0.17
(14) D3	69.43	1.00	69.43	1.07	0.36
(15) D4	57.40	1.00	57.40	0.89	0.40
Error	258.66	4.00	64.67		
Total SS	5986.55	19.00			

SS: Sum of Squares; df Degrees of freedom; MS Mean Squared; F: Calculated F value for the F-test.; p: p value

For this reason, MgSO_4 from 4 mM is not recommended, but lower concentrations might be necessary for other cell functions, like glycerol dehydratase reactivase, which needs magnesium ions (Mg^{2+}) as a cofactor to unbind vitamin B12 and glycerol from glycerol dehydratase in the 1,3-propanediol pathway. ANOVA results for dependent variable remaining glycerol are shown on table 5, and significant variables are shown in bold. Calculated F-value was higher than the F-value table.

Total yield was also calculated, and the effects of the variables are shown on figure 7. Several factors, *i.e.* inoculum concentration, ammonium sulfate, glycerol and ZnCl contributed significantly to this result. Fumaric acid, MgSO_4 , and MnSO_4 addition, had a negative effect on yield. Besides, vitamin B12, NaNO_3 , CaCl_2 and yeast extract had no significant influence on this dependent variable.

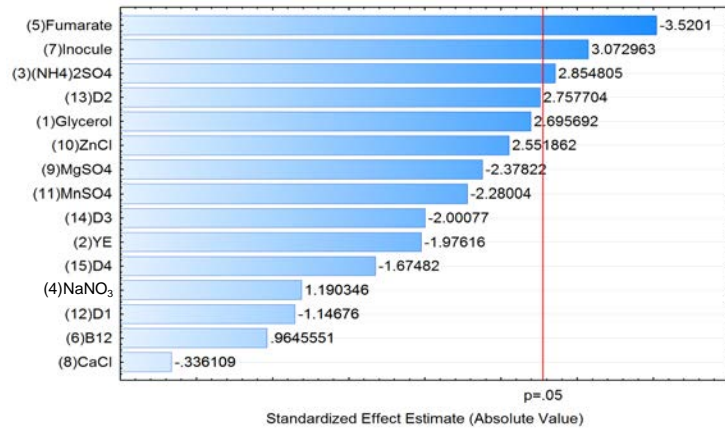


Figure 7 - Culture Optimizations of *Klebsiella pneumoniae* GLC29 - Pareto chart of effects for calculated yield in Plackett-Burman DoE. Variables with p-value <0.1 were considered significant.

Table 6 - ANOVA of the dependent variable Yield g_p/g_s; R² = 0.95; Adjusted R² = 0.76 (PE Plackett-Burman 11 var)

Variables	SS	df	MS	F	p
(1) Glycerol (g/L)	0.19	1.00	0.19	7.27	0.05
(2) Yeast Extract (g/L)	0.10	1.00	0.10	3.91	0.12
(3) (NH₄)₂SO₄ (g/L)	0.21	1.00	0.21	8.15	0.05
(4) NaNO ₃ (g/L)	0.04	1.00	0.04	1.42	0.30
(5) Fumarate (mM)	0.32	1.00	0.32	12.39	0.02
(6) Vitamin B12 (μg/L)	0.02	1.00	0.02	0.93	0.39
(7) Inoculum (%)	0.24	1.00	0.24	9.44	0.04
(8) CaCl ₂ mM	0.00	1.00	0.00	0.11	0.75
(9) MgSO ₄ (mM)	0.15	1.00	0.15	5.66	0.08
(10) ZnCl (mM)	0.17	1.00	0.17	6.51	0.06
(11) MnSO ₄ (mM)	0.13	1.00	0.13	5.20	0.08
(12) D1	0.03	1.00	0.03	1.32	0.32
(13) D2	0.20	1.00	0.20	7.60	0.05
(14) D3	0.10	1.00	0.10	4.00	0.12
(15) D4	0.07	1.00	0.07	2.81	0.17
Error	0.10	4.00	0.03		
Total SS	2.08	19.00			

SS: Sum of Squares; df Degrees of freedom; MS Mean Squared; F: Calculated F value for the F-test.; p: p value

From those data, it was possible to conclude that glycerol from 3% to 6% had a bad influence on total 1,3-propanediol and cell growth. Although it had a positive influence on total yield, this is not interesting since it had also a positive influence on remaining glycerol. Therefore, for further optimizations, glycerol was fixed only in the range from 1% to 3%.

Inoculum had a positive effect in almost every dependent variable, except for remaining glycerol, which was not significant. Therefore, using 10% inoculum is recommended and it was chosen for next optimizations.

Zinc chloride negatively influenced cell growth and glycerol consumption, so its removal is recommended from media formulation. Also, NaNO_3 , MgSO_4 and MnSO_4 had negative or no significant effect on each dependent variable, and their removal from next experiments were recommended as well. It's important to stress that concentrations used were obtained from BRENDA website, which describe optimum concentrations for the enzymes, but could not reflect the best concentrations for the cells. Therefore, the amount of ZnCl and MnSO_4 exhibited a toxic to the cells, but CaCl_2 had a positive effect with the concentration used.

Interestingly, vitamin B12 was not significant for most variables, and had negative effect on 1,3-propanediol production, which contradicts literature. This factor was maintained for further optimizations to confirm those data. Fumaric acid also had negative effect on several variables, including 1,3-propanediol production, contradicting literature and previous data obtained; therefore, it was kept as a variable for further analysis.

7.2.2 Box, Hunter & Hunter Full Factorial DoE

Five factors from the previous Design of Experiments (DoE) were chosen to screen variables, as glycerol concentration, yeast extract, ammonium sulfate, vitamin B12 and fumaric acid. Inoculum concentration was fixed at 10%, and CaCl_2 at 5 mM. Other variables, which had no significant effect were removed from the formulation.

Table 7 shows decoded variables for this DoE. It was collected samples after 24 h fermentation, and data analyzed were 1,3-propanediol (1,3-PDO g/L), acetate (g/L), propionate (g/L), 2,3-butanediol (g/L), and glycerol consumed. Due to the extent of those data and graphs generated we opted to show the most representative data.

Table 7 - Box, Hunter & Hunter Full Factorial Design: decoded levels of the independent variables.

Variable	Levels		
	-1	0	+1
(1) Glycerol (g/L)	10.0	20.0	30.0
(2) Yeast Extract (g/L)	3.0	5.0	7.0
(3) $(\text{NH}_4)_2\text{SO}_4$ (g/L)	0.0	1.5	3.0
(4) Vitamin B12 ($\mu\text{g/L}$)	0.0	250.0	500.0
(5) Fumarate (mM)	0.0	2.5	5.0

Table 8 shows ANOVA results for 1,3-PDO production after 24h in flasks. With Box, Hunter & Hunter full resolution DoE, it is possible to observe interactions between the variables. The interactions calculated are represented on the tables and graphs by the numbers of the independent variable by the other variable, *i.e.* 1by2 represents the interaction between

Glycerol (1) and Yeast Extract (2), 4by5 represents the interaction between Vit. B12 and Fumarate, and so on. Variables with p -value <0.05 were considered significant.

Table 8 - ANOVA; Var.: 1,3-propanediol at 24h; $R^2 = 0.93$; Adjusted R^2 : 0.89 (Results DoE Box, Hunter & Hunter 5 var. (Glycerol, YE, $(\text{NH}_4)_2\text{SO}_4$, Fumarate and Vit. B12);)

	SS	df	MS	F	p
(1)Glycerol	139.22	1.00	139.22	226.36	0.00
(2)YE	2.94	1.00	2.94	4.78	0.04
(3)$(\text{NH}_4)_2\text{SO}_4$	6.30	1.00	6.30	10.25	0.00
(4)B12	10.22	1.00	10.22	16.62	0.00
(5)Fumarate	3.30	1.00	3.30	5.37	0.03
1 by 2	7.27	1.00	7.27	11.82	0.00
1 by 3	1.94	1.00	1.94	3.16	0.09
1 by 4	12.37	1.00	12.37	20.10	0.00
1 by 5	3.38	1.00	3.38	5.49	0.03
2 by 3	0.08	1.00	0.08	0.13	0.72
2 by 4	0.80	1.00	0.80	1.31	0.27
2 by 5	0.00	1.00	0.00	0.00	0.99
3 by 4	0.39	1.00	0.39	0.63	0.44
3 by 5	1.13	1.00	1.13	1.84	0.19
4 by 5	3.90	1.00	3.90	6.33	0.02
Error	12.92	21.00	0.62		
Total SS	206.14	36.00			

SS: Sum of Squares; df Degrees of freedom; MS Mean Squared; F: Calculated F value for the F-test.; p: p value

ANOVA resulted on a satisfactory R^2 of 93% fit to the model, and ignoring the non-significant variables, the adjusted R^2 resulted in a 89% fit to the model. All independent variables selected from the Plackett-Burman DoE screening were significant to the experiment, and also some interactions among them. Figure 8 shows the calculated effect of each variables and respective interactions between them. Glycerol up to 30 g/L, Vitamin B12 up to 500 $\mu\text{g/L}$, Ammonium Sulfate up to 3 g/L, Fumarate up to 5 mM, Yeast Extract up to 7 g/L, and their interactions Glycerol with Vitamin B12, Glycerol and Yeast Extract, and Glycerol with Fumarate had a significant impact in 1,3-propanediol production.

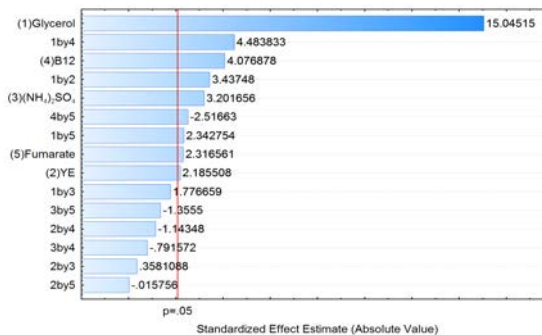


Figure 8 - Culture Optimizations of *Klebsiella pneumoniae* GLC29: 1,3-propanediol g/L at 24h cultivation Pareto chart of effects in Box, Hunter & Hunter Full DoE 5 var. (Glycerol, YE, $(\text{NH}_4)_2\text{SO}_4$, Fumarate and Vit. B12). Variables with p -value <0.05 were considered significant.

The interaction between Fumarate and Vitamin B12 (4by5 on figure 8) had a negative effect on production, which negative slope (-2.51) completely annulled fumarate effect (2.31). The negative effect of the Vitamin B12 on Plackett-Burman screening design could be explained by the negative effect it has when mixed with fumaric acid. Therefore, fumarate addition with vitamin B12 is not recommended, since it would have a higher cost in media formulation and the interaction between both components would result in the same or less production.

Calculated yield ($Y_{p/s}$) had only 3 significant variables, Glycerol, Ammonium Sulfate and the interaction of vitamin B12 and glycerol. Table 9 shows the ANOVA results for the Calculated Yield p/s. R^2 was 0.89 and the Adjusted R^2 , which ignores the effect of the non-significant variables, was 0.82. Variables with p-value<0.05 were considered significant and are shown in bold on Table 9.

Table 9 - ANOVA Calculated Yield p/s; $R^2 = 0.89$; Adjusted $R^2 = 0.82$. Results DoE Box, Hunter & Hunter 5 variables (Glycerol, YE, $(NH_4)_2SO_4$, Fumarate and Vit. B12).

	SS	df	MS	F	p
(1)Glycerol	0.11	1.00	0.11	151.69	0.00
(2)YE	0.00	1.00	0.00	1.29	0.27
(3)(NH4)2SO4	0.01	1.00	0.01	13.09	0.00
(4)B12	0.00	1.00	0.00	0.12	0.74
(5)Fumarate	0.00	1.00	0.00	0.09	0.76
1 by 2	0.00	1.00	0.00	2.61	0.12
1 by 3	0.00	1.00	0.00	0.21	0.65
1 by 4	0.00	1.00	0.00	6.67	0.02
1 by 5	0.00	1.00	0.00	0.11	0.74
2 by 3	0.00	1.00	0.00	0.07	0.79
2 by 4	0.00	1.00	0.00	2.54	0.13
2 by 5	0.00	1.00	0.00	0.44	0.51
3 by 4	0.00	1.00	0.00	0.20	0.66
3 by 5	0.00	1.00	0.00	3.38	0.08
4 by 5	0.00	1.00	0.00	1.29	0.27
Error	0.01	21.00	0.00		
Total SS	0.14	36.00			

SS: Sum of Squares; df Degrees of freedom; MS Mean Squared; F: Calculated F value for the F-test.; p: p value.

Calculated yield ($Y_{p/s}$) was enhanced by the addition of up to 3 g/L of Ammonium sulfate, and the glycerol interaction with Vitamin B12 had a positive effect on this variable (Figure 9). Other variables were not significant to $Y_{p/s}$, including the linear effect of vitamin B12, which is only positive by the interaction with glycerol.

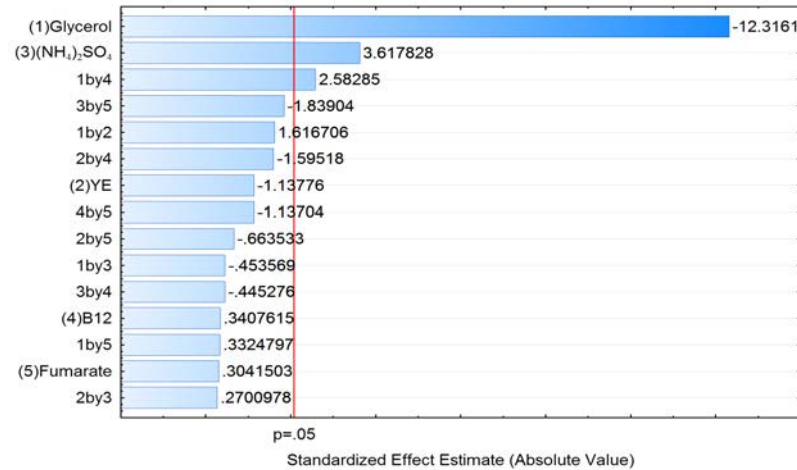


Figure 9 - Culture Optimizations of *Klebsiella pneumoniae* GLC29: 1,3-propanediol Yield_{p/s} at 24h cultivation Pareto chart of effects in Box, Hunter & Hunter Full DoE 5 var. (Glycerol, YE, (NH₄)₂SO₄, Fumarate and Vit. B12). Variables with p-value<0.05 were considered significant.

Data from other metabolites were collected, like ethanol, acetate, lactate and 2,3-propanediol and used as response variables. While byproducts formation was substantial, statistical correlation was only obtained for 2,3-butanediol production (Figure 10). Glycerol was the main factor to effect positively this byproduct formation. Interestingly, Fumarate, YE, and Vitamin B12 were also beneficial to the biosynthesis of this byproduct. But it is important to stress that the experimental design was performed in conical flasks and therefore in aerobic to micro aerobic conditions, with no pH control, which favors 2,3-butanediol formation.

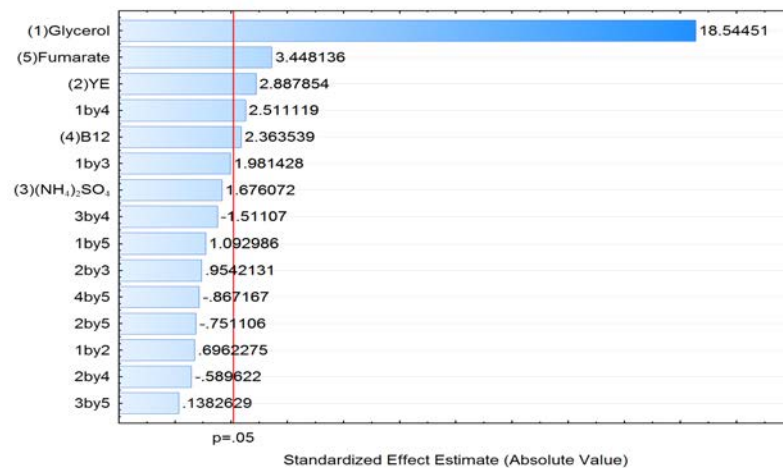


Figure 10 - Culture Optimizations of *Klebsiella pneumoniae* GLC29 - 2,3-butanediol at 24h cultivation Pareto chart of effects in Box, Hunter & Hunter Full DoE 5 var. (Glycerol, YE, (NH₄)₂SO₄, Fumarate and Vit. B12). Variables with p-value<0.05 were considered significant.

Table 10 shows the coded levels and results for Box, Hunter & Hunter Full Factorial Design used to perform the presented analysis. Figure 11 shows the plot between calculated values and observed values for each experiment.

Table 10 - Box, Hunter & Hunter Full Factorial Design: coded levels for screening culture media and results.

	Dependent Variables					Independent Variables				
	GlyOH g/L	YE g/L	(NH ₄) ₂ SO ₄ g/L	B12 mg/ L	Fumarate mM	GlyOH Remain g/L	1,3-PDO g/L	2,3- BTDO g/L	EtOH g/L	Yield p/s
1	-1	-1	-1	-1	-1	0.60	4.46	1.06	0.58	0.47
2	1	-1	-1	-1	-1	15.28	5.18	2.26	0.65	0.35
3	-1	1	-1	-1	-1	0.74	4.44	1.16	0.72	0.48
4	1	1	-1	-1	-1	15.96	5.20	2.64	0.72	0.37
5	-1	-1	1	-1	-1	0.82	4.94	1.19	0.57	0.54
6	1	-1	1	-1	-1	14.03	6.14	2.78	0.69	0.38
7	-1	1	1	-1	-1	0.78	5.12	1.30	0.76	0.56
8	1	1	1	-1	-1	6.86	9.77	4.11	1.05	0.42
9	-1	-1	-1	1	-1	0.71	4.59	1.25	0.80	0.49
10	1	-1	-1	1	-1	7.81	8.66	3.63	1.00	0.39
11	-1	1	-1	1	-1	0.70	4.42	1.31	0.91	0.48
12	1	1	-1	1	-1	3.11	10.57	4.10	1.13	0.39
13	-1	-1	1	1	-1	0.00	5.95	0.00	0.80	0.60
14	1	-1	1	1	-1	5.78	10.21	4.12	0.92	0.42
15	-1	1	1	1	-1	0.72	4.56	1.56	0.98	0.49
16	1	1	1	1	-1	4.39	10.93	4.02	1.09	0.43
17	-1	-1	-1	-1	1	0.57	5.07	1.25	0.64	0.54
18	1	-1	-1	-1	1	10.45	7.76	3.44	0.90	0.40
19	-1	1	-1	-1	1	0.83	4.65	1.34	0.79	0.51
20	1	1	-1	-1	1	3.86	9.43	3.96	1.44	0.36
21	-1	-1	1	-1	1	0.54	5.05	1.33	0.68	0.53
22	1	-1	1	-1	1	8.97	8.33	3.65	0.82	0.40
23	-1	1	1	-1	1	0.75	5.18	1.55	0.78	0.56
24	1	1	1	-1	1	3.20	10.52	4.64	1.43	0.39
25	-1	-1	-1	1	1	0.50	4.87	1.38	0.78	0.51
26	1	-1	-1	1	1	7.73	8.97	3.88	0.87	0.40
27	-1	1	-1	1	1	0.70	4.37	1.51	1.09	0.47
28	1	1	-1	1	1	3.67	10.54	4.22	1.28	0.40
29	-1	-1	1	1	1	0.54	4.91	1.46	0.88	0.52
30	1	-1	1	1	1	5.53	10.35	4.42	0.83	0.42
31	-1	1	1	1	1	0.62	4.32	1.56	1.09	0.46
32	1	1	1	1	1	4.54	11.10	4.34	1.14	0.44
33	0	0	0	0	0	0.57	8.28	3.02	0.96	0.43
34	0	0	0	0	0	3.04	7.47	2.71	1.00	0.44
35	0	0	0	0	0	0.53	8.07	2.96	1.06	0.41
36	0	0	0	0	0	0.41	7.96	3.07	1.03	0.41
37	0	0	0	0	0	0.00	8.18	2.75	1.42	0.41

GlyOH: Glycerol; YE: Yeast Extract; B12: Vitamin B12; GlyOH Remain: Remaining glycerol; 1,3-PDO: 1,3-propanediol; 2,3-BTDO: 2,3-butanediol; EtOH: Ethanol

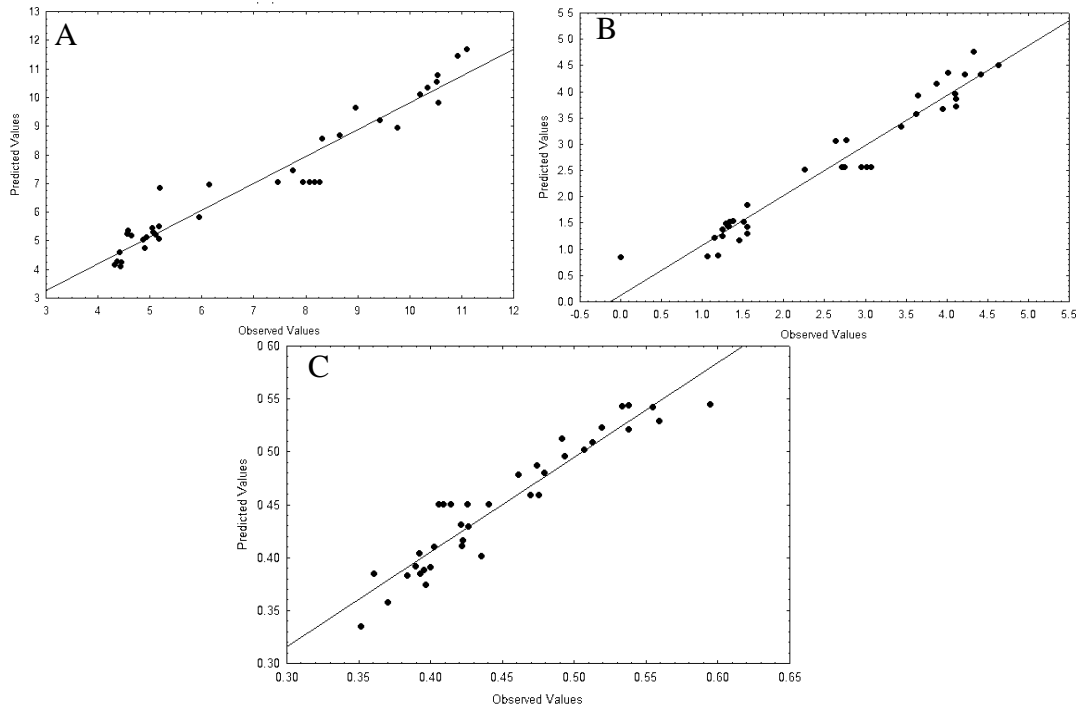


Figure 11 - Observed vs. Predicted plot from DoE Box Hunter & Hunter 5 var (Glycerol, YE, $(\text{NH}_4)_2\text{SO}_4$, Fumarate and Vit. B12). Dots represent observed values, line represents calculated values. A - 1,3-propanediol; B- 2,3-butanediol C- Yield p/s.

Based on the results obtained from Box, Hunter & Hunter design of experiments, Glycerol, Yeast Extract, Ammonium Sulfate and Vitamin B12 were chosen to be optimized by using Central Composite Rotational Design and surface response methodology. Since fumarate addition with vitamin B12 had negative interaction, fumaric acid was not used for the next optimization.

7.2.3 Central Composite, non-Factorial, Surface DoE

Four factors from the previous DoE (Box, Hunter & Hunter) were chosen to optimization: glycerol concentration, yeast extract, ammonium sulfate, and vitamin B12. Inoculum concentration was fixed at 10%, and CaCl_2 at 5 mM. Table 11 demonstrates decoded values for the first Central Composite non-factorial Surface response Design. Higher central levels were set up for ammonium sulfate and Vitamin B12 from last factorial designs, while glycerol and yeast extract were kept on the same levels as before.

Table 11 - First Central Composite non-factorial Surface response Design: decoded levels for optimizing culture media.

Variables	Levels				
	$-\alpha$	-1	0	+1	$+\alpha$
(1) Glycerol (g/L)	5.0	10.0	20.0	30.0	45.0
(2) Yeast Extract (g/L)	0.0	2.0	4.0	6.0	8.0
(3) $(\text{NH}_4)_2\text{SO}_4$ (g/L)	0.0	2.0	4.0	6.0	8.0
(4) Vitamin B12 (mg/L)	0.0	5.0	10.0	15.0	20.0

1,3-PDO production after 8 hours were benefited by higher amounts of glycerol, but a little but significant quadratic negative effect demonstrates that higher concentrations than 45 g/L of glycerol might inhibit 1,3-PDO. The addition from 5 mg/L to 20 mg/L of vitamin B12, production was negatively impacted because of its interaction with glycerol (Figure 12). As explained before, an irreversible binding of the vitamin B12 and glycerol with the enzyme, forms alkylcobalamines, and to avoid low activity of the enzyme, the amount of glycerol and vitamin should be controlled (YAMANISHI *et al.* 2012; NAKAMURA; WHITED, 2003; SHIBATA *et al.*, 2002; KAJIURA *et al.* 2001; DANIEL *et al.* 1998).

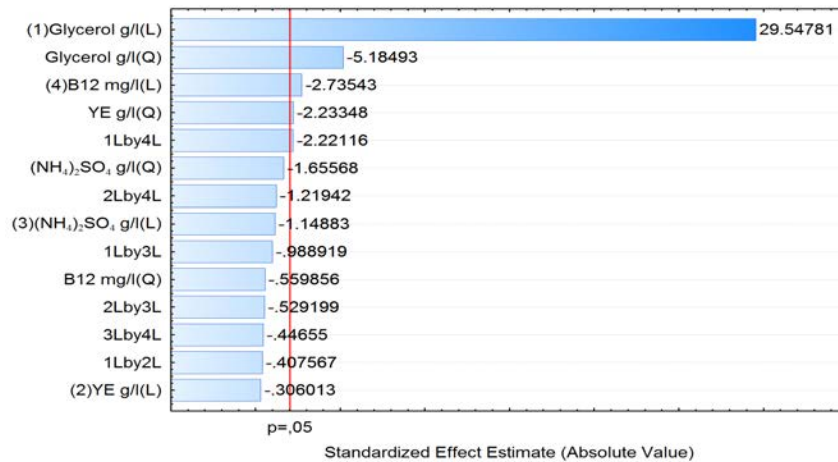


Figure 12 - Culture Optimizations of *Klebsiella pneumoniae* GLC29 - 1,3-propanediol at 8 h cultivation Pareto chart of effects – Central Composite, non-Factorial, Surface DoE. 4 Var.: Glycerol, YE, $(\text{NH}_4)_2\text{SO}_4$ and Vit. B12.

Both linear and quadratic significant effects were observed for glycerol, but since the linear effect was many times greater than the quadratic effect, this could mean an optimum region might not be found. Quadratic significance of yeast extract was observed, which means it was possible to obtain an optimum working region, but no significance was observed in ammonium sulfate using these concentrations, although it had a negative quadratic effect.

Surface response graphs are shown on Figure 13. Up to 12 g/L of 1,3-PDO was reached with this design. Higher concentrations, from 5 mg/L of vitamin B12 was not beneficial for 1,3-propanediol biosynthesis, especially when associated with glycerol, which

led to a loss of 2 g/L of 1,3-propanediol when 40 g/L of glycerol and 20 mg/L of vit. B12 are added. Lower levels of this vitamin were selected for a second DoE.

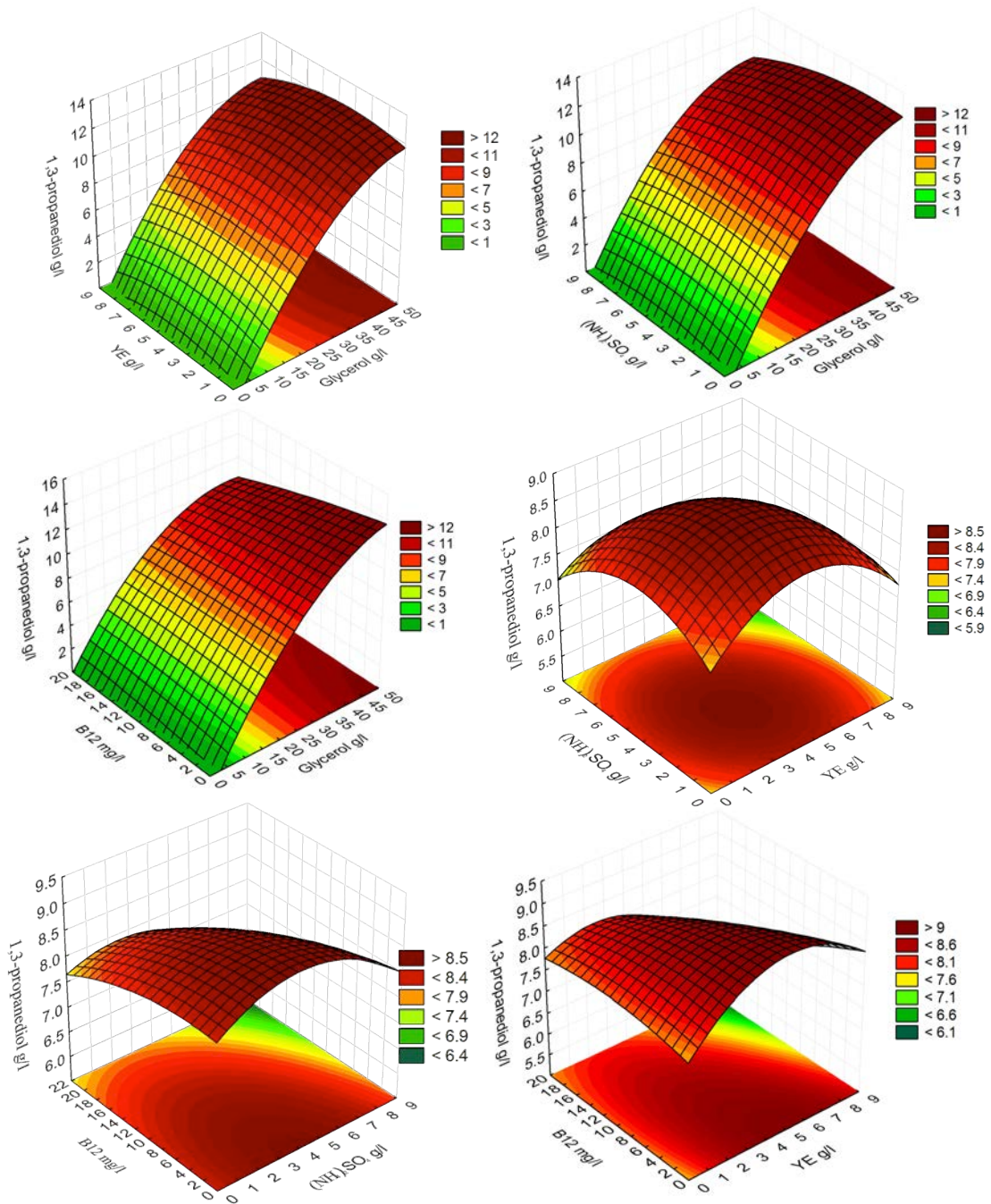


Figure 13 - Culture Optimizations of *Klebsiella pneumoniae* GLC29 –1,3-propanediol g/L production at 8 h cultivation in the First Central Composite, non-Factorial, Surface Response DoE. 4 Var.: Glycerol, YE, (NH₄)₂SO₄ and Vit. B12.

No other significant interaction was observed, which means most of the variables act independent on 1,3-PDO synthesis. An optimum region was observed when yeast extract was plotted against (NH₄)₂SO₄, in which 3 to 5 g/L of YE, and 2 to 4 g/L of (NH₄)₂SO₄ could be optimal for 1,3-propanediol production.

Table 12 shows the ANOVA results calculated for this experiment, where significant variables are shown in bold, which variables with p -value <0.05 were considered significant. Calculated F-value was higher than the tabled F-value. R^2 for the calculated model 0.95, which explains 95% of the results obtained. Figure 14 shows the experimental values obtained against the calculated model.

Table 12 - ANOVA: 1,3-propanediol g/L production at – First Central composite non-factorial surface design. 4 Variables: Glycerol, YE, $(\text{NH}_4)_2\text{SO}_4$ and Vit. B12 $R^2=0.95$; Adjusted $R^2 = 0.94$.

	SS	df	MS	F	p
(1)Glycerol g/L (L)	318.53	1.00	318.53	873.07	0.00
Glycerol g/L (Q)	9.81	1.00	9.81	26.88	0.00
(2)YE g/L (L)	0.03	1.00	0.03	0.09	0.76
YE g/L (Q)	1.82	1.00	1.82	4.99	0.03
(3) $(\text{NH}_4)_2\text{SO}_4$ g/L (L)	0.48	1.00	0.48	1.32	0.26
$(\text{NH}_4)_2\text{SO}_4$ g/L (Q)	1.00	1.00	1.00	2.74	0.11
(4) Vit. B12 mg/L (L)	2.73	1.00	2.73	7.48	0.01
Vit. B12 mg/L (Q)	0.11	1.00	0.11	0.31	0.58
1L by 2L	0.06	1.00	0.06	0.17	0.69
1L by 3L	0.36	1.00	0.36	0.98	0.33
1L by 4L	1.80	1.00	1.80	4.93	0.03
2L by 3L	0.10	1.00	0.10	0.28	0.60
2L by 4L	0.54	1.00	0.54	1.49	0.23
3L by 4L	0.07	1.00	0.07	0.20	0.66
Error	14.59	40.00	0.36		
Total SS	349.86	54.00			

SS: Sum of Squares; df Degrees of freedom; MS Mean Squared; F: Calculated F value for the F-test.; p: p value.

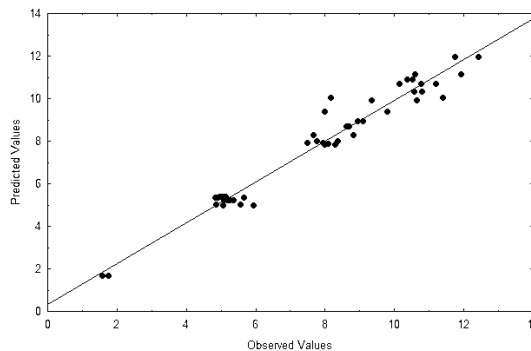


Figure 14 - Observed vs. Predicted plot from 1,3-propanediol g/L production at - First Central composite non-factorial surface design. 4 Var.: Glycerol, YE, $(\text{NH}_4)_2\text{SO}_4$ and Vit. B12. Dots represent observed values, line represents calculated values.

Fermentation results after 8-hour cultivation and other metabolites did not show significant R^2 values in the ANOVA's and were not considered. Table 13 shows the decoded values and the results obtained for this first Central Composite non-factorial Surface response Design.

Table 13 - First Central Composite non-factorial Surface response Design. Continues.

	Independent Variables				Dependent Variables				
	Glycerol g/L	YE g/L	(NH ₄) ₂ SO ₄ g/L	B12 mg/L	Acetate g/L	Remaining Glycerol g/L	1,3-PDO g/L	2,3-BTDO g/L	EtOH g/L
1	15.0	2.0	2.0	5.0	0.86	0.16	5.96	1.57	1.21
2	15.0	2.0	2.0	15.0	1.38	0.11	4.93	1.24	1.08
3	15.0	2.0	6.0	5.0	1.41	0.11	5.28	1.30	1.17
4	15.0	2.0	6.0	15.0	1.62	0.14	5.68	1.22	0.70
5	15.0	6.0	2.0	5.0	0.95	0.13	5.16	1.41	1.10
6	15.0	6.0	2.0	15.0	0.91	0.15	5.36	1.52	1.10
7	15.0	6.0	6.0	5.0	0.86	0.19	5.00	1.51	1.31
8	15.0	6.0	6.0	15.0	1.25	0.12	5.56	1.40	0.71
9	35.0	2.0	2.0	5.0	0.87	5.41	10.39	3.17	0.85
10	35.0	2.0	2.0	15.0	0.86	6.41	10.58	3.24	0.94
11	35.0	2.0	6.0	5.0	1.49	5.96	10.15	3.17	0.89
12	35.0	2.0	6.0	15.0	1.40	8.82	9.34	2.82	0.47
13	35.0	6.0	2.0	5.0	0.65	2.70	10.61	3.72	1.00
14	35.0	6.0	2.0	15.0	0.44	4.10	8.17	2.99	0.80
15	35.0	6.0	6.0	5.0	0.62	2.79	10.77	3.84	1.10
16	35.0	6.0	6.0	15.0	0.50	2.89	8.01	2.92	1.14
17	5.0	4.0	4.0	10.0	1.80	0.12	1.58	0.24	0.66
18	45.0	4.0	4.0	10.0	0.47	8.17	11.75	3.84	0.76
19	25.0	0.0	4.0	10.0	0.71	3.11	7.51	2.04	0.63
20	25.0	8.0	4.0	10.0	0.00	1.03	8.00	3.05	1.40
21	25.0	4.0	0.0	10.0	0.53	0.59	7.67	2.53	1.16
22	25.0	4.0	8.0	10.0	0.56	1.03	8.00	2.77	1.26
23	25.0	4.0	4.0	0.0	2.01	0.92	8.95	2.69	0.69
24	25.0	4.0	4.0	20.0	0.70	0.56	8.37	2.69	1.18
25	25.0	4.0	4.0	10.0	0.71	0.44	8.70	2.79	1.19
26	25.0	4.0	4.0	10.0	0.73	0.30	8.63	2.83	1.33
27	25.0	4.0	4.0	10.0	0.72	0.19	8.69	2.68	1.25
28	15.0	2.0	2.0	5.0	1.42	0.14	5.07	1.35	1.07
29	15.0	2.0	2.0	15.0	1.46	0.14	5.12	1.35	1.04

Table 13 – First Central Composite non-factorial Surface response Design. Continued.

	Independent Variables				Dependent Variables				
	Glycerol g/L	YE g/L	(NH ₄) ₂ SO ₄ g/L	B12 mg/L	Acetate g/L	Remaining Glycerol g/L	1,3-PDO g/L	2,3-BTDO g/L	EtOH g/L
30	15.0	2.0	6.0	5.0	1.26	0.11	5.09	1.41	1.08
31	15.0	2.0	6.0	15.0	1.40	0.12	4.86	1.28	1.04
32	15.0	6.0	2.0	5.0	0.75	0.14	4.96	1.43	1.04
33	15.0	6.0	2.0	15.0	0.84	0.13	5.19	1.49	1.07
34	15.0	6.0	6.0	5.0	0.80	0.14	5.08	1.50	1.12
35	15.0	6.0	6.0	15.0	0.79	0.10	4.88	1.29	1.06
36	35.0	2.0	2.0	5.0	0.67	5.48	10.54	3.43	0.89
37	35.0	2.0	2.0	15.0	0.65	4.67	10.79	3.41	0.92
38	35.0	2.0	6.0	5.0	1.45	4.31	11.22	3.62	0.96
39	35.0	2.0	6.0	15.0	1.36	5.84	10.66	3.48	0.90
40	35.0	6.0	2.0	5.0	0.66	2.02	11.93	4.17	1.12
41	35.0	6.0	2.0	15.0	0.68	3.49	11.42	4.03	1.05
42	35.0	6.0	6.0	5.0	0.66	3.69	10.78	3.86	1.14
43	35.0	6.0	6.0	15.0	0.61	1.62	9.80	3.50	1.26
44	5.0	4.0	4.0	10.0	1.86	0.10	1.78	0.29	0.68
45	45.0	4.0	4.0	10.0	0.93	10.34	12.45	4.03	0.76
46	25.0	0.0	4.0	10.0	0.79	3.08	7.96	2.19	0.64
47	25.0	8.0	4.0	10.0	0.80	0.64	8.31	2.93	1.46
48	25.0	4.0	0.0	10.0	0.62	0.76	8.83	2.88	1.25
49	25.0	4.0	8.0	10.0	0.69	1.02	8.10	2.69	1.30
50	25.0	4.0	4.0	0.0	2.06	0.47	9.10	2.71	0.81
51	25.0	4.0	4.0	20.0	0.67	2.38	7.77	2.54	1.20
52	25.0	4.0	4.0	10.0	0.70	1.75	8.62	2.67	1.19
53	25.0	4.0	4.0	10.0	0.95	0.37	8.66	2.44	1.22
54	25.0	4.0	4.0	10.0	0.79	0.89	8.65	2.89	1.19
55	25.0	4.0	4.0	10.0	0.92	0.67	8.70	2.70	1.23

YE: Yeast Extract; B12: Vitamin B12; 1,3-PDO: 1,3-propanediol; 2,3-BTDO: 2,3-butanediol; EtOH: Ethanol

A new Central Composite non-factorial Surface response design was then executed. New experimental levels were fixed. Table 14 shows media components and its decoded values for this design.

Table 14 - Second Central Composite non-factorial Surface Response Design: decoded levels for screening culture media.

Variable	Levels				
	$-\alpha$	-1	0	+1	$+\alpha$
(1) Glycerol (g/L)	5.0	25.0	45.0	65.0	85.0
(2) Yeast Extract (g/L)	0.0	2.5	5.0	7.5	10.0
(3) $(\text{NH}_4)_2\text{SO}_4$ (g/L)	0.0	2.5	5.0	7.5	10.0
(4) Vitamin B12 (mg/L)	0.0	1.0	2.0	3.0	4.0

Concentration was lowered from 1 to 3 mg/L of vitamin B12, and we have increased the concentrations for glycerol, yeast extract and ammonium sulfate.

Figure 15 demonstrates the significant effects on 1,3-PDO production. With this design, the quadratic effect of glycerol was increased. Also, the amount of $(\text{NH}_4)_2\text{SO}_4$ used had a negative effect on the production, but a quadratic effect for this variable was noticed. Interactions with glycerol and yeast extract (1Lby2L) showed a positive effect, while the interaction with glycerol and $(\text{NH}_4)_2\text{SO}_4$ (1Lby3L) showed a negative effect.

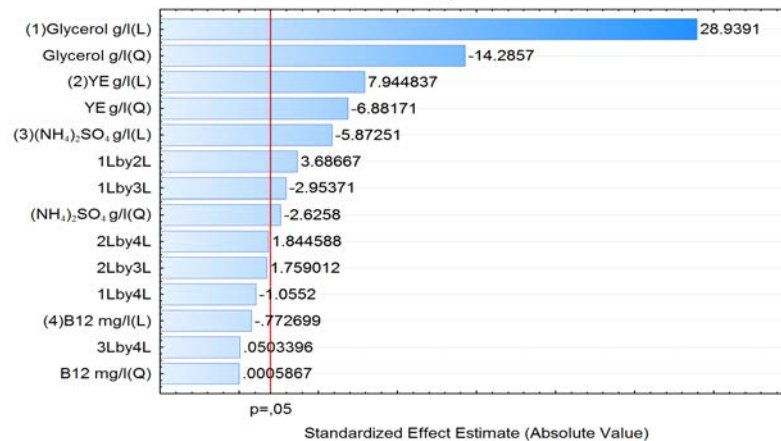


Figure 15 - Second Culture Optimizations of *Klebsiella pneumoniae* GLC29 - 1,3-propanediol at cultivation Pareto chart of effects - Central Composite, non Factorial, Surface DoE 4 Var.: Glycerol, YE, $(\text{NH}_4)_2\text{SO}_4$ and Vit. B12. Variables with p-value <0.05 were considered significant.

Table 15 shows the ANOVA results for this DoE. Variables with p-value <0.05 were considered significant. R^2 for the mathematical model was 0.95, which means the model explains 95% of the results. Calculated F-value for the variables was higher than the tabulated F-values, which confirms the significance level.

Table 15 - ANOVA from Second Central Composite Surface Response Design- 1,3-Propanediol g/L; 4 Variables: Glycerol, YE (yeast extract), $(\text{NH}_4)_2\text{SO}_4$ and Vit. B12. $R^2 = 0.95$; Adjusted $R^2 = 0.93$

	SS	df	MS	F	p
(1)Glycerol g/L(L)	568.30	1	568.30	837.47	0.00
Glycerol g/L(Q)	138.49	1	138.49	204.08	0.00
(2)YE g/L(L)	42.83	1	42.83	63.12	0.00
YE g/L(Q)	32.14	1	32.14	47.36	0.00
(3)(NH4)2SO4 g/L(L)	23.40	1	23.40	34.49	0.00
(NH4)2SO4 g/L(Q)	4.68	1	4.68	6.89	0.01
(4) Vit. B12 mg/L(L)	0.41	1	0.41	0.60	0.44
Vit. B12 mg/L(Q)	0.00	1	0.00	0.00	1.00
1L by 2L	9.22	1	9.22	13.59	0.00
1L by 3L	5.92	1	5.92	8.72	0.00
1L by 4L	0.76	1	0.76	1.11	0.30
2L by 3L	2.10	1	2.10	3.09	0.08
2L by 4L	2.31	1	2.31	3.40	0.07
3L by 4L	0.00	1	0.00	0.00	0.96
Error	43.43	64	0.68		
Total SS	875.84	78			

SS: Sum of Squares; df Degrees of freedom; MS Mean Squared; F: Calculated F value for the F-test.; p: p value.

Figure 16 shows that the experimental results are very fit to the calculated model, where the dots represent the obtained data, and the line represents the predicted values. Figure 17 shows the surface responses obtained from this experiment.

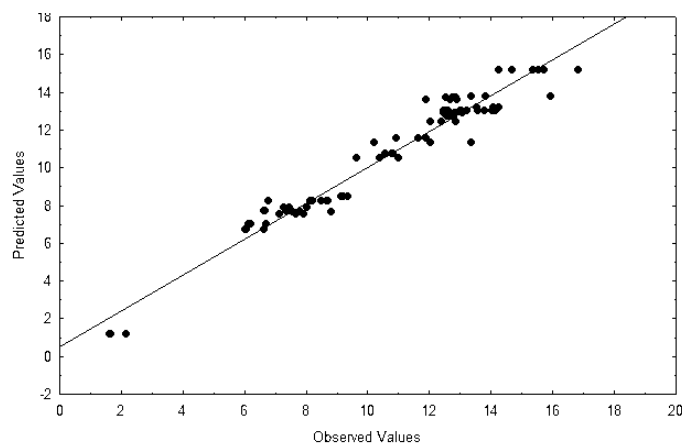


Figure 16 - Observed vs. Predicted plot from 1,3-propanediol g/L production at - Second Central Composite non-factorial surface design. 4 Var.: Glycerol, YE, $(\text{NH}_4)_2\text{SO}_4$ and Vit. B12.

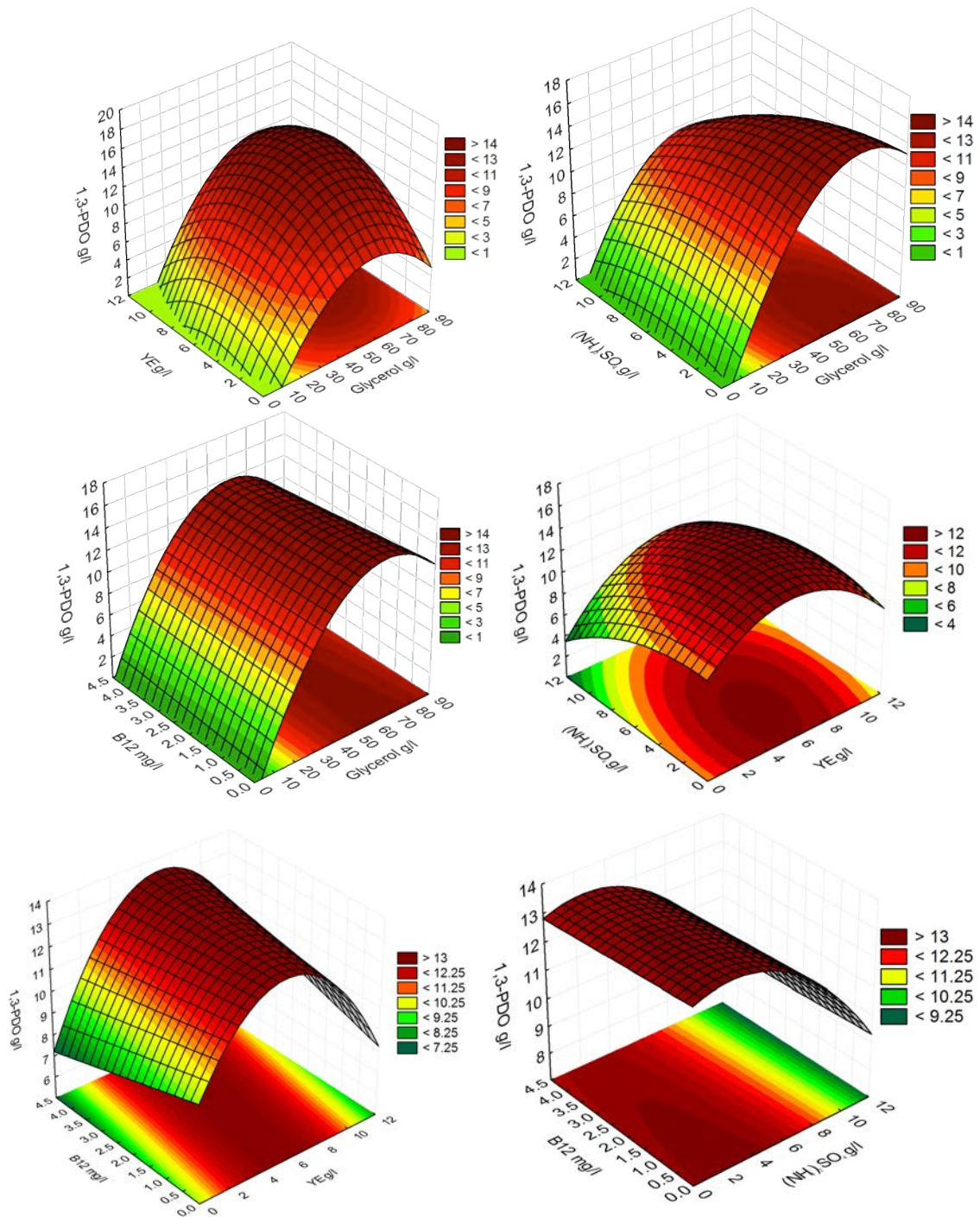


Figure 17 - Culture Optimizations of *Klebsiella pneumoniae* GLC29 – 1,3-propanediol g/L production at cultivation in Central Composite, non Factorial, Surface DoE. 4 Var.: Glycerol, YE, $(\text{NH}_4)_2\text{SO}_4$ and Vit. B12.

Based on the surface responses (Figure 17), it was possible to optimize glycerol and yeast extract concentration, which the addition of 50 g/L and 5 g/L of each respectively would result in maximum production. Also, based on the results obtained in Box Hunter & Hunter and compared with these two Central Composite DoE performed, we have observed that over 3 g/L of ammonium sulfate and 500 mg/L of vitamin B12 have negatively affected 1,3-

propanediol production. Thus, for further experiments in bioreactor, central points should be used, as 1,5 g/L ammonium sulfate and 0.25 mg/L of vitamin B12.

Ammonium sulfate could not be optimized by this design, but it is important to stress they were significant on Box Hunter & Hunter DoE in different levels (concentrations), and on the second central composite non factorial DoE, in which up to 2 g/L was favorable to 1,3-propanediol production. Therefore, higher concentrations used in the first and second central composite designs could have negatively affected the production.

On the second central composite non factorial DoE, the addition from 1 to 4 mg/L of Vitamin B12 had no effect on production, and the addition of higher concentrations, from 5 to 20 mg/L, as shown on first central composite non factorial DoE, had a negative effect on 1,3-propanediol production. Therefore, it is demonstrated that concentrations of 0.5 mg/L of vitamin B12 are more than enough to saturate the system.

Based on Box, Hunter & Hunter DoE, and further Central Composite non factorial DoE, the optimum media obtained was 250 µg/L of vitamin B12, 1,5 g/L of (NH₄)₂SO₄, 50 g/L of glycerol and 5 g/L of Yeast Extract. Fumarate should not be added with vitamin B12. Table 16 shows the decoded values used for the variables and results obtained for the second central composite non factorial surface response design.

Table 16 - Second Central Composite non factorial Surface Response Design: decoded levels for screening culture media and results obtained. Continues.

	Independent Variables				Dependent Variables			
	Glycerol g/L	YE g/L	(NH ₄) ₂ SO ₄ g/L	B12 mg/L	Remaining Glycerol g/L	1,3-PDO g/L	2,3-BTDO g/L	EtOH g/L
1	25.00	2.50	2.50	1.00	1.88	8.05	2.53	2.12
2	25.00	2.50	2.50	1.00	2.80	7.46	2.34	2.02
3	25.00	2.50	2.50	1.00	3.16	7.27	2.24	2.07
4	25.00	2.50	2.50	3.00	2.31	7.93	2.49	2.01
5	25.00	2.50	2.50	3.00	3.75	7.15	2.22	2.07
6	25.00	2.50	2.50	3.00	2.82	7.66	2.40	2.06
7	25.00	2.50	7.50	1.00	6.19	6.14	1.84	2.05
8	25.00	2.50	7.50	1.00	4.74	6.72	2.11	1.70
9	25.00	2.50	7.50	1.00	6.61	6.20	1.77	1.58
10	25.00	2.50	7.50	3.00	6.49	6.06	1.77	1.26
11	25.00	2.50	7.50	3.00	5.69	6.03	1.79	2.05
12	25.00	2.50	7.50	3.00	4.83	6.62	2.06	2.14
13	25.00	7.50	2.50	1.00	4.11	6.63	2.26	2.21
14	25.00	7.50	2.50	1.00	4.15	6.66	2.24	2.29
15	25.00	7.50	2.50	1.00	2.73	7.35	2.47	2.34
16	25.00	7.50	2.50	3.00	1.22	6.79	2.41	2.19
17	25.00	7.50	2.50	3.00	0.40	8.66	2.98	2.30
18	25.00	7.50	2.50	3.00	0.62	8.48	2.95	2.47
19	25.00	7.50	7.50	1.00	1.66	7.56	2.69	1.52

Table 16 – Second Central Composite non factorial Surface Response Design: decoded levels for screening culture media and results obtained. Continues.

	Independent Variables				Dependent Variables			
	Glycerol g/L	YE g/L	(NH ₄) ₂ SO ₄ g/L	B12 mg/L	Remaining Glycerol g/L	1,3-PDO g/L	2,3-BTDO g/L	EtOH g/L
20	25.00	7.50	7.50	1.00	1.52	7.80	2.80	2.52
21	25.00	7.50	7.50	1.00	0.27	8.81	3.17	2.79
22	25.00	7.50	7.50	3.00	0.09	8.19	2.81	2.79
23	25.00	7.50	7.50	3.00	0.05	8.72	2.90	3.03
24	25.00	7.50	7.50	3.00	0.00	8.15	2.78	2.79
25	65.00	2.50	2.50	1.00	26.87	12.70	4.29	2.43
26	65.00	2.50	2.50	1.00	29.88	11.89	3.40	1.85
27	65.00	2.50	2.50	1.00	28.85	12.89	4.62	2.12
28	65.00	2.50	2.50	3.00	29.61	12.56	4.46	2.61
29	65.00	2.50	2.50	3.00	27.46	12.79	4.40	1.89
30	65.00	2.50	2.50	3.00	30.28	12.60	4.01	1.81
31	65.00	2.50	7.50	1.00	29.55	12.04	4.15	1.05
32	65.00	2.50	7.50	1.00	32.91	10.21	3.52	1.08
33	65.00	2.50	7.50	1.00	29.21	13.35	4.45	0.92
34	65.00	2.50	7.50	3.00	33.21	11.01	3.79	1.07
35	65.00	2.50	7.50	3.00	33.62	10.39	3.57	1.09
36	65.00	2.50	7.50	3.00	33.71	9.66	3.21	1.08
37	65.00	7.50	2.50	1.00	22.91	15.38	5.51	0.86
38	65.00	7.50	2.50	1.00	18.93	16.83	6.27	0.96
39	65.00	7.50	2.50	1.00	21.43	15.54	5.75	0.92
40	65.00	7.50	2.50	3.00	24.08	14.68	5.54	1.05
41	65.00	7.50	2.50	3.00	28.07	14.27	4.85	0.83
42	65.00	7.50	2.50	3.00	24.76	15.71	5.55	0.88
43	65.00	7.50	7.50	1.00	27.93	12.75	4.62	0.91
44	65.00	7.50	7.50	1.00	27.21	12.85	4.73	0.93
45	65.00	7.50	7.50	1.00	29.52	12.54	4.58	0.92
46	65.00	7.50	7.50	3.00	23.73	15.94	5.84	1.04
47	65.00	7.50	7.50	3.00	29.36	13.35	4.88	0.99
48	65.00	7.50	7.50	3.00	27.07	13.81	4.97	0.96
49	5.00	5.00	5.00	2.00	0.46	2.18	0.41	0.85
50	5.00	5.00	5.00	2.00	0.06	1.64	0.27	0.78
51	5.00	5.00	5.00	2.00	0.00	1.66	0.27	0.77
52	85.00	5.00	5.00	2.00	44.55	12.41	3.65	0.63
53	85.00	5.00	5.00	2.00	44.55	12.87	3.63	0.61
54	85.00	5.00	5.00	2.00	47.56	12.03	3.37	0.60
55	45.00	0.00	5.00	2.00	19.48	9.16	2.29	0.66
56	45.00	0.00	5.00	2.00	19.54	9.36	2.29	0.66
57	45.00	0.00	5.00	2.00	18.87	9.23	2.40	0.69
58	45.00	10.00	5.00	2.00	11.81	10.93	4.28	1.34
59	45.00	10.00	5.00	2.00	9.54	11.89	4.64	1.36
60	45.00	10.00	5.00	2.00	10.64	11.64	4.41	1.45
61	45.00	5.00	0.00	2.00	7.93	14.05	4.84	1.14
62	45.00	5.00	0.00	2.00	9.94	13.00	4.64	1.07
63	45.00	5.00	0.00	2.00	7.61	14.16	5.02	1.16
64	45.00	5.00	10.00	2.00	12.79	10.80	3.96	1.35
65	45.00	5.00	10.00	2.00	12.32	10.56	3.83	1.29
66	45.00	5.00	10.00	2.00	12.97	10.82	3.90	1.32
67	45.00	5.00	5.00	0.00	9.50	13.55	4.94	0.93
68	45.00	5.00	5.00	0.00	8.25	14.06	5.15	1.04

Table 16 – Second Central Composite non factorial Surface Response Design: decoded levels for screening culture media and results obtained. Continued.

	Independent Variables				Dependent Variables			
	Glycerol g/L	YE g/L	(NH ₄) ₂ SO ₄ g/L	B12 mg/L	Remaining Glycerol g/L	1,3-PDO g/L	2,3-BTDO g/L	EtOH g/L
69	45.00	5.00	5.00	0.00	8.60	14.27	5.15	0.94
70	45.00	5.00	5.00	4.00	9.91	12.83	4.54	1.20
71	45.00	5.00	5.00	4.00	9.84	12.48	4.51	1.30
72	45.00	5.00	5.00	4.00	8.92	13.08	4.65	1.19
73	45.00	5.00	5.00	2.00	9.54	12.46	4.49	1.26
74	45.00	5.00	5.00	2.00	9.54	12.61	4.47	1.28
75	45.00	5.00	5.00	2.00	9.08	13.57	4.69	1.15
76	45.00	5.00	5.00	2.00	9.58	12.56	4.52	1.25
77	45.00	5.00	5.00	2.00	9.66	13.04	4.45	1.12
78	45.00	5.00	5.00	2.00	8.43	13.80	4.85	1.17
79	45.00	5.00	5.00	2.00	9.41	13.24	4.64	1.17

YE: Yeast Extract; B12: Vitamin B12; 1,3-PDO: 1,3-propanediol; 2,3-BTDO: 2,3-butanediol; EtOH: Ethanol

Glycerol concentration must be controlled because of 3-hydroxypropionaldehyde the inhibition, which is an intermediate metabolite in the conversion of glycerol to 1,3-propanediol. The 3-hydroxypropionaldehyde accumulation during fermentation has been described in anaerobic fermentation by *K. pneumoniae*, and when the initial glycerol concentration was superior to 430 mM, a high concentration of 3-HPA inhibited cell growth and production of 1,3-propanediol (BARBIRATO *et al.* 1996; HAO *et al.* 2008). Zheng *et al.* (2008) had demonstrated a productivity of 1.81 g/L.h from 50 g/L of glycerol, controlling pH, aeration rate and stirring in the bioreactor at 6,48, 0.6 VVM and 318 rpm, respectively, and the productivity and yield of 71.03 g/L, 2.37 g/L h and 0.6425 mol/mol (1,3-PDO/glycerol) at a fermentation of 30 h with the initial glycerol, rate of stirring, air aeration and pH of 50 g/L, 320 rpm, 0.6 VVM and 6.5 by fed-batch fermentation maintaining glycerol to 20-30 g/L.

Due to the different uses of pyruvate in different organisms, various by-products are formed during glycerol fermentation, *e.g.*, ethanol, lactic acid, succinic acid, and 2,3-butanediol (2,3-BTDO) are produced by *K. pneumoniae*. All of these byproducts are associated with a loss of 1,3-PDO in relation to particularly acetic acid, ethanol and butanol, which impairs the pool of NADH available to 1,3-PDO production (SONG *et al.* 2010). The production of 2,3-butanediol may be caused by the lack of pH control, since a pH below 5.5 drives *Klebsiella pneumoniae* metabolism towards producing this compound (BIEBL *et al.*, 1998). This way, flask cultures might have an increase in production on 2,3-propanediol

To optimize 1,3-propanediol synthesis, two stage processes are ideal, in which cells can be cultured first in a rich medium, and 1,3-propanediol production may be induced by

addition of glycerol on the stationary phase. Another strategy may be supplementing glycerol during fermentation. Both processes have increased the production of 1,3-propanediol during fermentation by *Klebsiella pneumoniae* over-expression of *dhaT* by Oh *et al.* (2012).

The boiling points of 2,3-butanediol, 1,3-PDO and glycerol are 184 °C, 214 °C and 290 °C respectively at normal pressure. In fact, it is a challenge to separate 1,3-PDO from a fermentation mixture. But in comparison with the chemical synthesis, which produces 1,2-propanediol, 1,3-PDO purification is even more difficult. Therefore, microbial production of 1,3-PDO may cost 50-70% of the total cost for production of 1,3-PDO. Consequently, the downstream plays an important role in the industrial microbial production (XIU; ZENG 2008).

7.2.4 Addition of Fumaric Acid

To optimize the addition of fumaric acid, a new experiment was performed, using only fumaric acid, since its interaction with vitamin B12 was not beneficial to the production of 1,3-propanediol. Table 17 shows the coded and real values used on the experimental design.

Table 17 - Third Central Composite non factorial Surface response Design: decoded levels for screening culture media with fumarate addition.

Variable	Levels				
	$-\alpha$	-1	0	+1	$+\alpha$
(1) Glycerol (g/L)	6.36	20	40	60	73.63
(2) Yeast Extract (g/L)	0.63	2	5	6	7.36
(3) Fumarate (mM)	0.05	1.25	3	4.75	5.95

As results, we had a strong interaction with fumarate and glycerol, but an even stronger negative effect with yeast extract and the fumaric acid, shown on figure 18. It was also observed a negative interaction between yeast extract and glycerol.

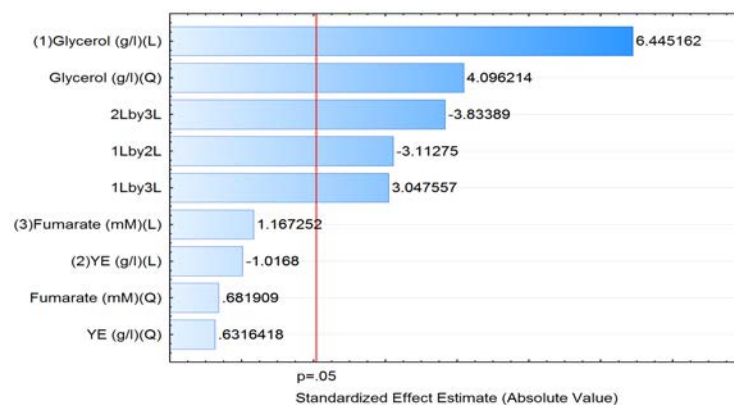


Figure 18 - Third Culture Optimizations of *Klebsiella pneumoniae* GLC29 - 1,3-propanediol at cultivation. Pareto chart of effects - Central Composite, non Factorial, Surface DoE. Variables with p-value<0.05 were considered significant.

Figure 19 shows the surface responses obtained for this experiment. Up to 12 g/L of 1,3-propanediol was produced, but no optimal region was obtained. The negative effect of fumaric acid and yeast extract might be shown here because of the vitamin B12 present on the yeast extract, since no vitamin B12 was added to the media. Table 18 shows the results for this experiment.

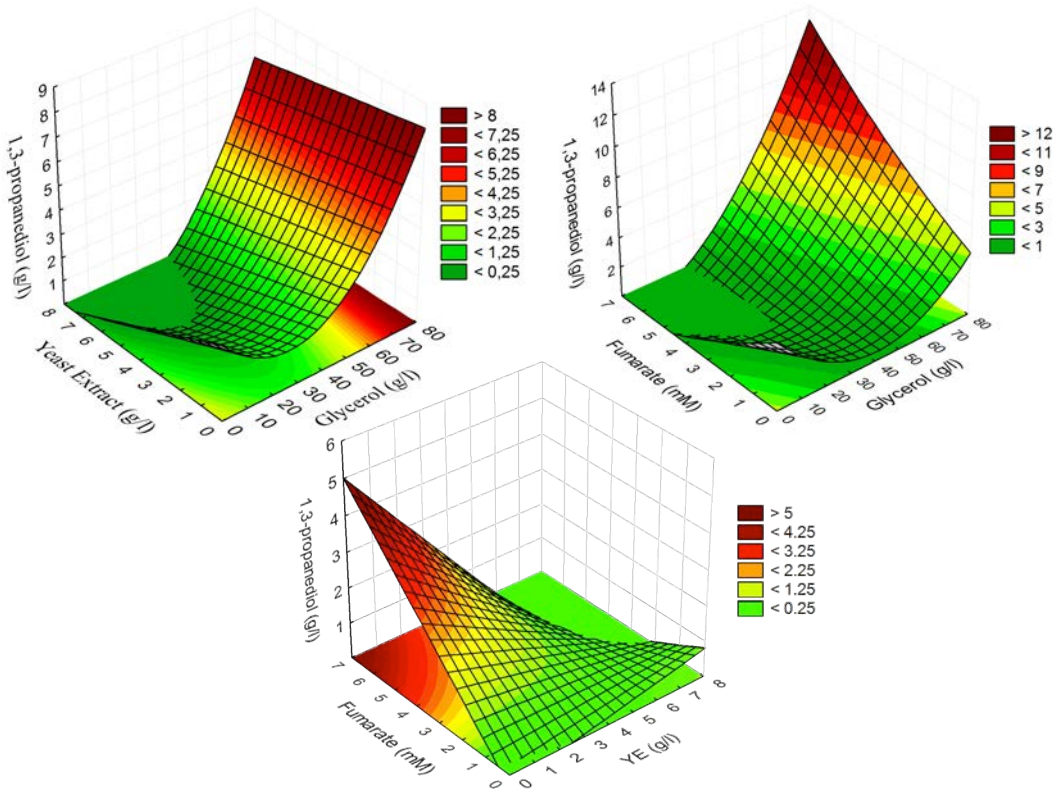


Figure 19 - Fumarate Addition - Culture Optimizations of *Klebsiella pneumoniae* GLC29 –1,3-propanediol (g/L) production at cultivation in Central Composite, non Factorial, Surface DoE.

Table 18 - ANOVA; Var.: 1,3-propanediol (g/L) production (); $R^2= 0.75905$; Adj.: 0.68677

	SS	df	MS	F	p
(1)Glycerol (g/L)(L)	62.56	1.00	62.56	41.54	0.00
Glycerol (g/L)(Q)	25.27	1.00	25.27	16.78	0.00
(2)YE (g/L)(L)	1.56	1.00	1.56	1.03	0.32
YE (g/L)(Q)	0.60	1.00	0.60	0.40	0.53
(3)Fumarate (mM)(L)	2.05	1.00	2.05	1.36	0.25
Fumarate (mM)(Q)	0.70	1.00	0.70	0.46	0.50
1L by 2L	14.59	1.00	14.59	9.69	0.00
1L by 3L	13.99	1.00	13.99	9.29	0.00
2L by 3L	22.14	1.00	22.14	14.70	0.00
Error	45.18	30.00	1.51		
Total SS	187.52	39.00			

SS: Sum of Squares; df Degrees of freedom; MS Mean Squared; F: Calculated F value for the F-test.; p: p value.

Since there is no evidence fumarate was benefic to the 1,3-propanediol production by *Klebsiella pneumoniae* GLC29, no further optimizations were performed. Table 19 shows the decoded values for each run and the results obtained to try to optimize fumaric acid addition.

Lin *et al.* (2005) describes that during the fermentation of 1,3-PDO, the addition of 5 mM fumarate increased glycerol consumption rate 34%, while 1,3-propanediol productivity increased 36% compared to the controls. It is believed that fumarate can significantly decrease the ratio $\text{NAD}^+ / \text{NADH}$ thus greater amount of NADH is available for conversion.

Table 19 - Experimental design results from Fumarate Addition – Central Composite Design – 8 hour fermentation, decoded values and obtained results. Continues

	Independent Variables			Dependent Variables										
	Glycerol (g/L)	YE (g/L)	Fumarate (g/L)	Citrate (g/L)	Succinate (g/L)	Lactate (g/L)	Formate (g/L)	Acetate (g/L)	Propionate (g/L)	Glycero l (g/L)	1,3-Pdo (g/L)	2,3-Btdo (g/L)	DCW (g/L)	EtOH (g/L)
1	20	2	1.25	1.10	0.30	0.62	0.00	2.35	0.22	6.99	0.44	1.20	0.61	0.39
2	20	2	4.75	0.14	0.51	0.62	0.00	0.18	0.20	7.82	0.00	0.69	0.62	0.20
3	20	6	1.25	0.03	0.19	0.79	0.10	0.59	1.08	4.37	1.00	1.33	0.84	0.30
4	20	6	4.75	0.06	0.60	1.32	0.21	6.90	2.00	8.22	0.49	1.81	1.08	0.55
5	60	2	1.25	0.16	2.70	0.72	0.13	0.40	2.20	34.07	1.61	1.49	0.84	0.02
6	60	2	4.75	1.50	0.26	0.70	0.13	0.35	0.21	31.91	9.60	1.10	0.73	0.00
7	60	6	1.25	0.07	0.45	1.20	0.23	6.30	1.45	28.05	2.42	2.12	1.32	0.12
8	60	6	4.75	0.06	0.70	1.31	0.28	0.72	1.80	33.91	1.10	1.39	1.07	0.16
9	6.36	4	3	0.02	0.30	0.10	0.21	0.64	0.64	0.00	0.66	0.00	0.86	0.14
10	73.64	4	3	0.38	1.30	0.51	0.21	0.84	1.10	47.80	5.45	4.50	1.08	0.00
11	40	0.64	3	0.29	0.55	0.48	0.15	0.23	0.21	16.30	0.01	0.50	0.52	0.09
12	40	7.36	3	0.04	0.52	1.28	0.21	0.70	1.89	11.39	1.69	2.10	1.38	0.39
13	40	4	0.06	0.09	1.60	1.04	0.12	0.63	0.84	15.20	1.12	2.09	0.96	0.33
14	40	4	5.94	1.30	0.66	9.60	0.20	3.20	1.07	14.64	0.13	0.98	0.76	0.23
15	40	4	3	0.08	0.42	0.98	0.14	1.30	1.08	16.68	0.00	3.14	0.92	0.32
16	40	4	3	0.38	0.48	0.97	0.24	0.33	1.07	15.79	0.72	2.15	0.95	0.50
17	40	4	3	0.09	0.42	1.02	0.16	0.37	0.96	14.21	0.41	1.26	0.95	0.30
18	40	4	3	0.10	0.43	0.97	0.16	0.34	0.96	14.14	0.52	1.64	0.97	0.34
19	40	4	3	0.07	0.39	1.02	0.17	0.45	1.09	16.27	0.66	1.24	0.88	0.30
20	40	4	3	0.08	0.42	1.02	0.16	0.40	1.06	15.67	0.79	1.79	0.93	3.60
21	20	2	1.25	1.20	0.35	0.68	0.00	2.50	0.21	7.81	0.41	1.11	0.70	0.34
22	20	2	4.75	0.16	0.52	0.69	0.17	0.23	0.19	10.01	0.00	0.46	0.59	0.11
23	20	6	1.25	0.06	2.80	1.26	0.03	0.60	1.30	7.17	0.59	1.51	0.88	0.47
24	20	6	4.75	0.05	0.52	0.86	0.17	0.66	1.49	6.13	0.45	1.58	1.06	0.34
25	60	2	1.25	0.21	0.51	0.52	0.13	0.33	0.20	33.84	0.75	1.44	0.81	0.06
26	60	2	4.75	1.46	0.25	0.75	0.13	0.32	0.19	33.00	9.00	1.03	0.74	0.05
27	60	6	1.25	0.08	0.44	0.83	0.20	6.30	1.26	27.63	2.65	2.39	1.38	0.04
28	60	6	4.75	0.06	0.62	1.11	0.23	0.42	1.64	29.68	1.19	1.43	1.09	0.19

Table 19 – Experimental design results from Fumarate Addition – Central Composite Design – 8 hour fermentation, decoded values and obtained results. Continued.

Independent Variables				Dependent Variables										
Glycerol (g/L)	YE (g/L)	Fumarate (g/L)		Citrate (g/L)	Succinate (g/L)	Lactate (g/L)	Formate (g/L)	Acetate (g/L)	Propionate (g/L)	Glycero l (g/L)	1,3-Pdo (g/L)	2,3-Btdo (g/L)	DCW (g/L)	Ethanol (g/L)
29	6.36	4	3	0.18	0.19	0.10	0.13	0.46	0.38	0.00	0.57	0.10	0.87	0.01
30	73.64	4	3	0.39	1.35	0.52	0.22	0.85	1.12	47.40	5.53	4.57	1.10	0.05
31	40	0.64	3	0.27	0.66	0.85	0.15	0.24	0.21	18.70	0.01	0.95	0.42	0.18
32	40	7.36	3	0.40	6.10	1.62	0.26	0.82	2.20	14.60	1.54	2.74	1.38	0.62
33	40	4	0.06	0.11	2.30	1.04	0.13	0.77	0.92	15.44	1.90	2.62	1.03	0.35
34	40	4	5.94	0.13	0.70	1.04	0.24	0.53	1.30	18.42	0.23	1.07	0.68	0.17
35	40	4	3	0.08	0.40	1.05	0.19	0.46	1.12	16.90	0.70	1.70	0.87	0.33
36	40	4	3	0.08	0.38	1.00	0.15	0.35	0.95	13.21	0.51	1.50	0.90	0.36
37	40	4	3	0.09	0.42	1.02	0.16	0.37	0.96	14.21	0.41	1.26	0.87	0.30
38	40	4	3	0.10	0.47	0.99	0.18	0.38	1.14	16.38	0.75	1.93	0.99	0.43
39	40	4	3	0.12	0.49	1.04	0.17	0.41	1.09	14.05	1.62	2.48	0.94	0.50
40	40	4	3	0.06	0.34	0.82	0.16	0.43	0.90	14.56	0.29	1.03	0.86	0.18

YE: Yeast Extract; 1,3-PDO: 1,3-propanediol; 2,3-BTDO: 2,3-butanediol; DCW: Dry Cell Weight; EtOH: Ethanol

7.3 Batch Fermentation of *K. pneumoniae* GLC29

Two batch fermentations were performed on a Multifors 2 Fermenter, at 37° C, 100 rpm, and pH control (pH 6.8), with a 300ml working volume, and nitrogen gas was added to purge the oxygen out of the vases.

Media components were optimized from the Experimental Design: Glycerol 50 g/L, YE 5 g/L, (NH₄)₂SO₄ 1,5 g/L, Vitamin B12 0.25 mg/L, KH₂PO₄ 5.0 g/L, K₂HPO₄ 1.5 g/L; NaCl 1.0 g/L, and 1 mL of trace elements stock solution added per liter of media prepared, which composition is (g/L): EDTA 0.5, CaCl₂.2H₂O 0.5, CoCl₂.6H₂O 0.16; MoNH₄.4H₂O 0.1, CuSO₄.5H₂O 0.16; FeSO₄.7H₂O 0.5, MnSO₄.H₂O 0.5, ZnSO₄.7H₂O 0.22, NiCl₂.6H₂O 0.03, H₃BO₃ 0.12.

Figure 20 shows the kinetics of 1,3-propanediol production using analytical grade glycerol and figure 21 shows the kinetics of 1,3-propanediol production using biodiesel glycerol, obtained from the biodiesel refinery Biocapital located in Charqueada – SP, Brazil.

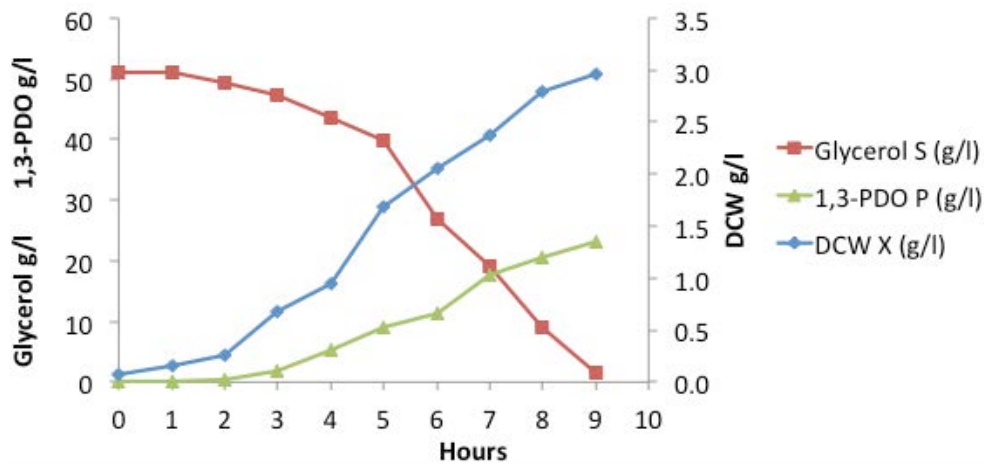


Figure 20 - Batch Culture of *K. pneumoniae* GLC29 in (A) Analytical grade Glycerol – 1,3-propanediol g/L production.

In some cases, initial glycerol was higher than weighted initially, and several analysis were performed on HPLC to check the calibration curve. Later, we found out that feeding glycerol was leaking into the media during sterilization in autoclave. Although in flasks, 50 g/L was not entirely consumed, it's noticeable that in 9 hours, analytical grade glycerol 51 g/L is completely consumed, and up 24.4 g/L of 1,3-propanediol was produced.

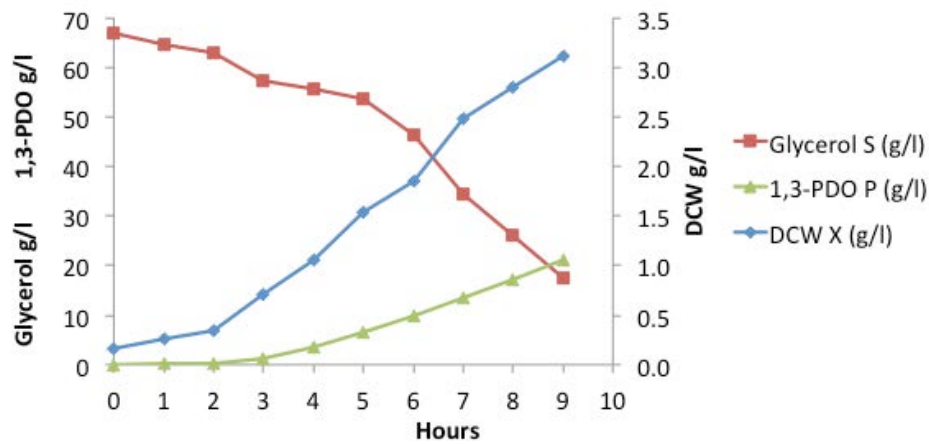


Figure 21 - Batch Culture of *K. pneumoniae* GLC29 in crude biodiesel glycerol – 1,3-propanediol g/L production.

In parallel, Biodiesel Glycerol, although had a longer fermentation (12 h), produced 27.6 g/L of 1,3-propanediol out of 66 g/L of biodiesel glycerol. These are an increase of 4 g/L and 7.2 g/L respectively since the last optimization of this strain by Da Silva (2014). Table 20 and 21 show the obtained data, specific growth, glycerol consumption, yields and productivity for each batch, respectively. Maximum productivity was 2.56 g/L.h using analytical grade glycerol and 2.3 g/L.h for biodiesel glycerol, and $Y_{p/s}$ was 0.46 g/g and 0.43 g/g respectively.

Comparing to Da Silva *et al.* (2014), which had a maximum productivity of 2.92 g/L.h and a yield of 0.51 from 40 g/L of glycerol, the results obtained from this work focused on maximum production, and although concentrations above 49 g/L inhibits the productivity (Da Silva *et al.*, 2014), and lower productivity was obtained, media can be re-adjusted and recalculated using the experimental design.

Table 20 - Batch Culture of *K. pneumoniae* GLC29 in Analytical grade Glycerol. DCW: Dry Cell Weight; 1,3-PDO; 1,3-propanediol.

Time (h)	Obtained Data			Specific Rates			Yields			Productivity	
	DCW X (g/L)	Glycerol S (g/L)	1,3-PDO P (g/L)	μ_X (h ⁻¹)	μ_S (h ⁻¹)	μ_P (h ⁻¹)	$A_{x/s}$ (g/g)	$A_{x/p}$ (g/g)	$Y_{p/s}$ (g/g)	P_p (g/L.h)	P_{ox} (g/L.h)
0	0.07	51.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	0.15	51.04	0.12	0.65	5.80	1.32	6.39	0.69	9.23	0.12	0.08
2	0.27	49.30	0.40	1.00	7.07	3.32	0.11	0.49	0.23	0.20	0.10
3	0.68	47.29	1.88	0.51	4.24	3.49	0.16	0.33	0.50	0.63	0.20
4	0.95	43.53	5.15	0.52	3.95	3.70	0.12	0.17	0.68	1.29	0.22
5	1.68	39.76	8.94	0.33	4.94	1.87	0.14	0.18	0.79	1.79	0.32
6	2.05	26.94	11.44	0.17	5.06	2.13	0.08	0.17	0.47	1.91	0.33
7	2.37	18.97	17.69	0.16	3.76	1.90	0.07	0.13	0.55	2.53	0.33
8	2.79	9.08	20.47	0.11	3.13	0.96	0.06	0.13	0.49	2.56	0.34
9	2.97	1.52	23.02	0.03	1.22	0.50	0.06	0.13	0.46	2.56	0.32

Table 21 - Batch Culture of *K. pneumoniae* GLC29 in Biodiesel Glycerol. DCW: Dry Cell Weight; 1,3-PDO; 1,3-propanediol.

Time (h)	Obtained Data			Specific Rates			Yields			Productivity	
	DCW X (g/L)	Glycerol S (g/L)	1,3-PDO P (g/L)	μ_X (h ⁻¹)	μ_S (h ⁻¹)	μ_P (h ⁻¹)	$A_{x/s}$ (g/g)	$A_{x/p}$ (g/g)	$Y_{p/s}$ (g/g)	P_p (g/L.h)	P_{ox} (g/L.h)
0	0.16	66.75	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	0.27	64.73	0.17	0.35	7.24	0.62	0.05	0.61	0.09	0.17	0.11
2	0.35	62.86	0.33	0.62	10.40	1.66	0.05	0.57	0.09	0.17	0.09
3	0.70	57.43	1.34	0.50	5.25	2.40	0.06	0.40	0.14	0.45	0.18
4	1.05	55.48	3.71	0.40	1.85	2.48	0.08	0.24	0.33	0.93	0.22
5	1.54	53.54	6.57	0.26	3.00	1.96	0.10	0.21	0.50	1.31	0.28
6	1.86	46.23	9.75	0.25	5.16	1.83	0.08	0.17	0.48	1.62	0.28
7	2.48	34.34	13.38	0.19	4.07	1.47	0.07	0.17	0.41	1.91	0.33
8	2.79	25.99	17.06	0.11	3.01	1.36	0.06	0.15	0.42	2.13	0.33
9	3.11	17.53	20.98	0.05	2.70	1.14	0.06	0.14	0.43	2.33	0.33
10	3.11	9.23	24.16	0.00	0.00	0.00	0.05	0.12	0.42	2.42	0.29
12	2.73	3.04	27.60	0.00	0.00	0.00	0.04	0.09	0.43	2.30	0.21

7.5 Exponential Fed-Batch Fermentation of *K. pneumoniae* GLC29

Fed-batch culture has often been used to achieve high cell density and high productivity of a desired bio product, and Fed-batch culture with exponential feeding allows cells to grow at constant specific growth rates, and have been successfully employed for high cell density culture (LEE *et al.*, 1997).

The fed-batch part of the fermentation operation depended on a model-based feeding strategy (EJIOFOR *et al.*, 1996). Fed-batch fermentation can be controlled according to the following equation for an exponential feed rate:

$$F(t) = \mu XV / (S_F - S) Y_{X/S} \cdot \text{EXP}(\mu t)$$

Which the Flow Rate at time t (l/h) = (Specific Growth Rate (μ) x Vessel Working Volume at time 0 (V_0) x cell concentration at time 0 [X_0] in DCW (g/L) / (Feed Substrate Concentration [S_F] g/L - Substrate concentration in culture [S] g/L) x Biomass yield on substrate [$Y_{X/S}$] g/g x $\text{EXP}(\mu t)$)

Using this model, feed started when the specific growth rate was maximum (2 h) and fed during 5 hours. We had experimented 2 different specific growth rates, being the two below the maximum specific growth rate to avoid the accumulation of substrate ($\mu_x=0.5$ and $\mu_x=0.4$). Figure 22 shows the kinetics of those fermentations using analytical grade glycerol.

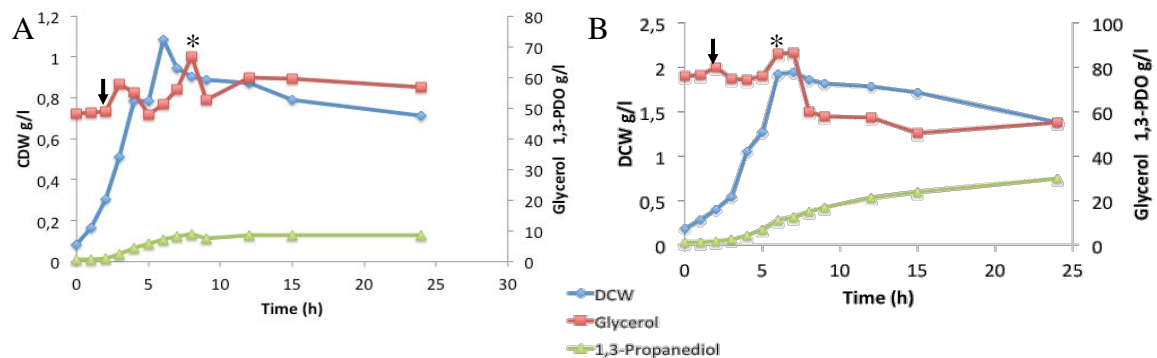


Figure 22 - Exponential Fed-Batch Culture of *K. pneumoniae* GLC29 in analytical grade glycerol– 1,3-propanediol g/L production. A. $\mu_x = 0.5$; B. $\mu_x=0.4$.

Exponential fed-batch using these parameters did not have better results compared to batch cultures. It can be observed there was glycerol accumulation after 5 hours, then, even after 24 h, glycerol was not totally consumed, and production was limited.

Other three exponential fed-batch fermentations, using biodiesel glycerol, were

performed (Figure 23). Fermentation did not start with the correct glycerol concentration weighted, (68 g/L) and we also notice an accumulation of glycerol. This might be because of substrate inhibition at the start of the fermentation or because of lower productivity as noticed in batch cultures.

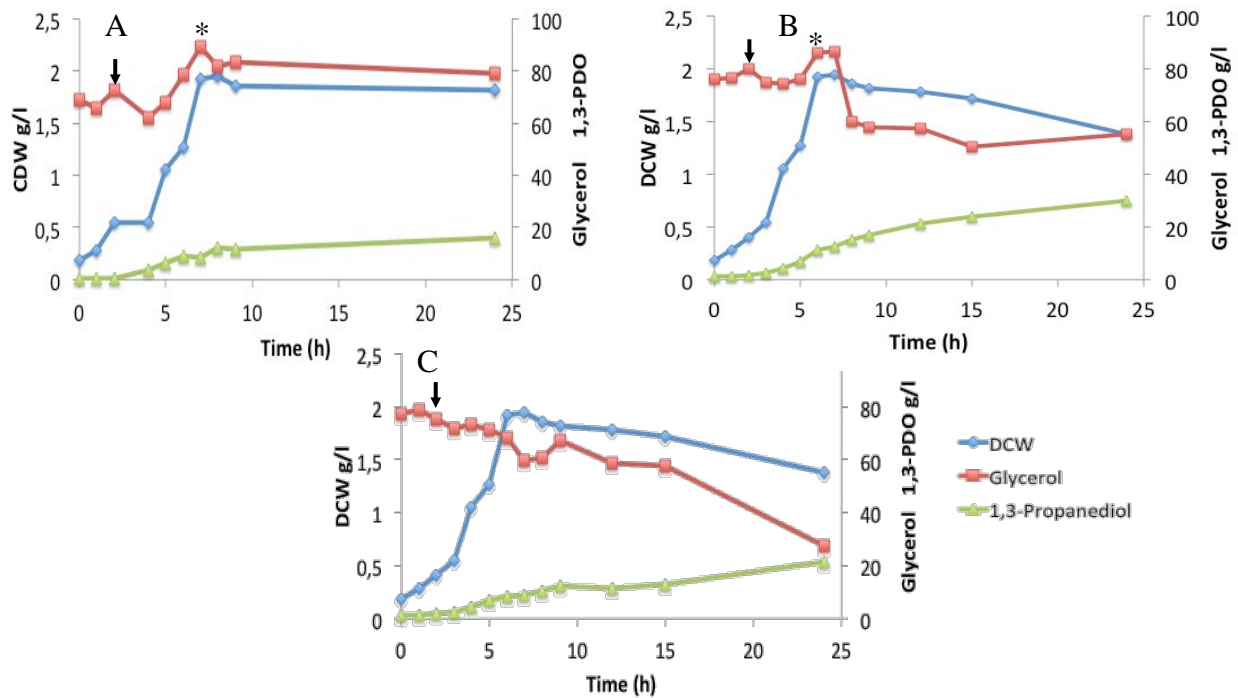


Figure 23 - Exponential Fed-Batch Culture of *K. pneumoniae* GLC29 in Biodiesel Glycerol– 1,3-propanediol production using (A) μ_x 0.9 h^{-1} ; (B) μ_x 0.5 h^{-1} ; (C) μ_x 0.4 h^{-1} .

It was observed that after 5 hours, there was glycerol accumulation using μ_x 0.9 h^{-1} and μ_x 0.5 h^{-1} , but using μ_x 0.4 h^{-1} glycerol was consumed. But still, no significant improvement was observed. This might be because of substrate inhibition, because glycerol measured at time zero was much greater than weighted. It was found glycerol was leaking inside the fermentation vessel during autoclave. The best production was achieved using μ_x 0.5 h^{-1} at 24 h with 29.9 g/L of 1,3-PDO.

A new fermentation using pure glycerol was performed. Batch started with 52 g/L of glycerol, and feeding started after 2 hours of fermentation, using μ_x 0.45 h^{-1} as the exponential reference. Figure 24 shows the kinetics of this experiment.

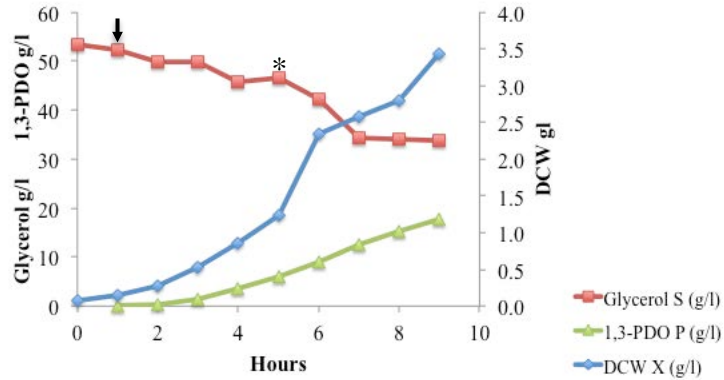


Figure 24 - Exponential Fed-Batch Culture of *K. pneumoniae* GLC29 in Biodiesel Glycerol–1,3-propanediol production using $\mu_x 0.45 \text{ h}^{-1}$.

We can observe that there was no glycerol accumulation, and although there was a higher growth compared to the previous experiment, reaching 3.43 g/L of DCW, not all glycerol was consumed after 9 hours fermentation, but also, 1,3-propanediol production was not enhanced using exponential fed-batch. Table 22 shows the data obtained and calculated yields and productivity for each fermentation time. Maximum productivity was 1,97 g/L.h of 1,3-propanediol.

New fermentations would be needed to evaluate the feasibility of fed-batch processes for 1,3-propanediol, in which there was no further time left to perform during this study. Variations on the feeding start time, type of feeding and flux would be necessary to completely describe the process.

Table 22 - Exponential fed Batch Culture ($\mu = 0.45$) of *K. pneumoniae* GLC29 in Analytical grade Glycerol.

Time (h)	Data obtained			Specific Rates		Productivity	
	DCW X (g/L)	Glycerol S (g/L)	1,3-PDO P (g/L)	μ_X (h^{-1})	μ_P (h^{-1})	Pp (g/L.h)	Px (g/L.h)
0	0.08	53.41	0.00	0.00	0.00	0.00	0.00
1	0.15	52.33	0.14	0.67	1.48	0.14	0.07
2	0.27	49.88	0.43	0.70	2.21	0.22	0.10
3	0.52	49.72	1.33	0.55	2.97	0.44	0.15
4	0.85	45.79	3.53	0.42	2.76	0.88	0.19
5	1.24	46.58	6.01	0.61	2.25	1.20	0.23
6	2.35	42.28	9.10	0.28	1.40	1.52	0.38
7	2.57	34.21	12.61	0.09	1.18	1.80	0.36
8	2.79	33.98	15.18	0.15	0.92	1.90	0.34
9	3.43	33.75	17.75	-	-	1.97	0.37

DCW: Dry Cell Weight; 1,3-PDO; 1,3-propanediol.

8. CONCLUSIONS

Eleven media components were screened using Plackett-Burman and Box, Hunter & Hunter experimental design. Five components from media were considered significant to 1,3-propanediol production, glycerol, yeast extract, ammonium sulfate, vitamin B12, and fumaric acid. Inoculum concentration was fixed at 10%, and CaCl_2 at 5 mM. According to the experimental design, 50 g/L of glycerol, 5 g/L of yeast extract, 1,5 g/L of ammonium sulfate and 0.25 $\mu\text{g/L}$ had the best results for 1,3-propanediol production. It is recommended the use of concentrations lower than 49 g/L of glycerol since the use of higher amounts resulted in lower concentrations and productivity. Batch fermentations had the best production, reaching 27.6 g/L of 1,3-propanediol, 0.46 $\text{yield}_{p/s}$ (g/g) and 2.6 g/L.h productivity using pure glycerol. Fed-batch did not improve production or productivity in this study, but also achieved 29.9g/L of 1,3-PDO in 24 h. New experimental designs can be performed using biodiesel glycerol and other nitrogen sources in order to make the production cheaper. Other experiments also need to be performed to achieve optimum productivity in fed batch fermentations.

CHAPTER II

Cloning and expression of DHA genes from *Klebsiella pneumoniae* in *Escherichia coli* strains for 1,3-propanediol production.

9. INTRODUCTION

For a large number of bacteria, including *Citrobacter*, *Clostridium*, *Enterobacter*, *Klebsiella*, and some *Lactobacillus* species, a consequence of anaerobic growth on glycerol is the generation of excessive reducers in the form of NADH. The regeneration of NAD requires the formation of a byproduct to serve as an electron sink. Enzymatic pathways for oxidation and reduction of glycerol were incorporated and used when glycerol is present and alternative carbon sources such as glucose are absent (NAKAMURA; WHITED, 2003).

The production of 1,3-propanediol is connected to an oxidative process of glycerol. Glycerol enters the cell by *glpF* (glycerol facilitated transport), or by diffusion (MAERVOET *et al.*, 2011). When it enters the cell, it can follow two routes. At the first, suffers oxidative dehydrogenation by a NAD⁺ dependent glycerol dehydrogenase, becoming dihydroxyacetone (DHA). DHA is next phosphorylated to dihydroxyacetone phosphate by an ATP-dependent DHA kinase. (OH *et al.* 2012; YAZDANI; GONZALES, 2007). Through the parallel process, glycerol is dehydrated to form 3-hydroxypropionaldehyde (3-HPA) by glycerol dehydratase (EC 4.2.1.30), in *K. pneumoniae* case, B12-dependent, composed of 3 peptides, encoded by *dhab1*, *dhaB2*, and *dhaB3*. Then, 3-HPA is reduced to 1,3-PDO by 1,3-PDO oxidoreductase (EC 1.1.1.202) linked to NADH (YAZDANI; GONZALES, 2007).

Using metabolic engineering, it is possible to manipulate the metabolic routes and obtain high value products, and reduce or eliminate the formation of undesirable byproducts (CHENG; LIU; LIU, 2005).

DuPont & Genencor International, Inc. modified *E. coli* genetically to produce 1,3-PDO from glucose. Since wild type *E. coli* can't efficiently produce glycerol from glucose, seven genes had to be introduced into the genome. Two of these genes (*dar1* and *gpp2*) originated from *Saccharomyces cerevisiae*, and five genes (*dhaB1*, *dhaB2*, *dhaB3*, *dhaBX* and *orfX*) from *Klebsiella pneumoniae*. Consequently, it was described a productivity of 1,3-propanediol of 3.5 g/L.h, a yield of 0.62 mol/mol, and a final concentration of 135 g/L (MAERVOET *et al.*, 2011).

Because *E. coli* has good growth on glycerol in aerobiosis, several researchers have been trying to genetically modify it to produce 1,3-PDO, thus enhancing glycerol as the carbon source. Ma *et al.* (2009) constructed a plasmid containing *dhaB* and *dhaT* genes in *E. coli* and expressing both genes in the same direction, successfully produced 11.3 g/L of 1,3-PDO from 40 g/L glycerol. Zhang *et al.* (2006) constructed a similar plasmid in *E. coli* with

genes from *Citrobacter freundii*, and using experimental design from fixed concentrations: 61.8 g/L glycerol, 6.2 g/L of yeast extract and 49 mg/L of vitamin B12, achieved a production of 41.3 g/L of 1,3-PDO. The maximum 1,3-PDO obtained with a mutant *E. coli*, had genes *dhaB1* and *dhaB2* from *C. butyricum*, which presents rapid conversion and is not dependent on vitamin B12. Two stage fermentation was performed, the first at 30 °C to obtain high cell density, and second, passing the culture to 42 °C and to totally anaerobic production, reaching a final 104 g/L of 1,3-PDO, and a productivity of 2.61 g/L.h (TANG *et al.*, 2009). Although it is not vitamin dependent, the enzyme is oxygen sensitive, requiring total anaerobic cultures (KAUR *et al.*, 2012).

10. OBJECTIVES

The objective of this study was to perform the cloning of the genes responsible for production of 1,3-PDO: *dhaB1*, *dhaB2*, *dhaB3*, *dhaT*, *dhaF* and *dhaG* from *K. pneumoniae* strain GLC29 into *E. coli* and verify the 1,3-propanediol biosynthesis.

Specific Objectives:

- 1) Construct a plasmid without reactivation factors and with genes *dhaB1*, *dhaB2*, *dhaB3*, and *dhaT*.
- 2) Construct a plasmid with *dhaB1*, *dhaB2*, *dhaB3*, and *dhaT* and the reactivation factors *dhaF* and *dhaG*.
- 3) Transform plasmids into strains TCS099 (Trinh Lab) and verify 1,3-propanediol production, comparing the two plasmids.
- 4) Perform fermentation in aerobic, micro-aerobic and anaerobic conditions.

11. MATERIAL AND METHODS

11.1 Strains studied and maintenance of isolates

The microorganism *K. pneumoniae* CLG29 was previously isolated by Da Silva (2014) and kept at -86 °C in 20% glycerol solution, and lyophilized kept under refrigeration. Samplings were performed to maintain the viability of the cultures regularly. Cultures were reactivated in test tubes with 5 mL of LB and incubated at 37° C for overnight. *E. coli*

transformants in this work were preserved in the same way. *E. coli* TCS099 (Δ msgA, Δ ldhA, Δ fdrA, Δ zwf, Δ ndh, Δ maeB, Δ pta, Δ poxB, Δ mhpF, Δ adhP and Δ adhE), described in Trinh and Srienc (2009) was tested at the University of Tennessee. *E. coli* SZ63 (W3110, Δ focA-pflB::FRT, Δ frdBC Δ adhE::FRT, ackA::FRT), described in Zhou *et al.* (2003) was obtained from University of Florida. Acetate mutants as described in Contiero *et al.* (2000), *E. coli* strains: W3100 (F^- gal hft), AJW1387 (W3100 Δ acs::Km), AJW1509 (W3100 iclR::Tn5), AJW1483 (W3100 Δ rpoS::Km) and *E. coli* Δ msgA were obtained from the Loyola University of Chicago,

11.2 Cloning

One plasmid was designed to include *dhaB1*, *dhaB2*, *dhaB3* (glycerol dehydratase), *dhaT* (1,3-propanediol oxidoreductase), to form pSB1C3-dhaB123T, and another to contain previous genes plus *dhaF* and *dhaG* (glycerol dehydratase reactivase), to form pSB1C3-dhaB123TFG (Figure 24).

Genes were obtained from *Klebsiella pneumoniae* GLC29 genomic DNA, new 1,3-PDO strain isolated from bryophytes grown on the base of leaf stalks of *Terminalia catappa* (DA SILVA, 2014) at UNESP – Universidade Estadual Paulista, in Rio Claro. Plasmid backbone pSB1C3 was assembled to contain BBa_R0010 promoter, sensitive to LacI and CAP protein, and BBa_B0015 double terminator (Figure 25). pSB1C3 is a high copy number plasmid carrying chloramphenicol resistance. The replication origin is a pUC19-derived pMB1 (copy number of 100-300 per cell). Additionally, a ribosome-binding site BBa_B0034 was designed within the primers which sequence was obtained from partsregistry.org, tested with a strong efficiency. Primers corresponding to genes were designed using known sequences from other *Klebsiella pneumoniae* strains 342, MGH78578, and KTCC 2242, which the complete genome is available at ncbi.nlm.nih.gov, and it was verified that in all 3 genomes homology regions were identical to the primers designed. All primers were designed for Gibson's Assembly (GIBSON *et al.*, 2009), with a 40 base pair overlap with the next sequence (Table 23), *dhaB1_F* was designed with a 40 base pair overlap with the promoter and *dhaT_term_R* was designed with an 40 base pair terminator overlap.

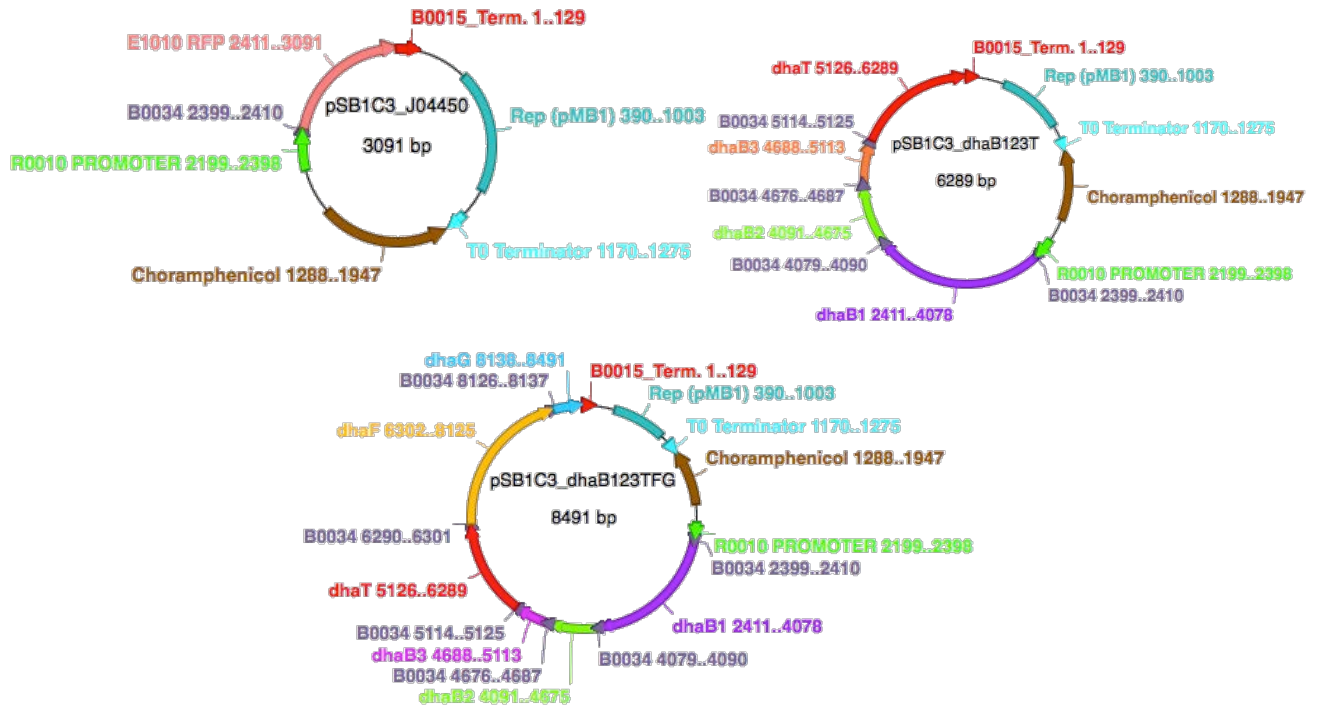


Figure 25 - Designed containing *dha* genes for 1,3-propanediol - Plasmid pSB1C3_JJ04450 used as Backbone template, pSB1C3_dhaB123T, and pSB1C3_dhab123TFG.

Table 23 - Primers designed for 1,3-Propanediol constructs - Gibson's Assembly: dotted underlined – Promoter overlap; Underlined –Ribosome Binding Site; Dot-dash underlined –Terminator overlap

Primer		Sequence
dhaB1_F	For	aattgtgagcggataacaatttcacacaaaagaggagaaaATGAAAAGATCAAACCGATT
dhaB1_R	Rev	GTTGTCTGTTGCATtctcctctttTTATTCAATGGTGTGTCAGGCTG
dhaB2_F	For	ACACCATTGAATAAaaagaggagaaaATGCAACAGACAACCCAAATTC
dhaB2_R	Rev	GTTTTCTCGCTCATtctcctctttTCACTCCCTTACTAAGTCGAC
dhaB3_F	For	TAGTAAGGGAGTGAAaagaggagaaaATGAGCGAGAAAACCATGCGCG
dhaB3_R	Rev	ATACGATAGCTCATtctcctctttTTAGCTTCCTTTACGCAGCTTATG
dhaT_F	For	GTAAAGGAAGCTAAaagaggagaaaATGAGCTATCGTATGTTTGATTATC
dhaT_R	Rev	GCTATTAACGGCATtctcctctttTCAGAATGCCTGGCGGAAAATC
dhaF_F	For	GCCAGGCATTCTGAaaagaggagaaaATGCCGTTAATAGCCGGGATTG
dhaF_R	Rev	GGTGAAGCGACATtctcctctttTTAATTCGCCTGACCGGCCAG
dhaG_F	For	GTCAGGCGAATTAaagaggagaaaATGTCGCTTTCACCGCCAGGCG
dhaG_R	Rev	cagtcttcgactgagcctttcgttttattgatcctggTCAGTTTCTCTCACTTAACG
dhaT term_R	Rev	cagtcttcgactgagcctttcgttttattgatcctggTCAGAATGCCTGGCGGAAAA
psB1C3_F	For	Ccaggcatcaataaaacgaaggctcag
psB1C3_R	Rev	Tctcctctttgtgtgaaattgtatcc

PCR reactions were performed in thermal cycler using high fidelity Phusion DNA polymerase for cloning or Taq polymerase for confirmations. Amplified products were visualized using gel electrophoresis (Agarose gel containing 0.01% ethidium bromide), and the bands sizes were determined by comparison with molecular weight marker. Target bands

from the amplified genes were excised from the gel and purified using Zymo Research[®] Zymoclean[™] Gel DNA Recovery Kit, cleaned and concentrated using Zymo Research[®] DNA Clean & Concentrator[™] and quantified using NanoDrop[™] Thermo[®]. Plasmids were extracted from *E. coli* cultures, after 6 h growth in LB media with 50 µg/mL chloramphenicol, using Zymo Research[®] - Plasmid Miniprep[™] - Classic, and quantified by NanoDrop[™]. Plasmid backbone proper for Gibson's Assembly was obtained using PCR reaction and gel purified. The plasmid constructs were performed using Gibson's Assembly (GA) isothermal protocol (GIBSON *et al.*, 2009) and assembled into pSB1C3. Negative controls were done using open plasmid backbone by itself into ligation mix, and submitted to transformations.

11.3 Transformations

Transformations (both target plasmids and negative controls) were performed adding 2 µL of plasmid (about 100 ng) to a falcon tube containing 50 mL of competent cells (*Escherichia coli*) incubated in ice for 20 minutes, then heat shocked for 50 seconds by placing the tube at 42° C in water bath, immediately cooling it on ice for 2 min. 1 mL of SOC broth at room temperature was added into the culture and incubated in rotatory shaker for 1:30 h at 37 ° C, and 200 rev/min⁻¹. After incubation, 100 µL was plated directly using spread plate technique with sterile glass beads on LB agar plates containing 50 mg/mL of chloramphenicol. The remaining culture was centrifuged, re-suspended in 100 µL SOC broth and plated on another agar plate.

Electroporation was performed when heat shock was not successful. Target cells were grown in LB to a O.D. of 0.5 to 0.7, then culture was chilled on ice for 10 min, centrifuged at 4000 *g* and 4 °C for 10 min, washed and centrifuged again three times with chilled ultrapure sterile water, re-suspended in 250 µl of sterile ultrapure water. Electroporation cuvettes were kept on ice prior to the reaction, then, 50 µl of cells and 1 µl of salt free plasmid were poured into the cuvettes. Transformation was performed applying 2.4 kV during 5 mSec. Right after transformation, 1 mL of SOC media at 37 °C was added to the cuvette and the solution was set to grow at for 60 minutes. After one hour incubation, 100 µl was transferred to agar plate with respective antibiotic of the plasmid.

11.4 Sequencing

After plasmids were constructed, they were sent to the Molecular Biology Resource Facility to be sequenced by capillary electrophoresis ABI 3730 genetic analyzers. Primers were sent and plasmid was treated with Big Dye Polymerase kit prior to sequencing. Sequences were aligned using software MEGA 5.0 and by ClustalW, and their codon translated, and they can be seen on Appendix I.

11.5 Characterizations

Confirmed plasmids were extracted from Top10 using plasmid mini-prep and transformed into competent *E. coli* TCS099 by heat shock. Transformed *E. coli* TCS099 pSB1C3+dhaB123T and pSB1C3+dhaB123TFG were cultivated into minimal media (Table 24) for 1,3-propanediol production and samples were taken every 24h. Experiments were performed in test tubes in aerobic (aerobic caps), anaerobic and in micro-aerobic conditions. Rubber stops sealed the tubes for anaerobic conditions. A needle with cotton on top was used to favor limited oxygen transfer through rubber stops when micro-aerobic conditions were used.

Table 24 - Media composition for characterization.

Composition	
KH ₂ PO ₄	3.50 g/L
K ₂ HPO ₄	5.00 g/L
(NH ₄) ₂ HPO ₄	3.50 g/L
MgSO ₄ .7H ₂ O	0.25 g/L
CaCl ₂ .2H ₂ O	0.015 g/L
Vitamin B12	0.25 mg/L
Cloramphenicol	30 µg/mL
Glycerol	20 g/L

11.6 Fermentations

Plasmids were extracted from Top10 using plasmid mini-prep and transformed into competent *E. coli* strains (W3100, AJW1483, AJW1509, AJW1387, ΔmsgA and DH10B) by electroporation. The transformed *E. coli* strains were cultivated into minimal media (Table 25) for 1,3-propanediol production and samples were taken every 24h. Experiments were performed in test tubes or Erlenmeyer flasks. Rubber stops sealed the tubes for anaerobic

conditions. Media components are shown on table 25 and 1 μM sodium selenite (Na_2SeO_3) was added when the experiment required.

Table 25 - Media composition for first and second *E. coli* strain selection respectively.

Composition 1		Composition 2	
KH_2PO_4	3.5 g/L	KH_2PO_4	0.136 g/L
K_2HPO_4	5.0 g/L	$(\text{NH}_4)_2\text{HPO}_4$	3.50 g/L
$(\text{NH}_4)_2\text{HPO}_4$	3.5 g/L	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.48 g/L
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	250.0 mg/L	CaCl_2	15 mg/L
CaCl_2	15.0 mg/L	Vitamin B12	10 mg/L
Vitamin B12	0.25 mg/L	Cloramphenicol	30 mg/mL
Cloramphenicol	30 mg/mL	Glycerol	20-45 g/L
Glycerol	20-45 g/L	Tryptone	10 g/L
Trace Metal Solution	0.1 mL/L	Sodium Selenite	1 μM
IPTG	200 mM	pH	6.3

11.7 Detection of produced metabolites

Periodically, samples of 1 mL were collected from the cultures and centrifuged at 10,000 G for 10 minutes. The cell-free supernatant was filtered (0.22 μm) and analyzed by high performance liquid chromatography (HPLC) using ion exchange column Phenomenex Rezex ROA (300 mm x 7.8 mm) at a temperature of 60 °C and 0.005 M of H_2SO_4 water solution as mobile phase at 0.5 mL/min as mobile phase. External standards used were ethanol, 1,3- propanediol, propionic acid, acetic acid, 2,3-butanediol and glycerol.

11.8 Cloning *dha* genes

Taq PCR was performed to determine best annealing temperature for the combinations of primers (Figure 26).

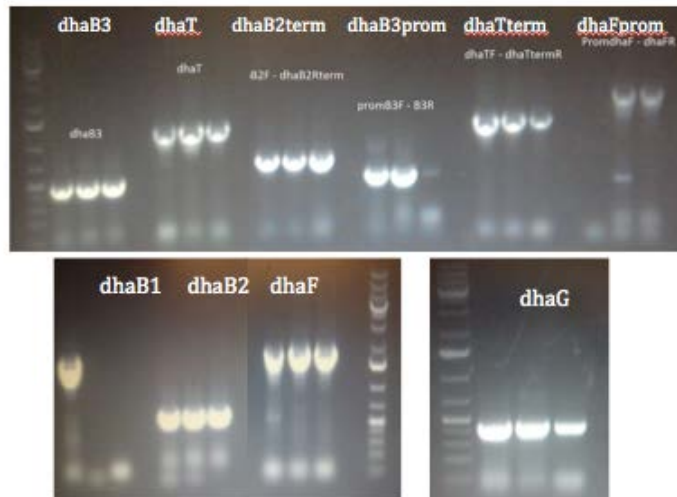


Figure 26 - Amplified genes to the different annealing temperature, 46, 56, and 65 °C respectively for each gene. Gene Ruler 1 kb Plus DNA Ladder 75 to 20,000 base pairs.

All genes used for cloning were amplified using the primers designed using Phusion polymerase high fidelity (Figure 27). The products were gel purified and used as template for overlapping PCR.

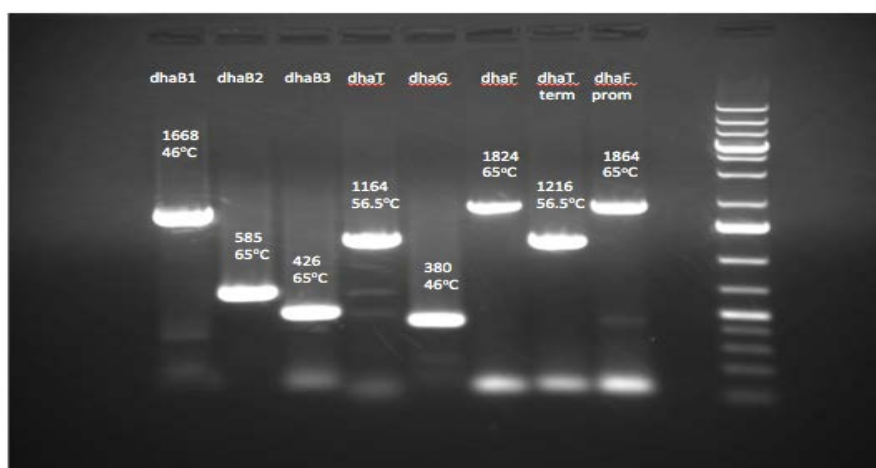


Figure 27 - Genes amplified with Phusion® polymerase. GeneRuler 1 kb Plus DNA Ladder 75 to 20,000 base pairs.

11.9 Construct 1 – pSB1C3_dhaB123T

Amplified genes *dhaB1*, *dhaB2* *dhaB3* and *dhaT_term* (Figure 27), were used as template to an overlapping PCR ligating *dhaB1B2* and *dhaB3T_term* (Figure 28). Those were gel purified and used for GA ligation. The reaction mix consisted of 15 μ l of GA master mix and 5 μ l of DNA pieces and water. The amount of DNA pieces varied according to the normalization according to the backbone , 5 ng or 10 ng was used. Calculations are shown on table 26.

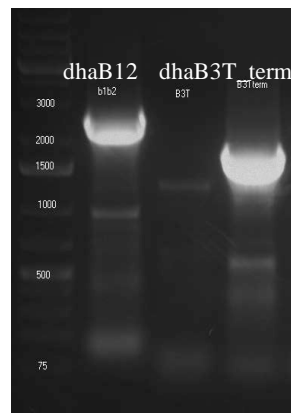


Figure 28 - Overlapping PCR's with *dhaB1B2*, *dhaB3T* and *dhaB3T_term*.

Table 26 - Concentrations and normalizations for GA of *dhaB12*, *dhaB3T_term*.

	Concentration	Size	Ratio	10 ng backbone
pSB1C3 Backbone	16.1 ng/ μ l	2409 bp	1	0.62 μ l
<i>dhaB1B2</i>	12 ng/ μ l	2331 bp	0.96	0.80 μ l
<i>dhaB3Tterm</i>	9.6 ng/ μ l	1668 bp	0.69	0.72 μ l
Water				2.85 μ l
GA Master Mix				15 μ l
Neg. Control	pSB1C3			0.62 μ l
Water				4.37 μ l
GA Master Mix				15 μ l

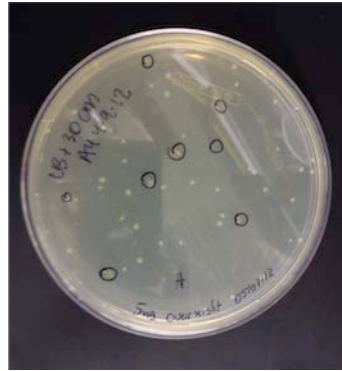


Figure 29 - Plate with positive colonies 5 ng of backbone and dhaB123T.

Colonies (Figure 29) were screened for inserts and confirmation was performed by PCR with all possible primer combinations to confirm the sequence of genes. Reaction mixes were visualized by gel electrophoresis (Figure 30). Confirmations showed the expected band sizes: dhaB123T 3.9 kb, dhaB123 2.7 kb, dhaB12 2.2 kb, dhaB23 1 kb, dhaB23T 2.2 kb, dhaB3T 1.6 kb, dhaB1 1.6 kb, dhaB2 585 bp, dhaB3 426 bp, dhaT 1.16 kb.

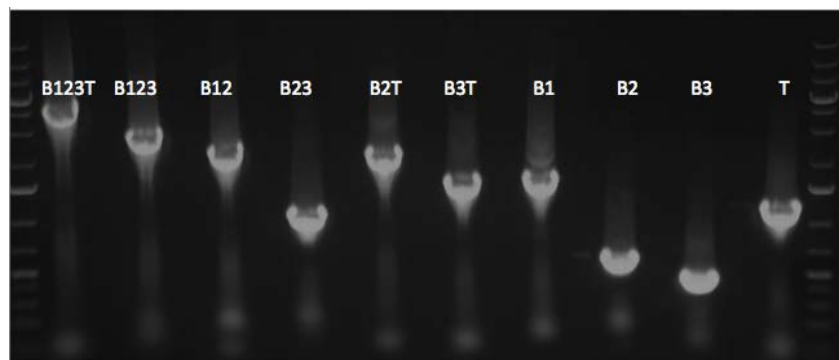


Figure 30 - Gel picture: Gel picture showing amplified sequences with combinations of primers from *dhaB1* to *dhaT*. GeneRuler 1 kb Plus DNA Ladder 75 to 20,000 bp.

11.10 Construct 2 - pSB1C3_dhaB123TFG

Genes *dhaF* and *dhaG* were amplified using Phusion[®] HF polymerase (Figure 31-A), and then gel purified and used as a template for overlapping PRC to obtain *dhaFG* (Figure 31-B). Using pSB1C3_dhab123T as a template, a backbone was obtained using primers pSB1C3_F and dhaT_R (figure 31-B). The reaction mixes were gel purified and used in GA protocol. It was found that genomic DNA was not working, and fresh new genomic DNA

was prepared. The successful approach was amplifying pSB1C3_dhaB123T, and the GA reaction is show in Table 27.

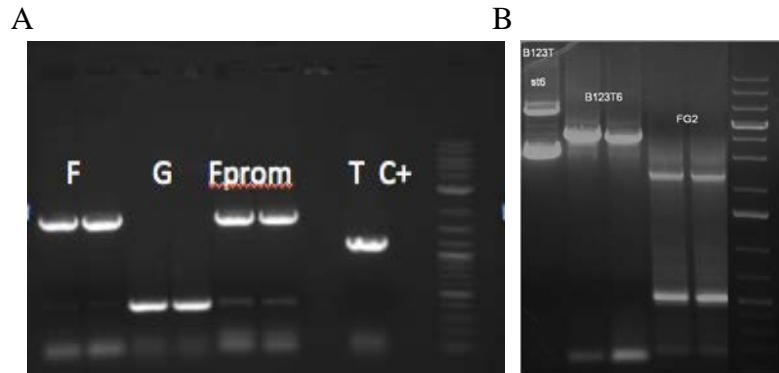


Figure 31 - Gel Pictures: A. Amplification of *dhaF*, *dhaG*, *dhaF_prom* and *dhaT* as positive control. B. Plasmid Backbone containing *dhaB123T*, *dhaB123T6* and Overlapping PCR *dhaF* and *dhaG*, 2.2kb. GeneRuler 1 kb Plus DNA Ladder 75 to 20,000 bp.

Table 27 - Concentrations and normalizations for GA insertion of *dhaF* and *dhaG*.

	Concentration	Size	Ratio	10 ng backbone
pSB1C3dhaB123T				
backbone	9.60 ng/ μ l	6366 bp	1.00	1.04 μ l
dhaFG	10.90 ng/ μ l	2256 bp	0.35	0.33 μ l
Water				3.63 μ l
GA Master Mix				15 μ l
			Negative Control	1.04 μ l
			Water	3.96 μ l
			GA Master Mix	15 μ l

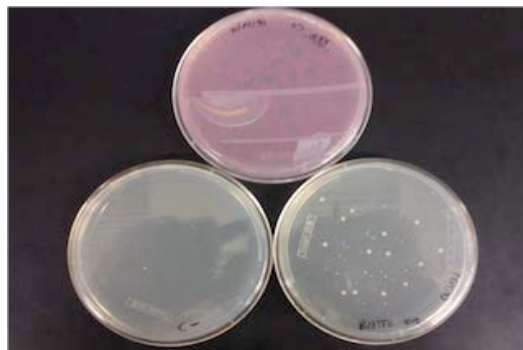


Figure 32 - Successful cloning: Top – Positive transformation control, with pSB1C3_RFP; bottom left - Negative control; Bottom Right – Colonies containing pSB1C3_dhaB123TFG.

Colonies (Figure 32) were screened for the presence of *dhaF* at first using colony PCR. Later, plasmids were extracted using mini-prep kit, quantified and used as template to screen for all genes and their sequence using Taq PCR, and gel imaging (Figure 33). Four colonies showed to have *dhaF*, and plasmids were extracted for other confirmations. Three colonies were confirmed to have all genes (F2, F3 and F4), and one colony didn't show all correct genes (F5) and was discarded.

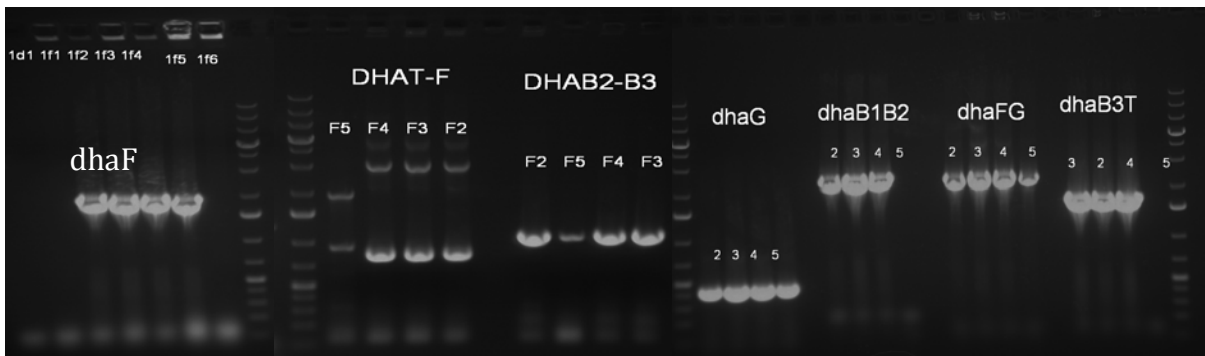


Figure 33 - Gel images confirming the presence of *dhaF* and *dhaG* into pSB1C3_dhaB123T.

12. RESULTS AND DISCUSSION

12.1 Characterizations

Transformed *E. coli* TCS099 pSB1C3+dhaB123T and pSB1C3+dhaB123TFG were characterized together for 1,3-propanediol production in minimal media, and how glycerol dehydratase reactivase (*dhaF* and *dhaG*) influenced in 1,3-propanediol production. We tested aerobic, micro-aerobic and anaerobic conditions, in triplicates and error bars indicate its standard deviation. Induction was performed at 48 h with IPTG, although it had already reached the maximum 1,3-PDO produced, we can see that it only was helpful for growth. Aerobic culture reached a max of 4.84 g/L of 1,3-propanediol produced, from 20 g/L glycerol, by TCS099+dhaB123TFG, and had better glycerol consumption (Figure 34). TCS099+dhaB123T only produced less than 250 mg/L of 1,3-PDO in these conditions, although growth was favored after IPTG induction.

Production was achieved also in anaerobic conditions (Figure 35), although growth and 1,3-propanediol was not favored, reaching less than 0.25 O.D. and 200 mg/L of 1,3-propanediol, probably because insufficient biomass. It is noticeable that error bars were

bigger than the anaerobic experiment, especially glycerol consumption, which might indicate cells were under stress. However, pSB1C3_dhaB123TFG had significant production against pSBC3_dhaB123T in anaerobic environment, where no 1,3-PDO was produced by pSBC3_dhaB123T.

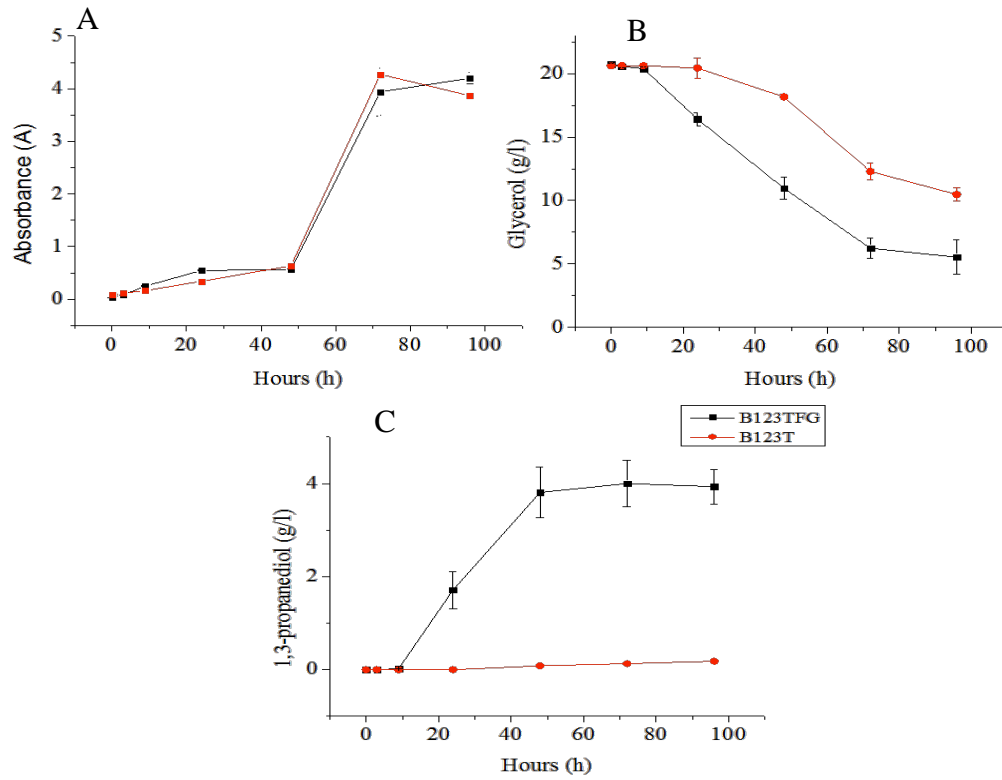


Figure 34 - Growth (A), Glycerol consumption (g/L) (B) and 1,3-propanediol (g/L) production (C) in aerobic conditions (induction at 48 h with IPTG 500 μ M).

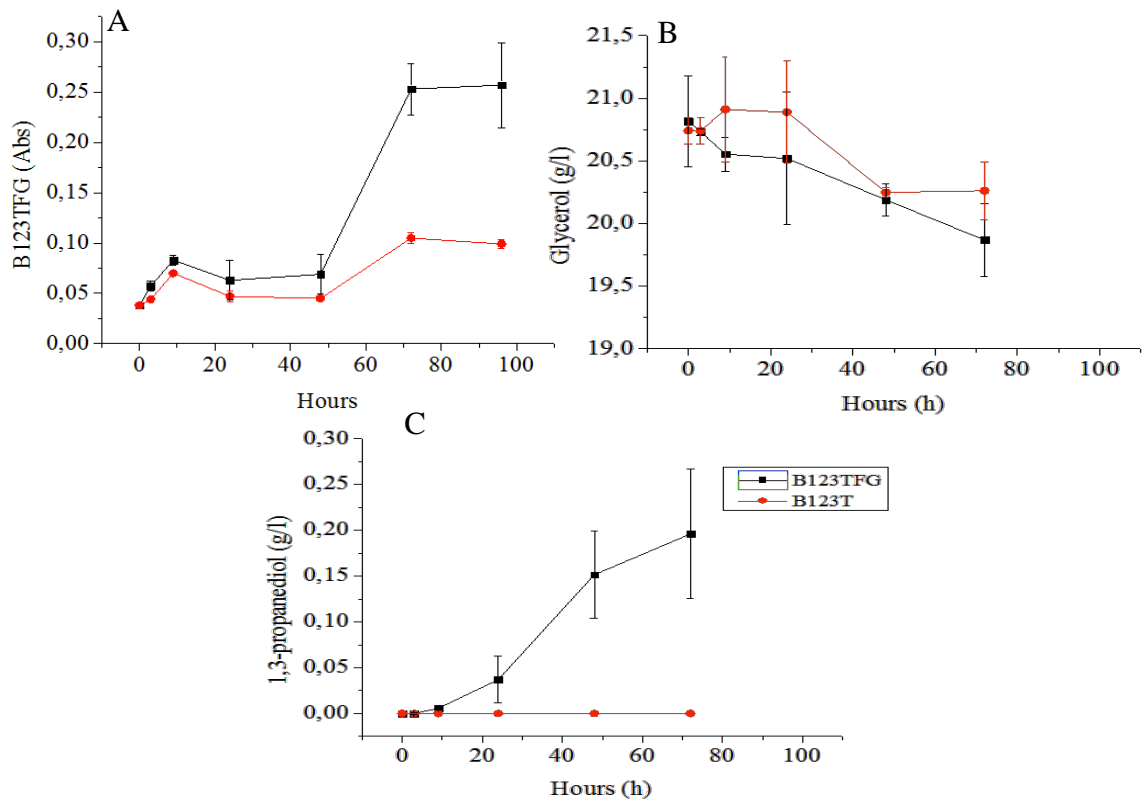


Figure 35 - Growth (A), Glycerol consumption (g/L) (B) and 1,3-propanediol production (g/L) (C) in anaerobic conditions (induction at 48 h with IPTG 500 μ M).

Another experiment was performed, including pSB1C3_RFP as a negative control and all strains were induced at inoculation. Production from anaerobic cultures (Figure 36) shows that there is not enough oxygen to support *E. coli* growth on glycerol and anaerobic conditions. As *E. coli* is not adapted to anaerobic fermentation of glycerol, it can't grow after oxygen is completely depleted from the test tubes. This affirmation is supported by the negative control, as we can see a discrete growth after 48 h. We can also see that dhaB1234TFG had the best and fastest growth, declining rapidly after oxygen depletion. In second, dhaB123T also had a faster growth then the negative control Red Fluoresce Protein (RFP).

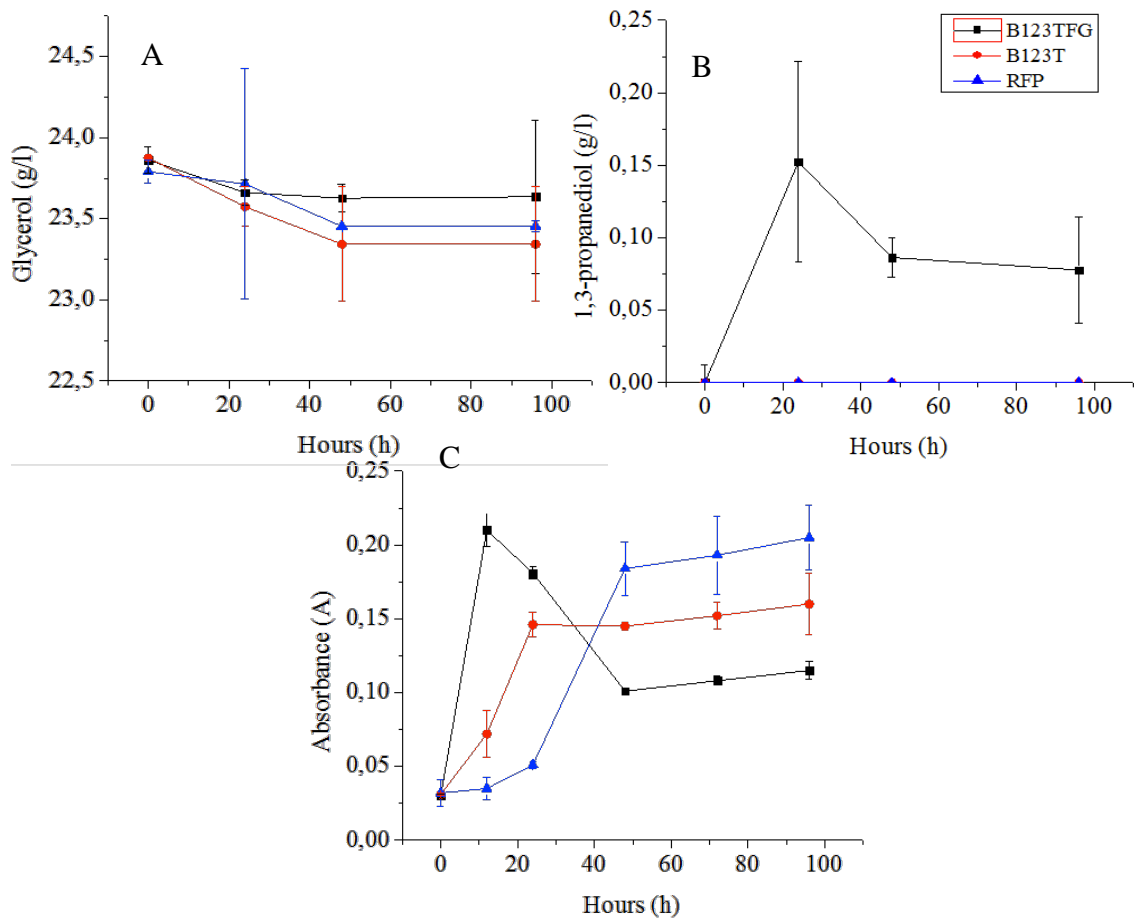


Figure 36 - Glycerol consumption (g/L) (A), 1,3-propanediol production (g/L) (B) and Growth (C) in anaerobic conditions (induction at time zero with IPTG 500 μ M).

Even though, little glycerol was consumed and little 1,3-PDO was produced only by dhaB123TFG. In the absence of oxygen or another electron acceptor, *E. coli* is not able to use glycerol as the only carbon source. In *E. coli*, there are two glycerol-3-phosphate dehydrogenases from *E. coli*, only one can use NAD⁺ as an electron acceptor (LIN, 1976), in glycerol degradation III. Figure 37 shows glycerol degradation in *E. coli*. Only one of these enzymes is able to donate electrons to the fumarate reductase complex, producing succinate from fumarate. Even though, Skraly *et al.* (1998) demonstrated that the quantity of succinate produced by their *E. coli* strain was only 4% of the glycerol metabolized, therefore not able to grow in anaerobic environment (SKRALY *et al.*, 1998).

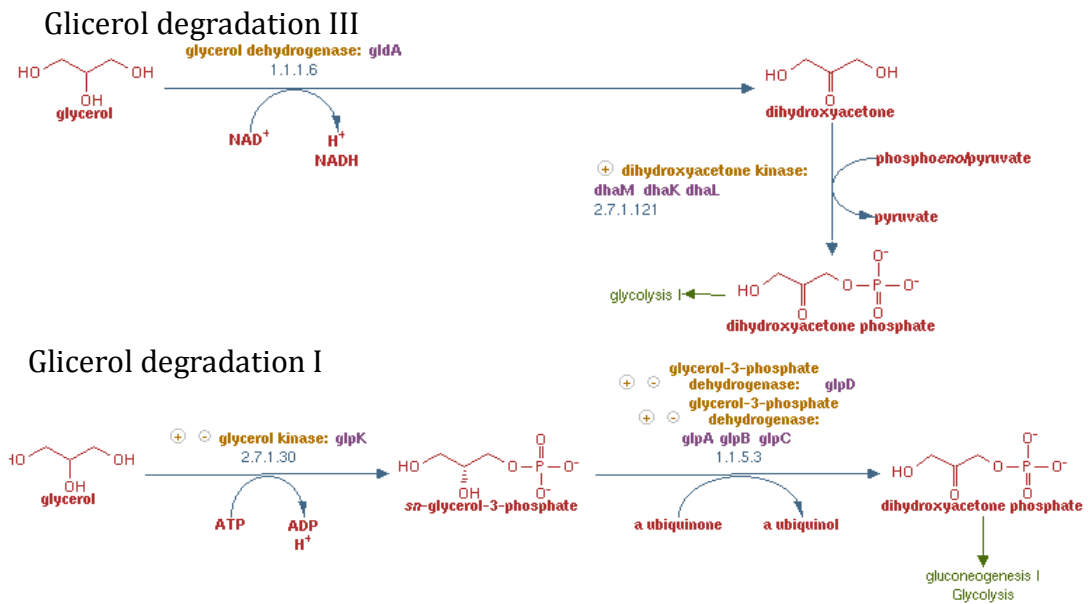


Figure 37 - Glycerol degradation in *E. coli*. (Adapted from ecocyc.org).

Using micro-aerobic conditions (Figure 38), growth and 1,3-propanediol production was better than anaerobic cultures, reaching up to 2.5 g/L of 1,3-propanediol from 6 g/L of glycerol consumed in 72 h from pSB1C3_dhaB123TFG. Glycerol consumption was more consistent and it is possible to see pSB1C3_dhaB123TFG consumed more glycerol than the others.

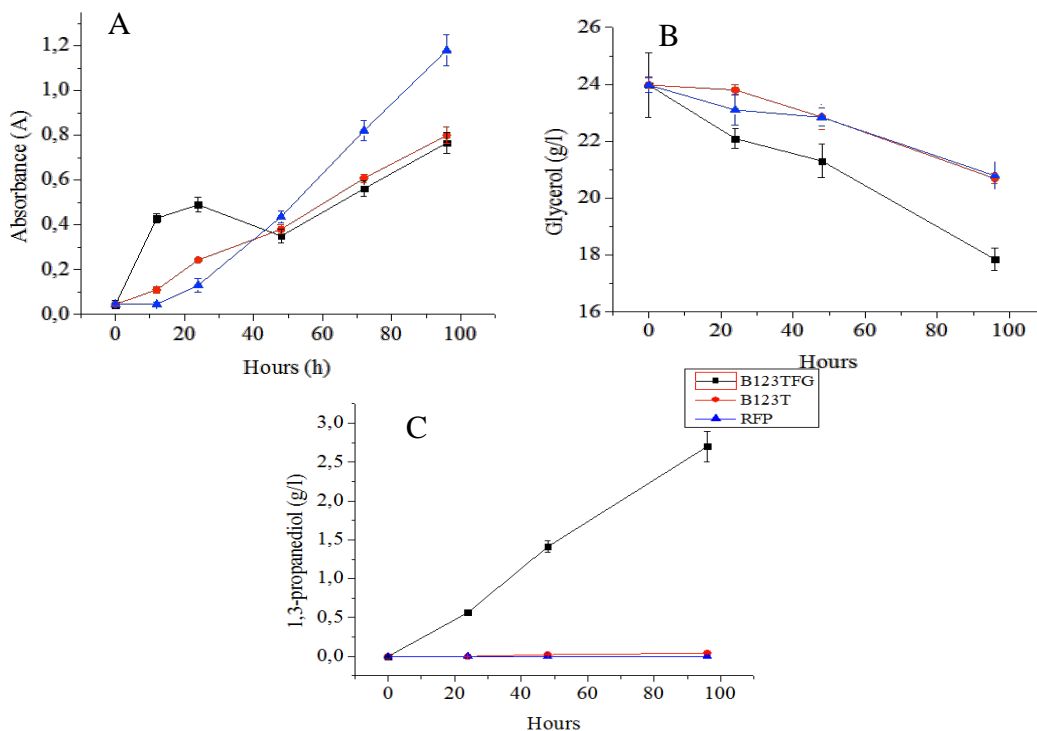


Figure 38 - Growth (A), glycerol consumption (g/L), (B) 1,3-propanediol production (g/L), and (C) in micro-aerobic conditions (induction at time zero with IPTG 500 μ M).

It is important to note that in this work, 250 µg/L of vitamin B12 was added to a minimal media, and fermentation strategies and media were not optimized. Skraly *et al.* (1998) demonstrated production up to 6.3 g/L of 1,3-PDO out of 9.33 g/L of glycerol in a 4-liter fed-batch *E. coli* AG1/pTC53 fermentation using 0.014 mg/L (or 10 nM) of vitamin B12. Using Statistical design, Zhang *et al.* (2006) constructed a novel *E. coli* recombinant using the complete *dhaB* gene set comprised of three different subunits *dhaBCE*, (2.8 kb) from *Citrobacter freundii* assembled into pHsh-yqhD. When used glycerol, yeast extract, vitamin B12 were set at 61.8 g/L, 6.2 g/L, 49 mg/L, respectively, and in a 5-l fermenter, 0.8 vvm aeration, 180 rev/min reached 43.8 g/L of 1,3-PDO produced. As the current high price of vitamin B12, using 49 mg/L of this vitamin is not feasible.

Glycerol can be oxidized to dihydroxyacetone (DHA) by enzyme GldA, a type II glycerol dehydrogenase, which is encoded by *gldA* in *E. coli*, however it's usually not expressed in wild type strains. Activation of this gene required inactivation of *glpK*, *glpR* and *glpD*, followed by mutagenesis and selection procedure, which resulted in a strain that recovered the ability to metabolize glycerol, however it did not provide the ability to ferment glycerol. (GONZALES, 2012).

Cameron *et al.* (1998) cloned from *K. pneumoniae* genes *dhaB* and *dhaT* to *E. coli*, including several ORFs (*dhaB3*, *dhaB3a*, *dhaB4*, and *dhaB4a*) from *dhaB* complex, knowing glycerol dehydratase had 3 subunits. A series of synthetic plasmids with *dhaB* and *dhaT* genes disposed in the same transcription direction and under the same promoter were built, and 1,3-PDO concentration reached over 70 g/L in 5 l fermenter, using fed batch fermentations, reaching an yield of 0.39 g_{1,3-PDO}/g_{glycerol} (0.48 mol/mol). The major byproduct was 2,3-butanediol, reaching nearly 20 g/L. No 2,3-butanediol was detected on fermentations using the plasmids and strains from this study.

12.2 Transforming pSB1C3_B123T and pSB1C3_B123TFG into other *E. coli* strains.

Confirmed plasmids pSB1C3_B123T (6259 bp) and pSB1C3_B123TFG (8491 bp) were extracted from Top10 using plasmid mini-prep and transformed by heat shock and electroporation into competent *E. coli* W3100, AJW1483, AJW1508, AJW1387, AJW1392, ΔmgsA⁻ and DH10B as competent cell control. pJet-Cm was used as transformation control. After heat shock, cells were cultivated in LB plates with 50 µg/mL of chloramphenicol.

Since methylglyoxal is a problem, and also 1,2 propanediol, we have acquired mutant *E. coli* Δ mgA⁻ (Kan⁺) from Prof^o. Dr. Alan J. Wolfe, Loyola University, Chicago, USA.

Transformations by electroporation occurred as expected; hundreds of colonies were obtained, and four colonies of each plate were isolated for colony PCR confirmation. pSB1C3_dhaB123T was successfully inserted in all strains, demonstrated by colony PCR of fragment dhaB3-T (Figure 39). As expected, fragment dhaF-G was not amplified, which means the plasmids inserted only contains genes *dhaB1*, *dhaB2*, *dhaB3* and *dhaT*. Although bands intensity varies, which is completely normal since colony PCR is a dirty and quick way to check, it is possible to see all strains amplified the desired fragment.

The same way, strains were screened to verify the correct insertion of pSB1C3_dhaB123TFG into the *E. coli* strains. Figure 40 shows colony PCR of strains transformed with pSB1C3_dhaB123TFG. As it can be observed, only *E. coli* AJW1508 did not transform correctly, being necessary to screen more colonies.

Later to the first strain selection, we have also acquired *E. coli* SZ63 (Causey *et al.*, 2003), which has the genotype Δ focA-pflB::FRT Δ frdBC Δ adhE::FRT ackA::FRT. This deletions had the purpose of increasing NADH₂, originally used to D-lactate production, but since 1,3-propanediol biochemical route is designed to recycle NADH₂, this strain is expected to produce more 1,3-propanediol when expressing genes from pSB1C3+dhaB123TFG. Transformations of *E. coli* SZ63 occurred by electroporation and confirmed by colony PCR (Figure 40 – 9).



Figure 39 - Colony PCR from transformed *E. coli* strains with pSB1C3+dhaB123T. Fragment dhaB3-T (1.6kb), fragment dhaF-G (2.2kb). C-: Negative control no template; 1. AJW1387; 2. AJW1508; 3. Δ mgA; 4. AJW1509; 5. W3100; 6. AJW1483; 7. DH10B; 8. Positive Control pSB1C3_dhaB123T. 1kb NEB Ladder.

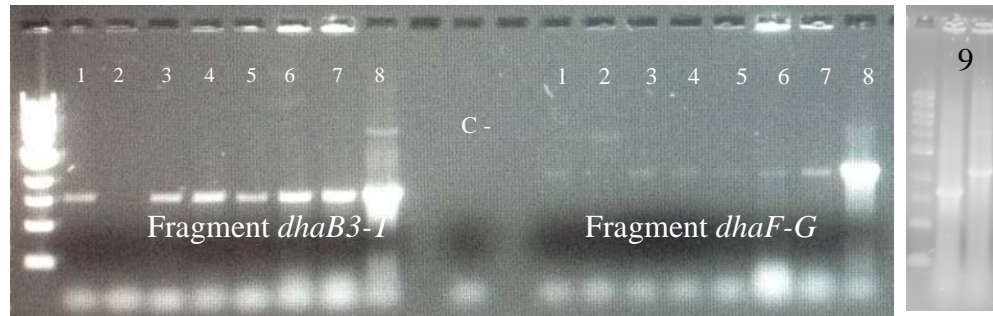


Figure 40 - Colony PCR from transformed *E. coli* strains. Fragment *dhaB3-T* (1.6kb), fragment *dhaF-G* (2.2kb). C-: Negative control no template; 1. AJW1387; 2. AJW1508; 3. Δ mgsA; 4. AJW1509; 5. W3100; 6. AJW1483; 7. DH10B; 8. Positive Control pSB1C3_dhaB123TFG; 9. SZ63.

Selected pSB1C3+dhaB123TFG transformed strains were then screened for 1,3-propanediol production.

12.3 Fermenting Glycerol to 1,3-propanediol with *E. coli*

Screening new strains were performed with the *E. coli* strains collection, using falcon 50 mL test tubes, in 25 mL working volume media as described in Material and Methods section. Figure 40 shows 1,3-propanediol production after 48 h.

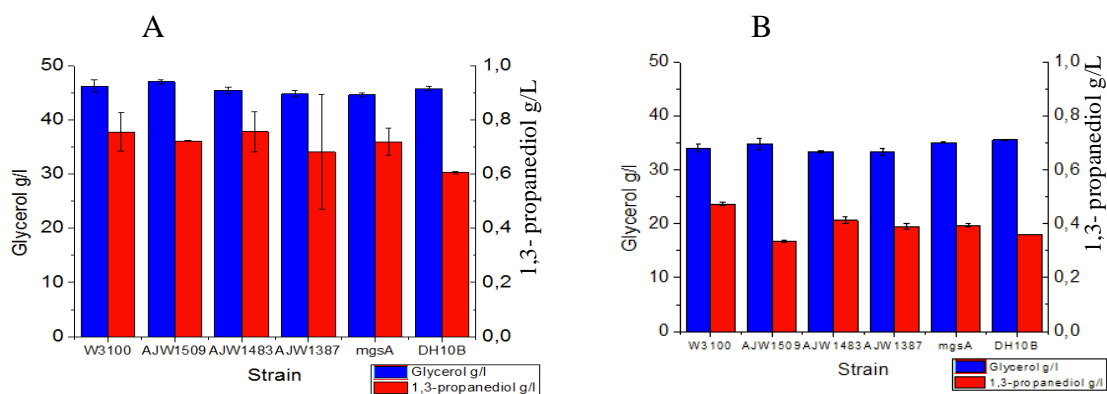


Figure 41 - First *E. coli* strain selection for 1,3-propanediol production. A. 50 g/L of initial glycerol. B. 50g/L of initial glycerol, with 5 g/L calcium carbonate addition.

1,3-propanediol production even after did not reach higher than 800 mg/L, and compared with *E. coli* TCS099, which reached 4.8 g/L of 1,3-PDO, production by these strains in the same media and conditions was not promising. Therefore, *E. coli* SZ63 (CAUSEY *et al.*, 2003) was acquired from Dr. Ingram's Lab at the Department of Microbiology and Cell Science, University of Florida. Figure 41 shows the second strain

selection after 24-hour cultivation.

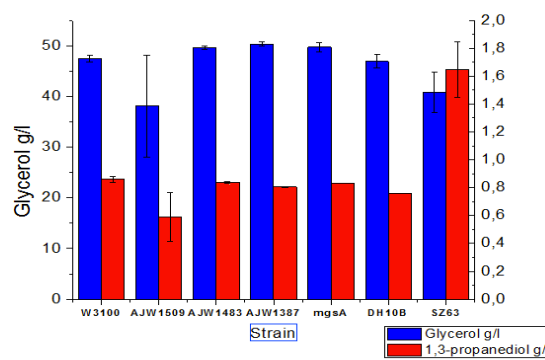


Figure 42 - Second *E. coli* strain selection for 1,3-propanediol production with 1 μM of sodium selenite and 10 g/L of triptone. 24h cultivation.

Just after 24 h, production of 1,3-propanediol was more than double than the other strains, meaning the NADH_2 pool of SZ63 favors the production. After selection of this strain, reactor fermentation was performed. Other strains were experimented for 1,3-propanediol production and even after 144 h did not produce higher amounts of 1,3-PDO (data not shown).

12.4 Reactor fermentation with *E. coli* SZ63

Five two stage fed-batch fermentations were performed, with different aeration conditions on the first stage for the first 24 hours. Then, one pulse of glycerol was fed at 24 h, which concentration of glycerol was calculated to be around 50 g/L. The second stage was later set into anaerobic conditions and a nitrogen gas was purged at flux of 0.05 L/min into the bioreactor. Figure 42 shows kinetics of these five fermentations.

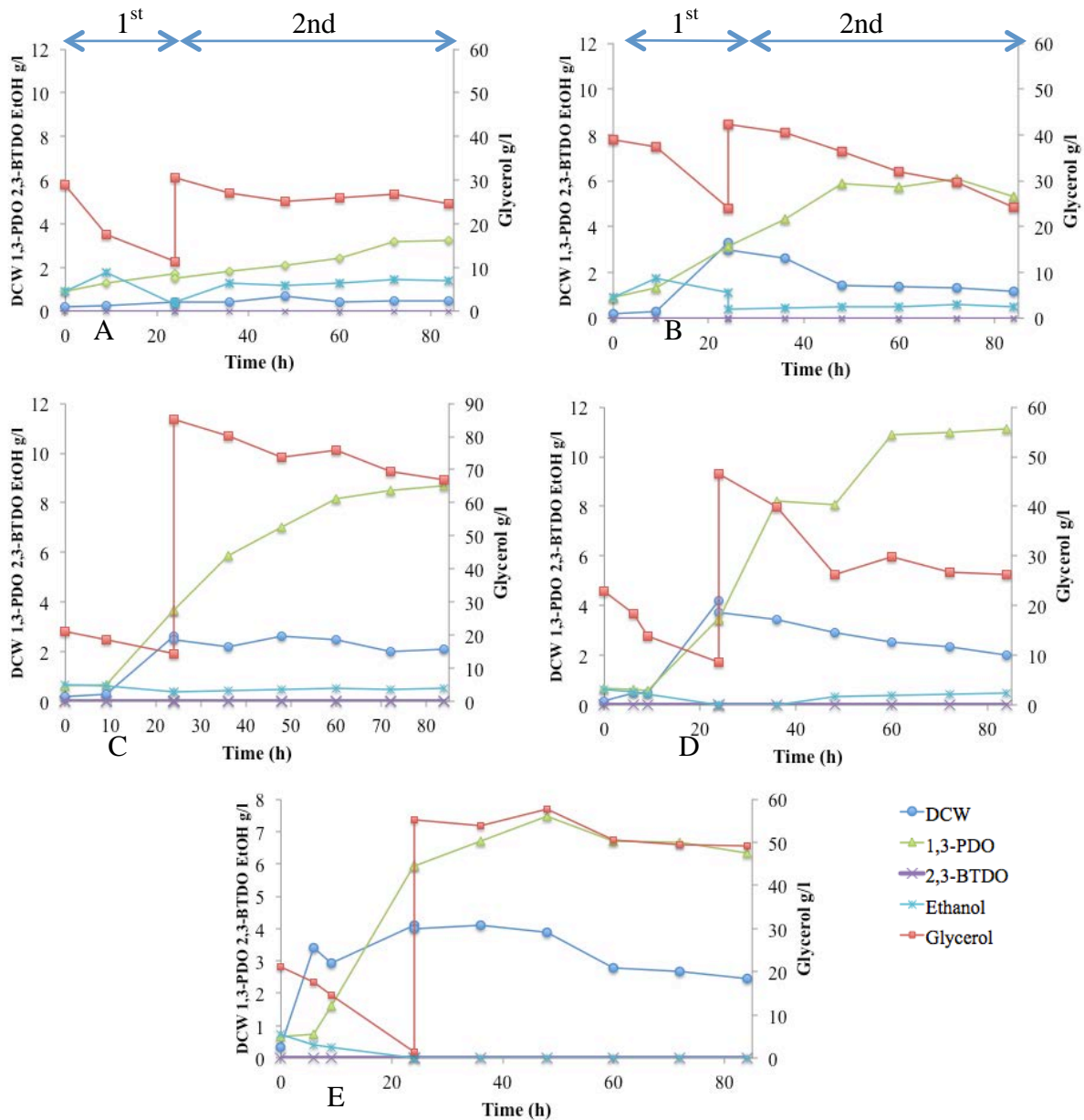


Figure 43 - Fed-Batch Culture of *E. coli* SZ63+pSB1C3dhaB123TFG – 1,3-propanediol g/L production in two stage fermentation. 1st - First Stage (0h-24h) A. Anaerobic; B. 5% pO₂; C. 10% pO₂; D. 15% pO₂; E. 20% pO₂; 2nd - Second Stage (24h-84h) Anaerobic. * Feed pulse at 24h.

Previous studies on D-lactate production have shown that an initial period of aeration in complex media can be used to boost the growth of D-lactate-producing strains of *E. coli* containing mutations in phosphoenolpyruvate carboxylase and phosphotransacetylase genes and shorten the time required for fermentation. Initial aeration of an SZ58 culture eliminated the lag in growth and resulted in a 10-fold increase in cell yield within the first 24 h, which accelerated glucose conversion to lactate and reduced the time required to complete fermentation (ZHOU *et al.*, 2003).

First fermentation started anaerobically, nitrogen gas was used to purge oxygen out of the fermenter prior to the start. Although about 18 g/L of glycerol was consumed in the first 24 h, little 1,3-propanediol was produced (1.69 g/L) and 0.41 g/L of DCW had grown. 18 g/L of glycerol was fed at 24 h, and even after 84h fermentation, only 3.23 g/L of glycerol was produced. Conversion rates were limited by the level of biocatalyst, due to slow growth and low cell density. No 2,3-butanediol was observed, but 1.4 g/L of ethanol was produced until the end of the fermentation.

Setting up to 5% pO₂ on the first 24h fermentation resulted in 15 g/L of glycerol consumed, 3.27 g/L of DCW and 3.15 g/L of 1,3-propanediol produced. This means more cells were able to produce more 1,3-propanediol. On the second stage, 18 g/L of glycerol was fed into the bioreactor, and after 60 hours, 6.1 g/L of 1,3-PDO was produced. This result could mean that *E. coli* SZ63 is not able to grow in anaerobic condition using glycerol as sole carbon source, even with the addition of sodium selenite, or producing 1,3-propanediol for NADH₂ recycling to NAD⁺. Micro-aerobiose conditions are necessary for it to grow using glycerol as carbon source.

A pO₂ set to 10% was also tested, but problems were observed with this fermentation. It started with 21 g/L of glycerol and after 24 hours, only 7 g/L of glycerol was consumed. It was also observed that DCW reached 2.5 g/L, and after 24 hours, 70 g/L of glycerol was fed into the bioreactor by a mistake with the pump calibration. Even though, on the second stage, in anaerobiose, 19 g/L of glycerol was consumed and 8.6 g/L of 1,3-propanediol was produced.

Using 15% pO₂, it was reached the highest production, but glycerol wasn't entirely consumed. It was produced 3.5 g/L of 1,3-PDO on the first 24 h. Production continued on second stage in anaerobiosis and reached 11 g/L after 60 h. Ethanol reached 0.5 g/L on this experiment, and no 2,3-butanediol was observed. Compared to the use of 20% pO₂, which depleted the initial glycerol after 24h, but produced 6.5 g/L, did not consume all the glycerol fed nor produced 1,3-propanediol substantially on second stage, which could mean cells were stressed from glycerol depletion on the first 24h. No 2,3-butanediol or ethanol was observed after 84 h.

We can also observe that 1,3-PDO accumulates on micro-aerobic conditions. Further fermentations should be performed to optimize these conditions. Unfortunately, with different strains tested, glycerol was not efficiently fermented to produce cell growth and 1,3-propanediol. Glycerol is a high-reduced substrate and maintaining redox balance is a challenge specially in anaerobic conditions, because the incorporation of this carbon source to

cell mass results in production of reducing equivalents, in which H₂ plays an important role, participating as electron donor for several reactions. If H₂ is decreased, this does not happen and fermentation proceeds optimally. Increasing headspace dilutes H₂, and also flushing it out with inert gas, such as Argon or Nitrogen, also CO₂, improves fermentation (GONZALES, 2012).

González (2012) describes that optimum glycerol fermentation by *E. coli* with acidic pH of 6.3, 10-20% or higher concentrations of CO₂, high concentrations of Glycerol, up to 100 g/L, 200 rpm, 37° C, 0.01 L/min argon or nitrogen, low potassium (less than 10 mM or even to less than 0.6 mM), and low phosphate concentrations (from 50 mM to less than 1.3 mM) are preferred, because high potassium and phosphate inhibits glycerol dehydrogenase and dihydroxyacetone kinase, and also increases methylglyoxal toxicity. Tryptone supplementation is also required when hydroxyacetone is not added. These conditions were replicated on the fermentation in bioreactors, but glycerol fermented did not result in cell growth and high productivity.

High concentration of glycerol is required to GldA, due to its low K_m, and acidic conditions favors reductive activity of GldA, while neutral to alkaline conditions increases oxidative activity. Also, alkaline conditions increase methylglyoxal toxicity. To prevent cytoplasmic acidification, *E. coli* produces CO₂ and H₂ from formic acid, but H₂ can be negative to glycerol fermentation. (GONZÁLES, 2012). But high concentrations of glycerol, over 49 g/L, is known to decrease 1,3-propanediol productivity, since it favors the inactivation of glycerol dehydratase (GDHt) (DA SILVA, 2014).

The over expression of GDHt leads to a serious growth deficiency of *K. pneumoniae*. Instability of the plasmids bearing the genes encoding GDHt and/or 1,3-PDO oxidoreductase, because of an imbalanced conversion of glycerol to 3-HPA and its toxicity of 3-HPA were responsible for the phenomena observed. 3-HPA limited the metabolic engineering because of its toxicity. Similar research was performed using resting cell systems, in which growth was stopped while metabolic activity was maintained, so disturbances associated with cell growth can be eliminated. Over expression of 1,3-PDO oxidoreductase led to a faster glycerol conversion and 1,3-PDO production. After a 12-h conversion process, it improved the yield of 1,3-PDO by 20.4%, and boosted yield p/s 50.8% to 59.8% (mol/mol) (SONG *et al.* 2010).

In an effort to maximize 1,3-PDO yield and at the same time minimize production time and byproduct production, Tang *et al.* (2009) established a two-stage two-substrate fermentation for producing 1,3-PDO by an engineered strain *E. coli* K-12 ER2925, which genotype or modifications were not disclosed in the article, and dhaB1 and dhaB2 from *C.*

butyricum SYU 20108 were cloned and expressed in the host strain. On the first stage from 0 to 10 h, dissolved oxygen was maintained above a the critical concentration, and glucose was added continuously to maintain 25 g/L, to a final DCW of 26 g/L. The second stage involved replacement of the glucose medium and byproducts of the first stage with fresh glycerol fermentation medium every 2 hours, shifting the temperature to 42 °C, 1,3-PDO reached a final concentration of 104.4 g/L.

13. CONCLUSIONS

It was possible to clone all genes responsible to 1,3-propanediol production. Two plasmids were compared, one with *dhaB1*, *dhaB2*, *dhaB3* and *dhaT* (pSB1C3+dhaB123T) and another with all these four genes and *dhaF* and *dhaG* responsible for the glycerol dehydratase reactivase (pSB1C3+dhaB123TFG). Only pSB1C3+dhaB123TFG was able to produce high amounts of 1,3-PDO in experiments in flasks, and 4.5g/L of 1,3-PDO was achieved in aerobiosis and 2.5g/L was achieved in microaerobiosis when *E. coli* TCS099 was used as a host. Also *E. coli* SZ63 was able to produce higher amounts of 1,3-PDO than other *E. coli* tested. Five fermentations were performed with diferent pO₂ in ther first 24h, then shifted to anaerobiosis. Aeration on the first 24h up to 15% was able to increase 1,3-propanediol production and productivity with *E. coli* SZ63, reaching 11 g/L of 1,3-PDO produced in 60 h. New fermentation strategies could be performed to improve productivity and production. Also, new plasmids could be engineered to contain different origin of replications, such as a low copy number plasmid, or diferent ribosome binding sites and promoters, because using high copy number plasmids and strong promoters and RBS as used in this study could be damaging to the cell balance and growth.

13. REFERENCES

ALMEIDA, M. M. T.; PINTO, G. A. S. Utilização do glicerol para a produção de biomassa por fermentação submersa de *Rhodoturula glutinis*. **VII Congresso Brasileiro de Engenharia Química em Iniciação Científica, UFSCar, São Carlos, 2007.** <http://www.ufscar.br/cobeqic07/pdf/poster_iii/piii40.pdf>

ARRUDA, P. V.; RODRIGUES, R. C. L. B; FELIPE, M. G. A. Glicerol: um subproduto com grande capacidade industrial e metabólica. **Revista Analytica**, N°26, 2007. <<http://www.biodieselbr.com/biodiesel/glicerina/biodiesel-glicerina.htm>>

BIEBL, H.; ZENG, A.-P.; MENZEL, K.; DECKWER, W.-D. Fermentation of glycerol to 1,3-propanediol and 2,3-butanediol by *Klebsiella pneumoniae*. **Applied Microbiology and Biotechnology**. V. 50, p. 24-29, 1998.

BRASIL. Lei 11.097 de 13 de janeiro de 2005. Dispõe sobre a introdução do biodiesel na matriz energética brasileira. **Diário Oficial da União**. 14 de janeiro de 2005.

BULHÕES, R. O Biodiesel e sua relação com a incorporação de novas áreas e produção de soja no Paraná. **Encontro de Economia Paranaense**. Curitiba, PR. Brasil, 2007. <http://www.ecopar.ufpr.br/artigos/a4_070.pdf>.

CAMERON, D. C.; ALTARAS, N. E.; HOFFMAN, M. L.; SHAW, A. J. Metabolic engineering of propanediol pathways. **Biotechnology progress**. V. 14, n. 1, p. 116–125, 1998. <<http://www.ncbi.nlm.nih.gov/pubmed/9496676>>.

CÁRDENAS, P.; PULIDO, C.; ARAGÓN, Ó. L.; ARISTIZÁBAL, F. A.; ROCÍO, S. Z.; MONTOYA, D. Evaluación de la producción de 1,3-propanodiol por cepas nativas de *Clostridium sp.* Mediante fermentación a partir de glicerol USP y glicerol industrial subproducto de la producción de biodiésel. **Revista Colombiana de Ciências Químico-Farmacéuticas**. N.35, p.120-137, 2006.

CARVALHO, G.D. A indústria dos hidrocarbonetos no Brasil: o problema com o caso gasoduto Bolívia- Brasil e a solução com os combustíveis renováveis. **Papel Político Bogotá (Colombia)**. V. 11, . pp. 739-760, 2006.

CHENG, K.-K.; Liu, DH Sun, Y.; Liu, W.B.; 1, 3-Propanediol production by *Klebsiella pneumoniae* under different aeration strategies. **Biotechnology letters**, V. 26, n. 11, p. 911–915, jun. 2004.

CHENG, K.-K.; LIU, H.-J.; LIU, D.-H. Multiple growth inhibition of *Klebsiella pneumoniae* in 1,3-propanediol fermentation. **Biotechnology letters**. V. 27, n. 1, p. 19–22, 2005. <<http://www.ncbi.nlm.nih.gov/pubmed/15685414>>.

CONTIERO, J.; BEATTY, C.; KUMARI, S.; DESANTI, C. L.; STROHL, W. R.; WOLFE, A. Effects of mutations in acetate metabolism on high-cell-density growth of *Escherichia coli*. **Journal of Industrial Microbiology and Biotechnology**. Vol. 24, n. 6, p. 421-430, 2000.

DA SILVA G.P.; BOLNER DE LIMA, C.J.; CONTIERO, J. Production and productivity of 1,3-propanediol from 31glycerol by *Klebsiella pneumoniae* GLC29. **Catalysis Today**. V. 10, p. 1-8, 2014.

DA SILVA, G.P.; MACK, M.; CONTIERO, J. Glycerol: a promising and abundant carbon source for industrial microbiology. **Biotechnology Advances**. V. 27, p. 30-39, 2009.

DANIEL, R.; BOBIK, T. A; GOTTSCHALK, G. Biochemistry of coenzyme B12-dependent glycerol and diol dehydratases and organization of the encoding genes. **FEMS microbiology reviews**. V. 22, n. 5, p. 553–66, 1998. <<http://www.ncbi.nlm.nih.gov/pubmed/9990728>>.

DHARMADI, Y.; MURARKA, A.; GONZALEZ, R. Fermentation of glycerol by *Escherichia coli*: A new platform for metabolic engineering. **Biotechnology and bioengineering**. V. 94, nº5, p. 821-829, 2006.

EJIOFOR, A.O.; CHISTI, Y.; MOO-YOUNG, M. Culture of *Saccharomyces cerevisiae* on hydrolysed waste cassava starch for production of baking-quality yeast. **Enzyme and Microbial Technology**. Vol 18, p. 519–525, 1996.

FARIA, R. C. M.; REZENDE, M. J. C.; REZENDE, C. M.; PINTO, A. C. Desenvolvimento e validação de metodologia de análise de misturas biodiesel:diesel utilizando cromatografia gasosa-espectrometria de massas. **Química Nova**. V. 30, No. 8, p. 1900-1905, 2007.

FELSENSTEIN J. Confidence limits on phylogenies: An approach using the bootstrap. **Evolution**. V. 39, p. 783-791, 1985.

FERRARI, R. A.; OLIVEIRA, V. S.; SCABIO, A. Biodiesel de soja – Taxa de conversão em ésteres etílicos, caracterização físicoquímica e consumo em gerador de energia. **Química Nova**. V. 28, n. 1, p.19-23, 2005.

FERREIRA, J.R.; CRISTO, C.M. O Futuro da Indústria: biodiesel. **Ministério do Desenvolvimento, Indústria e Comércio Exterior – MDIC Instituto Euvaldo Lodi – IEL/Núcleo Central**. p. 145, 2006.

FISCHER, G.; SCHRATTENHOLZER, L. Global bioenergy potentials through 2050. **Biomass & Bioenergy, Pergamon**, V. 20, p.151-159, 2005.

GANESH, I.; RAVIKUMAR, S.; HONG, S. H. Metabolically engineered *Escherichia coli* as a tool for the production of bioenergy and biochemicals from glycerol. **Biotechnology and Bioprocess Engineering**, V. 17, n. 4, p. 671–678, 2012.<<http://www.springerlink.com/index/10.1007/s12257-011-0446-3>>.

GERIS, R.; SANTOS, N.A.; AMARAL, B.A.; MAIA, I.D.; CASTRO, V.D.; CARVALHO, J.R. Biodiesel de soja: reação de transesterificação para aulas práticas de química orgânica. **Química Nova**. Vol. 30, p. 1369-1373, 2007.

GIBSON, D. G. Synthesis of DNA fragments in yeast by one-step assembly of overlapping oligonucleotides. **Nucleic acids research**, V. 37, N. 20, p. 6984–90, 2009. <<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2777417&tool=pmcentrez&rendertype=abstract>>.

GONZÁLEZ-PAJUELO, M.; ANDRADE, J. C.; VASCONCELOS, I. Production of 1,3-propanediol by *Clostridium butyricum* VPI 3266 using a synthetic medium and raw glycerol. **Journal of industrial microbiology & biotechnology**. V. 31, n.9, p. 442–6, 2004. <<http://www.ncbi.nlm.nih.gov/pubmed/15378388>>.

GONZÁLEZ-PAJUELO, M.; MEYNIAL-SALLES, I.; MENDES, F.; SOUCAILLE, P.; VASCONCELOS, I. Microbial conversion of glycerol to 1,3-propanediol: physiological comparison of a natural producer, *Clostridium butyricum* VPI 3266, and an engineered strain, *Clostridium acetobutylicum* DG1(pSPD5). **Applied and environmental microbiology**, V.

72, n. 1, p. 96–101, 2006. <<http://www.ncbi.nlm.nih.gov/pubmed/16391030>>.

GONZÁLEZ, R. Anaerobic Fermentation of Glycerol. WO Patent 2,007,115,228, 2012.

KAJIURA, H.; MORI, K.; TOBIMATSU, T.; TORAYA, T. Characterization and mechanism of action of a reactivating factor for adenosylcobalamin-dependent glycerol dehydratase. **The Journal of biological chemistry**. V. 276, n. 39, p. 36514–9, 2001. <<http://www.ncbi.nlm.nih.gov/pubmed/11470792>>.

LEE, J.; LEE, S.Y.; PARK, S. Fed-batch culture of *Escherichia coli* w by exponential feeding of sucrose as a carbon source. **Biotechnology Techniques**. Vol. 11, p. 59–62, 1997.

LIMA, S.A. Desenvolvimento Rural Biodiesel: combustível sustentável? **Revista Brasileira de Agroecologia**. V. 2, p. 359-362, 2007.

LIN, E. Glycerol dissimilation and its regulation in bacteria. **Annual Reviews in Microbiology**. Vol. 30, p.535-578, 1976. DOI: 10.1146/annurev.mi.30.100176.002535 <<http://www.annualreviews.org/doi/abs/10.1146/annurev.mi.30.100176.002535>>

MA, F; HANNA, M. A. Biodiesel production: A review. **Bioresource Technology**. V. 70, p. 1-15, 1999.

MA, Z.; RAO, Z. M.; XU, L. Y.; LIAO, X. R.; FANG, H. Y.; ZHUGE, B.; ZHUGE, J. Production of 1,3-propanediol from glycerol by engineered *Escherichia coli* using a novel co-expression vector. **African Journal of Biotechnology**. V. 8, nº 20, p.5500–5505, 2009.

MAERVOET, V.; MEY, M. DE; BEAUPREZ, J. Enhancing the microbial conversion of glycerol to 1, 3-propanediol using metabolic engineering. **Organic Process Research & Development**. V. 15, n. 1, p. 189–202, 2011.<<http://pubs.acs.org/doi/abs/10.1021/op1001929>>.

MU, Y.; TENG, H.; ZHANG, D.-J.; WANG, W.; XIU, Z.-L. Microbial production of 1,3-propanediol by *Klebsiella pneumoniae* using crude glycerol from biodiesel preparations. **Biotechnology Letters**. V. 28, p.1755–1759, 2006.

NAKAMURA, C. E.; WHITED, G. M. Metabolic engineering for the microbial production of 1,3-propanediol. **Current Opinion In Biotechnology**, V. 14, n. 5, p. 454–459,

2003. <<http://linkinghub.elsevier.com/retrieve/pii/S0958166903001265>>.

OH, B.-R.; SEO, J.-W.; HEO, S.-Y. *et al.* Optimization of Culture Conditions for 1,3-Propanediol Production from Glycerol Using a Mutant Strain of *Klebsiella pneumoniae*. **Applied Biochemistry and Biotechnology**. V. 166, n. 1, p. 127-37, 2012. doi: 10.1007/s12010-011-9409-62011. <<http://www.ncbi.nlm.nih.gov/pubmed/22072138>>.

PACHAURI, N., HE, B. Value-added Utilization of Crude Glycerol from Biodiesel Production. **Written for presentation at the 2006 ASABE Annual International Meeting**. Paper No. 06622. Portland, Oregon. 2006. <<http://www.webpages.uidaho.edu/~bhe/pdfs/asabe066223.pdf>>

PAPANIKOLAOU, S.; MUNIGLIA, L.; CHEVALOT, I.; AGGELIS, G.; MARC, I. *Yarrowia lipolytica* as a potential producer of citric acid from raw glycerol. **Journal of Applied Microbiology**. V. 92 p.737-744. 2002.

PARENTE, E.J. Uma aventura tecnológica num país engraçado. **TECBio**. Fortaleza, CE. 2003. 68p. <www.iadb.org/intal/intalcdi/PE/2008/01430.pdf>.

RATHMANN, R.; BENEDETTI, O.; PLÁ, J.; PADULA, A. Biodiesel: uma alternativa estratégica na matriz energética brasileira? **Seminário de gestão de negócios, II**. Curitiba – PR. V. 1, 2005.

RYMOWICZ, W.; RYWIŃSKA, A.; GŁADKOWSKI, W. Simultaneous production of citric acid and erythritol from crude glycerol by *Yarrowia lipolytica* Wratislavia K1. **Chemical Papers**. V. 62, p. 239-246, 2008.

RYMOWICZ, W.; Rywińska, A.; Żarowska, B.; Juszczak, P. Citric acid production from raw glycerol by acetate mutants of *Yarrowia lipolytica*. **Chemical Papers**, v. 60, n. 5, p. 391–394, out. 2006.

SAITOU N.; NEI M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. **Molecular Biology and Evolution**. V. 4, p. 406-425, 1987.

SCHOMBURG, I.; CHANG, A.; PLACZEK, S.; SÖHNGEN, C.; ROTHER, M.; LANG, M.; MUNARETTO, C.; ULAS, S.; STELZER M.; GROTE, A.; SCHEER, M.; SCHOMBURG, D.; BRENDA in 2013: integrated reactions, kinetic data, enzyme function data, improved disease classification: new options and contents in BRENDA. **Nucleic acids**

Research. V. 41, p.764-772, 2013.

SHIBATA, N.; MASUDA, J.; MORIMOTO, Y.; YASUOKA, N.; TORAYA, T. Substrate-Induced Conformational Change of a Coenzyme B₁₂-Dependent Enzyme: Crystal Structure of the Substrate-Free Form of Diol Dehydratase. **Biochemistry.** V. 41, n. 42, p. 12607–12617, 2002. <<http://pubs.acs.org/doi/abs/10.1021/bi026104z>>.

SKRALY, F. A.; LYTLE, B. L.; CAMERON, D. C. Construction and characterization of a 1,3-propanediol operon. **Applied and Environmental Microbiology.** V. 64, n. 1, p. 98–105, 1998.

SONG, Y.-Q.; XU, Y.-Z.; LIU, D.-H. 1,2 and 1,3-propanediol, microbial production methods. **Encyclopedia of Industrial Biotechnology: Bioprocess, Bioseparation, and Cell Technology.** n. 8, 2010.

TAMURA K.; NEI M.; KUMAR S. Prospects for inferring very large phylogenies by using the neighbor-joining method. **Proceedings of the National Academy of Sciences (USA).** V. 101, p11030-11035, 2004.

TAMURA K.; PETERSON D.; PETERSON N.; STECHER G.; NEI M.; AND KUMAR S. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. **Molecular Biology and Evolution.** V. 28 p. 2731-2739, 2011.

TANG, X. M.; TAN, Y. S.; ZHU, H.; ZHAO, K.; SHEN, W. Microbial Conversion of Glycerol to 1,3-Propanediol by an Engineered Strain of *Escherichia coli*. **Applied Environmental Microbiology.** V. 75, n° 6, p.1628– 1634, 2009.

TRINH, C. T.; SRIENC, F. Metabolic engineering of *Escherichia coli* for efficient conversion of glycerol to ethanol. **Applied and environmental microbiology,** v. 75, n. 21, p. 6696–705, nov. 2009.

VOLLENWEIDER, S.; LACROIX, C. 3-Hydroxypropionaldehyde: Applications and Perspectives of Biotechnological Production. **Applied Microbiology And Biotechnology.** V. 64, p. 16-27, 2004.

YAMANISHI, M.; KINOSHITA, K.; FUKUOKA, M. *et al.* Redesign of coenzyme

B(12) dependent diol dehydratase to be resistant to the mechanism-based inactivation by glycerol and act on longer chain 1,2-diols. **The FEBS Journal**. V. 279 n. 5, p.793-804, 2012 DOI: 10.1111/j.1742-4658.2012.08470.x <<http://www.ncbi.nlm.nih.gov/pubmed/22221669>>.

YAZDANI, S. S.; GONZALEZ, R. Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry. **Current Opinion in Biotechnology**. V. 18, n. 3, p. 213–9, 2007. <<http://www.ncbi.nlm.nih.gov/pubmed/17532205>>.

ZHANG, X. M.; LI, Y.; ZHUGE, B.; TANG, X. M.; SHEN, W.; RAO, Z. M.; FANG, H. Y.; ZHUGE, J. Construction of a novel recombinant *Escherichia coli* strain capable of producing 1,3-propanediol and optimization of fermentation parameters by statistical design. **World Journal of Microbiology and Biotechnology**. V. 22, n° 9, p. 945–952, 2006.

ZHENG, Z.; HU, Q.; HAO, J.; *et al.* Statistical optimization of culture conditions for 1,3-propanediol by *Klebsiella pneumoniae* AC 15 via central composite design. **Bioresource Technology**. V. 99, n. 5, p. 1052–6, 2008.

ZHOU, S.; CAUSEY, T. B.; HASONA, A.; SHANMUGAM, K. T.; INGRAM, L. O. Production of Optically Pure D -Lactic Acid in Mineral Salts Medium by Metabolically Engineered *Escherichia coli* W3110. **Applied and Environmental Microbiology**. V. 69, n. 1, p. 399–407, 2003.

14. APPENDIX

DNA sequencing results and translated codons for the *dha* genes cloned in pSB1C3 from *Klebsiella pneumoniae* GLC29 for 1,3-propanediol production.

>Consensus *dhaB1*

ATGAAAAGATCAAAACGATTTGCAGTACTGGCCCAGCGCCCCGTCAAT
 CAGGACGGGCTGATTGGCGAGTGGCCTGAAGAGGGGCTGATCGCCATGGACAGC
 CCCTTTGACCCGGTCTCTTCAGTAAAAGTGGACAACGGTCTGATCGTCGAGCTGG
 ACGGCAAACGCCGGGACCAGTTTGACATGATCGACCGGTTTATCGCCGATTACGC
 GATCAACGTTGAGCGCACAGAGCAGGCAATGCGCCTGGAGGCGGTGGAAATAGC
 CCGCATGCTGGTGGATATTCACGTCAGCCGGGAGGAGATCATTGCCATCACTACC
 GCCATCACGCCGGCCAAAGCGGTTCGAGGTGATGGCGCAGATGAACGTGGTGGAG
 ATGATGATGGCGCTGCAGAAGATGCGTGCCCGCCGGACCCCTCCAACCAGTGCC
 ACGTCACCAATCTCAAAGATAATCCGGTGCAGATTGCCGCTGACGCCGCCGAGGC
 CGGGATCCGCGGCTTCTCAGAACAGGAGACCACGGTTCGGTATCGCGCGCTACGC
 GCCGTTTAACGCCCTGGCGCTGTTGGTTCGGTTCGCAGTGCGGCCGCCCGGCGTG
 TTGACGCAGTGCTCGGTGGAAGAGGCCACCGAGCTGGAGCTGGGCATGCGTGGC
 TTAACCAGCTACGCCGAGACGGTGTTCGGTCTACGGCACGGAAGCGGTATTTACCG
 ACGGCGATGATACTCCGTGGTCAAAGGCGTTCCTCGCTTCGGCCTACGCCTCCCG
 CGGGTTGAAAATGCGCTACACCTCCGGCACCGGATCCGAAGCGCTGATGGGCTAT
 TCGGAGAGCAAGTCGATGCTCTACCTCGAATCGCGCTGCATCTTCATTACCAAAG
 GCGCCGGGGTTCAGGGGCTGCAAACGGCGCGGTGAGCTGTATCGGCATGACCG
 GCGCTGTGCCGTCGGGCATTCGGGCGGTGCTGGCGGAAAACCTGATCGCCTCTAT
 GCTCGACCTCGAAGTGGCGTCCGCCAACGACCAGACTTTCTCCCACTCGGATATT
 CGCCGCACCGCGCGCACCTGATGCAGATGCTGCCGGGCACCGACTTTATTTTCT
 CCGGCTACAGCGCGGTGCCGAACACTACGACAACATGTTTCGCCGGCTCGAACTTCGA
 TCGGGAAGATTTTGATGATTACAACATCCTGCAGCGTGACCTGATGGTTGACGGC
 GGCCTGCGTCCGGTGACCGAGGCGGAAACCATTGCCATTCGCCAGAAAGCGGCG
 CGGGCGATCCAGGCGGTTTTCCGCGAGCTGGGGCTGCCGCCAATCGCCGACGAG
 GAGGTGGAGGCCGCCACCTACGCGCACGGCAGCAACGAGATGCCGCCGCGTAAC
 GTGGTGGAGGATCTGAGTGCGGTGGAAAGAGATGATGAAGCGCAACATCACCGGC
 CTCGATATTGTCCGGCGCGCTGAGCCGCAGCGGCTTTGAGGATATCGCCAGCAATA
 TTCTCAATATGCTGCGCCAGCGGGTCACCGGCGATTACCTGCAGACCTCGGCCAT

TCTCGATCGGCAGTTCGAGGTGGTGAGTGCGGTCAACGACATCAATGACTATCAG
 GGGCCGGGCACCGGCTATCGCATCTCTGCCGAACGCTGGGCGGAGATCAAAAAT
 ATTCCGGGCGTGGTTCAGCCTGACACCATTGAATAA

>dhaB1 amino acid sequence translated

MKRSKRFAVLAQRPVNQDGLIGEWPEEGLIAMDSPFDPVSSVKVDNGLIV
 ELDGKRRDQFDMIDRFIADYAINVERTEQAMRLEAVEIARMLVDIHVSREEIIAITTAI
 TPAKAVEVMAQMNVVEMMMALQKMRARRTPSNQCHVTNLKDNPVQIAADAAEA
 GIRGFSEQETTVGIARYAPFNALALLVGSQCGRPGVLTQCSVEEATELELGMRGLTSY
 AETVSVYGTAVFTDGDTPWSKAFLASAYASRGLKMRYTSGTGSEALMGYSESKS
 MLYLESRCIFITKGAGVQGLQNGAVSCIGMTGAVPSGIRAVLAENLIASMLDLEVASA
 NDQTFSHSDIRRTARTLMQMLPGTDFIFSGYS AVPNYDNMFAGSNFDAEDFDDYNIL
 QRDLMVDGGLRPVTEAETIAIRQKAARAIQAVFRELGLPPIADEEVEEAATYAHGSNE
 MPPRNVVEDLSAVEEMMKRNITGLDIVGALSRSGFEDIASNILNMLRQRVTGDYLTQ
 SAILDRQFEVVS AVNDINDYQGGTGYRISAERWAEIKNIPGVVQPDTIE*

>Consensus *dhaB2*

ATGCAACAGACAACCCAAATTCAGCCCTCTTTTACCCTGAAAACCCGC
 GAGGGCGGGGTAGCTTCTGCCGATGAACGCGCCGATGAAGTGGTGATCGGCGTC
 GGCCCTGCCTTCGATAAACACCAGCATCACACTCTGATCGATATGCCCCATGGCG
 CGATCCTCAAAGAGCTGATTGCCGGGGTGAAGAAGAGGGGGCTTCACGCCCGGG
 TGGTGCGCATTCTGCGCACGTCCGACGTCTCCTTTATGGCCTGGGATGCGGCCAA
 CCTGAGCGGCTCGGGGATCGGCATCGGTATCCAGTCGAAGGGGACCACGGTCAT
 CCATCAGCGCGATCTGCTGCCGCTCAGCAACCTGGAGCTGTTCTCCAGGCGCCG
 CTGCTGACGCTGGAGACCTACCGGCAGATTGGCAAAAACGCCGCGCGCTATGCG
 CGCAAAGAGTCACCTTCGCCGGTGCCGGTGGTGAACGATCAGATGGTGCGGCCG
 AAATTTATGGCAAAGCCGCGCTATTTATATCAAAGAGACCAAACATGTGGTGC
 AGGACGCCGAGCCCGTCACCCTGCACGTCGACTTAGTAAGGGAGTGA

>dhaB2 amino acid sequence translated

MQTTQIQPSFTLKTREGGVASADERADEVVIGVGPFDKHQHHTLIDMP
 HGAILKELIAGVEEEGLHARVVRILRTSDVSFMAWDAANLSGSGIGIGIQSKGTTVIH
 QRDLLPLSNLELFSQAPLLTLETYRQIGKNAARYARKESPSPVPVNDQMVRPKFMA
 KAALFHIKETKHVVQDAEPVTLHVLDLVRE*

>Consensus *dhaB3*

ATGAGCGAGAAAACCATGCGCGTGCAGGATTATCCGTTAGCCACCCGC
 TGCCCGGAGCATATCCTGACGCCTACCGGTAAACCATTGACCGATATTACCTCG
 AGAAGGTGCTCTCTGGCGAGGTGGGCCCGCAGGATGTGCGGATCTCCCGCCAGA
 CCCTTGAGTACCAGGCGCAGATTGCCGAGCAGATGCAGCGCCATGTGGTGGCGC
 GCAATTTCCGCCGCGCGGGCGGAGCTTATCGCCATTCCTGACGAGCGCATTCTGGC
 TATCTATAACGCGCTGCGCCCGTCCGCTCCTCGCAGGCGGAGCTGCTGGCGATC
 GCCGACGAGCTGGAGCACACCTGGCATGCGACAGTGAATGCCGCCTTTGTCCGG
 GAGTCGGCGGAAGTGTATCAGCAGCGGCATAAGCTGCGTAAAGGAAGCTAA

>dhaB3 amino acid sequence translated

MSEKTMRVQDYPLATRCPEHILTPTGKPLTDITLEKVLSGEVGPQDVRISR
 QTLEYQAQIAEQMQRHVVARNFRRAAELIAIPDERILAIYNALRPFSSQAELLIAIDE
 LEHTWHATVNAAFVRESAEVYQQRHKLKGS*

>Consensus *dhaT*

ATGAGCTATCGTATGTTTGATTATCTGGTGCCAAACGTTAACTTTTTTG
 GCCCAACGCCATTTCCGTTAGTCGGCGAACGCTGCCAGCTGCTGGGAGGAAAGA
 AAGCCCTGCTGGTCACCGACAAAGGCCTACGGGCAATTAAGATGGCGCGGTGG
 AATAAACCTGCATTATCTGCGGGAGGCAGGGATCGAGGTAGCGATCTTTGACG
 ACGTCGAGCCGAACCCAAAAGACACCAACGTGCGCGACGGCCTCGCCGTGTTTC
 GCCGCGAACAGTGCGACATCATCGTCACCGTGGGCGGCGGCAGCCCGCACGACT
 GCGGCAAAGGCATCGGTATTGCAGCCACCCATGAGGGCGATCTGTACCAGTATG
 CCGGCATCGAGACCCTGACCAACCCGCTGCCGCCATCGTCGCCGTCAACACCAC
 CGCCGGCACCGCCAGCGAGGTCACCCGCCACTGCGTCCTGACCAACACCGAAAC
 CAAAGTGAAGTTTGTGATCGTCAGCTGGCGCAACCTGCCGTCCGTCTCCATTAAC
 GATCCGCTGCTGATGATCGGTAAACCGGCCGCCCTGACCGCGGCGACCGGGATG
 GATGCCCTGACCCACGCCGTAGAGGCCTATATCTCCAAGACGCTAACCCGGTGA
 CGGACGCCGCCCATGCAGGCGATCCGCCTCATCGCCCGCAACCTGCGCCAGGC
 TGTGGCCCTCGGCAGCAATCTGCAGGCGCGGGAAAACATGGCCTACGCCTCTCTG
 CTGGCCGGGATGGCCTTCAATAACGCCAACCTCGGCTACGTGCACGCCATGGCGC
 ACCAGCTGGGCGGCCTGTACGACATGCCGCACGGCGTGGCCAACGCTGTCCTGCT
 GCCGCATGTGGCCCGCTACAACCTGATCGCCAACCCGGAGAAATTCGCCGATATC

GCTGAACTGATGGGCGAAAATATCACCGGACTGTCCACCCTCGACGCAGCGGAA
 AAAGCCATCGCCGCTATCACGCGTCTGTTCGATGGATATCGGTATTCCGCAGCATC
 TGCGCGATCTGGGAGTAAAAGAGGCCGACTTCCCCTACATGGCGGAGATGGCTCT
 GAAAGACGGCAATGCGTTCTCGAACCCGCGTAAAGGCAACGAGCAGGAGATTGC
 CGCGATTTTCCGCCAGGCATTCTGA

>dhaT amino acid sequence translated

MSYRMFDYLVPNVNFPGNAISVVGERCQLLGGKKALLVTDKGLRAIKD
 GAVDKTLHYLREAGIEVAIFDDVEPNPKDTNVRDGLAVFRREQCDIIVTVGGGSPHD
 CGKGIGIAATHEGDLYQYAGIETLTNPLPIVAVNNTAGTASEVTRHCVLTNTETKVK
 FVIVSWRNLPVSINDPLLMIGKPAALTAATGMDALTHAVEAYISKDANPVTDAAM
 QAIRLIARNLRQAVALSGLQARENMAYASLLAGMAFNANLGYVHAMAHQLGGL
 YDMPHGVANAVLLPHVARYNLIANPEKFADIAELMGENITGLSTLDAAEKAIAAITR
 LSMDIGIPQHLRDLGVKEADFPYMAEMALKDGNAFSNPRKGNEQEIAAIFRQAF*

>Consensus *dhaF*

ATGCCGTTAATAGCCGGGATTGATATCGGCAACGCCACCACCGAGGTG
 GCGCTGGCGTCCGACGACCCGCAGGCGAGGGCGTTTGTGTCAGCGGGATCGTC
 GCGACGACGGGCATGAAAGGGACGCGGGACAATATCGCCGGGACCCTCGCCGCG
 CTGGAGCAGGCCCTGGCGAAAACACCGTGGTCGATGAGCGATGTCTCTCGCATCT
 ATCTTAACGAAGCCGCGCCGGTGATTGGCGATGTGGCGATGGAGACCATCACCG
 AGACCATTATCACCGAATCGACCATGATCGGTCATAACCCGCAGACGCCGGGCG
 GGGTGGGCGTTGGCGTGGGGACGACTATCGCCCTCGGGCGGCTGGCGACGCTGC
 CGGCGGCGCAGTATGCCGAGGGGTGGATCGTACTGATTGACGACGCCGTGATT
 CCTTGACGCCGTGTGGTGGCTCAATGAGGCGCTCGACCGGGGGATCAACGTGGTG
 GCGGCGATCCTCAAAAAGGACGACGGCGTGCTGGTGAACAACCGCCTGCGTAAA
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 GGCGATGAGCGCCTGCGCTCCGGTACGCGACATCCGCGGCGAACCAGGGCACTCA
 CGCCGGCGGCATGCTTGAGCGGGTGGCAAGGTAATGGCGTCCCTGACCGACCA

TGAGATGAGCGCGATATACATCCAGGATCTGCTGGCGGTGGATACGTTTATTCCG
 CGCAAGGTGCAGGGCGGGATGGCCGGCGAGTGCGCCATGGAAAATGCCGTCGGG
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 CTGAGCGCCCGACTGCAGACCGAGGTGGTGGTGGGCGGCGTGGAGGCCAACATG
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 ACCTCGGCGCCGGCTCGACGGATGCGGCGATCGTCAACGCGGAGGGGCAGATAA
 CGGCGGTCCATCTCGCCGGGGCGGGGAATATGGTCAGCCTGTTGATTAACCGA
 GCTGGGCCTCGAGGATCTTTCGCTGGCGGAAGCGATAAAAAAATACCCGCTGGC
 CAAAGTGGAAAGCCTGTTTCAGTATTCGTCACGAGAATGGCGCGGTGGAGTTCTTT
 CGGGAAGCCCTCAGCCCGGCGGTGTTTCGCCAAAGTGGTGTACATCAAGGAGGGC
 GAACTGGTGCCGATCGATAACGCCAGCCCGCTGGAAAAAATTCGTCTCGTGCGCC
 GGCAGGCGAAAGAGAAAGTGTTCACCAACTGCCTGCGCGCGCTGCGCCAGG
 TCTCACCCGGCGGTTCCATTCGCGATATCGCCTTTGTGGTGTGGTGGGCGGCTCA
 TCGCTGGACTTTGAGATCCCGCAGCTTATCACGGAAGCCTTGTCGCACTATGGCG
 TGGTCGCCGGGCAGGGCAATATTCGGGGAACAGAAGGGCCGCGCAATGCGGTGCG
 CCACCGGGCTGCTACTGGCCGGTCAGGCGAATTA

>dhaF amino acid sequence translated

MPLIAGIDIGNATTEVALASDDPQARAFVASGIVATTGMKGTRDNIAGTL
 AALEQALAKTPWSMSDVSRIYLNEAAPVIGDVAMETITETIITESTMIGHNPQTPGGV
 GVGVGTTIALGRLATLPAAQYAEGWIVLIDDAVDFLDAVWWLNEALDRGINVVAI
 LKKDDGVLVNNRLRKTLPVVDEVTLLEQVPEGVMAAVEVAAPGQVVRILSNPYGIA
 TFFGLSPEETQPIVPIARALIGNRSAVVLKTPQGHVQSRVIPAGNLYISGEKRRGEADV
 AEGAEAIMQAMSACAPVRDIRGEPGTHAGGMLERVRKVMASLTDHEMSAIYIQDLL
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 GGVEANMAIAGALTPGCAAPLAILDLGAGSTDAIVNAEGQITAVHLAGAGNMVS
 LLIKTELGLEDSLAEAIKKYPLAKVESLFSIRHENGAVEFFREALSPA VFAKV VYIKE
 GELVPIDNASPLEKIRLVRRQAKEKVFVTNCLRALRQVSPGGSIRDIAFVVLVGGSSL
 DFEIPQLITEALSHYGVVAGQGNIRGTEGPRNAVATGLLLAGQAN*

>Consensus *dhaG*

ATGTCGCTTTCACCGCCAGGCGTACGCCTGTTTTACGATCCGCGCGGG

CACCATGCCGGCGCCATCAATGAGCTGTGCTGGGGGCTGGAGGAGCAGGGGGTCC
 CCCTGCCAGACCATAACCTATGACGGAGGCGGTGACGCCGCTGCGCTGGGCGCC
 CTGGCGGCCAGAAGCTCGCCCCTGCGGGTGGGTATCGGGCTCAGCGCGTCCGGC
 GAGATAGCCCTCACTCATGCCAGCTGCCGGCGGACGCGCCGCTGGCTACCGGAC
 ACGTCACCGATAGCGACGATCATCTGCGTACGCTCGGCGCCAACGCCGGGCAGCT
 GGTTAAAGTCCTGCCGTTAAGTGAGAGAAACTGA

>dhaG amino acid sequence translated

MSLSPPGVRLFYDPRGHHAGAINELCWGLEEQGVPCQTITYDGGGDAAA
 LGALAAARSSPLRVGIGLSASGEIALTHAQLPADAPLATGHVTDSDHLRTLGANAGQ
 LVKVLPLSERN*

>Ribosome binding site BBa_B0034

aaagaggagaaa

>pSB1C3 backbone with terminator and promoter

ccaggcatcaataaacgaaaggctcagtcgaaagactgggcctttcgtttatctgttgttgcggtgaacgctctc
 actagagtcacactggctcaccttcgggtgggcctttcgtttataactagtagcgccgctcagtcggcaaaaaagggaaggt
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